Thesis
4296

THE EFFECTS OF PHOTOPERIOD MANIPULATION ON GROWTH AND REPRODUCTION IN RAINBOW TROUT

(*Oncorhynchus mykiss*)

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Abstract

Photoperiod is an important signal involved in the timing and co-ordination of many processes such as growth and reproduction in salmonids. Both growth and reproduction appear to be controlled by endogenous rhythms, which under natural conditions, is entrained by the seasonal changes in daylength, that is accurately reflected by the diel pattern of melatonin. This thesis investigates the influence of photoperiod on growth and reproduction in rainbow trout (*Oncorhynchus mykiss*), and examines the effect on plasma insulin-like growth factor-I (IGF-I). These studies aim to further our scientific understanding of the endocrine mechanisms controlling growth and reproduction and transfer this knowledge to commercial trout farming practices.

Studies at fry (<5g) and fingerling (>25g) stages investigated the effect of exposure to constant long-days (LD18:6) and constant light (LL) on growth performance. In all experiments, there was a significant growth enhancing effect following exposure to extended light regimes relative to those under ambient or constant short-day photoperiods. Growth enhancement appeared to be caused through direct photo-stimulation or a phase-shift in an underlying endogenous rhythm dependent on the timing of photoperiod application. Measurement of plasma IGF-I accurately reflected growth rate during juvenile development. Furthermore, a clear autumnal increase in IGF-I was observed that was apparently up-regulated by long-day photoperiods. Additionally, it was shown that melatonin implants significantly reduced growth rate below that of unimplanted controls irrespective of photoperiod, but did not reduce circulating IGF-I levels suggesting that elevated melatonin levels masked the perception of daylength but did not act directly on the somatotropic axis to control growth.

As size or one of its correlates (growth rate, energy balance or nutritional status) is regarded as an important determinant of the ability to undergo puberty, the interaction of natural (SNP) and advancing (ADV: long-short day) photoperiods with growth, IGF-I and reproduction was investigated in virgin female broodstock. Under SNP 63% of the population attained maturity while only 29% spawned in the ADV regime. Under SNP both size and growth rate in late spring/early summer appeared to determine whether an individual may initiate maturation. Conversely, under ADV, condition factor appeared to be a better predictor. A complete seasonal relationship between plasma IGF-I, daylength and temperature was demonstrated for the first time in rainbow trout under natural conditions, and provides direct evidence for the relationship between maturation and IGF-I. IGF-I levels showed a negative correlation with testosterone as fish initiated maturation. Furthermore, IGF-I levels accurately reflected growth rate prior to the initiation of vitellogenesis, suggesting that IGF-I may provide an endocrine signal between the somatotropic and reproductive axes that nutritional status, growth rate and/or size is sufficient to initiate maturation. In addition, maturing individuals under both photoperiods typically expressed higher circulating IGF-I levels than those that remained immature and may reflect a greater opportunity for IGF-I to act on the pituitary to stimulate gonadotropin production. In this sense, the observation of elevated levels for 3 months under SNP compared to only 1 month under ADV may reflect a reduction in the window of opportunity to initiate maturation under advancing photoperiods and hence explain the reduction in fish spawning. Finally, using a commercially available ELISA kit provided evidence of a possible detection method for measurement of a leptin-like peptide as

absolute levels differed significantly between mature and immature fish. This provides preliminary evidence for a possible involvement of a leptin-like peptide in rainbow trout reproduction and energy homeostasis.

Given the above evidence of a growth enhancing effect of photoperiod in covered systems, the transfer of constant light (LL) regimes to uncovered commercial trout farming practices was also examined. The application of LL regimes during either fry grow-out in tanks using floating lights or ongrowing to harvest size during winter in cages using submersible lights was investigated. In all trials conducted, the exposure to LL in autumn significantly enhanced winter growth rate and feeding efficiency by up to 30% and 25% respectively relative to those maintained under ambient light. Furthermore, the effect of light intensity was clearly shown to be an important requirement in successfully applying photoperiod regimes onto the ambient photoperiod in order to enhance commercial production in "uncovered systems".

These studies clearly indicate the importance of photoperiod in influencing growth and reproduction in rainbow trout. Furthermore, the use of plasma IGF-I as an indicator of growth provides a practical tool for studying growth-photoperiod-reproduction interactions in this species. However, further studies are necessary to further our understanding of the endocrine pathways governing physiological mechanisms, especially growth and reproduction. Overall, this work has provided important information to improve both scientific understanding and commercial development although it is clear that substantial research is still required.

Keywords: rainbow trout, photoperiod, growth, maturation, IGF-I, melatonin.

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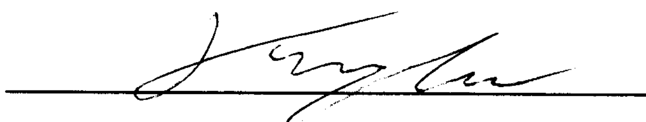
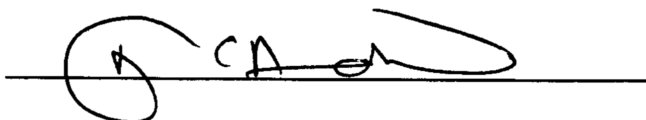
Declaration

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

Signature of candidate: 

Signature of supervisor: 

Date: 22/02/05.

Glossary of common and scientific names used within this thesis

rainbow trout	*Oncorhynchus mykiss*
pink salmon	*Oncorhynchus gorbuscha*
sockeye salmon	*Oncorhynchus nerka*
chum salmon	*Oncorhynchus keta*
coho salmon	*Oncorhynchus kisutch*
chinook salmon	*Oncorhynchus tshawytscha*
masu salmon	*Oncorhynchus masou*
amago salmon	*Oncorhynchus masou ishikawai*
Atlantic salmon	*Salmo salar*
brown trout	*Salmo trutta*
tilapia	*Oreochromis* various sp.
burbot	*Lota lota*
pike	*Esox lucius*
common carp	*Cyprinus carpio*
goldfish	*Carassius auratus*
European eel	*Anguilla anguilla*
Japanese eel	*Anguilla japonicus*
Japanese medaka	*Oryzias latipes*
zebrafish	*Brachydanio rerio*
sailfin molly	*Poecilia velifera*
Thai catfish	*Clarias macrocephalus*
channel catfish	*Ictalurus punctatus*
wallago catfish	*Wallago attu*
green sunfish	*Lepomis cyanellus*
bluegill sunfish	*Lepomis macrochirus*
white crappie	*Pomonix annularis*
largemouth bass	*Micropterus salmoides*
lamprey	*Petromyzon marinus*
Gulf killifish	*Funduls grandis*
common mummichog	*Fundulus heteroclitus*
red sea bream	*Pagrus major*
gilthead sea bream	*Sparus aurata*
European sea bass	*Dicentrarchus labrax*
Asian sea bass (barramundi)	*Lates calcarifer*
striped bass	*Morone saxatilis*
cod	*Gadus morhua*
haddock	*Melanogrammus aeglefinus*
Atlantic halibut	*Hippoglossus hippoglossus*
plaice	*Pleuronectes platessa*
turbot	*Scophthalamus maximus*
spotted sea trout	*Cynoscion nebulosus*
white grouper	*Epinephelus aeneus*
Iberian wall lizard	*Podarcis hispanica*
viperine snake	*Natrix maura*
African clawed frog	*Xenopus laevis*
red deer	*Cervus elaphus*

Chapter 1: General Introduction

1.0 General Introduction

The salmonids are members of the bony fish (Class Osteichthyes) which fall into the family Salmonidae which are characterised by the presence of a fleshy adipose fin. Within this family there are six genus, these being *Salmo*, *Oncorhynchus*, *Salvelinus*, *Hucho*, *Salmothymus* and *Brachymystax* (Phillips *et al.*, 1992). The Salmonidae are native to the freshwaters and oceans of the Northern Hemisphere. The greatest number of individuals are found in the Pacific basin which has two or more members of each genus, while the North Atlantic basin has two major species of *Salmo* and two chars (Ade, 1989). Basic habitat requirements are similar for all salmonids in that they are pollution sensitive, requiring pure, cool well-oxygenated water in which to grow and reproduce. A common feature shared by all salmonids is their dependence on freshwater for reproduction, many species migrating large distances from the oceans to their natal streams.

1.1 The Rainbow Trout

The rainbow trout was formerly known as *Salmo gairdneri* Richardson, but has now been recognised as a member of the Pacific Salmonidae, *Oncorhynchus*, being re-classified as *Oncorhynchus mykiss* Walbaum (Kendall, 1988). The species is naturally indigenous to the West Coast of North America, primarily a native to their coastal rivers. Its home range extends from the Kuskokwim River in Alaska, south through British Colombia to Baja in California. There is also a native population in the Rio Casa Grandes in the Mexican province of Chihuahua (Sedgewick 1992). Initially the species was introduced to other parts of North America to enrich fauna, and later in relation to its economic value as a food and sports fish (MacCrimmon, 1971). Nowadays it has been distributed globally with naturalised populations being found in South America,

Europe, Scandinavia and temperate regions of Asia and Australasia (Watson, 1993, Hershberger, 1992). One of the main reasons for such widespread distribution has been through the species adaptability to a wide range of environmental and culture conditions (Hershberger, 1992).

There are two major forms of the rainbow trout; the anadromous steelhead, which is the migratory form, spending a portion of its life at sea before returning to spawn in freshwater, and the non-migratory rainbow trout, which spends all its life in freshwater lakes, rivers and streams (Bromage & Cumaranatunga, 1988). Unlike most salmonids which spawn in autumn, the rainbow trout is regarded as a spring spawner in the Northern Hemisphere, typically February to June (Gall & Crandell, 1992), although selective breeding has led to the development of strains with different spawning times (Stevenson, 1987). Female rainbow trout never spawn in their first year and depending on size and growth rate some will spawn in their second year with the majority spawning by the end of their third year (Laird & Needham, 1988). As with the females, male rainbow trout will spawn by their second and third years, however, a proportion of the population will mature and be capable of spawning in their first year, with these fish being referred to as precocious males. Unlike in the natural environment where the female would build a redd (nest) in the gravel in which to deposit her eggs, in the confines of captivity eggs are ovulated but not oviposited, and therefore have to be artificially stripped and fertilised (Shepherd & Bromage, 1992). Hatching is related to the temperature and normally occurs after 400-degree days (Sedgewick, 1990). On hatching, the alevins (yolk-sac fry) are dependent on their yolk-sac reserves prior to swim-up and first feeding. At this stage the fry take on the distinctive vertical black banding referred to as parr marks. After a period of time, usually by the end of the first year, these markings fade at which point the typical adult colouration develops.

1.2 Rainbow Trout Industry

Due to its adaptability, the rainbow trout is cultured under a variety of aquaculture methods including freshwater ponds, raceways, tanks and cages, as well as in an ever increasing number of saltwater cages and tanks. Of these, there are farms specialising in hatchery and fry rearing, juvenile suppliers, ongrowers, as well as fully integrated systems with complete broodstock, hatchery and ongrowing facilities.

The most recent FAO figures reveal that global production of salmonids from culture reached approximately 1.8 million tonnes in 2002, of which four species dominated production, these being Atlantic salmon (*Salmo salar*) 61%, rainbow trout at 28%, coho salmon (*Oncorhynchus kisutch*) 6.3%, and chinook salmon (*Oncorhynchus tshawytscha*) 1.1%. Global rainbow production to 2002 was estimated at 508,000 tonnes from a total of 48 countries. In Europe, the rainbow trout is the second most economically cultured species other than Atlantic salmon, with a total production during 2001 of 233,950 tonnes. Within the United Kingdom total production for 2002 was 16,318 tonnes, with 74% (12,068 tonnes) of this produced for the table market and the remaining 26% (4,250 tonnes) for restocking purposes, which represented a 6.7% (1,076 tonnes) increase on the previous year's total production (Dunn 2003, SERAD 2003). During this period, Scottish production increased by 21%, whereas English and Welsh production fell by 3%. Figure 1.1 provides a summary and comparison of EU Atlantic salmon and rainbow trout production.

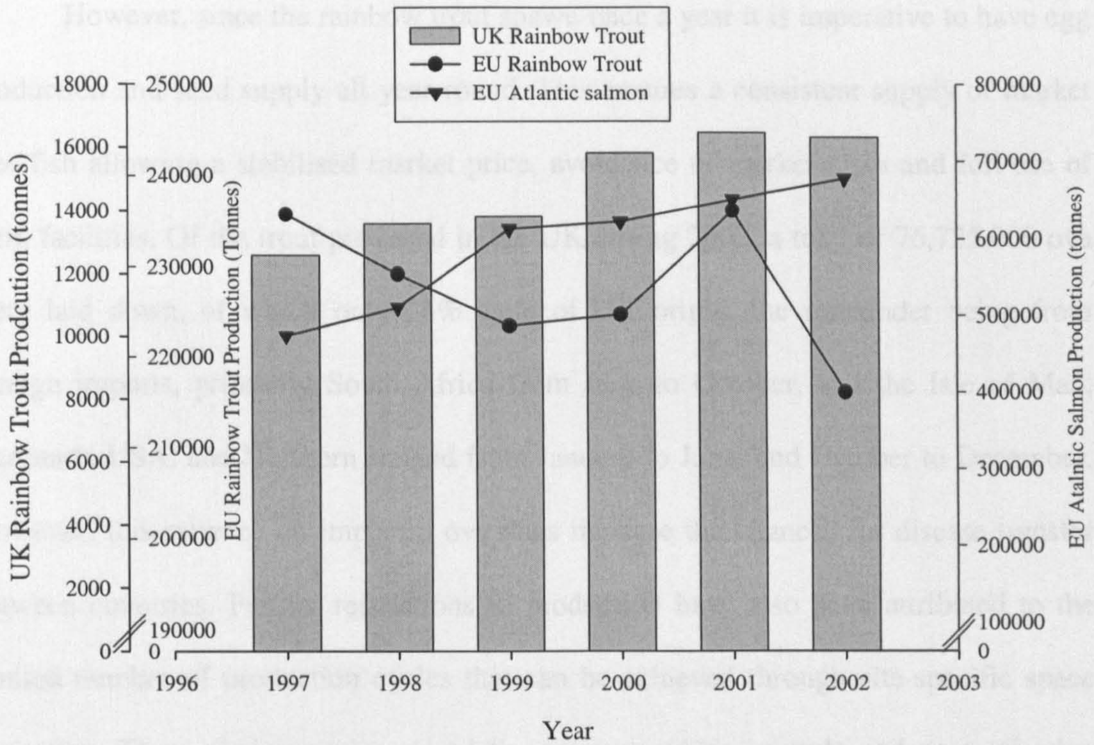


Figure 1.1 European production of rainbow trout and Atlantic salmon production from 1997-2002 and comparison with UK production of rainbow trout. (Figures collated from Dunn, 2003 & Winnard, 2003).

However, since the rainbow trout spawn once a year it is imperative to have egg production and seed supply all year round. This ensures a consistent supply of market size fish allowing a stabilised market price, avoidance of market gluts and full use of farm facilities. Of the trout produced in the UK during 2002, a total of 76,725,000 ova were laid down, of which only 28% were of UK origin, the remainder being from foreign imports, primarily South Africa from June to October, and the Isle of Man, Denmark, USA, and Northern Ireland from January to June, and October to December. However, this reliance on imported ova does increase the chances for disease transfer between countries. Further restrictions to production have also been attributed to the limited number of production cycles that can be achieved through site-specific space limitation. Thus, photoperiod manipulation may provide a simple and cost effective means of altering spawning times as well as providing the additional benefits of improving growth rates as has been observed in the Atlantic salmon industry.

1.3 Photoperiod and Reproduction

Reproduction in fish which inhabit temperate or higher latitudes is characteristically an annual event with the release of gametes programmed so that the progeny are produced in the spring when conditions are most favourable for their survival (Bromage *et al.*, 1994). As gonadal recrudescence to ovulation can take twelve months or more to complete in salmonids, with a further two to four months following fertilisation to first-feeding, then it is important that fish can synchronise their breeding time with the changing conditions in the external environment (Bromage & Cumaranatunga, 1988). Clearly then, gonadal recrudescence must be initiated many months earlier to ensure that production of first-feeding fry coincides with improving climatic and nutritional conditions (Bromage *et al.*, 2001).

As with other seasonally breeding animals, fish rely on cues from the external environment to achieve synchronisation of maturational events with the changing season. These cues are referred to as "proximate" which differentiates them from environmental or "ultimate" factors (Bromage *et al.*, 2001). In most temperate spawning species, including the salmonids, it is now widely accepted that the pattern of seasonally changing daylength is primarily responsible for the cueing and timing of reproduction (Bromage *et al.*, 1994).

Seasonally Changing Light Cycles

Modified seasonal light cycles have been shown to both advance and delay spawning in various salmonid species (Bromage *et al.*, 1992b, 2001). In the case of the rainbow trout, compression of the seasonal cycle into 6 or 9 months will produce 3-4 and 1-2 month advancements in spawning respectively, while an extended 18 month cycle can delay spawning by at least 3 months (Bromage & Duston, 1986). The time of first sexual maturation can also be advanced by photoperiod manipulation. Bromage (1987) showed that the exposure of fish of 4 months of age to two altered seasonal light regimes produced 3-4 month advancements in the time of puberty. However, it is now evident that the increasing and decreasing components of the seasonally changing daylength can be replaced by periods of constant daylength. Figure 1.2A provides examples of typical photoperiod regimes used to manipulate spawning time in rainbow trout.

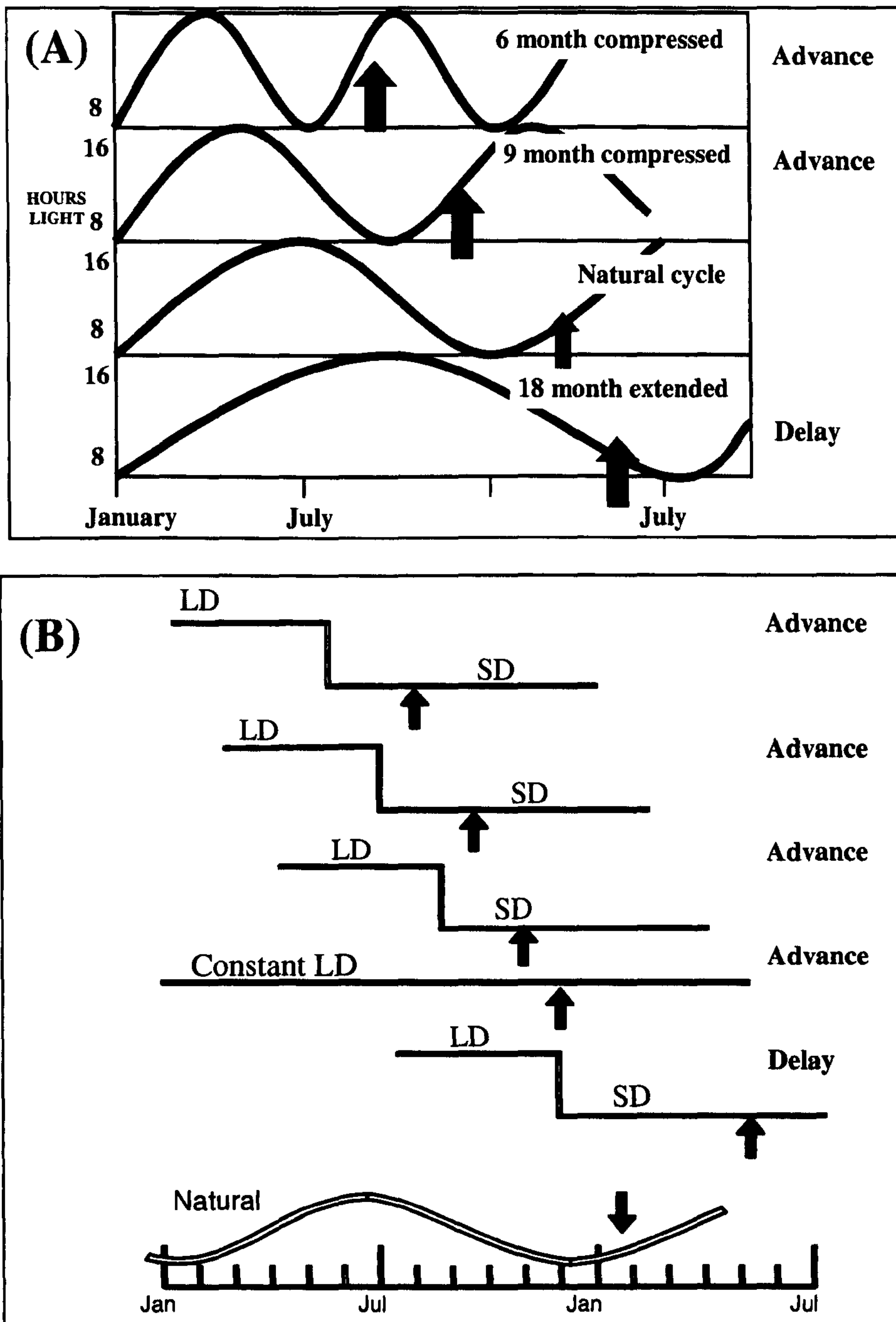


Figure 1.2 **(A)** Seasonally compressed and extended natural photo-cycles used to manipulate spawning time in relation to the natural spawning period. **(B)** The effect of timing and exposure to constant long (LD) to short (SD)-day photoperiods on spawning time in relation to the natural spawning period. In all regimes arrows indicate the occurrence of spawning (Adapted from Bromage & Cumaratunga, 1988).

Constant Photoperiods

Spawning of rainbow trout under photoperiods of constant duration has been well documented (Whitehead & Bromage, 1980; Bromage *et al.*, 1982a; 1982b, 1984; Bourlier & Billard, 1984; Bromage & Duston, 1986). Constant long days (LD 18:6) early in the year and short days (LD 6:18) 2-3 months before or around the summer solstice, both advance maturation, while short days in the first few months and long days after the summer solstice produce delays in maturation (Figure 1.2B). Similar observations following exposure to periods of constant light have also been made in Atlantic salmon, (Berg *et al.*, 1996; Oppedal *et al.*, 1997; Taranger *et al.*, 1998, 1999; Porter *et al.*, 1999a, 1999b), pink salmon (Beacham & Murray, 1993), masu salmon, *Oncorhynchus masou* (Takashima & Yamada, 1984) and European sea bass, *Dicentrarchus labrax* L. (Carrillo *et al.*, 1993). Randall *et al.*, (1991a) also demonstrated that as little as 2 months of exposure to constant light can advance or delay maturation in the rainbow trout.

When undertaking photoperiod manipulations it is important to consider the timing of the switch between photoperiod treatments as it determines the degree of advancement. The earlier the reduction in daylength, the more marked the advancement in spawning (Bromage *et al.*, 1994). This indicates that there is a period of photosensitivity in the first half of the annual cycle during which the trout can respond to long days (Bromage *et al.*, 1984). However, particular care needs to be taken when referring to long and short days in that daylength can act as a longer or shorter photoperiod, irrespective of its length, providing that it is longer or shorter than that to which the fish were previously exposed (Randall *et al.*, 1987). The variation in spawning response of fish in relation to when they are exposed to these periods of long and short days, suggests that the photoperiodic history, and the direction of change of

photoperiod is of greater importance in the timing of maturation rather than a critical daylength (Davies *et al.*, 1992).

Of further interest is that fish maintained for up to four years under constant short days have been shown to spawn with an annual periodicity, 3-4 months out of phase with the natural cycle, while those under constant light or long days spawn successively at 5-7 month intervals (Bromage & Duston, 1986, Duston & Bromage, 1986, 1987). These findings indicate that changes in the environmental photoperiod are not essential for maturation, and suggest that the timing of reproduction is being co-ordinated by endogenous processes (Bromage *et al.*, 1990).

Circannual Rhythms and the Endogenous Control of Reproduction

Under constant light conditions a truly endogenous rhythm would be expected to "free-run" with a period length which approximates, but is significantly different from one year, i.e. circannual; to be entrainable by an environmental zeitgeber; be temperature-compensated; and finally, maintain their periodicity for a number of cycles to establish that it is self-sustaining (Bromage *et al.*, 1990, Duston & Bromage 1991).

Evidence for the endogenous control of gonadal maturation was first provided by Duston & Bromage (1991), whereby female rainbow trout maintained on constant short days (LD 6:18) for a period of 51 months exhibited a free-running circannual rhythm for up to three cycles. During this period there was variation in the periodicity of the rhythm, both between individual fish, and also between successive spawnings of individual fish. Such desynchronisation of spawning time was also reported by Bromage *et al.*, (1984), which is typical of free-running endogenous rhythms as seen in other vertebrates. Further evidence for an endogenous rhythm was provided by Randall *et al.*, (1999), in which a seasonal-phase response-curve in spawning time was

described following exposure of groups of fish to long or short daylengths at a series of monthly intervals throughout the year. It is therefore proposed that long days, occurring earlier or later than they would be under a natural photoperiod, were perceived that the clock was running slow or fast, thus initiating corrective forward adjustments (advance phase-shifts) or backwards adjustments (delay phase-shifts), respectively (Randall *et al.*, 1998). The use of resonance and skeleton photoperiods has also indicated that daylength measurement is effected by endogenous circadian clocks rather than by hour-glass mechanisms (Duston, 1987; Duston & Bromage, 1986). Therefore, the response to photoperiod manipulation suggests that the reproductive cycle is controlled by a circannual rhythm, and that it is the entrainment of this rhythm by seasonally-different light cues (zeitgeber) which accurately co-ordinates reproductive development with the natural pattern of daylength change (Bromage & Duston, 1986). As a result, the precision and reliability of the reproductive response to specific environmental cues offers a mechanism by which spawning time can be adjusted to facilitate the production of off-season eggs and fry. However, photoperiod has not only been implicated in influencing reproduction in fish, it has also been demonstrated to have considerable effects on growth.

1.4 Photoperiod and Growth

Many of the photoperiodically induced effects on salmonid growth have been shown through the inhibition of maturation and subsequent reallocation of resources into somatic rather than gonadal development during later life stages. On the other hand, in juveniles, photoperiod manipulations are undertaken to promote greater growth and induce smoltification to ensure year round supply of salt-water adapted smolts. Certainly constant light and long day photoperiods have been shown to increase growth

in the juvenile and ongrowing stages of Atlantic salmon and are now general practice on salmon farms (Saunders & Harmon, 1988; Saunders & Henderson, 1988; Saunders *et al.*, 1989; Stefansson *et al.*, 1989; Clarke, 1990; Hansen *et al.*, 1992; Berg *et al.*, 1994; Saunders *et al.*, 1994; Taranger *et al.*, 1995; Oppedal *et al.*, 1997; Porter *et al.*, 1999b; Taranger *et al.*, 1999) but are not common practice in rainbow trout culture. While significantly decreasing the incidence of grilsing, Porter *et al.*, (1999b) observed an increase in growth of 30% in 1+ sea winter Atlantic salmon while maintained under constant light from November to July. In such studies, a decrease in specific growth rate is often observed following initial exposure to constant light from November, December, or January until July, followed by improved growth in the subsequent 4-5 months relative to controls (Endal *et al.*, 2000; Taranger *et al.*, 1999). Villarreal *et al.*, (1988) suggested that the delays observed in growth after daylength reduction may be related to the synchronising effect of an endogenous rhythm of appetite and growth. Atlantic salmon parr maintained on a longer daylength when the natural daylength should have been decreasing grew larger, but reached a smaller size than predicted. This provided evidence for the existence of endogenous appetite and growth rhythms which will assert themselves in the absence of cues from declining daylength.

However, how this photoperiodic information is transduced to initiate both growth and reproductive responses is unclear. A prime candidate for this is the pineal gland and the associated production of melatonin.

1.5 The Pineal Organ and Melatonin Synthesis

The pineal organ is a part of the central nervous system that is formed, like the retina, as an evagination from the embryonic primary forebrain. It is a complex structure composed of photoreceptor cells, interstitial cells, macrophages and neurons.

This organ is responsible for conveying photoperiodic information to the brain via neural pathways and by the release of indoleamines, primarily melatonin, into the circulation. It is currently agreed that the photoreceptor cells are the source of the melatonin produced by the pineal organ (Ekstrom & Meissl, 1997). Photoreceptor cells have been shown to respond to changes in photoperiod with morphological changes that may reflect changes in protein synthesis. However, throughout vertebrate evolution the pineal gland has undergone considerable changes in structure and function, from a directly photosensory pineal organ in "lower vertebrates" to a secretory gland in mammals (Ekstrom & Meissl, 2003).

Studies undertaking pinealectomy (PNX) have demonstrated that although the elevation of plasma melatonin levels during the night are significantly reduced, they are not completely abolished (Porter *et al.*, 1996). As yet the origin of this residual melatonin is unclear, although Gern *et al.*, (1978) identified *in vivo* rhythmic melatonin production in the retina of rainbow trout following PNX. Further studies using both PNX and enucleation have also suggested that the gastrointestinal tract may also be responsible for the production of extrapineal melatonin. However, whether this residual melatonin contributes to the diurnal rhythm in circulating melatonin has still to be clarified.

Melatonin Synthesis

The synthesis of melatonin by the pineal is regulated by the intensity of the ambient illumination and reaches the highest level in complete darkness, i.e. during the night. This pattern is apparent in all vertebrates, and seems to be the normal synthesis pattern.

In the pineal organ, melatonin is formed via a complex biosynthetic pathway (see Figure 1.3) from the essential amino acid L-tryptophan (reviewed in Ekstrom & Meissl, 1997). Four enzymes found sequentially in this pathway are tryptophan hydroxylase (TpH), aromatic amino acid decarboxylase (AADC), serotonin N-acetyl transferase (SNAT), and hydroxyindole-O-methyltransferase (HIOMT)). It is believed that TpH and SNAT are the most important with regard to the regulation of melatonin synthesis, and it is concluded that SNAT is the rate-limiting enzyme for generating rhythmic melatonin production.

Control of Melatonin Synthesis

Melatonin is synthesised by the pineal and retinal cells, and is associated with the light-dark cycle. The duration of this hormone message is directly proportional to the duration of darkness, and is considered as an internal zeitgeber of the organism. In fish, hormone biosynthesis is primarily controlled by light perception through intrapineal photoreceptors (Ekstrom & Meissl, 1997).

Melatonin secretion in many higher vertebrates appears to be under endogenous circadian control. In the case of mammals, melatonin secretion is regulated by photic information perceived by the eyes which is relayed to the pineal via a complex sympathetic neural pathway incorporating the superchiasmatic nuclei (SCN) of the hypothalamus (Ganguly *et al.*, 2002). Furthermore, throughout vertebrate evolution the mammalian pineal gland has regressed in its function, in that it has become a secretory gland rather than a direct photosensory organ. In this respect it has been shown that the SCN contains circadian oscillators that generate the rhythmic release of melatonin, rather than being located in the pineal gland itself (Simonneaux *et al.*, 2004). Similarly, in most teleosts studied to date, pineal photoreceptors which

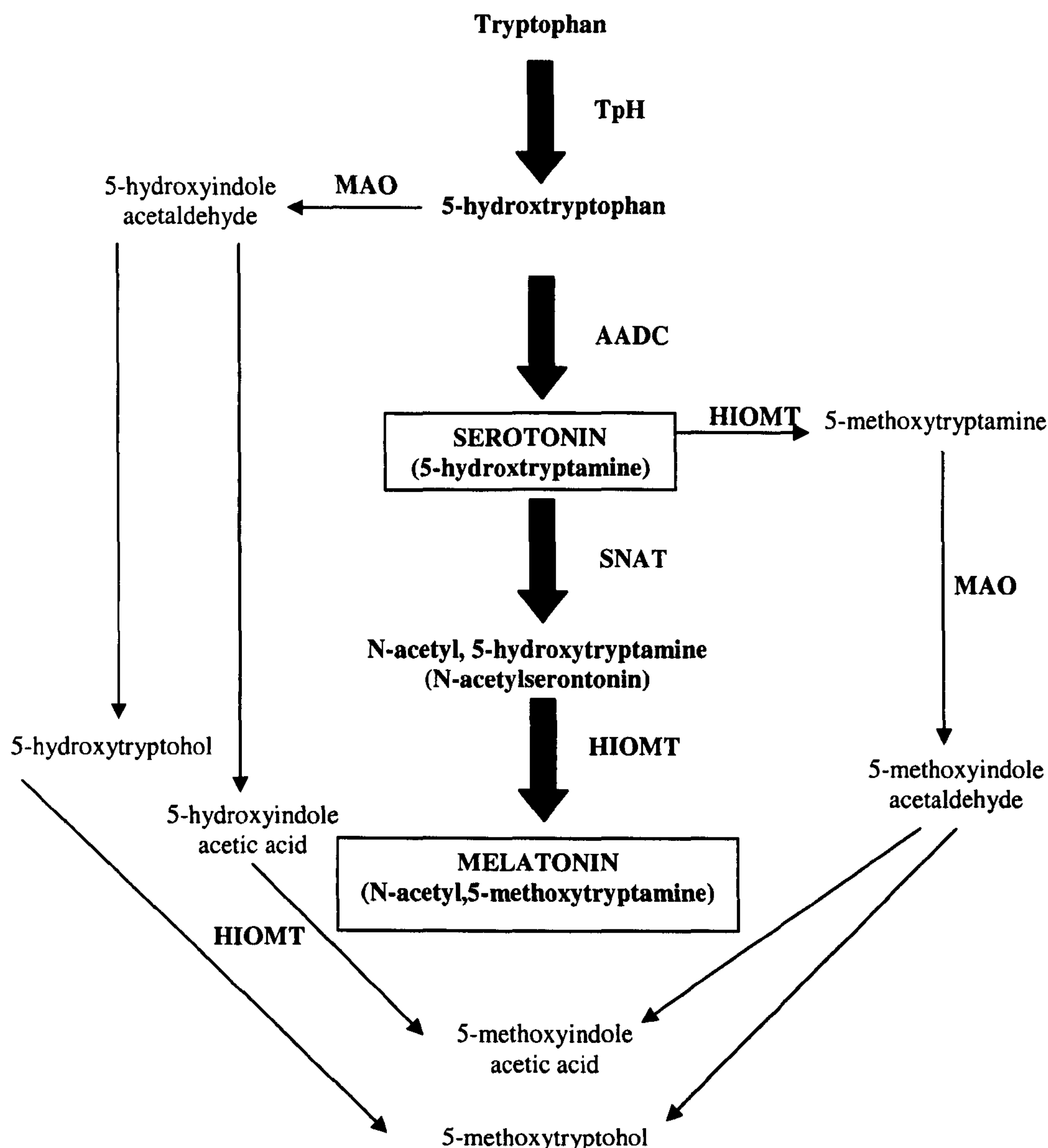


Figure 1.3 Pathways for synthesis and degradation of melatonin, methoxyindoles, and hydroxyindoles in the teleost pineal organ. TpH: tryptophan hydroxylase; AADC: aromatic amino acid decarboxylase; SNAT: serotonin N-acetyl transferase; HIOMT: hydroxyindole-O-methyltransferase; MAO: monoamine oxidase (After Ekstrom & Meissl, 1997).

contain a photopigment-based light transducer, embody a clock or endogenous circadian oscillator. The clock is synchronised to 24-hour daily cycles and creates the rhythm of melatonin secretion. Maintenance of pike (*Esox lucius*) pineal tissues in constant darkness have shown a persistent circadian rhythm in melatonin synthesis (Figure 1.4 A) (Falcon *et al.*, 1989). Consequently, these results support the idea that an endogenous circadian oscillator is located within the pineal. Similar intrapineal circadian oscillators have been suggested in the gilthead seabream (*Sparus aurata*) (Molina-Borja *et al.*, 1996), goldfish (Kezuka *et al.*, 1992), zebrafish (*Brachydanio rerio*) (Kazimi & Cahill, 1999) and sailfin molly (*Poecilia velifera*) (Okimoto & Stetson, 1999).

Experiments in both rainbow trout and Atlantic salmon demonstrated that circulating melatonin profiles always reflect the prevailing daylength (Randall *et al.*, 1994) exhibiting similar diel cycles of melatonin to those observed in higher vertebrates, defined as type "C" profiles (Figure 1.4 B). However, melatonin rhythms do not persist in trout or masu salmon maintained under constant darkness (DD) suggesting that the pineal gland in these species is not able to maintain an endogenous rhythm (Figure 1.4 A) (Randall *et al.*, 1991b,c; Iigo *et al.*, 1998). Additionally in both *in vivo* and *in vitro* studies, all detected changes in plasma melatonin content were directly associated with the light to dark or dark to light transitions (Alvarino *et al.*, 1993; Max & Menaker, 1992; Randall *et al.*, 1991b). Furthermore, dark phase light intensity has been shown to regulate circulating melatonin levels in both Atlantic salmon and brook trout, although the rhythm of production is maintained (Porter *et al.*, 2001; Zachmann *et al.*, 1992). Thus, melatonin production in the trout appears to be a direct response to darkness. Results of out-of-phase and solstice hold experiments further support that changes in the duration of and amplitude of the nocturnal increase

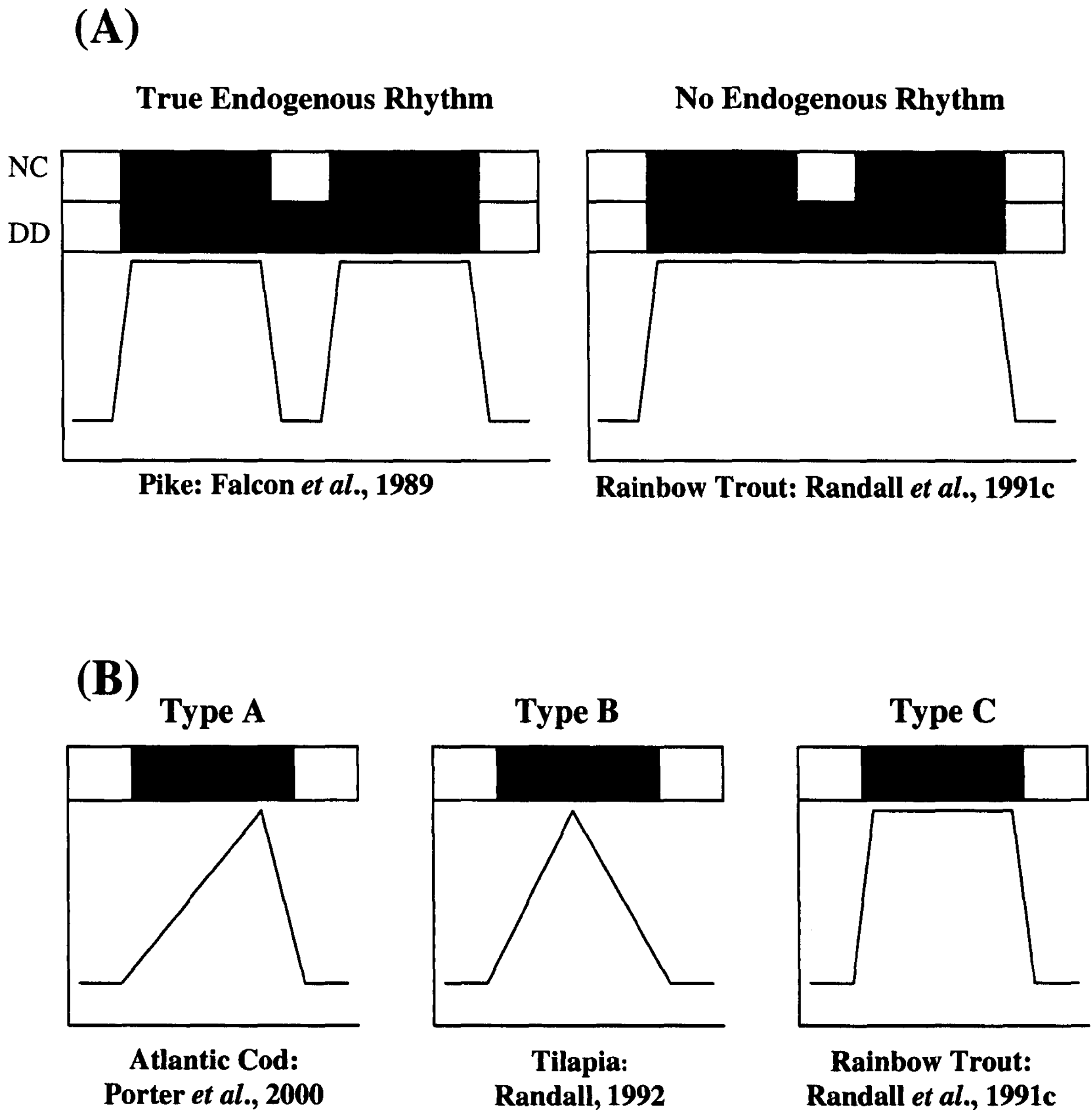


Figure 1.4 **(A)** Demonstration of the presence or absence of an endogenous rhythm of melatonin production in pike and rainbow trout respectively following the exposure to constant darkness (DD-bottom bar). White and black bars represent light and dark periods respectively, with the top bar representing the subjective natural light-dark cycle (NC). **(B)** Schematic representation of melatonin profiles reported in different fish species. White and black bars represent light and dark periods respectively.

in circulating melatonin is not attributable to an endogenous circannual variation in melatonin production (Randall *et al.*, 1995a). Thus it is proposed that the endogenous rhythms which control reproduction and growth in salmonid fish must be acting "down-line" from the information perceived by melatonin on daily and calendar time (Bromage *et al.*, 1995). This provides evidence that the trout pineal does not contain a circadian oscillator capable of regulating melatonin release, and that the pineal endocrine melatonin message mainly depends on the irradiance of the incident light. Hence this has the potential to provide the fish with accurate information on both daily and calendar time. Increasing or decreasing daylength would be the feature of a photoperiodic signal, responsible for the entrainment of the circannual clock, which may ultimately control growth, smoltification and reproduction (Randall *et al.*, 1995a). However, the link between these components in the various endocrine cascades and perception of light are still poorly understood and current evidence certainly does not support a central role of melatonin in the control of reproduction (Mayer *et al.*, 1997).

1.6 Endocrine Regulation of Reproduction: Hypothalamus-Pituitary-Gonadal Axis

Reproductive function in vertebrates is controlled by the hypothalamus-pituitary-gonadal (HPG) neuroendocrine axis (Figure 1.5). This process itself is submitted to the control of both external factors (physico-chemical, nutritional, social and environmental conditions) and internal factors (growth and metabolic conditions), all of which are transduced by the nervous system that ultimately acts on the HPG axis (Soma *et al.*, 1996).

The HPG axis comprises three component parts: (1) gonadotropin-releasing hormone (GnRH) neurons projecting from the hypothalamus of the brain to the

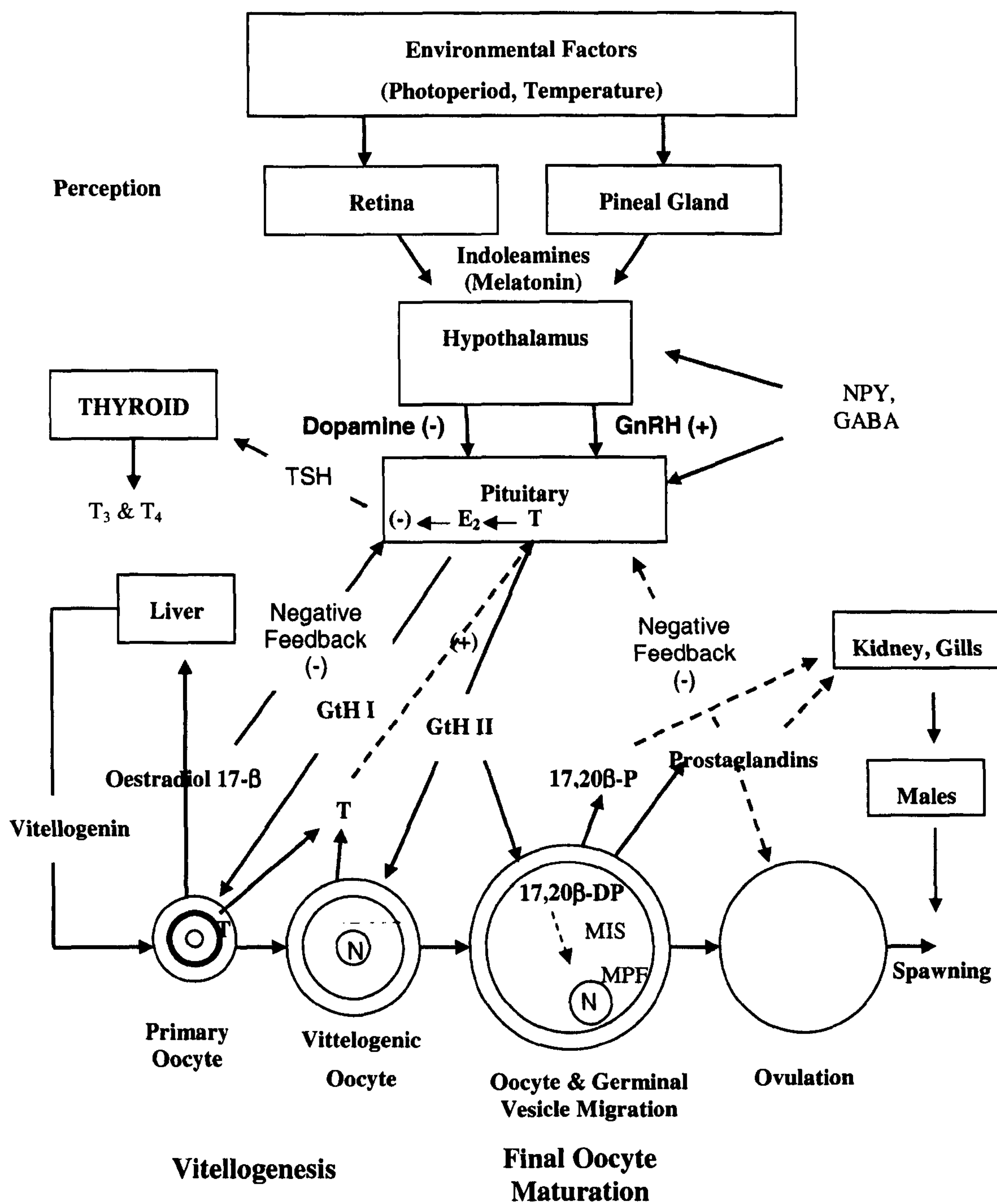


Figure 1.5 Summary of the hypothalamus-pituitary-gonadal axis and its interaction with environmental factors in the control and sequence of hormonal synthesis involved in gametogenesis in female salmonids. (Figure supplied by Dr. Herve Migaud).

pituitary. Unlike mammals, teleosts lack a hypothalamo-hypophysial portal system for the transport of neurohormonal regulators. Instead, the GnRH neurons directly innervate gonadotropin-producing cells (gonadotropes) through the hypohysial stalk and the *pars nervosa*; (2) gonadotropes in the anterior pituitary gland (*pars distalis*) which synthesis and secrete gonadotropins (GtH) (Lutenising hormone, LH, and follicle stimulating hormone, FSH, in tetrapods, and their homologues in fish, GtH II and GtH I); and (3) the somatic cells of the gonad (theca and granulosa cells in the ovary, Leydig and Sertoli cells in the testis) which are responsible for steroidogenesis and the production of growth factors (IGFs) involved in gametogenesis. These hormones are important for the regulation of reproduction, either directly on gonadal tissues in a paracrine or autocrine fashion, or through positive and negative feedback mechanisms on the hypothalamus and pituitary in an endocrine manner (Linard *et al.*, 1995; Weltzein *et al.*, 2004).

Gonadotropin Releasing Hormone (GnRH)

The number of GnRH family members identified in vertebrates has rapidly increased over the last decade, and to date a total of 14 variants have been categorised, of which 8 have been found in teleosts (Lethimonier *et al.*, 2004). As now shown in all vertebrate classes, the brain of teleosts contains at least two GnRH variants, but there is a growing number of species in which three GnRH variants have been found. Of these variants, chicken GnRH-II appears to be highly conserved across vertebrate evolution, while in many teleosts studied to date including the rainbow trout, the salmon GnRH is the most commonly detected second variant. In species expressing three GnRH forms, the third variant appears to be less conserved and varies considerably between species. In addition, the localisation and expression of these variants differs in relation to the

brain areas. Generally, cGnRH-II is consistently detected in the mid-brain, while mGnRH, sGnRH or sbGnRH are found in an anterior GnRH system (Kah *et al.*, 1999). Furthermore, the observation of only sGnRH fibres in the masu salmon pituitary while both sGnRH and cGnRH-II are observed in goldfish pituitary, would suggest that species specific GnRH profiles are involved in the regulation of pituitary function in teleosts (Kobayashi *et al.*, 1997). However, ultimately GnRH acts on the pituitary following the binding of GnRH to specific membrane-bound GnRH-R receptors to induce the release of gonadotropins.

Gonadotropins

Within the pituitary, two gonadotropins are released into circulation at different stages of gametogenesis and appear to be under regulation by gonadal synthesis of steroid hormones and growth factors (IGFs see chapter 4), and changes in the nature and distribution of gonadotropin receptors in the gonads during gametogenesis (Swanson *et al.*, 1995). FSH (GtH I) is generally associated with early oocyte growth and vitellogenic processes, while LH (GtH II) has a principal role in the final maturation and ovulation of oocytes in female fish (Swanson, *et* al., 1995; Breton *et al.*, 1998; Davies et al., 1999). In teleosts, an inhibitory control by dopaminergic neurons (Dopamine, GnRIH) is also exerted in addition to the stimulatory control by GnRH neurons. Dopamine has been shown to inhibit GtH release, while, treatment with dopamine antagonists such as pimozide, stimulates GtH release (Bromage & Duston, 1986).

Steroidogenesis: Oocyte Growth and Maturation

The process of oocyte growth and maturation in salmonids involves three principal steroid groups these being; androgens, principally testosterone and 11-ketotetosterone; oestrogens, 17β-oestradiol (E_2); and progesterones, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) (Nagahama, 1999). E_2 is known to be important for oocyte growth (vitellogenesis) while 17α,20β-DP is the principal maturation-inducing steroid (MIS) required for final oocyte maturation in salmonids (Nagahama, 1983). Production of both steroid hormones follows a classic two-cell type model (Figure 1.6) in which the precursor steroids testosterone and 17α-hydroxyprogesterone in the theca cell layer are converted to E_2 and 17α,20β-DP in the granulosa cells respectively (Nagahama *et al.*, 1995).

Oocyte Growth (Vitellogenesis)

Unlike mammalian oogenesis, oocytes of teleosts continue to grow while arrested in the first meiotic prophase. The principal event responsible for the significant increase in oocyte size is vitellogenesis, which involves the sequestration of the hepatically derived precursor, vitellogenin, into yolk protein (Nagahama, 1999). Plasma calcium is generally regarded as a good method for indirect assessment of vitellogenin (Norberg *et al.*, 1989). As noted earlier, the principal steroid responsible for the stimulation of hepatic synthesis and secretion of vitellogenin is E_2, following the conversion of testosterone (T) that diffuses into the granulosa cell layer by P-450arom. In this regard the principal target of FSH is the ovarian cytochrome P450 aromatase, which is considered to be one of the rate limiting enzymes for E_2 biosynthesis (Pankhurst *et al.*, 1996; Senthilkumaran *et al.*, 2004).

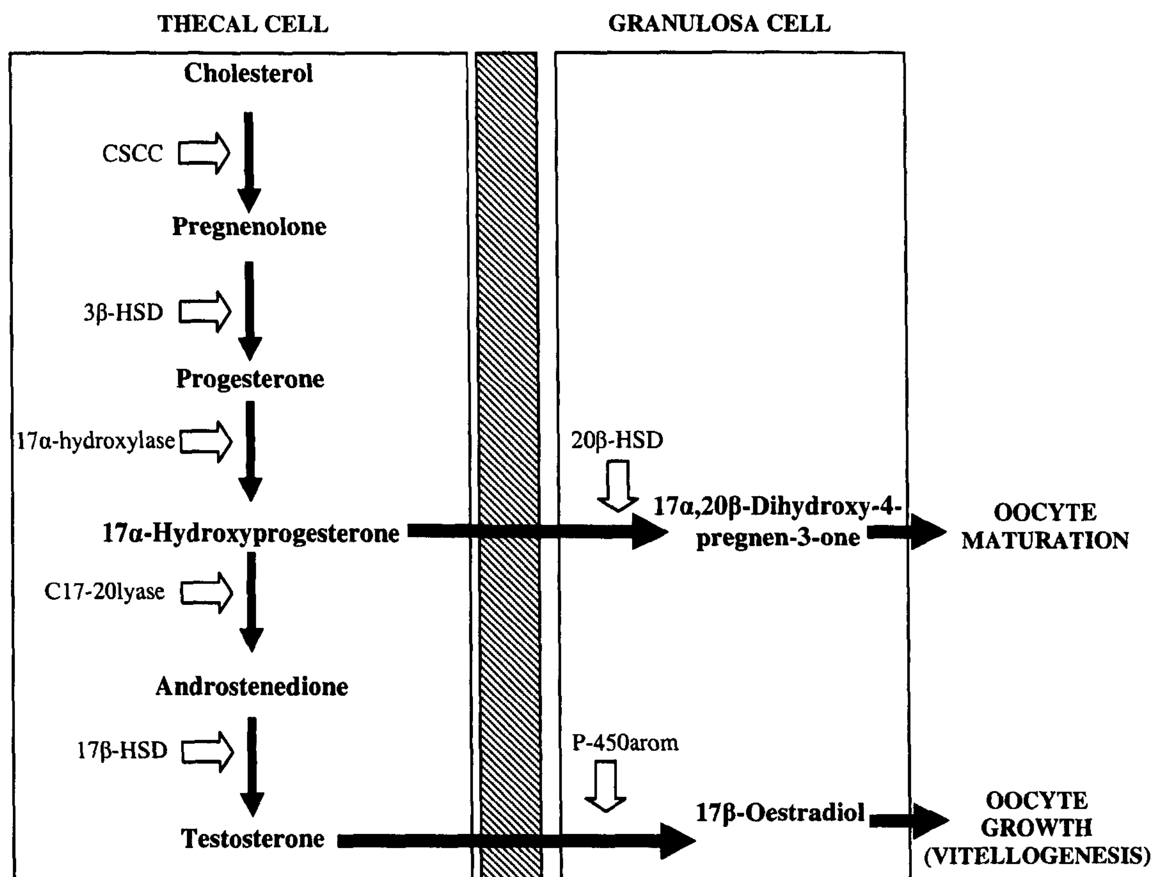


Figure 1.6 Pathway of steroid biosynthesis in the ovarian follicle of salmonids during oocyte growth and maturation, showing the relative contribution of thecal and granulosal cells in the production of 17β-oestradiol and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20 β-DP). CSCC, Cholesterol Side-chain Cleavage Cytochrome; 3β-HSD, 3β-hydroxysteroid dehydrogenase-isomerase; 17β-HSD, 3β-hydroxysteroid dehydrogenase; P-450arom, aromatase cytochrome P-450; 20β-HSD, 20β-hydroxysteroid dehydrogenase. (Adapted from Nagahama *et al.*, 1995).

Final Oocyte Maturation

On completion of vitellogenesis, the oocyte becomes ready for the resumption of meiosis, which is accompanied by several maturational processes in the nucleus and cytoplasm of the oocyte. This process, called oocyte maturation, is a prerequisite for successful fertilisation, and consists of germinal vesicle migration (GVM), followed by germinal vesicle breakdown (GVBD), chromosome condensation and assembly of the first polar body (Nagahama, 1997). Ultimately, when follicles reach postvitellogenesis, E_2 levels drop significantly, which subsequently removes the dopaminergic inhibition of LH secretion at the pituitary, that leads to a preovulatory surge of LH that is required for the induction of final maturation through a steroidogenic shift from the synthesis of T to E_2 to that of 17α,20β-DP (MIS) through the rapid expression of 20β-HSD in the granulosa cell layer in salmonids (Kah *et al.*, 1997; Nagahama, 1997). One of the features of 17α,20β-DP, is that its actions are mediated through receptors associated with the plasma membrane of the oocyte. Thus, one of the early steps following 17α,20β-DP action involve the formation of the major mediator of this steroid, maturation-promoting factor (MPF). MPF consists of a complex between cdc2kinase and cyclin B, with the appearance of the latter a crucial step for 17α,20β-DP-induced oocyte maturation in fish. MPF has a cyclical activity during oocyte maturation with the highest activity occurring at the first and second meiotic metaphase, and appears to be principally involved in the initiation of nuclear membrane breakdown.

<u>*Oocyte Recruitment*</u>

Many fish of commercial importance are typically iteroparous, in that they spawn more than once during their lives such as cod and Atlantic halibut. The rainbow trout is also included in this category and is the exception to the other pacific salmonids

(*Oncorhynchus sp.*) which are semelparous a die once they have spawned. However, many of theses species also exhibit significantly different reproductive strategies with regards to oocyte recruitment. Based upon the dynamics of the organisation of the ovary three types of ovarian development have been defined (Murua & Saborido-Rey, 2003). Firstly there is synchronous development, in which all the oocytes develop and ovulate at the same time, and no further replenishment from earlier stages takes place. The rainbow trout is categorised by such oocyte recruitment. The second group, which includes the Atlantic cod and halibut are group-synchronous. These are defined as having at least two recognisable populations of oocytes at one time, with one a fairly synchronous population of larger oocytes (defined as a clutch), and a second more heterogeneous population of smaller oocytes from which the clutch is recruited. Finally, there are the asynchronous, in which all stages of oocyte development are present without a dominant population. Only on hydration do clearly separate stocks of oocytes become evident. With regards to spawning pattern, two types have been defined these being total, in which all oocytes are ovulated at once, and batch spawners, in which only a portion of yolked oocytes are spawned in each batch.

Due to these different recruitment strategies, each species exhibits significantly different rhythms and levels of sex steroids involved in gonadal growth and development. For example, in the rainbow trout high testosterone levels (>30ng/ml) are associated with the complete recruitment of oocytes within the ovary (Whitehead *et al.*, 1983), while in Atlantic halibut, a group-synchronous batch spawner, testosterone levels regularly peak and trough with each group of oocytes recruited, with levels generally maintained at much lower levels <1.5ng/ml (Methven *et al.*, 1992).

1.6.1 Evidence for the action of photoperiod on components of the HPG Axis

In female rainbow trout, Davies *et al.*, (1995) reported that abrupt increases in daylength after spawning resulted in an increase in serum GtH I that coincided with an advance in the timing of vitellogenesis. Stimulatory long-short photoperiods increased GtH I levels during vitellogenesis compared to those on an ambient photoperiod, while GtH II levels rose just prior to and during the respective spawning period. Bon *et al.*, (1999) demonstrated similar GtH profiles under advanced photoperiod regimes but also observed a decrease in egg size. They concluded that this reduction in egg size was not due to a deficiency in GtH I levels as they were significantly higher under an accelerated regime, but was associated with an alteration in ovarian follicle growth during the later stages of vitellogenesis. These findings support the hypothesis that the early and middle stages of ovarian growth appear to be photosensitive periods, while later stages appear to be controlled by an endogenous clock synchronised by photoperiod.

Further down the endocrine cascade, Whitehead *et al.*, (1978a) demonstrated an increase in serum 17β-oestradiol, total calcium and phospho-protein phosphorous following a decrease in photoperiod. However, this effect was only observed in female and not male fish. Extensive reviews of changes in gonadal hormones (17β-Oestradiol, oestrone, testosterone, hydroxy-progesterone and vitellogenin), in response to photoperiod manipulation have been described (Whitehead *et al.*, 1978b; Bromage *et al.*, 1982a; Scott & Sumpter, 1983; Elliot *et al.*, 1984; Sumpter *et al.*, 1984; Duston & Bromage 1986, 1987, 1988; Bromage *et al.*, 1992a; Taranger *et al.*, 1998, 1999; Whitehead *et al.*, 1983).

However, although the mechanisms involved the reproductive endocrine cascade are relatively well known in teleosts, considerably less is known as to how an

individual assesses itself with regards to initiating the maturation process. Currently the most widely accepted hypothesis is that some threshold for size, rate of growth or energy storage must be surpassed during critical periods for sexual development to proceed (Silverstein *et al*, 1997; Silverstein *et al*, 1998). It is for this reason that growth factors (IGFs) and leptin have been proposed as possible mediators of growth and nutritional status, with a subsequent involvement in maturation and reproduction as observed in mammals.

1.7 Endocrine Regulation of Growth: GH-IGF Axis

Although growth is undoubtedly under the control of the genetic potential of the individual, in which food availability, nutrient utilisation and water temperature will control the rate of growth, the mechanisms underlying the process are ultimately controlled by endocrine functions.

1.7.1 Growth Hormone

Somatotropin or growth hormone (GH) is emerging as a multifunctional hormone in fish, having complex and intriguing interrelations between its different biological functions. As well as a principal regulator of somatic growth, GH has also been implicated as having important functions in the stimulation of protein synthesis and improvement of feed conversion during growth; the promotion of lipid and glycogen breakdown and mobilisation; a role in osmoregulation, including saltwater adaptation; increased appetite; increased swimming and anti-predator behaviour (reviewed in Bjornsson, 1997). The hormone was first characterised in chum salmon (*Oncorhynchus keta*) by Wagner *et al.*, (1985), and has since been detected in other species of *Oncorhynchus* and *Salmo*, revealing a high sequence similarity between the

species and different genus. Currently, two GH genes, sGH I and sGH II, have been isolated (Kawauchi *et al.*, 1986). However, as yet, no functional differences between the salmonid GHs has been observed, although this possibility should not be discredited.

1.7.2 GH Secretion

GH is known to originate from the anterior pituitary. The secretion itself appears to be primarily under inhibitory regulation. It appears that the major factor responsible for the inhibitory control is somatostatin (SRIF) which has been identified in the hypothalamus and pituitary of salmonid species. SRIF has been shown to inhibit GH release *in vitro*, and to lower circulating GH levels *in vivo* (Sweeting & McKeowen 1986; Luo *et al.*, 1990). In the goldfish, both norepinephrine and serotonin (5HT) have also been shown to have inhibitory actions on GH release *in vitro* in the goldfish (Peter *et al.*, 1990). More recently, insulin-like growth factor-I (IGF-I) has been shown to inhibit GH secretion by pituitary cells in a variety of teleosts (Perez-Sanchez *et* al., 1992; Fruchtman *et al.*, 2000) and reduce GH levels in cannulated fish (Blaise *et al.*, 1995). It is concluded that circulating GH exhibits a negative feedback control on its secretion indirectly through IGF-I. Recent studies have also suggested that GH may also have a direct negative feedback effect on its own secretion at the pituitary level (Agustsson & Bjornsson, 2000). Figure 1.7 provides a summary of the negative feedback control of GH secretion and the role of IGF in this system.

Much less is known about the possible GH secretagogues, although GH releasing factor (GRF) now identified as ghrelin, gonadotropin-releasing hormone (GnRH), sex steriods in particular oestradiol, dopamine, neuropeptide Y (NPY), thyrotropin-stimulating hormone (TSH), and cholecystokinins (CCKs) have all been

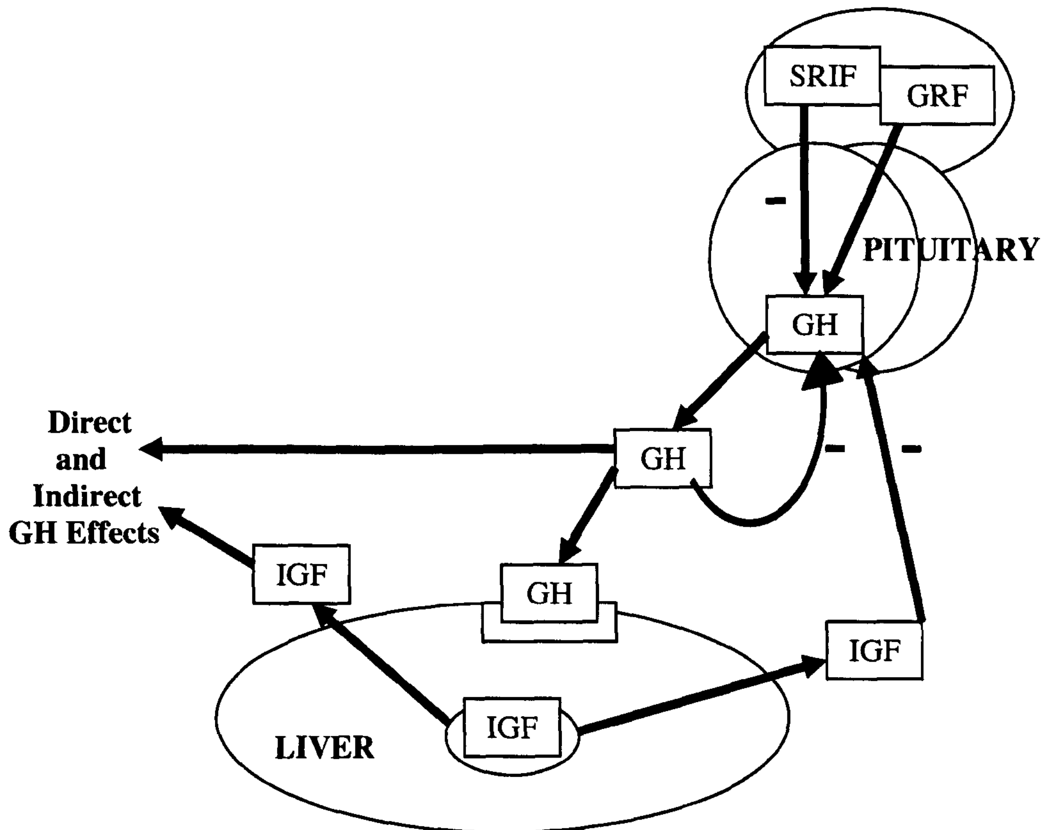


Figure 1.7 Hypothalamic and negative-feedback regulatory mechanism of pituitary GH secretion and possible direct and indirect mode of GH action through insulin-like growth factor-I (IGF-I). (After Bjornsson *et al.*, 1997).

demonstrated to have stimulatory effects on GH release both *in vitro* and *in vivo* (reviewed in Le Gac *et al.*, 1993; Peter & Marchant, 1995; Holloway & Leatherland, 1997; Yeung *et al.*, 2004).

1.7.3 GH Receptors

The initial step in GH action at the tissue level is the binding of GH to specific cellular membrane-associated receptors (reviewed in Kelly *et al.*, 1991). In mammals, it has long been known that the liver is the target organ of GH. Other tissues expressing GH receptor mRNAs include chondrocytes, adipose tissue, skeletal muscle, brain and gonadal tissues. Similarly, in teleosts, GH binding sites have also been localised in the liver, as well as the kidney, hypothalamus, and spleen (Munoz-Cueto *et al.*, 1996; Sun *et al.*, 1997; Lee *et al.*, 2001). This wide tissue distribution is in keeping with the pleiotropic nature of GH action (Peter & Marchant, 1995).

1.7.4 Insulin-like Growth Factor

The insulin-like growth factor (IGF) system plays an important role in controlling animal development and growth, with a large body of research devoted to the role in the mammalian system (Peter & Marchant, 1995). It is now widely believed that IGFs are responsible for mediating many of the growth promoting effects of GH (Duan, 1997). There are three components to the IGF system: ligands (IGF-I and IGF-II), receptors (Type I and Type II IGF receptors), and IGF-binding proteins (IGFBPs).

1.7.5 Ligand Structure

IGFs are members of a family of single-chain polypeptide growth factors, with a structural homology to proinsulin. The fish IGF-I sequence was first determined in coho

salmon, and the first IGF-II sequence in rainbow trout (Cao *et al.*, 1989; Shamblott & Chen, 1992). Since then, IGFs, in particular IGF-I have been detected in a variety of teleosts including Atlantic salmon, chinook salmon, common carp (*Cyprinus carpio*), Thai catfish (*Clarias macrocephalus*), European eel (*Anguilla anguilla*), turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) (Duguay *et al.*, 1992; Wallis & Delvin, 1993; McRoy & Sherwood, 1994; Duguay *et al.*, 1996; Liang *et al.*, 1996; Huang *et al.*, 1999; Duval *et al.*, 2002). Mature salmon IGF-I is a 70 amino acid basic peptide containing 3 intra-chain disulfide bridges, while trout IGF-II is a 70 residue neutral peptide similar in structure to IGF-I. IGFs are highly similar to insulin in the A and B chains, while the C domain is shorter and significantly different to that in proinsulin. Functional studies indicate that the biological potency of both IGFs and their very high degree of structural conservation between mammalian and salmonid IGF-I, as well as the complete conservation of the IGF-I structure between the salmonid species suggest that there has been strong evolutionary pressure to maintain the structure of IGF throughout vertebrate evolution (Duan, 1998; Moriyama *et* al., 1995; Peter & Marchant, 1995). Furthermore, IGF-II is thought to principally involved during early ontogeny and development due to its greater expression in developing embryos and appears to be independent of GH regulation. Conversley, IGF-I which is expressed in association with a subsequent reduction in IGF-II expression after hatching is thought to be involved in the later growth (Gentil *et* al., 1996; Mendez *et al.*, 2001a; Gabillard *et al.*, 2003a)

1.7.6 Tissue Distribution

IGF-I and II mRNA has been detected in a wide variety of salmonid tissues including the liver, brain, muscle, heart, intestine, pancreas, kidney, testes, ovary,

adipose tissue and spleen (Duan *et al.*, 1993; Gentil *et al.*, 1996). Of these, it appears that the liver contains the highest level of IGF-I mRNA which is in keeping with this organ as a major site of IGF-I synthesis in mammals (Duan *et al.*, 1993).

1.7.7 IGF Receptors

IGFs exert their biological actions by interacting with specific receptors localised on the cell membrane. Currently two IGF receptors have been characterised in mammals these being classified as type-I IGF receptor (IGF-I receptor) and type-II IGF receptor (IGF-II receptor). Despite their structural similarity, each receptor binds preferentially to its cognate ligand. The type-I receptor is similar in structure to the insulin receptor and binds both IGF-I and II with high affinity, and insulin with a lower affinity. In contrast, the type-II receptor of mammals shows no cross reactivity with insulin, and binds IGF-II with higher affinity than IGF-I (Duan, 1997).

Both the type-I and insulin receptor are present and functional in teleost fish sharing a structural similarity to that observed in mammals (Drakenberg *et al.*, 1993). Only recently have experiments detected an IGF-II receptor in fish (Mendez *et al.*, 2001a). However, the abundance of IGF-I receptors in fish skeletal muscle contrasts with the pattern observed in higher vertebrates, in which insulin receptors prevail over IGF-I receptors (Parrizas *et al.*, 1995). Currently it is believed that the biological actions of IGF-I and II are mediated primarily by the type-I IGF receptor, while the type-II/manose-6-phosphate IGF receptor may be responsible for clearance of IGF-II (Mendez *et al.*, 2001a).

More recently Planas *et al.*, (2000) detected for the first time the presence of both insulin and IGF-I receptors in the adipose tissue of brown trout. Their results suggest that adipose tissue may also be a target tissue for insulin and IGF-I and may

therefore have a possible role in regulating adipose tissue function in fish, particularly energy storage.

1.7.8 Binding Proteins (IGFBP)

The IGF system is unique among peptide hormone systems in that IGFs in the extracellular environment are bound to members of a group of high-affinity binding proteins (IGFBPs) (Duan, 1997). In the mammalian system at least 6 distinct but structurally similar proteins have been demonstrated, each being designated IGFBP-1 to 6, representing an individual gene product (Hwa *et al.*, 1999). IGFBPs prolong the half-life of IGFs, prevent their insulin like activity (possible acute hypoglycemic effects at high concentrations), and control their availability to target tissues. Each binding protein has been shown to inhibit IGF action when added to culture media, and it is proposed that these proteins exhibit their inhibitory effects by competing with the receptors for the ligand.

Gel filtration of human serum has shown three pools of IGFs, observed at 150, 40 and 7.5 kDa. The 150 kDa carries more than 80% of circulating IGFs and is saturated by endogenous IGFs. The 40 kDa complex includes IGFBPs that bind 20% of the circulating IGFs and contains unsaturated binding sites. Less than 1% of IGF circulates in the free form, which in this state is biologically active and is believed to be a more sensitive indicator of short-term changes than total IGF (Hwa *et al.*, 1999). However in the study of coho salmon, most IGF-I evolved from the 40 kDa complex which suggests the major form of bound IGF-I in circulation may be in the 40 kDa binary complex which is equivalent to IGFBP3 in mammals (Shimizu *et al.*, 1999), and is the primary BP mediating the anabolic effects of IGF-I (Beckman *et al.*, 2004). It was also demonstrated that about 0.3% of IGF-I circulates in the free form in salmon

plasma, which is significant in that this greatly reduces the probability of IGF-I acting on the insulin receptor for which it is well known (Shimizu *et al.*, 1999). Fasted fish also show higher binding affinity than did fed fish, suggesting induction of under-saturated binding protein by fasting. Furthermore, fasting has been shown to increase levels of 25-kDa IGFBP in striped bass, and is thought to be equivalent to IGFBP-I in mammals which is associated with growth inhibition (Siharath *et al.*, 1996). Thus it is apparent that depending on the type of IGFBP there would appear to be the capacity for both anabolic and catabolic effects (Kelly *et al.*, 2001).

1.7.9 GH-IGF Interactions

There is little doubt that endogenous GH is the principle hormone which regulates somatic growth in teleosts (Bjornsson, 1997). However, there is not always a precise relationship between blood GH levels and the rate of somatic growth. Transfer of salmon from freshwater to seawater prior to smoltification results in abnormal development (stunting). Despite retarded growth, such individuals exhibit increases in GH secretion and elevated plasma GH concentrations, while IGF-I mRNA expression and circulating IGF-I concentrations are significantly reduced (Duan *et al.*, 1995). These elevated plasma GH levels and reduced hepatic IGF mRNA expression suggest that stunted salmon may be GH resistant as previously suggested (Duan, 1998). The increased plasma GH levels may therefore be a result of increased GH release by the pituitary gland due to reduced negative feedback by IGF-I (Duan *et al.*, 1995). Hepatic GH receptors levels are also regulated during fasting and the subsequent reduction in growth in the gilthead seabream (Perrez-Sanchez *et al.*, 1995). Therefore, hepatic GH resistance and diminished IGF-I production may be the central endocrine defects leading to growth retardation in growth restricted fish.

Certainly at the tissue level *in vivo* injection of both coho salmon and rainbow trout with GH has been shown to significantly increase hepatic IGF-I mRNA expression above non-injected individuals (Duan *et al.*, 1993; Niu *et al.*, 1993; Shimizu *et al.*, 1999). This GH-induced increase in IGF-I mRNA expression is associated with an increase in circulating plasma IGF-I concentrations (Moriyama *et al.*, 1995). *In vitro* primary culture of salmon hepatocytes has also demonstrated increased IGF-I synthesis by GH stimulation (Duan *et al.*, 1993). Negative feedback on GH secretion by IGF-I has been shown in the rainbow trout (Blaise *et al.*, 1995). IGF-I was shown to inhibit GH release by pituitary cells, while bovine GH did not, thus suggesting a direct action of IGF at the pituitary level and indirect action of GH, possibly mediated by IGF-I. Injection of IGF-I has been shown to elevate *in vitro* sulphate uptake in the Japaneese eel branchial cartilage (*Anguilla japonica*) (Duan & Hirano, 1992). This suggests IGF-I may promote skeletal tissue growth through the stimulation of cartilage matrix protein synthesis. A role in skeletal growth has also been shown by an increase in length as well as weight gain in feed restricted coho salmon (McCormick *et al.*, 1992; Perrez-Sanchez & Le Bail, 1999), and enhanced protein synthesis in the Gulf killifish (*Fundulus grandis*) and tilapia (Negatu & Meier, 1995; Chen *et al.*, 2000). These findings support a role for endogenous IGF-I as an important mediator of some of the actions of GH in promoting and regulating growth in teleosts. However, it is also known that metabolic regulators, such as thyroid hormones (T4 and T3), cortisol, insulin, and somatostatins, act synergistically with GH to enhance body growth (Peter & Marchnat, 1995; Very *et al.*, 2001). Figure 1.8 provides a summary of how environmental and nutritional factors may be integrated with the GH-IGF axis to regulate growth in fish. Thus, in this respect, the GH-IGF endocrine axis may offer a potential system for supplying developmental cues to the organism. Plasma GH levels

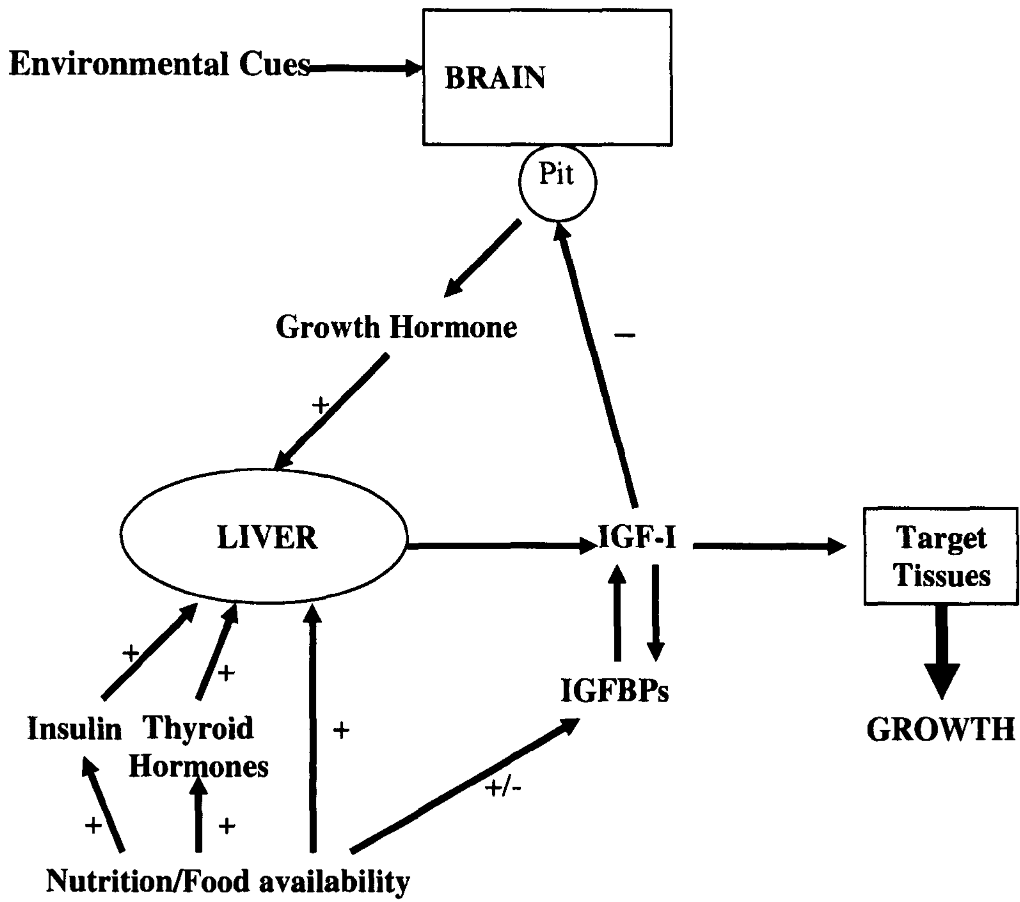


Figure 1.8 Endocrine axis controlling growth in teleost fish. Multiple hormonal and nutritional factors may stimulate (+) the production and/or modify (+/-) the activity of IGF-I. Negative feedback (-) of IGF-I inhibits growth hormone secretion by the pituitary (Pit). (After Duan 1997).

are known to change seasonally in response to photoperiod and temperature (Komourdjian *et al.*, 1976; Bjornsson *et al.*, 1995). Since plasma IGF-I levels are subject to regulation by GH and are directly related to nutritional and metabolic factors (Sumpter *et al.*, 1991; Duan & Hirano 1992; Duan *et al.*, 1993 1993), then the GH-IGF axis may provide an integrated signal with regard to season, temperature and food supply (Beckman *et al.*, 1998; Dickhoff *et al.*, 1997; Perrez-Sanchez & Le Bail, 1999). In addition, such a system may also offer a possible means of assessment of size or growth rate for the initiation of puberty.

