EFFECTS OF PHOTOPERIOD MANIPULATION ON GROWTH AND REPRODUCTION IN ATLANTIC COD (*Gadus morhua* L.)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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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.

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ABSTRACT

Sexual maturation during commercial culture of Atlantic cod (*Gadus morhua* L.) represents a significant production bottleneck restricting the profitability of the industry. Such problems in other species have traditionally been addressed by artificial manipulation of photoperiod cycles, however little research exists in this field in cod. This thesis therefore investigates the interactions between artificial photoperiod manipulation, sexual maturation and somatic growth in this species.

In the first experiment, populations of Atlantic cod (hatched, spring 1999) were maintained on either a simulated natural photoperiod (SNP) or continuous illumination (LL) from approximately 15 months post hatch (MPH) (July 2000) in an enclosed tank system. Growth performance was recorded monthly along with observations of reproductive activity over the subsequent 2 years (up to July 2002). At both 2 and 3 years of age the entire population raised under SNP matured and spawned, during which time mean weight reduced by 13% and 24% respectively. No spawning individuals were recorded at 2 years of age in the LL population and only 18% were observed to spawn at 3. However, observations of both changes in gonadal morphology (observed via ultrasound scanning) and a suppression in growth rate at 2 years of age in the LL population alluded to a maturation "dummy run" regulated by an endogenous clock. Despite this phenomenon, the LL treatment realised a 39% and 43% improvement in wet weight following 1 and 2 years of exposure to LL respectively. When the diel cycle of plasma melatonin was compared between the treatments in February 2001 (23MPH) the SNP population displayed an A-profile diel rhythm ranging between 20 and 50 pg/ml while the LL treatment did not display any rhythm.

In the second experiment of this work, two populations of cod (hatched, spring 2001) were reared in commercial open cage systems, one of which experienced

continuous additional artificial illumination between July 2002 (15MPH) and October 2003 (30MPH) provided by four, 400W submerged lighting units. Growth and maturation were assessed in both populations throughout. In March 2003 (24MPH) it was apparent that spawning individuals were present in both the SNP and LL populations though a significantly lower number of spawning individuals in the LL treatment suggested that the peak in spawning activity was delayed by about 1 to 2 months. With both populations apparently maturing at 2 years of age, there was no significant difference in weight between the populations at the end of the trial. In comparison to experiments I and IV of this work, these results would suggest that in comparison to salmonids for example, Atlantic cod appear to have a heightened sensitivity to light allowing individuals to differentiate the ambient photoperiod signal from the application of continuous artificial light.

In the third experiment, 6 populations of approximately 20 tagged individuals (hatched spring 1999) were maintained, from December 2000 to July 2002, under either SNP, LL or one of four, out of season "square wave" photoperiod regimes (repeating cycles with a 12 month period, consisting of a 6 month window of LL followed by six months of short day lengths [SD, 7L:17D] which had been staggered to start over a six month period). Each individual was monitored monthly for maturation status. Out of season "square wave" photoperiods were demonstrated to successfully entrain maturation and hence significantly alter the spawning profiles in these populations. Application of LL from December 2000 failed to inhibit maturation in the spring of 2001 and, in fact, advanced the spawning season by 1 month while those that experienced SD from the same date showed significant extension of the subsequent spawning season. Interestingly, the males maintained on LL throughout the experiment

matured both in the spring of 2001 and one year later in the spring of 2002 while females under the same treatment only matured and spawned in 2001.

In the fourth experiment, a total of 830 tagged individuals were raised either under SNP or one of 7 photoperiod treatments, consisting of 5 groups transferred from SNP to LL at 3 monthly intervals between 6 and 18 MPH where they remained and a further two groups maintained on LL from 6 to 15MPH and 6 to 21 MPH respectively before being returned to SNP. Both the gonadic and somatic axes were monitored at the physiological and endocrinological level at three monthly intervals from 6 to 27 MPH. The results demonstrated that it is the falling autumnal photoperiod signal after the summer solstice, more specifically after October, that is responsible for recruiting individuals to enter the sexual maturation cycle. Furthermore, in all treatments where this signal was masked i.e. those which experienced LL starting at or prior to 15MPH, except for some restricted spermatogenic activity in the males testis observed at 27MPH, there was no significant reproductive activity and growth was improved by up to 60% at 27 MPH. While providing evidence for direct photic stimulation of somatic growth, the growth results were also correlated with the measurement of plasma IGF-I and demonstrated its potential as a tool to assess growth rates in the species. Plasma melatonin measured at 15MPH, as in experiment I, was suppressed in all populations which were under LL photoperiods. By identifying the photoperiod "window of opportunity" which recruits individuals into the sexual maturation cycle, this work was able to conclude that the application of LL from the summer solstice prior to maturation is the most efficient photoperiod strategy to be adopted by the aquaculture industry to realise maximum growth potential from their cultured stocks.

Keywords: Atlantic cod, *Gadus morhua*, puberty, sexual maturation, growth, photoperiod.

GLOSSARY OF COMMON AND SCIENTIFIC NAMES USED WITHIN THIS THESIS.

Atlantic cod	*Gadus morhua* (Linnaeus, 1758)
haddock	*Melanogrammus aeglefinus* (Linnaeus, 1758)
Argentine hake	*Merluccius hubbsi* (Marini, 1933)
Atlantic (European) hake	*Merluccius merluccius* (Linnaeus, 1758)
pollack	*Pollachius pollachius* (Linnaeus, 1758)
whiting	*Merlangius merlangus* (Linnaeus, 1758)
brill	*Scophthalmus rhombus* (Linnaeus, 1758)
dab	*Limanda limanda* (Linnaeus, 1758)
flounder	*Platichthys flesus* (Linnaeus, 1758)
smooth flounder	*Liopsetta putnami* (Gill, 1864)
winter flounder	*Pseudopleuronectes americanus* (Walbaum, 1792)
Atlantic halibut	*Hippoglossus hippoglossus* (Linnaeus, 1758)
Queensland halibut	*Psettodes erumei* (Bloch & Schenider, 1801)
plaice	*Pleuronecetes platessa* (Linnaeus, 1758)
sole	*Solea solea* (Linnaeus, 1758)
turbot	*Psetta maxima* (Linnaeus, 1758)
barramundi	*Lates calcarifer* (Bloch 1790)
European seabass	*Dicentrarchus labrax* (Linnaeus, 1758)
Southern bluefin tuna	*Thunnus maccoyii* (Castelnau 1872)
Atlantic salmon	*Salmo salar* (Linnaeus, 1758)
chinook salmon	*Onchorhynchus tshawytscha* (Walbaum, 1792)
coho salmon	*Onchorhynchus kistuch* (Walbaum, 1792)
masu salmon	*Onchorhynchus masou* (Brevoort, 1856)
brook trout	*Salvelinus fontinalis* (Mitchell, 1814)
brown trout	*Salmo trutta* (Linnaeus, 1758.)
rainbow trout	*Onchorhynchus mykiss* (Walbaum, 1792)
barbel	*Barbus barbus* (Linnaeus, 1758.)
black bullhead	*Ameiurus melas* (Rafinesque, 1820)
channel catfish	*Ictalurus punctatus* (Rafinesque, 1818)

Indian catfish	*Heteropneustes fossilis* (Bloch, 1799)
dace	*Leuciscus leuciscus* (Linnaeus, 1758)
Nile tilapia	*Oreochromus niloticus niloticus* (Linnaeus, 1758)
silver perch	*Bidyanus bidyanus* (Mitchell, 1838)
pike	*Esox lucius* (Linnaeus, 1758)
stickleback	*Gasterosteus aculeatus* (Linnaeus, 1758)
Syrian hamster	*Mesocricetus auratus* (Waterhouse 1839)
Turkish hamster	*Mesocricetus brandti* (Nehring, 1858)
djungarian hamster	*Phodopus sungorus* (Pallas, 1773)
Mongolian gerbil	*Meriones unguiculatus* (Milne-Edwards, 1867)
eastern chipmunk	*Tamias striatus* (Linnaeus, 1758)
house mouse	*Mus musculus* (Linnaeus, 1758)
white foot mouse	*Peromyscus leucopus* (Rafinesque, 1818)
rat	*Rattus norvegicus* (Berkenhout, 1769)
domestic cat	*Felis silvestris* (Schreber, 1775)
Red deer	*Cervus elaphus* (Linnaeus, 1758)
sheep	*Ovis aries* (Linnaeus, 1758)
Human	*Homo sapiens* (Linnaeus, 1758)

GLOSSARY OF ABBREVIATIONS AND ACRONYMS USED WITHIN THIS THESIS

A	Anus
am	"ante meridian" (Latin)
ANOVA	Analysis of Variance
Ci	Curie
circa	"about" (Latin)
cm	Centimetre
conc.	Concentrated
CV	Coefficient of Variation
dl	Decilitre
dpm	Disintegrations per minute
et al.	"and others" (Latin)
Exp	Experiment
F	Female
FAO	Food and Agricultural Organisation of the United Nations
FOM	Final Oocyte Maturation
FRS	Fisheries Research Service
FSH	Follicle Stimulating Hormone
g	Gram
g	Gravitational constant
GH	Growth hormone
GLM	General Linear model
GnRH	Gonadotropin Releasing Hormone
GSI	Gonadosomatic Index
GtH	Gonadotropin
GVBD	Germinal Vesicle Breakdown
GVM	Germinal Vesicle Migration
hr	Hour
HPG axis	Hypothalamic, Pituitary and Gonad axis
HSI	Hepatosomatic Index
ICES	International Commission for the Exploration of the Seas
i.e.	"in other words" (Latin)
IGFBP	IGF-I binding protein

IGF-I	Insulin-Like Growth Factor –I
K	Foulton's Condition Factor
11-KT	11-Ketotestosterone
kDa	Kilo Dalton
kg	Kilogramme
km	Kilometer
L	Litre
L:D	Light:Dark
LH	Luteinising Hormone
LL	Continuous light
lux	Measure for luminous flux density at a surface.
M	Male
m	Metre
M^{100}	Date when 100% of a population are recorded as mature
MERL	Machrihanish Marine Environmental Research Laboratory
mg	Milligram
µl	Microlitre
µmoles	Micromoles
MHz	Megahertz
MIH	Maturation-Inducing Hormones
min	Minute
ml	Millilitre
mm	Millimetre
mmol	Millimole
MPF	Maturation Promoting Factors
MPH	Months Post Hatch
n	Number
n.b.	"take note" (Latin)
ng	Nanogram
nm	Nanometre
^{o}C	Degrees centigrade
PEG	Polyethylene glycol
Per se	"by itself" (Latin)
Pers comm.	Personal communication

pg	Picogram
Photo	Photoperiod
PIT	Pituitary
pm	“post meridian” (Latin)
QC	Quality Control
s	Second
SCN	Suprachiasmatic nucleus
SD	Short Day
SEM	Standard Error of the Mean
SGR	Specific Growth Rate
SGRl	Length Specific Growth Rate
SGRw	Weight Specific Growth rate
SNP	Simulated Natural Photoperiod
SOC	Supraoptic/suprashiasmatic nucleus
T	Testosterone
TGC	Thermal Growth Coefficient
UP	Urogenital Papillae
v/v	Volume/Volume
v/w	Volume/Weight
w/v	Weight/Volume
VTG	Vitellogenin
W	Watts

CHAPTER 1: GENERAL INTRODUCTION

The Atlantic cod (*Gadus morhua*, Linnaeus 1758) is a member of the family Gadidae within the order Gadiformes, which includes species such as haddock, whiting, and pollock. This predominately demersal marine species has a natural range extending over most of the northern Atlantic coastal region where it can be found over a wide range of salinities from almost fresh to full oceanic water, and an equally large temperature range (-2 to 20°C) (Cohen *et al.,* 1990). Cod have historically reached up to 1.8m long and a weight of approximately 95kg (Kurlansky 1999), they are however more usually found up to a maximum of approximately 1m in length and 15kg in weight. The flesh has a naturally low fat content and has been described as the "beef of the sea" due to its high protein content (Innis 1978). For this reason it has been the focus of much commercial fishery attention particularly in the last century. Consequently, wild stocks are presently classified as "outside safe biological limits" by the International Commission for the Exploration of the Seas (ICES, 2003) with the species itself being classified as vulnerable to extinction since 1996 by the World Conservation Union (Solbel, 1996).

The rapid expansion of aquaculture technology, primarily with salmonid species in northern Europe, has made it possible to realistically culture the species in the last 10 years. At present, the industry is poised for rapid expansion: however a few principal bottlenecks in production exist. These include problems in both the hatchery phase (e.g. poor broodstock performance accompanied with generally poor larval performance: low survival rates combined with high proportions of deformities) as well as in the on-growing phase (e.g. disease resistance [susceptibility to *vibrio sp.* outbreaks] and maturation control [maturation prior to the attainment of harvest weight]). This current investigation was designed to investigate the photoperiodic regulation of maturation in

Atlantic cod to better explain how this key physiological process in relation to growth, is regulated by the environment. The direct benefit of such knowledge would be the subsequent adoption of refined management practices, based on artificial photoperiod manipulation, which would allow the aquaculture industry to realise maximum grow-out potential of its stock.

1.1 Natural Range and Habitat

Atlantic cod are found across the Northern Atlantic Ocean from the Gulf of Maine, USA in the west, north past Greenland and Iceland to the Baltic Sea and south to the Bay of Biscay in the east (Cohen *et al.*, 1990) (Figure 1.1). Throughout the species' natural range there are many discrete 'stocks' that have their own physiological and behavioural characteristics, their names reflecting their chosen habitat e.g. Norwegian coastal cod (Ottera *et al.*, 1999), Arcto-Norwegian cod (Ottera *et al.*, 1999), North Sea cod (Cook *et al.*, 1999), West of Scotland cod (FRS, 2004), Icelandic cod (Baldursson *et al.*, 1996), Gulf of Maine cod and Grand Banks cod (Purchase and Brown, 2001).

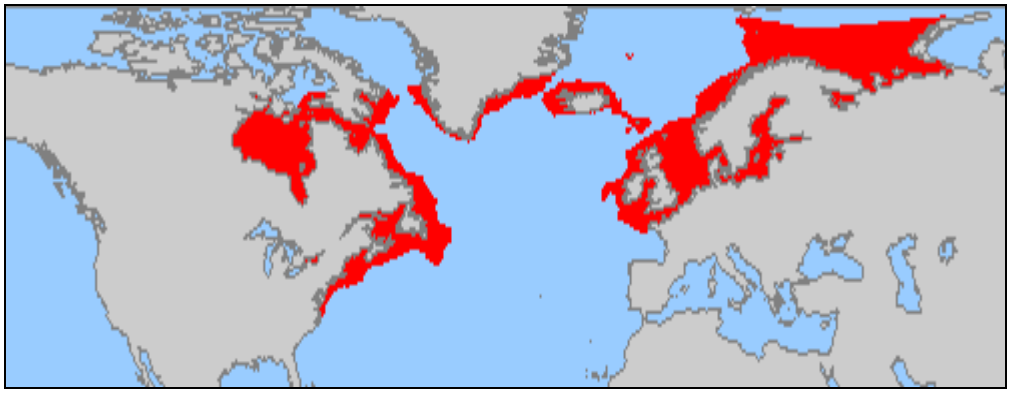


Figure 1.1: Natural range for Atlantic cod, Source: (Cohen *et al.*, 1990)

These stocks are widely distributed in a variety of habitats from the shoreline, across the continental shelf to depths over 600m, though they are generally found above 200m.

Cod are gregarious during the day forming shoals that swim approximately 30 to 80m above the seabed and disperse at night (Cohen *et al.*, 1990). With different stocks occupying widely varying locations, they experience differences primarily in seasonal temperature ranges, for example Arcto-Norwegian cod experience temperatures that range from sub-zero to 6 ^{0}C [*n.b.* northern strains seasonally produce an antifreeze protein in their blood serum to aid survival in winter water temperatures as cold as -2^{0}C (Fletcher *et al.*, 1997)] while Norwegian coastal cod experience a temperature range from 4-14 ^{0}C. Brander (1995) observed similar stock-specific thermal ranges off the North American coast which correlated with stock-specific growth rates. The same study reported that the mean weight of a 4 year old cod off Labrador (average temperature 2^{0}C) was 0.6 kg, whereas cod of the same age on Georges Bank (average temperature 8^{0}C) averaged 3.47kg. At present, temperature remains the principal defined regulator of growth rate in cod as long as the genetic contribution to growth performance remains unclear (Imsland and Jonsdottir, 2002). The different stocks not only experience temperature variations, there are also wide differences in salinity exposure (Lambert *et al.*, 1994). The same authors showed that Gulf of St. Lawrence (Canada) cod not only tolerate salinities as low as 7‰ but actually exhibited improved growth rates (up to 63% greater) under low salinity conditions (7 -14‰) in comparison with purely marine environments (34‰). However, the mechanism behind the improved growth and subsequent food conversion rates was not identified (Dutil *et al.*, 1997).

