

Environmental Influences on the Physiological and
Behavioural Growth Responses in Salmonids; with
Reference to the Growth-dip Phenomenon.

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Declaration

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

Signature of Candidate:

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Abstract

Photoperiod manipulations are widely used throughout the Atlantic salmon (*Salmo salar*) farming industry as a means of producing a product of uniform quality all-year round. However, farmers still remain sceptical over their effectiveness to regulate growth and maturation during the on-growing stage. Furthermore, reports of a characteristic growth-dip following light exposure suggest that light may negatively affect the physiological performance of fish in the short-term. Thus, this thesis investigates the effects of light characteristics (spectral quality, intensity and photoperiod) on growth and maturation of salmonid fish and addresses some of the uncertainties surrounding photoperiod use currently reported within the industry.

Rainbow trout (*Oncorhynchus mykiss*) are seemingly an ideal model species for examining photoperiod effects on growth. Consequently, the application of constant light exposure (LL) at two different intensities (28W and 16W) during two different thermal conditions (summer and winter) was examined on individually tagged fish. Feed intake and growth appeared to be related to the ambient water temperature and did not appear to be affected by intensity or photoperiod, although the onset of constant light did appear to initially affect growth rate. This may indicate that LL has a limiting effect on the growth of trout or that the prevailing water temperature at which light is applied may override the photoperiodic effect. Furthermore, the lack of enhanced growth in trout exposed to LL, unlike that demonstrated for other salmonids, suggest that there may be a species-specific response to environmental variables. Thus, questions regarding photoperiod effects should be limited to the species in question.

The main source of variation in results observed under photoperiod manipulations stems from the salmon industry. Atlantic salmon post-smolts were reared in seawater tanks and either maintained under a natural photoperiod (NP) or exposed to a simulated natural photoperiod (SNP), constant light superimposed on the natural light (NPLL) or constant light only (LL). Artificial light onset, irrespective of photoperiod, resulted in an apparent trend for a reduced appetite lasting up to 60 days. Furthermore, the onset of constant light resulted in a significant chronic elevation of plasma cortisol levels and changes to growth and thyroid hormone levels, providing direct evidence that constant light exposure induces stress. In addition, fish exposed to SNP failed to exhibit a stress response despite a low feed intake. However, differences in the plasma melatonin levels during twilight times, as compared to NP, suggest that gradual changes in the natural light intensity throughout the day, particularly around dawn and dusk, may be important for synchronizing daily events. No differences in growth were observed between the NP and NPLL regimes, although fish reared in an enclosed

regime (SNP and LL) exhibited a significantly lower weight gain than fish in an open environment (NP and NPLL). This further highlights the impact that the rearing environment has on the growth performances of fish and the need for commercially run trials.

Advances in lighting technologies and a greater understanding of how light is transformed through the water column have focussed research on the spectral sensitivity of fish. Therefore the lighting efficiency of novel blue narrow bandwidth LED lighting units through the water column and their effects on growth and maturation performances of salmon reared in commercial production cages were compared against the standard metal halide units currently utilized throughout the industry. LL application, irrespective of intensity or spectrum, reduced the numbers of fish maturing as compared to fish reared under a natural photoperiod. However, this was greatest under the standard metal halide units reflecting a greater light penetration and perception as determined by plasma melatonin levels. The metal halide groups exhibited the greatest relative weight gain over the trial period as compared to control fish. No evidence was observed for a growth-dip under metal halide light, although blue lit treatments exhibited an initial significant reduction in food consumption, suggesting a possible welfare issue. Nevertheless, the prototype blue LED units showed possible potential for commercial application by penetrating the water depth at half the distance of the metal halide units for only one eighth the power and one fifth the brightness. However, further tests of these prototype spectral units are required to examine the potential welfare and physiological growth and reproductive effects.

These studies have shown that the efficacy of artificial light regimes is largely dependent upon the effectiveness of the light source through the underwater environment and its perception by fish, providing a sufficient intensity is emitted exceeding the physiological threshold level for the species cultured. Moreover, whilst the onset of artificial light may elicit a stress response and demonstrate a trend for a suppression of appetite for salmon reared in experimental tanks, no compelling evidence for a suppression of appetite or growth was found under normal commercial cage conditions. This suggests that the growth-dip observed within the industry may in part be a combination of a physiological response to the onset of light further exaggerated by the farmer's perception and altered judgement in feeding. In addition, the results obtained from this study have helped to standardize the use of light regimes within the industry. Nevertheless, further studies are necessary to fully elucidate the underlying mechanisms which may govern growth and maturation in fish following the onset of light exposure.

Keywords: Atlantic salmon, *Salmo salar*, photoperiod, feed intake, growth, maturation, growth-dip.

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Glossary index of common and scientific names used within this thesis

Common name	Scientific name
Arctic charr	<i>Salvelinus alpinus</i>
Atlantic halibut	<i>Hippoglossus hippoglossus</i>
Atlantic salmon	<i>Salmo salar</i>
Australian snapper	<i>Pagrus auratus</i>
Baltic salmon	<i>Salmo salar</i>
Bluegill	<i>Lepomis macrochirus</i>
Brown trout	<i>Salmo trutta</i>
Chinnok salmon	<i>Oncorhynchus tshawytscha</i>
Coho salmon	<i>Oncorhynchus kisutch</i>
Eurasian perch	<i>Perca fluviatilis</i>
European sea bass	<i>Dicentrarchus labrax</i>
Gilthead seabream	<i>Sparus aurata</i>
Goldfish	<i>Carassius auratus</i>
Green sunfish	<i>Lepomis cyanellus</i>
Largemouth bass	<i>Micropterus salmoides</i>
Nile tilapia	<i>Oreochromis niloticus</i>
Pink salmon	<i>Oncorhynchus gorbuscha</i>
Plaice	<i>Pleuronectes platessa</i>
Rainbow trout	<i>Oncorhynchus mykiss</i>
Red drum	<i>Sciaenops ocellatus</i>
Sea lice	<i>Lepeophtheirus salmonis</i>
Syrian hamster	<i>Mesocricetus auratus</i>
Zebrafish	<i>Danio rerio</i>

‘Criticism is easy; achievement is difficult.’

[Sir Winston Churchill (1874-1965)]

Chapter 1: General Introduction

1.1. The UK Farming Industry

Aquaculture is currently the fastest growing sector of the world food economy, with almost half of the fish consumed coming from farmed sources (FAO, 2006). The farming of salmonids currently accounts for 2.7% of the total global production with Norway and Scotland leading production in European waters and Chile in the Americas. Within the UK, finfish production is largely dominated by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). At present, the annual tonnage of farmed salmon harvested within the UK stands at around 158,000 tonnes compared to 6,500 tonnes produced by the trout farming industry (Figure 1.1.; SEERAD, 2005).

The primary objective for the industry is to produce a uniform product of high quality and size in the shortest time possible. However, three principal factors which affect this objective are the physical environment, fish physiology and behaviour (Brett, 1979; Jobling, 1994). During the course of production, these factors may act independently or interact to determine growth and subsequently affect the final cost and quality of the product. As feed costs contribute around 50% of the total production cost (Sveier and Lied, 1998), understanding the factors which influence feeding behaviour, feed conversion and ultimately growth, is of prime concern to ensure that growth rates can be maximized and feed waste minimized to give production cycles which yield the greatest economic gain.

1.2. Atlantic Salmon

The Atlantic salmon is a migratory anadromous member of the Salmonidae family, a teleost group of fish comprised of three major genera: *Salmo*, which includes the Atlantic salmon and sea trout (*S. trutta*); *Oncorhynchus*, such as the rainbow trout and Pacific salmonids; and the *Salvelinus* group of charrs. As with most salmonids, the Atlantic salmon is well adapted to cold (2 - 9°C) oxygen rich waters reflecting their distribution throughout the temperate and Arctic zones of the northern hemisphere (68°N - 38°N,

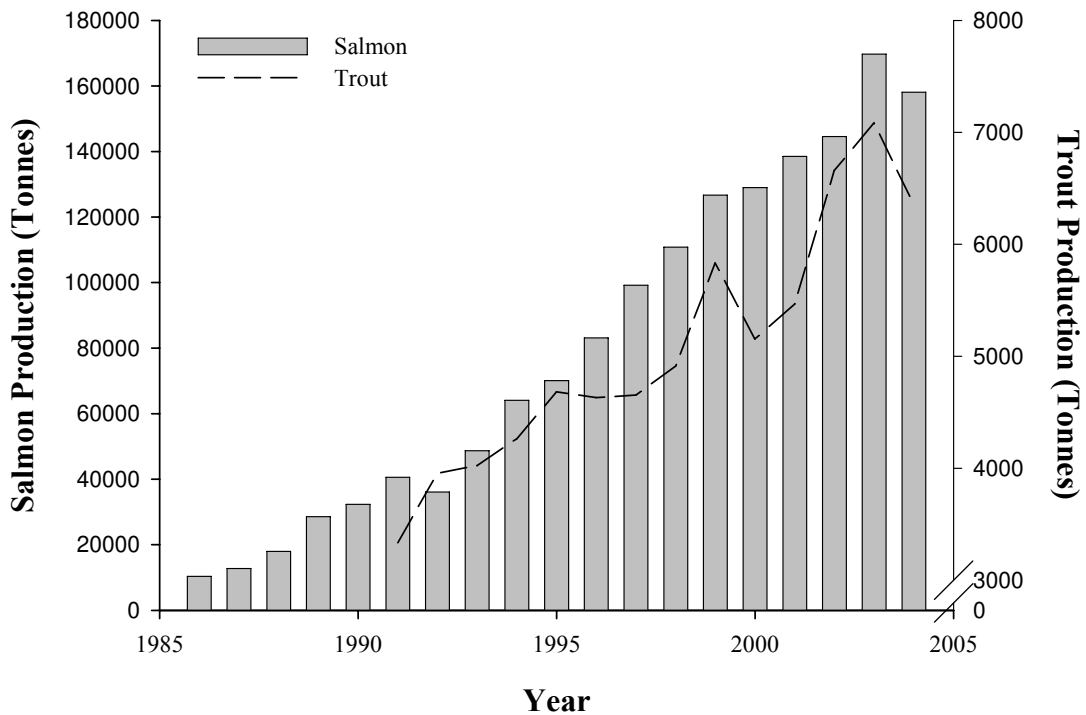


Figure 1.1. Annual production (tonnes) of Atlantic salmon and rainbow trout harvested in the UK during 1986-2004 (Source: SEERAD, 2005).

76°W - 55°E), ranging from the north of Canada to the Baltic States and extending as far south as northern Spain (MacCrimmon and Gots, 1979; Mills, 2001).

Occasionally, the Baltic salmon (*Salmo salar*) may also be referred within the scientific literature (e.g. Koskela *et al.*, 1997; Jobling *et al.*, 1998). However, this does not refer to a separate species of salmon rather an isolated stock found within the Baltic Sea (MacCrimmon and Gots, 1979).

Life history

Atlantic salmon have a relatively complex life history that extends from the spawning and juvenile growth in freshwater rivers through to the seaward migration where intensive feeding and reproductive development occurs in readiness for the homeward journey (Figure 1.2.; Mills, 2001).

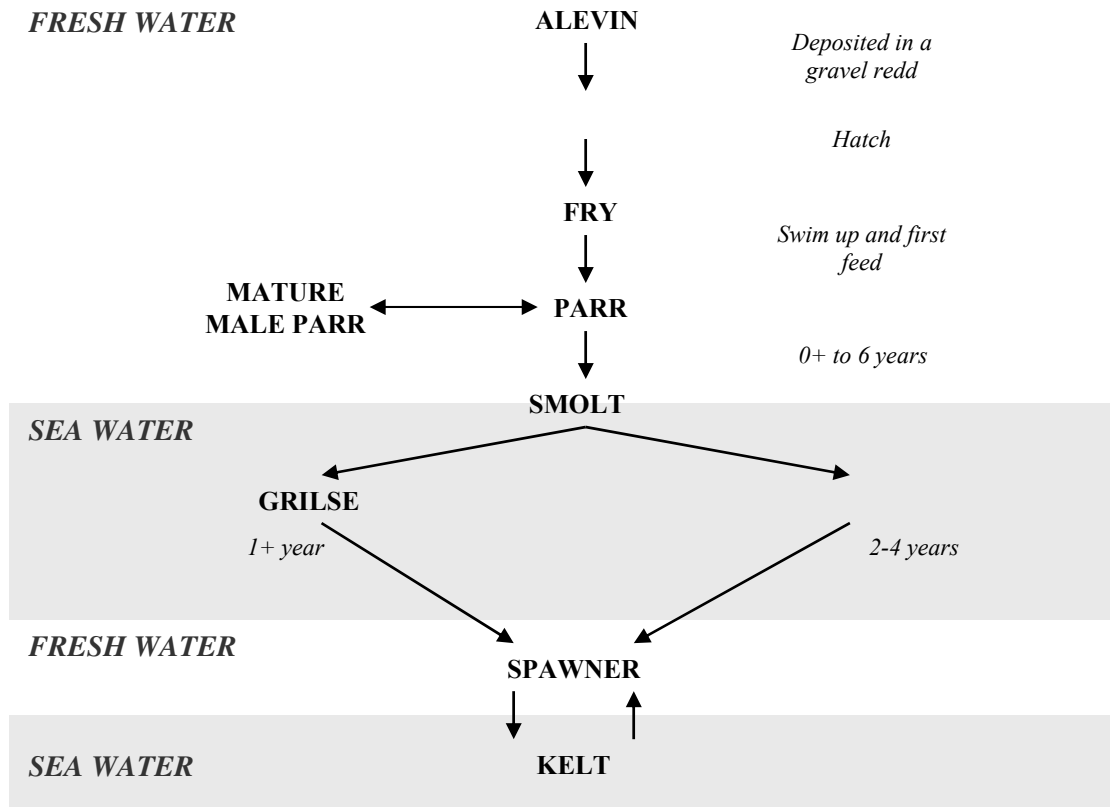


Figure 1.2. Schematic diagram outlining the life-cycle of the Atlantic salmon.

Wild salmon characteristically spawn between October and December, with the female preparing excavations (redds) in the gravel bed of the river into which the eggs are released and fertilized. The eggs then hatch during early spring and the alevins feed upon their yolk sacs before emerging through the gravel bed as fry. As the juveniles grow, often into two distinct weight classes, they develop the prominent brown-red markings of parr. The parr may remain within the freshwater for up to six years, with some males precociously maturing within this juvenile stage. Those parr reaching a critical size by spring undergo a series of physiological, morphological and behavioural changes that result in a silvery, streamlined appearance in a process known as smoltification in preparation for seaward migration during late spring. Smolts generally remain at sea for up to four years before returning to their native rivers to spawn, although a small proportion return as grilse having spent one winter at sea. During their

time at sea the salmon develop reproductively and feed intensively to accumulate fat reserves that serve as energy sources for gametogenesis and the strenuous return journey. However, unlike its Pacific cousin which dies following spawning, Atlantic salmon “kelts” may survive to migrate to sea and make subsequent spawnings.

Thus, the salmon aquaculture industry has evolved around both stages of the life-cycle to ensure that juveniles are reared and then on-grown under conditions most favourable for growth.

1.3. Growth and Feeding

The growth process for any animal is superficially simple, yet inherently complex. Three principal factors are known to influence the growth performance of fish: the physiological environment (*e.g.* nutrition, light, temperature); fish physiology (*e.g.* age, size, genetics, health); and fish behaviour (Brett, 1979; Jobling, 1994). These factors may act independently or interact to determine an individual’s growth. Since feed costs contribute towards 50% of the total production costs (Sveier and Lied, 1998), maintaining good growth rates whilst minimizing waste feed is vital to the farmer if they are to reap the best economic outcome and minimize the environmental effects.

The energy budget of an animal can be described in terms of growth as (Brett, 1979; Jobling, 1994):

$$G = I - (M + E)$$

where G is the surplus energy deposited as growth, I the energy ingested through consumption, M the energy used in metabolic processes (*i.e.* maintenance and activity such as swimming), and E the energy lost through excretion and heat loss (*e.g.* faeces, urinary, gill, body surface). Thus, a sustained growth increase can only be supported by an increased food consumption and/or food utilization.

Growth-ration relationship

Feed strategies employed within the aquaculture industry are designed specifically to nourish the animal to a desired level of productivity within the shortest available time and for the highest economic gain as practicably possible. Farmers should therefore aim for a low food conversion ration (FCR), the ratio of feed ingested to body weight gained. Generally, farmers look to attain an FCR of 1.0, *i.e.* for every 1 kg of dry weight food fed a 1 kg gain in wet body weight would result. However, as fish are able to efficiently convert high protein diets into somatic tissue an FCR below 1.0 is achievable (Lovell, 1998; Sinnott, 2001).

Growth monitoring is also an essential aspect of the farming process and is often expressed in simple terms as weight gain per unit of time. The most commonly used indicator of growth in the industry is the specific growth rate (SGR), the percentage body weight increase per day. The effect of ration on growth is best illustrated by the growth-ration curve, the relationship between growth rate and the rate of feeding (Figure 1.3.; Talbot and Hole, 1994). Underfeeding fish results in a deficiency of available energy for the basic metabolic requirements of an individual and subsequently leads to weight loss, a high FCR and negative SGR. Growth essentially occurs when the ration consumed by an individual exceeds the ration required for the metabolic requirements of the individual (R_{maint}). Further increases in ration level results in a linear increase in growth. Providing an optimal ration would therefore mean fish are fed to satiation resulting in maximal growth (R_{max}), where the growth rate plateaus, and a low FCR. Overfeeding on the other hand may yield a maximum growth rate but leads to waste feed and an increasing FCR. However, the growth-ration relationship is dependent upon several factors including fish physiology and environmental conditions which determine the maintenance ration required by effectively phase-shifting the curve due to either an exertion or reduction in metabolic expenditure (Staples and Nomura, 1976; Wurtsbaugh and Davis, 1977; Grove *et al.*, 1978). Thus, accurately judging the amount of ration fed is important both for economic and environmental gains.

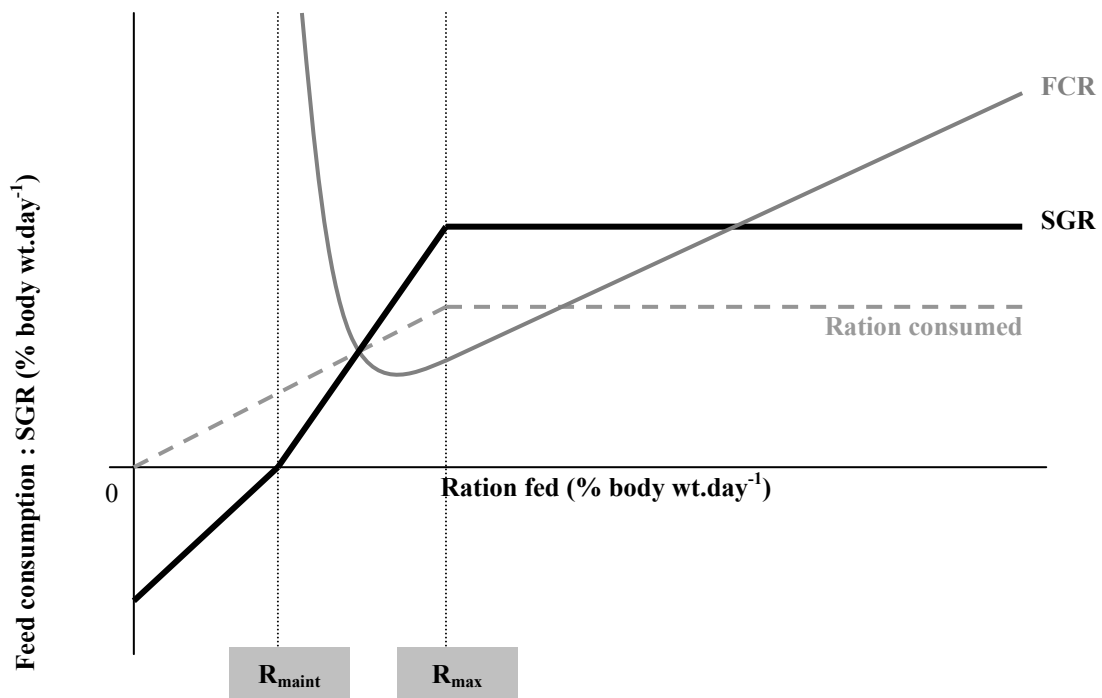


Figure 1.3. Generalized growth-ration curve and FCR relationship for salmonids. Fish fed a maintenance ration (R_{maint}) sustain body metabolism without affecting growth, whereas maximum growth (R_{max}) is achieved at the point where the fish's appetite is met (After Talbot and Hole, 1994).

Growth regulation

Growth has either been described in terms of the recruitment and hypertrophy of muscle fibres (see Johnston, 1999; Mommsen, 2001) or through protein turnover rates (Breier, 1999). An important component of being able to regulate growth and developmental state of an individual is to have the ability to recognize nutritional energy status. Growth generally increases with an increase in food consumption, however individuals can only grow to a pre-determined genetic size. Thus, a regulatory feedback mechanism controlling feed intake and growth must be in operation. Liopostatic models similar to those described for mammals have been proposed for fish (Shearer *et al.*, 1997; Jobling and Johansen, 1999; Jobling *et al.*, 2002; Johansen *et al.*, 2002), with several studies

designed to study the effects of adiposity on feeding and growth. Research shows that fish with greater adiposity levels grow much slower than leaner fish, indicating that adiposity exerts a negative feedback on feed intake and growth rate (Shearer *et al.*, 1997; Johansen *et al.*, 2001; Jobling *et al.*, 2002). This ability of fish to regulate their feed intake through adiposity levels is reasonably understood when conditions are present for growth, yet little is known about how fish respond when presented with unfavourable conditions for growth.

Compensatory or catch-up growth refers to a phase of rapid growth following a change from a period of unfavourable to favourable conditions for growth (see Broekhuizen *et al.*, 1994; Metcalfe and Monaghan, 2001). This period often results in a greater or equal growth rate as compared to normal growing fish, although incomplete or impartial gain has also been known to occur (Jobling and Johansen, 1999; Johansen *et al.*, 2001). This response is generally accompanied by hyperphagia and occasional improved growth efficiencies (Grove *et al.*, 1978; Talbot *et al.*, 1984; Johansen *et al.*, 2001), seemingly linked to the severity of feed restriction (Johansen *et al.*, 2001). Although generally associated with periods of feed restriction, compensatory growth responses also relate to situations where feeding and growth have been suppressed by unfavourable environmental conditions such as temperature (*e.g.* Mortensen and Damsgård, 1993; Koskela *et al.*, 1997) and salinity (*e.g.* Damsgård and Arnesen, 1998). However, the precise mechanisms that trigger the catch-up response are still unclear, although as previously discussed it is hypothesised that adiposity governs feeding in fish (Shearer *et al.*, 1997).

Variation in growth and feeding

Salmonid fish, particularly migratory anadromous species, generally show a certain degree of plasticity in growth mainly due to the variation in length at each stage of their life-cycle. One interesting observation on growth is the variability that occurs between and within populations held under identical rearing conditions, of which the bimodality of salmon parr is the most documented (Thorpe, 1977, 1989). Higgins and Talbot (1985)

found that the development of modality tended to occur as a result of differential feeding patterns, although they suggested that bimodality was probably not related to food availability but rather from biochemical reactions under internal control. Nevertheless, investigations studying fast- and slow-growing strains of trout fed different nutritional rations have failed to yield any differences in endocrine profiles (Valente *et al.*, 2003), indicating instead the involvement of an external component. As such, Villarreal *et al.* (1988) put forward the notion of environmental signals, in particular photoperiod, as a synchronizer of the internal rhythm of appetite and growth. However, it is more probable that a critical threshold size or energetic status is required resulting in a period of differential growth leading to upper and lower modal groups as has been proposed for both the parr-smolt transformation and the initiation of maturation (Duston and Bromage, 1988; Taranger *et al.*, 1998, 1999; Shearer and Swanson, 2000).

Food intake in fish varies both daily (Thorpe *et al.*, 1990b; Smith *et al.*, 1993; Blyth *et al.*, 1999) and seasonally (Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999; Oppedal *et al.*, 1999; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003). Such variations result from changes in light levels, water quality, stocking densities and stressful events. Long-term, seasonal variations in feed intake are thought to arise from changes in daylength rather than to other environmental variables (Smith *et al.*, 1993). Indeed, for a given temperature and body size, fish generally display a greater appetite and grow faster when the daylength is increasing than under a shortening daylength (Brett, 1979; Higgins and Talbot, 1985). However, since fish are ectotherms the ambient temperature itself exerts an influence on feed intake and growth, increasing with an increase in temperature, up to a species dependent maximum temperature (Staples and Nomura, 1976; Grove *et al.*, 1978), although behavioural and genetic factors will also invariably affect the feeding and growth responses in fish.

The formation of dominance hierarchies may also contribute to the development of size heterogeneity in cohorts of fish. Fish held in isolation generally show little variation in day-to-day feed intake, yet placed into groups dominance hierarchies are allowed to establish leading to increases in the intra- and inter variation of feed intake

and a gain or loss in weight (McCarthy *et al.*, 1992; Jobling and Baardvik, 1994). These social interactions can have a knock-on effect on the flesh quality (Hatlen *et al.*, 1997). However, through increases in ration both the variability and strength of the hierarchy is reduced allowing individual fish a greater opportunity to feed without the need for aggressive behaviour when competing for available feed (McCarthy *et al.*, 1992). A dominant status, however, does come at a cost. Aggressive and dominant fish obtaining a greater proportion of the meal have to repay a greater energy output to avoid incurring a negative growth rate (Øverli *et al.*, 1999). Subordinate fish on the other hand tend to make fewer foraging attempts and show lower signs of activity thereby saving their energy output (Metcalf, 1986). Moreover, sibling Arctic charr (*Salvelinus alpinus*) raised from one male and female from eggs of variable sizes resulted in large variances in body size but also produced a dominance hierarchy with different feeding strategies (Benhaïm *et al.*, 2003). These differences indicate a strong genetic component that may influence individual growth patterns.

With growth performances varying in individuals from within and between populations, the need for decreased variability and improved growth is of paramount concern to the commercial industry. Thus, the genetic component (strain) of fish used will have a limiting factor on production. Selected fish invariably have higher growth rates than wild strains (Valente *et al.*, 1998; Handeland *et al.*, 2003; Mambrini *et al.*, 2004), reflecting the trait they were specifically bred for. However, growth differences are ameliorated when fish are fed to satiation (Valente *et al.*, 1998). The slower growth of wild strains is normally accompanied by a greater variability in feed intake (Hatlen *et al.*, 1997), whereas the increased growth rate in selected strains is said to either occur through an increased feed intake (Mambrini *et al.*, 2004) or improved conversion efficiency (Handeland *et al.*, 2003). Furthermore, the improvement in growth between control and selected strains can be brought about by successful environmental manipulations that act as an additive component to the genetic effect which may be used to enhance the productivity rate (Handeland *et al.*, 2003).

Feeding regimes

Maximizing the growth rates and food conversion efficiency in commercial production depends upon matching the way in which the farmer makes food available to the fish with the physiological and behavioural mechanisms controlling the appetite and feeding activity (Talbot, 1993). Under commercial conditions, fish are either fed by hand or automatic feeders, of which there are various types. However, regardless of feeding method, some knowledge of the production state including the total biomass, stocking densities and environmental conditions is required. The majority of this knowledge is primarily based upon manufacturers feeding tables, assessed from calculations on growth rates (Austreng *et al.*, 1987) or energy requirements in relation to body weight and temperature (Cho, 1992a). However, this may not accurately reflect the real-time feeding behaviours of the fish. In-fact, fish fed on-demand exhibit lower levels of competitive behaviour during the feed process than fish fed using a standard feeding system, indicating a potential for improved growth and production efficiency through a lowering of energy normally expended in competing for available food (Boujard *et al.*, 2002). Thus, to minimize waste feed whilst maximizing growth performance fish should be fed to their behavioural feeding responses.

Salmonid fish are typically visual feeders displaying crepuscular or diurnal feeding behaviours with peaks in feed intake around dawn and dusk (Higgins and Talbot, 1985; Boujard and Leatherland, 1992a; Blyth *et al.*, 1999), although juvenile salmonids have been shown to switch survival strategies to nocturnal feeding patterns during the winter (Fraser and Metcalfe, 1997). As vision plays a crucial role in feed acquisition the lighting conditions and/or pellet contrast must be adequate to ensure high consumption rates (Pettrell and Ang, 2001). Thus, it is essential that the feeding behaviour of fish is monitored during the meal period.

Monitoring feed consumption in commercially produced fish is therefore an important aspect of the farming process, ensuring a sufficient ration is dispensed to feed the fish to satiation without wasting feed. However, unlike terrestrial agricultural animals the monitoring of feed intake in fish is a more demanding process as the

environment in which they reside creates difficulties in observing feeding behaviours. Surface observations of feeding responses are typically used as an assessment of appetite, yet this only permits the farmer to view a small proportion of the cage volume, most likely leading to underfeeding. Furthermore, the swimming behaviour of fish may be affected by various parameters such as the use of artificial light (Oppedal *et al.*, 2001; Juell *et al.*, 2003; Juell and Fosseidengen, 2004), and may consequently affect the depths at which fish may feed. In addition, depressions in feed intake are also common following the seawater transfer of smolts (*e.g.* Usher *et al.*, 1991, McCarthy *et al.*, 1996; Stead *et al.*, 1996; Arnesen *et al.*, 1998; Damsgård and Arnesen, 1998) and abrupt transfer between rearing temperatures (*e.g.* Mortensen and Damsgård, 1993; Koskela *et al.*, 1997; Arnesen *et al.*, 1998), indicating the need for rigorous monitoring of feeding behaviours within commercial practices. Several devices have been developed for the commercial industry incorporating feedback systems which allow the farmer to feed the fish to satiation more accurately, although it is the population rather than the individual that is fed to appetite. Such devices include automatic feeders, mechanical waste uplift systems, and video monitoring systems, but in all cases the farmers perceptual judgement is still required to ensure fish are neither under- nor over-fed.

Laboratory methods for studying feed intake in fish have been extensively reviewed (see Talbot, 1985; Jobling *et al.*, 1995, 1999). The simplest way for quantifying individual food intake is through stomach content analysis using either live or sacrificed fish. Stomach pumping and/or flushing techniques and even the use of radioisotopes incorporated into the diet have been employed previously. However, these techniques involve a starvation period prior to feeding the test meal, a factor which is known to induce hyperphagia (Talbot *et al.*, 1984). The use of a non-invasive procedure is therefore preferable. X-radiography is one such technique, whereby diets labelled with radio-opaque particles allow quantification of an individual's voluntary food consumption to be made (Talbot and Higgins, 1983). This allows for repeated measures to be made on the same fish throughout the trial, and as such has been employed in several studies to examine feeding behaviours and/or rhythms (*e.g.* Talbot *et al.*, 1984;

Koskela *et al.*, 1997) as well as for testing the palatability and/or efficiency of new diet formulations (Carter *et al.*, 1995). Moreover, as food consumption is directly measured the true growth efficiencies can be calculated as opposed to being based upon ration levels.

1.4. Endocrine Regulation of Growth

The endocrine regulation of growth is a complex process, centrally controlled by the brain-hypothalamus-pituitary axis which releases a cascade of hormones specific to growth and other physiological processes (Figure 1.4.; Björnsson, 1997; Le Bail and Boeuf, 1997; Very and Sheridan, 2002). Generally, growth hormone, insulin-like growth factors, insulin and the gonadal steroid hormones stimulate anabolic processes, whereas adrenocortical steroid hormones, glucagons and the catecholamines act predominantly to promote the mobilization of energy reserves (Leatherland, 1994).

The somatotropic axis

The somatotropic, or GH-IGF axis, is a multi-hormonal system involving growth hormone (GH), GH receptors (GHR), insulin-like growth factors (IGF's), their receptors (IGFR) and binding proteins (IGFBP). Essentially, GH influences growth through the stimulation of IGF production from the liver into circulation of the blood which targets tissues to enhance skeletal and muscle growth. Circulating IGF levels are closely associated with IGFBP's which are central in controlling the availability of IGF to target tissues and therefore an important factor in the GH-IGF axis.

Growth hormone, also termed somatotropin, is a 191 (~22 kDa) amino acid polypeptide that is secreted in a pulsative manner by the somatotroph cells of the anterior pituitary gland. Secretion is thought to be controlled by the hypothalamic production of GH-releasing factor (GRF) and somatostatin, an inhibitor of GH release (Very *et al.*, 2001; Very and Sheridan, 2002). GH is a multifunctional hormone and has been indicated in numerous physiological processes including the parr-smolt transformation (Komourdjian *et al.*, 1976; Björnsson *et al.*, 1998, 2000), sexual maturation (Björnsson *et*

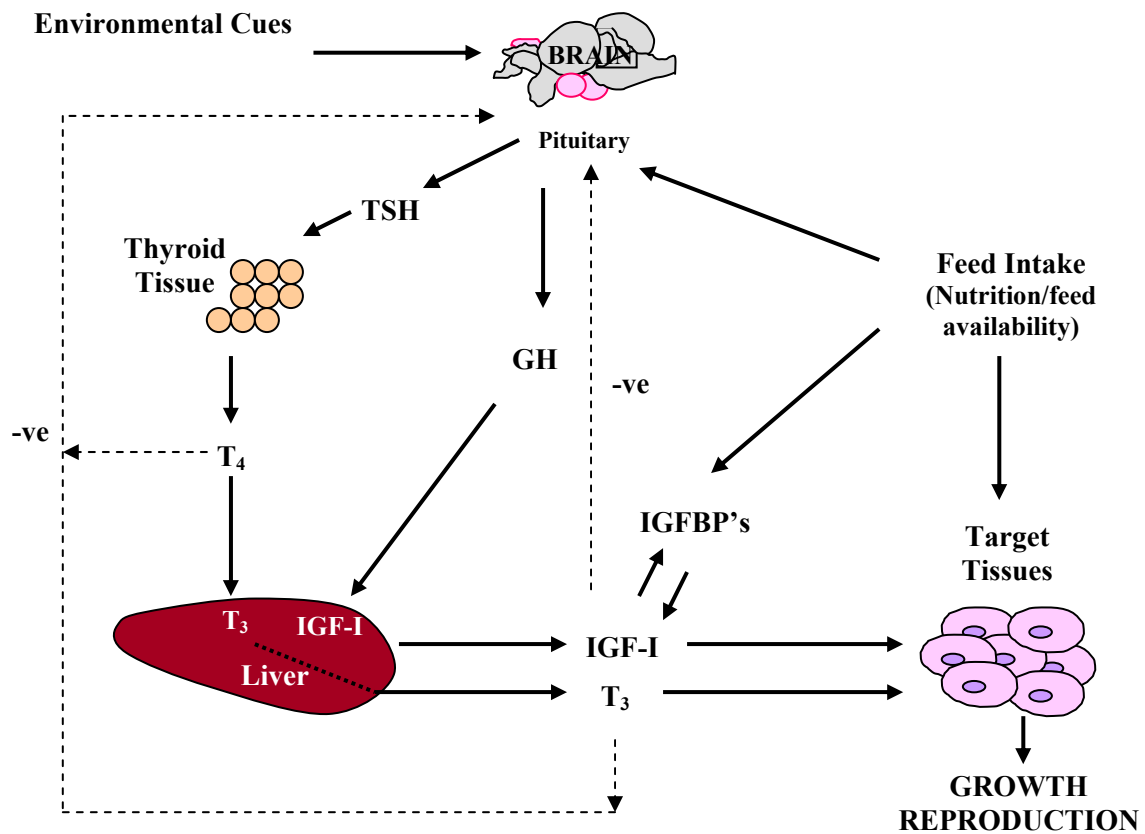


Figure 1.4. Schematic representation of the somatotrophic and thyroid axes controlling growth in fish. GH, Growth hormone; IGF-I, Insulin-like growth factor I; IGFBP's, Insulin-like growth factor binding proteins; TSH, Thyroid stimulating hormone; T₄ thyroxine; T₃ tri-iodothyronine. Negative feedback (-ve) indicated by dashed lines (Adapted from Leatherland, 1994, and Duan, 1997).

al., 1994) as well as affecting behavioural activities (Johnsson and Björnsson, 1994; Jönsson *et al.*, 1998). However, GH is best documented for increasing growth (see Björnsson, 1997) and can even induce skeletal growth under conditions of restricted feeding (Johnsson and Björnsson, 1994). The exact mechanisms underlying this cause remain unclear, although it is thought that GH exerts its growth influence by either increasing feed intake or improving conversion efficiency (Johnsson and Björnsson, 1994). Nevertheless, the majority of the biological effects of GH action appear to be mediated by IGF-I.

Insulin-like growth factor-I (IGF-I) is a 70 amino acid (~7.6 kDa) single chain polypeptide involved in the regulation and development of somatic growth in vertebrates. IGF's are classed as somatomedins, similar in structure to insulin, stimulating the growth of cartilage and the incorporation of sulphate into cartilage. However, unlike insulin IGF's circulate in the plasma complexed to a family of structurally related proteins. IGF mediates the biological effects of GH such that GH not only stimulates IGF secretion from the liver but also increases tissue sensitivity to IGF, stimulating cell differentiation, growth and proliferation (see Duan, 1997, 1998). As such, IGF increases with increases in GH levels, particularly during periods of high growth, *i.e.* smoltification (McCormick *et al.*, 2000) or following GH administration (Shimizu *et al.*, 1999). However, during periods of feed restriction there is a separation of the GH-IGF axis, whereby plasma GH levels steadily rise (Sumpter *et al.*, 1991; Leatherland and Farbridge, 1992) and IGF levels decrease (Duan, 1998; Pérez-Sánchez and Le Bail, 1999). Subsequently, plasma IGF levels appear to correlate well with both ration (Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004) and growth rate (Beckman *et al.*, 2001, 2004; Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004; Taylor *et al.*, 2005). Temperature (Larsen *et al.*, 2001; Gabillard *et al.*, 2003b) and photoperiod (McCormick *et al.*, 2000; Beckman *et al.*, 2004; Taylor *et al.*, 2005) have been suggested to exert an influence on IGF levels, primarily through an increased GH response (Gabillard *et al.*, 2003b). In particular, extended photoperiod regimes have been suggested to cause a direct photostimulation of growth through the up-regulation of IGF-I production (Taylor *et al.*, 2005), although the same authors also reported that growth may be maintained by an underlying endogenous mechanism phase-advanced by the change in photoperiod with IGF levels simply reflecting the growth rate. Thus, it would appear that the GH-IGF axis is an integral component of the growth axis, although other hormones will undoubtedly influence growth and development of fish as well.

Thyroid hormones

The thyroid hormones, triiodothyronine (T₃) and thyroxine (T₄), are products of the thyroid gland in all vertebrates and are essential for normal growth, development and

metabolism. In teleost fish, T_4 is predominantly secreted by the thyroid gland and undergoes deiodination in the peripheral tissue to the more biologically active derivative T_3 (Eales *et al.*, 1993). The thyroid hormones are best recognised in their involvement at various ontogenic developmental stages including larval growth, flatfish and amphibian metamorphosis and salmonid smoltification (Leatherland, 1982, 1994; De Pedro and Björnsson, 1999; Power *et al.*, 2001). Additionally, the thyroid hormones are also considered to play a permissive role in the growth process of fish by potentiating the anabolic effects of other growth promoting hormones (Leatherland, 1982, 1984; Macbride *et al.*, 1982; Sumpter, 1992). Administration of T_4 and/or T_3 enhances skeletal and somatic growth although their effects are more pronounced when acting synergistically with GH (Higgs *et al.*, 1982; Leatherland, 1982; MacLatchy and Eales, 1990). Daily T_3 concentrations appear to correlate well with growth rate (Gomez *et al.*, 1997) with $T_4:T_3$ ratios exhibiting seasonal variations reflecting the seasonal pattern of growth (Osborn *et al.*, 1978). As such, plasma T_3 levels have been shown to accurately reflect the nutritional status of the fish (Eales and Shostak, 1985a; Gabillard *et al.*, 2003a). Furthermore, diel variations in circulating thyroid hormone levels appear to be dependent upon feeding time and/or photoperiod (Eales *et al.*, 1981; Boujard and Leatherland, 1992a, 1992b; Gélineau *et al.*, 1996; Gomez *et al.*, 1997), although these are eliminated under periods of nutritional restriction (Eales *et al.*, 1981; Reddy and Leatherland, 1995).

1.5. Maturation

The ultimate 'life-goal' for any animal is to ensure its survival through reproduction. However, under commercial farming conditions early sexual maturation is undesirable since it interferes with production schedules and results in a lower growth performance and deterioration of flesh quality leading to the subsequent downgrading of fish at the processing plant. During 2004, around 27,000 tonnes of grilse¹ were harvested within the UK (SEERAD, 2005), equivalent to 17.5% of total salmon production for that year.

¹ Maturity status of fish not stated within farm survey data

Salmonid fish are characteristically annual spawners, with the reproductive cycle linked to the seasonally changing photoperiod (Bromage *et al.*, 1992, 2001). Like the parr-smolt transformation, a ‘critical period’ or ‘window of opportunity’ has been proposed to control the timing of sexual development (Duston and Bromage, 1988; Thorpe, 1989; Taranger *et al.*, 1998, 1999; Bromage *et al.*, 2001). This suggests that fish have to exceed developmental thresholds or be at a critical physiological state (*e.g.* age, size, growth rate, energy stores and stage of gonadal development) at a specific time of year, or during an ‘open gate’ period of a circannual rhythm entrained by photoperiod. Within the salmon farming industry, exposure to constant light has been suggested to advance the critical period, thereby preventing some of the fish exceeding the developmental threshold related to somatic growth and/or energy storage (Taranger *et al.*, 1999). Alternatively, the altered maturation rate may follow from the effects of constant light exposure on the growth performance of fish *per se* (Endal *et al.*, 2000), indicating that constant light exposure can increase or decrease the maturation rate as a consequence of the interactions between the effects of constant light exposure on the timing of the critical period and the resultant growth performance and/or energy storage. Shearer and Swanson (2000) further demonstrated the effects of lipid storage on maturation, with high dietary lipid levels significantly affecting the proportion of maturing male chinook salmon (*Oncorhynchus tshawytscha*) by influencing whole-body lipid levels (adiposity). Maturing fish therefore display a stronger feeding motivation from October to the following spring/summer, prior to maturation, to accumulate the necessary nutrient/energy reserves required to fuel reproduction (Kadri *et al.*, 1996). This results in maturing fish attaining a higher weight gain, through the anabolic affect of the sex steroid hormones, than immature fish and may allow a short period in which farmers can harvest the early maturing fish prior to the final onset of maturation. Applying nutritional restrictions at certain times of the year would therefore be expected to delay maturation without inhibiting growth, and have been successfully demonstrated in Atlantic salmon (Thorpe *et al.*, 1990a), female rainbow trout (Bromage *et al.*, 1992) and 2+ brown trout (Pirhonen and Forsman, 1999). However, it is through the manipulation

of photoperiod cycles that the reproductive status of fish can be advanced or delayed, depending upon the timing and direction of light onset, thereby giving the commercial on-grower a greater opportunity in controlling maturation rates.

Detecting grilse levels at an early stage in production is critical in ensuring early maturing fish are harvested prior to the onset of full maturation. Traditional methods usually rely on the farmer making a visual assessment of the external characteristics of fish stocks, *e.g.* skin colour, presence of kype, as the carotenoid pigments are redistributed from the flesh to the skin and gonads (Torrissen and Naevdal, 1988; Hatlen *et al.*, 1997; Bjerkeng *et al.*, 2000). However, this method is rather subjective and may result in non-maturing fish being wrongly identified. Furthermore, false maturation, a phenomenon in which fish briefly display the external characteristics of maturing fish without undergoing gonadal development, is widely reported throughout the industry in fish which have been exposed to artificial light. Thus, identifying and separating the maturing from the non- and false-maturing fish is key to ensuring an efficient production cycle. Increases in the levels of plasma sex steroids, such as testosterone, are generally associated with gametogenesis, gonadal development and maintenance of somatic tissue. Principal sex steroids are 17β -estradiol in females and 11-ketotestosterone in males, although it is testosterone which is the precursor hormone and the most commonly measured. Levels of circulating sex steroids often increase with higher growth rates and food consumption as energy reserves become replete (Ardnt, 2000), and are generally detectable up to 3-4 months prior to spawning (Taranger *et al.*, 1998; Oppedal *et al.*, 1999). Thus, this period between the increase in steroid hormone levels and spawning may provide a greater accuracy in predicting early maturing fish

1.6. Stress and its Effects on Growth

Stress is an energy-demanding process resulting in the mobilization of energy substrates that allow the fish to cope metabolically in response to the presence of a stressor. Increases in plasma cortisol concentrations are known to increase plasma glucose levels through a series of catabolic glycolytic and gluconeogenic effects that act as an important

energy source for the change in the energy demand of the fish (Schreck, 1982; Barton and Iwama, 1991; Pickering, 1993; Wedemeyer, 1996; Mommsen *et al.*, 1999). In addition to activating the HPI axis, the long-term physiology of the fish may also be affected in terms of suppressed immune function (Pickering and Pottinger, 1987; Harris and Bird, 2000), growth (McCormick *et al.*, 1998; Van Weerd and Komen, 1998; Gregory and Wood, 1999; Weil *et al.*, 2001) and reproduction (Schreck *et al.*, 2001). One of the first behavioural responses of fish to any form of stress is a cessation in feeding activity (Pickering, 1993; Wendelaar Bonga, 1997; Gregory and Wood, 1999).

Plasma cortisol levels are commonly measured as an indicator of stress in fish (Pickering, 1993). Typically, the stress response of fish is characterized by the release of the catecholamines, adrenaline and noradrenaline, from chromaffin tissue and corticosteroid hormones, primarily cortisol, from the interrenal tissue (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002). Cortisol release is mediated through the activation of the hypothalamic-pituitary-interrenal (HPI) axis, beginning when corticotrophin-releasing factor (CRF) is produced by the hypothalamus in turn releasing pituitary adrenocorticotrophic hormone (ACTH) and ending with cortisol synthesis by the interrenal cells of the head kidney. The duration and elevation of plasma cortisol levels are dependent upon the severity and length of the stressful event, either returning to basal levels within a couple of hours following an acute stress or remaining elevated for prolonged or repeated stressors. However, it is thought that fish can acclimate to some persisting stress events following an initial elevated stress response (Pickering and Pottinger, 1985, 1987; Pottinger and Pickering, 1992), with the rate of return to baseline levels suggested as a more important determinant of fish performance than the magnitude of the response (Weil *et al.*, 2001). Thus, one physiological approach to understanding fish growth requires an understanding of how environmental factors influence these endocrine mechanisms that promote growth and appetite (Beckman *et al.*, 2001).

1.7. Environmental Influences on Growth and Maturation

Physiological and behavioural growth patterns in animals tend to be modulated by their interactions with the environment through a range of biotic and abiotic factors. Fish, like any other animal, exhibit regularly repeated behaviours on a daily (*e.g.* feeding) and annual basis (*e.g.* spawning). These rhythmic behaviours are typically controlled by a strong endogenous rhythm, or biological clock, requiring an exogenous cue to act as a *zeitgeber* (*time giver*) in synchronizing the cycles. The environment therefore provides several proximate cues which may be used to coordinate life events. Photoperiod, temperature, rainfall, food availability and pheromones all have a role in cueing life-events in the majority of fish (Bromage *et al.*, 2001). However, for fish residing at higher latitudes, such as salmonids, it is the seasonally changing photoperiod that is the most likely responsible factor for cueing such events (Boeuf and Le Bail, 1999; Boeuf and Falcón, 2001; Bromage *et al.*, 2001).

1.7.1. Light

Light is characterized by its quantity (intensity), quality (spectral content) and duration (Sumpter, 1992; Boeuf and Le Bail, 1999). However, in order for light to affect the physiological functions of fish, light and/or dark must first be perceived. Fish like other vertebrates are sensitive to light and both the retina of the lateral eyes and the pineal gland receive and transduce this information into hormonal and neural signals. The teleost pineal gland (epiphysis) is a photoreceptive organ, located dorsal to the forebrain and is situated directly beneath or within the cranial roof (Ekström and Meissl, 1997). In Atlantic salmon, transparent tissue directly overlies the pineal and is commonly referred to as the “pineal window”. In response to light stimuli the pineal gland releases variable quantities of regulatory chemicals, primarily the indoleamine hormone melatonin, into the bloodstream as a function of daylength. Both *in vitro* (Yáñez and Meissl, 1996; Migaud *et al.*, 2006a) and *in vivo* (Randall *et al.*, 1995; Porter *et al.*, 1998, 2001; Bayarri *et al.*, 2002; Migaud *et al.*, 2006a) studies have demonstrated that melatonin synthesis varies inversely with the irradiance of the incident light. As melatonin levels accurately

reflect the prevailing light conditions, exhibiting low melatonin levels during the day and high at night (Randall *et al.*, 1995; Porter *et al.*, 1996, 1998, 2001), it has therefore been suggested that melatonin provides the fish with accurate information on the daily and calendar time to synchronize daily and seasonal events (Randall *et al.*, 1995; Boeuf and Le Bail, 1999; Boeuf and Falcón, 2001; Bromage *et al.*, 2001).

However, whereas light regimes have been applied successfully in tank conditions to manipulate physiological processes (*e.g.* Bromage *et al.*, 1992; Oppedal *et al.*, 2003; Nordgarden *et al.*, 2003) the superimposition of artificial light on the natural light, as occurs during the on-growing stages, poses greater problems. The quality and quantity of solar energy penetrating the water is altered in much the same way as in the atmosphere, through both absorption and scattering processes, although numerous predictable and unpredictable factors such as weather conditions, sea surface state, solar angle, time of day or season, planktonic blooms and land runoff will also exert an influence (Figure 1.5.). Water absorbs maximally in the far red (λ 700-800 nm) and infra-red (λ 750 nm to 1 mm) wavelengths, and as such these are rapidly absorbed and converted into heat energy. Blue light (λ 450 nm), on the other hand, has a higher energy content and is able to penetrate deeper through the water column reaching depths of up to 150 m in the clearest waters (Lobban and Harrison, 1994). Thus, understanding such concepts of light alteration is vital in terms of underwater light design, with respect to the spectral quality and intensity of the units, although knowledge of their effects on the physiological functions of fish is critical if such systems are to be used effectively.

Spectral and intensity effects

To date the effect of spectral quality and light intensity on growth remains largely unstudied. The most notable work on spectral composition stems from the work of Stefansson and Hansen (1989) who examined the effects of the colour temperature of lights on Atlantic salmon parr. However, in that investigation neither growth nor the parr-smolt transformation were affected by the various colour light treatments. Although this study used colour temperature rather than the spectral content of the light source, it

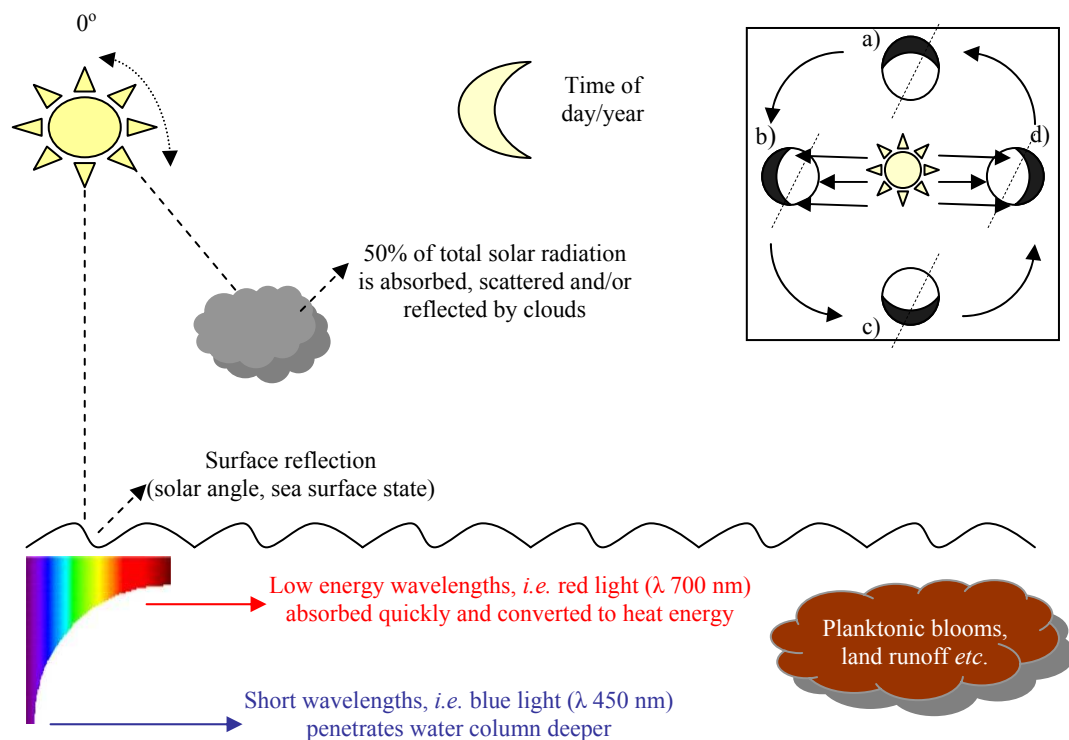


Figure 1.5. Schematic illustration of the factors affecting the passage of solar radiation through the atmosphere and sea surface. Inset: seasonal and latitudinal differences in solar radiation. Vernal and autumnal equinoxes (a and c respectively), equator faces the sun directly all regions experience the same daylength. Summer and winter solstices (b and d respectively), northern hemisphere tilts either towards or away from the sun providing increased or decreased daylengths. Note that irradiance levels are always highest at the equator, decreasing towards the poles to give the seasonal light variation.

still provides the most comparative study on the spectral quality of light on the fish physiology thus far. More recent studies however, have focussed on testing the spectral sensitivity of the pineal gland. Longer wavelengths of light (*i.e.* red λ 700 nm), at sufficient intensities, have been shown to suppress circulating melatonin levels (Bayarri *et al.*, 2002). However, it is the short wavelengths (*i.e.* blue light λ 450 nm) which have been found to be the most effective (Bayarri *et al.*, 2002; Migaud *et al.*, 2006a).

The available literature on light intensity effects on fish physiology is more plentiful although somewhat contradictory. Stefansson *et al.* (1993) and Oppedal *et al.*

(1999) for example, both noticed that whilst constant light regimes increased growth, compared to conspecifics reared under an ambient photoperiod, no overall differences were observed between the various light intensity groups. Contrastingly, Oppedal *et al.* (1997) found that higher intensities of light were most effective at increasing the growth rate and mean live body weights of salmon post-smolts, together with inhibiting the proportion of early maturing fish. These findings have led researchers to suggest that a threshold value of light intensity must exist in order to influence physiological functions in fish (Oppedal *et al.*, 1997; Porter *et al.*, 1999, 2001; Migaud *et al.*, 2006a). Recently, Migaud *et al.* (2006a) have calculated the light intensity threshold for Atlantic salmon to be around 0.016 W.m^{-2} , after allowing for the 2.4% of light lost during transmission through the cranium. This and the findings on spectral content therefore indicate the specific colour type and minimum intensity level that should be attained, when applying photoperiod regimes to production systems, in order for the additional illumination to be perceived to ensure the greatest possibility of eliciting physiological responses.

Photoperiod

The seasonally changing photoperiod has long been recognised to affect physiological developmental processes such as maturation and smoltification in fish. Under natural conditions salmonid growth appears to follow a seasonal pattern thought to be strongly influenced by changes in photoperiod (Higgins and Talbot, 1985; Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999; Oppedal *et al.*, 1999; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003). This change in daylength is thought to synchronize the endogenous clock to ensure that developmental events occur in time with favourable seasons. Salmonid reproduction is characteristically an annual event believed to be cued by photoperiodic signals (Bromage *et al.*, 2001). As previously stated, early maturation is problematic to the industry interfering with production schedules and leading to the downgrading of fish at the processing plant. Changes in photoperiod from short to long have been proposed to arrest sexual maturation by advancing the endogenous circannual rhythm (Duston and Bromage, 1988; Taranger *et al.*, 1999); thereby bringing forward the

decision period when only fish with the necessary energy reserves can initiate maturation. Thus, artificial light regimes have been used predominantly throughout the industry as a means of reducing the incidence of grilising. However, the expected reduction in the proportion of fish maturing (*e.g.* Hansen *et al.*, 1992; Porter *et al.*, 1999; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000) is not always found (*e.g.* Kråkenes *et al.*, 1991; Endal *et al.*, 2000; Johnston *et al.*, 2003). Björnsson *et al.* (1994) found that maturation could be advanced as well as delayed when exposed to constant light. This would suggest that both the timing, duration of exposure and direction of light change are important factors influencing maturational status. To demonstrate this effect, Endal *et al.* (2000) applied constant light regimes at various time intervals over the winter period. They found that exposure to constant light resulted in a higher growth performance as compared to fish reared under natural conditions, but also that the earlier the exposure the higher the proportion of sexually mature fish

In addition to inhibiting maturation, constant light regimes have been shown to enhance growth during the winter and spring period when growth depressions are normally observed (*e.g.* Forsberg, 1995; Porter *et al.*, 1999; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000), although growth deviations between control and lit groups appear only to be apparent following a minimum of 12-weeks light exposure (Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Taylor *et al.*, 2005). This has led to suggestions that constant light exposure applied over the winter period phase-shifts the seasonal growth pattern (Forsberg, 1995; Oppedal *et al.*, 1999; Endal *et al.*, 2000; Nordgarden *et al.*, 2003), although others indicate a possible direct photostimulation of light on growth (Komourdjian *et al.*, 1976). Generally, increased daylength accelerates growth in juvenile salmon in preparation for the parr-smolt transformation (Villarreal *et al.*, 1998). The production of out-of-season smolts through the application of photoperiod has increased steadily in recent years as a commercially important strategy for spreading the availability of market-sized salmon. Naturally produced smolts (1+) are commonly transferred to sea during early spring under an increasing photoperiod. However, underyearling (0+) salmon smolts can be transferred during late autumn under a

decreasing photoperiod, 7-8 months earlier than their natural counterparts (Duston and Saunders, 1995; Oppedal *et al.*, 1999). This is achieved by interrupting a continuous light regime with a short-day photoperiod when the parr display their smolt characteristics. This length of short-day exposure ultimately influences the survival, growth and smolting process as determined by gill Na⁺ K⁺-ATPase activity (Duston and Saunders, 1995; Duncan and Bromage, 1998). The prime concern arising from the production of out-of-season smolts is their performance in seawater. Nevertheless, despite displaying different patterns of growth, the overall growth performance and product quality of out-of-season smolts parallels that of regular spring smolts (Duncan *et al.*, 1998; Mørkøre and Rørvik, 2001).

Although there are clear benefits to using artificial light regimes, there still remains a high degree of scepticism within the industry over the benefits of lights on growth and maturation. One current source of controversy within the Scottish salmon industry relates to a growth depression, thought to be brought about by a reduction in appetite, which is commonly observed following the onset of lights (Figure 1.6.). This 'growth-dip' phenomenon has also been reported in several scientific studies for both salmonid (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005) and non-salmonid species (Simensen *et al.*, 2000) where constant light regimes have been applied. Endal *et al.*, (2000) suggest that these transient growth depressions are brought about by either an initial stress response to the change in rearing regime or by a phase advancement of a circannual growth pattern adjusted by photoperiod, although to date there is no clear understanding of this phenomenon. Thus, it is recommended that feeding practices should reflect changes in appetite related to the use of lights (Sinnott, 2002). However, the majority of the studies on feeding responses have predominantly focussed around the influence of temperature, as both the effects of photoperiod and temperature can often be confused due to the natural seasonal variation in temperature following similar cycles to photoperiod (Jobling, 1994).

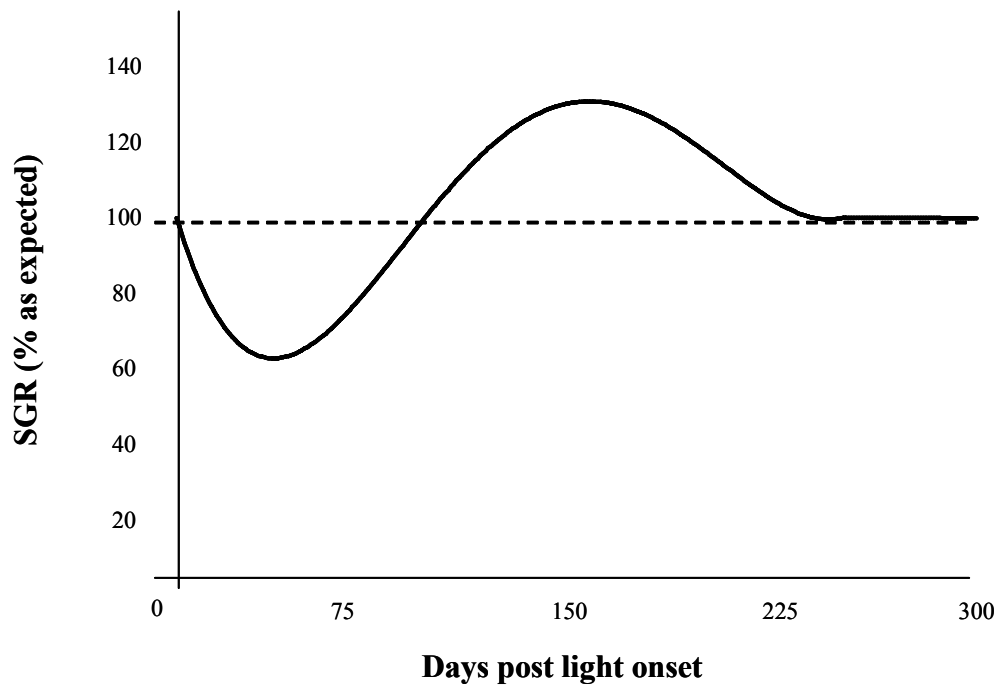


Figure 1.6. Graphical illustration of the highly contested growth-dip phenomenon based on farmers observations. Following the application of constant light, growth is reported to fall before undergoing compensatory growth responses and a return to expected levels (Courtesy of Dr. Clive Talbot; Marine Harvest, Stavanger, Norway).

1.7.2. Temperature

Fish are ectotherms and as such the ambient temperature has a pervasive effect on the rate of growth and food consumption. Increasing temperatures are known to increase feed intake and growth in a variety of species (Staples and Nomura, 1976; Grove *et al.*, 1978; Jonassen *et al.*, 2000), phase-shifting the maintenance ration required due to changes in metabolic expenditure (Wurtsbaugh and Davis, 1977). However, the growth-temperature relationship is characteristically bell-shaped with growth maximised at an optimal temperature for the species (Jobling, 1994). Above the thermal tolerance of the fish the growth rate is severely affected, regardless of the nutrition or availability of feed. The thermal growth coefficient (TGC) is another growth calculation used throughout the industry to model growth using past production records and current water temperatures and may also be used to make cross-site comparisons of growth performance where

temperature profiles differ. The TGC takes into account factors which the SGR fails to consider, incorporating changes in fish size and directly relates them to the sum of the daily water temperature over a known period of time (Cho, 1992a). Thus, fish may have a lower food intake and growth rate at lower temperatures, but when the TGC is compared to fish at higher temperatures their growth may be more favourable (Bendiksen *et al.*, 2002). However, the TGC itself is prone to error particularly when applied during conditions of high temperatures (Jobling, 2003).

Apart from growth, temperature also has a modulating role in other life processes. Although, photoperiod is known to affect the parr-smolt transformation (*e.g.* Duncan and Bromage, 1998; Oppedal *et al.*, 1999) and applied to produce out-of-season smolts (*e.g.* Duston and Saunder, 1995; Duncan *et al.*, 1999; Oppedal *et al.*, 1999), it has been shown that a thermal sum of approximately 400° days are necessary to help modify the rate of the response (Sigholt *et al.*, 1998). Similarly, advanced or delayed spawning times brought about by photoperiod manipulations are also known to be affected by temperature, with spawning either delayed until ideal temperatures are met otherwise egg fecundity may be severely affected (Davies and Bromage, 2002). However, whilst the control of temperature can be performed in indoor tank enclosures, photoperiod still provides the easiest alternative for improving production rates in outdoor systems.

1.7.3. Nutrition

As stated previously, both the nutritional quality and quantity of feed presented to fish is the ultimate factor in determining growth and developmental processes in fish. Commercial diets are generally comprised of protein, oil, carbohydrates, pigments, vitamins and minerals. The overall inclusion rates of feed ingredients are important for a balanced diet and essential for the health and subsequent growth performance in fish. Protein is an expensive component of fish feed, representing a source of essential amino acids and energy mostly utilized in salmonid fish to produce lean muscle growth. Like proteins, lipids also act as a source of energy storage and are mainly stored around the internal organs and muscle blocks (myosepta) in salmonids. As an alternative cheaper

component of commercial feeds increased lipid levels have been used to replace protein and, thus, allow protein sparing for use in muscle growth. However, diets with a high lipid inclusion rate have been shown to alter the proportion of sexually maturing fish by exceeding the threshold energy store levels required to initiate maturation (Shearer and Swanson, 2000). Thus, dietary lipid levels should replace protein concentration to a point where an optimal dietary balance and protein and energy is met, thereby yielding maximal growth and feed efficiency (Azevedo *et al.*, 2002).

The physical properties of the feed are also important in determining the growth performance of fish. Feed manufacturers produce feeding guidelines based on the type of diet and pellet size for a range of species of varying body weights (Austreng *et al.*, 1987; Cho, 1992a). However, it is not only the palatability of the diet but also the pellet contrast for detection under various light conditions (Pettrell and Ang, 2001) and the particle size (Wankowski and Thorpe, 1979) which are factors that can determine the feed consumption and, thereby, the growth rate of fish. Thus, every aspect of fish physiology and its environment should be taken into consideration both during the manufacturing of feeds and at the farming level.

1.8. Summary

Growth is a complex process involving the consideration of a multitude of abiotic (*e.g.* light, temperature, nutritional) and biotic (*e.g.* age, strain, size, social interaction) factors that interact to affect feeding, growth and the subsequent developmental strategies in salmonid fish. Moreover, these processes are mediated through a cascade of hormones operating through the brain-pituitary axis which appear to respond changes in the environment. As such, information about the environmental influences on feeding-growth-reproduction interactions are extremely important for almost every aspect of aquaculture. For salmonids, photoperiod manipulations have proven to be a useful tool in the management of production schedules. However, the use of photoperiod within the industry is still in its infancy and more knowledge on the effects of light characteristics

(spectrum, intensity and photoperiod) on feeding, growth, reproduction and their underlying mechanisms are needed.

1.9. Experimental Aim

Presently, the Scottish salmon farming industry remains indecisive over the benefits of light application within the on-growing stage of production, particularly with respect to its influence on growth and maturation. Moreover, reports of a characteristic feed and growth-dip have been purported to occur in 1+ Atlantic salmon post-smolts following the addition of continuous artificial light.

Thus, the objectives of this thesis were therefore to:

- Examine the effects of light characteristics (*i.e.* intensity, spectral quality and photoperiod) on the feeding, growth and maturational responses of farmed salmonids reared in experimental and commercial conditions.
- Investigate the underlying mechanisms through the assessment of endocrine changes that occur in response to light manipulation
- Elucidate the growth-dip phenomenon through the monitoring of feeding and growth responses following exposure to artificial light.

Chapter 2: General Materials and Methods

2.1. Fish Husbandry

Experimentation performed in all trials described within this thesis was carried out in accordance with the British Home Office Animals (Scientific Procedures) Act 1986 and, where applicable, with the standard operating procedures for husbandry practices within the commercial salmonid farming industry.

2.1.1. Experimental Animals

The study utilised both freshwater and marine species of the salmonid family. Rainbow trout (*Oncorhynchus mykiss*) from an all-female domesticated strain (Glen Wylln Trout Hatchery Ltd.; Isle of Man) were used in all freshwater experiments (Chapter 3). A commercial strain of 1+ Atlantic salmon (*Salmo salar*) post-smolts from a mixed-sex population of a medium grilising strain (LM20, Marine Harvest; Scotland) were used in the research and commercial trials performed in seawater (Chapters 4 and 5). Where possible, fish from the same source, age and size classes were used in related experiments and are further detailed in the methods section of each experiment.

2.1.2. Experimental Facilities

Since trials were carried out under research and commercial conditions fish were maintained in a variety of rearing environments appropriate to the experimental protocols. The rainbow trout freshwater experiments described in Chapter 3 were performed at the University of Stirling's Niall Bromage Freshwater Research Facility (Stirlingshire, Scotland; 56.02°N; 4.00°W). The tank-based salmon study documented in Chapter 4 was carried out at Marine Harvest's (Scotland) Lochailort Research Unit (Inverness-shire, Scotland; 56.52°N; 5.39°W). The commercial sea-cage trial described in Chapter 5 was conducted at Loch Leven salmon farm (Inverness-shire, Scotland; 56.41°N, 5.10°W) of Marine Harvest (Scotland). Further details regarding the rearing

systems, set-up and feeding regimes are detailed within the individual materials and methods section of the relevant experimental chapter.

2.2. Sampling Procedures

2.2.1. Anaesthesia

To minimise potential stress effects and injury, experimental animals were anaesthetised prior to any handling or experimental procedures. Rainbow trout (Chapter 3) were anaesthetised in a bath of 1:10,000 concentration of 2-phenoxyethanol (Sigma; Poole, UK) in fresh water. Atlantic salmon (Chapters 4 and 5) were anaesthetised in a 50 mg.l⁻¹ benzocaine solution (Sigma; Poole, UK). In all instances, loss of consciousness was generally induced within a 3 min period and fish recovered in fresh aerated water. Post-sampling mortalities were typically less than 0.1 %.

2.2.2. Fish Euthanasia

Where protocols involved the removal of tissue and/or organ samples for examination, experimental animals were sacrificed in accordance with the Home Office regulations for a Schedule 1 kill and outlined by Close *et al.* (1997). Experimental animals were placed in a lethal concentration of anaesthetic and following loss of consciousness, killed with a single blow to the dorsal surface of the head such that death was instantaneous.

2.2.3. Blood Sampling

Where blood samples were required, blood was withdrawn from the caudal vein of anaesthetised or sacrificed fish using 1 or 2 ml sterile syringes (Terumo N.V.; Leuven, Belgium) fitted either with a 23 or 21 gauge needle (Terumo N.V.; Leuven, Belgium) for fish under or over 250 g respectively. Syringes were rinsed with a 4 mg.ml⁻¹ solution of ammonium heparin salt (Sigma; Poole, UK) to prevent coagulation of blood. The blood was transferred to 1.5 ml eppendorfs and spun at 1200 g at °C for 15 min. The resulting plasma was aliquoted into 0.5 ml micro-eppendorfs and stored at -70°C until analysis.

2.2.4. Fish Identification

Where individuals from a population were continuously monitored throughout the course of an experiment (Chapter 3), passive integrated transponder (PIT) tags (Avid; Norco, USA) were employed. Tags were scanned with a tag reader (Avid; Norco, USA), ensuring they were functioning correctly, and inserted into the dorsal musculature of anaesthetised fish by making a 5 mm incision at the anterior of the dorsal fin and injecting the tag into the epaxial muscle so that the tag lay perpendicular to and just below the mid-rays of the dorsal fin. A 3:1 mix of Orahesive powder (Squibb & Sons; Hounslow, UK) and Cicatrin antibiotic (The Wellcome Foundation Ltd.; Middlesex, UK) was applied over the incision. To further aid with the identification of tagged individuals within sub-populations of untagged fish, the adipose fin was removed during the tagging process. Mortalities resulting from this procedure were generally less than 0.1 %.

2.3. Growth and Feed Intake Assessment

Length-weights of individual fish were recorded at the beginning of each trial and at regular intervals thereafter. In all cases, individual wet weights (± 0.1 g) were recorded using an electronic balance (Model QC7DCE-S, Sartorius AG; Göttingen, Germany) and fork lengths (± 1 mm) using a customised measuring board.

2.3.1. Specific Growth Rate

The specific growth rate (SGR) was calculated based on changes in weight (SGR_W) or length (SGR_L) over a known period of time:

$$SGR = (e^{g-1}) \times 100$$

where, g is the instantaneous growth rate:

$$g = (\ln X_2 - \ln X_1) / (t_2 - t_1)$$

and where, X_2 and X_1 are the weights or lengths of individuals at times t_2 and t_1 respectively.

2.3.2. Condition Factor

Condition factor (K) is often used as an index of the relationship between weight and length and as an indicator of a fish's energy reserves (Goede and Barton, 1990), which may reflect the level of feeding. Fish condition was calculated using Fulton's coefficient:

$$K = (W / L^3) \times 100$$

where, W is the wet weight (g) of the fish and L the fork length (cm).

2.3.3. Feed Intake

X-Radiography

The X-radiography technique is a non-invasive procedure developed by Talbot and Higgins (1983), whereby an inert radio-opaque marker is incorporated into a diet as a quantitative measure for estimating voluntary feed intake in fish.

A standard commercial diet, appropriate to the species and fish size, was ground to a fine dust and labelled with size 8 (0.4-0.52 mm diameter) lead glass ballotini beads (Jencons Scientific Ltd.; Leighton Buzzard, UK). The inclusion rate was calculated using in-house software kindly provided by Dr. Clive Talbot, which accounts for the expected feed intake together with fish size and pellet dimensions. The labelled diet was homogenised and repelleted using a California Laboratory Pellet Mill (California Pellet Mill Co.; San Francisco, USA). Radiography was performed using a G.E.C. MX2 series 7 portable X-ray unit (G.E.C. Ltd.; Wembley, UK), set up to expose X-ray plates for 3 seconds at 75 kV and 20 mA. Calibration curves were prepared (Figure 2.1.) by weighing known quantities (± 0.01 g) of labelled feed, in triplicate, X-raying and counting the amount of ballotini per unit weight of food.

Anaesthetised fish were placed directly on 35 x 43 cm Agfa DW ETE X-ray film (Agfa NDT Ltd.; Coventry, UK) and X-rayed. All X-ray films were developed under red light filter in a dark room using Agfa Industrex Manual Fixer and Agfa Industrex Manual Developer solutions (Agfa NDT Ltd.; Coventry, UK) and left to dry in hot air drying cabinets. The developed plates were viewed using a light box and the number of ballotini glass beads present in the gastrointestinal tract counted using a hand held counter (Figure 2.1.). The quantity of food consumed was determined using the linear regression equation of the relationship between the weight of the labelled feed and the number of ballotini. For each fish individual food consumption was expressed on a weight-specific basis (mg.g fish^{-1}) as outlined below.

Stomach Content Analysis

Where fish were sacrificed for experimental analysis, food consumption was measured from the gastrointestinal contents of individual fish.

An abdominal incision was made from the gills to the anal opening and the alimentary tract excised and sectioned according to the method of Austreng (1978). Gut contents were removed using the back of a knife, collected into tared foil trays and placed into a drying oven (Gallenkamp; Loughborough, UK) at 105°C for 20 h. The dried samples were weighed ($\pm 0.01\text{g}$) and the individual food consumption calculated, based on a weight-specific basis, as outlined below.

Calculation

For each individual fish, whether X-rayed or sacrificed, feed intake (FI) was expressed on a weight-specific basis in terms of mg dry food (g) per g of weight wet (mg.g fish^{-1}) using the following calculation:

$$\text{FI (mg.g fish}^{-1}\text{)} = (c \times 1000) / W$$

where, c is the amount of food consumed (g) and W body weight (g).

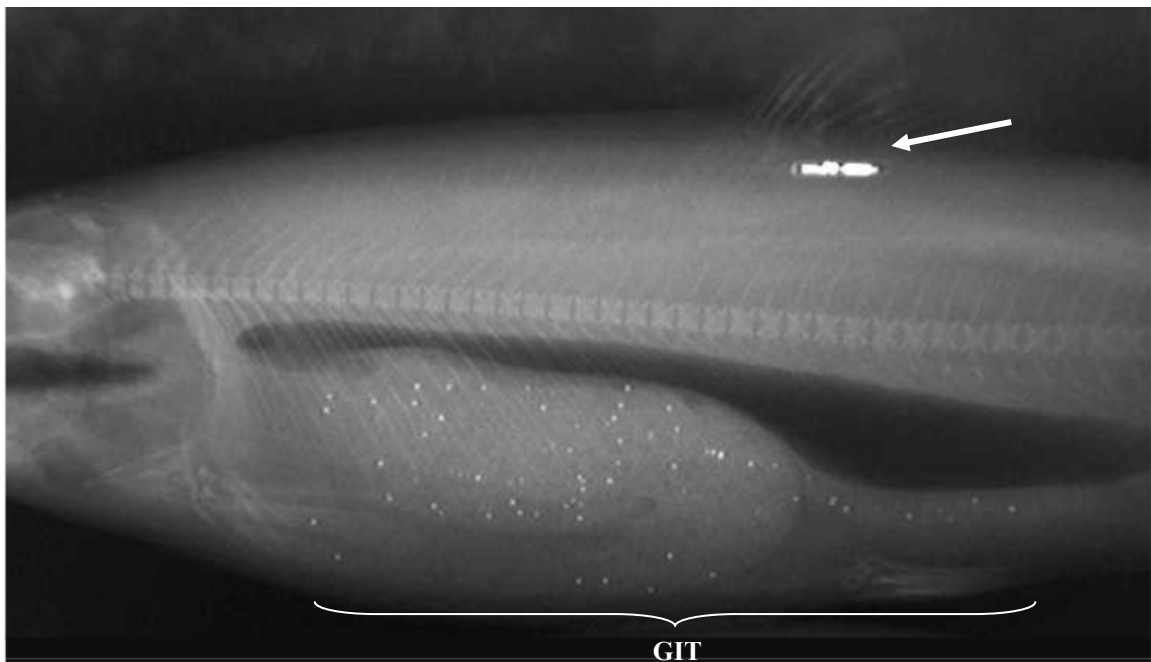
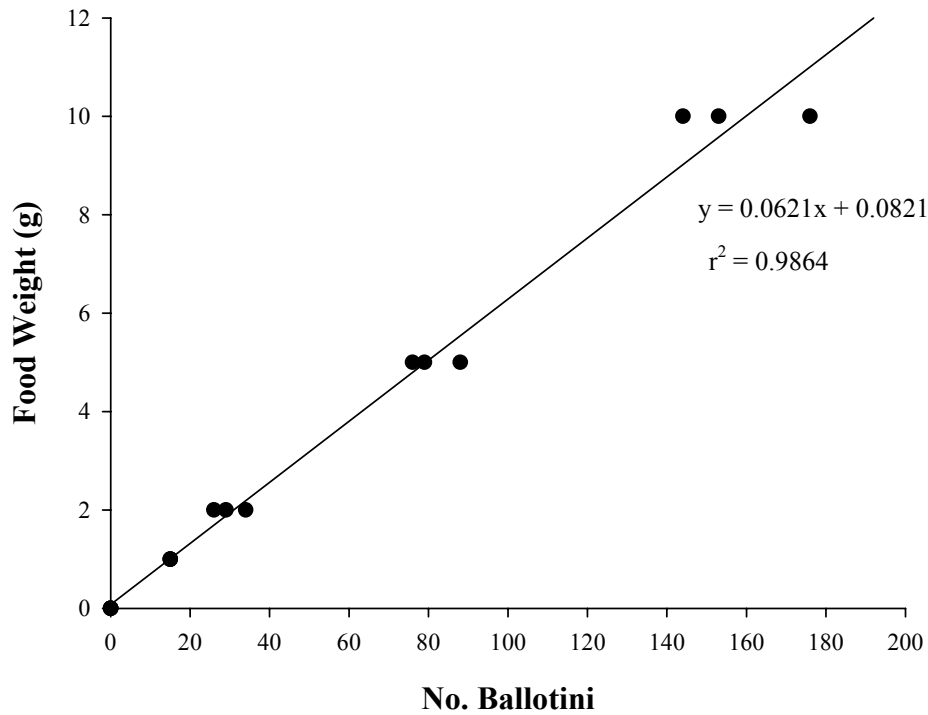


Figure 2.1. Top: Calibration curve for ballotini labelled diet. Food consumption was calculated using the linear regression of the relationship between the weight of the labelled feed and number of ballotini. Bottom: Typical radiograph of a rainbow trout fed labelled diet containing radio-opaque ballotini glass beads in the gastrointestinal tract (GIT). Note the presence of a PIT tag directly beneath the dorsal fin (indicated by arrow).