1.8 Leptin

In the animal kingdom one of the most remarkable observations in nutrition is the stability of body weight (Casanueva & Dieguez 1999). In mammals, the amount of body fat is tightly regulated by a feedback loop via circulating factors in order to maintain constancy of total body energy stores. To do so, circulating factors act in the brain to elicit compensatory changes in order to match energy intake to than that of expenditure. (Kennedy 1953; Coleman 1973; Friedman & Halaas 1998). In 1994, the *ob* gene was identified as one of these circulating factors. The *ob* gene encoded a 16-kDa protein, leptin, which was synthesised by the adipocytes and secreted into the bloodstream (Zhang *et al.*, 1994). Both leptin deficiency and leptin resistance lead to severe obesity in mice, strongly implicating leptin as a negative feedback signal critical to the normal control of food intake and body weight (Fruhbeck *et al.*, 1998). Consequently, leptin has been implicated in the regulation of food intake, energy expenditure and whole-body energy balance, attenuation of neuroendocrine response to stress and fasting, and as a permissive factor for the promotion of reproduction and maturation (Houseknecht *et al.*, 1998; Flier 1998; Casanueva & Dieguez 1999; Baker *et*

al., 2000b). However, only recently has a leptin-like substance been detected in a variety of teleosts including rainbow trout, brown trout, burbot, green sunfish, bluegill sunfish, largemouth bass, white crappie, channel catfish, and sea lamprey (Johnson *et al.,* 2000; Yagahoubian *et* al., 2001; Muruzabal *et* al., 2002; Mustonen *et al.*, 2002b; Bosi *et al.*, 2004). These findings have opened the possibilities of relating effects observed in mammalian species to those seen but previously unexplored in fish. The following review will now give an account of what is known in fish studied to date in relation to the mammalian system.

1.8.1 Leptin Structure

In mammals the *ob* gene encodes a 167 amino-acid sequence (16kDa) that appears to have been preserved well throughout evolution, sharing 84% homology in mouse and human leptin (Casanueva & Dieguez 1999). However, as yet a fish "leptin" has not been sequenced, although Johnson *et al.*, (2000) obtained a positive immunoblot at 16kDa using polyclonal anti-mouse leptin antibodies in a variety of teleosts. Conversely, in the sea lamprey four immunoreactive proteins (65, 100, 50 and 16kDa) were detected in serum and tissues samples, with the possibility that the higher molecular mass proteins may be phylogenetically limited to the lamprey and more ancient teleosts (Yagahoubian *et al.*, 2001).

1.8.2 Leptin Receptor

The helical structure of mammalian leptin implies that the leptin receptor would be similar in structure and function to the helical cytokine receptors (Samson *et al.*, 1996; Houseknecht *et al.*, 1998). In the mouse, two leptin receptors have been described, OB-Ra (OB-R-S) expressed in a large number of tissues, with the greatest

number of binding sites found in the choroid plexus and leptomeninges, and OB-Rb (OB-R-L) which is expressed at high levels in the hypothalamus (Tartaglia *et al.*, 1995; Lonnqvist *et al.*, 1999). Since the hypothalamus has a central role in the regulation of hunger and satiety, then this is probably the major target area for leptin, suggesting that the OB-Rb isoform is the functional receptor involved in leptin signalling via the JAK-STAT pathway (Tartaglia *et al.*, 1995). However, receptors are not just restricted to the brain, they have been detected in the liver, heart, kidneys, lungs, small intestine, testes, ovaries, spleen, pancreas and adipose tissue (Fruhbeck *et al*, 1998).

As with the structure of leptin, the presence of leptin receptors in fish has not been characterised, yet numerous tissues including blood, brain, heart, stomach, intestine and liver have shown the presence of leptin-like substances, but interestingly not the adipocytes (Johnson *et al.*, 2000; Yagahoubian *et al.*, 2001; Bosi *et al.*, 2004). Mustonen *et al.*, (2002a) suggested that the adipocytes may not be the main storage organs for lipid during the evolution of many teleosts. Certainly, the burbot used in their study principally stores lipid in the liver, which subsequently expressed the highest concentration of leptin relative to other tissues (Mustonen *et al.*, 2002b). Similarly, in the rainbow trout, leptin expression has been detected in the stomach and supports the idea of alternative sites of leptin production may have evolved in lower vertebrates (Muruzabal *et al.*, 2002). However, although leptin receptors have not been characterised in teleosts to date, there is growing evidence to suggest that mammalian leptin may in part be able to interact with fish leptin receptors and induce some of the physiological effects observed in mammals following leptin treatment.

1.8.3 Physiological effects

Energy Balance: Control of Food Intake and Body Weight

Studies in fish have shown that leptin-like protein is present in blood of several species and plasma concentration decreases with starvation (Johnson *et al.*, 2000), which is the commonly observed phenomenon in mammals under conditions of food deprivation (Boden *et al.*, 1996). Conversely, Kolaczynski *et al.* (1996) demonstrated a 300% increase in leptin concentration following a 10% increase in human body weight, while acute overfeeding which did not affect body weight, was still associated with a 40% rise in serum leptin levels. Thus under steady-state energy balance, leptin would act as an adipostat to signal the status of body energy stores to the brain, but could also function as a sensor of energy balance under conditions of imbalance (Tuominen *et al.*, 1997; Fruhbeck *et al.*, 1998).

Surprisingly, daily injections of murine leptin in green sunfish did not cause loss of total weight or body fat (Londraville & Duvall, 2002), trademark effects of leptin treatment in mammals, in which excess leptin levels not only inhibit food intake, but also stimulate thermogenesis and increased physical activity (Zhou *et al.*, 1997; Casanueva & Dieguez, 1999; Gardner *et al.*, 1998). However, the injections did increase fat metabolism in green sunfish (Londraville & Duvall, 2002). Since fat metabolism did increase which would ultimately lead to weight loss, it was postulated that the lower metabolic rate of fish may explain this apparent discrepancy between increased fat metabolism but no loss of weight, in the sense that whole animal effects may not manifest themselves over the short time period of this study (14 days) in ectothermic organisms.

In another study, goldfish central or peripherally administered murine leptin caused a reduction in food intake, in part through decreasing the orexigenic effects of

exogenous NPY and orexin A even at low doses 1ng/g (Volkoff *et al.*, 2003). Furthermore, leptin's actions on food intake appear to be mediated by potentiating the anorexigenic satiety effect of cholecystokinin (CCK). Similarly, in mammals it is known that leptin interacts with several other neuropeptides including neuropeptide-Y (NPY), cholecystokinin (CCK) and MCH (melatonin concentrating hormone) to control feed intake (Lonnqvist *et al.*, 1999; Le-Bail & Boeuf, 1997). Furthermore, it has been demonstrated recently that leptin receptors are actually located on the surface of NPY producing cells (Stephens *et al.*, 1995). Since NPY has a central role in the control of food intake (Fruhbeck *et al.*, 1999; Le-Bail & Boeuf, 1997), these findings clearly indicate that leptin activates areas of the hypothalamus and brain-stem that are known to regulate appetite and energy expenditure. Reduced hypothalamic NPY synthesis and release occurs following exogenous leptin administration which leads to the inhibition of appetite and food intake (Stephens *et al.*, 1995). In both chinook and coho salmon an increase in hypothalamic NPY mRNA and NPY secretion was demonstrated under conditions of food deprivation although leptin was not measured (Silverstein *et al.*, 1999). However, given the results observed in goldfish, then it could be hypothesised that the level of leptin was decreased, due to lower adiposity, as a result the inhibition of NPY synthesis may have been removed thus stimulating appetite. This provides further evidence for the possible role of leptin in the control of food intake.

Reproductive Axis

Immature coho salmon delivered human leptin via osmotic pumps for 14 days showed no effects on physiological parameters including growth, gonad weight, lipid content and hormone levels (FSH, IGF-I, insulin, GH & T_4), (Baker *et al.*, 2000), although the lack of effect may be due to the lower metabolic rate of leptin in

ectothermic animals. However, mammalian leptin did stimulate pituitary cells *in vitro* to secrete LH in sea bass (Peyon *et al.*, 2001) and LH/FSH in rainbow trout (Weil *et al.*, 2003), although the effect was dependent on sexual status. The direct action of leptin on FSH and LH release, evident only when gametogenesis has already started would suggest that leptin is not the unique signal for activation of the gonadotropic axis but requires a combined action with other promoting factors, and may hence have a more permissive role in reproduction. In sea bass similar results to those observed in trout have been demonstrated, but have also indicated that NPY is involved in potentiating the effects of leptin (Peyon *et al.*, 2001), and more recently somatolactin (SL) has also been implicated in the nutritional control of the onset of puberty through the stimulation of SL producing cells by leptin and NPY (Peyon *et al.*, 2003). Therefore, the effects of leptin and the co-involvement of other hormones and the fact that their effects are dependent on sexual status further support the idea that leptin may have a more permissive role in reproduction.

Certainly in mammals, inadequate nutrition is known to delay or prevent the onset of puberty. Undernutrition is often accompanied by hypogonadism and infertility, with a marked decrease in gonadotropin release (Houseknecht *et al.*, 1998), while leptin treatment has been shown to correct the sterility of both female and male adult *ob/ob* mice (Chehab *et al.*, 1996; Mounzih & Chehab., 1997). Furthermore, Ahima *et al.*, (1997) demonstrated that the treatment of normal mice with exogenous leptin advanced the onset of puberty. In humans, leptin levels have been shown to increase before the appearance of other reproductive hormones related to puberty in both girls and boys (Garcia-Mayor *et al.*, 1997; Mantzoros *et al.*, 1997), with boys always having lower levels than girls, with levels declining around the time of testosterone increase. These observations have led to the conclusion that leptin is the signal which informs the brain

that energy stores are sufficient to support the high energy demands of reproduction, and may therefore be a major determinant of the timing of puberty (Ahima *et al.*, 1997). However, until recently it was unclear whether the effect of leptin on reproduction was direct or indirect. Experimental work with rats has provided evidence that leptin may not be the primary signal that initiates the onset of puberty, but might act as a metabolic gate, to allow pubertal maturation to proceed, if and when metabolic resources are considered adequate (Cheung *et al.*, 1997). *In vitro* studies in mice by Yu *et al.*, (1997) demonstrated that leptin was acting on the hypothalamus to stimulate the release of lutenising hormone-releasing hormone (LHRH), thereby stimulating gonadotropin release, with subsequent release of FSH and LH. Release of these hormones would then stimulate gonadal steroid secretion which are then responsible for the development of the reproductive tract and the induction of puberty. Of significant importance was the finding that leptin receptors were also present in the anterior pituitary, for which there were no previous reports. This work confirmed earlier findings of significantly elevated serum LH and FSH levels following leptin treatment in female and male mice (Barash *et al.*, 1996).

1.9 Aims of Thesis

The work carried out in this thesis was funded by the British Trout Association and the NERC ROPA grant (GR3/R9827) awarded to Dr. Clive Randall. Considering the economic importance of the rainbow trout as an aquaculture finfish species within the UK, and the lack of knowledge regarding the influence of photoperiod on growth, reproduction and endocrine function within the species, the overall aims of the current thesis were to examine the influence of photoperiod manipulation on growth and the interaction with reproduction of rainbow trout. Chapter 3 aimed to examine the effect of

photoperiod on growth in juvenile rainbow trout. The use of plasma IGF-I as an indicator of growth was also examined to determine whether this hormone is suitable for use in rainbow trout as in other salmonids. Finally, the response of plasma IGF-I levels to photoperiod manipulation and melatonin implantation was investigated to determine how photoperiodic information may be conveyed to the somatotropic axis. Chapter 4 aimed to determine the possible mechanisms behind the reduction in the numbers of fish capable of undergoing puberty when the natural spawning cycle is advanced in rainbow trout; to examine the role of growth parameters as indicators of maturation; and finally, to assess the role of plasma IGF-I and leptin as potential indicators of growth and/or nutritional status to the reproductive axis. Finally, chapter 5 aims to examine the use of artificial lighting regimes as a means of increasing production efficiency under commercial farming conditions in tank and cage systems. The work within this chapter examines the influence of timing of application, light intensity and spectral composition of light on growth and feeding efficiency.

In summary, the experiments detailed in this thesis aimed to further our understanding of the photoperiodic influence on growth and reproduction in the rainbow trout, and whether the technologies can be applied to commercial practices.

Chapter Two: General Materials and Methods

2.1 Experimental Animals

In all experiments, with the exception of chapter 3 experiments 3.3 and 3.5, domesticated all-female rainbow trout (*Oncorhynchus mykiss*) strains were used. Detailed information regarding origin, age and size of fish is included in the methods section of each experiment. Further information is also given regarding diets fed during each experiment and how these were supplied.

2.1.1 Maintenance

Fish were maintained in a variety of flow-through systems designed to meet the specific requirements of the fish. All trials were conducted at the University of Stirling's Niall Bromage Freshwater Research Facility with the exception of experiments conducted in chapter 5. Commercial trials (chapter 5) were carried out at one of two sites (Sites 1 & 2). Experiments 5.3 & 5.5 were carried out at site 1 in an uncovered tank flow-through system, while experiments 5.4 & 5.6 were carried out at site 2 in cages. Full details of the systems are given in the relevant chapter materials and methods section.

2.1.2 Anaesthesia

All manipulative procedures were conducted under anaesthesia to facilitate easy handling and to minimise stress. An anaesthetic bath of 2-phenoxyethanol (Sigma Chemical Company Ltd., Dorset, UK.) was prepared at a concentration of 1:10,000 in water. Subjects achieved stage II plane 2 anaesthesia within 2 minutes of immersion. After completion of the necessary procedures subjects were placed into a well-aerated tank, with full recovery occurring within 5 minutes. Post-sampling mortalities were typically less than 0.1%.

2.1.3 Blood Sampling

Blood samples were taken via the caudal dorsal aorta of fully anaethetised fish. Fish deemed too small (typically <20g) to survive withdrawal of blood were firstly sacrificed by administration of a strong blow to the cranium following prolonged anaesthesia. For larger fish (typically >250g) and broodstock, blood was withdrawn into 2ml syringes (Terumo Europe N.V.; Leuven, Belgium) using 21 gauge sterile hypodermic needles (Terumo Europe N.V.; Leuven, Belgium). In the case of juvenile fish, blood was withdrawn into 1ml syringes (Terumo Europe N.V.; Leuven, Belgium) using either 23 or 25 gauge sterile hypodermic needles (Terumo Europe N.V.; Leuven, Belgium). For collection of plasma, syringes and needles were first rinsed with ammonium heparin (Sigma Chemical Company Ltd., Dorset, UK.) made up in distilled water at a concentration of 4 mg/ml. On collection, all blood samples were transferred to either sterile 2ml polystyrene tubes (LP3, Luckhams Ltd., Burgess Hill, Sussex, UK.) or 1.5 ml microcentrifuge eppendorffs (Life Sciences International UK Ltd.) and stored on ice prior to returning to the laboratory. Plasma samples were separated following centrifugation (CT 422 Chillspin, Jouan Ltd. Herts, UK.) at 2500 rpm for 15 minutes at 4°C. The resulting plasma was transferred to either new sterile 2ml polystyrene tubes (LP3, Luckhams Ltd.) or 1.5 ml microcentrifuge eppendorffs (Life Sciences Ltd.) and stored at –70°C for future analysis.

Blood samples required for melatonin profiling during the dark period were achieved by removing the fish from the tank in complete darkness and transferal to an anaesthesic bath. Blood samples were then collected under a dim red light (wavelength = 670-800nm, 0.2 lux at 0.5m).

2.1.4 Fish Identification

In order to distinguish individual fish or groups (Chapter 3, experiment 3.5 and chapter 4, experiment 4.1) within a population an intra-muscular passive integrated transponder (P.I.T.) tag (AVID Tags, Norco, Ca., USA) was inserted 10mm below the dorsal fin. Individual fish were anaethetised and a small 5mm incision was made below the dorsal fin. The tag was then injected anteriorly positioning it forward of the initial incision and 5mm beneath 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.; Middlesex, UK) and Cicatrin antibiotic (The Wellcome Foundation Ltd.; Middlesex, UK) was applied to the incision area.

2.1.5 Melatonin Implantation

Constant slow release melatonin implants (18mg melatonin) ("Regulin", Schering Agrochemicals, Alexandria, Australia) were used in experiment 3.5 to permanently elevate plasma melatonin in excess of night-time physiological rhythms. An implanter (Schering) was used to administer implants intra-muscularly 1 cm below the dorsal fin of anaethetised fish. Implanted fish were also adipose fin-clipped to aid identification.

2.1.6 Growth Measurement

Fish length and weight measurements were taken throughout the experiments. In all cases fork length was measured (±1mm) and weights (±0.1g) were recorded using an electronic balance (Model QC7DCE-S, Sartorius AG; Germany), with the exception of experiment 4.1 where weights (±5g) were recorded using another electronic balance (Model 8310 Indicator, Scanvaegt International A/S, Denmark).

2.1.6 Spawning Assessment

Fish were examined at monthly intervals outside the expected spawning period and at two-weekly intervals as they approached maturity (chapter 4). Ripe females were recognised by distension and softening of the abdomen and the extrusion of the urogenital papilla. Anaethetised females were lifted from the head upwards and then, if found to be ripe, the eggs expressed from the abdominal cavity by exerting gentle downward pressure from the pectoral fins towards the vent ("stripping"). The time of spawning of an individual fish was deemed as the point at which eggs could be stripped from the body cavity.

2.2 Plasma Melatonin Analysis

Melatonin present in plasma samples was measured using a direct radioimmunoassay adapted from Randall, *et al.* (1995).

Assay Buffer

The following buffer constituents were dissolved in 150ml nanopure water in a sterile polystyrene specimen container (Sterilin Ltd., Hounslow, Middlesex, UK) placed in a water bath at 50°C for 30 minutes. All chemicals were Analar grade supplied by BDH Chemicals Ltd.

Tricine [N-tris(hydroxymethyl)methylglycine]	2.688g
Sodium Chloride	1.350g
Gelatine	0.150g

Fresh tricine buffer was prepared the day before each assay and stored at 4°C.

Antibody

Sheep anti-melatonin antibody (Stockgrand Ltd., Guildford, Surrey, UK) was raised against N-Acetyl-5-methoxytryptophan conjugated through the side chain to

bovine thyroglobulin. All melatonin assays were carried out using batch number G/S/704-6483. Each vial of freeze-dried antibody was reconstituted in 2ml of nanopure water to provide an intermediate solution (1:10 dilution). This was then divided into 100μl aliquots and stored at –20°C in polystyrene tubes (Luckhams Ltd., UK). The working solution (1:2000 dilution) was prepared by adding one 100μl to 19.9ml assay buffer (sufficient for 96 tubes in duplicate).

Radiolabel

The label [O-methyl-^{3}H] melatonin, specific activity 70-85 Ci/mmol, was obtained in 250μCi batches from Amersham International Ltd. An intermediate stock "A" solution was prepared by diluting 20μl of the stock label in 2ml absolute ethanol (Analar Grade, Fisons Ltd.). The stock "A" solution was stored in glass vials () at –20°C. A working solution of approximately 4,000 dpm/100μl was prepared in assay buffer from the intermediate stock "A" solution (~21μl stock "A" in 10ml assay buffer, sufficient for 100 assay tubes).

Melatonin Standard

A stock standard solution of 1mg/ml melatonin was prepared by dissolving 10mg melatonin (N-acetyl-5-methoxytryptamine; Sigma Chemical Company Ltd., Dorset, UK) in 10ml absolute ethanol (Analar Grade, Fisons Ltd.). The stock solution was stored at –20°C. Standards were prepared fresh for each assay as follows;

(A) 100μl (1mg/ml) made up to 10ml with assay buffer	=10μg/ml
(B) 100μl solution **A** (100μg/ml) made up to 10ml with assay buffer	=100ng/ml
(C) 100μl solution **B** (1mg/ml) made up to 10ml with assay buffer	=1ng/ml
(D) 100μl solution **B** (1mg/ml) made up to 5ml with assay buffer	=2ng/ml

Six serial dilutions of 250μl aliquots of solution C (1ng/ml) with 250μl assay buffer were prepared to provide standards in the range of 3.9 to 250pg/tube. Solution D (2ng/ml) was used to provide a 500 pg/tube standard.

Assay Protocol

All unknown samples were assayed in duplicate using the following protocol:

1. Prepare a series of dilutions of melatonin standard with assay buffer in polystyrene assay tubes (LP3; Luckhams Ltd) to give a range 0-500pg/250μl.
2. Add 250μl assay buffer to each sample tubes and 450μl to non-specific binding tubes (NSBs).
3. Add 250μl assay buffer to standards and NSBs. Vortex tubes.
4. Add 250μl aliquots of unknown samples to sample tubes. Vortex tubes.
5. Add 200μl antibody to all tubes except NSB tubes, vortex tubes and incubate at 20°C for 30 minutes.
6. Add 100μl tritiated melatonin to all tubes. Include two scintillation vials as Totals, and one further vial containing scintillation fluid only for background count subtraction. Vortex all tubes and incubate at 4°C for 18 hours.
7. Dissolve 0.48g dextran-coated charcoal powder (Sigma Chemical Company Ltd., Dorset, UK) in 50ml assay buffer. Stir on ice for 30minutes.
8. Add 500μl charcoal to all tubes, vortex and incubate at 4°C for 15minutes.
9. Centrifuge at 200rpm at 4°C for 15 minutes.
10. Transfer 1ml of supernatant to 6ml polyethylene scintillation vials (Packard Biosciences; Groningen, The Netherlands) and add 4ml of scintillation fluid (Ultima Gold, Packard Biosciences; Groningen, The Netherlands).

11. Vortex the vials thoroughly and count the radioactivity for 5 minutes in a scintillation counter (1900TR LSA, Canberra Packard Ltd.; Berks., UK).

Quality Control and Validation

The sensitivity of the assay (i.e. the minimum amount of melatonin able to be distinguished from zero) was 3.9pg/tube. Pooled plasma collected from fish during the scotophase with a melatonin content of approximately 160pg/tube were used to check the reproducibility of measurements both between and within assays. From this the intra-assay coefficient of variation was calculated as 3.2% whereas the inter-assay coefficient of variation was 8.0%.

A serial dilution of pooled plasma samples (approx 250pg/ml) collected from 200 fish (250g) during the mid dark period (12am) were used to obtain an inhibition curve (Figure 2.1). When this inhibition plot was compared to the standard curve it was found that the two curves were parallel, with no statistical difference between the slopes of the plots ($t = 0.707 < t_{0.05\ (2),\ 12} = 2.179$). This validated that the melatonin measured in the samples was immunologically similar to that in the hormone standards.

2.3 Plasma Testosterone Analysis

Changes in the levels of plasma testosterone were used to investigate the development of maturation over time. Determination of plasma testosterone was measured by direct radioimmunoassay adapted from the method described by Duston and Bromage (1987):

Assay buffer

The following constituents were dissolved in 150ml of nanopure water and stirred at 35°C. This solution was then made up to 500ml with nanopure water and

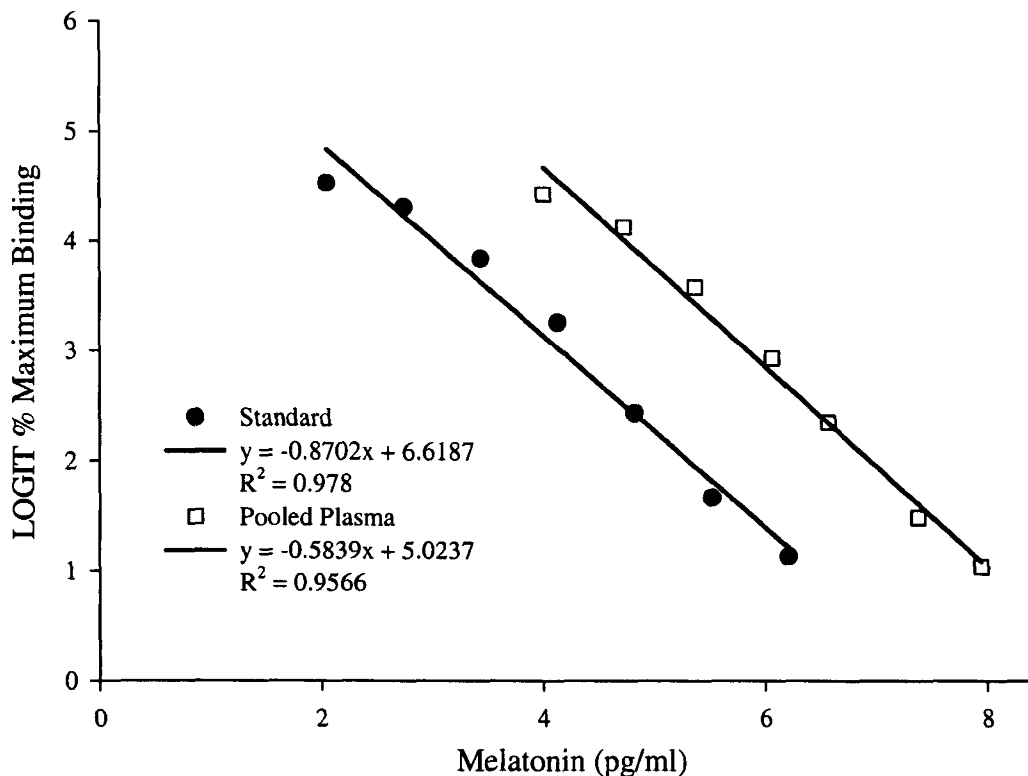


Figure 2.1 Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 250µl aliquots of pooled rainbow trout plasma collected during the scotophase, with the melatonin assay standard curve. Each point represents the mean of duplicate measurements. The curves have been linearised by using logit transformation, with the x-axis denoting the natural log of the melatonin content in the standards.

chilled to 4° prior to use. All chemicals were Analar grade supplied by BDH Chemicals Ltd. (Poole, England).

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

Antibody

1g freeze-dried anti-testosterone rabbit antiserum (Biogenesis; Poole, UK) was reconstituted in 1ml of nanopure water. This was then divided into 100µl aliquots and stored at -20°C until required. A working antibody solution was created by diluting 200µl of antibody in 19.8ml of assay buffer (sufficient for 200 assay tubes).

Radiolabel

The label [(1,2,6,7-^{3}H)] testosterone, specific activity 70-105 Ci/mmol, was obtained in 250µCi batches from Amersham International Ltd. An intermediate stock "A" solution was prepared by diluting 20µl of the stock label in 2ml absolute ethanol. This stock "A" solution was then used to prepare a working solution of approximately 20,000 dpm/100µl (~100µl stock "A" in 20ml assay buffer, sufficient for 200 assay tubes).

Testosterone Standard

A stock standard solution was produced by dissolving 1µg of freeze-dried testosterone (Sigma Chemical Company Ltd., Dorset, UK) in 10ml of Analar grade absolute ethanol (Sigma Chemical Company Ltd., Dorset, UK) to create a testosterone concentration of 100ng.ml^{-1}. This solution was stored at -20°C. A working standard

solution was produced by diluting 100μl of stock standard in 0.9ml of absolute ethanol to create a testosterone concentration of $10ng.ml^{-1}$.

Extraction

Prior to assaying it was necessary to extract the testosterone from the plasma samples according to the following protocol:

1. Thaw plasma samples and vortex thoroughly.
2. Transfer 50μl plasma into a polypropylene assay tube (LP3P tubes, Thermo Life Sciences; Hants, UK).
3. Add 1ml ethyl acetate (Sigma; Dorset, UK), stopper the tube and spin on a rotary mixer for 1 hr.
4. Centrifuge the tubes at 1500rpm for 10min. at 4°C.
5. Store at 4°C until assayed.

Assay Protocol

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

1. Prepare a series of dilutions of the standard testosterone hormone with absolute ethanol in polypropylene tubes (LP3P, Thermo Life Sciences; Hants, UK) to give a range of concentrations from 0-1000pg/100μl. Include a further tube containing 100μl ethanol which will be used to calculate the non-specific binding (NSB).
2. Add 50μl of each sample extract to the sample tubes.
3. Dry down the standards and sample extracts in a vacuum oven at less than 35°C.
4. Cool the dry tubes to 4°C.

5. Add 100µl of antibody to all standard and sample tubes except the NSB tubes.

6. Add 100µl of tritiated testosterone to each tube, vortex and incubate at 4°C for 18 hours. Additionally add 100µl of tritiated testosterone to two scintillation vials (Packard Biosciences; Groningen, The Netherlands) for the calculation of total radioactivity ("Totals"). Fill a third vial with scintillation fluid only to calculate the background radioactivity ("Blank").

7. Dissolve 0.48g dextran-coated charcoal (Sigma; Dorset, UK) in 100ml of assay buffer and stir on ice for 30min.

8. Add 500µl of charcoal solution to each tube, vortex and incubate for 10 min at 4°C.

9. Centrifuge at 2000rpm for 10 min at 4°C.

10. Transfer 400µl supernatant to 6ml polyethylene scintilation vials (Packard Biosciences; Groningen, The Netherlands) and add 4ml of scintillation fluid (Ultima Gold, Packard Biosciences; Groningen, The Netherlands).

11. Vortex the vials thoroughly and count the radioactivity for 5 minutes in a scintillation counter (1900TR LSA, Canberra Packard Ltd.; Berks., UK).

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

Quality Control and Validation

The sensitivity of the assay (i.e. the minimum amount of testosterone able to be distinguished from zero) was 1.9pg/tube. Pooled extractions collected from 50 maturing female fish with a testosterone content of approximately 152pg/ml were used to check the reproducibility of measurements both between and within assays. From this the

intra-assay coefficient of variation was calculated as 4.4% whereas the inter-assay coefficient of variation was 9.8%.

Serial dilutions of a pooled sample extract were used to obtain an inhibition curve (Figure 2.2). When this inhibition plot was compared to the standard curve and it was found that the two curves were parallel, with no statistical difference between the slopes of the plots ($t = 0.154 < t_{0.05\ (2),\ 14} = 2.145$). This validated that the testosterone measured in the samples was immunologically similar to that in the standards.

2.4 Determination of Total Plasma Calcium

Calcium analysis was undertaken to measure both total unbound and bound plasma calcium to equate normal basal levels and that involved in vitellogenesis. Plasma calcium levels were determined using an atomic absorption spectrophotometer (Model 2280 AAS, Perkin Elmer, Norwalk, USA). The following protocol was used at all times and carried out as follows;

Assay Buffer

A 1% nitric acid stock solution was prepared by adding 150ml of 69% nitric acid (Analar grade, BDH Chemicals Ltd., Poole, England) to 15 litres of distilled water. This was then stirred by magnetic flea for 15 minutes to ensure thorough mixing. 5 litres of the stock solution was then decanted into a sterile plastic barrel. To this, 50g of lanthium chloride (Sigma Chemical Company Ltd., Dorset, UK) was added to produce a working solution. Lanthium chloride prevents the interference from non-calcium ions during aspiration in the spectrophotometer.

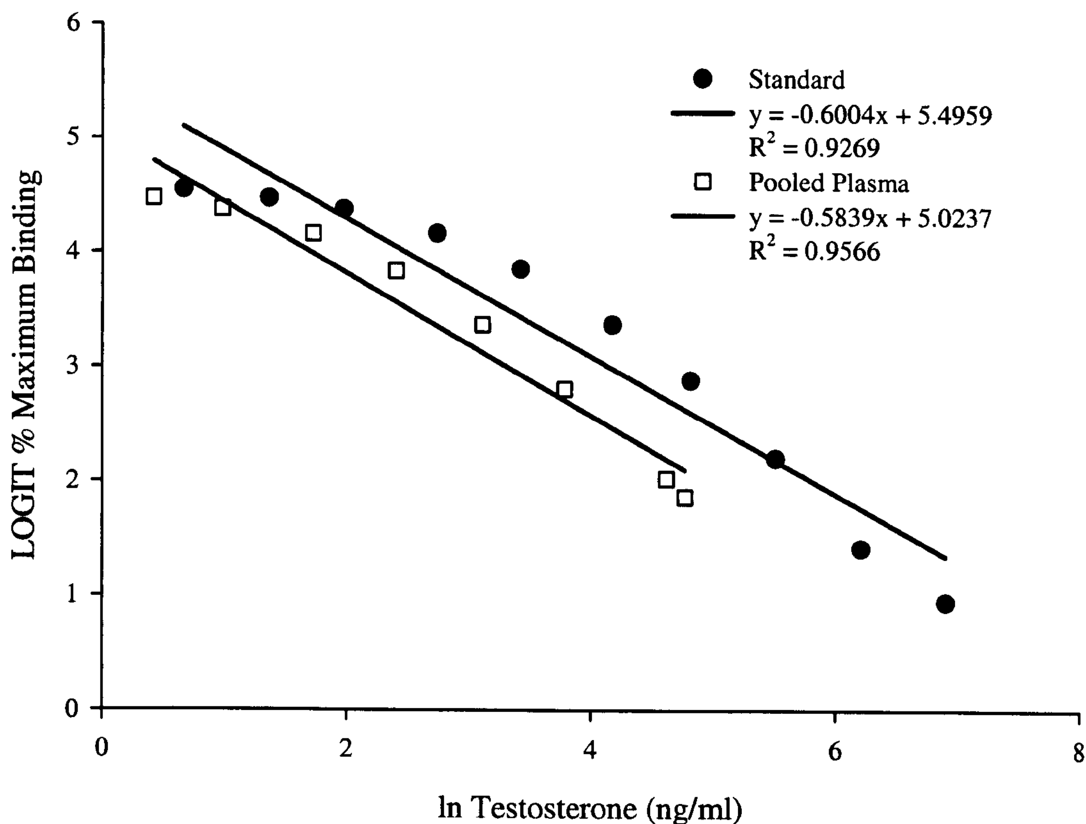


Figure 2.2 Parallelism of the inhibition curve obtained from the testosterone standard and a serial dilution of pooled maturing rainbow trout plasma extract (100μl aliquots). Each point represents the mean of duplicate measurements. The curves have been linearised by using logit transformation, with the x-axis denoting the natural log of the testosterone content in the standards.

Calcium Standards

Using the assay buffer, standards were prepared from a stock standard of calcium chloride (BDH Chemicals Ltd., Poole, England). Fresh standards were prepared for each assay. Two standards were prepared as follows;

(a) 2mg/l standard

200ml of working assay buffer solution was added to a volumetric flask. 400µl was removed using a pipette and replaced with 400µl of calcium standard. The flask was capped and then thoroughly mixed before transferring the solution to a sterile plastic bottle.

(b) 4mg/l standard

200ml of working assay buffer solution was added to a volumetric flask. 800µl was removed using a pipette and replaced with 800µl of calcium standard. The flask was capped and then thoroughly mixed before transferring the solution to a sterile plastic bottle.

Sample Dilutions

Sample dilutions were carried out dependent on the amount of calcium expected in the sample with respect to the stage of vitellogenesis. Samples from fish with low expected calcium levels were diluted to 1:150 in working solution. Fish samples in later stages of vitellogenesis were diluted to 1:250, with a 1:500 dilution carried out 1 month prior to spawning. All dilutions were carried out in polystyrene tubes (LP4 tubes, Thermo Life Sciences; Hants, UK).

Measurement of Plasma Calcium

Duplicate samples were aspirated at random in the flame spectrophotometer. Quality control was achieved by regularly recalibrating the flame spectrometer if assay drift was greater than 2%.

2.5 Plasma Insulin-like Growth Factor-I (IGF-I) Analysis

Plasma IGF-I analysis was carried out using commercially available fish kits obtained from GroPep Ltd. (Adelaide, Australia). The IGF-I RIA works on a double antibody-PEG principle. A limited amount of radioactive antigen, together with a known amount of corresponding antibody, is combined with the assay sample. The radiolabelled and unlabelled antigens compete for the same antibody binding sites. A second antibody precipitates the antibody-bound antigen, separating it from the unbound antigen which remains in solution. After careful removal of the supernatant, the precipitate is counted in a gamma counter. The amount of radiolabel present in the precipitate is directly related to the amount of unlabelled antigen present in the original sample.

Assay Buffer

Pour the 10x concentrate into 500ml sterile bottle and make up to a final volume of 500ml with nanopure water. Adjust to pH 7.5 with 5M HCl. The buffer solution can be stored at 4°C for seven days.

PEG Solution

Pour the 5x concentrate into 500ml sterile bottle and make up to a final volume of 500ml with nanopure water and store 4°C.

0.855M Tris Solution

51.8g Tris base, ULTROL® grade (Calbiochem, Canada) was dissolved in 350ml nanopure. This was then made up to a final volume of 500ml prior to storage at 4°C.

Acid-Ethanol Extraction Mix

In a fume cupboard, 62.5ml 2M HCl acid was added to 437.5ml 100% absolute ethanol. This was then mixed gently. Once cool, the solution was transferred to a sterile 500ml bottle and stored at -20°C.

Quality Control Serum

Each kit contained three vials of lyophilised fish serum which were reconstituted overnight with 40µl of assay buffer. Each vial was vortexed vigorously to ensure thorough mixing. The QC samples contained approximately 50 ng/ml and were used to validate intra- and inter-assay variation.

Antibody 1 Stock

Add 250µl assay buffer to the antibody 1 (Rabbit; lyophilised) vial and allow to reconstitute. This produces a stock solution equivalent to a 1:50 dilution which was stored at -20°C prior to use. Sufficient fresh working solution (~8ml for 60 samples in duplicate) was prepared immediately prior to assaying by diluting the stock solution 1:85.7 in assay buffer, giving a final dilution of 1:30,000 in the assay tube. 50µl is required for each tube.

Antibody 2 Stock

A stock solution was prepared by adding 1.1ml assay buffer to the antibody 2 (Sheep; lyophilised) vial and allowing it to reconstitute. After reconstitution the stock was transferred to an ependorff and stored at 4°C. Immediately prior to assaying sufficient working solution (~8ml for 60 samples in duplicate) equivalent to a 1:20 dilution was prepared from the stock solution. 50μl is required for each tube.

IgG Stock

The IgG solution is used for precipitation of the antigen-antibody bound complex. A stock solution was prepared by adding 225μl assay buffer to the IgG (Rabbit) vial and allowing it to reconstitute before storing at 4°C. Immediately prior to assaying sufficient working solution (~1.5ml for 60 samples in duplicate) equivalent to a 1:20 dilution in assay buffer was prepared from the stock solution. 10μl is required for each tube.

^{125}Iodine Radiolabel

80μl of assay buffer was added to the stock label provided and allowed to reconstitute prior to storage at -20°C. Immediately prior to assaying the dilution in assay buffer was calculated to produce a working solution with approximately 20,000 cpm/50μl. Due to the short half-life of iodine the starting activity was recalibrated using an iodine decay chart prior to making new working solution.

IGF-I Standard

Reconstitute one lyophilised IGF-I standard vial with 1.5ml assay buffer. This produces an IGF-I concentration equivalent to 70ng/ml. Nine serial 1/3 dilutions are

then made from the starting dilution, i.e. 350µl of standard and 700µl assay buffer, giving a final volume of 1.05ml. Each of these dilutions is one standard. A new vial of lyophilised standard was used for each assay.

Acid-Ethanol Extraction

IGF binding proteins are present in serum and plasma, which actively bind IGF-I and may interfere with the assay by sequestering the IGF-I present in the reaction mixture. Therefore IGF-binding proteins must be removed prior to assaying. The procedure is as follows;

1. Add 40µl of sample and QC serum to a sterile 1.5ml ependorff.
2. Add 160µl acid-ethanol extraction mix to all ependorffs.
3. Vortex and incubate at room temperature for 30 minutes.
4. Add 80µl 0.855M Tris to all tubes.
5. Vortex and spin in microfuge at 13,000 rpm (10,000g) for 10mins at 4°C.

Assay Protocol

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

1. Set up tubes: Totals, NSB, BO, Standards, QC, Unknown samples
2. Create an Acid-Ethanol Blank solution. Made up fresh for each assay.

    1ml assay buffer
    4ml acid-ethanol mixture
    2ml Tris solution

3. Create ^{125}I Label. Made up in a sterile glass vial.
4. Create Antibody-1. Made up in a sterile glass vial.
5. Create Standards over a range from 70 to 0.0036 ng/ml.

6. Add 50μl extracted samples to sample tubes

7. Add 50μl extracted QC to QC tubes

8. Add 50μl acid-ethanol blank solution to NSB, BO and Standard Tubes.

9. Add 200μl assay buffer to BO, QC and Sample tubes.

10. Add 250μl assay buffer to NSBs.

11. Add 200μl appropriate standard to standard tubes.

12. Add 50μl Antibody-1 to all tubes except totals and NSBs.

13. Add 50μl ^{125}I radiolabel to all tubes (Cap totals).

14. Vortex and incubate for 18hr at 4°C.

15. Create antibody-2 working solution. Made up in glass vial.

16. Create IgG working solution. Made up in an ependorff.

17. Add 50μl Ab-2 to ALL tubes EXCEPT TOTALS

18. Add 10μl IgG to all tubes except totals.

19. Vortex all tubes and incubate for 30 minutes @ 4°C.

20. Add 1ml cold PEG solution to all tubes except totals.

21. Vortex all tubes.

22. Centrifuge at 3000g for 30 minutes at 4°C.

23. Remove supernatant, invert tubes, and dry. Count tubes for 1 minute in a gamma counter (Wallac 1480 WIZARD® 3" Gamma Counter, Perkin Elmer, Life Sciences, Finland).

Assay counts per minute (cpm) values were converted to ng IGF/tube using the "Assayzap" computer program (Elsevier Biosoft) for the Apple Macintosh.

Quality Control and Validation

The sensitivity of the assay (i.e. the minimum amount of IGF-I able to be distinguished from zero) was 0.15ng/ml. QC samples containing an IGF-I content of approximately 50ng/ml were used to check the reproducibility of measurements both between and within assays. From this the intra-assay coefficient of variation was calculated as 4.4% whereas the inter-assay coefficient of variation was 13.9%.

Serial dilutions of a pooled sample extract were used to obtain an inhibition curve (Figure 2.3). When this inhibition plot was compared to the standard curve and it was found that the two curves were parallel, with no statistical difference between the slopes of the plots ($t = 0.290 < t_{0.05\ (2),\ 13} = 2.16$). This validated that the IGF-I measured in the samples was immunologically similar to that in the standards.

2.6 Plasma Leptin-like Peptide Analysis

2.6.1 Radioimmunoassay

For the purposes of identifying a leptin-like peptide from plasma samples in chapter 4, numerous commercially available mammalian RIA kits were used (Human, mouse, and multi-species; Linco Research, Missouri, USA). Serial dilutions of pooled plasma from 10 female broodstock (2.2kg, 10/11/00) were used to generate inhibition curves. However, samples were below the minimum detection (<1ng/ml) of any of the kits and failed to give any positive results.

2.6.2 Human Leptin ELISA

Due to the low levels of leptin possibly expected in fish plasmas a more sensitive approach was required. Plasma leptin analysis was carried out using commercially available Quantikine® human ELISA kits obtained from R&D systems (Oxon, UK).

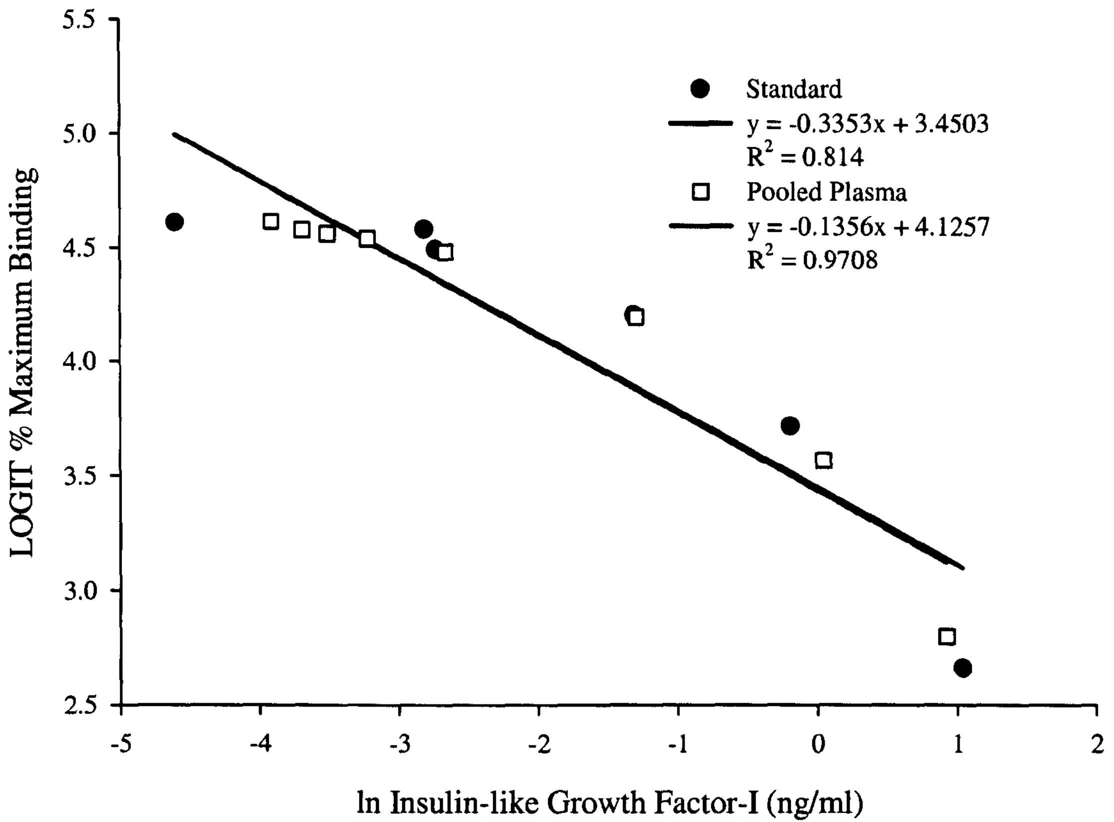


Figure 2.6 Parallelism of an inhibition curve obtained from a serial dilution of 50μl aliquots of pooled extracts of rainbow trout plasma with the IGF-I assay standard curve. Each point represents the mean of duplicate measurements. The curves have been linearised by using logit transformation, with the x-axis denoting the natural log of the IGF-I content in the standards.

Minimum sensitivity of the kits was 7.8pg/ml. All sample reagents and standards were prepared as directed in the kit. The protocol for the ELISA was carried out as follows:

1. Add 100μl of assay diluent added to each well.

2. 100μl of standard, quality controls and sample* added to respective wells.

(*100μl plasma used neat with no dilution)

3. Incubate at room temperature for 2 hours.

4. Aspirate and wash plate 4 times with assay buffer.

5. Add 200μl of enzyme conjugate to each well.

6. Incubate at room temperature for 1 hour.

7. Aspirate and wash plate 4 times with assay buffer.

8. Add 200μl of substrate solution to each well.

9. Cover plate with foil and incubate at room temperature for 30 minutes.

10. Add 50μl of stop solution to each well

11. Read plate at 450nm, at λ correction of 540 to 570nm.

12. Results were processed by automatic software to generate a calibration curve from which plasma results were extrapolated.

Details of gel electrophoresis, western blotting, and preliminary validation results of the Quantikine® human ELISA for use in detecting fish leptin-like peptide are provided in the chapter 4 section 4.2.

2.7 Analytical Calculations

2.7.1 Condition Factor

Condition factor has been used as a non-lethal measurement of body lipid content (Herbinger and Friars, 1991) with links being made to the condition of maturing individuals as well as growth performance. In all experiments, except 5.4 and 5.6,

condition factor was calculated from the measured length and weight of individual fish such that:

$$\text{Condition Factor} = [\text{Weight (g)} \times 100] / \text{length (mm)}^3$$

2.7.2 Specific growth rate (SGR)

Specific growth rate was calculated based on changes in weight or length over a known time such that:

$$\text{Specific growth rate (SGR)} = [\text{Ln } Wt_2 - \text{Ln } Wt_1] / (t_2 - t_1)$$

Where: Wt_1 = fish weight (g) at time t_1

Wt_2 = fish weight (g) at time t_2

2.7.3 Food Conversion Ratio

Food conversion (FCR) provides a crude estimate of how efficiently the food that is presented to the fish is converted into somatic growth over a period of time, such that:

$$\text{FCR} = \text{Mass of dry food provided (kg)} / \text{Wet mass increase of animals (kg)}$$

2.8 Statistical Analysis

The statistical principles used within this thesis are described in Zar (1999). Calculations were performed using either Minitab Statistical Package (version 13.1) or Instat version 3.0 (Graphpad Software Inc.). A significance level of 5% was used for all tests.

2.8.1 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 along with the standard error of the mean (SEM) to give a representation of the sample distribution. All basic statistics were calculated with the aid of Microsoft Excel 2000.

2.8.2 Parametric assumptions.

All parametric techniques are based on a number of fundamental assumptions. Firstly all observations must be derived randomly and the variation of these observations should be independently distributed. Furthermore, parametric tests require sample variations to be identically distributed (i.e. homogeneous) with a normal population structure. Therefore, all data were investigated to confirm normality and homogeneity of variance prior to detailed statistical analysis.

2.8.3 Testing for normality and homogeneity of variance

Where general linear models were performed n was typically large enough to allow normality and homogeneity of variance to be confirmed by the examination of the residual plots. However, where n was insufficient to allow this, or where other statistical techniques were performed, the following tests were used:

Normality

Normality was checked using the Kolmogorov-Smirnov test. This non-parametric test is typically used to compare two cumulative frequency distributions (F) but it can be adapted to compare the distribution of a known distribution with an expected distribution. This therefore allows sample populations to be compared to the ideal Gaussian distribution.

Homogeneity of variance

For the comparison of two sample variances the *F*-test was used. The F value was then compared to tabulated values such that if the calculated value was greater than or equal to the tabulated value, at the 5% level, the variances were considered as heterogeneous.

Bartlett's test (*B*) was used to compare more than two sample variances. The distribution of *B* is approximated by the chi-squared. Again if the calculated value was greater than or equal to the tabulated value, at the 5% level, the variances were considered as heterogeneous.

2.8.4 Parametric comparison of multiple samples

All parametric tests were performed using the analysis of variance technique (ANOVA). However, these calculations were manipulated by the use of General Linear Models (GLM). Through the Minitab Statistical Package it was possible to manipulate the ANOVA by constructing model formulae which accounted for a number of factor levels with replication and repeated measures sampling also included. As such it was possible to increase the robustness of each test for the particular parameters that were available. Furthermore, where replicate differences occurred, the GLM accounted for the variation within the replicates when presenting statistical significance levels. For post-hoc multiple comparisons Tukey tests were used. This method involves the pairwise comparison of group means to give the test statistic q. If the calculated q value was greater than the tabulated value, at the 5% significance level, the means of the two samples were considered to be significantly different.

2.8.5 Non-parametric comparison of multiple samples

Where the prerequisite assumptions for parametric analysis were not met, Kruskal-Wallis non-parametric multiple comparison test were performed in place of one way-ANOVA using the Instat statistical package. If a significant difference was observed ($p<0.05$), Dunn's post-hoc test was used for pair-wise comparison of samples.

2.8.6 Regression and Correlation

The degree of linear relationship between two variables was considered by calculating the Pearson correlation coefficient (r). If the calculated r value was greater than the tabulated r value, at the 5% level, the correlation between variables was considered as significant.

Linear relationships were assessed using simple linear regression of the two variables of interest, with Run's test used to check for departure from linearity (Instat version 3.0). Where Run's test failed, indicating a curvilinear relationship between variables, 2nd order polynomial regressions were plotted.

2.8.7 Comparing linear regression coefficients

Parallelism of inhibition curves used in the validation of the radioimmunoassay was obtained by comparing the gradients and assessing the equality of two population regression coefficients by means of the Student's t test.

Multiple comparison of linear regression gradients was carried out using analysis of covariance (ANCOVA) as described by Zar (1999).

Chapter 3: The Effects of Photoperiod Manipulation on Growth and Plasma Insulin-like Growth Factor-I in Juvenile Rainbow Trout

3.1 Introduction

The experiments conducted within this chapter were initiated by Dr. Clive Randall as part of the National Environmental Research Council (NERC ROPA GR3/R9827, January 2000) grant to investigate endocrine, growth and reproductive interactions in rainbow trout. The studies within the current chapter were designed to investigate photoperiodic manipulations of growth, and provide preliminary evidence of measurable endocrine changes in insulin-like growth factor-I in rainbow trout. The original design and set-up of experiments 1 and 2 was carried out in collaboration with Dr. Randall.

Growth of an organism integrates a host of biological processes which lead to a fertilised egg attaining the size, form and function of an adult as well as to the maintenance and modulation of adult structures appropriate to their function (Very *et al.*, 2001). Animal growth is influenced by genetic, environmental and nutritional factors. Extrinsic factors are particularly important in the growth of ectothermic vertebrates such as teleost fish, which rely on temperature, photoperiod and food availability to initiate developmental processes (Thorpe *et al.*, 1989; Boujard *et al.*, 1995; Imsland *et al.*, 1995; Jobling & Koskela, 1996). In the comprehensive review by Boeuf & Le Bail (1999) light induced effects on growth in a variety of species have been observed. In the case of salmonids, photoperiod is classified as a directive factor, controlling growth as a "zeitgeber" through its influence on endogenous rhythms (Porter *et al.*, 1998; Endal 2000), and acting through direct photostimulation of the somatotropic axis (Komojourdjian, 1976; Bjornsson, 1997). These effects are certainly evident in Atlantic salmon, where abrupt exposure to long photoperiods during naturally short-days (winter) has been shown to advance the natural growth rhythm

(Krakenes *et al.*, 1991; Hansen *et al.*, 1992) in a manner similar to that observed in advances in circannual spawning rhythms following photoperiod manipulation (Randall & Bromage, 1998; Randall *et al.*, 1999). However, in many studies in juvenile salmonids there is the inherent problem of dissociating photoperiod induced growth from smoltification (Stefansson *et al.*, 1991; Solbakken *et al.*, 1994; Berge *et al.*, 1995), since growth and size will determine whether a fish is of sufficient size to under go the transformation (Thorpe *et al.*, 1989; Skilbrei *et al.*, 1997). Furthermore, correct photoperiod application is important in ensuring successful completion of the process (Sigholt *et al.*, 1995). Similarly, in post-smolts photoperiod induced growth is the result of reallocation of energy reserves into somatic growth through photoperiodic induced maturational arrest (Krakenes *et al.*, 1991; Taranger *et al.*, 1998; 1999). With regards to the situation in rainbow trout even less literature exists, with current studies providing inconclusive and contradictory results (Skarphedinsson *et al.*, 1985; Mason *et al.*, 1992; Makinen, & Ruhonen, 1992; Solbakken *et al.*, 1999; Wildenhayn & Holtz, 1999). However, as the rainbow trout does not undergo parr-smolt transformation during its normal life history, the species may thus provide a simpler model for interpreting photoperiod induced changes in growth and endocrine parameters during juvenile development.

Thus, the integration of external and internal cues leads to a co-ordination of growth by endocrine mediators. A central component of growth co-ordination is the growth hormone (GH)-insulin-like growth factor-I (IGF-I) axis, with increasing evidence indicating that many of the growth promoting actions of GH in fish (Stefansson *et* al., 1991; Bjornsson, 1997) are mediated by IGF-I (Duan *et al.*, 1995; Duan, 1998). To date many studies on growth, IGF-I and its regulation have extensively examined the mechanistic effects of IGF-binding proteins (IGFBP) (Fukuzawa, *et* al.,

1995; Siharath *et al.*, 1996; Hwa *et al.*, 1999; Shinizu *et al.*, 1999; 2000; 2003), somatostatins (Very *et al.*, 2001), insulin (Shearer *et al.*, 1997b; Banos *et al.*, 1999), thyroid hormones (Schmid *et al.*, 2003), sex steroids (Ng *et al.*, 2001; Riley *et al.*, 2002), the external influence of temperature (Beckman *et al.*, 1998; Larsen *et al.*, 2001; Mingarro *et al.*, 2002; Gabillard *et al.*, 2003a) and nutrition (Shearer *et al.*, 1997b; Pierce *et al.*, 2001; Beckman *et al.*, 2004). However, as yet research has failed to explore other possible environmental influences on its regulation, including photoperiod. Since photoperiod is a commonly used tool in the aquaculture industry to maximise production through enhancement of growth rates in order to attain market sizes in as short a time period as possible and hence improve farming efficiency (Hansen *et al.*, 1999) this lack of research is surprising. Furthermore, since photoperiodic information is thought to be conveyed via melatonin secretion from the pineal gland to the gonado- and somatotropic axis no studies as yet have examined the influence on the GH-IGF axis to determine if melatonin may be acting directly on this system.

The effect of seasonally changing photoperiod on the IGF system has been most thoroughly studied in higher vertebrates, particularly in mammals like the red deer (*Cervus elaphus*). Several studies have shown increasing spring photoperiods result in increased appetite, growth, GH production and IGF-I levels (Webster *et al.*, 1996; Rhind *et al.*, 1998; Ditchkoff *et al.*, 2001). Furthermore, clear evidence has shown that plasma IGF-I increases are directly related to photoperiod, through sustained stimulation of IGF-I production even when feed is restricted during spring (Webster *et al.*, 2001). Thus it has been proposed that the rise in IGF-I is a component of the photoperiodically entrained seasonal drive of growth, and the increase in food intake is a response to satisfy the increased energy demand for maintaining growth (Webster *et*

al., 1996; 2001). Red deer also respond to decreasing photoperiods in autumn through decreased appetite, growth and IGF-I levels (Webster *et al.*, 1999).

Studies examining the effect of photoperiod on IGF-I production in lower vertebrates and especially fish are lacking in comparison to the many examining the dual role of nutrition and/or temperature. However, these studies have been of relatively short-term and may not reflect seasonal changes in growth. Plasma IGF-I has been shown to increase seasonally, with levels doubling from June to September regardless of feeding rate and under relatively constant water temperatures of 11°C in post-smolt coho salmon (Beckman *et al.*, 2004). Regression analysis showed that date had an overriding effect on IGF-I levels rather than growth rate. From these results, it was proposed that seasonally changing photoperiod may explain the changes observed during these experiments, in that the highest IGF-I levels were observed at the time of the autumnal equinox when daily photoperiod change was at it maximum. Similarly, IGF-I levels increased steadily between January and May under natural photoperiod when elevated water temperatures (10°C) were applied to under-yearling Atlantic salmon parr, while increases under the naturally changing ambient light and temperature occurred later in spring (April-May) (McCormick *et al.*, 2000). In contrast, exposure to LD 18:6 in combination with elevated temperatures caused an abrupt increase in IGF-I levels after 7 days. However, again the latter study examined the effect on smoltification, in which long-days applied in spring advanced the seasonal pattern of smoltification. Studies suggested that IGF-I is involved in osmoregulation through stimulation of $Na^{+}K^{+}$-ATPase activity in the gill and cortisol production which promote seawater tolerance (Reviewed in Dickhoff *et al.*, 1997 and Duan 1997; Shrimpton & McCormick, 2003). This highlights the differences that may occur between migratory and non-smolting salmonids, in that changes in IGF-I secretion may

also reflect later life history strategies in addition to growth. However, these studies do provide evidence of temperature regulation of photoperiod induced changes in GH and IGF-I production, and suggests that timing of photoperiod application may have important implications on the subsequent endocrine and growth response.

Thus considering the lack of knowledge regarding the possible effects of photoperiod on the IGF system this chapter aimed to determine the influence of photoperiod on growth and circulating plasma IGF-I in juvenile stages of rainbow trout. In this purpose three experiments were designed to:

Experiment 1: to examine the effects of photoperiod on growth and IGF-I during the earliest stages of development (IGF mRNAs are detectable at all developmental stages, but it is not clear when they are translated into functional peptides, i.e. when the light-GH-IGF axis becomes operative).

Experiment 2: to examine the effects of photoperiod on growth and IGF-I from fingerling stages of development (i.e. when the GH-IGF axis would be expected to be operative and circulating IGFs can be measured).

Experiment 3: to determine whether photoperiod affects growth directly via the GH-IGF axis, or whether its action may be mediated or influenced by melatonin (in this case by exogenous administration using slow-release implants).

3.2 General Materials and Methods

Experiments 1-3 were carried out in flow-through light-proof covered tanks with artificial lighting at the Niall Bromage Freshwater Research Facility. Figure 3.1 shows the layout and operation of a typical system utilised. Size of tanks used during the experiments varied according to developmental stages studied and maximum stocking density expected during grow-out. Full description of tank size and operation is given for each experiment in the relevant materials and methods section. All tanks were provided with inflow water from a nearby reservoir at ambient temperature with Figure 3.2 showing the fluctuations during the period of each experiment.

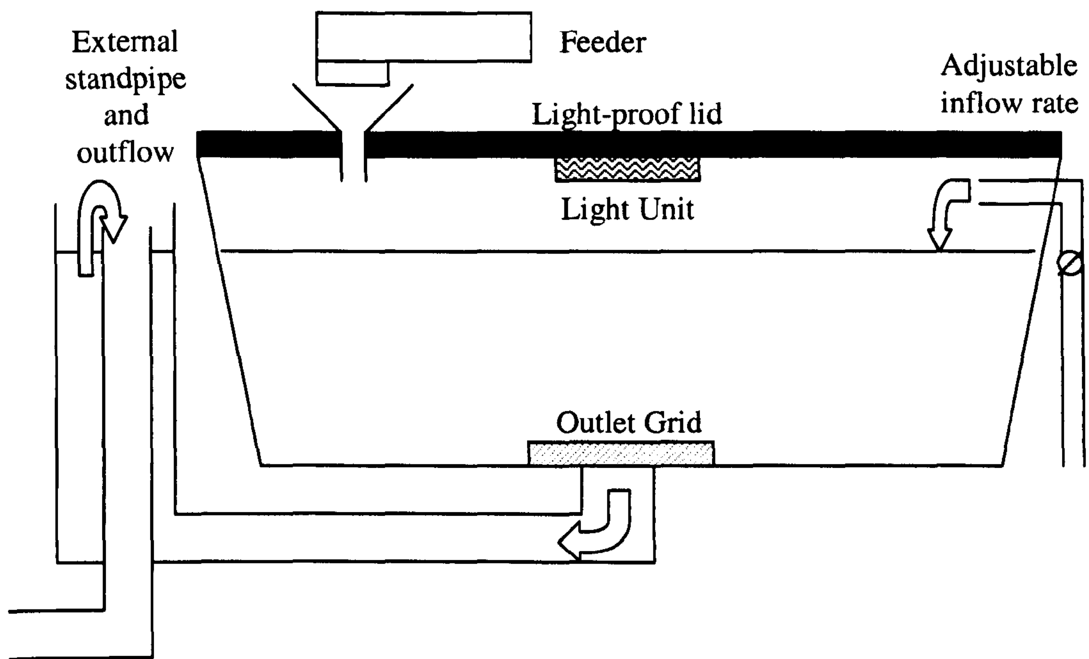


Figure 3.1 Schematic representation of a typical flow-through covered tank system used in experiments 1-3.

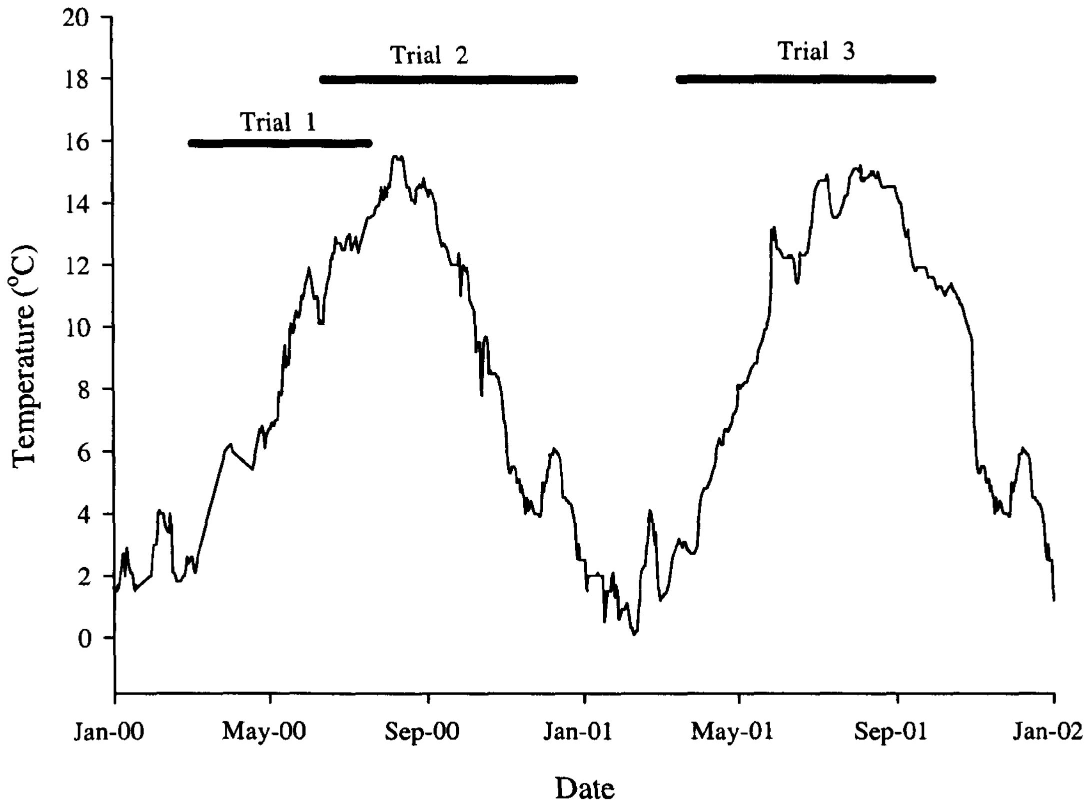


Figure 3.2 Daily inflow water temperature profile for the Niall Bromage Freshwater Research Facility during the period of 2000 to 2002. Horizontal bars indicate the period when experiments 1 to 3 were carried out.

3.3 Experiment 1: Effects of Exposure to Photoperiod from Fertilisation on Growth and Insulin-like Growth Factor-I Levels in First Feeding Fry

Aim: To examine the effects of exposure to different photoperiods on growth and IGF-I tissue production during the earliest stages of development in rainbow trout fry. This experiment aimed to (1) confirm preliminary evidence from non-replicated trials that long-day photoperiods improve growth rate in fry (Randall *et al.*, 2001) and (2) to analyse the ontogeny of IGF expression and production under different photoperiod regimes. Unfortunately due to limited funds and resources it was not possible to yet determine IGF-I expression and plasma IGF-I production during the time of this thesis. Egg and whole fry samples have been retained at -70°C for future analysis by in situ hybridisation techniques and RIA.

3.3.1 Materials and Methods

On March 20th 2000 fifteen 3-year old first spawning female rainbow trout (NBFRF broodstock, origin Selcoth Fish Farms, March 1997) were artificially stripped of ova. Eggs were fertilised using pooled milt from five second spawning male fish of the same origin. Fertilised eggs were laid down following water hardening in three discrete batches in separate rearing troughs and maintained under blackout covers. Post-fertilisation, eggs were exposed to one of three test photoperiod regimes in single; simulated natural photoperiod (SNP), constant short-day (LD 8:16) and constant long-day (LD 18:6) provided by 8 watt fluorescent strip lights positioned 30cm above the water surface (300 Lux). Hatching occurred on 12th May (407 degree days), with swim up and first feeding beginning on 31st May. On 2nd June, three groups of 5000 fry of mean weight 0.10 ± 0.02g (~1.7kgm^{-3} starting density) from each photoperiod regime

were stocked into twelve 0.3m^3 light-proof covered fiberglass tanks (1.7 kg m^{-3}) with an external standpipe adjusted to create a water depth of 0.3m and a flow rate of 1L/sec. Light was supplied by one 16 watt drum fitting light (RS Components Ltd.; Northants, UK) creating 1500 lux at the water surface and 300 lux at the tank floor. As of this date triplicate groups of first-feeding fry were exposed to the same photoperiods as in their respective rearing trough until 3rd August. SNP regimes (min/max hours light 7.5-17.25 hours light day^{-1}) were controlled using a photosensitive switch (RS components Ltd., Northants, UK) adapted by Alex Brewsters electrical contractors (Stirling, UK), while modified photoperiod regimes were controlled using electronic analogue timers (CPC UK.). Fish were fed Trouw Nutra Aminobalance™ crumb in various grades (Table 3.1) via clockwork belt feeders according to manufacturer's tables. Food in all tanks was presented during the hours of the shortest photoperiod for the duration of the experiment.

Table 3.1 Proximate analysis of diets presented during trial and size at which they were given.

Crumb Grade	Fry Size (mm)	Protein (%)	Lipid (%)	Carbo- (%)	Ash (%)	Moisture (%)	DE MJ/Kg
00	0.65	55	14	14	9	8	18.32
01,02,03	0.6-1.8	54	18	11	9	8	19.22
18	>1.8	54	18	11	9	8	19.22

Measurements for weight-length (n=100) were taken at 7-10 day intervals for each treatment with all fish removed being killed prior to measurement. At each sampling point a further 5-10 fry were sacrificed and frozen in liquid nitrogen for IGF-I analysis. In addition to whole fish samples, small batches of ova (20-30) and yolk-sac

fry (alevins) were also frozen in liquid nitrogen at regular intervals during egg development post-fertilisation for further IGF-I analysis.

SNP treatments were killed due to the development of rainbow trout fry syndrome (RTFS, bacterial agent, *Flavobacterium psychrophilum*) two weeks post-hatch, where large numbers of daily mortalities (>100 day^{-1}) were observed, with fry showing darkened colour, general lethargy and in some cases the development of lesions. Diagnosis was confirmed by the bacteriology unit of Stirling Aquaculture Vaccine. Short- and long-day photoperiod treatments were also tested but remained unaffected. General mortalities with in the remaining tanks for the duration of the trial was less than 0.5%.

Statistical Analysis

Growth performance parameters and plasma hormone levels were analysed by General Linear Models using time and treatment as categorical predictors, and replicate as a random factor. Homogeneity of variance and normality was checked using residuals plots. Where necessary data was log-transformed to ensure a linear relation was achieved. For all statistical tests a significance of $p<0.05$ was used.

3.3.2 Results

3.3.2.1 Effects of Exposure to Different Photoperiods on First Feeding rainbow Trout Fry Growth

On hatching no significant differences in alevin weight were found between short- and long-day treatments. Analysis of growth showed that following four weeks exposure to photoperiod from first feeding that fry maintained under long-days were significantly heavier than those under short-days (Figure 3.3). By the end of the experiment on 3rd August, fish maintained under long-days were 11% heavier than those under short-days. Similarly, fry under long-days reached a 4.3% significantly greater length (73.0 ± 0.5mm) than those under short-days (70.1 ± 0.5mm). Table 3.2 shows that fry exposed to long-days maintained a higher daily percentage gain in weight (SGRwt) and length (SGRL) compared to those under short-days for the period of the experiment.

Table 3.2 Total specific growth rates (% day^{-1}) for both weight (g) and length (mm) for fry maintained under short- (LD 8:16) and long-days (LD 18:6) for the duration of the experiment (62 days). Replicate data have been combined for each treatment (mean ± SEM). Statistical analysis was not performed as values are treatment averages based on mean weight changes between time points and therefore lack sufficient power to provide a statistical result.

Treatment	SGRwt g (% day^{-1})	SGRL mm (% day^{-1})
LD 8:16	**5.89 ± 0.04**	**1.74 ± 0.02**
LD 18:6	**6.23 ± 0.05**	**1.84 ± 0.02**

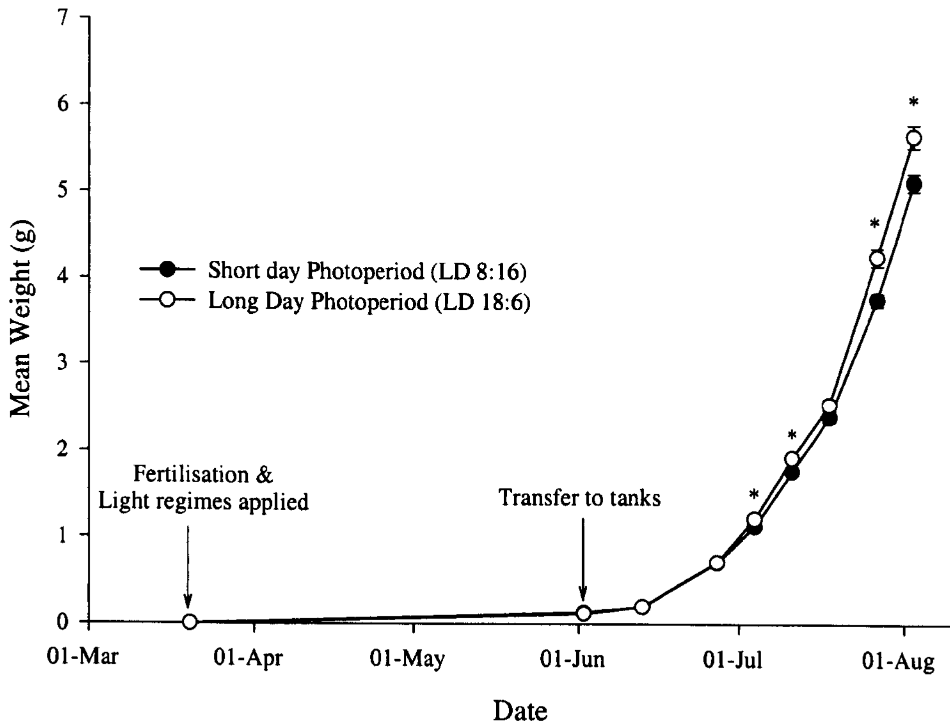


Figure 3.3 Effects of exposure to long- and short-day photoperiods from first feeding on growth (mean weight ± SEM of 3 replicates, n=300) of rainbow trout fry in covered tanks. Standard error bars are shown but in some cases are too small to be depicted. Asterisks (*) denote significant differences ($p<0.05$).

CF is only shown from hatching on 2nd June as length measurement during embryo development was not possible, as such CF could not be calculated. Following first feeding, fry condition factor increased steadily with time in both treatments (Figure 3.4). At the commencement of first feeding, fry exposed to short-days were of significantly higher CF than those under long-days. This difference was maintained until 27th June. Thereafter, no significant differences were apparent although short-day treatments did maintain a slightly higher mean CF. Sixty two days following the commencement of first feeding (final sample point 3rd August), short-day treatments continued to show an increase in CF while long-day treatments exhibited a decrease in CF attaining a significantly lower value than short-day fry despite being of a significantly greater weight and length, indicating that fry exposed to short-days were of a heavier weight for a given length compared to those maintained under long-day photoperiods.

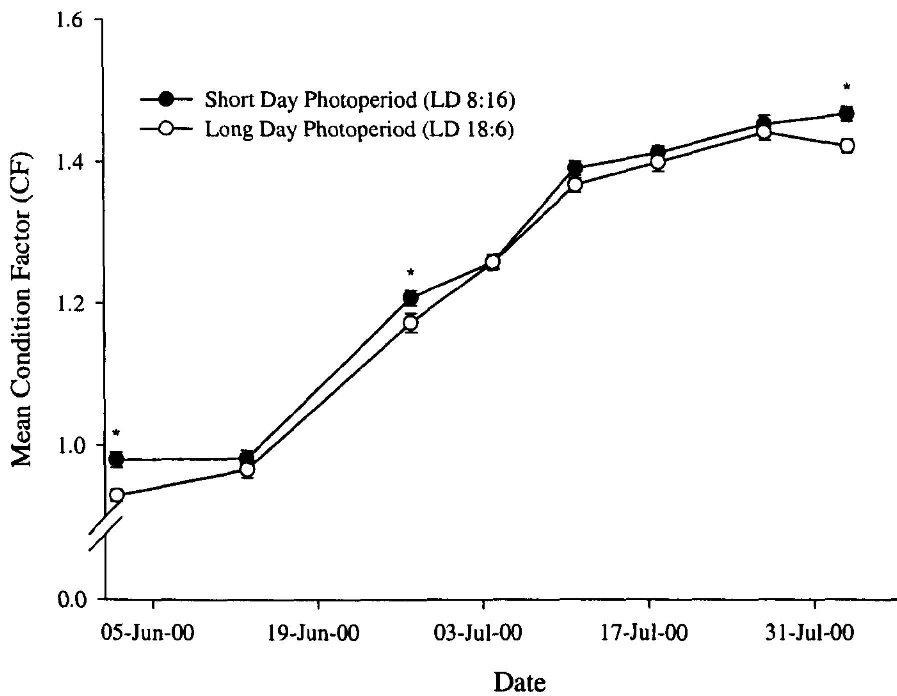


Figure 3.4 The influence of long- (LD 18:6) and short-day (LD 8:16) photoperiods from first feeding on condition factor variation (mean ± SEM of 3 replicates, n=100) of rainbow trout fry maintained in covered tanks. Standard error bars are shown but in some cases are too small to be depicted. Asterisks (*) denote significant differences ($p<0.05$).

3.4 Experiment 2: Effects of Photoperiod Regimes on Growth and Plasma Insulin-like Growth Factor-I Production in Fingerling Rainbow Trout

Aim: To examine the effects of exposure to different photoperiods on growth and plasma IGF-I production during on-growing of fingerling rainbow trout.

3.4.1 Materials and Methods

On 1st June 2000 groups of 142 all-female rainbow trout of mean weight 26.9 ± 2.8g (Selcoth Fish Farm, Glen Wyllin origin, reared under natural photoperiod from hatch in January 2000) were stocked into nine 0.3m^3 fiberglass tanks (12.7 kg m^{-3}) with an external standpipe adjusted to create a water depth of 0.3m and a flow rate of 1L/sec. Light was supplied by one 16 watt drum fitting light (RS Components Ltd.; Northants, UK) creating 1500 lux at the water surface and 300 lux at the tank floor. Photoperiod was maintained at LD 17:7. As of June 17th triplicate groups of fish were exposed to one of three photoperiod regimes; simulated natural photoperiod (SNP, range 7-17.5 hrs daylight day^{-1}), constant short-day (LD 8:16) and constant long-days (LD 18:6) from June 17th to 8th December 2000 under ambient water conditions (Figure 3.1, range 3-15.8°C). Short-day regimes were applied from 8am. Light regimes were controlled as described in **section 3.3.1**. Fish were fed Trouw Trout Aminobalance™ 40 and 50 pellets (Protein 45%, Lipid 30%, Carbohydrate 11%, Ash 8%, Moisture 6%, DE 21.46 MJ/kg) via clockwork belt feeders according to manufacturer's tables. Food was presented in all tanks during the hours of the shortest photoperiod for the duration of the experiment.