1.2 Life cycle

Like many fish at higher latitudes, the Atlantic cod life-cycle has a clear seasonal pattern the principal focus of which is the yearly mass spawning in early

spring. Then, in early summer, the recovering adults migrate to rich summer feeding grounds to replenish energy levels before gametogenesis begins again in mid-autumn which signals the start of the returning migration to arrive on the spawning grounds in early winter. Juveniles spend the first year on nursery grounds before joining the adult populations in their seasonal migrations and, eventually, joining in the complete maturation cycle from 2 to 3 years post hatch (Cohen *et al*., 1990).

Spawning

Spawning generally occurs between January and June, depending on the stock (Cohen *et al.*, 1990) however, some eastern Atlantic stocks are recorded as spawning from August to November (Gagne and O'Boyle, 1984). Cod is classified as a determinate multiple batch spawner (Kjesbu *et al.*, 1990), meaning that there is no recruitment of oocytes to the vitellogenic stage during spawning (Kjesbu, 1993). It is thought that potential fecundity, i.e. the number of vitellogenic oocytes recruited in that year, is determined at two periods of the year. For spring spawning stocks these occur firstly, post spawning (April-May) and then again prior to the first stages of vitellogenesis (late autumn). In the first period, the number of previtellogenic oocytes entering the circumnuclear ring phase (maturation commitment) is positively related to the post spawning weight of the fish (Waiwood, 1982). The later boost to previtellogenic oocytes is determined by the fishes' subsequent summer growth/condition. Fish showing greater growth increase their oocyte numbers before the initiation of vitellogenesis, after which stage recruitment is no longer possible (Kjesbu *et al.*, 1991). A comparable time scale has been demonstrated with virgin spawning females, where the adolescent body weight one year prior to spawning was the principle

determinant in fecundity followed by the growth rate in the subsequent autumn (Kjesbu and Holm, 1994).

Spawning occurs over a period of 2-3 months when individual females spawn over a period of approximately 50 to 60 days, during which time they will release between 9 to 20 egg batches at intervals of 60 to 70 hours (Kjesbu, 1989; Trippel, 1998). Cod are described as one of the world's most fecund fish, releasing approximately 500,000 eggs per kg over a typical spawning season (Cohen *et al.*, 1990). The act of spawning itself is preceded by a mating ritual in which females select displaying males. Female choice is based on a combination of the male drumming noise (generated by a muscle associated with the swim bladder) and fin display that is a reflection of their status and "quality". After selection, the pair enter a ventral mount which initiates spawning and ensures that the mating pair are the principal parents (Engen and Folstad, 1999).

Larval and juvenile stages

The size of the pelagic eggs varies between stocks (Kjesbu, 1993; Chambers and Waiwood, 1996) and time in the spawning season (Kjesbu, 1989; Trippel, 1998) although they are generally around 1 to 2 mm in diameter. Time to hatch varies with temperature with hatching occurring around 80 to 100 degree days post fertilisation (Laurence and Rogers, 1976; Brown *et al.*, 2003). The newly hatched larvae are 4-5mm in length (Pepin *et al.*, 1997) and have a limited yolk reserve (lasting up to 6 to 7 days post hatch: Walden, 2001) before exogenous feeding is required to maintain development (Brown *et al.,* 2003). These larvae are pelagic for the first 2 to 3 months, feeding continuously on pelagic zooplankton, during which time they pass through metamorphosis. By 4 to 5 months post hatch the juveniles adopt a more demersal

lifestyle where the diet changes to crustaceans and polychaete worms. As the juveniles grow, so the feeding becomes crepuscular and the diet focuses more on the larger crustaceans before fish become an increasingly important part of the diet (Moller 1978; Cohen *et al.*, 1990).

Adolescence and maturation

Although little is known about the movements of juvenile cod, it is believed they inhabit relatively shallow water nursery grounds with the individuals migrating offshore to the feeding grounds inhabited by the mature adults at around 1 year post hatch. Cod populations are highly mobile, displaying yearly migrations between discrete feeding and spawning grounds. These migrations have been recorded to cover distances of up to 1000km (Trout, 1957; Woodhead, 1959; Cohen *et al.*, 1990; Rose, 1993).

Onset of maturation is variable across stocks and reflects their growth rate, with eastern stocks starting maturation in their secound year while western stocks start in their fourth year (Cohen *et al.*, 1990). Gametogenesis (oogenesis and spermatogenesis in females and males respectively) begins approximately 6 months prior to spawning (Burton *et al.*, 1997). At this stage, the gonadosomatic index (GSI - relative proportion of gonad weight to total weight) starts to increase in maturing fish (Eliassen and Vahl, 1982a) for a period of approximately 4-5 months before spawning begins in early spring. Following spawning, spent adults return to the feeding grounds and subsequently mature every year as energy reserves allow (Rideout and Burton, 2000; Rideout *et al.*, 2000). Although individuals have been reported of up to 23 years of age (Beverton and Holt, 1959), the average age landed from commercial fisheries is between 4 and 8 years.

1.3 Commercial exploitation of Atlantic cod

1.3.1 Fisheries Interest

Cod have been a focus for Man's attention since the early 1400's due to its widespread distribution, large size, proliferation and high quality, protein rich flesh (Innis 1978). However, it was in the last century that cod stocks experienced the greatest fishing pressure due to the increased efficiency of fishing technology. Catches peaked in 1968 just below 4,000,000 tonnes (FAO 2004a). Since then, the cod fisheries of the northern Atlantic have seen a dramatic decline (Figure 1.2). This was first widely realised in the closure of the Canadian Grand Banks fishery in 1992 (Mackenzie, 1995) when these stocks were reduced to 2% of the levels recorded in the 1960's (Longhurst, 1998). In the eastern Atlantic, it was recommended that the fishing for Icelandic stocks ceased in 1996, as the populations were "far below benchmarks such as the maximum sustainable yield level" (Baldursson *et al.*, 1996). Soon after, it was predicted that North

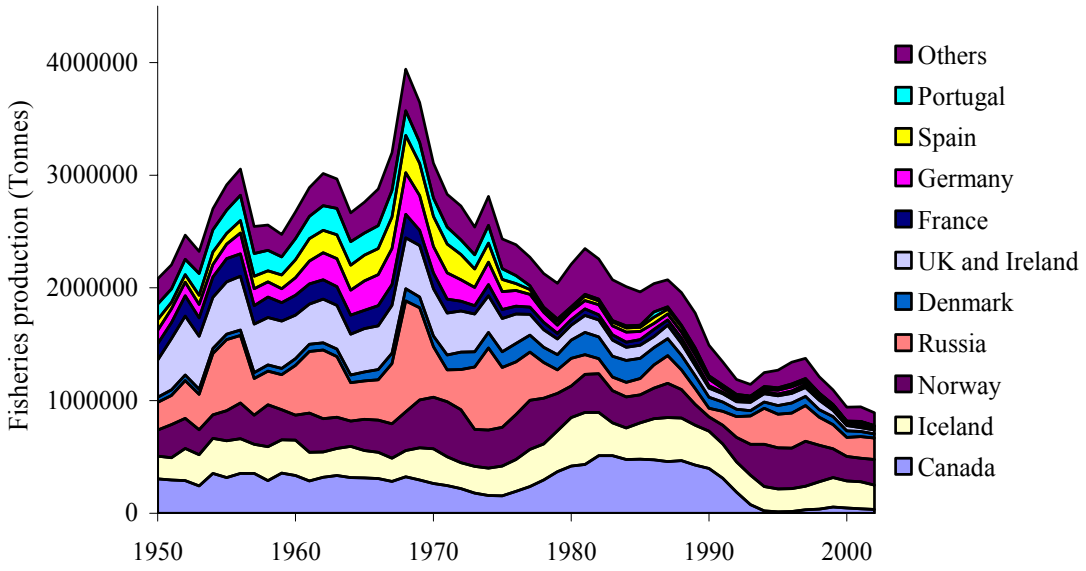


Figure 1.2: Total global catch of Atlantic cod from 1950 to 2002 (FAO 2004a).

Sea Cod stocks were in a similar situation (Cook *et al.*, 1997). At present, cod fishing is still closed in the western Atlantic, while in the eastern Atlantic, dramatic declines are continuing with the prospect of an outright ban likely to be proposed by the European Community (Borg, 2004). Many reasons for the dramatic decline have been offered however, the basis to all theories of cod stock decline is over-fishing supported by inaccurate modelling of the stocks (Cook *et al.*, 1997).

1.3.2 Fisheries Enhancement

Attempts to support the floundering wild stocks have been made. Throughout the last century there was some limited work on replenishing the natural populations with artificially reared individuals (for a review of "cod ranching" see Svasand *et al.*, 2000). From its early beginnings in 1880, on both sides of the Atlantic in Norway, America and Canada, the intention was to increase the potential wild harvest. During the 20th century more countries joined in with their own programmes, and the focus was more on saving stocks than boosting yield. However, there is no evidence to confirm that these programmes offered any aid to the ever declining stocks. Initial attempts were based around the release of newly hatched larvae, from captive spawning parents, while later attempts were based on rearing the larvae in artificially fertilised ponds or enclosures and then releasing them as juveniles (>10cm). A financial analysis (Svasand *et al.*, 2000) considering data such as cost of production, survival and recapture rates and market price, revealed that even the best case scenario was unprofitable, primarily due to the high production cost set against the subsequent poor recapture rate. These various schemes, however, formed the initial impetus for aquaculture development of this species.

1.3.3 Aquaculture

The interest in cod farming developed from three different perspectives. The various cod ranching programmes developed broodstock and juvenile rearing techniques (Oiestad *et al.*, 1985) while, in parallel, commercial fishermen realised that catching undersized fish and placing them in cages for on growing was reasonably profitable. Then, most significantly, the boom of Atlantic salmon farming in the 1990's led the various large multinational producers to consider product diversification to counteract their high reliance on a single income source (MacDubhghaill and Charron, 2000). Due to these various interests, most basic aspects of cod aquaculture have been researched and characterised. Currently, although still in its infancy, it has become a fully closed system and is the focus of rapid expansion primarily in the United Kingdom, Norway, Iceland and Canada. Production to date has been low, equating to less than 0.2% of the wild harvest (Figure 1.3). Despite much industry enthusiasm, the

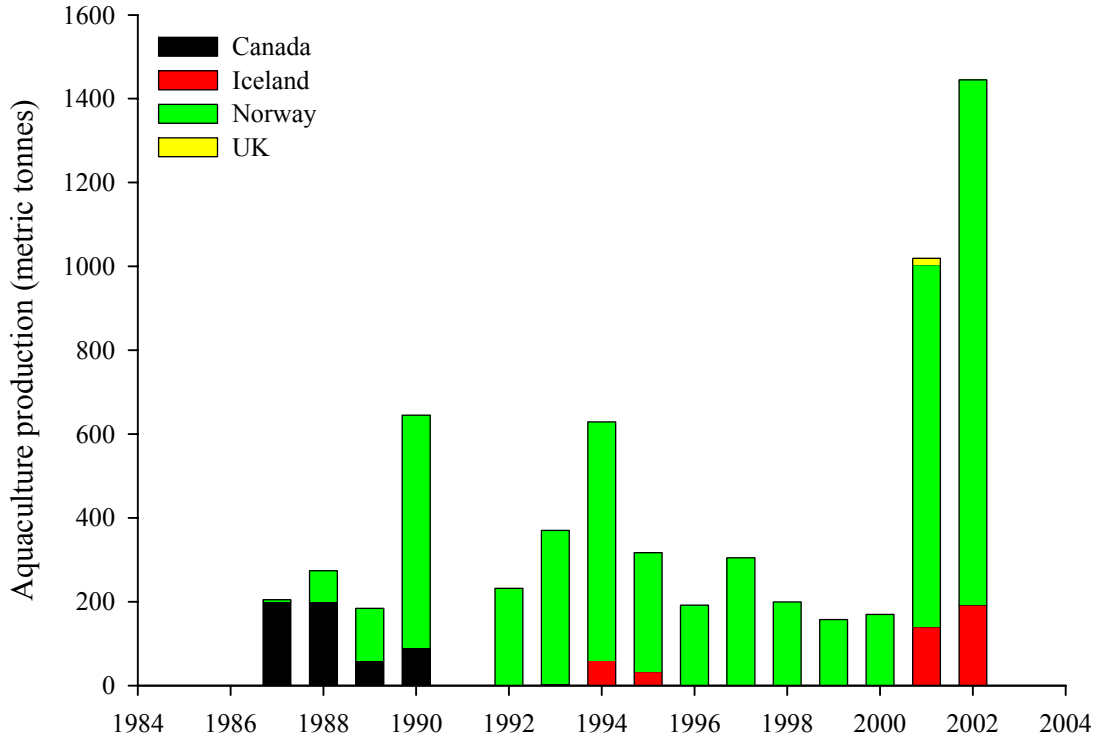


Figure 1.3: Total aquaculture production for Atlantic cod from 1987 to 2002 (FAO 2004b).

projected figures for 2003 and 2004, 2,340 and 4,030 tonnes respectively (Prickett, 2004), do not yet show the very significant rise in production that is required to meet the projected target of 175,000 tonnes (just under 20% of present consumption rates) by 2010 (Rosenlund and Skretting, 2004).

Such a rapid expansion in production is only achievable through extensive investment in juvenile production facilities in all countries which, for the first time, is allowing the mass rearing of juveniles in large scale commercial cage systems. Much of the short term projected production data (future 3-4 years) is based on a theoretical yield from fairly confidently predicted juvenile production numbers. Market demands are for a minimum harvestable individual of 2kg round weight (Slaski, 2003). Following favourable temperature and feeding conditions it is possible to get from egg to a 2kg fish in 24 months (Bjornsson *et al.*, 2001). Although acceptable, larger fish are preferred by the market. Unfortunately, this excellent growth rate is interrupted at 2 years post hatch when close to 100% of individuals mature in captivity (Johansen *et al.*, 2000; Walden, 2001). When fish mature there is a loss of somatic growth, condition and even a reduction in weight (Braaten, 1984; Pedersen and Jobling, 1989). Hence, farmers have to harvest prior to maturation at approximately 2kg, or allow the fish to mature and then recondition before harvesting at greater than 2kg. They may take a further 6 months to regain condition and weight and this represents a significant economic loss to the industry, leading in part to the shortfall between actual and projected harvest figures. Therefore, the prevention of maturation prior to the attainment of a suitable harvest weight during on-growing has been identified as a principle production bottleneck. Protocols to control maturation have already been adopted in a number of other cultured marine species (Bromage *et al.*, 2001). Their design is reliant on clear understanding of the environmental and endogenous regulation of this key physiological process.

1.4 Environmental regulation of sexual maturation in teleosts.

Maturation in cod, along with all temperate marine teleosts, is seasonal (Bye, 1990). The sole purpose of seasonal reproduction is to improve progeny survival through the coordination of larval abundance with favourable environmental conditions and an abundant food source. However, the time required for gonadal and subsequent embryonic development (>6 months in cod, see section 1.2) dictates that reproduction cannot be initiated by the detection of such 'ultimate' conditions. Instead, the individuals require the coordination of reproduction to be initiated by 'proximate' environmental factors which accurately predict the onset of these 'ultimate' environmental factors (Baker, 1938). Many environmental factors have been implicated as possible proximate cues, including photoperiod, temperature, rainfall and food supply (Bromage *et al.*, 2001). All such factors can provide timely indications of pending conditions and are often used in conjunction to provide the animal with specific entrainment to the prevailing local conditions. Of all the suggested environmental factors however, it is commonly believed that the daily changes in light intensity and the concomitant seasonal changes in daylength, commonly referred to as photoperiod, provide the most consistent "noise free" signal perceptible in almost all environments (Bromage *et al.*, 2001; Fleissner and Fleissner, 2002). Such fluctuations in photoperiod are a result of the tilted axis of the earth relative to the sun, and become more pronounced as the distance from the equator increases. Hence, in middle to high latitudes, light is the principal exogenous synchroniser (*zeitgeber; zeit=time, geber=giver*) that regulates many of the daily and seasonal biological rhythms (Foster, 2002), including reproduction (Bromage *et al.*, 2001).