2.4. Growth Hormone Analysis

2.4.1. Growth Hormone

Plasma growth hormone (GH) levels were determined using a double antibody homologous radioimmunoassay described by Le Bail *et al.* (1991). Hormone analysis was performed at INRA Scribe Research Station (Rennes, France) with the assistance of Dr. Jean-Charles Gabillard.

Stock solution

The following constituents were dissolved in 1000 ml of nanopure water in a volumetric flask with the aid of a magnetic stirrer to give a 50 mM Tris stock solution:

Tris-(hydroxymethyl)aminomethane	6 g
Magnesium chloride	2 g
Sodium azide	0.5 g

Once dissolved the solution was adjusted with HCl to give a final pH of 7.5 and could be stored for up to 1 month at 4°C. All chemicals were of Analar grade and supplied by Sigma or BDH Chemicals Ltd.

Assay buffer

The following constituents were dissolved in 100 ml of 50 mM Tris stock solution in a volumetric flask with the aid of a metallic stirrer:

BSA	1 g
Triton [®] X-100	100 µl

The pipette tip was truncated to allow the Triton[®] X-100 to fully dissolve in solution.

Antibody

Primary antibody

The primary antibody was prepared by reconstituting 5 µl of lyophilised rabbit anti-growth hormone antiserum (GroPep Ltd.; Adelaide, Australia) with 500 µl of 50 mM Tris stock solution (1:100 dilution) before storing in 25 µl aliquots at -20°C. A working solution was freshly prepared by further diluting 25 µl of frozen aliquot with 50 ml of assay buffer to achieve a final dilution of 1:200,000, sufficient for assaying 240 samples in duplicate.

Secondary antibody

The secondary antibody was freshly prepared by adding the following constituents to 40 ml of 50 mM Tris stock solution in a volumetric flask with the aid of a metallic stirrer:

Polyethyleneglycol 6000	3.3 g
Sheep serum anti-rabbit	6600 µl
Normal rabbit serum	137.5 µl

The volume was made up to 50 ml with assay buffer and remained stirring when dispensed.

Radiolabel

The [¹²⁵I]-labelled GH was prepared using the Chloramine-T method as described by Le Bail *et al.* (1991), using lyophilised receptor grade salmon/trout GH (GroPep Ltd.; Adelaide, Australia) and Na¹²⁵I (Amersham Biosciences; Buckinghamshire, UK) with a specific activity of 370 MBq (10 mCi). The radiolabelled GH was stored in 50% glycerol at -20°C and a working solution freshly prepared by diluting the label in assay buffer to give an activity of approximately 20,000 cpm/100 µl.

Growth hormone standard

Growth hormone standard (GroPep Ltd.; Adelaide, Australia) was stored at a solution of 100 ng / 40µl at -20°C. A working solution was freshly prepared by adding 960 µl of assay buffer to the stock standard to give 100 ng.ml⁻¹ used for the serial dilutions.

Assay protocol

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

1. Serial dilutions of the growth hormone standard were prepared with assay buffer in borosilicate glass assay tubes (Fisher Scientific; Leicestershire, UK) to give concentrations ranging from 2.6-25 ng.ml⁻¹. A further 2 tubes containing 200 µl of assay buffer were included as the non-specific binding tubes (NSBs) and 2 tubes containing 100 µl of assay buffer for the zero standard tubes (B₀)
2. 50 µl of sample were added to their respective assay tubes together with 50 µl of assay buffer
3. 100 µl of primary antibody was dispensed to all tubes, except NSBs and Total tubes, covered and left to incubate at room temperature overnight
4. 100 µl of [¹²⁵I]-labelled GH was added to all tubes, covered and left to incubate at room temperature overnight
5. 100 µl of the secondary antibody solution was dispensed into all tubes, except Totals, vortex mixed, covered and left to incubate at room temperature overnight
6. 3 ml of a 20 mM Tris stock solution was added to all tubes, except Totals, and centrifuged at 1500 g for 45 min at 4°C
7. The supernatant from all tubes, except Totals, was removed by inverting the tubes and blot drying
8. Tubes were counted for 1 min in a gamma counter (Packard Cobra II, PerkinElmer; Buckinghamshire, UK)

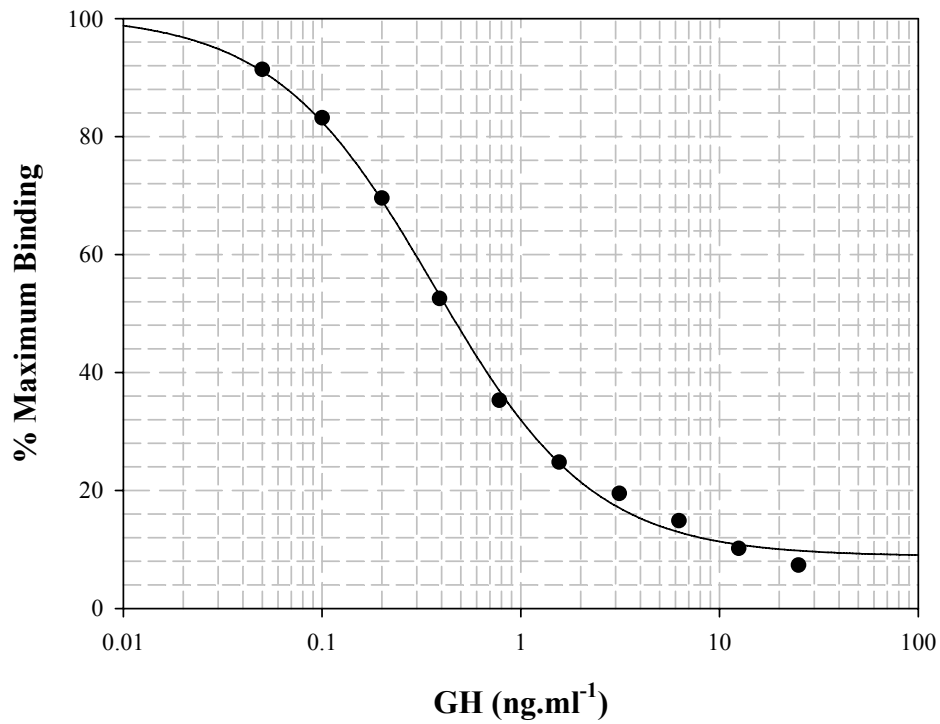


Figure 2.2. Typical standard curve from a growth hormone (GH) radioimmunoassay; the concentration of GH in the sample was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

The amount of GH present in the sample was obtained by intersecting the standard curve at the point corresponding to the percentage binding in the sample (Figure 2.2).

Quality control and validation

The sensitivity of the assay, *i.e.* the minimum amount of GH that is statistically distinguishable from zero, was 0.05 ng.ml^{-1} . Quality controls (QCs) with a GH content of approximately 0.5 ng.ml^{-1} were used to check the reproducibility of the measurements between assays. The intra- and inter-assay coefficient of variation was 7.9% and 16.1% respectively.

2.4.2. Insulin-Like Growth Factor I

Plasma insulin-like growth factor-I (IGF-I) was measured using a double-antibody homologous radioimmunoassay as outlined by Gentil *et al.* (1996). Hormone analysis was performed at INRA Scribe Research Station (Rennes, France) with the assistance of Dr. Jean-Charles Gabillard.

Stock solution and assay buffer

The Tris stock solution and assay buffer used within the IGF-I assay was the same as that described for the growth hormone radioimmunoassay (Section 2.4.1.).

Antibody

Primary antibody

The primary antibody was prepared by reconstituting a vial of lyophilised anti-fish IGF-I (GroPep Ltd.; Adelaide, Australia) with 500 µl of assay buffer (1:50 dilution) and storing at -20°C. A working solution was freshly prepared by further diluting 500 µl of frozen antibody with 42.85 ml of assay buffer to achieve a final dilution of 1:30000, sufficient for assaying 210 samples in duplicate.

Secondary antibody

The secondary antibody used was a purified sheep anti-rabbit IgG (Biogenesis Ltd.; Poole, UK) stored at 4°C. The antibody was mixed with the aid of a metallic stirrer and remained mixing when dispensed.

Radiolabel

The [¹²⁵I]-labelled IGF-I was prepared using the Chloramine-T method as described by Le Bail *et al.* (1991) using lyophilised receptor grade salmon/trout IGF-I (GroPep Ltd.; Adelaide, Australia) and Na¹²⁵I (Amersham Biosciences; Buckinghamshire, UK) with a specific activity of 370 MBq (10 mCi). The radiolabelled IGF-I was stored in 50%

glycerol at -20°C and a working solution freshly prepared by diluting the label in assay buffer to give an activity of approximately 20,000 cpm/100 μl .

IGF standards

Insulin-like growth factor-I fish receptor grade standard (GroPep Ltd.; Adelaide, Australia) was prepared by reconstituting 20 μg in 10 mM HCl to give a final concentration of 0.1 $\mu\text{g}\cdot\mu\text{l}^{-1}$. A 1 $\mu\text{g}\cdot\text{ml}^{-1}$ stock solution was prepared by adding 20 μl of the 0.1 $\mu\text{g}\cdot\mu\text{l}^{-1}$ solution to 1980 μl of assay buffer. A working solution was freshly prepared by adding 20 μl of the stock solution to 1980 μl of assay buffer to give 2 ng. 200 μl^{-1} used for the serial dilutions.

Sample extraction

Prior to analysis it was necessary to extract the IGF from the plasma sample using the following method:

1. 2 ml of 10 mM HCl was added to Centricon[®] YM-30 centrifugal filter units (Millipore; Massachusetts, USA)
2. 25 μl of sample was added to each vial, incubated at room temperature for 30 min and centrifuged at 1000 g for 40 min at 6°C
3. A further 2 ml of HCl was added to each vial and centrifuged at 1000 g for 60 min at 6°C
4. Vials were capped and stored at -70°C until frozen before placing into a lyophilizer (LyoLab A, Froilabo; Meyzieu, France) set at -37°C at 2 millibar until lyophilised
5. Lyophilised samples were reconstituted with 500 μl of assay buffer, vortex mixed and transferred to 1.5 ml eppendorfs

Assay protocol

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

1. Serial dilutions of IGF standard were prepared with assay buffer in borosilicate glass assay tubes (Fisher Scientific; Leicestershire, UK) to give concentrations ranging from 0.025-12.5 ng.ml⁻¹. A further 2 tubes containing 200 µl of assay buffer were included as the non-specific binding tubes (NSBs) and 2 tubes containing 100 µl of assay buffer for the zero standard tubes (B₀)
2. 25 µl of sample and control were added to their respective assay tubes together with 75 µl of assay buffer
3. 100 µl of primary antibody was dispensed to all tubes, except NSBs and Total tubes, covered and left to incubate at room temperature overnight
4. 100 µl of [¹²⁵I]-labelled IGF was added to all tubes, covered and left to incubate at room temperature overnight
5. 100 µl of the secondary antibody solution was dispensed into all tubes, except Totals, vortex mixed, covered and left to incubate at room temperature overnight
6. 3 ml of a 20 mM Tris stock solution was added to all tubes, except Totals, and centrifuged at 1500 g for 45 min at 4°C
7. The supernatant from all tubes, except Totals, was removed by inverting the tubes and blot drying
9. Tubes were counted for 1 min in a gamma counter (Packard Cobra II, PerkinElmer; Buckinghamshire, UK)

The amount of IGF-I present in the sample was obtained by intersecting the standard curve at the point corresponding to the percentage binding in the sample (Figure 2.3.).

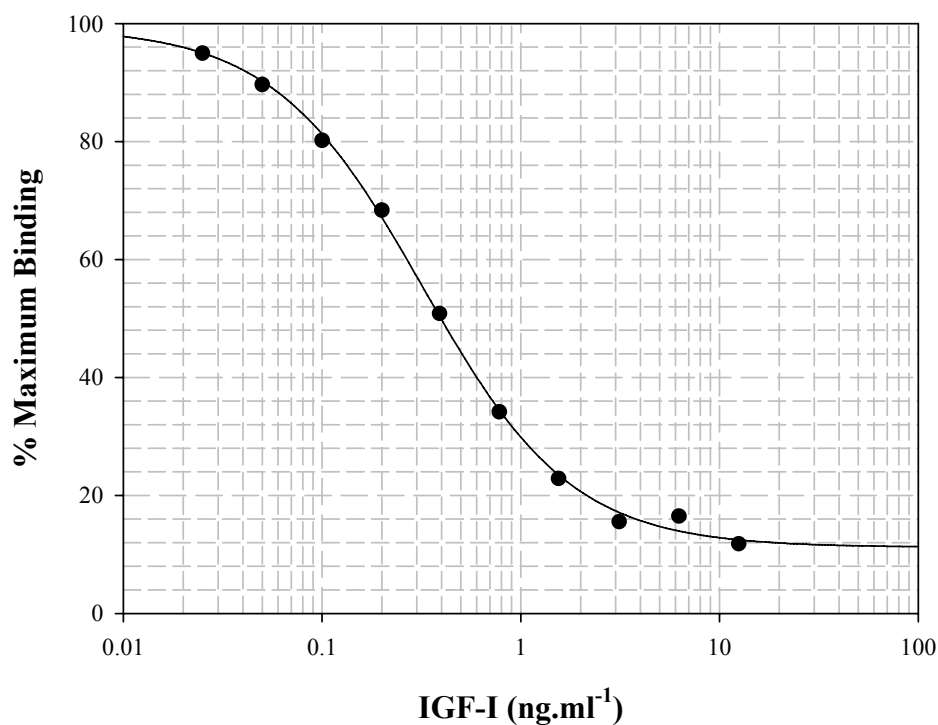


Figure 2.3. Typical standard curve from an insulin-like growth factor-I (IGF-I) radioimmunoassay; the concentration of IGF-I in the sample was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

Quality control and validation

The sensitivity of the assay, *i.e.* the minimum amount of IGF-I that is statistically distinguishable from zero, was 0.05 ng.m⁻¹. Quality controls (QCs) with an IGF-I content of approximately 4.5 ng.ml⁻¹ and 9 ng.ml⁻¹ were used to check the reproducibility of measurements between assays. The intra-assay coefficient of variation was 20.4%.

2.4.3. Thyroid Hormones

Free levels of the thyroid hormones, tri-iodothyronine (FT₃) and thyroxine (FT₄), were analysed by radioimmunoassay using commercially available kits (*AMERLEX-MAB*TM, Trinity Biotech plc.; County Wicklow, Ireland).

Assay reagents

All reagents (*i.e.* standards, labelled antibody and magnetic separation solution) were provided with the kits. Standards were reconstituted with 1 ml of nanopure water to give final concentrations ranging from 2.5 to 130 pmol.l⁻¹ and 2 to 40 pmol.l⁻¹, for FT₄ and FT₃ respectively.

Assay protocol

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

1. 50 µl of standard and sample were added to 3 ml borosilicate glass assay tubes (Fisher Scientific; Leicestershire, UK)
2. 500 µl of *AMERLEX-MAB*TM magnetic suspension solution was dispensed to all tubes except Total tubes
3. 500 µl of ¹²⁵I-labelled antibody was added to all tubes within a 5 min period of the magnetic solution being dispensed
4. Tubes were vortex mixed, covered with foil and left to incubate in a water bath set at 37°C for 30 min, except Total tubes
5. Following incubation, tubes were attached to an *AMERLEX-M*TM magnetic separator rack (Amersham Biosciences; Buckinghamshire, UK) and left to incubate at room temperature for 15 min
6. The supernatant from all tubes, except Total tubes, was removed by inverting the tubes and blot drying for 5 min

7. Tubes were counted for 1 min in a gamma counter (Wallac 1480 Wizard[®] 3” Gamma counter, PerkinElmer; Buckinghamshire, UK)

The amount of hormone present in the sample was obtained by intersecting the standard curve at the point corresponding to the percentage binding in the sample (Figure 2.4.).

Quality control and validation

The sensitivity of the kits, *i.e.* the minimum amount of FT₄ or FT₃ that is statistically distinguishable from zero, was 0.7 and 0.6 pmol.ml⁻¹ respectively. The inter and intra-assay coefficient of variations were 8.2% and 16.4% for FT₄ and 6.8% and 14.1% for FT₃. To ascertain whether the FT₄ and FT₃ in the standards were immunologically similar to that in the plasma sample, serial dilutions of pooled Atlantic salmon plasma were used to produce an inhibition curve (Figure 2.5.). No statistical difference ($P>0.05$) was found between the slopes of the standard curve and inhibition plot regression lines confirming that the FT₄ or FT₃ being measured in the samples were immunologically similar to that in the standards.

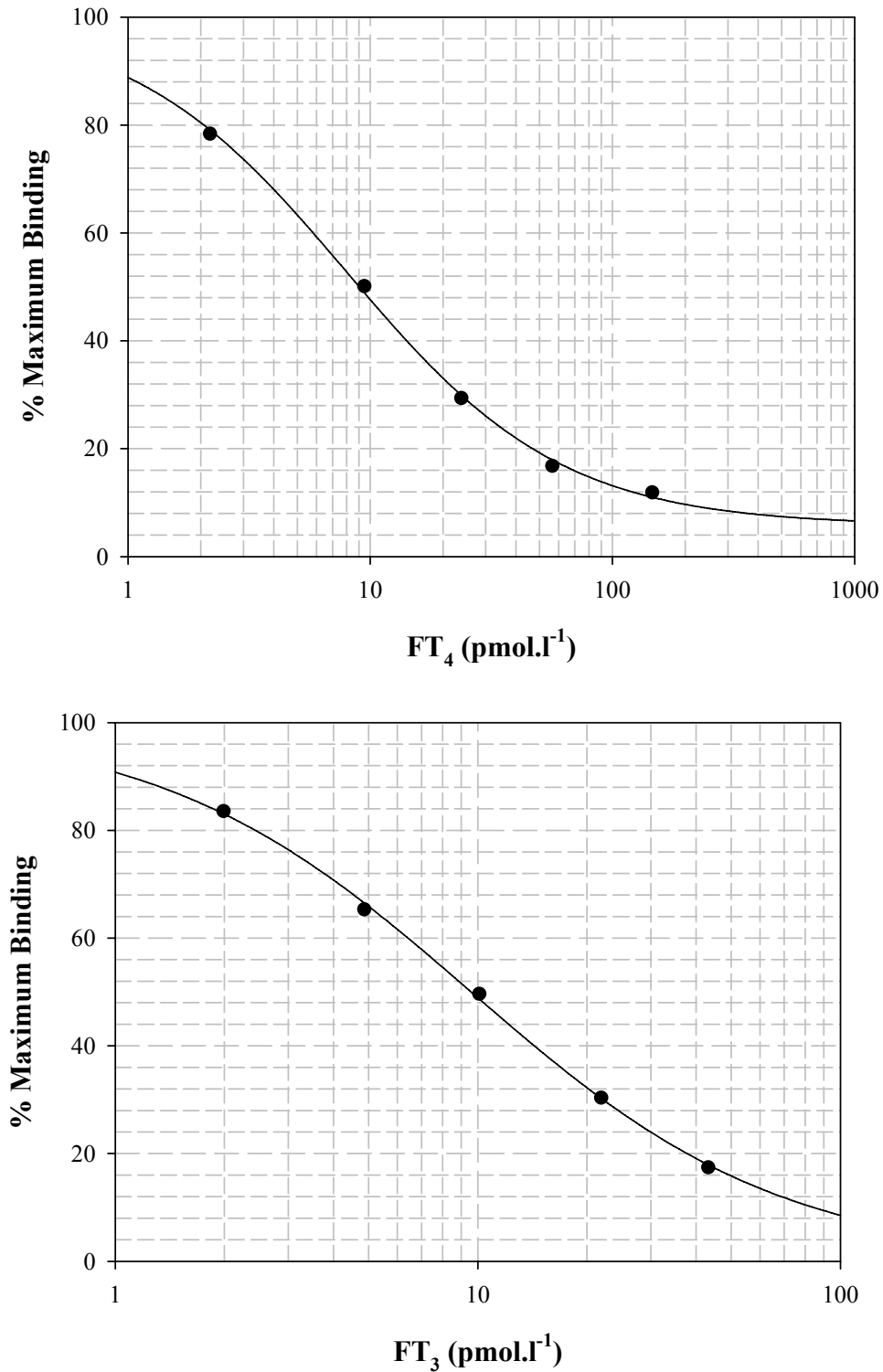


Figure 2.4. Typical standard curve of from a free thyroxine (FT₄; Top) and a free tri-iodothyronine (FT₃; Bottom) radioimmunoassay; the concentration of FT₄ or FT₃ in the sample was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

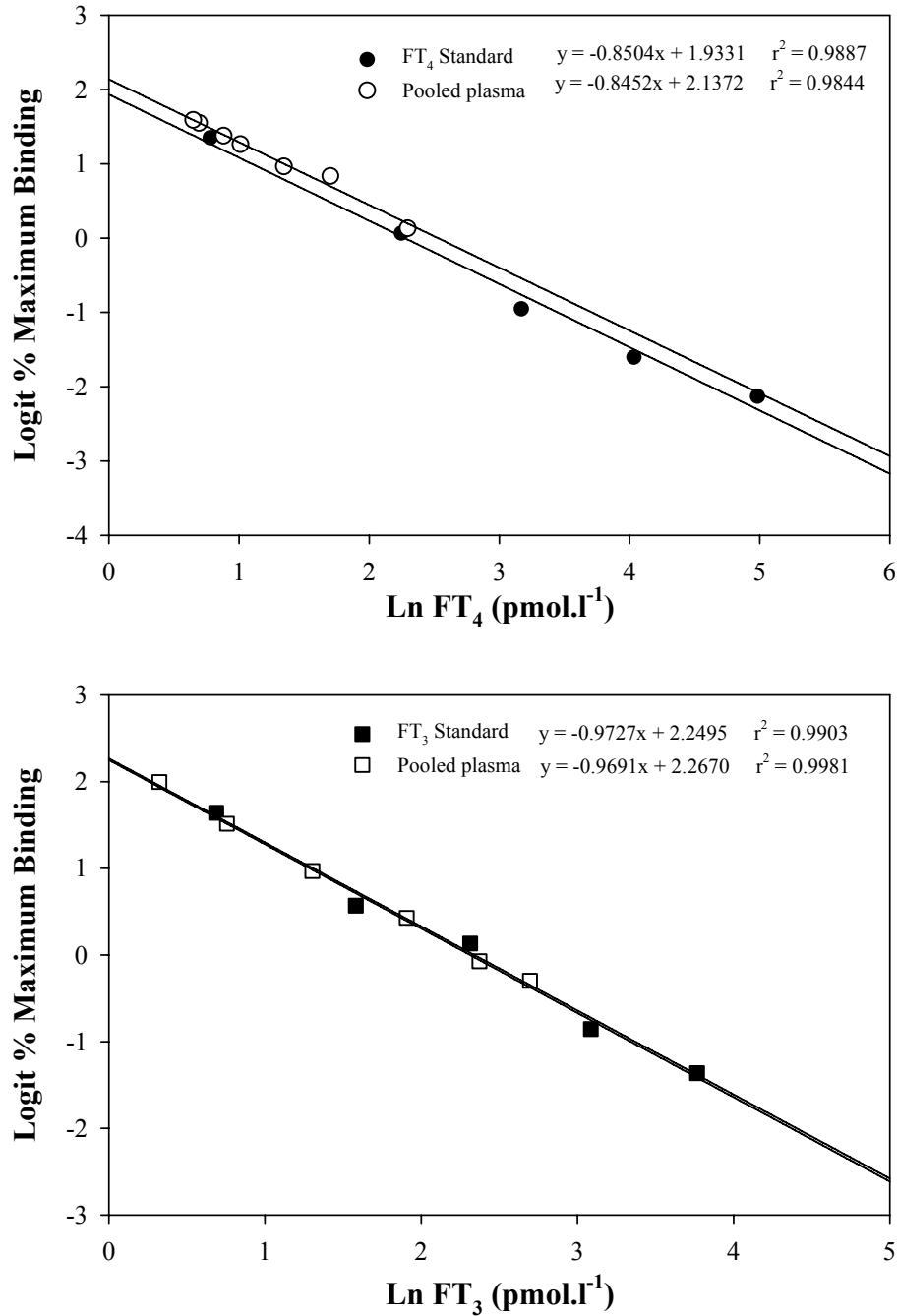


Figure 2.5. Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 100 μ l aliquots of pooled Atlantic salmon plasma with the free thyroxine (FT₄; Top) and free triiodothyronine (FT₃; Bottom) standards. Each point represents the mean of duplicate measurements; the X-axis denotes the natural log of the FT₄ or FT₃ content. The two curves have been transferred to a linear relationship using the logit transformation: $\text{logit } b = \ln(b/100-b)$ where b is the proportion of radiolabel bound to the antibody expressed as a percentage of that of the zero standard maximum binding).

2.5. Light Scanning and Perception

2.5.1. Light Scanning

Spectrum

The spectral quality of the natural light and artificial light penetrating the water column was measured using a portable spectroradiometer with fibre optic umbilical (StellarNet Inc. EPP2000, AstraNet Systems Ltd.; Cambridge, UK). Scans were made directly at the light source or water surface and at pre-determined intervals thereafter until no further readings were detectable by the equipment.

Light intensity

Light profiles of the various rearing systems used within the studies were mapped using illuminance (lux) and irradiance ($\text{W}\cdot\text{m}^{-2}$) readings recorded by photometric instruments (Skye Instruments Ltd.; Powys, UK) calibrated to National Physical Laboratory (UK) standards. Tank profiles were recorded by taking measurements from the centre of the tank and at the tank periphery at 4 compass bearings (0° , 90° , 180° and 270°) at both the waters surface and tank floor. Cage measurements were mapped by taking readings in a grid formation from below the water surface and repeating at pre-determined depths. Light distribution contour plots were produced graphical using SigmaPlot 8 (SPSS Inc.; USA).

2.5.2. Melatonin

Plasma melatonin levels were assayed using commercially available ELISA kits (IBL Ltd.; Hamburg, Germany).

Assay reagents

All reagents (*i.e.* standards, controls, buffer and antiserum) were supplied with the ELISA kits in lyophilised or concentrated forms and prepared according to the manufacturer's recommendations.

Sample collection

Blood samples taken from fish during the scotophase were withdrawn as detailed in Section 2.2.3. Fish were removed from their rearing environment under darkness and blood removed under dim red filter (λ 670-800 nm, 0.2 lux 0.5 m) in the shortest time possible.

Sample extraction

Prior to assay it was necessary to extract the melatonin from the standards, controls and samples using the following method:

1. 2 ml of absolute methanol (Fisher Scientific; Leicestershire, UK) was added to the extraction columns, provided with the kits, and placed in polystyrene assay tubes (LP4, Thermo Life Sciences; Hampshire, UK) and centrifuged at 200 g for 1 min at 4°C
2. 2 ml of nanopure water was added to the extraction columns and centrifuged at 200 g for 1 min at 4°C
3. 500 μ l of standards, controls and samples were added to the extraction columns and centrifuged at 200 g for 1 min at 4°C
4. 2 ml of 10 % methanol was added to the extraction columns and centrifuged at 500 g for 1 min at 4°C
5. 1 ml of absolute methanol was added into the columns and placed into clean assay tubes and centrifuged at 200 g for 1 min at 4°C
6. Tubes were dried in a vacuum evaporator (Genevac Ltd.; Suffolk, UK) at less than 37°C
7. Standards, controls and samples were reconstituted with 150 μ l of nanopure water, thoroughly vortexed and assayed immediately

Assay protocol

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

1. 50 μ l of standard, controls and sample were added to duplicate wells of a 96 well microplate, supplied with the kits
2. 50 μ l of melatonin biotin was added to all wells
3. 50 μ l of melatonin antiserum was added to all wells, the microplate covered with foil adhesive, gently shaken and incubated at 4°C for 18 h
4. The microplate was washed 3 times with 250 μ l of assay buffer before adding 150 μ l of enzyme conjugate to each well, covering with foil adhesive and incubating at room temperature for 2 h on an orbital shaker (500 rpm)
5. The microplate was washed 3 times with 250 μ l of assay buffer before adding 200 μ l of PNPP substrate solution to each well and left to incubate at room temperature for 30 min on an orbital shaker (500 rpm)
6. 50 μ l of PNPP stop solution was dispensed to each well, gently shaken and the optical density read at 405 nm using a Multiskan EX microplate reader (Labsystems; Hampshire, UK)

The amount of hormone present in the sample was obtained by intersecting the standard curve at the point corresponding to the optical density at (405 nm) of the sample (Figure 2.6.).

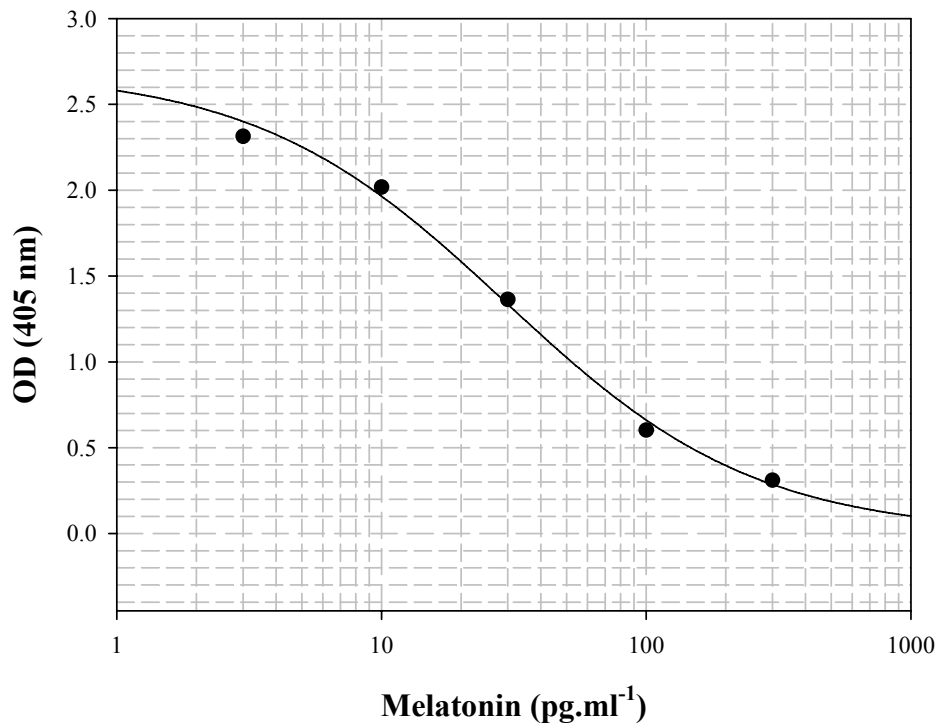


Figure 2.6. Typical standard curve from a melatonin ELISA; the concentration of melatonin in the sample was determined by intersecting the standard curve at the point corresponding to the optical density at 405 nm of the sample.

Quality control and validation

The sensitivity of the kits, *i.e.* the minimum amount of melatonin that is statistically distinguishable from zero, was 3.0 pg.ml⁻¹. Quality controls (QC's) were supplied with the kits with melatonin contents of 8.1 and 64.4 pg.ml⁻¹. Additionally, pooled samples of rainbow trout plasma, taken during the scotophase, with a melatonin content of approximately 60 pg.ml⁻¹ were also used as QC's to check the reproducibility of the assays. The inter-assay and intra-assay coefficients of variation were 3.8 and 10.7 % respectively.

2.6. Maturation Assessment

2.6.1. Gonadosomatic Index

Maturation status was assessed by calculating the gonadosomatic index (GSI), which expresses gonadal weight as a percentage of body weight:

$$\text{GSI} = [\text{gonadal weight (g)} / \text{body weight (g)}] \times 100$$

Individuals were identified as being mature if males had a gonadal weight of > 3 g and a GSI \geq 0.4 %, and females if the GSI \geq 0.8 %.

2.6.2. Histological Analysis

Histological examination of gonadal tissue was performed by removing a small transverse section from the mid-section of the gonad and fixing by placing into an excess of 10% buffered formalin solution. Fixed samples were sectioned, stained and mounted by the Veterinary Diagnostic Service, Institute of Aquaculture, University of Stirling. The mounted sections were examined under light microscopy using an Olympus BH-2 binocular microscope (Olympus Optical Co., London, UK) connected to an Olympus zoom lens (18-108/2.5; Olympus Optical Co., London, UK) which was linked to a computer using image capturing software (Image-Pro Plus[™] for Windows, Media Cybernetics[®]; USA). Oocytes were classified into stages of development as described by Taranger *et al.* (1999) and spermatogenesis was assessed using the classification of Dziewulska and Domagala (2003). Descriptions of the reproductive stages are indicated in Table 2.1.

Table 2.1. Stages of development of female (♀) ovaries and male (♂) testes as assessed by histological examination. Oocytes were classified according to Taranger *et al.* (1999) and stages of spermatogenesis as classified by Dziewulska and Domagala (2003).

Female (♀)		Male (♂)	
Stage	Description	Stage	Description
(i) Primary growth phase	Chromatin-nucleolus stage: Apparent nucleolus and chromatin threads. Perinucleolus stage: Multiple nuclei located around the periphery of the nucleus.	(i) Type A Spermatogonia (SG A)	Germ cells singular or in pairs, not connected with cytoplasmic bridges in the lobules among Sertoli cells
(ii) Secondary growth phase	Cortical alveoli stage: cortical alveoli (yolk vesicles) appear in the periphery of the oocyte Oil drop stage: oil droplets appear in the perinuclear area, and also periphery	(ii) Type B Spermatogonia (SG B)	Cysts formed surrounded by Sertoli cell processes forming a tight sheath. Young cells contain two and then four cells. Cells undergo incomplete cytokinesis. May be connected with cytoplasmic bridges
(iii) True vitellogenesis	Primary yolk stage: Yolk globules appear in the periphery of the oocyte Secondary yolk stage: more yolk globules throughout the oocyte Tertiary yolk stage: oocyte filled with yolk globules	(iii) Primary and secondary spermatocytes (SC)	More cysts observed. Contain cells undergoing first meiotic division. Some cysts still have SG B. SG A occur between cysts. Second meiotic division. Cysts show presence of spermatids.
(iv) Atresia	Dissolution of atretic oocyte, Phagocytosis by granulosa cells of follicular epithelium. Dissolution of granulosa cells	(v) Spermatozoa (SZ)	Advanced cysts show first spermatozoa. Connections between Sertoli cells and SZ broken down. Cyst wall breaks down,

2.6.3. Testosterone

Plasma testosterone levels were determined by radioimmunoassay using a method adapted from Duston and Bromage (1987).

Assay buffer

The following constituents were dissolved in 500 ml of nanopure water in a volumetric flask aided by a metallic stirrer and hotplate:

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

Assay buffer was prepared fresh on the first morning of each assay and allowed to chill at 4°C prior to use. All chemicals were of Analar grade and supplied by Sigma or BDH Chemicals Ltd.

Charcoal buffer

The following constituents were dissolved in 250 ml of assay buffer in a conical flask aided by a metallic stirrer:

Charcoal	5.0 g
Dextran	4.9 g

Charcoal buffer was freshly prepared and remained stirring continuously on ice for a minimum of 30 min prior to use.

Antibody

Antibody was prepared by diluting 100 μl of rabbit anti-testosterone antiserum (Biogenesis Ltd.; Poole, UK) with 990 μl of assay buffer (1:10 dilution) before storing in 200 μl aliquots at -20°C . A working solution was freshly prepared by further diluting 200 μl of frozen antibody with 20 ml of assay buffer to achieve a final dilution of 1:1000, sufficient for assaying 80 samples in duplicate.

Radiolabel

A primary stock of tritiated testosterone, [1,2,6,7- ^3H]-Testosterone (Amersham Biosciences; Buckinghamshire, UK), was supplied in quantities of 9.25 MBq (250 μCi). An intermediate stock solution was prepared by diluting 20 μl of the primary stock in 2 ml of Analar grade absolute ethanol (Fisher Scientific; Leicestershire, UK), which was stored in a high performance glass vial at -20°C . A working stock was freshly prepared by diluting the intermediate stock in assay buffer to give an activity of approximately 20,000 dpm/100 μl .

Testosterone standard

A standard stock solution of 100 $\text{ng}\cdot\text{ml}^{-1}$ of testosterone standard was prepared by dissolving 1 mg of testosterone (Sigma; Poole, UK) in 10 ml of absolute ethanol (Fisher Scientific, Leicestershire, UK). This intermediate solution was stored at -20°C in a high performance glass vial until required. A working solution of 10 $\text{ng}\cdot\text{ml}^{-1}$ was freshly prepared by diluting 100 μl of the intermediate stock in 0.9 ml of absolute ethanol.

Sample extraction

Prior to analysis, it was necessary to extract the testosterone from the plasma sample using the following method:

1. 100 μl of plasma sample was added to polypropylene assay tubes (LP3P, Thermo Life Sciences; Hampshire, UK)

2. 1 ml of ethyl acetate (BDH Chemicals Ltd.; Poole, UK) was dispensed to each tube and capped
3. Samples were attached to a rotary mixer for 1 h at room temperature and centrifuged at 430 g for 10 min at 4°C

The extracted sample could be assayed immediately or stored at 4°C until assay at a further date.

Assay protocol

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

1. Serial dilutions of the testosterone standard were prepared with ethyl acetate in polypropylene assay tubes (LP3P, Thermo Life Sciences; Hampshire, UK) to give concentrations ranging from 1.95-1000 pg.100 μl^{-1} . A further 4 tubes containing 200 μl of ethyl acetate only were included for the non-specific binding (NSBs) and zero standard (B_0) tubes
2. 50 μl of extracted sample and controls were added to their respective assay tubes
3. Standards, samples and controls were dried down in a vacuum evaporator (Genevac Ltd.; Suffolk, UK) at less than 35°C and allowed to cool to 4°C
4. 100 μl of antibody was dispensed to all tubes, except NSBs
5. 100 μl of tritiated testosterone label was added to all tubes, including 2 scintillation vials (Totals)
6. Tubes were vortex mixed, covered and incubated at 4°C for 18 h
7. 500 μl of freshly prepared charcoal buffer was added to all tubes and left to incubate at 4°C for 30 min
8. Tubes were centrifuged at 1270 g for 10 min at 4°C
9. From each standard, control and sample 400 μl of supernatant was transferred to 6 ml polyethylene scintillation vials (Packard Bioscience B.V.; Groningen,

Netherlands) and 4 ml of scintillation cocktail fluid (Ultima Gold™, PerkinElmer; Buckinghamshire, UK) added. A vial containing only 4 ml of scintillation fluid was included as a blank to calculate background radioactivity

10. Vials were capped, thoroughly vortex mixed and counted for 5 min in a scintillation counter (1900TR LSA, Canberra Packard Ltd.; Pangbourne, UK)

Calculation

The amount of testosterone ($\text{ng}\cdot\text{ml}^{-1}$) present in the sample was calculated in the following way:

- The concentration of testosterone per tube ($\text{pg}\cdot\text{tube}^{-1}$) was determined from comparing the disintegrations per minute (dpm) of the samples against the standard curve (Figure 2.7.) and multiplying by 1.75 to correct for the total reagent volume (700 μl) and the amount of supernatant added to the scintillation vial (400 μl).
- From this value, the concentration of testosterone in the extract ($\text{pg}\cdot\text{extract}^{-1}$) assayed was calculated by dividing the $\text{pg}\cdot\text{tube}^{-1}$ value by the amount of extract assayed (50 μl), and multiplying by the total extract (1100 μl).
- This value was then converted into $\text{ng}\cdot\text{ml}^{-1}$ by dividing by the amount of plasma sample originally extracted (100 μl).

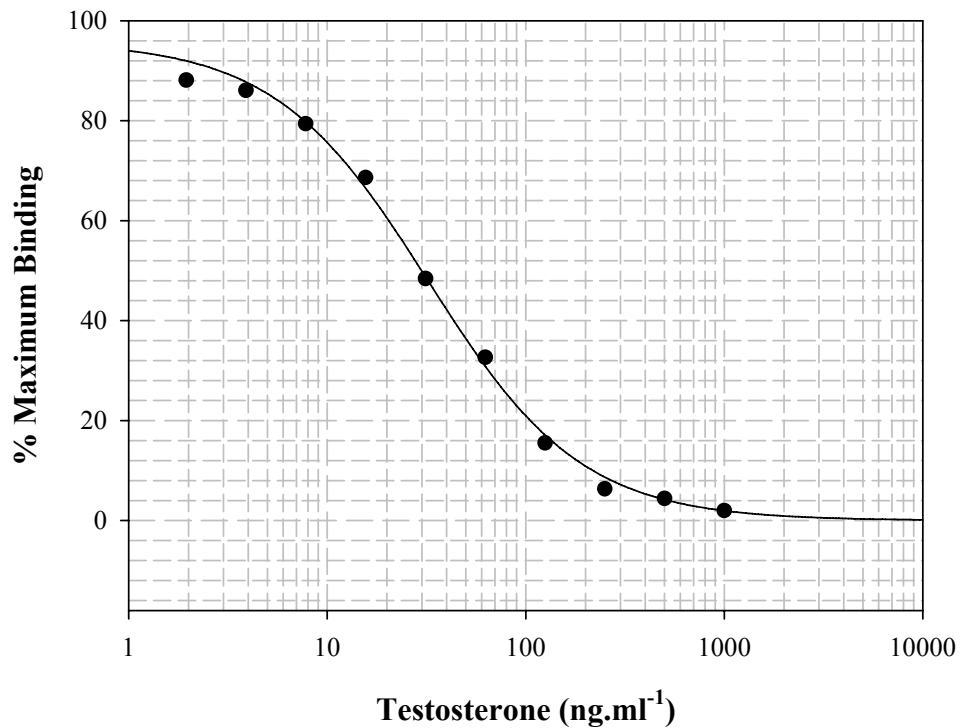


Figure 2.7. Typical standard curve from a testosterone radioimmunoassay; the concentration of testosterone in the sample was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

Quality control and validation

The sensitivity of the assay, *i.e.* the minimum amount of testosterone statistically distinguishable from zero, was (1.9 pg.tube^{-1}). Quality controls (QCs) with a testosterone content of approximately (100 pg.tube^{-1}) were used to check the reproducibility of measurements between assays. The inter-assay and intra-assay coefficient of variation were 8.2% and 14.8% respectively.

2.7. Stress and Welfare Indicators

2.7.1. Cortisol

Plasma cortisol levels were measured using a radioimmunoassay as described by Ellis *et al.* (2004).

Assay buffer

The following constituents were dissolved in 250 ml of nanopure water in a volumetric flask with the aid of a metallic stirrer and hotplate:

Sodium dihydrogen phosphate	0.74 g
Disodium hydrogen orthophosphate	2.88 g
BSA	1.0 g
Sodium chloride	4.0 g
EDTA	0.15 g
Sodium azide	0.5 g

Assay buffer was prepared fresh on the day of use and stored at 4°C, although the addition of sodium azide permitted the buffer to be stored for up to 7 days. All chemicals were of Analar grade and supplied by Sigma or BDH chemicals Ltd.

Charcoal buffer

The following constituents were dissolved in 200 ml of nanopure water in a conical flask with the aid of a metallic stirrer and hotplate:

Sodium dihydrogen orthophosphate	0.37 g
Disodium hydrogen orthophosphate	1.44 g
Gelatine	0.25 g

Once the gelatine was in solution, the following constituents were added to the buffer and the volume made up to 250 ml and stirred on ice with the aid of a metallic stirrer:

Activated charcoal	1.25 g
Dextran	0.25 g

Charcoal buffer was freshly prepared and remained stirring on ice for a minimum of 1 h prior to use.

Antibody

Antibody was prepared by hydrating 1 g of freeze-dried sheep anti-serum (Diagnostics Scotland; Edinburgh, UK) with 20 ml of fresh assay buffer (1:20 dilution) before storing in 1ml aliquots at -20°C. A working solution was freshly prepared by further diluting 1 ml of frozen antibody with 20 ml of assay buffer to achieve a final dilution of 1:400, sufficient for assaying 90 samples in duplicate.

Radiolabel

A primary stock of tritiated cortisol, [1,2,6,7-³H]-Cortisol (Amersham Biosciences Ltd.; Buckinghamshire, UK), was supplied in quantities of 9.25 MBq (250 µCi). An intermediate stock solution was prepared by diluting 20 µl of the primary stock in 2 ml of Analar grade absolute ethanol (Fisher Scientific; Leicestershire, UK), which was stored in a high performance glass vial at -20°C. A working stock was freshly prepared by diluting the intermediate stock in assay buffer to give an activity of approximately 5000 dpm/100 µl.

Cortisol standard

A standard stock solution of 50 µg.ml⁻¹ (Stock A) was prepared by dissolving 1 g of hydrolysed powdered hydrocortisone (Sigma; Poole, UK) in 20 ml of absolute ethanol

(Fisher Scientific; Leicestershire, UK). Two further intermediate solutions, Stock A and Stock B, were prepared as follows:

Stock B: 100 μl of Stock A in 10 ml absolute ethanol ($5 \mu\text{g}\cdot\text{ml}^{-1}$ solution)

Stock C: 100 μl of Stock B in 10 ml absolute ethanol ($50 \text{ng}\cdot\text{ml}^{-1}$ solution)

These intermediate solutions were stored at -20°C in high performance glass vials until required. A working solution of $4 \text{ng}\cdot\text{ml}^{-1}$ was freshly prepared by diluting 400 μl of Stock C ($50 \text{ng}\cdot\text{ml}^{-1}$) in 4.6 ml of ethyl acetate.

Sample extraction

Prior to analysis, it was necessary to extract the cortisol from the plasma sample using the following method:

1. 200 μl of plasma sample was added to polypropylene assay tubes (LP3P, Thermo Life Sciences; Hampshire, UK)
2. 1 ml of ethyl acetate (BDH Chemicals Ltd.; Poole, UK) was dispensed to each tube and capped
3. Samples were attached to a rotary mixer for 1 h at room temperature and centrifuged at 430 g for 10 min at 4°C

The extracted sample could be assayed immediately or stored at 4°C until assay at a further date.

Assay protocol

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

1. Serial dilutions of the cortisol standard (4 ng.ml^{-1}) were prepared with ethyl acetate in polypropylene assay tubes (LP3P, Thermo Life Sciences; Hampshire, UK) to give concentrations ranging from $12.5\text{-}800 \text{ pg.tube}^{-1}$. A further 4 tubes containing $200 \text{ }\mu\text{l}$ of ethyl acetate only were included for the non-specific binding (NSBs) and zero standard (B_0) tubes
2. $200 \text{ }\mu\text{l}$ of extracted sample and controls were aliquoted to their respective assay tubes
3. Standards, samples and controls were dried down in a vacuum evaporator (Genevac Ltd.; Suffolk, UK) at less than 35°C and allowed to cool to 4°C
4. $100 \text{ }\mu\text{l}$ of chilled assay buffer was added to all tubes
5. $100 \text{ }\mu\text{l}$ of antibody was dispensed to all tubes, except NSBs to which $100 \text{ }\mu\text{l}$ of assay buffer was added
6. $100 \text{ }\mu\text{l}$ of tritiated cortisol label was added to all tubes, including 2 scintillation vials (Totals)
7. Tubes were vortex mixed, covered and incubated at 4°C for 18 h
8. 1 ml of freshly prepared charcoal buffer was added to each tube, vortex mixed and left to incubate at 4°C for 30 min
9. Tubes were centrifuged at 1270 g for 12 min at 4°C
10. From each standard, control and sample $1000 \text{ }\mu\text{l}$ of supernatant was transferred to 6 ml polyethylene scintillation vials (Packard Bioscience B.V.; Groningen, Netherlands) and 4 ml of scintillation cocktail fluid (Ultima Gold™, PerkinElmer; Buckinghamshire, UK) added. A vial containing only 4 ml of scintillation fluid was included as a blank to calculate background radioactivity
11. Vials were capped, thoroughly vortex mixed and counted for 5 min in a scintillation counter (1900TR LSA, Canberra Packard Ltd.; Pangbourne, UK)

Calculation

The amount of cortisol ($\text{ng}\cdot\text{ml}^{-1}$) present in the sample was calculated in the following way:

- To correct for the difference between total reagent volume ($1300\ \mu\text{l}$) and the amount of supernatant added to each scintillation vial ($1000\ \mu\text{l}$), the average disintegrations per minute (dpm) of all standards and samples was multiplied by $1/1.3$
- The average dpm of the NSB's was subtracted from all standards and samples
- The percentage binding (percentage radiolabel bound to antibody) of the standards and samples were calculated relative to the total counts:

$$\% \text{ binding} = (\text{standard or sample dpm} / \text{total dpm}) \times 100$$

- The pharmacology feature in SigmaPlot 8 (SPSS Inc.; USA) was used to plot the standard curve and calculate the cortisol concentration for unknown samples (Figure 2.8.).
- The concentration of cortisol per tube ($\text{ng}\cdot\text{tube}^{-1}$) was determined from the curve and multiplied by 0.03 to correct for the volume of extract assayed (*i.e.* $200\ \mu\text{l}$ from $1.2\ \text{ml}$; $\times 6$), volume of plasma extracted ($200\ \mu\text{l}$; $\times 5\ \text{ml}$), and converted to $\text{ng}\cdot\text{ml}^{-1}$ ($\times 1/1000$).

Quality control and validation

The sensitivity of the assay *i.e.* the amount of cortisol that is statistically distinguishable from zero, was $12.5\ \text{pg}\cdot\text{tube}^{-1}$. Pooled plasma samples from rainbow trout with a cortisol content of approximately $30\ \text{ng}\cdot\text{ml}^{-1}$ were used as quality controls (QCs) to check the reproducibility of the measurements between assays. The intra-assay and inter-assay coefficients of variation was 6.4 and 14.8 % respectively.

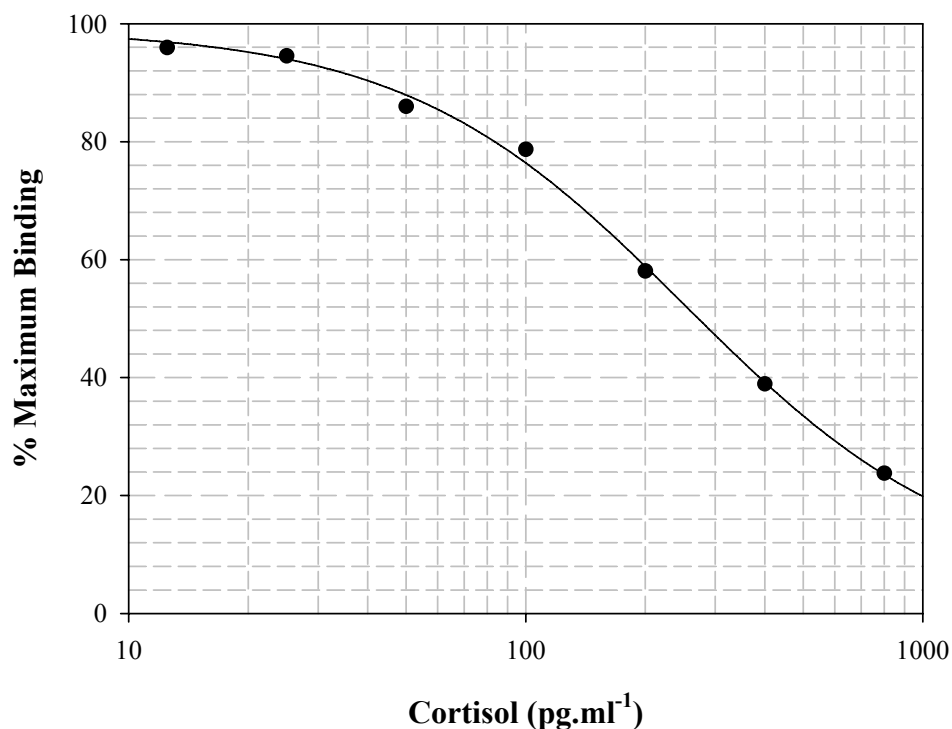
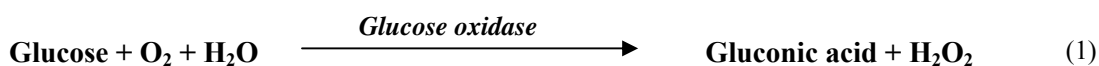
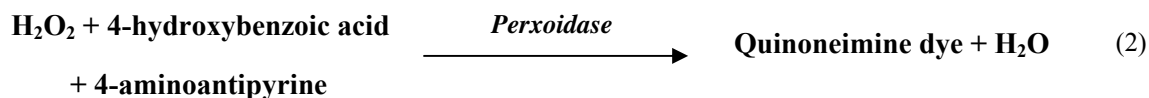


Figure 2.8. A typical standard curve from a cortisol radioimmunoassay; the concentration of cortisol in the sample was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

2.7.2. Glucose

Plasma glucose levels were determined colorimetrically using Glucose (Oxidase) Infinity™ kits (Alpha Labs Ltd.; Hampshire, UK). The assay is based on the hydrogen peroxide indicator reaction as first proposed by Trinder (1969). Glucose is oxidised by glucose oxidase to gluconic acid and hydrogen peroxide (1) which reacts in the presence of peroxidase with 4-hydroxybenzoic acid and 4-aminoantipyrine to form a red quinoneimine dye (2). The intensity of the colour formed is proportional to the glucose concentration and can therefore be measured photometrically between 460 and 560 nm.





Sample collection

To prevent the red blood cells from metabolising the glucose, blood samples were spun in a micro-centrifuge (Sigma 1-15, Sartorius AG; Göttingen, Germany) within 30 min of collection and the plasma aliquoted and stored at -70°C until assay.

Assay protocol

1. 2.3 µl of plasma sample were added to 4 replicate wells of a 96 well microplate (Nunc A/S; Roskilde, Denmark) from columns 1 to 10
2. 2.3 µl of 13.2 mmol.l⁻¹ glucose standard was added to all 8 wells of column 11, and 2.3 µl of nanopure water (blank) was added to all 8 wells of column 12
3. 350 µl of glucose reagent was added to all wells and the microplate incubated at 37°C for 5 min
4. The absorbance was read at 505 nm using a Dynex MRX 1.2 microplate reader (Labsystems; Hampshire, UK) and the glucose levels (mmol.l⁻¹) calculated using the following formula:

$$\left[\frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{standard}} - \text{Abs}_{\text{blank}}} \right] \times \text{Glucose standard concentration (mmol.l}^{-1}\text{)}$$

where, Abs_{sample}, Abs_{blank} and Abs_{standard} are the absorbance readings at 505 nm for the sample, blank and standard respectively.

2.7.3. Lysozyme

Plasma lysozyme activity was assessed using a turbidimetric assay adapted from Lygren *et al.* (1999). The assay measures the lytic activity of the plasma sample against a *Micrococcus lysodeikticus* bacterial solution through changes in absorbance.

Assay buffer

A 0.04 M (pH 5.8) sodium phosphate assay buffer was prepared by mixing 92 ml of Stock A with 8 ml of Stock B to achieve a 0.2 M solution before performing a 1:5 dilution with nanopure water.

Stock A: 15.6 g sodium dihydrogen phosphate in 500 ml nanopure water (0.2 M)

Stock B: 17.8 g sodium hydrogen phosphate in 500 ml nanopure water (0.2 M)

A 0.2 $\mu\text{g}\cdot\text{ml}^{-1}$ *Micrococcus lysodeikticus* (Sigma; Poole, UK) bacterial solution was then prepared using the assay buffer.

Assay protocol

1. 10 μl of plasma sample was added to 4 replicate wells in a 96 well microplate (Nunc A/S; Roskilde, Denmark)
2. 190 μl of a 0.2 $\mu\text{g}\cdot\text{ml}^{-1}$ *Micrococcus lysodeikticus* bacterial solution was added to each well using a multi-channel pipette
3. The absorbance at 540 nm was read using a Dynex MRX 1.2 microplate reader (Labsystems; Hampshire, UK) after 1 and 5 min, following the addition of the *M. lysodeikticus* solution
4. Lysozyme activity ($\text{U}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$) was calculated using the following formula:

$$\left[\frac{(\text{Abs}_1 - \text{Abs}_2)}{(t_2 - t_1)} \right] / 0.001 \times 100$$

where, Abs_1 and Abs_2 are the absorbance readings of the samples at 540 nm at times t_1 and t_2 respectively.

2.8. Flesh Quality Analysis

The flesh quality analysis described in this section was performed at ‘*Lochailort Fish Health and Quality Laboratory*’ of Marine Harvest (Scotland) Ltd.

2.8.1. Sample Preparation

Salmon fillets were prepared using the Norwegian quality cut (NQC) from the left-hand side of sacrificed fish. The NQC is a standardized muscle section used in flesh quality determination corresponding to the region of flesh posterior to the dorsal fin to the anterior of the anal fin. Sample cuts were placed into labelled polypropylene bags, immediately frozen in a liquid nitrogen shipper and stored at -70°C until analysis. Prior to analysis, samples were thawed at 4-8°C for a period of 20 h under lightproof sheeting to prevent discolouration. Thawed samples were skinned and deboned, leaving as much brown muscle as was possible.

2.8.2. Roche Colour Score

The visual colour of the salmon fillets were assessed using the Roche *SalmoFan*TM lineal colour card for salmonids, scale 20-34 (Hoffman-La Roche Ltd.; Basel, Switzerland). Fillets were placed into a neutral grey coloured light cabinet fitted with a D65 daylight fluorescent light source with a colour rendering index (Ra) >90 and a colour temperature of 6500 K to allow accurate colour matching. Two independent scorers measured the colour of the salmon fillets by placing the colour card alongside the dorsal, midline and belly regions of the fillet and selecting the closest colour score (Figure 2.9.). Since the *SalmoFan*TM score is a visually subjective evaluation of flesh colour, the same individuals and light source were used for all samples evaluated to avoid any inconsistencies.

2.8.3. Colorimetric Analysis

The colour composition of the flesh was also measured instrumentally using a tristimulus colorimeter (Minolta Chroma Meter, CR-310, Minolta Corporation; Osaka, Japan), which measures the reflectance of light from the flesh in relation to a standard calibration

tile (number 12133332). Flesh samples were measured in triplicate, once at each of the fillet regions (see Figure 2.9.), with the average value of the three regions recorded. The colorimeter measures the lightness (L^*), red/green chromaticity (a^*) and yellow/blue chromaticity (b^*) in accordance with the International Commission on Illumination (CIE, 1976). From the a^* and b^* values, the chroma (C^*_{ab}) and the hue (H°_{ab}) were calculated (Hunt, 1977):

$$(C^*_{ab}) = a^* \times b^*$$

$$(H^{\circ}_{ab}) = [\tan^{-1} (b^* / a^*)]$$

The chroma is an expression of intensity and clarity, whereas the hue is expressed as the relationship between the redness and the yellowness of the fillet in the form of an angular measurement where 0° indicates a red hue and 90° yellow.

2.8.4. Total Pigment and Lipid Levels

Total pigment and lipid levels of individual flesh samples were analysed by near-infrared (NIR) spectroscopy, which measures the reflectance of light from the sample and calculates the pigment (mg.kg^{-1}) and lipid (%) levels using a predictive equation. Quality control (QC) checks were performed on the day of use with a calibration check cell (number 20635) and a QC flesh sample. The QC of the predictive equation was by comparison of the NIR output with primary analysis (“wet chemistry”) results performed on a quarterly basis at Nutreco Aquaculture Research Centre, Stavanger, Norway.

The flesh sample was homogenized using a Braun food processor (Braun GmbH; Frankfurt, Germany), placed into a sample cup cell and loaded into a FOSS 6500 NIR analyser (Foss NIRSystems, Foss UK Ltd.; Didcot, UK). The cell was scanned and the total pigment and lipid values calculated. To prevent contamination of the samples between scans, the sample cell was washed thoroughly with warm soapy water before each use.

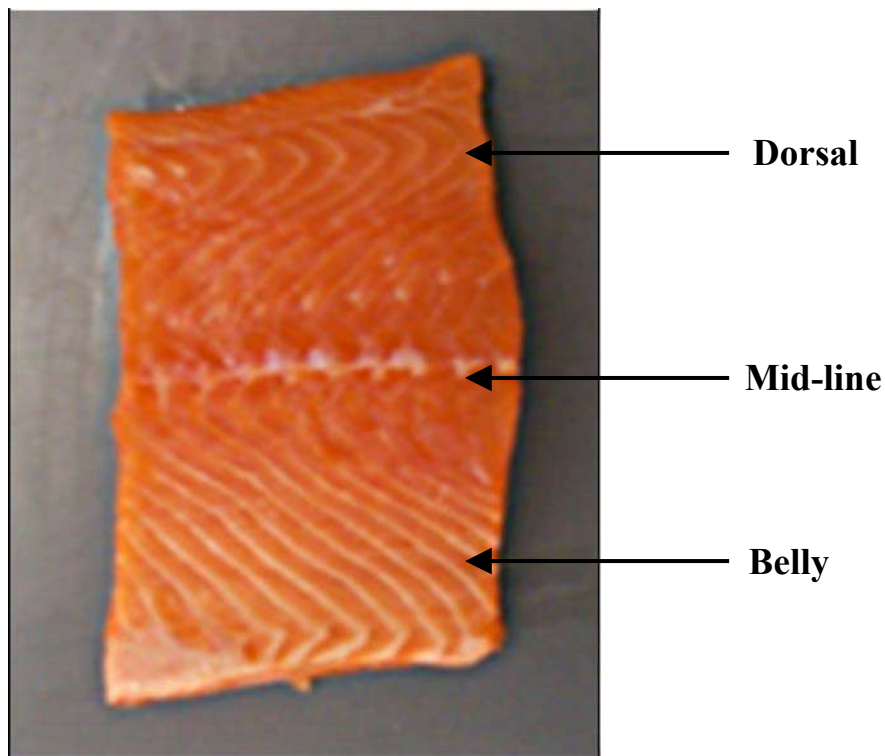


Figure 2.9. Example of a prepared Atlantic salmon flesh sample outlining the dorsal, midline and belly regions used for the Roche *SalmoFan*TM and Minolta colour scores.

2.8.5. Moisture Content

Moisture content (%) was determined by weighing 5 g of homogenized flesh sample into tared foil trays and placing into a drying oven (Gallenkamp; Loughborough, UK) at 105°C for 20 h. The dried sample was then reweighed and the moisture content calculated using the following equation:

$$\text{Moisture (\%)} = [(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100$$

2.9. Statistical Analysis

The statistical methods used within this thesis are described within Zar (1999). All statistical analyses were performed using the Minitab Statistical software package (Version 14.1, Minitab Inc.; Pennsylvania, USA), unless otherwise stated. In some cases data sets were first compiled using Microsoft Excel[®]. A significance level of 5% ($P < 0.05$) was used for all tests performed.

2.9.1. Basic calculations

Arithmetic mean

The arithmetic or sample mean (\bar{X}) was used to provide as an estimate of the population mean (μ) together with the standard error of the mean (SEM) to represent sample distribution.

Coefficient of variation

The coefficient of variation (CV) is a measure of relative variability, expressing sample variability relative to the mean of the sample. The CV is often multiplied by 100 in order to express as a percentage, and is defined as:

$$\text{CV (\%)} = (\sigma / \bar{X}) \times 100$$

where σ is the standard deviation

2.9.2. Parametric Testing

Parametric tests were performed based on the assumptions that the observations were made at random and the test variances independent. Furthermore, the sample variances must be homogenous and the data normally distributed. Where data failed to meet these requirements, non-parametric tests were employed (Section 2.9.6.).

2.9.3. Normality Testing and Homogeneity of Variance

A Kolmogorov-Smirnov test was used to determine the normality of a distribution. This test compares the cumulative distribution of the data with that of an ideal Gaussian distribution, basing its P value on the largest discrepancy. Homogeneity of variance was tested using the F-test for comparison of two samples and Bartlett's test for three or more samples. If the calculated F-test value was less than the tabulated value at $P=0.05$, then the variance was treated as homogenous and if greater as heterogeneous.

Where data was examined by a general linear model (Section 2.9.5.), and n was typically large, normality and homogeneity of variance were assessed through examination of the residual plots.

2.9.4. Comparison of Two Samples

Providing samples passed the assumptions for parametric testing, the means of two samples were compared using the Student's t -test with a pooled estimation of variances. Means were considered significant if the calculated value was greater than the tabulated t value at $P=0.05$ (5%) or less. Data which failed parametric assumptions were instead examined using a non-parametric Mann-Whitney U -test, where medians were considered significant if the calculated U value was less than the tabulated value at $P=0.05$ (5%) or less.

2.9.5. Multiple Comparisons

Data involving three or more samples, and which met the assumptions set out for parametric testing, were analyzed using a one-way analysis of variance (ANOVA). In addition, the General Linear Model (GLM) feature was applied to analyze appropriate data sets. The GLM incorporates a number of different statistical models accounting for numerous factor levels including replication and repeated sampling measures. Where data differed significantly ($P<0.05$), Tukey's multiple comparison *post-hoc* test were applied.

2.9.6. Nonparametric Testing

Data failing to meet the assumptions for parametric tests were analyzed using non-parametric statistical methods. A Kruskal-Wallis test was performed using the InStat statistical package (InStat version 3.0.; GraphPad Software Inc., California, USA). Means bearing significant differences ($P < 0.05$) were further tested using Dunn's multiple comparison *post-hoc* test.

2.9.7. Linear Regression

Linear relationships between two variables were calculated using the Pearson product moment correlation coefficient (r). Run's test was used to check for linearity using the InStat statistical package (InStat version 3.0.; GraphPad Software Inc., California, USA), with data failing the test indicating a curvilinear relationship. Multiple comparisons of linear regression gradients were made by analysis of covariance (ANCOVA; Zar, 1999), using formulated spreadsheets in Microsoft Excel[®].

Chapter 3: The effects of constant light exposure and light intensity on the growth and feeding responses of juvenile rainbow trout (*Oncorhynchus mykiss*).

3.1. Introduction

The environment in which an animal resides will inevitably exert an influence on an individual's developmental and growth processes. Photoperiod, temperature, rainfall, food availability and pheromones all have some role in cueing life events in the majority of fish (Bromage *et al.*, 2001). For higher latitudinal fish, such as the salmonids, the seasonally changing photoperiod is considered as the primary entraining factor (Boeuf and Le Bail, 1999; Boeuf and Falcón, 2001; Bromage *et al.*, 2001), providing reliable “noise free” information on the time of year. Long day photoperiods and constant light regimes have been shown to significantly enhance the growth rates of salmonid fish when applied during the winter period (*e.g.* Saunders and Harmon, 1988; Villarreal *et al.*, 1988; Hansen *et al.*, 1992; Taranger *et al.*, 1995; Oppedal *et al.*, 1999; Endal *et al.*, 2000; Taylor *et al.*, 2005, 2006), suggesting either a phase advancement of endogenous growth rhythms (Endal *et al.*, 2000) or the direct photostimulation of growth (Komourdjian *et al.*, 1976; Johnston *et al.*, 2003), relating to changes in behaviour, hormonal profile and/or appetite. However, the interaction of temperature on the photoperiod entrainment of biological rhythms in fish remains unclear.

Fish are ectothermic animals and as such the ambient temperature will have a pervasive effect on growth and food consumption. Feeding and growth both vary seasonally in fish (Higgins and Talbot, 1985; Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999; Nordgarden *et al.*, 2003), often making it difficult to distinguish between the photoperiodic and temperature related effects. Smith *et al.* (1993) reported that the seasonal variation in feed intake of Atlantic salmon (*Salmo salar*) was related to changes in daylength rather than to water temperature, whereas Kavadias *et al.* (2003) found that the growth rates of European sea bass (*Dicentrarchus labrax*) exhibited a strong correlation with the water temperature and average daily food consumption as opposed to

photoperiod. Indeed increasing temperatures are known to increase feeding and growth in a variety of fish species (Staples and Nomura, 1976; Grove *et al.*, 1978; Koskela *et al.*, 1997; Jonassen *et al.*, 2000). However, the growth enhancement frequently reported in response to constant light exposure has been attributed to either a stimulated feed intake (Handeland *et al.*, 2003; Petit *et al.*, 2003), improved conversion efficiency (Boeuf and Le Bail, 1999; Jonassen *et al.*, 2000; Taylor *et al.*, 2006), or as a result of both (Nordgarden *et al.*, 2003).

The vast majority of the published literature relating to photoperiodic effects on growth performances in salmonids has focussed mainly on various life-stages of the Atlantic salmon (*e.g.* Saunders and Harmon, 1988; Hansen *et al.*, 1992; Solbakken *et al.*, 1994; Oppedal *et al.*, 1999; Endal *et al.*, 2000). However, the application and timing of light in salmon generally occurs around crucial developmental processes (*i.e.* smolting and maturation). This may lead to misguided or false interpretation of growth data, particularly with respect to the question of whether photoperiod directly stimulates growth. In addition, the salmon farming industry also reports the occurrence of a characteristic growth-dip following exposure to constant light, which has also been suggested within the scientific literature (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005). Furthermore, this phenomenon has yet to be observed in freshwater in photoperiod-manipulated smolts. The rainbow trout, *Oncorhynchus mykiss*, is therefore a seemingly ideal model species to use in growth studies, particularly during the juvenile stage where no growth transitions occur (*i.e.* parr-smolt transformation). Moreover, the rainbow trout is the second most cultured salmonid species in Europe, with around 5,550 tonnes currently produced for the UK table market (SEERAD, 2005). Recent research suggests that rainbow trout exposed to constant light under commercial farming conditions can reduce the time to produce a marketable sized product by as much as two months through an improvement of growth performance and feed conversion efficiency (Taylor *et al.*, 2006). However, in order to fully examine these effects, controlled experimental trials are required.

Finally, the underwater light environment varies considerably between freshwater and marine environments. For example, shorter wavelengths (*i.e.* red λ 750 nm) tend to be absorbed and attenuated in the upper water column in a marine environment, whereas longer wavelengths (*i.e.* blue λ 450 nm) tend to penetrate further (Lobban and Harrison, 1994; Taylor *et al.*, 2006). However, in freshwater light attenuation and penetration is highly variable due to the greater long-term fluctuations in water quality (*e.g.* peaty water *etc.*). Thus, ensuring adequate light conditions is important if photoperiod responses are to be observed. Light intensity has been studied little in salmonids, with the majority of work performed in salmon (Stefansson *et al.*, 1993; Oppedal *et al.*, 1997; Oppedal *et al.*, 1999) than in trout (Cho 1992b; Taylor, 2006). However, Cho (1992b) found that trout exposed to constant light at higher intensities (1600 lux) grew significantly better than those at lower intensities (100 lux). This is in accordance with results from Taylor *et al.* (2006) which demonstrated greater increase in growth with an increase in light intensity. Therefore, if the interactions of photoperiod and temperature on feeding and growth responses are to be studied, it is vital that the intensity of light is sufficient to induce a photoperiodic response.

Thus, the present study examines the interaction of photoperiod and temperature on the growth and feeding responses of individually tagged juvenile monosex (♀) rainbow trout reared in enclosed freshwater tanks. Specifically, the application of constant light exposure and its supposed growth promoting effects were addressed at two different light intensities set at two different periods of the year (summer/winter), characterized by two different water temperature profiles.

3.2. Materials and Methods

The trial was conducted at the University of Stirling's 'Niall Bromage Freshwater Research Unit' (Stirlingshire, Scotland) between February 2003 and August 2004. In order to examine constant light application at different temperatures the trial was separated into two experiments: Experiment I investigating the effect of summer temperatures; and Experiment II examining the effect of winter temperatures on feeding and growth responses.

To maintain standards in line with those of the UK trout farming industry, an all-female diploid stock of rainbow trout (Glen Wyllin Trout Hatchery Ltd., Isle of Man) was used in both experiments. All fish were hatchery reared and maintained under a natural photoperiod prior to the stocking of experimental tanks. Experiments were performed in 1.5 m (0.85 m³, 0.80 m water depth) circular lightproof fibreglass tanks supplied with flow-through freshwater from a local reservoir of ambient temperature (Figure 3.1.). Light was supplied by either a 16W or 28W opal round drum lamp fitted with a 2700 K colour temperature bulb (RS Components Ltd.; Northants, UK), and positioned at the centre of the tank 20 cm above the water surface. Irradiance levels (W.m⁻²) were measured just below the water surface and on the tank floor using light sensors calibrated to National Physical Laboratory UK standards (Skye Instruments; Powys, UK). Light readings were taken at night with one measurement at the centre of the tank and further readings at four compass bearings (0°, 90°, 180°, 270°) around the periphery of the tank, with readings at the water surface and tank floor

Feed intake

Feed intake was examined by X-radiography (Talbot and Higgins, 1983) using a commercial diet labelled with radio-opaque ballotini glass beads (refer to Section 2.3.3.). For each batch of labelled diet, standard curves were prepared by X-raying known weights of the labelled feed. Food consumption was then calculated using the linear regression of the relationship between the weight of the labelled feed and number of ballotini (see Table 3.1. and 3.2.). Feed measurements were made at pre-arranged

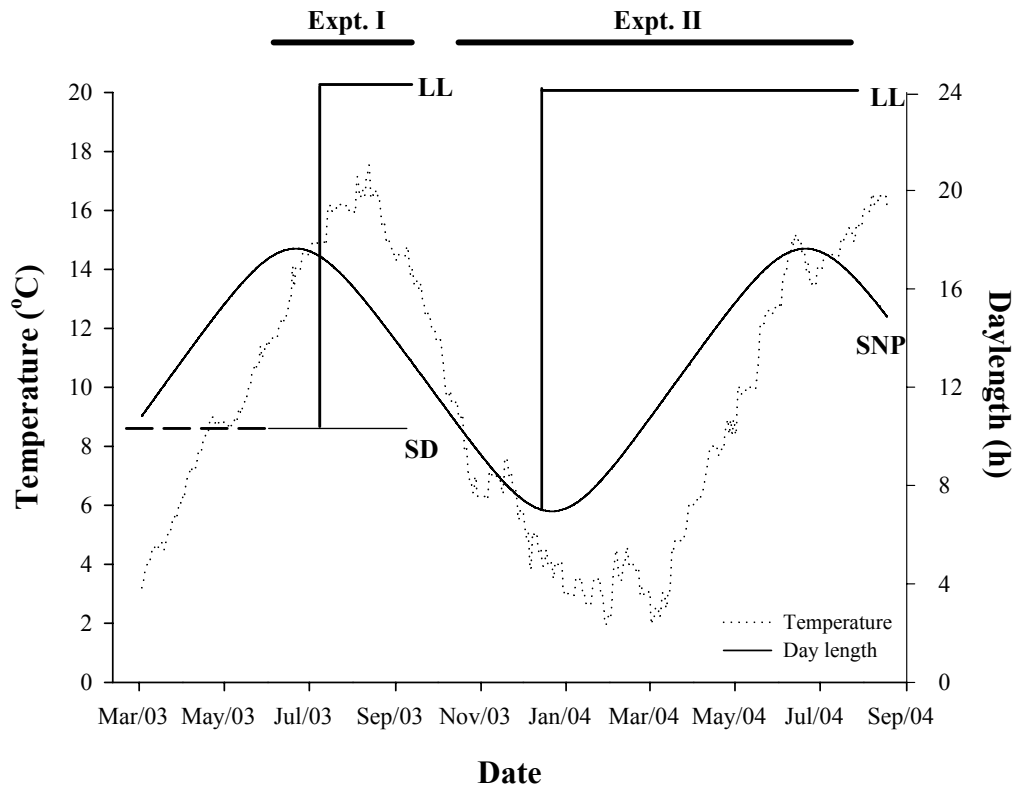


Figure 3.1. Ambient water temperature ($^{\circ}\text{C}$) profile and experimental photoperiod (h) regimes used throughout the trial. Photoperiod regimes are indicated as 9L:15D short-day photoperiod (SD), simulated natural photoperiod (SNP) or constant light (LL). Horizontal bars indicate duration of experiments.

intervals (see Sampling regimes) to assess the acute and chronic feeding responses following exposure to constant light. On the morning of sampling fish were fed the labelled diet to the point of satiation, determined by a build up of feed on the tank floor and changes in feeding behaviour. Tagged fish were anaesthetized in a 0.1 ml^{-1} solution of 2-phenoxyethanol, X-rayed (G.E.C. MX2 Series 7 portable X-ray unit; Agfa DW ETE film), individually recognized by tag-reading and length-weighed. X-ray plates were developed and the individual food consumption estimated from the amount of ballotini present in the gastrointestinal tract. Individual feed intake was calculated on a weight-specific basis (mg.g fish^{-1}). Fish which died or where feed intake could not be quantified at any of the time points were removed from analysis.

3.2.1. Experiment I: Summer Growth

The experiment was conducted between the 3rd June and 17th September 2003. In order to prevent fish from perceiving an increase in the natural daylength, 320 all-female diploid rainbow trout (2002 hatch) were transferred to experimental tanks and held under a 16W intensity 9L:15D artificial photoperiod (Control) from the 29th February until the start of the investigation. This was to ensure that the greatest response, if applicable, to constant light exposure could be seen when light was applied.

Experimental design

On the 3rd June 2003, 320 fish were equally split amongst eight tanks (40 fish per tank). From each tank population, 20 fish were randomly selected, individually length-weighed and injected with a PIT tag into the dorsal muscle (Section 2.2.4.). Tagged individuals were additionally fin-clipped (adipose) to make identification clearer for following individual feeding and growth responses throughout the trial.

On the 9th July, duplicate tanks either remained under a 16W light intensity or were transferred to a 28W rearing intensity and were maintained under a 9L:15D short-day photoperiod (SD) or exposed to a constant light regime (LL). Thus, the four photoperiod treatments used in the trial were: 16W SD, 28W SD, 16W LL and 28W LL. Tank placement was randomised in all instances. Fish were fed to excess with a standard commercial dry diet (Excel, pellet size 4.0 mm; Skretting, UK) distributed by clockwork belt feeders between 0800-1530 h.