Measurements for weight-length (n=60) were taken at two weekly intervals, with blood samples taken on 17th June, 27th June, 27th July, 1st September, 10th October,

11th November and 8th December (n=10/tank/treatment). All fish were returned to the tanks following sampling. Only 5 bloods per replicate were assayed for IGF-I due to financial restrictions. As fish grew and density increased, a weight equivalent proportion of the population was randomly sacrificed in order to maintain density below 40 kg/m^3. Mortalities in all tanks were less than 0.5% for the duration of the trial.

Statistical Analysis

Differences in growth parameters (weight, length and condition factor) where tested using GLM procedures using time and treatment as categorical predictors with replicate as a random factor. Where no replicate differences were apparent replicates were pooled. Relationships (semi-log plots) between IGF-I and temperature, SGRwt and SGRL were tested using Pearson product moment correlation coefficient. Preliminary analyses prior to regression analysis were performed to ensure normality, linearity and homoscedasticity (variability in scores for x similar at all values of Y). Relationships were linearised to allow statistical tests. Tests for common slopes (gradient) and elevation (y intercept) between treatments were compared using ANCOVA. Multiple regression analysis was not carried out due to multicollineaity within the independent variables (temperature, SGRwt and SGRL were highly correlated with one another, $r^2>0.9$). As such no factor could be used to determine which was having the greatest influence/variance on IGF-I levels.

3.4.2 Results

3.4.2.1 Effects of Exposure to Different Photoperiod Regimes on Fingerling Rainbow Trout Growth Performance

For the purpose of clarity of interpretation simulated natural, LD 8:16 and LD 18:6 photoperiods will be referred to as SNP, short- and long-day treatments respectively.

Weight

All treatments increased weight steadily from the beginning of the experiment with the long-day photoperiod reaching a significantly heavier mean weight than SNP and short-day treatments by late-September and mid-October respectively, 121 days post-exposure (Figure 3.5). A significantly heavier weight was maintained from this period onwards. No significant differences were found between the SNP and short-day treatments at any given time point. Weight gain in all treatments was greatest between June and late September, with weights at each time point significantly greater than the previous month. By the end of the experiment fish under long-days were 17.5 and 11.1% heavier than fish under SNP and short-days respectively.

Although statistical analysis was not performed due to lack of statistical power (SGR based on mean weight of each replicate, n=3/treatment), SGRwt suggests that fish exposed to long-days maintained a higher growth rate than other treatments between mid-August and late September (Figure 3.6). All treatments showed an increase in SGR between June and Mid August, with long-days showing the quickest and greatest relative increase achieving 1.97 % day^{-1}, albeit slightly lower than fish under SNP which reached 2.05 % day^{-1}. This was then followed by a steady decrease in growth rate until December at which point all groups ranged between 0.11 and 0.25% day^{-1}.

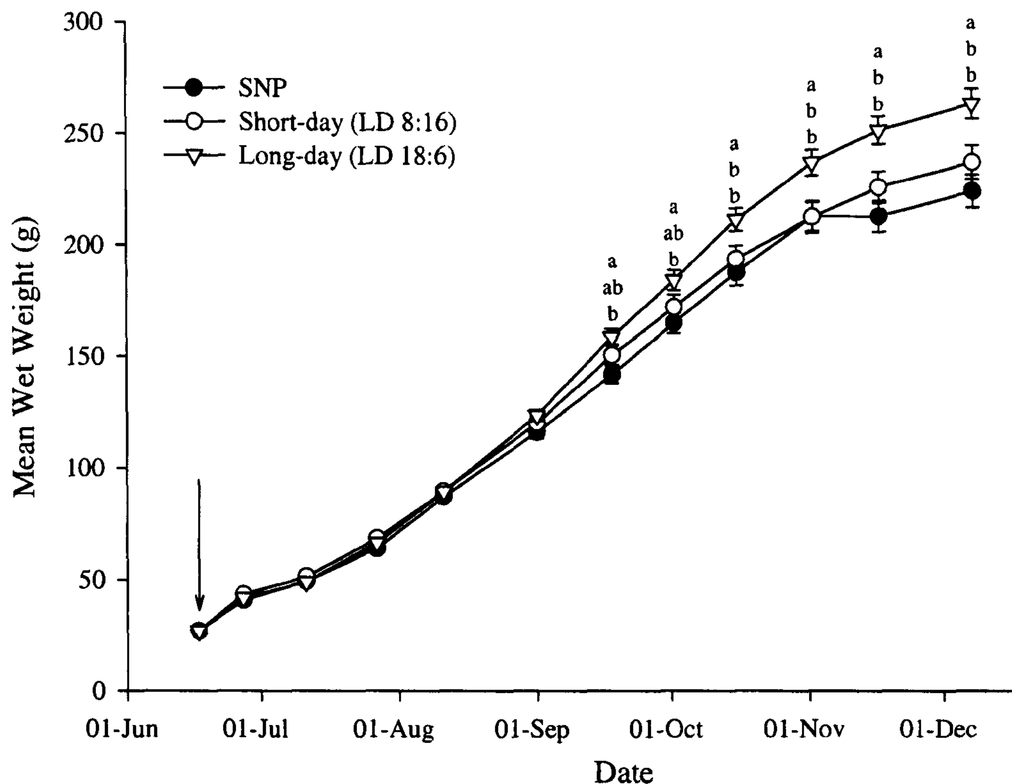


Figure 3.5 The effects of exposure to different photoperiod regimes (SNP, LD 8:16, or LD 18:6) on growth of juvenile rainbow trout maintained in covered tanks (mean ± SEM of 3 replicates, n=180). Standard error bars are shown but in some cases are too small to be depicted. Treatments sharing different superscripts at a given time point are significantly different ($p<0.05$). Lettering is stacked in the same order as graph lines. Arrow indicates where photoperiod regimes were applied.

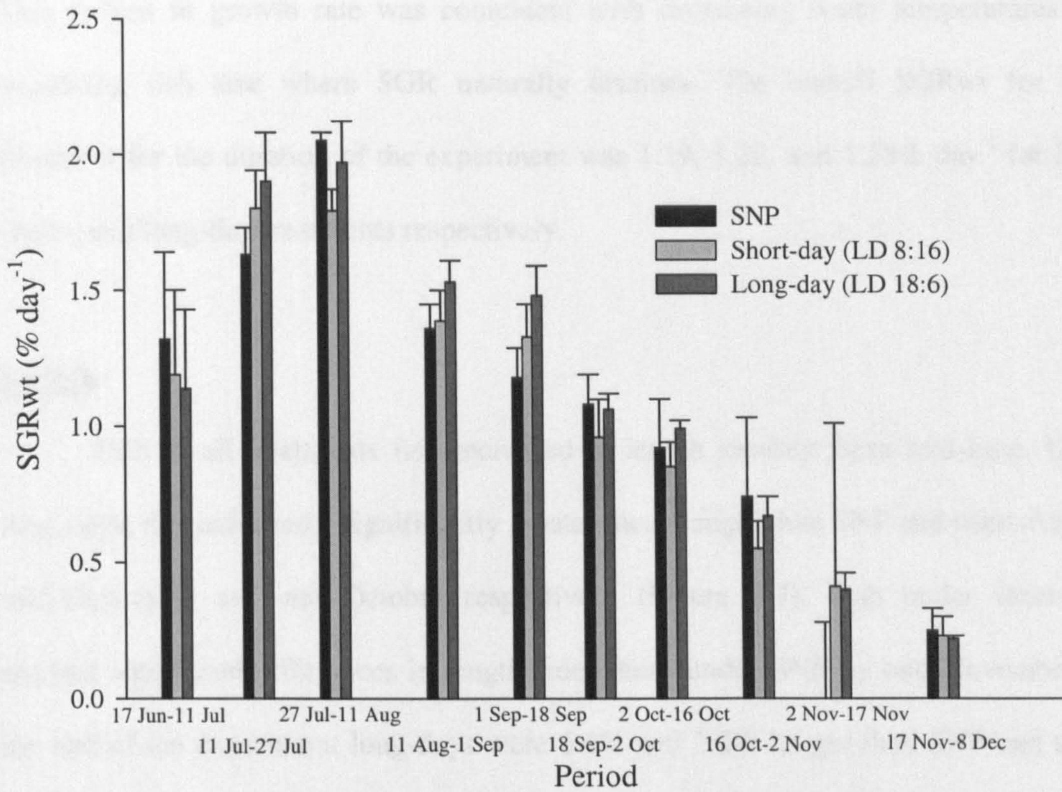


Figure 3.6 The effects of exposure to different photoperiods (SNP, LD 8:16, or LD 18:6) on rate of daily weight gain (SGRwt) of juvenile rainbow trout maintained in covered tanks between sample points (mean ± SEM of 3 replicates).

This pattern in growth rate was coincident with decreasing water temperatures and increasing fish size where SGR naturally declines. The overall SGRwt for each treatment for the duration of the experiment was 1.19, 1.22, and 1.28% day^{-1} for SNP, short-, and long-day treatments respectively.

Length

Fish in all treatments fish increased in length steadily from mid-June. Under long-days, fish achieved a significantly greater mean length than SNP and short-days by mid-September and mid-October respectively (Figure 3.7). Fish under short-days showed significant differences in length from those under SNP by mid-November. At the end of the experiment long-days were 6.1% and 3.2% longer than SNP and short-day treatments respectively, with short-days achieving a 2.8% greater mean length than SNP.

In terms of growth rate short-day treatments maintained a relatively constant SGRL of 0.47% to 0.49% day^{-1} between mid-July and late August, before steadily decreasing to 0.12% day^{-1} by December (Figure 3.8). In contrast, SNP and long-day exhibited an increase in SGRL, both reaching 0.6% day^{-1} by mid-August, then SGRL declined steadily to 0.12% day^{-1} by December. Although statistical analysis was not performed long-days generally maintained a higher SGRL than the other treatments between late August and early November despite a declining rate of gain. The overall SGRL for each treatment for the duration of the experiment was 0.35, 0.36, and 0.38% day^{-1} for SNP, short-, and long-days respectively.

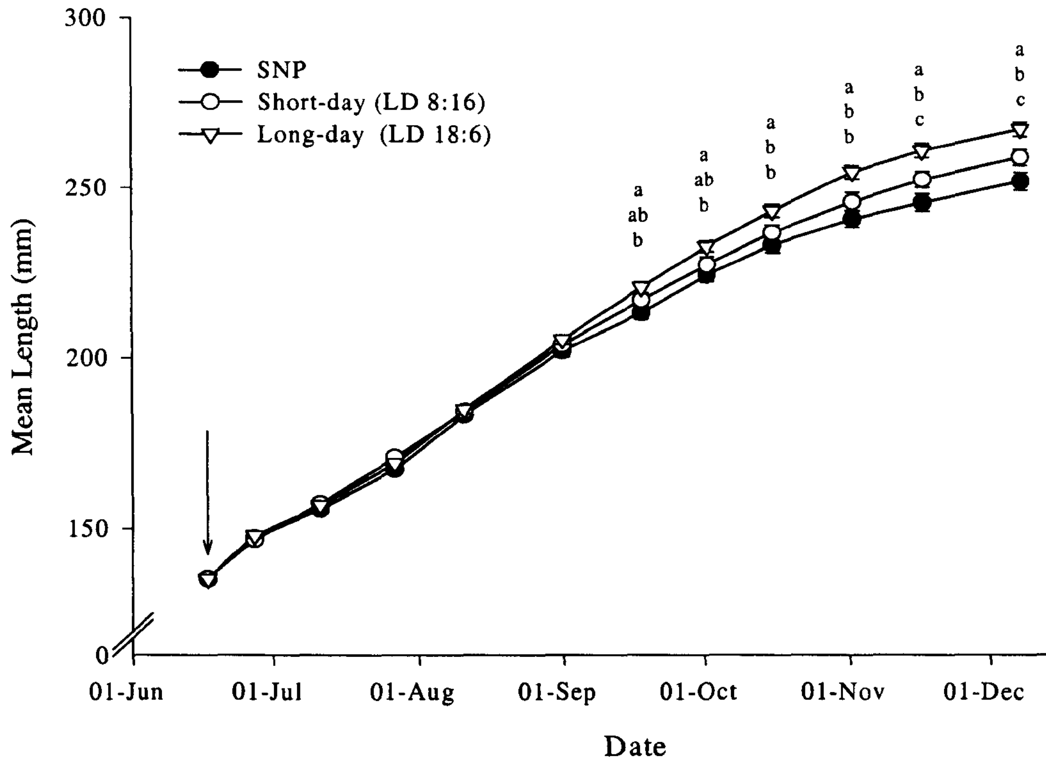


Figure 3.7 The effects of exposure to different photoperiod regimes (SNP, LD 8:16, or LD 18:6) on length increase (mean ± SEM of 3 replicates, n=180) of juvenile rainbow trout maintained in covered tanks. Standard error bars are shown but in some cases are too small to be depicted. Treatments sharing different superscripts at each time point are significantly ($p<0.05$). Lettering is stacked in same order as graph lines. Arrow indicates where photoperiod regimes were applied.

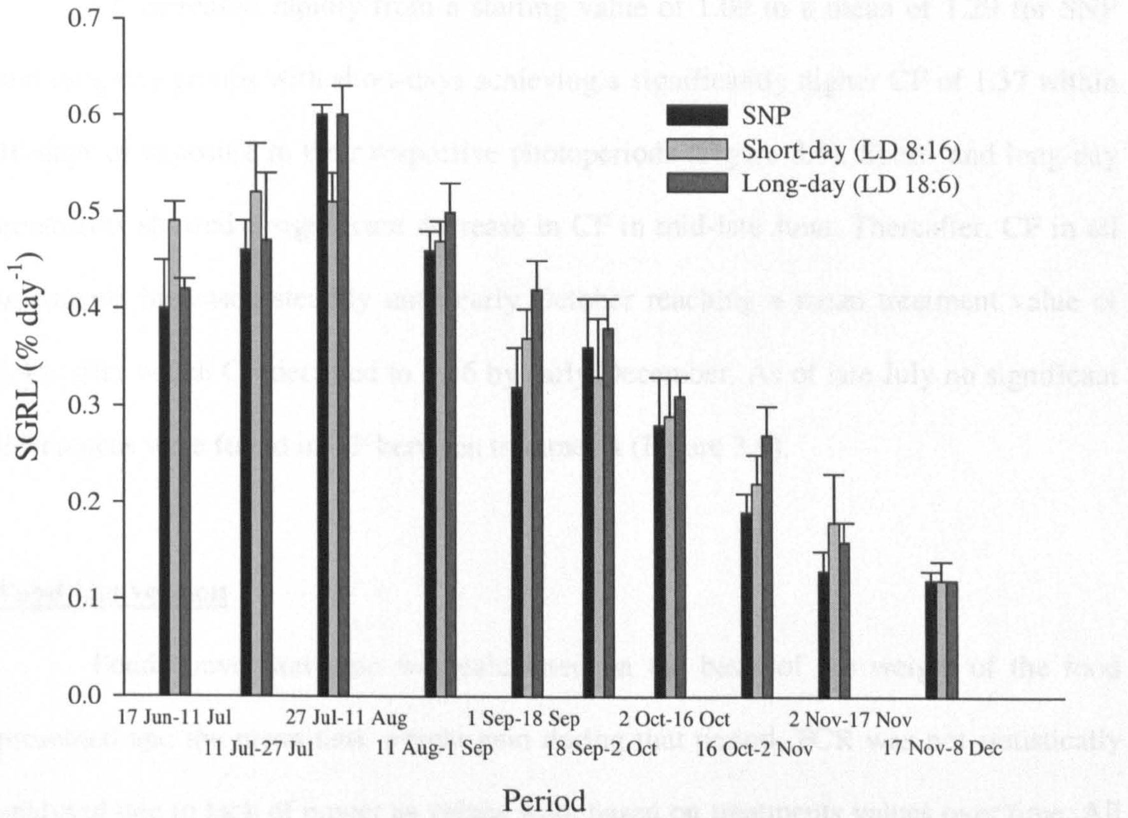


Figure 3.8 The effects of exposure to different photoperiod regimes (SNP, LD 8:16, or LD 18:6) on rate of daily length gain (SGRL) of juvenile rainbow trout maintained in covered tanks between sample points (mean ± SEM of 3 replicates).

Condition Factor (CF)

CF increased rapidly from a starting value of 1.09 to a mean of 1.29 for SNP and long-day groups with short-days achieving a significantly higher CF of 1.37 within 10 days of exposure to their respective photoperiods (Figure 3.9). Short- and long-day treatments showed a significant decrease in CF in mid-late June. Thereafter, CF in all treatments increased steadily until early October reaching a mean treatment value of 1.45, after which CF declined to 1.36 by early December. As of late July no significant differences were found in CF between treatments (Figure 3.9).

Food Conversion

Food conversion ratio was calculated on the basis of the weight of the food presented and the mean tank weight gain during that period. FCR was not statistically analysed due to lack of power as values were based on treatments values over time. All treatments showed a general improvement in feeding efficiency from mid-July to mid-August following the onset of photoperiod treatment and higher water temperatures (Figure 3.10). SNP exhibited a marked decrease in feeding efficiency from mid-August to mid-September, thereafter FCR improved. Short-day groups exhibited a similar pattern to SNP between mid-august and Mid-October, although short-days did maintain a more efficient FCR in mid-September. From mid-October to mid-November all treatments exhibited a reduction in feeding efficiency. Nevertheless long-days maintained a lower and more efficient FCR than the other treatments from late August. Total FCR was 1.04, 1.07 and 0.92 for SNP, short-, and long-day treatments respectively, representing an 11.5% and 14% improvement in FCR of long-days over SNP and short-day treatments.

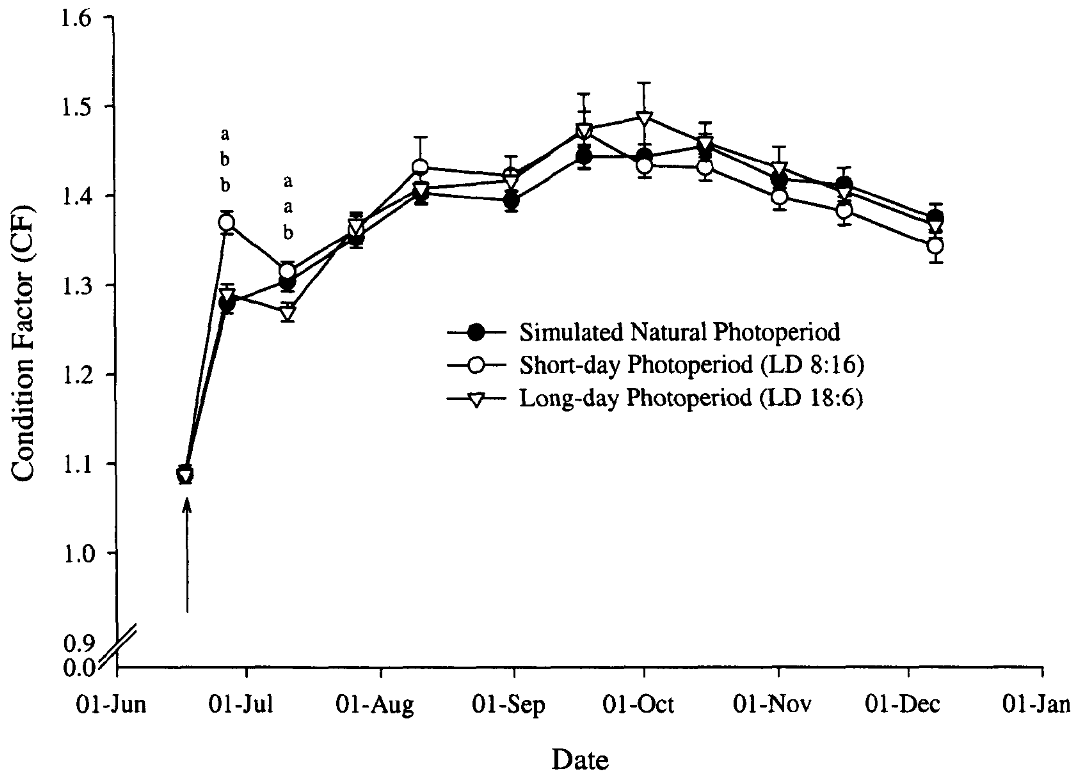


Figure 3.9 Changes in condition factor of juvenile rainbow trout exposed to different photoperiod regimes (SNP, LD 8:16, or LD 18:6) maintained in covered tanks (mean ± SEM of 3 replicates, n=180). Standard error bars are shown but in some cases are too small to be depicted. Treatments sharing different superscripts at each time point are significantly different ($p<0.05$). Lettering is stacked in same order as graph lines. Arrow indicates where photoperiod regimes were applied.

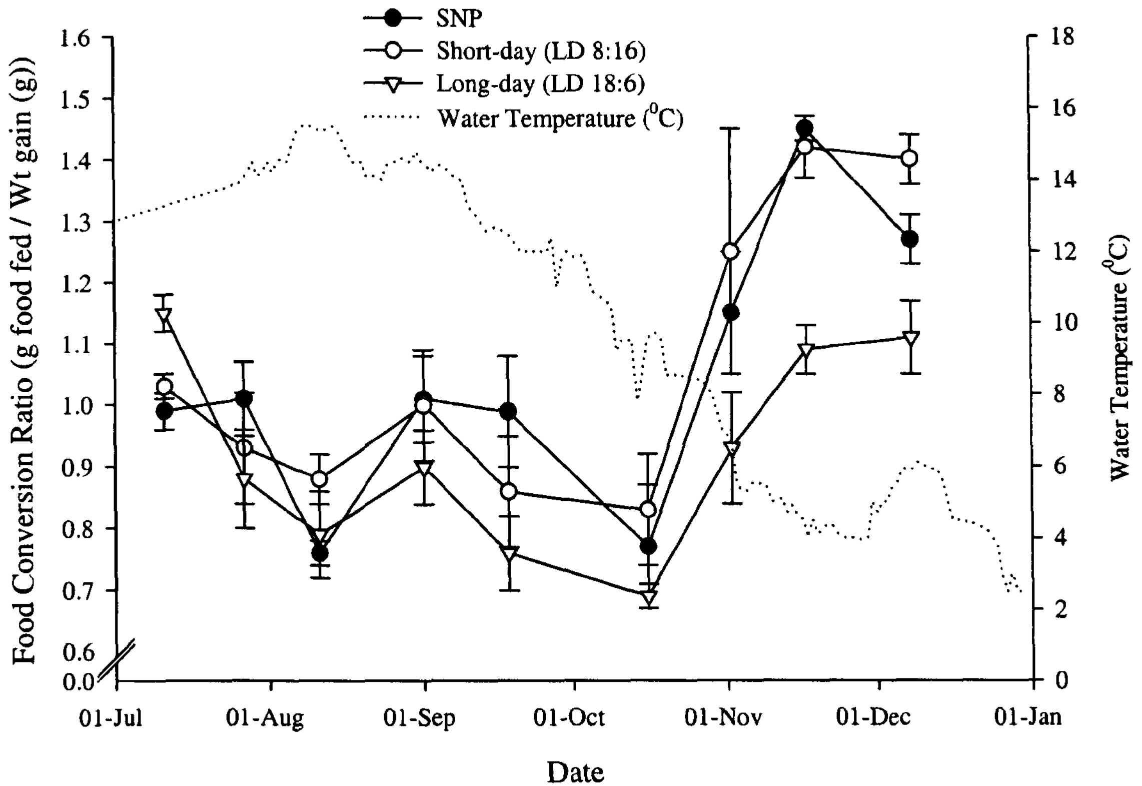


Figure 3.10 The effects of exposure to different photoperiod regimes (SNP, LD 8:16, or LD 18:6) on food conversion ratio (FCR; food presented:wet weight gain) of juvenile rainbow trout maintained in covered tanks (mean ± SEM of 3 replicates). FCR during the first period of the experiment 17th June to 7th July is represented by the first point.

3.4.2.2 Effects of Exposure to Different Photoperiods on Fingerling Rainbow Trout Plasma IGF-I Concentration

Circulating IGF-I levels

Following the exposure to their respective photoperiods all treatments showed a progressive increase in IGF-I levels from a mean level of 39.9 ± 3.5ng/ml in June to a range between 86.9 and 93.4ng/ml by late-July (Figure 3.11). An abrupt increase was then observed, with peak levels recorded in all groups on 1st September, 75 days following the onset of their respective photoperiod treatments. Long-days exhibited a significantly higher concentration (298.7 ± 32.2ng/ml) than short-day and SNP groups (155.2 ± 15.7ng/ml and 179.7 ± 18.4ng/ml respectively). No significant differences were found between short-day and SNP treatments. This peak in IGF-I levels was also coincident with the period of highest water temperatures. Thereafter, plasma levels in all declined rapidly to a 44.6 ± 4.4ng/ml (mean of all 3 treatments) by early October and no difference between treatments was then observed. At the final sample point (8th December), long-day fish showed an increase in IGF-I levels from 15.2 ± 1.2 to 32.1 ± 4.5ng/ml, a significantly higher concentration than the SNP group which remained at 17.2 ± 2ng/ml. This increase coincided with a small but significant increase in ambient water temperature during this period.

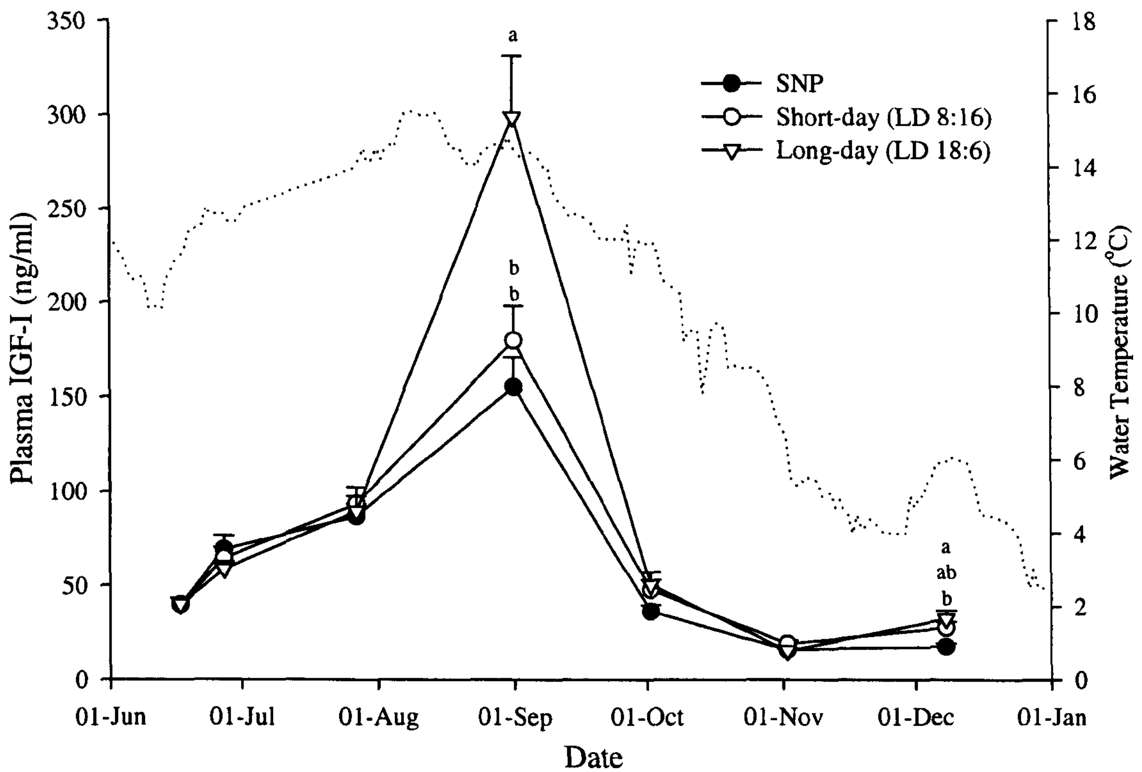


Figure 3.11 The effects of exposure to different photoperiod regimes (SNP, LD 8:16, or LD 18:6) on plasma IGF-I concentrations in juvenile rainbow trout maintained in covered tanks (mean ± SEM of 3 replicates, 5 bloods assayed/tank, n=15/treatment). Standard error bars are shown but in some cases are too small to be depicted. Treatments sharing different superscripts at a given time point are significantly different ($p<0.05$). Lettering is stacked in same order as graph lines. The broken line indicates changes in ambient water temperature during the period of the experiment.

The Interactions Between IGF-I level, Growth and Water Temperature

Simple Regression Relations

In all treatments, strong positive correlations were found between plasma IGF-I levels and water temperature (r^2=0.85, n=36), SGRL (r^2=0.79, n=36) and SGRwt (r^2=0.78, n=36) between July and November (Figure 3.12 a-c).

Mean plasma IGF-I levels were significantly related to mean water temperature from July to December in all treatments but showed a curvilinear relationship, with levels remaining constant between 4.4 and 8.9°C, and increasing thereafter (Figure 3.12a). Regression coefficients (r^2) varied between 0.90 (Long-day) and 0.85 (SNP) Table 3.3a). Removal of the December sample point increased linear regression coefficients significantly to a range between 0.83 (SNP) and 0.88 (Short-day) in all treatments (Table 3.3b). No differences in slope or elevation were found between treatments when December was included or excluded from analysis, showing that temperature had the same effect on IGF-I levels irrespective of photoperiod treatment.

Mean plasma IGF-I levels were also significantly related to SGRwt from July to December in all treatments (Table 3.3c). Regression coefficients (r^2) varied between 0.63 (LD 18:6) and 0.73 (LD 8:16). Removal of the December sample point again increased regression coefficients significantly to a range between 0.70 (SNP) and 0.92 (Long-day) in all treatments (Table 3.3d). The gradient of the long-day fish was significantly steeper than in SNP and short-day groups when December was excluded, associating higher IGF-I levels for a given rate of weight gain following exposure to long-day photoperiods between June and November. Slopes of weight gain did not differ between SNP and short-day treatments.

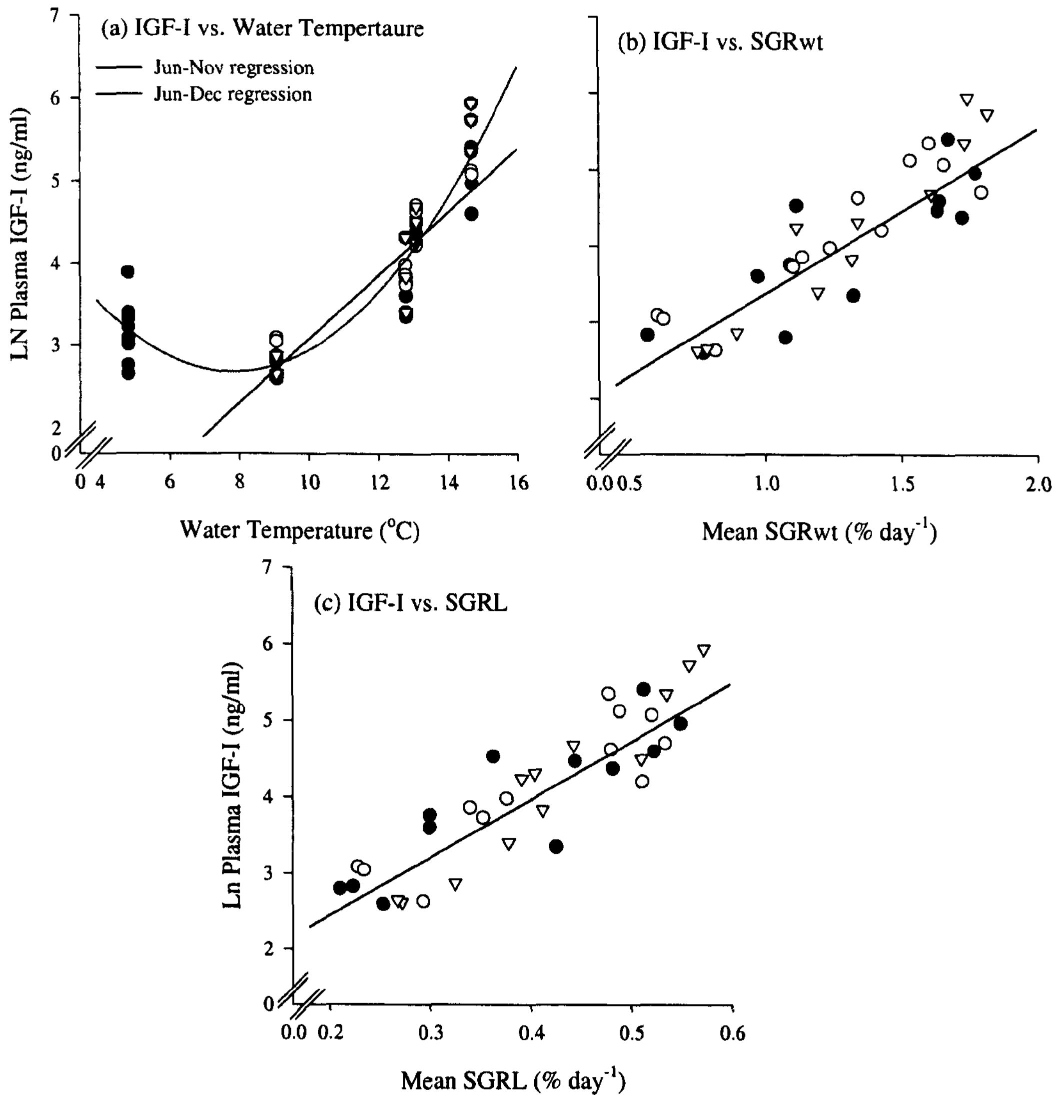


Figure 3.12 Correlations between plasma IGF-I and (a) water temperature, (b) weight specific growth rate (SGRwt), and (c) length specific growth rate (SGRL) for fish maintained under different photoperiods (● SNP, ○ LD 8:16, and▽ LD 18:6) between June and November (No August blood sample point). Regression lines shown for all treatments combined (n=36) with each point representing a tank. ● represents all data points for temperature regression Jun-Dec (n=42). Regression statistics (a) Jun-Nov: $y=0.385x-0.78$, $r^2=0.85$, $p<0.0001$; Jun-Dec: $y=0.056x^2-087535x+6.105$, $r^2=0.88$, $p<0.001$ (b) $y=2.162x+1.21$, $r^2=0.78$, $p<0.0001$, (c) $y=7.566x+0.93$, $r^2=0.79$, $p<0.0001$.

Table 3.3 Correlations between *(a)* IGF-I and water temperature from July to December, *(b)* IGF-I and water temperature excluding December sample period, *(c)* IGF-I and weight specific growth rate (SGRwt) from July to December, *(d)* IGF-I and weight specific growth rate (SGRwt) excluding December sample, *(e)* IGF-I and length specific growth rate (SGRL) from July to December, *(f)* IGF-I and length specific growth rate (SGRL) excluding December sample. Gradients sharing common superscripts are not significantly different from each other for respective regressions (ANCOVA, p>0.05).

Treatment	Correlation coefficient	F	p	r^2	Gradient	Intercept
(a) Ln IGF-I vs Temperature (all months)						
SNP		28.5	<0.0001	0.85		
Short-day		20.3	<0.0001	0.87		
Long-day		13.0	<0.0001	0.90		
(b) Ln IGF-I vs Temperature (exc. December)						
SNP	0.911	49.0	<0.0001	0.83	0.389 [a]	-0.90
Short-day	0.939	75.0	<0.0001	0.88	0.389 [a]	-0.71
Long-day	0.927	61.1	0.001	0.86	0.494 [a]	-1.96
(c) Ln IGF-I vs SGRwt (all months)						
SNP	0.830	28.8	<0.0001	0.69	1.32 [a]	2.33
Short-day	0.852	34.5	<0.0001	0.73	1.40 [a]	2.49
Long-day	0.791	20.6	0.001	0.63	1.60 [a]	2.25
(d) Ln IGF-I vs SGRwt (exc. December)						
SNP	0.834	22.9	0.001	0.70	1.89 [a]	1.53
Short-day	0.915	51.5	<0.0001	0.84	2.05 [a]	1.57
Long-day	0.960	105.3	<0.0001	0.92	2.94 [b]	0.32
(e) Ln IGF-I vs SGRL (all months)						
SNP	0.876	43.1	<0.0001	0.77	5.37 [a]	1.95
Short-day	0.842	31.6	<0.0001	0.71	4.93 [a]	2.22
Long-day	0.796	22.5	0.003	0.63	5.67 [a]	1.95
(f) Ln IGF-I vs SGRL (exc. December)						
SNP	0.870	31.0	<0.0001	0.76	6.48 [a]	1.47
Short-day	0.888	37.4	<0.0001	0.79	7.00 [a]	1.29
Long-day	0.965	136.2	0.001	0.93	10.50 [b]	-0.27

Finally, mean plasma IGF-I levels were significantly related to SGRL from July to December in all treatments (Table 3.3e). Regression coefficients (r^2) varied between 0.63 (Long-day) and 0.77 (SNP). Removal of the December sample point increased regression coefficients significantly to a range between 0.76 and 0.93 in all treatments (Table 3.3f). The gradient of the long-day fish was significantly greater than in SNP and short-day groups when December was excluded ($p<0.05$), associating higher IGF-I levels with length gain under long-day photoperiods between June and November. No difference in SGRL and IGF-I levels was found between SNP and short-day treatments when gradients were compared.

3.5 Experiment 3: Effects of Photoperiod Regime and Melatonin Implant on Growth and IGF-I levels in Juvenile Rainbow Trout

Aim: To determine how artificial melatonin administration interacts with photoperiod to affect growth and plasma IGF-I production. Currently, it is not known whether light affects growth directly via the GH-IGF system or its action could be mediated or influenced by melatonin. It is widely accepted that melatonin produced in the pineal gland accurately reflects the prevailing daylength, thus melatonin may transmit photoperiodic information to the somatotropic axis.

3.5.1 Materials and Methods

On March 6th 2001, six 2m diameter light-proof covered circular tanks were stocked with groups of 90 1-year old mixed sex rainbow trout of mean weight 101.5g ± 22.1g and maintained under SNP. Fish were reared from eggs fertilised by first spawning broodstock (origin Selcoth Fish farms, 1997) as used in experiment 1. Mixed sex fish were used to due to facility restrictions of importing fish on site at NBFRF during 2001. External standpipes were adjusted to create a water depth of 0.57m, giving a tank volume of 1.8m^3. Light was supplied by four 9 watt equivalent G23 bulbs housed in two aluminium alloy bulkhead fittings (Alex Brewsters, Stirling, UK.) creating 334 lux at the water surface and 97.3 lux centrally at the tank floor. Prior to experimental set up fish were maintained under simulated natural photoperiod (SNP).

On 19th April all fish were individually P.I.T. tagged as described in **chapter 2 section 2.1.4** to allow individual group identification. At this point three groups of 30 fish within each tank received one of the following; slow release 18mg melatonin implant (IMP) (described in **chapter 2, section 2.1.5**), sham implant (SHAM) or left

intact as a control (CTRL). At the same time duplicate tanks were then exposed to one of three photoperiods; simulated natural photoperiod (SNP), constant short-day (LD 8:16), or constant light (LL), thus giving three groups within three light treatments in duplicate. The LL regime was chosen to remove the natural day-night cycle of melatonin. Light regimes were controlled as described in **section 3.3.1**. Hours of light ranged from 12 to 17.5 for SNP regimes during the trial. Fish were fed the same diet (Trouw Aminobalance) as described in section **3.4.1** via clockwork belt feeders according to manufacturer's tables. Food was presented to all tanks during the hours of the shortest photoperiod (LD 8:16 8am-4pm) for the duration of the experiment (19th April to 21st September). Mortalities were less than 0.1% for all tanks with the exception of LL rep 2 in which a blocked standpipe lead to the loss of 8 implanted fish and 6 controls in August.

All fish were measured for weight-length at monthly intervals (n=30), and ten random blood samples taken per group (i.e. implant, sham or control) for plasma melatonin and IGF-I production. Bloods were pooled in pairs to ensure sufficient plasma for melatonin analysis from five samples (**chapter 2, section 2.2).** Baseline samples were taken in April prior to implanting. P.I.T tagged fish were not serially sampled as tags were primarily used to distinguish groups due to limited tank availability for this trial.

Statistical Analysis

Differences in growth parameters (weight, length, condition factor, SGRwt and SGRL) of individually P.I.T. tagged fish where tested using GLM procedures with time, treatment and group (Imp, Sham, and Ctrl) as categorical predictors incorporating replicate and fish as random factors. Due to heterogeneous of variance shown by

examination of residuals plots, plasma IGF-I levels were compared using a non-parametric statistical test (Kruskal-Wallis) with differences between pairs tested by Dunn's multiple comparison procedures. Relationships (semi-log plots) between plasma IGF-I and temperature, SGRwt and SGRL were tested using Pearson product moment correlation coefficient and regression analysis. Preliminary analyses were performed to ensure normality, linearity and homoscedasticity (variability in scores for x similar at all values of Y). Relationships were linearised to allow statistical tests. Tests for common slopes and elevation between treatments were compared using ANCOVA. Water temperature and plasma IGF-I relationships were also analysed using 2nd order polynomial regression where curvilinear relations existed.

No significant differences were found between sham-implants and controls with respect to growth parameters or plasma IGF-I levels, as such only data for melatonin implants and controls are presented in the results.

Prior to the presentation of results although a mixed sex population was used in this experiment, maturation was not expected. However, 100% of males were in the process of maturing. This only became apparent during the final sample point (September) by which point blood samples had been pooled for each respective group (i.e implant or control) under treatment, as such both male and female plasmas were combined in most instances. Thus the IGF-I data presented is the combined mean of both sexes, as such, the growth data has also been analysed in the same manner.

3.5.2 Results

3.5.2.1 Effect of Slow Release Melatonin Implants on Plasma Melatonin Concentration

Figure 3.13 illustrates the melatonin release profile of implanted fish from May to September following administration in April. One month post-implantation plasma concentrations in implanted fish were elevated to between 8731 and 7922pg/ml in SNP and LL treatments, significantly higher than short-day at 6969pg/ml. From June to September plasma concentrations decreased steadily in all treatments reaching between 1250 and 1400pg/ml on the final sample point. During this period no significant differences were found between photoperiod treatments at any time point. On all sampling points, plasma melatonin concentrations were significantly higher in implanted fish ($p<0.001$) than in controls and sham implants in all treatments.

3.5.2.2 Effects of Melatonin Implantation and Exposure to Different Photoperiods on Rainbow Trout Growth Performance

Weight

Weight increased over time in all treatments with all controls reaching significantly heavier mean weights than their respective implanted fish by September (Figure 3.14 a-c). Relative to their implants, control fish under SNP, short-days and LL achieved 7.6, 7.3 and 18.6% heavier mean weights respectively. Significant differences in weight between controls and implants were observed by mid-June, mid-July and mid-August in LL, short-day and SNP treatments respectively.

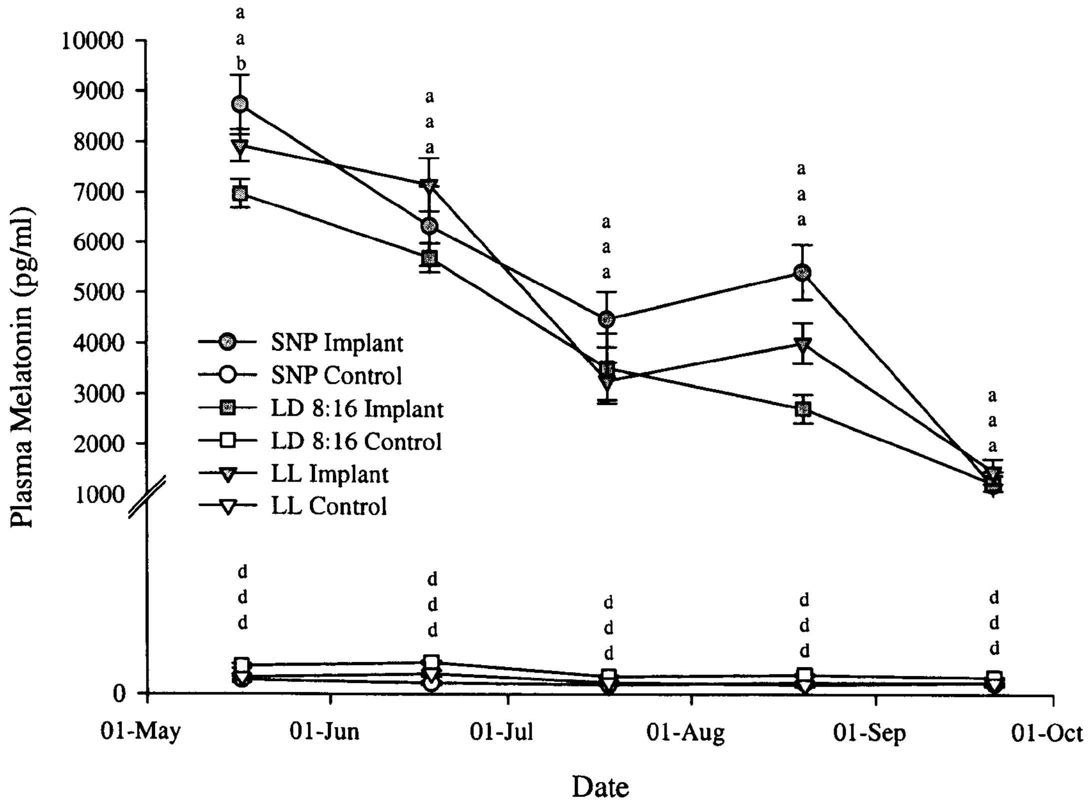


Figure 3.13 Comparison of changes in plasma melatonin concentration (mean ± SEM, n=10/treatment) of individually P.I.T. tagged implanted fish (grey symbols) over time and their levels relative to their respective controls (open symbols) in fish exposed to different photoperiod regimes (SNP, LD 8:16 and LL). Points with different superscripts are significantly different ($p<0.05$). Lettering is stacked in the same order as the graph lines. All blood measurements were taken at 12noon on the respective day (10 bloods pooled in pairs per replicate).

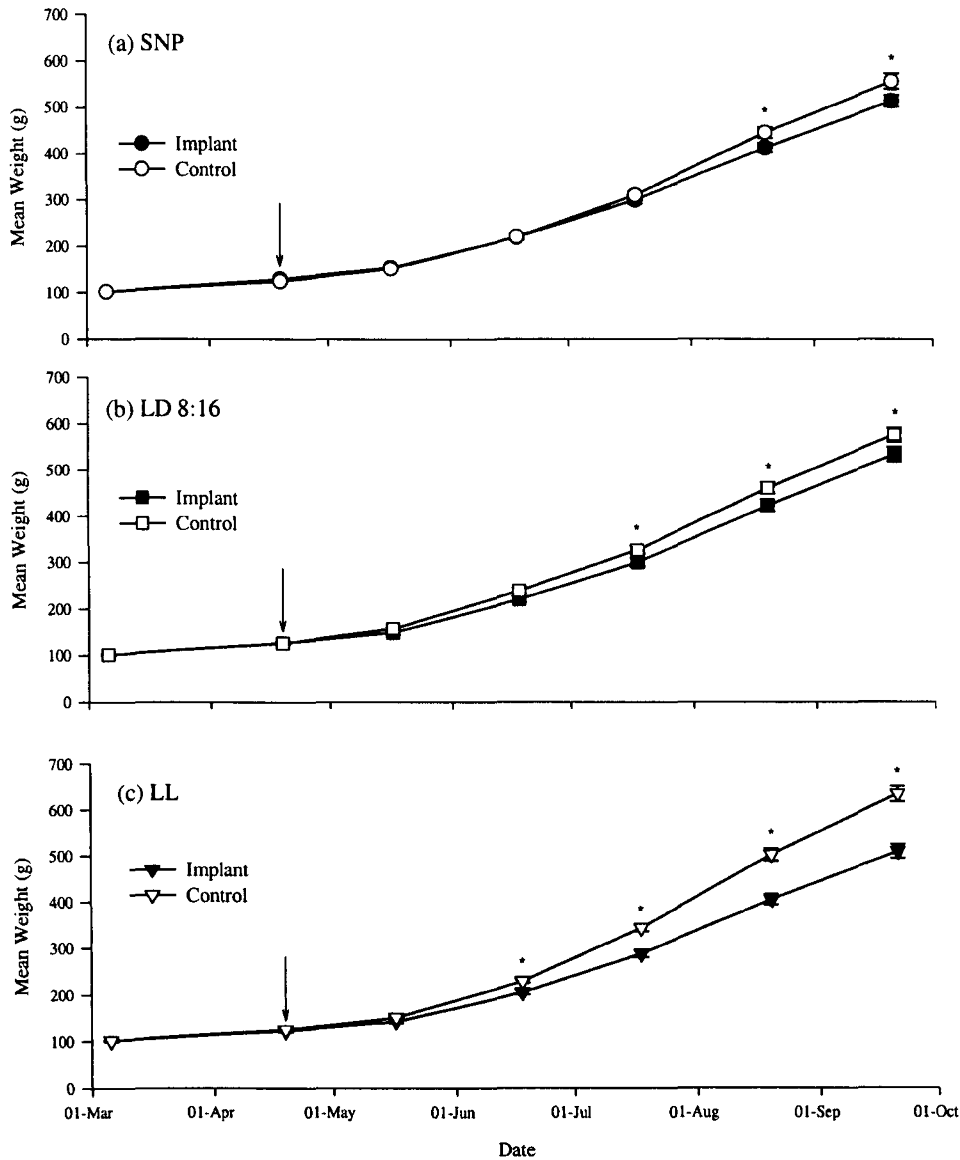


Figure 3.14 Weight gain of individually P.I.T. tagged melatonin implanted and intact fish (controls) exposed to 3 different photoperiod regimes (a) simulated natural photoperiod (SNP), (b) constant short-days (LD 8:16) or (c) constant light (LL) (mean ± SEM of replicates, n=30). In some cases standard error bars are too small to be depicted. Asterisks (*) denote significant differences between points (p<0.05). Arrow denotes start of photoperiod and melatonin treatment.

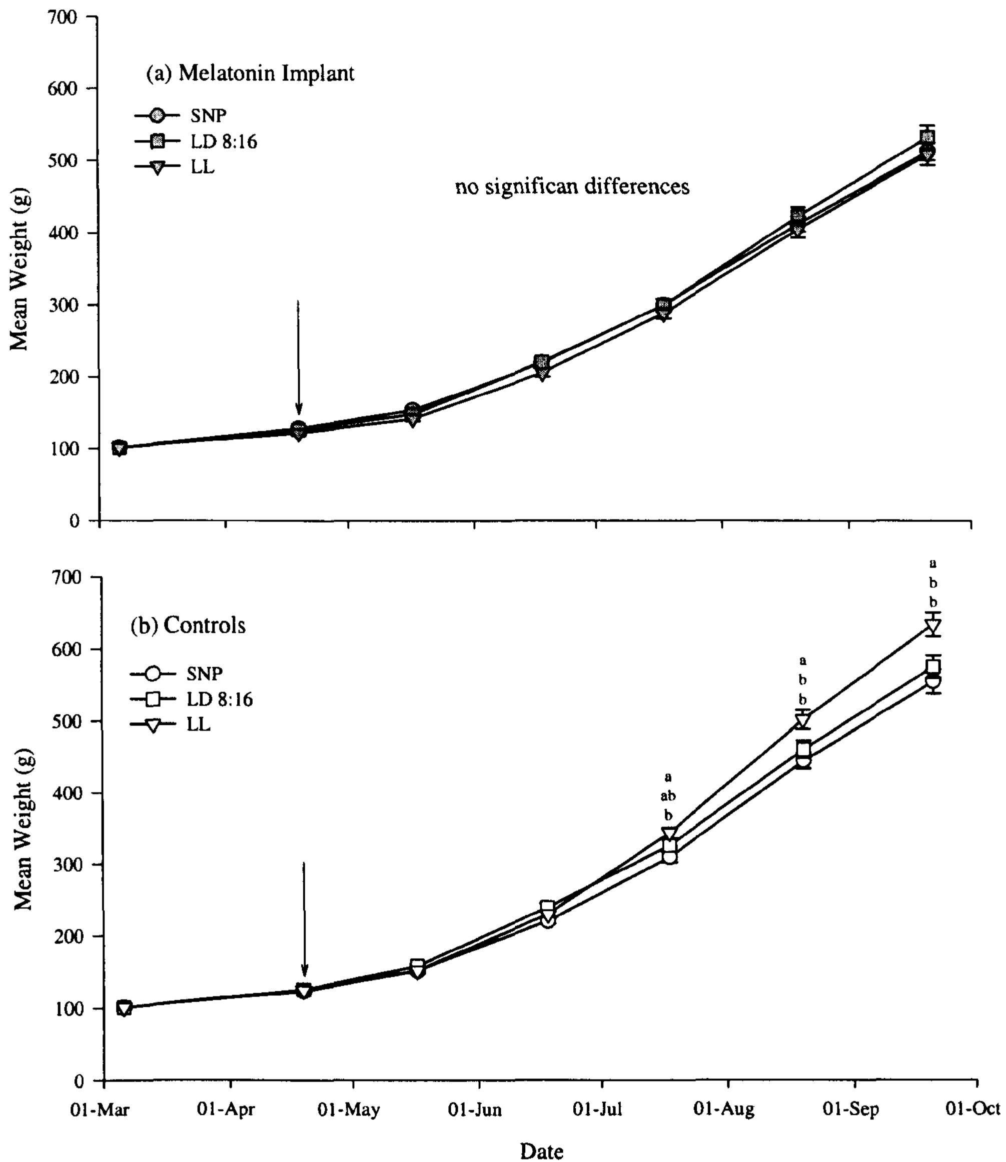


Figure 3.15 Comparison of weight gain variation of individually P.I.T. tagged melatonin implanted fish (a) and intact fish (controls) (b) when maintained under 3 different photoperiod regimes (SNP, LD 8:16 or LL) (mean ± SEM of replicates, n=30/hormone treatment). In some cases standard error bars are too small to be depicted. Points with different superscripts are significantly different ($p<0.05$). Lettering is stacked in the same order as the graph lines. Arrow denotes start of photoperiod and melatonin treatment.

Irrespective of photoperiod treatment, no significant differences in weight of implanted fish were observed at any time, with fish achieving an overall mean weight of 521.1 ± 19.1g by September (Figure 3.15a).

LL controls achieved a 12.5 and 9.2% significantly heavier weight than SNP and short-days by September, with initial significant differences in weight occurring by mid-July and mid-August respectively (Figure 3.15b). No significant differences in weight were observed between SNP and short-days at any time point.

Length

Length increased over time in all treatments with all controls reaching significantly longer mean lengths than their respective implanted fish by mid-May, mid-June and mid-July in LL, short-day and SNP treatments respectively (Figure 3.16 a-c). Relative to their implants, control fish under SNP, short-days and LL achieved 4.8, 5.1 and 8.5% greater mean lengths respectively.

One month post-implant (mid-May), SNP treated fish achieved a significantly greater length than LL implant, with short-days neither significantly different from LL or SNP implants (Figure 3.17a). From mid-June to mid-September short-days maintained a significantly greater length than LL implanted fish. Only during September did short-days achieve a significantly longer length than SNP implanted fish.

LL and short-day controls achieved a 3.9 and 2.4% significantly longer length than SNP controls by September, with no significant differences found between LL and short-day fish by this point (Figure 3.17b). Significant differences in control fish length

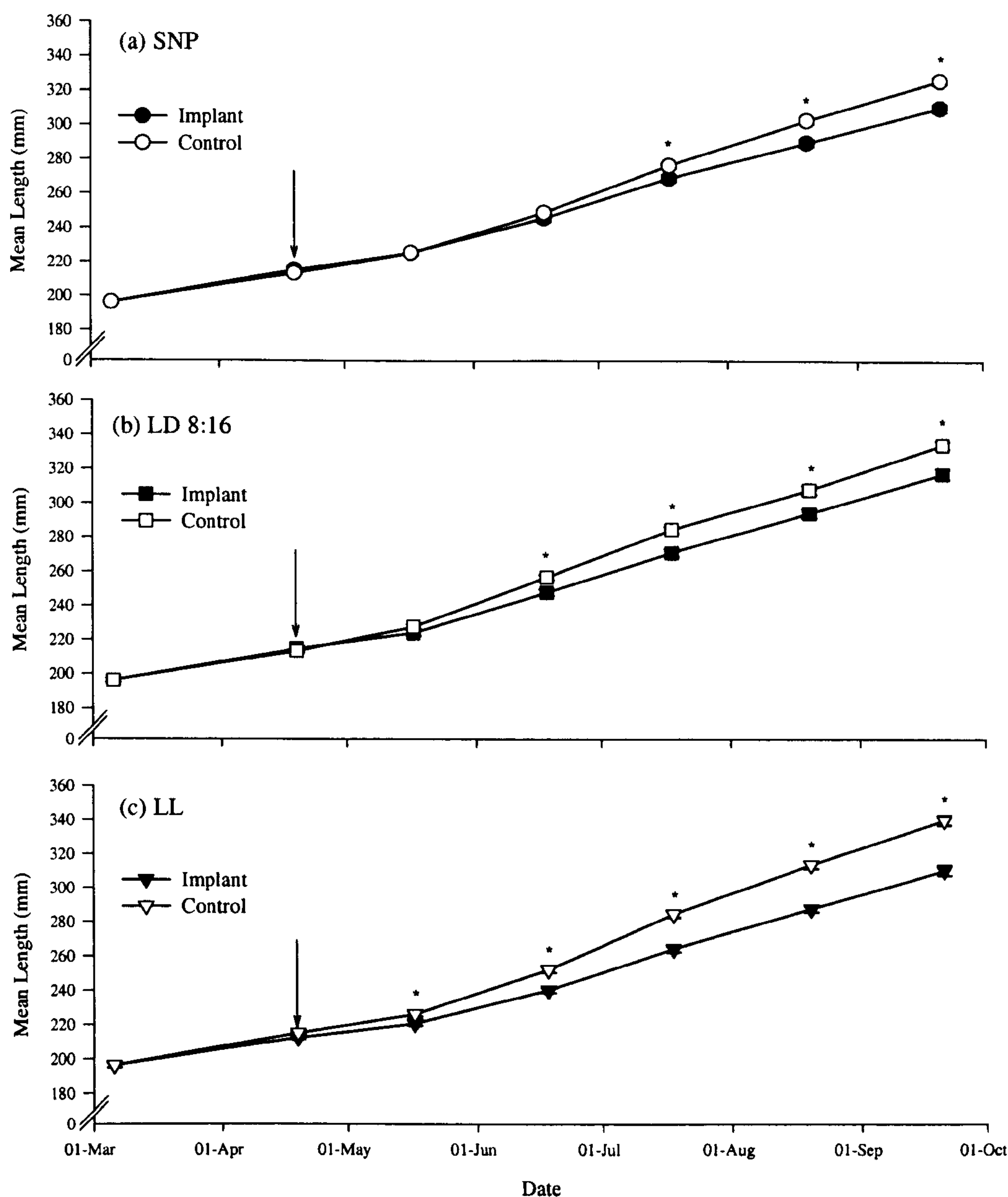


Figure 3.16 Length gain of individually P.I.T. tagged melatonin implanted and intact fish (controls) (mean ± SEM of replicates, n=30/hormone treatment) exposed to 3 different photoperiod regimes (a) simulated natural photoperiod (SNP), (b) constant short-days (LD 8:16) or (c) constant light (LL). In some cases standard error bars are too small to be depicted. Asterisks (*) denote significant differences between points ($p<0.05$). Arrow denotes start of photoperiod and melatonin treatment.

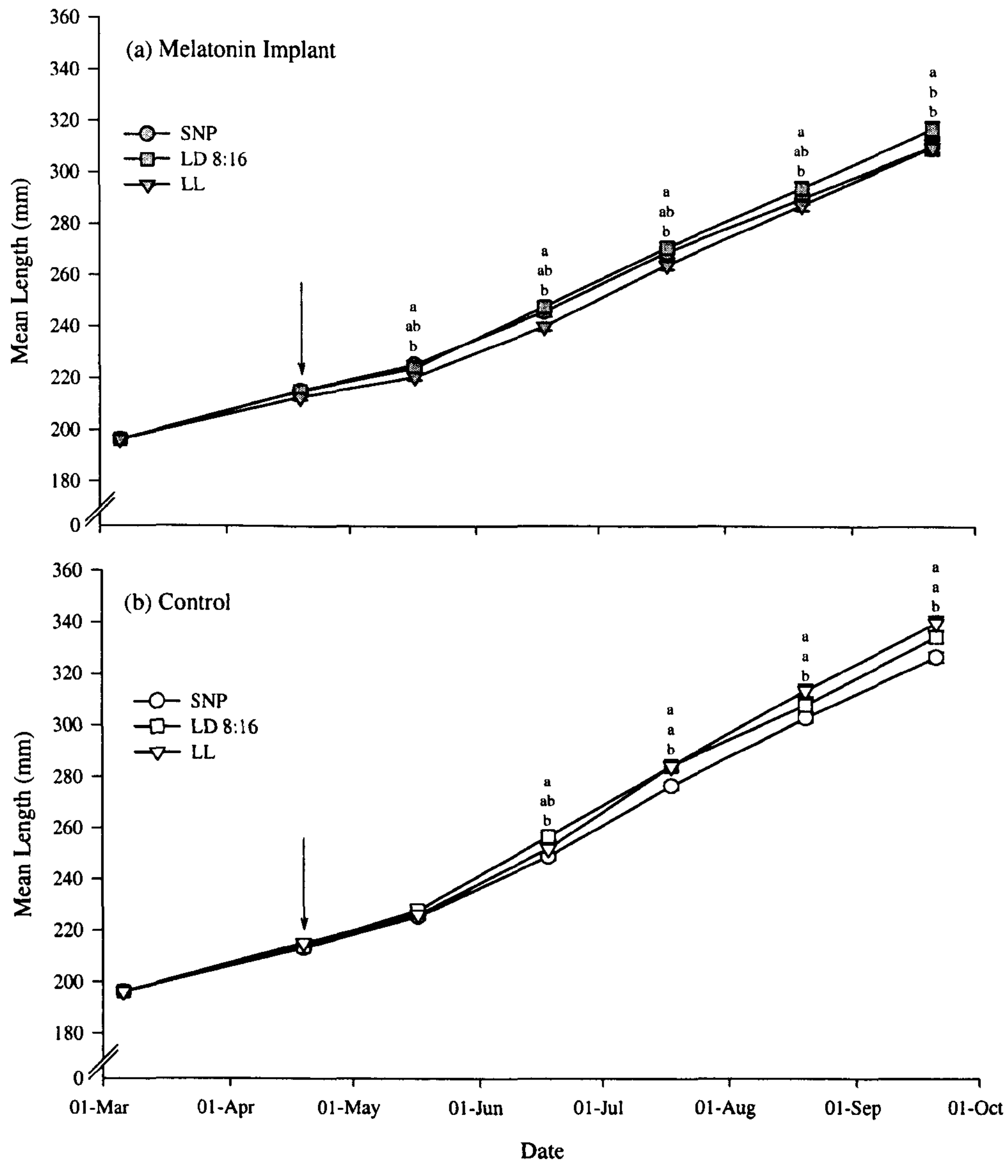


Figure 3.17 Length variation (mean ± SEM of replicates, n=30) of individually P.I.T. tagged melatonin implanted fish (a) and intact fish (controls) (b) when maintained under 3 different photoperiod regimes (SNP, LD 8:16 or LL). In some cases standard error bars are too small to be depicted. Points with different superscripts are significantly different ($p<0.05$). Lettering is stacked in the same order as the graph lines. Arrow denotes start of photoperiod and melatonin treatment.

between short-days and LL relative to SNP controls were first observed by mid-June and mid-July respectively.

Condition Factor

Irrespective of photoperiod treatment, a significantly higher CF was achieved in implanted fish by mid-July relative to their controls, and was maintained until the end of the experiment in September (Figure 3.18a-c). The greatest increase in CF of was observed between mid-May and mid-August for both implants and controls coincident with increasing water temperatures, while remaining steady between mid-August and mid-September.

No significant differences in CF were observed in implant fish between photoperiod treatments, with CF increasing steadily over time from 1.27 in April to 1.69 by September (Figure 3.19a).

Comparison of control fish showed that those exposed to LL and SNP maintained a significantly greater CF than short-day groups from mid-July to mid-September (Figure 3.19b).

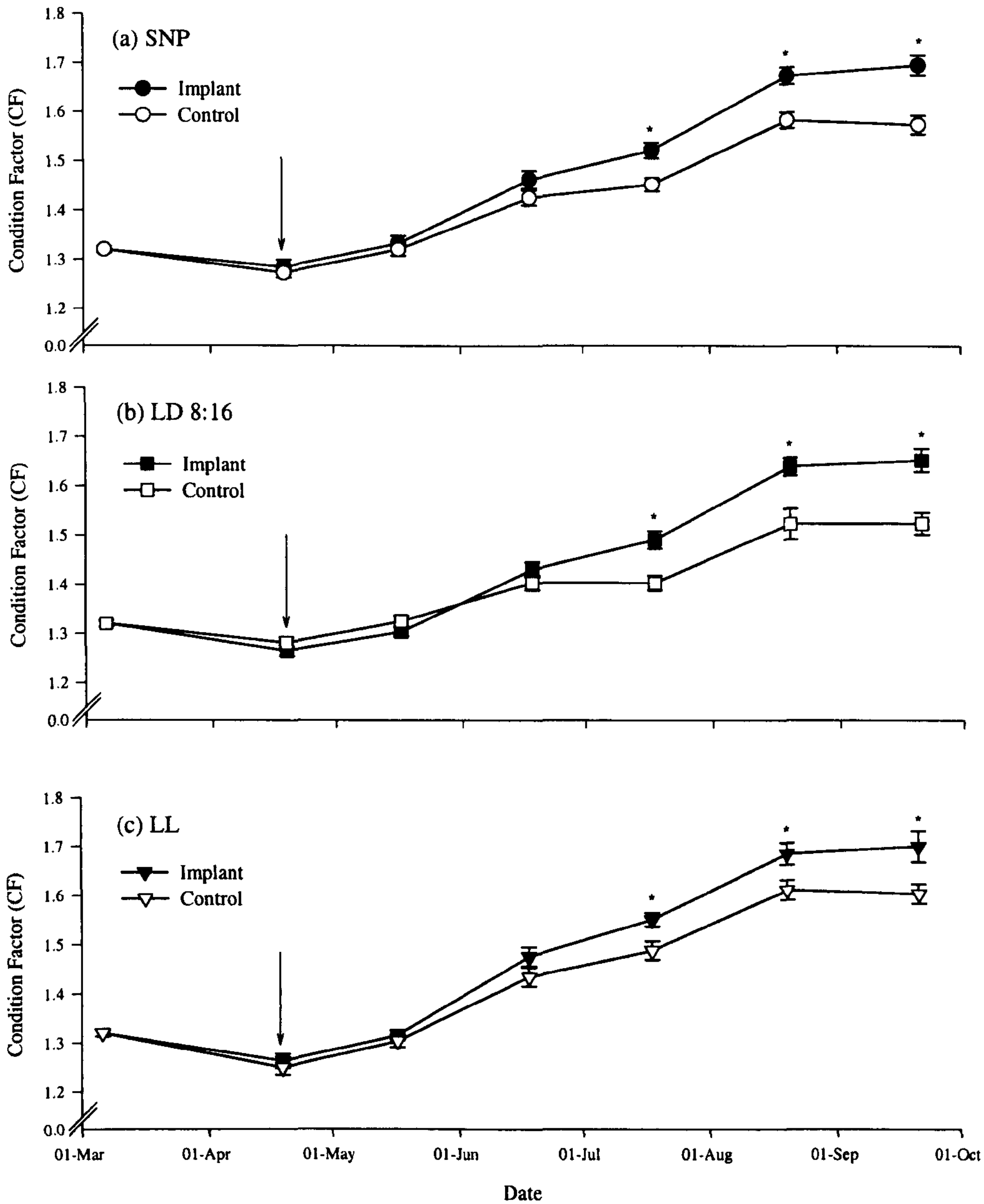


Figure 3.18 Change in condition factor of individually P.I.T. tagged melatonin implanted and intact fish (controls) (mean ± SEM of replicates, n=30) exposed to 3 different photoperiods regimes (a) simulated natural photoperiod (SNP), (b) constant short-days (LD 8:16) or (c) constant light (LL). In some cases standard error bars are too small to be depicted. Asterisks (*) denote significant differences between points ($p<0.05$). Arrow denotes start of photoperiod and melatonin treatment.

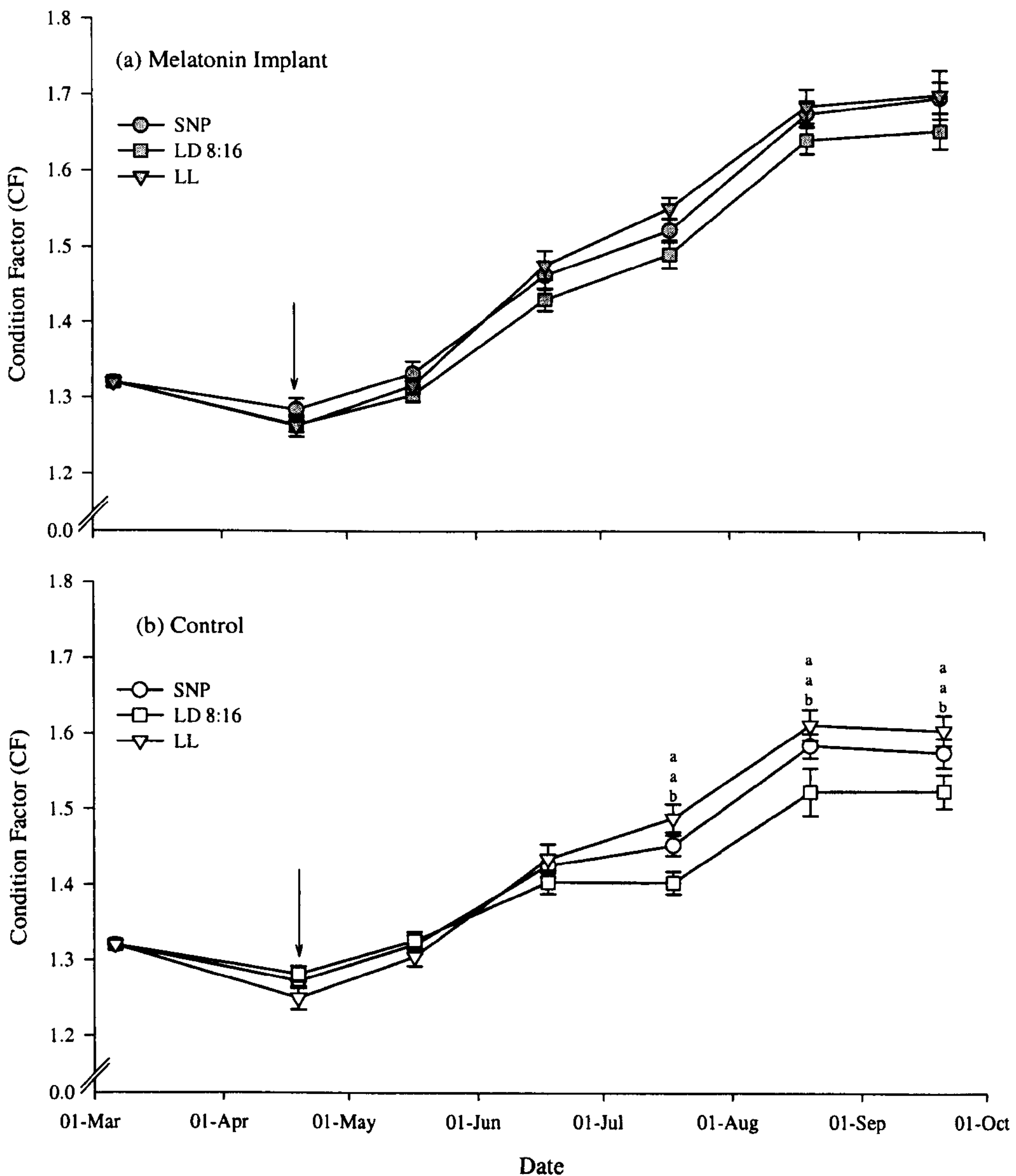


Figure 3.19 Condition factor variation (mean ± SEM of replicates, n=30) of individually P.I.T. tagged melatonin implanted fish (a) and intact fish (controls) (b) when maintained under 3 different photoperiod regimes (SNP, LD 8:16 or LL). In some cases standard error bars are too small to be depicted. Points with different superscripts are significantly different ($p<0.05$). Lettering is stacked in the same order as the graph lines. Arrow denotes start of photoperiod and melatonin treatment.

3.5.2.3 Effects of Melatonin Implantation and Exposure to Different Photoperiods on Plasma IGF-I Concentration

No significant differences in plasma IGF-I levels were found between implants and controls under each photoperiod treatment at any given time point ($p>0.05$) (Figure 3.20 a-c). Time significantly affected all treatments with both implants and controls showing a significant increase in IGF-I levels between May and June coincident with increasing water temperature (8.8 to 11.5°C).

Both groups under SNP continued to show an small increase in IGF-I levels between June and July reaching a maximum of 116.2ng/ml. Although decreasing between mid-July and mid August, plasma levels did not differ significantly between mid-July and September (Figure 3.20a).

Under short-days, IGF-I levels did not change significantly between June and mid-August in either group. Only during September did controls show a significant time increase reaching 116.7 ± 5.2ng/ml, although this was not significantly higher than implants (95.6 ± 4.7ng/ml).

Levels in both controls or implants under LL did not change significantly between July and September (Figure 3.20c). Both groups achieved approximately 102ng/ml by mid-September, significantly higher than in April or May ($p<0.05$).

Comparison of plasma IGF-I levels between implants showed that SNP achieved a significantly higher levels than short-days but not LL in mid-July (Figure 3.21a). At no other time point where significant differences observed. Comparison of plasma IGF-I levels between controls showed that SNP achieved a significantly higher levels ($p<0.05$) than short-days but not LL in mid-July, while LL maintained a significantly higher level ($p<0.05$) than both SNP and short-days in mid-August (Figure 3.21b). At no other time point where significant differences observed.

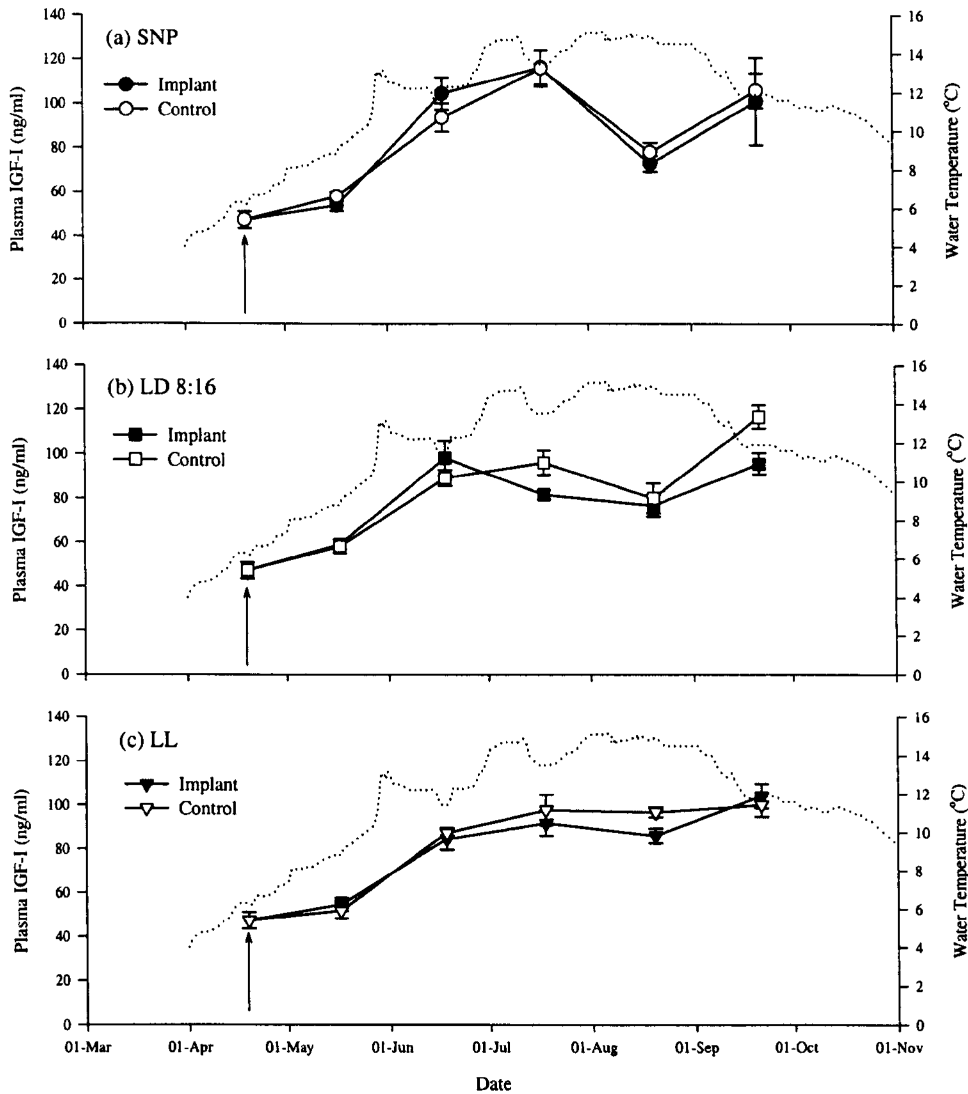


Figure 3.20 The effects of slow release melatonin implants on rainbow trout plasma IGF-I levels (mean ± SEM of replicates, 5 bloods assayed/replicate, n=10/treatment) relative to those left intact as controls following exposure to 3 different photoperiods (a) simulated natural photoperiod (SNP), (b) constant short-days (LD 8:16) or (c) constant light (LL). No significant differences found at any given time point between implants and controls (Kruskal-wallis test, p>0.05). The broken line indicates water temperature during the period of the experiment. Arrow denotes start of photoperiod and melatonin treatment.