The physiological basis for 'photoperiodism' is believed to be common to all vertebrates and is rooted in a "central circadian axis" (Menaker *et al.*, 1997) comprising

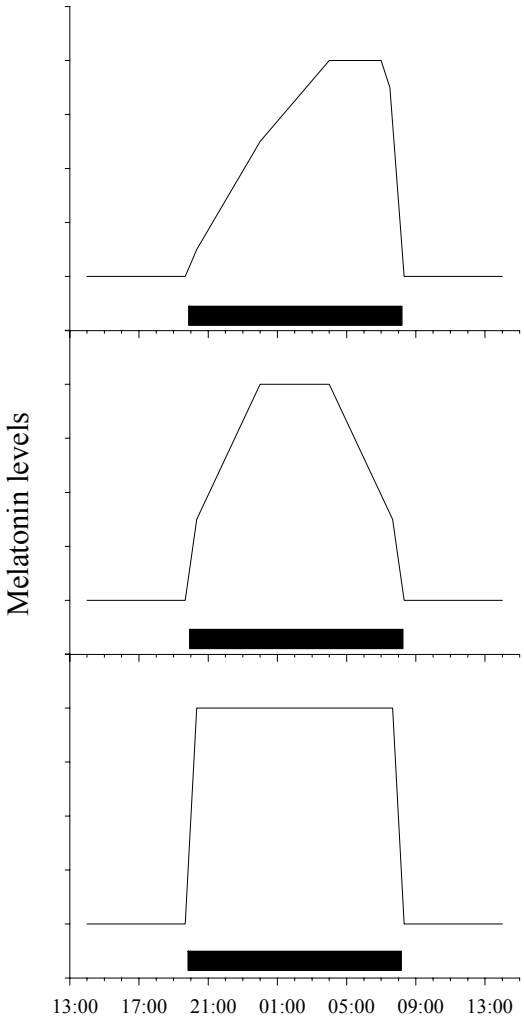
the retina, the suprachiasmatic nucleus (SCN) of the hypothalamus and the pineal complex. These have each been shown to be involved to varying degrees in the control and regulation of these photoperiod entrained rhythms (Menaker *et al.*, 1997; Falcon, 1999). In all vertebrates this 'circadian axis' is based around an oscillating mechanism or clock, that runs on a period approximating to 24 hours (i.e *circadian*) which is subjected to entraining light signals from photoreceptors. These signals are translated into neuroendocrinological signals that transmit information to target tissues determining the physiological responses. These components can be based in single cells, as is the case with lower vertebrates including teleosts (Falcon, 1999; Whitmore *et al.*, 2000) or as in mammals they may be sited in different discrete tissues (photoreceptors = retina, SCN = clock mechanism, pineal complex = melatonin generation) with communication networks linking them (Morse and Sassone-Corsi, 2002).

In teleosts, at present, it is proposed that the pineal gland or epiphysis, which is located on the dorsal surface of the brain is the principal photoperiod interpreting organ (Bromage *et al.*, 2001). For a comprehensive review of the role of the pineal gland see Ekstrom and Meissl (1997). The gland's epithelial layer contains a large number of photoreceptor cells that react to light stimulation and release neural signals. It has been shown that the photoreceptors response is proportional to light intensity (Falcon and Meissl, 1981; Meissl and Ekstrom, 1988). With this being the case, a simple theory of a luminance response has been offered, where the change in light intensity as the sun rises or sets acts as a gradual stimulus until the threshold level is attained when stimulation remains constant. However, spectrally sensitive pigments, opsins (Section 3.6), have been detected in the photoreactive layer. These have optimum sensitivities described as λ_{max} indicating a wavelength of light to which they are most sensitive. For example, in rainbow trout, dependent on age and season, suggestions have been made for

sensitivities in the region of 460-500nm and 520-560nm (Ekstrom and Meissl, 1997) or just 520-530nm by Meissl and Ekstrom (1988). In pike, it has been suggested (again dependent on conditions) that there are peaks at either approximately 550nm or two located in the regions of 620-640nm and 530-540nm (Falcon and Meissl, 1981). Related to this, the same authors have offered a chromatic response hypothesis that allows the distinction of dusk and dawn periods. It is proposed that short wavelength sensitive pigments, in the blue to ultraviolet wavelengths, cause an inhibitory response, while the longer wavelength sensitive pigments elicit a stimulatory response. As such, these two systems work as antagonists either within similar photoreceptor cells or as isolated groups that have a common neural synapse. As the wavelengths of natural light change in the twilight periods (i.e. dusk and dawn), this interaction provides the switching mechanism for stimulation. Similar models have been offered for other vertebrates including lizards and frogs (Ekstrom and Meissl, 1997).

The principal output of the pineal gland is the indoleamine hormone, melatonin, which, released into the cerebrospinal fluid and blood plasma, has a secretion profile that follows day length (*n.b.* the pineal is not the only source of circulating melatonin: both the retina and gastrointestinal tract have been demonstrated in different species to contribute to the levels recorded [Porter *et al.*, 1996; Bubenik and Pang, 1997; Ekstrom and Meissl, 1997; Bayarri *et al.*, 2003]). In the plasma of teleosts during the light phase, melatonin is maintained at basal levels but becomes elevated during the dark phase as melatonin is synthesised by the pineal (Gern *et al.*, 1978). The presence of this rhythm in cod has been demonstrated by Porter *et al.* (2000b). The rhythm is termed an A-type profile (Reiter, 1988) which is characterised by a delay after the start of the dark phase before melatonin rises to its peak towards the end of the dark phase (Figure 1.4). This report by Porter *et al.* (2000b), was the first of an A-type profile in teleosts (*n.b.* a

similar profile has recently been confirmed in a close relative, the haddock [Davie *et al* unpublished]). The authors provide a list of other teleosts, freshwater species and migratory salmonids, which have been studied and all show a C-type profile. This more common profile is characterised by a rapid rise in melatonin at the onset of the dark



Description of rhythm

Type A: Discrete peak in late dark phase

Species: Syrian Hamster
Mongolian Gerbil
House Mouse
Cod
Haddock

Type B: Discrete peak in mid dark phase

Species: Rat
Eastern Chipmunk
Turkish Hamster
Human
Nile Tilapia

Type C: Prolonged peak through the majority of the dark phase

Species: White - Foot Mouse
Djungarian Hamster
Domestic cat
Sheep
Atlantic salmon
Rainbow trout
Atlantic halibut

Figure 1.4: Diagrammatic representation of the different melatonin profiles recorded in vertebrates. Examples of species which express such patterns of plasma melatonin for each profile are listed. Horizontal black bar denotes subjective dark period. (Figure adapted from Reiter R.J. 1988)

phase to a maximum, which remains until the start of the light phase when there is a rapid fall back to the basal levels (Reiter, 1988). The significance of these different profiles is not at present well understood.

The synthesis and hence level of circulating melatonin has been shown to be influenced by both the period and intensity of light (Porter *et al.*, 2000a; Futter *et al.*, 2000; Porter *et al.*, 2001; Migaud, *et al.,* 2004a,b), as well as by other environmental variables like temperature (Ekstrom and Meissl, 1997; Bromage *et al.*, 2001; Porter *et al.*, 2001) and physiological variables like animal size (Porter *et al.*, 2000b). Furthermore, in all teleosts studied to date, with the exception of salmonids, the secretion of melatonin in the absence of an entraining photoperiod signal is maintained by the endogenous clock mechanism, a central component of the circadian axis outlined above (Iigo *et al.*, 1991; Bromage *et al.*, 1995; Cahill, 1996; Bolliet *et al.*, 1996; Ekstrom and Meissl, 1997; Okimoto and Stetson, 1999). Importantly, these diel rhythms also have a seasonal element to their expression, as the seasonal cycle in day length will be reflected by the diel rhythm of melatonin production. Winters have long periods of melatonin production while summers have short periods of melatonin production, as shown in Atlantic salmon (Randall *et al.*, 1995). Hence, the natural photoperiod cycle is transferred into a hormonal rhythm providing timing information on both daily and seasonal scales.

In mammals, it has been clearly established that melatonin acts in discrete hypothalamic areas of the brain to control seasonal reproduction (Malpaux *et al.*, 1997). Although numerous studies have been conducted, as yet there is little direct evidence of melatonin playing a role in the photoperiodic control of reproduction in teleosts (Ekstrom and Meissl, 1997; Mayer *et al.*, 1997). However, it continues to be measured as an assessment of the light perception in fish, especially in this field of aquaculture

research, to elucidate how an individual perceives an applied photoperiod manipulation (Section 1.6) and will be employed in such a manner in experiments presented in this thesis.

1.5 Endogenous regulation of sexual maturation in teleosts.

Sexual maturation in teleosts comprises a group of processes initiating and maintaining gametogenesis, spawning and gonadal recrudescence. As discussed above (Section 1.4), exogenous environmental signals (principally photoperiod) are interpreted by the circadian axis and entraining neuroendocrine signals (although unconfirmed at present, presumed to be based in the circulating melatonin signal) passed to the hypothalamus. Sexual maturation *per se* is under the direct regulation of the hypothalamic, pituitary and gonad axis (HPG axis, Redding and Patino, 1993: Figure 1.5). The hypothalamus, located at the base of the brain in close proximity to the pituitary gland, releases one or more of five forms of gonadotropin releasing hormone (GnRH: Kah *et al.*, 2000). These short oligopeptides are passed on to the pituitary, either through a specific blood supply or direct neural innervation originating in the hypothalamus and terminating in the pituitary (Redding and Patino, 1993), where they are believed to stimulate gonadotropin (GtH) release. Gonadotropins are larger glycoproteins produced by gonadotropic cells in the pituitary (Bromage, 1988) consisting of two subunits (α and β chains) both 100 amino-acid residues long. There are two distinct forms of GtH found in teleosts, as is the case with most other vertebrates (Redding and Patino, 1993), these are Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH). They perform separate functions and are found at different stages of the maturation process. FSH is found in the pituitary and blood of fish undergoing initial gonadal growth and gametogenesis while LH is predominant

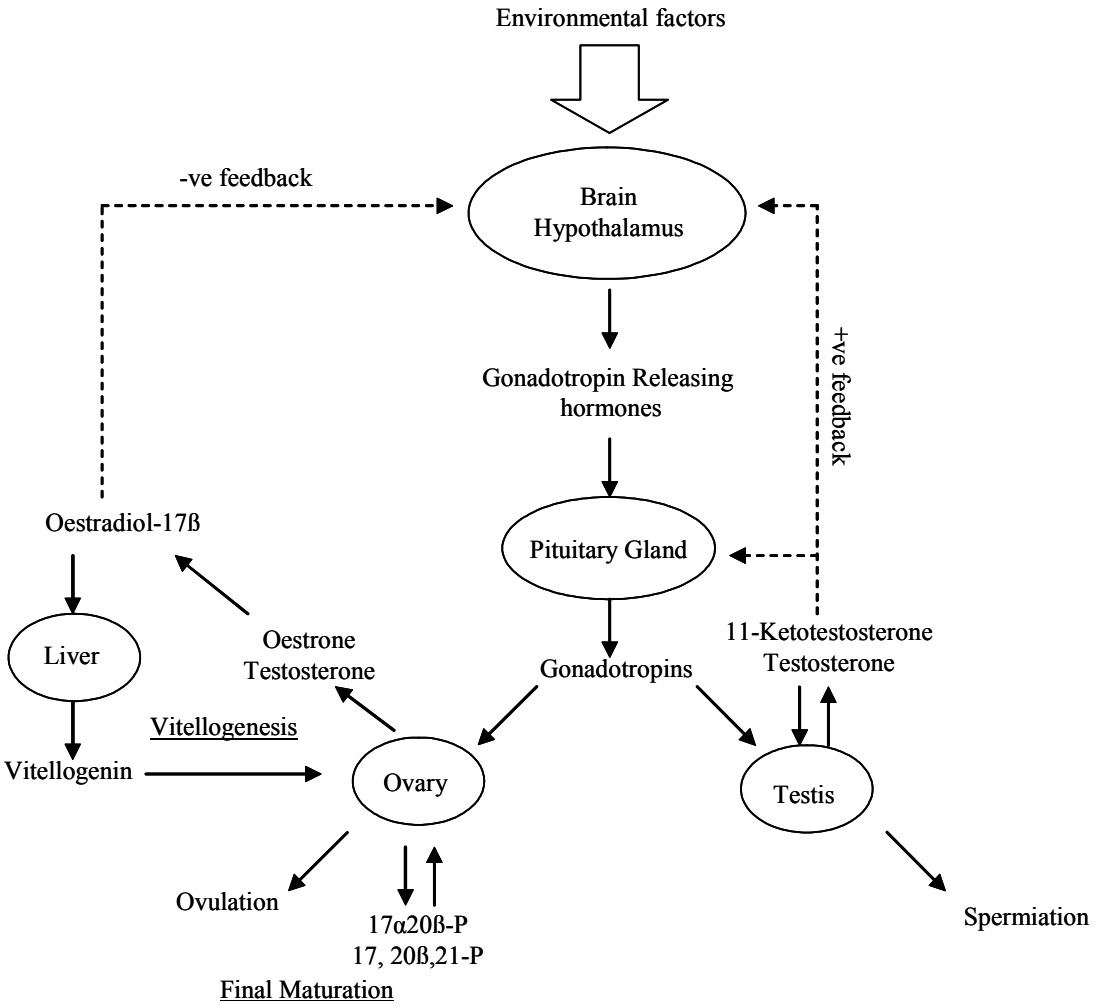


Figure 1.5: Outline of the neuroendocrine basis for the HPG axis in teleosts (adapted from Bromage, 1988).

during the final stages of reproduction, i.e. final oocyte maturation (FOM) and ovulation (Nagahama *et al.*, 1995). The gonadotropins are transferred in the bloodstream and stimulate specific receptors on the gonads.

Ovaries

In the females these gonadotropins stimulate the theca and granulosa cells of the ovarian follicle to secrete steroids (Redding and Patino, 1993), of which there is a wide array including the androgens (e.g. testosterone), oestrogens (e.g. 17β-oestradiol) and

progestagens (e.g. 17α-20β-dihydroprogesterone). Their presence is linked to the stage of ovarian development. Nagahama *et al.* (1995) summarised sex steroid production in two parts. During early ovarian growth and vitellogenesis, FSH stimulates the thecal cells of the oocyte follicle to produce testosterone that in turn is converted by the granulosa cells into oestradiol-17β. This stimulates the hepatic system to create vitellogenin (VTG) which is then passed through the blood system back to the ovaries and is sequestered by the growing oocytes. Calcium is an integral component of the vitellogenin complex acting as a blood carrier and hence, calcium levels rise concomitantly with those of vitellogenin (Elliot *et al.*, 1984; Norberg *et al.*, 1989). Norberg *et al.* (1995) showed the natural seasonal changes of oestradiol-17β and its precursor, testosterone (T), in female Atlantic cod in relation to seasonal cycles of maturation. At the end of vitellogenesis, oestrogen levels fall rapidly, removing their negative feedback on the hypothalamus and pituitary, causing a second surge of GtH's, this time LH. This stimulates final maturation through the secretion of progestagens (17α20ßP or 17,20ß,21-P) which act as maturation-inducing hormones (MIH) (Nagahama, 2000). However, the specific MIH in Atlantic cod remains undetermined (Kjesbu *et al*., 1996a). MIH receptors on the oocyte membranes release maturation promoting factors (MPF) into the cytoplasm which stimulate germinal vesicle migration (GVM) towards the micropyle, whereupon the germinal vesicle breaks down (GVBD) prior to hydration and subsequent ovulation. In Atlantic cod, each spawning interval represents the passage of a batch of eggs from the vitellogenic oocyte stage through final maturation to ovulation and consequently the levels of the various endocrine components remain elevated throughout the spawning season, as demonstrated by Kjesbu *et al.* (1996a).