Table 3.1. Linear regressions of the relationship between weight of labelled feed and number of ballotini, as used throughout Experiment I (summer growth).

		r^2	Date used
Feed in stomach/intestine (g) =	$0.0903 \times (\text{ballotini beads}) - 0.0691$	0.9981	24/06-21/07
	$0.1528 \times (\text{ballotini beads}) + 0.247$	0.9992	22/07-18/08

Sampling regime

In order to ascertain feeding levels of tagged individuals prior to the onset of treatments, a baseline sample was performed on 24th June (-14 days). Thereafter, measurements were obtained on the 10th (+1), 16th (+7), 22nd (+13) and 29th July (+19), 7th (+28) and 18th August (+39), whereby the onset of light (9th July) represented day 0. All sampling was performed during the light phase of the shortest photoperiod (*i.e.* 9L:15D), between 1000-1600 h. A malfunction with the X-ray machine on the 22nd July and 7th August resulted in only one replicate tank from each treatment being examined.

An initial length-weight sample was performed on the 3rd June for all tagged individuals. Thereafter, individual growth performance was determined at each of the feed intake sample points, with an additional length-weight sample made at the final sample point on the 17th September. To improve the analysis of growth performance, selected sampling points which occurred within a few days of one another were removed to minimize sampling errors. Thus, growth performance was determined on the 3rd (-35 days) and 24th June (-14), 16th (+7) and 29th July (+19), 18th August (+39) and 17th September (+69). For each individual the condition factor (K) and the daily weight gain (SGR_W) were calculated (Section 2.3.). Fish which died or were missed from any sampling points were removed from analysis.

3.2.2. Experiment II: Winter Growth

The experiment was conducted between 1st October 2003 and 18th July 2004 using a 2003 hatch of all-female diploid rainbow trout. All fish were maintained under a simulated natural photoperiod (SNP) at 16W intensity prior to treatment onset.

Experimental design

On the 1st October 2003, 1200 fish were selectively hand graded and equally distributed amongst eight experimental tanks. A total of 35 fish from each replicate tank were randomly selected, individually length-weighed and tagged by injecting a PIT tag into the dorsal musculature (Section 2.2.4.). The tagged individuals were additionally fin

clipped (adipose fin) to make identification easier for following individual feeding and growth responses throughout the trial.

On the 1st December 2003, replicate tanks under each of the intensity treatments either remained under a simulated natural photoperiod (SNP) or were exposed to LL. Thus, the four photoperiod regimes used within the experiment were: 16W SNP (control), 28W SNP, 16W LL and 28W LL. Light in the SNP treatments were controlled by clockwork timers (Kingshield timer, Powerbreaker PLC.; Essex, UK), which were adjusted in accordance with sunrise and sunset under a natural photoperiod on a weekly basis. Fish were fed to excess with a standard commercial dry diet (Royal Optima, pellet size 4.0 mm; Skretting, UK) distributed by clockwork belt feeders between 0800-1530 h.

Sampling regime

In order to ascertain feed consumption levels prior to the onset of treatments, a baseline sample was performed on 28th November 2003 (-2 days). Thereafter, measurements were obtained on the 3rd (+2), 8th (+7) and 16th December 2003 (+15), 8th (+38) and 15th January (+45), 15th (+105) and 18th March (+108) and the 6th (+127) and 26th April 2004 (+147), whereby the onset of light (1st December 2003) represented day 0. All sampling was performed during the light phase of the shortest photoperiod (*i.e.* SNP), between 0900-1500 h.

Table 3.2. Linear regressions of the relationship between weight of labelled feed and number of ballotini, as used throughout Experiment II (Winter growth).

		r²	Date used
	0.0629 x ballotini beads + 0.0354	0.9995	28/11/03-16/12/03
Feed in stomach/intestine (g) =	0.0517 x ballotini beads + 0.0017	0.9979	17/12/03-17/03/04
	0.0665 x ballotini beads + 0.0922	0.9891	17/03/04-26/04/04

An initial length-weight sample was performed on the 1st October 2003 for all tagged individuals. Thereafter, individual growth performance was determined at each of the feed intake samples points, with additional length-weight samples performed on the 27th May, 28th June and at the final sample point on the 18th July 2004. To improve the analysis of growth performance, selected sampling points which occurred within a few days of one another were removed to minimize sampling errors. Thus, growth performance was determined on the 1st October (-60 days), 28th November (-2), 16th December (+15), 15th January (+45), 18th March (+108), 6th (+127) and 26th April (+147), 27th May (+178), 28th June (+210) and 18th July 2004 (+230). For each individual the condition factor (K) and the daily weight growth rate (SGR_{W}) were calculated (Section 2.3.). Fish which died or were missed from any sampling points were removed from analysis.

In addition to assessing the growth performance, the endocrine profile of fish was also examined. At each sample point eight untagged fish per replicate tank were randomly selected, anaesthetized and killed by a single blow to the head. Blood was withdrawn from the caudal peduncle, centrifuged and the plasma stored at -70°C until analysis. Plasma growth hormone (GH) levels were measured using a double-antibody homologous radioimmunoassay (Section 2.5.1.) as previously described by Le Bail *et al.* (1991).

3.3.3. Statistical Analyses

Growth and feeding data, with exception to the feed intake data from Experiment I, was analysed by ANOVA using the General Linear Model (GLM) feature, where time and treatment were used as the categorical predictors and replicate nested within the treatment factor. Tukey's *post hoc* comparison tests were applied to determine differences between treatment groups. A malfunction with the X-ray machine in Experiment I resulted in only one replicate tank from each treatment being examined on the 22nd July and 7th August. Thus, feed data from Experiment I was analysed using a one-way ANOVA with treatment as the dependent factor. All data were tested for normality of distribution and homogeneity of variance by the Kolmogorov-Smirnov test

and from examination of the residual plots. Where necessary, data was transformed using the natural logarithm, square root or arcsine transformation to improve normality conformation. Replicate tanks were found not to differ significantly ($P>0.05$) for all parameters measured and were therefore pooled. A minimum significance level of $P<0.05$ was applied to all tests performed. All data are presented as mean \pm SEM.

3.3. Results

3.3.1. Light Distribution

Light readings recorded during the trial clearly showed a distinct difference between the two intensity treatments (Figure 3.2.). For both treatments, irradiance levels were greatest just below the water's surface at the centre of the tank directly beneath the light source with 8 W.m^{-2} (4200 lux) recorded for the 28W treatment compared to 1.15 W.m^{-2} (520 lux) in the 16W treatment tanks. Irradiance levels decreased through an increase in water depth with tank floor values of 0.31 W.m^{-2} (117 lux) and 0.05 W.m^{-2} (18 lux) measured in the 28W and 16W treatment tanks respectively. Similarly, regardless of depth, light intensity values decreased from the centre to the periphery of the tank. No light was detected during the dark phase in the SD (Experiment I) or SNP (Experiment II) tank groups.

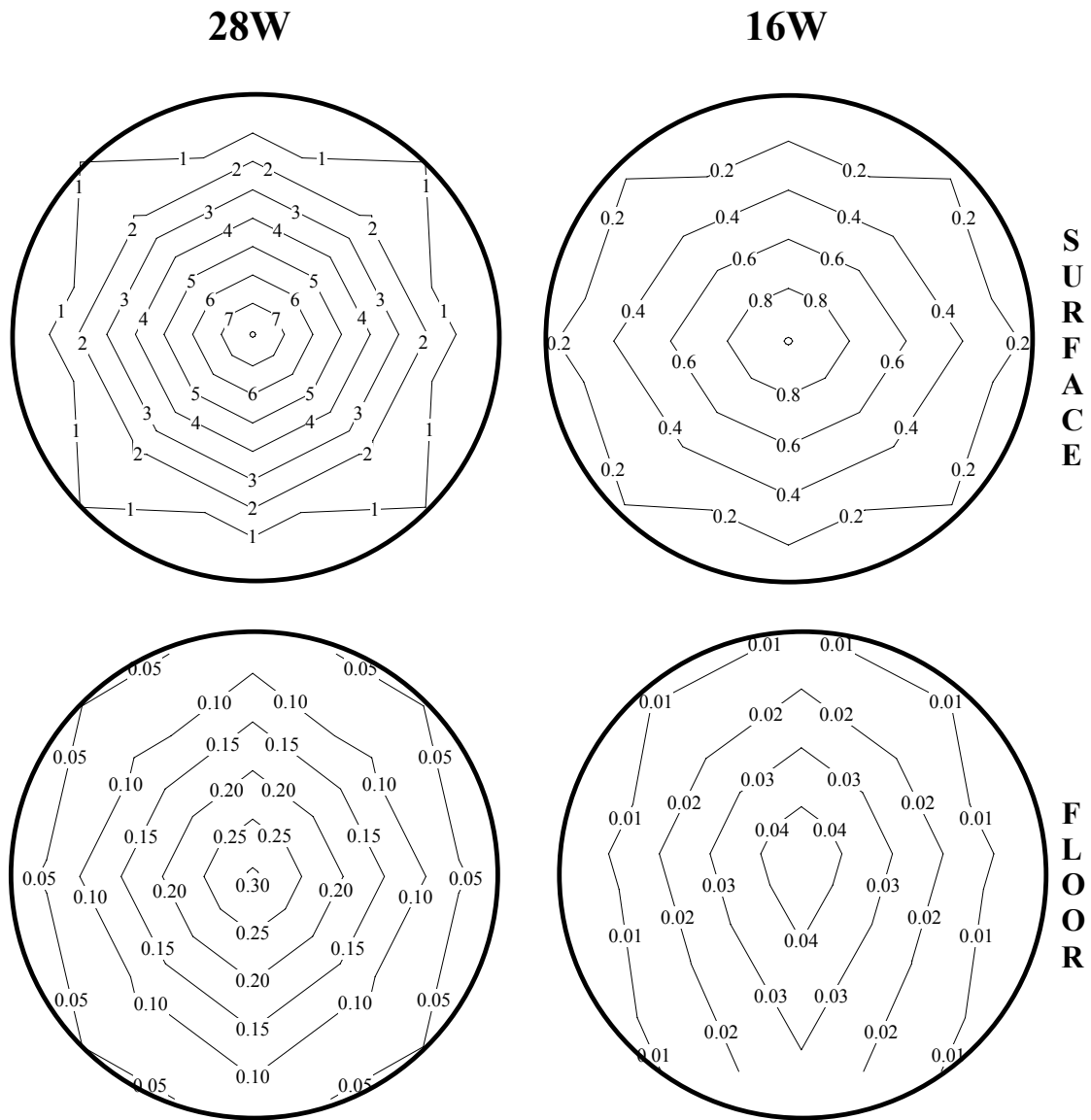


Figure 3.2. Irradiance levels ($\text{W}\cdot\text{m}^{-2}$) for the different intensity tank treatments (16W and 28W). Light readings are taken from just below the water surface and on the tank floor. Light source positioned at the centre of the tank, 20 cm above water surface.

3.3.2. Experiment I: Summer Growth

Feed intake

The initial mean feed intake of fish in all treatments was around 26 mg.g fish⁻¹ (Figure 3.3.). At the first sampling point, one day following light onset, feed intake for all treatments remained unchanged. However, at the 16th July sampling interval food consumption decreased in all treatments, although fish in the 28W LL treatment (22.4 ± 1.8 mg.g fish⁻¹) consumed significantly more than fish reared under 16W LL (22.4 ± 1.8 mg.g fish⁻¹). Feed intake then remained stable within the control (16W SD) at around 18-20 mg.g fish⁻¹. However, food consumption in the 28W LL treatment decreased on the 29th July resulting in a significantly lower food consumption than the 16W SD. Feed intake in the 28W SD treatment also showed a gradual decline, whereas the 16W LL exhibited an increase from the 16th July (4.1 ± 0.2 mg.g fish⁻¹) to the 7th August (6.3 ± 0.6 mg.g fish⁻¹). This resulted in both the 16W SD and 16W LL treatments having a significantly higher food consumption than the 28W SD. At the final sample point on the 18th August, mean feed intake levels for all groups were between 16-18 mg.g fish⁻¹.

Growth

At the start of the experiment fish had statistically similar mean wet weights, ranging between 166 g to 187 g (Figure 3.4.; Table 3.3.). Growth increased in a linear fashion throughout the experiment resulting in final wet weights of 408.9 ± 12.6 g (16W SD), 401.1 ± 20.0 g (28W SD), 384.8 ± 11.2 g (16W LL) and 348.9 ± 20.5 g (28W LL). No significant differences between treatment groups were observed at any of the sampled time points throughout the trial. However, the relative weight gain for the trial period illustrates a better growth performance for fish maintained under a SD photoperiod, with a 10-20% improvement over the LL regimes (Table 3.3.). Furthermore, when the final weight data was analysed by one-way ANOVA the 28W LL treatment weighed significantly less than both the SD photoperiods.

No significant differences were observed in the weight specific growth rate (SGR_w) of tagged individuals (Figure 3.5.), although both constant light treatments

appeared to exhibit a lower SGR_W than their control counterparts. Mean SGR_W of individually tagged fish from the 16W SD (control group) remained relatively constant during the first four sampling periods to August before decreasing with the decrease in temperature. However, within the 28W SD and in particular the LL regimes the mean SGR_W fell from $1.0 \text{ bw}\cdot\text{day}^{-1}$ at the start of the trial to around $0.4\% \text{ bw}\cdot\text{day}^{-1}$ during late July.

Condition factors (K) of the individually tagged fish at the start of the trial were between 1.12 and 1.18 (Figure 3.6.). Mean K generally increased over the course of the trial with all treatments exhibiting a similar pattern, except between the 24th June and 16th July when both the LL treatments failed to show any increase in K . Condition factors at the final sample point on the 17th September were 1.34 ± 0.02 (28W LL), 1.35 ± 0.01 (16W LL), 1.35 ± 0.02 (16W SD) and 1.39 ± 0.05 (28W SD) respectively. However, no significant differences were observed between treatments at any of the sampled intervals, although one interesting observation to note is that during the July sample periods fish condition appeared to be in a photoperiod and intensity order (e.g. 16W SD, 28W SD, 16W LL and 28W LL).

Table 3.3. Treatment weight gain (g) and relative weight gain (%) of rainbow trout reared at an intensity of 16W or 28W under a 9L:15D short-day photoperiod (16W SD and 28W SD respectively) or exposed to constant light (16W LL and 28W LL respectively) between 3rd June and 17th September 2003. Values in parenthesis are from replicate tanks. Values with identical superscripts in the same column are not statistically different ($P>0.05$; ANOVA).

Treatment	Trial Weight Gain			Relative weight gain (%)
	Initial weight (g)	Final weight (g)	Weight gain (g)	
16W SD	186.1 ^a (178.8, 193.1)	408.9 ^a (389.6, 427.1)	226.6 (210.8, 234.0)	119.7 (117.9, 121.2)
28W SD	172.0 ^a (170.2, 173.8)	401.1 ^a (400.0, 403.6)	229.1 (229.4, 229.8)	133.2 (134.8, 132.2)
16W LL	187.4 ^a (183.6, 191.5)	384.8 ^{ab} (372.0, 398.0)	182.4 (189.3, 206.5)	105.3 (103.1, 107.8)
28W LL	166.5 ^a (161.0, 171.7)	348.9 ^b (335.4, 361.5)	197.4 (174.4, 189.9)	109.5 (108.4, 110.6)

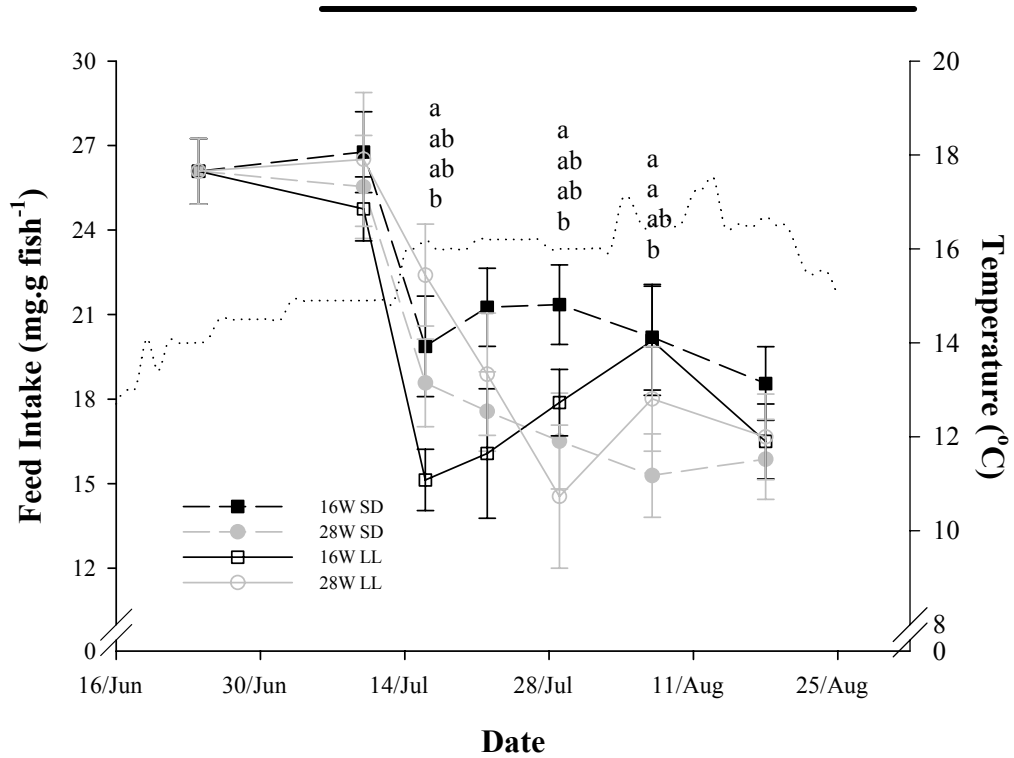


Figure 3.3. Mean feed intake ($\text{mg.g fish}^{-1} \pm \text{SEM}$) of rainbow trout reared at an intensity of 16W or 28W under a 9L:15D short-day photoperiod (16W SD and 28W SD respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 9th July 2003). Means bearing identical lettering are not statistically different ($P > 0.05$; ANOVA) between treatments at given time points. Due to an X-ray malfunction, only one replicate of each treatment was examined on the 22nd July and 7th August.

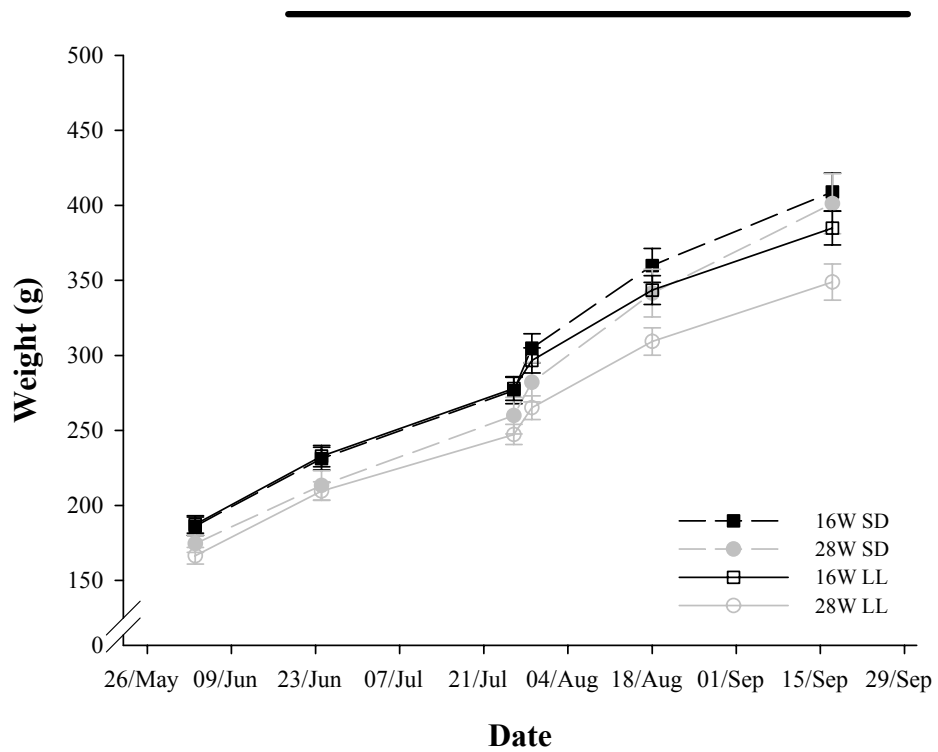


Figure 3.4. Mean wet body weights ($g \pm SEM$) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a 9L:15D short-day photoperiod (16W SD and 28W SD respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 9th July 2003). No statistical differences were evident between treatments at given time points ($P > 0.05$; GLM).

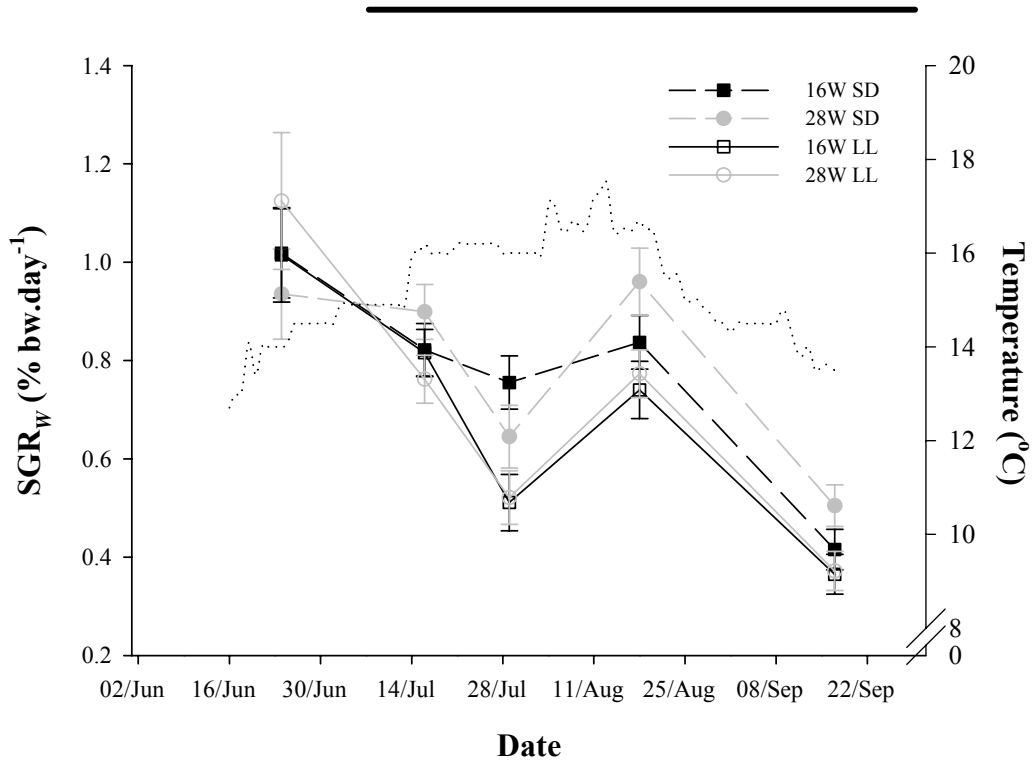


Figure 3.5. Mean specific growth rate (SGR, % bw.day⁻¹ ± SEM) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a 9L:15D short-day photoperiod (16W SD and 28W SD respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 9th July 2003). No statistical differences were evident between treatments at given time points ($P>0.05$; GLM). Broken line indicates ambient water temperature (°C).

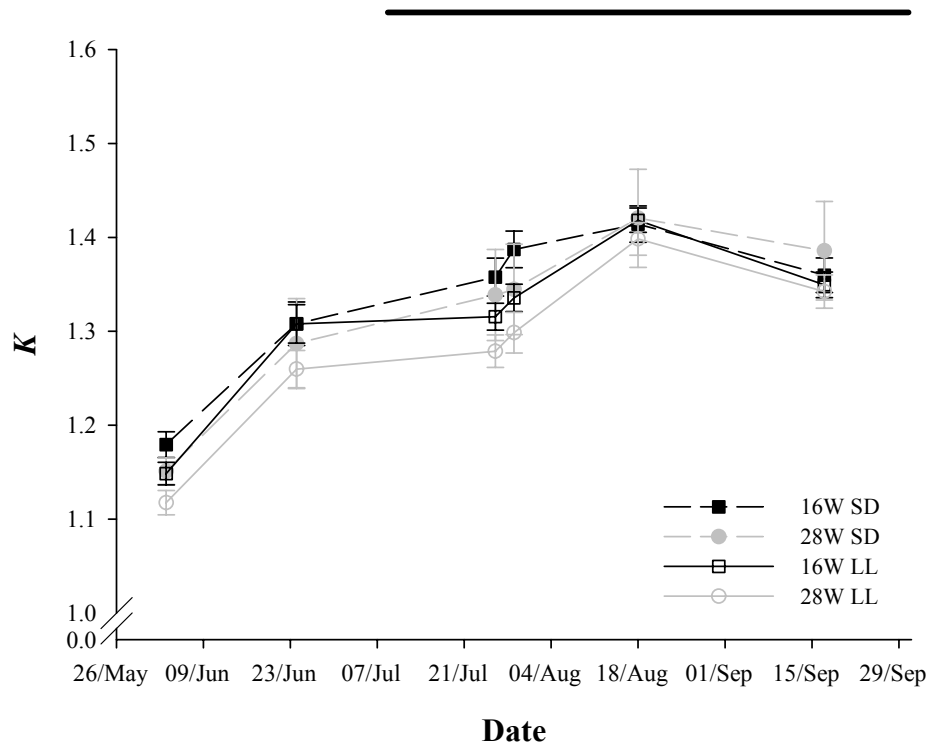


Figure 3.6. Mean condition factor ($K \pm \text{SEM}$) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a 9L:15D short-day photoperiod (16W SD and 28W SD respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 9th July 2003). No statistical differences were evident between treatments at given time points ($P > 0.05$; GLM).

3.3.3. Experiment II: Winter Growth

Feed intake

The mean food consumption of individually tagged fish generally followed the ambient water temperature (Figure 3.7.). Prior to the onset of light, the mean feed intake level for all groups ranged from 11-13 mg.g fish⁻¹. However, at the 3rd December sample point, feed intake in the 16W SNP (control) decreased to less than 6 mg.g fish⁻¹, with the decrease in water temperature, whereas both the LL regimes and the 28W SNP exhibited a minor decrease and were significantly higher than the control. Food consumption in the 28W LL regime remained significantly higher than the 28W SNP group at the 16th December and 8th January sampling points. Thereafter, feed intake levels for all groups fell to around 2 mg.g fish⁻¹ at the 15th January sample. Feed intake then gradually increased towards the end of the trial reaching 6.6 ± 0.5 mg.g fish⁻¹ (28W SNP), 6.7 ± 0.7 mg.g fish⁻¹ (16W SNP) and 6.9 ± 0.6 mg.g fish⁻¹ (16W LL) at the final sample point in late April. However, feed intake of fish in the 28W LL regime reached 11.9 ± 0.9 mg.g fish⁻¹, resulting in a significantly higher feed consumption than all other treatments.

Growth performance

Mean wet body weights of individually tagged rainbow trout exhibited a similar pattern of growth (Figure 3.8.), increasing from 76 g in October to 534.8 ± 12.4 g (28W SNP), 550.7 ± 12.7 g (16W SNP), 570.0 ± 11.3 g (28W LL) and 583.3 ± 18.9 g (16W LL) at the final sampling point in July. Consequently, no significant differences were found between treatments, irrespective of intensity or photoperiod, at any of the sampled time points throughout the trial. However, the relative weight gain of treatment fish for the trial period showed that both the LL treatments (647.1% and 661.5%, 28W and 16W LL respectively) had a better growth performance than their SNP (600.8% and 629.4%, 28W and 16W SNP respectively) counterparts (Table 3.4.). Additionally, when the weight data of the final sample point was analysed by one-way ANOVA, as compared to the general linear model, the 16W LL treatment was found to be significantly heavier than the 28W SNP group.

Significant differences were however found in the mean weight specific growth rates (SGR_W) of fish. Initial SGR_W for all treatments, prior to the addition of treatments, were around 0.9% $bw.day^{-1}$ (Figure 3.9). At the first sample point, following constant light exposure, the SGR_W for all treatments fell although both LL groups displayed the greatest decline falling to around 0.10 $bw.day^{-1}$. The SGR_W for the 28W SNP group however, decreased to only $0.29 \pm 0.03\%$ $bw.day^{-1}$, a significantly higher growth rate than measured for the 28W LL regime ($0.12 \pm 0.02\%$ $bw.day^{-1}$) regime. At the subsequent sample point, both LL treatments appeared to exhibit a higher growth rate ($\sim 0.4\%$ $bw.day^{-1}$) than the control treatments ($\sim 0.27\%$ $bw.day^{-1}$), although these differences were not significant. Growth rates then stabilized around 0.30% $bw.day^{-1}$ between January and March. At the late April sample point the SGR_W of the 16W LL regime ($0.55 \pm 0.04\%$ $bw.day^{-1}$) was significantly higher than the 16W SNP group ($0.42 \pm 0.04\%$ $bw.day^{-1}$). The SGR_W increased towards the end of the trial, peaking in June at around 1.10 % $bw.day^{-1}$, with the growth rate significantly higher in the 16W SNP group as compared to the 28W LL regime, before decreasing to around 0.7% $bw.day^{-1}$ for all treatments. Similar to feed intake, growth rates also showed a pattern to follow the seasonal ambient water temperature.

Table 3.4. Treatment weight gain (g) and relative weight gain (%) of rainbow trout reared at an intensity of 16W or 28W under a simulated natural photoperiod (16W SNP and 28W SNP respectively) or exposed to constant light (16W LL and 28W LL respectively) between 1st December and 18th July 2004. Values in parenthesis are from replicate tanks. Values with identical superscripts in the same column are not statistically different ($P>0.05$; ANOVA).

Treatment	Trial Weight Gain			Relative weight gain (%)
	Initial weight (g)	Final weight (g)	Weight gain (g)	
16W SNP	75.5 ^a (74.8, 76.2)	550.7 ^{ab} (540.9, 560.6)	475.2 (451.1, 484.4)	629.4 (623.0, 635.7)
28W SNP	76.3 ^a (75.8, 76.9)	534.7 ^b (522.1, 547.6)	458.4 (446.4, 470.7)	600.8 (589.1, 612.2)
16W LL	76.6 ^a (76.3, 77.0)	583.3 ^a (579.9, 588.3)	506.7 (503.6, 511.3)	661.5 (659.6, 664.0)
28W LL	76.3 ^a (74.7, 77.9)	570.0 ^{ab} (554.2, 586.4)	493.7 (479.6, 508.5)	647.1 (642.2, 652.7)

Mean condition factor (K) of tagged individuals appeared to be different at the start of the trial, ranging from 1.36 to 1.42, although differences were not significant (Figure 3.10.). As with growth all treatments exhibited a similar pattern in K , decreasing to around 1.40 between late December and May and increasing to around 1.50 towards the end of the trial in September 2004. However no significant differences were evident between treatments at any of the sample time points, although there was a tendency for 28W LL fish to remain higher than all other treatments, especially for April 2004.

Growth hormone

Plasma growth hormone (GH) levels from the two extreme treatments, *i.e.* 16W SNP and 28W LL, were analysed from pre-selected sample points for differences (Figure 3.11.).

Plasma GH profiles exhibited a similar pattern between treatments, increasing from around 0.2 ng.ml⁻¹ in December to around 0.7 ng.ml⁻¹ at the end of the trial in August. However, no significant differences were found between treatments at any of the analysed time points.

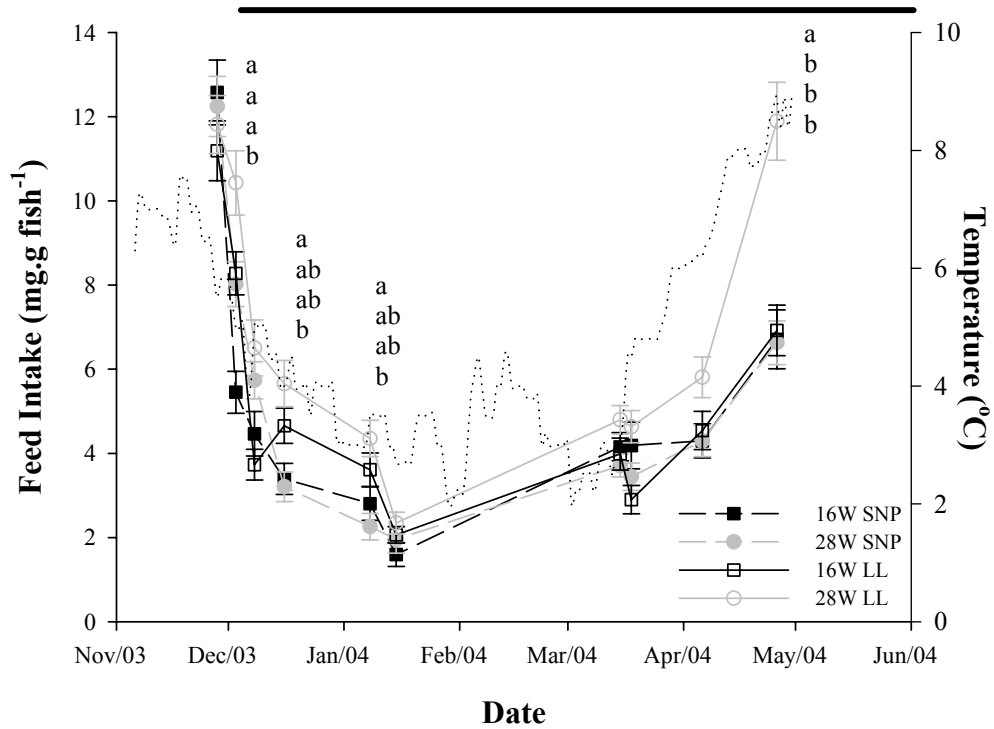


Figure 3.7. Mean feed intake ($\text{mg.g fish}^{-1} \pm \text{SEM}$) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a simulated natural photoperiod (16W SNP and 28W SNP respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 1st December 2003). In some instances error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$; GLM) between treatments at given time points. Broken line indicates ambient water temperature ($^{\circ}\text{C}$).

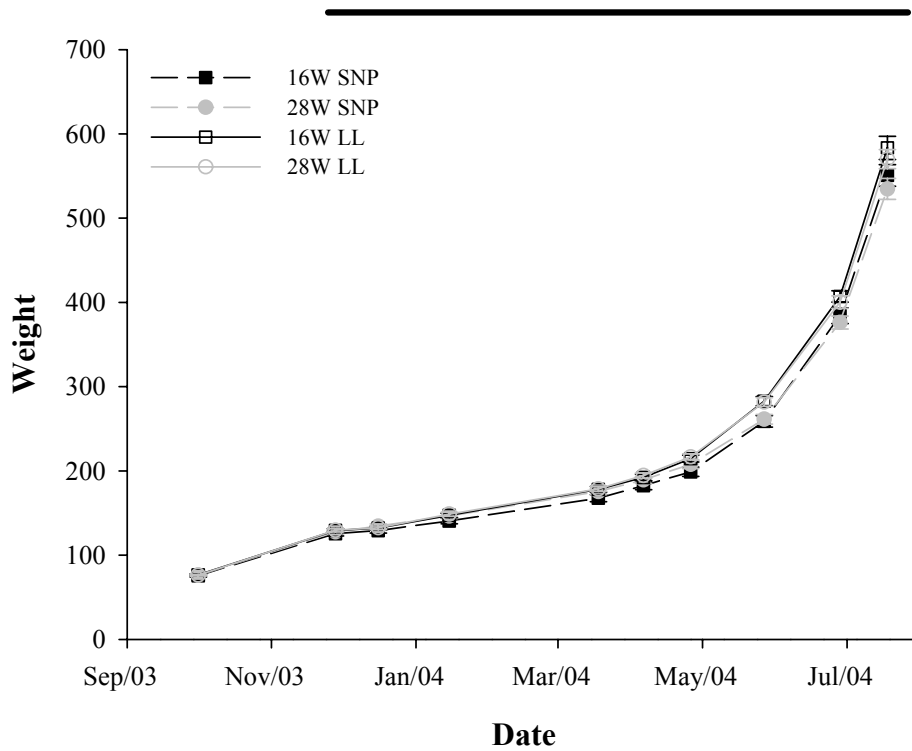


Figure 3.8. Mean body weights ($g \pm SEM$) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a simulated natural photoperiod (16W SNP and 28W SNP respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 1st December 2003). In some instances error bars are too small to be depicted. No statistical differences were evident between treatments at given time points ($P > 0.05$; GLM).

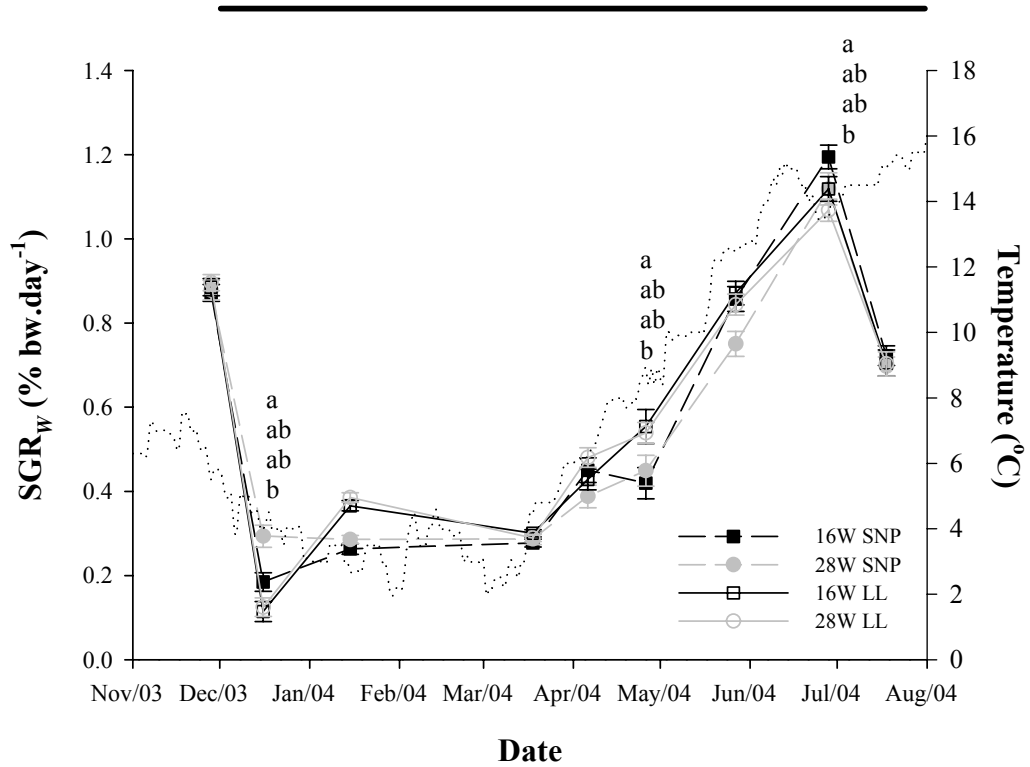


Figure 3.9. Mean specific growth rate (SGR, % bw.day⁻¹ ± SEM) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a simulated natural photoperiod (16W SNP and 28W SNP respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 1st December 2003). In some instances error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P>0.05$; GLM) between treatments at given time points. Broken line indicates ambient water temperature (°C).

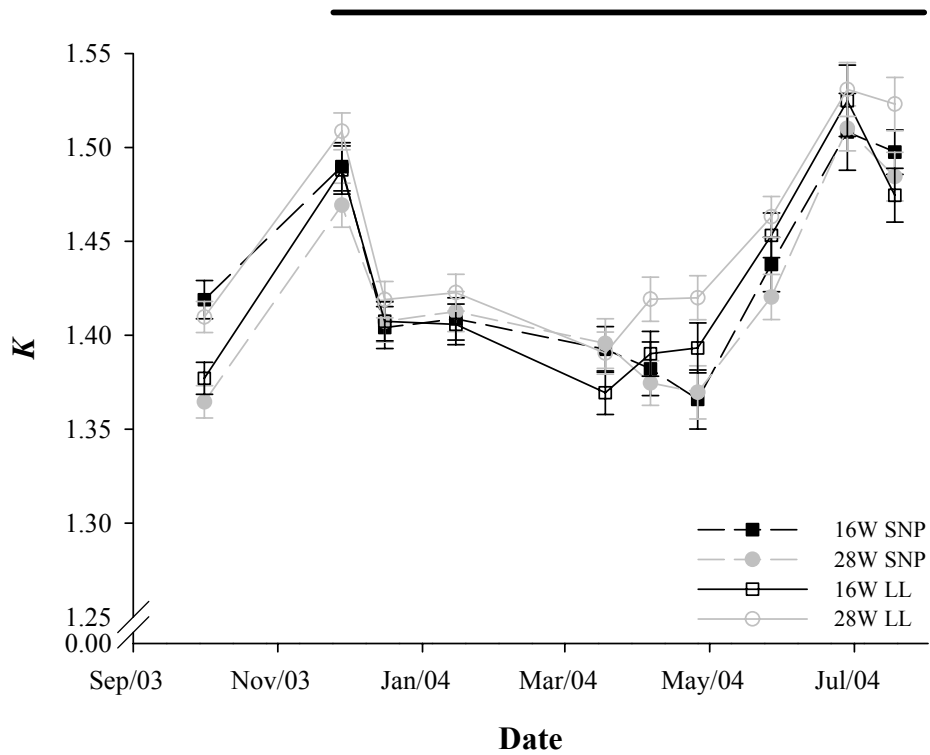


Figure 3.10. Mean condition factor ($K \pm \text{SEM}$) of individually tagged rainbow trout reared at an intensity of 16W or 28W under a simulated natural photoperiod (16W SNP and 28W SNP respectively) or exposed to constant light (16W LL and 28W LL respectively). Horizontal bar indicates duration of constant light exposure (from 1st December 2003). In some cases error bars may be too small to depict. No statistical differences were evident between treatments at given time points ($P > 0.05$; GLM).

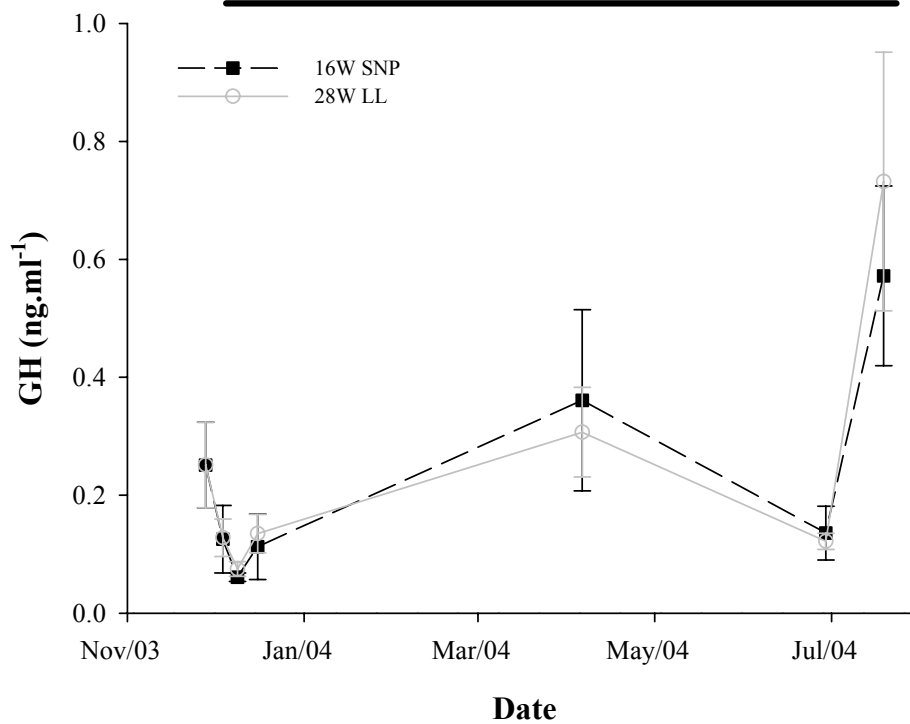


Figure 3.11. Mean plasma growth hormone levels ($\text{ng.ml}^{-1} \pm \text{SEM}$) of rainbow trout reared under a 16W simulated natural photoperiod (16W SNP) or exposed to 28W constant light (28W LL). Horizontal bar indicates duration of constant light exposure (1st December 2003). No statistical differences were evident between treatments at given time points ($P > 0.05$; GLM). $N = 16$ fish per treatment per time point.

3.4. Summary of Results

In summary, the results from the experiments demonstrate the following:

- Feeding and growth appeared to follow the seasonal water temperature profile.
- Fish exposed to LL exhibited a lower feed intake and appeared to have a lower growth rate than control fish (16W SD) in Experiment I. Additionally, fish exposed to 28W LL in Experiment II exhibited a significantly lower growth rate than their 28W SNP counterparts following the onset of light.
- No significant effect of constant light on growth was observed throughout the trial when examined by GLM. However, one-way ANOVA on final weight data revealed that 28W LL fish weighed significantly less than SD fish following a 12-week exposure period (Experiment I), whereas 16W LL fish were significantly heavier than 28W SNP fish after 7 months LL exposure (Experiment II).
- The relative weight gain for fish exposed to constant light showed a reduction over the trial in Experiment I, whereas an increase was observed in Experiment II.
- Light intensity, irrespective of photoperiod, had no overall effect on growth, although feed intake of the 28W LL appeared highest throughout Experiment II.
- Plasma growth hormone levels of fish reared under the different photoperiod regimes tested in Experiment II (*i.e.* 16W SNP and 28W LL) demonstrated similar profiles with no significant differences detected.

3.4. Discussion

Seasonally changing photoperiod and temperature profiles are known to influence the feeding and growth patterns of salmonid fish. Photoperiod has primarily been applied to the aquaculture industry as a means of controlling egg production (*e.g.* Duston and Bromage, 1988), producing out-of-season smolts (*e.g.* Solbakken *et al.*, 1994; Duston and Saunders, 1995; Oppedal *et al.*, 1999), to inhibit maturation (*e.g.* Hansen *et al.*, 1992; Endal *et al.*, 2000), or enhance growth (*e.g.* Saunders and Harmon, 1988; Villarreal *et al.*, 1988; Kråkenes *et al.*, 1991; Taylor *et al.*, 2006). However, the timing and application of photoperiod manipulations tend to occur around the significant life-stages of fish (*e.g.* smoltification and maturation processes), often making it difficult to distinguish between the photoperiodic and natural physiological effects. In particular is the question of light manipulation on growth enhancement as commonly reported in Atlantic salmon, either as a result of a direct stimulus of the photoperiod treatment or a consequence of an altered physiological response. Moreover, appetite and growth depressions are commonly reported following exposure to constant artificial light (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjelldal *et al.*, 2005). Since both photoperiod and temperature exhibit similar seasonal profiles it is difficult to establish the interaction of temperature on the photoperiod entrainment rhythms of fish. Thus, the current investigation sought to examine the application of constant light at two different times of the year on the feeding and growth responses of juvenile freshwater rainbow trout. In addition, photoperiod treatments were further examined at two different light intensities on feed and growth performance.

3.4.1. Feed intake

The feed consumption and subsequent growth of the fish generally showed a trend to follow the ambient water temperature profile, irrespective of photoperiod. This would agree with the findings of Kavadias *et al.* (2003) who found that the growth rate of European sea bass was related to feed intake and water temperature. However, constant

light exposure has been reported to stimulate feed intake in Atlantic salmon (Handeland *et al.*, 2003; Nordgarden *et al.*, 2003) and largemouth bass, *Micropterus salmoides* (Petit *et al.*, 2003) or improve the feed conversion efficiency in trout (Taylor *et al.*, 2006), salmon (Nordgarden *et al.*, 2003) and Atlantic halibut, *Hippoglossus hippoglossus* (Jonassen *et al.*, 2003). In Experiment II, the 28W LL regime exhibited a higher food consumption throughout the trial than all other treatments. However, unlike the other investigations where feed intake was stimulated, no improved growth response was observed. This increase in food consumption may compensate for a higher metabolic rate and greater locomotor activity (Imstrand *et al.*, 1996; Petit *et al.*, 2003). Godin (1981) for example, found that the mean swimming speed of pink salmon, *Oncorhynchus gorbuscha*, increased significantly with an increasing intensity of constant light. Thus, despite exhibiting a higher feed consumption no differences in growth were observed possibly due to an altered activity pattern.

Within the seawater stage of the salmon industry there are frequent reports of a characteristic growth dip following the addition of constant artificial light. In the current trial no real evidence for a feed dip was observed, although during Experiment I fish under constant light, irrespective of intensity, exhibited a sharp decline in feed intake and were found to differ significantly from the 16W control. However, it is perhaps more likely that these differences were due to the normal day-to-day variation in feed intake, also experienced in the 28W SD treatment. In fact, in both experiments the 28W LL group had the highest feed intake following the onset of constant light, and was found to consume significantly more than the 16W LL treatment in both cases. If a growth-dip were to be observed under constant light, then the greatest effect would be expected to occur in the 28W regime since light intensity was higher within this group. It has been postulated that the growth-dip is an initial stress response to the onset of light (Endal *et al.*, 2000). It is therefore possible that there is a species-specific reaction to stress response. McCarthy *et al.* (1993) reported that a handling stress resulting from the X-ray procedure does not affect the total amount of food eaten by a sampled group of trout following radiography, but does however suppress the appetite in Atlantic salmon

examined by the same procedure. Similarly, Jobling and Koskela (1996) found that rainbow trout return to normal feeding behaviours following a stress response. It is therefore possible that if the onset of light does result in a stress response that may lead to appetite suppression then rainbow trout may not be a suitable model. Thus, further studies should be performed on Atlantic salmon to examine the feeding responses to the onset of constant light exposure.

3.4.2. Growth

Although it has been well documented that the use of extended photoperiod regimes is beneficial to growth in Atlantic salmon (*e.g.* Saunders and Harmon, 1988; Villarreal *et al.*, 1988; Hansen *et al.*, 1992; Oppedal *et al.*, 1999; Endal *et al.*, 2000) and rainbow trout (*e.g.* Taylor *et al.*, 2005, 2006), no growth improvement was observed in any of the constant light regimes in the present study relative to control fish. The relatively short exposure period (8 weeks) to constant light in Experiment I may explain the lack of enhanced growth. Generally, growth deviations between lit and unlit populations of fish have been reported to occur following 12 to 17 weeks of constant light exposure (Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Taylor *et al.*, 2005), although Taylor *et al.* (2006) suggest that this appears to be dependent upon the timing of exposure to constant light and also the genetic strain of fish used. However, in Experiment II no significant growth enhancement was observed, despite an exposure period to constant light of around 8 months, although the relative weight gain would suggest otherwise. The timing of the onset of extended light is considered critical to altering physiological responses in fish. In an experiment performed on coho salmon, *Oncorhynchus kisutch*, McCormick *et al.*, (1992) exposed fish to constant light from late March. They failed to find any difference in the overall growth performance, suggesting that fish had already perceived the naturally increasing photoperiod prior to the application of light. The same may be true for the winter trial (Experiment II), whereby fish had already perceived the natural decrease in photoperiod prior to the onset of constant light. The onset of light in the present experiment (winter) was one to two months later than used in the trout industry,

where growth enhancement has been shown (Taylor *et al.*, 2005, 2006). However, in the summer investigation (Experiment I), fish were held under a 9L:15D photoperiod from the 29th February specifically to prevent them from perceiving a change in daylength. It is possible that this prolonged exposure to a short-day photoperiod resulted in fish altering their entrainment rhythms from daylength changes to changes in the ambient water temperature instead. Furthermore, constant short day exposure has been shown to delay rhythms such as maturation and spawning (*e.g.* Taranger *et al.*, 1998; see also Bromage *et al.*, 2001). Thus, in Experiment I of the present investigation the holding of fish under a constant short-day photoperiod (9L:15D), including those later exposed to LL, may have conceivably resulted in the delay of any endogenous growth rhythms and hence the lack of growth differences.

The interaction between temperature and photoperiod has been studied on numerous occasions, although there is still some controversy as to the exact role of temperature on fish physiology. Solbakken *et al.* (1994) and McCormick *et al.* (2000) both noticed a limited photoperiodic control of the smolting process at low temperatures, whereas Clarke *et al.* (1978) found that the changes in growth rate by photoperiod were more apparent at higher temperatures due to the rate-controlling effect of temperature. However, no photoperiodic effects were found under either of the temperature regimes. The lack of a growth response shown by the rainbow trout may be related to the fact that growth enhancements arising from photoperiod manipulations normally occur from the alteration of other physiological functions. In Atlantic salmon, where light manipulations are predominantly used, light application is typically applied around the time of developmental changes, such as smoltification and maturation. In maturing salmon for example, increases in sex steroid levels result in an anabolic effect of a faster growth rate and greater food consumption, fuelling reproduction, subsequently leading to fish attaining a greater weight than non-maturing fish (Kadri *et al.*, 1996). Whilst light application is used to inhibit maturation (*e.g.* Hansen *et al.*, 1992; Endal *et al.*, 2000), the increased growth rates observed under constant light exposure may be explained by the energy diverted away from reproductive into somatic growth. Nevertheless, Taylor *et al.*

(2005, 2006) have shown constant light exposure to enhance the growth of rainbow trout similarly to the current study. Thus, further studies are required in rainbow trout to fully examine the effects of light application on growth.

With respect to the growth-dip phenomenon frequently reported in the Atlantic salmon industry (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005), there appears only to be circumstantial evidence for a growth-dip in trout. Certainly, with respect to the relative weight gain observed between treatments in Experiment I the constant light treatments appeared to show a deficit in growth over the trial period as compared to the short-day (control) groups. However, one aspect of the growth-dip is that following the initial reduction there appears to be a period of rapid growth, through the manifestation of a compensatory growth response. Whilst the exposure time in Experiment I was too short to demonstrate any positive growth effects, the specific weight gain (SGR_w) of fish in Experiment II appeared to show signs of a dip and recovery. At the first sample point post light onset, both the constant light treatments exhibited a significantly lower SGR_w than the 28W SNP group. However, at the subsequent sampling point in January both the constant light groups appeared to be higher than the controls, although the differences in SGR_w were not significant. These types of observations are in accordance with those made by farmers within the salmon industry (Clive Talbot, personal communication). Endal *et al.* (2000) postulate that this depression may be the result of a direct stress response to the onset of light or as a phase advancement of a circannual growth pattern adjusted by photoperiod. However, stress generally results in a loss of appetite and all groups appeared to display a similar feed intake pattern following light onset, with the 28W LL regime consuming significantly more over the experimental period than any other treatment. Furthermore, the similarities in growth patterns and condition factor suggest against a phase advancement or direct photostimulation of growth. Thus, further studies using constant water temperatures are required to fully understand the extent of the photoperiodic effect on feed intake and growth. Such studies were not possible within the current facilities

used in this trial. In addition, trials should be conducted on Atlantic salmon where observations for lower growth and feeding responses are commonly observed.

It must be noted that the type of statistical test applied appeared to affect the result. In the present investigation, a General Linear Model (GLM) was employed to investigate the difference in the feeding and growth responses of individually tagged fish. The GLM is a powerful statistical test incorporating all the collected data and accounting for numerous factor levels. This was used to investigate time and treatment effects as well as to examine the interaction between time and treatment over the trial period. The ANOVA however, only compared the mean values of data for each tested time point, consequently pulling out more differences. This was shown using the final sample weight data from both experiments, where significant differences were found by using the ANOVA but not from the GLM. The decision to use the GLM in the current trial was to follow the individually tagged fish throughout the trial, examining both the changes over time as well as between treatments. Thus, even though both tests were applied at the same significance level ($P < 0.05$), the type of statistical test applied was shown to affect the results which may have subsequent implications for the comparison and analysis of published data.

Light intensity

Throughout both experiments, no effect of light intensity on growth was evident, supporting earlier studies performed on salmon (Stefansson *et al.*, 1993; Oppedal *et al.*, 1999). Nevertheless, the current results are contrary to previous studies performed on rainbow trout (Cho, 1992b; Taylor *et al.*, 2006). Cho (1992b) for example, found that trout reared in enclosed tanks grew better with a light intensity of 1600 lux at the water surface compared to fish exposed to an intensity of 100 lux. In the present investigation, light intensity recorded at the water's surface was 4200 lux (8 W.m^{-2}) and 520 lux (1.15 W.m^{-2}) for the 28W and 16W treatments respectively. Recent studies have proposed that a light intensity threshold must exist in fish which must be exceeded for physiological functions to be affected (Oppedal *et al.*, 1997; Porter *et al.*, 1999, 2001; Migaud *et al.*,

2006a). In the case of the current trial, it is possible that the difference between the highest and lowest intensity treatments (8 times) was either above this threshold or that the difference in intensity between treatments was insufficient to induce any growth responses, whereas in Cho's study the difference in intensity (16 times) may have been sufficient or alternatively above and below the threshold level. It is of relevance to mention that the photometric (lux) measurement of light intensity is based on the same spectral sensitivity as the human eye and is therefore inappropriate for underwater light measurements due to the dynamic changes that occur in the spectral quality of light. It is more practical to measure the irradiance of light, expressed as watts per metre square ($\text{W}\cdot\text{m}^{-2}$), which measures the energy content of light arriving at given area surface. Migaud *et al.* (2006a) have recently calculated the intensity threshold level to be around $0.016 \text{ W}\cdot\text{m}^{-2}$ in Atlantic salmon and between $3.8 \times 10^{-5} \text{ W}\cdot\text{m}^{-2}$ and $3.8 \times 10^{-6} \text{ W}\cdot\text{m}^{-2}$ for the European sea bass. Applying the salmon intensity threshold as an indicator for trout in the current investigation, then the lowest intensity readings obtained from the tank floor, $0.31 \text{ W}\cdot\text{m}^{-2}$ and $0.05 \text{ W}\cdot\text{m}^{-2}$ for the 28W and 16W intensities respectively, would suggest that both the intensity treatments used in the current trial were above this threshold value explaining the lack of growth differences between intensity treatments. Furthermore, one further problem associated with light studies is that the spectral content of the light emitted by the artificial light systems is altered depending on the water conditions, with shorter wavelengths not penetrating freshwater well due to peatiness (Taylor *et al.*, 2006). However, as previously suggested the effect of the ambient water temperature on the growth of trout may again have overridden any photoperiodic effect, regardless of intensity applied. Thus, further studies are required using constant water temperatures.

A further indicator of the perception of light by fish would be through the assessment of plasma melatonin levels, a hormone which responds to the prevailing light conditions. Porter *et al.* (2001) demonstrated that melatonin levels were affected by both light intensity and water temperature, although the role of melatonin on the physiological axis remains to be elucidated. However, unfortunately within the current experiments no

melatonin samples were taken leaving an unclear picture as to how light was perceived in the treatments. Thus, it is suggested that further studies examining the effect of light (intensity, spectrum or photoperiod) on the physiology of fish should include melatonin as an indicator of the level of light perception.

3.4.3. Summary

The present investigation was undertaken to examine the effects of photoperiod manipulation on the feeding and growth responses of juvenile rainbow trout when applied at two different temperature profiles. However, constant light exposure failed to induce any significant growth effects, regardless of temperature, indicating that extended photoperiods may have a limited effect on the growth of rainbow trout, or that the prevailing water temperature at which photoperiod manipulations are applied may override the photoperiodic effect. However, this was dependent upon the statistical test used, with the ANOVA finding differences in body weights at the final time points whereas GLM's did not. Additionally, the onset of constant light did appear to at least initially affect the growth rate of individually tagged fish indicating a possible growth-dip, and was further reflected through changes in appetite (decrease Experiment I, increase Experiment II). However, body weights and hormonal profile remained unaffected. Light intensity, irrespective of photoperiod, similarly had no major affect on growth. This may be a result of applying intensities above the intensity threshold levels of rainbow trout or due to the lack of photoperiodic response by an override of temperature.

The main body of uncertainty surrounding photoperiod use (*e.g.* growth-dip, effect of light on maturation) stems from the Atlantic salmon industry. Although both the rainbow trout and Atlantic salmon are members of the same family, the differences in the rearing environment (*e.g.* fluctuation in temperatures, water quality *etc.*) and the possibility of species-specific responses to different environmental variables suggest that investigations related to questions on photoperiod manipulations should be performed on the species in question.

Chapter 4: The effects of artificial light regimes on the appetite, growth and stress responses in 1+ Atlantic salmon post-smolts reared in seawater tanks

4.1. Introduction

The environmental light-dark cycle provides a potential means through which daily and seasonal rhythms may be entrained (Ekstrom and Meissl, 1997; Bouef and Le Bail, 1999; Falcón, 1999; Boeuf and Falcón, 2001; Bromage *et al.*, 2001). Information on the daily and seasonal calendar time in salmonid fish is conveyed through the pineal gland and its shifting pattern of melatonin secretion in response to the prevailing light conditions (Randall *et al.*, 1995; Porter *et al.*, 1998, 1999, 2001; Bromage *et al.*, 2001). Throughout the winter period of the Atlantic salmon (*Salmo salar*) on-growing stage, constant light regimes are routinely applied as a tool for reducing the incidence of early maturation (Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1998, 1999; Oppedal *et al.*, 1997; Porter *et al.*, 1999; Endal *et al.*, 2000) and to increase the rate of growth (Saunders and Harmon, 1988; Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Forsberg, 1995; Oppedal *et al.*, 1997, 2003; Porter *et al.*, 1999, 2000; Endal *et al.*, 2000; Nordgarden *et al.*, 2003; Fjellidal *et al.*, 2005). In practice however, the outcomes of photoperiod manipulations are variable and unpredictable due to many reasons including the timing and duration of exposure to artificial light, the intensity of the light source, and genetic and environmental interactions. Recently, some salmon farmers throughout the industry have reported the occurrence of a characteristic ‘growth-dip’ following the superimposition of continuous artificial illumination in sea cages, resulting in a suppressed appetite and growth for up to 12 weeks. Thereafter, a compensatory growth response is said to occur such that fish held under constant light may be equal to or larger than their conspecifics maintained under a natural photoperiod. Similar observations have also been reported in salmonids under experimental conditions for both cage (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Fjellidal *et al.*,

2005) and tank-based populations (Taranger *et al.*, 1995; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003).

Although the appetite and growth of fish are linked to the seasonal changes in the natural photoperiod and temperature (Higgins and Talbot, 1985; Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999), their influence will most likely be mediated through the endocrine system (Duan, 1998; Company *et al.*, 2001). The endocrine regulation of growth is a complex process centrally mediated by the somatotropic axis involving growth hormone (GH), insulin-like growth factor-I (IGF-I), their specific receptors and a series of binding proteins (Brier, 1999; Company *et al.*, 2001). Growth hormone, also termed somatotropin, is involved in the regulation and maintenance of somatic growth and other metabolic processes (Björnsson, 1997) and can act both directly on target tissues but also by stimulating the liver to synthesize IGF-I. GH is a key regulator of growth in salmonids (Björnsson, 1997). Exogenous GH administration for example, has been shown to lead to higher growth rates (Johnsson and Björnsson, 1994; Johnsson *et al.*, 1996; Björnsson, 1997; Silverstein *et al.*, 2000) through increased food consumption as well as improving the food conversion efficiency (Johnsson and Björnsson, 1994; Johnsson *et al.*, 1996; Jönsson *et al.*, 1998). Furthermore, GH is also affected by nutritional status with periods of fasting or feed restriction elevating circulating levels (Sumpter *et al.*, 1991; Farbridge and Leatherland, 1992; Leatherland and Farbridge, 1992; Pottinger *et al.*, 2003). Elevated plasma GH levels during the salmonid parr-smolt transformation are important for the subsequent hypoosmoregulatory ability in seawater (Komourdijan *et al.*, 1976; Björnsson *et al.*, 1998). This observation of increased GH levels during smolting has led to the suggestion that GH is under the influence of a seasonally changing photoperiod (Björnsson *et al.*, 1995; Björnsson, 1997). This has been demonstrated through comparisons of GH levels under photoperiod manipulations used in spring (1+, yearling) and autumn (0+, underyearling) smolt production (Björnsson *et al.*, 2000), although spring increases have also been detected in adult Atlantic salmon (Björnsson *et al.*, 1994; Nordgarden *et al.*, 2005). These effects are likely to be most effective when working in synergy with IGF.

Insulin-like growth factor-I, a 70 amino acid (~7.6 kDA) single chain polypeptide, is involved in the regulation and development of somatic growth of all vertebrates. IGFs are somatomedins and are similar in structure to insulin. However, unlike insulin, IGFs circulate in the blood plasma complexed to a family of structurally related binding proteins that protect the IGFs from degradation as well as modulating its actions (Jones and Clemmons, 1995). IGF-I is involved in the regulation of development and growth in fish by mediating the biological effects of GH for cell growth differentiation and metabolism (Duan, 1997, 1998). Plasma IGF-I levels appear to correlate well with ration (Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004) and as such correlate well with the growth rate (Beckman *et al.*, 2001, 2004; Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004; Taylor *et al.*, 2005). However, temperature is believed to exert the greatest effect on IGF-I levels (Larsen *et al.*, 2001; Gabillard *et al.*, 2003b), primarily through an increased GH response (Gabillard *et al.*, 2003b). Nonetheless, under constant temperatures plasma IGF-I levels appear to reflect the changes in photoperiod (McCormick *et al.*, 2000; Beckman *et al.*, 2004). Therefore it appears that the GH:IGF-I axis is an integral component of the growth axis, dependent upon a multitude of external and internal factors, although other hormones will undoubtedly also influence the growth and development of fish.

The thyroid hormones, thyroxine (T₄) and triiodothyronine (T₃), are also considered to play a permissive role in the growth process of fish by potentiating the anabolic effects of other growth promoting hormones (Leatherland, 1982, 1994; Macbride *et al.*, 1982; Sumpter, 1992). The salmonid thyroid predominantly secretes T₄ which undergoes deiodination in the peripheral tissues to the more biologically active T₃. Both T₄ and T₃ bind reversibly to plasma proteins leaving a small percentage of the total hormone in a free and more physiologically reactive form, which is highly correlated to the total hormone level (Eales and Shostak, 1985b). However, temperature and pH alter the proportions of plasma T₄ and T₃ *in vitro* (Eales and Shostak, 1986) but remain unaltered during the parr-smolt transformation (Boeuf *et al.*, 1989). Thyroid hormones

are best recognized for their involvement at various ontogenic developmental stages including larval growth, flatfish and amphibian metamorphosis and salmonid smoltification, but are also involved in regulating growth and metabolism (Leatherland, 1982, 1994; De Pedro and Björnsson, 1999; Power *et al.*, 2001). Administration of T₄ or T₃ enhances skeletal and somatic growth although its effects are more pronounced when acting synergistically with GH (Higgs *et al.*, 1982; Leatherland, 1982; MacLatchy and Eales, 1990). Daily T₃ concentrations correlate well with the growth rate in fish (Gomez *et al.*, 1997) and both T₃ and T₄ exhibit seasonal variations with the T₄:T₃ ratio highest during the winter and lowest in summer (Osborn *et al.*, 1978), reflecting the seasonal growth pattern. As such, plasma T₃ levels accurately reflect the nutritional status of fish (Eales and Shostak, 1985a; Gabillard *et al.*, 2003a). Diel variations in circulating thyroid hormone levels appear to be dependent upon both the feeding time and/or photoperiod (Eales *et al.*, 1981; Boujard and Leatherland, 1992a, 1992b; Gélinau *et al.*, 1996; Gomez *et al.*, 1997) and are eliminated under periods of nutritional restriction (Eales *et al.*, 1981). However, the effect of constant photoperiods remains unstudied. One physiological approach to understanding fish growth requires an understanding of how environmental factors influence the endocrine mechanisms that promote growth and appetite (Beckman *et al.*, 2001).

The manipulation of environmental parameters inevitably results in an abrupt change in the rearing conditions, a challenge to which the individual will make appropriate physiological adjustments in order to survive. Endal *et al.* (2000) suggested that the transient growth depression following exposure to constant artificial light is brought about by either an initial stress response to the change in rearing regime or by a phase advancement of a circannual growth pattern adjusted by photoperiod, although to date there is no clear understanding of this phenomenon. The stress response in fish is typically characterized by the release of the catecholamines, adrenaline and noradrenaline, and through the activation of the hypothalamic-pituitary-interrenal (HPI) axis leading to alternations in the circulating levels of the corticosteroid hormone cortisol (Schreck, 1982; Barton and Iwama, 1991; Pickering, 1993; Wedemeyer 1996; Wendelaar

Bonga, 1997; Barton, 2002). Environmental stressors may be classified as either acute or chronic. Acute stressful encounters are of short duration, lasting either minutes or hours, and result in short-term endocrine and metabolic changes primarily through the activation of the HPI axis and the subsequent short-lived elevations in the circulating levels of plasma cortisol (Barton and Iwama, 1991; Pickering, 1993; Wedemeyer, 1996; Wendelaar Bonga, 1997). Chronic stress events are a continuous form of stress from which there may be no escape. In addition to activating the HPI axis, which may remain elevated for several days or weeks, the long-term physiology of the fish may also be affected in terms of suppressed immune function (Pickering and Pottinger, 1987; 1989; Harris and Bird, 2000), growth (McCormick *et al.*, 1998; Van Weerd and Komen, 1998; Gregory and Wood, 1999; Weil *et al.*, 2001) and reproduction (Schreck *et al.*, 2001). One of the first behavioural responses of fish to any form of stress is a cessation in feeding activity (Pickering, 1993; Wendelaar Bonga, 1997; Gregory and Wood, 1999). Fish appetite has commonly been reported to be suppressed following an environmental manipulation, most notably the seawater transfer of salmon smolts (Usher *et al.*, 1991; McCarthy *et al.*, 1996; Stead *et al.*, 1996; Arnesen *et al.*, 1998; Damsgård and Arnesen, 1998) as well as following abrupt changes to the rearing temperature (Mortensen and Damsgård, 1993; Koskela *et al.*, 1997; Arnesen *et al.*, 1998). However, it is thought that fish are able to acclimate to persisting stress events with plasma cortisol returning to basal levels following the initial stress response (Schreck, 1982; Pickering and Pottinger, 1985, 1989).

Under commercial production conditions fish are exposed to a variety of stressors, *e.g.* netting, handling, transportation, grading, social hierarchies and water quality to name but a few (Schreck, 1982; Barton and Iwama, 1991; Pickering, 1993; Wedemeyer, 1996 Wendelaar Bonga, 1997), but little is known on the effects of light and photoperiod on the stress axis. Recently, Leonardi and Klempau (2003) demonstrated that the application of constant artificial light chronically increased plasma cortisol levels and resulted in changes to the non-specific immune function of rainbow trout,

Oncorhynchus mykiss. However, to date no such studies have been performed in Atlantic salmon.

In addition to the possibility that the addition of artificial light might suppress appetite through some form of physiological response, there may also be some form of husbandry-related issues which contribute to the growth depression. Specifically, changes in fish behaviour by application of artificial light (Oppedal *et al.*, 2001; Juell *et al.*, 2002; Juell and Fosseidengen, 2004; Marchesan *et al.*, 2005) may influence the farmer's decisions about how much food to give. Juell and Fosseidengen (2004) for example, found that salmon reared in lit cages swam deeper than fish in unlit cages; this may result in an impression that the fish are not feeding and the subsequent low growth may be the outcome of underfeeding (*i.e.* an artefact of husbandry practices rather than a physiological response by the fish). Maximizing the growth rates and food conversion efficiency in commercial production depends upon matching the way in which the farmer makes food available to the fish with the physiological and behavioural mechanisms controlling the appetite and feeding activity (Talbot, 1993). Many farmers use apparent satiation and feed tables based on temperature and fish weight to feed their fish (Austreng *et al.*, 1987; Storebakken and Austreng, 1987; Cho, 1992a). However, the assessment of feeding motivation is difficult and very subjective and feeding tables do not take into consideration any photoperiod manipulations.

Thus the aim of this chapter was to examine the feeding, growth and stress responses in relation to the somatotrophic axis in Atlantic salmon post-smolts following exposure to artificial light, with particular emphasis on confirming or refuting the anecdotal growth-dip and elucidating the possible underlying mechanisms. A greater understanding of this phenomenon is important in managing the effective use of artificial lights, and to improve productivity within the farming industry by minimizing the uncertainty in the outcomes of photoperiod manipulations.

4.2. Materials and Methods

The trial was performed at Marine Harvest's (Scotland) Lochailort Research Unit (Inverness-shire, Scotland) between the 19th January and 23rd July 2004. A commercial strain of 1+ Atlantic salmon mixed-sex post-smolts (LM20; Loch Garry, Inverness-shire, Scotland), utilized in the trial, were reared under a natural photoperiod from hatch (January 2002) and transferred to seawater during March 2003.

Experimental design

On the 19th January 2004, 1200 individual fish with an initial mean wet weight of 1202 ± 15 g (\pm SEM; 16th February) were transferred from a commercial cage site at Loch Duich (Inverness-shire, Scotland) and stocked equally into eight 4 m diameter (10.05 m^3) circular fibreglass tanks, with a water depth set at 0.9 m. Tanks were supplied with a constant flow of pumped ashore seawater ($\sim 100 \text{ l.min}^{-1}$) of ambient temperature supplied from Loch Ailort adjacent to the experimental site. Water temperature varied throughout the trial between 8-10°C during the 60-day feeding study and upwards of 15°C at the end of the growth study by mid July (Figure 4.1). Tanks were covered with lightproof polyethylene canopies with zippered openings at either side to allow or exclude the natural light. Duplicate tanks were used for each treatment, initially stocked with 150 fish per tank.

On 1st March 2004 (day 0), replicate tanks were subjected to one of four photoperiod regimes, either remaining under a natural photoperiod² (NP, control) or exposed to: continuous light superimposed on the natural light (NPLL); a simulated natural photoperiod (SNP), with an absence of twilight phases; or a continuous light regime (LL). The latter two regimes were fully enclosed to exclude the natural light. Artificial illumination was provided by a single asymmetric metal halide lamp (Osram, HQI-T, 400 W/N; Norselights) fixed at the side of the tank, approximately 1.5 m above the water surface, providing irradiance (W.m^{-2}) and illuminance (lux) readings that varied according to time of day and light regime (refer to Figures 4.2 and 4.3, Skye Instruments;

² NP denotes fish exposed to the natural changes in photoperiod and light intensity

Powys, UK, calibrated to National Physical Laboratory UK standards). Light onset and offset within the SNP regime was controlled using a photocell (RS Components Ltd.; Corby, UK) modified to switch lights-on and -off when the ambient light intensity exceeded or fell below a threshold of approximately 70 lux, respectively.

Since both feeding and growth responses were assessed, fish in all treatments were fed in excess of the manufacturers recommended feeding rates with a standard commercial diet (MHS Orion, Skretting UK, pellet size 8.5 mm) using clockwork belt feeders set to dispense feed between the hours of 0800-2000 h, which were supplemented with an additional hand feed in the morning and afternoon periods.

Sampling regime

In order to determine the effects of artificial light on the feeding and stress responses in fish, a pre-treatment sample was performed for all tanks on the 27th February (day -2). The acute and chronic responses were then evaluated on days 2, 5, 10, 21, 32, 45 and day 60 post light onset, with sampling occurring between 1000-1100 h. At each sample point, six fish per tank (12 fish per treatment) were randomly netted and killed by anaesthesia in a lethal dose of Benzocaine. Blood was immediately withdrawn from the caudal vein and the plasma separated by centrifugation (1300 g, 15 min) and aliquoted into separate vials and stored at -70°C until analysis. The time taken to remove blood from individuals was always within a 5 min period, netting to bleeding, to prevent an increased cortisol response from a handling stress.

Sacrificed fish were individually marked for future identification, length and weight recorded and placed on ice until dissection. Following evisceration, the sex of the fish was noted and the gonads removed, weighed and the gonadosomatic index (GSI) determined. Fish were deemed to be maturing according to the method of Endal *et al.* (2000), if males had a gonadal weight ≥ 3 g and a GSI $\geq 0.4\%$ and females if the GSI $\geq 0.8\%$. The gastrointestinal tract was then excised and the digesta collected, oven dried and weighed (Section 2.4.2). The individual feed consumption was calculated on a weight-specific basis (mg.g fish^{-1}). The inter-individual variability in feed intake (CV_F)

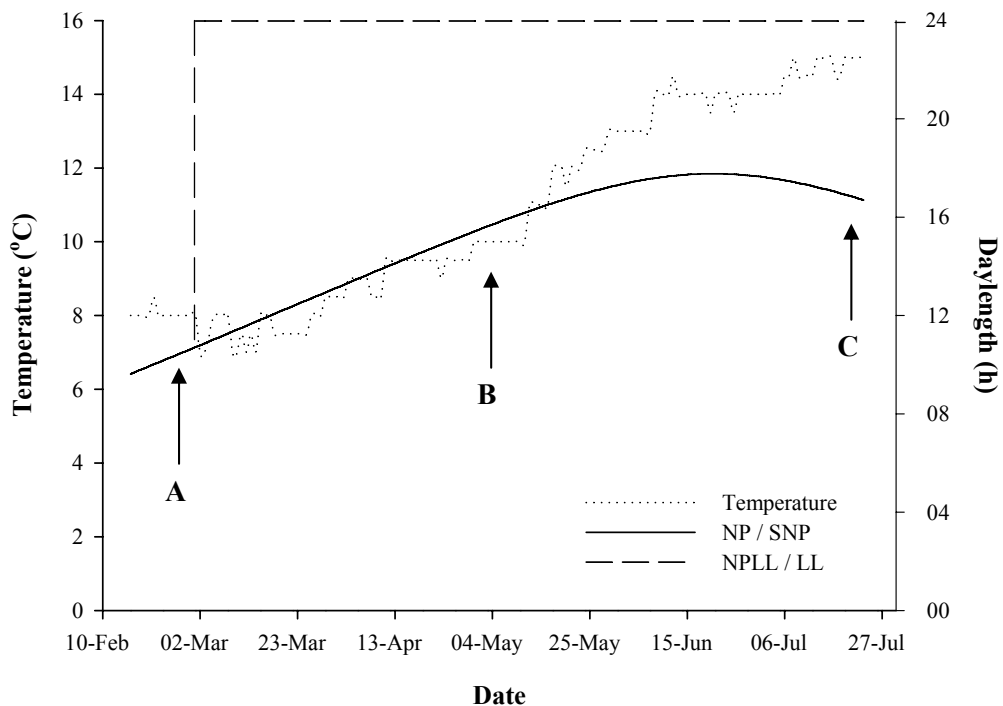


Figure 4.1. Experimental photoperiod regimes used throughout the trial and the daily temperature profile ($^{\circ}\text{C}$) recorded during the experimental period. Arrows indicate pre-treatment sample (A), end of 60 day feed study (B), and end of growth study (C).

within treatments was examined by the calculation of the coefficient of variation.