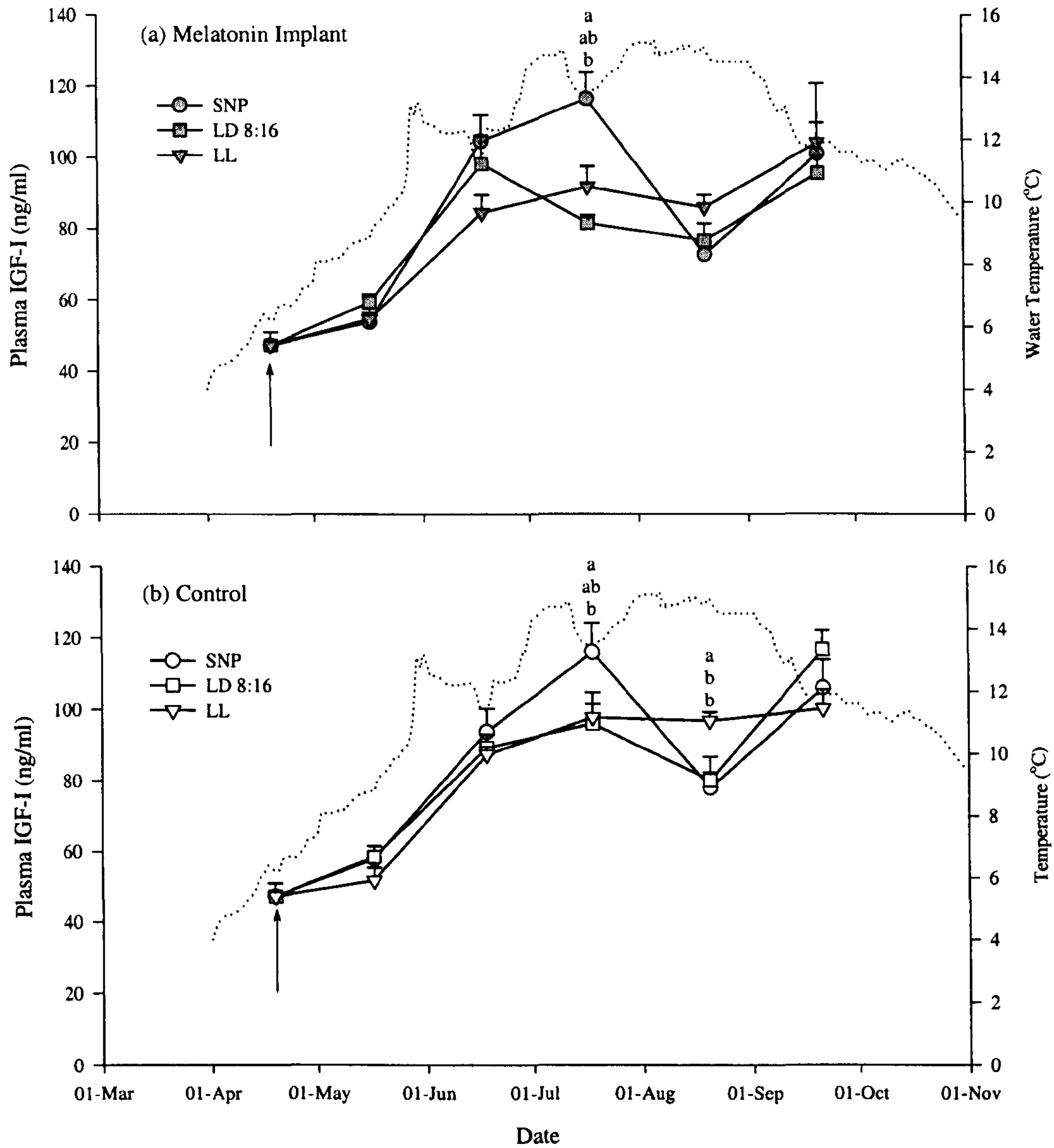


Figure 3.21 Effects of exposure to 3 different photoperiod regimes (SNP, LD 8:16, or LL) on plasma IGF-I levels in (a) melatonin implanted fish and (b) control fish (mean ± SEM of replicates, 5 bloods assayed/replicate, n=10/treatment). Treatments sharing different superscripts are significantly different (Kruskal-Wallis test, $p<0.05$). Lettering is stacked in the same order as the graph lines. The broken line indicates water temperature during the period of the experiment. Arrow denotes start of photoperiod and melatonin treatment.

3.5.2.4 Relationships Between Photoperiod, Melatonin, Growth and Plasma IGF-I Concentration

Mean Treatment Relationships over Time

Significant positive linear correlations were found between mean plasma IGF-I levels and daily weight gain (SGRwt, $r^2=0.57$, $p<0.0001$) or length gain (SGRL, $r^2=0.58$, $p<0.0001$) in all treatments from April to August (Figure 3.22 a-b). No difference in slopes were found between treatments or groups for SGRwt or SGRL (Table 3.4 a&b), with the exception of SNP implants which had a significantly steeper gradient than all other treatments when IGF-I was plotted against SGRL (Table 3.4 b). Only short-day controls showed no relation of IGF-I to either SGRwt or SGRL from April to August, with SNP controls showing no correlation with SGRL during this same period but did exhibit a strong correlation with SGRwt.

A significant curvilinear relation (2nd order polynomial) was found between plasma IGF-I and water temperature ($r^2=0.72$, $p<0.0001$) for all treatments (Figure 3.23), with plasma levels increasing with temperatures between 6.4 and 11.5°C, reaching a plateau between 11.5 and 13.6°C, and thereafter decreasing. When individual treatments were plotted with temperature r^2 values ranged between 0.7 and 0.85 ($p<0.0001$).

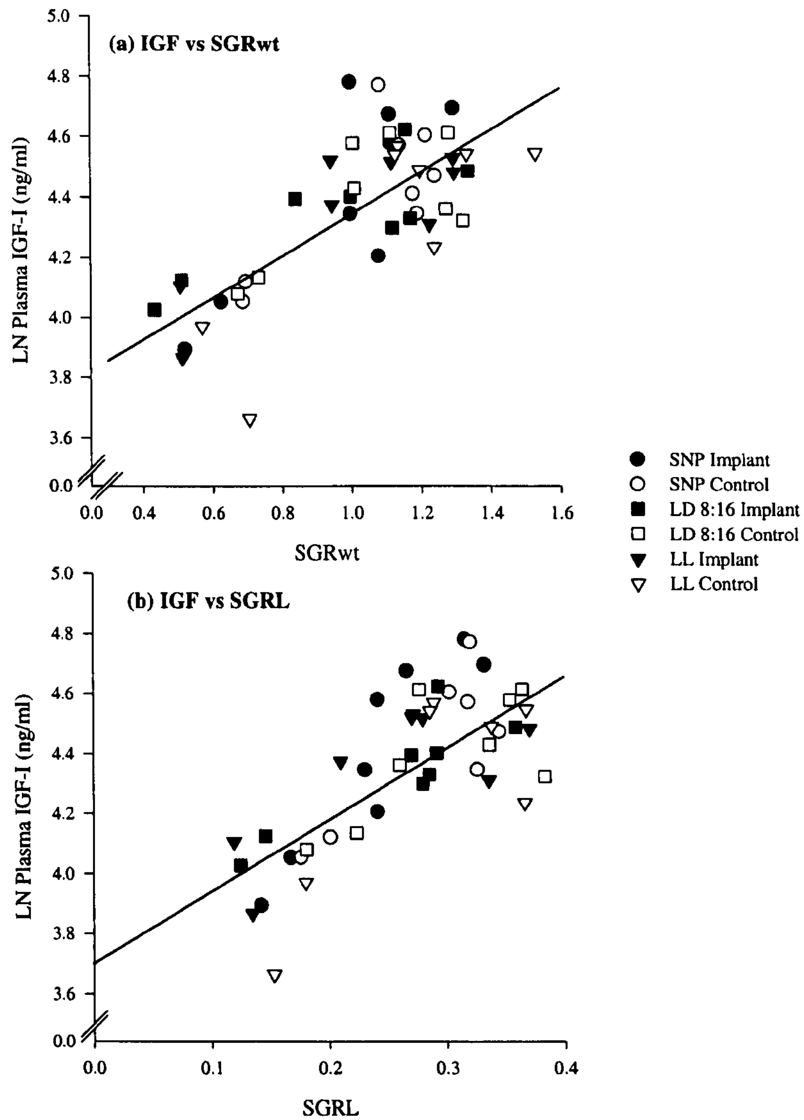


Figure 3.22 Linear regression relations found between natural log-transformed plasma IGF-I and **(a)** daily weight gain (SGRwt) and **(b)** daily length gain (SGRL) for fish maintained under different photoperiods and administered melatonin implants or left intact (controls). Regression lines shown for all photoperiod treatments combined (SNP, LD 8:16 and LL). Each treatment point is represented by 10 pooled bloods, with SGRs calculated as the average of the fish bloods pooled. Regression statistics (a) $y=3.70x+2.39$, $r^2=0.57$, $p<0.0001$, (b) $y=3.65x+0.70$, $r^2=0.58$, $p<0.0001$.

Table 3.4 Correlations between *(a)* IGF-I and weight specific growth rate (SGRwt) from April to August, and *(b)* IGF-I and length specific growth rate (SGRL) from April to August. Regression analysis performed on pooled bloods (2 fish/pool, n=5/replicate/time) versus the mean SGR of all fish pooled. Gradients sharing common superscripts are not significantly different from each other for respective regressions (ANCOVA, p>0.05).

Treatment	Pearson Correlation coefficient	F	p	r^2	Gradient	Intercept
(a)Ln IGF-I vs SGRwt						
SNP Implant	0.821	12.41	0.012	0.67	1.03^{a}	3.41
SNP Control	0.754	7.91	0.031	0.57	0.80^{a}	3.57
Short-day Implant	0.826	12.89	0.011	0.68	0.48^{a}	3.88
Short-day control	0.636	4.08	0.090^{ns}	0.41	0.54^{a}	3.83
LL Implant	0.827	13.03	0.011	0.69	0.62^{a}	3.73
LL Control	0.816	11.95	0.014	0.67	0.87^{a}	3.36
(b) Ln IGF-I vs SGRL						
SNP Implant	0.921	33.76	0.001	0.85	4.64^{a}	3.28
SNP Control	0.665	4.76	0.072^{ns}	0.44	2.14^{b}	3.78
Short-day Implant	0.869	18.46	0.005	0.76	2.09^{b}	3.80
Short-day Control	0.684	5.27	0.061^{ns}	0.47	1.94^{b}	3.81
LL Implant	0.747	7.58	0.033	0.56	2.01^{b}	3.84
LL Control	0.798	10.49	0.018	0.64	2.92^{b}	3.44

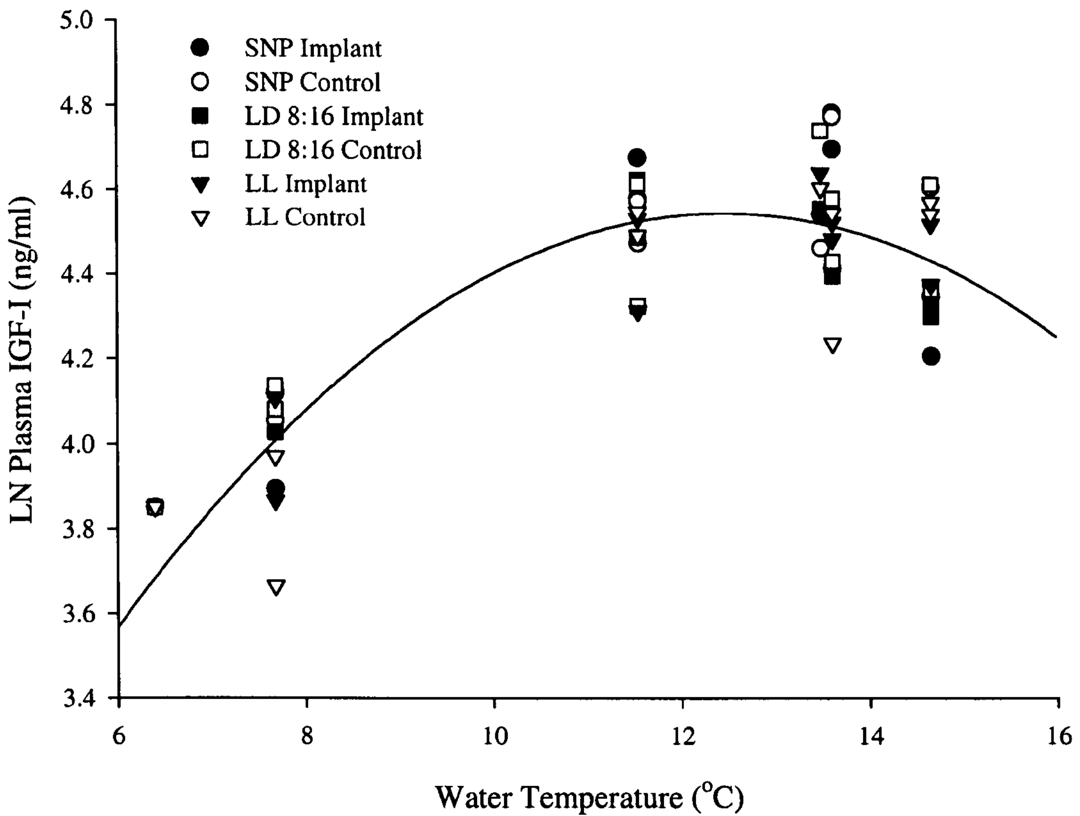


Figure 3.23 Relationship between plasma IGF-I and water temperature for fish maintained under different photoperiods and administered melatonin implants or left intact (controls) between April and August. Regression line shown for all photoperiod treatments combined (SNP, LD 8:16 and LL). Each treatment point is represented by 5 pooled bloods per replicate. Regression statistics $y = -0.23x^2 + 0.582x + 0.9145$, $r^2 = 0.72$, $p<0.0001$. Some data points may overlap and are not visible.

3.6 Summary of Results

Experiment 1

- No differences in hatching weight were observed following exposure to photoperiod from fertilisation although condition factor was significantly higher in LD 8:16 fry.
- Exposure of fry to LD 18:6 significantly enhanced growth rate and subsequent weight gain relative to those exposed to short-day photoperiods 4 weeks after hatching.
- Plasma IGF-I pre and post-hatching were not analysed.

Experiment 2

- Exposure of fingerling all-female rainbow trout to LD 18:6 significantly enhanced growth rate and subsequent weight gain relative to those exposed to SNP or short-day photoperiods from June to December.
- Feeding efficiency (FCR) was significantly improved in juvenile trout exposed to LD 18:6.
- Significantly higher plasma IGF-I levels were measured in trout under LD 18:6 in September and December relative to those maintained under SNP or short-days.
- Strong positive linear correlations ($r^2 > 0.7$) were observed between plasma IGF-I and SGRwt, SGRL, or water temperature between July and November.
- Regression analysis indicated higher plasma IGF-I levels for a given weight or length gain (SGRwt or SGRL) in trout exposed to LD18:6 between July and November relative to those maintained under SNP or short-days, but not in December.

Experiment 3

- Exposure of juvenile trout to LL significantly enhanced growth rate and subsequent weight gain relative to those exposed to SNP or short-day photoperiods from April to September.
- Slow release melatonin implants (18mg) significantly reduced growth rate of fish relative to intact controls. Photoperiod (SNP, LD 8:16, or LL) had no effect on growth rate of implanted fish.
- Exposure to LL lead to significantly higher IGF-I levels in control fish in mid-August than in SNP or LD 8:16 fish, with SNP achieving significantly higher levels than LD 8:16 in mid-July.
- Administration of exogenous melatonin appeared to have no effect on circulating plasma IGF-I levels irrespective of photoperiod when implant levels were compared with controls.
- Strong positive linear correlations were found between plasma IGF-I levels and SGRwt and SGRL over time in all treatments, except SGRwt in control fish under constant short-days (LD 8:16) and SNP and LD 8:16 controls for SGRL.
- A strong curvilinear relation was found between plasma IGF-I and water temperature in all treatments over time.

3.7 Discussion

3.7.1 Effects of Photoperiod Manipulation on Growth Performance

Numerous studies have demonstrated that both Atlantic and Pacific salmonid growth is enhanced by either exposure to long-day (Clarke *et al.*, 1978; Stefanson *et al.*, 1989) or LL regimes (Taranger, *et* al., 1999; Endal *et al.*, 2000) applied in both freshwater (Solbakken *et al.*, 1994; Sigholt *et al.*, 1995) and seawater environments

(Oppedal, *et al.*, 1999; Porter *et al.*, 1999) and in both juvenile (Clarke *et al.*, 1981, Saunders *et al.*, 1985; Saunders & Henderson, 1988) and post-smolt/adult (Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Taranger *et al.*, 1998) stages of development.

Similarly, the results of experiments presented in this chapter showed a strong growth enhancing effect following exposure of fry and juvenile rainbow trout to LL or constant long-day photoperiods (LD 18:6) with both significant increases in weight and length observed relative to those ongrown under SNP or constant short-day regimes (LD 8:16). However, in the current chapter, experiments were carried out during the summer/autumn periods, with photoperiod applied typically from late spring/early summer, whereas the literature regarding salmonid growth enhancement by photoperiod manipulation has typically applied light regimes during the autumn through to spring, with the principal aim being to inhibit maturation (Bromage *et al.*, 2001). As a consequence of delayed or arrested maturation, energy is diverted into somatic rather than gonadal growth. Furthermore, use of photoperiod during earlier life stages of salmon has principally looked at the effects on growth and subsequent parr/smolt transformation. However, the freshwater rainbow trout does not undertake such a process during its typical development and as such photoperiodic influences on growth patterns and development has received relatively little attention. Noel & Le Bail (1997) demonstrated a 4-5 week cyclicity of growth rate in rainbow trout under SNP and constant environmental conditions, while a 6 month cycle of growth was observed in Arctic charr under constant light and temperature (Jobling, 1987), suggesting the presence of endogenous growth rhythms.

Endogenous Control of Growth

In all three present experiments significantly greater weight gains were observed approximately 12-14 weeks after the onset of long-days or LL in both fry and juveniles relative to those under SNP or short-days. Such findings support the theories proposed for the endogenous control of growth rhythms in addition to direct photostimulation (Komojourdjian *et al.*, 1976; Eriksson & Lundqvist, 1982; Saunders & Harmon, 1988; Krakenes *et al.*, 1991). The current results in this chapter suggest that there is a lag/adjustment-phase before photoperiod is capable of inducing growth enhancement, an observation that is well documented in Atlantic salmon culture (Oppedal *et al.*, 1997; Taranger *et al.*, 1999). Clarke (1990) showed that the application of LD 17:7 (daylength equivalent of longest daylength in British Columbia) from late June to February could be used to extend the growing season of coho salmon in sea cages by maintaining daylengths after the summer solstice. Fish exposed to artificial lighting continued to grow rapidly during October and November while those under natural light ceased growing at the end of September. Thus it was postulated there may be a "critical period" just after mid-summer, in which if salmon had stored enough energy above a certain threshold rate, growth could be maintained. Certainly in experiment 2 fingerlings exposed to LD 18:6 first showed significant weight increases relative to those under SNP and short-days only from mid-September, with increasing weight gain maintained throughout October. Similarly in trial 3, fish exposed to LL showed the first significant increases in weight between mid-August and September relative to those under SNP or LD 18:6. During these time periods in both experiments the natural photoperiod was rapidly decreasing (17.5 to 12hrs daylight), and it has been shown that growth rate in juvenile salmon reduces in accordance with decreasing daylength (Stefansson *et* al., 1989b; Berge *et al.*, 1995). Thus the long-day (LD 18:6) and LL

regimes in experiments 2 and 3 are likely to be extending the natural daylength from the summer solstice and altering perception of the summer period, and hence potentially entraining a new growth rhythm. Such a model is possible as the application of LL for as little as 2 months from the summer solstice was shown to delay the natural spawning time of rainbow trout through the delay of a circannual spawning rhythm (Randall *et al.*, 1991). However, direct photostimulation cannot be ruled out as clear influences on GH secretion have been observed (Komojourdjian *et al.*, 1989).

Direct Photostimulation of Growth: Influence of Season and Temperature

Since growth enhancing effects were not observed under extended photoperiod regimes in trials 2 and 3 until daylengths decreased then an extended period of growth opportunity through direct photostimulation may also have been possible. Similarly in juvenile halibut little difference in growth was observed following exposure to LL from May to early-July suggesting that sensitivity to continuous light is less pronounced under naturally increasing photoperiods, while out-of-season exposure to LD 8:16 (March-May) followed by LL did promote a significant enhancement of growth during the same period (May-July) (Simensen *et al.*, 2000). This suggests that photoperiodic history and the direction of change are having important influences on the subsequent growth response, with photoperiod acting as a zeitgeber to entrain natural growth rhythms after a period of time as well as having a potential role for direct photostimulation.

Furthermore, sensitivity to photoperiod manipulation has been found to change with season (Clarke *et al.*, 1981; Imsland *et al.*, 1995) and temperature (Saunders *et al.*, 1985; Solabakken *et al.*, 1994; Jonassen *et al.*, 2000). In both experiments 2 and 3, fish under long-days and LL first exhibited significant growth enhancements relative to SNP

when daylengths were decreasing. Since temperatures were still relatively high during this period in both experiments the sensitivity to photoperiod may have been maintained and hence the potential for direct stimulation of growth by extended daylength may have been possible. The growth response of juvenile Atlantic salmon exposed to LD 16:8 from August to November was rapid (41 days) compared to those under natural light even when temperatures fell from 17 to 5°C (Saunders *et al.*, 1989). Similarly, in both underyearling coho and sockeye salmon the response to photoperiod manipulation was greater at higher temperatures in Autumn (Clarke *et al.*, 1978) although chinook salmon and Arctic charr show no consistent growth response to photoperiod treatments irrespective of temperature suggesting species differences (Clarke *et al.*, 1981, Mortensen & Damsgard, 1993). Thorpe *et al.*, (1989) showed the greater the opportunity for growth as represented by degree-daylight hours in mid to late summer the greater the proportion of juvenile salmon that would maintain rather than arrest growth. Thus extended photoperiods used in experiments 1-3 may be directly stimulating growth rather than altering an endogenous rhythm.

It has been clearly shown in Atlantic salmon that the longer the exposure to LL, the longer the period that higher growth rates will be maintained (Endal *et al.*, 2000). However, it was also shown that enhanced growth was maintained after salmon were returned to natural photoperiod following LL application suggesting that photoperiod is adjusting a circannual growth rhythm, rather than as a consequence of direct photostimulation. If direct photostimulation of growth does occur then the stimulatory effect would last only as long as additional light was applied. Taking the above into account, the growth patterns exhibited by fish exposed to constant short-days (LD 8:16) in experiments 2 and 3 provide further evidence that there may also be an underlying endogenous control of growth in rainbow trout.

Influence of Constant Short-days on Growth: Further Evidence of Endogenous Control of Growth

Interestingly exposure of trout to constant short-days (LD 8:16) in either experiments 2 or 3 did not reduce growth rates below that of those under SNP as may have been expected, since it has been shown in juvenile Atlantic salmon that exposure to constant short-days or a decrease in photoperiod causes a growth depressing effect (Duncan & Bromage, 1988; Skilbrei *et al.*, 1997). In the current studies, several explanations are possible.

Firstly considering trial 2, photoperiod regimes were applied from around the time of the summer solstice where daylengths would have been at their maximum (~LD 17.5:6.5). Thus as the experiment progressed (June-December) daylengths would have been naturally decreasing in the SNP treatment (17.5 to 7.8 hours daylight). Therefore, the similar growth pattern observed in short-day (LD 8:16) fish relative to those under SNP suggests that it is the direction of change rather than an absolute daylength that is more important in influencing growth as has been shown in the control of reproduction in rainbow trout (Bourlier & Billard 1984; Davies *et al.*, 1992; Randall & Bromage, 1998). The constant short-day regime may have thus been perceived as the "expected" natural photoperiod decrease after the solstice and the endogenous growth rhythm may have remained unaltered, hence potentially explaining why the growth rhythm and weight gain were similar between SNP and LD 8:16 treatments. Therefore, if photoperiod was directly stimulating growth, then lower growth rates would have been expected under LD 8:16 relative to those under SNP until daylengths were of equal duration, after which similar growth rates could have been expected. Furthermore, the overriding effect of high water temperatures rather than photoperiod may have been driving growth during this period.

In experiment 3 photoperiod regimes (SNP, LD 8:16 and LL) were applied from mid-April (14 hours daylight), two months earlier than in trial 2, however, again no differences in growth were observed between LD 8:16 and SNP despite the earlier application of the photoperiod regimes. Hypothetically, the fish had previously been reared under SNP prior to exposure to constant short-days and may have thus perceived the naturally increasing daylength and were hence unresponsive to a reduction in daylength at this stage of development. Certainly, timing of photoperiod application requires careful consideration to manipulate spawning in rainbow trout in that the same photoperiods applied at different stages of reproductive development can advance or delay spawning (Whitehead & Bromage, 1980; Bromage *et al.*, 1982; 1984), suggesting that critical decision periods are present and will determine whether maturation will proceed or be arrested, and that photoperiod manipulation outside these periods will not elicit a response. Growth patterns may thus have similar critical decision periods.

Alternatively, and more plausible is that although an increasing daylength would have been perceived prior to a reduction in daylength in the LD 8:16 treatment, the time required to adjust an endogenous rhythm may have been too short (treatments applied 8 weeks prior to summer solstice) prior to the decrease in the natural daylength and hence the natural growth pattern was maintained. It has been shown that fish are unable to synchronise their endogenous rhythms under rapidly increasing and decreasing artificial photoperiods (Clarke *et al.*, 1978; Villarreal *et al.*, 1988). Hence fish under LD 8:16 in experiment 3 may have "expected" the natural decrease in daylength as perceived by those under SNP. These findings provide further support for the endogenous control of growth in rainbow trout. However, it has recently been proposed that poor growth under LD 9:15 in 0+ Atlantic salmon smolts is caused by reduced photostimulation rather than

an alteration in an endogenous rhythm although the presence of such a rhythm was not dismissed (Duncan *et al.*, 1999).

Evidence of Endogenous Growth Patterns: Change in Condition Factor

Looking at changes in length and condition factor (CF) in all three experiments may also provide further support of the existence of endogenous growth patterns in rainbow trout. Bjornsson *et al.*, (2000) has proposed that skeleton elongation in winter in Atlantic salmon undergoing smoltification creates the potential for rapid weight gain following transfer to seawater in spring under increasing daylength and temperature. Although freshwater rainbow trout do not undergo the parr-smolt transformation such a mechanism of winter growth cannot be ruled out. Therefore exposure to constant short-day light regimes (LD 8:16) may have advanced such a winter growth phenomenon in the current chapter and provides further evidence of endogenous control of growth in rainbow trout.

In trial 3 photoperiod regimes were applied as of mid-April under increasing daylengths, and although control fish under LD 8:16 had a significantly lower mean weight than those under LL, both treatments exhibited a similar gain in length with no significant differences. Furthermore, although both treatments maintained a significantly greater length than SNP from mid-July onwards, no difference in weight was observed between SNP and LD 8:16 treatments. Taken together this suggests that a circannual rhythm of growth may have been advanced under the LD 8:16 regime, and that this regime was perceived as "winter" and a switch to skeletal rather than muscle growth occurred. Such a growth model is supported by the fact that CF in LD 8:16 groups was maintained at a significantly lower level than LL and SNP from mid-July. This observation is typical of smoltification in Atlantic salmon prior to seawater

migration/transfer (Eriksson & Lundqvist, 1982; Berg *et al.*, 1994; Berge *et al.*, 1995; Sigholt *et al.*, 1995) in which the longer skeletal frame provides the potential to take advantage of the greater feeding opportunities and weight gain in the marine environment (Bjornsson *et al.*, 2000).

However, such a pattern in length and CF was not apparent in trial 2 despite the use of similar photoperiod regimes. It is possible that as the photoperiod regimes were applied as of the summer solstice both SNP and LD 8:16 were ongrown during the period of natural daylength decrease, then no phase adjustment in the natural growth rhythm may have occurred, however, LD 8:16 did maintain a significantly higher length than SNP during the Nov-Dec period. The fact that length gain did not occur immediately (~17 weeks after LD 8:16 onset) suggests that a period of time is necessary in order to re-entrain a circannual growth rhythm.

In experiment 1, fry CF was significantly greater at hatching in LD 8:16 than in LD 18:6 suggesting that early development may have also been affected by photoperiod. Taking the previously discussed views of Bjornsson *et al.*, (2000) with regards to skeletal elongation providing an increased frame for rapid weight gain, it is plausible that embryo skeletal elongation in response to LD 18:6 may have occurred in trial 1. A similar effect was apparent in trial 3 in that length significantly increased one month prior to significant increases in weight. Thus, the net of effect of this may explain why a significantly greater weight was achieved 4 weeks after first feeding in fry (equivalent to 12 weeks total photoperiod exposure) and concurs with the growth enhancement effects of LD 18:6 and LL observed in later juvenile stages in experiments 2 and 3 after similar periods of exposure to photoperiod (12-14 weeks). Further studies during early ontogeny are certainly warranted to determine if such a growth pattern could exist with regards to photoperiod manipulation.

The Effect of Photoperiod Manipulation on Feeding Efficiency (FCR)

The significant growth enhancement was accompanied by improvements in FCR in juvenile female trout exposed to LD 18:6 in experiment 2, and agrees with those observed in juvenile salmon (Krakenes *et al.*, 1991; Berg *et al.*, 1992) and gilthead seabream (Kissil *et al.*, 2001). In the current study, feed intake itself was not measured directly, thus it is difficult to determine whether extended photoperiods enhanced growth through greater feed intake. Handeland *et al.*, (2003) showed that enhanced growth of Atlantic salmon smolts under LL was due to increased feed intake, although genetically selected strains did exhibit more efficient feeding efficiency and that the effect of increased daylength was "additive" to the genetic improvement. However, in the case of trial 2, all fish were of the same strain and were presented a body weight equivalent ration throughout the shortest photoperiod (LD 8:16), and thus had the same period of opportunity to feed, which opposes the view of Mason *et al.*, (1992) whereby extended photoperiods were proposed to increase growth and feeding efficiency through increasing the time of food acquisition. In both juvenile sockeye and coho salmon offered similar feeding opportunities increases and decrease in appetite were directly related to photoperiod (Clarke *et al.*, 1978; 1981). Therefore, it is possible that increased feeding motivation through greater swimming activity (i.e. increased metabolic costs) (Petit *et al.*, 2003) and entrainment of appetite rhythms (Villarreal *et al.*, 1988) rather than visual feeding time that may explain the more efficient feed conversion observed under LD 18:6 in trial 2. Boujard & Leatherland (1992b) found crepuscular feeing patterns in demand-feeding rainbow trout under photoperiod regimes of 12 and 16 hours of light per day, while fish exposed to LD 8:16 showed no such clear peaks in feeding activity. Therefore, short-day photoperiods may be altering natural endogenous feeding rhythms, the presence of which have been clearly

demonstrated in rainbow trout (Boujard *et al.*, 1995; Bolliet *et al.*, 2001; Chen *et al.*, 2002) as seems to be the case for endogenous rhythms of growth. This was certainly evident from examination of total FCRs for the duration of experiment 2, where constant LD 8:16 treated fish expressed the poorest FCR of 1.07, while those under long-days exhibited the most efficient feeding at 0.92.

3.7.2 Relationship Between Photoperiod Manipulation, Temperature, Growth, and Circulating IGF-I Levels During Juvenile Growth

In the discussion of the following results care must be taken when referring to absolute values and comparison of IGF-I values between studies. Failure to find relationships in past studies have been attributed to disturbing effects of IGF binding proteins (IGFBP), which are known to inhibit or potentiate IGF-I actions (Duan, 1997;). Furthermore, most IGF RIAs including that used in these studies measure "total" IGF rather than "free" IGF. In coho salmon approximately 0.3% of IGF-I circulates as the free form, with IGFBPs believed to control it biological activity (Shimizu *et al.*, 1999). However, it is widely accepted that for the assessment of growth potential of an organism the determination of total IGF-I provides useful information (Plisetskaya, 1998).

Prior to the discussion of photoperiod effects on IGF-I levels two important findings from experiments 2 and 3 will be presented with regards to growth rate and temperature. These findings will have particular relevance to the later discussion of photoperiodic effects on growth and circulating IGF-I levels.

Plasma IGF-I and Growth Rate

In both experiments 2 and 3 plasma IGF-I levels were positively correlated with both weight and length growth rates over time (r^2 values 0.57 to 0.93, $p<0.05$) with the exception of LD 8:16 controls in experiment 3, the reasons behind which will be discussed later. Given this exception, the findings are in accordance with those observed in gilthead sea bream (Mingarro *et* al., 2002), Nile tilapia (Uchida *et al.*, 2003) Pacific salmonids (Beckman & Dickhoff, 1998; Beckman *et al.*, 1998; Pierce *et al.*, 2001; Beckman *et al.*, 2004) and Atlantic salmon (Dyer *et al.*, 2004a), and to my knowledge is the first reported evidence for a direct correlation between growth rate and plasma IGF-I levels in rainbow trout.

However, care must be taken when comparing results of these experiments with those reported in the literature since the method of statistical analysis, time of sampling, and the experimental remit has provided conflicting evidence in many past experiments leading to suggestions that relations between IGF-I and growth are highly dependent on season. For example Beckman *et al.*, (2001) found a positive linear relation between individual plasma levels and growth rate at one specific time point in. In contrast, Silverstein et al (1998) found no relationship when mean IGF-I levels were regressed against mean growth rates at monthly intervals between April and September. However these approaches ask two different questions in that do individual IGF levels relate to individual growth rates at one time or, do seasonal changes in hormone level relate to seasonal changes in growth rate. In support of the latter, Beckman *et al.*, (1998; 1999) found a strong positive relationship between seasonal changes in mean plasma IGF-I and growth in yearling chinook salmon sampled monthly from January to May using similar statistical analysis to that in the current chapter. In experiments 2 and 3 mean

plasma levels were regressed against mean SGRs for the previous month, i.e. May plasma vs. Apr-May SGR.

With regards to the observations made by Beckman *et al.*, (1998; 1999) it was proposed that the period studied encompassed a large and naturally occurring seasonal change in growth rate, while the studies of Silverstein *et al.*, (1998) occurred between April and September, in which seasonal changes in environmental parameters were less pronounced and may explain the lack of seasonal correlation of IGF with growth. However smoltification did occur that spring and changes in hormone levels may also have been affected by this process. Many studies have looked at changes in GH and IGF-I during spring in salmonids with the principal emphasis on understanding endocrine changes during smoltification. However, clear evidence has shown that these springtime elevations are a seasonal phenomenon associated with increasing growth rate, water temperature and photoperiod and not just smoltification (Duan *et al.*, 1995). Consequently, it has been proposed that growth-controlling hormones are likely to integrate photoperiod, temperature and nutritional information and regulate smoltification and growth (Dickhoff *et al.*, 1997). Thus under both changing photoperiod and water temperatures as in the current experiments it may be difficult to clearly separate the individual effects of each and their subsequent effect on IGF-I levels.

On a final note, as bloods were pooled from maturing males and immature females unknowingly in experiment 3 then differences in growth patterns and IGF-I levels between the sexes could not be elucidated. Higher IGF-I levels have been found in maturing amago and male chinook salmon compared to immature fish (Moriyama *et al.*, 1997; Shearer & Swanson, 2000), but it could not be determined if the differences were due to higher growth rates as a result of the larger size of mature individuals, or

due to maturation itself (Shearer & Swanson, 2000). However, in chinook salmon, plasma IGF-I was found to correlate equally well with body weight in both immature and preciously maturing males during spring, suggesting that IGF-I plays a key role in controlling growth in immature fish as it does mature fish (Shimizu *et al.*, 2000). Therefore, although the fact that combined sexes were used in plasma analysis in experiment 3, strong correlations were still achieved between IGF-I and SGR suggesting that early male maturation did not cause a loss in the seasonal pattern of IGF production in relation to growth in the rainbow trout.

Plasma IGF-I and Temperature

In both experiments plasma IGF-I levels were found to correlate strongly with water temperature irrespective of photoperiod (r^2 values 0.5 to 0.88, $p<0.05$), suggesting that the effect of temperature on IGF-I was similar between photoperiod treatments. The effect of temperature on circulating IGF-I differed between the two experiments depending on the time period in which the data were analysed. This was highlighted by the difference in regression relation between trials 2 and 3 when comparing IGF-I levels and water temperature. In trial 2 a linear relationship was found over time (July-November, falling water temperature), while in trial 3 a curvilinear relation was observed (April-September, rising then constant water temperature). However, inclusion of December (lowest temperatures) in analysis of experiment 2 resulted in a curvilinear relation indicating that between 4.5 and 8.9°C IGF-I levels remained relatively constant. Thus IGF values would appear to accurately reflect falling water temperatures between July and November, but below a possible threshold achieved between Nov-Dec IGF-I levels tended to remain relatively constant. Likewise, in experiment 3, IGF-I levels rose in a linear manner with temperature before a plateau

above 11.5°C, that was accompanied by a decrease in growth rate during this period in all treatments suggesting that the temperature optima for growth (Piper *et* al., 1992; Sumpter, 1992) may have been exceeded, and was hence reflected in circulating IGF-I levels. These findings provide further support for temperature-growth optimas in which IGF-I may reflect the prevailing conditions such that under rising and falling temperatures linear relationships will be found, while temperatures exceeding (both above and below) the preferred range for optimal growth show a plateauing of IGF-I levels which may be associated with subsequent retardation or cessation of growth.

In rainbow trout high temperature has been shown to increase plasma GH irrespective of whether fish are fed ad libitum or feed restricted, while pituitary GH levels were affected more by nutritional status than temperature (Gabillard *et al.*, 2003b). In a concurrent study it was shown that the growth promoting effect of temperature was not mediated by autocrine/paracrine expression of IGF-I in muscle (Gabillard *et al.*, 2003a). In fact, at the endocrine level, environmental temperature promoted growth by stimulating plasma IGF-I most likely through the direct action on GH secretion as previously observed (Gabillard *et al.*, 2003b).

In experiment 2, significantly higher IGF-I levels were evident in December in LD 18:6 relative to SNP treatments. This is surprising since temperature is regarded as rate limiting to endocrine function (McCormick *et al.*, 2000). However, the increase in IGF-I levels coincided with a small increase in water temperature (3.9 to 6°C in 10 days), suggesting that the GH-IGF system is sensitive to even slight changes in water temperature. Similarly, Larsen *et al.*, (2001) showed that a rise from to 2.5 to 10°C resulted in an increase in IGF-I levels within 1 week of the temperature change in coho salmon. Furthermore, IGF-I levels in fed but not fasted fish increased from January to March, proposed to be in association with increasing daylength acting through GH, thus

implying that feeding during winter may be important to maintain the sensitivity of the GH-IGF axis. Therefore in experiment 2, a more efficient FCR and/or greater feed intake induced by LD 18:6 could certainly improve the nutritional status of an individual even under decreasing temperatures, and thus maintain the sensitivity of the GH-IGF system to promote greater growth though upregulation of IGF-I synthesis. However, the trial was terminated at this point and the subsequent effect on growth and IGF-I cannot be ascertained, but may warrant further research.

Plasma IGF-I, Growth and Environmental Interactions

Although similar photoperiod treatments were used in both experiments, the timing of application was significantly different, thus the subsequent discussion will aim to consider the effect of each separately. With regards to experiment 3, the influence of melatonin on IGF and growth will be discussed in section 3.7.3, as such only photoperiod-growth differences in control fish will be considered in the following discussion.

Direct Photostimulation of IGF-I and Growth

In experiment 2, growth and plasma IGF-I were analysed from the summer solstice onwards (i.e. decreasing photoperiod and rapidly falling temperatures), and therefore seems that the seasonal changing growth patterns were accurately reflected by changes in IGF-I under natural photoperiod conditions. Similar findings were shown in the comprehensive study in red deer (*Cervus elaphus*) under natural photoperiod regimes, but exposure to "summer hold" photoperiods from the solstice delayed the natural changes (Webster *et al.*, 1999). Similarly, IGF-I levels in experiment 2 were significantly higher under LD 18:6 than SNP or LD 8:16 treatments during early-

September and was coincident with the first significant differences in weight and length gain. This may provide evidence for direct endocrine stimulation of growth by photoperiod, rather than a change in growth rhythm. If the latter were the case then a subsequent shift in the IGF-I profile may have been expected (Webster *et al.*, 1999). However, all three treatments showed the same pattern of change over time, with only the relative levels differing between treatments. A similar autumnal peak has been observed in chinook salmon, with the phenomenon related to the natural decrease in daylength and the possible removal of GH regulation of IGF-I production (Pierce *et al.*, 2002). However, in experiment 2, this peak was also apparent under constant daylengths (either short- or long-day) and the reason behind this peak remains unclear. It is possible that this natural peak occurred after only 8 weeks of exposure to the regimes and may have been too short a period to adjust a possible underlying endogenous control of growth, and thus the fish were growing in a "normal" manner at this period. Furthermore, the fact that IGF-I levels were greater for a given weight and length growth rate under LD 18:6 (as indicated by a significantly steeper regression) compared to SNP or LD 8:6 over the same time period and under the same temperature conditions, further suggest that photoperiod was directly stimulating IGF-I production and subsequently affecting growth. Thus these two observations with regard to IGF-I production would suggest that LD 18:6 applied as of the summer solstice may be causing direct stimulation of growth at the endocrine level. However, higher IGF-I may simply be reflecting greater growth rates rather than the driving mechanism behind growth, with larger fish expressing higher levels of IGF. This seems unlikely since no relationship between body size and IGF-I is readily found (Beckman *et al.*, 1998). Furthermore, in both coho salmon and tilapia, injection of IGF-I induced significantly greater gain in both weight and length (McCormick *et al.*, 1992; Chen *et al.*, 2000a).

Since plasma GH levels are known to change seasonally in response to both photoperiod and temperature (Bjornsson *et al.*, 1995), and plasma IGF-I levels are subject to regulation by GH, with levels also directly related to nutritional factors (Duan, 1997; 1998) then the GH-IGF axis may provide an integrated signal with regard to season, temperature and food supply. Beckman *et al.*, (1998) proposed that conditions found in spring (increasing photoperiod, temperature and food supply) could up-regulate the GH-IGF system, which in an organismal sense, could suggest that environmental conditions are positive and favourable for development. Conversely, decreasing photoperiod, temperature, and scarce food supplies would down-regulate such an axis and signal winter conditions, thus retarding development. Therefore in experiment 2, exposure to LD 18:6 after the solstice may have signalled an "extended summer period". This in conjunction with favourable temperatures and food supply, in theory could have promoted or maintained growth rates above those under SNP and LD 8:16, which may have perceived the decreasing photoperiod as the period entering winter and down-shifted growth rates.

However, in experiment 3, exposure to LL significantly increased growth yet did not induce higher IGF-I levels than SNP or short-days treatments as was observed in experiment 2, suggesting that an alternative mechanism was involved. The difference in IGF-I and subsequent growth response between the fish exposed to LL or LD 18:6 may be related to the perception of the regimes as continuous (LL) or changing (LD 18:6). Certainly under LL, endogenous reproductive rhythms are known to free-run in the absence of a changing photoperiod (zeitgeber), with subsequent phase advances or delays dependent on when the regimes are applied in relation to the ambient photoperiod cycle (Randall *et al.*, 1992). Therefore, those under LL may have perceived an increase in daylength, but without the day-night cycle believed that endogenous

clock was running behind time and subsequently phase shifted a growth response. This difference between experiment 2 and 3 particularly in relation to the timing of photoperiod application provides further support that there may be an underlying endogenous and endocrine control of growth in rainbow trout entrained by photoperiod.

Endogenous Control of IGF-I and Growth

In experiment 3 highest IGF-I levels were found over a greater period between mid-June and mid-September rather than a discernable peak as observed in experiment 2, and may reflect when the regimes were applied as previously discussed. Similar patterns in plasma IGF-I have been observed in juvenile gilthead sea bream, with highest growth rate and IGF-I titres achieved during summer (Mingarro *et al.*, 2002). Furthermore, in experiment 3, IGF-I levels in all treatments increased significantly between April and June most likely in association with increases in water temperature (6.6 to 11.2°C) rather than photoperiod since those under shorter photoperiods (LD 8:16) showed a similar rate of increase to those under LL or increasing photoperiod (SNP), despite being significantly smaller than LL. However, in yearling chinook salmon exposed to cooler water temperatures (2.5°C) than would have been naturally experienced during spring failed to prevent an increase in plasma IGF-I, with photoperiod proposed as the driving mechanism through its actions on GH (Larsen *et al.*, 2001). The opposing growth and IGF-I results of LD 8:16 fish in experiment 3 to those of Larsen *et al.*, (2001) may be explained by an underlying endogenous control of growth in rainbow trout as previously discussed (**section 3.7.1**), with these differences reflected in IGF-I levels.

McCormick *et al.*, (1992) failed to see any effects of LL application on growth or IGF-I levels when applied from late-March proposing that photoperiods were already

perceived as increasing prior to light treatment. Likewise in experiment 2, LD 8:16 showed no change in the pattern of IGF-I relative to SNP, again suggesting that a decreasing photoperiod was "expected" through an underlying rhythm. The second possibility is that the time required to adjust such a growth rhythm was too short prior to the decrease in the natural photoperiod at the time of the summer solstice, and the subsequent pattern of IGF-I production remained unaltered. Either of these possibilities would certainly suggest that photoperiod is the driving force behind endocrine changes in GH and IGF-I as generally accepted in the literature (Bjornsson *et al.*, 1995; Duan *et al.*, 1995) and that higher temperatures could further upregulate the photoperiodic response (Gabillard *et al.*, 2003a). Failure to find a correlation between LD 8:16 plasma IGF-I levels and SGRwt or SGRL in experiment 3 also provides further support that the "natural" growth rhythm may have been disrupted or became free-running in the absence of the seasonally changing photoperiod, and subsequently a loss of synchrony between IGF-I and growth developed. It is known that during short winter days, photoperiodic stimulation of growth hormone is low and as a result GH stimulation of IGF-I production is lower (Larsen *et al.*, 2001). Therefore, the significantly lower IGF-I levels under the LD 8:16 regime relative to SNP during July may reflect a down regulation of IGF production after a phase adjustment to the new photoperiod regime.

Unlike the observations of Silverstein *et al.*, (1998) in chinook salmon, experiment 3 showed significant correlations between growth and IGF-I levels during the same time period in SNP and LL treatments (April to August). It is likely that species differences exist, and that different life history strategies may explain the opposing results. In the study of chinook salmon the fish were preparing to enter seawater and may have subsequently altered their growth pattern which was not reflected in circulating IGF-I levels, while the rainbow trout in the current study

continued to grow in a "normal" manner, hence IGF-I levels accurately reflected growth and seasonal changes (Duan *et al.*, 1995).

As with LD 18:6 in experiment 2, LL controls in experiment 3 exhibited significantly higher plasma levels during mid-August than those under SNP or LD 8:16, again coincident with the period of highest water temperatures. This could provides further support of direct photostimulation of growth by extended photoperiod regimes under falling photoperiods. However, the patterns of IGF-I production did differ somewhat between LL and the other two regimes indicating that the earlier photoperiod application may have phase-shifted a growth rhythm (Eriksson & Lundqvist, 1982; Krakenes *et al.*, 1991) which was then reflected in the circulating IGF-I levels. In support of this was that significant growth differences (both weight and length gain) following LL application (**section 3.7.1**) occurred prior to any significant changes in IGF-I levels between the treatments. Furthermore, the fact that no significant differences were found in regression relations between LL and SNP control photoperiod treatments suggest that direct photostimulation of IGF-I by LL was not the cause of enhanced growth as in experiment 2. Considering all of the above, it would therefore be plausible to conclude that an advance in the endogenous clock controlling growth that then "free-ran" in the absence of a day-night signal may have initiated this response. As such IGF-I levels were simply reflecting growth rather than enhanced growth rates through stimulation of higher IGF levels by photoperiod. Since it is widely accepted that slower growing fish express lower hepatic mRNA and plasma IGF-I levels (Duan *et al.*, 1995), the fact that no differences in plasma levels were observed between LL and SNP in experiment 3 before weight separation occurred further support the idea that the growth rhythm was phase-shifted.

In summary, the findings from experiments 2 and 3 suggest that the timing of photoperiod application certainly affects growth, although how growth responds may be affected through the perception of light, and that its effects may be reflected in the endocrine response of IGF-I.

3.7.3 The Influence of Melatonin on Plasma IGF-I and Growth

To date the mechanisms that convey photoperiodic information to the reproductive and somatotropic axis are not clearly understood. Certainly the clear effects of photoperiod on the timing of reproduction and the corresponding diel and seasonal patterns of melatonin provide strong circumstantial evidence that melatonin may be an intermediary in the process (Bromage *et al.*, 2001). In this respect, melatonin levels may act to provide a signal to entrain endogenous rhythms including those involved in reproduction and growth. However, existing evidence does not support the concept that melatonin plays an important physiological role in the photoperiodic control of reproduction in non-mammals (Mayer *et al.*, 1997).

Melatonin Signalling: Endogenous Control of Growth

Experiment 3 clearly showed that supraphysiological levels of melatonin (up to 10,000pg/ml) inhibited growth rate in implanted rainbow trout relative to their intact controls irrespective of photoperiod treatment. However, plasma IGF-I levels were not significantly different between controls and implants, suggesting that melatonin does not act directly on the GH-IGF axis to control growth. Furthermore, IGF-I levels also correlated significantly with growth rate in all implanted fish in each photoperiod treatment (SNP, LD 8:16, LL). However, controls (no implants) under constant-short-days (LD 8:16) did not show a significant correlation between IGF-I and SGRwt or

SGRL, and as suggested earlier (section 3.7.2) may be a reflection of the desynchronisation of growth through down-regulation of IGF-I production in response to a phase adjustment in the endogenous clock controlling growth. Thus the fact that LD 8:16 implants showed a significant correlation between IGF-I and SGR, as did LL and SNP implants, coupled with the same growth rate and weight gain observed in each treatment would suggest that the photoperiod regimes were not perceived. If the regimes were perceived, then a similar down shift in growth and desynchrony relative to IGF-I production in LD 8:16 implants may have been expected as observed in LD 8:16 controls.

It is known that exposure to constant long-days (LD 18:6) from January to May, followed by constant short-days (LD 8:16) will advance maturation in rainbow trout through photoperiod induced phase shifts of the endogenous clock controlling reproduction (Randall *et al.*, 1998). Thus it has been proposed that artificially elevating plasma melatonin levels using melatonin implants may mimic short-day photoperiods. However, implants administered to rainbow trout under LD 18:6 or increasing natural photoperiod in May failed to advance maturation, suggesting that artificially elevated melatonin levels do not mimic short-day photoperiods (Randall, 1992; Nash *et al.*, 1999). Similarly, pinealectomy (PINX), which has been shown to abolish the normal diel pattern of melatonin (Porter *et al.*, 1996), did not influence either the incidence or timing of sexual maturation in male Atlantic salmon parr (Mayer, 2000). These findings indicate that a light-dark cycle of melatonin production via the pineal gland may be required in order to entrain the endogenous clocks controlling reproduction. Similarly, in Atlantic salmon it has been shown that supraphysiological levels of melatonin mask the natural melatonin profile, and that desynchronisation of smoltification may result from an endogenous clock "free-running" in the absence of a light-dark cycle (Porter *et*

al., 1998). Therefore, the fact that all implanted fish in experiment 3 grew at the same rate irrespective of photoperiod suggests that the level of melatonin provided by the implants also masked the natural diel cycle of melatonin and the subsequent perception of the light regime. This also supports the idea that perception of light and subsequent melatonin production by the pineal organ may be more important in conveying photoperiodic information than visual perception and ocular melatonin production in salmonids (Porter *et al.*, 1995).

Thus the findings from experiment 3 would further support the idea that implants do not mimic short-days, but rather abolish the perception of a day-night cycle, and that growth will be maintained via an underlying endogenous mechanism. Again this is supported by the failure to demonstrate significant differences in circulating IGF-I levels between controls and implants, whereby IGF-I levels simply reflected growth rate rather than photoperiod enhanced growth in controls. Overall these results may provide further evidence for the endogenous control of growth and support the idea that circulating IGF-I levels reflect growth rate in rainbow trout.

However, the current study does differ from the few studies examining the effects of melatonin on growth rates in fish. Following melatonin implantation (10,000 pg/ml) of Atlantic salmon parr in June, Porter *et al.*, (1998) observed an alteration in population structure and a significant growth increase in individuals one month post-implant. Weber (1999), observed similar effects in rainbow trout six weeks post implantation, although the differences were not significant there was a tendency for implanted individuals towards a larger mean weight (+10g above control mean weight). Similarly, in the goldfish (*Carassius auratus*), a positive relationship between growth and melatonin dose has been observed, while PINX induced a reduction in growth (De Vlaming, 1980). In Atlantic salmon parr, Mayer, (2000) also observed that PINX

significantly affected growth, although its effect was strongly dependent on the season. Under increasing photoperiods until the summer solstice pinealectomised fish exhibited lower specific growth rates (SGR). Thereafter, these fish exhibited higher SGRs under decreasing photoperiods. The differences between the work carried out in experiment 3 and those of previous studies may therefore relate to seasonal differences between the studies. However, to date, clear evidence between melatonin and the central control of physiological processes such as reproduction does not exist (Mayer *et al*, 1997).

Regulation of Food Intake and Growth

Recently, it has been shown that peripherally administered melatonin inhibited food intake in the goldfish (Pinillos *et al.*, 2001), as a result, lowered food intake could be another possibility for the reduced growth observed in trial 3 in implanted fish. However, actual feed intake was not measured in this study. Certainly, plasma IGF-I levels have been shown to decrease in starved fish as a result of reduced hepatic IGF mRNA expression (Duan 1998), yet in experiment 3 plasma IGF-I levels in melatonin implanted fish were not significantly lower than intact controls as would have been expected if reduced feed intake were the cause of the lower growth. However, in Atlantic salmon, acute stress and reduced intake caused low growth rates, yet plasma IGF-I levels were unaffected relative to controls (McCormick *et al.*, 1998).

Furthermore, in experiment 3 condition factor did not decrease in implanted fish as would have been expected in feed restricted/starved fish (Sumpter *et al.*, 1991; Uchida *et al.*, 2003). In fact quite the opposite was observed in experiment 3 in that that melatonin implanted fish were of a greater expected weight for a given length, indicated by a disproportionately high condition factor relative to intact controls. Weber (1999) also observed such a response in rainbow trout following melatonin implantation. Thus

it is plausible that weight gain was less restricted than length increase, and that melatonin may be altering some other developmental process in normal physiological growth.

Melatonin and Skeletal Development

To date, the effects of melatonin on physiological development in fish have not been widely studied, with the only demonstrable effect being an inhibitory action causing down-regulation of estrogen receptor and IGF-I mRNAs in osteoclastic and osteoblastic cells in fish scales (Suzuki & Hattori, 2002). Since osteoclast (resorptive) and osteoblast (formative) cells are known to function in mammalian bone formation through calcium homeostasis then an inhibitory action of melatonin on fish skeletal development may explain the apparent inhibition of length gain in the present study. However, the presence of osteoclastic and osteoblastic-like cells in fish bone are still widely debated.

In contrast to higher vertebrates, IGF-I receptors have been shown to predominate over insulin receptors in skeletal muscle in both coho salmon and brown trout (*Salmo trutta*) (Parrizas *et* al., 1995b; Mendez *et al.*, 2001). Furthermore, IGF-I has been shown to elevate *in vitro* sulfate uptake in the Japaneese eel branchial cartilage (*Anguilla japonica*) (Duan & Hirano, 1992), suggesting IGF-I may promote skeletal tissue growth through the stimulation of cartilage matrix protein synthesis. A role in skeletal growth has been shown by an increase in length as well as weight gain in coho salmon (McCormick *et al.*, 1992; Perrez-Sanchez & Le Bail, 1999), enhanced protein synthesis in the Gulf killifish (*Fundulus grandis*) and tilapia (Negatu & Meier, 1995; Chen *et al.*, 2000). Therefore, elevated melatonin may function to inhibit the autocrine and paracrine function of IGF-I through down regulation of mRNA tissue expression

during development. However, as this was not the main objective of the thesis, these observations suggest that further research into the effect of melatonin and photoperiod on developmental physiology may be warranted.

3.7.4 Summary

The findings from the present experiments suggest that there is an underlying endogenous control of growth in rainbow trout, and that LD 18:6 and LL regimes may also be having direct stimulatory effects on growth by extending the natural daylength from the summer solstice and hence increasing the period of growth opportunity when temperatures remain relatively high. However, the initial delays before growth is significantly enhanced under extended photoperiod regimes suggest that a period of time is required for trout to adjust to a change in photoperiod again providing further support for endogenous control of growth rather than as a consequence of direct photostimulation. Further studies will be required in order to separate the two effects.

The results also provide substantial evidence to indicate that plasma IGF-I levels accurately reflect growth rates in rainbow trout, and that it can be used as a long-term growth rate assessment tool. Furthermore, long-day photoperiods (LD 18:6) would appear to have direct stimulatory effects on growth through increased stimulation of IGF-I production when applied as of the summer solstice, while earlier application provides further evidence for photoperiod (LL) induced phase-shifts in growth rhythms in which IGF-I levels reflect growth rather than stimulated IGF production leading to enhanced growth. Thus the initial findings from experiments 2 and 3 suggest that IGF-I measurement may provide a useful tool for examining growth in rainbow trout, and that it may be capable of providing an endocrine signal to reflect growth performance and season. As such the rainbow trout may provide a simpler model to understand the

underlying endocrine mechanisms behind the regulation of growth in salmonids. However, due care should be given to sampling regime, seasonal influences, and analysis of growth relations in future studies.

Finally, artificially elevated melatonin levels achieved through slow release implants significantly reduced growth in rainbow trout. However, although both weight and length gain were significantly reduced, circulating IGF-I levels were not. Furthermore, IGF-I levels correlated equally with growth rate in both implanted and intact controls irrespective of photoperiod, suggesting that melatonin does not act directly on the GH-IGF axis to control growth, but functioned to remove the natural light-dark cycle possibly used to entrain growth. Significantly higher condition factors in implanted fish indicated that melatonin may also act on developmental control of growth via an alternative mechanism. Taken as a whole the results would suggest a functional relationship between the pineal organ, melatonin production and the control of somatic growth in the rainbow trout.

3.7.5 Conclusions

- Photoperiod is an important environmental parameter influencing the growth of rainbow trout fry and fingerlings.
- Growth rates can be significantly enhanced during development following exposure to constant long-day (LD 18:6) and LL photoperiod regimes.
- Growth appears to be under the control of an endogenous rhythm, with extended photoperiods capable of directly stimulating growth as well as entraining altered patterns of growth.
- Plasma IGF-I levels accurately reflect growth rate and water temperatures under natural photoperiod conditions in rainbow trout.

- An autumnal peak in plasma IGF-I was apparent in September
- Timing of photoperiod application determines the response of IGF-I production and subsequent effect on growth. Extended photoperiods (LD 18:6) applied as of the solstice appear to directly stimulate greater IGF-I production leading to enhanced growth. In contrast, earlier application of LL appears to phase-shift an underlying endogenous control of growth in the absence of a light-dark signal, and circulating IGF-I levels simply reflect growth rate.
- Supraphysiological levels of melatonin administration significantly reduced growth rate irrespective of photoperiod.
- Artificially elevated melatonin levels appeared to mask the perception of daylength.
- Exogenous melatonin administration does not appear to influence circulating plasma IGF-I levels between April and September.
- Supraphysiological levels of melatonin appear to alter "normal" body development.

Chapter 4: The Effects of Photoperiod, Growth, and Plasma IGF-I on Reproduction in First-time Spawning Female Rainbow Trout

4.1 Introduction

The experiment within this chapter was conducted as part of the National Environmental Research Council (NERC ROPA GR3/R9827, January 2000) grant awarded to Dr. Clive Randall to investigate endocrine, growth and reproductive interactions in rainbow trout and provide preliminary seasonal profiles of circulating IGF-I and a possible leptin-like peptide in first time spawning rainbow trout.

4.1.1 Photoperiodic Control of Salmonid Reproduction

In most temperate spawning species, including salmonids, it is now widely accepted that the pattern of seasonally changing daylength is primarily responsible for synchronising the timing of reproduction (Bromage *et al.*, 1994). Although modified light regimes can be used successfully to compress (advance) or extend (delay) the spawning period (Bromage & Duston, 1986; Bromage, 1987; Bromage *et al.*, 1992b), the increasing and decreasing components of the seasonally changing daylength can also be replaced by periods of constant daylength (Whitehead & Bromage, 1980; Bourlier & Billard, 1984; Randall *et al.*, 1991a; Beachham & Murray, 1993).

When undertaking photoperiod manipulations to alter spawning times it is important to consider the timing of the switch between photoperiod treatments as it determines the degree of advancement. In this respect, the earlier the reduction in daylength (LD 18:6 to LD 8:16), the more marked the advancement in spawning (Bromage *et al.*, 1994). Of considerable interest is that varying the position of the continuous light period in relation to the phase of the reproductive cycle not only affects the time of spawning, but also produces marked differences in the proportion of fish that spawn out of season (Randall *et al.*, 1998). This has been observed in

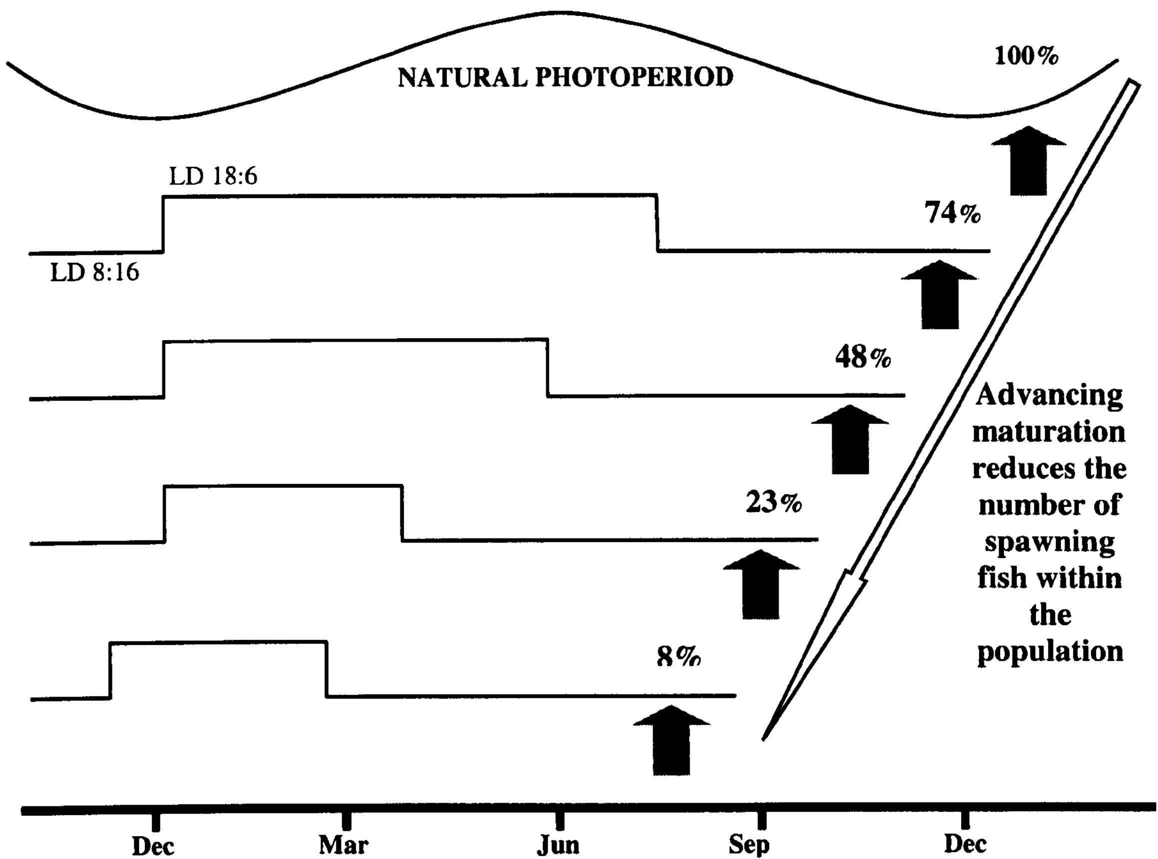


Figure 4.1 Diagram of the typical advancement in spawning time (indicated by black arrows) and the reduction of the population maturing and spawning (percentage) following long- to short-day switches in photoperiod applied at different times of the year relative to the natural reproductive-photo cycle in female rainbow trout. (Modified from Bromage *et al.*, 2001).

compressed photoperiod cycles where the greater the advancement in spawning time the fewer the fish that will respond (Figure 4.1). Duston & Bromage (1988) proposed that these variations could be explained by a "gating mechanism", whereby fish will only mature (undergo puberty) in a particular year if they had reached a certain threshold stage of reproductive development or size while the circannual clock is at a specific (gate-open) phase of the circannual cycle. In contrast, those failing to reach this threshold before the gate closes remain immature until the following year.

4.1.2 Physiological Regulation of Teleost Reproduction

To become sexually active all animals must undergo puberty. Puberty is described as the period in which a juvenile acquires for the first time the capacity of reproduction. It is characterised by various morphological, physiological and behavioural changes. In teleosts, initiation of puberty starts with the appearance of the first spermatocytes in the male and the beginning of vitellogenic processes in the female. Puberty is regarded as complete with the first ovulation in females and the first spermiation in males (Dufour *et al.*, 1999). The process is a kinetic phenomenon during which juveniles are preparing to become mature organisms capable of successful reproduction. In terms of endocrinology, the most important event is the activation of the production of gonadotropins which are a central component to the hypothalamus-pituitary-gonadal (HPG) axis that are responsible for initiating main stages of gonadal activity (Davies *et al.*, 1999). In all vertebrates, puberty occurs when individuals have reached a certain age and size and accumulated enough energy reserves to ensure the success of reproduction.

Hormones play a central role in the regulation of growth and nutrient utilisation in fish (Mackenzie *et al.*, 1998). In the case of salmonids there is a correlation between

growth, adiposity and early maturation (Rowe & Thorpe, 1990b; Silverstein *et al.*, 1998; 1999). Rapid growth and delayed maturity are desirable characteristics for commercially grown salmonids, however, evidence suggests that more rapidly growing individuals mature at younger ages leading to the problem of early maturity at small body size (Skilbrei 1989). It has been well demonstrated in a variety of salmonids that restricted feed intake during certain months slows growth and energy storage, leading to the reduced incidence of sexual maturation (Berglund 1995; Silverstein & Shimma 1994; Bromage *et al.*, 1992a; Silverstein & Shimma, 1994; Silverstein *et al.*, 1998). During these studies it has also been shown that maturing fish had higher fat levels than non-maturing fish. However, the physiological mechanisms by which maturity is triggered in fish are not clear (Silverstein *et al.*, 1999). Currently the most widely accepted hypothesis is that some threshold for size, rate of growth or energy storage must be surpassed during critical periods for sexual development to proceed (Silverstein *et al.*, 1997; Silverstein *et al.*, 1998). Therefore, hormones implicated in the control of growth (IGFs, growth hormone) and energetic metabolism (leptin) are likely to play a key role in the onset and time course of puberty as seen in mammals (Bromage *et al.*, 2001; Dufour *et al.*, 1999).

4.1.3 IGFs and Reproduction

Over the last decade studies have focused on the involvement and important roles of IGFs in fish reproductive systems. However, most studies have focused on direct actions of IGF on gametogenesis, with little research examining the relationship between IGF-I, growth and reproduction. Numerous studies have examined the relationships between body growth and IGF-I as introduced in chapter 3, finding significant correlations, leading to the proposed idea that IGF-I may be an accurate

indicator of growth performance. However, to date little research has focused on the role of growth-IGF interactions and the subsequent effect on reproduction. Certainly if IGF-I accurately reflects growth, particularly at "critical" decision periods, then this hormone may be capable of providing a signal to the reproductive axis that growth and/or size is sufficient in order to successfully undergo puberty. Furthermore, as IGF-I is known to be nutritionally regulated it may also provide further information on energetic status.

4.1.3.1 IGFs: A Direct Role in Reproduction

Growing evidence has indicated that IGF-I has a direct role in both spermatogenesis and oogenesis in teleost fish. From numerous studies, two modes of action of IGF on gametogenesis have been identified, acting through localised sites within the gonads through paracrine/autocrine mechanisms, and also via endocrine stimulation of the brain-pituitary-gonadal axis.

Paracrine/Autocrine Functions

Evidence for GH binding to specific receptors in the trout testes and GH involvement in the regulation of fish gonadal functions have been obtained both *in vivo* and *in vitro* (Le Gac *et al.*, 1992, 1993). However, whether these GH effects are mediated through modification of IGF-I production and/or IGF binding in fish gonadal tissues remains to be clarified. Insulin-like growth factors have been implicated in the physiology of mammalian ovary and testis (reviewed in Lackey *et al.*, 2000) but as yet have only recently been studied in the fish gonadal system (Duan, 1997).

Current research has demonstrated that fish ovaries express IGF-I mRNA and its peptide, and that specific binding sites for IGF-I and insulin are also present (Kagawa *et*

al., 1995). In membranes of both granulosa cells and theca-interstitial layers, IGF-I receptors have a higher binding, number, affinity and specificity than insulin receptors (Maestro *et al.*, 1995). Also shown are two peaks of maximum binding during the reproductive cycle, one during primary oocyte growth and the second appearing prior to ovulation. Following binding to the fish ovary, IGF-I has been shown to activate the IGF-I receptor tyrosine-kinase activity which increases GtH II stimulated steroidogenesis in salmon granulosa cells, while inhibiting this effect in theca-interstitial layers in preovulatory ovaries prior to germinal vesicle breakdown (GVBD). *In vitro* studies in red sea bream (*Pagrus major*) and striped bass (*Morone saxatilis*) have provided evidence for a role of IGF-I in final oocyte maturation (FOM) through stimulation of GVBD (Kagawa *et al.*, 1994; Weber & Sullivan, 2000). Administration of IGF-I was shown to act directly on *Fundulus heteroclitus* and spotted sea trout (*Cynoscion nebulosus*) oocytes to induce both GVBD and maturation induced steroid production (Negatu *et al.*, 1998; Thomas *et al.*, 2003). More importantly, IGF-I administration has been found to initiate oocyte maturation in a dose-dependent manner even more rapidly than 17α,20β-dihydroxy-4-pregnene-3-one (DHP), which is the naturally occurring maturation-inducing steroid in striped bass, thus suggesting a more important and direct role in oocyte maturation than first believed (Weber & Sullivan, 2000; Sullivan *et al.*, 2003).

Fish testicular cells have also been shown to express IGF-I and II and that they bind to a type-I IGF receptor, with these being responsible for the stimulation of premeiotic germ cell proliferation (Le Gac *et al.*, 1993, 1994; Le Gac *et al.*, 1996; Perrot *et al.*, 2000). Since GH specific receptors effects have been observed both *in vitro* and *in vivo* on gonadal functions, then it seems probable that IGF-I is a potential mediator of GH action in the trout testis particularly due to the relative abundance of

IGF-I mRNA (Le Gac & Loir, 1995). However, only recently has it been demonstrated that IGF-I expression in the trout testicular cell is more related to GtH I and androgen plasma levels than to GH or testicular GH-receptor changes (Le Gac *et al.*, 1999). Significant increases in plasma IGF-I at spermiation suggest a possible further role during final stages of spermatogenesis (Campbell *et al.*, 2003).

Endocrine Functions

In the European eel, incubation of pituitary cells with IGF-I caused a dose-dependent stimulation of GtH II synthesis and release, while initiating an inhibitory feedback on GH secretion. IGF-II was also demonstrated to have the same dose-dependent effects on GH and GtH II. In contrast, GH itself had no direct effect on GtH II production (Huang *et al.*, 1999). More recently, *in vitro* studies in coho salmon have shown that IGF-I strongly increased intracellular levels of GtH I and II and GnRH-induced GtH I release (Baker *et al.*, 1999, 2000a). As seen in previous studies the effects of IGF-I on GnRH-induced release and cell content are concentration dependent, and the effects were more potent than seen with insulin. The action of IGF-I on the sensitivity to GnRH was found to differ between GtH and GH cells. To be responsive to GnRH, GH cells were found to require longer exposure to IGF-I, with the efficiency decreasing with gonad maturation. Blaise *et* al., (1997) observed a similar effect in rainbow trout where a GnRH effect occurred at the pituitary level to induce GH release following long term (48 hr) exposure to IGF-I. In contrast, GtH cells required shorter incubation, and that sensitivity was independent of sexual stage. This suggests that IGF-I may be responsible for increasing GtH sensitivity to GnRH (Weil *et al.*, 1999).

The effect of IGF-I on GnRH-induced GH release by pituitary cells has been detected in immature rainbow trout or those at the beginning of gametogenesis, but not

in mature fish. *In vitro* IGF-I inhibition of GH release during preincubation also varied with sexual stage, being greater in immature than in mature fish. This permissive effect of IGF-I seems specific to somatotrophs since IGF-I does not modify GnRH action on GtH II release (Blaise *et al.*, 1997). This effect may be associated with an increase in GH synthesis which could lead to a more visible effect of GnRH. Van der Kraak *et al.*, (1990) provided evidence for GH effects during gonadal growth and development. Injection of immature rainbow trout with recombinant rainbow trout and salmon GH have both been shown to cause elevation of oestradiol 17-β and testosterone. Thus increased pituitary responsiveness to GH secretagogues such as GnRH may be responsible for gonadal steroid priming in immature fish.

In similar studies, fasting resulted in a marked suppression of pituitary content of GtH I relative to fed fish (Baker *et al.*, 1999). GtH I increased marginally in fasted fish, despite loss in body weight, while levels rose several-fold in fed fish, indicating that levels are not dependent on body weight. This data suggests that nutritional status can alter the gonadotropin axis, and hence the effect of fasting on pituitary GtH I may be associated with decreased IGF-I.

Nutritional Regulation of GH-IGF Expression

Considerable research is now being devoted to the possible nutritional regulation of the GH-IGF axis in fish. In salmonids, prolonged starvation causes cessation of growth but also causes significantly elevated levels of plasma GH, while fed fish have low levels of GH and continue to grow steadily (Niu *et al.*, 1993; Sumpter *et al.*, 1991). Starvation induced rises in plasma GH concentrations appear to be associated with a significant decrease in the hepatic binding sites for GH, suggesting a possible resistance to GH at tissue levels (Duan 1998). Conversely, starvation has been

shown to reduce circulating IGF levels in various fish species, while re-feeding results in a subsequent rise in hepatic IGF mRNA levels (Duan & Hirano 1992; Duan *et al.*, 1993). These findings suggest that nutritional status regulates IGF production at the mRNA level. As yet no evidence exists for nonhepatic IGF mRNA regulation through starvation. Taken together, this data and that previously discussed demonstrate that IGF-I stimulates GtH synthesis and release in teleosts by a direct pituitary action, suggesting that IGF-I action at the pituitary may be a mechanism by which energy balance influences reproductive maturation (puberty) in salmonids (Huang *et al.*, 1998; Baker *et al.*, 1999).

4.1.3.2 Permissive Role of Circulating IGFs as a Growth Indicator in Reproduction

Given the extensive knowledge with regards to the action of IGFs within the gonads, the involvement in pituitary function, and potential nutritional signaling, it would seem likely that IGF-I may also be capable of providing a signal of growth status. To my knowledge only four papers exist to date looking at the effect of circulating plasma IGF-I as an indicator of maturation in fish or salmonids for that matter, three being in the spring chinook salmon and one devoted to the amago salmon, and have provided conflicting results. As yet no research has focused on such a role in the rainbow trout.

Plasma IGF-I levels significantly increased from March to August in both male and female amago salmon, with a subsequent gradual decline in plasma levels in early maturing males and females, with lower levels maintained during the spawning period in November (Moriyama *et al.*, 1997). In contrast plasma IGF-I levels in immature fish remained elevated throughout September, peaking in October before gradually

declining in November. At no point were differences in weight observed between maturing and immature individuals.

Similarly, higher plasma IGF-I levels were found in early maturing 1+ yr chinook salmon than non-maturing individuals and showed distinct seasonal patterns of change (Shearer & Swanson, 2000; Campbell *et al.*, 2003). In both studies male chinook salmon tended to have higher levels of plasma IGF-I as they approached maturity (March-September), with highest levels in September coincident with a decrease in primary A spermatogonia, increases in 11-KT and pituitary FSH, which are consistent with IGF-I's stimulatory role in the early mitotic stages of spermatogenesis. Lowest levels were recorded in February (Campbell *et al.*, 2003).

However in the former study (Shearer & Swanson, 2000), early maturing males were significantly larger than non-maturing fish, and the difference in IGF-I levels may have been attributed to differences in growth or due to maturation. Certainly, anabolic steroids such as androgens that increase during maturation may further stimulate growth in maturing fish. In tilapia 17α-methyltestosterone stimulated the GH-IGF axis and subsequent growth (Riley *et al.*, 2002), while no effect on growth was observed in the rainbow trout following in-feed treatment with 17α-MT (Sower *et al.*, 1983). However in the study by Shearer & Swanson (2000), the differences in growth only occurred after the initiation of maturation, supporting the idea that the decision to mature is initiated prior to later increases in growth observed in maturing fish. Yet, prior to the initiation of maturation there was a tendency for individuals to be of a larger size, suggesting that higher growth rate may be a factor involved in the initiation of maturation.

In a further study of yearling chinook salmon parr plasma levels of IGF-I reflected growth at certain time points, but were not regarded as a good indicator of

sexual maturation 1 year prior to sexual development in autumn, nor in spring 6-7 months prior to sexual maturity (Silverstein *et al.*, 1998). Size and to some degree fat content 1 year prior to maturation were better indicators of fish that attained maturity.

Thus, the differences observed between studies in terms of finding relationships between IGF-I and growth and maturation highlight the need for further research in order to clarify the possible interactions. Differences in plasma IGF levels may also reflect different species life history strategies including early maturation preceded by smoltification as in the chinook salmon, or smoltification after precocious maturation in the amago salmon. Furthermore, in many salmonids, fish cease feeding at maturation, and this may also reflect changes in circulating IGF levels, although maturing male chinook salmon continued to feed in the study by Shearer & Swanson (2000) and had already undergone smoltification the previous year.

4.1.4 Evidence for a Possible Role of a Leptin-like Peptide in Fish Reproduction

Only very recently has the expression of a leptin-like peptide been detected in fish including the rainbow trout (Johnson *et al.*, 2000). However, unlike the growing evidence for leptin's involvement in the control of mammalian reproduction, the mechanisms behind the role of leptin in fish reproduction has received very little attention primarily due to the difficulty in confirming the presence of such a peptide in fish. In the burbot (*Lota lota* L.), high concentrations of a leptin-immunoreactive peptide have been found in the liver suggesting that it may be secreted by the hepatocytes (Mustonen *et* al., 2002a). This differs from the mammalian system in which the adipocytes are the main production sites of circulating leptin (Zhang *et al.*, 1994). However, in the burbot the liver is the main lipid-storing organ, suggesting that the original site of leptin production in early vertebrates could have been the liver. Further

evidence in support of alternative sites of leptin production were confirmed by Johnson *et* al., (2000) when fish adipocytes failed to show positive expression unlike the strong staining observed in the brain, blood, liver and heart. Studies in other non-mammalian vertebrates including the frog *Xenopus laevis*, snake *Natrix maura*, lizard *Podarcis hispanica*, and the rainbow trout have also shown expression of leptin-like proteins in the stomach tissues (Muruzabal *et al.*, 2002).

To date two studies, both in burbot, have suggested a possible role of a leptin-like peptide in fish reproduction (Mustonen *et al.*, 2002a, Mustonen *et al.*, 2002b). Liver leptin levels were found to be high before and during spawning (600-800ng $liver^{-1}$) despite a decreasing liver size associated with gonadal development, followed by a significant decrease in liver concentration after spawning (258ng $liver^{-1}$). In contrast plasma levels of leptin were low prior to and during spawning, with a subsequent increase post-spawning that was proposed to be a seasonal event in order to help the animals recover from the exertion of reproduction. Taken together these data may indicate that high liver leptin concentration and low plasma levels of leptin pre-spawning may reflect good nutritional status. Certainly in burbot preparation for the natural winter spawning (Jan-Mar) occurs in summer through the storage of lipids within the liver. The subsequent decrease in liver leptin levels post-spawning and the subsequent increase in plasma levels may reflect energy depletion and nutrient mobilisation involved in gonadal development and spawning itself. However, the functions of leptin in fish are not understood and are not consistent with their known function in mammalian physiology and reproduction. Mice deficient in the *ob* gene express low levels of leptin and are sterile and incapable of undergoing puberty, yet injection of leptin restores fertility (Chehab *et al.*, 1996; Mounzih *et al.*, 1997).