Testis

In the testis, as with the ovaries, the two GtH's (FSH and LH) perform different functional roles with FSH being elevated during spermatogenesis while LH levels peak during spermiation. Two gonadotropin receptor types have been proposed, one specific to LH located on the Leydig cells and a second, showing affinity to both FSH and LH, has been localised to both the Leydig and Sertoli cells. The dual function of the testis is to produce spermatozoa and hormones, amongst which the sex steroids form a prominent group (Schultz and Goos, 1999). With both FSH and LH being steroidogenic (Schultz and Goos, 1999), gonadotropin stimulated testicular development is accompanied by elevated plasma levels of T and 11-Ketotestosterone (11-KT) (Borg, 1994). 11-KT is reported as being generally more effective than T in stimulating secondary sexual characters, reproductive behaviour and spermatogenesis (Borg, 1994). However, T plays a further important role in stimulating hypothalmic and pituitary activity, hence further stimulating testicular activity (Kah *et al.*, 2000). Norberg *et al.* (1995) have recorded peaks in plasma T in male cod prior to maturation, while Cyr *et al.* (1998) recorded peaks in plasma T and 11-KT at the same time as the gonadosomatic index (GSI) peaked in male cod.

Several of the parameters (testosterone and calcium) outlined in this brief overview have been monitored in experiments presented in this thesis to provide an assessment of the rate of sexual maturation under the various experimental conditions.

1.6 Artificial control of sexual maturation in teleosts.

The issue of maturation during production is not limited to the farming of cod, it is a common problem faced in many production systems. Hence, a number of control tactics have already been tried in other species. These have included ploidy

manipulation and direct or indirect sex reversal of stocks (Bromage 1988; Johnstone, 1993). However, the application of such techniques in cod culture would require extensive research investment and are not necessarily applicable or appropriate to the cod production system. This is principally because the established industry is reliant on mass spawning broodstock systems. Such a culture technique prevents the commercial application of ploidy manipulation by temperature or pressure shocking due to the techniques necessity of treating homogenous batches of eggs in very specific time frames (Johnstone, 1993). The production of single sex stocks could be attractive, however at present little production performance evidence exists to support the adoption of one sex over the other. As the industry develops, such evidence may be gathered however, in the meantime, there remains an extensive range of basic research to be performed before such a technique can be applied in cod. The most cost effective management technique which has the potential to provide solutions for the industry, in the short term, is the use of artificial photoperiod manipulation. As was explained above (Section 1.4), photoperiod is the natural *zeitgeber* that regulates many physiological processes in mid to higher latitude animals, including reproduction. The first demonstration of artificial control (hence management of maturation) was reported by Hoover (1937), who demonstrated that brook trout exposed to artificial seasonal cycles lasting less than one year spawned up to 4 months earlier than their ambient photoperiod siblings. Since then, similar findings have been demonstrated in a wide range of teleosts (for a review see Bromage *et al.*, 2001) including the Atlantic cod. Norberg *et al.* (1995) subjected groups of cod to seasonally changing photoperiod cycles adjusted from the norm by compression (i.e. a full cycle of photoperiod from winter solstice through summer back to winter solstice reduced to last less than 12 months e.g. 6 or 9 months) or extension (i.e. a full cycle extended to last more than 12

months e.g. 18 months). These authors demonstrated that these cod spawned naturally, reflecting the photoperiod cycle they were subjected to and that the steroid profiles were adjusted accordingly, i.e. photoperiod was entraining the endocrine system and so controlling the onset of spawning. In the most extreme situation, fish spawned only six months after their previous spawning season after experiencing a complete photoperiod cycle in that time. Van der Meeren (2001) also demonstrated that a delay of 6 months, i.e. an 18 month cycle, was possible with the use of an out of phase photoperiod.

In their simplest sense, seasonal photoperiod cycles could be viewed as the continuous gradual transition between long (summer) and short (winter) day lengths. Therefore, the use of constant day length photoperiods was first investigated in rainbow trout culture in the early 1980's. Combinations of short day (6 hours light:18 hours dark, 6L:18D) and long days (18L:6D) were able to control spawning, providing advanced and delayed spawning times (Bromage *et al.*, 1984). The change between treatments simulates abruptly the more gradual change perceived under natural conditions. Similar constant photoperiods have been used with success on a range of salmonids, including masu salmon (Takashima and Yamada, 1984), Atlantic salmon (Taranger *et al.*, 1998, 1999, 2000; Duncan *et al.*, 2000), brook trout (Henderson, 1963) and brown trout (Bromage *et al.*, 1990) as well as dace (Brook, 1988) and European seabass (Zanuy *et al.*, 1991). As part of these experiments some groups were subjected to continuous long or short days with no subsequent change. After an initial delay or advance depending on the photoperiod they were subjected to, these fish continued to spawn at approximately yearly intervals despite having no perceptible photoperiod changes. This is a suggestion of an endogenous *circannual* rhythm or "clock" that will cue spawning on a yearly cycle in the absence of changing photic information. This will be discussed further later (Section 3.6).

With regards to the entrainment of maturation cycles, the previous photoperiodic history is of great importance as this is what has governed development to a given point. Subsequent development is dependent on the developmental state at that stage in relation to the subsequent photoperiod administered. In order that an individual matures they must pass through a gate (Duston and Bromage 1987) or "window of opportunity" (Bromage *et al*., 2001) which represents a period of stimulatory photoperiod signal in conjunction with a suitable physiological state (either a threshold developmental or energetic level). Should either condition not be realized then sexual maturation will not proceed. Hence, the response of a fish to any given photoperiod manipulation clearly depends on whether it is applied during a period when the physiological condition is suitable. This explains why some photoperiod regimes will not affect maturation as the subjects are not in the correct state when the photoperiod window is open. It is through an understanding of this system that photoperiod manipulation can be applied to delay maturation. If a fish perceives a reproductively stimulating photoperiod but is not within the appropriate physiological status, then it will skip a spawning. i.e. delay by a year (if it is an annual spawner). An example of this was clearly demonstrated in Atlantic salmon (Taranger *et al.*, 1999). Commercial on-growing was realising a problem of early maturation after 1 year in sea cage rearing, termed "grilsing". Early maturation before harvest weight is achieved, led to loss of product quality and increased mortality. However, the authors demonstrated that the application of continuous light (LL) in the winter or early spring (January or March) would stop maturation during the following autumn in the majority of the experimental group (91:74 % maturing females:males under ambient photoperiod conditions compared to 67:57% F:M after March application and 9:16% F:M after January application). The proportion of fish that did not mature was related to the timing of the light application. Earlier application resulted

in fewer maturing individuals. This suggests that the light application effectively opened the reproductive gate earlier than in natural photoperiod groups and thus such fewer fish were in a suitable physiological state to mature and the majority would skip that year's spawning. Similar findings have been reported in rainbow trout (see review by Bromage *et al*. [2001]).

It was felt the employment of such tactics should provide the cod farming industry with the most rapid and cost effective farming strategy to regulate maturation and in turn improve their stock growth potential. The limited research to date in this field with cod is reviewed later (Section 3.1) and has served to inform the research strategy adopted in this thesis. What was apparent from the outset was the lack of understanding of the photoperiod signals that recruit individual cod to mature. Such knowledge would be required to define the timing and style of photoperiod management to be adopted in the species.

1.7 Experimental aims

At present, the profitable commercial production of Atlantic cod is inhibited by significant production loss associated with maturation during the grow out cycle. While it is felt that artificial photoperiod manipulation will be an appropriate management approach to counteract these losses, the processes by which environmental parameters (namely photoperiod) entrain and regulate maturation and subsequently growth in the species are poorly understood and these are investigated in this thesis where the overall aims are as follows:-

1.) To investigate the impact on maturation and growth of long term exposure to a continuous illumination photoperiod applied from the summer solstice prior to expected first maturation in a closed production system.

2.) To investigate the impact on maturation and growth of continuous illumination photoperiods applied from the summer solstice prior to expected first maturation in an open production system.
3.) To investigate the significance of timing of the application of phase shifted constant daylength photoperiod regimes as well as continuous illumination photoperiod regimes on first and subsequent maturation.
4.) To investigate the link between photoperiod treatment and somatic growth.
5.) To provide the aquaculture industry with optimised guidelines (specifically timing and duration) for the photoperiod inhibition of maturation.

In summary, the experiments outlined in this thesis aim to elucidate further the photoperiod mechanisms that regulate the important physiological processes of both maturation and growth in Atlantic cod, one of the most commercially important marine species in the northern hemisphere. This knowledge can then be applied to improve productivity of the rapidly expanding aquaculture sector which, in turn, will serve to reduce pressure on the wild stocks which are at present at perilously low levels.

CHAPTER 2: GENERAL MATERIALS AND METHODS

[*n.b. For a list of equipment and reagents used along with supplier details see Appendix I*]

2.1 Fish Husbandry

All animals were held in accordance with, and experiments performed under, the licensing of the Home Office Animals (Scientific Procedures) act (1986).

2.1.1 Maintenance

All fish used in experiments were from hatchery reared sources as described in the specific experimental methodology. In experiments I, III and IV animals were held in enclosed tank facilities where seawater, filtered to 60 microns, was supplied at a flow rate of approximately 50 $l.min^{-1}$ and drained to waste. Dissolved oxygen was monitored daily at midday, in each tank and additional aeration provided. Lighting was supplied by artificial illumination (detailed in specific methodology) and was checked each morning. Failed bulbs were replaced immediately. All lights were connected to digital timers (±1min) that were checked weekly and adjusted as required.

2.1.2 Anaesthesia

Experimental animals were anaesthetised prior to any regular handling or experimental procedure; all fish were starved for 24 hr prior to administration of anaesthetic. They were immersed in a bath of 1:10,000 concentration of 2-phenoxyethanol that induced loss of equilibrium within 3 minutes. Following the experimental procedure, the fish were placed in clean aerated seawater and allowed to fully recover, which was within 5 minutes, before being returned to their original housing.

2.1.3 Fish sacrifice

In experiment IV, where individuals were sacrificed prior to removal of tissue samples, fish were anaesthetised in a 1:10,000 solution of 2-phenoxyethanol and killed with a strong blow to the dorsal surface of the head such that death was instantaneous.

2.1.4 Blood sampling

Blood was collected from the caudal dorsal aorta using 1 ml or 2 ml syringes, dependent on the sample volume required, with 25G, 23G or 21G sterile hypodermic needles dependent on the size of the fish. All syringes and needles were flushed with a 4 $mg.ml^{-1}$ solution of heparin ammonium salt. In experiment III, where individuals were sampled on a monthly basis, sample volumes never exceeded 0.2% body weight (v/w). The blood samples were transferred into new Eppendorf tubes and stored on ice prior to centrifugation at 1200 g for 15 minutes at 4 ^{o}C. The plasma was then pipetted off, placed in new Eppendorf tubes and stored at –70 ^{o}C until analysis.

For blood samples withdrawn during the hours of darkness, fish were removed from their housing and placed in a light proof container with anaesthetic, under complete darkness. The fish were sampled where the only light source was a dim red light (670-800 nm) with an intensity of 0.0004 $W.m^{-2}$ (equivalent to 0.2 lux or 0.0021 $\mu moles.m^{-2}s^{-1}$) at 0.5 m.

2.1.5 Identification

When individual identification of fish was necessary (experiments III and IV), the fish had a passive integrated transponder tag inserted subcutaneously. The tag was inserted anteriorly via a 5 mm incision, 30 mm below the first fin ray of the dorsal fin. The tag was placed 30 mm in front of the initial incision, approximately 5-10 mm below

the skin. The incision was then coated in a 3:1 mixture of orahesive powder and cicatrin antibiotic. No mortalities were experienced following this procedure. Failure rate of the tags was less than 3%. The tags were read when required by passing an extended range reader within 10 cm of the inserted tag.

2.2 Growth assessment

Measurements of fish length and weight were made throughout the experiments. Once anaesthetised, total length was measured to the nearest millimeter and fish weighed to the nearest 0.1 g in all experiments except II where weight was measured to 10 g.

2.3 Maturation Assessment

2.3.1 Ultrasound scanning

The development of gonads was monitored non-invasively by ultrasound imagery using an adaptation of the techniques described in Karlsen and Holm (1994). A 7.5 MHz portable ultrasound scanner fitted with a submersible horizontal probe was used throughout. Anaesthetised fish were placed in a container of fresh seawater large enough to allow free movement of the fish. While under the influence of the anaesthetic the fish was held inverted and the scanning head was passed along the ventral surface of the fish starting at the uro-genital vent, moving slowly anteriorly. This technique allowed up to 3-5 minutes of investigation before the fish recovered from anaesthesia. There were no mortalities as a result of using this technique.

2.3.2 Visual Inspection

To record spawning time the fish were also examined for external indications of

maturation. First indications were a darkening of the skin and swelling of the abdomen. Prior to spawning time, the genital papilla became more pronounced in the male and allowed sexing of the fish (Plate 2.1, Figure 2.1). While anaesthetised, fish were checked for spawning by applying gentle pressure to the posterior end of the abdomen. Spawning time was defined as the time period when eggs or milt were freely released due to stripping.

2.3.3 Gross gonadal morphology

In experiment IV, gonads were removed and weighed to the nearest 0.01 g which was then expressed as a percentage of the whole body wet weight, referred to as Gonadosomatic index (GSI), as follows:

$$GSI = (\text{gonad weight (g)}/\text{whole body weight (g)}) \times 100$$

The gonad developmental stage was then assessed on a 5 point visual scale taken from (Morrison, 1990) a summary of which is shown in Table 2.1.

2.3.4 Histology

In experiment IV, the removed gonads were placed in an excess of 10% buffered formalin solution and stored for later analysis. This solution was prepared as follows:

disodium hydrogen phosphate	6.5 g
sodium dihydrogen phosphate	3.5 g
Formalin (40% formaldehyde)	100 ml
Deionised water	900 ml

The fixed gonad samples were embedded, sectioned, stained and mounted by Diagnostic Services, Institute of Aquaculture, University of Stirling, UK, using the following procedures:

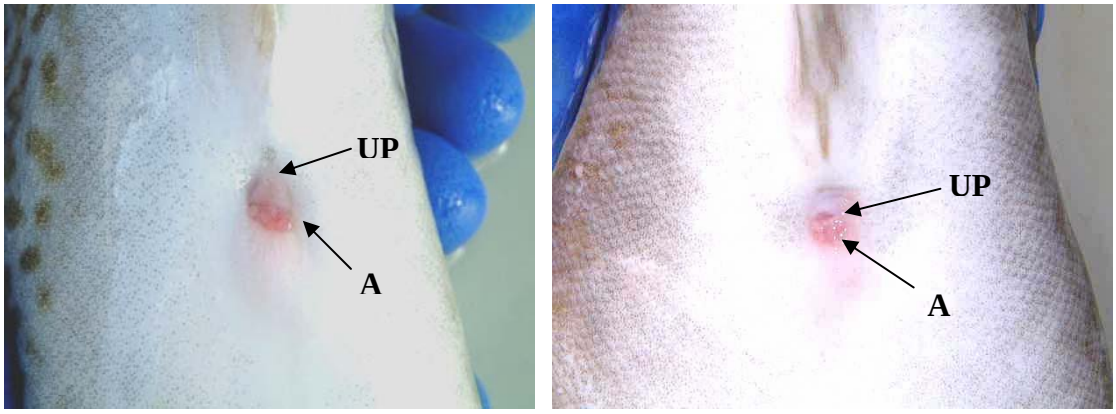


Plate 2.1: The anus (A) and urogenital papillae (UP) of male (left) and female (right) Atlantic cod during the spawning season showing the pronounced UP of the males.