On 16th February 2004, an initial length-weight sample was carried out using a random sample of 50 fish per tank (100 fish per treatment). Thereafter, growth performance was measured on a monthly basis, whereby 50 randomly selected fish per tank were weighed under anaesthesia, except in June and July when all fish per tank were length-weighted. From the biometric values, condition factor (K) of the individual fish was determined and the daily weight (SGR_W) and length (SGR_L) gain for each tank population calculated.

Light perception

To assess light perception by the fish reared under the various light treatments, a 24-hour melatonin profile was performed between the 22nd and 23rd July 2004. A random sample

of 6 fish per tank (12 fish per treatment) were removed from the tanks and bled at the following times: 1200, 2230 (dusk), 0030 (mid-dark), 0430 (dawn) and 1200 h. The resulting plasma was stored at -70°C until analysis. Plasma melatonin was assayed by ELISA (Section 2.5.2.) using commercially available kits (IBL Ltd.; Hamburg, Germany). In addition, at each sampling interval the light intensity, in terms of irradiance ($\text{W}\cdot\text{m}^{-2}$) and illuminance (lux), was measured from just below the water's surface and from the tank floor of each treatment tank (Skye Instruments; Powys, UK, calibrated to National Physical Laboratory UK standards).

Plasma analysis

The plasma collected from all individually sacrificed fish during the course of the 60-day feeding study was analysed for various parameters using a variety of techniques. Plasma cortisol levels were determined by radioimmunoassay (Section 2.7.1.), using extracted plasma samples, according to the method of Ellis *et al.* (2004). Glucose levels were measured colorimetrically (Section 2.7.2.) using Infinity™ Glucose Oxidase kits (Alphalabs; Hampshire, UK) adapted for use in a micro-well plate. Plasma lysozyme activity was assessed using a turbidimetric assay (Section 2.7.3.) adapted from Lygren *et al.* (1999), where one unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min. Circulating levels of plasma growth hormone (GH) were measured using a double-antibody homologous radioimmunoassay (Section 2.4.1) as described by Le Bail *et al.* (1991).

Further, plasma samples from selected fish at specific time points over the 60 day feeding study were analysed for insulin-like growth factor I (IGF-I) by homologous double-antibody radioimmunoassay (Section 2.4.2.) according to Gentil *et al.* (1996), and for free levels of the thyroid hormones, thyroxine (T_4) and tri-iodothyronine (T_3), by radioimmunoassay (Section 2.4.3) using commercially available kits (Trinity Biotech Plc.; County Wicklow, Ireland).

Statistical analysis

Feed intake, growth performance and plasma parameters (GH, IGF-I, free T₄ and T₃, glucose and lysozyme) were all analysed by one-way ANOVA with replicate nested within the dependent factor treatment. Plasma cortisol and melatonin were analysed by ANOVA using a General Linear Model, where time and treatment were used as the categorical predictors and replicate nested within the treatment factor. All data were tested for normality of distribution and homogeneity of variance by the Kolmogorov-Smirnov test and from examination of the residual plots. *Post hoc* comparisons were made using Tukey's test. Where necessary, data was transformed using the natural logarithm, square root or arcsine transformation to improve normality conformation. Replicate tanks were found not to differ significantly ($P>0.05$) for all parameters measured and were therefore pooled. Due to a lack of statistical power, based on the mean of the replicates ($n=2$), no statistical analyses were performed for the CV_F , SGR_W and SGR_L data. Linear relationships between measured variables were assessed using Pearson's correlation coefficient (r). Run's test was used to check for linearity, with data failing the test indicating a curvilinear relationship. Multiple comparisons of linear regression gradients were compared by ANCOVA (Zar, 1999). A minimum significance level of $P<0.05$ was applied to all tests performed. All data are presented as mean \pm SEM.

4.3. Results

4.3.1. Light Distribution

Light intensity, recorded as irradiance (W.m^{-2} ; Figure 4.2.) and illuminance (lux; Figure 4.3.), varied accordingly between treatments and time of day. Due to the positioning of the light source, light distribution for all lit treatments was asymmetric. All readings given were recorded from the tank floor of each treatment tank.

The amount of ambient light entering the NP tank was at its highest during the day with approximately $4.\text{Wm}^{-2}$ recorded nearest the light openings, situated either side of the tank, reducing to 1 W.m^{-2} towards the centre of the tank. Similarly, intensity levels at dusk were highest nearest the light openings (0.2 W.m^{-2}), decreasing to around 0.05 W.m^{-2} at the centre of the tank.

The superimposition of constant light on the natural light in the NPLL group resulted in daytime intensities of around 5 W.m^{-2} directly below the light source, 3 W.m^{-2} towards the centre of the tank and between $1\text{-}2 \text{ W.m}^{-2}$ at the opposite side of the light source and tank periphery. However, during dusk light intensities decreased to around 2 W.m^{-2} beneath the light source, 0.9 W.m^{-2} towards the tank centre and 0.3 W.m^{-2} at the edges and furthest point from the light source.

Intensities in both the LL and SNP treatments were analogous, since both were subjected to the same light source, a 400 W metal halide light positioned 1.5 m above water surface, and both were excluded from any ambient light. Directly beneath the light source, intensity was around 3 W.m^{-2} , decreasing to around 2.5 W.m^{-2} at the centre of the tank and 0.5 W.m^{-2} at the furthest point from the light source and the perimeter of the tank. These intensity profiles remained constant throughout the 24-hour period in the LL treatment, whereas in the SNP regime no intensities were recorded during the twilight (*i.e.* dawn and dusk) or dark phases owing to the light source switching off as part of the rectangular daily light-dark cycle.

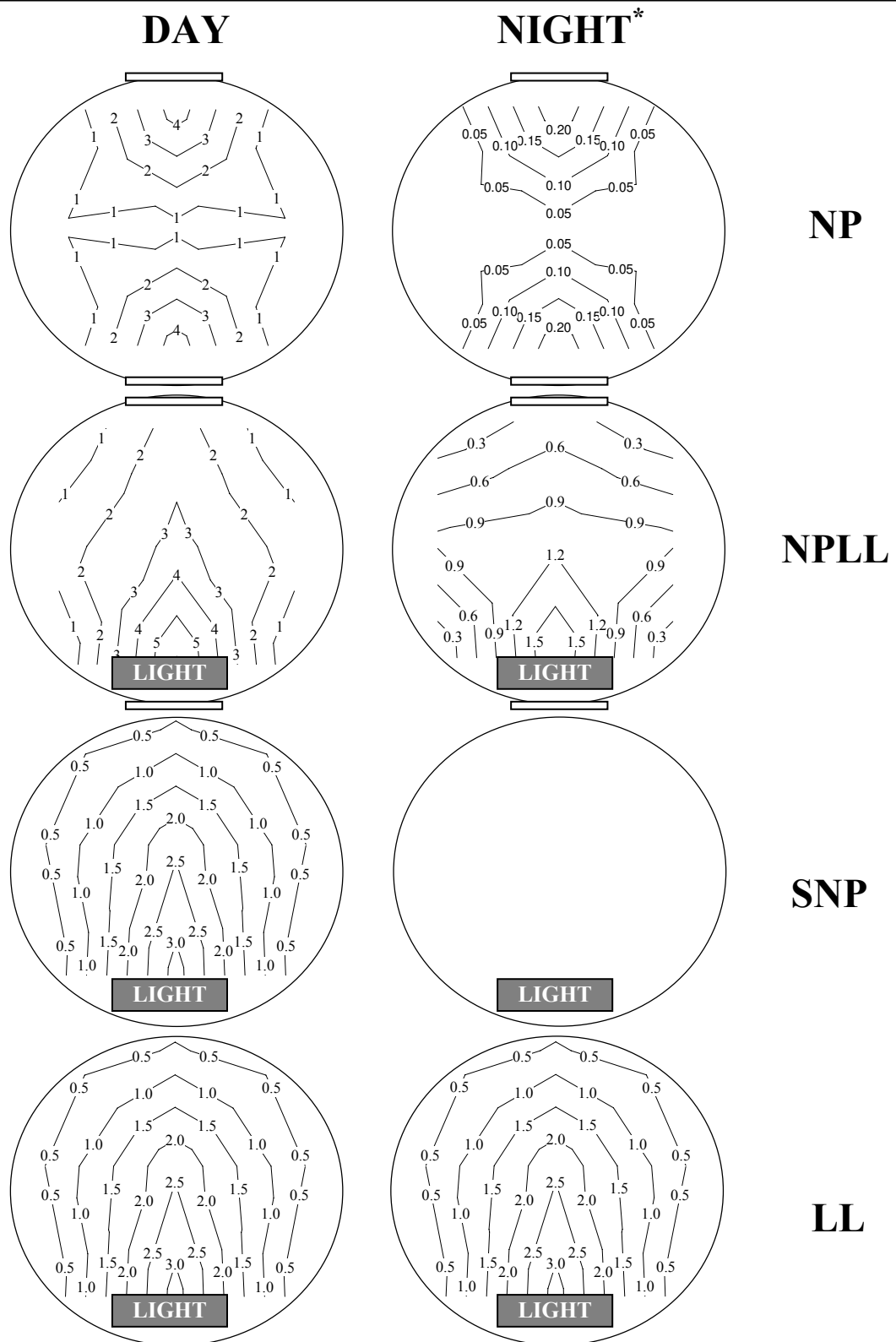


Figure 4.2. Irradiance (W.m^{-2}) contour plots recorded from the tank floor during the day and night*. Diagram illustrates the asymmetric positioning of the light source, where applicable, and location of the 'window' openings (\equiv) in the NP and NPLL regimes. *Night values were recorded around dusk (2230 h; 22nd July 2004); light in SNP regime at this time was switched-off.

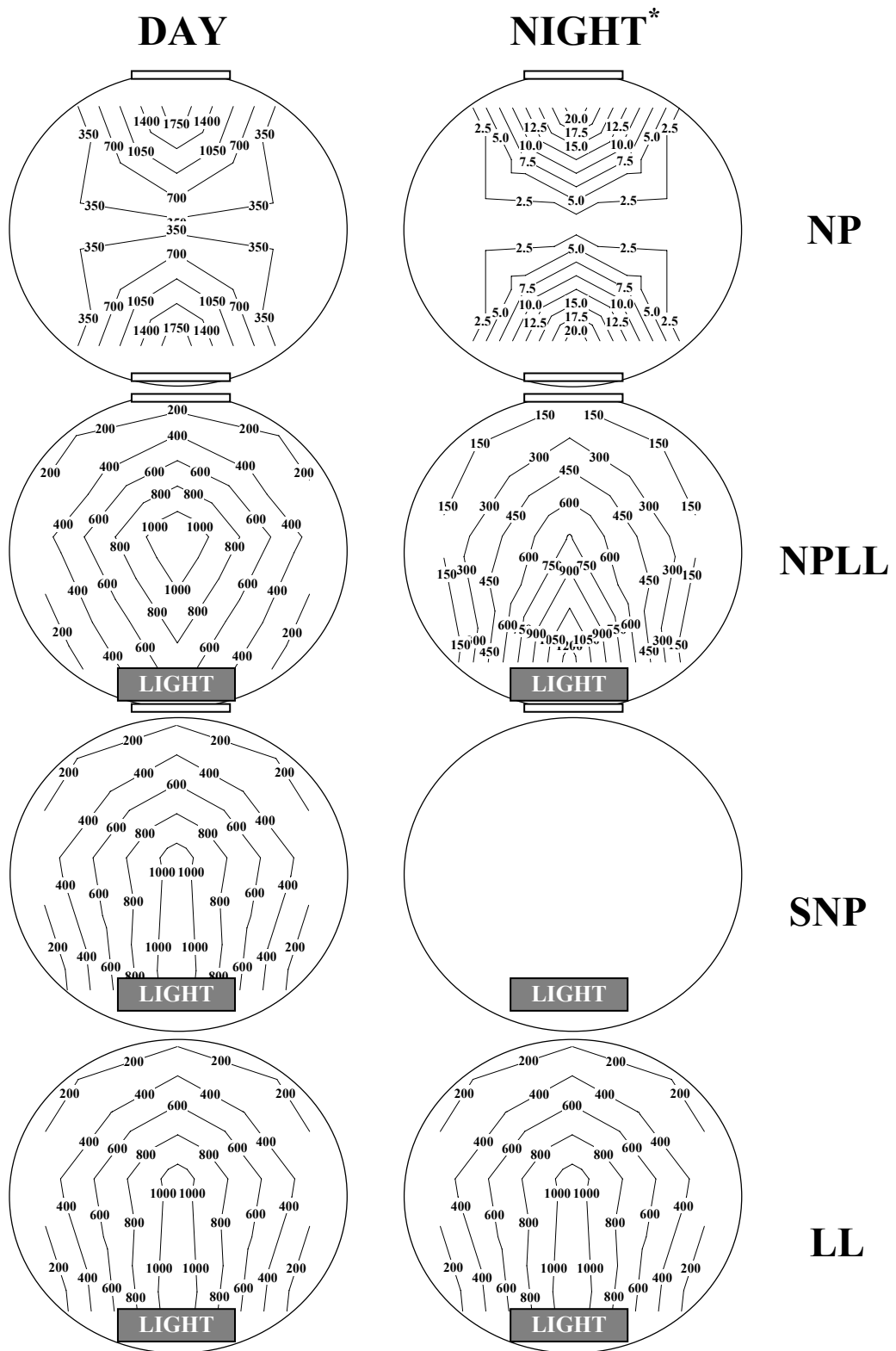


Figure 4.3. Illuminance (lux) contour plots recorded from the tank floor during the day and night*. Diagram illustrates the asymmetric positioning of the light source, where applicable, and location of the ‘window’ openings (▬) in the NP and NPLL regimes. *Night values were recorded around dusk (2230 h; 22nd July 2004); light in SNP regime at this time was switched-off.

4.3.2. Melatonin

Plasma melatonin levels accurately reflected the photoperiod under which the fish were reared (Figure 4.4), *i.e.* low plasma levels throughout the 24-hour period under constant light conditions (NPLL and LL), whereas fish held under NP and SNP displayed elevated levels for the duration of the scotophase ($\sim 200 \text{ pg.ml}^{-1}$). At dusk (2230 h) both the NP and SNP treatments were significantly higher than the constant light regimes. In addition, the NP treatment ($79.0 \pm 0.2 \text{ pg.ml}^{-1}$) was significantly lower than the SNP regime ($196.5 \pm 13.5 \text{ pg.ml}^{-1}$) in which the dark phase had already commenced, *i.e.* artificial light switched off. During dawn, plasma melatonin levels were not significantly different between the NPLL, LL and SNP treatments, the latter regime entering the light phase. The NP group, on the other hand, had significantly higher levels than the NPLL and LL treatments. However, plasma melatonin levels in the NP group were lower than the levels observed during dusk since sampling occurred towards the latter part of the dawn period when daylight was breaking and the natural light intensity increasing (Table 4.1).

Table 4.1. Irradiance readings (W.m^{-2}) recorded at each sampling interval for all experimental treatments during the 24-hour melatonin profile. Values given are recorded from the tank floor at the centre of each treatment tank.

Time (h)	NP (W.m^{-2})	NPLL (W.m^{-2})	SNP (W.m^{-2})	LL (W.m^{-2})
1200	0.90	3.05	2.20	2.23
2230	0.02	1.46	0.00	2.23
0030	0.00	1.50	0.00	2.23
0400	0.04	1.80	2.20	2.23
1200	1.00	3.50	2.20	2.23

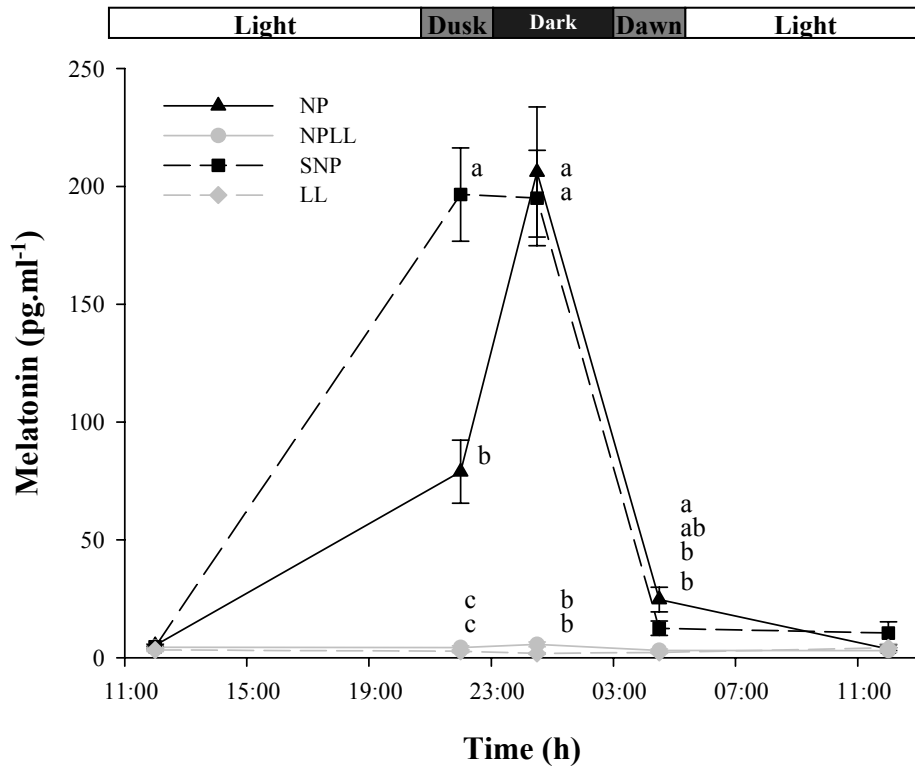


Figure 4.4. 24-hour mean plasma melatonin profile ($\text{pg.ml}^{-1} \pm \text{SEM}$) of 1+ Atlantic salmon reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Horizontal bar indicates the light/dark phase, including twilight phases, under ambient photoperiod. Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. In some cases, error bars are too small to be depicted. $n = 12$ fish per treatment per time point.

4.3.3. Feed Intake

Statistical analyses showed an overall significant effect of treatment on food consumption, with fish in the NP group ($43.36 \text{ mg.g fish}^{-1}$) consuming more over the 60-day feed study than fish reared under SNP ($28.78 \text{ mg.g fish}^{-1}$) or LL ($26.73 \text{ mg.g fish}^{-1}$) treatments (Table 4.2.).

Initial feed intake of all treatments was similar, increasing in the control group (NP) from $1.9 \pm 0.3 \text{ mg.g fish}^{-1}$ on the 27th February to $9.4 \pm 2.1 \text{ mg.g fish}^{-1}$ by the end of the 60-day feeding study on the 2nd May (Figure 4.5.). The application of artificial light resulted in the mean feed intake of all lit groups displaying a trend for a reduced appetite, relative to the control, such that within 10 days of light onset (11th March) food consumption was reduced by 56%, 54% and 34% in the SNP, NPLL and LL treatments respectively as compared to fish in the NP group. Further, on the 22nd March fish in the SNP regime ($2.3 \pm 0.6 \text{ mg.g fish}^{-1}$) were consuming a significantly smaller meal compared to fish in the NP treatment ($7.0 \pm 1.3 \text{ mg.g fish}^{-1}$). Following this initial suppression, feed intake in the NPLL and SNP treatments increased to reach similar levels to the NP group by the 2nd May, 60 days after light onset. However, feed intake in the fish reared under LL appeared to remain consistently lower throughout the duration of the trial (range of $2\text{-}6 \text{ mg.g fish}^{-1}$), although differences were not significant, such that by the end of the feeding study feed intake was still lower by 27% compared to the controls.

Table 4.2. Mean cumulative amount of food consumed during the 60-day feeding study of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or continuous light.

	Cumulative amount food consumed (mg.g fish^{-1})	Food consumed (%) relative to NP	<i>P</i> value
NP	43.36	-	-
NPLL	35.73	82.4%	ns
SNP	28.78	66.4%	0.0110
LL	26.63	61.4%	0.0034

Inter-individual variability in feed intake

Due to a lack of statistical power ($n=2$ replicates per treatment), no statistical analysis was performed for the inter-individual variability in feed intake (CV_F). The CV_F for all treatments was initially high, $104 \pm 7\%$. Over the course of the trial the CV_F in the NP treatment gradually decreased from $116 \pm 0.5\%$ on the 27th February to around 60% on the 22nd March maintaining this level for the remainder of the study (Figure 4.6.). This decrease in CV_F coincided with an increase in the mean feed intake. Comparable patterns within the NPLL and SNP regimes were also observed around the 22nd March and 2nd April respectively, when the mean feed intake in these groups also began to increase. However, the CV_F in the LL treatment remained consistently high throughout the experimental period, with a CV_F of around 100% except on the 6th and 11th March when CV_F temporarily decreased to between 80-90%.

Proportion of fish feeding

The initial amount of food consumed individually was uniformly low for all treatments with approximately 80-100% of the fish sampled from their respective treatment populations consuming a meal $< 5\text{mg.g. fish}^{-1}$. This pattern of feed intake changed over the course of the trial, with around 50% of fish sampled from the NP, NPLL and SNP treatments consuming a meal $> 10\text{mg.g fish}^{-1}$ by the end of the trial in May (Figure 4.7.). This increase in meal size was concomitant with the decline in the CV_F observed within the NP, NPLL and SNP treatments, decreasing from the 11th March, 22nd March and 2nd April respectively. However, the proportion of fish consuming a meal $< 5\text{mg.g fish}^{-1}$ in the LL treatment remained relatively unchanged for the duration of the trial. At the final sample point of the feed study (2nd May), 60 days after light onset, the proportion of fish consuming $< 5\text{mg.g fish}^{-1}$ in the NP, NPLL and SNP treatments was less than 30% compared to over 70% observed within the LL treatment.

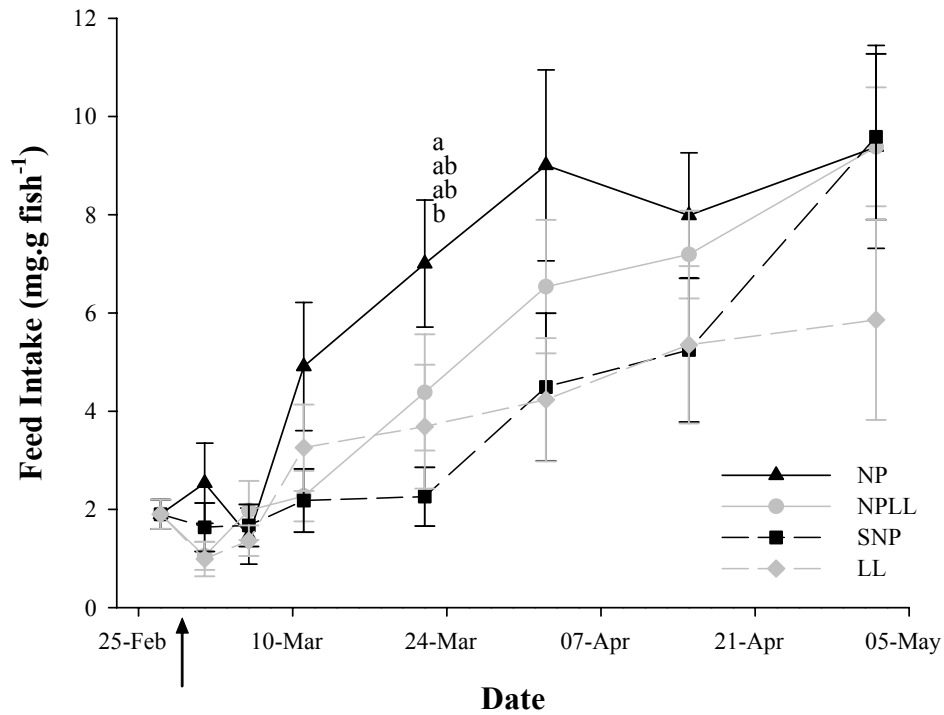


Figure 4.5. Change in the mean feed intake ($\text{mg.g fish}^{-1} \pm \text{SEM}$) of 1+ Atlantic salmon post-smolts maintained under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Arrow denotes period of light onset (1st March). $n = 12$ fish per treatment per time point.

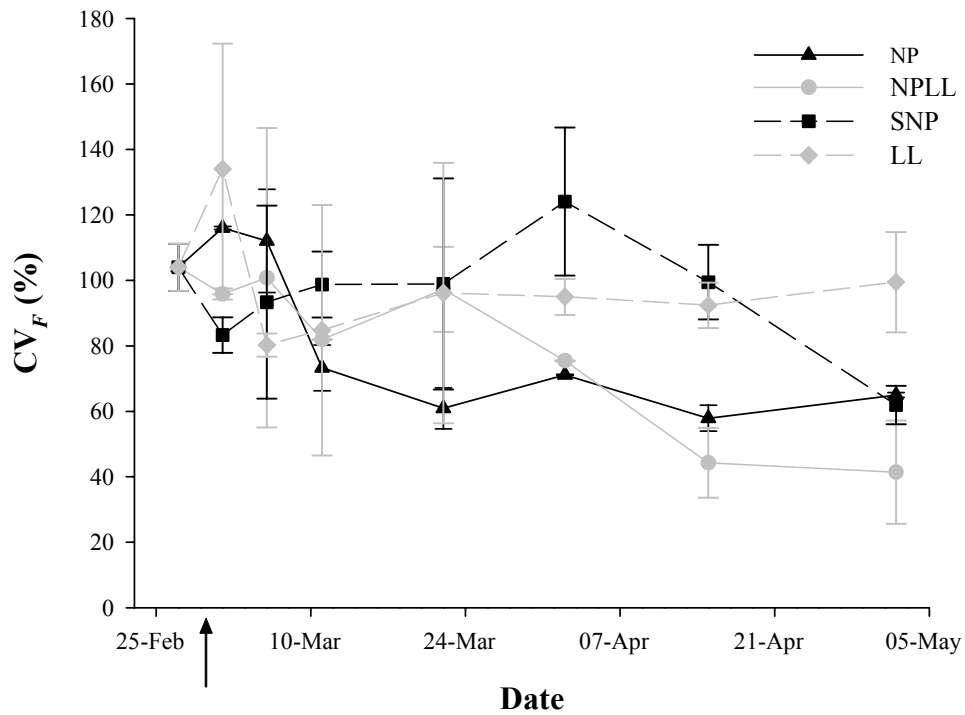


Figure 4.6. Change in the mean coefficient of variation in feed intake (CV, % \pm SEM) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod of exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Arrow denotes period of light onset (1st March). $n = 12$ fish per treatment per time point).

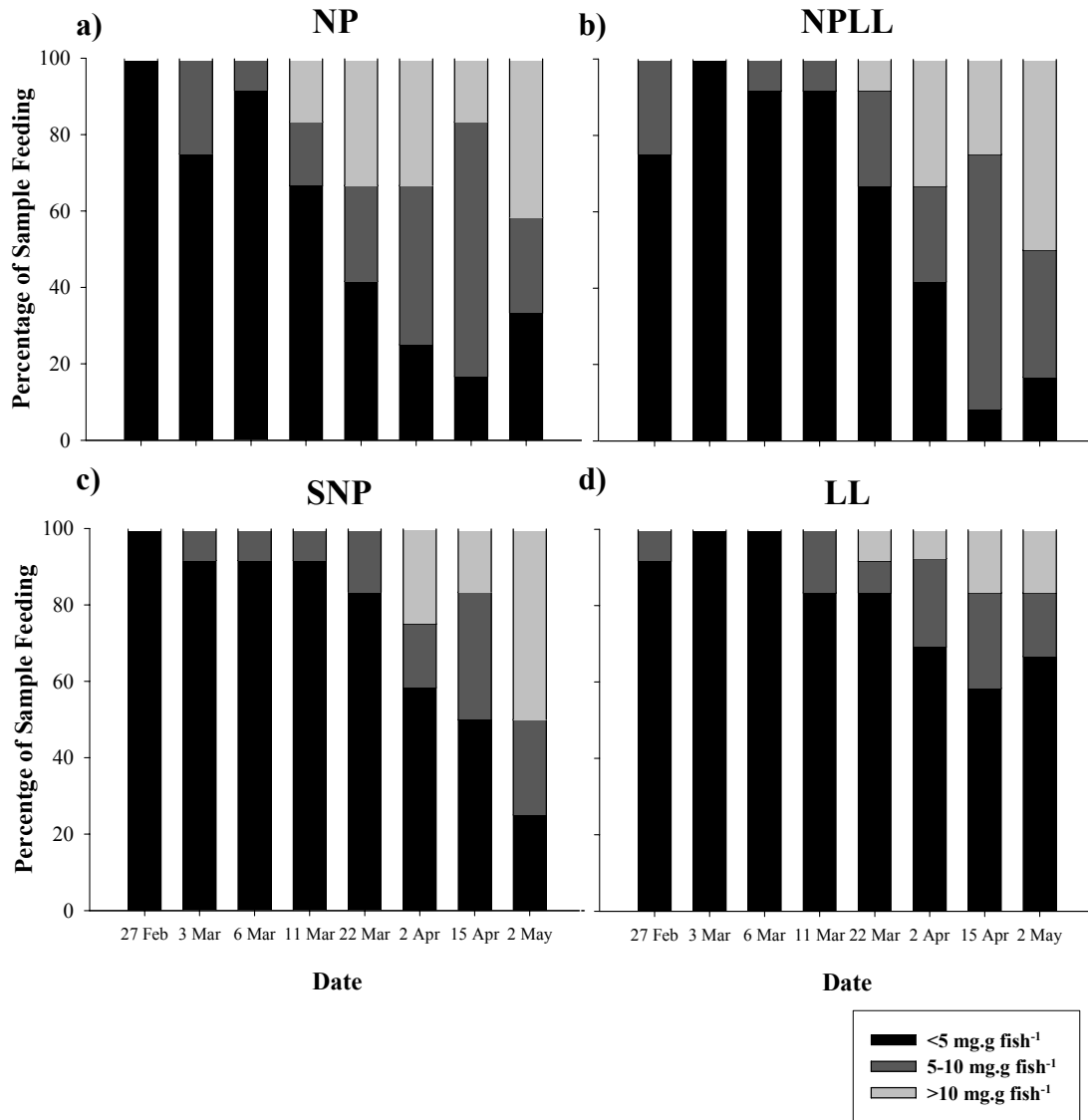


Figure 4.7. Relative proportion of fish sampled consuming a meal < 5 mg.g fish⁻¹, 5-10 mg.g fish⁻¹ or > 10 mg.g fish⁻¹ under a natural photoperiod (a), continuous light superimposed on the natural light (b), simulated natural photoperiod (c) or a continuous light regime (d). Date of light onset 1st March. $n = 12$ fish per treatment per time point.

4.3.4. Growth Performance

Weight gain

Initial body weights were statistically similar amongst treatments, although fish in the LL regime appeared to be heavier (1266 ± 32 g) than all other treatments (NP, 1186 ± 29 g; NPLL, 1151 ± 36 g; SNP, 1205 ± 29 g). Fish in all treatments steadily increased in weight over the course of the investigation, with the NP group attaining a significantly heavier weight by May than fish reared under LL (Figure 4.8.). By June both the NP and NPLL treatments were significantly heavier than fish reared under LL. From May, weight gain in the SNP group appeared to plateau such that at the final sample point in July, fish reared in the ‘open’ systems, *i.e.* NP (2057 ± 54 g) and NPLL (2093 ± 46 g) were significantly heavier than their counterparts reared in the ‘enclosed’ systems, *i.e.* SNP (1792 ± 48 g) and LL (1822 ± 42 g).

No statistical analysis was performed for the weight specific growth (SGR_w) data since there was a lack of statistical power ($n=2$ replicates per treatment). The SGR_w observed for the growth period February-April, during which light was applied, appeared to be highest in the NP ($0.63\% \text{ day}^{-1}$) and NPLL ($0.57\% \text{ day}^{-1}$) groups in comparison to the SNP ($0.38\% \text{ day}^{-1}$) and LL ($0.16\% \text{ day}^{-1}$) treatments (Figure 4.9.). At the April-May period, SGR_w decreased sharply in the NP, NPLL and SNP treatments but only slightly under LL where growth was around 30% lower than that under NP. Additionally, during the same period, growth was approximately 50% higher under NPLL and SNP relative to the control. Throughout the May-June period, daily growth rates were similar between treatments (0.3% to $0.4\% \text{ day}^{-1}$). However, from June-July to the end of the experiment both the constant light regimes displayed higher rates of growth of around 10-40% higher than that in the NP, whereas growth in the SNP treatment appeared to be half of that observed for NP.

Length gain

Initial fork lengths of fish in the LL group (476 ± 32 mm) were significantly longer than fish reared under NPLL (458 ± 36 mm) reflecting their higher initial weight. Following

the application of artificial light fish reared under LL lacked any real length gain, such that by May a reversal in the order of lengths had occurred with NPLL significantly longer than LL fish (Figure 4.10.). From June through to the end of the trial in July, length gain was higher in the NPLL group such that fish reared in this group were significantly longer than their conspecifics reared under SNP and LL.

Due to a lack of statistical power no analysis was performed ($n=2$ replicates per treatment) on the length specific growth (SGR_L) data. The SGR_L of the control (NP) group remained consistent at around $0.10\% \text{ day}^{-1}$ for the duration of the trial (Figure 4.5.). SGR_L peaked at around $0.20\% \text{ day}^{-1}$ in the lit treatments during the April-May growth period, 20-70% higher than in the NP regime. During the May-June and June-July periods length gain was reduced by up to 70% in the SNP with a similar SGR_L observed between the NP and SNP during July-August. From June-July to the end of the trial SGR_L in the constant light regimes were equal to or greater than that recorded in the NP group.

Condition factor

Condition factor (K) of fish reared under NP remained consistently higher throughout the duration of the trial than fish reared under any of the artificially lit treatments (Figure 4.6.). Following light application, all treatments with the exception of the LL regime whose condition remained unchanged, displayed an increase in K between February and April leading to a significantly higher K in the NP, NPLL and SNP treatments compared to the LL regime. Between April and May, K in all treatments exhibited a decrease, although this decline was more pronounced in the lit treatments. This subsequently resulted in a significantly lower K in the SNP (1.10 ± 0.02) and LL (1.05 ± 0.02) treatments relative to the NP (1.18 ± 0.02). During the May and June sampling periods, control fish exhibited a significantly higher K compared to fish reared under LL. The NP was also significantly higher than both the 'enclosed' treatments (*i.e.* SNP and LL) at the final sample point in July.

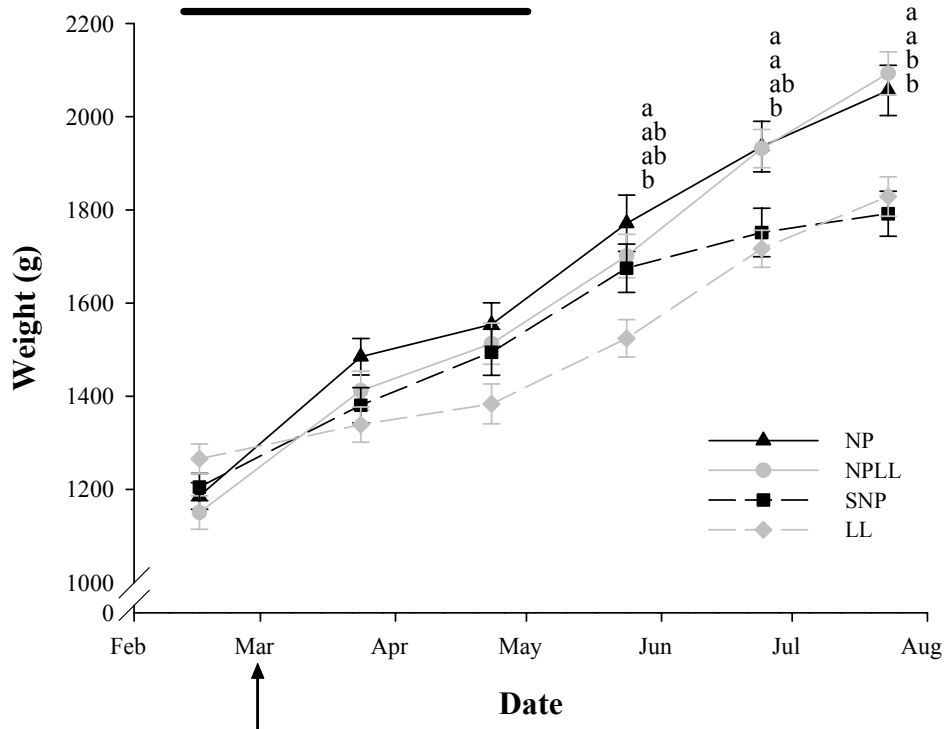


Figure 4.8. Change in the mean wet weight ($g \pm SEM$) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Horizontal bar indicates period where feed intake was studied (22nd February to 2nd May). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Arrow denotes period of light onset (1st March). $n = \text{min } 100$ fish per treatment per time point.

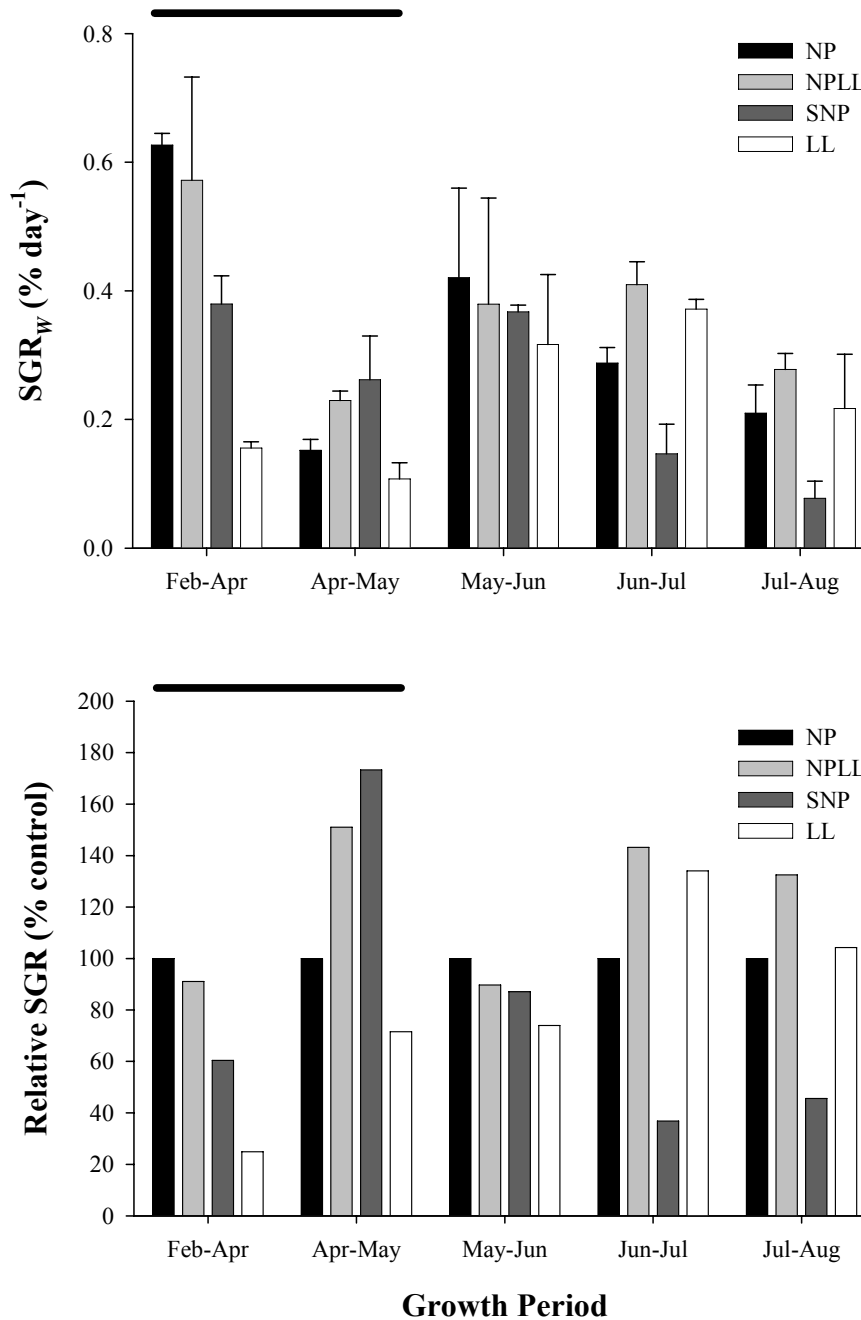


Figure 4.9. Change in the mean weight specific growth rate (SGR_w, % day⁻¹ ± SEM; a) and the SGR_w relative to the control (b) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Date of light onset 1st March. Horizontal bar indicates period where feed intake was studied (27th February to 2nd May). *n* = 2 replicates per treatment per time point.

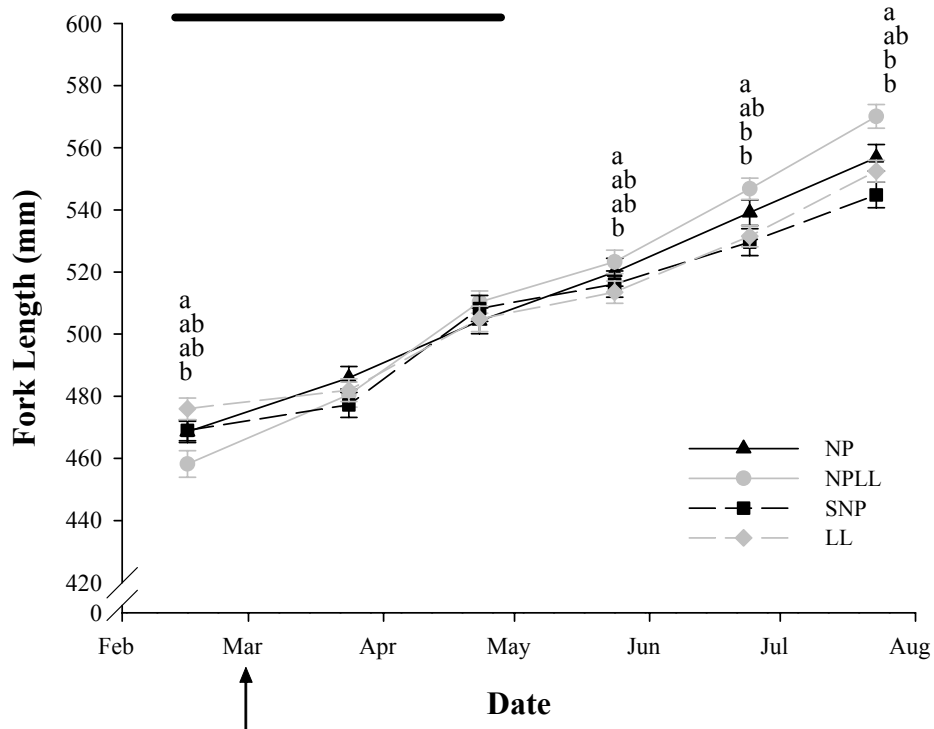


Figure 4.10. Change in mean fork length (mm \pm SEM) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Horizontal bar indicates period where feed intake was studied (27th February to 2nd May). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. In some cases, error bars are too small to be depicted. Arrow denotes period of light onset (1st March). $n = \text{min } 100$ fish per treatment per time point.

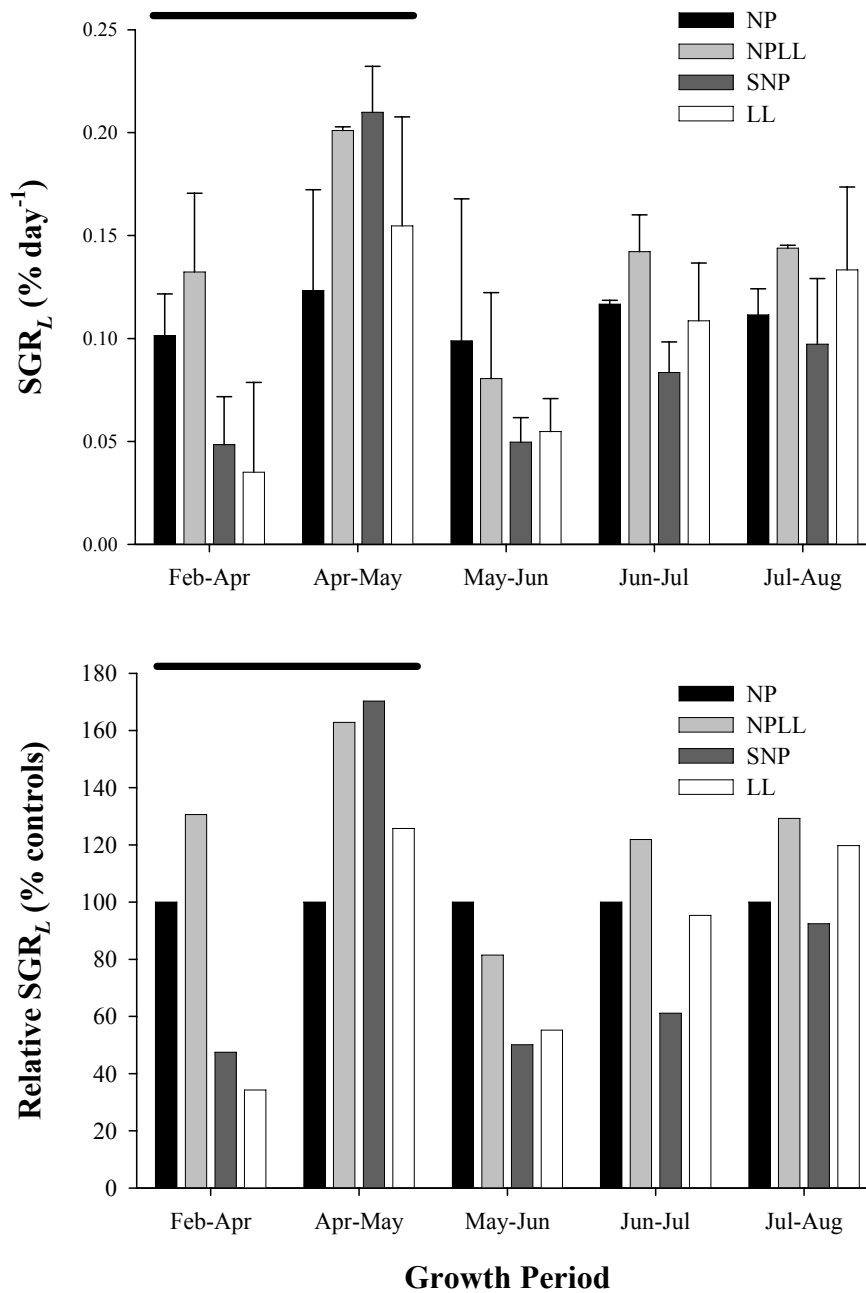


Figure 4.11. Change in the mean length specific growth rate (SGR_L , % day⁻¹ ± SEM; a) and the SGR_L relative to the control (b) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Date of light onset 1st March. Horizontal bar indicates period where feed intake was studied (27th February to 2nd May). $n = 2$ replicates per treatment per time point.

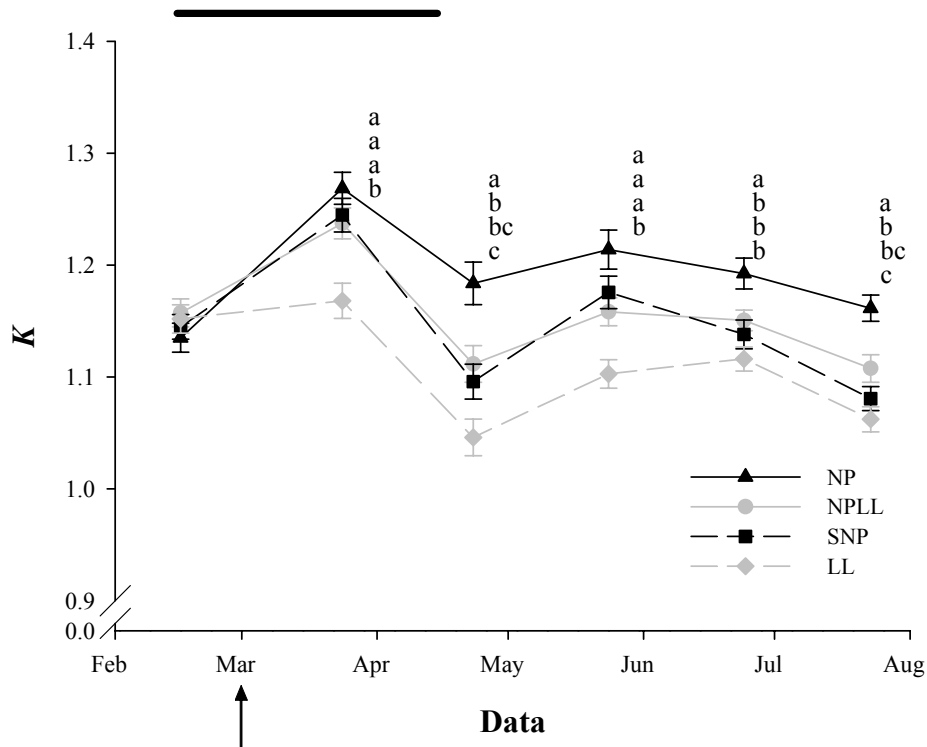


Figure 4.12. Change in mean condition factor ($K \pm \text{SEM}$) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Horizontal bar indicates period where feed intake was studied (27th February to 2nd May). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Arrow denotes period of light onset (1st March). $n = \text{min } 100$ fish per treatment per time point.

4.3.5. Cortisol

The onset of constant artificial light resulted in a significant ($P < 0.05$) elevation of plasma cortisol from basal levels of $< 5 \text{ ng.ml}^{-1}$ to a peak of 9.0 ± 1.4 and $24.0 \pm 7.2 \text{ ng.ml}^{-1}$ in the NPLL and LL treatments respectively (Figure 4.13.), which remained elevated for a period of up to 4 weeks. Plasma cortisol concentrations in the NP and SNP treatments remained at basal levels of around $0\text{-}5 \text{ ng.ml}^{-1}$ for the duration of the study, although on the 3rd March mean cortisol levels in the NP group reached $8.58 \pm 2.98 \text{ ng.ml}^{-1}$ although this increase was not significant ($P > 0.05$), relative to pre-treatment levels. On the 6th and 20th March mean plasma levels in the LL treatment were significantly higher than both the NP and SNP groups. Additionally, plasma cortisol concentrations in the NPLL regime were significantly higher than the SNP treatment on both the 3rd and 11th March. Thereafter, plasma cortisol levels measured in the NPLL and LL treatments decreased to basal levels, on the 22nd March and 3rd April respectively, similar ($P > 0.05$) to levels observed in the NP and SNP treatments. No significant differences between treatments in plasma cortisol levels were observed from the 3rd April to the end of the experimental period in May.

4.3.6. Glucose and Lysozyme

Plasma glucose levels (mmol.l^{-1}) and lysozyme activity ($\text{Umin}^{-1}\text{ml}^{-1}$) are presented in Table 4.3.

Glucose

Mean plasma glucose levels within all treatments remained relatively stable for the duration of the trial ranging from 4.4 to 5.9 mmol.l^{-1} . On the 6th March all treatments displayed a minor decrease in glucose levels. However, no significant differences were observed between treatments at any of the sampled time points.

Lysozyme activity

Initial lysozyme activity was similar between all treatments prior to the onset of light. On the 3rd March, 2 days after exposure to artificial light, all groups exhibited an increase in plasma lysozyme activity resulting in significantly higher levels in the NP group ($1600 \pm 108 \text{ Umin}^{-1}\text{ml}^{-1}$) than those measured in the NPLL treatment ($1283 \pm 76 \text{ Umin}^{-1}\text{ml}^{-1}$). Thereafter no further significant differences were observed between treatments, although on the 2nd April lysozyme activity decreased to as low as $833 \pm 77 \text{ Umin}^{-1}\text{ml}^{-1}$ in the NP regime and $1197 \pm 113 \text{ Umin}^{-1}\text{ml}^{-1}$ in the NPLL group.

Table 4.3. Mean plasma glucose levels ($\text{mmol}\cdot\text{l}^{-1} \pm \text{SEM}$) and lysozyme activity ($\text{Umin}^{-1}\text{ml}^{-1} \pm \text{SEM}$) of 1+ Atlantic salmon post-smolts reared under the various light treatments. Onset of light 1st March 2004. Means bearing identical superscripts in the same row are not significantly ($P > 0.05$) different ($n = 12$ fish per treatment).

	NP		NPLL		SNP		LL	
	Glucose (mmol L^{-1})	Lysozyme ($\text{Umin}^{-1}\text{ml}^{-1}$)	Glucose (mmol L^{-1})	Lysozyme ($\text{Umin}^{-1}\text{ml}^{-1}$)	Glucose (mmol L^{-1})	Lysozyme ($\text{Umin}^{-1}\text{ml}^{-1}$)	Glucose (mmol L^{-1})	Lysozyme ($\text{Umin}^{-1}\text{ml}^{-1}$)
27/02/04	4.8 ± 0.3	1144 ± 97	4.1 ± 0.3	1081 ± 92	3.9 ± 0.3	1202 ± 95	4.0 ± 0.3	1283 ± 90
03/03/04	4.8 ± 0.1	1606 ± 108^a	5.2 ± 0.3	1283 ± 76^b	4.9 ± 0.3	1321 ± 54^{ab}	4.7 ± 0.3	1453 ± 57^{ab}
06/03/04	3.5 ± 0.4	1387 ± 144	4.4 ± 0.3	1297 ± 167	3.5 ± 0.4	1314 ± 141	4.0 ± 0.4	1216 ± 169
11/03/04	4.5 ± 0.5	1433 ± 111	5.9 ± 0.5	1121 ± 101	5.1 ± 0.4	1401 ± 63	4.7 ± 0.4	1265 ± 106
22/03/04	5.1 ± 0.2	1417 ± 87	4.4 ± 0.2	1615 ± 147	5.2 ± 0.2	1540 ± 162	4.8 ± 0.2	1377 ± 117
02/04/04	4.8 ± 0.2	833 ± 77	5.9 ± 0.2	1197 ± 113	4.6 ± 0.2	955 ± 90	5.0 ± 0.2	1006 ± 102
15/04/04	5.3 ± 0.2	1219 ± 121	5.2 ± 0.3	1240 ± 91	5.0 ± 0.4	1267 ± 101	4.7 ± 0.4	1211 ± 110
02/05/04	4.6 ± 0.2	1438 ± 116	5.2 ± 0.1	1576 ± 93	4.6 ± 0.3	1455 ± 73	4.7 ± 0.2	1597 ± 97

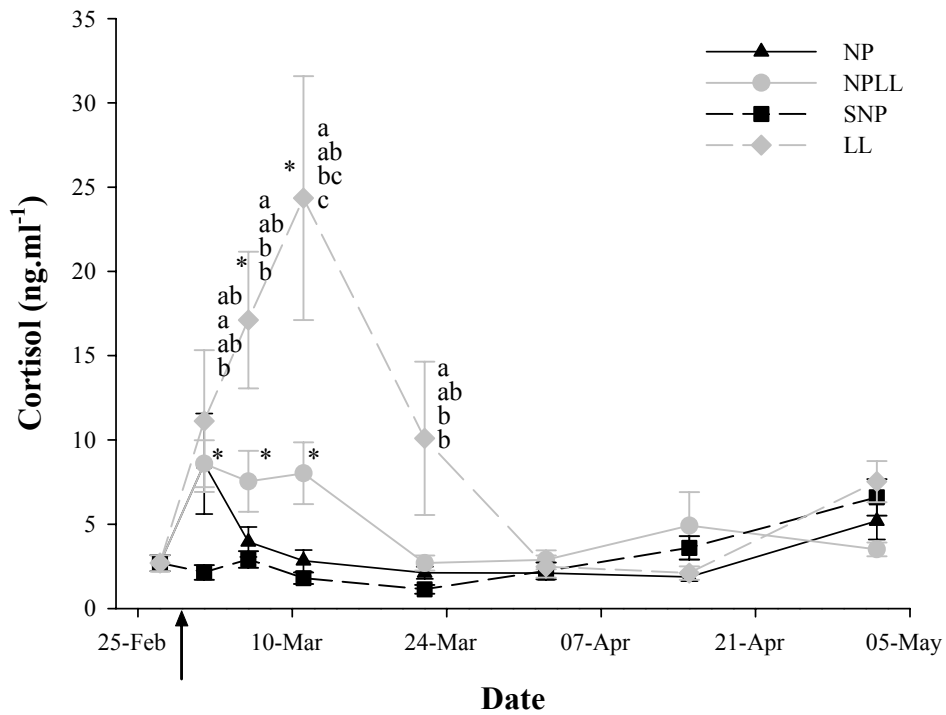


Figure 4.13. Change in mean plasma cortisol levels ($\text{ng.ml}^{-1} \pm \text{SEM}$) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Asterisk (*) next to treatment symbol signifies value is significantly higher ($P > 0.05$) than pre-treatment level ($< 5 \text{ ng.ml}^{-1}$). In some cases, error bars are too small to be depicted. Arrow denotes period of light onset (1st March). $n = 12$ fish per treatment per time point).

4.3.7. Growth Hormone

Following the application of light, plasma growth hormone (GH) levels in the NPLL ($2.23 \pm 0.3 \text{ ng.ml}^{-1}$) and LL ($2.49 \pm 0.2 \text{ ng.ml}^{-1}$) treatments increased whereas GH concentrations in the SNP ($0.97 \pm 0.3 \text{ ng.ml}^{-1}$) decreased resulting in significantly lower levels on the 6th March (Figure 4.14. a). Additionally, plasma GH levels in the control group ($1.44 \pm 0.3 \text{ ng.ml}^{-1}$) also increased for the same period but remained significantly lower than the LL treatment. Plasma GH concentration then decreased for all treatments reaching a low of 0.3 ng.ml^{-1} in the NP group on the 22nd March. Thereafter, GH levels followed a similar pattern in all treatment groups stabilising between 0.60 to 1.08 ng.ml^{-1} towards the end of the experimental period in May.

4.3.8. Insulin-like Growth Factor I

Plasma insulin-like growth factor I (IGF-I) of the NPLL treatment increased from $29.3 \pm 1.9 \text{ ng.ml}^{-1}$ on the 27th February to $36.5 \pm 3.7 \text{ ng.ml}^{-1}$ on the 6th March, five days after exposure to constant light, whereas plasma IGF-I concentrations in all other treatments decreased to between 20 to 25 ng.ml^{-1} (Figure 4.14. b). This resulted in significantly higher levels of plasma IGF-I in the NPLL group compared to fish reared under NP ($20.8 \pm 3.2 \text{ ng.ml}^{-1}$). Thereafter, no further differences were recorded between treatments, although IGF-I levels in both the NP ($36.7 \pm 3.7 \text{ ng.ml}^{-1}$) and NPLL ($42.2 \pm 4.9 \text{ ng.ml}^{-1}$) groups appeared to be higher than conspecifics reared under SNP ($31.8 \pm 3.8 \text{ ng.ml}^{-1}$) and LL ($27.9 \pm 3.3 \text{ ng.ml}^{-1}$) on the 2nd May.

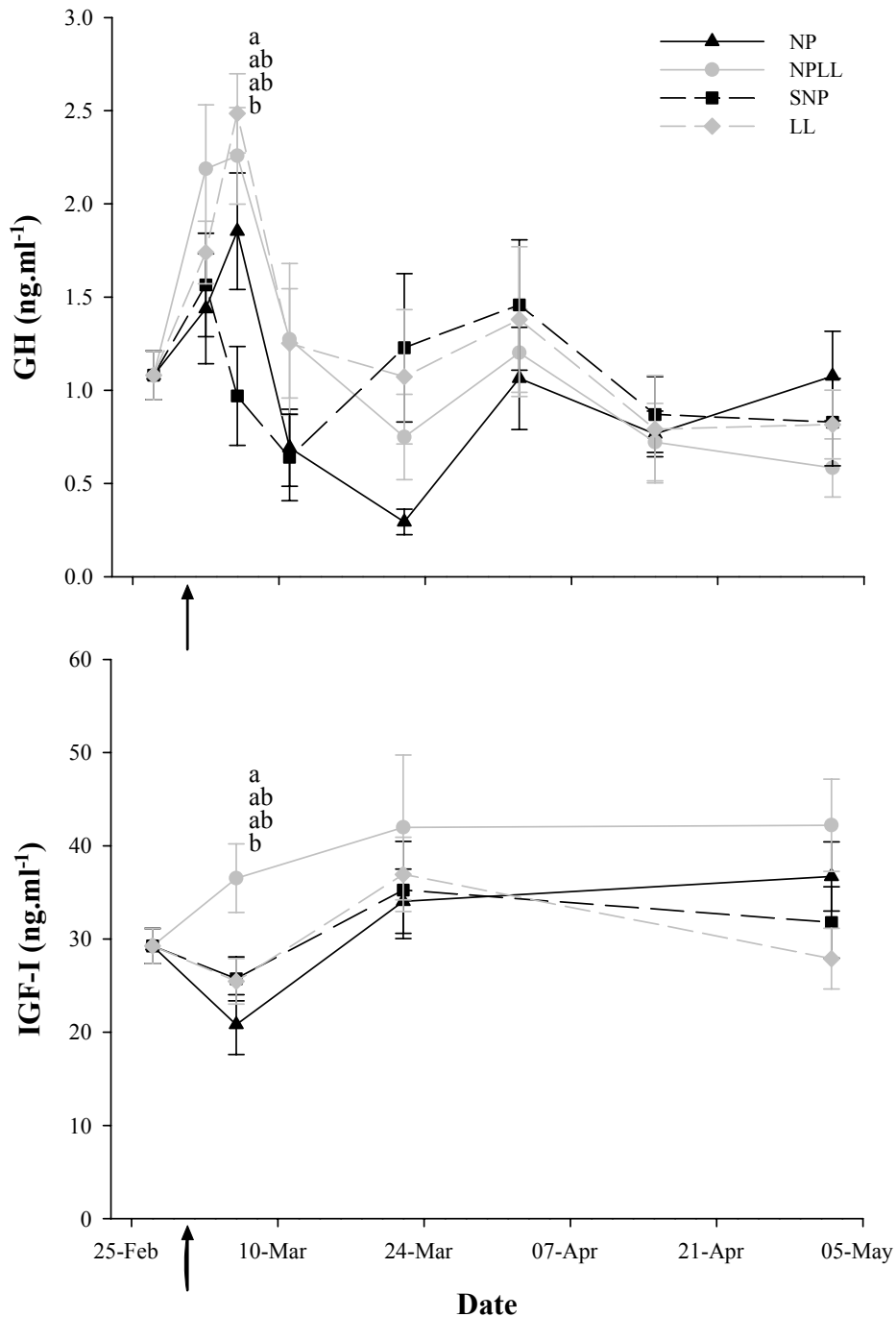


Figure 4.14. Plasma growth hormone (GH, $\text{ng.ml}^{-1} \pm \text{SEM}$; a) and insulin-like growth factor I (IGF, $\text{ng.ml}^{-1} \pm \text{SEM}$; b) levels of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Arrow denotes period of light onset (1st March). $n = 12$ and 8 fish per treatment per time point for GH and IGF-I respectively.

4.3.9. Thyroid Hormones

Free plasma levels of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) are presented in Figure 4.15.

Thyroxine (T_4)

Exposure to constant light resulted in significantly higher levels of free plasma T_4 in both the NPLL ($7.6 \pm 0.6 \text{ pmol.l}^{-1}$) and LL ($7.2 \pm 0.7 \text{ pmol.l}^{-1}$) groups compared to the NP ($4.6 \pm 0.4 \text{ pmol.l}^{-1}$) and SNP ($4.6 \pm 0.3 \text{ pmol.l}^{-1}$) treatments on the 3rd March sample, two days after the onset of treatments (Figure 4.15. a). Additionally, on the 6th March free T_4 levels in the LL regime were significantly higher than levels measured in the SNP, whereas free T_4 concentrations in the NPLL group decreased to similar levels recorded in the NP and SNP treatments. Free plasma T_4 levels for all treatments then decreased to reach a trough of around 3 pmol.l^{-1} at the 22nd March sample, before a secondary peak was observed with levels in both the continuously lit treatments being significantly higher than the NP and SNP treatments. Free T_4 levels then decreased in the constant light treatments, although this reduction was greater in the NPLL regime resulting in significantly lower levels than in the LL treatment. Plasma free T_4 concentrations continued to decrease in all treatments culminating around $3\text{-}4 \text{ pmol.l}^{-1}$ at the final sampling point on the 2nd May.

Triiodothyronine (T_3)

The application of constant light resulted in free plasma T_3 concentrations in both the continuously lit regimes exhibiting a similar increase to that observed in free T_4 . This resulted in significantly higher levels of plasma T_3 in the NPLL ($18.1 \pm 1.1 \text{ pmol.l}^{-1}$) and LL ($17.3 \pm 2.8 \text{ pmol.l}^{-1}$) groups compared to the NP ($12.1 \pm 0.9 \text{ pmol.l}^{-1}$) and SNP ($13.5 \pm 1.5 \text{ pmol.l}^{-1}$) regimes on the 3rd March (Figure 4.15. b). Free plasma T_3 concentrations briefly decreased in the constant light regimes on the 6th March, after which levels in the NP and NPLL treatments gradually increased to around 18 pmol.l^{-1} where they remained for the rest of the study. However, free plasma levels of T_3 in the LL regime decreased

from $19.1 \pm 2.1 \text{ pmol.l}^{-1}$ on the 11th March to a level comparable to that measured in the SNP regime, approximately 13 pmol.l^{-1} , on the 2nd April. This resulted in significantly lower levels in the SNP and NP treatments compared to NPLL ($18.4 \pm 1.8 \text{ pmol.l}^{-1}$). Thereafter, free plasma T_3 levels increased in the SNP and LL treatments to between 16 to 18 pmol.l^{-1} , analogous to levels observed in the NP and NPLL groups.

T₄:T₃ ratio

Changes in the relative representation of free T_4 and T_3 over time in the blood plasma of fish reared under the various experimental treatments are presented in Figure 4.16. Mean values of the $T_4:T_3$ molar ratio were comparatively similar between the NP and NPLL treatments throughout the course of the study, ranging from 0.5 to 0.2. The $T_4:T_3$ ratio in both the SNP and LL treatments appeared to be higher than the NP and NPLL treatments on the 2nd April and was significantly higher in the LL treatment (0.59 ± 0.10) as compared to the NPLL group (0.31 ± 0.05) on the 15th April.

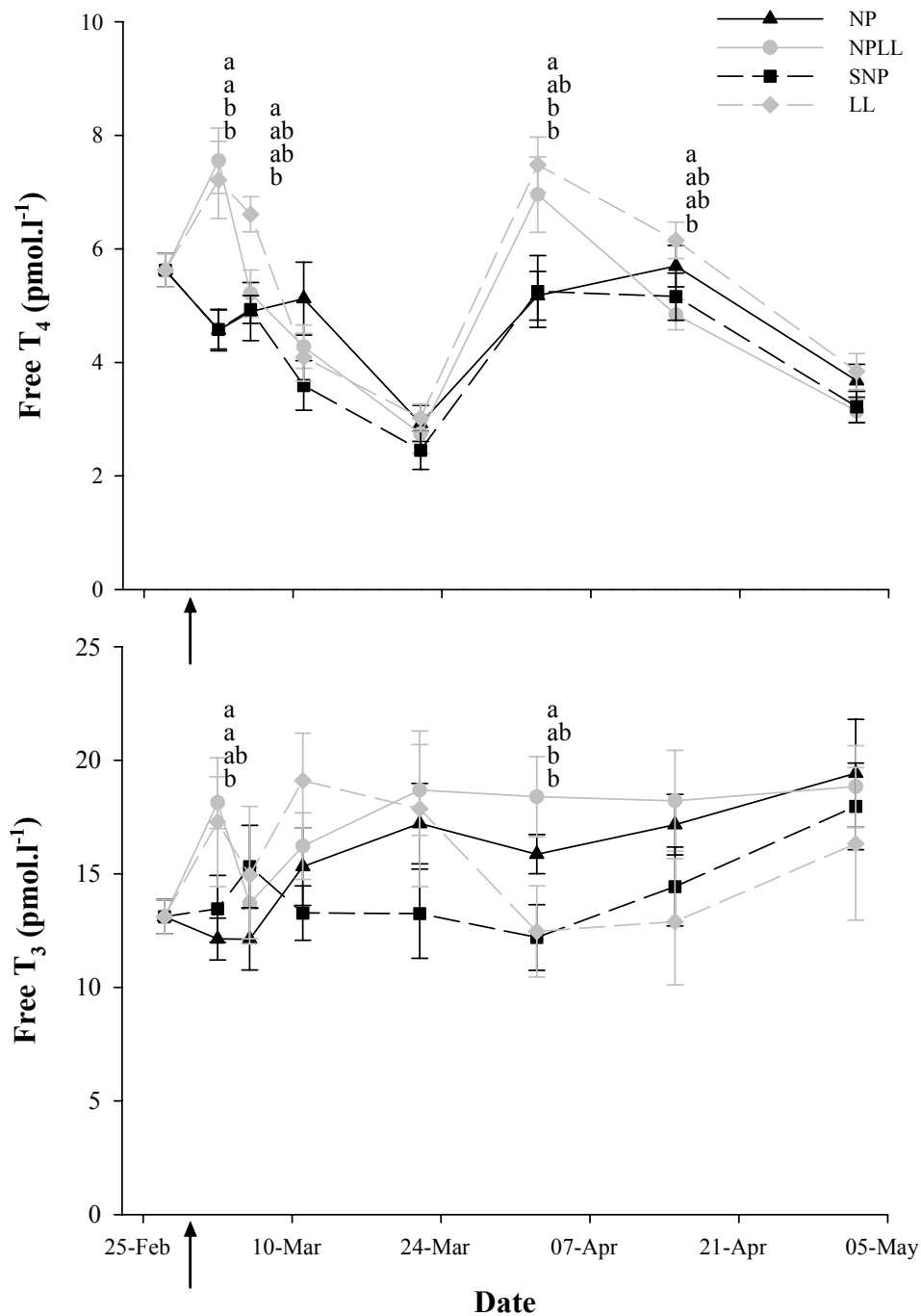


Figure 4.15. Plasma levels of free thyroxine (T_4 , $\text{pmol.l}^{-1} \pm \text{SEM}$; a) and free triiodothyronine (T_3 , $\text{pmol.l}^{-1} \pm \text{SEM}$; b) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Arrow denotes period of light onset (1st March). $n = \text{min } 10$ fish per treatment per time point).

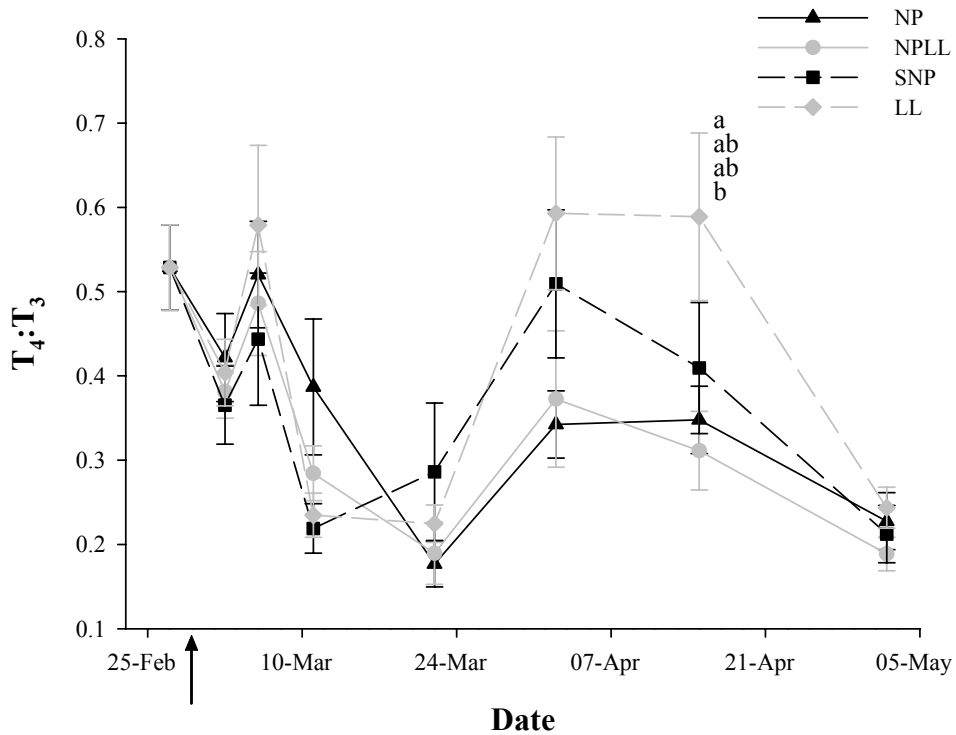


Figure 4.16. Mean $T_4:T_3$ ratios (mean \pm SEM) of 1+ Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to continuous light superimposed on the natural light (NPLL), simulated natural photoperiod (SNP) or a continuous light regime (LL). Means bearing identical lettering are not significantly different ($P > 0.05$) between treatments at given time points. Arrow denotes period of light onset (1st March). $n = \text{min } 10$ fish per treatment per time point).

4.3.10. Maturation

Overall, from a total number of 384 fish sampled during the 60-day feeding study, 41 fish were deemed to be maturing of which 39 were males and 2 females. No correlations were observed between maturational status and any of the measured parameters, although fish with a higher gonadosomatic index (GSI) appeared to have higher growth hormone levels. As such these fish were removed from analysis from this specific hormone.

The proportion of fish deemed to be maturing, based upon a gonadal weight > 3 g and a GSI $\geq 0.4\%$ for males and a GSI $\geq 0.8\%$ for females (Endal *et al.*, 2000), was similar among treatments with 9.4% displaying signs of maturing under NP, 11.5% under NPLL, 10.4% under SNP and 11.5% under LL (Figure 4.17.).

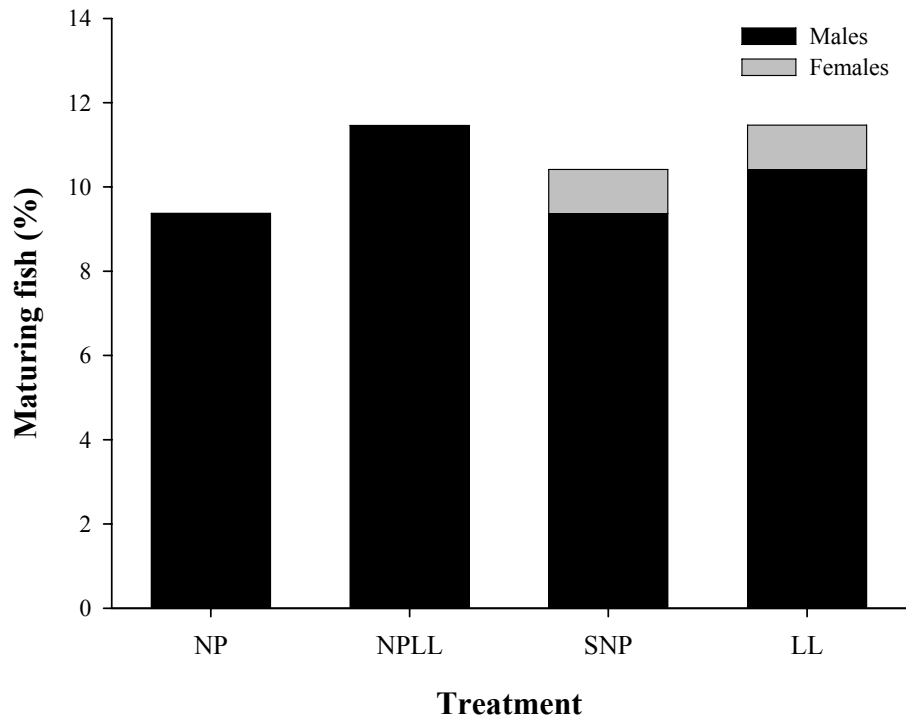


Figure 4.17. Proportion of 1+ Atlantic salmon deemed to be maturing (March to May 2005), based on the gonadosomatic index described by Endal *et al.* (2000), reared under a natural photoperiod (NP); continuous light superimposed on the natural light (NPLL); simulated natural photoperiod (SNP); or constant light (LL). $n = 96$ fish per treatment.

4.3.11. Relationships and Regression Analyses

Linear and non-linear relationships between mean replicate data of the various parameters studied, for all treatments and treatment data combined (overall), are presented in Table 4.4.

All treatments exhibited a significant positive linear relationship between water temperature and feed intake (overall $r^2=0.48$, $P<0.0001$). However, ANCOVA revealed no significant differences in the slope or elevation between treatments. Additionally, both the NP ($r^2=0.68$, $P=<0.0001$) and SNP ($r^2=0.82$, $P=<0.0001$) treatments displayed a strong positive relationship between photoperiod (daylength) and feed intake, with significant differences between the treatments for intercept values but not for the slope of the relationships.

With exception to the SNP regime, no significant linear relationships, non-linear in the NPLL group, were found between plasma cortisol levels and feed intake for any of the treatments. Conversely, the NP ($r^2=0.31$, $P=0.0255$), NPLL ($r^2=0.49$, $P=0.0027$) and LL ($r^2=0.29$, $P=0.0318$) treatments all exhibited linear correlations between GH and feed intake whereas the SNP regime exhibited a non-linear relationship. However, the slope of the relationship was similar amongst all treatments ($P>0.05$).

IGF-I levels did not correlate well with either GH or mean feed intake levels, but showed positive linear relationships with T_3 for the NP ($r^2=0.51$, $P=0.0453$), NPLL ($r^2=0.71$, $P=0.0083$) and LL ($r^2=0.57$, $P=0.0307$) treatments. No differences between slopes and intercepts were observed.

Plasma T_3 concentrations displayed significant negative correlations with GH under NP ($r^2=0.34$, $P=0.0183$) and SNP ($r^2=0.28$, $P=0.0343$) and a strong positive relationship for the same treatments with feed intake.

Table 4.4. Correlations between mean replicate data ($n = 2$ replicates) of measured parameters for each treatment. Slopes and intercepts bearing identical lettering are not significantly different, ns denotes not significant ($P > 0.05$). Treatments without slope and intercept values indicate non-linear relationships.