However, such differences in function may be expected given the extremely different environments and physiology of fish and mammals

Currently very few experiments regarding direct physiological effects of leptin in fish have been reported. Immature coho salmon delivered human leptin via osmotic pumps for 14 days showed no effects on physiological parameters including growth, gonad weight, lipid content and hormone levels (FSH, IGF-I, insulin, GH & T_4), (Baker *et al.*, 2000). Similarly, daily injections of murine leptin in green sunfish (*Lepomis cyanellus*) did not cause loss of total weight or body fat, trademark effects of leptin treatment in mammals, although injections did increase fat metabolism (Londraville & Duvall, 2002). However, in goldfish central or peripheral administration of murine leptin caused a reduction in food intake, in part through modulatory effects on NPY, and that its actions appear to be mediated by cholecystokinin (Volkoff *et al.*, 2003). Numerous hypotheses have been proposed to explain the lack of effect of mammalian leptin in fish including incompatibility of mammalian leptin with fish receptors, certainly different isoforms of immunoreactive proteins have been found in the lamprey, *Petromyzon marinus* L. (Yaghoubian *et al.*, 2001). Additionally, use of inadequate or non-physiological doses of leptin and that leptin's actions may be slower in ectotherms due to their lower metabolic rate have also been suggested. Thus the action of a leptin-like substance in fish may act as an indicator of energy/nutritional status rather than through the direct endocrine control of reproduction. However, evidence particularly from *in vitro* studies also exists to support a direct action of leptin in fish reproduction especially at the pituitary level (Weil *et al.*, 2000).

In rainbow trout, Weil *et al.*, (2000) demonstrated that high doses (10^{-6} M) of rhleptin were efficient in inducing increased release of FSH and LH from pituitary cultures, with the effect dependent on sexual status. However, only females in

endogenous and advanced exogenous vitellogenesis and males in early spermatogenesis or those that were spermiating were responsive to leptin administration, while increasing doses were ineffective in inducing FSH release in immature fish whatever the sex. Furthermore, no effect on gonadotropin-releasing hormone (GnRH) was observed. The direct action of leptin on FSH and LH release, evident only when gametogenesis has already started suggest that leptin is not the unique signal for activation of the gonadotropic axis but requires a combined action with other promoting factors, and may hence have a more permissive role in reproduction. In sea bass similar results to those observed in trout have been demonstrated, but have also indicated that NPY is involved in potentiating the effects of leptin (Peyon *et al.*, 2001), and more recently somatolactin (SL) has also been implicated in the nutritional control of the onset of puberty through the stimulation of SL producing cells by leptin and NPY (Peyon *et al.*, 2003). Therefore, the effects of leptin and the co-involvement of other hormones and the fact that their effects are dependent on sexual status further support the idea that leptin may have a more indirect role in reproduction. These observations would suggest that leptin is a metabolic signal to the neuroendocrine reproductive system and that under conditions of inadequate energy levels, low leptin levels may act as a metabolic "gate" to inhibit the activity of the neuroendocrine axis (Cunningham *et al.*, 1999). Such modes of action are generally accepted in mammals in that leptin is known to stimulate hypothalamic-pituitary function shown through the presence of leptin receptors in the anterior pituitary (Garcia-Mayor *et al.*, 1997; Yu *et al.*, 1997) in addition to informing the brain that energy reserves are sufficient to support the high energy demands of reproduction (Barash *et al.*, 1996; Ahima *et al.*, 1997).

Thus far, the actions of leptin on reproductive mechanisms in fish are still poorly understood and will require further studies in order to elucidate possible

functions and mode of action. However, the function of leptin in fish may not be characterised until the issue of its structural variation in vertebrate phylogeny is resolved and homologous proteins and antibodies are made available.

4.1.5 Chapter Aims

Thus considering the above information, the aim of this chapter was to determine how photoperiod, growth, IGF-I and leptin-like peptide may potentially interact with one another to determine whether reproduction will proceed under natural and advancing photoperiod regimes. Furthermore, such interactions if they exist may help further the understanding of the underlying physiological mechanism that control puberty. To this extent such knowledge may help explain why fewer fish are capable of undergoing puberty when the natural spawning time is artificially advanced by environmental manipulation.

Experimental objectives were:

- To manipulate the natural spawning time in virgin spawning rainbow trout using an advancing photoperiod and alter the percentage of fish attaining maturity.

Experimental aim:

- To determine the possible mechanisms behind the reduction in the numbers of fish capable of undergoing puberty when the natural spawning cycle is advanced in rainbow trout.
- Examine the role of growth parameters as indicators of maturation.
- To assess the role of insulin-like growth factor-I as a potential indicator of growth and/or nutritional status to the reproductive axis.
- To assess whether a plasma leptin-like peptide is measurable in rainbow trout, and whether such a substance is involved in reproduction.

4.2 Materials and Methods

4.2.1 Experimental Protocol and Sampling

One hundred and twenty 465g ±35g Danish 2-year old virgin female rainbow trout (supplied by Selcoth Fisheries Ltd.) with an expected natural spawning time of February/March 2001 were stocked into a 23.5m^3 circular tank on the 6th January 2000 and maintained on a simulated natural photoperiod (SNP) (7L:17D) until the end of the month. On the 31st January all fish were individually tagged using AVID passive integrated transponder (P.I.T) tags and randomly split between two 23.5m^3 tanks into two groups of 60 fish. Each group was exposed to one of two photoperiods (Fig 4.2); SNP or an advancing photoperiod regime (ADV), with constant long-days (LD 18:6) until 15th May then constant short-days (LD 8:16). Each tank was covered by a blackout tent with lighting provided by four 16watt fluorescent strip lights positioned 1m above the water surface giving a mean light intensity of 100 lux at the water surface and 10 lux on the tank floor. Water flow rates to the tanks were maintained at 10 L/sec and at ambient temperature (Fig 4.2). Fish were fed according to manufacturer's guidelines via an automated hopper set at 15 minute intervals using 6.5 and 8.5mm Trouw AminoBalance™ pellets (Protein: 45%, Lipid: 30%, Carbohydrate: 11%, Ash: 8%, Moisture: 6%, Astaxanthin: 40mg/kg; DE: 21.46 MJ/kg). Food was presented during the daylight hours of the shortest photoperiod for both tanks.

Weight-length measurements were taken from each fish at monthly intervals. Monthly blood samples were also taken from each individual for assessment of plasma IGF-I, calcium, testosterone and "leptin-like" peptide. Only 10 bloods from the same individuals per cohort per treatment were assayed for plasma IGF-I from all time points, while only 5 bloods and 8 time points were assayed for leptin-like peptide

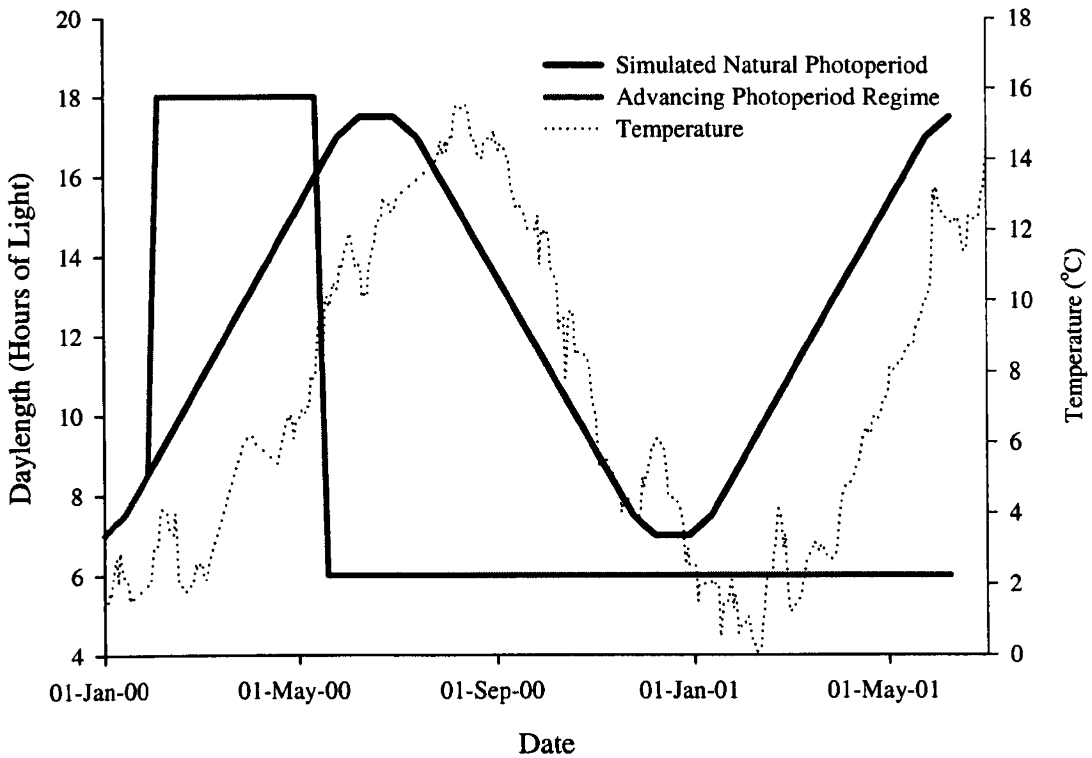


Figure 4.2 Photoperiod regimes used to produce a natural spawning time population (black) and an out-of-season advance spawning population (grey). The ambient temperature regime (broken line) of the water supply during the period of the study is also shown.

due to the high cost in using the Gropep Ltd. and R&D Systems kits. As fish approached maturity they were examined at two-week intervals and the spawning time of each individual recorded. A total of 9 fish from each tank were lost during the first five months of the experiment, thereafter no further mortalities were recorded, leaving 51 fish per tank. All mortalities were disregarded from analysis.

4.2.2 Preliminary Validation of Leptin ELISA

Validation and verification of the protein-antibody detection were carried out after assaying of the selected blood samples using the ELISA kit.

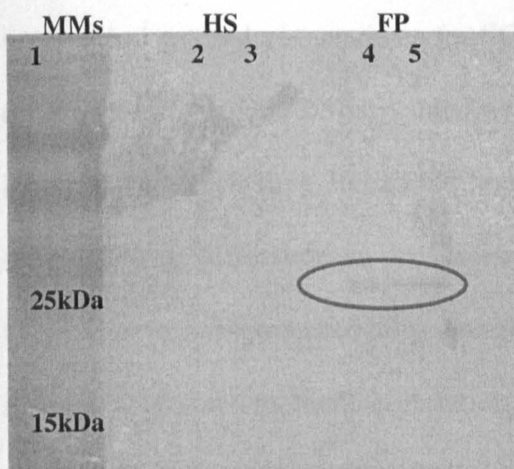
Gel Electrophoresis

Pooled plasmas collected from 10 female broodstock (2.2kg) on 26/11/00 were used to create a 1:1 dilution (100µl sample:100µl DTT/SDS sample buffer) for gel electrophoresis. Human leptin standard (10,000pg/ml) was supplied by R&D systems and was also used to make a 1:1 dilution with sample buffer. Before loading, samples were boiled for 2 minutes at 80°C. Duplicate groups of 20µl diluted sample and standards were run. Samples were separated on a 15% polyacrylamide separating and stacking gel (constituents, Sigma Chemical Company Ltd., Dorset, UK) made as below. The gel was run for 1 hour at 200 volts.

	Separating Gel	**Stacking Gel**
Separating gel buffer	5ml	-
Stacking gel buffer	-	2.5ml
Distilled Water	5ml	6.1ml
Acrylamide	10ml	1.34ml
Temed	150µl	10µl
ASP	70µl	50µl

Immunoblot

Proteins were transferred to a nitrocellulose membrane with a semi-dry electro blotter at 60 volts for 1 hour. Blots were blocked in 3% Marvel (w/v) in 1x PBS solution for 2 hours. Following blocking, a 1:250 dilution of mouse-anti-human leptin detection antibody solution in PBS was prepared (supplied by R&D Systems, 2μg/ml dilution). The membrane was then incubated in 10ml of detection antibody dilution overnight (16 hours) at room temperature on an orbital shaker. After incubation the membrane was washed 3 times in 1xPBS. The membrane was then incubated for 1 hour in a 1:100 dilution of anti-mouse horseradish peroxidase conjugate solution (3mL). To visualise the proteins a peroxidase substrate solution (4.5ml PBS/DAB, 5μl hydrogen peroxide) was incubated with the membrane. The results of the blot are shown below.



Immunoblot Results

Channels
1- Molecular Weight markers (MMs)
2- Human standard (HS)
3- Pooled Fish Plasma (FP)
Red circle highlights positive immunoblot

A single positive blot was obtained at 25kDa in the pooled fish plasmas, indicating that the mouse anti-human leptin antibody was specific to the protein of this size as no other positive blots were obtained. Unfortunately, no positive blots were obtained for the human leptin standard. This difference may be due to the slower passage of whole plasma through the separating gel during the 1 hour separating period, in which the purified leptin standard may have traveled completely through the gel and was therefore not detected. These preliminary results suggest that the R&D system kits may be suitable for the detection of a leptin-

like peptide in fish plasmas although further validation and sequencing of the identified protein will be necessary in order to determine the structure of the protein that the antibody is complexing. This is highlighted by the fact that mammalian leptin has a molecular weight of 16kDa, while the protein-antibody complex of the pooled trout plasma was around 25kDa.

Statistical Analysis

Growth performance parameters and plasma hormone levels were analysed by General Linear Models using time, treatment, maturational status and fish as categorical predictors. Homogeneity of variance and normality was checked using residuals plots. Where necessary data was log-transformed to ensure a linear relation was achieved. SGR data was arcsine transformed, with initial weight and length for the start of each time period entered as covariates for SGRwt and SGRL analysis. This compensates for differences in growth rate associated with examining fish of different starting weight or length during each time stanza. Differences in spawning dates were tested using a 2-sample T-test. Differences in peak hormone levels were tested using the Mann-Whitney U-test due to non-parametrically distributed data. Linear relations were assessed using Pearson's product moment correlation, while 2nd order polynomial regressions were used to examine the relationship between water temperature and plasma IGF-I over time. For all statistical tests a significance of $p<0.05$ was used.

4.3 Results

In the presentation of results, photoperiod treatments will be referred to as SNP or ADV regimes for the simulated natural and the advancing photoperiod regimes respectively. For clarity of interpretation, figures for comparison of cohort differences within treatments and between treatments will be presented separately.

4.3.1 Population Structure and Changes in Spawning Time in Relation to Photoperiod Regime

Within the 51 fish in the SNP regime 32 were classified as maturing following stripping of eggs, with 19 remaining immature (Figure 4.3 a). In contrast, of the 51 fish in the ADV regime 15 were classified as maturing following stripping of eggs, with 36 remaining immature (Figure 4.3 a).

Under the SNP regime 63% of the population attained maturity (n=32) and spawned over a 141 day period beginning on the 10th January until the 9th May 2001 (Fig 4.3 b). Peak spawning was attained on the 6th April with 39.6% of the maturing population ovulating. No spawning was recorded from the 7th February until 6th April.

A significant 6 month advancement in spawning time was achieved under the ADV regime ($p<0.0001$, 2-sample T-test) with only 29% of the population maturing (n=15). Spawning occurred over a 63 day period from 10th October to 12th December 2000, although only one fish ovulated in December. Peak spawning was attained on the 23rd October with 15.4% of the maturing population ovulating.

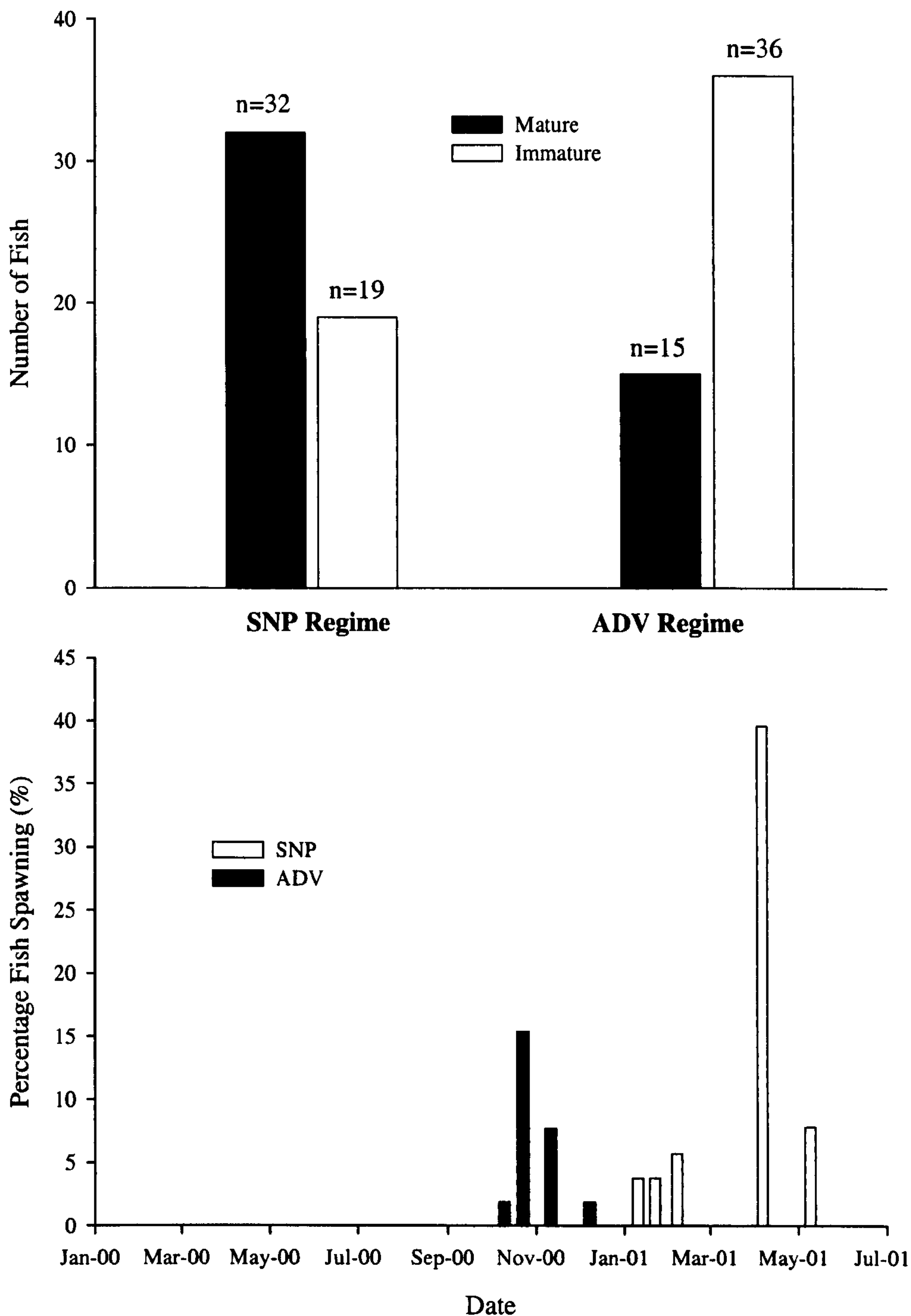


Figure 4.3 (**a**) Population structure of two groups of female rainbow trout maintained under two different photoperiod regimes. (**b**) The effect of an advancing (ADV) photoperiod regime (long-short day) on spawning time and the percentage of the population maturing with respect to the same stock maintained under a simulated natural photoperiod (SNP).

4.3.2 Growth Performance in Relation to Photoperiod Regime

Weight

There were no differences in fish weight (465g ± 35g) at the start of the experiment between treatments as fish were split at random into experimental tanks. No relationship between initial weight and maturational status was found. However, time significantly affected weight gain with differences observed between cohorts within treatments (Figure 4.4 a-b), and between photoperiod treatments (Figure 4.5 a-b). Furthermore, care must be taken when comparing cohort weights around the time of gonadal development and spawning as that total wet weight was measured and will not reflect a true comparison of somatic growth between the cohorts.

Cohort Differences within Treatments

SNP Regime

Fish that remained immature showed a steady increase in weight from January 2000 to May 2001 (time 1-16) (Figure 4.4 a), although a significantly lower mean weight than SNP mature was maintained from September '00 to March '01. Only during the final month (May-June '01) was there a significant increase in the rate of weight gain ($p<0.0002$), with immature fish reaching a statistically larger final weight of 2771 ± 252g relative to those that matured, 2445 ± 61g. Fish which matured under SNP showed a significantly different growth pattern (Figure 4.4 a). From January to July, weight gradually increased reaching a slightly higher mean weight than

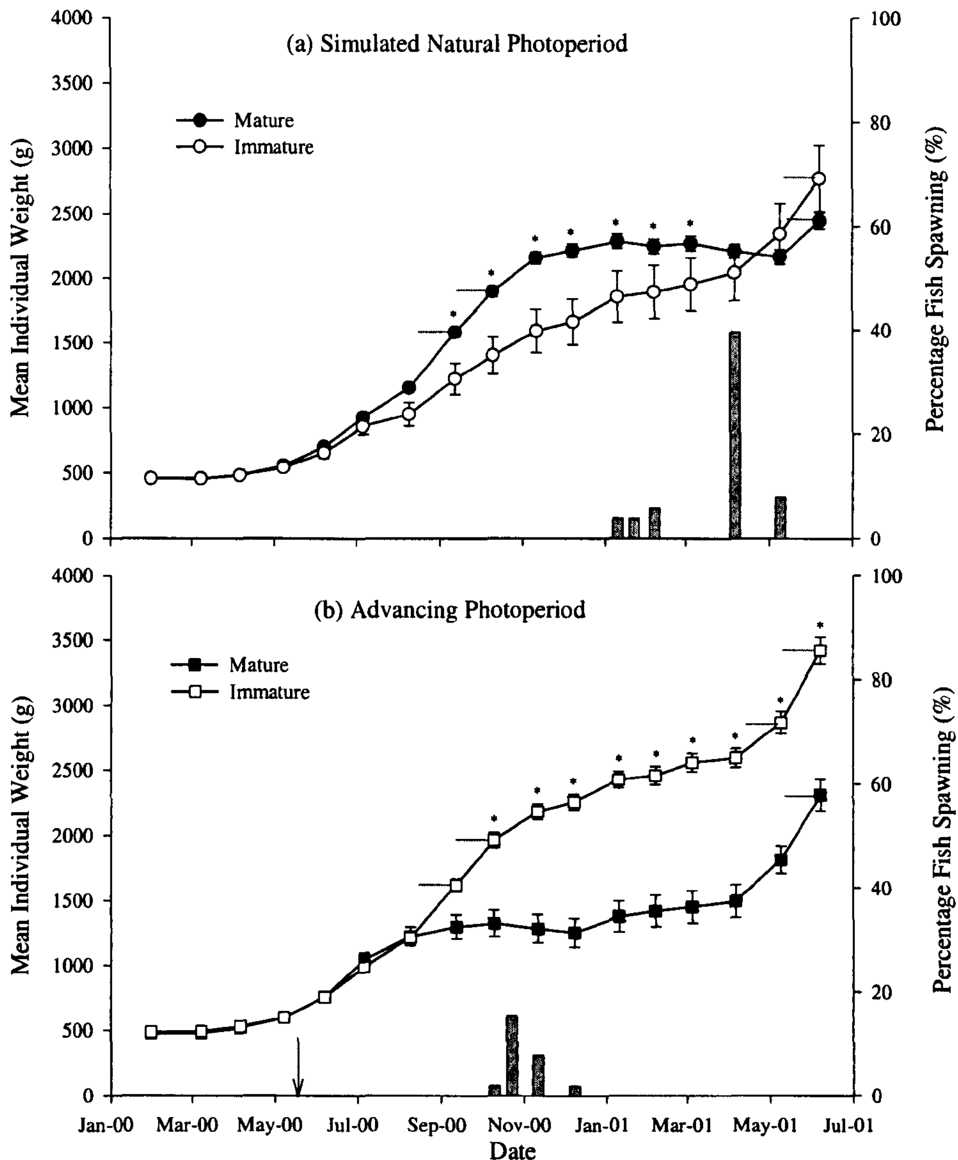


Figure 4.4 Changes in total wet weight of maturing and immature individuals during the maturation cycle of fish maintained under (**a**) simulated natural and (**b**) advancing photoperiod regimes (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). Asterisks (*) denote significant differences between maturational status within the respective photoperiod treatment ($p<0.05$, GLM), while red horizontal bars indicate a significant increase to the previous time point. Vertical bars represent the percentage of the total population spawning at a given time point. Arrow denotes long-short day change in photoperiod of ADV regime.

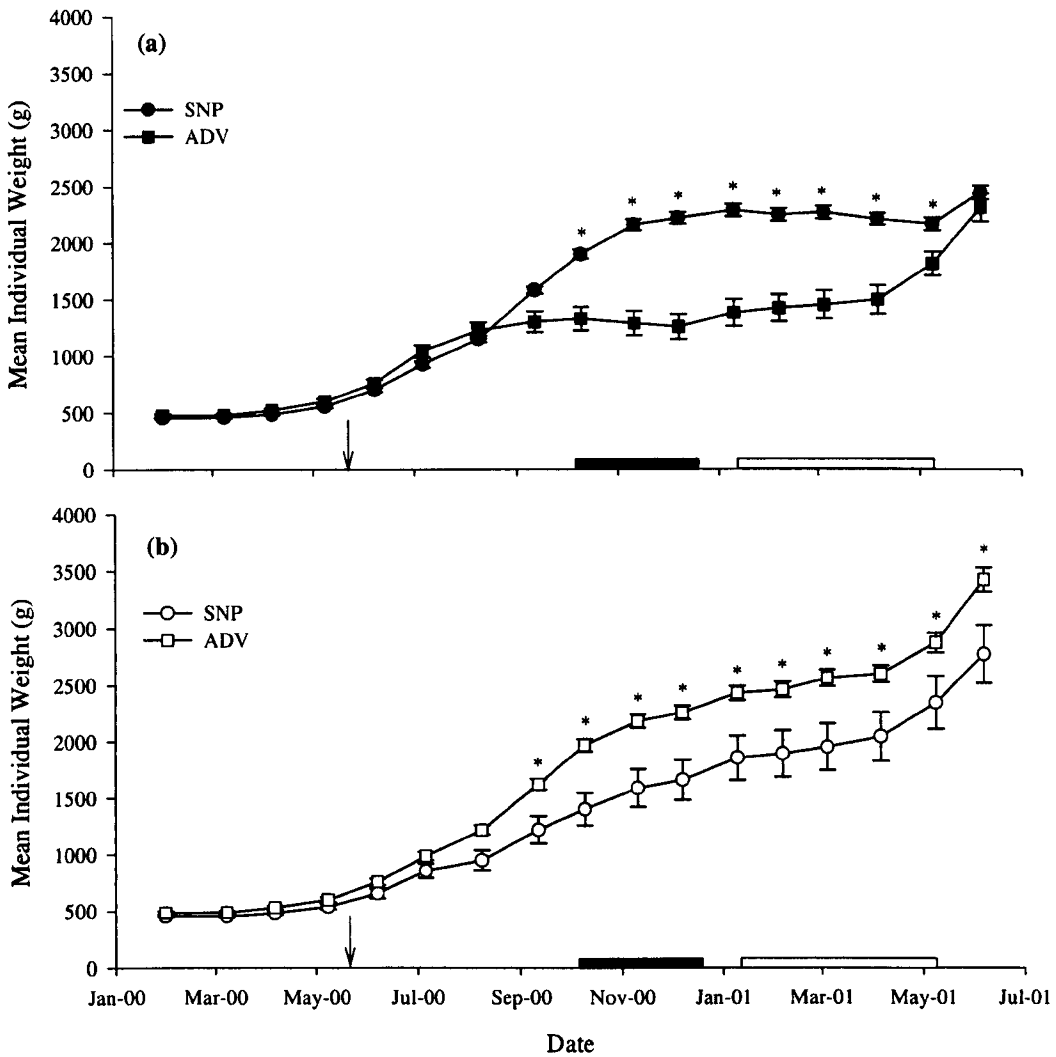


Figure 4.5 Comparison of changes in cohort weight between treatments (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). (**a**) Mature cohorts, (**b**) immature cohorts. Asterisks (*) denote significant differences ($p<0.05$). Black and white horizontal bars indicate ADV and SNP spawning periods respectively. Arrow denotes long-short day change in photoperiod of ADV regime.

immature fish. From July to August (time 6-7) weight increased more rapidly, followed by a two month period (time 7-9) where weight increased at a significantly greater rate ($p<0.002$) to the previous months. As of October 2000 weight reached a plateau of approximately 2285g ± 54g, with further weight gain not observed until the final month following the completion of spawning, June 2001. SNP mature only maintained a significantly greater weight than SNP immature until March '01.

Advancing Photoperiod Regime

As with the SNP regime, mature and immature individuals under the ADV regime exhibited significantly different growth patterns from each other (Figure 4.4 b). Maturing fish showed a gradual increase in weight from January to June 2000 at a similar rate to that of immature fish. From June to July (time 5-6) weight increased at a slightly greater rate than that of the immature fish, with mature fish reaching a mean weight of 1042 ± 55g and immature fish 987 ± 37g ($p>0.05$). Thereafter, maturing fish weight reached a plateau of 1328g ± 103g as of August (time 7). The cessation of weight increase was maintained for 8 months until April 2001 (time 8-16). During the final month (time 17), a significant increase in weight was observed in ADV mature reaching 2313 ± 122g, statistically smaller than ADV immature (3423 ± 104g).

Immature fish under ADV exhibited a gradual increase in weight between January and August 2000, followed by a significant increase in the rate of weight gain ($p<0.0003$) between August and October (time 7-9). From October onwards (time 9-17) ADV immature fish maintained a significantly greater weight than ADV mature. Immature fish weight then increased gradually each month until April '01, after which followed a two month period of significantly greater weight increase achieving a final weight of 3423 ± 104g by June 2001.

<u>*Cohort Differences between Treatments*</u>

Mature Cohorts

From January to August maturing individuals in both treatments increased weight at a similar rate (Figure 4.5 a). ADV mature reached a weight plateau two months earlier than SNP mature, and at a weight significantly smaller than those that matured under SNP. In addition, this plateau lasted two months longer than that observed in SNP mature before weight increase was resumed. Between April and June 2001, two months of rapid weight gain allowed ADV mature to reach a similar weight to SNP mature following completion of spawning in the latter.

Immature Cohorts

Weight increase between the treatments was similar between January and August '00 (Figure 4.5 b). ADV immature exhibited significantly greater weight increase than SNP immature between August and October, with a significantly greater mean weight maintained from September '00 until the conclusion of the experiment in June 2001. Both cohorts showed a slower increase in weight between November '00 and April '01.

Rate of Daily Weight Gain (SGRwt)

Cohort Differences within Treatments

SNP Regime

In both cohorts the changes in SGRwt accurately reflected rising and falling water temperatures (Figure 4.6 a). Between January and July SGRwt in both cohorts increased steadily, with mature individuals showing a significant increase in the rate of gain between May and June ($p<0.0002$), an increase absent in those that remained immature. Highest SGRwt was achieved in both cohorts during July with mature and immature individuals attaining 0.94 and 0.72 % day^{-1} respectively. Both cohorts then exhibited a decrease in SGRwt between July and August, with immature fish expressing a significant decrease in the rate of gain. This decrease was coincident with water temperatures exceeding 14°C during this period. After this period, SGRwt in both cohorts increased to a rate similar to that previously observed in July, before the rate of gain decreased steadily by December concurrent with falling water temperatures. Between December and April '01 no significant differences were observed between cohorts, although mature individuals had a tendency towards a lower SGRwt during the spawning period. At a water temperature of 6°C, immature individuals showed a significant increase in SGRwt between April and May. Following the completion of spawning in May, SGRwt of mature fish increased significantly to a rate similar to immature individuals ($p>0.05$).

Advancing Photoperiod Regime

As with the SNP regime, both cohorts under the advancing regime showed a steady increase in SGRwt between January and July achieving their highest growth rates of 1.06 and 0.91 % day-1 for mature and immature fish respectively (Figure

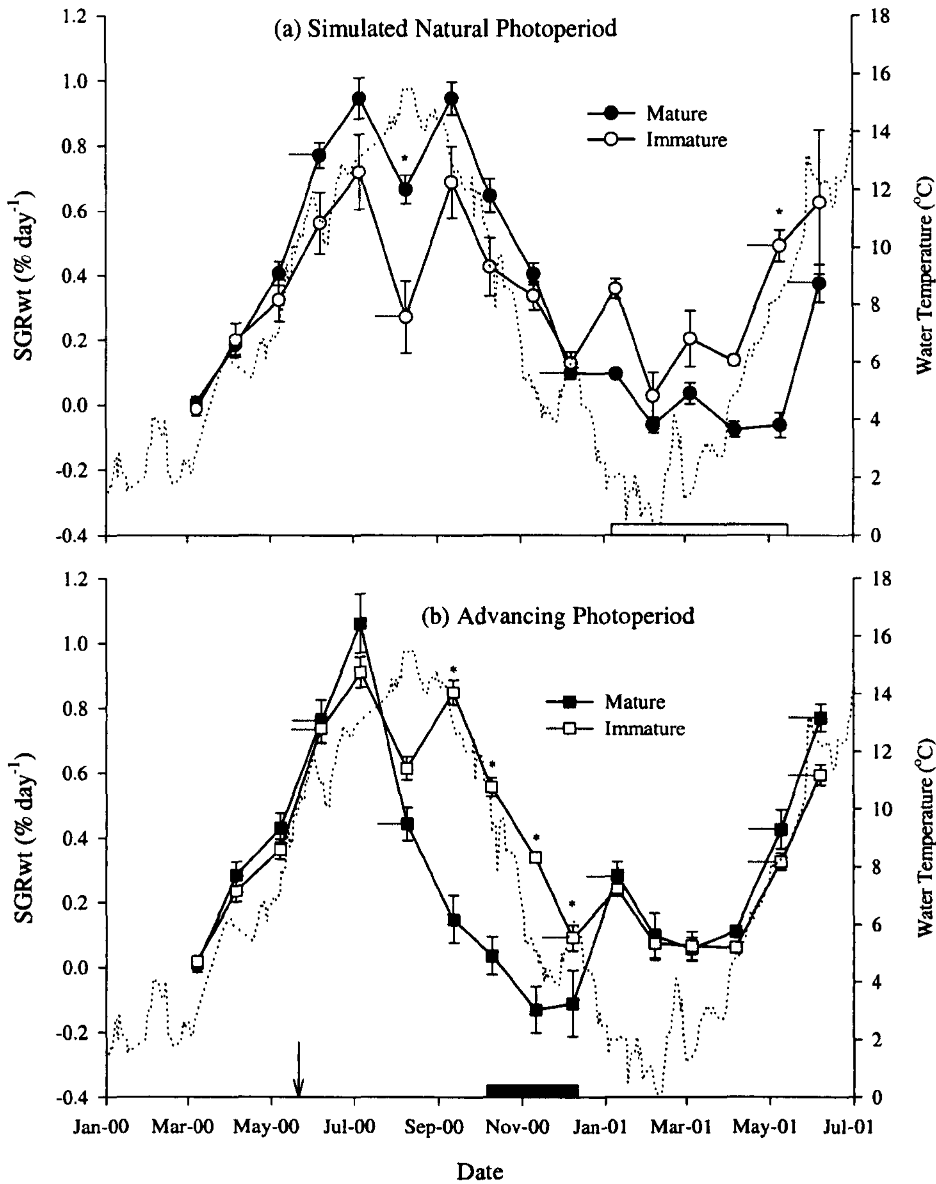


Figure 4.6 Rate of daily weight gain (SGR) of mature and immature individuals during the maturation cycle of fish maintained under (**a**) simulated natural and (**b**) advancing photoperiod regimes (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). Asterisks (*) denote significant differences between maturational status ($p<0.05$), while red horizontal bars indicate significant differences to the previous time point. Black and white horizontal bars indicate ADV and SNP spawning periods respectively. Broken line represents ambient water temperature (°C). Arrow denotes long-short day change in photoperiod of ADV regime.

4.6b). Both cohorts also exhibited a significant increase in the rate of gain between May and June. As of July maturing individuals showed a significant decrease in SGRwt by August, with the decrease continued until the completion of spawning in December. In contrast, those that remained immature increased their rate of gain during August and September, with the SGRwt then decreasing in association with falling water temperatures until December. During this period immature fish maintained a significantly higher SGRwt than mature fish. Following the completion of spawning in December, mature individual SGRwt increased significantly to a rate similar to immature fish ($p>0.05$). As of January '01 both cohorts exhibited a similar increase in SGRwt until the conclusion of the experiment.

Cohort Differences between Treatments

Mature Cohorts

Between January and July SGRwt increased at an identical rate for both cohorts, with both exhibiting the same significant increase in the rate of gain between May and June (Figure 4.7 a). From September to December SNP mature maintained a significantly higher SGRwt than ADV mature, the period in which the latter entered the final stages of maturation. From April to June coincident with the main spawning period of SNP mature, ADV mature maintained a significantly greater SGRwt, with the rate increasing in relation to rising water temperatures.

Immature Cohorts

No significant differences were observed in the pattern of SGRwt in immature cohorts between treatments over time, although SNP immature did exhibit a greater decrease in SGRwt during July and August (Figure 4.7 b).

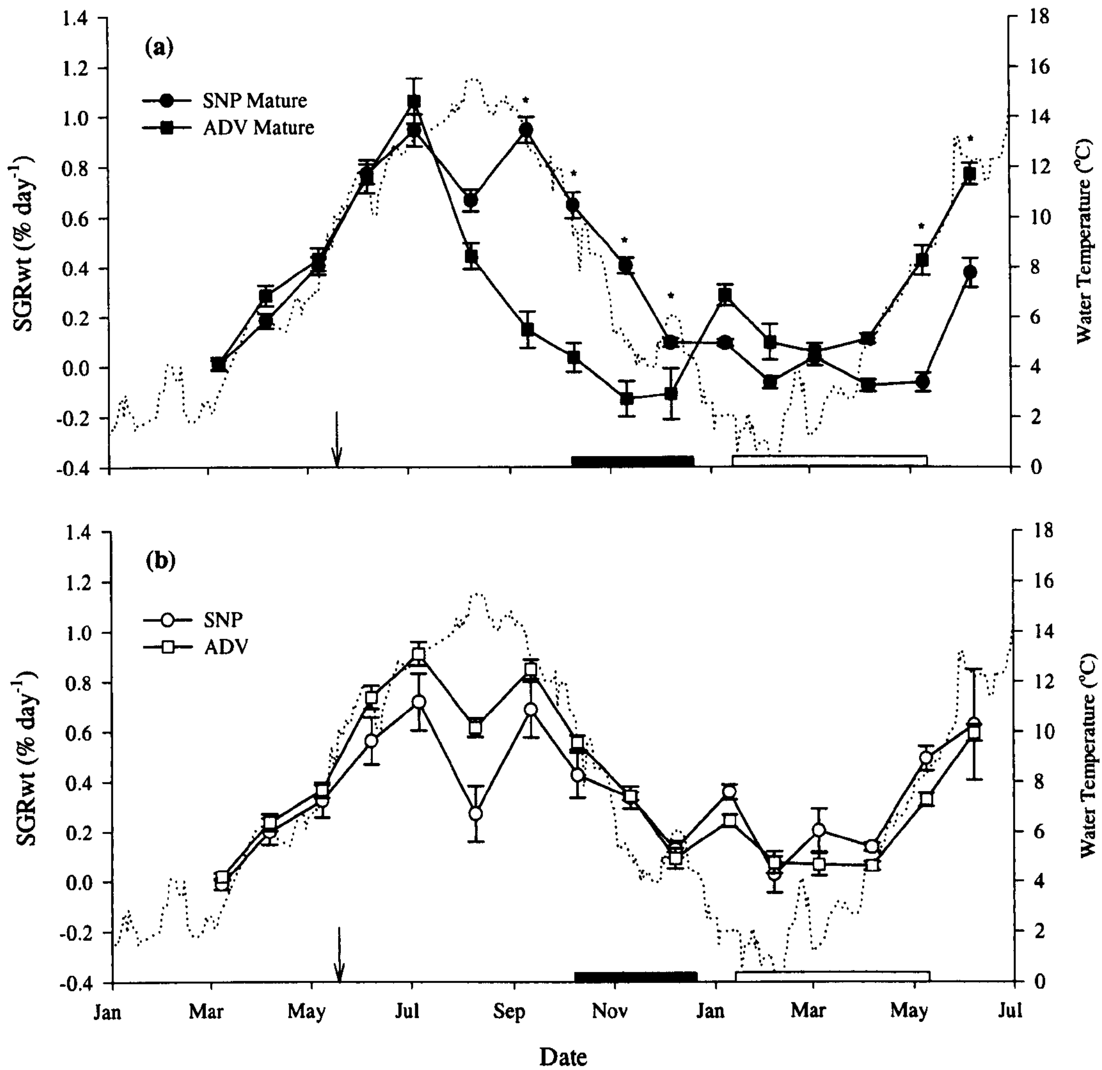


Figure 4.7 Comparison of cohort growth rate (SGRwt) between treatments (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). (**a**) Mature cohorts, (**b**) immature cohorts. Asterisks (*) denote significant differences ($p<0.05$). Black and white horizontal bars indicate ADV and SNP spawning periods respectively. Broken line indicates ambient water temperature (°C). Arrow denotes long-short day change in photoperiod of ADV regime.

Rate of Daily Length Gain (SGRL)

Cohort Differences within Treatments

SNP Regime

SGRL in both cohorts appeared to accurately reflect changes in water temperature, although both groups did express some significant differences in the pattern of growth (Figure 4.8 a).

Mature individuals showed a significant time increase in SGRL between May and July ($p<0.0002$), while immature SGRL only increased significantly from June to July. However, no significant differences were observed between the cohorts from January to July, although maturing fish had a tendency towards maintaining a higher rate of gain. Mature fish then maintained a three month period of growth with a significantly higher SGRL than immature fish, which preceded a significant decrease in SGRL between October and November. Between December and April both cohorts maintained a similarly low SGRL in association with cooler water temperatures, with a tendency for mature fish to have a naturally lower rate of gain during the spawning period. Immature SGRL then increased in association with rising water temperatures during the final two months, while mature fish exhibited a significant increase in SGRL post-spawning maintaining a daily rate of gain similar to immature fish ($p>0.05$).

ADV Regime

SGRL in both cohorts increased steadily in conjunction with rising water temperatures between January and July '00, with no significant differences observed at any time point (Figure 4.8 b). Mature individuals then showed a significant

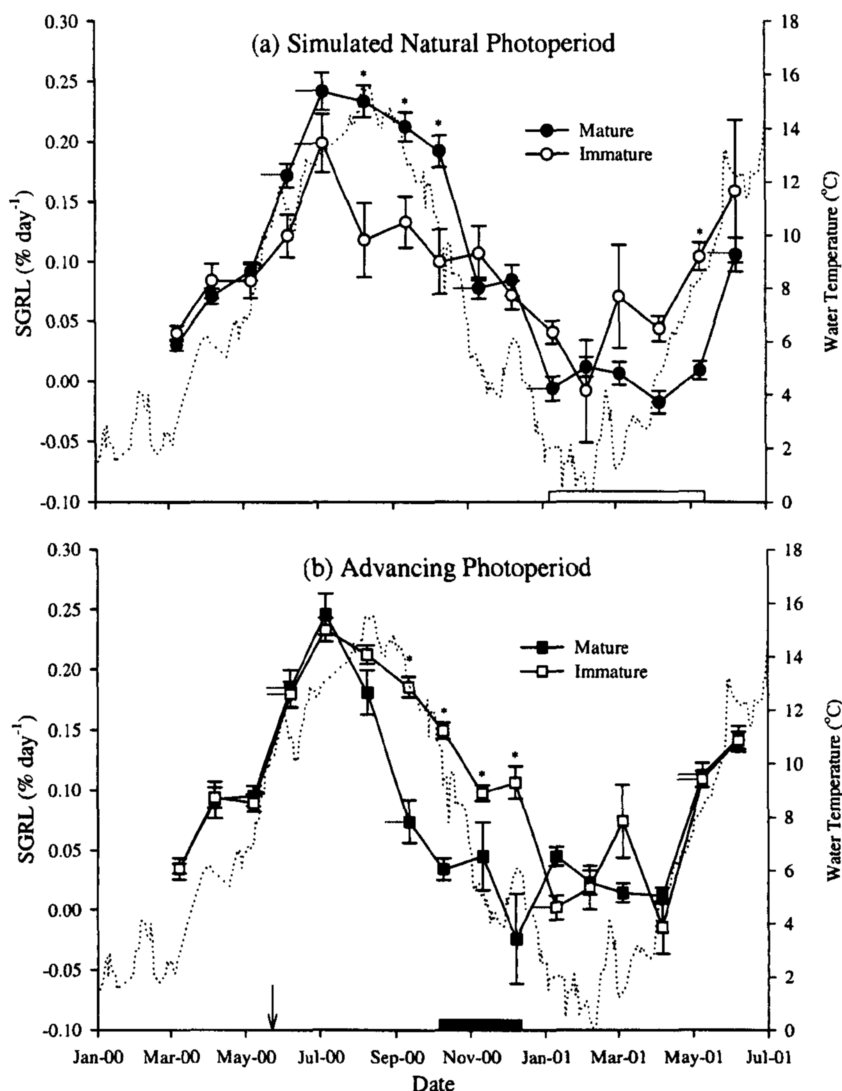


Figure 4.8 Rate of daily length increase (SGR) of mature and immature individuals during the maturation cycle of fish maintained under (**a**) simulated natural and (**b**) advancing photoperiod regimes (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature).. Vertical bars represent the percentage of the total population spawning at a given time point. Asterisks (*) denote significant differences between maturational status ($p<0.05$), while red horizontal bars indicate significant differences to the previous time point. Black and white horizontal bars indicate ADV and SNP spawning periods respectively. Broken line represents ambient water temperature (°C). Arrow denotes long-short day change in photoperiod of ADV regime.

decrease in the rate of gain during the next two months, with SGRL continuing to decline to 0% day^{-1} by December '00 following completion of the spawning period.

Similarly, the immature cohort showed a similar decline in SGRL albeit at a more gradual rate, in which a significantly higher rate of gain relative to mature fish was maintained from September to December. From December until the conclusion of the experiment no further differences in SGRL were observed, with rates increasing significantly in relation to rising water temperatures in spring.

<u>*Cohort Differences between Treatments*</u>

Mature Cohorts

Significant differences between the cohorts were only observed in relation to their entry into their main spawning periods (Figure 4.9 a). SNP individuals maintained a higher SGRL than the ADV regime between September, October and December, but not November most likely in association with the rapid decrease in water temperature from 6 to 2°C (Figure 4.9 a). Only as water temperatures rose in spring did ADV mature individuals increase SGRL to a significantly greater rate than SNP individuals, until the latter completed spawning, at which point no difference in SGRL were observed.

Immature Cohorts

Only during August did fish under SNP maintain a significantly higher SGRL than those under the ADV regime (Figure 4.9 b). At no other time pint were differences observed, with both cohorts exhibiting an identical change in SGRL over time.

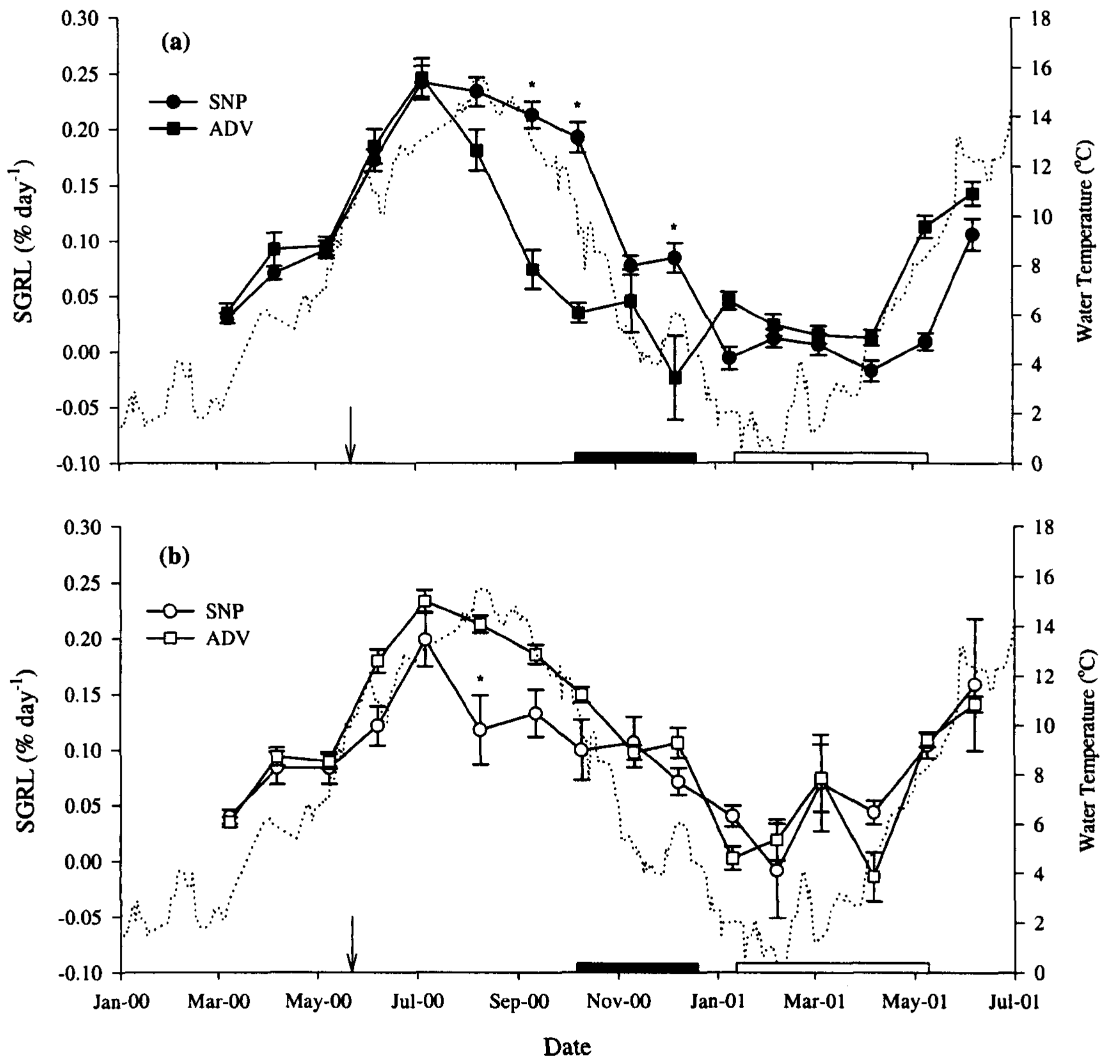


Figure 4.9 Comparison of cohort growth rate (SGRL) between treatments (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). (**a**) Mature cohorts, (**b**) immature cohorts. Asterisks (*) denote significant differences ($p<0.05$). Black and white horizontal bars indicate ADV and SNP spawning periods respectively. Arrow denotes long-short day change in photoperiod of ADV regime.

Condition Factor (CF)

Cohort Differences within Treatments

SNP Regime

Both cohorts showed a significant increase in CF over time throughout the experiment ($p<0.0002$) (Figure 4.10 a). In general, maturing individuals showed a tendency towards having a higher CF from June until January, although the differences were not significant with the exception of the November and December sample points. The higher CF at this point may be an artefact of increased gonadal growth prior to the commencement of spawning in January.

However, maturing fish showed significant time increases in CF between June-July and August-September (periods prior to expected significant gonadal growth). From September to the conclusion of the experiment, CF did not change significantly in maturing fish, maintaining a range between 1.62 and 1.67 ($p>0.05$).

Immature fish only showed a significant time increase in CF between August-September. Thereafter, CF increased steadily, achieving a significantly higher CF than maturing individuals during the final two months (1.83) of the experiment as the latter completed spawning (Figure 4.10 a).

ADV Regime

As observed under the SNP regime, maturing individuals showed a tendency towards maintaining a higher CF than immature fish, albeit from an earlier time period of May until July (Figure 4.10 b). Thereafter, CF declined steadily from 1.57 to 1.33, no different to that at the start of the experiment as maturation progressed and spawning was completed. Post-spawning CF gradually increased with time, showing a significant increase during the final month to 1.72.

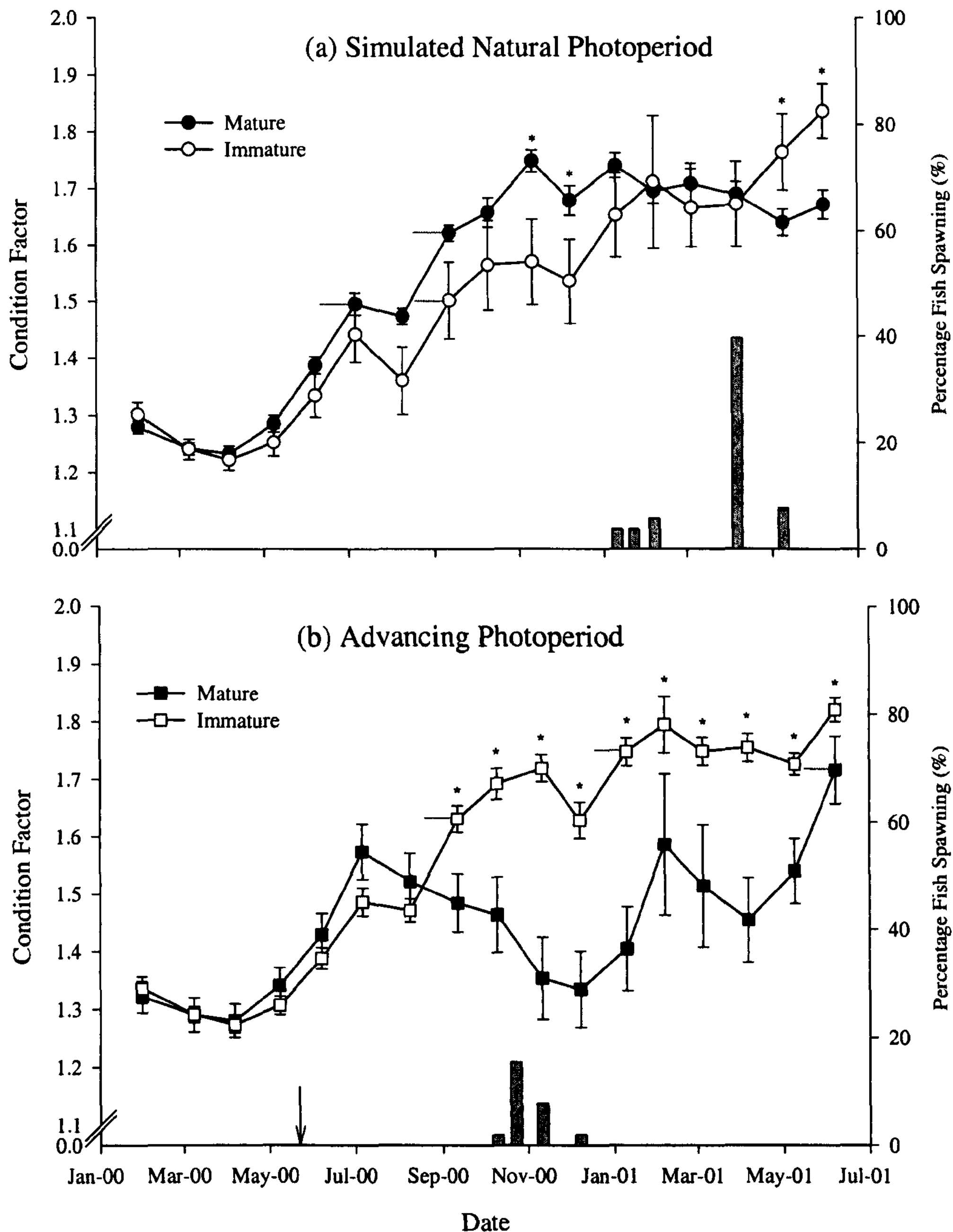


Figure 4.10 Change in condition factor of mature and immature individuals recorded throughout the maturation cycle of fish maintained under (**a**) simulated natural and (**b**) advancing photoperiod regimes (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). Vertical bars represent the percentage of the total population spawning at a given time point. Asterisks (*) denote significant differences between maturational status ($p<0.05$). Arrow denotes long-short day change in photoperiod of ADV regime.

In contrast, immature fish showed a steady increase in CF over time (1.33 to 1.82), maintaining a significantly higher CF than mature individuals from September until the conclusion of the experiment (Figure 4.10 b).

Cohort Differences between Treatments

Mature Cohorts

Significant differences in CF between the two cohorts were only observed during entry and throughout the spawning periods of the two treatments (Figure 4.11 a). SNP individuals maintained a steady increase in CF from August until December, while ADV CF declined steadily as spawning approached and progressed. On completion of spawning CF then increased, achieving a similar CF as SNP fish by May following completion of spawning of the latter. SNP mature maintained a significantly higher CF than ADV fish from September to April (1.62-1.67, $p>0.05$), but did not show a decrease in CF as ADV did on entry into their spawning period.

Immature Cohorts

Both cohorts showed a significant increase in CF over time, with ADV individuals achieving a significantly higher CF between September and November (Figure 4.11 b). At no other time points were consistent differences observed.

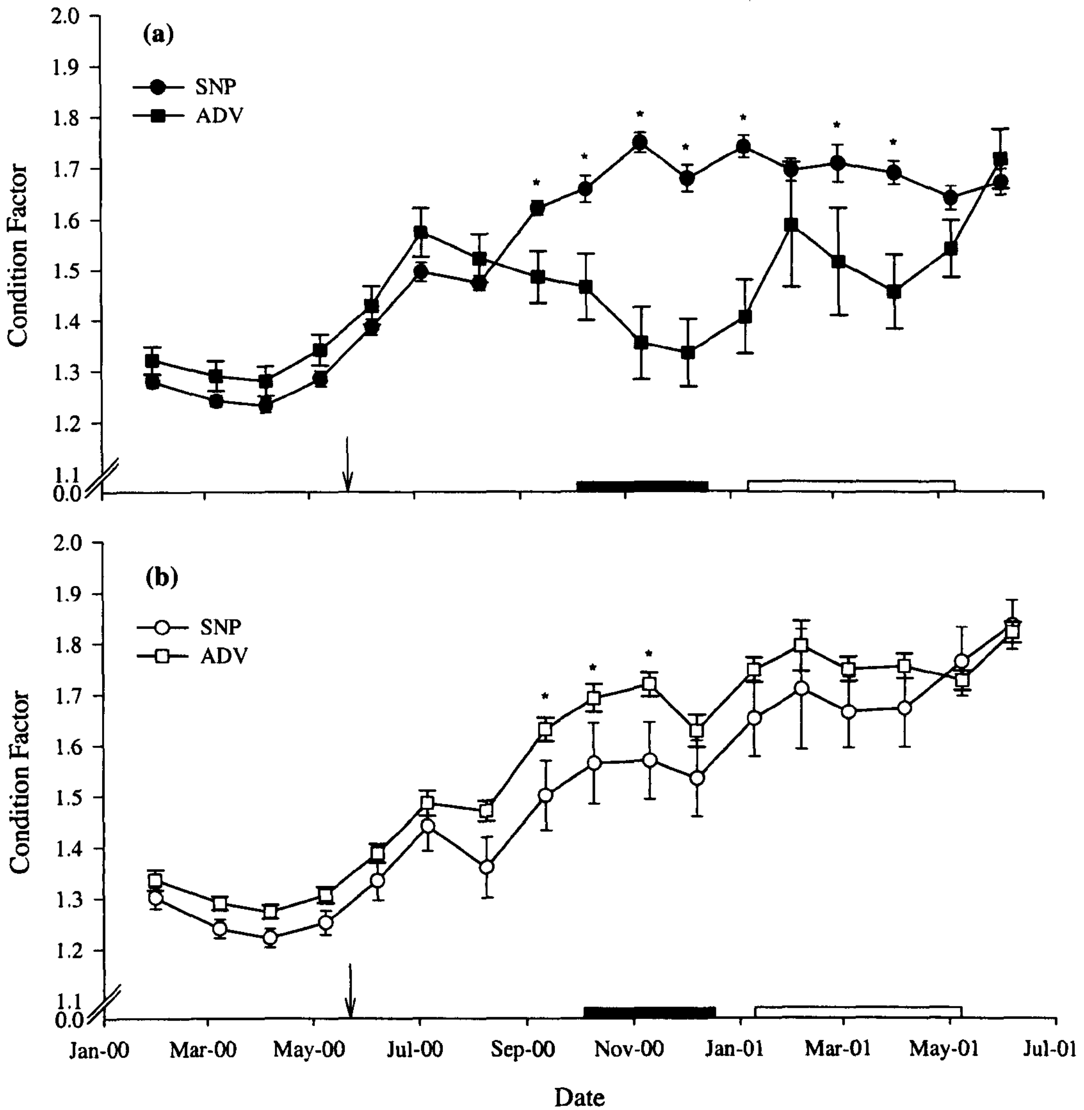


Figure 4.11 Comparison of change in cohort condition factor between treatments (mean ± SEM; n=32 for SNP mature, n=15 for ADV mature, n=19 for SNP immature, and n=36 for ADV immature). (**a**) Mature cohorts, (**b**) immature cohorts. Asterisks (*) denote significant differences ($p<0.05$). Black and white horizontal bars indicate ADV and SNP spawning periods respectively. Arrow denotes long-short day change in photoperiod of ADV regime.

4.3.3 Hormone Profiles in Relation to Photoperiod Regime

Plasma Testosterone

Immature fish in both photoperiod treatments showed no increase in plasma testosterone above basal levels (4.5ng/ml) throughout the reproductive cycle (Figure 4.12 a & b). However, of the 19 immature fish under SNP on the basis of a significant increase in plasma testosterone (Figure 4.12a) but failure to spawn 4 individuals were reclassified as having arrested maturation (Figure 4.13). Similarly on the basis of significant increases in plasma testosterone (Figure 4.12b) but failure to spawn 5 individuals of the 36 immature fish were reclassified as having arrested maturation in the ADV regime (Figure 4.13). However, as sampling was carried out at monthly intervals this may not represent the total amount of fish that arrested maturation as testosterone increases may have been missed.

From this point onwards the three cohort classes will be referred to as mature, immature and arrested. Following this observation immature growth parameters were re-evaluated, but no significant differences in growth parameters were found between immature and arrested cohorts.

In fish that were classified as having arrested maturation, plasma testosterone increased significantly above basal levels between August and October and from November to January for ADV and SNP regimes respectively (Figure 4.14 a & b). At no other time point did levels differ from immature fish under their respective regimes.

Maturing fish in both treatments exhibited significant increases in plasma testosterone levels during the reproductive cycle ($p < 0.001$). However, there were significant differences in the timing of these changes associated with the differences in spawning time (Figure 4.12 c).

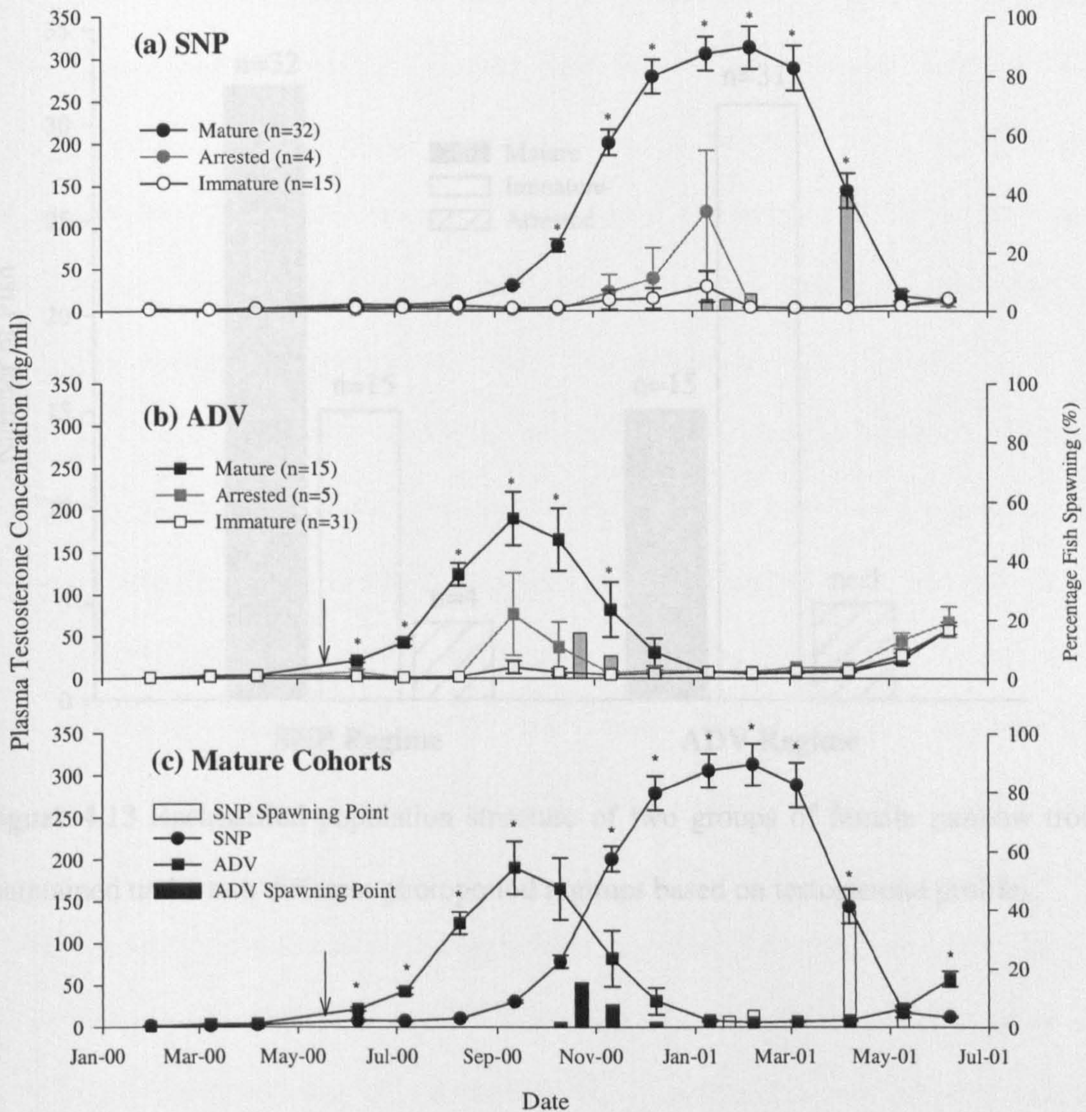


Figure 4.12 Plasma testosterone levels (ng/ml, mean ± SEM) of mature, arrested and immature cohorts exposed to **(a)** simulated natural photoperiod or **(b)** an advancing photoperiod regime (ADV), with asterisks (*) denoting significant differences between mature and arrested/immature cohorts (p<0.05). **(c)** Comparison of changes in timing of plasma testosterone increases in maturing individuals maintained under SNP and ADV photoperiod regimes. Asterisks (*) denote significant differences between treatments (p<0.05). In all figures vertical bars represent the percentage of the total population spawning at a given time point. Arrow denotes long-short day change in photoperiod of ADV regime.

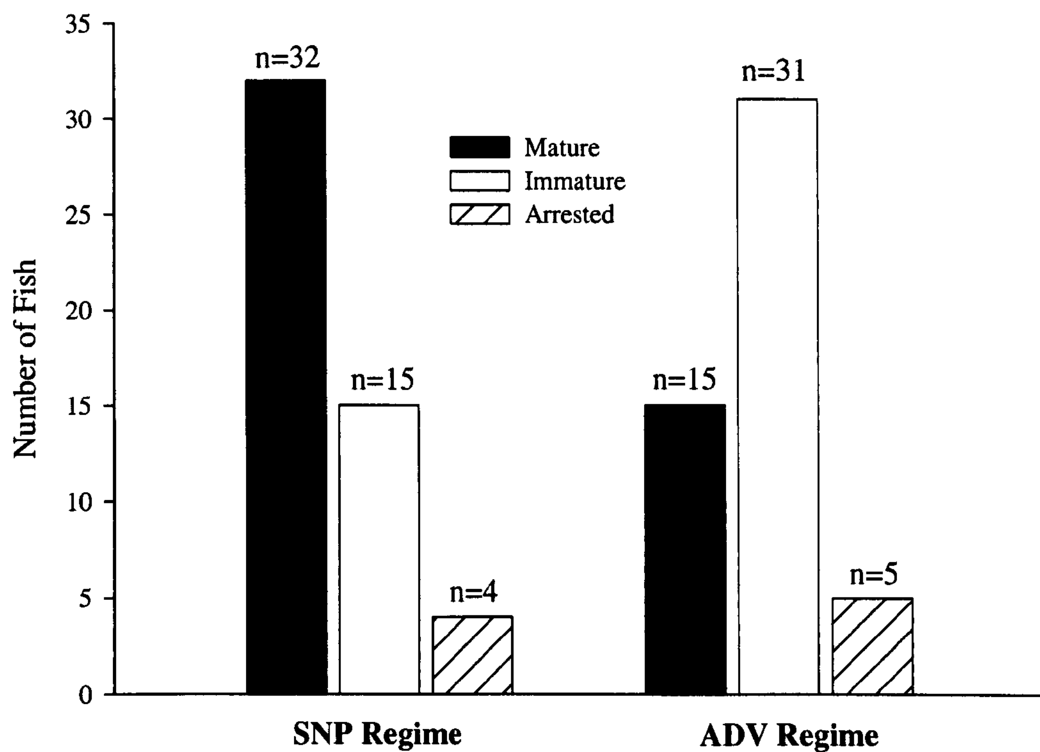


Figure 4.13 Reclassified population structure of two groups of female rainbow trout maintained under two different photoperiod regimes based on testosterone profiles.

Under SNP, a significant increase was observed between August and December, with plasma levels remaining elevated until March 2001 (Figure 4.12 a). Peak concentrations (313 ± 25ng/ml) were measured in February, with no significant differences were observed in plasma levels between December and March. This peak was followed by a rapid decrease to basal levels from March to May 2001 in association with the commencement of the main spawning period.

In contrast, maturing individuals under the ADV regime exhibited a gradual increase in plasma testosterone from April to July 2000, followed by a significant and rapid rise until September in which peak levels (190 ± 32ng/ml) were achieved (Figure 4.12 b). Peak concentrations were significantly lower than that achieved by fish under the SNP regime ($p<0.005$, Mann-Whitney U-Test). Levels remained elevated until October, after which plasma concentration decreased to basal levels by December 2000 and remained low thereafter. However, towards the end of the experiment, mature fish under the ADV regime exhibited a significant increase in testosterone (58 ± 9ng/ml) above basal levels (7.8 ± 0.5ng/ml) from May 2001 until the culmination of the experiment. This trend was also evident in the immature fish and fish that arrested maturation (Figure 4.12 b).

Plasma Calcium

Immature fish in both photoperiod treatments showed no increase in plasma calcium above basal levels (13mg/dL) throughout the reproductive cycle (Figure 4.14 a & b).

Under SNP fish that were classified as having arrested maturation showed a slight but non-significant elevation in plasma calcium between December and January

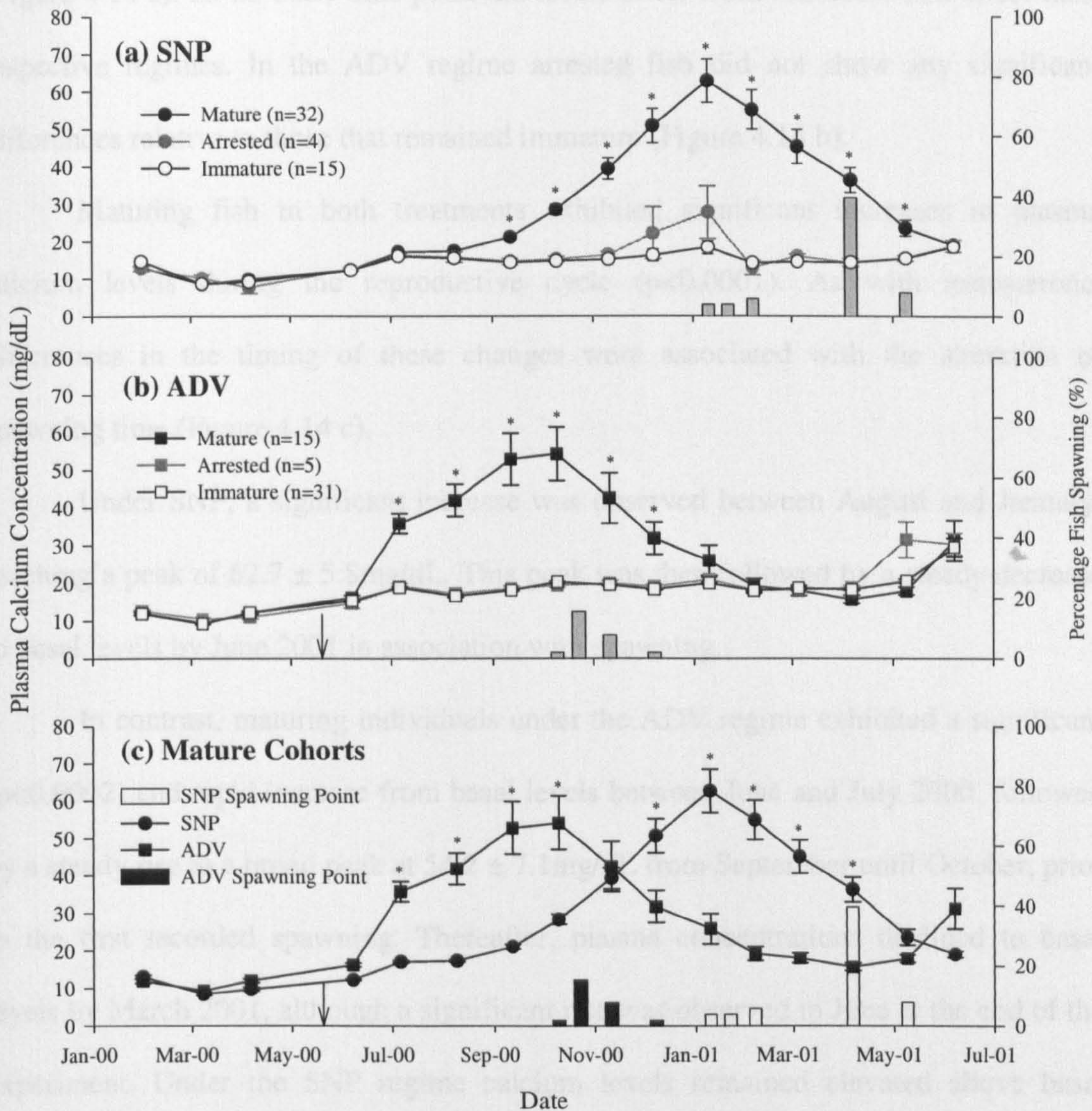


Figure 4.14 Plasma calcium levels (mg/dL, mean ± SEM) of mature, arrested and immature cohorts exposed to (**a**) simulated natural photoperiod or (**b**) an advancing photoperiod regime (ADV), with asterisks (*) denoting significant differences between mature and arrested/immature cohorts ($p<0.05$). (**c**) Comparison of changes in timing of plasma calcium increases in maturing individuals maintained under SNP and ADV photoperiod regimes. Asterisks (*) denote significant differences between treatments ($p<0.05$). In all figures vertical bars represent the percentage of the total population spawning at a given time point. Arrow denotes long-short day change in photoperiod of ADV regime.

(Figure 4.14 a). At no other time point did levels differ from immature fish under their respective regimes. In the ADV regime arrested fish did not show any significant differences relative to those that remained immature (Figure 4.14 b).

Maturing fish in both treatments exhibited significant increases in plasma calcium levels during the reproductive cycle ($p<0.0001$). As with testosterone, differences in the timing of these changes were associated with the alteration of spawning time (Figure 4.14 c).

Under SNP, a significant increase was observed between August and January, reaching a peak of 62.7 ± 5.8mg/dL. This peak was then followed by a steady decrease to basal levels by June 2001 in association with spawning.

In contrast, maturing individuals under the ADV regime exhibited a significant ($p<0.0002$) and rapid increase from basal levels between June and July 2000, followed by a steady rise to a broad peak at 54.2 ± 7.1mg/dL from September until October, prior to the first recorded spawning. Thereafter, plasma concentrations declined to basal levels by March 2001, although a significant rise was observed in June at the end of the experiment. Under the SNP regime calcium levels remained elevated above basal concentrations for a period of 9 months compared to only 6 months under the ADV regime.

Total Plasma Insulin-like Growth Factor-I

Cohort Differences within Treatments

SNP Regime

Both mature and immature cohorts exhibited a similar change in plasma IGF-I over time, with both having low levels between January and June, followed by significantly higher levels from July to October (Figure 4.15 a). Only in July '00 did

mature fish achieve a significantly higher IGF-I level relative to immature fish (24.3 ± 4ng/ml cf. 14 ± 2ng/ml) principally due to a large individual variability. At this point cohorts were not significantly different in terms of weight (Figure 4.4 a) although, SGRwt, SGRL and condition factor was generally greater in maturing individuals (Figure 4.6 a, 4.8 a & 4.10 a). A similar, but non-significant time increase in IGF-I levels was also observed in immature fish during this period. Thereafter levels decreased significantly, and remained low between November and May, followed by a significant increase from May to June '01 in both cohorts. A significant curvilinear relationship (2nd order polynomial) was found between mean plasma IGF-I and water temperature throughout the experiment in both cohorts irrespective of maturation (r^2 0.76-0.79, $p<0.0002$) (Figure 4.16 a & b). A positive linear relationship (r^2 0.61, $p<0.004$) was also found between daylength and plasma IGF-I in both cohorts (4.16 c & d).

As the experiment was carried out under the NERC ROPA award the provision of plasma IGF-I profiles was necessary for the first deliverable, as such plasma testosterone was assayed after IGF-I. In this respect 10 bloods were randomly selected for IGF analysis from each cohort and photoperiod treatment, and unfortunately only included one immature fish that was later classified as having arrested maturation following testosterone analysis. Remaining bloods have been retained for future analysis. This individual showed a substantial increase in plasma levels between June and July as observed in maturing individuals (Figure 4.15 a). This rapid increase was then followed by a sharp decrease during the following month to levels similar to those in June. Thereafter, plasma IGF-I followed an identical pattern to immature individuals with the exception of a large peak recorded in October.