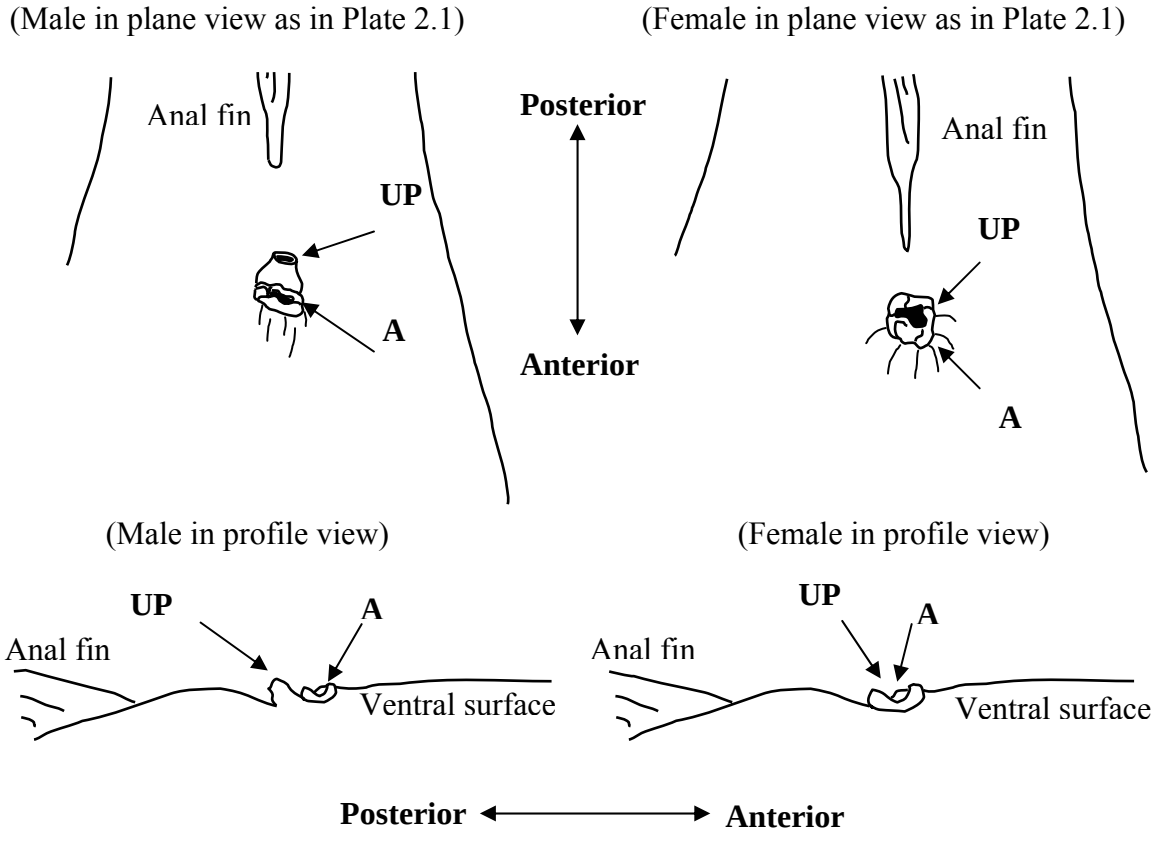


Figure 2.1: Line drawings of anus (A) and urogenital papillae (UP) of male (left) and female (right) Atlantic cod during the spawning season showing the pronounced UP of the males in plane view as in Plate 2.1 (above) and in profile view (below).

Table 2.1: Summary of gross morphology of reproductive stages of Atlantic cod gonads (taken from Morrison, 1990).

Reproductive stage	Female	Male
Stage 1 (Immature)	Ovary small; restricted to posterior part of body cavity. Translucent in small fish, more opaque in larger fish.	Testis small and translucent, flat, proximal to mesorchium, forming frill distally.
Stage 2 (Ripening 1)	Ovary becomes firm. Still small at beginning of stage, then starts to enlarge. Blood vessels visible.	Testis becoming larger and pink in colour.
Stage 3 (Ripening 2)	Ovary continues to enlarge, occupying about half the body cavity. Becomes opaque and cream in colour. Individual eggs can be seen with the naked eye, giving the ovary a granular appearance. Blood vessels prominent.	Testis occupies about half ventral cavity and beginning to turn white. In later part of stage proximal testis white, distal translucent.
Stage 4 (Ripe and Spawning)	Ovary fills most body cavity. Translucent as well as opaque eggs can be seen. Eggs easily released from vent at end of stage.	Testis white. Spermatozoa easily released from vent in later part of stage.
Stage 5 (Spent)	Ovary shrunken, soft and flabby with whitish cast.	Proximal testis white and flabby. Distal testis translucent border as ripe spermatozoa are removed.

Sectioning

Fixed gonad samples were dehydrated in a series of methylated spirits baths followed by ethanol prior to being 'cleared' in chloroform and then embedded in paraffin wax, as listed below:

1.	50% Methylated spirit	30 min
2.	80% Methylated spirit	90 min
3.	100% Methylated spirit	90 min (x3)
4.	100% Absolute ethanol	105 min, 90 min
5.	Chloroform	50 min (x2)
6.	Molten wax	105 min, 90 min (x2)

The embedded tissues were then cut in 5 μm thick sections using a Supercut automatic retracting microtone.

Staining

The sections were stained with Mayer's haematoxylin and eosin Y in a procedure modified from Bancroft and Stevens (1991) as follows:

1.	Xylene	3 min, 2 min
2.	Absolute ethanol	2 min
3.	Methylated spirit	1 min
4.	Wash in water	0.5 min
5.	Haematoxylin	5 min
6.	Wash in water	0.5 min
7.	1% Acid alcohol	3 quick dips
8.	Wash in water	0.5 min
9.	Scotts tap water substitute	1 min
10.	Wash in water	0.5 min
11.	1 part 1% Eosin (aq) to 8 parts Putts's Eosin	5 min
12.	Wash in water	0.5min
13.	Methylated spirit	1 min
14.	Absolute alcohol	2 min, 1 min
15.	Xylene	5 min

The slides were held in xylene until cover slipping using Pertex mountant. The mounted sections were examined using an Olympus BH-2 binocular microscope with images being captured using an Olympus zoom lens linked to a computer using image capture software.

2.4 Hormone Analysis

2.4.1 Radioimmunoassays

Plasma melatonin, testosterone and IGF-I were measured using the radioimmunonassay technique. The first radioimmunoassay (RIA) was developed to measure insulin in humans (Yalow and Berson, 1960) and since then the technique has become an essential tool for the accurate measurement of small concentrations of biologically potent compounds. An RIA is dependent on the coming together of an antigen and antibody to form the antigen – antibody complex, which after a period of incubation will settle at a level of equilibrium between both free (unbound) antigen/antibody and the bound antigen/antibody complex. The technique is dependent on determining the percentage of the total amount of antigen (free plus bound) that is present in the bound fraction. This amount is dependent upon three factors:

- It is directly related to the total amount of antibody present;
- It is directly related to the avidity with which the antibody binds the antigen and;
- It is inversely related to the total amount of antigen present.

In an RIA, the same concentration of the same antibody is present in each tube i.e. factors 1 and 2 are kept constant, so that the only factor that influences the percentage of bound antigen is the total amount present. In order to calculate the amount of the bound fraction, a fixed concentration of radioactively labelled antigen (label) is

also added to all tubes. The percentage of the total counts present in the bound fraction can then be determined and will accurately reflect, and be inversely related to, the total amount of antigen present. An unknown amount of the compound to be assayed can thus be quantified by comparing the distribution of the tracer with the distributions produced by a number of known standards. For further details of the basis for RIA techniques see Chard (1995).

2.4.2 Melatonin Assay

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

Assay Buffer

Fresh buffer was made the day before each assay and stored overnight at 4^{o}C prior to use. The following chemicals were dissolved at 50^{o}C for 30 minutes in 150 ml of nanopure water:

2.688 g tricine [N-tris(hydroxymethyl)methylglycine]

1.350 g sodium chloride

0.150 g gelatine

Radiolabel

A primary stock of tritiated melatonin, [*O-methyl-*^{3}*H*]melatonin, was supplied in 250 μCi quantities with a specific activity of 70-85 Ci.mmol^{-1}. This primary stock was used to create an intermediate stock by diluting 20 μl in 2 ml absolute ethanol. This was stored at –20^{o}C in 20 ml high performance glass vials. A fresh working stock was then made for each assay from this intermediate stock. The intermediate stock was diluted in

assay buffer to give an approximate activity of 4000 disintegrations per minute (dpm)/100 µl (this was approximately 21 µl intermediate stock in 10ml of assay buffer).

Antibody

Freeze dried sheep anti-melatonin antiserum was reconstituted in 2 ml of nanopure water. This was then aliquoted in 100 µl volumes into stoppered 3 ml polystyrene tubes and stored at –20^{o}C until used. The working solution was used by reconstituting one 100 µl aliquot in 20 ml of fresh assay buffer.

Standards

A stock solution of 1 mg.ml^{-1} of melatonin, N-acetyl-5-methoxytryptamine, was prepared by dissolving 10 mg of melatonin in 10 ml of absolute ethanol. This stock solution was stored in a high performance glass vial at –20^{o}C until required. For each assay two working solutions of 1 ng.ml^{-1} and 2 ng.ml^{-1} were prepared from this stock by dilution in assay buffer to provide the working standards for the preparation of the assay standard curve. The standard curve was prepared with a range from 500 pg per tube to 0 pg per tube in duplicate tubes as follows: 250 µl of the 2 ng.ml^{-1} standard were placed in the first pair (i.e. 500 pg per tube standard) followed by 250 µl of the 1 ng.ml^{-1} standard in the second pair (i.e. 250 pg per tube standard) and thereafter a 1:1 serial dilution was performed with fresh assay buffer to produce a range of paired standards from 125 pg per tube to 3.9 pg per tube (Figure 2.2).

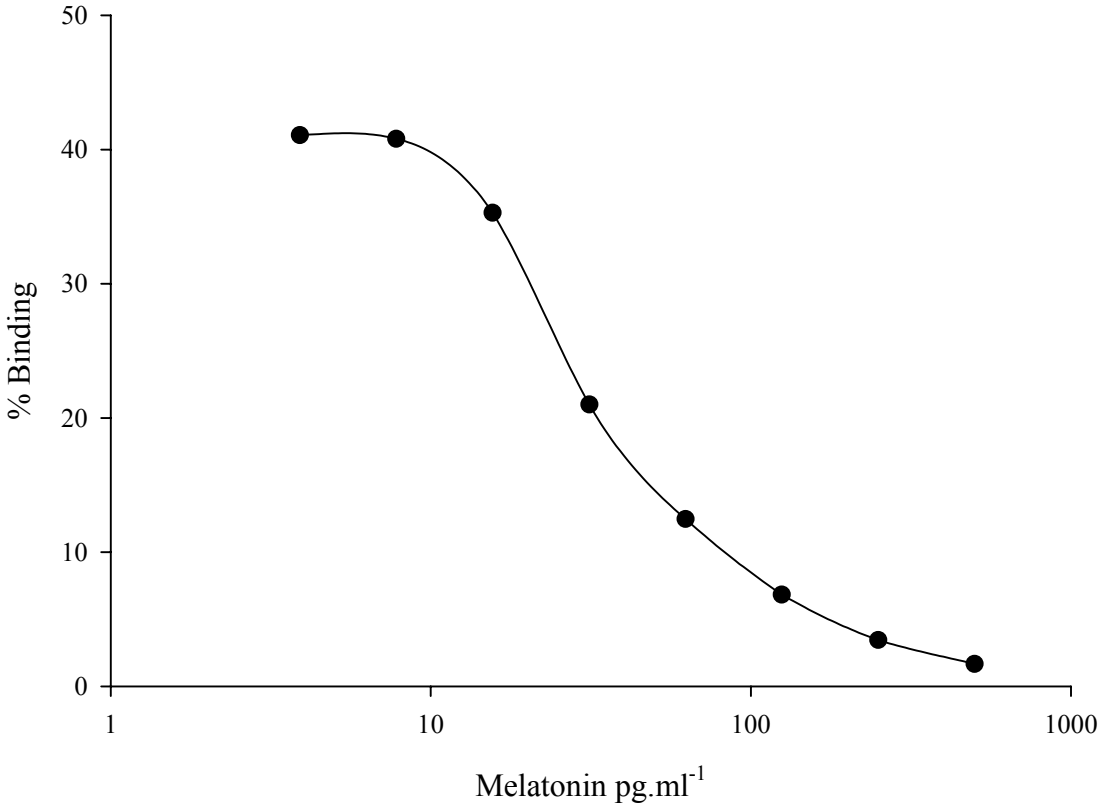


Figure 2.2: A typical standard curve obtained from a radioimmunoassay of melatonin. The hormone concentration within a sample is obtained from the intersect of the percentage binding in the sample.

Assay Protocol

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

1. Prepare a series of melatonin standards in 3 ml polystyrene tubes to give a range of dilutions from 0-500 pg / 250 µl. Then add a further 250 µl of assay buffer to these tubes.
2. Add 700 µl of buffer to two tubes which are used to calculate the non-specific binding (NSB) and add 500 µl of sample to sample tubes.
3. Add 200 µl of antibody to all tubes except the NSB's, vortex and incubate at room temperature for 30 minutes.

4. Add 100 µl of tritiated melatonin to all tubes, vortex and incubate at 4°C for 18 hr.
5. Dissolve 0.48 g of dextran coated charcoal in 50 ml of assay buffer and stir on ice for 30 minutes. Add 500 µl of this dextran/charcoal suspension to each tube, vortex and incubate at 4°C for 15 minutes.
6. Centrifuge at 1730 g for 15 minutes at 4°C.
7. Transfer 1 ml of supernatant to 6 ml polyethylene scintillation vials and add 4 ml of scintillation fluid.
8. Place 4 ml of scintillation fluid into 3 extra vials. Into 2 of these place 100 µl of tritiated melatonin to calculate total radioactivity. Use the final vial to calculate background radioactivity.
9. Vortex all vials and count the radioactivity for 10 minutes in a scintillation counter.

The unknown samples dpm values were compared to the standard curve dpm values and hence converted to pg melatonin per tube using Riasmart software. This value was subsequently converted to $pg.ml^{-1}$ plasma in an Excel spreadsheet using the following formula:

$$\left[\left(\frac{pg\ \ per\ tube}{1000}\right)\times 1300\right]\times 2 = \text{ pg.ml}^{-1} \text{ of melatonin in plasma}$$

Quality Control & Validation

The sensitivity of the assay, i.e. the minimum amount of melatonin statistically distinguishable from zero, was 3.9 pg per tube. Aliquots of pooled plasma with a melatonin content of approximately 200 $pg.ml^{-1}$ stored at –70°C, were used to check the reproducibility of measurements both within and between assays. The intra-assay coefficient of variation was 5.6% (n=4) and the inter-assay coefficient of variation was

8.9% (n=8). Serial dilutions of pooled Atlantic cod plasma were used to obtain inhibition curve (Figure 2.3). When plotted against the standard curve it was observed that both curves were parallel and no statistical difference in the gradient was found (ANOVA), indicating that the samples were immunologically similar to the standards. A fuller description of the validation of this assay for the detection of melatonin in cod plasma has been reported by Porter *et al.* (2000b).

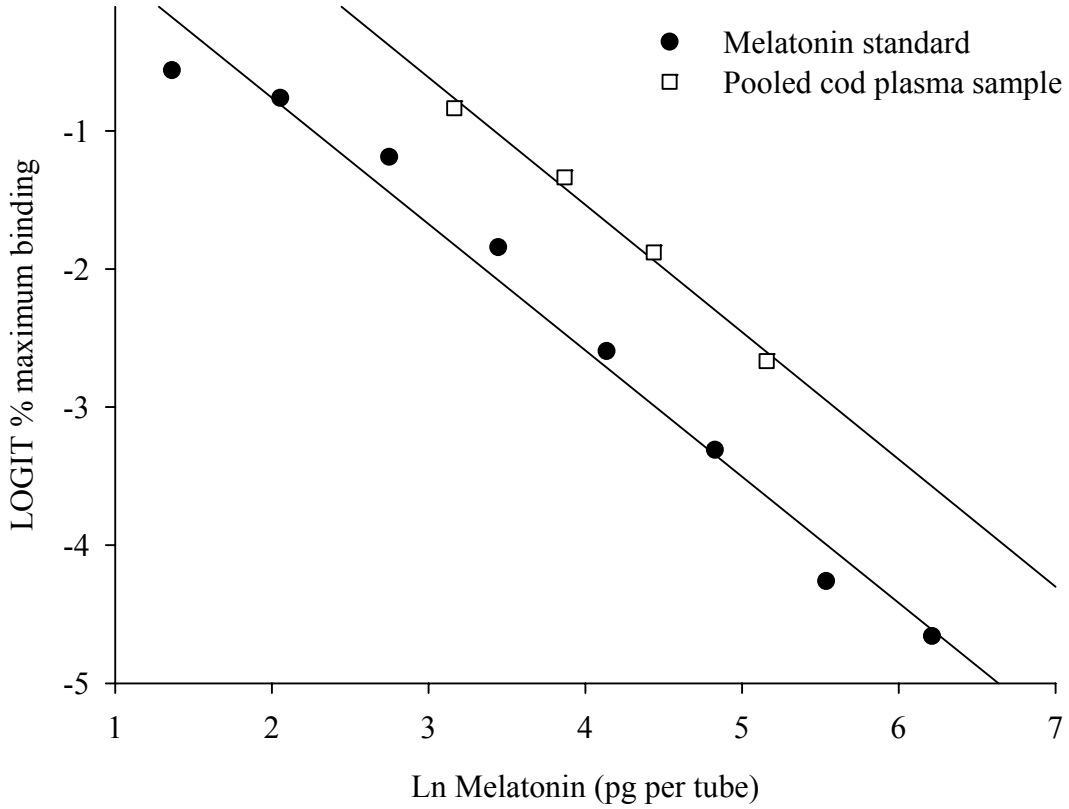


Figure 2.3: The parallelism of the inhibition curves obtained from the melatonin standard and a serial dilution of cod plasma. Each point represents the mean of duplicate measurements. The x-axis denotes the natural logarithm of the melatonin content in the standards.