	Pearson	r²	P value	F value	Slope	Intercept
Temp v FI						
Overall	0.6919	0.4788	<0.0001	56.946	0.5155	-2.334
NP	0.5477	0.3000	0.0281	6.000	0.4712 ^a	-1.714 ^a
NPLL	0.8314	0.6912	<0.0001	31.343	0.6178 ^a	-3.110 ^a
SNP	0.8780	0.7710	<0.0001	47.123	0.5889 ^a	-3.076 ^a
LL	0.6522	0.4253	0.0062	10.361	0.3843 ^a	-1.438 ^a
Photo v FI						
NP	0.8217	0.6752	<0.0001	29.105	0.5285 ^a	10.800 ^a
SNP	0.9082	0.8249	<0.0001	65.961	0.7512 ^a	10.962 ^b
Cortisol v FI						
Overall		0.0580	ns	1.890		
NP	-0.3217	0.1035	ns	1.616	-0.4619	2.720
NPLL		0.5690	ns	5.280		
SNP	0.6558	0.4300	0.0058	10.562	0.5954	1.327
LL	-0.2058	0.0424	ns	0.6194	-0.1204	1.948
GH v FI						
Overall		0.314	<0.0001	13.95		
NP	-0.5553	0.3084	0.0255	6.242	-0.8073 ^a	3.042 ^a
NPLL	-0.6970	0.4857	0.0027	13.224	-0.7522 ^a	2.908 ^a
SNP		0.1950	ns	1.570		
LL	-0.5375	0.2889	0.0318	5.688	-0.5407 ^a	2.461 ^a
GH v IGF						
Overall	-0.1559	0.0243	ns	0.7475	-1.897	33.965
NP	-0.5609	0.3146	ns	2.754	-6.347	36.761
NPLL	0.1384	0.0192	ns	0.1172	1.796	34.766
SNP		0.5250	ns	2.760		
LL	-0.2392	0.0572	ns	0.3642	-2.040	33.379
GH v T3						
Overall		0.0550	ns	1.780		
NP	-0.5808	0.3373	0.0183	7.126	-2.044 ^a	11.990 ^a
NPLL	-0.1922	0.0369	ns	0.5369	-0.4948	11.679
SNP	-0.5310	0.2819	0.0343	5.497	-1.937 ^a	11.284 ^a
LL	0.0260	0.0007	ns	0.0095	0.0716	10.209
T₃ v FI						
Overall		0.1870	0.0020	7.040		
NP	0.7423	0.5510	0.0010	17.180	0.3066	-0.8282
NPLL		0.2950	ns	2.720		
SNP		0.4020	0.0350	4.370		
LL	-0.2579	0.0665	ns	0.9971	-0.09414	2.703
IGF v FI						
Overall	0.4407	0.1942	0.0116	7.230	0.0449	0.4491
NP	0.6896	0.4756	ns	5.441	0.0842	-0.5318
NPLL	0.5452	0.2972	ns	2.537	0.0442	0.4072
SNP	0.3268	0.1068	ns	0.7176	0.0572	0.0759
LL		0.3270	ns	1.210		
IGF v T3						
Overall	0.6636	0.4403	<0.0001	23.603	0.1673	4.815
NP	0.7171	0.5142	0.0453	6.351	0.2382 ^a	2.694 ^a
NPLL	0.8450	0.7141	0.0083	14.986	0.1850 ^a	3.795 ^a
SNP	0.1504	0.0226	ns	0.1388	0.0518	8.052
LL	0.7538	0.5683	0.0307	7.897	0.1339 ^a	6.463 ^a

4.3.12. Summary of Results

The results from the current experiment indicate the following:

- Under enclosed conditions light intensity remained constant within the LL regime and switched abruptly from light to dark under SNP, whereas the open groups were subjected to the natural changes in light intensity, including under the superimposition of artificial light in the NPLL treatment.
- Plasma melatonin levels accurately reflected the photoperiod under which the fish were reared, *i.e.* low under constant light and elevated during the dark phases in the NP and SNP treatments. Additionally, melatonin concentrations were significantly different between the NP and SNP regimes during dusk.
- Cohorts of fish exposed to artificial light, irrespective of photoperiod, tended to display an initial reduction in appetite compared to conspecifics reared under a natural photoperiod; fish reared under SNP and LL had an overall lower mean food consumption than control fish (NP).
- The growth performance of fish reared in an enclosed light system (*i.e.* SNP and LL) was significantly lower than their counterparts reared in the open light systems (*i.e.* NP and NPLL).
- Fish exposed to constant light exhibited a significant 4-week elevation of plasma cortisol compared to pre-treatment levels. However, no significant correlations between plasma cortisol levels and feed intake were evident in these treatments.
- Plasma GH and IGF-I increased in the NPLL and LL groups immediately following light onset. No significant correlations between hormones were found for any of the treatments.
- Thyroid hormone levels, T₄ and T₃, in the constant light regimes (NPLL and LL) displayed significant differences in the period immediately following the onset of light and again at the beginning of April.
- Maturation, based upon the gonadosomatic index, was similar amongst all treatments regardless of photoperiod regime.

4.4. Discussion

The reports of reduced appetite and growth following the application of constant artificial light are currently of major concern for the on-growing stage of the salmon industry as it results in feed management concerns, lower growth performances and may also interfere with production schedules. Furthermore, the use of such lighting regimes under commercial conditions may also have implications for the welfare of farmed fish through a stress response. Fish behaviour has already been studied in Atlantic salmon in response to artificial light, with respect to the density and swimming depth of the fish (*e.g.* Oppedal *et al.*, 2001; Juell *et al.*, 2003; Juell and Fosseidengen, 2004). However, to my knowledge the present study is the first of its kind to solely focus on the ‘growth-dip’ phenomenon and investigate the growth endocrine axis, stress and immune responses following exposure to artificial light. Thus, the aim of the current chapter was first; to determine whether a ‘growth-dip’ occurred and, if so, whether this phenomenon was a consequence of a physiological and/or behavioural reaction by the fish to the onset of light rather than the result of husbandry-related factors (*i.e.* feed management) and second; to examine the underlying mechanisms in the form of the interaction between the stress, growth and thyroid axes. Only by gaining a greater understanding of these processes can artificial lighting regimes and feed guidelines be improved.

The present trial has been organized according to three principal questions: 1.) Is there a ‘growth-dip’ and, if present, is it a result of physiological and behavioural responses to the onset of light? 2.) Does the application of artificial light and/or photoperiod regimes influence the stress and/or non-specific immune responses in fish? and 3.) What are the acute responses of the growth endocrine axis following light application? Finally, the relevance of the results from the current trial to the on-growing stage of the salmon industry shall also be addressed.

4.4.1. Is there a ‘growth-dip’ and, if present, is it a result of physiological and behavioural responses to the onset of light?

Transient growth depressions have often been reported following manipulations to the rearing environment. The seawater transfer of salmon smolts, for example, has been recognized to suppress appetite and growth for up to 30 days (Usher *et al.*, 1991; McCarthy *et al.*, 1996; Stead *et al.*, 1996; Arnesen *et al.*, 1998; Damsgård and Arnesen, 1998), with similar observations recorded for several salmonid species following an abrupt change in the rearing temperature (Mortensen and Damsgård, 1993; Koskela *et al.*, 1997; Arnesen *et al.*, 1998). In the present investigation the feeding levels for all treatments studied were initially low, increasing steadily in the control group (NP) during the course of the experiment. This is suggested to be a reflection of a seasonal pattern of appetite, with the increase in feed consumption related to the changes in the ambient photoperiod and temperature (Higgins and Talbot, 1985; Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999), which during the course of the 60-day feeding study increased from 10.5 to 15.5 hours and 8°C to 10°C respectively. Furthermore, the level of feed intake observed in the current trial is supported by previous studies where Atlantic salmon of a comparable age and size were studied at a similar time of year (*cf* Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003). In contrast, fish reared under artificial light, irrespective of photoperiod, failed to display a similar increase in feed intake as recorded in the control group. Instead, these fish appeared to exhibit an initial reduction in appetite which persisted for up to 60 days in the NPLL and SNP treatments before recovering to similar feeding levels observed under NP. However, fish exposed to LL failed to recover over the same period. This noticeable reduction in appetite, particularly with respect to the constant light regimes, is in accordance with the reports currently made within the industry together with those previously documented under experimental conditions (Taranger *et al.*, 1995; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003).

However, unlike the studies of Nordgarden *et al.* (2003) and Oppedal *et al.* (2003), no significant differences in the levels of food consumption were detected between fish maintained under a natural daylength photoperiod (*i.e.* NP and SNP)

compared to fish reared under constant light in the present study. This may be associated with the contrasting style of methodologies employed in these studies. Feeding, both in terms of frequency, duration and quantity will determine the availability of food at any instant in time and hence the instantaneous levels of hunger and feeding motivation in individual fish (Talbot, 1993). In the present investigation feed was continuously supplied throughout the day, whereas in the Norwegian studies fish were presented with two discrete daily meals lasting up to 60 minutes. Noeske and Spieler (1984) remarked that providing multiple daily feeds increases the likelihood that food will be given at a time which coincides with the maximum appetite of the fish. Given that feeding, though often of an endogenous nature, can be synchronized by the light-dark cycle (Kadri *et al.*, 1991; Boujard and Leatherland, 1992a, 1992b; Bolliet *et al.*, 2001), it may be that the onset of constant light resulted in the displacement or phase-shift of the feeding rhythm. On that basis, it is possible that the two daily meals fed in the earlier studies were dispensed at times out of synchrony with the altered appetite of the fish, thereby inducing the significantly lower feeding levels measured under constant light. Equally, the different methods used to quantify feed intake may also have contributed to the differences reported. In the Norwegian studies feed intake was indirectly measured by assessing the feeding responses of the tank populations through the collection of waste feed, whereas in the current investigation the individual feeding responses were examined. Whilst waste feed collection provides a valuable means for measuring the daily feed intake in groups of fish held in tanks (Helland *et al.*, 1996), the individual feeding responses, as studied in the present trial, are more likely to differ than the feeding responses between populations. As such, it could be that the variability in the level of food consumption between individuals within the same population may have masked any significant differences in feed intake from being detected. Nevertheless, although feed intake was not significant at a statistical threshold of $P < 0.05$, it is felt that the two to three fold variations in the mean values of food consumption between the lit treatments in comparison to fish maintained under NP are of importance, particularly as a drop in

mean feed intake by up to 50% would cause concern within a commercial production environment.

On a cautionary point of note, it is accepted that the single greatest limitation to the current trial was that the sample size ($n=6$ fish per replicate per sample point) may have been too small to detect any significant differences between the mean feeding levels. This is one of the main constraints of such studies in which killing large numbers of fish over an extended period is impracticable. The estimation of sample size has traditionally been based upon the statistical significance of the outcome measure by taking into account the smallest effect size to detect, the *type I* (*i.e.* which hypothesis, null or alternative, is most likely to be true) and *type II* (*i.e.* probability of accepting the null hypothesis when it is false) error rates, and the design of the study (Ruohonen *et al.*, 2001). Applying Altman's nomogram (Altman, 1982) to the present study to determine the sample size for an 80% chance of detecting a 4 mg.g fish^{-1} difference in mean feed intake at $P=0.05$ would have required 30 fish from each treatment to be sampled per sample point. In this study, the 12 fish sampled from each treatment per sample point only corresponded to a 40-45% chance of detecting a significant difference of 4 mg.g fish^{-1} in mean feed intake. Unfortunately, due to the limitations for experimental procedures at this particular site (*i.e.* Home Office), sampled fish had to be sacrificed at each sample point. Furthermore, whilst the analysis of gut contents may provide an accurate assessment of feed intake it is acknowledged that the results are from different fish at each sampling period, which is less desirable than serial measurements on individual fish (Talbot, 1985). Future trials of a similar nature should therefore try to use an increased sample size as well as employing repeated serial measurements on individually tagged fish using a non-invasive technique such as X-radiography.

The inter-individual variability in feed intake (CV_F) was initially high for all treatments, and gradually decreasing as the trial progressed. High variability in feed intake has been attributed to aggressive behaviour amongst individuals competing for the same available food source (McCarthy *et al.*, 1992) or alternatively as a lack of acclimatization to the experimental conditions (Koskela *et al.*, 1997). Competitive

behaviour would have probably been vastly reduced in the current trial, as providing fish with an increased ration has been shown to reduce aggression due to a greater food availability (McCarthy *et al.*, 1992; Jobling and Koskela, 1996). Hatlen *et al.* (1997), found that maximal growth and appetite took up to two months to establish following the formation of groups of Arctic charr, *Salvelinus alpinus*.

Under artificial light the eventual decrease in CV_F , with exception of the LL regime, appeared to be phase-shifted from that observed under NP. This decrease in CV_F coincided with the sample point at which feed intake began to increase as a greater proportion of fish consumed a larger meal. This pattern of recovery is similar to the earlier findings of Usher *et al.* (1991) who found that the percentage of salmon smolts feeding after seawater transfer slowly increased, suggesting that the fish were regaining their appetite following the environmental manipulation. The consistently lower feed intake and higher CV_F recorded in the LL treatment during the 60-day feed study would therefore imply that some fish in this particular treatment were taking longer to adjust to the changed conditions. Indeed, the proportion of fish consuming a smaller sized meal of less than 5 mg g fish^{-1} repeatedly remained above 60%, whereas in all other treatments this fell below 20% by the end of the feed study. Koskela *et al.* (1997) commented that poorly acclimatized fish, wherein appetite was uniformly suppressed, would be expected to result in a low CV_F . However, in the present investigation as with previous studies involving changes to the rearing conditions (*e.g.* Hatlen *et al.*, 1997; Koskela *et al.*, 1997), the CV_F was at its highest at the beginning of the trial and progressively decreased over time. These findings have previously been interpreted as providing evidence that fish were acclimatizing to the changed rearing conditions at different rates (Koskela *et al.*, 1997). Since nearly all-experimental studies acclimatize the fish prior to the start of the experiment, it therefore seems logical to suggest that the observed depression in feed intake following the application of light is one of adaptation to the new rearing environment.

As previously mentioned, the onset of artificial light may have altered the behavioural feeding responses by physiologically shifting the feeding rhythm. Rawlings

et al. (1991) observed a single peak in the feeding activity of salmon smolts recently transferred to seawater, concluding that the fish were still developing their feeding pattern to suit their new environment. It is well known that many species of fish demonstrate circadian rhythms in feeding activity synchronized by the light-dark cycle (Kadri *et al.*, 1991; Boujard and Leatherland, 1992a, 1992b; Bolliet *et al.*, 2001), although the regular timing of feeding *per se* can also entrain the feeding activity in fish by overriding the natural feeding rhythm (Boujard and Leatherland, 1992a; Sánchez-Vázquez *et al.*, 1995; Bolliet *et al.*, 2001; Chen and Tabata, 2002). Since the feeding period (0800-2000 h) remained unaltered for all treatments throughout the current study and that appetite was originally synchronized by the light-dark cycle, the onset of constant illumination possibly resulted in an alteration of feeding activity with respect to periods food availability. Indeed, under constant light conditions the demand-feeding rhythm has been shown to shift to the period of food availability (Sánchez-Vázquez *et al.*, 1995; Bolliet *et al.*, 2001), although Sánchez-Vázquez and Tabata (1998) found that rainbow trout, *Oncorhynchus mykiss*, were still capable of maintaining their demand-feeding rhythms under free-running conditions of constant light. It may be that different species of fish will display different feeding behaviours when presented with the same environmental change.

Fish reared under a SNP regime also exhibited an initial reduction in appetite despite experiencing a similar daylength to that under NP, albeit without a dawn and dusk period. As with most laboratory-based studies, fish reared under SNP were subjected to a rectangular light-dark cycle where an abrupt alternation between light and dark was provided by means of lights-on and -off. However, under natural conditions the environmental light intensity gradually changes throughout the day, with the greatest changes occurring around the twilight phases of dawn and dusk. Plasma levels of melatonin, the light perception hormone, clearly demonstrated that fish maintained under natural light perceived the gradual transition in light intensity during dusk compared with the SNP regime, where artificial light had already switched off. The absence of any noticeable differences at dawn can easily be explained by the lateness of sampling during

this period, where the light phase had already commenced in the SNP regime. Since salmonid fish display crepuscular activity in feeding, with the highest activity around dawn and dusk (Higgins and Talbot, 1985; Kadri *et al.*, 1991, 1997; Blyth *et al.*, 1999) it may be assumed that the gradual transition in light intensity which occurs throughout the day, particularly between light and dark, is important for the entrainment of feeding rhythms in fish. In the Syrian hamster, *Mesocricetus auratus*, artificial twilights have been shown to expand the upper limit of entrainment compared to conspecifics reared under a rectangular light-dark cycle (Boulos *et al.*, 2002), suggesting that the twilight transition strengthens the phase-shifting ability of the light-dark cycle. Other studies have similarly discussed the importance of twilight periods on fish physiology and the entrainment of rhythms. In the Eurasian perch, *Perca fluviatilis*, it has been suggested that dawn plays an important role in the synchronization of spawning since significantly fewer fish spawned under a simulated natural photoperiod compared to those under ambient conditions (Migaud *et al.*, 2006b). Nonetheless, little is still known about the role of illuminance changes in the entrainment of vertebrate circadian rhythms (Usui, 2000; Boulos *et al.*, 2002) and further investigations should be performed to examine the effects of twilight transitions on melatonin production and its subsequent influence on the physiological performance in fish.

Growth performance

In addition to the observation for a suppressed appetite, a transient reduction in the growth rate of fish has also been reported following the onset of constant light (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1999; Endal *et al.*, 2000; Nordgarden *et al.*, 2003; Fjellidal *et al.*, 2005). The present study illustrates a similar trend with the weight specific growth rate (SGR_w) for all lit treatments lower, as compared to the control, during February to April, the period in which artificial light was applied. This reduction in growth appears to be linked to the initial lower feed intake recorded in these treatments over the same period. However, the SGR_w for the April to May growth period would seemingly contradict this point, with apparent higher growth rates and

lower feeding levels recorded for the NPLL and SNP treatments than observed in the NP group. This would suggest a compensatory growth response, a reaction that is normally accompanied by hyperphagia (Grove *et al.*, 1978; Talbot *et al.*, 1984; Johansen *et al.*, 2001). If compensatory growth had occurred, then the sampling strategy may have been insufficient in 'capturing' the accurate feeding responses of the fish. Whitley and Hayward (2000) observed that high levels of environmental variation resulted in a greater variation in the daily feed intake of the green sunfish, *Lepomis cyanellus*, and bluegill, *Lepomis macrochirus*. Using Monte Carlo simulations, they calculated that for precise *in situ* estimates of cumulative food consumption, sampling should be conducted at least every five days, although this is suggested to vary depending on the species and size of fish examined. This would indicate that the sampling performed post 22nd March (day 21) probably does not accurately reflect the overall feeding responses of the fish, since sampling was conducted at 10 to 15 days intervals. Moreover, the 60-day feeding study only lasted until the 2nd May, whereas growth for the April to May period was taken from the 23rd April to the 24th May, respectively. As feed intake was not assessed for the last 22 days of the April to May growth period it is not known whether food consumption in fish reared under the NPLL and SNP treatments increased above those in the control, hence accounting for the apparent higher growth rates observed in these treatments during this particular growth period.

The reduction in the condition of fish reared under artificial light probably reflects the initial lower feed intake. Fish condition is a relative expression of the weight to length ratio with alterations in either of these biometric parameters affecting the overall condition of the fish. The change in condition observed in the NP treatment throughout the trial for instance was related to the varying rates of weight gain as the rate of length gain remained relatively stable at 0.10% day⁻¹ across the experimental period. Fish reared under NPLL exhibited similar body weights to fish maintained under NP yet the condition of these fish were significantly lower than the controls. This is most likely to be the result of a consistently higher rate of length gain, relative to control fish, giving the fish a leaner appearance. This pattern of growth is analogous to the changes that

occur in juvenile salmon undergoing the parr-smolt transformation with skeletal elongation believed to create the potential for a rapid weight gain (Björnsson *et al.*, 2000). This may account for the greater growth rates typically observed in fish exposed to constant light, occurring first through a possible photostimulation of vertebral growth (Fjelldal *et al.*, 2005) before a 'bulking' out phase in which weight gain may possibly occur through an increased food consumption or enhanced feed conversion rate. Although no such pattern of weight gain was observed, the rates of weight gain were highest in the constant light regimes relative to the NP treatment from the June to July growth period onwards, indicating that a compensatory weight gain in terms of body mass may have occurred had the trial been prolonged.

Interestingly, cohorts of fish reared under an open system (NP and NPLL) performed significantly better (*i.e.* higher SGR_M) than their counterparts reared in the enclosed systems (SNP and LL). This again may relate to the level of feeding, since mean food consumption levels recorded over the 60-day study was significantly lower for the enclosed groups relative to the control. With both lower feeding levels and growth in comparison to their counterparts reared under an open system, this would suggest that the fish in these treatments were either taking longer to adapt to the changed rearing conditions or that the constant light intensities were having an adverse affect on the physiological growth performance of the fish. Nevertheless, these findings raise possible questions about the extent to which results from laboratory studies, where fish are reared under artificial photoperiods in enclosed conditions (*e.g.* SNP or LL), can be generalized to commercial conditions.

Constant light regimes are frequently reported to enhance the growth of Atlantic salmon reared in seawater (Saunders and Harmon, 1988; Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Forsberg, 1995; Taranger *et al.*, 1995; Oppedal *et al.*, 1997, 2003; Endal *et al.*, 2000; Porter *et al.*, 1999; Nordgarden *et al.*, 2003; Fjelldal *et al.*, 2005). However, in the present study no such growth enhancement was observed. This lack of improved growth under constant light may have been a consequence of the timing of application of light. In the present study light onset began in March, due to technical constraints,

whereas throughout the industry light is generally applied between October to January when growth is naturally low. Endal *et al.* (2000) employed a modelling study to examine the effects of the timing and duration of constant light exposure on growth and maturation in Atlantic salmon post-smolts reared in sea cages. They found that exposure to constant light from November, December or January resulted in enhanced growth compared to control fish maintained under a natural photoperiod. Moreover, the same authors also noted that fish exposed to light from November had the largest weight gain over the experimental period, concluding that a longer exposure period maintains a higher growth rate. As with the present study, McCormick *et al.* (1992) found no overall difference in the growth performance of Coho salmon, *Oncorhynchus kisutch*, when light was applied in late March. They suggested that the fish had already perceived the naturally increasing photoperiod prior to the application of light and as such did not recognize the onset of constant light as an increase in photoperiod. Alternatively, as growth is seasonally linked to the changes in the natural photoperiod and temperature (Higgins and Talbot, 1985; Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999) and with the application of light around the time of the vernal equinox, when the rate of change in daylength is at its highest, the growth rate of the fish in the NP group would have been greater than when light is normally applied during the winter, thereby masking any growth effects.

Conversely, the duration of exposure to light may also be a significant factor in governing the rate of growth. Oppedal *et al.* (1997) for example, found no significant differences in growth between fish reared under constant light compared to controls during the first 11 weeks of exposure. Similar observations have also been previously observed in Atlantic salmon (Hansen *et al.*, 1992) and rainbow trout (Taylor *et al.*, 2005), with these authors suggesting that a period of 17 weeks should elapse before growth deviations between lit and unlit populations of fish are detectable. In the current investigation fish were exposed to light for a period of 20 weeks before the trial concluded. However, whilst no differences in growth was observed during the experimental period it is of interest to note that the body weights in both the NPLL and

LL treatments crossed over their respective control groups at exactly the same time, at the end of June. Correspondingly, growth rates in both the NPLL and LL treatments were higher than controls after 12 weeks of exposure to constant light, from the June to July growth period until the end of the trial. Thus, it is probable that the exposure period coupled with the late light onset may have been insufficient to show a growth response within the time period of the current trial.

The results presented thus far indicate that there is a trend for a decreased appetite and reduced growth rate in Atlantic salmon exposed to artificial light, irrespective of photoperiod. This implies that the observed growth-dip phenomenon following the application of light could be the consequence of a physiological process rather than an artefact of husbandry-related factors (*i.e.* change in feed management). However, unlike the previous studies where the growth-dip has been reported and the reasons for its occurrence speculated upon (*e.g.* Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005) the current study has specifically focussed on the growth-dip phenomenon and the possible underlying mechanisms associated with the onset of photoperiod regimes.

4.4.2. Does the application of artificial light and/or photoperiod regimes influence the stress and/or non-specific immune responses in fish?

With regards to the results discussed thus far, it appears that the growth-dip is related to physiological and behavioural responses to the application of light that enable the fish to adapt to the changed rearing regimes. Acclimatization is often a standard process to any experimental study as the transportation, anaesthetization, tagging and handling of fish during the initial set-up may induce stress (Jobling *et al.*, 2001). Thus, fish require time before they become accustomed to the new environment. It therefore seems logical that the reduced feeding and growth rate observed in the present study was most probably a consequence of stress due to the experimental lighting regimes.

Stress in fish is typically characterized by a series of behavioural and physiological responses involving the activation of the hypothalamic-pituitary-interrenal (HPI) axis which may result in an initial reduced period of feeding and, in the long-term lead to lower growth (Pickering, 1993; Wedemeyer, 1996; Wendelaar Bonga, 1997; McCormick *et al.*, 1998; Gregory and Wood, 1999; Bernier and Peter, 2001). Endal *et al.* (2000) suggested that the initial growth and appetite depression observed in cohorts of fish exposed to continuous light was either a consequence of an initial stress response to the changed rearing conditions or as a phase advancement of a circannual growth pattern adjusted by photoperiod. The results from the current study would support the former of Endal's views with the fish exposed to constant light exhibiting a significant elevation in plasma cortisol concentrations above basal levels ($0-5 \text{ ng.ml}^{-1}$), which was maintained for up to four weeks. This characteristic response has been commonly reported in salmonids following other forms of chronic and repeated stressors (Pickering and Pottinger, 1985, 1987, 1989; Pottinger and Pickering, 1992), with the abating stress response viewed as an interrenal acclimation to the stressor. The findings reported here are also consistent with the earlier results of Leonardi and Klempau (2003) who similarly observed an increase in the plasma cortisol levels of rainbow trout following exposure to constant light. These studies therefore suggest that constant illumination as opposed to the onset of light itself is capable of inducing a chronic stress response in fish.

In contrast, fish reared under SNP did not exhibit any signs of plasma cortisol elevation even though they displayed an apparent initial reduction in appetite. One possibility is that the daily onset of the light phase may have triggered an acute stress response in the fish which would have resulted in short-lived amplified levels of plasma cortisol returning to resting levels after a couple of hours. Rance *et al.* (1982) studied the circadian cortisol profiles in rainbow trout reared under commercial conditions as well as in trout housed in laboratory tanks. They noticed that in addition to a midnight peak, trout reared in tanks also exhibited a 1-2 hour period of raised cortisol concentrations in the early morning coinciding with the onset of artificial light concluding that the abrupt switch-on of light was the most likely cause. Similar peaks have also been reported in

sea bass, *Dicentrarchus labrax*, at both the light onset and offset (Cerdá-Reverter *et al.*, 1998) as well as in studies which incorporated a simulated dawn and dusk into the photophase (Reddy and Leatherland, 2003). Since sampling periodically occurred several hours after the onset of the light phase in the SNP regime, it is not known whether these fish experienced an acute daily stress. Nevertheless, the reduced food consumption in the SNP group by 68% and 34% following 21 and 45 days of exposure, relative to the control, is coherent with the study of McCormick *et al.* (1998). They subjected Atlantic salmon parr to a repeated handling stress, in which cortisol levels were temporarily elevated, and found that food consumption was reduced by 61% and 37% following 17 and 37 days of exposure as compared to unstressed fish. The similarity of the results between the studies suggest that fish reared under SNP were most probably experiencing an acute stress response possibly associated with the daily onset of light and that the eventual increase in food consumption was most likely due to an interrenal acclimation.

Although both the constant light regimes revealed an elevation in plasma cortisol concentrations, the magnitude of the stress response was much greater in the LL treatment than for that observed under NPLL. The differences in the rearing conditions between the two treatments (*i.e.* constant photic conditions under LL and superimposition of an ambient rhythm under NPLL) may have been attributable to the results obtained. Fish reared under LL were 'enclosed' from the ambient photoperiod and exposed to a constant light intensity of approximately 2.5 W.m^{-2} , whereas under NPLL the continuous light was superimposed on to the natural light with diurnal changes in intensity ranging from around 3 to 1.2 W.m^{-2} between day and night respectively. This would suggest that fish reared under NPLL were still able to perceive the changes in the ambient daylight. Even so, plasma concentrations of the light perception hormone melatonin were not found to differ significantly at any of the sampled time points over the 24-hour period, with levels in both treatments around 4 pg.ml^{-1} . Recently, it has been proposed that salmon have a light intensity threshold of around 0.016 W.m^{-2} (Migaud *et al.*, 2006a), indicating that the fish can perceive light at and above this level. Since the

irradiance levels were above this intensity threshold in both the constant light groups this would suggest that melatonin levels do not discriminate between higher intensities of light, implying instead that some other perceptory mechanisms were most probably involved such as retinal or deep brain photoreceptors. Additionally, Migaud *et al.* (2006a) have recently shown that the light sensitivity of the pineal examined *in vitro* differs somewhat from the sensitivity of the pineal in *in vivo* fish. Thus, it is reasonable to suggest that the fish were still visually recognizing the changes in the time of day which may have possibly buffered the amplitude of the stress response to the exposure of constant light. This may still have provided the fish with the information needed to synchronize their feeding rhythms, explaining the minor amplitude in plasma cortisol concentrations and accounting for why the appetite of fish reared under NPLL recovered during the feeding trial unlike the fish under LL.

Furthermore, the amplitude of the stress response recorded in the NPLL treatment was found not to differ significantly from the levels measured in the control group. Nevertheless, whilst this difference may not be of any statistical importance the levels observed in the NPLL may still have a biological relevance. For example, it has previously been shown that a two to four week elevation in plasma cortisol levels from a resting state of 0-4 ng.ml⁻¹ to a peak of just 9 ng.ml⁻¹ is sufficient to increase the susceptibility of trout to disease (Pickering and Pottinger, 1985, 1989). In addition the raised plasma cortisol levels recorded in the control group on the 3rd March would suggest that these fish were 'stressed' since the cortisol levels measured were higher than the 0-5 ng.ml⁻¹ resting levels thought to be representative of unstressed salmonid fish (Pickering and Pottinger, 1985, 1989). However, this increase was not significantly elevated relative to pre-treatment levels and is suggested to have most likely arisen through an acute stress response to handling.

Increased plasma cortisol concentrations have previously been suggested to have a negative impact on appetite (Gregory and Wood, 1999). However, no significant correlations were found between feed intake and cortisol levels in the present study, although it is worth noting that at the same time at which plasma cortisol returned to

baseline levels in the NPLL and LL treatments the percentage number of fish consuming a meal greater than 5 mg.g fish⁻¹ began to increase. This lack of correlation is presumably related to the fact that the measurement of feed intake and cortisol is just a 'snapshot' of the fish's appetite and stress levels at a particular point in time. Nevertheless further studies are recommended to demonstrate which factor is cause and which is effect or whether both are secondary responses to some other factors such as a change in the growth-promoting hormones. However, it appears most likely that the onset of light rather than the lower feed consumption led to the higher levels of plasma cortisol as both feed restriction and starvation have been found to have no direct effect on circulating cortisol levels (Sumpter *et al.*, 1991; Pottinger *et al.*, 2003).

Current information within the scientific literature surrounding the relationship between feeding and plasma cortisol concentrations is somewhat contradictory. Cortisol implants for example, have been found to either result in a negative impact on appetite, growth and condition (Gregory and Wood, 1999) or lead to a higher feeding activity (Lyytikäinen and Ruohonen, 2001). Similarly, Bernier *et al.* (2004) found that the impact of cortisol had marked variations on the food consumption and growth in goldfish, *Carassius auratus*, with moderate elevations stimulating feed intake without promoting growth and high levels of cortisol inhibiting growth without affecting the overall appetite. Therefore, it appears that cortisol can exhibit both anabolic and catabolic actions on fish performance most likely through intermediary metabolic processes.

Metabolic and immune response

The primary activation of the HPI axis is known to lead to changes in the metabolism, hydromineral balance and innate immune function as part of the secondary stress response (Schreck, 1982; Barton and Iwama, 1991; Pickering, 1993; Wedemeyer, 1996; Wendelaar Bonga, 1997; Van Weerd and Komen, 1998; Harris and Bird, 2001; Barton, 2002). In the present study, neither plasma glucose levels nor lysozyme activity

exhibited any major noticeable differences between the treatments throughout the course of the investigation suggesting no evidence of a stress effect.

Stress is an energy-demanding process resulting in the mobilization of energy substrates that allow the fish to cope metabolically with the presence of the stressor. Plasma glucose levels are commonly measured as an indicator of the possible metabolic changes that occur in response to a stressor. The predominant site for glucose production is in the liver. The stress hormones in conjunction with other glucoregulatory hormones, *i.e.* insulin, are suggested to have an important role in the regulation of glucose production (Mommsen *et al.*, 1999). Increases in plasma cortisol concentrations are known to increase plasma glucose levels through a catabolic glyconeogenic or gluconeogenic effect (Schreck, 1982; Barton and Iwama, 1991; Pickering, 1993; Wedemeyer, 1996; Mommsen *et al.*, 1999). Thus, the maintenance of similar plasma glucose concentrations in all treatments is therefore quite surprising; especially as fish in both the NPLL and LL regimes exhibited significantly raised levels of the stress hormone cortisol following the onset of constant illumination. Furthermore, plasma glucose concentrations generally increase following a meal. These post-prandial increases from the feeding fish within the control group may well have masked any gluconeogenic effect resulting from the chronic stress response observed in the constant light groups as well as that from the suggested repeated acute stress previously discussed for the SNP regime. Moreover, increased gluconeogenesis only tends to occur when the supply of carbohydrate is insufficient to meet the fish's energy requirements (Wedemeyer, 1996). As food contents were still present in the gastrointestinal tract of the majority of fish sampled, including those fish exhibiting high cortisol levels, it could be that the fish still had sufficient amounts of carbohydrate available which would have possibly inhibited a gluconeogenic effect. This suggestion can be further supported by the work of Aas-Hansen *et al.* (2005) who found that exogenous exposure of salmonid hepatocytes to cortisol significantly increased glucose production from the hepatocytes of fasted fish but had no effect on the hepatocytes of fed fish.

The non-specific immune system in fish is characterized by the microbicidal activity due to the lysozyme and complement systems together with macrophage phagocytosis (Wedemeyer, 1996). Plasma lysozyme activity varies in response to a stressor with either lowered (Fevolden *et al.*, 2002) or increased activity (Demers and Bayne, 1997) reported depending upon the type of stressor encountered. In the current study plasma lysozyme activity was only found to be significantly reduced in the NPLL treatment in comparison to control fish on the 3rd March, two days after light was applied. Again, one would have expected the LL treatment to exhibit the greatest change in lysozyme activity as this treatment displayed the greatest elevation in circulating plasma cortisol concentrations. As previously discussed for glucose, this may be related to the fact that the fish were still able to meet their immunological requirements and maintain homeostasis. Alcorn *et al.* (2003) examined ration level on the immune function in salmonids. They noticed that although the salmonid immune system may be fairly robust with regard to the available metabolic energy, the significant changes observed in the phagocytotic cell activity suggest that some cellular immune functions may be affected by feed level

Conversely, lysozyme activity has been shown to exhibit strong seasonal differences (Tort *et al.*, 1998; Bowden *et al.*, 2004; Morgan, 2004) with the lowest values reported between March to June (Morgan, 2004), the period across which the trial was conducted. Any noticeable differences in activity may therefore have been masked by the fact that lysozyme activity was at its lowest levels during the experimental period in which plasma samples were taken. Furthermore, the application of artificial photoperiods has been shown not to exert any significant influence on lysozyme activity in Atlantic halibut, *Hippoglossus hippoglossus* (Bowden *et al.*, 2004) and rainbow trout (Morgan, 2004). This suggests that photoperiod alone is ineffective at mimicking the influence of season on lysozyme activity and that a combination of environmental factors such as photoperiod and temperature may be required (Bowden *et al.*, 2004). Similarly, Leonardi and Klempau (2003) observed that not all immune parameters were affected by

the application of artificial photoperiods, although in that particular trial lysozyme was not amongst the measured parameters.

Selecting the most appropriate parameters to measure is of vital importance when undertaking stress studies. The stress response in fish may be polymorphic depending upon the species, strain, maturity and dominance status of the fish as well as the type and severity of stressor encountered (Schreck, 1981; Pickering and Pottinger, 1987, 1989; Barton and Iwama, 1991; Pottinger *et al.*, 1995; Wendelaar Bonga, 1997; Pottinger and Carrick, 2001; Schreck *et al.*, 2001). Differences in the primary and secondary stress responses have previously been shown to exist between species (*e.g.* Pickering and Pottinger, 1987, 1989; Ruane *et al.*, 1999; Barton, 2000), strain (*e.g.* Weil *et al.*, 2001) and season (*e.g.* Tort *et al.*, 1998; Bowden *et al.*, 2004; Morgan, 2004) when challenged with the same stressor. Barton (2000) for example, found that different species of juvenile salmonid fish exhibited different cortisol and glucose responses when challenged with a variety of acute and chronic stressors. These differences in the stress response between fish would therefore suggest that extreme care should be taken when selecting stress indicators to examine treatment effects on the primary and secondary stress responses. The suitability of cortisol alone for instance has often been questioned regarding its reliability as an indicator in stress measurement (Pickering and Pottinger, 1987; Van Weerd and Komen, 1998; Barton, 2000). Van Weerd and Komen (1998) make a valid point by commenting that individual fish may employ different strategies to cope with stressful conditions whereby some fish may display low glucose levels, for instance, whereas others may show an opposite reaction.

Notwithstanding this, the reduction in appetite coupled with increases in plasma cortisol levels is perhaps sufficient enough to suggest that the application of constant artificial light represents a stressful situation requiring adaptation. It is most probable that the changes in physiology to stressful rearing conditions will be mediated through complex endocrine pathways brought about to return the fish to a homeostatic state.

4.4.3. What are the acute responses of the growth endocrine axis following light application?

Information from both internal and external stimuli are processed and integrated for the appropriate regulation of growth through hormonally mediated pathways (Duan, 1998; Company *et al.*, 2001). Thus, in the present investigation the observed differences in feeding and growth may have been determined in the period immediately following the environmental stimulus, *i.e.* the application of light and/or photoperiod, and would therefore have been expected to exert changes in circulating hormone levels through the somatotrophic and other associated growth hormone axes. Growth hormone (GH), insulin-like-growth factor-I (IGF-I) and the thyroid hormones have all been linked to a central role in growth and metabolic processes in fish (Leatherland, 1994; Björnsson, 1997; Duan, 1998), and were therefore judged as suitable hormonal indicators in the present study.

Throughout the experimental period plasma GH levels generally remained below 2 ng.ml⁻¹, levels which are consistent with those previously measured in adult Atlantic salmon reared under various photoperiod regimes (Björnsson *et al.*, 1994; Nordgarden *et al.*, 2005). Of particular interest is the brief period immediately following the onset of light when plasma GH levels in both the constant light regimes significantly increased to around 2.5 ng.ml⁻¹. This minor increase could be associated with the decreased levels of feed intake observed after the onset of light, since GH levels are known to increase during periods of fasting (Sumpter *et al.*, 1991; Farbridge and Leatherland, 1992; Leatherland and Farbridge, 1992; Pottinger *et al.*, 2003). However, the SNP regime displayed no such increase despite experiencing an initial reduction in feed intake. Furthermore, these raised levels were not sustained during the period in which feed intake was suppressed, and it seems highly unlikely that the circulating levels of GH would have increased so rapidly in such a short space of time following the voluntary cessation in feed consumption. In overwinter fasting fish for instance, plasma GH levels increased eight weeks after the fish ceased feeding (Pottinger *et al.*, 2003), whereas GH receptors have been reported to decrease after just 3 weeks of fasting (Fukada *et al.*,

2004). However, transient increases in the concentrations of circulating GH have been reported following an abrupt transfer from SNP to constant light in other studies (Stefansson *et al.*, 1991; Björnsson *et al.* 1995). Thus, it may appear that the brief elevation of plasma GH is photoperiod-induced.

Plasma GH levels are under the strong influence of a seasonally changing photoperiod (Björnsson *et al.*, 1995; Björnsson, 1997) and are best associated with the parr-smolt transformation in salmonids (Boeuf *et al.*, 1989; Stefansson *et al.*, 1991; Björnsson *et al.*, 1995, 1998, 2000; McCormick *et al.*, 2000; Arnesen *et al.*, 2003; Handeland *et al.*, 2003), although spring increases have also been detected in adult salmon maintained under a natural or simulated natural photoperiod (Björnsson *et al.*, 1994; Nordgarden *et al.*, 2005). However, circulating GH levels in smolting salmon have also been observed to increase with a 24-hour period following seawater transfer which may be sustained for up to 10 days (Boeuf *et al.*, 1989; Handeland *et al.*, 2003). Additionally, Björnsson *et al.* (1998) reported minor transient increases in the plasma GH levels of photoperiod-inhibited smolts, maintained under constant light, following salinity exposure with maximal levels observed between 12 and 48 hours. In smolting salmon in freshwater the increase in the circulating levels of plasma GH is seen as having an important role in the hyperosmoregulatory ability of fish in seawater (Komourdijan *et al.*, 1976; Björnsson *et al.*, 1995, 1998; Handeland *et al.*, 2003). Thus it is speculated that the increase in GH levels measured in the constant light regimes in the present experiment may act as an adaptive mechanism to adjust growth/metabolism. This brief surge could for example produce a compensatory reaction by stimulating the competitive ability of fish and increase the appetite and feed conversion efficiency as has previously been demonstrated in trout through GH administration (Johnsson and Björnsson *et al.*, 1994; Johnsson *et al.*, 1996; Silverstein *et al.*, 2000). However, the current results are only preliminary findings and further studies should focus on the short-term changes on GH levels and its influence on the GH-IGF-I axis.

IGF-I

The actions of plasma GH are mediated to some degree through the secretion of IGF-I (Duan, 1998). In that respect this appears to be true with IGF-I levels remaining more or less consistent throughout the experimental period. At the 6th March sample point plasma IGF-I levels in the NPLL regime increased whereas in the other treatments IGF-I levels decreased. This may be a result in the increase of GH levels at this time or could alternatively be due a decreased clearance rate or a down-regulation of binding proteins or receptor sites. Nevertheless, plasma levels were in a comparable range at the following time point on the 21st March.

Plasma IGF-I has been shown to correlate well with ration levels (Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004) and as such correlates well with the growth rate (Beckman *et al.*, 2001, 2004; Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004; Taylor *et al.*, 2005). However, in the present study IGF-I was not significantly correlated with feed intake for any of the treatments and it is impossible to determine whether plasma IGF-I levels represented a true reflection of the growth rate as there were too few samples, corresponding to just two growth periods, where growth rate could be compared.

Although nutritional status influences IGF-I levels in fish (Larsen *et al.*, 2001; Pierce *et al.*, 2002; Gabillard *et al.*, 2003b; Dyer *et al.*, 2004) temperature is thought to exert the greatest influence on IGF-I secretion (Larsen *et al.*, 2001; Gabillard *et al.*, 2003b). Larsen *et al.* (2001) for example noticed that a reduction in temperature decreased circulating IGF-I regardless of whether fish were fed or fasted. However, the same authors also acknowledged that their work was performed under winter conditions when growth is low and GH production is also likely to be low. However, Gabillard *et al.* (2003b) suggest that temperature promotes growth by stimulation of IGF-I levels through its direct effect on GH secretion. Notwithstanding this, under constant temperature IGF-I levels appeared to reflect changes in photoperiod (McCormick *et al.*, 2000; Beckman *et al.*, 2004).

To date few studies have examined the effects of photoperiod on IGF-I. In a similar trial to that performed here, Nordgarden *et al.* (2005) examined the effects of an SNP and LL photoperiod on GH and IGF-I levels in adult Atlantic salmon over a year, but failed to observe any differences in IGF-I levels between the two light regimes. In addition, the levels of IGF-I reported in that study were two-fold higher than those reported in the current trial, although this may be the result of differences between the assays and the various extraction methods used (Shimizu *et al.*, 2000). In both this study and Nordgarden *et al.* (2005) constant photoperiods were employed to replicate the light regimes employed within the industry in reducing maturation rates and promoting growth. This is suggested to affect the growth performance in fish by either phase-shifting an endogenous growth pattern (Eriksson and Lundqvist, 1982; Endal *et al.*, 2000) or through a direct photostimulatory effect (Komourdijan *et al.*, 1989). Taylor *et al.* (2005) examined various photoperiod regimes on growth and IGF-I secretion in rainbow trout. They found that the application of extended photoperiods (*e.g.* 18L:6D) appeared to cause direct stimulation of growth through an up-regulation of IGF-I. However, under a constant photoperiod (*i.e.* LL), where the diel melatonin signal is abolished, the growth pattern was entrained through an underlying endogenous rhythm explaining why plasma IGF-I levels reflect the growth rate.

The possible effects of the elevated plasma cortisol levels on IGF-I production cannot be excluded. Aquaculture related stressors have recently been shown to affect the circulating levels of IGF-I (McCormick *et al.*, 1998; Dyer *et al.*, 2004). However, current information within the literature is limited and somewhat contradictory as both increases (McCormick *et al.*, 1998) and decreases (Dyer *et al.*, 2004) have been reported following exposure to a stressor. Unfortunately, the current results do nothing to add to this debate with no significant correlations found for any of the treatments between IGF-I and plasma cortisol levels. In addition, unlike GH where plasma levels for all fish were analysed over the 60-day feed intake and hormone study, only a limited number of randomly selected plasma samples from specific sample points, based on the most

interesting data points from feed intake results, were assayed for IGF-I due to economic restrictions.

In addition to measuring IGF-I, circulating IGF-II concentrations were also assessed using a similar radioimmunoassay technique (Gabillard *et al.*, 2001b). IGF-II is structurally similar to IGF-I and is believed to have derived from a common ancestral molecule (Jones and Clemmons, 1995). Although IGF-II has been found to decrease during food restriction (Gentil *et al.*, 1996), and is suggested to be involved in the control of plasma GH levels independent of temperature (Gabillard *et al.*, 2003b), its exact biological potency still remains unknown. Despite the omission of IGF-II data from the current chapter, it was still felt necessary that results should be briefly mentioned. Circulating levels of IGF-II measured from fish in the present experiment were two- to five-fold higher than the IGF-I levels measured, a similar finding to that reported for trout (Gabillard *et al.*, 2003b), and displayed a similar profile to the IGF-I. Further studies are required to elucidate the role of IGF-II in regulating growth.

It must be noted however that in the present investigation both the total levels of GH and IGF-I were measured. The majority of plasma IGF circulating in the blood is bound to specific binding proteins leaving a small fraction in the free and more physiologically reactive form (Jones and Clemmons, 1995; Shimizu *et al.*, 1999). In coho salmon 0.3% of the total amount of circulating IGF-I is in the free form (Shimizu *et al.*, 1999). However, the measurement of total levels of IGF-I has generally been accepted by fish physiologists as a representative measure of the level of IGF-I in the free form (Plisetskaya, 1998). Both GH administration and fasting for example have been shown to cause changes to the circulating levels of IGF-I without altering the ratio of total to free IGF-I (Shimizu *et al.*, 1999). Nevertheless, this does not exclude the possibility that other environmental factors such as photoperiod may alter the proportion of total and free hormone levels circulating in the plasma. Factors which may alter the blood levels of the binding proteins or binding hormones to proteins will inevitably cause changes to the total hormone level without necessarily altering the concentration of the free hormone level (Leatherland, 1994). The most commonly referred example is the

changes that occur in the total thyroid hormone concentrations in pregnant women. The increased levels of oestrogen increases the amount of thyroid hormone binding protein subsequently leading to a shift in the free:bound hormone ratio which raises the total hormone level (Ingbar and Woeber, 1981). This would give the impression of hyperthyroidism, although in reality the physiologic free levels remain at normal levels indicating normal thyroidal function. Thus, further studies should perhaps at least focus on the measurement of free hormone levels or wherever possible on the GH and IGF-I binding proteins, binding affinity and/or receptor levels in response to environmental stimuli.

Thyroid hormones

The thyroid hormones are also known to be involved in the development and growth processes of fish (Leatherland, 1982, 1994; Macbride *et al.*, 1982; Sumpter, 1992; Power *et al.*, 2001). In the majority of teleostean thyroidal studies the total thyroid hormone level is often measured in preference to the more physiologically relevant free fraction. This is based upon the early work of Eales and Shostak (1985b) who demonstrated that the proportion of total hormone correlates to the free levels in the plasma of Arctic charr. However, both environmental pH and temperature have been shown to affect the proportion of total T₄ to the levels of T₄ in the free form (Eales and Shostak, 1986), whereas no differences were found during the parr-smolt transformation (Boeuf *et al.*, 1989). Thus, to avoid the possibility of a photoperiodic effect on the proportion of thyroid hormone levels the free fraction of hormone was measured in the current investigation.

Free levels of plasma T₄ varied between 2-8 pmol.l⁻¹ throughout the experimental period, a range which is comparable to the previously reported levels in Arctic charr (Eales and Shostak, 1985b). In contrast, free levels of the extrathyroidally produced T₃ were approximately three fold higher than estimated for charr, implying that the free levels of thyroid hormones may vary between species. Eales and Shostak (1987) similarly found that the range of free thyroid hormone levels varied by up to three orders of magnitude within and among 16 different species of tropical fish. Additionally,

plasma levels of free T₃ exceeded free T₄. This contrasts with other studies where free (*e.g.* Eales and Shostak, 1985b) and total (*e.g.* Boujard and Leatherland, 1992c; Gélinau *et al.*, 1996; Gomez *et al.*, 1997) levels of T₄ were higher than T₃ in salmonid species. Nonetheless, serum levels of T₃ have been found to exceed T₄ levels in rainbow trout (Cyr *et al.*, 1998), in red drum *Sciaenops ocellatus* (Leiner *et al.*, 2000; Leiner and MacKenzie, 2001), and to occur at approximately the same levels in plaice, *Pleuronectes platessa* (Osborn *et al.*, 1978).

In both the constant light regimes, free T₄ and T₃ were significantly elevated, relative to the SNP and NP treatments, shortly after light onset. This may have been brought about by the increase in GH levels at this time. Intraperitoneal injection of human GH increases plasma T₃ levels in rainbow trout through an increase in 5' monodeiodinase, responsible for the peripheral conversion of T₄ to T₃ (MacLachy and Eales, 1990). This increase in hormone levels may indicate that GH and free T₄ and T₃ may be involved in regulating the physiological response to environmental stressors and in maintaining/restoring homeostasis. Given that the LL regime exhibited the highest cortisol elevation and also suffered an overall reduction in food consumption relative to the NP group it is interesting to note that, with the exception of the 6th March sample, free T₃ levels decreased at the same time as when cortisol levels returned to basal.

Plasma levels of T₃ have been found to correlate with both ration and growth, supporting the idea that increased T₃ production induced by feed consumption may exert some role in promoting growth (Eales and Shostak, 1985a; Gabillard *et al.*, 2003a). Thus it is possible that, since feed intake was similar amongst groups at the start of the trial, the initial high levels of T₃ measured in the constant light regimes were probably due to the stress response rather than to feed consumption. The subsequent lower levels of free T₃ in the LL group from the beginning of April may be representative of the lower nutritional status in these fish. This is reflected in the SNP group whereby both food consumption and free T₃ levels remained consistently low for the majority portion of the 60-day feed/hormone study before increasing towards the end of the study.

A secondary peak in free T_4 levels was also observed in both constant light treatments at the beginning of April. However, unlike the initial peak the LL regime failed to exhibit higher free T_3 concentrations. This resulted in a higher $T_4:T_3$ ratio. The $T_4:T_3$ molar ratio is often used as a sensitive index of the change in thyroidal status, which may not necessarily be detected by changes in T_4 or T_3 alone (Osborn *et al.*, 1978; Eales and Shostak, 1985a). During the two sample points in April the $T_4:T_3$ ratio in the SNP and LL treatments appeared higher than that measured for the NP and NPLL regimes. The ratio of $T_4:T_3$ has been found to be sensitive to ration level and is often elevated in starved fish (Eales and Shostak, 1985a). This may reflect the seasonal changes in appetite and growth, and indeed the $T_4:T_3$ ratio has been observed in trout to be highest during the summer and lowest in the winter when growth is at its highest and lowest respectively (Osborn *et al.*, 1978), indicating a decrease in peripheral utilization or hepatic degradation of T_4 . This may suggest a decreased sensitivity in the target tissue leading to a decrease in the 5' monodeiodinase in converting T_4 to T_3 . Thus, an increase in thyroidal T_4 release may still be taking place without affecting the T_4 to T_3 conversion (Eales and Shostak, 1985a).

The lack of significantly strong relationships between the hormones and various parameters examined within this trial (*e.g.* GH v FI) can probably be explained by the pleiotropic nature of the hormones, which affect and are themselves affected by several physiological processes. Plasma GH levels for example, have been shown to vary with nutritional status (Sumpter *et al.*, 1991; Farbridge and Leatherland, 1992; Leatherland and Farbridge, 1992; Pottinger *et al.*, 2003), stress levels (Pickering *et al.*, 1991; Farbridge and Leatherland, 1992), and maturational status (Sumpter *et al.*, 1991; Björnsson *et al.*, 1994; Holloway *et al.*, 1999). Thus, hormonal status is invariably dependent upon the physiological status of the fish at the time of sampling. As adult Atlantic salmon were used in the current trial, coupled with the small number of fish sampled at each time point, the differences between individual fish in terms of feeding, maturational status, stress and growth rate would probably account for some, if not most, of the ambiguity of the results presented.

On a final point, hormones are secreted in a pulsatile manner or through changes in the blood chemistry brought about by altered activity (*e.g.* feeding), rather than in a continuous manner. Since most of the samples were collected serially between 1000 and 1100 h, it cannot be ruled out that some of the changes observed in the plasma hormone levels were as a result of a shift in the diel cycle. Leatherland (1994) comments that one of the major flaws in comparative endocrinological studies is that researchers tend to justify sampling at set times of the day as eliminating any circadian influence. As previously discussed, both photoperiod and changes in feed activity could entrain circadian rhythms (Boujard and Leatherland, 1992a; Leiner *et al.*, 2000; Leiner and MacKenzie, 2001). Thus, it is conceivable that the samples obtained in the present study may have been collected during the acrophase of one or more of the treatments and during the nadir in the others. Nonetheless, whilst it is freely accepted that the treatment application may have possibly affected the diel hormone rhythm, monitoring such a response in the current investigation would have resulted in a continuous disturbance of the fish populations. This in turn could have induced a stress response and affected the feeding behaviour in fish, thereby influencing the main objectives of the study. Therefore, further studies are suggested to examine the effect of photoperiod-induced changes on the diel hormone cycle.

These results provide a preliminary insight into the acute hormonal changes that occur following the application of artificial photoperiod regimes and demonstrate the difficulties in interpreting such an intricate system. Nevertheless, further studies are necessitated to examine the effect of light and photoperiod treatment on the GH-IGF-I and other associated growth-promoting axes, both in the short- and long-term period and its role in the control of homeostasis and growth.

4.4.4. Relevance to the Industry

The present study was performed to elucidate the growth-dip phenomenon frequently reported within the commercial industry and to draw on the research findings to improve husbandry practices (*e.g.* feeding guidelines) at the production level for Atlantic salmon

reared under photoperiod regimes. However, it is often difficult to interpret the results from such studies and apply them to a commercial situation where the rearing environment will markedly vary from that tested under laboratory-controlled conditions. This point of view is supported by the differences observed in the growth performance between fish reared in the open- and enclosed-light systems, which may cast doubts over previous work where artificial photoperiods such as SNP and LL have replaced the natural equivalent (NP and NPLL) that are most likely to be involved in the majority of rearing regimes utilized within the industry. The trend for the observed feeding and growth depression in the present study for example may have been attributed to a higher light intensity (400 W) than what fish would be subjected to in lit commercial production pens. Under commercial conditions, a fish would have to remain continuously within a 0.5 m radius from the standard submersible 400 W metal halide light unit, commonly used throughout the salmon industry, to experience an intensity of $2.5 \text{ W}\cdot\text{m}^{-2}$ recorded for the LL treatment. That said, behavioural studies conducted in commercial cages have shown that Atlantic salmon tend to position themselves at the depth of the submersible lighting units resulting in a shoaling behaviour around the units, particularly during the night (Oppedal *et al.*, 2001; Juell *et al.*, 2003; Juell and Fosseidengen, 2004). Only by studying fish in their natural varying environment, or under typical commercial conditions, will the physiological and behavioural responses be made clear and their adaptive significance fully understood.

Notwithstanding this, the current study was designed to confirm or refute the disputed 'growth-dip' phenomenon and clarify whether its existence was a feature of a physiological response rather than the by-product of a farmer's ill-judged perception of the fishes feeding behaviour under constant light. Maximizing the growth rates and food conversion efficiency in fish in intensive culture depends upon matching the way in which the farmer makes food available to the fish (Talbot, 1993). Irrespective of whether conditions were similar or not to those found in the commercial cage setting this trial has clearly demonstrated an apparent trend for an initial reduction in appetite as well as revealing that artificial light application may invoke a stress response. Thus this

investigation may be viewed as a test or pilot study, first illustrating the cause under controlled conditions where direct effects can be ascertained (*e.g.* photoperiod-induced changes) before modifying and applying the trial design to conditions realistic of those found under commercial conditions to confirm whether the same results are replicated.

With respect to commercial production, the use of artificial light and photoperiod regimes are widely accepted as a tool for enhancing the productivity at all levels within the salmon industry. The primary use for artificial light at the on-growing stage is to reduce the numbers of fish maturing as grilse, mature after one sea winter (Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1998, 1999; Oppedal *et al.*, 1997; Porter *et al.*, 1999; Endal *et al.*, 2000). The incidence of maturation in the present study, based upon the gonadosomatic index (GSI), was similar amongst all treatments with the majority of males deemed to be maturing. Taranger *et al.* (1999) found a similar trend when light was applied in March, with more males maturing than females and only a slight reduction in the overall number of fish maturing compared to when constant light was applied from January. Thus, as with growth, the timing of the onset of light appears to play a pivotal role in determining sexual maturation. Duston and Saunders (1992) suggested that maturation was reliant upon a crucial decision period based on the energy reserves of the individual. The application of constant light would therefore alter the positioning of the critical period affecting gonadal development, depending upon when the light was applied (Taranger *et al.*, 1999). As previously mentioned, Endal *et al.* (2000) found that the growth rate of salmon reared in sea cages was greater when light was applied in November as compared to January, although both onset dates resulted in a greater weight gain than fish reared under ambient conditions. However, the same authors also noted that the earlier onset resulted in higher numbers of maturing fish than when light was applied in January, implying some form of trade-off between growth and maturation. Similarly, a February onset has also been shown to advance maturation (Kråkenes *et al.*, 1991), supporting the idea for a window of opportunity in which maturation can be suppressed. Nonetheless, although providing a trend for maturation in Atlantic salmon the gonadosomatic index does not represent an accurate indication of

gonadal activity (De Vlaming *et al.*, 1982). Since no histological analyses were performed on gonadal tissue samples in the current study it is impossible to determine whether the application of constant light had any inhibiting (*e.g.* atretic oocytes) or enhancing affect on gonadal development. As gonadal samples were removed from sacrificed fish during the 60-day feeding study (March to May), it is suggested that this was too early to be able to detect major changes in gonadal development and would not represent an accurate portrait of the levels of maturing fish during the trial. The effectiveness of light application on reducing the numbers of grilse has previously been attributed to the occurrence of the growth-dip (Hansen *et al.*, 1992; Taranger *et al.*, 1999). This could possibly hold true since restricting feed during certain months of the year has been shown to reduce the proportion of fish maturing (Thorpe *et al.*, 1990a; Bromage *et al.*, 1992). Since maturation was not the major aim of the current study, further studies are suggested to examine the effects of light application on the growth-dip in terms of a reduction in appetite and its subsequent effects on maturation.

At present, the way lights are used within the industry is impromptu with sites applying light as and when they are available. This makes the comparison of production data virtually worthless and may also explain why reports of the growth-dip and the effectiveness of constant light regimes on maturation levels widely varies both between and within sites year-on-year. Only through the standardization of lighting regimes with regards to the intensity, spectral quality, the number of light units per pen and the timing and duration of exposure to light can the comparisons and potential benefits of such regimes be realized. However, prior to the implementation of such regimes in commercial scale trials, pilot studies such as that performed here must first be conducted to minimize the economic risk to the industry as well as to evaluate the potential risks and/or benefits to the production cycle.

4.4.5. Summary

This study has examined numerous physiological and behavioural parameters to primarily confirm or refute the disputed ‘growth-dip’ phenomenon commonly reported

within the salmon farming industry. The results from the current study clearly indicate that Atlantic salmon tend to demonstrate a trend for a reduced appetite following the application of artificial light, irrespective of photoperiod. Furthermore, a chronic stress response was clearly demonstrated in fish exposed to constant illumination, implying that the acclimation to the changed rearing regimes is in part related to stress. The growth performance of fish was significantly affected by the type of rearing environment under which the fish were reared, and differences in the melatonin profiles between individuals under a natural and simulated natural photoperiod suggest a role for diel changes in light intensity in entraining circadian rhythms. No role for the GH-IGF-I axis could be concluded although the thyroid axis is suggested to have some metabolic role in maintaining homeostasis following the application of constant light. Finally, although the results presented here clearly demonstrate physiological and behavioural differences in fish reared under various light regimes, the light conditions experienced within the tank-systems are not reflective of those perceived in cages. Thus, further studies are required under commercial conditions to assess the impact on welfare and overall growth performances.

Chapter 5: The effect of light characteristics (spectral quality and intensity) on the growth and maturation of 1+ Atlantic salmon reared in commercial sea-cages

5.1. Introduction

Ensuring an all-year round supply of Atlantic salmon (*Salmo salar*) of uniform size and quality is an important objective for the farming industry. However, the unpredictable rates of grilising (mature after one sea winter) reported during the on-growing phase still represent a major constraint. Early maturation not only interferes with production schedules but also results in a lower growth performance as well as a deterioration of flesh quality that subsequently leads to the downgrading of fish at the processing plant. During 2004, approximately 27,000 tonnes of grilse were harvested within the UK (SEERAD, 2005), equivalent to 17.5% of the total salmon production for that year. Although artificial photoperiod regimes have been used to improve growth and reduce the proportion of early maturing fish (Hansen *et al.*, 1992; Taranger *et al.*, 1995; Oppedal *et al.*, 1997; Porter *et al.*, 1999; Endal *et al.*, 2000; Bromage *et al.*, 2001), there is still a lack of information regarding light characteristics and their effects on the physiological responses of fish under commercial operations.

Light is characterized by its quantity (intensity), quality (spectral content) and duration (Sumpter, 1992; Boeuf and Le Bail, 1999). However, the quality and quantity of wavelengths penetrating the water are altered in much the same way as in the atmosphere, through both absorption and scattering processes. Water absorbs maximally in the far red (λ 700-800 nm) and infra-red (λ 750 nm to 1 mm) wavelengths, and as such these are rapidly absorbed and converted into heat energy. Blue light (λ 450 nm), on the other hand, has a higher energy content and is able to penetrate deeper through the water column reaching depths of up to 150 m in the clearest waters (Lobban and Harrison, 1994). Understanding light alteration is vital in terms of underwater light design, with respect to the spectral quality and intensity of the units, although knowledge of their

effects on the physiological functions of fish is critical if such systems are to be used effectively.

To date, relatively few studies have examined the effects of light characteristics on physiological responses in fish. The most notable work on spectral composition stems from the study of Stefansson and Hansen (1989) who investigated the effects of various colour temperatures³ of light on Atlantic salmon parr. However, in that particular investigation neither growth nor the parr-smolt transformation were affected by the various light sources. Although, this study used colour temperature rather than spectral content, it still provides the most comparative study thus far on the spectral quality of light on fish growth and development. In contrast, the available literature on light intensity effects of fish physiology is more plentiful but also somewhat contradictory. Stefansson *et al.* (1993) and Oppedal *et al.* (1999) for example, both noticed that whilst the continuous light regimes increased growth, compared to conspecifics reared under a natural photoperiod, no differences were observed between the various light intensity groups. Conversely, Oppedal *et al.* (1997) found that higher intensities were the most effective at increasing the specific growth rate and mean live body weight as well as inhibiting the rate of maturation in salmon post-smolts. These findings have led researchers to suggest that a threshold value of light intensity must exist in order to influence physiological functions (Oppedal *et al.*, 1997; Porter *et al.*, 1999, 2001; Migaud *et al.*, 2006a).

The salmonid pineal gland and its hormone, melatonin, are believed to utilise photoperiodic information to synchronise daily and seasonal events (Boeuf and Le Bail, 1999; Boeuf and Falcón, 2001; Bromage *et al.*, 2001). Both *in vitro* (Yáñez and Meissl, 1996; Migaud *et al.*, 2006a) and *in vivo* (Randall *et al.*, 1995; Porter *et al.*, 2001; Bayarri *et al.*, 2002; Migaud *et al.*, 2006a) studies have demonstrated that melatonin synthesis varies inversely with the irradiance of the incident light. Recently, Migaud *et al.* (2006a) reported the light intensity threshold for Atlantic salmon to be around 0.016 W.m⁻², allowing for the 2.4% of light transmitted through the cranium. In addition to the light

³ different spectral mix bulbs (colour temperature ranging from 2000 K to 10,000 K)

sensitivity, the teleost pineal also exhibits a spectral sensitivity to light. Although longer wavelengths of light (*i.e.* red λ 700 nm) at sufficient intensities were found to suppress circulating melatonin levels (Bayarri *et al.*, 2002), shorter wavelengths (*i.e.* blue light λ 450 nm) were found to be the most effective (Bayarri *et al.*, 2002; Migaud *et al.*, unpublished data). These findings therefore indicate the colour type and minimum intensity that should be attained, when applying photoperiod regimes to production systems, for the additional illumination to be perceived as a continuous daylength thus ensuring the greatest possibility of eliciting physiological responses.

Although there are clear benefits to be gained from the use of photoperiod regimes there still appears to be some degree of uncertainty concerning their effects on the overall performance of farmed fish. One controversy within both the Scottish and Norwegian salmon industries relates to the depression in growth thought to be brought about by a reduction in appetite that sometimes appears to occur in response to the onset of lights. This ‘growth-dip’ phenomenon is also reported in several scientific studies where continuous light regimes have been applied for both salmonid (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005; see also Chapter 4) and non-salmonid species (Simensen *et al.*, 2000). Whilst this event raises concerns on the welfare of farmed fish reared under light regimes, from a farming perspective any negative impact on the growth performance would be expected to affect productivity and profitability, and may also affect the final product quality. Nevertheless, despite the importance of quality (texture, colour, proximate composition) to the salmon farming industry, relatively few studies have investigated the effects of photoperiod regimes on the flesh characteristics.

The quality of the flesh of farmed salmon is by far the most important criteria to the retailer and consumer. It is generally accepted that the colour of the salmon product is one of the most important quality parameters, since colour plays a decisive role when consumers are evaluating the product at the point-of-sale. The characteristic pink/red colour of salmonid flesh is a result of the deposition of the carotenoid pigments

astaxanthin and canthaxanthin. Fish are unable to synthesise these carotenoids *de novo* and as such they are included in the diets of farmed fish, accounting for around 15-20% of the total feed cost or 6-8% of the total production cost (Torrissen, 1995). Flesh quality is known to vary seasonally (Mørkøre and Rørvik, 2001; Roth *et al.*, 2005), although it is the maturation process, through the redistribution of carotenoid pigments and lipids from the flesh to the skin and gonads, that has the most detrimental affect on the flesh characteristics of salmonids (Aksnes *et al.*, 1986; Torrissen and Naevdal, 1988; Hatlen *et al.*, 1997; Bjerkeng *et al.*, 2000). With the seasonally adjusting photoperiods used to control physiological responses in fish, through endogenous rhythms, it has been suggested that artificial light regimes may induce compositional changes in the flesh during growth (Johnston, 1999). For instance, continuous light regimes have been shown to advance lipid and carotenoid deposition (Oppedal *et al.*, 2006) and alter the pattern of muscle fibre recruitment resulting in a higher fibre density and firmer flesh (Johnston *et al.*, 2003, 2004). Since carotenoid pigments are deposited within the muscle fibres (Johnston, 1999), it is not known how any such change in the composition and size distribution of these muscle fibres will affect the overall colour and quality of the flesh. Furthermore, fast growth responses, typically associated with the use of constant light regimes, can promote flesh softening in Atlantic salmon (Mørkøre and Rørvik, 2001). Thus, there is a strong case for the evaluation of photoperiod regimes and lighting characteristics on the flesh quality of farmed salmon to be performed.

In addition to the physiological effects, there are also the costs associated with the supply, maintenance and running of the lighting units. It is currently estimated that the total power cost of running the extended light regimes over the standard six-month period of the production cycle stands at between 0.25 and 1 euro per fish, depending upon the size of the production system (Clive Talbot, personal communication). With increasing global energy costs and the unpredictable nature of the price of salmon, it is vital that the ambiguous growth and maturation performances currently reported within the industry are addressed in order to make the use of artificial photoperiod regimes an economically viable option. At present, no such standardization over the use of lights

(*i.e.* timing and duration of onset, no. of lighting units *etc.*) exists within the industry with companies failing to implement any form of standard operating procedures (SOPs). Furthermore, the particular problem associated with superimposing the artificial illumination on to the ambient light may also account for the main uncertainty in the outcome of photoperiod manipulations.

The emergence of new lighting technologies, such as light emitting diodes (LEDs) and cold cathode units, offers the potential for a more effective control over the spectral and intensity output from the lighting units whilst also reducing the associated running costs. LEDs for instance, emit light of an intended colour without the use of traditional colour filters. As such, these low-voltage, low-current devices are more efficient with power savings of between 50-80% over conventional lighting systems, and have an estimated life-span of 15 years compared to 3 years with the standard metal halide units under normal operating conditions. With that in mind, this advancement in lighting technology provides the opportunity for light systems within commercial production systems to be designed to the light specificity of the species of fish being farmed.

Therefore, the purpose of this study was to test the effects of the spectral quality of the novel 50W blue narrow bandwidth LED lighting units on the growth and reproductive performance of salmon reared in sea cages and compare against the 400W standard metal halide units presently employed throughout the industry. Furthermore, the effects of light intensity on physiological functions were further examined by the number of units used per production pen. Finally, to follow on from the pilot study described in Chapter 4, the purported 'growth-dip' was monitored through the assessment of the acute and chronic feeding responses following light onset under current commercial production conditions.

5.2. Materials and Methods

The trial was performed at Marine Harvest's Loch Leven commercial salmon farm (Inverness-shire, Scotland) between the 11th January and 31st July 2005. A commercial low-maturing strain of 1+ Atlantic salmon mixed-sex post-smolts (LM20 strain, Inverness-shire, Scotland), identical to the strain used in Chapter 4, were reared under a natural photoperiod from hatch (January 2003) and transferred to seawater during March 2004.

Experimental design

All fish were initially held under ambient conditions in three holding pens prior to the commencement of the trial. On the 15th January 2005 fish were transferred from their holding pens into and randomly stocked into six 4000 m³ (20 x 20 x 10 m) commercial production cages. Ambient water temperature varied throughout the investigation between 7-18°C (Figure 5.1.).

On the 22nd January 2005 four cages were exposed to one of four continuous light regimes provided by two different types of submersible lighting units: 400 W standard metal halide Aquabeam Pisces 400 units (BGB Engineering Ltd.; Lincolnshire, UK) or 50 W light emitting diode (LED) narrow bandwidth 'blue' spectral lights (Idema Aqua UK Ltd.; Aberdeen, UK). In addition, two types of light intensities were tested for each of the two different light types. This was achieved by positioning two units per cage at a depth of 4.5 m (Figure 5.2.a.) or 6 units per cage set out with three units at a 3 m depth in a triangular arrangement and a further 3 units at 6 m depth in an inverse triangular formation (refer to Figure 5.2.b.). Thus, a multifactorial design consisting of four light treatments used within the trial were: two 50W blue LED units per cage (2B); six blue LED units per cage (6B); two metal halide, 'white light', units per cage (2W); and 6 White units per cage (6W). Two further cages remained under a natural photoperiod (NP)⁴ for the duration of the trial acting as a control. From the 21st June until the trial completion in July all fish were reared under ambient conditions. Treatment cages were

⁴ NP denotes fish exposed to the natural changes in photoperiod and light intensity

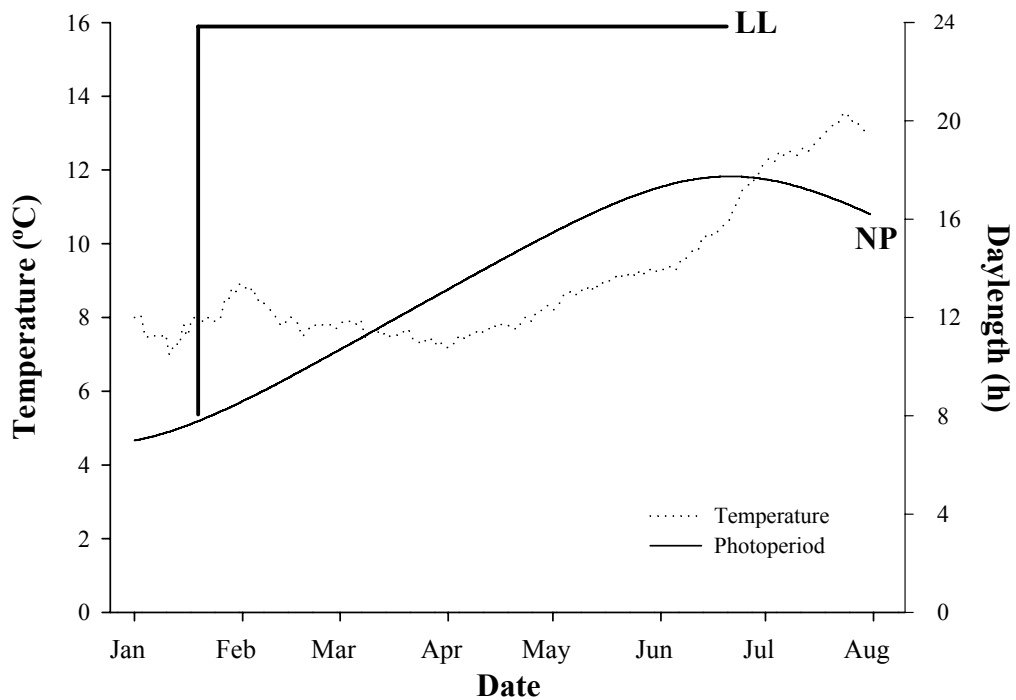


Figure 5.1. Experimental photoperiod regimes used within the trial and the daily water temperature profile ($^{\circ}\text{C}$) recorded during the experimental period. NP indicates natural photoperiod and LL denotes the period when constant light was applied, 22nd January to 21st June.

separated by unoccupied or unlit production pens to prevent spill-over of light between the treatment pens. Although treatment pens were randomly stocked, an overestimation of initial biomass and fish numbers in one of the holding pens resulted in significant differences between treatments in the initial mean weight and the numbers of fish per pen as follows: NP⁵ (1958 g; 15,212), NP⁵ (1972 g; 17,423), 2B (1780 g; 17,837), 6B (1433 g; 17,649), 2W (1662 g; 18,027) and 6W (1663 g; 18,166).

Fish were fed a standard commercial diet (MHS Atlantic, Skretting, UK; pellet size 9.0 mm) according to the manufacturer's recommended feeding tables throughout the trial. Feed was delivered using automatic feeders during daylight hours to all

⁵ NP treatment duplicated throughout trial

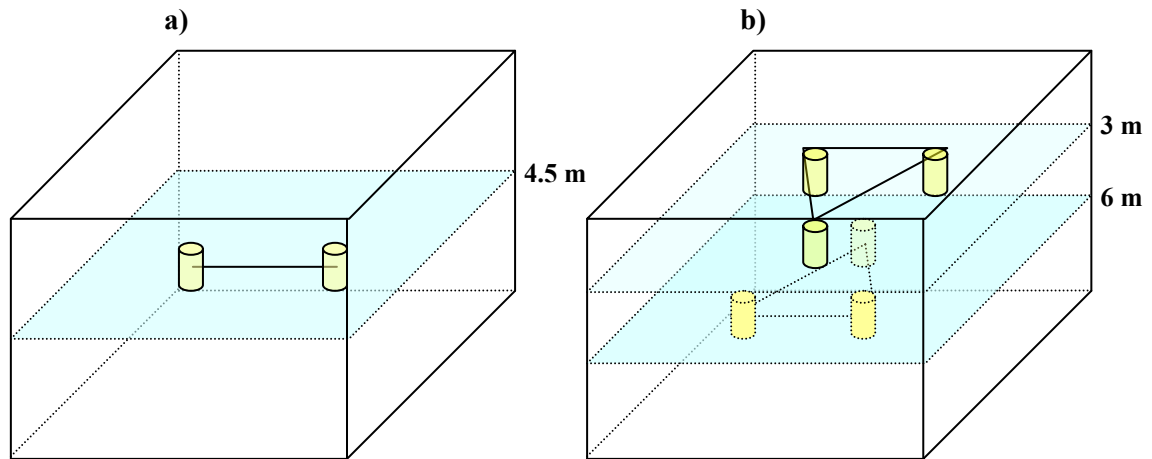


Figure 5.2. Schematic representation of the cage set-up regarding the positioning and depth of the light units within the low intensity (2 units, a) and high intensity (6 units, b) treatments. Cage dimensions 20 x 20 x 10 m (length x width x depth).

treatments. To account for the earlier times at which feed delivery commenced, sampling times varied ensuring that the quantity of feed supplied to the fish (% biomass) remained consistent throughout the experimental period. To control sea lice infestation, fish were fed SLICE[®] (Schering-Plough Ltd.; Middlesex, UK) at a dose of 0.5% biomass per day for one week from the 21st February.

Light characteristics and perception

In order to determine the lighting properties of the various light units through the water column, the spectrum and intensity were measured using a portable fibre optic spectroradiometer (StellarNet Inc. EPP2000, AstraNet Systems Ltd.; Cambridge, UK). Measurements were taken during the scotophase at the light source (0 m) and at 0.5 m increments thereafter until no further readings were detected. In addition, irradiance ($\text{W}\cdot\text{m}^{-2}$) readings were recorded using a photometric instrument (Skye instruments Ltd.; Powys, UK) calibrated to National Physical Laboratory (UK) standards. The light distribution profile of each lit treatment cage was mapped by taking readings at 12

positions in a grid formation below the water surface and then by repeating the measurements for each plane at 2 m interval depths.

To assess the effectiveness of the various light regimes, the light perception hormone melatonin was analysed. On the 22nd March 2005 a random sample of 15 fish per treatment cage were removed at 1130 h and then again at 2330 h for assessment of day and night-time levels of melatonin. Blood was withdrawn from the caudal vein and the resultant plasma stored at -70°C until analysis. Plasma melatonin was assayed by ELISA (Section 2.5.2.) using commercially available kits (IBL Ltd.; Hamburg, Germany).

Sampling regime

Feeding and growth

On the 11th January 2005, prior to fish being split into their respective treatment pens, a total of 60 randomly selected fish were removed from the holding cages and sacrificed in order to establish a baseline measurement representative of the population feeding levels. Following the onset of light on the 22nd January 2005, the acute and chronic feeding responses were further assessed on days 9, 17 and 32 post-light onset and then at monthly intervals thereafter. In June no sampling was conducted due to the grading of treatment cages. A final sample was performed on the 21st July 2005, one month after the switch-off of lights. At each sample point 25 fish per treatment cage were randomly netted and killed by a single blow to the dorsal surface of the head.