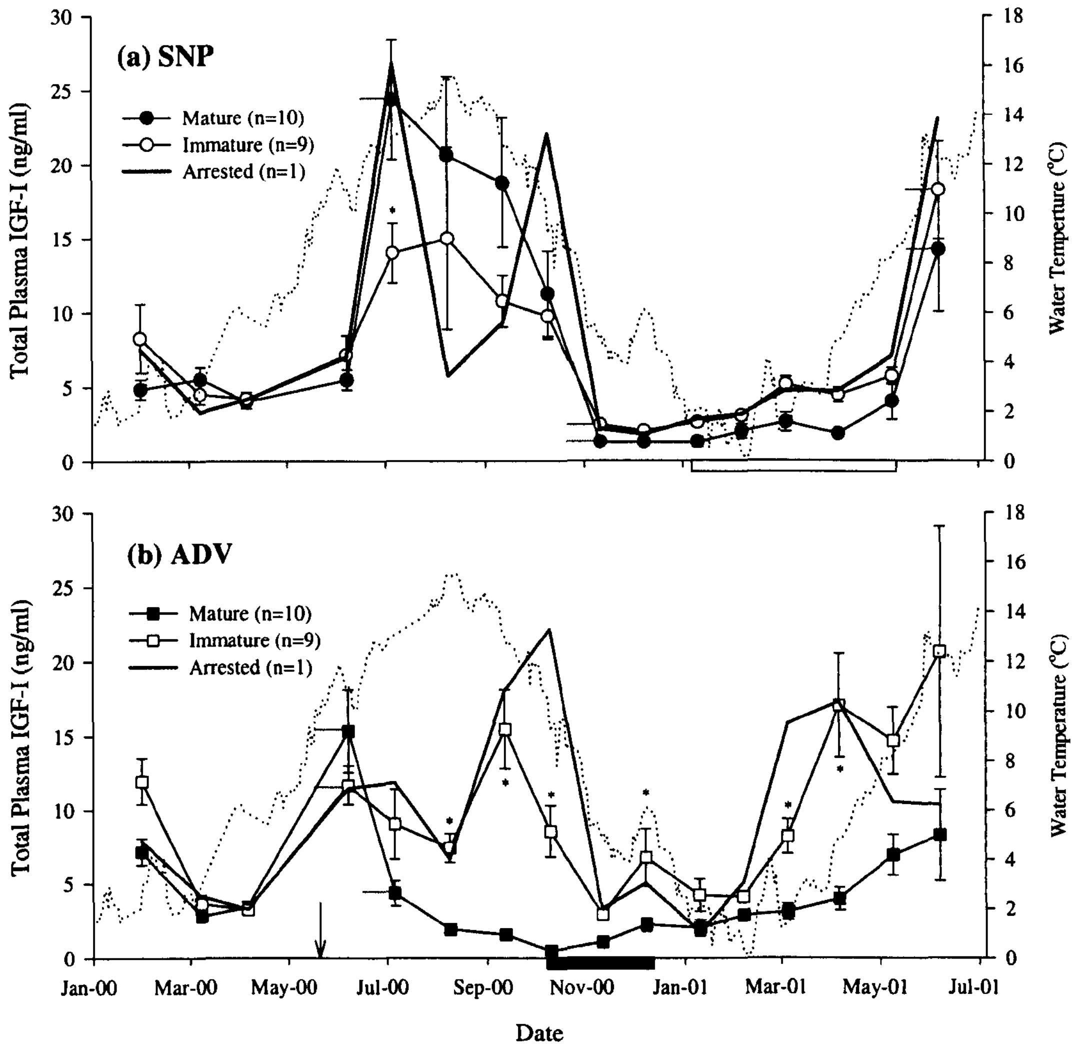


Figure 4.15 Change in total plasma IGF-I (ng/ml) of mature, maturation arrested and immature individuals recorded throughout the maturation cycle of fish maintained under (**a**) simulated natural and (**b**) advancing photoperiod regimes (mean ± SEM; n=10 for SNP and ADV mature, n=9 for SNP and ADV immature, n=1 for SNP and ADV arrested). Asterisks (*) denote significant differences between mature and immature individuals only ($p<0.05$). White and black horizontal bars represent spawning periods. Red horizontal bars indicate significant differences to the previous time point. Broken line represents ambient water temperature (°C). Arrow denotes long-short day change in photoperiod of ADV regime.

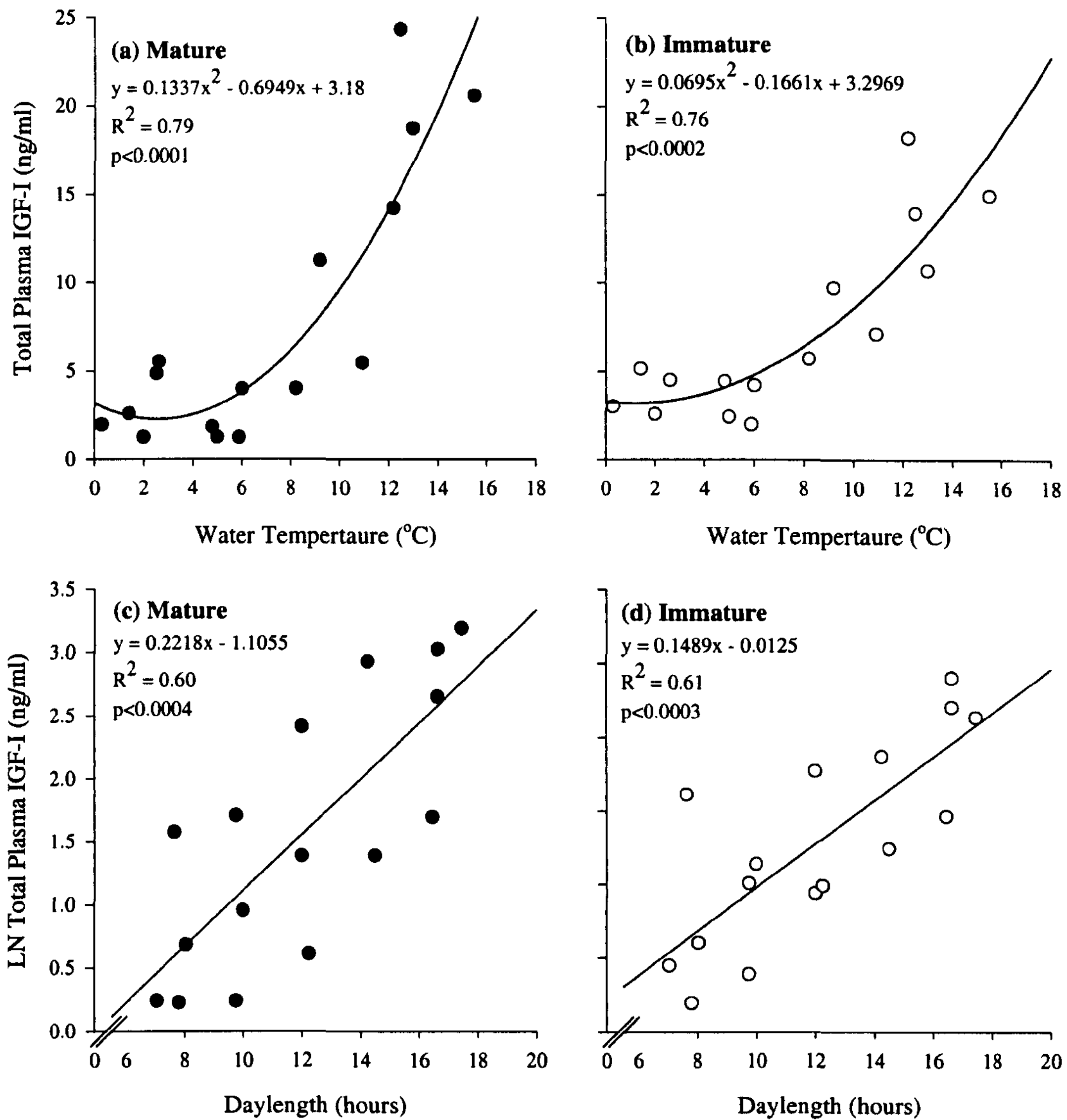


Figure 4.16 Relationship between total plasma IGF-I and water temperature in (**a**) mature and (**b**) immature fish under the SNP regime over the duration of the experiment. Correlation between total plasma IGF-I (log transformed to improve clarity) and daylength in (**c**) mature and (**d**) immature fish under the SNP regime over the duration of the experiment. Each point represents the mean plasma IGF-I of 10 and 9 fish for mature and immature fish respectively.

ADV Regime

No significant differences were found between the mature and immature cohorts from January to April '00. Thereafter, the two cohorts showed significantly different patterns of plasma IGF-I production (Figure 4.15 b).

Maturing fish showed a significant increase in plasma levels from 3.5 ± 0.3 to 15.3 ± 2.3ng/ml between April and June. As in the SNP regime cohorts were not significantly different in terms of weight at this time period (Figure 4.4 b) although SGRwt and condition factor was generally greater in maturing individuals (Figure 4.6 b & 4.10 b). This peak, was then followed by a significant decrease to 4.4 ± 0.8ng/ml during the following month. From August '00 to April '01, levels remained low (1.1 to 3.9 ng/ml) and did not differ significantly over time with the exception of October (0.5 ± 0.01ng/ml) when spawning commenced ($p<0.0002$). Post spawning, plasma levels gradually increased reaching 8.9 ± 3ng/ml by June '01.

Similarly, immature individuals showed a significant increase in plasma levels between April and June, reaching 11.7 ± 1.2ng/ml, statistically similar to maturing individuals. Plasma levels also decreased between June and August, before increasing to 15.5 ± 2.6ng/ml in September '00. IGF-I levels then decreased and increased in a manner similar to the change in ambient water temperatures (Figure 4.15 b). Over time immature fish maintained significantly higher plasma levels than maturing individuals from August to October, during December, and from March to April '01.

No significant relationships between either water temperature or daylength was found relative to plasma IGF-I in either cohort over time when all time points were included. However, removal of July to November points from mature cohorts, coincident with significantly elevated plasma testosterone (equivalent to initiation of final maturation and spawning period), showed a significant curvilinear relationship

(2nd order polynomial, r^2 0.69, $p<0.018$) between water temperature and plasma IGF-I (Figure 4.17 a). A similar relationship (r^2 0.57, $p<0.018$) was also found in immature cohorts with the exception of July and August time points (Figure 4.17b).

As with the SNP regime, again only one maturation arrest individual was included for IGF analysis. However, plasma levels followed a very similar pattern to immature individuals, again with the exception of the presence of a large peak in October (Figure 4.15 b).

<u>*Cohort Differences between Treatments*</u>

Mature Cohorts

From January to April '00 plasma concentrations did not differ significantly between the treatments. ADV mature fish showed a significant increase in plasma levels one month earlier than SNP mature (June cf. July), 4 months prior to spawning (Figure 4.18 a). SNP mature reached peak (higher than ADV) levels 7 months prior to commencement of spawning and maintained significantly higher plasma concentrations than ADV mature from July to October. At no other time point were significant differences observed. In both cohorts the decrease in plasma IGF-I from peak levels coincided with the first significant increase in plasma testosterone (Figure 4.18 a).

Immature Cohorts

Plasma levels did not differ significantly between the two treatments from January to November. ADV fish showed significantly higher levels in December following a 2°C rise in water temperature (Figure 4.18 b). Plasma levels then

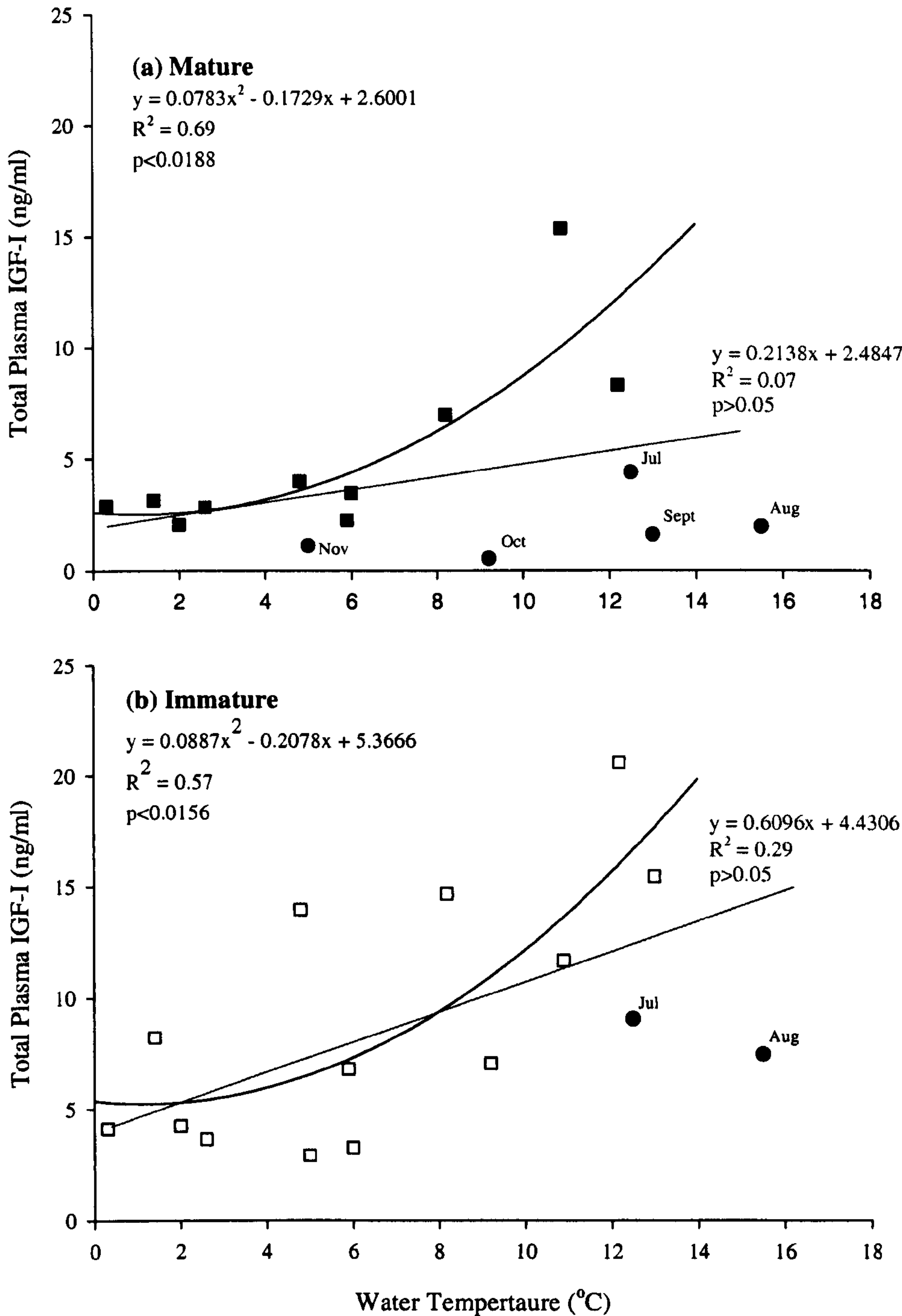


Figure 4.17 Relationship between mean total plasma IGF-I and water temperature in (**a**) mature and (**b**) immature fish under the ADV regime over the duration of the experiment. Each point represents the mean plasma IGF-I of 10 and 9 fish for mature and immature fish respectively. Red regression line represents all points included, while black regression line represents relationship with excluded points. Red circles represent time points excluded from analysis.

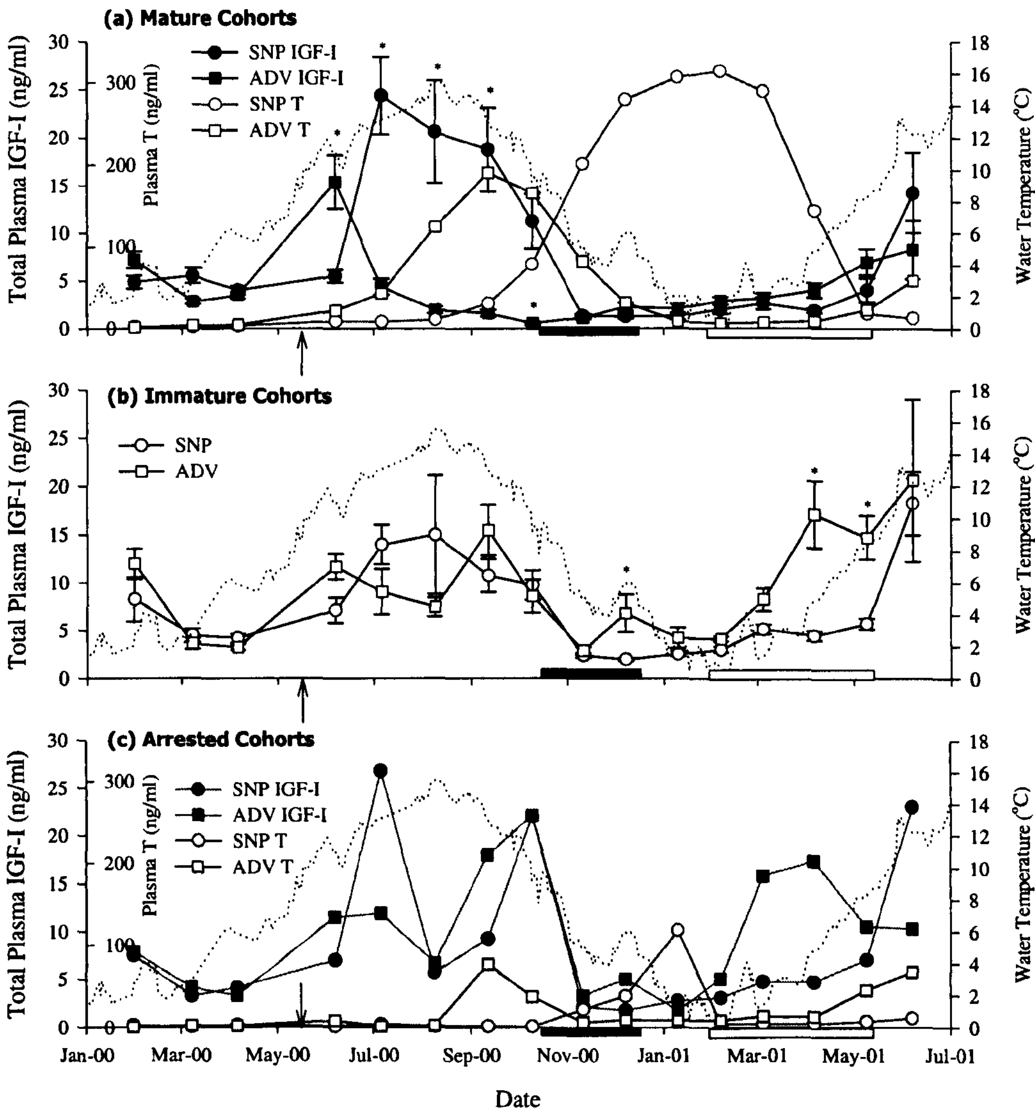


Figure 4.18 Comparison of plasma IGF-I profiles (mean ng/ml ± SEM) of (**a**) maturing, (**b**) immature and (**c**) maturation arrested fish under simulated natural (SNP) and advancing (ADV) photoperiod regimes (n=10). Asterisks (*) denote significant differences between IGF-I levels ($p<0.05$). Blue and red lines with open symbols illustrate change in plasma testosterone (T) levels in ADV and SNP mature and arrested cohorts. Black and white horizontal bars indicate ADV and SNP spawning periods respectively, with the broken line representing ambient water temperature (°C). Arrow denotes long-short day change in photoperiod of ADV regime.

remained similar from January to March '01, before ADV exhibited a significant elevation during the next two months. Only between May and June '01 did SNP fish increase plasma levels significantly, reaching a statistically similar concentration to ADV individuals at the last sample point.

Maturation Arrested Individuals

IGF-I levels in the individual under SNP showed a substantial decrease between October and November (22 to 2.1ng/ml), prior to an increase in testosterone levels (38.1ng/ml) in December (Figure 4.18 c). Plasma IGF-I levels remained at their lowest when the highest testosterone level were recorded in January. Thereafter, testosterone decreased, with IGF-I levels remaining low until the final month in which a rapid increase was observed. Similarly, the individual under the ADV regime showed an increase in IGF-I levels from September to October as testosterone levels decreased. Thereafter levels followed a similar pattern to immature fish (Figure 4.15 b). However, during the final two months (April to June '01) IGF-I levels declined following a second increase in plasma testosterone (Figure 4.18 c).

Plasma IGF-I and Growth Rate

To avoid the influence of gonadal weight on growth rate, plasma IGF-I relationships with growth rate were examined using length-specific growth rate (SGRL). Within the SNP treatment of all sampling dates individual plasma IGF-I levels were significantly related to individual SGRL in maturing fish only in September (r^2=0.59, p<0.025), while a weaker but significant relationship (r^2=0.41, p<0.046) was found in immature fish in July '00 only. Within the ADV regime maturing fish did not show any relationship between individual IGF-I and SGRL at any of the sampling

points, while immature fish showed significant relationships in April '00 (r^2=0.51, p<0.02) and September '00 (r^2=0.57, p<0.02).

When mean monthly plasma IGF-I levels were plotted against mean monthly SGRL for the duration of the experiment (January 2000 to June 2001) both cohorts under SNP showed a significant relationship, while only maturing fish under ADV showed a similar relationship (Table 4.1). ANCOVA revealed no significant differences in slope or elevation between cohorts or treatments.

Table 4.1 Relationship between mean plasma IGF-I (log transformed) and mean SGRL from January 2000 to June 2001 for each cohort under treatment. Gradients and intercepts with common superscripts are not significantly different, while ns signifies not significant (p>0.05)

Cohort	Pearson	F	p	r^2	Gradient	Intercept
SNP Mature	0.799	23.00	0.0004	0.64	9.492[a]	0.66[a]
SNP Immature	0.777	18.29	0.0010	0.60	9.030[a]	0.69[a]
ADV Mature	0.582	6.66	0.0230	0.34	6.631[a]	0.53[a]
ADV Immature	0.201	6.09	ns	0.04	0.021	0.08

To examine the relationship between growth rate prior to testosterone increase, and exclude the possible effect of testosterone on growth rate (Shearer & Swanson, 2000), individual plasma IGF-I was plotted against individual SGRL for March to August 2000 and March to June 2000 for SNP and ADV treatments respectively. Significant relationships were found however ANCOVA revealed no significant differences in slope or elevation between cohorts or treatments (Table 4.2).

Table 4.2 Relationship between individual plasma IGF-I (log transformed) and individual SGRL prior to testosterone increase for each cohort under treatment. Gradients and intercepts with common superscripts are not significantly different.

Cohort	Pearson	F	p	r^2	Gradient	Intercept
SNP (March-August 2000)						
Mature	0.652	27.5	0.0001	0.42	0.069[a]	0.001[a]
Immature	0.703	37.2	0.0001	0.49	0.095[a]	-0.040[a]
ADV (March-June 2000)						
Mature	0.795	44.7	0.0001	0.63	0.068[a]	0.001[a]
Immature	0.768	38.8	0.0002	0.59	0.088[a]	-0.020[a]

Plasma Leptin-like Peptide

As part of the ROPA grant a preliminary investigation into the detection of a leptin-like peptide was made. For each treatment only eight time points and 5 blood samples per cohort were assayed using R&D systems human leptin ELISA kit. Due to the small sample size GLM statistical analysis could not be used to test differences over time, as such t-tests were used to compare specific time points.

Within Treatment Differences

SNP Regime

Immature fish showed no significant change in leptin-like levels over time maintaining a range between 13.2 and 30.8ng/ml, but had significantly lower plasma levels in February and April '01 relative to mature individuals (Figure 4.19 a). Maturing individuals did exhibit a pattern of change over time but it was not statistically significant, possibly due to the small sample size (n=5). However, from August to December ''00, plasma levels showed a gradual decline prior to the spawning period, before exhibiting a significant increase from December to April as

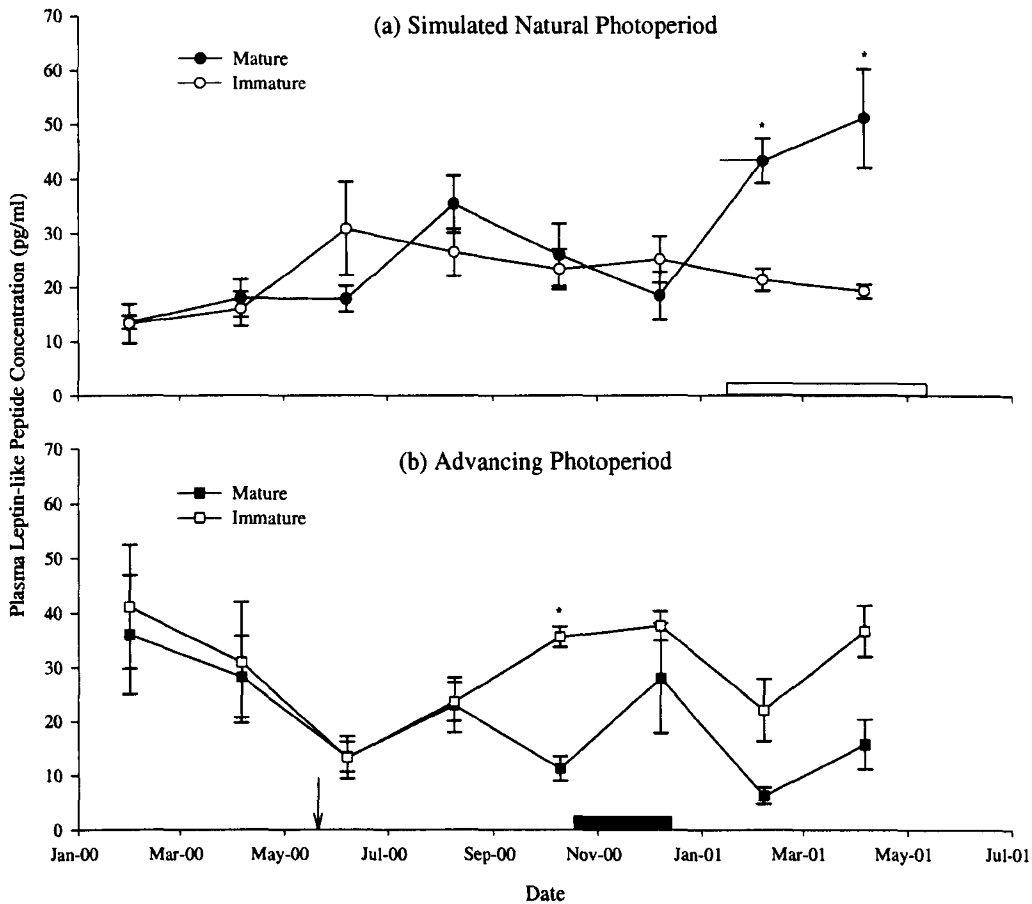


Figure 4.21 Plasma leptin-like peptide concentration (pg/ml) of mature and immature individuals recorded throughout the maturation cycle of fish maintained under (a) simulated natural (SNP) and (b) advancing (ADV) photoperiod regimes (mean ± SEM; n=5 per cohort). Asterisks (*) denote significant differences between cohorts ($p<0.05$). White and black horizontal bars represent spawning periods. Arrow denotes long-short day change in photoperiod of ADV regime.

spawning commenced.

ADV Regime

Both cohorts showed the same decrease in plasma levels from January to June, followed by an increase by August (Figure 4.21 b). Thereafter, immature fish levels continued to increase until December, during which period a significantly higher plasma concentration was seen in October relative to mature individuals. In contrast, mature fish showed a decrease in plasma levels from August to October as the spawning period approached. On completion of spawning in December, plasma levels then rose to statistically similar levels to immature fish. Thereafter, both cohorts followed a similar fall and rise in plasma levels between December and April '01, with a tendency for fish which had matured to have lower plasma concentrations.

Using condition factor as an indirect measurement of total body lipid (Herbinger & Friars, 1991) no correlation was found with plasma leptin levels in both photoperiod treatments and cohorts at any sampling point or over time (r^2<0.12). Similarly no correlations were found when body weight was used.

4.4 Discussion

4.4.1 Effect of Long- Short Photoperiods on Spawning Time

As anticipated the photoperiod regimes used within this chapter produced two populations with significantly different spawning times and numbers of fish spawning and confirms the importance of photoperiod as the principal environmental cue in the entrainment of the reproductive cycle in rainbow trout. Although 7 fish spawned in January and February under SNP which may reflect a modulatory role of fluctuating

water temperature during this period (Davies & Bromage 2002), the main spawning season was observed between late April and early-May (no. spawners = 25). When the median spawning intervals (mid-Oct vs. mid-Apr) were compared, the ADV regime resulted in a 6 month advancement in spawning time coincident with a reduction from 32 to 15 fish maturing relative to the SNP regime from a starting population of 51 fish in each treatment. These results are consistent with previous reports on manipulation of spawning time and reduction in individuals spawning following exposure to long-short photoperiods (Whitehead & Bromage, 1980; Bromage *et al.*, 1992b; Randall, *et* al., 1998; Davies *et al.*, 1999).

Furthermore, the advancement in spawning under the ADV regime was reflected by a subsequent phase shift in plasma testosterone and calcium levels and concurs with other studies on reproductive function in trout (Whitehead *et al.*, 1983; Elliot *et al.*, 1984; Duston & Bromage, 1986, 1987). As only plasma testosterone and calcium (as an index of vitellogenin) were measured in the current study and not circulating LH and FSH it is difficult to determine exactly when ovarian maturation was initiated. However, using a similar long- to short-day regime, photoperiod failed to alter the timing of peaks in pituitary sGnRH or plasma FSH levels during the initial stages of vitellogenesis relative to individuals maintained under ambient photoperiod, but did enhance levels of plasma FSH during secondary oocyte growth (exogenous vitellogenesis) in post-spawned female rainbow trout (Davies *et al.*, 1999). In this respect, it was concluded that the effect of a stimulatory long-short photoperiod is to advance the peaks in 17β-oestradiol, testosterone, calcium and LH and prolong the elevation of FSH during secondary oocyte growth. Similar observations have been recorded in Atlantic salmon subjected to abrupt changes in photoperiod (Taranger *et al.*, 1998). However, to date the function of high levels of testosterone during maturation in

female trout is still unclear, although it is widely known that testosterone is a precursor to 17β-oestradiol through the conversion by aromatase in the ovary, which is subsequently involved in stimulating production of vitellogenin by the liver which is then incorporated into the oocytes (Nagahama *et al.*, 1995). As such, elevated testosterone levels may be an artefact of increased 17β-oestradiol production. Furthermore, such elevated levels may also be a reflection of the single synchronous recruitment of all oocytes in salmonids reproductive development. Unlike many marine species, in which small batches of oocytes are recruited at different rates, salmonids recruit all oocytes for one spawning rather than continual batches over a spawning season. In the current experiment, both maturing cohorts exhibited an increase in plasma testosterone one month prior to an increase in plasma calcium which would be indicative of the beginning of exogenous vitellogenesis. Thus using testosterone and calcium as an indicator of initiation of oogenesis and subsequent vitellogenin uptake in the current study would seem suitable in providing an accurate indicator of the induction of the later stages of ovarian development.

In this respect, some attention should be drawn to the observation of individuals in both treatments that increased testosterone but failed to spawn. In the SNP regime this increase occurred between November and January, with the initial increase in plasma testosterone appearing two months later than the mature cohort. Similarly, under the ADV regime individuals classified as arrested also showed increased testosterone levels in September, but again two months later than in which this increase was observed in the mature cohort in June 2000. The occurrence of an increase in testosterone but failure to spawn suggest that as in other salmonids there may be a second permissive period in which maturation is allowed to proceed or will be arrested if specific thresholds are not met (Thorpe, 1986). Unfortunately, from the available data

in this chapter it is impossible to conclude why these individuals appeared to arrest maturation, or explain why the occurrence of testosterone rises occurred later than in fish which successfully matured. However, evidence of a seasonal cycle of vitellogenesis that does not lead to maturity does exist and has been termed as a "dummy-run" and may represent an inbuilt natural rhythm used in practice for spawning the following year. This developmental process is characterised by vitellogenesis that is arrested before reaching the final stages, followed by follicular atresia and oocyte reabsorption (Hassin *et al.*, 1997). Dummy-runs are frequently detected by minor seasonal changes in the gonadosomatic index, and they have been described in numerous teleosts including rainbow trout (Elliot *et al.*, 1983), white grouper (Hassin *et al.*, 1997), Atlantic halibut (Bjornsson *et al.*, 1998), sole (Ramsay & Witthames 1996), plaice (Bromley, 2000), and Atlantic salmon (Dodd *et al.*, 1978 cited in Hassin *et al.*, 1997). Currently what is not clear, is why this process operates in some fish within a population but not others (Ramsay & Whitthames, 1996). Certainly in both photoperiod treatments the increased testosterone levels may reflect the start of vitellogenesis. Consequently, individuals within the SNP regime also exhibited an increase in serum calcium above basal levels during December and January 2001 before returning to basal levels thus showing an initiation of vitellogenesis. However, under the advancing regime such an increase in calcium was not apparent and may be a reflection of the reduction in the window of opportunity following a phase-shift in the endogenous circannual rhythm controlling reproduction. Undoubtedly, further research into this apparent phenomenon is required to clarify its biological significance.

Given the above account the following discussion will attempt to examine the role of the growth parameters studied and the changes in endocrine hormones (IGF-I

and leptin) prior to testosterone increases in the context of the ability to successfully complete maturation and spawn relative to those individuals that remained immature.

4.4.2 Effect of Size and Growth Rate on Maturation

On a cautionary note prior to the main discussion, one of the problems to date is that much of the literature has focused on early maturation in male salmonids in relation to growth and/or body energy stores since precocious maturation in commercial culture can represent a significant loss to the industry. Extensive studies have suggested that large size and growth (Adams & Thorpe, 1989; Rowe & Thorpe 1990b; Silverstein *et al.*, 1997), higher condition factors as an index of lipid/energy reserves (Rowe *et al.*, 1991; Simpson, 1992: Silverstein *et al.*, 1997), and sufficient feeding opportunity in spring (Rowe & Thorpe 1990b; Thorpe *et al.*, 1990; Berglund 1995; Hopkins & Unwin, 1997) are key aspects in the regulation and initiation of precocious parr maturation. However, precocious male maturation may reflect an alternative life history strategy. In this respect, maturation is viewed as a positive developmental decision in underyearling salmonids if genetically determined thresholds are met (Thorpe *et al.*, 1983), while failure to meet these requirements will result in the activation of smoltification as a mode of negative developmental function (Thorpe & Metcalfe, 1998). Considering the above, the underlying mechanisms or physiological signals behind precocious maturation may not necessarily reflect the processes or decision periods that are involved in the initiation of puberty and the acquisition of maturational competence in female salmonids, which typically do not undergo early maturation with the exception of amago salmon (Moriyama *et al.*, 1997). Consequently, there is a considerable lack in the literature of the underlying mechanisms that are responsible for triggering maturation in female salmonids, particularly with regards to rainbow trout. However, it

has been demonstrated that good growth opportunity in non-maturing female Atlantic salmon parr in spring will result in an increase in reproductive investment by increasing oocyte size (Adams & Thorpe, 1989). Yet, there is also the inherent problem in that many studies have focused on Atlantic salmon which typically spawn in autumn, while the rainbow trout is a winter/spring spawner and the identification and timing of the aforementioned "critical periods" may not be applicable to the rainbow trout. Furthermore, there are also considerable differences in life history strategies between the anadromous Pacific salmonids and the non-anadromous rainbow trout.

Body Size and Growth Rate

Under natural photoperiod conditions (SNP) and ambient water temperatures individual body weight, and growth rates (both SGRwt and SGRL) would appear to have a role in determining whether a fish would undergo maturation. In this respect, maturing fish tended towards a greater body weight from July 2000, with a significant increase observed from August until October. Although gonadosomatic index (GSI) was not measured in this experiment, higher body weight due to gonadal development during this period, certainly from June/July to September, is unlikely since an ongoing trial utilising a similar spring spawning rainbow trout strain did not observe an initial increase in GSI (0.5 to 1.9) under ambient photoperiod until late-September (Migaud & Taylor 2004, data unpublished to date). However, from August to September, maturing fish in the experiment did show an increase in plasma testosterone above basal levels and may explain the subsequent increase in weight since anabolic steroids such as androgens are known to stimulate growth (Shearer & Swanson, 2000; Riley *et al.*, 2002). Yet, rate of weight gain (SGRwt) increased significantly in maturing fish between May and June 2000 several months prior to the rise in testosterone and

coincided with the tendency towards a higher body weight achieved in July. In this respect, higher growth rate in late-spring to early summer may reflect an initial decision period in which maturation is initiated on the basis of weight gain and/or body size in the female rainbow trout. Certainly, previous studies in other salmonids have suggested that there are two "critical decision periods" during the process of maturation, the first up to 1 year prior to maturity that is involved in the primary initiation, and a second permissive phase (6 months prior) where maturation is permitted to proceed if environmental conditions and a threshold size or energy status are sufficient to support gonadal development (Thorpe, 1991; Berglund, 1995). Furthermore, it has been clearly demonstrated that long-days or increasing daylength (spring through to summer) are known to entrain the earlier stages of ovarian recrudescence in rainbow trout, while short-days or decreasing daylengths in autumn are involved with the co-ordination and synchronisation of the later stages of ovarian development (Bromage & Duston, 1986). Given the above account, Thorpe (1986) argued that size may be more a measure of past growth, while SGR measures current performance and could provide a better basis for making developmental decisions. As such, a high SGR in spring/early summer would be associated with maturation, whereas rates below a genetically fixed threshold would inhibit it (Thorpe *et al.*, 1983). Certainly, this would fit with the concept of a "gating" mechanism whereby maturation will only be initiated or allowed to proceed under the photoperiodic cue of increasing daylengths if certain genetically determined physiological signals of growth and/or energy status have been surpassed when the endogenous clock is at a specific "gate open" phase of the circannual cycle (Duston & Bromage, 1987, 1988). Consequently, the greater increase in growth rate observed between May and June 2000, 10-11 months prior to the main spawning period and may reflect such a decision period in rainbow trout.

As with the pattern of weight gain and SGRwt, examination of length also showed a distinct separation between the cohorts under SNP occurring as early as June 2000. Immature fish showed a steady increase in length from June to October 2000, while those individuals destined to mature showed a 4 month period of significantly greater length increase during the same time frame. This rapid increase in length was also reflected in a significant increase in the rate of length gain (SGRL) from May to July, after which mature fish maintained a significantly higher SGRL than immature fish from July to October 2000 prior to the first significant elevation in plasma testosterone. Interestingly immature individuals also exhibited a significant increase in the rate of length gain but only from June to July, which may reflect an attempt to initiate but subsequent failure to surpass a set growth rate requirement, and may thus provide further evidence of a possible decision period.

Concurrently, long- to short-day photoperiods applied before the summer solstice are widely accepted to initiate corrective forward adjustments (advance phase-shift) of the endogenous circannual clock controlling maturation (Randall *et al.*, 1998), as a result the "window of opportunity" (gate open phase) for the initiation of maturation is reduced and dependent on the degree of advancement it will determine the number of fish capable of initiating puberty in relation to whether they have surpassed predetermined thresholds of development. In this respect individual body size (both weight and length) and growth rate (SGRwt and SGRL) appeared to be poor predictors of whether fish would initiate maturation under the ADV regime as no significant differences in any of the aforementioned parameters were observed between cohorts prior to the first recorded increase in plasma testosterone (June-July 2000), although there was a tendency towards a higher monthly SGRwt for maturing fish from March-May 2000 relative to the immature cohort. Although not significant, this earlier

appearance of growth rate differences (cf. May-June under SNP) may reflect a phase shift in the natural growth rhythm and circannual cycle of reproduction induced by the early exposure to long-days, and hence a possible decision period was also naturally advanced. However, as no clear differences were apparent in this study other parameters other than body size and growth rate not studied within this experiment would appear to be more important in relation to determining whether an individual is capable of maturing under stimulatory long-short photoperiods. In Atlantic salmon parr monthly specific growth rates did not differ between maturing and non-maturing parr between January and July yet maturation rates were strongly correlated with increases in mean condition factor during April (Rowe & Thorpe, 1990a).

Condition Factor (CF)

In both treatments mature cohorts showed a tendency towards a higher condition factor (CF) prior to increased plasma testosterone than individuals that remained immature during spring. Under SNP fish which attained maturity exhibited a higher CF from May to September with a significant increase between June and July, while those under the ADV regime maintained a higher CF from May to July. Furthermore, there was also a tendency towards maturing individuals under the ADV regime to maintain a higher CF than those under SNP from January to July 2000 and may represent a differential resource allocation induced by the exposure to a stimulatory photoperiod. Moreover, since CF is generally known to correlate strongly with whole body lipid in salmonids (Herbinger & Friars, 1991) the observed trend towards a higher condition factor in the current experiment may indicate that energy stores (lipid) were greater in fish which subsequently under went maturation. Although not statistically different such subtle differences in condition cannot be discounted as having a significant

biological effect, in that the accumulation of sufficient energy reserves in spring and early summer may have a role in the "initiation" of maturation in rainbow trout. Both body size and fat content have been shown to be important determinants of maturation in Atlantic, amago and spring chinook salmon up to 1 year prior to maturation (Rowe & Thorpe, 1990b; Moriyama *et al.*, 1997; Silverstein *et al.*, 1998; Shearer & Swanson, 2000) and that restricted feeding will reduce the incidence of female maturation (Silverstein & Shimma, 1994).

Muscle fat is known to decrease in female rainbow trout during the most active period of gonadal formation and during spawning, with the greatest mobilisation of phospholipids and fatty acid reserves observed in order that the final stages of oocyte development can proceed (Tveranger, 1985; Nassour & Leger, 1989). Therefore, a second critical period of maturation may occur during autumn in the rainbow trout, and may equate to a permissive period as proposed by Thorpe (1986). However, the use of CF to identify a period in which the latter stages of maturation are allowed to proceed is more problematic as higher CF may be an artefact of rapid gonadal growth during this period. This may account for the significantly higher CF observed during November and December in mature individuals relative to those that remained immature under SNP. Thus it is difficult to identify a so-called "permissive decision period" in which maturation could be arrested if reserves are not sufficient to permit final ovarian development. However, as previously discussed the presence of individuals that appeared to initiate maturation and then arrest (dummy-run) by December under SNP, and by September under ADV may indicate that such a decision period exists one month prior to final oocyte maturation and ovulation in rainbow trout. However, the proximity of this period to ovulation would seem a little too close as significant

investment in reserves may have already been placed into oocyte development. Thus given the current data it is difficult to conclude if such a period exists or when it occurs.

Yet, in both cohorts under the ADV regime CF increased steadily from April to July, thereafter maturing fish CF decreased steadily as spawning approached. Interestingly, immature fish showed a halt in the rise in CF between July and August, before significantly increasing over the subsequent months. This apparent cease in CF may reflect a second decision period in which investment in oogenesis is allowed to proceed, but since CF was lower the decision to mature was halted possibly on the basis of insufficient energetic status. Certainly between July and August the first significant increase in testosterone in the maturing cohorts became apparent, and may thus reflect such a permissive decision period in which the latter stages of vitellogenesis were allowed to proceed.

Thus under natural photoperiod conditions it would seem plausible to conclude that a decision period may occur in spring under increasing daylength in which the "decision" to initiate maturational development is made on the basis of a size/growth rate-energy storage threshold as previously suggested (Rowe *et al.*, 1991; Simpson, 1992). However, identification of a second decision period is difficult given the current data available. In contrast, under a stimulatory photoperiod regime, the initiation of maturation may be taken on the basis of condition factor in spring rather than growth rate or size, while a second permissive period may exist up to 3 months prior to spawning assuming CF reflects surplus energy reserves required to complete ovarian development (Adams & Huntingford, 1997). Furthermore it has been demonstrated recently that higher mean size does not always translate into a higher proportion of mature parr (Aubin-Horth & Dodson, 2004) and supports the hypothesis that thresholds may vary across habitats within the same population, and that there is a high level of

complexity in the proximate mechanisms governing life-history strategies in relation to both genetic and environmental control of maturation (Baum *et al.*, 2004).

4.4.3 Plasma IGF-I and Maturation

With regards to plasma IGF-I levels this experiment aimed to assess whether IGF-I could be a useful predictor of growth rate in rainbow trout as a possible means of signaling growth performance to the reproductive-endocrine axis by studying seasonal changes in IGF-I under natural and advancing photoperiods. The results from this experiment to my knowledge are the first to provide a complete seasonal profile of circulating IGF-I in rainbow trout under ambient conditions, and provides strong evidence for the role of IGF-I in maturation in relation to photoperiod manipulation.

Seasonal Changes in Plasma IGF-I in Relation to Maturation

Under SNP little difference in the patterns of plasma IGF-I were observed between maturing and non-maturing individuals, with levels generally increasing from spring into summer, and then declining in Autumn and remaining low in winter before increasing during the following spring. The only notable differences were in relation to the absolute levels which were generally higher between July and September in maturing fish. In contrast, a significantly different pattern of plasma IGF-I was observed under the ADV regime between the cohorts, that appeared to be a direct response to exposure to a stimulatory long-short day photoperiod. Plasma levels in both cohorts increased significantly between June and July, one month earlier than under SNP, thereafter, maturing individuals showed a significant decrease in plasma levels which then remained low over of the next 9 months in the period preceding and during spawning. Conversely, immature females also showed a decline in levels between June

and August, but maintained significantly higher plasma IGF-I levels over this period, with a distinct peak in IGF-I recorded in September, before steadily declining in autumn.

Irrespective of maturational status the seasonal change in plasma IGF-I levels under SNP are consistent with observations reported in other salmonids in relation to seasonally changing environmental conditions, and to my knowledge is the first reported evidence of a complete seasonal profile of IGF-I in rainbow trout. The observation of a strong positive correlation with both daylength (r^2=0.61) and water temperature (r^2=0.79) supports the idea that circulating IGF-I may provide an integrated signal with regards to season, temperature and daylength (Beckman *et al.*, 1998). In this respect, IGF-I levels under SNP in the current experiment increased in spring in parallel with increasing daylength and rising water temperatures (Beckman *et al.*, 1998), remained elevated during summer at highest water temperatures (Larsen *et al.*, 2001; Mingarro *et al.*, 2002) and maximal daylength (McCormick *et al.*, 2000), and then subsequently decreased in conjunction with decreasing daylength and water temperatures in autumn and winter (Webster *et al.*, 1999; McCormick *et al.*, 2000; Larsen *et al.*, 2001), before again increasing in May-June 2001.

Conversely, failure to demonstrate a seasonal relationship between daylength and IGF-I levels in either cohort under the ADV regime is not surprising since the photoperiods were held constant under changing water temperature, and IGF-I levels may have thus been driven by temperature-growth interactions (Larsen *et al.*, 2001). However, the current results would indicate a clear relationship between maturation and IGF-I, as under SNP, plasma levels in mature fish were high when water temperatures were at their highest and may thus reflect the normal timing of the reproductive cycle. Conversely, under ADV the drop in IGF-I levels in mature fish occurred when

temperatures were only 10°C, suggesting that the natural IGF-I pattern was advanced by the stimulatory long-short day photoperiod regime and reflected maturation rather than changes in ambient water temperature.

Given the absence of a relationship of IGF-I with daylength under ADV, no relationship was also found between plasma IGF-I and water temperature in either cohort when all sampling points were included. However, the removal of IGF-I levels measured during spawning (July to November 2000) in the ADV mature cohort resulted in a strong relationship with water temperature (r^2=0.69). Similarly, in immature females the removal of July and August sample points resulted in a strong relationship between temperature and plasma IGF-I (r^2=0.57). The removal of these two time points coincided with a decrease in IGF-I levels which may have been related to a "decision" period in which maturation may have been taken, but was then halted due to the failure to achieve some growth/energy threshold stage of development. Thus, these results would provide strong evidence that IGF-I production is not only driven by water temperature, but is more strongly influenced by photoperiod under ADV. These findings would also support the direct relationship between IGF-I and maturation. However, separating the importance of temperature or daylength on IGF-I under SNP cannot be determined since the two factors were highly correlated.

The observation of significantly lower plasma IGF-I levels in mature fish in the three months preceding spawning and during the three month spawning period itself under ADV are consistent with those reported in amago salmon (Moriyama *et al.*, 1997). Consequently, it is has been demonstrated that 17β-oestradiol (E_2) has inhibitory action on hepatic IGF-I mRNA synthesis in amago salmon, and may thus be responsible for the decline in plasma IGF-I levels. In the current experiment, in both photoperiod treatments maturing cohort plasma IGF-I levels seemed to decrease in association with

increasing plasma testosterone, which is likely to be a reflection of increasing E_2 production during vitellogenesis. Similarly, the individuals in each photoperiod regime which appeared to initiate then arrest maturation also showed a decline in IGF-I levels as testosterone increased. Certainly, under the advancing regime the apparent negative correlation between IGF-I and the onset of vitellogenesis as indicated by increasing testosterone would fit such a model. Thus it would appear than steroid production has a stronger effect on IGF-I than water temperature, since IGF-I levels decreased even at high temperatures in relation to increasing testosterone under the ADV regime. This again provides further evidence for a direct relationship between IGF-I and maturation, and that photoperiod is responsible for entraining the reproductive cycle. Thus in this respect, under SNP declining IGF-I levels in maturing fish may also be associated with increasing testosterone levels, which may be a reflection of the known entrainment of the latter stages of the natural reproductive cycle under the falling daylengths of autumn rather than decreasing water temperatures (Duston & Bromage, 1988).

Studies carried out under constant temperatures and changing photoperiod have shown that IGF-I levels appeared to accurately reflect manipulations in photoperiod (McCormick *et al.*, 2000; Pierce *et al.*, 2002; Beckman *et al.*, 2004). Of interest in the current experiment was that under the ADV regime the first recorded increase in IGF-I levels occurred one month (June 2000 cf. July 2000) earlier than that observed under SNP. Moreover, this increase occurred within 4 weeks of the change from LD 18:6 to LD 8:16 in May. This supports the idea of a phase-advance in a natural autumn growth/reproductive endocrine rhythm in response to an abrupt reduction in photoperiod (Bromage & Duston, 1986). In this sense although ADV immature females showed a significant increase in IGF-I levels in September, there was also an initial depression in IGF-I levels in July and August 2000 which may relate to such a proposed

photoperiod induced shift. Taken at this level, these observations would concur with previously suggested ideas that photoperiod is a key environmental factor influencing both maturation and plasma IGF-I levels. In this respect both amago and chinook salmon exhibit a clear autumnal peak which has been proposed to be a natural phenomenon associated with decreasing daylengths (Moriyama *et al.*, 1997; Beckman & Dickhoff, 1998; Beckman *et al.*, 2000; Pierce *et al.*, 2002; Beckman *et al.*, 2004) although this relationship is not always found and may relate to differences between the studies in relation to feeding, maturation or smoltification (Shearer & Swanson, 2000). Thus far, the biological significance of this apparent autumnal IGF-I increase has yet to be clarified, and whether it is under regulation by GH or not (Moriyama *et al.*, 1997; Pierce *et al.*, 2002). Conversely, no discernable autumnal IGF-I peak was observed in either cohort under SNP in autumn with highest plasma levels observed in summer apparently more related to ambient water temperatures. However, as shown in chapter 3 a peak in plasma IGF-I does appear to occur in underyearling rainbow trout, although, as previously suggested by Plisetskaya (1998) such relationships may not necessarily be reflected in later stages of development. If there is a true "peak" that was apparently phase-advanced under ADV in response to daylength reduction such a peak would have been expected under the SNP regime in autumn, yet appeared to be absent. However, the effect of water temperature cannot be ruled out as both daylength and temperature changed in unison. Therefore, the current observations highlight the complexity of differential regulation and possible interactions of IGF-I with constant and changing environmental conditions, and suggest that further investigations into the seasonal aspects of changes in IGF are required in order to separate the key environmental components that may be influencing circulating IGF-I levels and how these may interact with growth.

Plasma IGF-I as an Indicator of Growth Rate

The aim of this experiment was to determine whether seasonal changes of circulating IGF-I could be used as an indicator or predictor of growth, in that if such a relationship exists then plasma levels may provide a link between the somatotropic and reproductive axes to provide information on growth status. In several studies in salmonids a strong correlation has been found between IGF-I levels and growth (Beckman *et al.*, 1998, 1999; Shimizu *et al.*, 2000; Pierce *et al.*, 2001; Uchida *et al.*, 2003; Beckman *et al.*, 2004; Dyer *et al.*, 2004) although this relationship is not always found (Silverstein *et al.*, 1998; Nankervis *et al.*, 2000; Devlin *et al.*, 2004).

Within this chapter three major findings were observed with regards to the IGF-I vs. SGRL relationships examined. Firstly, mean monthly plasma IGF-I correlated strongly with mean monthly SGRL throughout the duration of the experiment in both cohorts under SNP, but only in maturing fish under the ADV regime, with no significant differences between the regressions. These observations confer with the previous studies presented above, and indicate that measurement of total plasma IGF-I is a useful indicator of growth rate in rainbow trout. However, the fact that ADV immature females showed a relationship from March to June, but no relation over the whole duration of the experiment suggests that the exposure to constant short-days, which were applied as of mid-May, may somehow be altering the subsequent relationship between IGF-I levels and growth. Similarly in chapter 3, a failure to find a relationship between IGF-I levels and SGR in juvenile rainbow trout exposed to constant short days (LD 8:16) was also demonstrated. Thus, there may be a possible desynchrony between circulating IGF-I and growth that occurs when fish are held under constant short-days through a phase-shift of an endogenous rhythm governing growth than then became "free-running" in the absence of a changing light cycle.

Secondly, no consistent relationships were found when specific time periods were examined (i.e. May IGF-I vs. Apr-May SGRL). Under SNP significant correlations were only found in September in mature females and July for immature fish, while ADV immature showed two relationships one, in April and the other in September, with maturing fish not showing any such relation. However, care should be taken when comparing these relationships since the slope of IGF-I to growth relationships are known to change from season to season (Beckman *et al.*, 2004). In this respect, Plisetskaya (1998) has speculated that the correlation between plasma IGF-I and growth rate in fish may vary seasonally and with developmental stage, and that these factors and the characteristics of IGF-I assays and IGFBP extraction techniques used may explain the lack of correlation in some studies. Thus, IGF-I probably reflects both past growth and predicts future growth. This view is supported by Beckman *et al.*, (2001) in the sense that different IGF-I vs. growth rate relationships may well be expected when differing biological situations and seasons are examined. Thus further work will be necessary in order to determine the time period and growth measurement which gives the strongest and most biologically significant correlations.

The final observation examined the relationship between IGF-I and growth before testosterone increased, (in the sense that 17β-oestradiol is known to down-regulate IGF-I synthesis in the liver), in order to explore whether different IGF-SGR relations may exist prior to the initiation of maturation. In both cohorts under each photoperiod treatment a significant relationship between individual IGF-I and SGRL was observed (SNP: March-August; ADV: March-June), although the regressions did not differ. Thus, it would appear that IGF-I levels accurately reflected growth prior to the initiation of the latter stages of maturation, and may thus provide a link between the somatotropic and reproductive axis to convey information on both growth and

nutritional status. From the current results it would appear that measuring total plasma IGF-I is a useful tool in relation to studying growth rate in rainbow trout, but at what stage of development it may provide a useful signal on growth performance to the reproductive axis remains to be clarified.

In this regard total plasma IGF-I levels in maturing fish under SNP were significantly higher than immature individuals in July 2000, and were also generally maintained at higher but non-significant levels during August and September. Similarly, under the ADV regime there was a tendency towards maturing fish expressing higher circulating levels albeit one month earlier in June 2000. These observations concur with several studies in which maturing fish appear to express higher IGF-I levels although differentiating the effect of larger size or maturation on plasma levels has proven difficult (Moriyama *et* al., 1997; Shearer & Swanson, 2000). However, Beckman *et al.*, (1998) found little relationship between mean IGF-I and mean body size independent of growth, and suggested that body size alone has little influence on plasma IGF-I levels. Thus differences in body size between the cohorts, particularly with respect to the SNP regime during August and September may not have had an effect on circulating plasma levels. Furthermore, as the relationship between IGF-I and SGRL did not differ between cohorts or treatments prior to the first recorded testosterone increase it would suggest that differential interactions between growth rate and IGF-I would not explain the elevated levels of IGF-I observed in maturing females. Potentially, the elevation of circulating IGF-I may be attributable to the regulatory action of IGFBPs which may dictate the availability of "free" IGF-I (Siharath *et* al., 1996; Shimizu *et al.*, 2000; Kelley *et al.*, 2001), although this cannot be directly determined from measurements of total IGF-I.

Nonetheless, elevated total IGF-I levels in peripheral circulation of maturing fish, in conjunction with the known direct effect on pituitary FSH content (Huang *et al.*, 1999; Baker *et al.*, 2000a) are consistent with IGF-I acting as a metabolic trigger for puberty in fish (Shearer & Swanson, 2000). In this respect, the fact that under SNP plasma IGF-I levels were maintained at elevated levels for 3 months prior to an increase in testosterone while under the ADV regime elevated levels were only observed for one month may reflect an advancement and reduction in the "window of opportunity" for IGF-I to act on the pituitary to initiate the release of gonadotropin and subsequent steroidogenesis, and hence explain in part why fewer fish initiated the final stages of oogenesis and successfully spawned under the advancing photoperiod regime. In support of this hypothesis, not only IGF-I but also testosterone levels differed significantly between maturing cohorts under each photoperiod. Under SNP, fish attained levels of 24.3 and 313ng/ml for IGF-I and testosterone, while those under ADV only achieved 15.3 and 190ng/ml. This may reflect the fact that as maturation is advanced the processes required to initiate the later stages of oocyte maturation have to occur more rapidly (Whitehead *et al.*, 1983), and thus do not allow sufficient time for steroid levels to increase before the next stages of oogenesis have to occur. In this respect there may be significant effects on egg quality of lowered testosterone levels, and may explain in part the reduction in egg size observed under advancing photoperiod regimes, due to the shortened period of stimulated vitellogenesis. These findings again provide evidence to suggest that the window of opportunity to induce maturation is reduced in response to a phase-shift in the reproductive cycle under the stimulatory long-short day photoperiod. Overall these results would highlight the complexity of the IGF system in relation to both growth and reproduction.

4.4.4 Effect of Plasma Leptin-like Peptide on Maturation

The principal aim of this experiment with regards to leptin was in identifying a possible detection method for measuring a leptin-like substance in rainbow trout, and thus provide preliminary evidence for a possible role in maturation. The detection of a leptin-like peptide in rainbow trout plasma in the current study provides an interesting finding that should certainly warrant further research. The observation of differences between maturing and immature cohorts with regards to absolute levels would suggest that changes are occurring at the endocrine level with regards to the reproductive cycle. The observation of a decrease in plasma levels as the spawning period approaches in maturing fish followed by a subsequent increase in plasma levels towards the end of spawning are consistent with those reported in spawning burbot (Mustonen *et al.*, 2000a, 2000b). However, as samples were not assayed post-spawning in the SNP regime it cannot be determined whether plasma levels would have decreased as observed under the ADV regime. Given the lack of knowledge and conflicting evidence that exists to date regarding the involvement of leptin in fish metabolism and reproduction and the apparent opposite function relative to those known in the mammalian system it is difficult to elucidate the biological significance of such observations. Certainly the ideas proposed by Mustonen *et al.*, (2000 a&b), would seem plausible in that decreasing and/or low plasma levels pre-spawning in maturing fish may represent energy conservation and storage required for latter investment in oogenesis, while increasing levels towards the end of- and post-spawning could reflect nutrient/energy mobilisation required during the spawning process itself. In this respect, the observation of significantly higher plasma levels in immature fish relative to pre-spawning maturing fish under ADV in October may reflect a reallocation and investment of possible energy reserves into growth rather than reproduction in autumn

prior to entry into winter. Yet, under the SNP regime plasma levels did not appear to vary throughout the experiment, but in this sense may reflect some level of energy homeostasis or steady-state energy reserves as has been suggested in goldfish (Volkoff *et al.*, 2003) in relation to continued growth rather than investment in reproduction. Furthermore, as whole-body lipid or liver lipid assessments were not possible a correlation between adiposity and leptin cannot be determined (Johnsson *et al.*, 2000), which may have provided an endocrine index of condition or energy status. Thus, until homologous antibodies are made available against fish "leptin" it is impossible to draw conclusive evidence that a such a peptide may have a role in reproduction, although the data from the current study would suggest that leptin may play a role in relation to energy storage and mobilisation of reserves during spawning.

4.5 Conclusions

Under natural photoperiod conditions it would appear that body size, growth rate and possibly lipid status as indicated by condition in the spring one year prior to maturation may be responsible for allowing fish to undergo maturation. However, under an advancing photoperiod regime the window of opportunity is reduced and it would appear that body size and growth rate were not the principal determinants of whether maturation can proceed. Of all performance parameters examined condition factor in spring would appear to be the most useful indicator of whether an individual was capable of initiating maturation under either natural or advancing photoperiods.

The results of the current experiment clearly demonstrated a direct relationship between IGF-I and maturation, and that IGF-I levels were more strongly influenced by changes in photoperiod than water temperature. Furthermore, a complete seasonal profile of plasma IGF-I in rainbow trout was demonstrated for the first time. Plasma

IGF-I levels were generally higher in maturing individuals prior to the first increases in plasma testosterone but was generally a poor indicator of growth rate at specific time points. However, the demonstration of a significant relationship between IGF-I and SGRL preceding testosterone increases in both treatments suggest that plasma IGF-I levels accurately reflect growth rate in rainbow trout, and may have some role in conveying information from the somatotropic axis to the reproductive axis in order to allow maturation to proceed. Finally, the identification of a possible leptin-like substance in rainbow trout and the differential pattern of circulating peptide observed in the current study between maturing and immature fish may suggest a possible role in energy storage and mobilisation rather than a direct role in reproduction. Further studies are undoubtedly required.

Chapter 5: Commercial Application of Photoperiod Regimes

5.1 Introduction

The use of artificial light in order to improve growth rate, reduce sexual maturation and induce smoltification has been one of the most significant changes in Atlantic salmon farming procedures during the last 5-10 years (Hansen, *et al.*, 1999). For example, the Norwegian salmonid industry has shown a large increase in productivity, with faster growth, shorter production time, higher production per man-year, a lower cost and lower mortalities during the recent years. This has been attributed to the use of artificial light in conjunction with larger sites/cages, better feed and feeding regimes.

In the case of European trout production, there has been a gradual increase from 296,048 tons in 1996 to 333,950 by 2001 (SERAD, 2003). However, in terms of UK production of rainbow trout the current figures show a general stagnation in production at around 16,000 tonnes per annum since 1996 (Dunn 2003, SERAD 2003). A variety of reasons have been implicated in this including lowered market prices over recent years. However despite this, a recent survey of the UK industry showed that most of the main farm types (cages, tanks and raceways) are performing well with good profit margins of over 20% except for medium sized table trout producers (50-200t) which appear to be making a net loss of 6% (Winnard, 2003). This is primarily because they cannot compete with large sites (>200t) mass producing for the table market due to increased production costs, while similarly they cannot compete with small farms (<50t) who often supply local markets direct to the customer through a shorter supply chain. However, farms in the 50-200t category represent a significant proportion of the total number of sites in the UK, and if they do not have the capacity to increase their production they will need to improve production efficiency in order to remain cost effective.

Within the UK industry, Scottish production increased by 21% (1,193 tonnes) from 2001-2002, while production in England and Wales has fallen slightly by 3%. The increase was associated with an increase in production from freshwater cages for the table trade. In 2002, 52 % of total Scottish production was from 9 freshwater cage sites, of which 7 were large scale operation production (>100 ton/p.a.). In contrast, 39.2% of production was from ponds and raceways, but involved 30 sites, 5 more than in 2001. Thus if cage production is increasing, then farmers may be wish to improve the efficiency of their current production by adopting new farming strategies. Using photoperiod techniques to potentially enhance trout growth and improve feeding efficiency may be one such approach.

In the comprehensive review of light effects on fish growth (Boeuf & Le Bail, 1999) numerous considerations for the application of light regimes were proposed including; light quality (spectrum), quantity (intensity), photoperiod, fish age, and the synergistic effects of temperature.

5.1.1 Light Quality (Spectrum)

To date very few studies have examined the effects of spectral composition on fish growth, although growing interest is now focusing on this aspect of photoperiod manipulation. Larval growth and survival was significantly improved using red light of continuous duration in the catfish, *Wallago attu*, compared to LL, 12L:12D or 0L regimes provided by a 40Watt bulb (Giri, *et al.*, 2002). High intensity illumination in predatory catfish has been shown to exert more stress and increase aggression. Furthermore, predatory and nocturnal species have been shown to possess dichromatic vision based on green-sensitive rods and red-sensitive cones providing visual discrimination at low light levels (Kusmic & Gualtieri, 2000). Conversely, in European

sea bass, blue light (434-477nm) was more effective than red light (610-687nm) in suppressing ocular and plasma melatonin, although effects did vary with intensity (Bayarri, *et al.*, 2002). In contrast to the above studies, Stefansson & Hansen (1989) found no significant differences in growth or smoltification capacity in Atlantic salmon parr exposed to illumination of different spectral composition (2-10,000K) and intensity. Therefore, since each species is known to have a different and unique photoreceptor mosaic array and grouping adapted specifically for their natural environment, their response may subsequently differ following exposure to light of different spectral composition (Kusmic & Gualtieri, 2000). The lack of information and contrasting results highlight the importance of giving due care and attention to the consideration of the characteristics of light regime to be utilised.

5.1.2 Light Quantity (Intensity)

In larval stages, many studies have examined the influence of light intensity and photoperiod on growth, with the general consensus being that a minimal threshold intensity is required to achieve normal development and growth, and their response to changes in light intensity and duration have more often been related to efficiency of prey visualisation and capture rather than as a direct stimulation of growth through photoperiod (Puvanendran & Brown, 2002, Dou, *et al.*, 2003, Moustakas, *et al.*, 2004, Trotter, *et al.*, 2003). However, in salmonids there is not a true larval stage and the effects of light intensity are far from clear. Furthermore, commercial production typically uses inert pellets rather than live feed in salmonid culture, and as long as sufficient light is supplied, growth and feeding is not deliriously affected. Although, Kim, *et al.*, (1996) showed that visual cues (live feed) are important triggers for a

feeding response in juvenile coho salmon, olfactory function was shown to be of equal importance in rainbow trout alevins (Valentincic *et al.*, 1999).

Furthermore, fish light perception has been shown to change with age, in addition to different developmental stages showing preferences for different light intensities (Kusmic & Gualtieri, 2000). In juvenile haddock (*Melanogrammus aeglefinus*) a reduction in light intensity from 100 to 30 lux, more characteristic of their natural habitat, applied continuously led to improved juvenile growth (Trippel, *et al.*, 2003). It was concluded that the requirement of bright visual feeding conditions associated with planktivory appears to diminish as they shift from the pelagic to demersal zone. Similarly, in post-metamorphic halibut and barramundi (*Lates calcarifer*), further exposure to continuous light did not enhance growth above that exposed to natural photoperiods (Barlow, *et al.*, 1995, Solbakken *et al.*, 2004), although feed intake was higher in juvenile barramundi under LL, most likely in association with higher metabolic costs through increased activity. In older fish, light intensity effects are not so clear, and it is proposed that specific thresholds may not be such an important factor on growth. In Atlantic salmon lower light intensities were required to improve growth, while higher intensities were required in order to inhibit maturation (Oppedal *et al.*, 1997; 1999). This suggests that different thresholds may exist and are dependent on what the proposed use of the artificial light regime is. Certainly what is important is the relative threshold between day and night intensity in order that the fish perceive the potential photoperiod to be applied as continuous or changing (Kissil, *et al.*, (2001). Thus in outdoor systems such as ponds, tanks and cages it could be argued that higher light intensities are needed to compete with natural sunlight intensities in order that the fish perceive additional artificial illumination, rather than the intensity itself which is

having the direct effect on fish growth. Therefore, it has been proposed that daylength (photoperiod) itself appears to be the more important factor (Boeuf & Le Bail, 1999).

5.1.3 Photoperiod

Numerous studies have observed growth enhancing effects of extended and constant light photoperiod regimes (LL) in a variety of species including Atlantic salmon (Saunders & Henderson, 1988, Villarreal *et al.*, 1988, Saunders & Harmon 1988, Saunders *et al.*, 1989, Stefansson *et al.*, 1989, Clarke, 1990, Krakenes *et al.*, 1991, Hansen *et al.*,1992, Oppedal *et al.*, 1999), largemouth bass (Beauchaud & Buisson, 2003), Japanese medaka, *Oryzias latipes* (Davis, *et al.*, 2002), juvenile Atlantic halibut, *Hippoglossus hippoglossus*, (Jonassen *et al.*, 2000, Norberg *et al.*, 2001), juvenile turbot, *Scophthalmus maximus*, (Imsland *et al.*, 1995, 1997), juvenile haddock, *Melanogrammus aeglefinus* (Trippel & Neil, 2003), male European sea bass, *Dicentrarchus labrax*, (Rodriguez *et al.*, 2001) and gilthead sea bream, *Sparus aurata*, (Kissil *et al.*, 2001). However, although the second most commonly cultured salmonid in Europe, very little information regarding the effect of extended photoperiod regimes exists with regards to rainbow trout growth and performance. Solbakken *et al.*, (1999) observed no effect of LL application on winter growth rate of seawater cage reared rainbow trout. Contrary evidence suggests that rapid growth was not associated with maturation and that exposure to long photoperiods can improve growth rate (Skarphedinsson *et al.*, 1985), during which, exposure to LD 14:10 produced the best growth relative to SNP or short-day regimes. Similarly, in freshwater reared rainbow trout it has been demonstrated that under natural photoperiod cycles a reduction in the rate of decreasing daylength is favourable for growth and feed conversion efficiency (Makinen & Ruhonen, 1992). Mason *et al*, (1992) related this observation to the longer

photophase providing more favourable conditions for an increase in food intake. The timing of the daily meal has also been shown to have considerable effect on the growth of trout. Those fed at dawn were shown to have the greatest performance while those fed around midnight exhibited significantly poorer performance (Boujard *et al.*, 1995), suggesting a natural appetite rhythm dictates feeding motivation (Chen, *et al.*, 2002). In addition, compensatory growth has been observed in many salmonid species following periods of feed restriction, or transfer from cold to warmer temperatures while maintained under long day photoperiods (Jobling & Koskela, 1996; Mortensen & Damsgard, 1993) or from natural daylength to constant light. Therefore, when investigating the effects of photoperiod on growth it is essential to maintain as many outside parameters as possible constant, and an important factor to be certain of is that light affects fish growth through a better food conversion efficiency and not just through stimulated food intake (Boeuf & Le Bail, 1999).

In Atlantic salmon studies a decrease in specific growth rate is often observed following initial exposure to constant light from November, December, or January until July, followed by improved growth in the subsequent 4-5 months relative to controls. Furthermore, it was also shown that longer exposure maintains a higher growth rate for a longer period (Endal *et al.*, 2000; Taranger *et al.*, 1999). In contrast, exposure of juvenile salmon parr to periods of short days has shown that growth opportunity is reduced with fewer fish being able to maintain a period of high growth (Duncan & Bromage, 1998; Skilbrei *et al.*, 1997). However, it must be noted that rapid growth is associated with smoltification in Atlantic salmon. This complicates the understanding of how photoperiodic information is being transduced, as it is difficult to dissociate growth from smoltification (Porter *et al.*, 1998). However, by maintaining juvenile Atlantic and chinook salmon on constant light regimes (24hr light and LD 16:8) growth

rates were improved although smoltification was not completed, probably due to the absence of important changing photoperiod cues (Eriksson & Lunqvist, 1982; Berge, *et* al., 1994; Hoffnagle & Fivizzani, 1998; Rottiers, 1992). Of interest is that growth is influenced more on the ascending than on the descending phase of the annual photoperiod cycle in the case of Atlantic salmon (Thorpe *et al.*, 1989). Villarreal *et al.*, (1988) suggested that the delays observed in growth after daylength reduction may be related to the synchronising effect of an endogenous rhythm of appetite and growth. Atlantic salmon parr maintained on a longer daylength when the natural daylength should have been decreasing grew larger, but reached a smaller size than predicted. This provides evidence for the existence of endogenous appetite and growth rhythms which will assert themselves in the absence of cues from declining daylength. By using 1 hour pulses of light within the dark period (9L:9D:1L:5D or 24L:0D), Thoransen *et al.*, (1989) demonstrated that it is the time of day when light is experienced rather than its accumulation which is influencing growth. Furthermore, slower growth rates were observed in fish exposed to nocturnal illumination which suggests that a period of total darkness may be required to obtain maximum growth.

However, it has been suggested that growth enhancement under long photoperiods and continuous light is either due to a photoperiodic alteration of seasonal growth patterns, or to a direct photostimulation of growth (Endal *et al.*, 2000). It is thus possible that photoperiod is acting as a zeitgeber adjusting a circannual growth rhythm, whereby a change from short to long days earlier in the season is advancing the rhythm as seen in circannual phase-advances of spawning time (Bromage *et al.*, 2001). However, although not directive, water temperature has been shown to have a modulating effect on the time of maturation and ovulation, with female rainbow trout showing the ability to delay final maturation and ovulation when temperatures were at

the high or low end of the natural range (Davies & Bromage, 2002). In addition to modulating maturation, temperature has been shown to act synergistically with photoperiod in a rate-controlling manner on growth in response to photoperiod manipulation in numerous species (Clarke *et al.*, 1978; Saunders *et al.*, 1985; Solbakken *et al.*, 1994; Hallaraker, *et al.*, 1995; Jonassen, *et al.*, 2000). Thus the timing of photoperiod application and the subsequent response should be given careful consideration with regards to ambient temperatures.

Finally, in addition to entraining circannual growth rhythms, reasonable evidence exists to support the existence of a light-pituitary axis, whereby photoperiods that enhance growth do so by stimulating increased somatotroph activity (cells within the pituitary associated with the synthesis and secretion of growth hormone), leading to increased plasma growth hormone levels (Komourdjian *et al.*, 1989; Bjornsson, 1997). However, how the photoperiodic information is transduced to initiate both growth and reproductive responses is unclear. A prime candidate for this is the pineal gland and the associated production of melatonin (see review chapter 1).

5.1.4 Aims of the Chapter

Since little work with regards to the use and characteristics of photoperiod manipulation to enhance growth in rainbow trout under commercial conditions exists to my knowledge, a series of four experiments were initiated to determine:

- At what life stage(s) of development could photoperiod application be applied?
- In what systems could photoperiod manipulation be applied?
- The effect of photoperiod manipulation on production performance in terms of growth and feeding efficiency.
- Did different strains show differing responses to photoperiod manipulation?

- What is the effect of light intensity and spectral composition on response to photoperiod manipulation?
- The effect on circulating melatonin levels by photoperiod treatment.
- Briefly examine any negative effects of photoperiod treatment on product quality.

Four experiments are presented within this chapter:

1. The effects of exposure to constant light on growth and feeding in rainbow trout fry during winter on-growing.

2. The effects of submersible lighting providing constant illumination on growth of two strains of cage reared rainbow trout during winter on-growing.

3. The effects of constant light provided by different numbers of lights on growth of rainbow trout fry during winter on-growing.

4. The effects of constant light of different colour temperature bulbs and intensity on the growth of cage reared rainbow trout during winter on-growing.

5.2 General Materials and Methods

Within this chapter, trials were conducted at one of two commercial freshwater on-growing sites. For each trial a full description of the system, stock, diet and experimental protocol is provided within each section. Site 1 was a tank and raceway site supplied by local river water located at 55.6 °N, 2.8 °W, while site 2 was a cage site located at 55 °N, 1.8 °W. Anaesthesia and length-weight procedures were carried out according to those described in section 2.1.2 and 2.1.6. Where blood sampling was carried out this was undertaken according to fish size as described in section 2.1.3. Assessment of plasma melatonin concentration was undertaken following the protocol in section 2.2. Colour scoring and fillet quality assessment was carried out by Scot Trout Ltd.

5.3 Trial 1: The effects of exposure to constant light on growth and feeding in rainbow trout fry during winter on-growing

5.3.1 Materials and Methods

On 6th November 2000 four outdoor 4m circular tanks (15.2m^3) were stocked with equal numbers (approx 50,000) of all-female rainbow trout fry (mean weight 5.1±0.1g) (Aquazure Hatcheries, South Africa) to a total biomass of 250kg per tank. These fish had previously been reared from hatch (12th August 2000) in hatchery raceways under low level constant illumination (30 Lux). Water was supplied by a nearby river at variable rate with ambient temperature conditions (Figure 5.1.). Indoor tanks received additional water from a borehole supply, mean temperature 6°C. On the 17th January 2001 stock from each tank were split evenly between two tanks providing

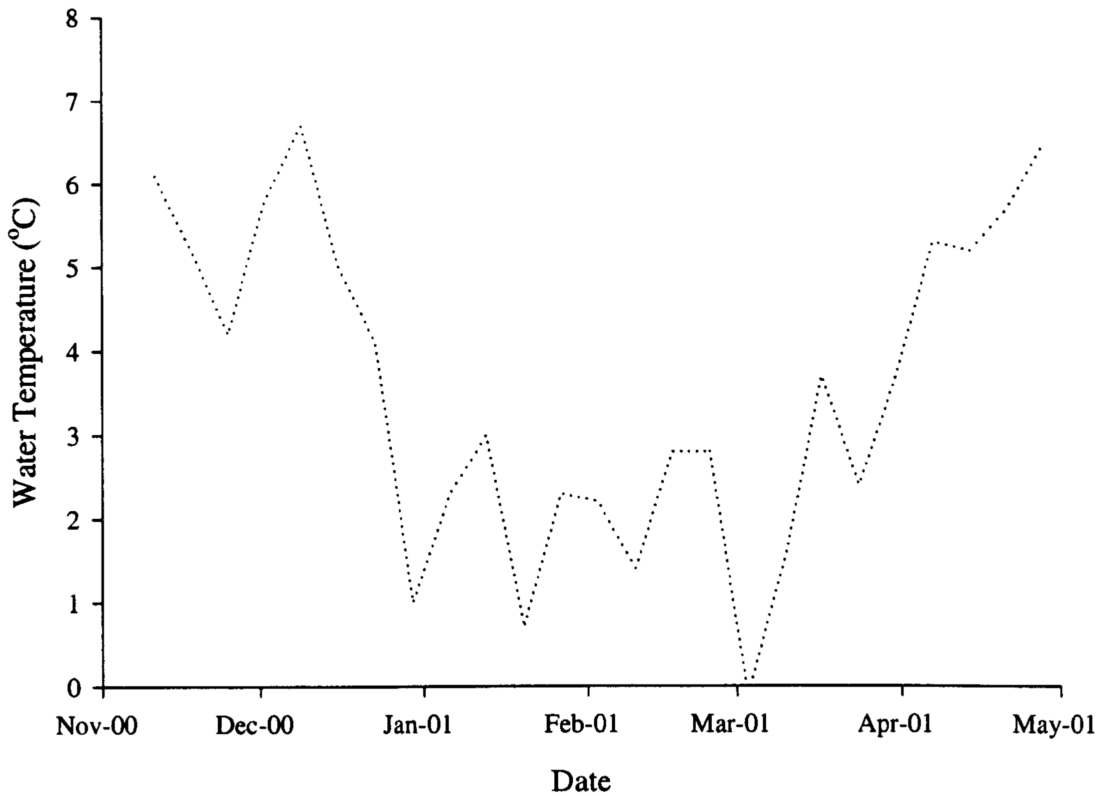


Figure 5.1 Site 1 mean weekly inlet water temperature profile supplying trial tanks from November 2000 to May 2001.

duplicate treatments. Top grades (17.5 to 19g, ~200kg/tank) were subsequently removed from each tank on the 20th March 2001 prior to completion of the trial on 3rd May 2001.