2.4.3 Testosterone Assay

Plasma samples were analysed for testosterone using a protocol adapted from Duston and Bromage (1987) as follows:

Assay Buffer

Fresh buffer was made the day before each assay and stored overnight at 4°C prior to use. The following chemicals were dissolved at 50°C for 30 minutes in 250 ml of nanopure water:

4.44 g disodium hydrogen phosphate

2.91 g sodium dihydrogen phosphate

2.25 g sodium chloride

0.25 g gelatin

Radiolabel

A primary stock of tritiated testosterone, [1,2,6,7-^{3}H]testosterone, was supplied in 250 μCi quantities with a specific activity of 70-105 Ci.mmol^{-1}. This primary stock was used to create an intermediate stock by diluting 20 μl in 2 ml absolute ethanol. This was stored at –20°C in 20 ml high performance glass vials. A fresh working stock was then made for each assay from this intermediate stock. The intermediate stock was diluted in assay buffer to give an approximate activity of 20,000 dpm/100 μl (50 μl intermediate stock in 10 ml of assay buffer).

Antibody

Freeze dried rabbit anti-testosterone antiserum was reconstituted in 1 ml of assay buffer mixed and 100 µl aliquots were transferred into stoppered 3ml polystyrene tube and stored at –20^{o}C until used. The working solution was prepared by reconstituting one 100 µl aliquot in 9.9 ml of fresh assay buffer.

Standards

A stock solution of 100 ng.ml^{-1} of testosterone was prepared by dissolving 1 mg testosterone in 10 ml of absolute ethanol. This intermediate solution was stored in a high performance glass vial at –20^{o}C until required. A working solution of 10 ng.ml^{-1} was prepared by diluting 100 µl of intermediate stock in 0.9 ml of absolute ethanol. A standard curve was prepared from the working standard solution with a range of standards from 1000 pg per tube to 1.95 pg per tube. The 1000 pg per tube standard was prepared from 100 µl of the working solution with all subsequent standards being prepared from a series of 1:1 serial dilutions (Figure 2.4).

Steroid Extraction

Prior to assaying the testosterone must be extracted from the plasma samples by suspending the organic phase in a solvent (ethyl acetate) as follows:

1. Add 200 µl of plasma sample to 3ml polypropylene tubes.
2. Add 1 ml ethyl acetate to each tube and stopper.
3. Spin tubes on a rotary mixer for 1 hr.
4. Centrifuge tubes at 430 g for 10 minutes at 4^{0}C.

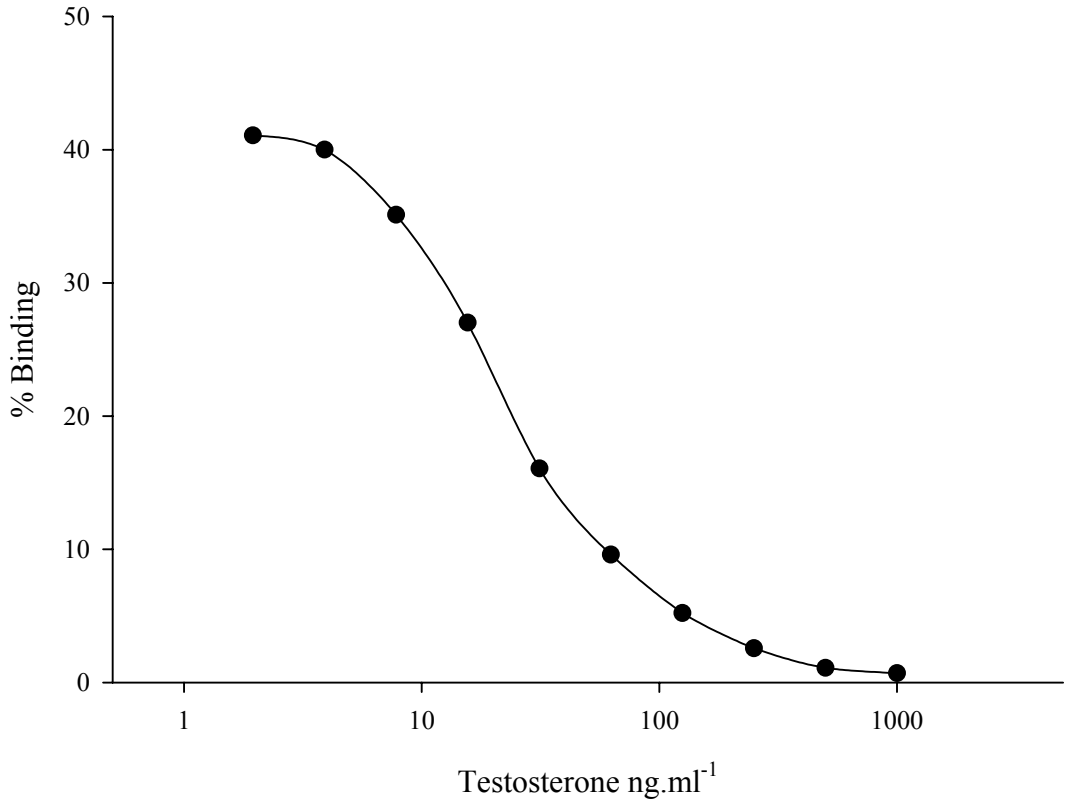


Figure 2.4: A typical standard curve obtained from a radioimmunoassay of testosterone. The hormone concentration within a sample is obtained from the intersect of the percentage binding in the sample

5. Remove and store supernatant (containing organic phase) in clean tubes at 4^0C until to be used for assay.

Assay Protocol

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

1. Add 200 µl extract to each 3 ml polypropylene tube.

2. Prepare standard curve as listed above creating a range of standards from 0-1000 pg/100 µl in 3 ml polypropylene tubes. Include two tubes containing only 100 µl absolute ethanol which are used to calculate non-specific binding (NSB's).
3. Dry down all tubes in a vacuum oven at less than 35^0C (approximately 45 minutes), then cool to 4^0C.
4. Add 100 µl antibody solution to all tubes except NSB's (to these add 100 µl of assay buffer).
5. Add 100 µl of ^{3}H-Testosterone solution, to all tubes.
6. Vortex and incubate at 4^0C for 18 hr.
7. Dissolve 0.48 g of dextran coated charcoal in 100 ml of assay buffer and stir on ice for 30 minutes, add 500 µl of the dextran/charcoal suspension to each tube, vortex and incubate at 4^oC for 15 minutes.
8. Centrifuge at 770 g for 15 minutes.
9. Transfer 400 µl of supernatant to 6 ml polyethylene scintillation vials and add 4 ml of scintillation fluid.
10. Place 4 ml of scintillation fluid into three extra vials. Into two of these place 100 µl of tritiated testosterone to calculate total radioactivity. The final vial is used to calculate background radioactivity.
11. Vortex all vials and count the radioactivity for 10 minutes in a scintillation counter.

The unknown sample, dpm values, were compared to the standard curve dpm values and hence converted to pg testosterone per tube using Riasmart software. This value was subsequently converted to pg.ml^{-1} testosterone in plasma in an Excel spreadsheet using the following formulae:

Step 1.) $\left[\left(\frac{pg\ \ per\ tube}{400}\right)\times 700\right]$ = pg of testosterone per 200 µl of extract (Equation 1)

Step 2.) $\left[\left(\frac{Equation\ 1}{200}\right)\times 1200\right]\times 5$ = pg .ml^{-1} of testosterone in plasma

Quality Control & Validation

The sensitivity of the assay i.e. the minimum amount of testosterone statistically distinguishable from zero, was 1.95 pg per tube. Testosterone standard, stored in absolute ethanol at -20^{o}C at a concentration of 94pg.ml^{-1}, was used to check the reproducibility of measurements both within and between assays. The intra-assay coefficient of variation was 7.7% (n=4) and the inter-assay coefficient of variation was 14.4% (n=80). Serial dilutions of pooled Atlantic cod plasma were used to obtain an inhibition curve (Figure 2.5). When plotted against the standard curve, it was observed that both curves were parallel to the standard curve and no statistical difference in the gradient was found (ANOVA), indicating that the samples were immunologically similar to the standards.

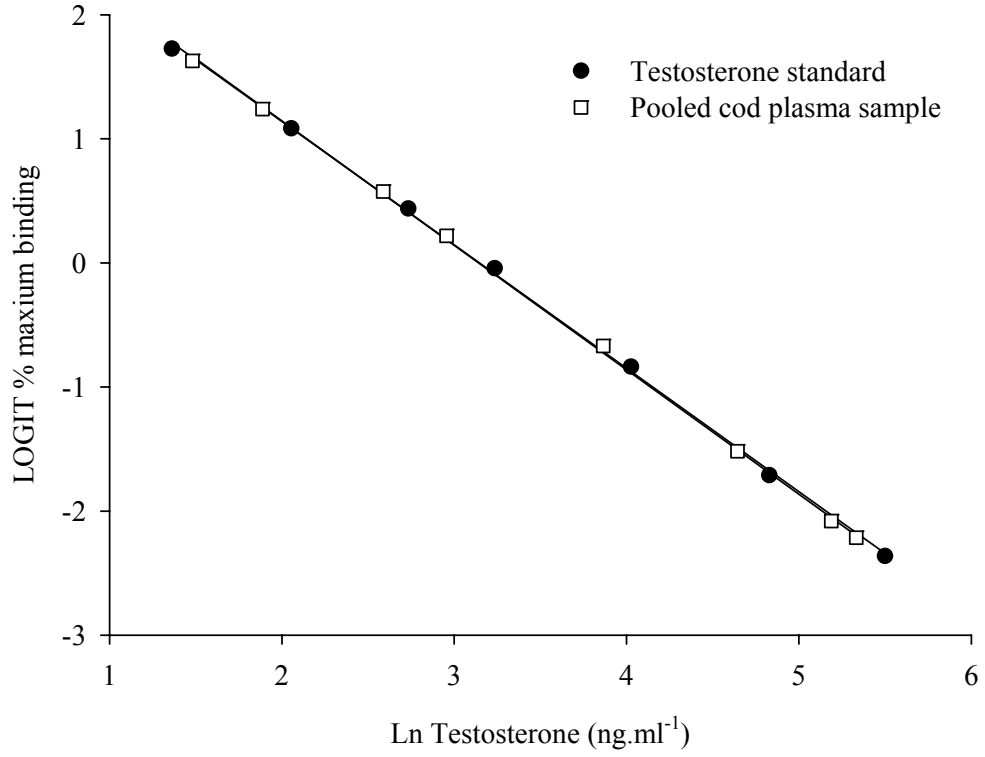


Figure 2.5: The parallelism of the inhibition curves obtained from the testosterone standard and a serial dilution of cod plasma. Each point represents the mean of duplicate measurements. The x-axis denotes the natural logarithm of the testosterone content in the standards.

2.4.4 IGF Assay

Plasma samples were analysed for total IGF-I using a commercially available RIA kit which has recently been validated for the measurement of fish IGF-I (Dyer *et al.*, 2004b). The protocol (Appendix II) can be summarised as follows:

Assay Buffer/ Radiolabel/ Antibodies/ Standards

Unless otherwise stated, all constituents were provided in predetermined quantities requiring dilution or rehydration with nanopure water or RIA buffer to set volumes as outlined in the manufacturers' protocol. Assay buffer was reconstituted with nanopure water and pH adjusted if necessary to pH 7.5 with 5 M HCl. ^{125}I-Fish IGF-I

tracer was reconstituted in RIA buffer with an approximate activity of 20,000 cpm/50 µl and stored at -20°C. Antibodies 1, 2 and the IgG were reconstituted to 1/85.7, 1/20 and 1/20 dilutions respectively in RIA buffer. Lyophilised, recombinant barramundi IGF-I standard, stored at 4°C was reconstituted overnight at 4°C in 1.5 ml RIA buffer to create a stock solution of 70 ng.ml^{-1}. This was diluted in 9 serial, 1/3 dilutions in RIA buffer to provide a standard range from 0.0036-70 ng.ml^{-1} (Figure 2.6).

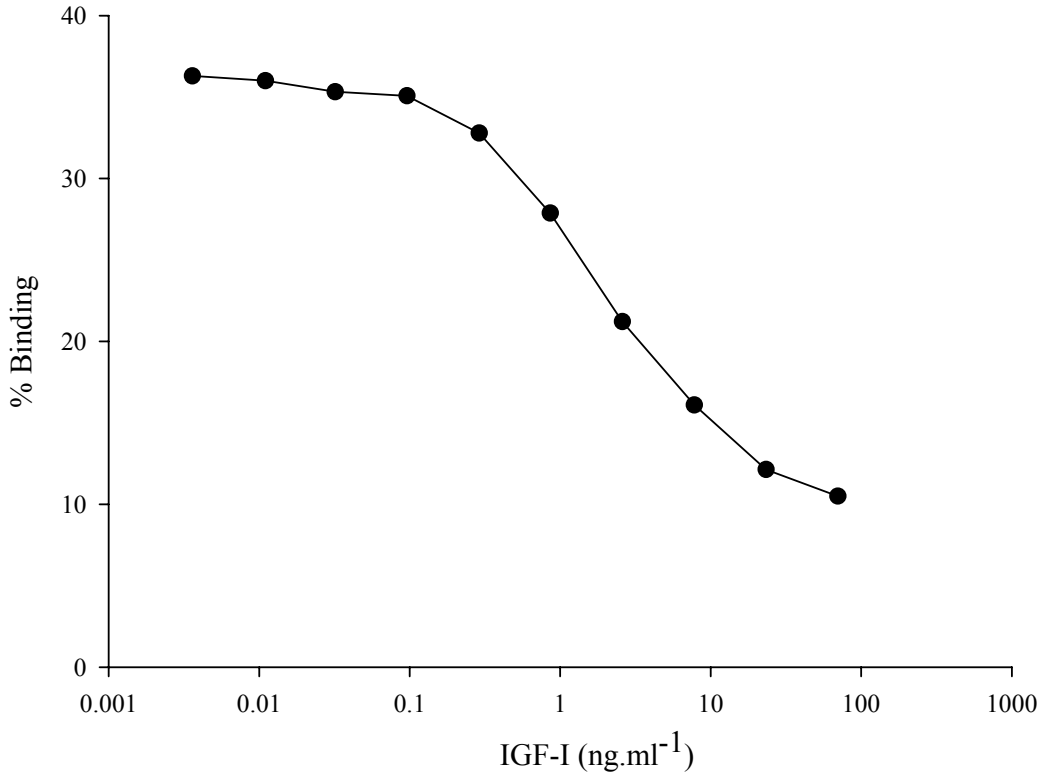


Figure 2.6: A typical standard curve obtained from a radioimmunoassay of IGF-I. The hormone concentration within a sample is obtained from the intersect of the percentage binding in the sample

Acid ethanol extraction

All plasma samples and quality control samples (QC: reference samples provided by manufacturer containing a predetermined quantity of IGF-I) require acid ethanol extraction prior to analysis. This extraction was necessary to remove IGFBP's so that total IGF-I content could be measured. The extraction was performed as follows:

1. Acid ethanol extraction mix: carefully add 62.5 ml of 2 M HCl to 437.5 ml of absolute ethanol. Mix gently and, when cool, transfer to a sterile 500 ml bottle and store at -20^{o}C.
2. Place 40 µl of plasma or QC in a clean 1.5 ml Eppendorf tube and add 160 µl of acid ethanol extraction mix.
3. Vortex and incubate at room temperature for 30 min.
4. Add 80 µl of 0.855 M tris(hydroxymethyl)methylamine (Tris) and vortex.
5. Centrifuge at 10,000 g for 10 min at 4^{o}C.

Samples were assayed immediately following extraction.