Sacrificed fish were individually marked for future identification, length and weight recorded and placed on ice until dissection. Following evisceration, the sex of the fish was noted and the gonads removed, weighed and the gonadosomatic index (GSI) determined (Section 2.6.1.). Fish were deemed to be maturing according to Endal *et al.* (2000), if males had a gonadal weight ≥ 3 g and a GSI $\geq 0.4\%$ and females if the GSI $\geq 0.8\%$. The gastrointestinal tract was excised and the digesta collected, oven dried and weighed (Section 2.3.3.). Individual food consumption was calculated on a weight-

specific basis (mg.g fish^{-1}). Inter-individual variability in feed intake (CV_F) within treatments was examined by calculation of the coefficient of variation.

Growth performance was assessed on a monthly basis from January through to June 2005 when all treatment cages were size-graded, in line with normal commercial operating procedures. At each sampling point a minimum sample of 200 randomly selected fish per treatment cage were individually length-weighed under anaesthesia. In addition, manual growth data were compared with farm data obtained using the Vaki Biomet System (Vaki Aquaculture Systems Ltd.; Kópavogur, Iceland). From the biometric measurements, the condition factor (K) of individual fish and the daily weight (SGR_W) and length (SGR_L) gain for each treatment cage were calculated.

Maturation

In addition to the determination of the GSI of individual fish, the gonads of 10 fish per treatment from the April and May sampling points were removed and a small transverse section from the middle region of the largest gonad fixed in a 10% buffered formalin solution. Fixed samples were sectioned, stained and mounted by the Institute of Aquaculture's Diagnostic Services department (University of Stirling; Stirling, UK). The sections were examined under a light microscope and classified for maturational status. Oocytes were classified into stage of development as described by Taranger *et al.* (1999); (i) primary growth phase, (ii) secondary growth phase, (iii) true vitellogenesis and (iv) atresia (Section 2.6.2.). Additionally, the oocyte diameters were measured using image-capturing software (Image-Pro Plus[™] for Windows, Media Cybernetics[®]; USA). Stages of spermatogenesis were assessed using the classification of Dziewulska and Domagala (2003); (i) Type A spermatogonia, (ii) Type B spermatogonia, (iii) primary and secondary spermatocytes, (iv) spermatids, and (v) spermatozoa (Section 2.6.2.).

During June 2005, treatment cages were graded as part of normal farming practices. During this period blood samples were taken from fish of the various size grades and analysed for testosterone levels as a further indication of maturity status. From each treatment pen, blood was withdrawn from a random sample of 50 large sized

individuals which formed the top grade, and 25 samples from both medium and small sized fish making up the bottom grade. Plasma was separated by centrifugation and stored at -70°C until analysis by radioimmunoassay (Section 2.6.3.) according to the method previously described by Duston and Bromage (1987). Fish were classified as maturing by assigning an arbitrary testosterone threshold value of $3 \text{ ng}\cdot\text{ml}^{-1}$, based upon the assessment of previous results (Taranger *et al.*, 1998) where plasma testosterone had been measured.

Flesh analysis

All flesh analyses were performed at Marine Harvest's (Scotland) Lochailort Fish Health and Quality Laboratory. The Norwegian quality cut (NQC) from the left-hand side of 10 sacrificed fish per treatment per sample point was removed for flesh analysis. The NQC is a standardized muscle cutlet corresponding to the region of flesh posterior to the dorsal fin to the anterior of the anal fin. Samples were immediately frozen in liquid nitrogen following removal and stored at -70°C until further analysis.

The visual colour of the salmon cutlets was evaluated using the Roche *SalmoFan*TM Lineal colour card for salmonids (Hoffmann-La Roche Ltd.; Switzerland) by two independent scorers (Section 2.8.2.). The colour composition of the flesh was also measured instrumentally using a tristimulus colorimeter (Minolta Chroma Meter, CR-310, Minolta Corporation; Osaka, Japan). This provided an objective evaluation of lightness (L^*), red/green chromaticity (a^*) and yellow/blue chromaticity (b^*), in accordance with the International Commission on Illumination (CIE, 1976). From the a^* and b^* values, the chroma (C^*_{ab}) and the hue (H°_{ab}) were calculated (Hunt, 1977):

$$(C^*_{ab}) = a^* \times b^*$$

$$(H^{\circ}_{ab}) = [\tan^{-1}(b^*/a^*)]$$

The chroma is an expression of the intensity and clarity of the flesh, whereas the hue represents the relationship between the redness and yellowness of the cutlet and is expressed as an angular measurement where 0° indicates a red hue and 90° yellow (Section 2.8.3.).

Total pigment (mg.kg^{-1}) and fat content (%) of homogenized flesh samples were determined using near-infrared (NIR) spectroscopy (model Foss 6500, Foss Ltd.; Didcot, UK) (Section 2.8.4.). Moisture content was estimated by drying 5 g of homogenised flesh in a drying oven (Gallenkamp; Loughborough, UK) at 105°C for 20 hours before reweighing the dried sample (Section 2.8.5.).

Statistical analysis

Feed intake, growth performance and flesh quality were all analysed by one-way ANOVA with treatment as the dependent factor. All data were tested for normality of distribution and homogeneity of variance by the Kolmogorov-Smirnov test and from examination of the residual plots. *Post hoc* comparisons were made using Tukey's test. Condition factor failed to meet the assumptions for normality testing and was instead analysed using non-parametric tests (Kruskal-Wallis) and, where applicable, for *Post hoc* multiple comparisons using Dunn's test. Where required, sample data was transformed using either the natural logarithm or arcsine transformation to improve normality conformation. Linear relationships between measured variables were assessed using Pearson's correlation coefficient (r). Run's test was used to check for linearity, with data failing the test indicating a curvilinear relationship. Multiple comparisons of linear regression gradients were compared by ANCOVA (Zar, 1999). A significance level of $P < 0.05$ was applied to all statistical tests performed. Replicate control cages were found not to differ for any of the parameters measured and were therefore pooled in all instances. All data are presented as mean \pm SEM.

5.3. Results

5.3.1. Light Characteristics

Natural light

To further compare the artificial light characteristics to light naturally penetrating the water column, natural light scans recorded from a cage site in Lønningdal, Norway, during March and June 2004 are presented.

Natural light intensity penetrating the water column during March ranged from 83.5 to 0.15 W.m⁻² (>2000 to 60 lux) from the water surface to 14 m depth respectively (Figure 5.3.). Light intensity was significantly higher during June than recorded in March, with readings of >200 to 13.6 W.m⁻² (>20000 to 6600 lux) from the water surface to 14 m depth respectively. Profiles of the spectral content of the natural light in relation to the depth are presented in Figure 5.4. The water environment was most transparent to light in the range of λ 470 nm to λ 580 nm (blue green) during ambient illumination, with the shorter and longer wavelengths (blue and red respectively) being significantly reduced below 6 m depths. No shift in the water absorptive properties were found between the March and June 2004 readings.

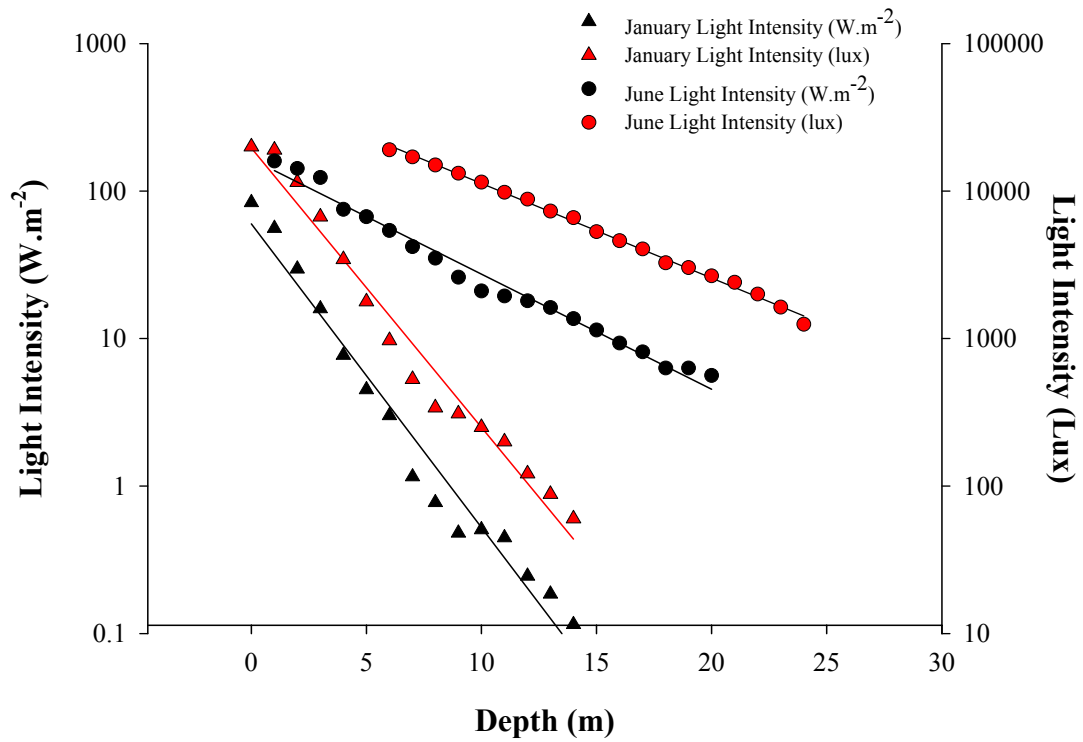


Figure 5.3. Linear regressions of the irradiance ($W.m^{-2}$; black) and illuminance (lux; red) intensity variation in relation to the depth from the water surface on the 10th March 2004 and 8th June 2004 at a cage site in Lønningdal, Norway.

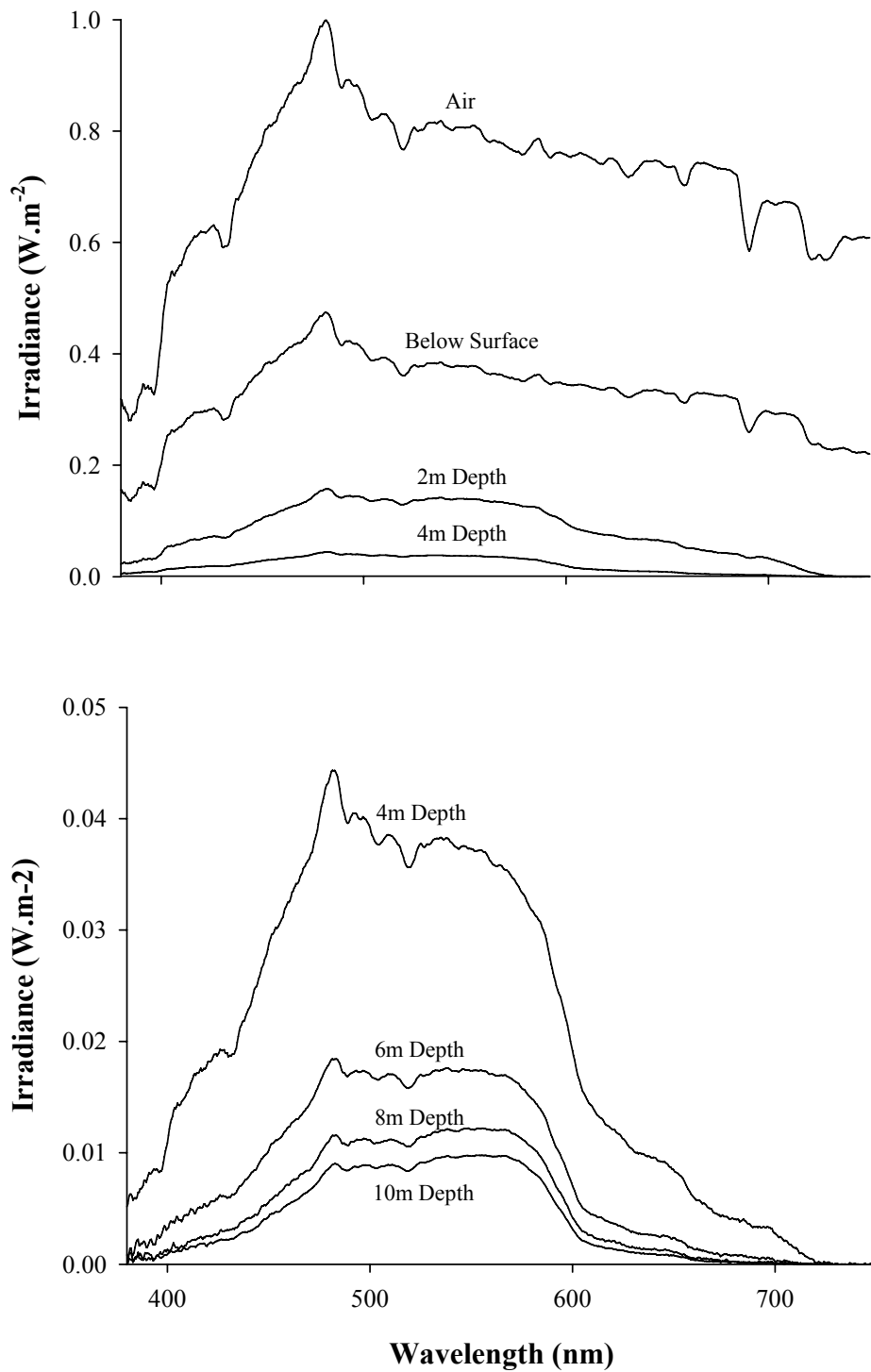


Figure 5.4. Profiles of light spectral content in relation to depth from the water surface. Above surface to 4 m depth (a) and 4 m to 10 m depth. Readings taken from a cage site in Lønningdal, Norway on 10th March 2004.

Artificial light

The characteristics of the different lighting units, based upon the intensity and spectral quality of light emitted through the water column, are presented in Figures 5.5. through to Figure 5.7.

Intensity

Total irradiance at the light source was greater for the Aquabeam metal halide unit (46.9 W.m⁻²) compared to the Idema blue LED spectral unit (9.8 W.m⁻²). Light was detectable up to 6 m from 400 W metal halide unit where intensity was recorded at 0.0019 W.m⁻², whereas light from the 50 W blue LED spectral unit could only be detected up to 3 m from the light source giving an intensity reading of 0.0007 W.m⁻². At distances greater than these light could not be differentiated from background levels measurable by the equipment. Thus, the intensity of light emitted by the blue spectral LED units degraded at a greater rate than the light from the standard metal halide units, as indicated by the regression slopes in Figure 5.5.

Recently Migaud *et al.* (2006a) proposed a light intensity threshold value for Atlantic salmon of 0.016 W.m⁻², above which plasma melatonin levels were suppressed to daytime levels. The horizontal dashed line depicted in Figure 5.5. estimates the distances from the various light sources from which the suggested intensity threshold value penetrates the water column. The 'required' 0.016 W.m⁻² intensity was detected at approximately 4 m from the metal halide unit and around 1.5 m from the blue LED unit.

Spectrum

Principle peak emissions for the Aquabeam metal halide units were at λ 592, 548 and 510 nm (Figure, 5.6.), whereas a solitary peak at λ 470 nm was recorded for the Idema blue spectral LED units (Figure 5.7.). All peaks persisted throughout all depths where light was detectable.

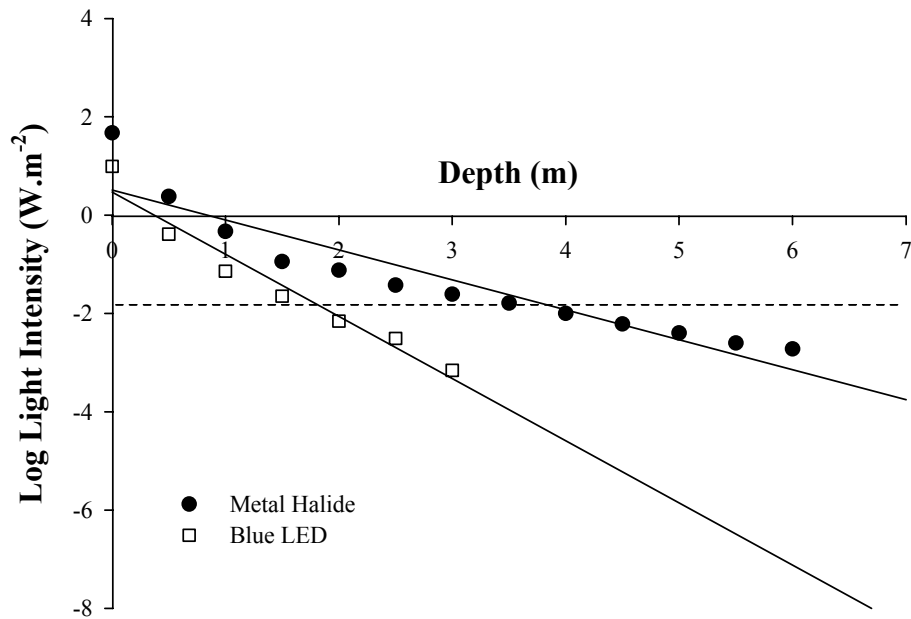


Figure 5.5. Comparison of the log of light intensity (W.m^{-2}) in relation to the distance from the light source through seawater for the Aquabeam 400 W standard metal halide and Idema 50 W blue spectral LED light units. Horizontal dashed line indicates the distance from the various light sources from which the 0.016 W.m^{-2} salmon light intensity threshold is estimated to be detected.

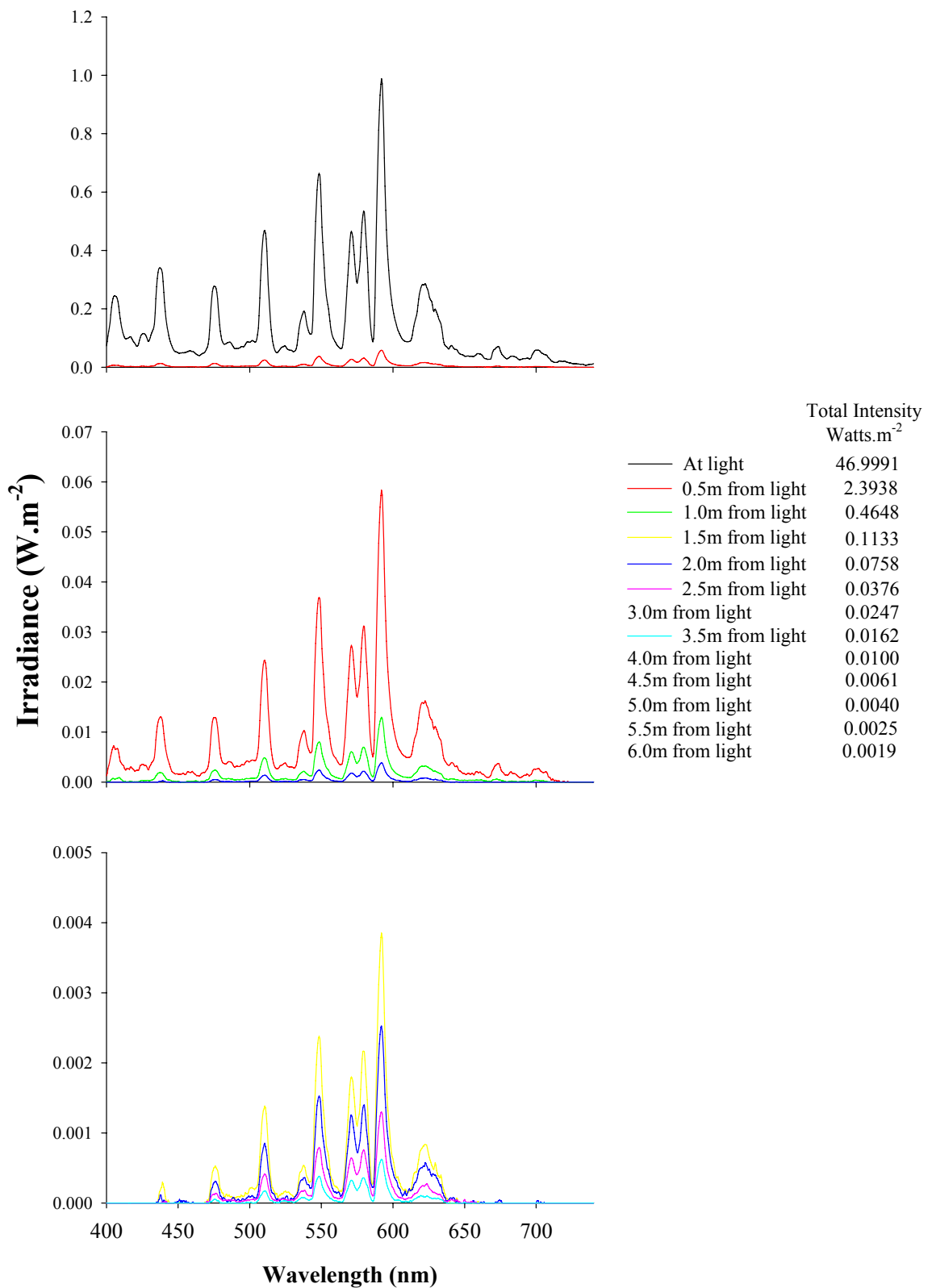


Figure 5.6. Emission spectral profiles for the Aquabeam 400 W standard metal halide units through the water column in relation to the distance from the light source.

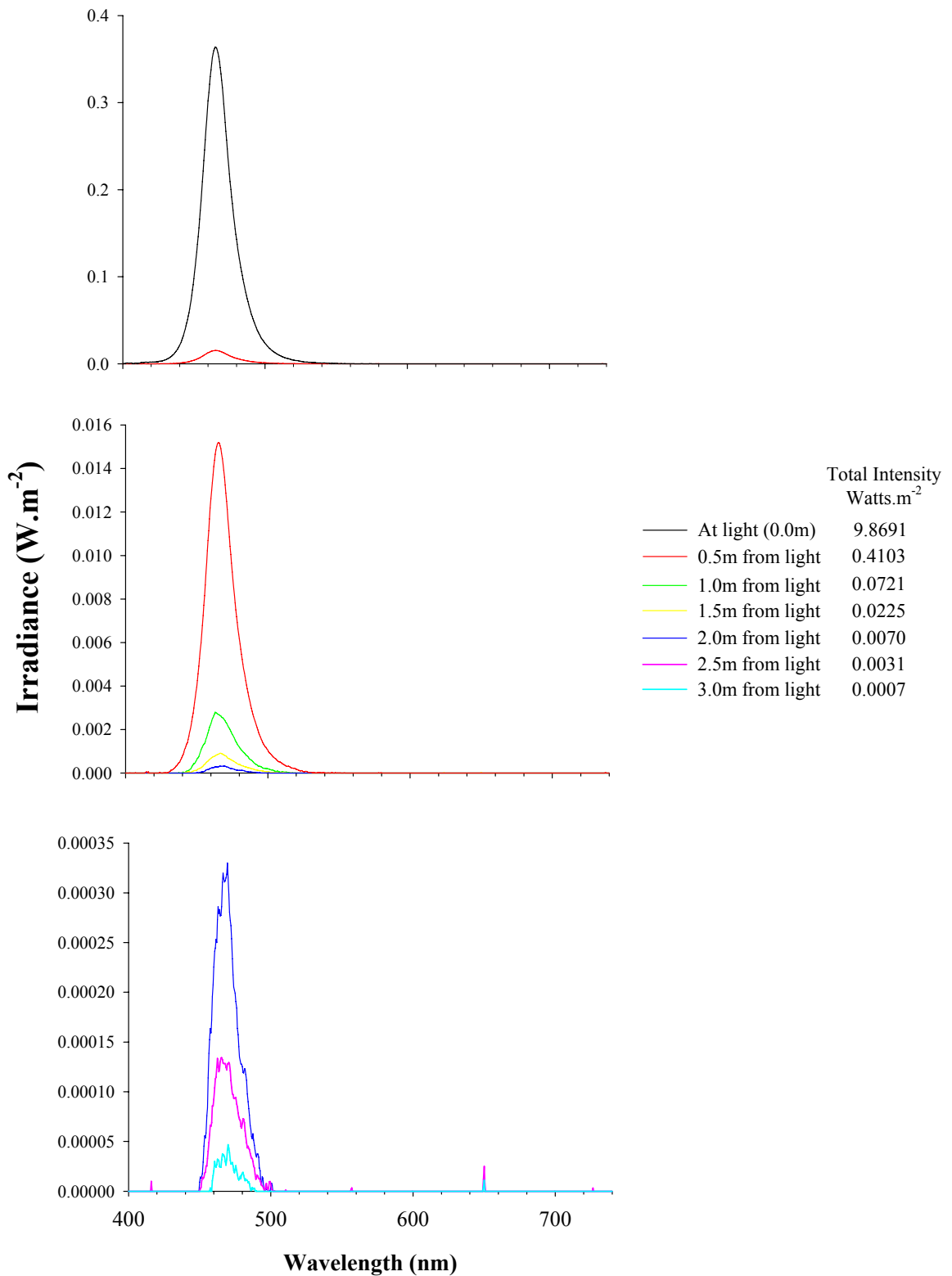


Figure 5.7. Emission spectral profiles for the Idema 50 W blue LED units through the water column in relation to the distance from the light source.

5.3.2. Cage Lighting Profiles

The cage light profiles for each lit treatment group, shown as intensity contour plots at the various plane depths measured within each cage, are presented in Figures 5.8. through to Figure 5.11.

Light distribution was greater in the metal halide cages, irrespective of intensity, relative to the blue LED treatments as determined by the maximum recorded intensity level for each depth. As expected, the highest intensity plots were at the approximate depth of plane as the light source(s).

Table 5.1. summarizes the distances at which light intensities emitted from the various light treatments were above the suggested salmon intensity threshold level of 0.016 W.m^{-2} , after allowing for transmission through the pineal window (Migaud *et al.*, 2006a). The 50 W blue narrow bandwidth LED light units failed to emit a sufficient intensity of light, irrespective of the number of units per cage, above the 0.016 W.m^{-2} threshold at any of the measured plane depths. However, the 400 W standard metal halide units produced a satisfactory amount of intensity such that at both 6 m and 8 m in the 2W group, and for all depths measured in the 6W treatment light was calculated as being above the threshold value.

Table 5.1. Distances (m) at which light emitted from the various lighting units per treatment cage were above the adjusted salmon light threshold of 0.016 W.m^{-2} (Migaud *et al.*, 2006a), as indicated by X. Fish reared under 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively).

Water Depth	Treatment			
	2B	6B	2W	6W
4 m	–	–	–	X
6 m	–	–	X	X
8 m	–	–	X	X
10 m	–	–	–	X

Similarly, knowing the distance from the various light sources to be above the salmon light intensity threshold and using a simulation model kindly provided by Dr. Clive Talbot of Marine Harvest (Stavanger, Norway), the percentage volume of each lit treatment cage (4000 m³) estimated to be above the 0.016 W.m⁻² threshold was calculated and are presented in Table 5.2. Cages lit by the metal halide lighting units had the greatest proportion of total cage volume lit above the 0.016 W.m⁻² salmon light intensity threshold, with over three quarters (3141 m³) of the total cage volume in the 6W treatment and around a quarter (1047 m³) of the cage volume of the 2W treatment estimated to be above the threshold. In comparison, only 2% of the highest intensity blue lit cage (6B) was estimated to be above the light intensity threshold and less than 1% for the 2B treatment.

Table 5.2. Percentage volume of the treatment cages (4000 m³) estimated to be above the 0.016 W.m⁻² salmon light intensity threshold. Fish reared under 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively).

Treatment	Volume of cage (4000 m ³) above 0.016 W.m ⁻² intensity threshold	
	(%)	(m ³)
2B	0.7%	28 m ³
6B	2.1%	85 m ³
2W	26.2%	1047 m ³
6W	78.5%	3141 m ³

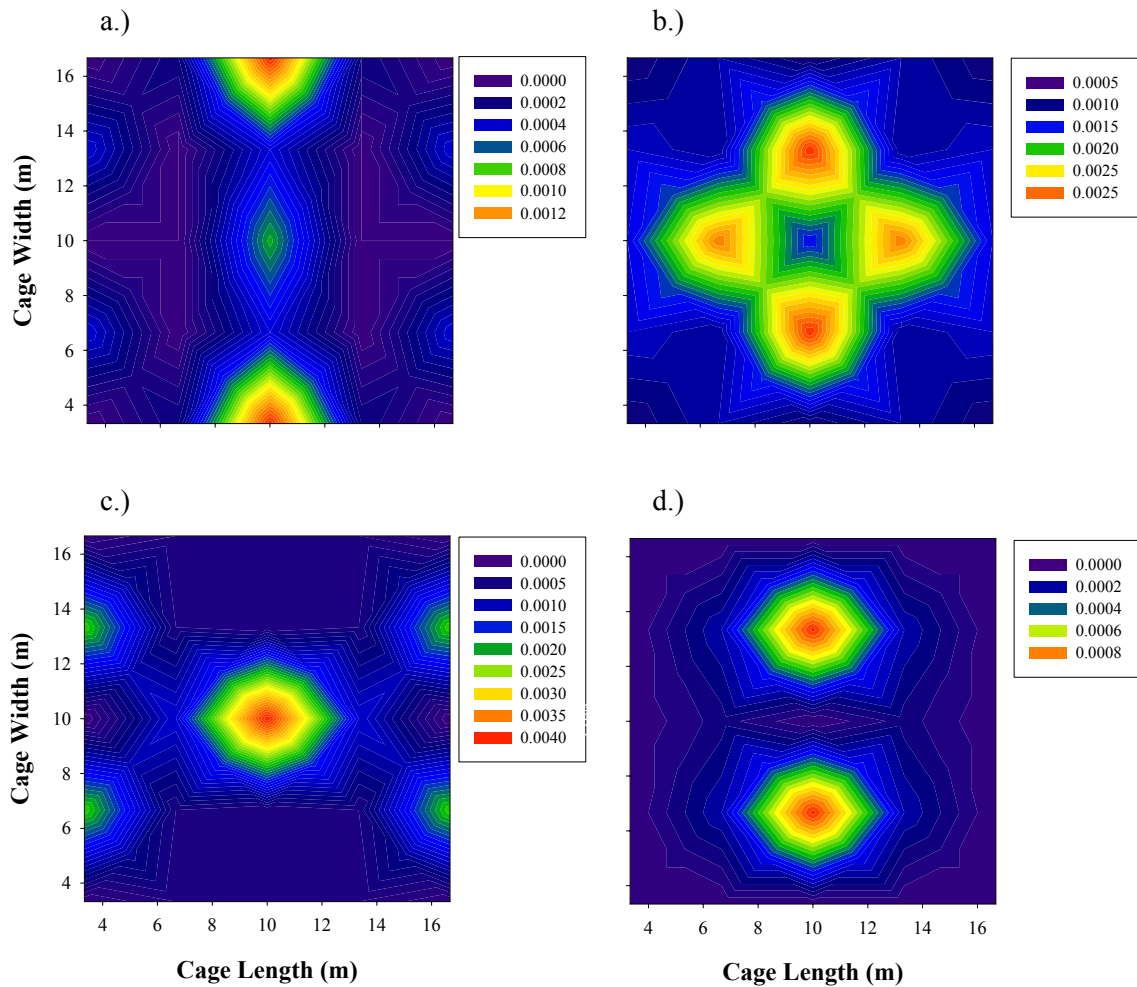


Figure 5.8. Contour plots for the cage light distribution profile for the two blue (2B) LED unit treatment at 4 m (a), 6 m (b), 8 m (c) and 10 m (d) depths. Light units positioned at 4.5 m depth. Note that brightness scales may vary between plots.

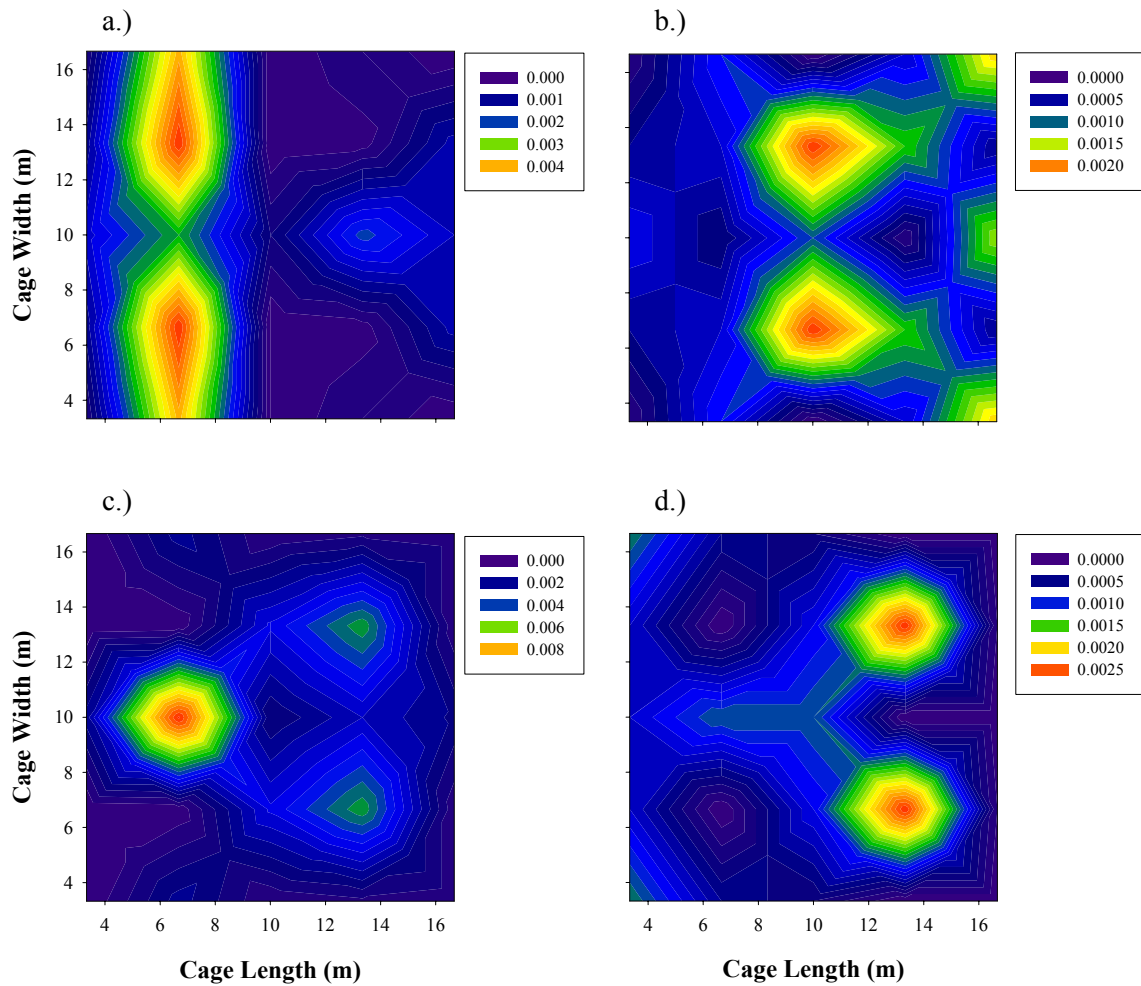


Figure 5.9. Contour plots for the cage light distribution profile for the six blue (6B) LED unit treatment at 4 m (a), 6 m (b), 8 m (c) and 10 m (d) depths. Light units positioned at 3 m and 6 m depths respectively. Note that brightness scales may vary between plots.

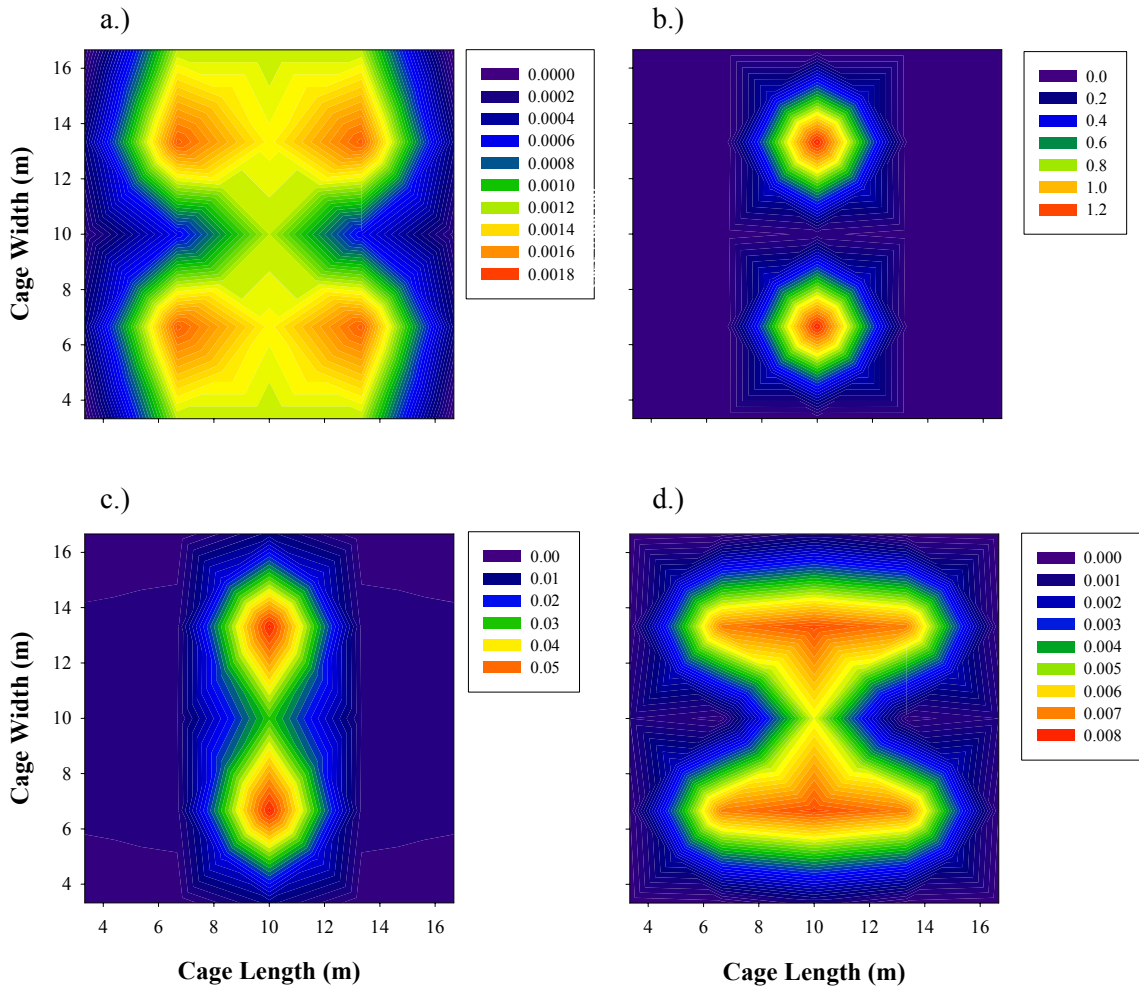


Figure 5.10. Contour plots for the cage light distribution profile for the two white (2W) standard metal halide unit treatment at 4 m (a), 6 m (b), 8 m (c) and 10 m (d) depths. Light units positioned at 4.5 m depth. Note that brightness scales may vary between plots.

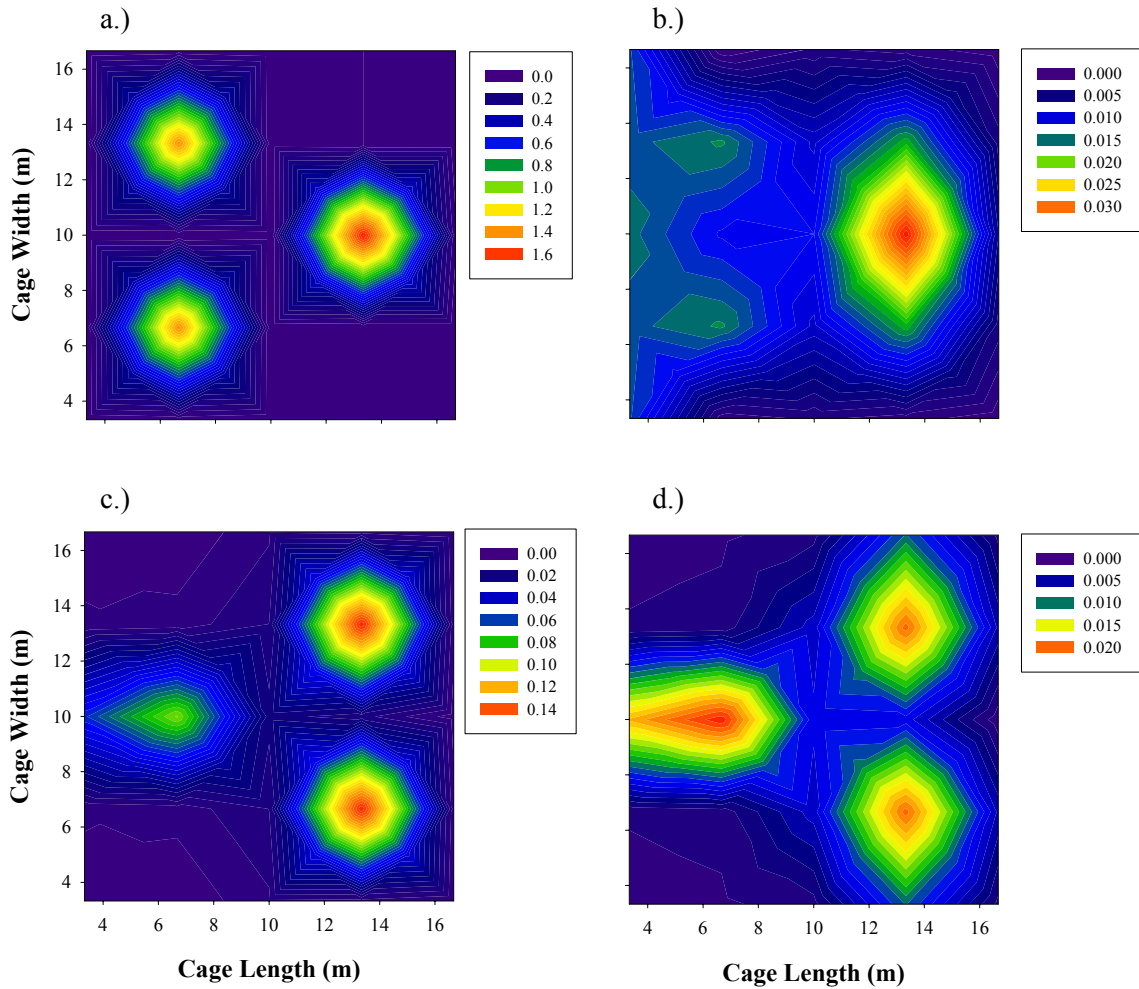


Figure 5.11. Contour plots of the cage light distribution profile for the six white (6W) standard metal halide unit treatment at 4 m (a), 6 m (b), 8 m (c) and 10 m (d) depths. Light units positioned at depths of 3 m and 6 m respectively. Note that brightness scales may vary between plots.

5.3.3. Melatonin

To assess the effectiveness of the various light sources and treatments, plasma melatonin levels were measured from individual treatment fish as a biological indicator of light perception. Daytime plasma melatonin levels were similar among all treatments ($P>0.05$) and were therefore pooled to simplify comparisons between nocturnal melatonin levels of the various treatments and daytime values.

Under a natural photoperiod, fish displayed the typical elevation in plasma melatonin levels resulting in significantly higher concentrations at night than during the day (Figure 5.12.). Similarly fish reared under constant light, with exception of the 6W treatment, exhibited significantly higher levels of plasma melatonin at night than during the day ($8.8 \pm 1.3 \text{ pg.ml}^{-1}$) which was statistically similar to nocturnal levels produced by the NP treatment. The higher intensity treatment of the standard metal halide light units (*i.e.* 6W) resulted in a significantly lower nocturnal melatonin concentration ($16.4 \pm 2.5 \text{ pg.ml}^{-1}$) than fish reared under NP ($44.9 \pm 8.8 \text{ pg.ml}^{-1}$) and both 2B ($65.2 \pm 3.1 \text{ pg.ml}^{-1}$) and 6B ($37.1 \pm 3.4 \text{ pg.ml}^{-1}$) light regimes, but were statistically similar to melatonin levels produced under 2W ($30.4 \pm 3.4 \text{ pg.ml}^{-1}$). Additionally, nocturnal concentrations of plasma melatonin in the 6B treatment were statistically similar to 2W but significantly lower than levels produced under 2B.

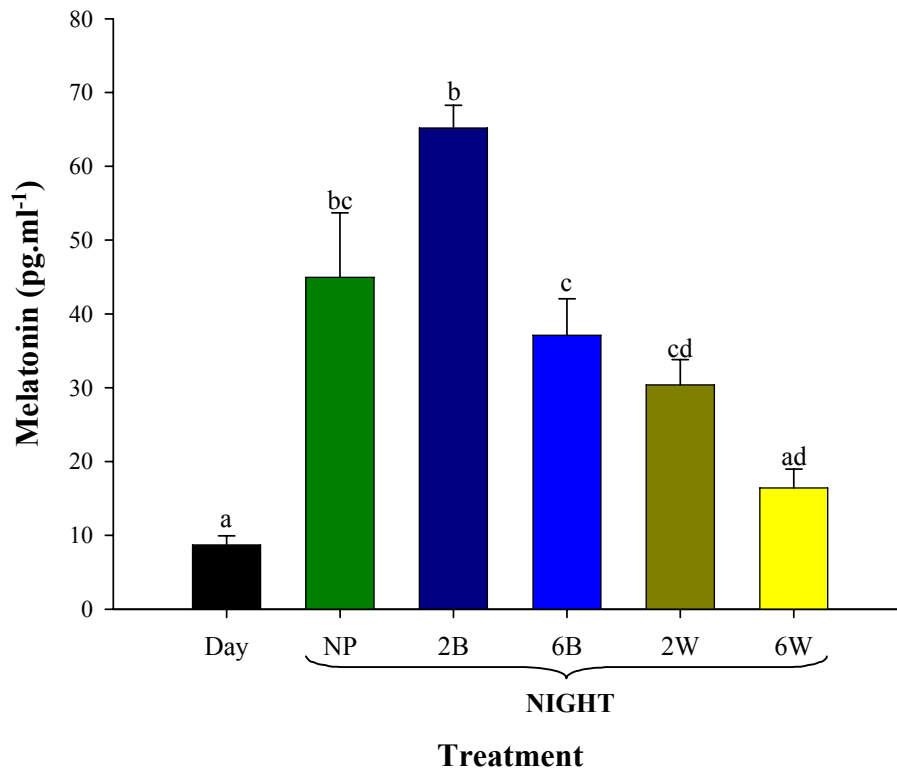


Figure 5.12. Mean night-time plasma melatonin levels ($\text{pg.ml}^{-1} \pm \text{SEM}$) of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively). For easier comparison daytime melatonin values of all treatments are pooled ($N = 75$). Melatonin sample performed on 22nd March 2005, night-time samples taken during mid-dark (1130-0300 h). Means bearing identical lettering are statistically similar ($P > 0.05$). $n = 15$ fish per treatment for nocturnal levels.

5.3.4. Feed intake

The mean relative feed intake taken from the pooled population on the 11th January (7°C, 1557.7 ± 83.9 g) prior to the onset of constant light was 4.0 ± 0.7 mg.g fish⁻¹ (Figure 5.13.). Following the application of continuous artificial illumination feed intake in the NP, 2W and 6W treatments increased to around 5 to 6 mg.g fish⁻¹, whereas food consumption in both the blue light treatments (2B and 6B) remained between 3 to 4 mg.g fish⁻¹. This resulted in significant differences between the 2B treatment and the NP, 2W and 6W regimes, as well as a difference between the NP and 6B treatments. At the following sampling point on the 8th February, feed intake remained within similar levels resulting in a significantly higher consumption in the NP group (5.0 ± 0.5 mg.g fish⁻¹) as compared to both blue treatments. In addition, the 6W treatment (5.6 ± 0.5 mg.g fish⁻¹) also consumed significantly more feed than fish reared under 6B (2.8 ± 0.4 mg.g fish⁻¹) at this time. Thereafter, feed intake declined slightly in all treatments such that at the 21st March sampling period mean feeding levels were significantly lower in the blue treatments as compared to the 2W and was also lower in the 2B group relative to the control. Between April and May feeding remained relatively stable for all treatments, in the region of 2 to 4.5 mg.g fish⁻¹, although mean food consumption in the 2W treatment decreased from around 4.5 mg.g fish⁻¹ to 3 mg.g fish⁻¹. At the final sample point in July, following the switch-off of lights at the summer solstice, feed intake in the 6B treatment increased to 6.2 ± 1.1 mg.g fish⁻¹ which was significantly higher than that recorded in the 2W (3.2 ± 0.4 mg.g fish⁻¹) and NP (3.1 ± 0.3 mg.g fish⁻¹) groups.

To address the feeding/growth dip, feed consumption was compared between NP fish (control) and their conspecifics exposed to two 400 W metal halide units (2W), typical to that routinely used throughout the Scottish farming industry for a similar cage size (Figure 5.14.). Although feed intake in the 2W treatment appeared to be lower during April and May, both treatments demonstrated a similar trend in the feeding response throughout the trial period with no significant differences observed.

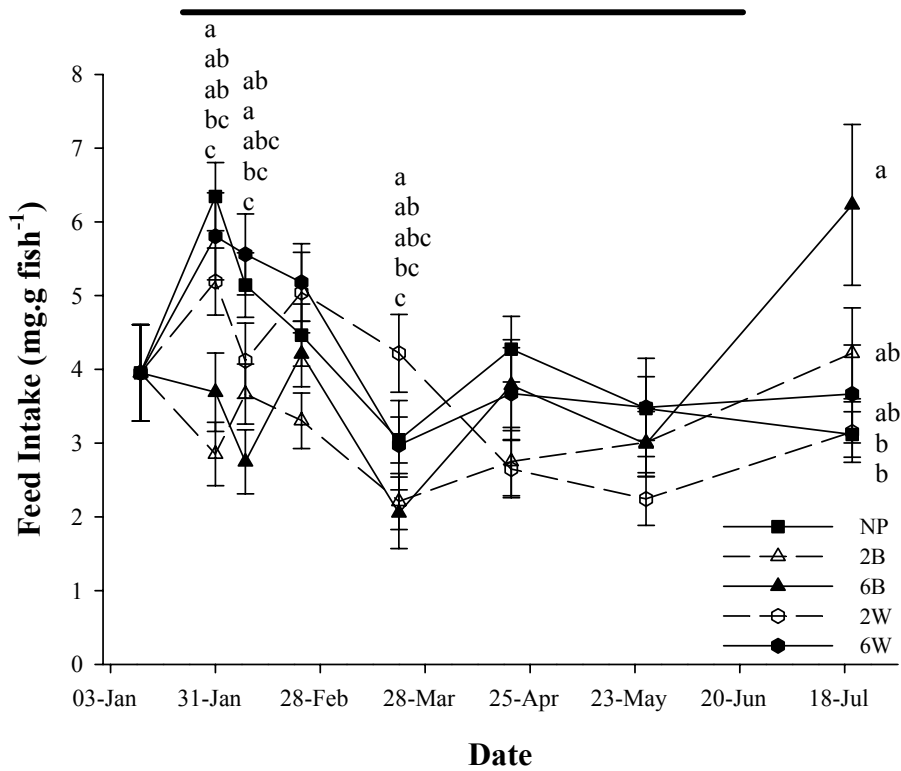


Figure 5.13. Mean feed intake levels* ($\text{mg.g fish}^{-1} \pm \text{SEM}$) of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. $n = 25$ fish (50 for NP) per treatment per sample point. *Sampling occurred after 60% of the daily ration had been fed to the fish.

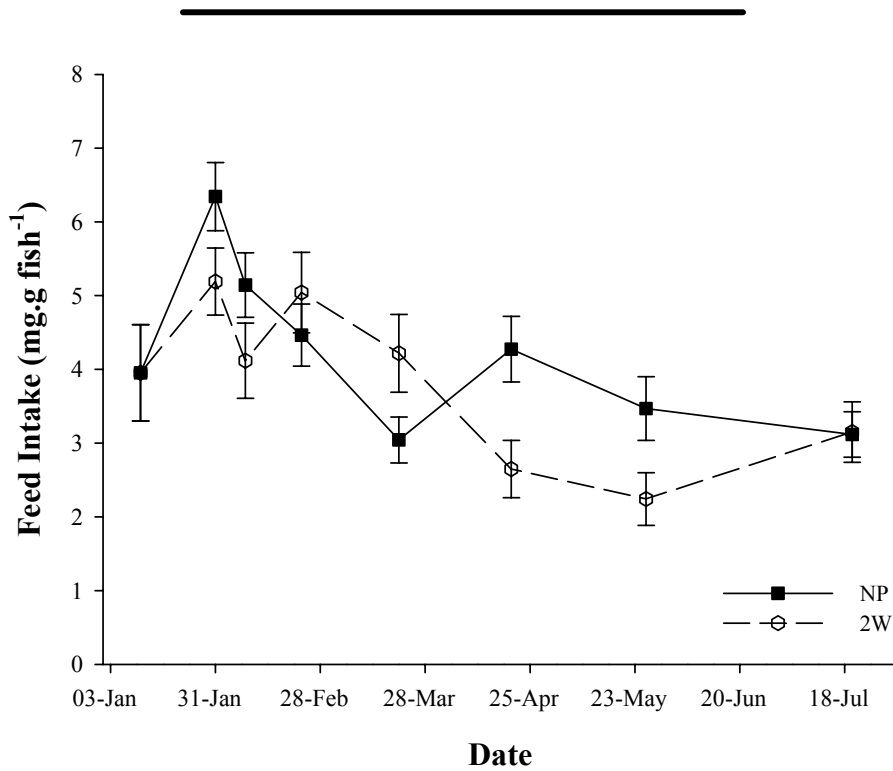


Figure 5.14. Comparison of the mean feed intake* (mg.g fish⁻¹ ± SEM) between fish reared under a natural photoperiod (NP, control) or exposed to 2 metal halide units (2W) as currently used throughout the Scottish salmon farming industry. Horizontal bar indicates period of light (22nd January to 21st June 2005). No statistical differences were evident between treatments at given time points ($P > 0.05$). $n = 25$ fish (50 for NP) per treatment per sample point. *Sampling occurred after 60% of the daily ration had been fed to the fish.

5.3.5. Growth Performance

No differences in the monthly mean length-weights of fish were recorded between data collected manually and that obtained using the Vaki Biomest Systems. Results presented here are therefore based on the individually length-weighed data and are taken as an accurate representation of the treatment cage population.

Weight gain

Initial weights were found to differ significantly between the control group (NP) and all lit treatment groups. This resulted in the NP remaining consistently heavier than the other treatments throughout the course of the trial (Figure 5.15.). In addition, mean body weights of fish exposed to 2B were also significantly heavier than the 6B, 2W and 6W treatments at the initial sample and both 2W and 6W were also heavier than the 6B regime. These differences remained present throughout the experimental period, with exception of the March sampling point when the weight of fish exposed to 6W (2269 ± 42 g) was similar to the 2B (2249 ± 46 g) treatment. At the final sampling period in June, the NP group (3678 ± 62 g) was significantly heavier than all lit treatments. No differences were observed between the standard metal halide treatments, although the 6W (3058 ± 59 g) was significantly heavier than the 2B (2820 ± 67 g) regime. In addition, the 6B (2251 ± 58 g) regime had significantly lower body weights than the 6W, 2W (2955 ± 59 g) and 2B treatments.

The weight specific growth rate (SGR_w) of the NP group remained relatively constant around 0.3 to 0.4% day^{-1} throughout the experimental period (Figure 5.16). Fish exposed to 6B grew at around 0.4 to 0.5% day^{-1} throughout the duration of the trial, although this briefly fell to 0.2% day^{-1} during the April-May growth period. A similar pattern of growth was observed under 2W with the growth rate also falling to 0.2% day^{-1} during the March-April growth period. Under 6W the daily growth rate increased from 0.3% day^{-1} to a peak of 0.7% day^{-1} during March-April before decreasing to a trough of 0.2% day^{-1} in April-May and finally increasing to around 0.5% day^{-1} at the final growth period during May-June. Fish exposed to 2B exhibited the greatest oscillations in

growth, decreasing from $0.5\% \text{ day}^{-1}$ to just above maintenance growth during the March-April growth period and increasing dramatically to $0.8\% \text{ day}^{-1}$ during April-May before falling to negligible growth ($-0.1\% \text{ day}^{-1}$) at the final growth period.

The overall relative weight gain for the growth period January to June is presented in Figure 5.17. In terms of comparative weight gain relative to the control, as indicated by the dashed line, fish reared under the standard metal halide light units exhibited the greatest increase in weight, with the higher intensity (6W) displaying a 10% overall increase in weight and the lower intensity group (2W) a 6% increase in weight. Fish reared under the 2B exhibited a 20% reduction in growth whilst a similar growth gain to fish held under NP was observed under 6B.

Length gain

Mean fork lengths exhibited a similar pattern to that seen for weight, with fish from the NP treatment significantly longer than all other treatments throughout the experimental period (Figure 5.18.). Initial mean fork lengths were also different between the 2B and the 2W, 6W and 6B treatments as well as between the standard metal halide treatments (2W and 6W) and the 6B group. Throughout the course of the trial no differences in length were observed between fish reared under 2W or 6W. Fish exposed to 6B were consistently the shortest from January to June. However, despite the differences between the 2B ($54.2 \pm 0.3 \text{ cm}$) and 6B ($50.9 \pm 0.3 \text{ cm}$) treatments at the beginning of the trial no differences in length were evident at the end in June, although both treatments were significantly shorter than their conspecifics reared under the metal halide lighting units.

The length specific growth rate (SGR_L) of the control group remained around $0.15\% \text{ day}^{-1}$ throughout the experimental period, with fish exposed to 6B displaying a similar SGR_L growth pattern (Figure 5.19.). As with weight gain, fish exposed to 2B exhibited the greatest oscillations in length gain ranging from around $0.025\% \text{ day}^{-1}$ in the March-April and May-June periods and peaking at $0.25\% \text{ day}^{-1}$ during the April-May growth period. Under 2W, length gain during the first two growth periods was comparable with that in the NP group, before decreasing to around 30% of that measured

under NP in the April-May growth period and approximately 50% higher than NP during the May-June period. Fish exposed to 6W exhibited the greatest rate of length gain during the March-April growth period, but remained otherwise similar to that recorded in the control.

Condition factor

During the trial period K decreased in all treatments from as high as 1.26 ± 0.01 for NP fish at the start of the trial to a low of 1.04 ± 0.01 recorded in 6B fish at the final sampling point in June (Figure 5.20.). As with other growth parameters, the initial condition factor (K) varied significantly between treatments with the control group significantly higher than all other treatments. Additionally, the 2B and 6W regimes were significantly higher than the 6B and the 6W was also different from the 2W group. With exception to the April sampling point, when K was statistically similar between the NP (1.15 ± 0.01) and 2B (1.14 ± 0.01) treatments, the K of fish in the control treatment (NP) remained highest throughout the course of the investigation. At the February sample point all treatments had a significantly higher K than the 6B group. Between February and March the K of fish in the 2W treatment decreased from 1.14 ± 0.01 to 1.10 ± 0.01 resulting in a similar K to fish in the 6B regime, although both treatments were significantly lower than all other treatments. Fish exposed to 6W displayed a rapid decrease in K between the March and April sampling points culminating in a similar condition as fish in both the 2W and 6B treatments. At the final sampling point in June, K factor was similar between all treatments except for the 6B regime where the K was statistically lower than all other groups.

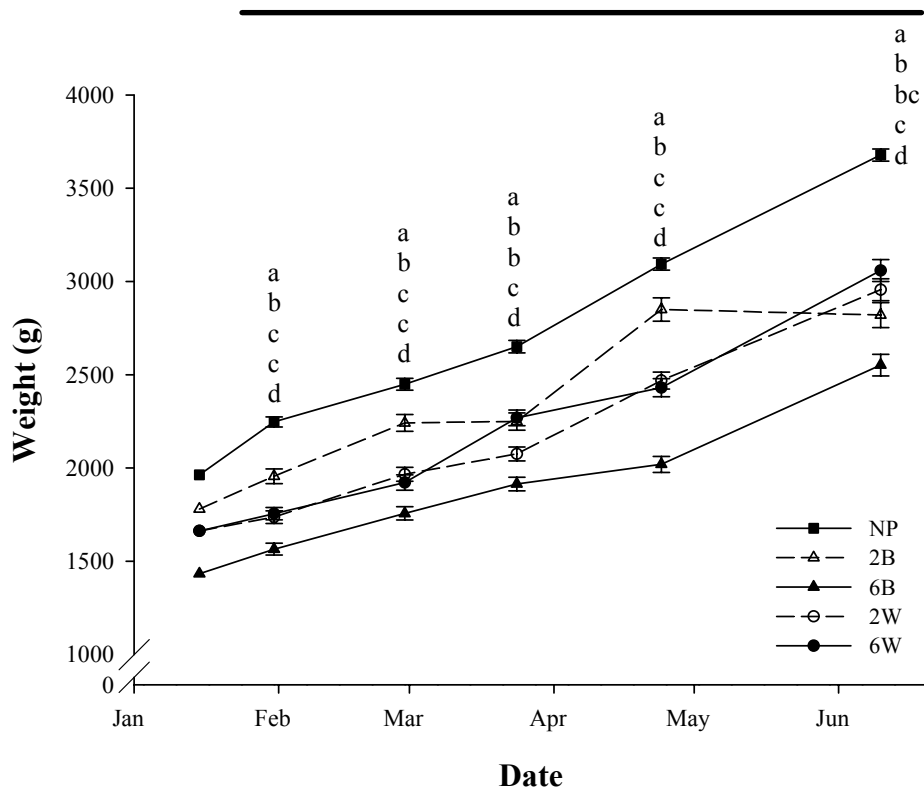


Figure 5.15. Mean body weights ($g \pm SEM$) of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. n = minimum of 200 fish (400 for NP) per treatment per sample point. Statistics were unable to be performed on initial weights, as individual weights were not available (mean values only).

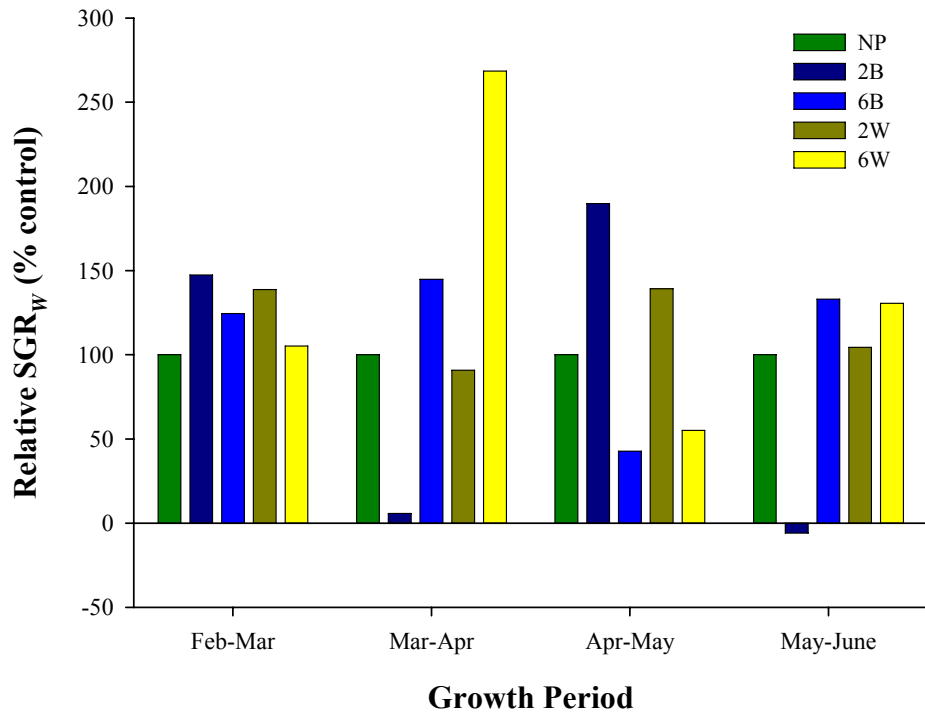


Figure 5.16. Weight specific growth rate (SGR_w) relative to the control of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Period of light duration, 22nd January to 21st June 2005.

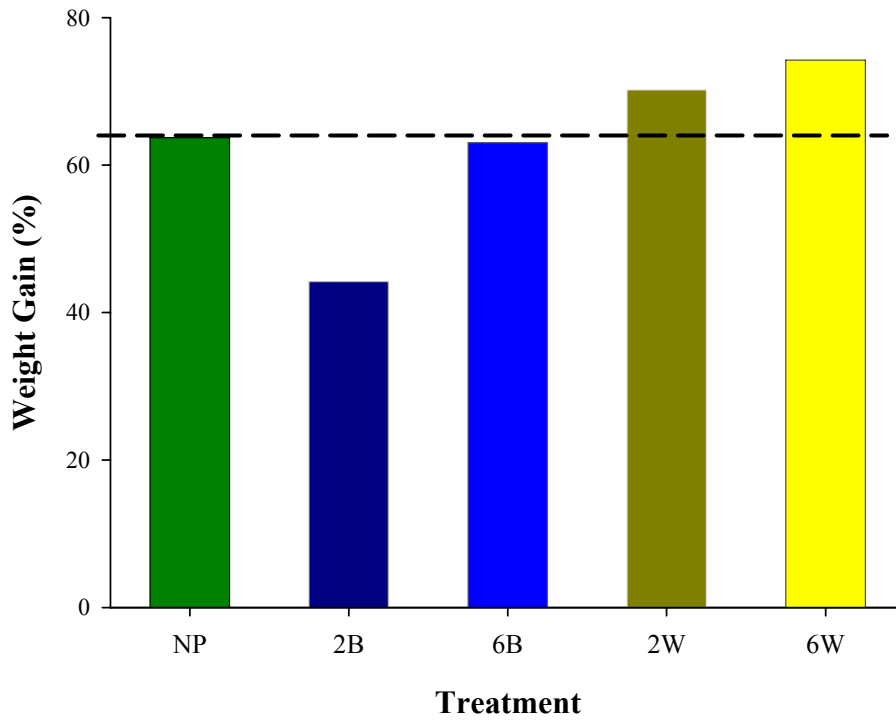


Figure 5.17. Relative weight gain over the January to June growth period of Atlantic salmon post-smolts reared under natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). The horizontal dashed line indicates the benchmark growth comparable to the control (NP).

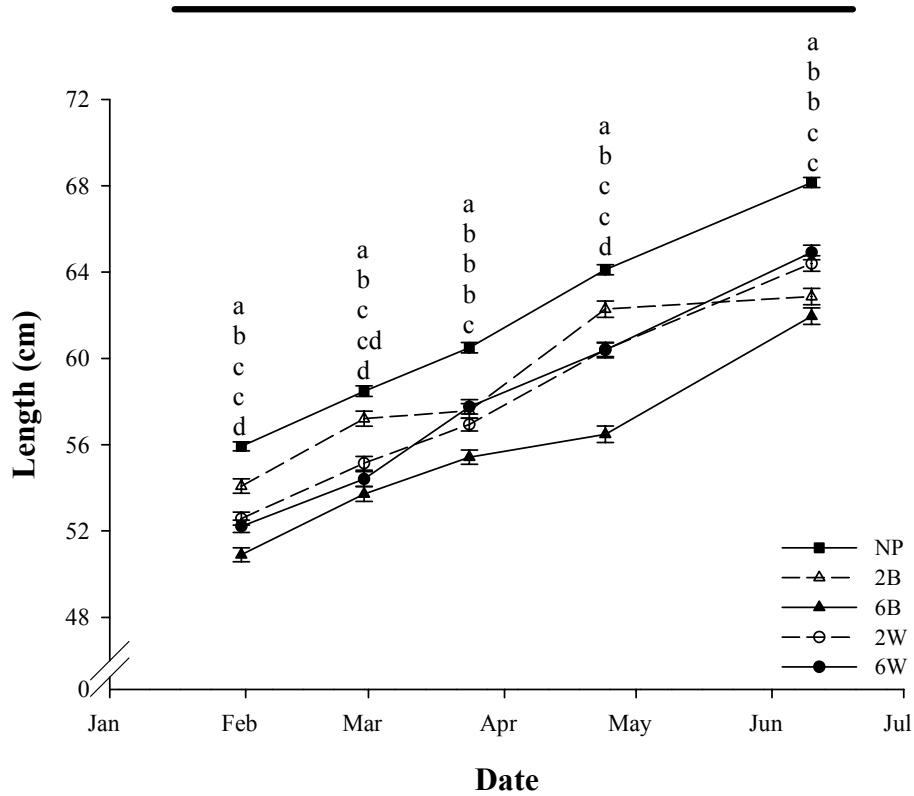


Figure 5.18. Mean fork length (cm \pm SEM) of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are statistically different ($P > 0.05$) between treatments at given time points. n = minimum of 200 fish (400 for NP) per treatment per sample point.

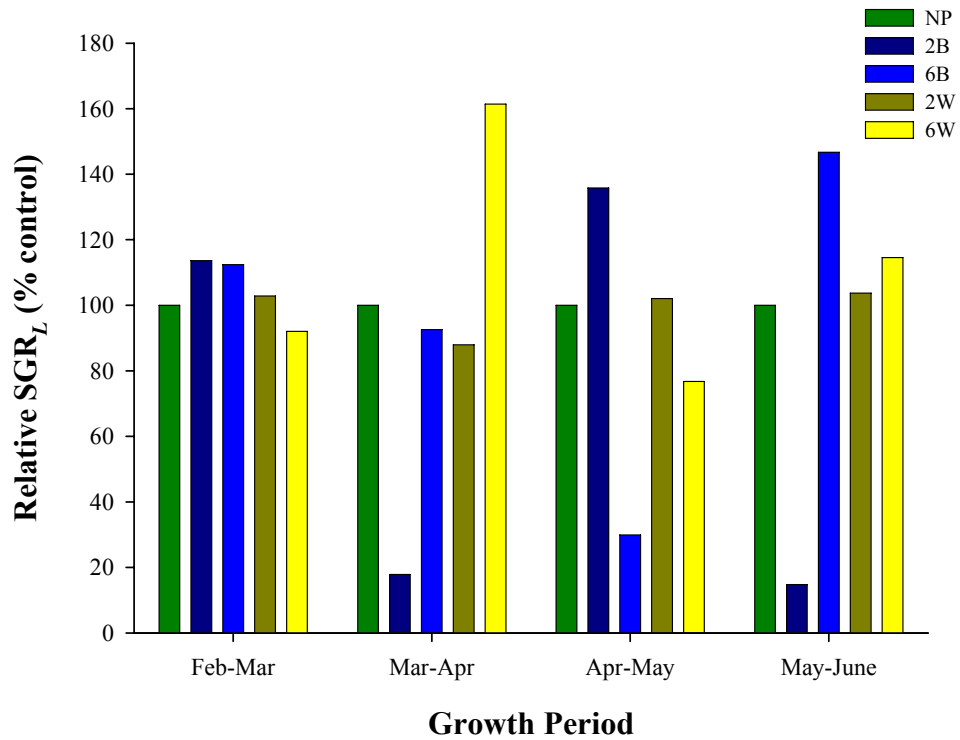


Figure 5.19. Length specific growth rate (SGR_L) relative to the control of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Period of light duration, 22nd January to 21st June 2005.

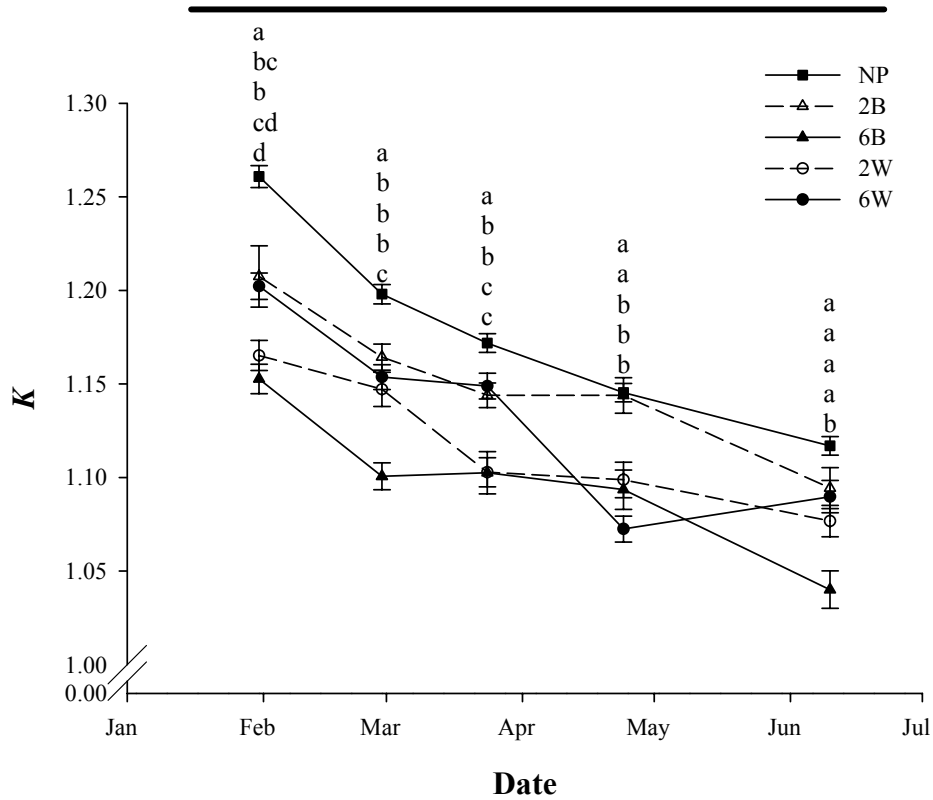


Figure 5.20. Condition factor (K ; mean \pm SEM) of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. n = minimum of 200 fish (400 for NP) per treatment per sample point.

5.3.6. Maturation

Gonadosomatic index

The mean gonadosomatic index (GSI) for male (♂) fish remained constant over the trial in the 6B, 2W and 6W treatments and increased to around 0.08% in the NP and 2B regimes prior to grading in June (Figure 5.21.a). During this period, the GSI was significantly ($P < 0.05$) higher at the March sampling point in fish reared under NP as compared to the 2B, 6B and 6W treatments, although no other differences were recorded. Following the June grade, GSI of the NP and 2B treatments reached $0.16 \pm 0.09\%$ and $0.11 \pm 0.09\%$ respectively, compared with around 0.07% in the 6B, 2W and 6W treatments.

Mean GSI of female (♀) fish was similar ($P > 0.05$) between all treatments, increasing from $0.13 \pm 0.01\%$ at the initial sample to around 0.3% at the final sample point in July (Figure 5.21.b).

Whilst the mean treatment GSI did not indicate maturation, individually sampled fish were found to exhibit signs of maturation. From a total number of 1090 fish sampled throughout the trial (January to July), only 15 individuals were deemed to be maturing (NP: 1 ♂, 4 ♀; 2B: 1 ♂, 1 ♀; 6B: 2 ♀; 2W: 2 ♀; 6W 1 ♂, 3 ♀), based upon a gonadal weight > 3 g and a (GSI) $\geq 0.4\%$ for males and a GSI $\geq 0.8\%$ for females (Endal *et al.*, 2000).

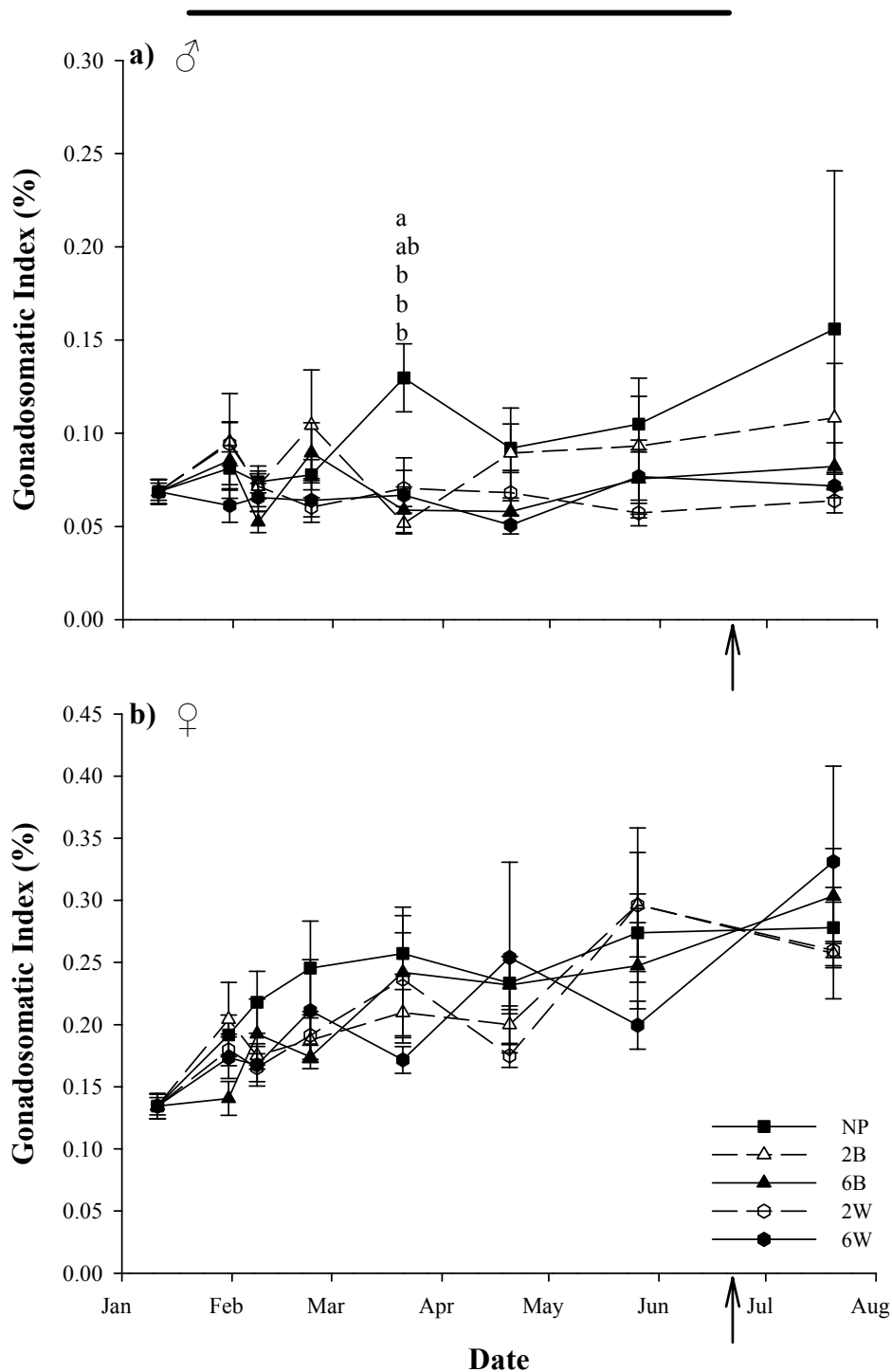


Figure 5.21. Gonadosomatic index (GSI; mean \pm SEM) of male (σ , a) and female (ρ , b) Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. Arrow denotes time at which cages were graded. $n = 25$ (50 NP) fish per treatment per sample point.