To examine the effects of photoperiod and feeding regime on growth, four treatments were chosen as follows:

Outside: Ambient Photoperiod (7 to 14 hours Light) and Ambient Feeding (AA)

Outside: Constant Light and Ambient Feeding (LA)

Outside: Constant Light and Extended Feeding (LE)

Inside: Constant Light and Extended Feeding (IL)

Lighting in each outdoor tank was provided by two Aquabeam Pisces floating 400W lamps (4000K), positioned each side of the central standpipe and screen. Lighting in the indoor tank was provided by two 48 Watt fluorescent tubes positioned 3 metres above the tank. Light intensity readings (Skye Instruments, Oxon, UK) were taken during both day and night to assess the relative differences between treatments (Table 5.1). Daytime light intensity readings were taken between 11am and 12pm, with night readings taken between 7.30 and 8.30pm on 16th February 2001. Water quality during this period was good with minimal suspended solids. Readings were taken at the bottom of each tank from 12 locations around the tank. An outdoor air intensity reading of >20,000 Lux was recorded under bright, clear conditions with a light breeze, while the intensity indoors in the partly covered building was 300 Lux.

Table 5.1. Mean light intensity (Lux) readings ± SEM recorded in each tank under ambient photoperiod (AA) and constant light (LL) treatments during day (11am-12pm) and night (7.30-8.30pm).

Treatment	Day	Night
AA	>20,000 Lux	0 Lux
LA	>20,000 Lux	3419 ± 2139 Lux
LE	>20,000 Lux	3370 ± 2105 Lux
IL	37 ± 6 Lux	36 ± 7 Lux

From first feeding to 5g, fry diets were supplemented with Ergosan (AquaVac™) at 0.5% inclusion to improve resistance against rainbow trout fry syndrome. Until fish reached 8g, Trouw Aminobalance™ in 1.8mm pellets were provided (Protein: 54%, Lipid: 18%, Carbohydrate: 11%, Ash: 9%, Moisture: 8%, Astaxanthin: 5mg/kg; DE: 19.22 MJ/kg). Thereafter, fry were fed a larger pellet of 2.3mm (Protein: 47%, Lipid: 24%, Carbohydrate: 12%, Ash: 9%, Moisture: 8%, Astaxanthin: 5mg/kg; DE: 19.79 MJ/kg). Food was initially presented by clockwork belt feeders and later (1st March) in conjunction with demand feeders. Rations were maintained at the same quantity with the rate adjusted to that of the tank with the lowest feed intake, and fed throughout the natural daylight hours for ambient feeding treatments (AA and LA), while extended feeding regimes (LE and IL) received an additional ration spread over the extended photophase by means of clockwork belt feeders.

To ensure a homogenous sample, twenty sweeps of a net were taken at random positions around each tank, with fish caught being mixed in a large container prior to measurement. A total of 200 individual length-weight measurements were taken per

treatment with an additional 2000 fish batch weighed in eight groups of 250 fish, to verify representation of the individual weight measurements.

The indoor tank was included in analysis despite different lighting and temperature regimes as this tank is used to produce top grade fish for first cycle stocking on this particular site. This tank was used as a reference mark to see what potential improvements in production could be achieved by photoperiod application in the outdoor tanks, the applied aim being to produce more fish in the top grade for earlier stocking into ongrowing raceways.

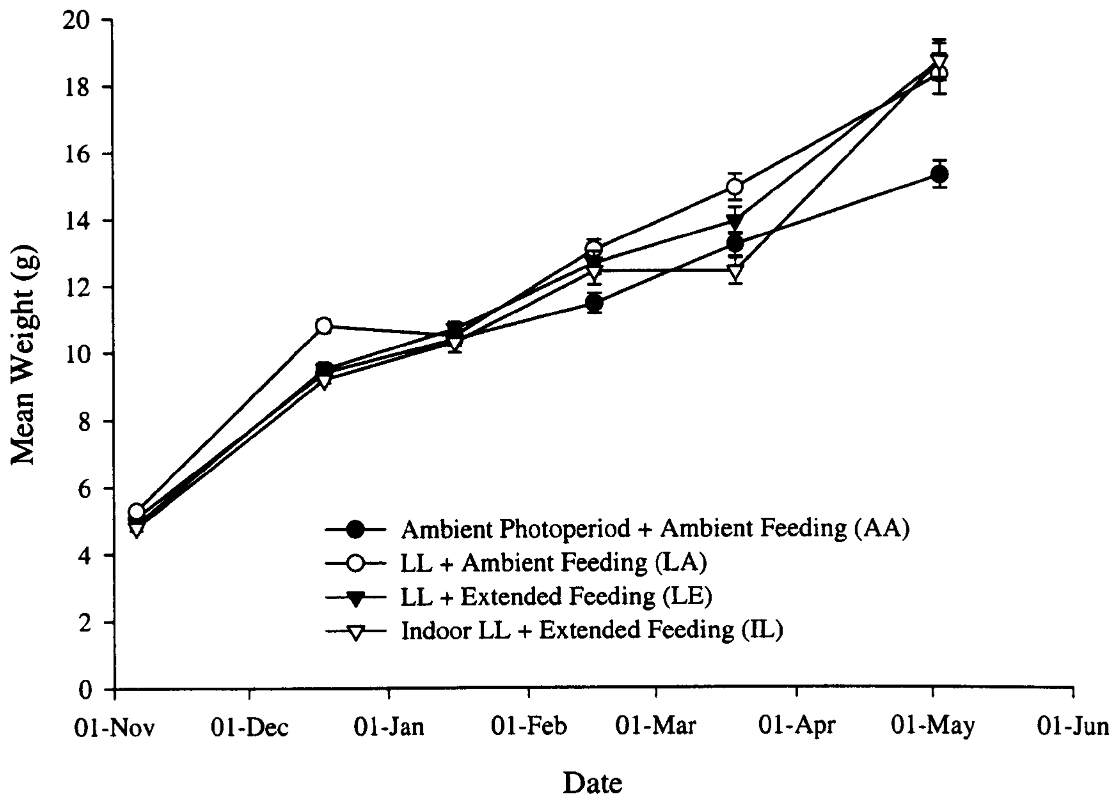
5.3.2 Results

At the start of the trial, fish under each treatment were not significantly different in terms of weight, length and condition factor.

Effects of Constant Light on Growth Parameters

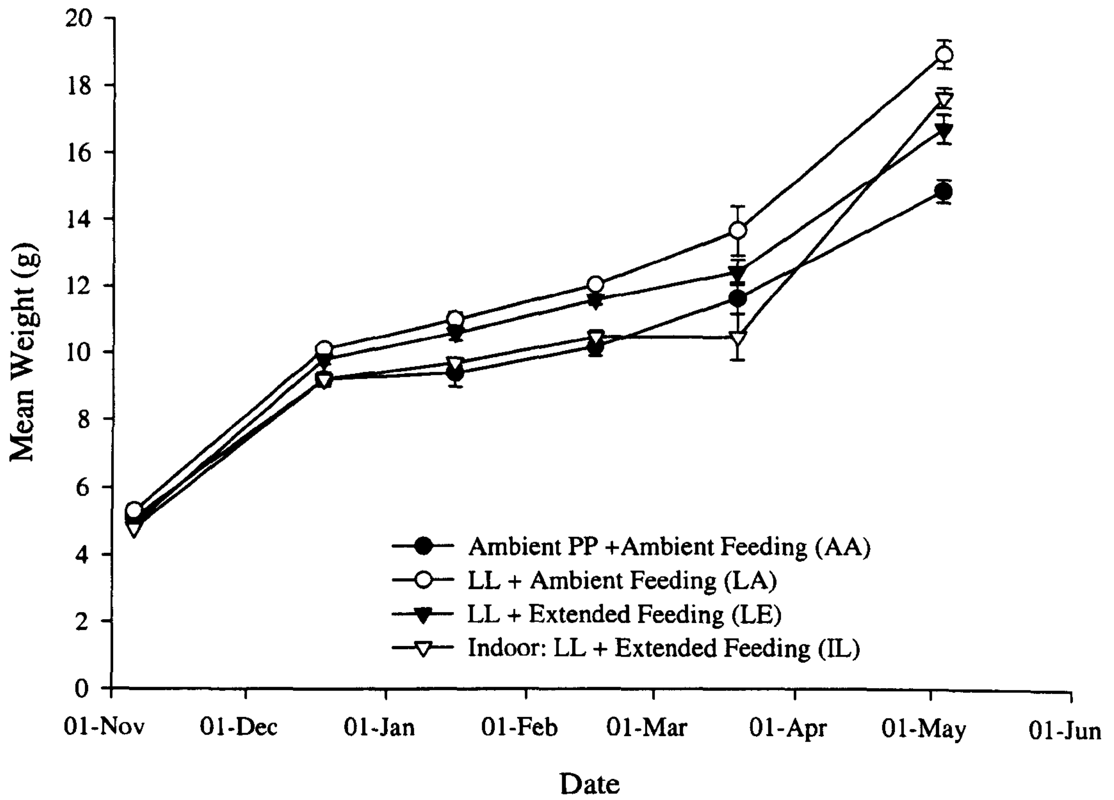
Weight

Following one month exposure to constant light, LA fish were significantly heavier than all other treatments (Figure 5.2). During the following month (Dec-Jan), growth rate was reduced in all groups, most likely in association with rapidly falling temperatures (Figure 5.1). Over the subsequent months constant light groups continued to increase weight at a greater rate than those under ambient photoperiod, with LA and LE groups reaching a significantly higher weight by February. This was more evident with batch weight measurements, which showed a clear distinction in mean weight gain in both LA and LE groups over IL and AA groups (Figure 5.3). This pattern was evident



Time Treat	1	2	3	4	5	6
AA	a	a	a	a	ac	a
LA	a	b	a	b	b	b
LE	a	a	a	b	a	b
IL	a	a	a	ab	c	b

Figure 5.2 Influence of constant light and feeding regime on individual mean weight gain ± SEM in rainbow trout held in tanks (n=200). Superscripts denote significant differences between treatments at given time points ($p<0.05$). Numbers in table represent specific time points.



Time / Treat	1	2	3	4	5	6
AA	a	a	a	a	a	a
LA	a	a	a	b	c	b
LE	a	a	a	b	b	b
IL	a	a	a	a	ab	b

Figure 5.3 Influence of constant light and feeding regime on mean weight gain ± SEM in rainbow trout held in tanks as calculated from batch weights. Superscripts denote significant differences between treatments at given time points ($p<0.05$). Numbers in table represent specific time points.

throughout the entire period of the trial following one month exposure to constant light. By Mid-March LA fish had reached a significantly greater weight than all other groups, with no significant difference observed between LE and AA groups. Clearly evident during this period is a stagnation in weight in the IL group, which was also evident in batch weight measurements (Figure 5.3) By the end of the trial, 3rd May 2001 both individual measurements and batch weights showed that all groups on constant light were significantly heavier than those under ambient conditions (AA). During the final month IL fish exhibited a rapid increase in weight gain (+68.6%) compared to LA (+38.8%), LE (+34.8%) and AA (+28.0%) groups.

Specific Growth Rate (SGRwt)

The observed improved growth rate of the fish under constant light is well illustrated in the relative weight gain and growth rate (SGRwt, % day^{-1}) between the start and end of the trial (Table 5.2). It is evident that a significantly lower weight gain was achieved in the AA group compared to all other treatments. Although reaching a lower final weight than LA, IL fish showed the greatest weight gain between all treatments, an increase of 261.2% from the original starting weight. Of interest is a 16.1% greater weight gain in the ambient feeding (LA) group compared to those on extended feeding (LE). Although seemingly small, this represents a significant increase in such small fish.

Total SGRwt for the period of the trial shows that LL led to a higher SGRwt compared to those maintained under ambient conditions (AA) allowing the attainment of stock-out weight for ongrowing one month earlier than under natural photoperiod.

Table 5.2 The effect of photoperiod and feeding regime on relative weight gain (%) and total growth rate (SGRwt) in tank reared fry from 6th November 2000 to 5th May 2001. AA: Outside: Ambient Photoperiod and Ambient Feeding; LA: Outside: Constant Light and Ambient Feeding; LE: Outside: Constant Light and Extended Feeding; IL: Inside: Constant Light and Extended Feeding.

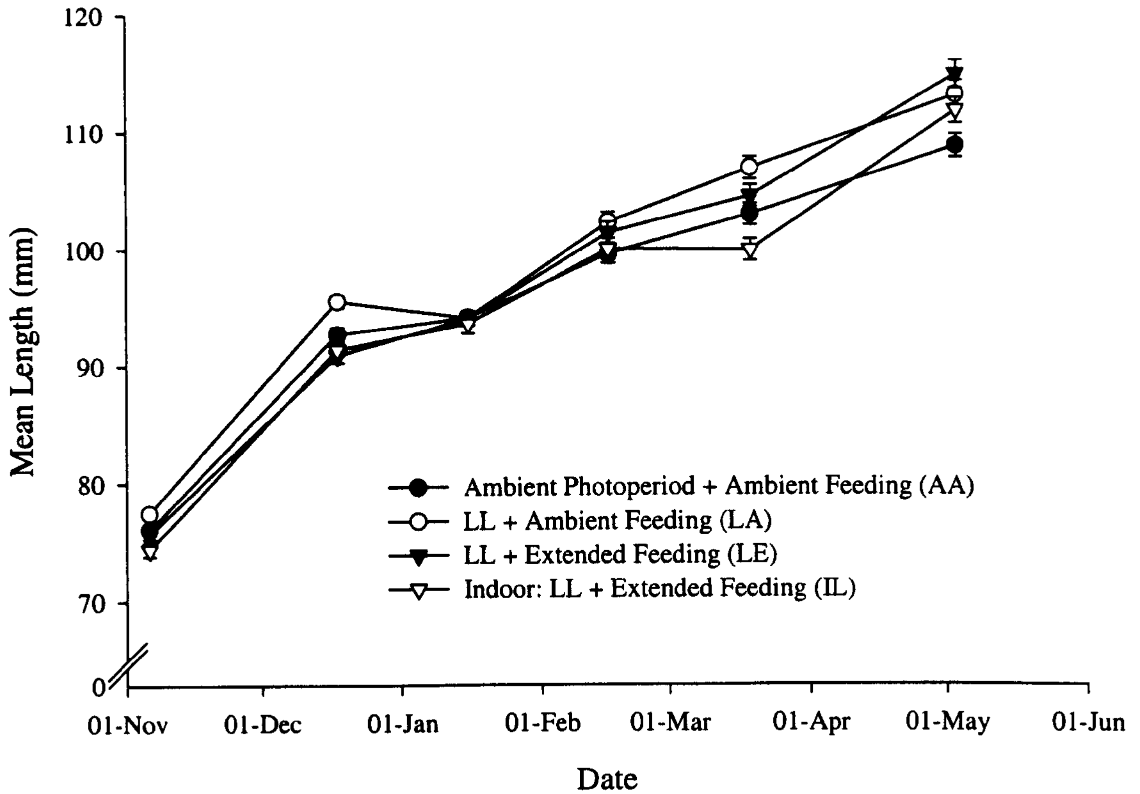
Treatment	Weight (g)		% Weight Gain	Total SGRwt (% day^{-1})	% improvement SGR relative to control
AA	Start	5.1	+ 192.2 %	0.60	-
	End	14.9			
LA	Start	5.3	+ 258.5 %	0.72	+ 20 %
	End	19			
LE	Start	4.8	+ 250 %	0.69	+ 15.7 %
	End	16.8			
IL	Start	4.9	+ 261.2 %	0.73	+ 21.7 %
	End	17.7			

Length

Length increase during the trial followed a similar pattern to that observed in weight increase. By the end of the trial all constant light groups (LA and LE) except IL were significantly longer than AA fish (Figure 5.4).

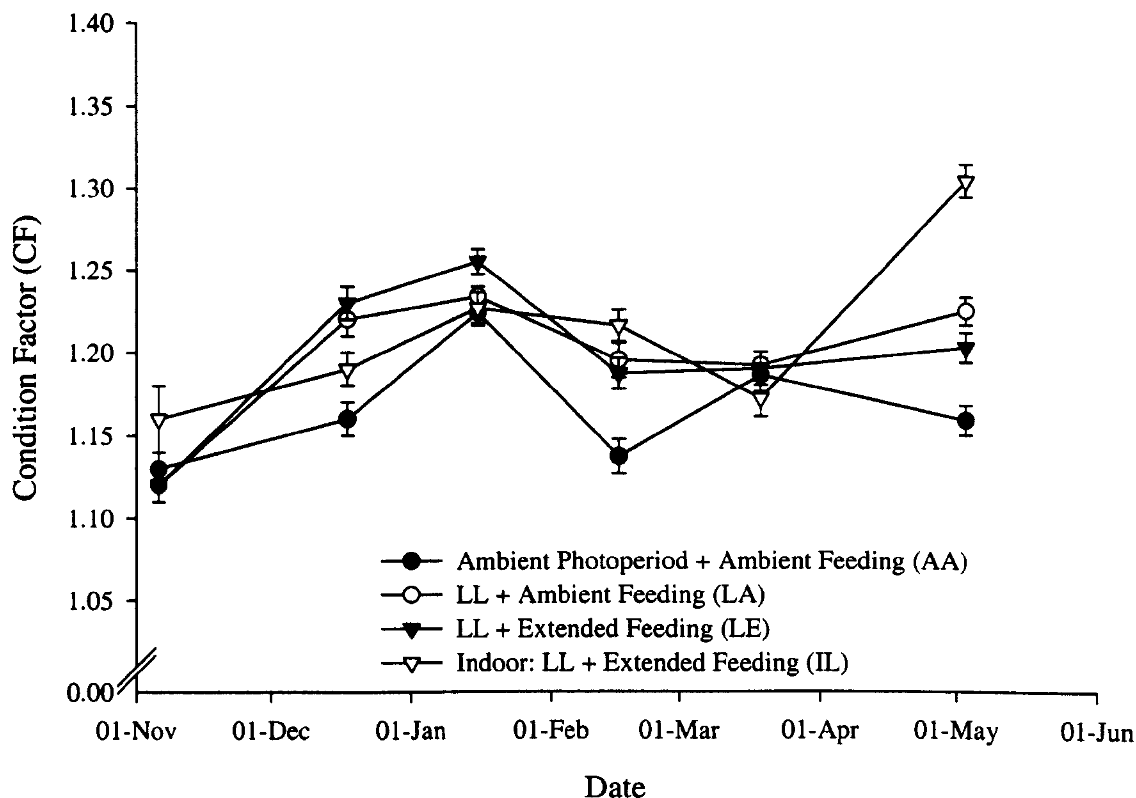
Condition Factor (CF)

During the trial CF showed a similar pattern of increase and decrease in all groups during the first four months, ranging between 1.1 and 1.24 (Figure 5.5). Subsequently, IL fish maintained a significantly higher CF than all other groups. CF in both LA and LE were not considered significantly different, but were significantly higher than those under ambient photoperiod (AA). Throughout the period of the trial AA fish maintained the lowest condition at each sample point, with the exception of mid-March.



Time / Treat	1	2	3	4	5	6
AA	a	a	a	a	a	a
LA	a	b	a	a	b	b
LE	a	a	a	a	ab	b
IL	a	a	a	a	c	ab

Figure 5.4 Influence of constant light and feeding regime on length increase (mean ± SEM) in rainbow trout fry held in tanks (n=200). Superscripts denote significant differences ($p<0.05$). Numbers in table represent specific time points. Numbers in table represent specific time points.



Time Treat	1	2	3	4	5	6
AA	a	a	a	a	a	a
LA	a	bc	a	b	a	b
LE	a	b	b	b	a	b
IL	a	c	a	b	a	c

Figure 5.5 Condition factor variation (mean ± SEM) of rainbow trout fry held in tanks on different photoperiod and feeding regimes (n=200). Superscripts denote significant differences at given time points (p<0.05). Numbers in table represent specific time points.

Feed Conversion

No statistical analysis was performed as values were based on single values between time points. Although fluctuating throughout the trial, feed conversion (FCR) was clearly maintained at a lower and more efficient ratio in LA fish than those under the other treatments (Figure 5.6). Following two months exposure to constant light it was also evident that LE fish maintained a more efficient FCR, although AA FCR improved towards the end of the trial. Observation of the total FCR for the entire period of the trial (Table 5.3) shows that constant light alone (LA) exhibited the most efficient FCR, which was significantly lower than those receiving extended feeding (LE and IL) or those maintained under ambient photoperiod (AA).

Table 5.3 Mean feeding efficiency (FCR) of rainbow trout fry from stocking in October to grading on 5th May 2001, and the relative differences between those maintained under natural photoperiod (NL) and those under constant illumination (LL). AA: Outside: Ambient Photoperiod and Ambient Feeding; LA: Outside: Constant Light and Ambient Feeding; LE: Outside: Constant Light and Extended Feeding; IL: Inside: Constant Light and Extended Feeding.

Treatment	**Biomass (kg)**		**Biomass Gain (kg)**	**Food Presented (kg)**	**FCR**	**% Improvement Relative to Control**
	Start	**End**				
NL	212.8	524.1	311.3	650	2.08	-
LA	222.8	735.1	512.3	609.5	1.19	+42.8 %
LE	206.2	687	480.8	800	1.66	+ 20.2 %
IL	241.1	958.3	717.2	1000	1.39	+ 33.2 %

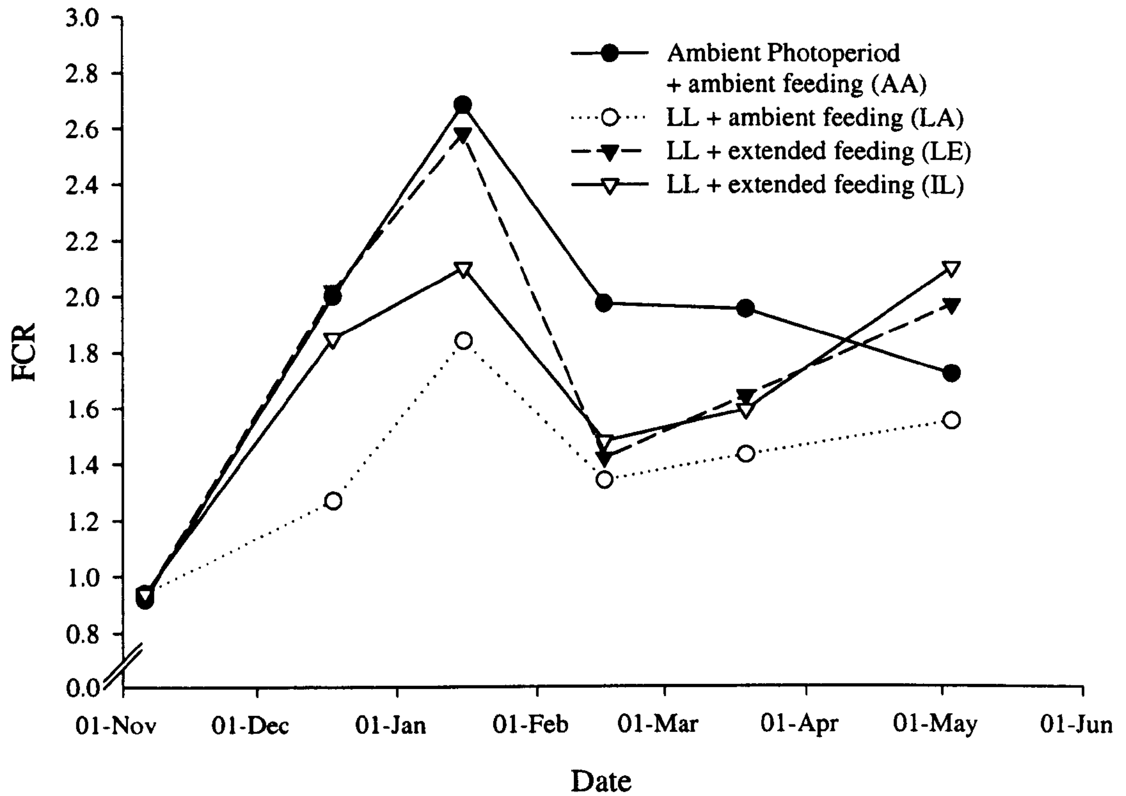


Figure 5.6 Influence of constant light and feeding regime on the ratio of food fed to weight gain (FCR) of rainbow fry trout held in tanks.

5.3.3 Summary of Results

- Significant effects on fry weight gain were observed 14 weeks after LL application.
- Fish exposed to LL had a higher condition factor than those under ambient photoperiod at grading.
- LL application and feeding only during the natural daylight hours significantly improved growth rate and feed conversion efficiency relative to ambient photoperiod exposure, resulting in greater biomass and individual weight gains.
- Extended feeding under LL did not improve growth rate and led to a less efficient FCR than feeding during the natural ambient day length only.
- Overall, LL application in outdoor tanks improved specific growth rate and feeding efficiency, leading to greater biomass gains than those exposed to ambient photoperiod. Fish under LL in outdoor tanks were also ready to be stocked for ongrowing one month earlier than those under ambient photoperiod.

5.4 Trial 2: The effects of submersible lighting providing constant illumination on growth of two strains of cage reared rainbow trout during winter on-growing

5.4.1 Materials and Methods

On 25th October 2001 four 64m^3 (4 x 4 x 4m) cages were stocked with one of two commercially available all-female rainbow trout strains. Strain 1 (-1) was supplied by Glen Wyllin Hatcheries, Isle of Man and strain 2 (-2) was a selected fast-growing strain supplied by Trend, Denmark. Both strains were reared from hatch, January 2001, at the same hatchery under similar conditions until stocking at 8 months of age. For each strain one cage was exposed to natural photoperiod (NL) and the other to constant light (LL). Each cage was set up as a treatment in single as shown in table 5.4 and maintained under ambient water temperatures (Figure 5.7).

Table 5.4 Mean starting weights and experimental set up for two strains of rainbow trout exposed to natural or constant light photoperiod regimes.

Strain	Photoperiod	Mean Weight (g)	Fish Number	Biomass (kg)
One	Natural Light (NL-1)	71.4 ± 3.7	23,431	1673
	Constant Light (LL-1)	79.9 ± 4.5	19,011	1519
Two	Natural Light (NL-2)	57.9 ± 2.1	13,972	809
	Constant Light (LL-2)	50.8 ± 2.4	17,795	904

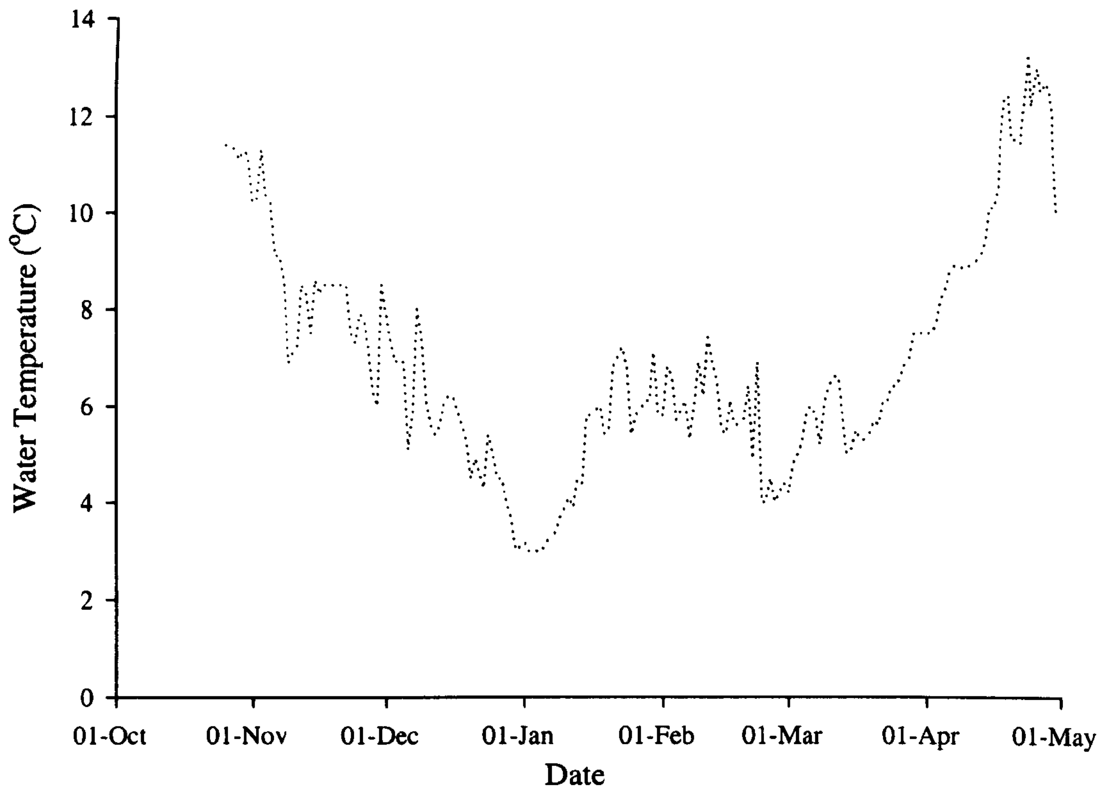


Figure 5.7 Site 2 daily water temperature profile for period of trial 25th October 2001 to 30th April 2002.

Due to increased stocking density both strain 1 stocks were transferred to larger 256m^3 (8 x 8 x 4m) cages on 16th January 2002, while strain 2 stocks were transferred on 18th February 2002.

Lighting was provided by two submersible Aquabeam Pisces 5 submersible 400W lamps (4000K) positioned mid-cage depth (2m) and 2 metres apart either side of the central feed hopper. Light intensity readings were taken at 12pm and 12am on 2nd November 2001 and 1st April 2002 (Table 5.5). A daytime air intensity of 2900 Lux was measured during November under conditions of low cloud and heavy rainfall, while 13,580 Lux was recorded under bright, clear conditions with a light breeze during April. Water quality during November was poor, with high levels of suspended solids observed following flood conditions, while April saw minimal suspended solids giving exceptionally clear conditions. Readings were taken at approximately 1m intervals across and along the cage at the surface, mid depth and bottom of each cage during both day and night.

Table 5.5 Mean day-night light intensities (Lux ± SEM) measured during November and April at the water surface, 2m (mid-cage) and 4m (cage bottom) in cages with ambient photoperiod (NL) and constant illumination (LL).

		2nd November 2001		1st April 2002	
Treatment	**Depth (m)**	**Day**	**Night**	**Day**	**Night**
	0	1292 ± 92.9	0	5100 ± 450	0
NL	2	52 ± 8.4	0	240 ± 80	0
	4	1.3 ± 0.3	0	0.1± 0.01	0
	0	1223 ± 74.2	0.35 ± 0.16	4258 ± 556	13.1 ± 5.7
LL	2	201.3 ± 89.4	98.8 ± 39	618 ± 259	101.6 ± 28
	4	1.72 ± 0.15	4.55 ± 1.9	2.4 ± 0.11	4.3 ± 1.3

Under both ambient and artificial lighting conditions there was a clear reduction in intensity with depth during both day and night. However, mid-water intensities were significantly higher under LL than ambient conditions during the day in both November

and April, with similar levels maintained during the night at both time points. Higher intensities were also achieved on the cage floor in lit cages during the night than during the day at both time points.

Fish were fed identical rations according to manufacturer's tables during the daylight hours by means of a centrally positioned automated vibro-spin hopper set at 15 minute intervals. Trouw Aminobalance™ in 3mm pellets were provided (Protein: 45%, Lipid: 30%, Carbohydrate: 11%, Ash: 8%, Moisture: 6%, Astaxanthin: 50mg/kg; DE: 21.46 MJ/kg), followed by Trouw Royal Optima™ in 3 and 4mm pellets (Protein: 44%, Lipid: 30%, Carbohydrate: 12%, Ash: 7%, Moisture: 7%, Astaxanthin: 50mg/kg; DE: 21.38 MJ/kg) as of 12th December 2001.

To facilitate easier sampling all fish were crowed by raising the cage nets. A total of 200 individual length-weight measurements and 20 random batch weights (approx. 10kg / batch weight) were taken per cage at monthly intervals. All fish measured were returned to the opposite side of the crowded net to avoid the possibility of weighing fish twice. Fish were blood sampled for melatonin analysis on 21st November 2001 and 1st April 2001, with a total of 20 fish per cage killed and sampled at 2 time points (12pm and 12am). The trial ended on the 30th April 2001 following grading of all cages with the exception of LL-1 which was graded on 22nd April. At grading a random sample of 50 fish from strain 2 harvest grades under each photoperiod treatment were processed for fillet pigmentation using a tristimulus colorimeter (Minolta Chroma Meter.) This analysis was carried out courtesy of Scot Trout Ltd. The measured colour parameters were fillet red/green chromaticity (a*), yellow/blue chromaticity (b*), and lightness (L*). The a* value represents fillet redness and the b* value the yellowness. From a* and b* values, chroma (Cab*) and Hue (Hab*) were calculated. Chroma is an expression of the intensity and clarity of the colour, while hue

is the relationship between the redness and the yellowness of the fillet, where hue is an angular measurement (0° indicates red and 90° indicates a yellow hue).

5.4.2 Results: Effect of Constant Light on Growth Parameters

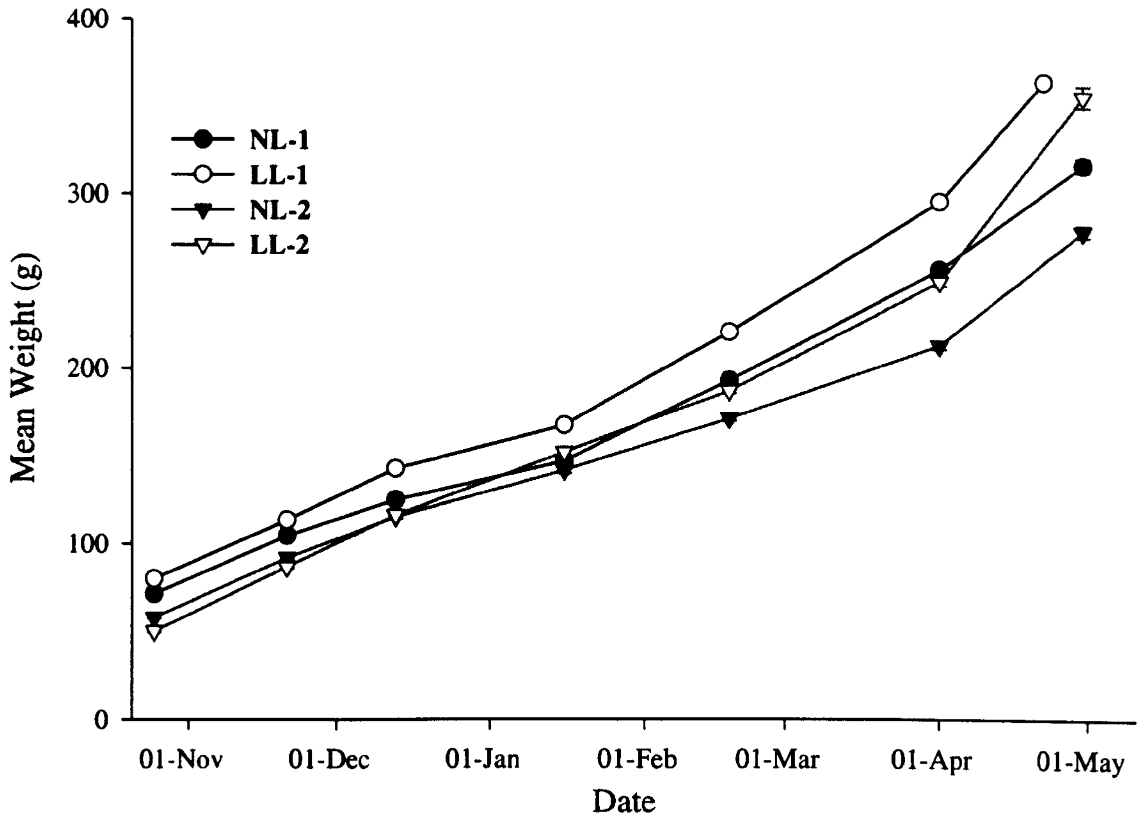
Weight

At the beginning of the trial no significant differences were observed in starting weight between treatments in strain 1 and 2. Strain 1 was significantly larger than strain 2 (Figure 5.8). Under LL both strains grew steadily reaching significantly higher mean weights than their respective controls by the culmination of the trial. However, both strains exhibited different growth patterns (SGRwt) in response to light treatment.

Specific Growth Rate (SGRwt)

Strain 1

After 8 weeks exposure to constant light (Dec '01), LL-1 attained a significantly higher mean weight, 167.3 ± 1.3g, relative to their control, NL-1, 146.9 ± 2.7g (Figure 5.8). This separation in weight coincided with a period of growth between November and December where LL-1 maintained a higher SGRwt (1 % vs. 0.77 day^{-1}, NL-1) despite a natural decrease in the rate of gain associated with decreasing temperatures (Figure 5.9). Thereafter, SGRwt for both LL-1 and NL-1 exhibited a similar rate and pattern for the remainder of the trial, with the exception of the final month where LL-1 showed a rapid increase in SGRwt to 0.99 % day^{-1} compared to 0.72 % day^{-1} for NL-1. The period of higher growth maintained the weight difference until grading of LL-1 on 22nd April 2002, where end weights of 316.3g and 364g for NL-1 and LL-1 were achieved.



Time Treat	1	2	3	4	5	6	7
NL-1	a	a	a	ac	a	a	a
LL-1	a	a	b	b	b	b	-
NL-2	b	b	c	a	c	c	b
LL-2	b	b	c	c	a	a	c

Figure 5.8 The effect of constant light on weight gain (mean ± SEM, n=200) in two different strains of rainbow trout held in cages exposed to natural photoperiod and constant illumination. Superscripts denote significant differences between treatments and strain. In some cases SE bars are too small to be depicted.

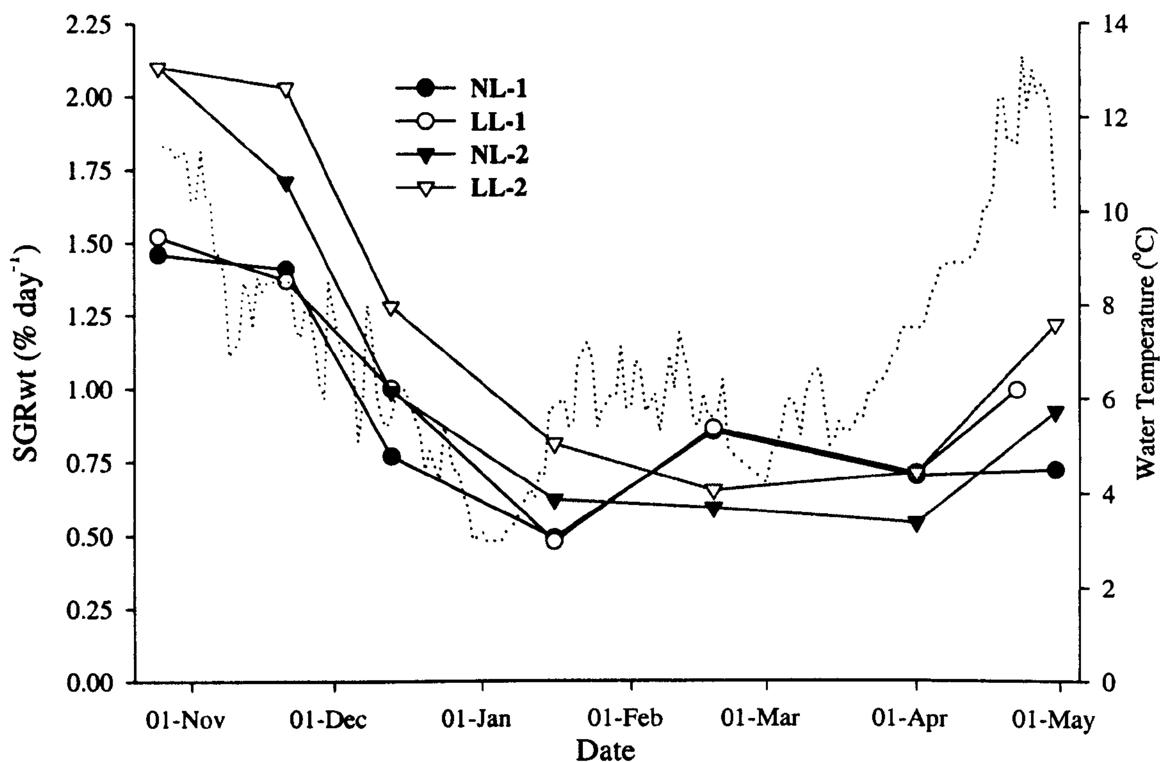


Figure 5.9 The effects of constant light on daily weight gain (SGRwt) between two different strains of rainbow trout relative to those maintained under natural photoperiod. The broken line represents ambient water temperature.

Strain 2

In contrast, LL-2 exhibited a greater weight gain relative to NL-2 after only 4 weeks exposure to constant light (Figure 5.8). Thereafter, weight gain in LL-2 continued at a steady rate reaching a significantly greater weight, 151.9g ± 1.6 SEM, than NL-2 (141.6g ± 1.6 SEM) by January 2002 (12 weeks exposure). From this point onwards, the weight advantage increased until grading on 30th April 2002. Examination of the SGRwt showed that LL-2 maintained a consistently higher rate than NL-2 throughout the trial, with a strong positive increase in growth rate during April (Figure 5.9).

Furthermore, LL-2 also exhibited a higher SGRwt than LL-1 and NL-1 with the exception of the period between January and February 2002. As a result, LL-2 not only achieved a significantly larger weight, 356 ± 6.1g, than NL-2 (278 ± 3.1g), but also grew to a significantly larger mean weight than NL-1 (316.3 ± 3.9g) despite starting the trial at a significantly smaller weight. As LL-1 was graded before the final sample point comparison of end weight achieved relative to LL-2 is not possible. Table 5.6 provides a summary of start and end weights achieved and the average growth rate throughout the trial indicating the improvements of LL treatment relative to their respective controls. A greater total SGRwt was maintained by LL-2 compared to LL-1 resulting in a greater percentage weight gains in the former relative to its natural light control.

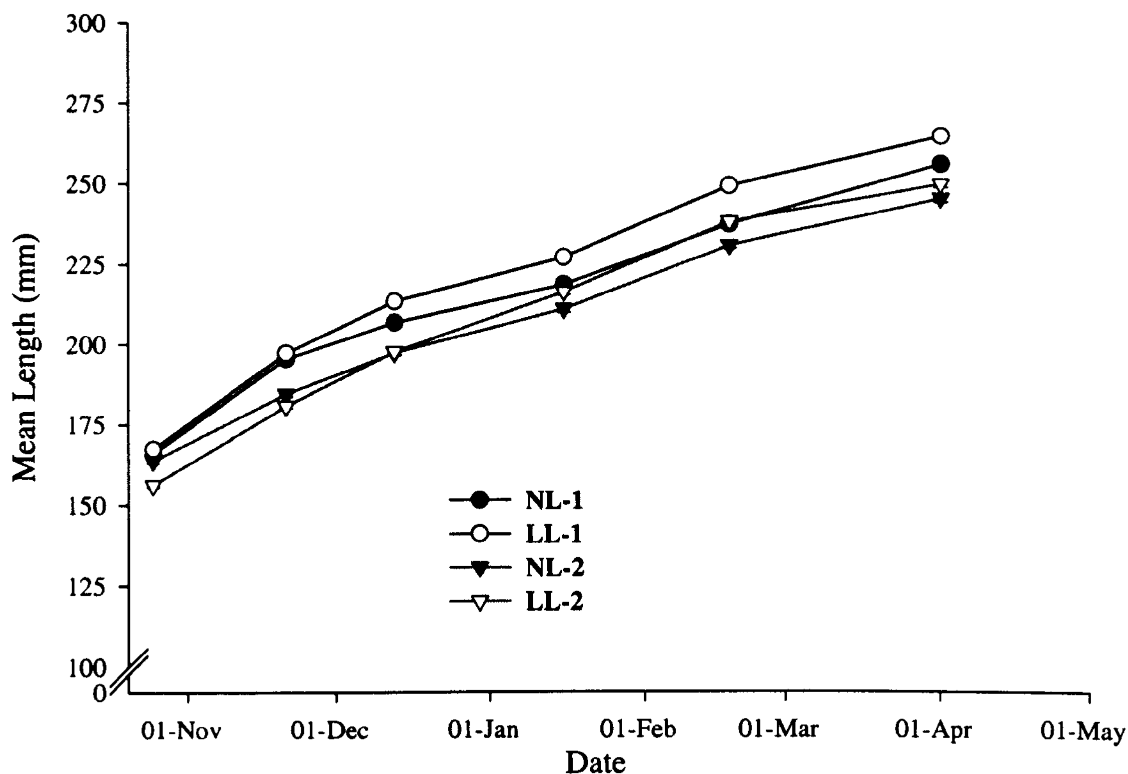
Table 5.6 Comparison of relative weight gain (%) and total growth rate (SGRwt) between fish exposed to natural photoperiod and constant light in two commercial strains of rainbow trout from 25th October 2001 to 30th April 2002.

Treatment	Weight (g)		% Weight Gain	Total SGRwt (% day^{-1})	% improvement SGRwt relative to control
NL-1	Start	71.4	343.0 %	0.82	
	End	316.3			
LL-1	Start	78.2	365. 5%	0.89	+ 8.5%
	End	364			
NL-2	Start	57.9	380.7 %	0.87	
	End	278.3			
LL-2	Start	50.1	609.6 %	1.08	+ 24.1%
	End	355.5			

Length

At the start of the trial NL-1, LL-1 and NL-2 were of similar length ranging from 163.7 to 167.4mm, and LL-2 significantly smaller with a mean length of 156.3mm. Length increased steadily in all treatments with LL fish reaching significantly greater mean lengths than their respective controls by the end of the trial, although the pattern of length increase was different in each strain (Figure 5.10).

In strain 1, length of NL-1 and LL-1 increased rapidly during the first 8 weeks (Dec '01), exceeding that of NL-2 and LL-2. At this point LL-1 achieved a significantly longer mean length of 213.4 ± 0.8mm than NL-1, 206.0 ± 0.8mm, with this advantage maintained until 1st April where final lengths of 264.6 ± 1.1 mm and 256 ± 1.0 mm were achieved (Figure 5.10). By February 02, LL-1 were approximately 5% longer than NL-1, however, although longer by 1st April, the difference reduced to 3.4%.



Time Treat	1	2	3	4	5	6
NL-1	a	a	a	a	a	a
LL-1	a	a	b	b	b	b
NL-2	a	b	c	c	c	c
LL-2	b	b	c	a	a	d

Figure 5.10 The effect of constant light on length increase (mean ± SEM, n=200) in two different strains of rainbow trout held in cages. No length measurements were taken on 30th April as grading had commenced. Superscripts denote significant differences between treatments and strain. In some cases SE bars are too small to be depicted.

In strain 2, after 4 weeks exposure to LL, LL-2 showed a greater length gain reaching 180.7 ± 0.7 mm, similar to that of NL-2 at 184.5 ± 0.7mm. By mid-January LL-2 length significantly exceeded that of NL-2 reaching 216.2 ± 0.9mm, a length statistically similar to NL-1 at 218.5 ± 0.9mm. By mid-February, LL-2 attained a length of 237.8 ± 0.8mm, 3.1% longer than NL-2 which reached 230.5 ± 0.9mm. As observed in strain 1, length advantage was reduced during April to 1.8%.

Length Specific Growth Rate (SGRL)

Strain 1

The achievement of a significantly longer length by LL-1 after 8 weeks exposure to constant light coincided with a period of higher rate of daily length gain (SGRL) (Figure 5.11). SGRL then followed the same pattern and rate as NL-1 until February 02, at which point LL-1 SGRL showed a marked reduction from 0.29 to 0.15 % day^{-1} compared to 0.26 to 0.19 % day^{-1} for NL-1 during the March-April period.

Strain 2

In terms of SGRL, LL-2 maintained a higher daily rate of gain than NL-2 after 4 weeks exposure to LL, which remained substantially higher until February 02 (Figure 5.11). Thereafter, as seen in LL-1, LL-2 showed a significant reduction in the rate of length gain during the mid-February-April period, decreasing from 0.3 to 0.12 % day^{-1}, compared to NL-F which decreased from 0.28 to 0.15 % day^{-1} during the same period.

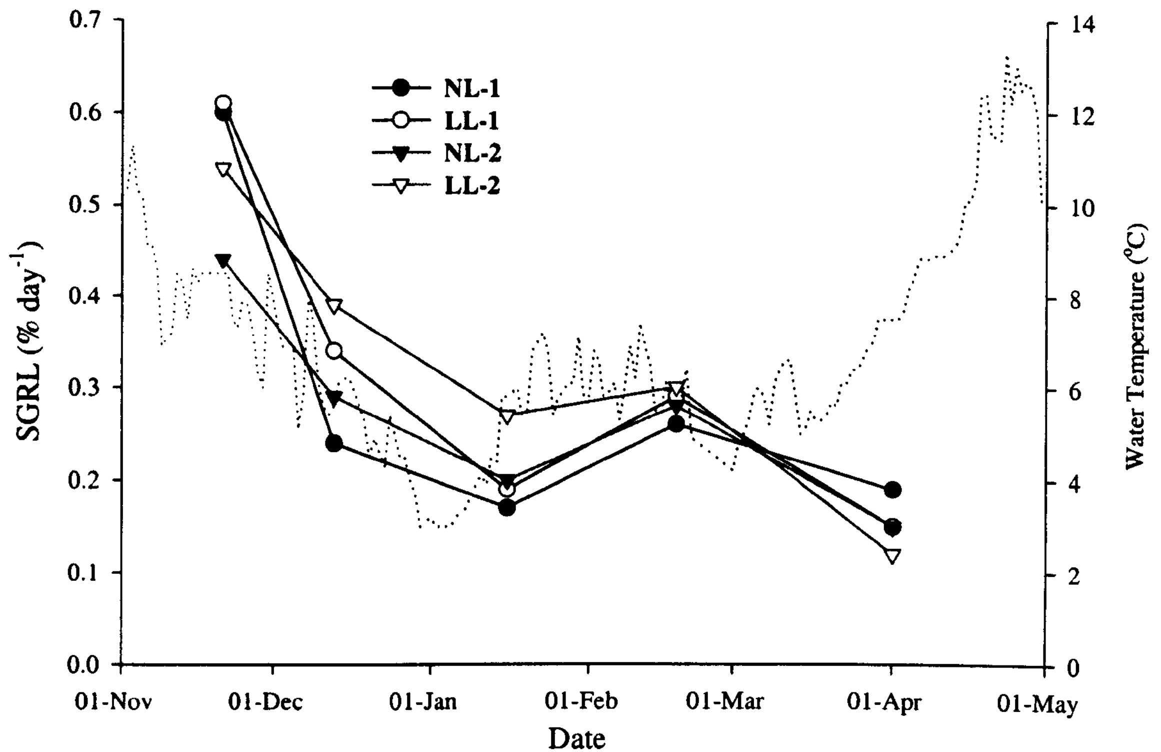


Figure 5.11 The effects of constant light on daily length gain (SGRL) between two different strains of rainbow trout relative to those maintained under natural photoperiod. No starting SGRLs are available for the period prior to 25th October 2001 as length measurement is not standard farm practice at this site. The broken line represents ambient water temperature.

Condition Factor (CF)

CF changed throughout the period of the trial, with both strain and treatments exhibiting different responses, although final CFs were higher than at first stocking for all treatments (Figure 5.12). CF in NL-1 remained relatively constant from October to January, ranging from 1.39 to 1.41, with subsequent months seeing a steady increase in CF, reaching 1.53 by 1st April. In contrast, LL-1 showed an increase in CF during the first 4 weeks of LL treatment rising from 1.44 to 1.48. Thereafter, condition decreased steadily to 1.43 by 18th February. During the February-April period, CF then showed a significant increase reaching a value of 1.59.

In strain 2, CF rose steadily between October and December (8 weeks exposure) in both NL-2 and LL-2 to 1.51, and remained constant until January. CF at this point was considerably higher than either treatment in strain 1 during the same period. The statistical difference could not be assessed as CF was calculated from mean batch weight and mean individual length measurements. Over the next month, CF decreased in both treatments, falling to 1.39, substantially below that of NL-1 and LL-1. Between February and April, both strain 2 treatments showed a positive increase in condition, with LL-2 achieving 1.60, similar to that recorded in LL-1. NL-2 expressed the lowest condition factor of all treatments at 1.45 during the final sample.

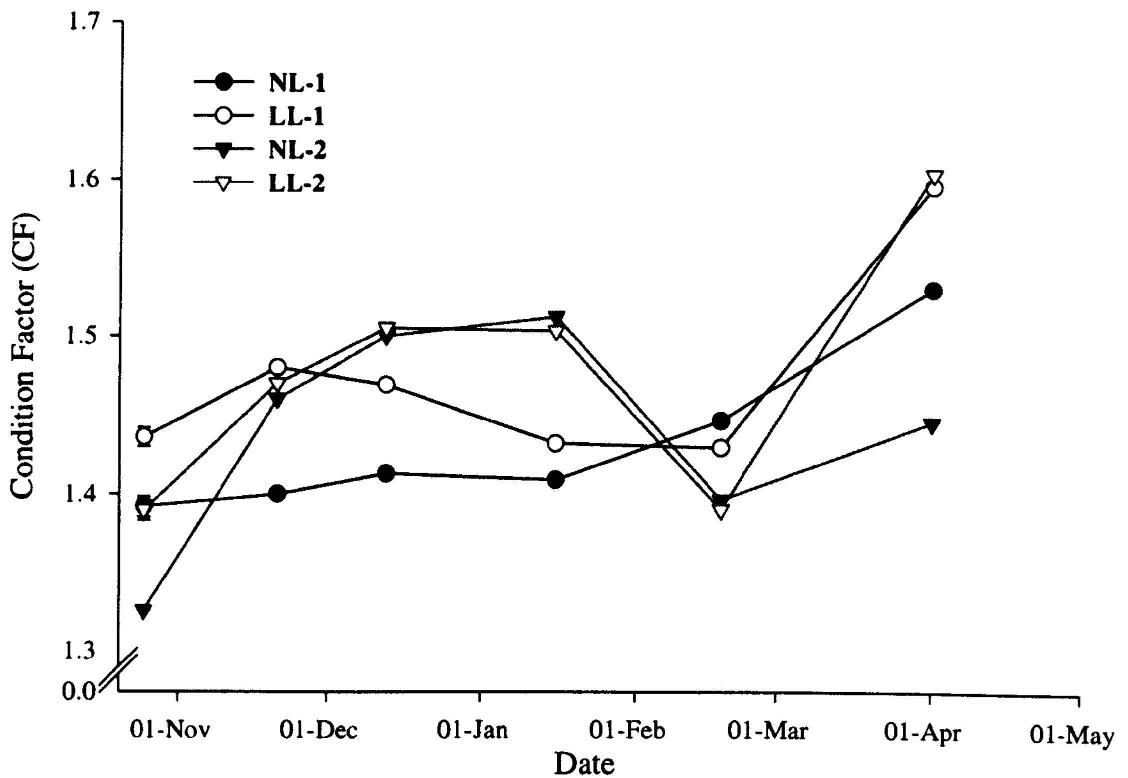


Figure 5.12 The effect of constant light on condition factor in two different strains of rainbow trout held in cages. As length measurements were not taken during grading, condition factor could not be calculated for the final time points in any treatment. Statistical analysis was not performed as CF was calculated from mean batch weight and mean individual length measurements.

Feeding Efficiency

In both strains, total food conversion ratio (FCR) was more efficient in fish under LL than ambient photoperiod for the period of the trial (Table 5.7). Of all treatments, LL-2 exhibited the most efficient FCR, followed by marked improvements in LL-1, with little difference in feeding efficiency observed between NL-1 and NL-2.

Table 5.7 Total feeding efficiency (FCR) of two strains of rainbow trout from stocking in October to harvest on 30th April 2002, and the relative differences between those maintained under natural photoperiod (NL) and those under constant illumination (LL).

Treatment	Biomass (kg)		Biomass Gain (kg)	% Weight Gain	Food Presented (kg)	FCR	% Improvement Relative to Control
	Start	End					
NL-1	1673	7204	5531	+331%	6916	1.25	
LL-1	1519	6838	5319	+350%	5623	1.06	+ 15.2 %
NL-2	809	3792	2983	+369%	3614	1.21	
LL-2	904	6185	5281	+584%	4782	0.91	+ 24.8 %

5.4.3 Effects of Submersible Cage Lighting on Pattern of Melatonin Secretion

Figure 5.13 (a) and (b) provide day-night variation in plasma melatonin levels for each treatment and strain taken on the falling (November '01) and rising phase (April '02) of the natural photoperiod. During November, photophase plasma levels did not differ between treatments or strain ranging from 36.4 to 41.7pg/ml. During the scotophase, all treatments showed significant elevations ($p<0.001$) in plasma melatonin above that during the photophase. As with photophase concentrations there were no significant differences between treatments or strain during the night, with LL-1 and

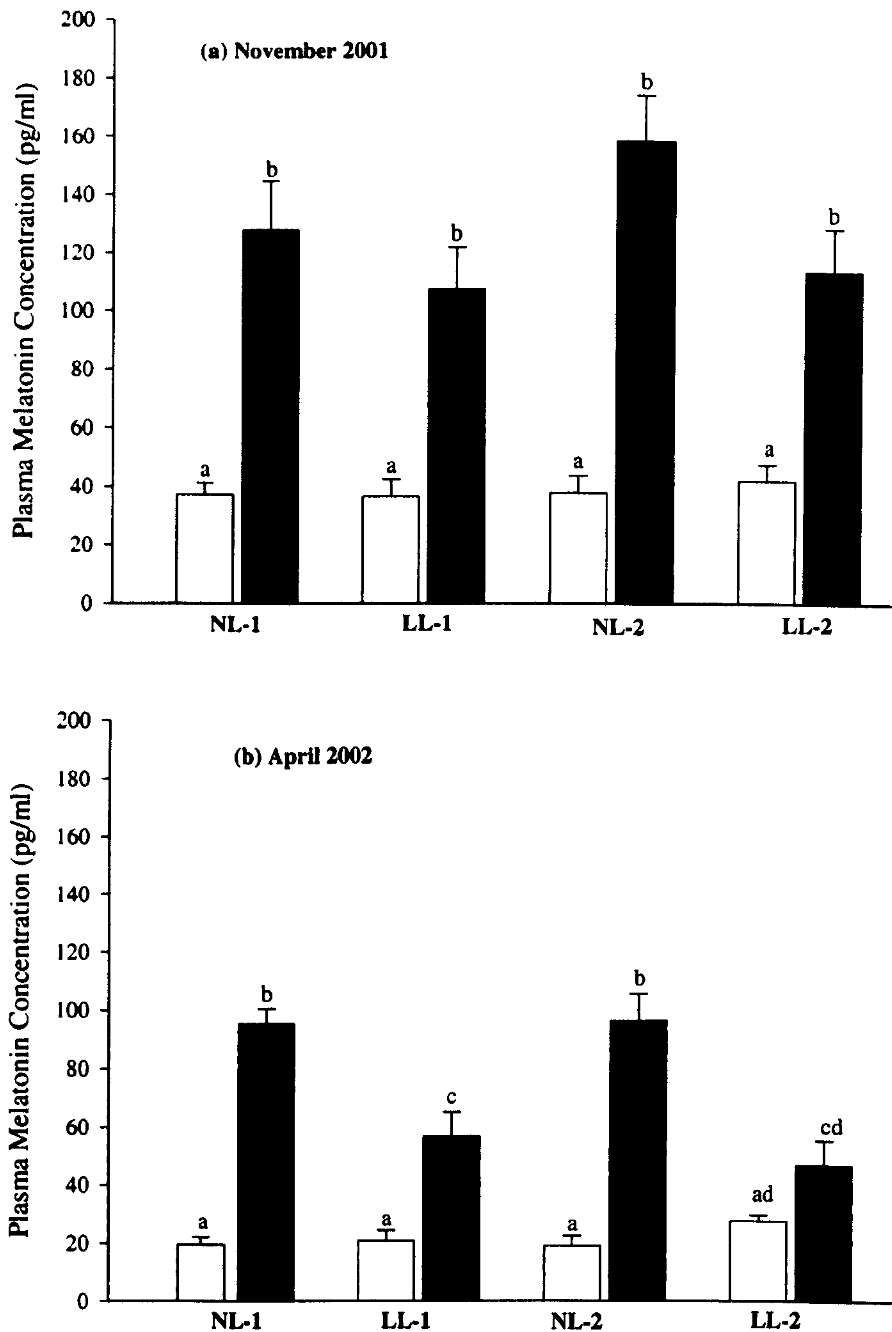


Figure 5.13 Day-night variation in plasma melatonin levels (mean ± SEM, n=20) for each treatment and strain taken on the **(a)** falling 21st November 2001 and **(b)** rising phase 1st April 2002 of the natural photoperiod. White bars represent photophase and black bars scotophase. Bars sharing common superscripts are not significantly different (p>0.05).

LL-2 having plasma levels of 107.3 ± 14.5pg/ml and 113.4 ± 14.7pg/ml compared to their respective controls NL-1 (127.7 ± 16.7pg/ml) and NL-2 (157.9 ± 15.9 pg/ml).

Melatonin profiles taken during the photophase in April showed no significant differences between treatments or strain ranging from 18.9 to 27.5pg/ml. During the scotophase, all treatments showed significant elevations ($p<0.001$) in plasma melatonin above that during the photophase. However, in contrast to that observed in April, scotophase concentrations in constant light treatments (56.7 ± 8.4pg/ml and 46.9 ± 8.3pg/ml, LL-1 and LL-2 respectively) were significantly lower than their respective controls (95.3 ± 5.3pg/ml and 96 ± 9.4pg/ml, NL-1 and NL-2 respectively). Furthermore, scotophase levels were reduced to 46.9 ± 8.3pg/ml in LL-2 and were not significantly different in comparison to that during the photophase (27.5 ± 2.0pg/ml). Of note is that plasma concentrations during both photo- and scotophase during April were lower than those measured in November.

5.4.4 Effects of Constant Light on Fillet Pigmentation

No fillet pigment data was provided for strain 1 as fish were harvested at different times, therefore only results from strain 2 are provided (Table 5.8). No significant differences were found in yellow/blue chromaticity (b*) or fillet lightness (L*) between treatments. LL treatment resulted in significantly higher fillet redness (a*), $p<0.016$, greater red hue (Hab*), $p<0.036$, and stronger intensity and clarity of colour (Cab*) scores, $p<0.048$, in fillet pigmentation.

Table 5.8 Comparison of Minolta colorimetry values (mean ± SEM, n=50) of fillets from strain 2 exposed to natural photoperiod (NL) or constant illumination (LL). Values in columns sharing different superscripts denote significant differences ($p<0.05$).

	Minolta Chroma Meter Scores				
Treatment	**a* Score Red/Green Chromaticity**	**b* Score Yellow/Blue Chromaticity**	**L* Score Fillet Lightness**	**Hab* Score Hue**	**Cab* Score Chroma**
NL-2	24.8 ± 0.20 [a]	24.02 ± 0.20	49.92 ± 0.22	0.7478 ± 0.003 [a]	34.54 ± 0.25 [a]
LL-2	25.52 ± 0.21 [b]	24.44 ± 0.31	50.04 ± 0.21	0.7386 ± 0.003 [b]	35.36 ± 0.32 [b]

5.4.5 Summary of Results

- Successful application of an artificial photoperiod was demonstrated by the reduction of plasma melatonin during the scotophase in fish exposed to constant light.
- Differences in perception of light between strains were observed in April as indicated by plasma melatonin profiles.
- Significant differences in growth rates and response time to LL application were observed between strains.
- LL application significantly enhanced weight and biomass gain relative to those maintained under ambient photoperiod.
- Enhanced performance under LL was achieved through significantly greater specific growth rates and improved feed efficiency (FCR).
- Fish of both strains exposed to LL showed a reduction in length gain and a greater increase in weight gain in spring, leading to higher condition factors than

those under ambient conditions, which maintained weight and length gain at similar rates.

- No significant effects on pigmentation were observed in fish exposed to constant illumination.

5.5 Trial 3: The effects of constant light provided by different numbers of lights on growth of rainbow trout fry during winter on-growing

5.5.1 Materials and Methods

On 7th November 2001 four 4m circular tanks (15.2m^3) at site 1 were stocked with equal numbers (approx 46,500) of all female rainbow trout fry (4.5 ± 0.05g, Aquazure Hatcheries, South Africa) giving a total biomass of 210kg. These fish had previously been reared from hatch (27th August 2002) in hatchery raceways using borehole water (6°C) under low level constant illumination (30 Lux). Water to the tanks was supplied by a nearby river with temperature dependent on ambient conditions (Figure 5.14).

To examine the effects of light intensity on growth, four treatments were chosen as follows:

Outside: Ambient Photoperiod (NL)

Outside: Constant Light provided by one light (LLx1)

Outside: Constant Light provided by two lights (LLx2)

Inside: Constant Light (IL)

Lighting in two outdoor tanks was provided by either one or two Aquabeam 400w Pisces floating light units (LLx1 and LLx2 respectively), positioned each side of the central standpipe and screen. Lighting in the indoor tank (IL) was provided by two 48 Watt fluorescent tubes positioned 3 metres above the tank. Light intensity readings were taken during both day and night to assess the relative differences between treatments (Table 5.9).

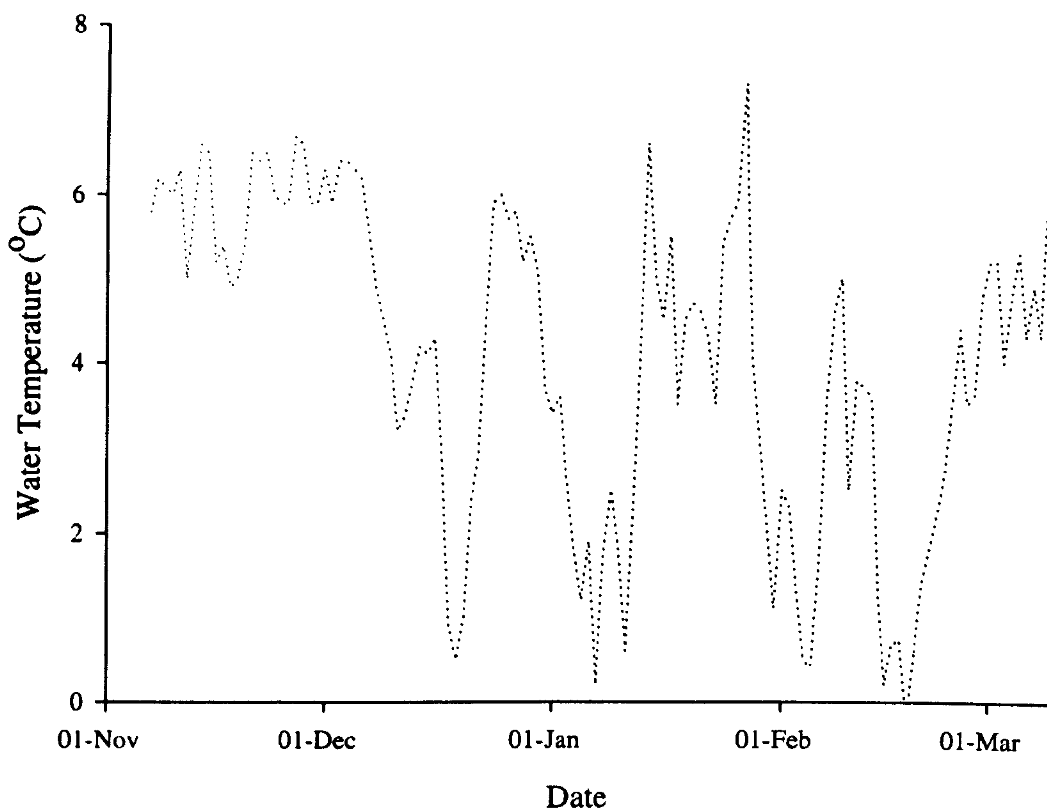


Figure 5.14 Site 1 daily water temperature profile for period of trial 7th November 2002 to 10th March 2003.

Daytime light intensity readings were taken between 11am and 12pm, with night readings taken between 7 and 8pm on 12th December 2002. Water quality during this period was good with minimal suspended solids. Readings were taken at the bottom from 12 locations around each tank. An outdoor air intensity reading of 5,500 Lux was recorded under cloudy, overcast conditions with a light breeze, while the intensity indoors in the partly covered building was around 74.5 Lux. Higher light intensities were provided by two submersible lights compared to one, although these were not significantly different ($p = 0.053$). Indoor light intensities were significantly lower ($p<0.001$) than all other treatments during both day and night time samples.

Table 5.9. Mean light intensity readings (±SE) recorded in each tank under treatment during daylight (11am-12pm) and night time (7-8pm). Superscipts denote significant differences between treatments.

Treatment	Day	Night
NL	1325 ±108.2 Lux ^{a}	0 Lux ^{b}
LLx1	1292.1 ± 138.7Lux ^{a}	143.3 ± 68.9 Lux ^{d}
LLx2	1648.7 ± 140.7 Lux ^{a}	255.3 ± 78.7 Lux ^{d}
IL	11.1 ± 0.9 Lux ^{c}	10.5 ± 0.7 Lux ^{c}

From first feeding to 4.5g, fry diets were supplemented with Ergosan (AquaVac™) at 0.5% inclusion. Diets fed were the same as those described in section 5.3.1. Food was presented by clockwork belt feeders set to deliver feed during the hours of the natural daylength. Fish were presented the same ration adjusted to the rate of the tank with the lowest feed intake.

Fish were sample for weight-length measurement as described in section 5.4.1.

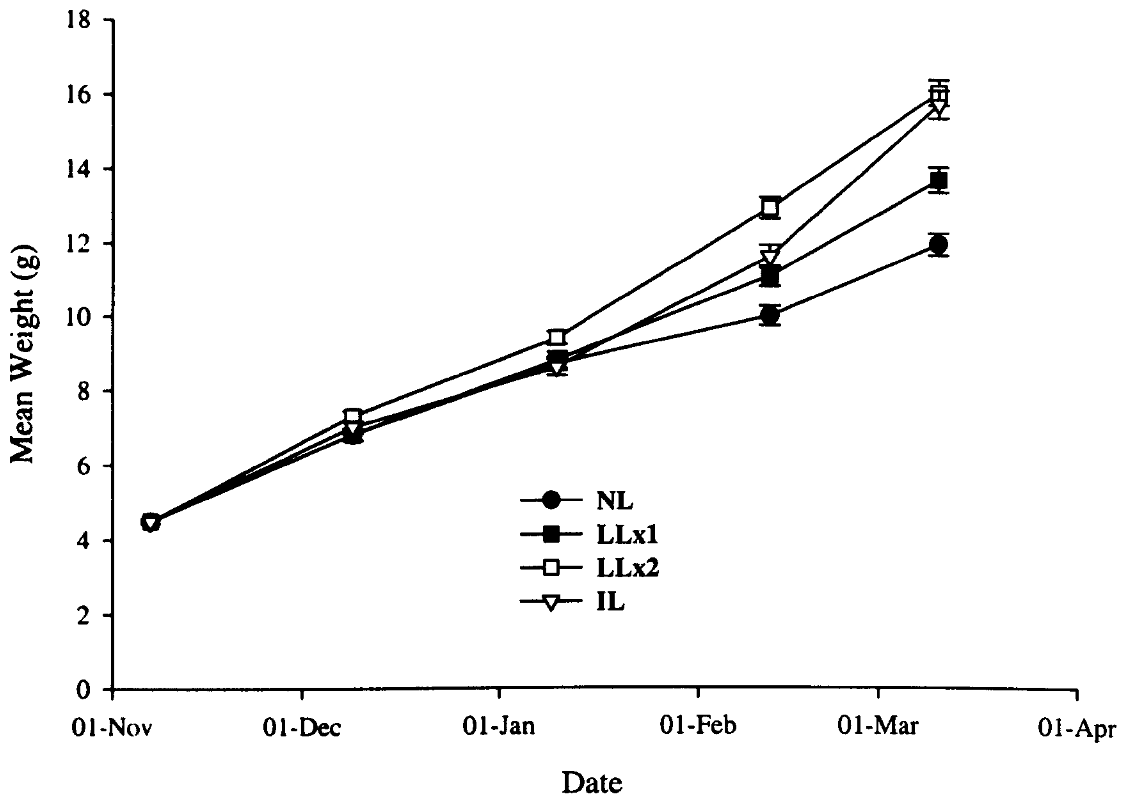
5.5.2 Results: Effects of Constant Light on Growth Parameters

Weight

All groups grew steadily following exposure to their respective treatments reaching significantly higher mean weights at grading on 10th March 2003 (Figure 5.15). Of all the treatments, LLx2 showed the greatest weight gain, attaining a significantly higher weight by mid-February compared to LLx1 and NL, with this advantage maintained until 10th March. Similarly, NL, LLx1 and IL grew at a steady rate from November to January, with LLx1 and IL showing a substantial increase in weight during February attaining a significantly higher mean weight than NL. Thereafter, IL showed a significant increase in weight gain, attaining a weight of 15.7 ± 0.3g, similar to that of LLx2 at 16 ± 0.3g, and significantly larger than LLx1 at 13.7 ± 0.3g. Of all treatments NL showed the lowest weight gain over the course of the trial reaching 11.2g ± 0.2, with a significantly lower mean weight relative to LL treatments maintained from February onwards. The final weight achieved by LLx2 allowed stocking for grow-out approximately 8 weeks earlier than those under NL.

Weight Specific Growth rate (SGRwt)

Table 5.10 provides a summary of the percentage weight gain and relative daily growth rate (Total SGRwt) for the period of the trial for each treatment. In all cases, exposure to LL significantly improved SGRwt relative to fish maintained under ambient conditions, with LLx2 and IL achieving 30% higher growth rates than NL, resulting in a significantly higher final weight. LLx1 also achieved a higher SGR of 0.9 % day^{-1} compared to NL, representing a 13.9% improvement, approximately half that of LLx2 and IL.



Time Treat	1	2	3	4	5
NL	a	a	a	a	a
LLx1	a	a	a	b	b
LLx2	a	a	a	c	c
IL	a	a	a	b	c

Figure 5.15 The effects of exposure to constant light provided by different numbers of lights on rainbow trout fry weight gain (mean ± SEM, n=200) relative to those maintained under ambient photoperiod (NL). Superscripts denote significant differences at given time points ($p<0.05$).

Table 5.10 The effect of exposure to constant light of different intensity on relative weight gain (%) and total growth rate (SGRwt) in tank reared fry from November to 10th March 2003 relative to those maintained under natural photoperiod (NL).

Treatment	Weight (g)		% Weight Gain	Total SGR (% day^{-1})	% improvement SGR relative to control
NL	Start	4.5	+ 164 %	0.79	-
	End	11.9			
LL-LOW	Start	4.5	+ 204 %	0.90	+ 13.9 %
	End	13.7			
LL-HIGH	Start	4.5	+ 256 %	1.03	+ 30.4 %
	End	16			
IL	Start	4.5	+ 249 %	1.02	+ 29.1 %
	End	15.7			

Length

All treatments increased length at a steady rate over the course of the trial with LLx2 and IL achieving a significantly higher mean length than LLx1, which were significantly longer than NL by March (Figure 5.16). LLx2 achieved the longest length of all treatments after 4 weeks exposure to LL with this difference becoming significant in February. IL and LLx1 also achieved a greater mean length than NL by this time point, with the difference becoming significant by March.

Condition Factor (CF)

Following exposure to their respective treatments all groups showed a significant decrease in CF from November to December, with IL maintaining a significantly higher CF than outdoor treatments (Figure 5.17). IL CF continued to decrease from December to January reaching 1.22, before remaining constant until March. LLx1 and LLx2 showed a similar decrease in CF from December to January,

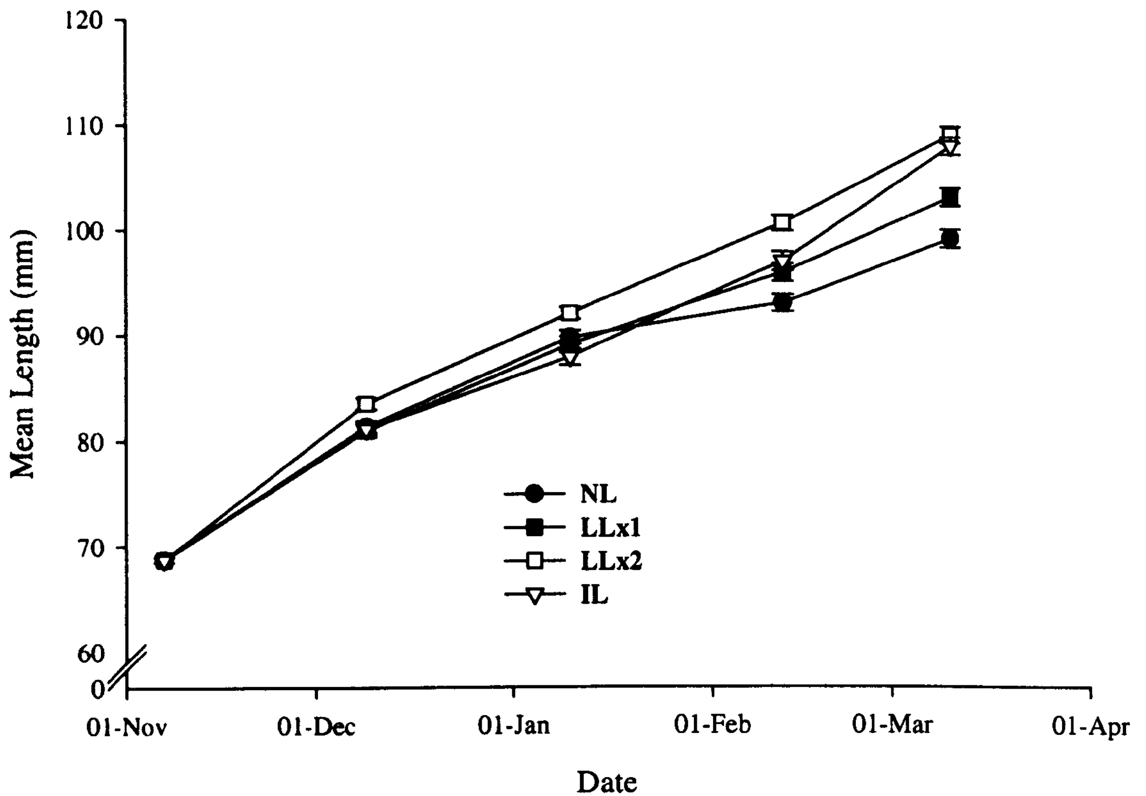
before LLx2 exhibited a significant increase in February. Although fluctuating over time, CF did not change significantly between January and March for all treatments, with the exception of LLx2. By grading all LL treatments achieved a similar CF of 1.20 but were significantly higher than NL at 1.18.

Feeding Efficiency

Table 5.11 provides details of biomass gain and total feeding efficiency (FCR) for each treatment. In all cases, exposure to LL significantly improved FCRs relative to those maintained under ambient photoperiod (NL). The most efficient FCR was achieved by LLx2 at 0.98, resulting in the greatest biomass gain of 472.4kg, which is equivalent to a 25.7% and 7.7% higher production than LLx1 and IL respectively. Similarly, LLx2 achieved a 20.3% and 4.9% more efficient FCR than LLx1 and IL. IL maintained a significantly more efficient FCR than LLx1 over the course of the trial.