Assay Protocol

1. Into duplicate, 3 ml, borosilicate glass tubes add 50 µl of acid ethanol extracted sample/QC or to the standard, reference and blank tubes add 50 µl of acid ethanol blank solution [A/E blank solution = 1ml RIA buffer, 4 ml acid ethanol extraction mix and 2 ml 0.855 M Tris solution].
2. Add 200 µl of the appropriate standard to the standard tubes.
3. Add 200 µl of RIA buffer to the sample, QC and reference tubes and 250 µl to the two blank tubes.
4. Add 50 µl of antiserum-1 to all tubes except blanks and totals.

5. Add 50 µl diluted radiolabel to all tubes.
6. Vortex all tubes and incubate at 4°C overnight.

   (For optimal performance ensure that all following steps are performed on ice.)
7. Add 50 µl of diluted antiserum-2 and 10 µl of diluted IgG to all tubes except 'totals'.
8. Vortex all tubes and incubate at 4°C for 30 min.
9. Add 1 ml of cold (4°C) polyethylene glycol (PEG) solution to all tubes except 'totals' and vortex.
10. Centrifuge all tubes (except totals) at 3000 g for 20 min at 4°C.
11. Remove supernatant and count radioactivity using a Gamma Counter.

The unknown sample, dpm values, were compared to the standard curve dpm values and hence converted to pg of IGF-I per ml plasma using "Assayzap" software for the Apple MacIntosh.

Quality control and Validation

The sensitivity of the assay, i.e. the minimum amount of IGF-I statistically distinguishable from zero, was 3.6 pg per tune. QC standards (provided by the manufacturer) with a content of approximately 50 $ng.ml^{-1}$ were used to check the reproducibility of measurements both within and between assays. The intra-assay coefficient of variation was 6.5% (n=2) and the inter-assay coefficient of variation was 18.4% (n=16). Serial dilutions of pooled Atlantic cod plasma were used to obtain an inhibition curve (Figure 2.7). When plotted against the standard curve it was observed that the curve was parallel to the standard curve and no statistical difference in the

gradient was found (ANOVA), indicating that the samples were immunologically similar to the standards.

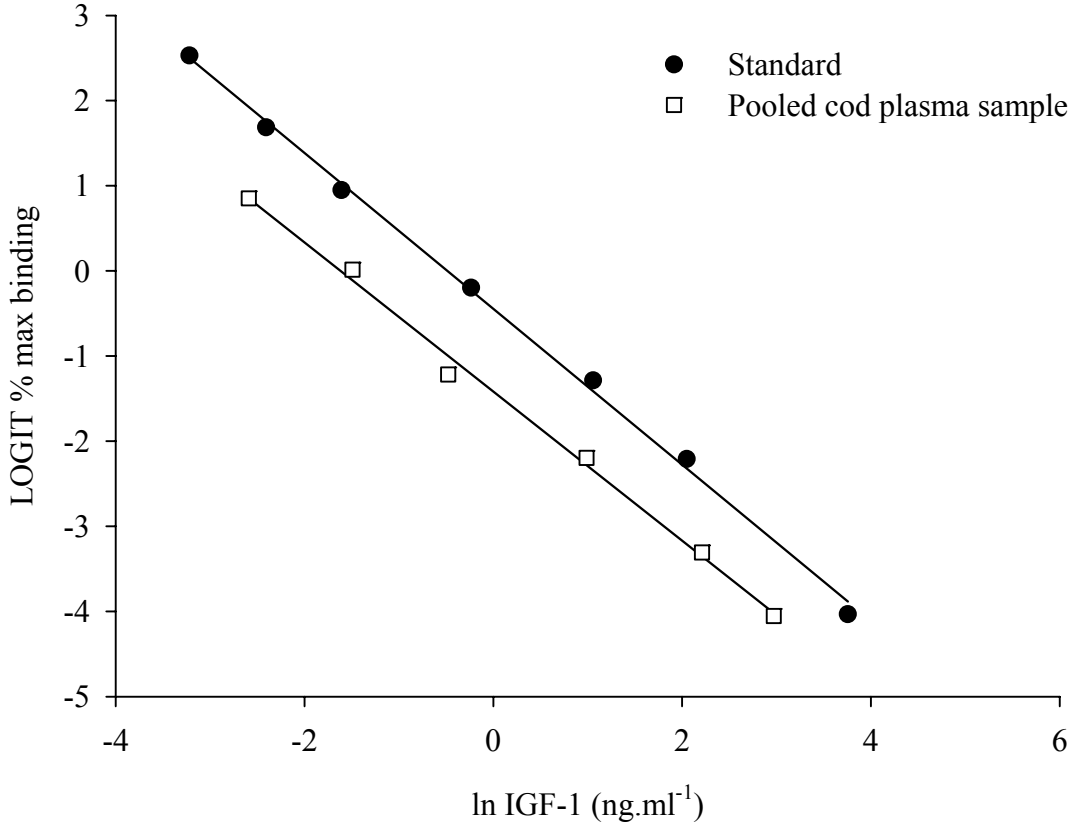


Figure 2.7: The parallelism of the inhibition curves obtained from the IGF-I standard and a serial dilution of cod plasma. Each point represents the mean of duplicate measurements. The x-axis denotes the natural logarithm of the IGF-I content in the standards.

2.5 Calcium Measurement

The protocol described below measures total unbound and bound calcium in plasma; respectively these equate to normal basal levels of calcium and the calcium bound to vitellogenin (Elliot *et al.*, 1984), therefore giving a total value. Plasma calcium levels were determined using atomic absorption, flame spectrophotometry.

Diluent solution

A 1% (v/v) Nitric acid solution was made by mixing concentrated nitric acid with deionised water. To this 1% nitric acid solution, 1% (w/v) lanthanum chloride was added. The lanthanum chloride is added to prevent interference from non calcium ions while aspiration is taking place in the spectrophotometer.

Standards

Two calcium standards were prepared by diluting a 1000 $mg.l^{-1}$ stock standard of calcium chloride with diluent. Fresh standards were prepared for each run. Standards of 2 $mg.l^{-1}$ and 4 $mg.l^{-1}$ were prepared:

2 $mg.l^{-1}$: 400 µl stock standard was made up to 200 ml with diluent.

4 $mg.l^{-1}$: 800 µl stock standard was made up to 200 ml with diluent.

Sample Dilutions

The sample dilutions used depended on the amount of calcium expected in the sample which is directly related to the stage of vitellogenesis. Because of this wide seasonal variation, a range of dilutions of samples were prepared with diluent from 1:150 to 1:500 (v/v).

Measurement of plasma calcium levels

Samples were measured in duplicate. The samples were aspirated in an atomic absorption spectrophotometer with readings taken at 423 nm (slit width set at 0.7 nm) through an oxidising flame using an air-acetylene gas mixture. Measurement drift was monitored by testing the prepared standards. The spectrophotometer was recalibrated if the reading drift exceeded 2.5%.

2.6 Proximate compositional analysis

Tissue samples (muscle and liver) in experiment IV were taken for protein and/or lipid determination. Both muscle and liver samples were taken from standardised locations. The muscle was sampled in line with the first ray of the dorsal fin above the lateral line on the left side (looking towards the anterior end of the dorsal profile). The liver samples were taken from the common anterior mass of the liver structure. Samples were stored at -20^{o}C until analysis.

2.6.1 Moisture content

Both protein and lipid levels were initially calculated as a percentage of dry weight. Hence, approximately 15 g samples in triplicate were weighed to nearest 0.1 g and dried to constant mass (approx. 24 hr) in a drying oven held at 100^{o}C. Dried samples, once weighed to the nearest 0.1 g, were ground into a coarse powder using a mortar and pestle and then stored in air tight containers prior to analysis.

2.6.2 Lipid analysis

Lipid determination was performed by the soxhlet extraction method using a Soxtec HT 6 extraction unit as follows:

1. Approximately 1-3 g of dried sample was weighed into an extraction thimble and the extraction thimbles were fitted to the Soxtec unit.
2. An extraction cup containing 5 glass beads was weighed to the nearest 0.001 g.
3. 50 ml of petroleum ether was added to each extraction cup and the cups were fitted to the Soxtec unit.
4. The extraction thimbles were lowered into the boiling petroleum ether for 20 min.
5. The thimbles were then rinsed for 1.5 hr after which the petroleum ether was evaporated from the extraction cup for 15 min.
6. The extraction cup was placed into an oven for 1hr at 100^{o}C.
7. The cooled extraction cup was then re-weighed to the nearest 0.001 g.
8. Percentage dry weight lipid was calculated as follows:

   % lipid = (extracted lipid weight/sample weight) x 100
9. These values were then converted to % wet weight lipid content as follows:

   % wet wt lipid = (% dry wt)/100 x % dry weight lipid

2.6.3 Protein analysis

Protein determination was performed by the Kjeldahl analysis technique using a Tecator Kjeltec system as follows:

1. Place dried weighed samples (approx 200 mg) into a digestion tube.
2. Include 3 standard tubes containing 1 urea standard tablet (50 mg) and 3 blank tubes.
3. To each tube add 2 mercury Kjeltabs and 5ml concentrated sulphuric acid.
4. Place sample tubes in digestion block at 400^{o}C for 1 hr with a vacuum extractor hood in a fume cupboard.
5. Remove tubes from digestion block and allow to cool for 30 min in fume cupboard.

6. To each tube add 20 ml of deionised water and 5 ml sodium thiosulphate and vortex to mix.
7. Distill tubes using Kjeltec Autoanalyser.

Protein content was calculated as follows:

$$\text{Protein content (\%)} = \frac{(sample \quad titre - blank \quad titre) \times 1750.875 *}{sample \quad weight\ (mg)}$$

* = Multiplication factor to convert titre vol. to % protein based on standardised protein factor.

2.7 Analytical calculations

2.7.1 Condition Factor

Condition factor can provide an indication as to the "relative well being" of the individual fish or group by comparing the weight to the length. Although a number of formulae have been proposed (Bolger and Connoly, 1989), Foulton's condition factor (K) (Foulton, 1911) is the most consistently applied in the literature. In studies in Atlantic cod, K has been demonstrated to accurately reflect energetic reserves (Lambert and Dutil, 1997) which have in the past been used to predict likelihood of survival (Dutil and Lambert, 2000). K presumes isometric growth, i.e. growth with unchanged body proportions with the length-weight relationship having a regression coefficient of 3, which is not always the case (Bolger and Connoly, 1989). Hence prior to use, the length to weight regression coefficient of all data sets was checked following which, K was calculated from the measured length and weight of individuals as follows:

$$\text{Condition factor (K)} = \frac{Weight\ (g) \times 100}{Total\ length\ (cm)^3}$$

2.7.2 Hepatosomatic index

In experiment IV livers were removed and weighed to the nearest 0.01 g (Model: BFS-242-020C, Sartorius, UK). The liver weight was then expressed as a percentage of the whole body wet weight referred to as hepatosomatic index (HSI) as follows:

$$\text{HSI} = \frac{Liver\,weight\,(g)}{Whole\,body\,weight\,(g)} \times 100$$

2.7.3 Specific Growth Rate

The rate of growth, be it in weight or length, over a given time period was expressed as Specific Growth Rate (SGR) using the following calculations:

$$\text{SGR}_{\text{weight}} = \frac{Ln(weight_{end}) - Ln(weight_{start})}{Time\,(No.of\ days)} \times 100$$

or

$$\text{SGR}_{\text{length}} = \frac{Ln(length_{end}) - Ln(length_{start})}{Time\,(No.of\ days)} \times 100$$

2.7.4 Thermal Growth Coefficient

The rate of growth, weight gain (g) per degree day, was expressed as Thermal Growth Coefficient (TGC) using the following calculation:

$$\text{TGC}_{\text{weight}} = \frac{\left(\sqrt[3]{weight_{end}} - \sqrt[3]{weight_{Start}}\right) \times 1000}{degree\ days}$$

As with condition factor, TGC assumes the length to weight relationships has a regression coefficient of 3 (Jobling, 2003). In all cases where TGC was used, the

regression coefficient of length to weight for all data was calculated to ensure this assumption was not violated allowing for cross comparison of data.

2.8 Statistical Analysis

The statistical techniques applied within this thesis are described in Sokal and Rohlf (1995) and Zar (1999). The majority of calculations were performed using the Minitab™ statistical package (release 13.1). Where statistical analyses were calculated by hand, Microsoft® Excel 2002 was used to aid data manipulation.

2.8.1. Estimation of the mean

The arithmetic or sample mean, ($\overline{X}$) was used to provide an estimation of the populations mean μ.

Arithmetic mean $\overline{X} = \frac{\sum X}{n}$ Where $\sum X$ = the sum of observed samples

n = the number of observations

Arithmetic mean was presented in association with the standard error of the mean (SEM) unless otherwise stated ± 1 SEM, to give a representations of the sample distribution.

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

$$\text{Where } s = \text{sample standard deviation} = \sqrt{\frac{\sum X^2 - \frac{\left(\sum X\right)^2}{n}}{n-1}}$$

2.8.2 Coefficients of variation

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

$$\text{CV} = \frac{(s \times 100)}{\overline{X}}$$

2.8.3 Parametric assumptions

In order that parametric analytical techniques can be applied, some fundamental assumptions must be adhered to. Observations must be taken at random and their variance must be independent. Furthermore, they require the data to be normally distributed (Gaussian distribution) and with homogenous variance. Therefore all data were examined for normality and homogeneity of variance before further statistical analyses were performed.

2.8.4 Testing for normality and homogeneity of variance

Where General Linear Models were performed (section 2.8.6) *n* was typically large enough to allow normality and homogeneity of variance to be assessed through careful scrutiny of the plots of the residuals. In situations where *n* was inadequate to allow this or where other statistical techniques were utilised the preliminary analysis was performed as follows:

Normality

To assess normality of distribution the Kolmogrov-Smirnov test was applied. This test quantifies the discrepancy between the distribution of the experimental data and an ideal Gaussian distribution.

Homogeneity of variance

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

$$F_s = \frac{S_1^2}{S_2^2}$$

Where S_1^2 and S_2^2 are the greater and lesser variances respectively.

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

If the calculated value of F was less than the tabulated value of F at p = 0.05, it was concluded that the variance was homogenous, while if greater it was concluded that it was heterogeneous.

2.8.5 Comparison of two samples

When two sample popualtions were normally distributed and the variances were homogeneous, then means were compared by a Students' t-test which utilised a pooled estimation of the variance when they were homeogeneous, however a separate estimate of each variance was incorporated when the variances were heterogeneous:

$$t = \frac{\overline{X} - \mu}{\sigma / \sqrt{n}}$$

Where μ is the sample mean

σ is the variance

$\overline{X}$ is the variable to be tested

2.8.6 Multiple Comparisons

All parametric tests which compared three or more samples were performed using the analysis of variance technique (ANOVA). Where appropriate, these

calculations were manipulated by the use of General Linear Models (GLM). Model formulae which take into account numerous factor levels including replication and repeated measures sampling were constructed.

For post-hoc multiple comparisons, Tukey tests were used in all significant factor/interaction levels. This method utilises the pairwise comparison of group means to give the test statistic *q* as follows:

$$q = \frac{\overline{X}_B - \overline{X}_A}{\sqrt{\frac{s^2}{2}\left(\frac{1}{n_A} + \frac{1}{n_B}\right)}}$$

Where $\overline{X}_A$ and $\overline{X}_B$ are sample means

n_A and n_B are the number of observations in each sample

s^2 is the error mean square (calculated by the ANOVA)

If the calculated *q* value was greater than the tabulated value when p = 0.05, the means of the two samples were considered to be significantly different.

2.8.7 Analysis of proportions

Where differences in proportions were to be investigated, the 95% confidence limits were calculated for the respective proportions in accordance with the methods outlined in Fowler and Cohen (1987) as follows:

$$95\% \text{ confidence limits} = 1.96\left(\sqrt{\frac{p(1-p)}{n-1}}\right)$$

Where: p = the sample proportion

n = number of sample units

When the upper and lower confidence limits of the respective proportions were not found to overlap the proportions were considered to be statistically different at the 5 % level (p = 0.05)

2.8.8 Correlation coefficient

Where the degree of linear relationship between two variables was to be investigated the Pearson product moment correlation coefficient (r) was calculated as follows:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{(n-1)s_x s_y}$$

Where: $\bar{x}$ and $\bar{y}$ are the means of the variables

s_x and s_y are the standard deviation of the variables

If the calculated r value was greater than the tabulated r value at the 5% level, the correlation between the variables was considered to be significant.

CHAPTER 3: EFFECTS OF PHOTOPERIOD MANIPULATION ON GROWTH AND REPRODUCTION: THE IMPACT OF EXPOSURE TO CONSTANT ILLUMINATION.