Histological examination

From a total of +100 gonad samples, 20 per treatment, from randomly selected fish during the April and May sampling periods, a near equal ratio (1:1) of male to females per treatment group were examined.

Female histology

Histological examination of female gonad samples (Figure 5.22.) revealed that fish exposed to continuous light, irrespective of light treatment, all exhibited signs of atresia with 10%, 20%, 70% and 50% of the fish examined from the 2B, 6B, 2W and 6W treatments respectively undergoing atresia (Figure 5.23.). From the fish sampled from the control group (NP), 50% were at the early vitellogenic stage whilst the remainder were at late vitellogenesis. Comparisons between lit treatment types revealed that a greater proportion of fish reared under blue light were undergoing late vitellogenic stages as compared to their conspecifics reared in the metal halide groups, 50% and 30% for 2B and 6B treatments respectively relative to 20% in the 2W regime. No late stage vitellogenesis was recorded for any of the 6W fish examined.

Mean oocyte diameters measured from all female fish examined show that fish from all treatments had oocytes at the primary oocyte (range 175-216 μm) and perinucleolus (range 532-755 μm) stages (Figure 5.24.). In addition, one fish from the NP treatment ($1016 \pm 35 \mu\text{m}$) and two from the 2B regimes ($802 \pm 127 \mu\text{m}$) exhibited oocytes in the early vitellogenic stage. Three fish from the NP group ($1264 \pm 41 \mu\text{m}$), four from 2B ($1167 \pm 24 \mu\text{m}$), two from 6B ($1113 \pm 44 \mu\text{m}$) and one fish from the 2W regime ($1531 \pm 87 \mu\text{m}$) all had oocytes at the late vitellogenic stages.

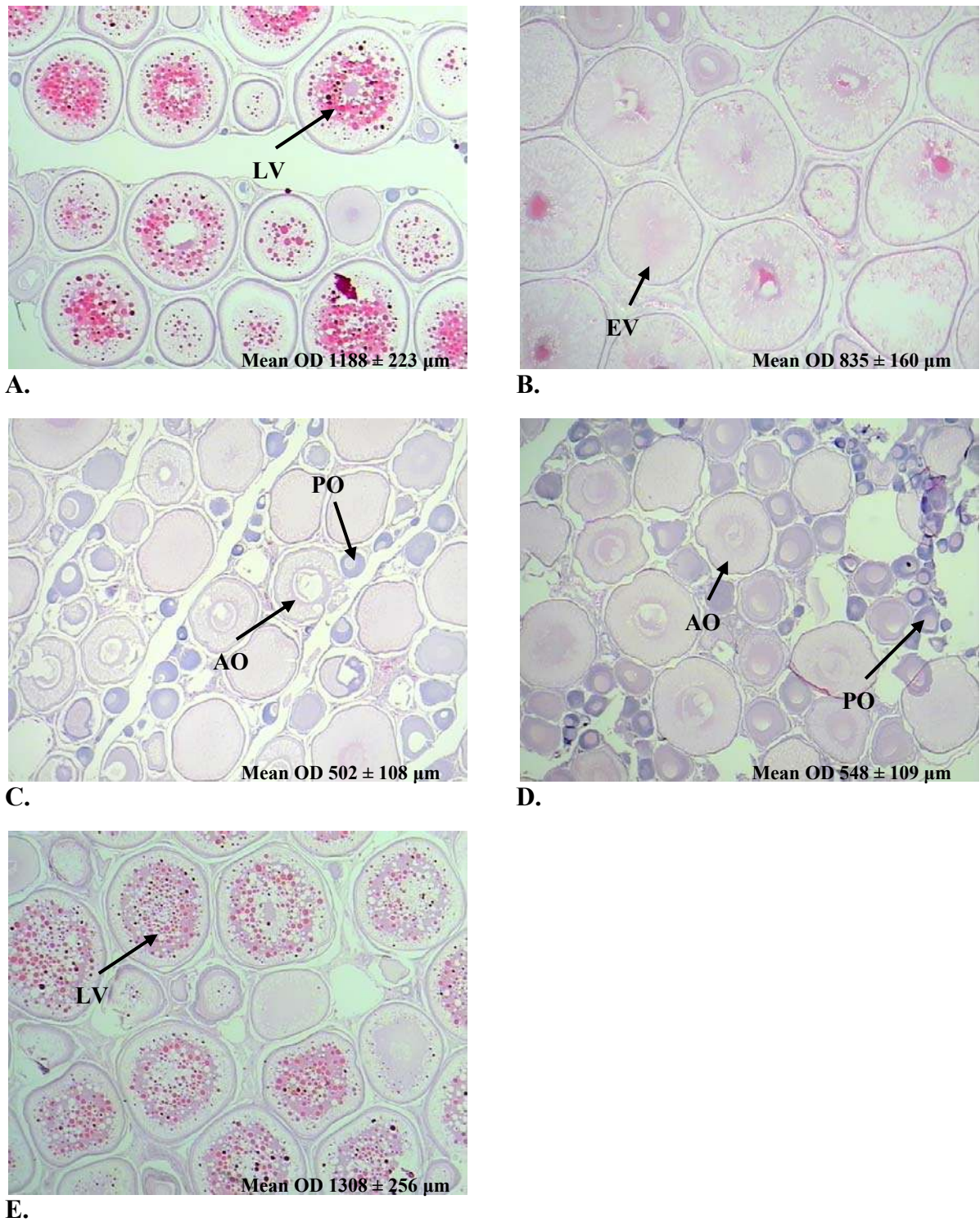


Figure 5.22. Photomicrographs of sections of Atlantic salmon ovaries removed for examination during April and May 2005. **A.** 2 Blue **B.** 6 Blue **C.** 2 White **D.** 6 White **E.** Natural Photoperiod. PO, Primary growth oocyte; EV, Early vitellogenesis; LV, Late vitellogenesis; AO, Atretic oocyte. Subscripts for each photomicrograph denote the mean \pm SEM oocyte diameter.

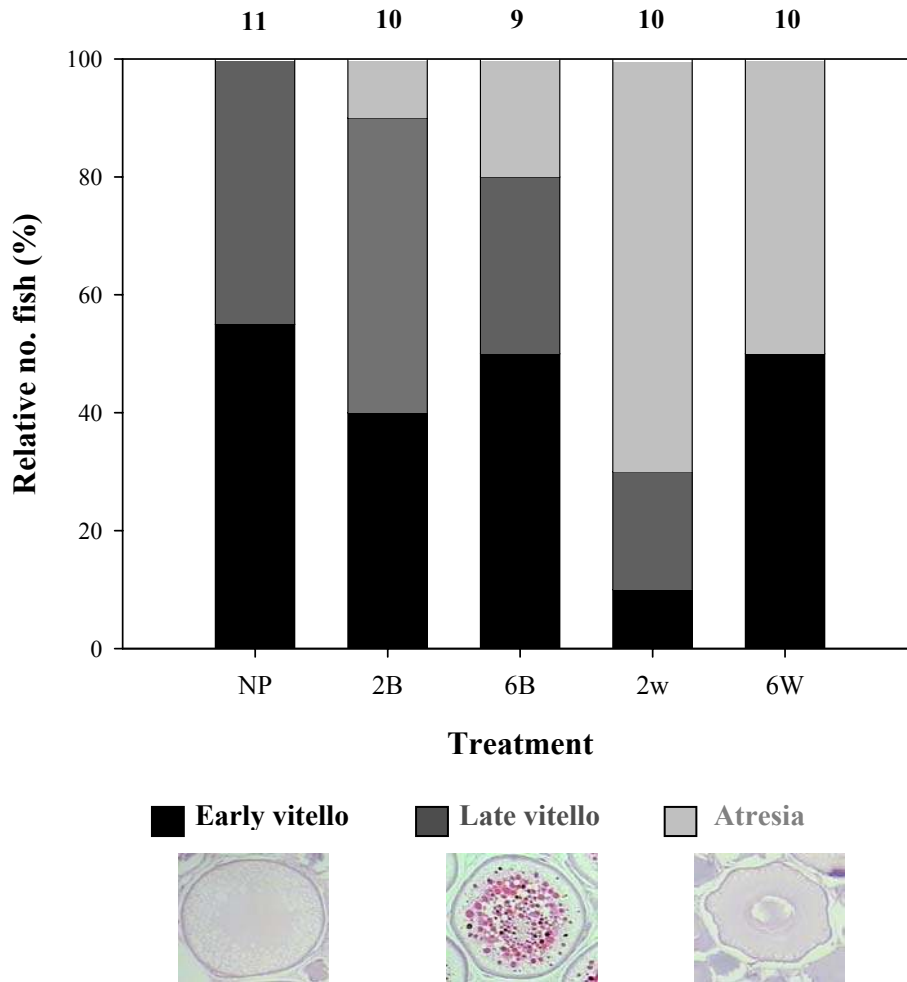


Figure 5.23. Relative proportion of female Atlantic salmon at various stages of oocyte development removed for examination during April and May 2005. Fish reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively) between the 22nd January and 21st June 2005. Numbers above bars indicate the number of female gonad samples examined.

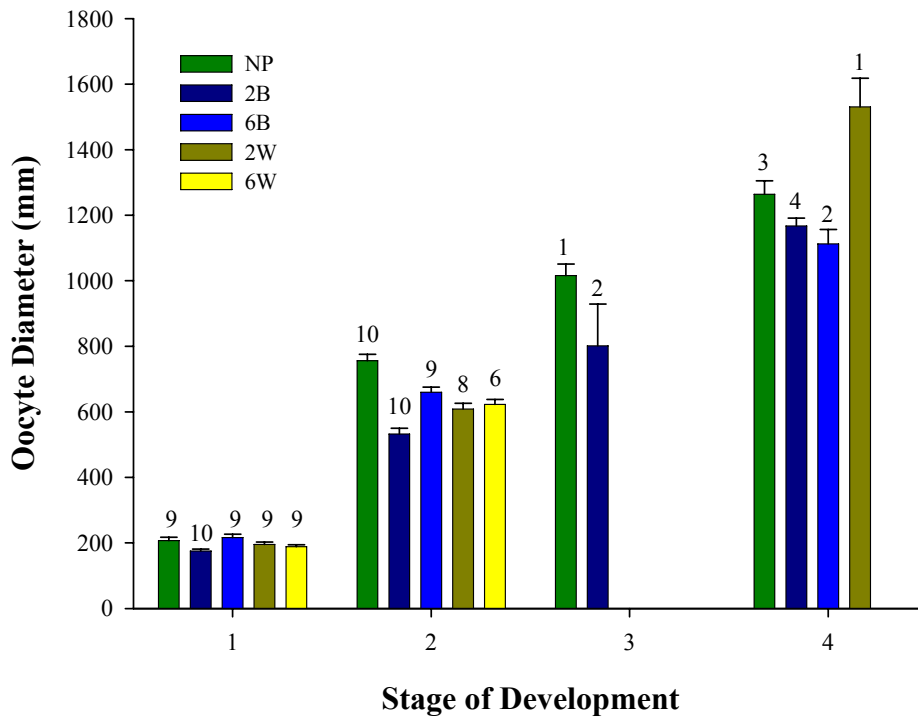


Figure 5.24. Mean oocyte diameter ($\mu\text{m} \pm \text{SEM}$) of 1+ female Atlantic salmon reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Oocytes removed for examination during April and May 2005. Stage 1 indicates primary oocytes; Stage 2, perinucleolus; Stage 3, early vitellogenesis; and Stage 4, late vitellogenesis. Numbers above columns indicate the number of females at each development stage.

Male histology

Histological changes in the testes development of male fish were different between the various treatments studied (Figure 5.25.). Under the ambient photoperiod (NP), males displayed an advanced maturational status with many of the fish observed to have spermatids present within the observed gonadal section. In total, 50% of the males examined from this treatment were deemed as initiating maturation (Table 5.3.). Fish from both the 2B (33%) and 6W (20%) treatments also exhibited signs of initiating maturation, although development was not as advanced as seen in the control group. Instead, spermatogonia B and primary spermatocytes were observed. However, no signs of testes development were found in any of the 6B and 2W fish examined, with these 'resting' testis containing spermatogonia A and sertoli cells only. No significant differences in the body weights or condition factor of males examined for histology analysis was found between treatments.

Table 5.3. Percentage number of male Atlantic salmon deemed as initiating maturation, as determined by histological analysis (April and May). Fish reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively) between 22nd January and 21st June 2005. Fish numbers examined per treatment are indicated in parenthesis. Mean body weights (g \pm SEM) and condition factor ($K \pm$ SEM) of sampled fish also presented, no significant differences between biometric measurements was evident ($P > 0.05$).

Treatment	Male Maturation (%)	Weight (g)	K
NP	50% (6)	3146 \pm 180	1.12 \pm 0.04
2B	33% (10)	2989 \pm 237	1.06 \pm 0.05
6B	0% (9)	2504 \pm 251	1.02 \pm 0.02
2W	0% (13)	2701 \pm 267	1.05 \pm 0.04
6W	20% (10)	2628 \pm 251	1.10 \pm 0.05

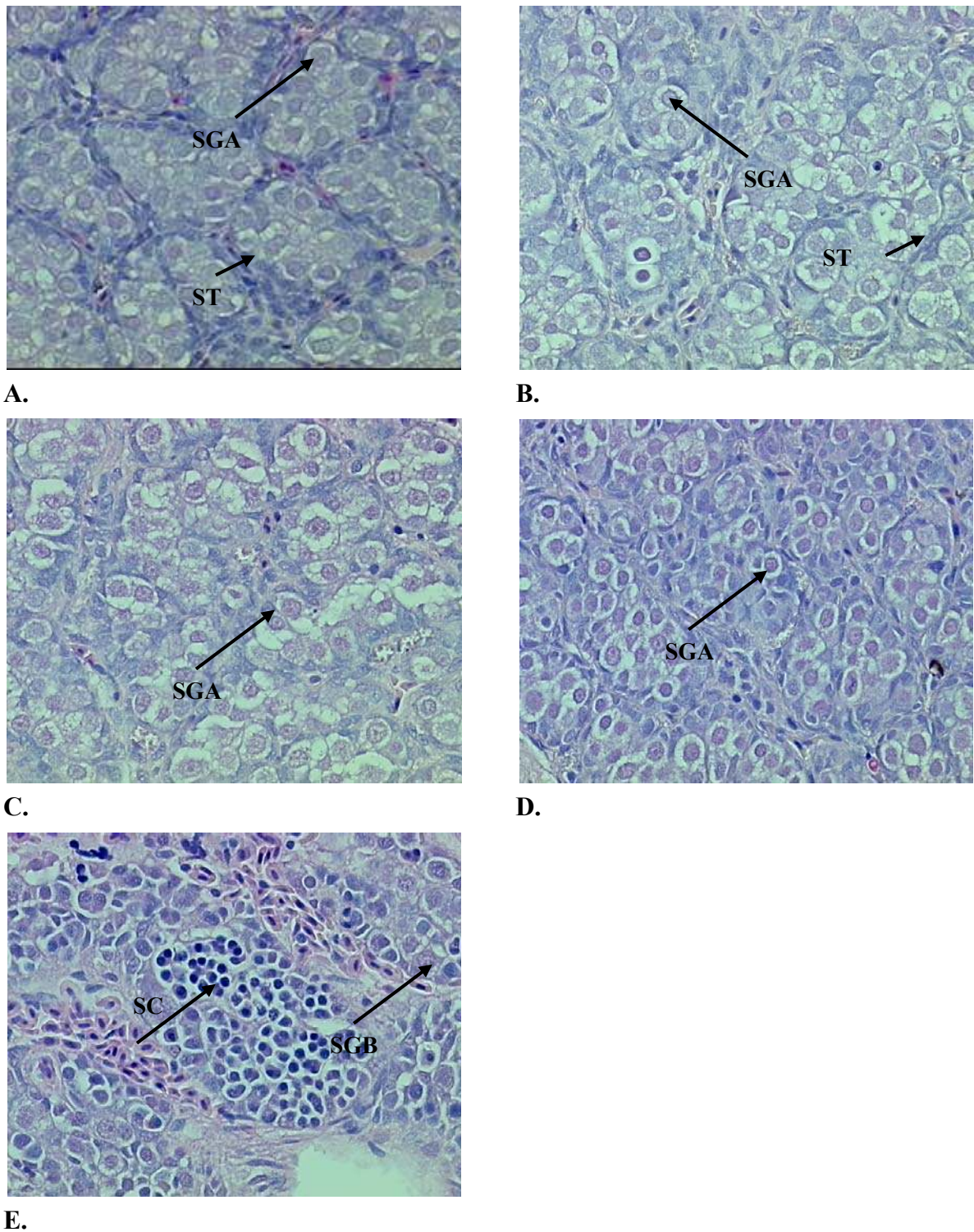


Figure 5.25. Photomicrographs of sections of Atlantic salmon testes removed for examination during April and May 2005. **A.** 2 Blue **B.** 6 Blue **C.** 2 White **D.** 6 White **E.** Natural Photoperiod. ST Sertoli cells; SG A Spermatogonia type A; SG B Spermatogonia type B; Spermatocytes SC.

Testosterone

Plasma testosterone levels taken from fish during the grading period in June were highest amongst the larger-size grade of fish (Table 5.4.). From the medium/small grade of fish only two individuals had a plasma testosterone level greater than the 3 ng.ml⁻¹ arbitrary threshold; one fish from the 6W treatment in the medium grade (equivalent to 4% of the sampled size grade), and one from the control group in the small grade (2% of sampled size grade).

Overall, the proportional frequency of fish with a plasma testosterone level greater than the 3 ng.ml⁻¹ threshold was highest amongst fish maintained under ambient conditions (NP), with 46% of the total sampled population above the threshold compared with 30% and 26% in the 2B and 6B treatments respectively (Figure 5.26.). However, fish exposed to the standard metal halide units, irrespective of intensity, had the least percentage number of fish with high testosterone levels with only 4% above the 3 ng.ml⁻¹ threshold.

Table 5.4. Proportion of 1+ Atlantic salmon post-smolts from each grade size deemed to be maturing, based upon a testosterone threshold level > 3 ng.ml⁻¹. Number of fish from sample size indicated in parenthesis.

Grade Size	Treatment				
	NP	2B	6B	2W	6W
Overall	24% (47/200)	15% (15/100)	13% (13/100)	2% (2/100)	3% (3/100)
Large	46% (46/100)	30% (15/50)	26% (13/50)	4% (2/50)	4% (2/50)
Medium	–	–	–	–	4% (1/25)
Small	2% (1/50)	–	–	–	–

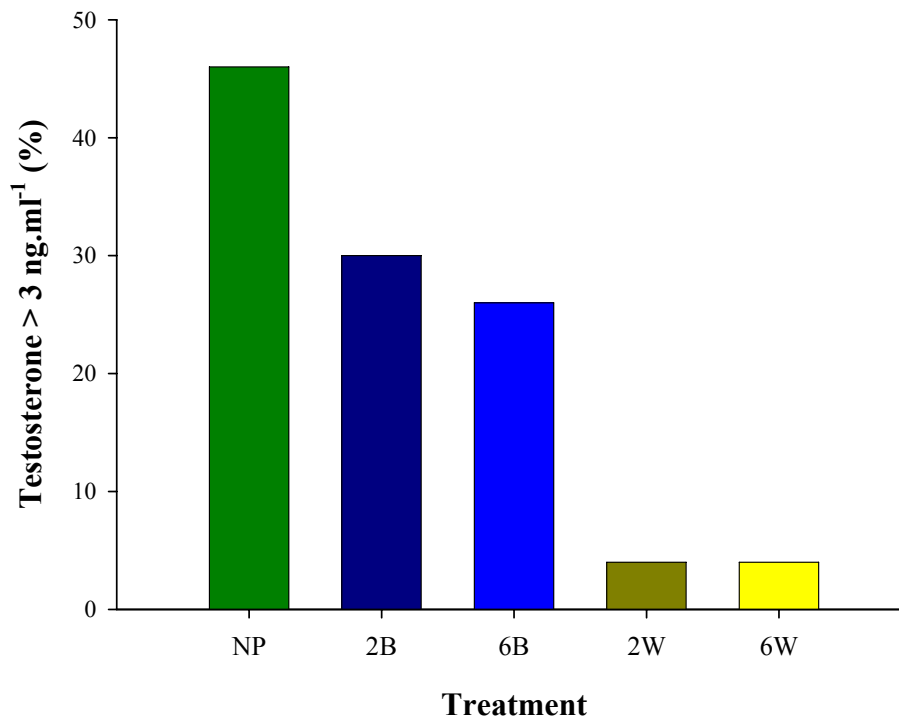


Figure 5.26. Proportion of Atlantic salmon post-smolts sampled from the large grade during June with a plasma testosterone level greater than the 3 ng.ml⁻¹ arbitrary threshold level. Fish were reared under a natural photoperiod (NP) or exposed to 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively). $n = 100$ fish sampled (200 for NP) per treatment.

5.3.7. Flesh Quality

Roche Colour Score

The mean range of Roche *SalmoFan*TM colour scores from the various regions of the Norwegian quality cut (NQC) flesh samples for the experimental period, January to July, are presented in Table 5.5. Since the flesh colour was also determined instrumentally using the Minolta colorimeter, no statistical analyses were performed for the visually subjective score.

For all treatment groups the mid-line region had the highest colour score of the three fillet areas assessed, whereas the dorsal and belly regions exhibited a similar colour score. The variable range of colour score measured for the dorsal region was lowest in the NP and 2B treatments and highest for the remainder of the groups. In addition, the lowest and highest mean colour scores recorded for the dorsal area from the flesh of 6W fish was on average lower than the other treatments by a score of 1.0 and 0.6 respectively. A similar pattern was noticeable for both the mid-line and belly regions of the NQC fillet samples, with the 6W regime scoring the lowest lower and upper range of mean colour score.

Table 5.5. Mean range of Roche *SalmoFan*TM colour scores for the trial period, January to July, of the various regions of the NQC. 1+ Atlantic salmon reared under a natural photoperiod (NP) or exposed to 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively). *n* = total of 60 fish per treatment sampled.

Treatment	NQC Fillet Area		
	Dorsal	Mid-line	Belly
NP	27.20 – 28.50	28.40 – 29.50	26.80 – 28.50
2B	27.00 – 28.40	28.00 – 29.60	26.20 – 28.10
6B	26.30 – 28.40	27.20 – 29.80	26.10 – 27.50
2W	26.80 – 28.50	27.80 – 29.40	26.20 – 27.80
6W	25.80 – 27.84	26.90 – 28.95	25.20 – 27.16

Minolta Colorimetric Analyses

The red/green chromaticity (a^*) decreased from 28.0 ± 0.4 at the initial sample in January to 27.2 ± 0.8 (6B), 27.0 ± 0.5 (2B) and 25.8 ± 0.4 (2W) at the February sample, whereas a^* increased in the NP group to 29.1 ± 0.6 (Figure 5.27.a.). This resulted in a significantly higher level in the control group than both the 2B and 2W regimes. During March the a^* increased in the 2W treatment but decreased in both the 2B and 2W groups. This led to significant differences between the NP and the 2B, 2W and 6B treatments as well as between the 6W and 6B regimes. At the April sample point a^* chromaticity values decreased sharply in both the NP and 6W groups such that all treatments exhibited a similar value. From then on no further differences were observed with a^* chromaticity values increasing in all treatments to between 27 and 29 at the end sampling point in July.

The blue/yellow chromaticity (b^*) of the control group increased from 27.5 ± 0.4 at the initial sample to 30.3 ± 0.4 in February, which resulted in this group having a significantly higher b^* value than the 2W (26.6 ± 0.6) treatment (Figure 5.27.b.). The b^* of the flesh from 6W fish increased to a statistically similar level as that of the NP group during March, such that both treatments were significantly higher than both blue light regimes. Between March and April, the b^* value of the NP regime decreased unlike the 6W treatment which remained stable. However, no differences between treatments were observed at this time. At the May sample, the b^* level of the 6W treatment continued to increase leading to a higher value than measured in both the 6B and 2B regimes. The b^* of the 6W treatment remained around 31 at the final sample point in July, whereas this increased to around 30 in the NP and 2B regimes. However, in the 6B regime the b^* decreased to 27.4 ± 0.7 resulting in a significantly lower value as compared to the 6W treatment.

Lightness (L^*) of all fillets, irrespective of treatment, increased over the trial period (Figure 5.28.). Following the onset of constant illumination, the flesh from fish reared under the artificial photoperiod regimes all exhibited similar L^* values (ca. 47 to 48). However, the NP (50.2 ± 0.5) group displayed a rapid increase in the L^* of flesh

which was significantly higher than that measured for the 6B. From March until the end of the trial period in July the L^* of the flesh from all groups, with exception to 6W, remained constant around 50 to 51. Conversely, the L^* values of the flesh from fish reared under 6W increased from 48.1 ± 0.4 in February to 53.3 ± 0.5 at the March sampling resulting in the 6W group being significantly higher than all other treatments. Thereafter, the L^* of the flesh from 6W fish remained the highest for the remainder of the trial, although no further differences were detected until the final sampling point in July when both the 2B and 2W treatments were significantly lower than the 6W regime.

Table 5.6. displays mean body weights and condition factor of fish examined for flesh quality parameters. Generally, NP fish were found to be significantly heavier than lit treatment fish in February (NP v 6W), March (NP v 2B, 6B and 6W), April (NP v 6B and 2W) and May (NP v all). With exception to April, where NP fish had a higher condition factor than 6B, no significant differences in fish condition were found.

Table 5.6. Mean body weights ($g \pm SEM$) and condition factor, in parenthesis, of Atlantic salmon sampled for flesh quality parameters, reared under a natural photoperiod (NP) or exposed to 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively). Means bearing identical superscripts in the same row are not significantly ($P > 0.05$) different ($n = 10$ fish per treatment per sample point).

Date	Treatment				
	NP	2B	6B	2W	6W
11/01/05	1645 ± 92 g (1.20 ± 0.02)	1645 ± 92 g (1.20 ± 0.02)	1645 ± 92 g (1.20 ± 0.02)	1645 ± 92 g (1.20 ± 0.02)	1645 ± 92 g (1.20 ± 0.02)
08/02/05	2454 ± 150 g ^a (1.20 ± 0.04)	2170 ± 156 g ^{ab} (1.16 ± 0.03)	1832 ± 198 g ^{ab} (1.10 ± 0.03)	1970 ± 173 g ^{ab} (1.10 ± 0.03)	1764 ± 178 g ^b (1.14 ± 0.04)
21/03/05	3088 ± 286 g ^a (1.28 ± 0.09)	2165 ± 159 g ^b (1.16 ± 0.03)	1876 ± 136 g ^b (1.05 ± 0.04)	2547 ± 159 g ^{ab} (1.24 ± 0.08)	2146 ± 148 g ^b (1.09 ± 0.02)
21/04/05	3189 ± 142 g ^a (1.22 ± 0.07) ^a	2938 ± 186 g ^{ab} (1.12 ± 0.04) ^{ab}	2377 ± 231 g ^b (0.99 ± 0.05) ^b	2390 ± 127 g ^b (1.04 ± 0.04) ^{ab}	2550 ± 251 g ^{ab} (1.10 ± 0.04) ^{ab}
26/05/05	3980 ± 232 g ^a (1.20 ± 0.07)	2689 ± 249 g ^b (1.10 ± 0.04)	2507 ± 140 g ^b (1.05 ± 0.02)	2973 ± 146 g ^b (1.10 ± 0.04)	2902 ± 167 g ^b (1.14 ± 0.04)
21/07/05	3808 ± 189 g (1.02 ± 0.02)	3444 ± 175 g (1.06 ± 0.04)	3126 ± 211 g (1.06 ± 0.03)	3633 ± 123 g (1.03 ± 0.02)	3281 ± 156 g (1.05 ± 0.02)

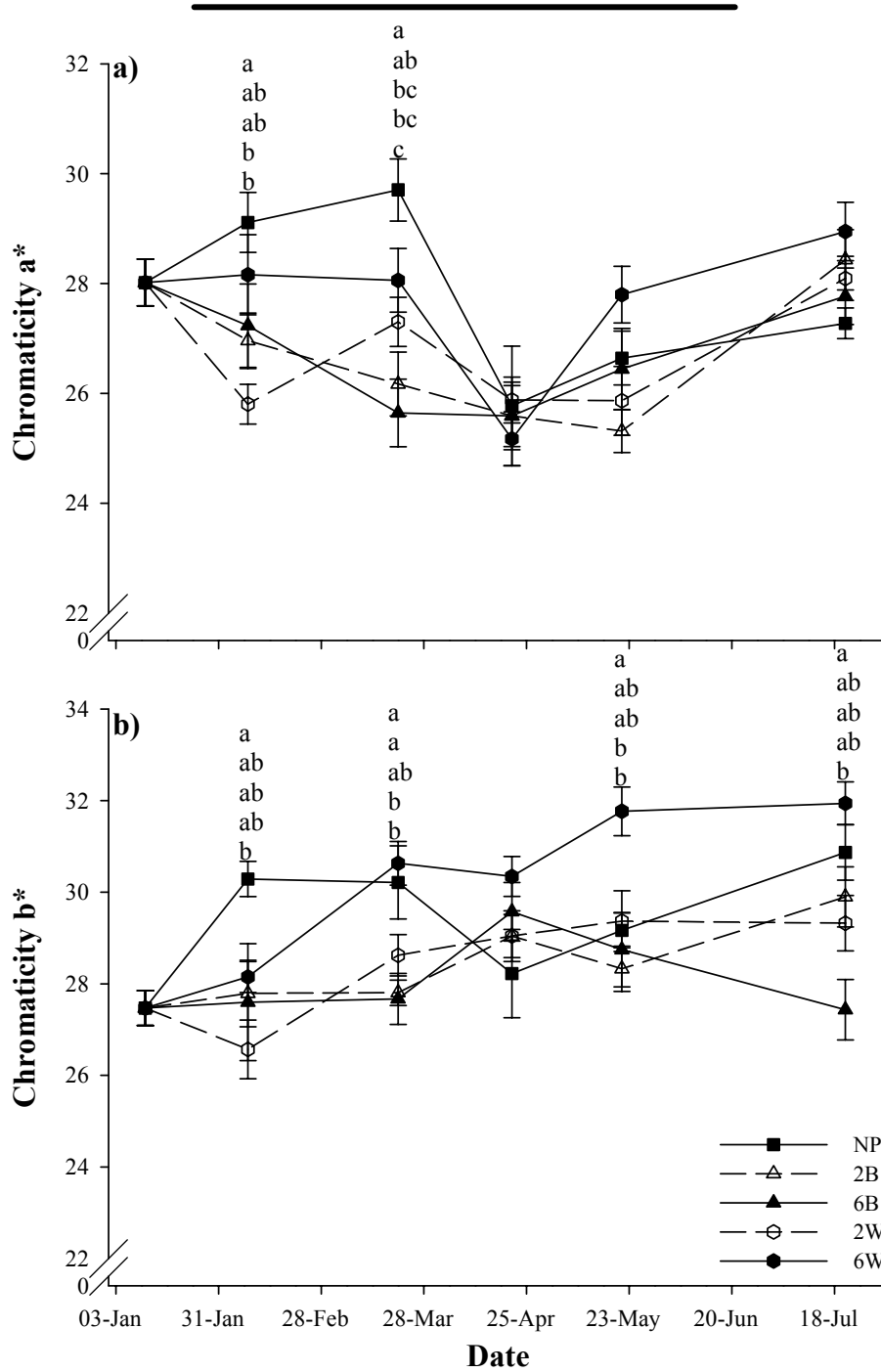


Figure 5.27. Change in red/green chromaticity (a*) values (mean \pm SEM; a) and yellow/blue chromaticity (b*) values (mean \pm SEM; b) over time in the flesh of fish reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. $n = 10$ fish per treatment per sample point.

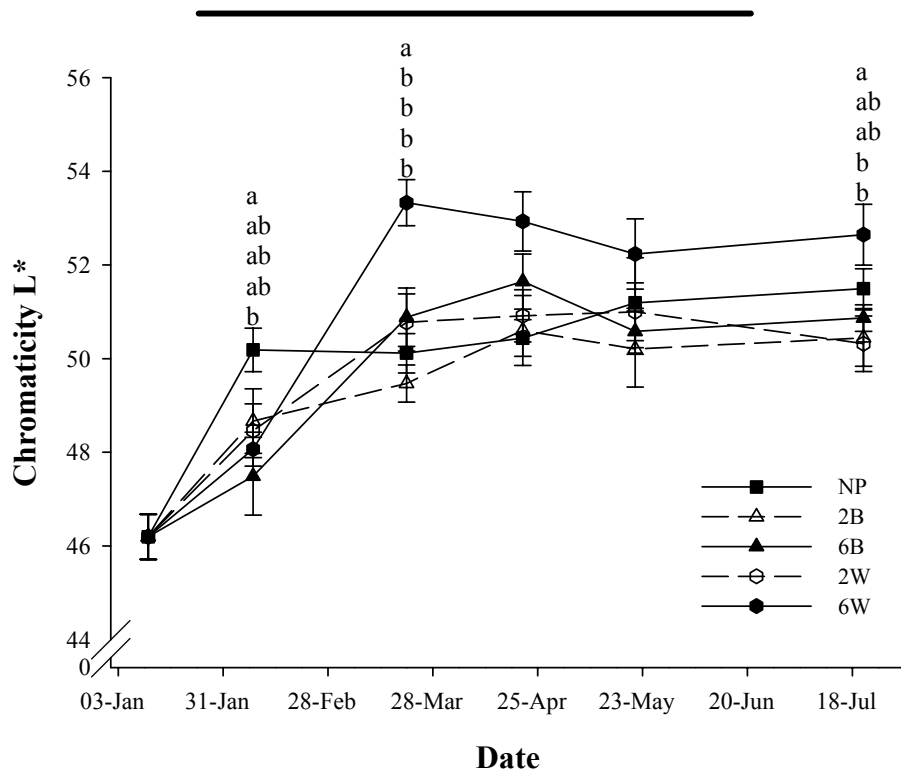


Figure 5.28. Change in lightness (L^*) values (mean \pm SEM) over time in the flesh of fish reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. $n = 10$ fish per treatment per sample point.

The chroma (C^*_{ab}), an expression of the intensity/clarity of the salmon cutlets, increased in the NP group from 39.3 ± 0.5 in January to 42.0 ± 0.6 at the February sample point and was significantly higher than both the 2B (38.7 ± 0.8) and 2W (37.1 ± 0.7) treatments (Figure 5.29.a.). At the March sample point the NP and 6W treatments were significantly different from both blue lit treatments. During April, the chroma value from the flesh of fish from the NP and 6W treatments decreased to around 39, similar to the chroma values of the other treatment groups. Between April and May the intensity/clarity of the 6W flesh increased from 39.4 ± 0.6 to 42.2 ± 0.7 resulting in a significantly higher chroma value than the 2B regime. Following grading, no differences in flesh chroma values were detected although the intensity/clarity of flesh from fish reared under 6W appeared highest.

The hue (H°_{ab}), a measure of the redness/yellowness of the flesh, remained relatively stable throughout the course of the trial, increasing from $44.5 \pm 0.4^{\circ}$ for all treatments at the start of the trial to $48.5 \pm 0.4^{\circ}$ in the NP group at the final sampling point in July (Figure 5.29.b.). At the March sample point, significant differences were evident between the 6W ($47.5 \pm 0.3^{\circ}$), 6B ($47.2 \pm 0.6^{\circ}$) and 2B ($46.8 \pm 0.6^{\circ}$) treatments compared to the hue of the control flesh ($45.5 \pm 0.3^{\circ}$). Between March and April the hue of the flesh from fish from all treatments increased, although flesh samples from the 6W treatment were significantly more yellow in colour than the 2B, 2W and NP groups. During May, significant differences in the hue of the flesh were detectable between 2W fish and fish reared under NP and 6B as well as between the 6W and 2B treatments compared to 6B fish. At the final sampling point in July, following grading and the switch off of lights, the hue of the NP group had increased such that it was significantly higher than both the 2B and 2W treatments which had both exhibited a decrease in hue to around 46° .

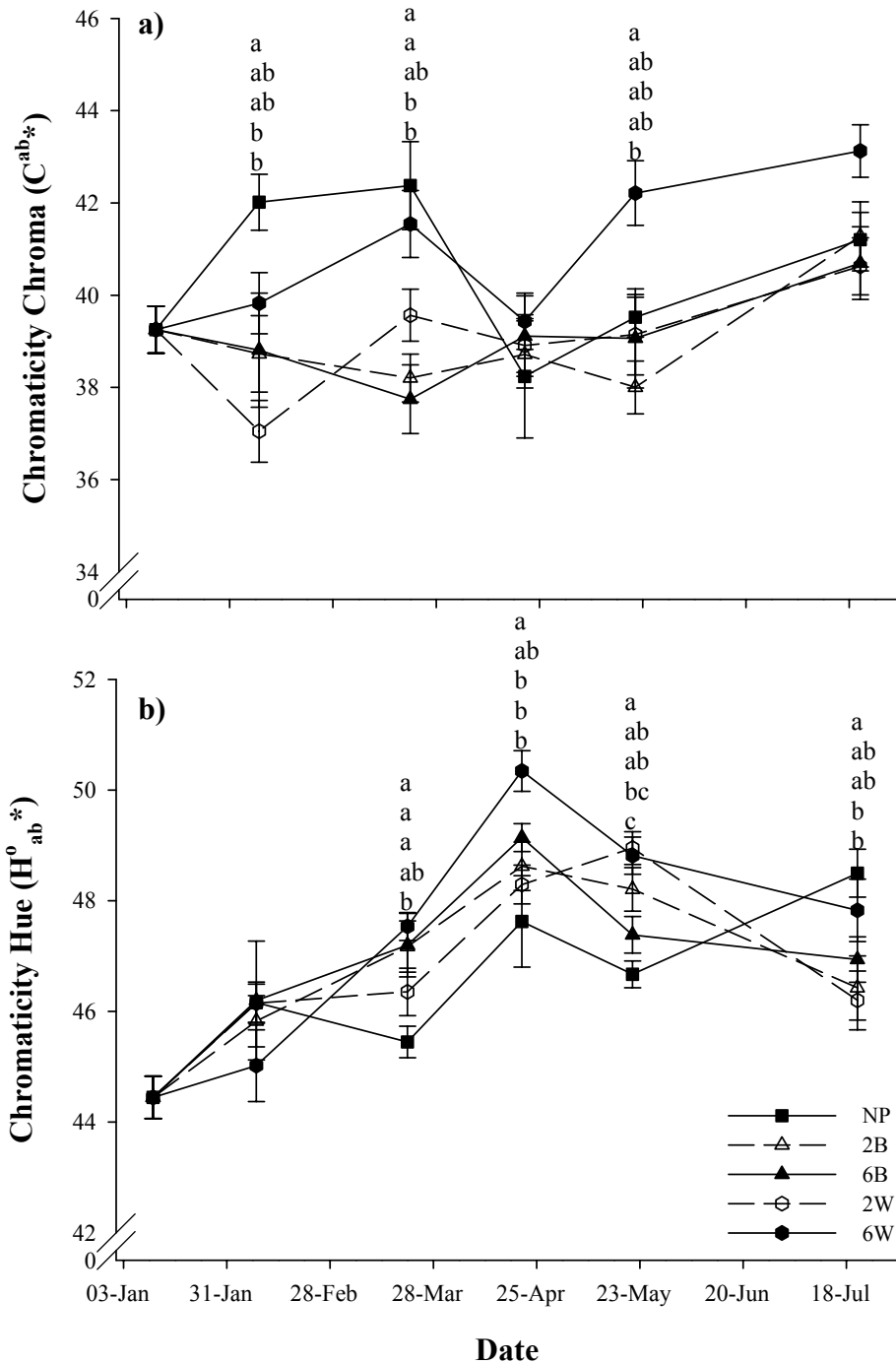


Figure 5.29. Change in mean chromaticity chroma (C_{ab}^*) values (mean \pm SEM; a) and chromaticity hue (H_{ab}^*) values (mean \pm SEM; b) over time in the flesh of fish reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. $n = 10$ fish per sample point.

Pigmentation

Total pigment levels from the flesh of salmon from all treatment groups were similar from the trial start through to the end of March (Figure 5.30.). Thereafter, mean flesh pigment levels for all groups increased with the control group exhibiting a greater increase. This resulted in a significantly higher pigment content, at the April sample point, in fish sampled from the NP group as compared to fish from the 6W and 6B treatments. In addition, the 2W and 2B treatments were also more significantly pigmented than the 6B regime. At the May sample point, total pigment levels were significantly higher in the NP group compared to the 2B and 6B regimes. Following grading in June, no differences were observed in the total pigment levels with all treatments around 9.6 to 10.9 mg.kg⁻¹.

Significant linear and non-linear relationships existed between body weight and the total flesh pigment content irrespective of treatment (refer to Table 5.7.). However, slopes from the linear correlations, *i.e.* 2B and 6W, were found not to differ significantly indicating that the relationships were similar.

Lipid

Lipid levels in the flesh of fish sampled from the control group increased from 10.7 ± 0.5% in January to 13.3 ± 0.8% in February (Figure 5.31.a), resulting in a significantly higher lipid content than measured in the 2W and 6W treatments (9.9 ± 0.7% and 9.6 ± 0.7% respectively). In March, lipid levels in the NP treatment (13.1 ± 0.7%) were significantly higher than the 6W (10.1 ± 0.7%), 2B (9.1 ± 0.6%) and 6B (7.5 ± 0.4%) treatments as well as differing between the 2W (11.7 ± 0.4%) regime and both the blue light treatments. At the April sample point the lipid content for all treatments decreased, which led to the NP, 2B and 2W treatments having a significantly higher lipid level than the 6W regime. Thereafter, the lipid content in the flesh from fish of all treatments stabilized between 6.4% and 6.9% until the final sampling period in July.

Significant non-linear relationships were found for all treatments between body weight and flesh lipid levels (refer to Table 5.7.).

Moisture

Moisture levels from the flesh of fish from all treatments inversely reflected the pattern of flesh lipid levels, increasing throughout the experimental period from 57.0% in all treatments to 66.5% in the control group (Figure 5.31.b). As such, moisture levels demonstrated a significantly strong non-linear relationship with the lipid level of the individual flesh samples (refer to Table 5.7.).

In the period following light onset (8th February) moisture levels were significantly higher in the metal halide groups relative to the control. This changed during March when the moisture in the flesh of 6B fish increased, whereas all other treatments remained unchanged, resulting in a difference between the flesh of fish from the 6B treatment with 6W, 2W and NP as well as between 2B and the 6W, 2W and NP treatments and a significant difference between the flesh of 6W and the control group. Between March and April, moisture levels in the flesh of fish reared in the 2B and 2W regimes increased dramatically, as compared to the other treatments, such that levels were significantly higher than the remaining groups. During May, all lit treatments had significantly higher moisture levels than the NP group, although differences were also observed between both blue light treatments and the 6W regime. From May to the final sampling period, post-grading, in July moisture levels in the 6B and 6W treatments remained stable at around 65% and 62% respectively, whereas the moisture levels in the flesh from fish reared under 2B and 2W fell from around 65% to approximately 60% and levels in the control group rose from 58% to 66%. This resulted in both the NP ($66.5 \pm 0.5\%$) and 6B ($65.7 \pm 0.5\%$) regimes having a significantly higher moisture level than the 6W ($62.4 \pm 0.9\%$), 2B ($60.5 \pm 0.4\%$) and 2W ($60.0 \pm 0.5\%$) as well as a reported difference between the 6W and 2W groups.

As with flesh lipid, moisture levels also demonstrated significant non-linear relationships with fish body weight (refer to Table 5.7.).

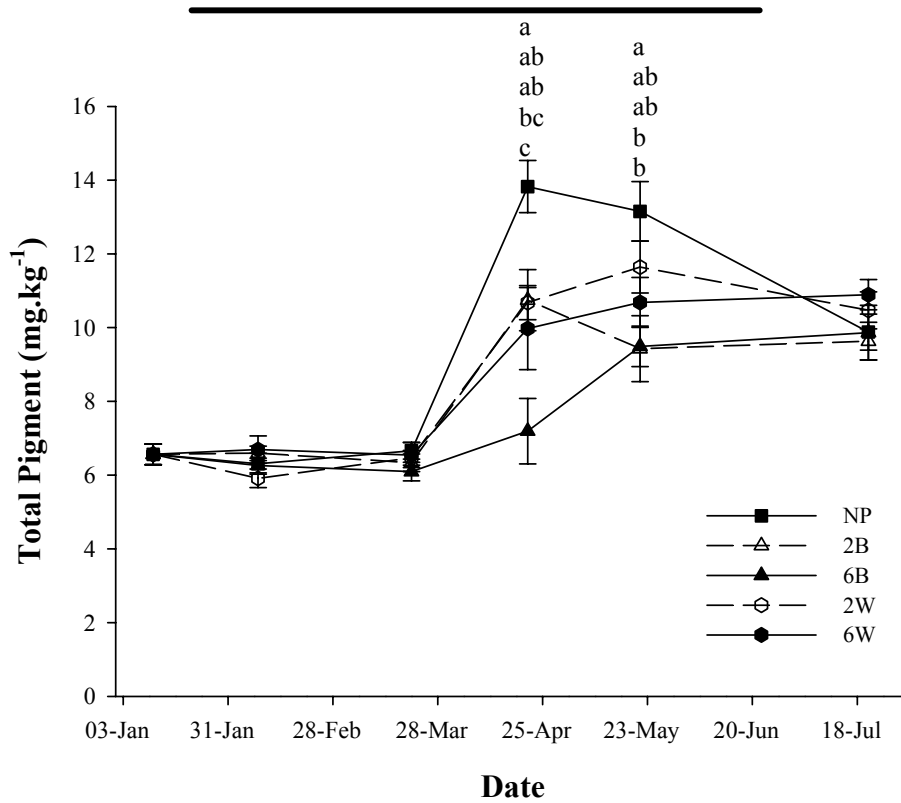


Figure 5.30. Mean total pigment content ($\text{mg.kg}^{-1} \pm \text{SEM}$) from the Norwegian Quality Cut of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. $n = 10$ fish per treatment per sample point.

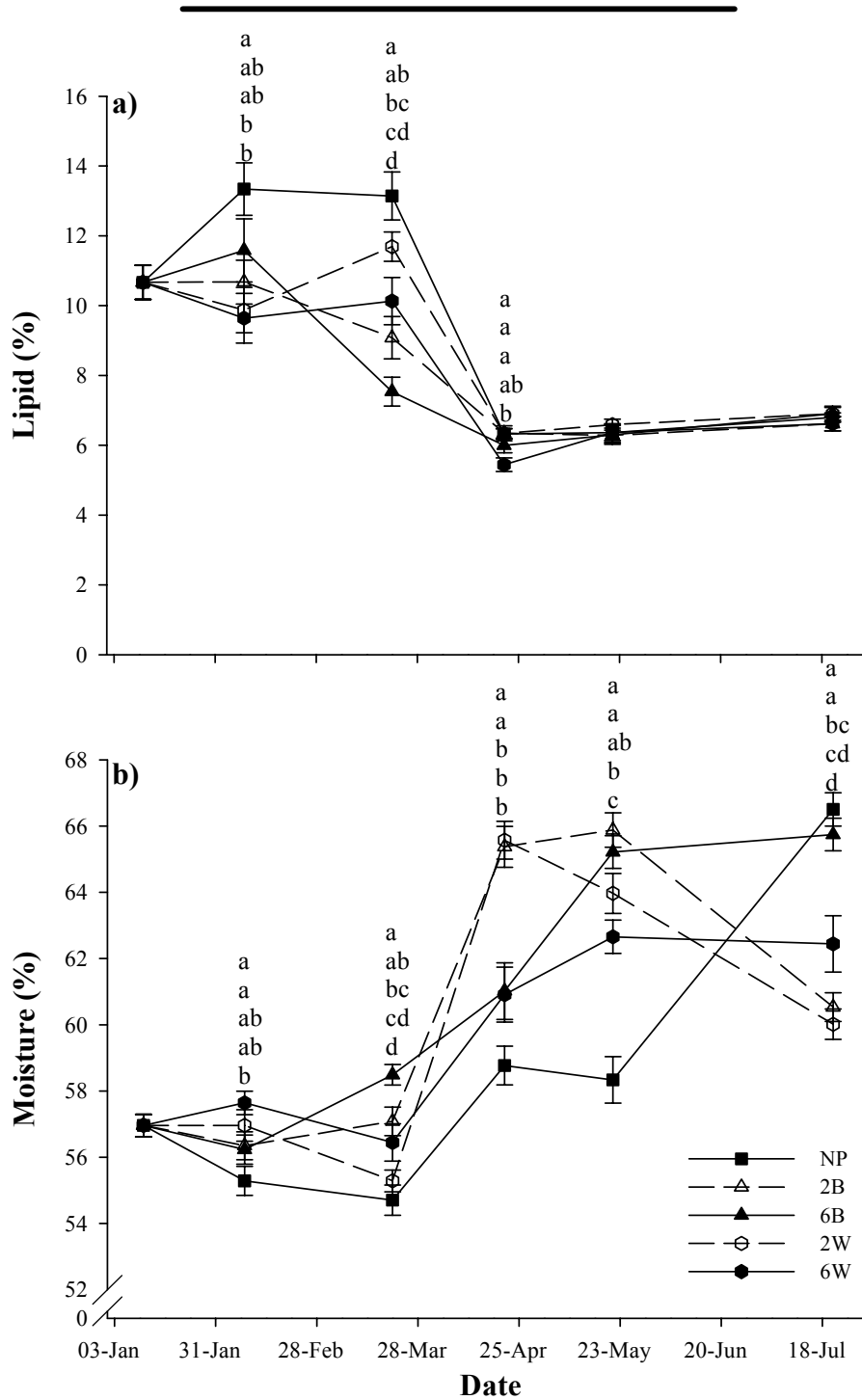


Figure 5.31. Mean lipid (% \pm SEM; a) and moisture (% \pm SEM; b) levels from the Norwegian quality cut of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Horizontal bar indicates period of light onset (22nd January to 21st June 2005). In some cases error bars are too small to be depicted. Means bearing identical lettering are not statistically different ($P > 0.05$) between treatments at given time points. $n = 10$ fish per treatment per sample point.

Table 5.7. Correlations between body weight and various flesh characteristics of fish reared under a natural photoperiod (NP) or exposed to 2 or 6 blue LED light units (2B and 6B respectively) or standard metal halide units (2W and 6W respectively). Treatments without slope and intercept data indicate a non-linear relationship.

	Pearson	r²	P Value	F Value	Slope	Intercept
<i>Weight v Pigment</i>						
Overall		0.4460	<0.0001	135.45		
NP		0.4040	<0.0001	24.04		
2B	0.6932	0.4806	<0.0001	61.991	0.0021	3.055
6B		0.4500	<0.0001	28.87		
2W		0.3970	<0.0001	23.36		
6W	0.7283	0.5304	<0.0001	75.666	0.0024	2.784
<i>Weight v Lipid</i>						
Overall		0.1180	<0.001	23.35		
NP		0.1330	0.003	6.22		
2B		0.2080	<0.001	9.95		
6B		0.0730	0.030	3.69		
2W		0.1570	0.001	7.35		
6W		0.1950	<0.001	9.22		
<i>Weight v Moisture</i>						
Overall		0.1020	<0.001	19.90		
NP		0.0660	0.039	3.40		
2B		0.1330	0.003	6.20		
6B		0.2610	<0.001	12.98		
2W		0.0770	0.027	3.82		
6W		0.1470	0.002	6.85		
<i>Lipid v Moisture</i>						
Overall		0.5590	<0.001	212.99		
NP		0.4370	<0.001	27.36		
2B		0.6420	<0.001	62.07		
6B		0.5420	<0.001	41.21		
2W		0.7540	<0.001	105.45		
6W		0.4970	<0.001	34.55		

5.3.8. Summary of Results

The results from the current investigation indicate the following and are further summarized in Table 5.8.:

- Light emitted by the 400 W standard metal halide units penetrated the water column up to 6 m away from the light source, whereas light from the 50 W blue LED units was only detectable up to 3 m from the light source. However, this was achieved by one eighth of the power wattage.
- The percentage volume of the treatment cage estimated to be lit above the suggested salmon light intensity threshold of $0.016 \text{ W}\cdot\text{m}^{-2}$ was over 75% for the 6W regime compared to around 26% in the 2W, 2% in the 6B and less than 1% for the 2B treatment.
- Nocturnal plasma melatonin levels were lower in the metal halide treatments as compared to the blue lit groups. However, only the 6W treatment resulted in a suppression of melatonin levels statistically similar to those produced during the day.
- No evidence of a growth-dip or reduced feed intake was evident for fish reared under the standard metal halide lights, although both the blue light treatments exhibited signs of a suppressed appetite in the period immediately following the onset of artificial illumination.
- Despite differences in the initial body weights, no differences in the overall growth were evident (SGR_W , weight gain). However, in terms of the relative weight gain for the trial period fish reared under metal halide units outperformed their NP counterparts, whereas cohorts of fish reared under the blue units had an equal or lesser weight gain relative to the control.
- Continuous light, irrespective of intensity or spectrum, showed signs of inhibiting maturation in both male and female Atlantic salmon, as seen with oocyte diameters, relative to the control (NP).

- Both the metal halide treatments were more effective than the blue light treatments at reducing plasma testosterone levels below the 3 ng.ml⁻¹ threshold.
- Flesh characteristics showed considerable differences between treatments throughout the trial, with lipid and moisture levels decreasing and increasing respectively over the experimental period.
- Flesh colour also varied between treatments during the investigation, although the overall range remained relatively unchanged. Total flesh pigment levels were found to exhibit significant linear and non-linear relationships with the body weight of individual treatment fish.

Table 5.8. Trial summary of growth and maturation of Atlantic salmon post-smolts reared under a natural photoperiod (NP) or exposed to 2 or 6 units of blue LED light (2B and 6B respectively) or standard metal halide (2W and 6W respectively) between January and July 2005. Values in the same row bearing identical superscripts are not significantly different ($P>0.05$).

	TREATMENT				
	NP	2 Blue	6 Blue	2 White	6 White
Weight at January (g)	2247 ^a	1955 ^b	1565 ^c	1737 ^c	1755 ^d
Weight at June (g)	3672 ^a	2820 ^b	2551 ^{bc}	2955 ^c	3058 ^d
Relative Weight Gain (%)	64	44	63	70	74
SGR _w (% day ⁻¹) January to June	0.383	0.282	0.377	0.413	0.428
% Maturation* (June grade)	48	32	28	4	4

*based on fish with a plasma testosterone level > 3 ng.ml⁻¹ sampled from the top grade during June

5.4. Discussion

Improving the growth performance of fish, whilst also reducing the proportion of grilse is of prime importance to the commercial salmon farmer. The application of artificial photoperiod regimes has in part helped the industry to realize this aim. However, whilst growth benefits are commonly reported, the expected reduction in the proportion of fish maturing (*e.g.* Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Porter *et al.*, 1999; Endal *et al.*, 2000) is not always found (*e.g.* Kråkenes *et al.*, 1991; Endal *et al.*, 2000; Johnston *et al.*, 2003). The lack of standardization regarding the use of light regimes (*i.e.* type of light units, timing and duration of onset, number of units per pen *etc.*) is prominent within the industry, with no standard operating procedures (SOPs) currently in place. There is, therefore, a need for the development of lighting regimes which are specific to the light sensitivity of the fish, both in terms of the spectral content and intensity level of light, whilst also reducing the associated running costs. With that in mind, the aim of the current investigation was to first; compare the efficiency of the novel blue narrow bandwidth LED units in seawater against the metal halide systems, currently used within the industry. Second; to examine the effects of light intensity and spectral content of light on melatonin profiles as a mean to determine how light generated by the different systems tested is perceived by the fish affecting the growth, maturation and flesh characteristics of 1+ Atlantic salmon post-smolts reared under commercial production conditions. Ultimately, the potential for the use of this new lighting technology shall also be discussed.

5.4.1. Light characteristics and perception

Designing a lighting system for use within commercial production systems requires a thorough understanding of light characteristics, both with regards to the aquatic environment and species sensitivities. Such systems are dependent upon the efficacy of the artificial light through the aquatic environment and how well the light is perceived and utilized in coordinating physiological responses in fish. Results from current research on the spectral sensitivity of fish (*c.f.* Bayarri *et al.*, 2002; Migaud *et al.*, 2006a),

together with our knowledge of how the natural light is transformed through the water column, all suggest that the blue spectral area of light may provide the most effective lighting source.

The 'blue' narrow bandwidth LED lighting units (λ 450 nm) used within the current trial however, were less efficient, in terms of energy (W.m^{-2}), at penetrating the water column compared to the standard metal halide units currently employed throughout the industry. This may be explained by the fact that the blue light units (50 W) were considerably less powerful than the metal halide units (400 W) and is clearly evident from the overall intensity readings taken at the source of light: 9.8 W.m^{-2} compared to 46.9 W.m^{-2} for the LED and metal halide lighting units respectively. One problem identified with the design of the prototype blue LED units was that the base of the light units were capped with no light emitted below the units. Nevertheless, whilst the energy output generated by one blue LED unit was eight times lower than that of the metal halide units (*i.e.* 50 W versus 400 W), light penetration through the water column by one blue unit was only half of that emitted by the metal halide with light detectable at 3 m and 6 m respectively. Consequently, the blue LED lighting units may offer a more cost effective alternative, in terms of the overall running cost, than the more energy consuming metal halide units, although at this stage it appears apparent that a more powerful LED unit is required to equal the penetrative distance of the metal halides.

The intensity profiles (contour plots) mapped for each lit treatment group provided a clearer understanding as to how light emitted from the various units was distributed within each cage. This further confirmed the low efficiency of the blue light units in comparison to the standard metal halide. Furthermore, the intensity profiles also revealed that almost the entire volume of water within the lit cage of the 6W treatment was above the proposed light intensity threshold value of 0.016 W.m^{-2} suggested by Migaud *et al.* (2006a). This is further supported by the 'lit cage volume' model kindly provided by Dr. Clive Talbot of Marine Harvest (Stavanger, Norway), which shows that over 75% (3000 m^3) of the total cage volume (4000 m^3) for the 6W treatment used in the current trial was estimated to have been lit above the salmon light intensity threshold.

This compares to around 25% (1000 m³) for the 2W treatment with lower values of just 2% (80 m³) and less than 1% (40 m³) for the 6B and 2B regimes respectively. This would suggest that even if fish in the blue treatments swam at the depths of the light units, as has previously been demonstrated for standard submersible lighting units (Oppedal *et al.*, 2001; Hevrøy *et al.*, 2003; Juell *et al.*, 2003; Juell and Fosseidengen, 2004), very few fish would have been within the range of the suggested intensity threshold. Moreover, as a further indication of the low light output, it is of interest to note that a total of 74 blue LED units would be required to light an equivalent cage volume as was achieved in the 2W regime. From the natural light scans taken at the Lønningdal site in Norway, it would appear that the shorter wavelengths of light (*i.e.* blue) were significantly reduced below depths of 6 m, whereas the blue green area of light penetrated well. Thus, it is probable that the spectral region of light used in this study may not be in the specific bandwidth for high water penetration resulting in light being absorbed within a short distance from the units. However, although these light scans are representative of the marine environment it is still important to consider the changes in the local conditions between sites (water clarity, visibility) that may affect the spectral quality of light. Nevertheless, further studies focussing on other areas of the light spectrum based on natural light plots should be investigated. It is important to realize nonetheless, that whilst the intensity of the light source(s) may be of some significance to the management of underwater lighting systems, it is the positioning of the light sources within the pen (depth, formation *etc.*) that may elicit the greatest responses. Furthermore, regardless of how efficiently or poorly the light penetrates the water column, it is how well the light is perceived and transduced by the individual fish that is of greatest interest to the salmon farmer and fish biologist alike.

Light perception

The light perception hormone, melatonin, was used as a biological indicator to assess how the different light treatments were perceived by the fish. Under natural conditions (NP), plasma melatonin levels followed the 'classical' rhythm with low levels measured

during the day ($8.7 \pm 1.3 \text{ pg.ml}^{-1}$) and increasing at night ($45.0 \pm 8.8 \text{ pg.ml}^{-1}$). However, it must be noted that these nocturnal levels were much lower than the expected 200 pg.ml^{-1} levels previously observed in an identical strain, age and size of salmon (refer to Chapter 4.). To facilitate the night-time sampling of this group, fish were crowded into a corner of the pen during the early evening. However, all the lit treatment cages were split and crowded at the time of sampling since the artificial light from the treatment pens was used to provide a sufficient level of 'working light' for the safe operation of machinery to lift the nets. In addition, on the night of the melatonin sample the sky was clear with a full moon. These factors could have resulted in the suppression of plasma melatonin levels of fish within the NP group resulting in a false measure. Indeed, Migaud *et al.* (2006a) have demonstrated that a light intensity as low as 0.1% of the daylight value is sufficient in lowering nocturnal melatonin levels by up to 45%, and increasing intensities in *in vivo* studies has been shown to significantly decrease nocturnal plasma melatonin levels measured in juvenile salmon (Porter *et al.*, 2001).

The lack of suppression in nocturnal melatonin production observed within the blue treatments is most likely explained by the relatively low intensity of light produced by the LED units. Previous investigations with Atlantic salmon and European sea bass, *Dicentrarchus labrax*, have demonstrated a decreasing rate of melatonin synthesis with an increasing intensity of night-time illumination (Yáñez and Meissl, 1996; Porter *et al.*, 2001; Bayarri *et al.*, 2002; Migaud *et al.*, 2006a). Further evidence of this can be seen within the current results whereby the highest light intensity within each colour treatment was the most successful at suppressing melatonin levels (*i.e.* 2B < 6B and 2W < 6W), although this difference was only found to be significant within the blue treatment groups. As previously mentioned the distribution of light throughout the majority of the 6W cage and also at both 6m and 8 m depths within the 2W cage were above the salmon light intensity threshold. This statistically similar level of suppressed melatonin production between the two metal halide groups may have been brought about by a change in the swimming behaviour of fish. Numerous studies have shown that Atlantic salmon are attracted to light and position themselves in the cage with the depth of the

artificial lights (Oppedal *et al.*, 2001; Hevrøy *et al.*, 2003; Juell *et al.*, 2003; Juell and Fosseidengen, 2004). Thus, it is possible that a high proportion of fish in the 2W regime may have continuously shoaled around the lights, thereby remaining within the intensity threshold levels to effectively reduce the melatonin secretion. Moreover, only the 6W treatment produced night-time melatonin levels statistically similar to those produced during the day. In this case, the increased percentage of the cage volume lit above the salmon intensity threshold may have resulted in a decrease in the inter-fish variation in melatonin levels that may be needed to avoid the perceived transition from day to night.

The comparatively lower intensity output from the blue light units may have masked any differences between the colour treatments. Despite the blue emission peak (λ 470 nm) of the metal halide units bearing an identical irradiance level (0.4 W.m^{-2}) to that measured from the LED units at the light source, the green (λ 550 nm) and yellow peaks (λ 600 nm) from the metal halide units emitted a much higher irradiance level (0.6 and 1.0 W.m^{-2} respectively) that may have ultimately affected how this light was perceived by the fish. Bayarri *et al.* (2002) for example, noted that whilst blue light is the most efficient at reducing plasma melatonin, other wavelengths at sufficient intensities were also capable of suppressing circulating levels of melatonin. Furthermore, the pineal window of salmonids, the translucent tissue which directly overlies the pineal gland, has a greater tendency for the transmission of higher wavelengths (Nordtug and Berg, 1990; Migaud *et al.*, 2006a). An ideal solution would have been to ‘cap’ the irradiance levels of all wavelengths produced from the metal halide units to a maximum of 0.4 W.m^{-2} which was emitted by the solitary blue spectral wavelength of the LED unit, possibly through the use of neutral density filters for example. This poor irradiance output may account for the contradiction between the current data and that of earlier studies where blue spectral light was most effective at suppressing the melatonin levels (Bayarri *et al.*, 2002; Migaud *et al.*, unpublished data). However, these studies either involved the *in vitro* culture of the pineal gland or housing experimental fish in small tanks, rather than the commercial production cages used in the present investigation. There were, therefore, marked variations in the culture

environments (*e.g.* enclosed compared to an open environment) which makes it difficult to compare the results between the previous and current studies. Therefore, for a more comprehensive understanding of how the available light is conveyed by the fish, comparative physiological performances such as growth and maturation should be considered.

5.4.2. Growth and Feed Intake

The application of continuous light regimes as a tool for increasing the growth of fish did not appear to be apparent when comparing the monthly growth-performance of the fish (*e.g.* body weights, specific growth rates), contradicting previous studies which have demonstrated the enhanced growth of post-smolt salmon under extended or continuous photoperiod regimes (Saunders and Harmon, 1988; Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Forsberg, 1995; Oppedal *et al.*, 1997, 2003; Porter *et al.*, 1999; Endal *et al.*, 2000; Nordgarden *et al.*, 2003). The initial differences in the body weights and other growth parameters between the various treatment groups confounded the data. This was apparently caused by an overestimation of the biomass and numbers of stock fish in one of the holding pens, resulting in the discrepancy when the treatment pens were split. This highlights the difficulties encountered when conducting scientific studies under commercial conditions, although this should not detract from the significance of this investigation as relatively few studies of this kind exist. Nevertheless, when the relative weight gain for the trial period is considered the metal halide treatments showed a positive increase in weight gain relative to the control group (NP). This would therefore suggest that continuous lights do have an influence on the growth rate. Conversely, fish reared under the blue LED light regimes exhibited a similar weight gain to the controls or, in the case of the 2B treatment a 20% deficit in weight gain. However, it seems highly unlikely that, if the specific light spectrum were to affect growth performances the lower of the intensity groups would exhibit the more severe effects. Moreover, since the blue light units were only shown to illuminate a small percentage of the cage volume above the salmon intensity threshold, one would clearly expect to see similar results to

fish reared under the natural photoperiod. Thus, it would appear that the discrepancy in the weight of fish from the 2B regime may in part be explained by sampling error or a pen effect due to the positioning of the pen in the group. This is particularly evident when reviewing the weight and SGR_w data for the 2B group which clearly showed an oscillatory pattern between monthly samples, whereas all other treatments demonstrated a gradual increase in weight gain. These inconsistencies are commonplace within the farming industry and often obscure treatment effects observed under controlled laboratory conditions. Endal *et al.* (2000) for example, studied the effects of photoperiod regimes on growth and maturation using 90.75 m³ cages, as compared to the 4000m³ cages used in the present trial. In that particular trial, light was provided by means of a 1000 W above cage metal halide unit which could have easily led to a more even distribution of light than that obtainable using a similar light source in production sized cages. Furthermore, the same authors also stocked their cages with equal numbers of fish of similar size, whereas within a commercial environment the variation in size and numbers of fish is not so easily controlled.

The period of exposure to the extended light may have been a significant factor in determining the rate of growth. It has previously been suggested that a period of 12-17 weeks of exposure to constant light is required before any growth deviations would be expected to appear between lit and unlit populations (Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Taylor *et al.*, 2005). In the present study, fish were exposed to constant light for around 18 weeks. This might imply that the fish may still have been adapting to the change in photoperiod and that the growth enhancement may have been more apparent had the fish been exposed to light for a longer period or had further weight measurements been taken. However, due to the occurrence of the grilse grade during June no further measurements were performed since the removal of larger sized fish from the treatment pens would have confounded the data, making the comparison and interpretation of the data impossible.

Throughout the course of the investigation, condition factor of fish from all treatments decreased suggesting a reflection of the seasonal variation in growth. This is

particularly evident between the body weight and condition factor data of the NP and 6B groups, which maintained a similar weight/condition difference for the duration of the trial period. Nonetheless, at the start of the trial the condition of fish maintained under NP was significantly higher than all other treatments, but by the end was similar to all others except the 6B regime. This change is suggested to have occurred through changes in the weight gain (SGR_W) and length gain (SGR_L) of the metal halide lit fish and further reflects their increase in the relative weight gain over control fish over the trial period, *i.e.* greater weight gain than length. However, as previously mentioned the similar condition seen in fish exposed to 2B may well reflect the oscillations in growth, most probably through sampling errors.

The effect of both spectral quality and light intensity on the growth performance of salmonids remains largely unstudied. The work of Stefansson and Hansen (1989) is still the most notable to date regarding spectral content, although in that study no differences were observed. In the present study, the metal halide treatments outperformed the blue lit pens in terms of weight gain. However, if due to lighting conditions, then these differences are most likely explained by the low lit volume of the blue LED treatment pens and the differences in perception of the light. Light intensity on the other hand, has been studied more often although this is somewhat contradictory with no differences between treatments (*e.g.* Stefansson *et al.*, 1993; Oppedal *et al.*, 1999) or improved growth under higher intensities (*e.g.* Cho, 1992b; Oppedal *et al.*, 1997) reported. Within the current investigation, body weights of fish exposed to the metal halide lights were statistically similar at both the start and termination of the trial. However, when the relative weight gain for the period January to June is compared, fish under 6W exhibited an extra 4% weight gain over the 2W regime. This may be of some interest to the industry, although it is important to consider whether the cost gained from this minor increase outweighs the cost associated with purchasing and running the extra four lights units rather than the increased growth gained with two metal halide units. Similarly, the blue light treatments remained significantly different throughout the trial duration, with 2B significantly heavier than the 6B regime. Nonetheless, when the

relative weight gain is considered, fish exposed to 6B gained 20% more weight than fish exposed to 2B. However, this particular result needs to be interpreted with care since, as previously mentioned, this may simply be a result of the sampling error of fish sampled from the 2B pen as demonstrated through higher growth rate fluctuations.

Feed intake

Contrary to the industry-held belief, fish exposed to constant light in the standard metal halide treatments failed to exhibit a characteristic 'growth-dip'. This contradicts previous studies (e.g. Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995, 1999; Endal *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005) where a feeding/growth-dip have been reported. Furthermore, this result is different from the findings observed in Chapter 4, where Atlantic salmon of a similar age, size and strain displayed a trend for a reduced appetite following the onset of artificial light in tanks. However, it must be noted that the light conditions experienced within the tank-systems were generally much higher than the irradiance levels recorded in the lit treatment cages in the current study. Nevertheless, during the first four sampling periods post light-onset, the appetite of fish in the blue lit treatments was found to be significantly lower than the feed intake measured for both the NP and metal halide treatments. This brief depression in appetite may be the result of reversible retinal damage caused by the light, since shorter wavelengths of light (*i.e.* λ 450 nm) are known to be more harmful than longer wavelengths (*i.e.* λ 700-800 nm), with blue light shown to cause damage in the retina of mammals (Dawson *et al.*, 2001). Furthermore, Vihtelic *et al.* (2006) found that high intensities of light induced rod and cone cell death (apoptosis) in adult albino Zebrafish, *Danio rerio*. However, no retinal damage was observed from any of the histological eye samples examined from fish removed during the acute sampling periods, which may possibly be a consequence of a small sample size. It is also possible that the blue light may have invoked a stress response, although studies involving Nile tilapia, *Oreochromis niloticus*, would actually suggest that blue light prevents stress rather than inducing stress responses in fish

(Volpato and Barreto, 2001). The results obtained from Chapter 4 however, would suggest that higher intensities of light, in this case the metal halide treatments, could elicit a stronger stress response. In the present study it was not possible to measure plasma cortisol levels since no special equipment (*i.e.* throw nets) was available at the time of sampling to ensure that fish were removed and blood sampled within the required time without eliciting a stress response from the handling. Therefore, further studies are required to investigate the effects of the spectral properties of light on various other aspects of fish physiology (*e.g.* cortisol response, eye damage) which may affect the welfare and thus the overall growth performance of farmed fish.

One possible theory concerning the ‘growth-dip’ phenomenon reported in commercial practices is that changes in feeding behaviour are wrongly interpreted as a decrease in appetite by the farm workers, and as such the farmer reduces the ration fed. The “growth dip” might therefore be caused by underfeeding. In the present study however, fish were fed to the manufacturer’s recommended feeding guidelines throughout, even when a possible appetite depression by fish exposed to constant light was perceived by the farmer. In this case the duration of feeding was prolonged throughout daylight hours without affecting the quantity of feed presented. Although, no behavioural measurements were made (*e.g.* feed capture time), this observation may suggest that fish take longer to feed rather than ceasing feeding altogether. Thus prolonging the feeding period would allow any ‘stressed’ fish a greater opportunity to feed throughout the day.

Throughout the duration of the trial, feed intake in all groups remained at a consistent level, generally around 3-6 mg.g fish⁻¹. This was unexpected since the appetite of fish is known to reflect the changes in the seasonal photoperiod and temperature (Higgins and Talbot, 1985; Smith *et al.*, 1993; Forsberg, 1995; Blyth *et al.*, 1999), and one would have expected a rise in the feeding level. This may partly be explained by the timing of the sampling period which occurred prior to the majority of fish consuming their daily meal; this was demonstrated at our baseline sample in January when one of the batches of fish sampled during the early morning period were found to

have empty stomachs as compared to fish sampled later on during the day. As a consequence, sampling always occurred once 60% of the daily ration had been fed, and when the rate at which fish were fed had been dramatically reduced. Additionally, a sea lice (*Lepeophtheirus salmonis*) infection noted during February may also have affected feeding levels as has previously been shown (Dawson *et al.*, 1999). However, no data is available on the intensity of the sea lice infestation, other than hearsay, and would otherwise not account for the low level of feeding throughout the trial.

5.4.3. Maturation

The application of artificial light as a means of reducing early maturation was evident in all lit groups, supporting previous studies where continuous artificial illumination successfully inhibited early maturation as compared to fish maintained under ambient conditions (Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Porter *et al.*, 1999; Taranger *et al.*, 1998, 1999; Endal *et al.*, 2000). However, the blue light treatments were not as efficient in reducing the proportion of fish initiating gonadal development as was observed with the metal halide groups. Once again this is expected to relate back to poor efficiency of the LED light units and the subsequent perception (*i.e.* high nocturnal melatonin) by the fish.

Throughout the experimental period, the gonadosomatic index (GSI) for the respective treatments showed no overall increase, although some individual fish were identified as maturing within treatments based on the conditions set out by Endal *et al.* (2000). The GSI for Atlantic salmon has been suggested to only indicate trends in maturation and hence, does not provide an accurate indication of gonadal activity (De Vlaming *et al.*, 1982). Histological examination of gonadal tissue samples clearly identified that fish reared under NP were at more advanced stages of gonadal development, with a greater proportion of females at the late vitellogenic stage and a higher number of males initiating spermatogenesis. In contrast, the application of artificial light generally resulted in atresia in females and fewer males undergoing spermatogenesis. However, the proportion of female fish showing signs of regressed

maturation was greatest under the metal halide units, with fish exposed to blue light and in particular 2B exhibiting signs of vitellogenic oocytes. This was further evident from the examination and measurement of female oocytes where the metal halide treatments were at the primary stages of development, giving a further indication of regressed maturation. However, the number of fish analysed by histological examination was low and only gave a sample assessment of the developmental stage. Further techniques including looking at cell death (apoptosis) and regeneration would give clearer information in characterizing the stage of maturation. Where later stages of maturational development were shown to occur in the lit treatments, such as the onset of spermatogenesis in some of the 6W fish, it is suggested that individuals had initiated and undergone development prior to the onset of light. It has previously been proposed that a 'gating' or 'critical period' exists which controls the timing of sexual development in salmonid fish (Duston and Bromage, 1988; Taranger *et al.*, 1998, 1999; Bromage *et al.*, 2001). This suggests that fish have to be at a minimum or critical physiological state (*e.g.* age, size, growth rate, energy stores and stage of gonadal development) in order for them to respond to photoperiodic cues (Taranger *et al.*, 1999; Bromage *et al.*, 2001). Thus, the timing of the onset of continuous light is therefore vital to ensure that it occurs within the so called window of opportunity, when individuals undertake the decision to initiate maturation (Duston and Bromage, 1988; Bromage *et al.*, 2001). In the current study the decision to switch lights on during January was based upon the consultation of previously published research. Taranger *et al.* (1998) and Endal *et al.* (2000) both found that a greater proportion of fish matured when exposed to constant light earlier or later than when exposed to constant light in January. Furthermore, Endal *et al.* (2000) also demonstrated that a trade-off existed between the growth and maturation performance according to the onset of light with January being the optimal timing.

To further analyse the maturational status of fish reared under the various treatments during the trial period, plasma testosterone levels from individuals were assessed during grading in June. Increases in the levels of plasma sex steroids such as testosterone, is generally associated with gametogenesis and gonadal development and

can be detected up to 3-4 months prior to spawning (Taranger *et al.*, 1998; Oppedal *et al.*, 1999). To determine the level at which fish were deemed as 'hormonally' mature, a threshold value of 3 ng.ml⁻¹ was arbitrarily selected based on the results of Taranger *et al.* (1998). However, in that particular study only the testosterone levels of female fish were analyzed. It is therefore possible that male fish may have a lower or higher threshold than females. Since blood samples were collected from live fish in the current study, it is not known whether the testosterone levels measured were sampled from male or female fish and are as such subject to question. However, it can probably be assumed that the estimation of maturation rates are very safe and that rates are probably higher, as if all fish > 3 ng.ml⁻¹ can be confidently classified as maturing fish, a proportion below this threshold will probably also be maturing. Nonetheless, the fact that fish showed signs of maturation, hormonally, within the top grade is testament to fact that grading serves its purpose in removing grilse from the population. Whilst this decision may be beneficial to fish reared under a natural photoperiod (46% mature), and to some extent under the blue LED lights (~30% mature), analysis of the current results appears to suggest that the removal of larger sized fish from the metal halide treatments (4% mature) results in the removal of non-maturing fish that may be on-grown for a longer period of time if required.

In the present study, the decision to undertake grading was brought about by the farmer's concerns over grilising as determined from a visual assessment of the external characteristics of the fish, *e.g.* skin colour, presence of kype. During the maturational process the carotenoid pigments are redistributed from the flesh to the skin and gonads (Torrissen and Naevdal, 1988; Hatlen *et al.*, 1997; Bjerkeng *et al.*, 2000), resulting in the overall deterioration of the flesh quality and subsequent marketability. However, an unusual occurrence commonly referred to as false maturation or 'dummy run', in which individuals display the external characteristics of maturing fish that have initiated maturation but failed to go through with the process of gonadal development, is widely reported throughout the industry in fish which have been exposed to artificial light. At the time of grading, the farmer was asked to identify and select five fish which they

considered to be maturing based on their normal assessment of external characteristics. Furthermore, from the five fish selected only one exhibited any sign of advanced gonadal development when dissected, although this was based on a visual assessment of the gonad rather than a histological examination. One possibility for this phenomenon might be that the addition of constant artificial light may affect the pigments in the skin. The chromatophores of the skin contain pigments or light-scattering or -reflecting organelles. These pigments consist of melanins, responsible for the dark colouration of fish; the carotenoids which give a yellow to red colouration; pteridines which are similar to the carotenoids; and the purines. It may be that these pigments have a protective role in the skin and combine in response to the increased light conditions brought about by the presence of artificial light to give the individual the appearance of a maturing fish. In immature gilthead seabream, *Sparus aurata*, for example the luminosity of the skin has been shown to increase when exposed to constant light (Ginés *et al.*, 2004), whereas in the Australian snapper, *Pagrus auratus*, increasing the amount of shading from light decreases the redness and increases the luminosity of the skin (Booth *et al.*, 2004). However, the current study was not designed to address this phenomenon and further studies are therefore suggested to examine the effect of constant artificial light application on skin pigmentation of farmed salmon.