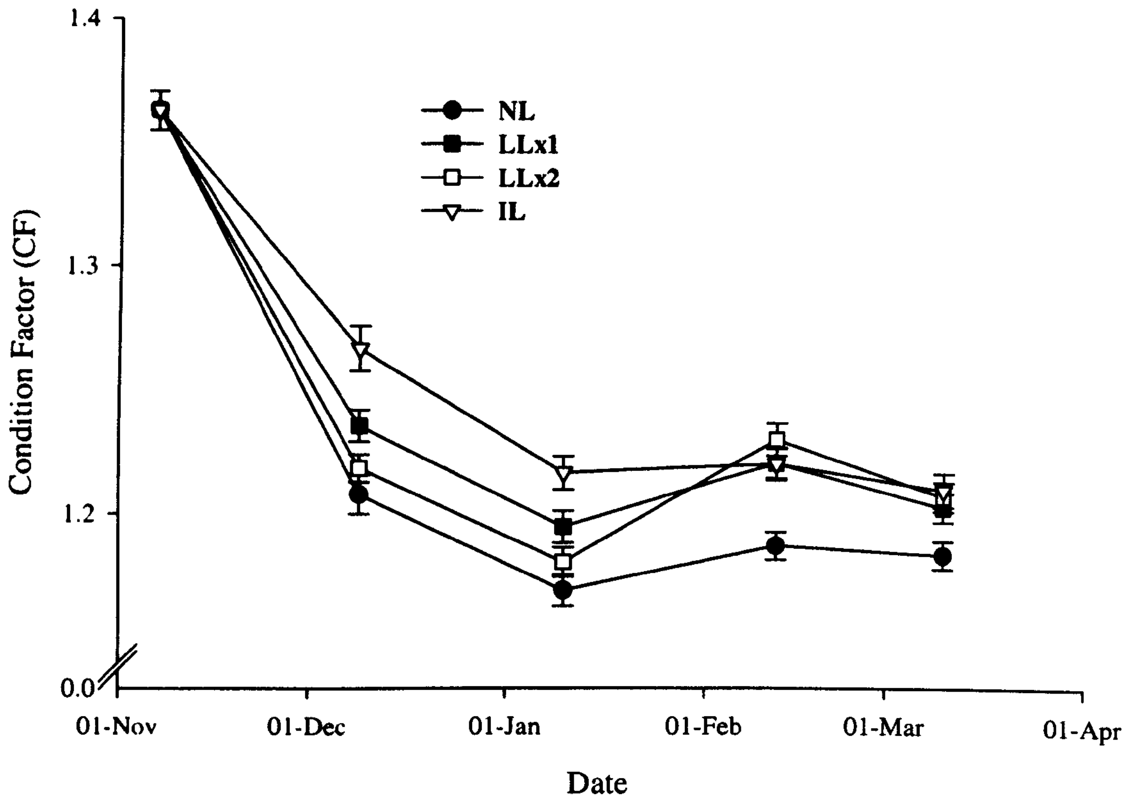
Table 5.11 Total feeding efficiency (FCR) of rainbow trout fry exposed to constant light provided by different numbers of light from stocking in November to 10^h March 2003, and the relative differences between those maintained under natural photoperiod (NL).

Treatment	Biomass (kg)		Biomass Gain (kg)	Food Presented (kg)	FCR	% Improvement Relative to Control
	Start	**End**				
NL	209.3	501	291.8	451	1.55	-
LLx1	209.3	585.2	375.9	462	1.23	+ 20.6 %
LLx2	209.3	681.8	472.4	465	0.98	+ 36.8 %
IL	209.3	647.9	438.6	452	1.03	+ 33.5 %



Time / Treat	1	2	3	4	5
NL	a	a	ab	a	a
LLx1	a	a	ab	ac	b
LLx2	a	a	a	b	c
IL	a	a	b	c	c

Figure 5.16 The effects of exposure to constant light provided by different numbers of lights on rainbow trout fry length gain (mean ± SEM, n=200) relative to those maintained under ambient photoperiod (NL). Superscripts denote significant differences at given time points ($p<0.05$).



Treat \ Time	1	2	3	4	5
NL	a	a	a	a	a
LLx1	a	a	ab	b	b
LLx2	a	a	a	b	b
IL	a	b	b	b	b

Figure 5.17 The effects of exposure to constant light provided by different numbers of lights on rainbow trout fry condition factor variation (mean ± SEM, n=200) relative to those maintained under ambient photoperiod. Superscripts denote significant differences at given time points ($p<0.05$).

5.5.3 Summary of Results

- Constant illumination provided by two floating units promoted significantly greater individual weight and biomass gain in outdoor tanks.
- This was achieved through a 30% greater specific growth rate and a 37% improvement in feeding efficiency (FCR) relative to ambient controls (NL).
- Constant illumination provided by one floating unit also promoted significantly greater biomass gain in outdoor tanks relative to ambient controls, although performance enhancement was approximately 50% less than that achieved using two units.
- LLx2 application in outdoor tanks produced a greater performance enhancement than indoor illumination, but LLx1 outdoor application resulted in a lower performance.

5.6 Trial 4: The effects of constant light of different colour temperature bulbs and intensity on the growth of cage reared rainbow trout during winter on-growing

5.6.1 Materials and Methods

5 month old 25g all-female rainbow trout (Faufing Danburg, Denmark) were first stocked into two 320 m^3 (9x9x4m) cages on 1st September 2002 and reared under natural photoperiod and temperature (Figure 5.18) at site 2 until 17th November. These fish were hatched in April 2002 from and reared in raceways at the nearby hatchery. As of the 18th November stocks were split between four 320m^3 (8x8x5m) cages as shown in Table 5.12. Due to a faulty electronic fish counter, LL-STD was under stocked by approx 4000 fish. As a result, fish were overfed from November to December, when fish numbers were recounted.

Each cage was then subjected to one of four light regimes provided by submersible Aquabeam Pisces 5 submersible 400W light units; natural light (NL), constant light provided by two 4000K bulbs (LL-STD), constant light provided by one 10,000K bulb (LLx1) and constant light provided by two 10,000K bulbs (LLx2). The spectral quality and irradiance of the two different bulbs are shown in Figure 5.19. Lighting units were positioned mid-cage depth (2m) and 2 metres apart either side of the central feed hopper for LL-STD and LLx2 or centrally in the cage for LLx1. Mid-water night time light intensities recorded for cages were 0, 15.2 ± 7.7, 9.5 ± 11.5, and 19.1 ± 3.6 Lux for NL, LL-STD, LLx1 and LLx2 respectively.

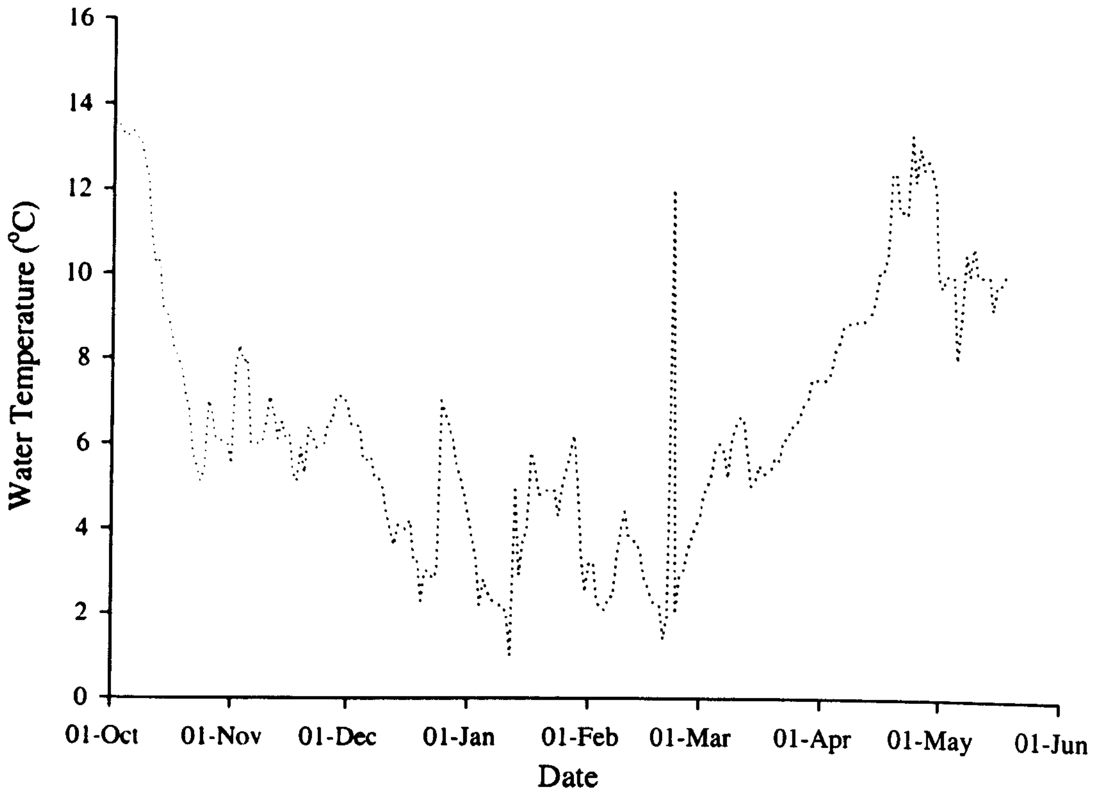


Figure 5.18 Site 2 daily water temperature profile for period of trial 18th November 2002 to 5th May 2003.

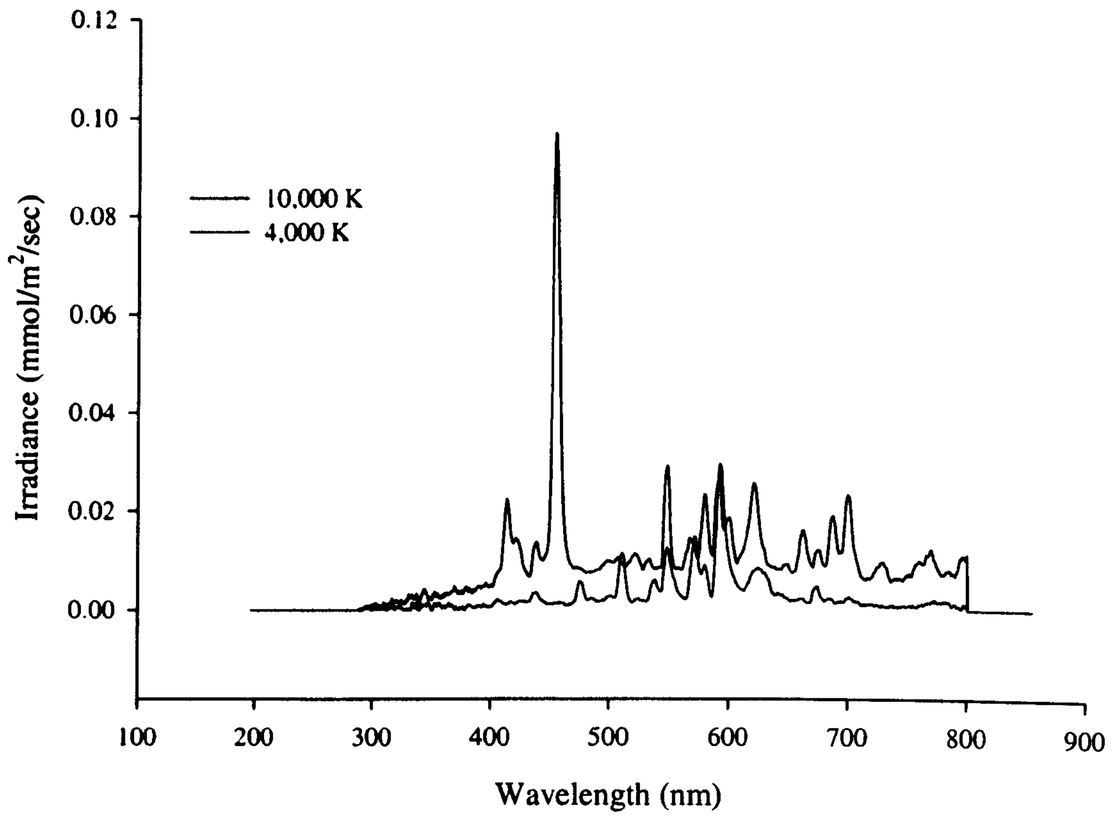


Figure 5.19 Comparison of irradiance and spectrum profile of 4000 and 10,000 Kelvin Aquabeam Pisces 5 400watt bulbs used in trial 4.

Table 5.12 Experimental set up of cages and treatments used in trial 4. * denotes actual numbers of fish stocked and not 25, 885 as expected on initial stocking.

Photoperiod	Mean Weight (g)	Fish Number	Biomass (kg)
Natural Light (NL)	74.1 ± 2.8	26,389	1955
LL 2x 4000K (LL-STD)	69.2 ± 3.7	21,235*	1469
LL 1 x 10,000K (LLx1)	74.1 ± 2.8	26,643	1974
LL 2x 10,000 K (LLx2)	69.2 ± 3.7	26,129	1808

Fish were presented numerous diets as fish size increased and a specially formulated winter diet for use below water temperatures of 5°C (Neo Prima Hiver^{TM}). All diets were supplied by Le Gouessant, with Table 5.13 providing details of proximate analysis and the periods in which each diet was presented.

Table 5.13 Proximate analysis of diets presented and timing of provision during trial 4.

	Period Diet Provided			
	Nov- Jan	Jan-Feb	Feb-mid Mar	mid-Mar-May
Inclusion Rate	Neo Start 3mm	Neo Nova 5mm	Neo Prima Hiver 5mm	Neo Nova 5mm
Protein (%)	47	40	45	40
Lipid (%)	17	30	19	30
Carbohydrate (%)	2	2	14	2
Ash (%)	10	9	10	9
Moisture (%)	10	10	10	10
Astaxanthin (mg/kg)	50	50	50	50
Digestible Energy (MJ/kg)	18	21	18.5	21

All cages were fed percentage body weight day^{-1} rations according to manufacturer's tables presented during the natural daylight hours of the ambient photoperiod treatment by means of automated feed hoppers set at 15 minute intervals.

Fish were sampled for growth performance as described in trial 2, section 5.4.1. Fish were blood sampled for melatonin analysis on 18th February 2003, with a total of 20 fish terminally sampled at 7 time points throughout the 24 hour light cycle (12pm, 4pm, 8pm, 1am, 6am, 8am and 12pm). The trial ended on the 5th May 2003.

5.6.2 Results

5.6.2.1 Light Intensity and Spectrum

Figure 5.20 (a) shows the natural reduction in ambient sunlight irradiance through the water column with depth during the day at this particular site. Within 0.01m of the water surface irradiant light was reduced by 50% from 6 to 3mmol/m^2/sec. As depth increased the irradiance also decreased with significant changes in the spectrum also evident. Within 0.5m of the surface much of the spectrum in the 400 to 500m region was absent. From 2.5m onwards (Figure 5.20 b), natural irradiance continued to reduce by approximately 50% for every 0.5m increase in depth, and that the majority of irradiant light was in the range of 600-690nm, with a prominent peak around 650nm. On the cage bottom irradiance reached 0.005mmol/m^2/sec.

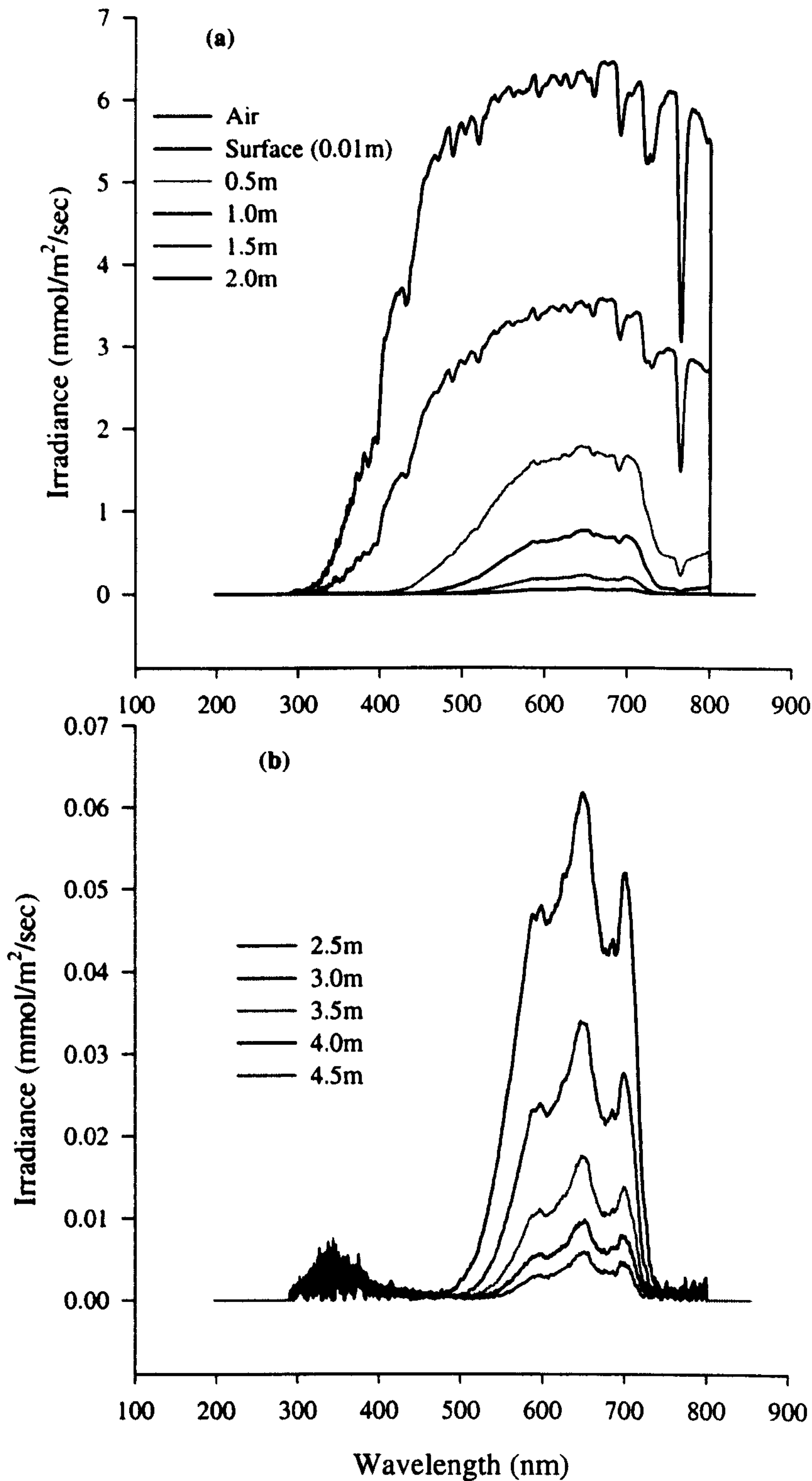


Figure 5.20 Change in irradiance and spectral content of ambient sunlight with depth through the water column at site 2. (a) surface to 2m depth, (b) 2.5m to 4.5m depth.

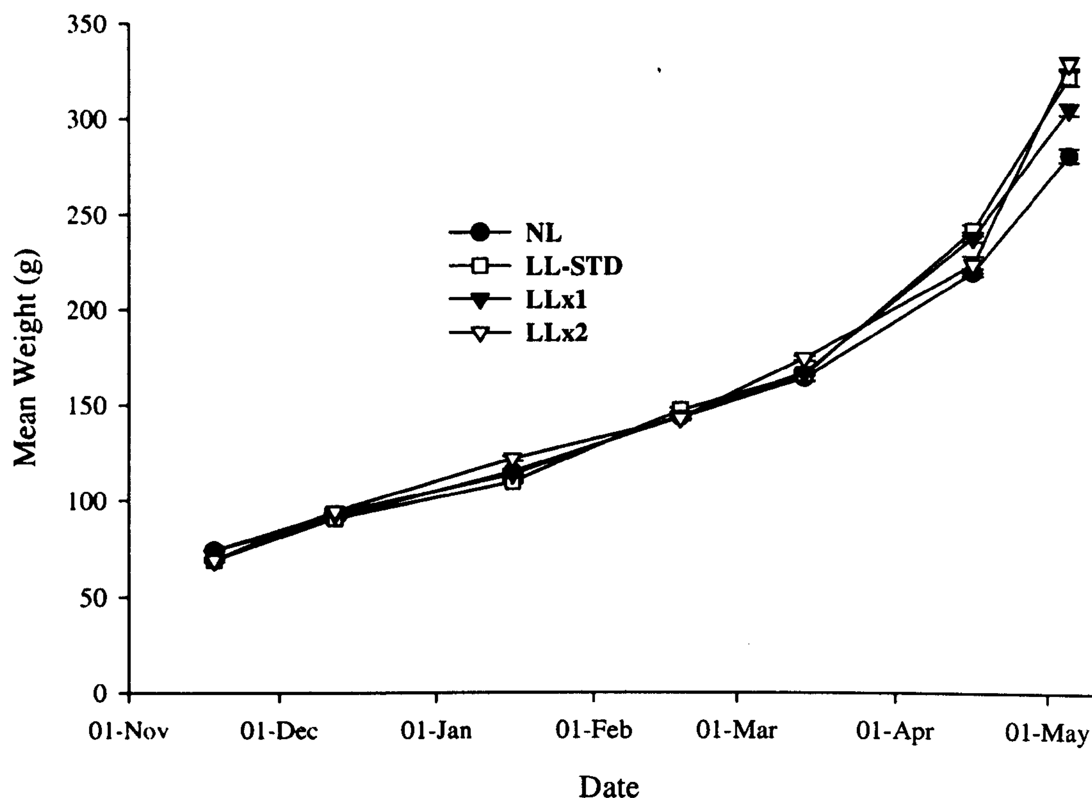
5.6.2.2 Effects on Growth Parameters

Weight

All treatments grew at a steady rate following exposure to their respective light regimes, with LL-STD and LLx1 reaching a significantly higher mean weight than LLx2 and NL by mid-April (Figure 5.21). LLx2 then exhibited a rapid gain in weight between mid-April and May. All LL treatments achieved significantly higher final weights than NL, with LL-STD and LLx2 reaching a significantly greater mean weight than LLx1.

Weight Specific Growth Rate (SGRwt)

SGRwt fluctuated in all treatments but generally showed the same pattern over time with LL treatments maintaining a higher rate than NL. Table 5.14 shows that LLx2 achieved the highest growth rates during the trial and were greater than those under NL thus achieving the greatest weight gain. LLx1 also achieved a higher SGR than NL but not to the same degree as LLx2. Due to the overfeeding of LL-STD it is not possible to determine whether SGRwt is a representative of the true growth rate. However, since overfeeding was restricted to the first 4 weeks of the trial such a substantial increase in SGRwt through feeding alone seems unlikely.



Time Treat	1	2	3	4	5	6	7
NL	a	a	a	a	a	a	a
LL-STD	a	a	a	a	ab	b	b
LLx1	a	a	a	a	ab	b	c
LLx2	a	a	b	a	b	a	b

Figure 5.21 The effects of exposure to constant light of different intensity and colour temperature on weight gain (mean ± SEM, n=200) of cage reared rainbow trout relative to those maintained under ambient photoperiod (NL). Superscripts denote significant differences at given time points ($p<0.05$).

Table 5.14 The effect of exposure to constant light of different colour temperature and intensity on relative weight gain (%) and total growth rate (SGRwt) in cage reared rainbow trout from 18th November to 5th May 2003 relative to those maintained under natural photoperiod (NL). No statistical analysis was performed as SGRwt are single values based on weight change between two time points.

Treatment	Weight (g)		% Weight Gain	Total SGRwt (% day^{-1})	% improvement SGR relative to control
NL	Start	74.1	+ 280 %	0.79	-
	End	281.2			
LL-STD	Start	69.2	+ 366 %	0.92	+ 16.5 %
	End	322.4			
LLx1	Start	74.1	+ 312 %	0.84	+ 6.3 %
	End	305.2			
LLx2	Start	69.2	+ 376 %	0.93	+ 17.7 %
	End	329.2			

Length

All treatments started at a similar length of 174 ± 0.6mm, and showed steady increase in length over time with NL, LL-STD, LLx1 and LLx2 achieving lengths of 275.4mm, 281.7mm, 277.7mm and 278.8mm respectively in May. Only LL-STD was considered significantly longer than NL at this point.

Condition Factor (CF)

CF was calculated from mean batch weight and individual length measurements, as a result no statistical analysis could be performed. NL showed a drop in CF between November and December, which then remained constant at 1.33 to 1.34 for two months, before a further two month decrease to 1.26 was observed (Figure 5.22). Only during the final month did CF rise reaching 1.35, although lower than that at stocking, 1.42.

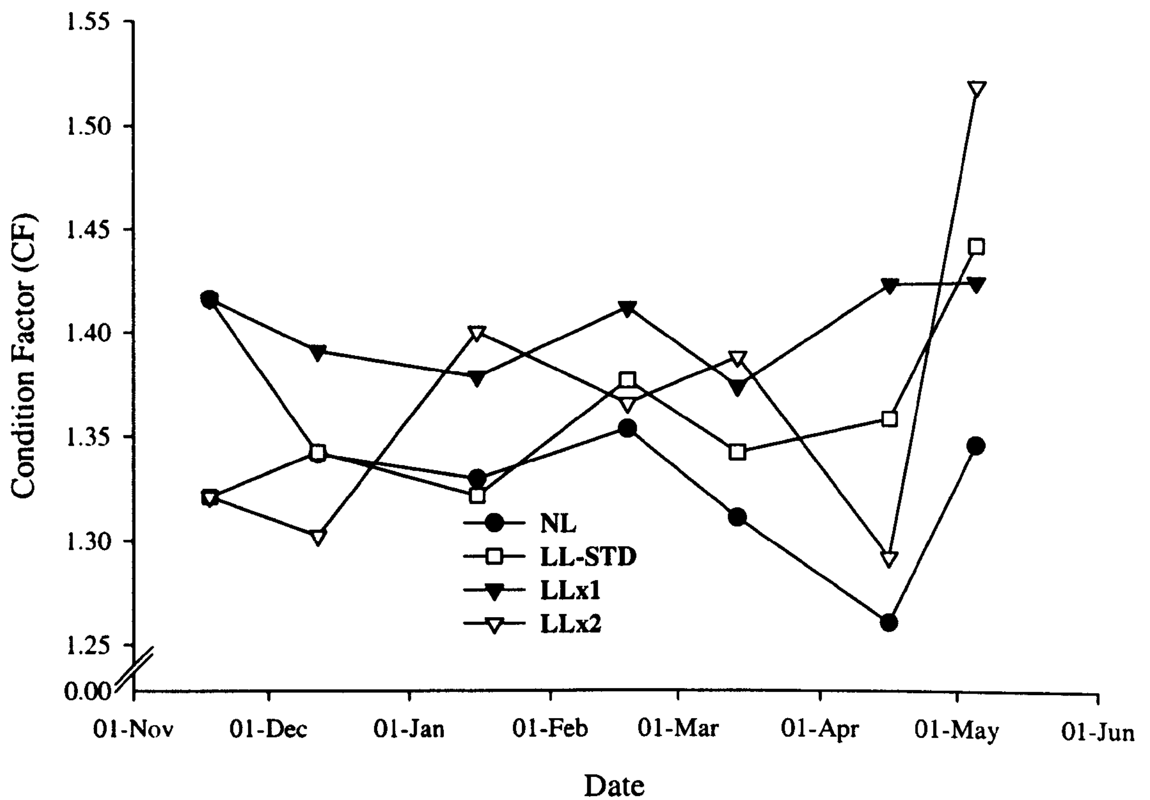


Figure 5.22 The effects of exposure to constant light of different intensity and colour temperature bulb on condition factor variation of cage reared rainbow trout relative to those maintained under ambient photoperiod (NL).

LLx1 maintained a more or less consistent CF throughout the trial following LL exposure, ranging between 1.37 and 1.42. In all cases LL treatment resulted in fish with higher CF in May than those under ambient photoperiod (NL), with LLx2 achieving the highest at 1.52, and LL-STD and LLx1 having a CF of 1.44 and 1.42 respectively. LLx2 and LLx1 also achieved a higher CF in May than at stocking, with both demonstrating increases during April.

Feeding Efficiency (FCR)

FCR was calculated from mean weight change between two time points relative to the food presented during that period, as such no statistical analysis could be performed as points are single values. All treatments exhibited feeding efficiencies within acceptable commercial ranges (Table 5.15). Of all treatments LLx2 showed the most efficient FCR at 0.96, closely followed by LLx1 at 1.05, both values lower than those under NL. As LL-STD was overfed, a true estimation of FCR could not be ascertained, with this treatment showing the same FCR as NL at 1.12.

Table 5.15 Total feeding efficiency (FCR) of rainbow trout exposed to different colour temperatures and intensities of constant light from stocking in November to grading on 5^h May 2003, and the relative differences between those maintained under natural photoperiod (NL). * Fish overfed due to over estimation of fish numbers at the start of the trial.

Treatment	Biomass (kg) Start	Biomass (kg) End	Biomass Gain (kg)	% Weight Gain	Food Presented (kg)	FCR	% Improvement Relative to Control
NL	1955	7197	5242	+268%	5849	1.12	-
LL-STD	1469	6625	5156	+351%	5784*	1.12	+ 0 %
LLx1	1974	7839	5865	+297%	6167	1.05	+ 6.3 %
LLx2	1808	8111	6303	+349%	6073	0.96	+ 14.3 %

5.6.2.3 Plasma Melatonin Levels

Figure 5.23 provides the diurnal variation in plasma melatonin levels for each of the treatments sampled on 18th February 2003. During the photophase plasma levels did not differ between treatments ranging from 46.2 to 48.1pg/ml. During the scotophase, all treatments showed significant elevations in plasma melatonin above that during the photophase, returning to basal levels within 1 hour of sunrise. During the mid-dark period plasma concentrations were significantly higher in NL at 157.9 ± 16pg/ml than LL treatments. No significant differences were apparent between LL treatments at any point during the scotophase with LL-STD and LLx1 ranging between 96.9 and 106.6pg/ml, and LLx2 ranging from 80.7 to 83.4pg/ml.

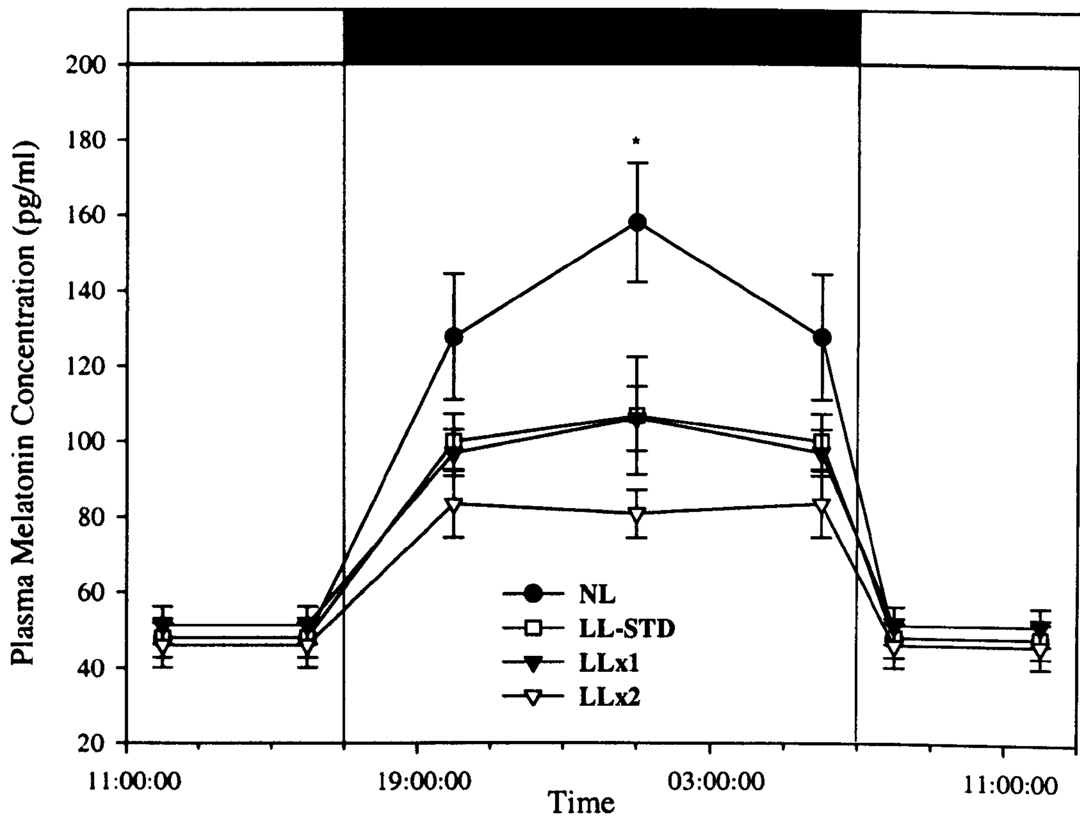


Figure 5.23 Diurnal changes in plasma melatonin concentrations (mean ± SEM, n=20) of trout exposed to constant light of different colour temperature bulb and intensity relative to those under ambient photoperiod (NL). Asterisk (*) denote significant differences between treatments ($p<0.05$).

5.6.2.4 Effects on Flesh Quality

Table 5.16 provides a summary of carcass quality assessment of top grade portion-sized fish at harvest. CF in LL-STD and LLx2 were considerably higher than LLx1 and NL. The greatest slaughter loss (carcass yield: weight minus visceral weight) was observed in LLx2 and the least in LLx1. LL treatment resulted in higher visual colour score using the SalmoFan™ (visual assessment of fillet pigmentation) than those under NL, with all treatments above the commercially accepted minimum score of 28-29 (Pers com., Skretting). Minolta a* scores indicated a greater fillet redness in LL-STD than all other treatments at 14.3. NL and LLx2 expressed similar scores with LLx1 having the lowest score at 15.7. Flesh pigment concentration was greatest in LL-STD at 8.9 mg/kg, with the other three treatments varying between 7.8 and 8.1mg/kg. Percentage lipid was similar in all treatments. All treatments were within commercially acceptable ranges.

Table 5.16 Carcass quality assessments of fish exposed to ambient photoperiod (NL) or constant light of different intensity and colour temperature. Values calculated from 10 fish at harvest and results provided by Scot trout Ltd, sample processing by Skretting UK.

Treatment	Weight (g)	Condition Factor	Slaughter Loss (%)	SalmoFan Score	Minolta a*	Pigment (mg/kg)	Lipid (%)
NL	334	1.46	15.8	29.4	14.9	7.9	9.6
LL-STD	411	1.64	16.4	32.1	14.3	8.9	9.6
LLx1	350	1.40	14.7	32	15.7	8.1	9.0
LLx2	379	1.61	19.6	30	15.2	7.8	9.1

5.7.3 Summary of Results

- At site 2, natural sunlight irradiance was reduced by 50% for every 0.5m increase in depth. Furthermore, wavelengths below 500nm were rapidly attenuated with depth. Highest irradiant light was found between 600-700nm.
- LL-STD was overfed during the first month of the trial and is difficult to make comparisons with the other treatments.
- Fish exposed to LL provided by two 10,000K bulbs enhanced production performance. One bulb did improve production above that of ambient photoperiod, but was 50% less than that by two bulbs.
- LL resulted in the maintenance of a higher specific growth rate and a substantially more efficient feeding efficiency (FCR) throughout the trial.
- Fish under LL were of a significantly higher weight for a given length relative to ambient controls in spring.
- LL significantly reduced scotophase melatonin levels below that of ambient controls, with two 10,000K bulbs reducing melatonin levels below that of two 4000K or one 10,000K bulbs, however this difference was not significant ($p>0.05$).
- No negative effects on pigmentation or carcass quality were observed at harvest following exposure to LL. Pigment scores were above commercially acceptable minimums.

A summary of the results from all 4 trials is presented in Table 5.19.

Table 5.17 Summary of growth performance parameters following four field trials examining the effect photoperiod manipulation on rainbow trout growth in uncovered tanks and cages. Bold type in brackets indicate percentage improvements relative to controls.

Treatment	Start Weight (g)	Final Weight (g)	Biomass Gain (%)	Total SGRwt (% day^{-1})	Total FCR
Photoperiod Manipulation During Fry Ongrowing in Tanks					
(a) Trial 1					
Ambient photoperiod & ambient feeding (AA)	5.1	14.9	192%	0.60	2.08
LL & ambient feeding (LA)	5.1	19.0	273%	0.72 (**+20%**)	1.19 (**+43%**)
LL & extended feeding (LE)	5.1	16.8	229%	0.69 (**+16%**)	1.66 (**+20%**)
IL & extended feeding (IL)	5.1	17.7	261%	0.73 (**+22%**)	1.39 (**+33%**)
(b) Trial 3					
Ambient photoperiod (NL)	4.5	11.9	139%	0.79	1.55
LL by 1 Light (1xLL)	4.5	13.7	180%	0.90 (**+13.9%**)	1.23 (**+20.6%**)
LL by 2 Lights (2xLL)	4.5	16.0	226%	1.03 (**+30.4%**)	0.98 (**+36.8%**)
Indoor LL (IL)	4.5	15.7	249%	1.02 (**+29.1%**)	1.03 (**+33.5%**)
Photoperiod Manipulation During Cage Ongrowing					
(c) Trial 2					
Strain 1					
Ambient photoperiod (NL-1)	74.8	316.3	331%	0.82	1.25
LL & ambient feeding (LL-1)	74.8	364.0	350%	0.89 (**+8.5%**)	1.06 (**+15.2%**)
Strain 2					
Ambient photoperiod (NL-2)	54.0	278.3	369%	0.87	1.21
LL & ambient feeding (LL-2)	54.0	355.5	584%	1.08 (**+24.1%**)	0.91 (**+24.8%**)
(d) Trial 4					
Ambient photoperiod (NL)	71.7	281.2	268%	0.79	1.12
LL 2x4000K (LL-STD)	71.7	322.4	351%	0.92 (**+16.5%**)	1.12 (na)
LL 1x10,000K (LLx1)	71.7	305.2	297%	0.84 (**+6.3%**)	1.05 (**+6.3%**)
LL 2x10,000K (LLx2)	71.7	329.2	349%	0.93 (**+17.7%**)	0.96 (**+14.3%**)

5.7 Discussion

The aim of this chapter was to examine whether artificial photoperiod regimes could be successfully superimposed onto the natural ambient photoperiod to potentially enhance production in commercial outdoor systems used in rainbow trout culture. Trial 1 (section 5.3) was designed to examine the effect of LL application on tank reared fry production, while trial 2 (section 5.4) was used to assess the use of submersible lighting on on-growing of cage reared juvenile rainbow trout. Trials 3 and 4 (sections 5.5 and 5.6) assessed the importance of light intensity and bulb colour temperature in tank and cage culture respectively.

5.7.1 The Effect of LL Application on Production Performance in Outdoor Systems

In both tank and cage rearing units, artificial light was successfully applied onto the ambient photoperiod cycle to significantly enhance production performance in all trials undertaken. Furthermore, it was clearly demonstrated that the techniques can be applied to both fry (<5g) and juvenile (>35g) rainbow trout. The growth enhancing effect of continuous light from these trials is to my knowledge the first reported in rainbow trout in outdoor uncovered systems.

Growth and Weight Gain

Increased growth and higher final weights were observed under LL photoperiod regimes in both tanks and cages compared to their respective controls exposed to an ambient photoperiod only. The growth enhancing effect is in accordance with those observed with the application of extended photoperiods to freshwater stages of Atlantic salmon parr (Saunders & Henderson, 1988, Villarreal *et al.*, 1988, Saunders *et al.*,

1989, Stefansson *et al.*, 1989), seawater cage rearing of post-smolts (Saunders & Harmon 1988, Clarke, 1990, Krakenes et al., 1991, Hansen *et al.*,1992, Oppedal *et al.*, 1999), covered tank rearing of rainbow trout (Mason *et al.*, 1992), largemouth bass (Petit *et al.*, 2003), juvenile Atlantic halibut (Jonassen *et al.*, 2000, Norberg *et al.*, 2001), juvenile turbot (Imsland *et al.*, 1995, 1997), juvenile haddock (Trippel & Neil, 2003), European sea bass (Rodriguez *et al.*, 2001) and gilthead sea bream (Kissil *et al.*, 2001). The current results oppose those suggested by Solbakken et al., (1999), where LL had no effect on winter growth rate of seawater cage reared rainbow trout.

Furthermore the current trials showed that although LL treatment initiated a higher growth rate, analysis of flesh quality was maintained within commercially acceptable ranges. This contradicts observations made by Morkore & Rorvik, (2001) in which breaking strength of Atlantic salmon fillets correlated negatively with specific growth rate. However, as this was not the main objective of the current studies it could warrant further more detailed research into aspects of flesh quality and photoperiod enhanced growth.

Growth and Feeding Efficiency

The similar growth rate and subsequent weight gain achieved in fry fed during the daylight hours or receiving an extended feeding regime (trial 1) indicates that growth rate in rainbow trout is not enhanced by extending the feeding outside the natural daylight hours when held under continuous illumination. Similar responses to those observed in the current study have been observed in first feeding Atlantic salmon parr (Berg *et al.*, 1992) and post-smolts (Krakenes *et* al., 1991) where no advantage to 24 hour feeding under constant light was found relative to those fed for only 12 hours.

Furthermore, FCR in trial 1, based on food presented to wet weight gain, indicated that not only was growth enhanced, but that feeding efficiency was markedly improved in fry exposed to constant light over those maintained under ambient photoperiod. In addition, those receiving an extended ration under LL had a poorer FCR than those fed during the natural daylight hours, which is most likely through waste feed as the natural feeding rhythm would dictate appetite and feeding period (Kadri *et al.*, 2001, Krakenes *et* al., 1991, Boujard *et al.*, 1995). However, this contrasts with observation made by Mason *et al.*, 1992 where rainbow trout exposed to a LD 16:8 and fed for 16 hours from November to March, had a more efficient FCR than those fed during the natural daylight hours only. It was concluded that the extended time of feed availability increased the efficiency of feed input to that expended in standard metabolic activity. Although extended daylength provides fish with more daylight hours to feed, it is more likely that enhanced growth is the consequence of physiological changes resulting in increased appetite and/or food conversion efficiency (Saunders *et al.*, 1994). Kadri *et al.*, 1991 has shown that automated feeding regimes dispensing food at low rates throughout the daylight hours may be failing to provide enough food when the fish are hungry, and causing substantial feed wastage the rest of the time. Feeding motivation and pattern has also been shown to be influenced by time of feeding, season (Kadri, *et al.*, 1997a), fish size and social hierarchy (Jobling & Koskela, 1996, Kadri, *et al.*, 1997b). Certainly studies examining food intake under photoperiod regimes could resolve these conflicting views with regards to feeding efficiency in rainbow trout.

In both tank and cage trials carried out for this chapter there were significant improvements in feed conversion ratio in fish exposed to LL during the winter period in both fry and juvenile ongrowing relative to their ambient controls. Handeland *et al.*, (2003) found LL application from December to May to enhance growth of Atlantic

salmon smolts through the stimulation of food intake, although genetically selected strains did exhibit a more efficient feed conversion. However, in juvenile Atlantic halibut, gilthead sea bream and haddock, faster growth of fish exposed to continuous light and long-days was accompanied by greater feeding efficiency rather than feed intake (Jonassen, *et al.*, 2000, Kissil, *et al.*, 2001, Trippel, *et al.*, 2003), with feed intake only increasing in association with rising temperatures in the case of halibut (Jonassen, *et al.*, 2000). In largemouth bass, exposure to LL significantly enhanced growth relative to those under 12:12 LD, through both greater feed intake and a more efficient feed conversion (Petit, *et al.*, 2003). Therefore, it is clear that there are species differences in the response to photoperiod application. Some of these differences may be explained through metabolic costs and physiological responses. Bass were found to be more active under LL, with increased locomotor activity being shown to improve food conversion (Petit, *et al.*, 2003). Increased feed consumption may be compensating for a higher metabolic rate and activity under continuous light as observed in turbot (Imsland, *et al.*, 1995). Berg *et al.*, (1996) found that in order to influence energy reserves, swimming velocity of Atlantic salmon must be maintained above 0.5 body lengths per second. However, despite numerous studies in a variety of species it is still not clear how photoperiod is acting to influence growth. Further studies are required in order to elucidate whether growth is affected through direct photostimulation or functions as a zeitgeber for circannual endogenous rhythms including appetite. An important factor for the aquaculture industry is to be certain that light affects fish growth through a better food conversion efficiency and not just through stimulated food intake (Boeuf & Le Bail, 1999).

Strain Differences in Relation to Photoperiod Application

In all trials where growth was enhanced through photoperiod treatment each stock of rainbow trout exhibited a different response time to the onset of LL application. In trials 1 and 3, tank reared fry (both of Aquazure origin) exposed to LL during the first week of November showed significant improvements in weight gain 14 weeks after application. In trial 2, cage reared juveniles exposed to LL provided by submersible lighting in late October showed significant improvements in weight gain within 8 weeks for strain 1 (Glen Wyllin origin) and 4 weeks for strain 2 (Trend origin), while fish in trial 4 (Faufing Danburg origin) exposed to LL in mid-November did not show a significant weight gain until 21 weeks, 4 weeks prior to harvest in May.

Valente *et al.*, (1999) demonstrated that rainbow trout strains selected for fast and slow growth exhibited different capacities for muscle fibre recruitment and subsequent muscle growth. The better growth performance of faster growing strains has also been attributed to differences in metabolic activity and feeding capacity (Valente *et al.*, 1998). Greater swimming activity was observed in first feeding rainbow trout selected for fast growth, with these fish exhibiting more rapid feeding, a higher number of snapping responses and occupation of more rewarding positions in tanks in relation to the site of feed delivery (Valente *et al.*, 2001b). Under conditions of self-feeding fast growing strains of rainbow trout have been shown to have a higher voluntary food intake, greater feeding motivation, a bigger appetite and better feed conversion (Valente *et* al., 2001a). In contrast, restricted feeding by means of automated feeders, reduced feeding efficiency principally through provision of feed out with their preferred feeding period. However, to date, little evidence exists in relation to comparing the effects of photoperiod manipulation between genetically selected strains. Handeland *et al.*, (2003) did report improved growth performance of a selected strain of Atlantic salmon smolt

over wild strains following exposure to LL during the winter period. Results from trial 2 support this observation, as clear differences in strain performance under LL were evident in rainbow trout. Although both strains grew faster than their respective controls reaching significantly greater weights at harvest, strain 2 exhibited a higher SGRwt than strain 1. Furthermore, strain 2 also exhibited a more efficient FCR than strain 1. In contrast, SGRwt and FCR were similar under natural light in both strains, suggesting that strain response to photoperiod may differ, and certainly warrants further research.

In addition to response time, fish exposed to LL also exhibited different patterns of growth in relation to their respective controls. In trial 2, rainbow trout exposed to LL in cages exhibited a tendency for greater length elongation (SGRL) during late autumn and winter followed by a reduction in the rate of length gain and a concurrent rapid increase in daily weight gain in April. Although strains were different, both groups under LL showed a similar decline in condition factor from January to March, followed by a sharp increase during April. Similarly, in trial 4, fish exposed to LL tended to be of a greater mean weight despite being of equivalent length to those maintained under natural photoperiod by late April/early May, and subsequently exhibited a higher condition factor than their ambient control. Bjornsson *et al.*, (2000) speculated that the possible functional importance of such a selective resource allocation for skeletal growth is that the elongated skeleton creates the potential for a rapid weight gain after smolts have entered the marine environment where food is usually more plentiful. The same may be true in rainbow trout whereby they are preparing their bodies for spring increases in water temperature in anticipation of a more plentiful food supply in the freshwater environment. However, it has been suggested that although in the same family there are significant differences in response to photoperiod between Atlantic and

Pacific salmonids during the freshwater stages (Solbakken *et al.*, 1994), although these differences have mainly been in association with the successful completion of smoltification. Since the rainbow trout does not undergo this process there may be underlying differences in growth patterns within the pacific salmonids. In Atlantic salmon parr LL application from November to late February enhanced growth for two months post exposure, thereafter those maintained under natural photoperiod grew faster (Saunders *et al.*, 1985). This response was related to the advancement of the natural endogenous growth pattern in salmon by LL, with those under natural light exhibiting a period of faster growth at a later but natural time point in the growing season. The application of LL may have advanced such a growth pattern in rainbow trout, however as the trials were not continued past this period due to grading or harvest, it is not possible to determine whether those under ambient conditions may have under gone a similar response.

Regarding other species, an enhanced growth response of largemouth bass to LL following an initial acclimatisation to 12:12 LD was not observed until 6 weeks post exposure (Petit *et al.*, 2003), while juvenile halibut showed a 21 day delayed response to exposure to LL following a short-day 8:16 LD cycle, suggesting that fish require some time to acclimate to a change in photoperiod (Simensen *et al.*, 2000). Such varied responses in the current studies could also be related to the different strains utilised in each trial, as Henryon *et al.*, (2002) demonstrated an additive genetic effect for growth rate and feeding efficiency within farmed populations of rainbow trout. However, temperature regimes between each trial varied significantly and will have undoubtedly played a significant role in the response to photoperiod treatment.

The Effect of Temperature on Response to Photoperiod

Since fish are ectothermic, then many of their physiological processes are regulated by the thermal regime, with optimum ranges for a variety of freshwater and marine species (Saunders *et al.*, 1985, Solbakken *et al.*, 1994, Hallaraker, *et al.*, 1995, Jonassen, *et al.*, 1999). Temperature is considered to be a rate-controlling factor, whereas light is classified as a directive factor that stimulates the endocrine system through changes in endogenous rhythms (Bromage, *et al.*, 1994). Clarke *et al.*, (1978, 1981) showed that temperature controlled the rate of response of underyearling coho and sockeye salmon to photoperiod, whereby changes in growth rate caused by photoperiod treatment were apparent sooner at higher temperatures than at lower ones. Similar responses have been observed in Atlantic salmon whereby increasing daylengths did not enhance growth when temperatures were low, while elevated temperatures during late winter and early spring in association with exposure to LL successfully enhanced growth (Saunders *et al.*, 1985, Solbakken *et al.*, 1994). Therefore, the positive increase in growth in response to LL of fry in trials 1 and 3, and cage reared juveniles in trials 2 and 4, which were all initiated between late October and early December, suggest that temperatures were suitable under the falling photoperiod of autumn to allow an endocrine response to photoperiod treatment.

The Effect of Timing of Photoperiod Application

In order to respond to long daylength, several species of salmonids first have to be exposed to short photoperiods for some minimum period of time. An abrupt increase in and maintenance of a long photoperiod may be acting as a 'zeitgeber' to entrain endogenous circannual rhythms and lead to phase changes in these rhythms for the capacity for growth, smoltification and reproduction (Clarke *et al.*, 1978; Stefansson *et*

al., 1989; Berg *et al.*, 1992; Sigholt *et al.*, 1995, Bromage, *et al.*, 1994).. In juvenile turbot, continuous light was stimulatory for growth in early spring, while growth was faster in fish exposed to natural photoperiod in late spring (Imsland *et* al., 1995). Similarly, long-days applied under increasing daylengths have been found to produce poorer growth in Atlantic salmon compared to those maintained under natural conditions, while the opposite was true under decreasing daylengths (Komourdjian *et al.*, 1976). Clarke *et al.*, (1978) also found that growth responses were not regulated by accumulated number of hours of exposure to daylight, but more importantly by direction and rate of change of the daylength. Accelerated rates of increase in daylength were less effective in promoting growth in both sockeye and coho salmon from March to May than the normal rate of increase, suggesting that the fish were unable to synchronise their endogenous rhythm with such a rapidly changing signal, whereas a growth promoting effect was observed under long-days between October and December. As previously postulated with temperature, the timing of application may explain the positive increase in growth in response to LL application of fry in trials 1 and 3, and cage reared juveniles in trials 2 and 4, all of which were initiated between late October and early December, suggesting rainbow trout are more responsive to photoperiod application on the descending arm of the natural changing photoperiod.

Finally, as fish exposed to LL provided by two submersible lights in cage trial 2 and 4 exhibited significantly improved performance above ambient controls irrespective of strain, it suggests that in trial 4, where light was provided by one submersible unit, that light intensity and distribution may not have been sufficient to enhance production. A similar response to the use of two lights relative to one light was also observed in fry trial 3. This will now be discussed in more detail.

5.7.2 The Effect of Light Intensity and Bulb Colour Temperature on Production Performance in Outdoor Systems

Care must be taken when referring to light intensity, particularly in trials 3 and 4 which examined numbers of lights. Although two lights were used in both trials they did not produce significantly higher intensities than one light unit, suggesting that a better term for discussion may be light distribution.

Light Intensity and Distribution

In trial 3, one and two lights provided additional night time illumination intensities equivalent to 7% and 9% of the relative day time intensity respectively. A concurrent performance response to increasing light intensity was observed in rainbow trout fry in outdoor tanks. Fry exposed to LL provided by two floating units (255 lux) exhibited significant enhancements in growth rate and subsequent weight gain, as well as marked improvements in feeding efficiency relative to those under natural photoperiod. Significant improvements were also observed in LL application using one floating unit per tank (143 lux), although the response for both growth and feeding efficiency was approximately 50% less than that of the high intensity treatment. Stefansson *et al.*, (1991) showed decreasing growth responses in post smolts when reared under day/night ratios of 1400lux/1400 lux, 1400lux/27lux, and finally 1400lux/dark. However, in freshwater Stefansson et al., (1993) found no effect on growth rate of salmon parr exposed to 27, 335 or 715 lux at daytime and 0 lux at night. These two findings support the idea that growth rate is influenced by the relative difference between day and night intensity rather than absolute intensity (Oppedal, *et al.*, 1997). Cho, (1992) reported improved growth was obtained under conditions of high (1600 lux) rather than of low (100 lux) light intensity in rainbow trout, where fish

under high intensity appeared to be more active, with a subsequently greater energy balance, although no effect on feed conversion was observed. In contrast, Oppedal, *et* al., (1999) found no difference in growth rate of underyearling Atlantic salmon exposed to continuous light of different intensity. It was hypothesised that only very low intensities were needed to affect changes in growth patterns of fish of small size, and that the threshold for larger salmon may be higher. However, this study only exposed fish for a short period of time, 12 weeks, to LL and may have been too little to induce any growth differences, whereas a previous study did not observe any effect of light intensity until 18 weeks post exposure (Oppedal, *et al.*, 1997). As low a level as 0.0001 lux additional illumination during the dark phase was shown to interfere with the completion of smolting in coho salmon under laboratory conditions (Thorarensen *et al.*, 1989). Furthermore, it was shown that transmission of light at 500nm by the skin and the skull over the pineal was 25% in fry compared with 10% in yearling postsmolt coho salmon. It may therefore be argued that small fish are more sensitive to light than their elder siblings simply through the greater transmission of light through the pineal window, as yet this remains to be clarified. Randall (1992) showed that underyearling rainbow trout (<100g) exhibited significantly higher night time plasma melatonin than larger two year-old fish (2kg+), and thus the amplitude of change in response to photoperiod manipulation may be greater in smaller fish. However, in trial 3 the fish were too small to obtain sufficient quantities of plasma for melatonin analysis, and the perception of light between LL and natural light could not be determined.

Interestingly, although it is argued that higher intensities appear to promote greater growth, in both fry trial 1 and 3, low level indoor illumination (11-30 lux) promoted a similar effect on growth rate and feeding efficiency to that achieved in the higher intensity outdoor tanks. However, this is unsurprising as the fry were previously

maintained within the covered hatchery building and received constant illumination at the same level of intensity from hatch, and therefore perceived a continuous photoperiod with no diurnal change in daylength or intensity as would have been the case in the outdoor tanks under naturally changing conditions. Furthermore, higher intensities will be naturally required in outdoor uncovered systems in order to successfully superimpose an artificial photoperiod onto the natural photoperiod in order to effectively mask the diel change from day to night intensity (Oppedal *et al.*, 1997).

Shading of pens during summer and early autumn was shown to have an enhanced effect on Atlantic salmon growth, while reducing growth in winter and spring (Huse *et al.*, 1990). This could be explained by the naturally low illumination levels in winter being further reduced by shading, thus inhibiting feeding, and prolonging the typically low appetite pattern of winter feeding. On the other hand, high illumination in summer may have inhibited ingestion and growth through greater surface avoidance, while shaded pens provide more favourable conditions. Salmon in sea cages exposed to LL with different intensities will experience a different day/night light intensity ratio dependent on the ambient level at daytime and the constant level at night, and the intensity perceived will change with the water characteristics and swimming depth (Juell, *et al.*, 2003).

In trial 2 it was evident that the intensity provided by submersible cage lighting changed with season, ambient intensity and water conditions. However, in both November and April, submersible light units positioned at mid-cage depth created intensities greater than that provided by natural sunlight penetration during the day (52 vs. 201 Lux; 240 vs. 618 Lux. ambient:LL ratios). Furthermore, day and night time intensities under LL did not differ significantly. Since the majority of Atlantic salmon within a cage have shown a preference to swim at the depth of the artificial lights

(Oppedal *et al.*, 2001; Juell *et al.*, 2003; Juell & Fosseidengen, 2004), then the intensities provided by the artificial units may have allowed the lighting regime to be perceived as continuous rather than changing and hence removed the natural light-dark cycle as suggested by Kissil, et al., (2001). This could not be confirmed in trial 4 as a faulty light meter did not allow assessment of the daytime intensities between treatments. However, since the natural rhythms of growth and smoltification are known to be entrained by the seasonally changing photoperiod (Bromage *et al.*, 2001), the provision of equivalent or higher intensities within the cage at night relative to day time levels to remove the light/dark cycle may have resulted in the fish in trial 2 to subsequently adjust an endogenous growth rhythm in accordance with this. Thus under the conditions experienced in this study two lights appear to be the minimum required in order to achieve an intensity and distribution capable of promoting a growth response in rainbow trout at site 2. In support of this, the use of one light at site 2 in trial 4 showed substantially poorer growth performance than the use of two submersible lights irrespective of spectrum.

It has also been suggested that food intake and resulting growth is controlled by a seasonal endogenous rhythm, advanced by exposing fish to additional continuous light from mid-winter to mid-summer, only if sufficient light intensity is applied (Oppedal *et al.*, 1997). Oppedal *et al.*, (2001) related this observation to feeding motivation, with preferences for maximum temperatures and darkness acting as modifying factors. Primarily, light intensity was found to alter vertical distribution of Atlantic salmon at different times of the season, in association with changing temperature. It has been suggested that there is a trade-off between avoidance of surface light (predation) and hunger level (Juell *et al.*, 2003). An advanced seasonal ascent has been observed if salmon are exposed to high intensity lighting in early spring, and has

been interpreted as a light-induced shift in a seasonal rhythm of feeding motivation (Oppedal *et al.*, 2001). Therefore, the greater feeding efficiencies observed in trials 3 and 4 in two light versus single light treatments may reflect such a shift in feeding response.

The mechanism that is responsible for detecting daylength in fish must somehow discriminate between day levels of light intensity and the perceived night, and that there is a threshold intensity at which the transmission between the subjective day and night is transduced (Thorarensen *et al.*, 1989; Porter *et al.*, 1999c). Numerous studies have indicated that the light/dark cycle is used to entrain various endogenous rhythms relating to growth and reproduction in temperate species of fish, and that plasma melatonin may be the intermediary in the transduction of information on daylength to the appropriate axis, as scotophase plasma levels accurately reflect the duration of the night period (Alvarino *et al.*, 1993; Randall *et al.*, 1995a; Porter *et al.*, 1996; Bromage *et al.*, 2001). As yet direct evidence for a mode of action is still lacking.

Effects of LL Application in Cages on Plasma Melatonin

Melatonin profiles taken during the mid-day and -dark periods in trial 2 showed that additional night time illumination significantly reduced plasma concentrations during the dark phase below that of fish exposed to natural light in April, but not November. Similarly, mid-dark phase levels were significantly reduced in trial 4 below that under ambient photoperiod. A combination of water clarity and temperature (Porter *et al.*, 2000, 2001) may have explained the differences in response between the November and April sample points. Higher water temperatures in November, 10.3 °C, are certainly likely to explain the generally higher levels of melatonin during both day and night compared to those measured in April when lower temperatures were

experienced (7.5°C). In Atlantic salmon scotophase plasma melatonin concentrations were shown to decrease in response to increasing additional night time light intensity (Porter, *et al.*, 2001). The difference between amplitude of the light and dark decreased with increased light intensity. Salmon subjected to additional night time illumination in cages from November to July demonstrated significantly lower scotophase phase melatonin levels relative to control fish under natural light (Porter, *et al.*, 1999b). In addition to successfully reducing grilsing from 63% to 6%, growth was increased by 30% (Porter, *et al.*, 1999c). It was proposed that plasma melatonin must therefore be reduced below a threshold before the artificial photoperiod was capable of altering the timing of reproductive and growth patterns. Oppedal, *et al.*, (1997) postulated that there may be different threshold values of light intensity required to alter either sexual maturation or growth.

Melatonin and Daylength Perception

It has been suggested that increased melatonin secretion in conjunction with increased temperatures may enhance the ability of fish to discriminate between seasons (Max & Menaker, 1992). As such when daylengths are of equivalent length in autumn (falling temperature) and spring (rising temperature), then through up or down-regulation of melatonin secretion may serve to provide further information of season rather than what could be regarded as an ambiguous signal if photoperiod alone were used. Thus it is possible that although greater reduction of melatonin was achieved in spring under LL in trial 2, the endogenous growth pattern was advanced following LL application in November in response to a reduced dark phase melatonin concentration. Furthermore, if melatonin was reduced below a threshold in autumn, it could be argued that continued photoperiod application in association with lower temperatures in April,

in which natural suppression of melatonin production occurs, may have removed dark phase inhibition on growth. Certainly if melatonin does play a role in growth regulation, then the known inhibitory effect of LL on arylakylamine-N-acetyltransferase biosynthesis of melatonin as described by Falcon *et al.*, (1987) may have significant biological relevance and warrant further research in relation to the control of growth.

Interestingly in trial 2, strain 2 exhibited the greatest reduction in melatonin, to the extent that day and night levels were not considered significantly different in April. In conjunction with this, strain 2 also showed greatest enhancement of growth and improvement in feeding efficiency in response to photoperiod application. In contrast, LL treated fish of strain 1 showed a significant reduction in dark phase melatonin relative to their control, but not to the same degree as strain 2. Therefore, it is plausible that strain differences in perception of light may exist, and hence differentially effect their subsequent growth responses to light treatment.

Bulb Colour Temperature

Rainbow trout exposed to continuous illumination provided by two submersible units with bulbs of different colour temperature (4000K and 10,0000K) showed no significant differences in production performance during cage rearing in trial 4, although weight gain and feeding efficiency were significantly improved relative to ambient controls. Similarly, Stefansson & Hansen (1989) found no differences in growth or smoltification capacity in Atlantic salmon parr exposed to illumination of different spectral composition. However, the characteristics of the water at site 2 indicated complete absorption of wavelengths below 500nm within 50cm of the water column, and may have therefore negated any effects of spectrum of the 10,000K bulb in the 450nm region.

Confirmation of the successful application of the light treatments onto the natural photoperiod cycle was shown through the reduction of mid-dark phase plasma melatonin to significantly lower levels than those under natural light in all light treatments. This suggests that threshold levels of melatonin and intensity were reached in order to evoke a change in the growth response as has been suggested in Atlantic salmon (Porter *et al.*, 1999c). However, in the current trial, fish exposed to LL provided by a single 10,000K bulb also exhibited a significantly higher growth performance than those under natural light, although performance was poorer relative to those supplied by two lights. Interestingly, a single 10,000K bulb was equally effective as two 4000K bulbs in suppressing dark phase melatonin levels. This is surprising considering the poorer growth performance observed utilising one bulb relative to two. However, as previously discussed, the intensity or light distribution provided by a single bulb may not have been sufficient in order to compete with the ambient photoperiod cycle in order for the light treatment to be perceived as continuous, therefore the natural growth pattern was not sufficiently advanced in autumn rather than a consequence of spectrum. Unfortunately, as no blood samples to assess melatonin could be taken in autumn it is impossible to conclude whether this was the case for the single light, although such an effect seems likely.

With regards to the results of trial 4 it may be concluded that spectrum appears to have little effect on growth and performance of rainbow trout, and that relative light intensity and distribution is more important in order that the fish perceive the artificial additional illumination. This conclusion is also supported by the graded response of fry to increasing numbers of light units observed in trial 3 and the subsequent enhancement of growth.

5.8 Conclusions

Artificial constant light regimes can be applied in autumn (mid October-early November) to significantly enhance commercial production in both fry and fingerling rainbow trout. Successful application of constant light regimes and the subsequent perception was confirmed through the reduction of dark phase melatonin levels. The outcome of these studies has been the ability to increase growth rates by up to 25%, improve feeding efficiency by up to 30%, allow as much as 2 months reduction in production time and alter stock out times. The ability to alter the timing of final product availability may help avoid market gluts, in addition to allowing maximal use of facilities, increased production efficiency and production per man-hour. Furthermore, improved growth performance in fry hatchery stages facilitated earlier stock out times as well as reducing time to first intraperitoneal vaccination. In addition to significantly enhanced growth rates, since feed itself typically accounts for 40-60% of farm production costs then such improvements in feeding efficiency represent a significant reduction in farm expenditure. Overall, under the conditions in which these studies were performed it has been shown that a relatively simple tool can be applied to commercial trout ongrowing with significant benefits.

Chapter 6: General Discussion

6.0 General Discussion

The overall aim of this doctoral study was to investigate the influence and interaction of photoperiod with growth and reproduction in rainbow trout. Chapter 3 examined the influence of photoperiod on growth at two different developmental stages (fry and fingerling), and how these effects may be reflected in changes in circulating IGF-I levels, an indicator of growth rate already reported in other teleosts. A final experiment examined the role that melatonin may have in signalling photoperiodic information to the somatotropic axis using slow release melatonin implants. Chapter 4 investigated the effect photoperiod on the interactions between growth, plasma IGF-I and reproduction in first-time spawning rainbow trout. This experiment also examined the use of a detection method for the presence of a "leptin-like" substance in rainbow trout. Chapter 5 examined whether photoperiod techniques could be successfully transferred to commercial rainbow trout farming practices, with the key aim being to enhance winter grow-out and production. Four experiments were undertaken to assess the effects of photoperiod in relation to; growth and feeding regime in fry; growth and feeding efficiency in cages; light intensity and growth in fry; and the use of different lighting technologies in cages. Thus, this final chapter reviews the main conclusions arising from the present study and emphasises the need for future work.

6.1 Influence of Photoperiod on Growth and Plasma IGF-I (Chapter 3)

The results from this chapter clearly demonstrated that exposure to constant long-days (LD 18:6) or constant light (LL) significantly enhanced growth in both fry (<25g) and fingerling (>25g) in covered systems, and supports the observations by Skarphedinsson *et*

al.., (1985). Furthermore, there would appear to be an underlying endogenous rhythm controlling growth, although the timing of photoperiod application may directly photo-stimulate growth rather than phase shift such a rhythm. In this respect, plasma IGF-I levels accurately reflected growth rate, and suggests that using IGF-I to study and evaluate growth in relation to environmental manipulations is a powerful tool that has not been widely used in studies on rainbow trout to date. Exposure to long-days as of the summer solstice appeared to directly up-regulate the natural autumnal production of IGF-I relative to those under ambient or constant short-day photoperiods. This increase in IGF-I preceded a significant increase in growth rate and suggests that photoperiod was directly photostimulating growth at the endocrine level. In contrast, earlier application of LL regimes under increasing daylengths (April) did significantly enhance growth rate but did not change circulating IGF-I levels relative to fish under ambient and short-day regimes, supporting the idea that growth in rainbow trout is controlled by an underlying endogenous mechanism. These findings highlight the importance of the timing of light application in order to enhance growth. In this respect, there is now a requirement to study changes in IGF-I levels at different seasons (spring, summer, autumn and winter) under both changing and constant environmental conditions (photoperiod-temperature) in order to provide a more complete understanding of the interaction of growth and IGF-I during development. Furthermore, since the rainbow trout does not smolt, this species could be an ideal model for studying salmonid growth, in that the direct influence of environmental manipulation on growth-IGF-I interactions could be more clearly ascertained.

Of major importance is the finding within this chapter that artificially elevated melatonin levels significantly reduced growth rate in rainbow trout, but did not affect

circulating IGF-I levels relative to unimplanted controls. This suggests that melatonin is acting upstream of the liver-IGF system to control growth. However, caution must be taken when analysing these data as melatonin was artificially increased to non-physiological levels. Although GH was not measured in the current study it seems unlikely that melatonin would act directly on the pituitary to influence GH synthesis/IGF-regulation since the pituitary has been found to be devoid of 2-[^{125}I]iodomelatonin binding, and melatonin receptor gene expression was not detectable in rainbow trout (Mazurias *et al.*, 1999). This would further support the idea that melatonin is not acting centrally on the endocrine GH-IGF system controlling growth, but may act through an alternative route or hormonal mechanism. In support of this, experiment 3 demonstrated that melatonin did affect the weight to length gain ratio, in that implanted fish had a disproportionately high condition factor (i.e. greater weight for a given length). Since IGF-I is known to be involved in skeletal formation in vertebrates, and that prolonged exposure to supraphysiological levels of melatonin has been shown to inhibit [^{3}H]leucine uptake and skeletal cell growth in chicks (Lamosova *et al.*, 1997), *in vitro* studies examining IGF-receptors and mRNA expression during skeletal cell formation in response to melatonin treatment may elucidate the potential role that melatonin plays in teleost morphological development.

6.2 Effect of Photoperiod, Growth and Plasma IGF-I on Reproduction (Chapter 4)

The results of this experiment to my knowledge have provided for the first time a complete seasonal profile of IGF-I in rainbow trout. Furthermore, the results provided direct evidence for a relationship between maturation and circulating IGF-I levels, and that

the latter is regulated by seasonally changing daylength rather than a direct influence of water temperature. This was clearly demonstrated by the failure to find a relationship between water temperature and IGF-I under the ADV regime, with the pattern of IGF-I more related to phase-shifts in the endogenous rhythms controlling growth, reproduction and steroid production in response to a stimulatory long-short day photoperiod.

This experiment confirmed the importance of photoperiod as the principal cueing and synchronising mechanism controlling reproduction in the rainbow trout. Under natural photoperiod conditions it would appear that body size, growth rate and possibly lipid status as indicated by condition factor in the late-spring one year prior to maturation may have a role in determining whether a fish would initiate maturation. Conversely, under the advancing photoperiod regime the "window of opportunity" is naturally reduced and it would appear that body size and growth rate were not the principal determinants of whether maturation can proceed. However, plasma IGF-I did correlate strongly with growth rate in both treatments prior to the first indication of the initiation of maturation (testosterone increase) and may thus provide an endocrine link between the somatotropic and reproductive axes that growth rate, size or nutritional status is sufficient to allow latter stages of maturation to proceed. Furthermore, maturing fish typically expressed higher IGF-I levels relative to their non-maturing counterparts during this period and is in keeping with the known direct actions of IGF-I on pituitary-GtH stimulation. Nonetheless, failure to demonstrate a consistent relationship over time between growth rate and plasma IGF-I in maturing and immature fish suggests that a standardised approach to using and evaluating IGF-I as a growth signal is required, in order that we can determine the period in which such relationships provide the most useful biological information. Both the anabolic and

catabolic effects of IGFBPs and their regulation of circulating IGF need to be further elucidated. With regards to understanding the processes underlying the initiation of puberty in rainbow trout one of the principal objectives should be a thorough investigation of possible critical periods of decision making for the initiation of puberty in relation to known important endocrine changes.

Of all performance parameters examined in the current study, condition factor in late-spring would appear to be the most useful indicator of whether an individual would initiate maturation under either natural or advancing photoperiods. Whether this was related to whole-body lipid levels could not be determined in this study. However, in this respect, use of a combination of varying lipid diets to create populations of equal size and/or growth rate but different levels of adiposity prior to a possible "decision period" could be useful to determine if lipid/energy status has a significant role in reproductive success/signalling in female rainbow trout as has been suggested in other salmonids, albeit precociously maturing males (Rowe *et al.*, 1991; Silverstein *et al.*, 1998; Shearer & Swanson, 2000). If this were the case, then the use of high fat diets early in the reproductive cycle may be possible to increase numbers of fish spawning under advancing regimes to improve production efficiency of broodstock farmers. Certainly it is known that limiting ration early in the reproductive cycle reduces not only subsequent fecundity but also the percentage of female rainbow trout capable of maturing (Bromage & Jones, 1991). However, whether this is simply related to a perceived poor feeding opportunity that season or is reflected in lowered lipid status was not determined and certainly warrants further research. In this sense, further investigation of leptin and the emerging role of ghrelin as indicators of nutritional status and energy signalling will be of importance. Short-term

feeding trials utilising diets of different lipid content and measuring circulating leptin levels may provide useful information regarding energetic endocrine and signalling with regards to lipid status and feeding opportunity.

Overall, the current results concur with the already extensive knowledge that photoperiod is undoubtedly the principal cueing mechanism involved in maturational control of rainbow trout as in other salmonids, yet the physiological mechanisms underlying the initiation of puberty have not been resolved. To date extensive research has been devoted to understanding the mechanisms controlling precocious maturation in male salmonids, which is essential in resolving the potential economic losses associated with early maturation in commercial production. However, the physiological processes underlying the initiation of puberty in female salmonids are still not clear and a concerted effort should be made to resolving this lack of understanding. Although the endocrine changes have been widely researched and documented, the interaction between these endocrine processes and growth and development have not been ascertained. Such information will be particularly useful not only to scientific research but also with regards to the larger table market rainbow trout producers (>1kg-3kg), since photoperiod regimes used to date have been unsuccessful in preventing maturation of diploid stocks currently used. This has have resulted in large economic losses principally through increased mortality, loss of final product quality and extended production time required to recondition fish which have matured (pers. com. Stuart Cannon, Kames Aquaculture).

6.3 Commercial Application of Photoperiod Regimes (Chapter 5)

Given that the rainbow trout is the second most economically important finfish cultured in the UK, the lack of research regarding the use of photoperiod in on-growing stages is surprising given the now almost standard practice in Atlantic salmon farming. The results from the current chapter clearly demonstrate for the first time that relatively simple constant light photoperiod regimes can be successfully applied in 'open/uncovered' commercial rainbow trout culture systems to significantly improve grow-out performance (both growth and feeding conversion) during the autumn-spring production cycle. The ability to significantly enhance growth rates will allow farmers a means of reducing production time, both harvest and fry stock out times, and thus increase output per man hour. Ultimately this should allow sites to reduce production costs per unit, capitalise on achieving better market prices and improve their competitiveness.

One of the major problems associated with the application of photoperiod regimes in open rearing systems is successfully applying light onto the natural photoperiod. Of major interest in the current studies, was the importance of intensity or more correctly, light distribution, which was highlighted by the graduated enhancement in growth in response to increasing numbers of light units. In this respect, there was a more even lighting field provided by two units relative to one, and that the photoperiod regimes may have been perceived as continuous rather than changing. Such findings could have significant interest in applying the technologies used in this chapter to raceway systems which present an altogether entirely different problem with regards to lighting regimes due to the nature of these systems, their typically long but narrow and shallow design. Furthermore, although bulb spectrums tested appeared to have no effect on growth performance, the possibility

does not seem unrealistic since site specific water characteristics and irradiance will determine the natural penetration of specific wavelengths through the water column. In this respect, spectral content of bulbs could be tailored to site-specific requirements to optimise penetration of light. Recently, it has been shown that specific wavelengths have differential penetration rates of the cranium and pineal window (Migaud *et al.*, 2004, data unpublished), suggesting that specific wavelengths could have a greater stimulatory effect on the pineal organ.

In order to optimise the use of LL regimes in commercial practice an examination of the timing and duration of exposure to LL is required. This could determine whether the growth advantage is maintained after periods of short exposure. Since growth enhancing effects were only apparent after 12-14 weeks exposure in the current experiments, the question arises as to whether this growth advantage would be maintained following a return to ambient photoperiods. Such experiments would also help clarify whether photoperiod effects are directly photo-stimulating growth or whether they are truly altering an underlying endogenous rhythm controlling growth. Earlier application, say September instead of October, may also be able to take advantage of slightly higher water temperatures, and thus capitalise on a reduced rate-limiting effect of temperature on the physiological response to photoperiod. However, whether earlier application is applicable will ultimately be dependent on each site's specific stocking/harvest production cycles.

One final approach could be the evaluation of different strains in response to photoperiod manipulation. The results of experiment 2, would certainly suggest that there may be a differential response of strains to photoperiod exposure. Certainly, within the UK trout industry many sites are keen to evaluate and adopt the use of alternative strains that

may be better suited to their specific site and farming practice (per com. Mark Davies, British Trout Association). However, such evaluation of strains would have to be carried out at one site to exclude water quality and temperature differences experienced between different sites.

Finally, photoperiod significantly enhanced feed conversion efficiency in all experiments with respect to the current feeding practices used at these sites. Simple experiments in the future utilising in-feed ballotini and X-radiography could prove extremely useful in determining whether this observed improved feeding is due to stimulated feed intake and/or more efficient feed conversion. The outcome of such experiments could help farmers tailor their feeding regimes to their specific sites and thus optimise their feeding rates under LL and obviously reduce the significant costs associated with waste feed and/or overfeeding.

6.4 Summary

This thesis has expanded our knowledge of the importance of photoperiod in the control of growth and the interaction with reproduction in rainbow trout. There is now substantial evidence that extended photoperiods have a significant enhancing effect on the growth of rainbow trout at various developmental stages which has generally received little attention in the literature. Furthermore, the apparent relationship between growth rate, plasma IGF-I and maturation provides strong evidence for an endocrine link between the somatotropic and reproductive axes. Overall, this work has provided new and useful information to improve both scientific understanding and potential commercial development, although it is clear that substantial research is still required to provide a more complete understanding of the interactions of photoperiod with growth and reproduction.

List of Referred Publications

(1) Taylor, J.F., Porter, M.J.R., Randall, C.F. & Bromage, N. R. (2003). The interactions of photoperiod and insulin-like growth factor-I (IGF-I) in the control of rainbow trout reproduction. *Fish Physiology and Biochemistry*, 28, 449-450.

(2) Taylor, J.F., Migaud, H., Davies, M., Yonge, N., Porter, M.J.R. & Bromage, N.R. (2004). Photoperiod can be used to enhance growth rate and improve feeding efficiency in commercially farmed rainbow trout: A UK Perspective. *Aquaculture International* (submitted for publication)

(3) Taylor, J.F., Migaud, H., Porter, M.J.R. & Bromage, N.R. (2004). Photoperiod influences growth rate and insulin-like growth factor-I (IGF-I) levels in juvenile rainbow trout. *General and Comparative Endocrinology* (submitted for publication)

List of Non-Referred Publications

(1) Taylor, J.F., North, B., Porter, M.J.R. & Bromage, N.R. (2002). Photoperiod manipulation can be used to improve growth rate and feeding efficiency in rainbow trout. *Trout News*, **33**, 20-23.

(2) Davie A. & Taylor J.F. (2003). Light manipulation helps achieve market size sooner. *Aquaculture Risk (Management) Ltd Annual Newsletter.*

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