For the farmer, maturation of the population can be good in certain cases for subsequent growth boosting effects. Maturing fish display a greater appetite prior to maturation to accumulate the necessary energy reserves to fuel reproduction (Kadri *et al.*, 1996). This results in maturing fish attaining a higher weight gain than immature fish, primarily through the effects of an increase of the anabolic sex steroid hormones. Thus, identifying and removing maturing fish prior to the final stages of maturation is important. However, the growth enhancement and low maturation rates observed under constant light may be the result of a higher percentage of fish displaying atresia, as seen in the metal halide treatments. This potential sex steroid surge in the false maturing fish may explain why the metal halide treatment both grew well and displayed a similar rate of maturation.

The main objective behind studying the effects of photoperiod and lighting characteristics within commercial production systems was that controlling the rates of maturation is an important component of the farming process in avoiding interference with production schedules and the loss of revenue through the downgrading of fish. From the results discussed above, there is a clear indication that continuous light, irrespective of spectrum or intensity, was to some degree able to inhibit the maturational process compared to fish maintained under a natural photoperiod. Furthermore, whilst there was an intensity effect on reproductive development within the blue lit treatments, the metal halide regimes were far superior in reducing the numbers of fish that initiated maturation. This further demonstrates the effectiveness of the metal halide units and the lack of penetration of light from the blue LED units through the cage volume and the subsequent perception of the light by the treatment fish. Whilst it is difficult to compare the different spectral units, based on the poor performance of the blue LED units, what is interesting to note is that the metal halide regimes, irrespective of the intensity, both resulted in an identical ‘hormonal’ maturation rate (4%) with similar observations viewed from the histological examination of the gonad samples. This indicates that the light emitted by the 2W regime was sufficient for altering the physiological responses of fish as was observed under 6W, a hypothesis previously suggested by Oppedal *et al.* (1997). This result therefore has potential implications for energy saving costs within production cages of this size, in terms of the number of lights employed, as well as from the costs saved through the reduced numbers of downgraded fish. Obviously, if LED units are to be implemented within the salmon farming industry, an increase in the energy output and possible shift in the spectral content (blue to blue green) in order for the light to be well perceived.

5.4.4. Flesh quality

Flesh quality characteristics of the final product are an important aspect of the farming process of salmonids. Reducing the variation in pigment content and other quality traits is therefore of great importance to the industry in producing a uniform product which is

acceptable to the processor, retailer and consumer. Of all the features of the flesh, the final colour is by far the most significant quality parameter assessed by the consumer at the point-of-sale. Flesh quality is known to vary seasonally in Atlantic salmon (Mørkøre and Rørvik, 2001; Roth *et al.*, 2005). The use of photoperiod manipulations to alter endogenous rhythms and seasonal growth patterns has also been shown to advance lipid and carotenoid deposition, such that advancing light regimes result in higher lipid and pigment levels in June in parallel with higher condition factors (Oppedal *et al.*, 2006). In the present investigation however, the total pigment content of fish in the NP group was higher than the majority of the lit treatments during April and March. It is possible that this lower pigment level in the constant light treatments may have been the result of poorly growing and/or feeding fish, indicating the possibility of a growth-dip. Although, no differences were observed in feed intake, possibly due to the snapshot feature of feed intake measurements, the chemical composition of the flesh from the uptake of pigment in the diet may prove a better indicator of feeding and growth. Moreover, the initial differences in weights and condition, a factor noted to be related to lipid and carotenoid deposition (Oppedal *et al.*, 2006), may have masked any potential photoperiodic effect on flesh quality. The carotenoid pigment content of the flesh samples for example, was found to show a significantly strong relationship with the wet body weight of the fish (Overall $r^2 = 0.4460$, $P < 0.0001$). This agrees with numerous other studies which have shown that as body weight increases the flesh becomes increasingly pigmented (Torrissen, 1986, 1995; Torrissen and Naevdal, 1988; Forsberg and Guttormsen, 2006). Indeed the order of the body weights at the April sample point for example, corresponded with the order of pigment levels at the same time point (NP > 2B, 2W, 6W > 6B). Thus, the differences observed in the pigment levels of the flesh within the current study are most likely explained by the differences in fish biometry (weight, condition) brought about during the initial set-up of the trial.

Chemical analyses of the flesh, whether it be through high performance liquid chromatography (HPLC) or near-infrared (NIR) spectroscopy as measured in the current investigation, give a measure of the actual levels of carotenoid in the flesh. In contrast,

the preferred colour scoring method employed by the industry gives a measure of the perceived colour of the flesh regardless of whether it is scored by visual assessment or by instrumental analysis. The objective measurement of the flesh colour, as measured instrumentally with the Minolta colorimeter, showed considerable variation between treatments with respect to the lightness (L^*), red/green (a^*) and the yellow/blue (b^*) chromaticity. However, although both the calculated Hue (H°_{ab}) and Chroma (C^*_{ab}) values, which define the intensity and clarity (saturation) of the flesh colour, exhibited minor but nonetheless significant changes during the trial although the overall range of these values remained relatively unchanged. This would therefore indicate that the colour of the fillet was unaffected by light treatment. Nevertheless, from a consumer perspective the visually perceived colour of the fillet is the most important factor when assessing and selecting the final product at the point-of-sale. To that effect, the Roche *SalmoFan*TM is heavily relied upon by farmers, processors and retailers alike in assessing the visual hue redness of the fillet. Visual colour scores recorded in the present trial showed, as with previous studies (e.g. Norris and Cunningham, 1996), that the midline area of the fillet exhibited the highest colour score. This unequal distribution of carotenoid is not unusual in Atlantic salmon since muscle colour has been shown to lighten from the tail to the head, as well as from the midline to the dorsal and ventral regions of the fish (No and Storebakken, 1991; Forsberg and Guttormsen, 2006). Generally, the minimum acceptable colour score set by the industry is around 26 and 28. The measured regions of the fillet flesh from fish of all treatments were above this value range, although samples from fish in the 6W treatment exhibited a greater variation in colour score as well as an overall lower score range as compared to the control treatment. The reason for this variation is unclear, although it is possible that a faster growth rate or a quicker gut passage time may have prevented the deposition of astaxanthin. Johnston *et al.* (2003) found that constant light exposure immediately following the seawater transfer of out-of-season salmon smolts altered muscle recruitment resulting in a higher fibre density and firmer flesh, although Roche colour score was unaffected. However, the effect on normal 'spring' smolts remains unknown. Nonetheless, the wider variation

in the visual colour may influence the consumer preference and cause concern amongst the farmers.

The assessment of flesh colour using both the instrumentally objective and visually subjective methods has its drawbacks. In particular, the visual scoring of flesh involves a high degree of professionalism requiring an individual to score large quantities of samples in an unbiased manner. Furthermore, the perceived colour is highly dependent upon the viewing conditions such as the illuminating source and varies from person to person. Thus, for the present study the same two individual scorers were used throughout the trial, and fillets were measured in a neutral grey coloured light cabinet fitted with a daylight fluorescent light source to allow accurate colour matching. However, the perceived colour may also change depending upon the water and fat content in the flesh as this affects the reflection of colour from the fillet sample (Norris and Cunningham, 2004) and will also be influenced by developmental processes such as maturation.

Maturation is known to affect flesh colour through the migration of carotenoids from the flesh muscle to the skin and gonads (Aksnes *et al.*, 1986; Torrissen and Naevdal, 1988; Hatlen *et al.*, 1997; Bjerkeng *et al.*, 2000). In the present study only two of the fish sampled for flesh characteristics were deemed as maturing, as determined from a gonadosomatic index sex threshold set out by Endal *et al.* (2000). However, these samples were found not to differ from the average treatment values at the respective sampling times and were therefore included in the data sets. Rye and Gjerde (1996) previously demonstrated that maturational status will not influence the flesh colour providing the maturation process had not reached a critical stage, *i.e.* the transfer of flesh carotenoids. Thus, it has been suggested that late-maturing fish will not necessarily result in a more pigmented fish rather a later maturing fish with the same pigmentation level (Norris and Cunningham, 2004).

Lipid and moisture levels in the flesh from all treatments decreased and increased respectively over the trial period, demonstrating a significantly strong relationship (Overall $r^2 = 0.5590$, $P < 0.0001$). Lipid in particular halved over the trial duration,

decreasing from around 12% at the start of the trial in January to 6% in July. These levels were lower than what might be expected for the time of year but were of little concern to the farmer. A decline in the lipid content of the flesh has been suggested to reflect an elevated maintenance requirement or that muscle growth surpassed that of fat accumulation (Mørkøre and Rørvik, 2001). Constant light regimes have been shown to alter muscle recruitment (Johnston *et al.*, 2003, 2004), and advance lipid deposition (Oppedal *et al.*, 2006). Thus, although all treatments including that of the control exhibited a similar pattern it is not known whether the muscle cell recruitment differed. However, flesh lipid levels tend to maximize during early spring and autumn. Thus, it is possible that what was observed was a normal seasonal effect, with the higher summer water temperatures allowing a more efficient burning off of fat during the period of rapid growth (J.G. Bell, personal communication). Further evidence is provided by the condition factor, an individual's energy reserves, which decreased in all treatments over the experimental suggesting a reflection of a seasonal growth pattern. Indeed, body weight demonstrated a weak but significant relationship with the moisture (Overall $r^2 = 0.1180$, $P < 0.0001$) and lipid (Overall $r^2 = 0.1020$, $P < 0.0001$) content of the flesh.

Lipid storage levels have been found to significantly affect the proportion of male-maturing salmon (Shearer and Swanson, 2000). The initial higher lipid levels observed within the NP group may relate to an initial higher growth rate coupled with a higher level of maturing fish. Certainly, the NP group was found to have a higher rate of maturation, as determined both from histological examination of the gonads and hormonally. Furthermore, following the grading process when the majority of maturing fish would have theoretically been removed from the population, all treatments displayed similar lipid levels. This is interesting as it would appear that constant light reduces lipid levels, most possibly through the inhibition of anabolic steroid hormones, which ultimately affect maturation. Nevertheless, further studies are required.

The lipid and moisture content of flesh is also known to differ depending on the region of fillet analysed (Kaitkou *et al.*, 2001). In the present study flesh samples were taken using the 'Norwegian Quality Cut', a standardized muscle section from the rear of

the dorsal fin to the anterior of the anal fin, which may differ from other cuts taken for analysis. Furthermore, both the total pigment and lipid content were analysed using near-infrared (NIR) spectroscopy rather than the conventional solvent extraction method commonly used. Although both are deemed as chemical analysis, the NIR technique is based upon an equation calibrated by the standard chemical analyses. However, the NIR technique has been successfully demonstrated in numerous studies as a useful tool for determining the crude fat content (*e.g.* Downey, 1996; Isaksson *et al.*, 2001; Solberg *et al.*, 2003; Nielsen *et al.*, 2005) and total pigment content (Norris and Cunningham, 2004) in fish. These results therefore show that the addition of constant light, regardless of the spectrum or intensity, has little impact on the flesh quality parameters (*i.e.* colour, lipid and moisture) of Atlantic salmon thus eliminating any concern that the farmer may have over the effect of light and photoperiod.

5.4.5. Summary

The current study was designed to address the effect of lighting characteristics, spectral quality and light intensity, on the reproductive and growth performances of Atlantic salmon. The initial set-up resulted in differences between treatment groups in the biometry of fish which persisted through the trial. This made comparisons on growth much more difficult to compare. However, it must not be forgotten that the trial was specifically designed to investigate light effects at a commercial production level and as such, the results supplement small scale trials which do not reflect the conditions experienced in commercial production systems.

The results clearly demonstrated that the metal halide units were the most effective at enhancing growth and reducing the proportion of maturing fish. This was achieved through a higher penetration of light through the water column, resulting in a greater volume of the cage being lit above the suggested salmon light intensity threshold. Furthermore, the 2W treatment resulted in an identical growth and maturation response as observed under 6W. This not only demonstrates the effect that the 2W had on reducing the melatonin levels below the putative physiological threshold level, but also

demonstrates the effects that the effective positioning of submersible light units can have on fish production, subsequently affecting the associated running costs through a lower number of lighting units.

In contrast, the blue LED light units failed to illuminate any of the cage volume above the salmon light intensity level, and had little effect on the growth and a slight improvement in inhibiting maturation compared to controls. Furthermore, whilst no 'feeding'- or 'growth-dip' was detected in fish reared under the metal halide lights, cautiously dismissing the contested growth dip phenomenon, fish under blue light exhibited a significant reduction in feeding levels for the first four sampling points suggesting a possible stress/welfare issue. However, whilst it is easy to criticize the weakness of the blue lighting systems, it is important to remember that the units used in the present study were prototypes. Of particular interest is the penetration through the water column, which was half that of the metal halide units (3 m and 6 m respectively) for only one eighth the power and one fifth the brightness. Thus, the data provided by this trial must now be used to further develop and modify the prototype lighting systems to one capable of emitting the same intensity of light as the metal halides, but at the fraction of the cost. Furthermore, prior to the introduction of the new technology to commercial farming, the spectral quality of light must be examined on the welfare and physiological growth and reproductive performances of farmed salmon.

Finally, the present study set a precedent within Marine Harvest (Scotland) Ltd. by standardizing the use of lights within commercial cage culture (*i.e.* timing and duration of light and no. units per pen) for the first time. Moreover, Marine Harvest have recently implemented a Standard Operating Procedure (SOP) for the use of photoperiod regimes during the salmon on-growing phase. It is therefore hoped that this will reduce some of the ambiguity surrounding the results when making cross-farm comparisons.

Chapter 6: General Discussion

The overall objective of this research thesis was to further investigate the effects of lights on growth and maturation of salmonid fish and address some of the uncertainties surrounding the use of photoperiod regimes, currently reported within the commercial salmon farming industry. In particular, experiments primarily focussed on the feeding and growth responses following exposure to constant light, to either confirm or refute the disputed growth-dip phenomenon, and also determine the underlying mechanisms (*i.e.* growth axis) governing these responses. However, since light is characterized by its spectral quality, intensity and duration (Sumpter, 1992; Boeuf and Le Bail, 1999), various lighting parameters were applied to a range of rearing systems in order to assess light perception and its subsequent effects on the feeding, growth and maturational responses in fish. This involved the use of new lighting technology specifically developed for this research, designed to meet the light specificity of the fish. Thus, the results from the respective experiments are discussed in relation to Figure 6.1., summarizing the factors investigated throughout this study, with suggestions for further studies.

6.1. Light characteristics and Perception

For light to be an effective factor in the manipulation of physiological responses, it must first be perceived by the individual. Therefore, measurements of the light perception hormone melatonin were used as a biological indicator as to how well light treatments were perceived by the fish. The 24-hour profile performed on Atlantic salmon, *Salmo salar*, reared in tanks (Chapter 4) clearly demonstrated that plasma melatonin levels accurately reflected the prevailing light conditions under which the fish were reared, *i.e.* low levels during the day and high at night or continuously suppressed under constant light exposure. This confirms with previous studies performed in salmonids suggesting that this photoperiodic information may be involved in the physiological timing of functions of fish (Randall *et al.*, 1995; Porter *et al.*, 1998, 2001). Furthermore, the

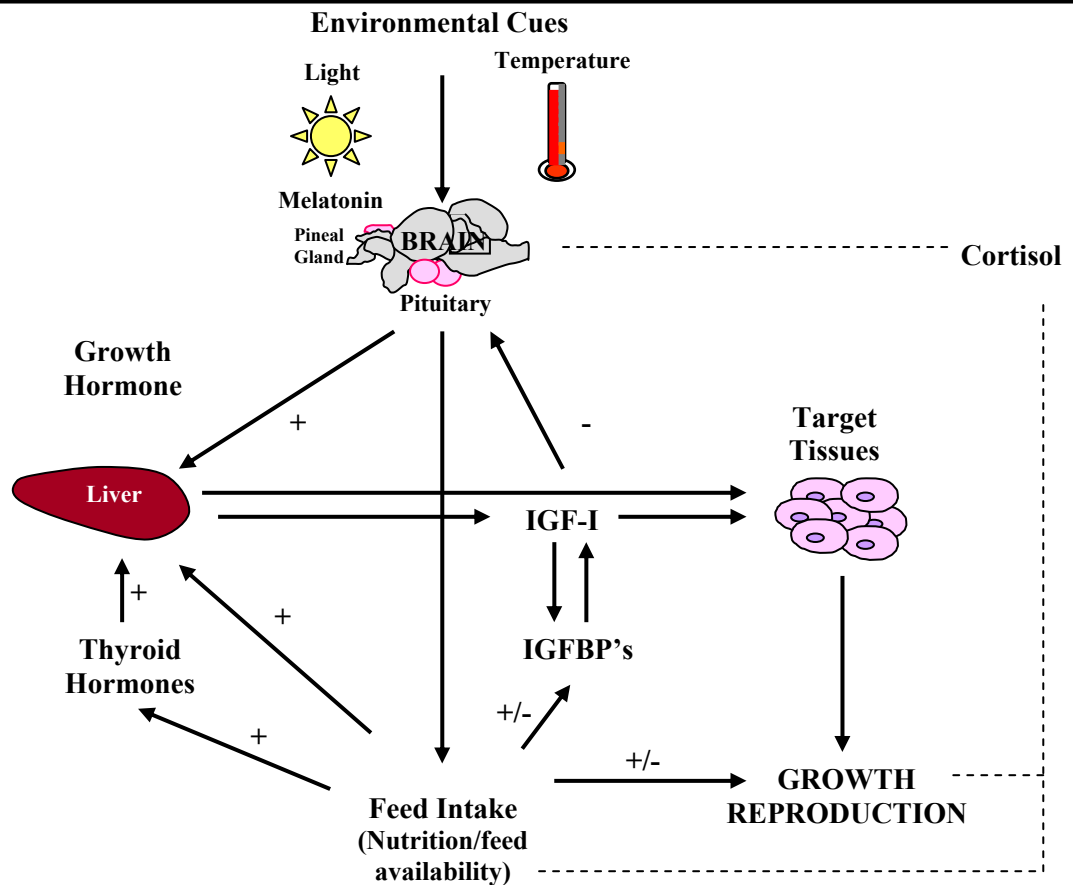


Figure 6.1. Schematic representation of the factors investigated within this research thesis and their interactions on growth and maturation (Figure adapted from Duan, 1997). Dotted line represents a hypothesised pathway.

inclusion of a simulated natural photoperiod within the same study also revealed that the gradual transition in the intensity of light throughout the day, particularly around the times of dawn and dusk, also are reflected in the melatonin profile and may provide an important role in synchronizing daily events. Such knowledge is now becoming increasingly evident within mammalian studies (Usui, 2000; Boulos *et al.*, 2002). Nevertheless, further studies in fish are necessitated to examine the effects of twilight transitions on melatonin production and its subsequent influence on the physiological performance in fish.

One of the major problems encountered within the industry remains the superimposition of artificial light upon the natural light. Metal halide units are the standard source of underwater light units presently used within the farming industry. The

variation in growth and maturation responses which are often obtained in photoperiod manipulated experiments has led researchers to suggest that a light intensity threshold must exist in order for the physiological functions of the fish to be affected (Oppedal *et al.*, 1997; Porter *et al.*, 1999, 2001; Migaud *et al.*, 2006a). However, light intensity failed to have an overall effect on the feeding or growth of rainbow trout (Chapter 3), contrasting a previous reported study (Cho, 1992b). Although the timing of the application of constant light may have limited the intensity effect on growth (see Section 6.3.), the recent hypothesised calculation of the light intensity threshold for Atlantic salmon and European sea bass, *Dicentrarchus labrax* (Migaud *et al.*, 2006a) in addition to the light readings taken from the two intensity treatments indicate that the intensities used within that particular study may have been above the threshold level. Nevertheless, higher intensities have been shown to further enhance growth (Cho, 1992b; Taylor *et al.*, 2006). Thus, it is not only appears to be a threshold but a cumulative effect of intensity on growth (*i.e.* direct photostimulation). This highlights the importance of obtaining light readings from the rearing systems and measuring plasma melatonin levels, ensuring both an adequate distribution of light and also that the light emitted is perceived by the fish.

Because of the way in which light is altered by absorption and scattering processes in the underwater environment, a shift from the focus of light perception to the sensitivity of the pineal gland has occurred. Recent evidence has found that shorter wavelengths of light (*i.e.* blue light λ 450 nm) are more effective at suppressing circulating melatonin levels (Bayarri *et al.*, 2002; Migaud *et al.*, unpublished work). The introduction of new lighting technologies in commercial farming, such as LED or cold cathode, not only represents possible economic savings due to the efficiency of the light sources, but also provides the opportunity for light systems to be designed to the light specificity of the species farmed. Thus, the current designs of LED and cold cathodes units have focussed primarily on the blue and green areas of spectral light since these wavelengths penetrate the marine environment well (Lobban and Harrison, 1994). In the commercial trial described in Chapter 5, both the spectral quality and intensity (no. lights

per pen) of novel blue narrow bandwidth LED lights were tested against the standard metal halide units currently used on production farms. However, the blue light only penetrated half the distance of the metal halide units, although it must be noted that this was for only one eighth of the power (50W versus 400W). Nevertheless, when the percentage volume of the cage estimated to be above the intensity threshold was calculated, the blue lights only covered around 2% of the total cage volume, irrespective of intensity, compared with 25 and 75% from the metal halide sources. This resulted in lower night-time plasma melatonin levels within the metal halide treatments, and as a consequence a small percentage of fish under the metal halide treatments were found to be maturing as compared to ambient counterparts, confirming a previous study by Porter *et al.* (1999). These results therefore suggest that melatonin has a role in the physiological functions of fish, although its exact role remains unknown.

6.2. The Growth-Dip Phenomenon

One of the most contentious issues within the salmon farming industry at present, and that which the research is principally focussed on, is the purported occurrence of a growth-dip following exposure to constant light. Numerous studies have often reported observing such a depression in appetite and growth (Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1999; Endal *et al.*, 2000; Simensen *et al.*, 2000; Mørkøre and Rørvik, 2001; Nordgarden *et al.*, 2003; Oppedal *et al.*, 2003; Fjellidal *et al.*, 2005). However, the present research is the first to date to have specifically investigated this phenomenon in both tank and commercial conditions.

The Atlantic salmon study performed in seawater tanks (Chapter 4) provided the clearest indication of a possible appetite depression. Whilst no significant differences were recorded between the ambient control and constant light groups the apparent trend for a suppressed feed intake is of particular interest to the commercial industry, particularly with respect to accounting for changes in feeding behaviour and managing future feeding regimes. Similarly, all lit groups displayed a transient reduction in the rate of growth as compared to the control group before exhibiting what appeared to be a

compensatory growth response with higher growth rates. Additionally, the inclusion of the simulated natural photoperiod treatment within the same study was of particular interest as, although displaying a similar light-dark cycle to the natural photoperiod, an initial reduction in food consumption and growth rate was also found. The absence of twilight phases (dawn and dusk) within this treatment may have altered the feeding rhythms, since the greatest feeding activity is observed around these times (Higgins and Talbot, 1985; Kadri *et al.*, 1997; Blyth *et al.*, 1999). This was further evident from the melatonin profile performed which showed a direct change from low to high (light to dark) rather than the gradual transition observed under natural light. Moreover, this highlights the possible problems associated with using experimental photoperiods normally applied to laboratory studies which may not always be reflective of the changes that occur under ambient conditions. Nevertheless, further replication of this study is required using tagged individuals and the application of non-invasive techniques to monitor feed intake, such as X-radiography.

The growth depression has been attributed to a possible initial stress reaction following the onset of light (Endal *et al.*, 2000). Indeed, in the same experiment (Chapter 4) a significant chronic elevation of plasma cortisol levels was observed following exposure to constant light, although surprisingly no significant effects on plasma glucose or lysozyme was observed. This is in accordance with the results of Leonardi and Klempau (2003) who also reported a chronic elevation of plasma cortisol levels in rainbow trout following constant light application. However, in the trout experiments (Chapter 3) no depression of appetite was observed, although trout in Experiment I (summer temperature) did display a reduction in growth over the eight week exposure period. This may argue against the case for a growth-dip, but could be explained by a possible species-specific response to the onset of light. McCarthy *et al.*, (1993) for example, found that rainbow trout return to normal feeding conditions much quicker than in Atlantic salmon following a handling stress. For the simulated natural photoperiod group which also displayed a lower feed intake relative to the control, it is suggested that if a stress response had occurred then it may have been acute rather than

chronic and associated with the daily onset to the photophase as previously reported (Rance *et al.*, 1982; Cerdá-Reverter *et al.*, 1998; Reddy and Leatherland, 2003). Rubio *et al.* (2004) demonstrated that the oral administration of melatonin significantly reduced the feed intake in European sea bass, although exogenous administration of melatonin often results in non-physiological levels (Mayer *et al.*, 1997). If light inhibits melatonin, then the onset of constant light would have expected to have increased feeding. Therefore, it is hypothesized that the increases in plasma cortisol levels were directly related to the onset of artificial light. Thus, the depression in appetite and growth suggest that fish must acclimate to their new rearing conditions as with other situations when an environmental manipulation have been applied, *e.g.* the seawater transfer of smolts (Usher *et al.*, 1991; McCarthy *et al.*, 1996; Stead *et al.*, 1996; Arnesen *et al.*, 1998; Damsgård and Arnesen, 1998). These results therefore appear to confirm an acute adverse effect of light on the feed intake of Atlantic salmon. Since feeding is typically based on manufacturers feeding tables, determined from calculations of growth rates (Austreng *et al.*, 1987) or energy requirements (Cho, 1992a), particular attention may be needed with respect to the use of photoperiod manipulations to take into account the possible altered feeding behaviours of the fish. Nevertheless, further studies are still needed to corroborate this study.

Under commercial conditions (Chapter 5) however, there was no compelling evidence to suggest a growth-dip. Irrespective of the number of units per pen, the metal halide treatments, which are normally applied within the industry, demonstrated a similar pattern of feed intake to the natural photoperiod group. However, it was observed that fish reared under constant light appeared to be eating more slowly, thus feeding regimes were prolonged to allow fish to feed over a longer period. Unfortunately, this is only based upon observations and no monitoring of feeding response time was assessed. It is therefore speculated, that whilst this growth-dip may in part be a physiological response to the onset of constant artificial light it is more likely to be exaggerated by the farmers decision to restrict feed at the first observational sign of a reduced appetite or slower reaction to food delivery. Nevertheless, fish exposed to the blue LED narrow bandwidth

light exhibited a significantly lower feed intake over the initial sampling points than the control. It can be hypothesized that this brief depression in appetite may be the result of reversible retinal damage caused by the lighting units. Vihtelic *et al.* (2006) for instance found that high intensities of light induced rod and cone cell death (apoptosis) in adult albino Zebrafish, *Danio rerio*. Furthermore, shorter wavelengths of light (*i.e.* λ 450 nm) are known to be much more harmful than longer wavelengths (*i.e.* λ 700-800 nm), with blue light shown to cause damage in the retina of mammals (Dawson *et al.*, 2001). Since salmon are behaviourally attracted to light (Oppedal *et al.*, 2001; Juell *et al.*, 2003; Juell and Fosseidengen, 2004), further investigations on the effects of artificial light (blue light) exposure on eye damage (*e.g.* histological examination of retina) together with other welfare parameters (*e.g.* cortisol) should be conducted before the new lighting technologies are introduced into the industry.

6.3. Light and Growth

The effects of light on growth have mainly focussed around the various life-stages of the Atlantic salmon. However, developmental processes such as smolting and maturation which often occur in relation to the timing of the onset of light can often be misleading, questioning whether light directly stimulates growth or whether it is the consequence of the manipulated physiological response. Thus, in Chapter 3 juvenile rainbow trout were used as a model species for growth assessment. Application of constant light, regardless of temperature, failed to have an enhancing effect on growth. Furthermore, in Experiment I fish exposed to constant light exhibited a lower weight gain than those reared under a short-day, whereas in Experiment II no such growth patterns were observed. Furthermore, plasma levels of growth hormone, the somatotropic hormone best documented for increasing growth (Björnsson, 1997), exhibited a similar pattern between treatments, contradicting previous studies where constant light exposure enhanced the growth of trout (Cho, 1992b; Taylor *et al.*, 2005, 2006). It is suggested that either the timing of the exposure to light or the change in the ambient water temperature may have affected the growth outcome. Temperature has been shown to

limit the effects of photoperiod manipulations in salmonid fish (Solbakken *et al.*, 1994; McCormick *et al.*, 2000). Thus, the lack of growth responses in rainbow trout to the photoperiod manipulation may have arisen from the ambient water temperature overriding the potential of any possible photoperiodic effects. This gives a further indication that photoperiod may have species-specific effects. Similarly, light intensity also failed to have any effect on the growth of rainbow trout, which once again may be explained by the timing of light onset or the role of temperature in modulating the growth response. However, as previously stated it is likely that the intensities used were above the light intensity threshold for trout, if similar as in salmon.

Despite an initial appetite and growth rate suppression, constant light exposure had little effect on the growth of salmon reared in seawater tanks as compared to fish under ambient light (Chapter 4), although the rearing environment significantly affected growth. The later onset of constant light may have meant that fish had already perceived the change in daylength, as suggested in a previous study performed on coho salmon, *Oncorhynchus kisutch* (McCormick *et al.*, 1992). However, a longer exposure time may also have been required before growth deviations became evident. Taylor *et al.* (2006) found that the date of onset of light affected the time taken for growth deviations to become apparent, although a strain effect was also evident. Fish were exposed to constant light for 20 weeks and despite no growth differences being observed, growth rates were higher in the constant light treatments after 12 weeks exposure than in the control. Thus, timing the onset of light appears to be critical in achieving maximal growth rates.

Of particular interest was that growth performance appeared to be significantly affected by the type of rearing regime applied (Chapter 4). Fish reared under an open system, where access to the natural light was permitted regardless of whether light was applied, demonstrated greater wet body weights than fish reared in an enclosed system where the natural light was kept out. This suggests that fish either took longer to adapt to the changed rearing conditions or that the constant light intensity provided by the light source were having an adverse affect on the physiological growth performance of the

fish. Moreover, these findings raise questions about the possible extent that laboratory studies using artificial photoperiods can be generalized to commercial conditions. Thus, a further understanding of rearing regimes is required.

In addition to elevating plasma cortisol levels, constant light exposure also appeared to have a direct influence on the growth hormone axis (Chapter 4). Both the circulating levels of growth hormone and free thyroid hormone levels were significantly elevated following constant light exposure. These increases may have a role in regulating the physiological response to environmental stressors and could be involved in maintaining and restoring homeostasis. Growth hormone in particular has been shown to affect the behavioural activities of fish, increasing the competitive ability (Johnsson and Björnsson, 1994; Jönsson *et al.*, 1998). However, no role for the GH-IGF-I axis could be concluded from the study most likely due to the multifunctional nature of the hormone and the various physiological states of the individually sampled fish. Nevertheless, these findings were preliminary and it is suggested that further work should be performed at investigating the acute effects of light onset on the circadian hormonal profiles, together with a greater emphasis at the molecular level with regards to binding and receptor sites.

The initial differences in the weights of fish reared in commercial sea cages (Chapter 5) highlights the difficulties associated with trying to perform experimental studies in a commercial practice. Nevertheless, the study is the most relevant to the industry since it is representative of the conditions normally experienced on salmon farms, and further supplements the small scale trials (pilot studies) which are not always reflective of intensive commercial production systems. To deal with the weight differences, the relative weight gain for the trial period was used. This revealed that fish exposed to constant light emitted by the metal halide units experienced a higher weight gain (~ 4-10%) than fish maintained under a natural photoperiod giving further support to previous studies where light enhanced growth (Saunders and Harmon, 1988; Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Forsberg, 1995; Oppedal *et al.*, 1997, 2003; Porter *et al.*, 1999; Endal *et al.*, 2000; Nordgarden *et al.*, 2003). However, no such gain was seen for fish reared under the blue spectral light units. The differences in the spectral quality of

light on growth are probably explained by the low lit volume of the blue LED treatment pens and the differences that occurred in the perception of light. Thus, if blue lights are to be effective then higher energy units should be tested.

The flesh quality characteristics of the final product are an important aspect of the farming process of salmonids. Light exposure, irrespective of intensity or spectrum, had little effect on the flesh quality measured instrumentally, with differences which did occur found to relate to the weight of the fish. However, fish reared under the highest intensity of light of the standard metal halide group exhibited a greater variation in the visually subjective colour, as measured by the Roche *SalmoFan*TM score. Whilst this variation remains unclear the colour as perceived by the farmer, retailer and consumer alike may cause concern. Furthermore, since constant light exposure has been shown to alter muscle recruitment in fish (Johnston *et al.*, 2003, 2004), further studies should be conducted on the use of lights on muscle recruitment and the effects on flesh quality.

6.4. Light and Maturation

Photoperiod regimes are primarily used during the on-growing stage of the Atlantic salmon industry as a means of inhibiting early maturation. The commercial study detailed in Chapter 5 examined different light characteristics on the growth and maturation performance of salmon reared in sea cages. Although the highest percentage of maturing fish was found amongst the largest size of fish, a testament to the current grading practices used within the industry, exposure to constant light appeared to effectively reduce the numbers of fish maturing when assessed hormonally. However, fish exposed to the novel blue LED narrow bandwidth lighting units showed higher rates of maturation than fish reared under the standard metal halide units currently used on production sites, although rates were still lower under blue light than ambient. This was related back to the poor efficacy of the light from the blue LED units through the water column, resulting in a limited volume of the cage being lit above the intensity threshold level for salmon. This was further confirmed by the poor perception of light through higher nocturnal plasma melatonin levels. Nevertheless, these findings support those of

Porter *et al.* (1999) where additional night-time illumination suppressed plasma melatonin levels and resulted in a reduction of grilse. This would suggest that melatonin has a role in controlling maturation development in salmon, although this is dependent upon the light intensity threshold being attained.

Interestingly, fish exposed to the metal halide units, irrespective of intensity, exhibited a similar grilse rate. This demonstrates that the distribution of the light within the cage rather than the intensity of light is itself important in ensuring as much as the cage volume is lit above the intensity threshold for salmon. These findings could be of significant importance to the industry as it may allow decisions to be made regarding the deployment of lights (*i.e.* the number of units to use to have the required effect), thereby providing economic gains from the energy cost associated with the running of lights. Furthermore, since the trial, Marine Harvest (Scotland) Ltd. have now produced a standard operating procedure regarding the use of underwater lights to control maturation in sea cages which has now been implemented across all farm sites. This has set a precedent within the industry by standardizing light application which aims to reduce some of the variation and ambiguous results often associated with the individual farms

One further area for investigation is the ‘dummy run’ or false maturation commonly observed in fish populations exposed to constant light. Individuals typically display the external characteristics of maturing fish without undergoing the latter stages of gonadal development. Histological examination of gonads from fish exposed to constant light showed signs of atresia, indicating that fish had initiated maturation before halting the process part way through. This was further clarified from the oocyte stage of development from female fish, showing that individuals maintained under a natural photoperiod were at a more advanced stage as compared to those exposed to constant light. Throughout maturation, the carotenoid pigments are redistributed from the flesh to the skin and gonads (Torrissen and Naevdal, 1988; Hatlen *et al.*, 1997; Bjerkeng *et al.*, 2000) resulting in an overall deterioration of the flesh quality and subsequent marketability. One possibility for the dummy run may be that the addition of light may affect the pigments in the skin. Whilst no differences were observed in the flesh quality

parameters, which could not be attributed to weight, the visually subjective colour score of the flesh showed the greatest variation under constant light. However, further examination of the false maturation phenomenon and the effects of artificial light should be further performed.

6.5. Summary

This research thesis has dealt with the effects of light on the feeding and growth responses in fish, and relating results to improving the use of photoperiod manipulations within the commercial salmon farming industry. The effectiveness of artificial light regimes appears to be wholly dependent upon the efficacy of the light source through the underwater environment and its perception by the individual. Thus, the intensity or distribution of light throughout the rearing system must be sufficient to exceed the physiological threshold level for the species cultured. In addition, whilst the spectral quality of light appeared at first to have little effect on the growth and maturational performance of farmed Atlantic salmon it should be noted that the units were prototypes of a new lighting technology. Furthermore, the potential of the new light sources was evident in that they penetrated half the distance of the water column as compared to the conventional standard metal halide units which was achieved through one eighth of the power supply. Thus, further studies which investigate the effects of new lighting technologies including cold cathode units should first be tested for their effectiveness in penetrating the water column.

Although there was no overwhelming evidence to support the purported growth-dip phenomenon under commercial conditions, it does appear that the onset of artificial light results in an initial chronic stress response. Furthermore, changes to the somatotropic and thyroid axes following exposure to constant light may indicate some form of compensatory or adaptive mechanism. However, further studies specifically focussing on the changes in hormone levels to photoperiod regimes should be performed with respect to the diel rhythm to elucidate whether changes in hormone levels are increased or phase-shifted due to photoperiod. Whilst a trend for a suppressed appetite

may be observed, the growth and appetite suppression reported within the industry may be a combination of a physiological response to the onset of artificial light further exaggerated by the farmer's perception of the dip and their decisions in restricting feed. Nevertheless, this still raises questions concerning the potential stress and welfare related issues to photoperiod manipulations. Further studies using non-invasive techniques such as X-radiography should be performed using a combination of experimental pilot studies together with commercial scale trials to fully explore the possible effects of photoperiod applications. Overall, it is still necessary to investigate the acute changes at the endocrine and molecular levels following the onset of light exposure to fully examine and understand the underlying mechanisms governing growth and maturation in fish.

Finally, the research conducted within this thesis was primarily designed to help establish a standardized use of light regimes throughout the industry. The successful application of constant light exposure on reducing the percentage of early maturing fish in cages has helped Marine Harvest (Scotland) Ltd. to create a standard operation procedure regarding the timing and number of lights for application of artificial photoperiod regimes in commercial farming practices.

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AMERLEX-MAB* FT3 Kit

FOR INFORMATION USE ONLY
Not to be used for performing the assay.
Refer to insert accompanying kit

Intended Use

For the *in vitro* quantitative measurement of free triiodothyronine (FT3) in human serum and plasma (EDTA or heparin) to aid in the differential diagnosis of thyroid disease. Measurement range 0 to 40 pmol FT3/l.

Summary and Explanation of the Test

The free fraction of circulating triiodothyronine (T3) is considered to exert the main influence on metabolic control. In hyperthyroidism the FT3 concentration is generally elevated and gives efficient discrimination at the euthyroid/toxic borderline, providing an effective method for the confirmation of hyperthyroidism and monitoring of its treatment. In hypothyroidism FT3 concentrations tend to be lower, although the decrease is insufficient to give clear diagnostic information⁽¹⁻⁴⁾. FT3 concentrations are independent of the concentration of binding proteins (mainly TBG and albumin). Measurement of FT3 may thus be carried out on patients with reduced or elevated levels of these binding proteins⁽⁵⁾. FT3 determinations should be used as part of a thyroid test strategy, which may include free thyroxine (FT4) and high sensitivity TSH immunoassay.

Principles of the Procedure

The AMERLEX-MAB* FT3 Kit utilizes a direct, labelled antibody, competitive radioimmunoassay technique. FT3 present in the sample competes with a separation suspension for a limited number of binding sites on an ¹²⁵I-labelled mouse monoclonal anti-T3 antibody. The separation suspension, present in excess, contains magnetizable polymer particles chemically modified to act as a ligand for uncombined tracer. The assay design, together with optimal reagent concentrations, ensures minimal disturbance of the T3/binding protein equilibrium in the sample. Separation of the antibody-bound fraction is effected by magnetic separation, followed by decanting of the supernatant. The amount of tracer bound is inversely proportional to the concentration of FT3 present.

Warnings and Precautions

For *In Vitro* Diagnostic Use Only

Caution - Radioactive Material

This radioactive material may be received, acquired, possessed and used only by authorized persons in clinical laboratories or hospitals and only for *in vitro* clinical or laboratory tests not involving internal or external administration of the material or the radiation therefrom to humans or animals. Its receipt, acquisition, possession, use, transfer and disposal are subject to the regulations and a general licence of Atomic Energy Agencies or of the state/national body responsible for the exercise of such regulatory authority.

Warning - Potentially Infectious Material
Human blood products provided as components of this pack have been obtained from donors who were tested individually and who were found to be negative for human immunodeficiency virus (HIV-1) antibody and hepatitis B surface antigen using approved methods (enzyme immunoassays). These components have also been tested using approved methods (enzyme immunoassay) and found to be negative for Hepatitis C Virus (HCV) antibody and HIV-2 antibody.

Care should be taken when handling material of human origin. All samples should be considered potentially infectious. No test method can offer complete assurance that hepatitis B virus, HCV, HIV 1+2 and other infectious agents are absent. Handling of samples and assay components, their use, storage and disposal should be in accordance with the procedures defined by the appropriate national biohazard safety guideline or regulation.

Warning - Contains Azide

Some components contain sodium azide (0.1% w/v). The total azide content is 112 mg (100 tests) or 448 mg (400 tests). Use copious amounts of water for disposal.

Materials Provided

- 1/2[†] sets FT3 standards (freeze-dried human serum, nominal values A-F: 0, 2, 5, 10, 20, 40 pmol/l, exact values stated on standard value label) with Antimicrobial Agent. Reconstitution volume 1 ml.
 - 1/4[†] bottles AMERLEX-MAB* separation suspension (magnetizable polymer-ligand particle suspension) in buffer with Antimicrobial Agent (55 ml).
 - 1/4[†] bottles tracer (¹²⁵I-labelled mouse monoclonal anti-T3, <370 kBq/bottle, binds ≥7.13 pmol T3/bottle) in buffer with Antimicrobial Agent (55 ml).
 - Package insert.
- [†] 100/400 Tests.

Note: Contains bovine serum albumin, bovine gamma globulin and bovine gelatin.

Materials Required but not Provided
Precision pipettes, repeating dispenser (optional), control sera, 5 ml assay tubes and racks, plastic or metal film (to cover tubes), vortex mixer, water bath, AMERLEX-M separators, gamma scintillation counter, distilled water.

Reagent Preparation and Storage

Do not use reagents beyond the expiration date stated on the package label. Store unopened reagents at 2-8 °C. Do not freeze.

Standards: reconstitute with 1 ml distilled water. Store at 2-8 °C for up to 2 weeks, or at -20 °C for 4 weeks.

All other reagents are supplied ready for use, store at 2-8 °C after opening.

Sample Collection, Preparation and Storage

Serum or plasma (EDTA or heparin) samples may be used. Store samples at 2-8 °C for up to 7 days or at -20 °C for up to 4 weeks. Avoid repeated freezing and thawing.

Quality Control and Procedural Notes

1. Reagents from the same lot number may be combined in a clean glass vessel. Samples and reagents should be brought to 18-28°C and thoroughly mixed (avoiding excessive foaming) before use. All reagents should be dispensed without interruption. The dispensing times of the AMERLEX-MAB* separation suspension and tracer should not exceed 15 and 5 minutes respectively. Dispense the tracer immediately after completing dispensing of the separation suspension.
2. Run a separate standard curve, in duplicate, for each assay. Controls and samples should be assayed in duplicate. Good laboratory practice requires that controls be run to verify the performance of the assay. Radioassay controls with high medium and low levels of FT3 should be run.
3. Use of a 37 °C dry air incubator is not recommended. Determination of total counts is optional. There is no requirement for non-specific binding determination in this assay. Ensure all tubes contact the separator base during separation. Do not remove rack from separator base when decanting and draining tubes. Do not reinvert drained tubes once they have been turned upright.

Test Protocol

Procedure
1. Assemble and label assay tubes.
2. Pipette 50 µl standard, control or sample into appropriate tubes.
3. Dispense 500 µl AMERLEX-MAB* separation suspension into all tubes (except total count tubes).
4. Dispense 500 µl tracer into all tubes. Set aside total count tubes (optional).
5. Vortex, cover and incubate at 37 °C for 30 minutes.
6. Attach the rack to the separator base, leave for 15 minutes. Decant and drain for 5 minutes with blotting.
7. Count tubes so as to accumulate at least 2 000 counts in the F standard tubes.

Results

Plot standard curve, either manually or using an RIA curve fit programme. Results may be calculated using logit-log or linear plotting.

Logit-log Plotting

Correct counts for background (if measured). Calculate percentage bound (%B/Bo) relative to the zero standard mean (Bo) for each standard and unknown (B) i.e. (B/Bo) x 100. Plot %B/Bo against standard concentration on logit-log graph paper. Draw the best straight line through the mean of

duplicate points, rejecting grossly aberrant counts. Read the concentration of the unknowns from the standard curve.

Linear-linear Plotting

Plot standard counts (corrected for background if required) against concentration on linear graph paper. Draw the best curve through the mean of duplicate points, rejecting grossly aberrant counts. Read the concentration of the unknowns from the standard curve.

Tube Number	Sample	Standard Conc	Counts/min X1	Counts/min X2	%B/Bo X1	%B/Bo X2	Mean FT3 Conc
1-2	Totals		97588	97760			
3-4	Std A	0	26600	26776	27.23	27.41	
5-6	Std B	1.9	24000	24236	24.57	24.82	
7-8	Std C	5.2	20026	20036	20.50	20.52	
9-10	Std D	12.9	13900	13942	14.23	14.27	
11-12	Std E	22.5	9800	9854	10.03	10.09	
13-14	Std F	47.7	5640	5672	5.77	5.81	
15-16	U1*		22850	22900	23.39	23.45	2.81
17-18	U2		19600	19700	20.07	20.17	5.62
19-20	U3		13150	13250	13.46	13.57	14.14

* Unknown

Limitations of the Procedure

1. The results obtained from this or any other diagnostic kit should be used and interpreted only in the context of an overall clinical picture.
2. Lipemic, haemolyzed and icteric samples may be used up to levels of 34 mmol/l triolein, 10 g/l haemoglobin and 0.2 mmol/l bilirubin. Do not use turbid samples.
3. Heat treated samples give elevated FT3 values due to protein denaturation and disturbance of the FT3/T3 equilibrium. In highly processed sera, e.g. some quality control material, the thyroid binding capacity may be reduced. In such sera the AMERLEX-MAB* FT3 value may be elevated in line with theoretical prediction.
4. Heterophilic antibodies in serum or plasma samples may cause interference in immunoassays⁽⁶⁾. These antibodies may be present in blood samples from individuals regularly exposed to animals or who have been treated with animal serum products. Results which are inconsistent with clinical observations indicate the need for additional testing.

Expected Values

It is recommended that each laboratory establish its own reference interval. As a guide, a normal reference interval of 3.4 to 7.2 pmol FT3/l with a median of 5.1 pmol/l was obtained from 1236 patients of euthyroid status, not on thyroid treatment (untransformed 1st and 99th percentiles). Of 262 hyperthyroid patients tested 89% had FT3 concentrations >7.2 pmol/l. Samples from 43 women in the third trimester of pregnancy showed an observed FT3 range of 4.3 to 6.1 pmol/l.

Performance Characteristics

1. Calibration

The assay was calibrated against a reference FT3 assay using patient samples. A correlation between the AMERLEX-MAB* FT3 Kit and an FDA cleared assay has been obtained by measuring a panel of 564 patient samples from a variety of clinical categories. The range of samples used covered 1.71 to 37.86 pmol/l in the

AMERLEX Assay and 0.48 to 37.64 pmol/l in the FDA cleared assay:
 AMERLEX-MAB* FT3 Kit = 1.00 x FDA cleared assay + 0.13 (pmol/l), with a correlation coefficient of 0.944.

FT3 concentrations are quoted in units of pmol/l or pg/ml. Conversion of units may be made using the formula:

Result in pg FT3/ml = result in pmol FT3/l x 0.651

2. Reproducibility

Three freeze-dried control sera were assayed in 10 replicates to determine within-assay reproducibility. The between-assay data presented are representative of the performance found over the shelf life of the product.

Within-assay		Between-assay	
Mean	CV(%)	Mean	CV(%)
2.92	5.8	2.75	9.8
5.93	4.0	5.60	6.4
13.8	3.5	13.8	6.5

3. Sensitivity

Sensitivity is defined as the concentration 2 standard deviations from the zero standard when 20 replicates are determined. The sensitivity of the AMERLEX-MAB* Free T3 Kit is typically 0.7 pmol/l.

4. Specificity

The percentage cross-reactivity on a molar basis was <0.7% for L-thyroxine, <0.42% for L-3,3',5'-triiodothyronine and <35% for L-3,3',5'-triiodothyroacetic acid. Cross-reactivity of <0.01% was exhibited for 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, Diphenylhydantoin, phenylbutazone, sodium salicylate and o-acetylsalicylic acid.

Note: Bio-Rad controls, Catalogue number 370, are commercially available to use as controls for this product. Please contact Trinity Biotech Technical Services at (353) 1 276 9800, for expected values.

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RISK AND SAFETY



Sodium azide

- R22: Harmful if swallowed.
- R32: Contact with acids liberates very toxic gas.
- S36: Wear suitable protective clothing.

Xn

Prepared in accordance with requirements for EEC label.

EINECS 247-852-1

Key Guide to Symbols



Lot



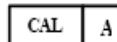
Use by



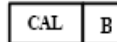
For *In Vitro* Diagnostic Use



Caution, consult accompanying documents



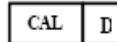
Calibrator A



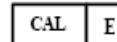
Calibrator B



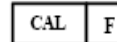
Calibrator C



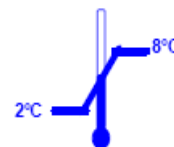
Calibrator D



Calibrator E



Calibrator F



Store at 2-8°C

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 IM.5104 (400 Tests)

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 Web: www.trinitybiotech.com

242-005 02/02



AMERLEX-MAB* FT4 Kit

FOR INFORMATION USE ONLY
Not to be used for performing the assay.
Refer to insert accompanying kit

Intended Use

For the *in vitro* quantitative measurement of free thyroxine (FT4) in human serum and plasma (EDTA or heparin) to aid in the differential diagnosis of thyroid disease. Measurement range 0 to 130 pmol FT4/l.

Summary and Explanation of the Test

The free fraction of circulating thyroxine (T4) is considered to exert the main influence on metabolic control⁽¹⁻³⁾. Consequently, measurement of FT4 is believed to be the most direct indicator of the thyroid status of an individual. In hypothyroidism FT4 concentration is generally depressed and in hyperthyroidism it is generally raised. FT4 measurement thus provides an aid to the differential diagnosis of thyroid disease. FT4 concentrations are independent of the concentration of thyroid hormone binding proteins⁽⁴⁾. Measurement of FT4 may thus be carried out on patients with elevated or reduced levels of these binding proteins⁽⁵⁻⁶⁾ without the need for additional tests of binding capacity. In borderline cases of suspected thyroid malfunction, additional tests such as free T3, or TSH immunoassay may be necessary.

Principles of the Procedure

The AMERLEX-MAB* FT4 Kit utilizes a direct, labelled antibody, competitive radioimmunoassay technique. FT4 present in the sample competes with a separation suspension for a limited number of binding sites on an ¹²⁵I-labelled mouse monoclonal anti-T4 antibody. The separation suspension, present in excess, contains magnetizable polymer particles chemically modified to act as a ligand for uncombined tracer antibody. The assay design, together with optimal reagent concentrations, ensures minimal disturbance of the T4/binding protein equilibrium in the sample. Separation of the antibody-bound fraction is effected by magnetic separation, followed by decanting of the supernatant. The amount of tracer bound is inversely proportional to the concentration of FT4 present.

Warnings and Precautions

For *In Vitro* Diagnostic Use Only

Caution - Radioactive Material

This radioactive material may be received, acquired, possessed and used only by authorized persons in clinical laboratories or hospitals and only for *in vitro* clinical or laboratory tests not involving internal or external administration of the material or the radiation therefrom to humans or animals. Its receipt, acquisition, possession, use, transfer and disposal are subject to the regulations and a general licence of Atomic Energy Agencies or of the state/national body responsible for the exercise of such regulatory authority.

Warning - Potentially Infectious Material

Human blood products provided as components of this pack have been obtained from donors who were tested individually

and who were found to be negative for human immunodeficiency virus (HIV-1) antibody and hepatitis B surface antigen using approved methods (enzyme immunoassays). These components have also been tested using approved methods (enzyme immunoassay) and found to be negative for Hepatitis C Virus (HCV) antibody and HIV-2 antibody.

Care should be taken when handling material of human origin. All samples should be considered potentially infectious. No test method can offer complete assurance that hepatitis B virus, HCV, HIV 1+2 and other infectious agents are absent. Handling of samples and assay components, their use, storage and disposal should be in accordance with the procedures defined by the appropriate national biohazard safety guideline or regulation.

Warning - Contains Azide

Some components contain sodium azide (0.1% w/v). The total azide content is 110 mg (100 tests) or 440 mg (400 tests). Use copious amounts of water for disposal.

Materials Provided

- 1/2* sets FT4 standards (freeze-dried human serum, nominal values A-F: 0, 2.5, 10, 25, 60, 130 pmol/l, exact values stated on standard value label) with Antimicrobial Agent. Reconstitution volume 1 ml.
- 1/4* bottles AMERLEX-MAB* separation suspension (magnetizable polymer-ligand particle suspension) in buffer with Antimicrobial Agent (55 ml).
- 1/4* bottles tracer (¹²⁵I-labelled mouse monoclonal anti-T4, <370 kBq/bottle, binds >5 fmol T4/bottle) in buffer with Antimicrobial Agent (55 ml).
- Package insert.

* 100/400 Tests.

Note: Contains bovine serum albumin and bovine gamma globulin.

Materials Required but not Provided

Precision pipettes, repeating dispenser (optional), control sera, 5 ml assay tubes and racks, plastic or metal film (to cover tubes), vortex mixer, water bath, AMERLEX-M separators, decanting racks, gamma scintillation counter, distilled water.

Reagent Preparation and Storage

Do not use reagents beyond the expiration date stated on the package label. Store unopened reagents at 2-8 °C. Do not freeze.

Standards: reconstitute with 1 ml distilled water. Store at 2-8 °C for up to 4 weeks.

All other reagents are supplied ready for use, store at 2-8 °C after opening.

Sample Collection, Preparation and Storage

Serum or plasma (EDTA or heparin) samples may be used. Store samples at 2-8 °C for up to 7 days or at -20 °C for up to 3 weeks. Avoid repeated freezing and thawing.

Quality Control and Procedural Notes

1. Reagents from the same lot number may be combined in a clean glass vessel. Samples and reagents should be brought to 18-28 °C and thoroughly mixed (avoiding excessive foaming) before use. All reagents should be dispensed without interruption. The dispensing times of the AMERLEX-MAB* separation suspension and tracer should not exceed 10 and 5 minutes respectively. Dispense the tracer immediately after completing dispensing of the separation suspension.
2. Run a separate standard curve, in duplicate, for each assay. Controls and samples should be assayed in duplicate. Good laboratory practice requires that controls be run to verify the performance of the assay. Radioassay controls with high medium and low levels of FT4 should be run.
3. Ensure all tubes contact the separator base during separation. Do not remove rack from separator base when decanting and draining tubes. Do not reinvert drained tubes once they have been turned upright.

Test Protocol

Procedure

1. Assemble and label assay tubes.
2. Pipette 50 µl standard, control or sample into appropriate tubes.
3. Dispense 500 µl AMERLEX-MAB* separation suspension into all tubes.
4. Dispense 500 µl tracer into tubes.
5. Vortex, cover and incubate at 37 °C for 30 minutes.
6. Attach the rack to the separator base, leave for 15 minutes. Decant and drain for 5 minutes with blotting.
7. Count tubes so as to accumulate at least 2 000 counts in the F standard tubes.

Results

Plot standard curve, either manually or using an RIA curve fit programme. Results may be calculated using logit-log or linear plotting.

Linear-linear Plotting

Plot standard counts (corrected for background if required) against concentration on linear graph paper. Draw the best curve through the mean of duplicate points, rejecting grossly aberrant counts. Read the concentration of the unknowns from the standard curve.

Logit-log Plotting

Correct counts for background (if measured) then calculate percentage bound (%B/Bo) relative to the zero standard mean (Bo) for each standard and unknown (B) i.e. (B/Bo) x 100. Plot %B/Bo against standard concentration on logit-log graph paper. Draw the best straight line through the mean of duplicate points, rejecting grossly aberrant counts. Read the concentration of the unknowns from the standard curve.

Tube Number	Sample	Standard Conc.	Counts/min X1	Counts/min X2	Mean FT4 Conc.
1-2	Totals		110092	113598	
3-4	Std A	0	53013.2	52046.4	
5-6	Std B	2.5	41268.9	40607.6	
7-8	Std C	10.0	23573.1	22617.5	
9-10	Std D	25.0	11737.2	11478.7	
11-12	Std E	60.0	5239.8	5008.6	
13-14	Std F	130	2389.6	2300.9	
15-16	U1 [†]		44681.1	44875.1	1.6
17-18	U2		17497.1	16710.4	14.8
19-20	U3		6917.8	7023.3	45.5

[†] Unknown

Limitations of the Procedure

- The results obtained from this or any other diagnostic kit should be used and interpreted only in the context of an overall clinical picture.
- Lipemic, haemolyzed and icteric samples may be used up to levels of 33.9 mmol/l triolein, 5 g/l haemoglobin and 1.7 mmol/l bilirubin. Do not use turbid samples.
- Heat treated samples give elevated FT4 values due to protein denaturation and disturbance of the FT4/FT4 equilibrium.
- Heterophilic antibodies in serum or plasma samples may cause interference in immunoassays⁽⁹⁾. These antibodies may be present in blood samples from individuals regularly exposed to animals or who have been treated with animal serum products. Results which are inconsistent with clinical observations indicate the need for additional testing.

Expected Values

It is recommended that each laboratory establish its own reference interval. As a guide, a normal reference interval of 11 to 24 pmol FT4/l with a mean of 16.1 pmol/l (95% confidence interval using a log transform) was obtained from 483 patients of euthyroid status, not on thyroid treatment. No differences in FT4 concentrations were observed between males and females and there was no correlation with age. Of 87 hyperthyroid patients tested, 95.4% had FT4 concentrations >24 pmol/l. Of 59 hypothyroid patients tested, 93.2% had FT4 concentrations <11 pmol/l.

A study of apparently euthyroid pregnant women showed a progressive reduction in FT4 concentration as pregnancy advances. The means and ranges (95% confidence intervals) for the first, second and third trimesters were 15.4 pmol/l (11.6 to 19.2 pmol/l, 42 samples), 12.8 pmol/l (9.3 to 16.3 pmol/l, 44 samples) and 11.6 pmol/l (8 to 15.2 pmol/l, 43 samples) respectively. In patients with non-thyroidal illness FT4 concentrations were normal or occasionally depressed. For example, the mean FT4 concentration in 32 apparently euthyroid patients with renal failure was 15.3 pmol/l, with a range of 9.9 to 24.3 pmol/l.

Performance Characteristics

1. Calibration

The assay was calibrated indirectly against equilibrium dialysis. A correlation between the AMERLEX-MAB* FT4 Kit and an FDA cleared assay has been obtained by measuring a panel of 623 patient samples from a variety of clinical categories:

AMERLEX-MAB* FT4 Kit = 1.05 x FDA cleared assay - 0.23 (pmol/l), with a correlation coefficient of 0.96.

FT4 concentrations are quoted in units of pmol/l or ng/dl. Conversion of units may be made using the formula:

Result in ng FT4/dl = result in pmol FT4/l x 0.078

2. Reproducibility

Four freeze-dried control sera were assayed in 10 replicates to determine within-assay reproducibility. To determine between-assay reproducibility 16 assays were performed by 6 operators using different reagent batches. The data presented are representative of the performance found over the shelf life of the product.

Within-assay		Between-assay	
Mean	CV(%)	Mean	CV(%)
5.6	6.5	5.3	7.5
14.2	3.7	13.9	5.9
17.8	3.8	17.8	6.2
46.8	3.7	47.6	5.0

3. Sensitivity

Sensitivity is defined as the concentration 2 standard deviations from the zero standard when 20 replicates are determined. The sensitivity of the AMERLEX-MAB* Free T4 Kit is typically 0.6 pmol/l.

4. Specificity

The percentage cross-reactivity on a molar basis was 1.2% for L-3,3',5'-triiodothyronine, 15.6% for L-3,3',5'-triiodothyronine (reverse T3), >100% for D-thyroxine and <0.02% for mefenamic acid. Cross-reactivity of <0.01% was exhibited for 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine, diphenylhydantoin, phenylbutazone, sodium salicylate and o-acetylsalicylic acid, fenclofenac, methimazole, 6n-propyl-2-thiouracil and frusemide.

Note: Bio-Rad controls, Catalogue number 370, are commercially available to use as controls for this product. Please contact Trinity Biotech Technical Services at (353) 1 276 9800, for expected values

References

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- Robbins J & Rall JE. In Grady CH & Bacharach AL (eds) *Hormones in Blood volume 1*, London Academic Press: 383-490 (1967).
- Lewis M. As reference 2: 167-180 (1979).
- Franklyn JA *et al.* *Clin Chem* 29: 1527-1530 (1983).
- Anderson BG. *JAMA* 203: 577-582 (1968).
- Dussault JH *et al.* *J Clin Endocrinol Metab* 43: 232-235 (1976).
- Levinson SS. *J Clin Immunoassay* 15: 108-115 (1992).

RISK AND SAFETY



Sodium azide

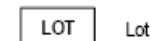
- R22: Harmful if swallowed.
R32: Contact with acids liberates very toxic gas.
S36: Wear suitable protective clothing.

Xn

Prepared in accordance with requirements for EEC label.

EINECS 247-852-1

Key Guide to Symbols



Lot



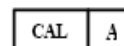
Use by



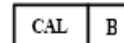
For *In Vitro* Diagnostic Use



Caution, consult accompanying documents



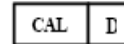
Calibrator A



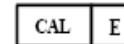
Calibrator B



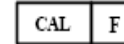
Calibrator C



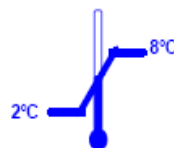
Calibrator D



Calibrator E



Calibrator F



Store at 2-8°C

IM.5051 (100 Tests)
IM.5054 (400 Tests)

Trinity Biotech plc,
IDA Business Park,
Bray,
Co. Wicklow,
Ireland.
Tel: (353) 1 276 9800,
Fax: (353) 1 276 9888,
Web: www.trinitybiotech.com

242-008 02/02



Instructions for Use

Melatonin ELISA

Enzyme immunoassay for the in-vitro diagnostic quantitative determination of melatonin in human serum and plasma.

REF **RE54021**

 **96**

   **2-8 °C**

EU: **IVD**  U.S.: *For research use only.
Not for use in diagnostic procedures.*



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1. INTENDED USE

Enzyme immunoassay for the *in-vitro diagnostic* quantitative determination of melatonin in human serum and plasma.

2. SUMMARY AND EXPLANATION

The pineal gland ("corpus pineale") has been called a neuroendocrine transducer because of its important role in photoperiodism. The major hormone of the pineal gland is N-acetyl-5-methoxy-tryptamine or melatonin which is synthesized from the amino acid tryptophane. Melatonin has its highest levels in plasma during nighttime. Its characteristic nocturnal surge appears to encode temporal information such as length of night. Regulation of the melatonin secretion is under neural control. Sympathetic innervation seems to play a major role via its release of noradrenaline. Altered patterns and/or levels of melatonin secretion have been reported to coincide with sleep disorders, "jet lag", depression, stress, schizophrenia, hypothalamic amenorrhea, pregnancy, anorexia nervosa, some forms of cancer, immunological disorders as well as control of sexual maturation during puberty.

Most of the circulating melatonin is metabolized in the liver to 6-hydroxymelatonin and subsequently to 6-sulfatoxymelatonin which is excreted into the urine.

The concentration of 6-Hydroxymelatonin Sulfate in urine correlates well with the total level of melatonin in the blood during the collection period.

3. TEST PRINCIPLE

The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of anti-biotin alkaline phosphatase as marker and p-nitrophenyl phosphate as substrate. Quantification of unknowns is achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards.

4. WARNINGS AND PRECAUTIONS

1. For in-vitro diagnostic use only. For professional use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the IBL-Homepage.
3. In case of severe damage of the kit package please contact IBL or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details. Material Safety Data Sheets for this product are available on the IBL-Homepage or upon request directly from IBL.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
8. Avoid contact with Stop solution. It may cause skin irritations and burns.
9. All reagents of this kit containing human serum or plasma have been tested and were found negative for HIV I/II, HBsAg and HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

5. STORAGE AND STABILITY

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.

The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2-8°C.

After elution with methanol the Extraction Columns may be used for extraction of the next samples or stored at 2-8°C protected from dust. Extraction Columns may be re-used up to 4 times.


6. SPECIMEN COLLECTION AND STORAGE

Serum, Plasma (EDTA, Heparin)

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	≤ -20°C (Aliquots)	≤ -70°C (Aliquots)	Keep away from heat or direct sun light. Avoid repeated freeze-thaw cycles.
Stability:	24 h	3 mon	1 y	

7. MATERIALS SUPPLIED

 The reagents provided with this kit are sufficient for single determinations in the sample preparation (extraction) and duplicates in the assay. Additional reagents are available upon request.

Quantity	Symbol	Component
1 x 12x8	MTP	Microtiter Plate Break apart strips. Coated with anti-rabbit IgG (goat, polyclonal).
3 x 2 mL	BIOTIN LYO	Melatonin Biotin , lyophilized Contains: stabilizers.
3 x 2 mL	ANTISERUM LYO	Melatonin Antiserum lyophilized Contains: Antiserum (rabbit, polyclonal), stabilizers.
1 x 250 µL	ENZCONJ CONC	Enzyme Conjugate, Concentrate (80x) . Contains: anti-Biotin antibodies (goat), conjugated to alkaline phosphatase, Tris buffer, stabilizers.
1 x 6 x 2 mL	CAL A-F LYO	Standard A-F , lyophilized Contains: stabilizers. For exact concentrations see vial labels or QC Certificate.
1 x 2 x 2 mL	CONTROL 1+2 LYO	Control 1+2 , lyophilized Contains: stabilizers. Concentrations / acceptable ranges see vial labels.
1 x 50 mL	ASSAYBUF CONC	Assay Buffer, Concentrate (10x) Contains: phosphate buffer, Tween, stabilizers.
1 x 9x	PNPP SUBS	PNPP Substrate Tablets In one foil packet. Contains: p-nitrophenyl phosphate (PNPP).
1 x 27 mL	PNPP BUF	PNPP Substrate Buffer Ready to use. Contains: diethanolamine, water.
1 x 15 mL	PNPP STOP	PNPP Stop Solution Ready to use. Contains: 1 M NaOH, 0.25 M EDTA.
2 x 10	EXTRCOL	Extraction Columns Ready to use. C18 RP. 1 cm ³ /100 mg. Additional extraction columns can be ordered separately from IBL under REF KEME761.
3 x	FOIL	Adhesive Foil

8. MATERIALS REQUIRED BUT NOT SUPPLIED


1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV). Volumes: 50; 500 µL
2. Disposable glass test tubes or round-bottom polystyrene test tubes (12 x 75 mm)
3. Orbital shaker (400-600 rpm)
4. Vortex mixer
5. 8-Channel Micropipettor with reagent reservoirs
6. Wash bottle, automated or semi-automated microtiter plate washing system
7. Centrifuge (preferably refrigerated); 200-500 x g
alternatively: Vacuum manifold (e.g. Mallinckrodt-Baker or Waters)

8. Methanol (HPLC grade)
9. Evaporator centrifuge (Speed-Vac)
alternatively: Sample concentrator by use of nitrogen (e.g. Techne)
10. Microtiter plate reader capable of reading absorbance at 405 nm (reference wavelength 600-650 nm)
11. Bidistilled or deionised water
12. Paper towels, pipette tips and timer

9. PROCEDURE NOTES

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each component and specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. It is advised to determine samples in duplicate to be able to identify potential pipetting errors.
5. Use a pipetting scheme to verify an appropriate plate layout.
6. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
7. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
8. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.
9. The relative centrifugal force (g) is not equivalent to rounds per minute (rpm) but it has to be calculated depending on the radius of the centrifuge.

10. PRE-TEST SETUP INSTRUCTIONS

	The contents of the kit for 96 determinations can be divided into 3 separate runs. The volumes stated below are for one run with 4 strips (32 determinations).
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10.1. Preparation of lyophilized or concentrated components

Dilute/dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
15 mL	Assay Buffer	ad 150 mL	bidist. water	1:10		2-8°C	4 w
	Standards, Controls	with 2.0 mL	bidist. water		Let stand for 15 min. Mix without foaming.	≤ -20°C (Aliquots)	until Exp. date
	Melatonin Biotin	with 2.0 mL	Assay Buffer (diluted)		Let stand for 15 min. Mix without foaming.	Prepare freshly and use only once.	
	Melatonin Antiserum	with 2.0 mL	bidist. water		Let stand for 15 min. Mix without foaming.		
70 µL	Enzyme Conjugate	with 5.6 mL	Assay Buffer (diluted)	1:81			
3	PNPP Substrate Tablets	with 8 mL	PNPP Substrate Buffer				

Melatonin ELISA (RE54021)

ENGLISH

10 mL	Methanol (undiluted)	ad 100 mL	bidist. water	10 % (v/v)		
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If a larger volume is needed, vials can be pooled. Avoid repeated freeze-thaw cycles.


10.2. Dilution of Samples

Samples suspected to contain concentrations higher than the highest standard have to be diluted with diluted Assay Buffer prior to extraction step.

10.3. Extraction of Samples, Standards and Controls (Extraction Column)

The yield of extraction with this procedure is approx. 90 - 100 %.

Filter or centrifuge the samples prior to extraction in order to avoid clogging of the columns.

	<p>Each sample, Standard and Control has to be extracted. Extraction may be performed in advance. The dried extracts (after evaporation of methanol) may be stored at 2-8°C or ≤ -20°C for up to 24 h.</p> <p>After elution with methanol the Extraction Columns may be used for extraction of the next samples or stored at 2-8°C protected from dust. Extraction Columns may be re-used up to 4 times. In case of re-use, start again with A.1 (Column Conditioning).</p>
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A. Standard version: Procedure for Centrifuge and Evaporator Centrifuge**1. Column Conditioning:**

1.	Place the Extraction Columns into polystyrene or glass tubes (12 x 75 mm).
2.	Add 2 x 1 mL of methanol (undiluted) to the columns. Let the solvent pass through the column by centrifugation for 1 min at 200 x g. Discard eluate.
3.	Add 2 x 1 mL of bidist. water to the columns. Let the solvent pass through the column by centrifugation for 1 min at 200 x g. Discard eluate.
4.	Proceed with sample application without delay in order to avoid the columns getting dry.

2. Sample Application:

5.	Place the Extraction Columns into correspondingly marked polystyrene or glass tubes (12 x 75 mm).
6.	Add 0.5 mL of Standards, Controls and samples to the columns. Let pass through the column by centrifugation for 1 min at 200 x g. Discard eluate.

3. Washing:

7.	Add 2 x 1 mL of 10 % methanol in bidist. water (v/v) to the columns. Let the solvent pass through the column by centrifugation for 1 min at 500 x g. Discard eluate.
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4. Elution of Extract:

8.	Place the Extraction Columns into new, correspondingly marked polystyrene or glass tubes (12 x 75 mm).
9.	Add 1 mL of methanol (undiluted) to the columns. Let the solvent pass through the column by centrifugation for 1 min at 200 x g.
10.	Remove columns from the tubes. Avoid drops to be left at the columns. Use columns for extraction of the next samples or store at 2-8°C protected from dust. Extraction Columns may be re-used up to 4 times.

5. Evaporation and Reconstitution of Extract:

11.	Evaporate the methanol to dryness by use of evaporator centrifuge.
12.	Reconstitute samples with 0.15 mL of bidist. water .
13.	Vortex at least 1 min and assay immediately.

B. Alternative version: Procedure for Vacuum Manifold instead of a Centrifuge

For the extraction scheme follow points A 1-5 accordingly. The volumes remain unchanged.
Let the **solvent** pass through the column using vacuum and a flow rate of ≤ **5mL/min**.
For the **samples and extracts** use a flow rate of ≤ **2 mL/min**.
The evaporation of the solvent may be performed by using an evaporator centrifuge or by nitrogen.

11. TEST PROCEDURE

1.	Pipette 50 µL of each <u>extracted Standard</u> , <u>extracted Control</u> and <u>extracted sample</u> into the respective wells of the Microtiter Plate.
2.	Pipette 50 µL of Melatonin Biotin into each well.
3.	Pipette 50 µL of Melatonin Antiserum into each well.
4.	Cover plate with adhesive foil. Shake plate carefully. Incubate 14-20 h at 2-8°C.
5.	Remove adhesive foil. Discard incubation solution. Wash plate 3 x with 250 µL of diluted Assay Buffer . Remove excess solution by tapping the inverted plate on a paper towel.
6.	Pipette 150 µL of freshly prepared Enzyme Conjugate into each well.
7.	Cover plate with new adhesive foil. Incubate 120 min at RT (18-25°C) on an orbital shaker (500 rpm).
8.	<i>Approx. 10 min before end of incubation prepare PNPP Substrate Solution.</i>
9.	Remove adhesive foil. Discard incubation solution. Wash plate 3 x with 250 µL of diluted Assay Buffer . Remove excess solution by tapping the inverted plate on a paper towel.
10.	For adding of Substrate and Stop Solution use, if available, an 8-channel Micropipettor. Pipetting should be carried out in the same time intervals for Substrate and Stop Solution. Use positive displacement and avoid formation of air bubbles.
11.	Pipette 200 µL of freshly prepared PNPP Substrate Solution into each well.
12.	Incubate 20 - 40 min at RT (18-25°C) on an orbital shaker (500 rpm).
13.	Stop the substrate reaction by adding 50 µL of PNPP Stop Solution into each well. Briefly mix contents by gently shaking the plate.
14.	Measure optical density with a photometer at 405 nm (Reference-wavelength: 600-650 nm) within 60 min after pipetting of the Stop Solution.

12. QUALITY CONTROL

The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All kit controls must be found within the acceptable ranges as stated on the vial labels. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

It is recommended to participate at appropriate quality assessment trials.

13. CALCULATION OF RESULTS

The obtained OD of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) either on semi-logarithmic graph paper or using an automated method. A good fit is provided with cubic spline, 4 Parameter Logisitics or Logit-Log.

For the calculation of the standard curve, apply each signal of the standards (one obvious outlier of duplicates might be omitted and the more plausible single value might be used).

The concentration of the samples can be read from the standard curve.

In case of diluted samples the values have to be multiplied with the corresponding dilution factor.

Samples showing concentrations above the highest standard have to be diluted as described in PRE-TEST SETUP INSTRUCTIONS and reassayed.

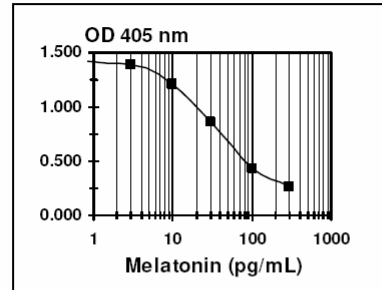
Conversion:

Melatonin (pg/mL) x 4.30 = pmol/L

Typical Calibration Curve

(Example. Do not use for calculation!)

Standard	Melatonin (pg/mL)	Mean OD	OD/OD _{max} (%)
A	0.0	1.517	100.0
B	3.0	1.383	91.1
C	10	1.214	80.1
D	30	0.867	57.1
E	100	0.434	28.6
F	300	0.260	17.1

**14. EXPECTED VALUES**

The results themselves should not be the only reason for any therapeutical consequences. They have to be correlated to other clinical observations and diagnostic tests.

A study with apparently healthy subjects has shown that the melatonin levels in humans have a marked circadian rhythmicity characterised by very low levels during day-time and high levels during night-time, and show a considerable inter-individual variation. Furthermore, the melatonin concentration is age dependent. The highest concentrations were found in samples of infants (up to 3 years).

In a group of six healthy volunteers the circadian rhythm of melatonin was studied. The mean value reaches a minimum of about 4.6 pg/mL during daytime at 4 p. m. and a maximum of about 77.5 pg/mL during night-time at 4 a.m.

The nocturnal melatonin peak among healthy individuals varies significantly.

It is recommended that each laboratory establishes its own range of normal values.

15. LIMITATIONS OF THE PROCEDURE

Specimen collection has a significant effect on the test results. See SPECIMEN COLLECTION AND STORAGE for details.

For cross-reactivities, see PERFORMANCE.

The following blood components do not have a significant effect (+/- 20 % of expected) on the test results up to the concentrations stated below:

Hemoglobin	8.0 mg/mL
Bilirubin	0.36 mg/mL

16. PERFORMANCE

Analytical Specificity (Cross Reactivity)	Substance	Cross Reactivity (%)	Cross-reactivity of other substances tested < 0.01 %		
	5-Methoxy-Tryptophole	1.2			
	N-Acetyl-Serotonin	1.2			
	5-Methoxy-Tryptamine	2.5			
Analytical Sensitivity (Limit of Detection)	1.6 pg/mL	Mean signal (Zero-Standard) - 2SD			
Precision	Range (pg/mL)	CV (%)			
	Intra-Assay	8.8 – 151.7	3.0 – 11.4		
Inter-Assay	5.6 – 134.3	6.4 – 19.3			
Linearity	Range (pg/mL)	Serial dilution up to	Range (%)		
	80.7 – 191.4	1:16	73 - 135		
Recovery	Mean (%)	Range (%)	% Recovery after spiking		
	102.4	83 - 125			
Method Comparison versus IBL RIA	IBL ELISA = 1.01 x IBL RIA + 4.6		r = 0.98; n = 50		
Method Comparison versus other RIA	IBL ELISA = 0.86 x other RIA + 5.33		r = 0.96; n = 46		


17. PRODUCT LITERATURE REFERENCES

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Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός-Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazena a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!
<p align="center">Symbols of the kit components see MATERIALS SUPPLIED. Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben. Voir MATERIEL FOURNI pour les symbôles des composants du kit. Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS. Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS. Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT. Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.</p>	

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LIABILITY: Complaints will only be accepted in written and if all details of the test performance and results are included (complaint form available from IBL or supplier). Any modification of the test procedure or exchange or mixing of components of different lots could negatively affect the results. These cases invalidate any claim for replacement. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the kit during transportation is not subject to the liability of the manufacturer.

List of Publications

PUBLICATIONS IN PEER-REVIEWED JOURNALS

- Sprague, M.**, Talbot, C., Gabillard, J.-C., Bell, J.G., Bromage, N.R. and Migaud, H. (2006). The effects of artificial light regimes on the growth endocrine axis in 1+ Atlantic salmon post-smolts (Submitted to Aquaculture).
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