3.1 Introduction

As with many species of northern latitudinal origin, it is believed that to assist the profitable culture of Atlantic cod, the development and application of artificial photoperiod management strategies to regulate or inhibit maturation will be essential. Of greatest present concern is the delay or prevention of maturation during the on growing cycle. It has been reported that cod in culture will mature at two years of age, prior to harvest weight being attained. At the start of this programme of research, limited work on the impact of exposure to continuous illumination prior to maturation had been reported from Norway (Hansen *et al.*, 1995; Karlsen *et al.*, 2000; Dahle *et al.*, 2000) (Figure 3.1). Hansen *et al.* (1995) tested 3 different experimental photoperiod treatments in comparison to a simulated natural photoperiod control (SNP) in an enclosed tank environment. The fish studied were hatched in spring 1992 with the trial beginning in July 1993, i.e. when fish were approximately 15 months post hatch (MPH). The test photoperiods consisted of: 1.) continuous illumination from July 1993 (LL treatment); 2.) continuous illumination from July 1993 to December 1993 then return to the simulated natural photoperiod (LL/SNP treatment); and 3.) simulated natural photoperiod to December 1993 then continuous illumination (SNP/LL treatment). The authors reported that both the SNP and SNP/LL spawned between January and April 1994 while the LL/SNP were delayed 3 months and the LL group did not mature, with oocytes being held at the cortical alveoli stage. For the following year, summer 1994 to summer 1995, (i.e. from 2-3 years of age) only the LL/SNP and LL groups were maintained. The authors reported that "two year old cod that spawned three months

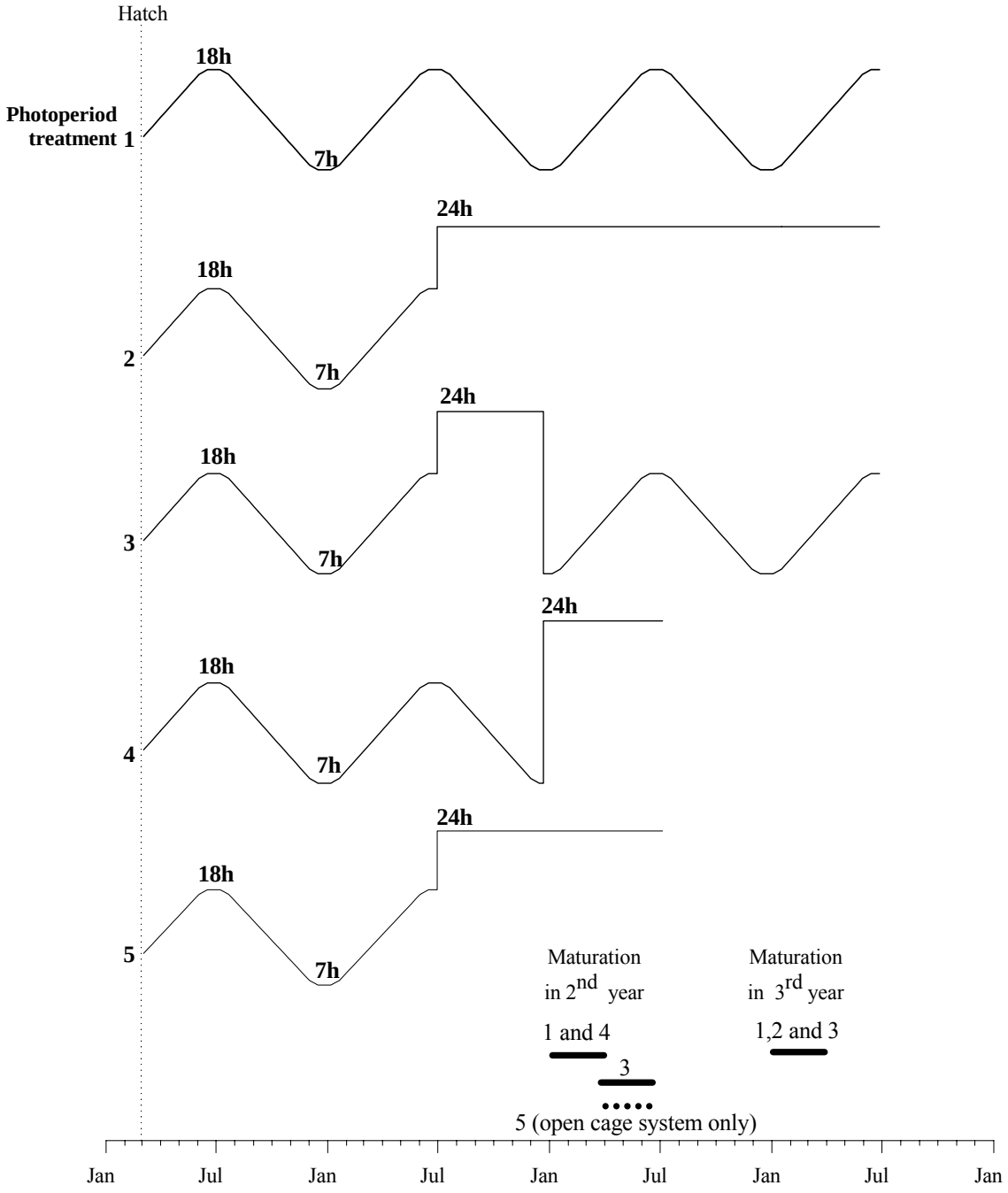


Figure 3.1: Diagrammatic summary of previously reported photoperiod treatments designed to inhibit maturation in Atlantic cod. The range of daylight hours that populations were exposed to are indicated in each plot. The reported spawning periods for each treatment are collated at the base of the diagram. Photoperiod 1 is a simulated natural photoperiod to which all experimental treatments (2 to 5) were compared. Experimental photoperiods 2 to 4 were reported in Hansen *et al*. (1995), photoperiod 5 was reported in both Dahle *et al*. (2000) and Karlsen *et al.* (2000).

delayed (LL/SNP) spawned again at their normal spawning time (as three year olds) when reared under a natural photoperiod. A few of the females reared under continuous light ovulated at an age of three years."

Subsequent work published by Dahle *et al.* (2000) and Karlsen *et al.* (2000) focused on the application of LL from summer solstice and reported, without providing details of the trials, that such application based in open cage systems did not prevent maturation as in the report of Hansen *et al.* (1995), but rather caused a delay. Karlsen *et al.* (2000) suggested that this may be due to different utilisation of energy reserves in cage systems. Hence, they tested simulated natural and constant light regimes with varied flow rates in tanks. They concluded that constant light in tanks stopped maturation as before, however exercise had no significant effect on maturation. Meanwhile, Dahle *et al.* (2000), considering the same problem, concluded that the difference between an open cage system and enclosed tanks may be the overlying natural photoperiod experienced by the cage cultured fish which cued maturation irrespective of the continuous artificial illumination. Hence, they compared in an open tank system the application of constant illumination at high (1600 lux) and low (100 lux) intensities superimposed over a natural photoperiod regime (no intensity levels presented) between October and July. Prior to this the fish were held in an open cage with additional artificial light from the summer solstice to October. Only the ambient photoperiod stock spawned during the trial period, with the authors concluding "The different light intensities have so far been equally effective in delaying maturity". This data suggests that the apparently most effective photoperiod manipulation to inhibit maturation is the application of continuous illumination from the summer solstice prior to maturation. (*n.b.* continuous illumination is the only practicable manipulation that can be applied in the commercial production systems as the open nature of cages does

not allow for the exclusion of ambient light). This was therefore accepted as the first manipulation which should be further examined in this investigation.

With regards to growth performance, all trials reported significant improvement in somatic growth in populations where maturation was prevented by LL application with, in one case, over a 60% improvement being recorded (Hansen *et al.*, 1995). However, the work so far has only reported growth up to 26 months post-hatch by which stage the maximum mean weight was 2.5 kg, representing the lowest acceptable harvestable weight (Section 1.3.3). Hence, the longer term impacts of photoperiod manipulation on growth performance needed to be addressed.

While the prevention of maturation during on-growing is the principal goal of this work, it should not be forgotten that artificial photoperiod management is also consistently applied to entrain maturation to provide year round spawning broodstocks (Bromage *et al*., 2001) which will spawn at set times of the year to supply the ongrowing industry with year round production of juveniles. Such management strategies will also benefit from a clear understanding of the photoperiod signals that regulate and entrain maturation. While such managed stocks have been reported (Van der Meeren, 2001), little work has been published in this field in cod. As previously discussed, Norberg *et al.* (1995) demonstrated adjustment of spawning with extended and compressed seasonal photoperiod cycles. While effective, such strategies are labour intensive for on farm application. The use of constant or "square wave" photoperiods however, i.e. the maintenance of stocks on, and switching between, fixed long and short day photoperiods, is simpler for farm application (Bromage and Duston, 1986). These have also been demonstrated, most clearly in salmonids, to be more versatile in altering maturation and, equally, more insightful in explaining the underlying signals that entrain this process (Randall *et al.*, 1991; Randall, 1992; Randall *et al.*, 1998). Hence,

since the application of such regimes is not apparent to date in Atlantic cod they were examined at this stage due to both their potential scientific and commercial value.

All of the above described previously reported trials were performed in Norway using the Norwegian coastal cod strain. No published literature on such work in the UK with cod from any of the reported UK strains (Brander, 1994) were available. As was explained earlier (Section 1.1 & 1.2), the numerous geographically discrete "strains" of Atlantic cod exhibit variable growth and maturation performances. Therefore, as the culture of cod within the UK will initially be reliant on locally sourced broodfish, of greatest importance is the demonstration that such locally collected and subsequently reared strains perform in a comparable manner to those sourced and reared in the Norwegian environment. This will then allow the placement of the limited existing work in context with the likely UK culture cycle.

AIMS

Commercial cod farming in the UK will be reliant on photoperiod management of maturation to both improve product quality and profitability as well as generate a year round supply of eggs. Fundamentally, the mechanisms by which photoperiod regulates maturation in cod are poorly characterised. Furthermore, the significance of difference in genotype and/or environmental conditions are unknown in this respect. Therefore the aims of this chapter are:

- To investigate the impact of LL photoperiods applied from the summer solstice prior to likely maturation at 2 years on both maturation and growth performances of a UK origin stock held in both enclosed tank and open cage systems.
- To investigate the impact of long term exposure to LL on growth.

To investigate the application of out of phase constant day length photoperiods on maturation to elucidate the underlying signals of photoperiod regulation.

3.2 EXPERIMENT I: THE IMPACT OF EXPOSURE TO CONTINUOUS ILLUMINATION FROM 15 MPH ON MATURATION AND GROWTH IN AN ENCLOSED TANK SYSTEM

3.2.1 Objectives

This experiment was designed to investigate the influence of continuous illumination, applied between 15 and 36 months post hatch (MPH), on the growth and maturation rates of a spring spawning native strain of Atlantic cod in an enclosed tank system.

3.2.2 Materials and Methods

3.2.2.1 Fish stocks, rearing conditions and photoperiod regimes

Atlantic cod juveniles were produced at Port Erin Marine Laboratory, Isle of Man, UK (54:05^{o}N, 4:45^{o}W) during the spring of 1999 from a broodstock of Irish Sea origin where they were maintained under ambient conditions (photoperiod and temperature) for this location. The fish were brought to the Marine Environmental Research Laboratory (MERL), Machrihanish, Argyle, Scotland (55:44^{o}N, 5:44^{o}W) in the autumn of 1999 where they were maintained under ambient conditions (photoperiod and temperature) until the start of the experiment.

On the 2nd of June 2000, 621 cod (Weight: 561 ± 6 g, mean ±SEM) were randomly divided into 4 experimental tanks. The experimental tanks, located indoors at MERL, were circular 7 m^3 tanks (3 m diameter, running depth approximately 1 m) with light proofed lids. Artificial light was provided by two 9 W fluorescent bulbs located centrally under the tank cover approximately 0.5 m above the water surface. Light intensity at the water surface was 0.32 Wm^{-2} (1.705 µmoles m^{-2}s^{-1} or 105 lux) when illuminated and 0 Wm^{-2} (0 µmoles m^{-2}s^{-1}, 0 lux) in the dark phase [light measurements recorded using an energy sensor or lux sensor calibrated to N.P.L. UK standards (Skye

Instruments Ltd, Powys, UK)]. Throughout the experimental period, water temperature ranged from 5^{O}C to 15^{O}C (Figure 3.2) and the salinity ranged between 29 ‰ and 34 ‰ with an average of 33 ‰ (Figure 3.3). During the experimental period, the fish were held within a stocking density range of 10 to 30 kgm^{-3}. Fish were fed a commercial dry pellet (EWOS, Bathgate, UK) to satiation 3-5 times per day between 9 am and 5 pm, 7 days a week.

On the 1st of July 2000 two experimental tanks were placed onto continuous illumination (LL). The remaining two tanks were left on the simulated natural photoperiod (SNP) for the trial duration (Figure 3.4). In order to maintain the populations within the set stocking densities, on the 6th of February 2001, fifty fish from each tank were randomly removed. On the 26th of June 2001, one tank from each treatment was harvested by the industrial partners leaving one tank of approximately 100 fish from each treatment to be maintained until the end of the experimental period. On the 11th of February 2002 50% of each population were randomly removed to maintain the population within the stocking density limits. The trial ended on the 10th of June 2002.

3.2.2.2 Sampling

Each month, 30 individuals from each tank were randomly collected, anaesthetised and their weights to 0.1 g and total lengths to 1 mm recorded. Their sex and maturation status were assessed by visual external inspection and ultrasound scanning (Section 2.3), before returning them to the experimental setup. Between July 2001 and October 2001 only weight was recorded. On the 15th and 16th of February 2002 a sampling regime was performed to examine the diel melatonin profile in both treatments. A total of 7 sample points were performed at 1 pm, 3 pm, 7 pm on the 15th,

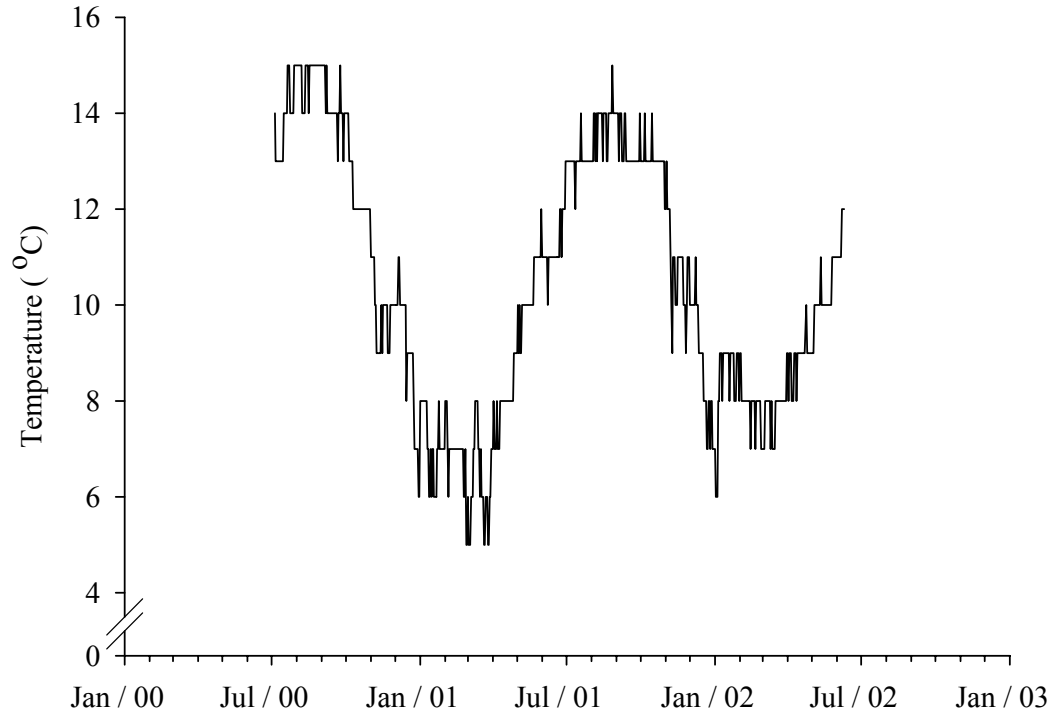


Figure 3.2: Daily temperature (°C) of seawater at Machrihanish from 1st July 2000 to 10th June 2002.

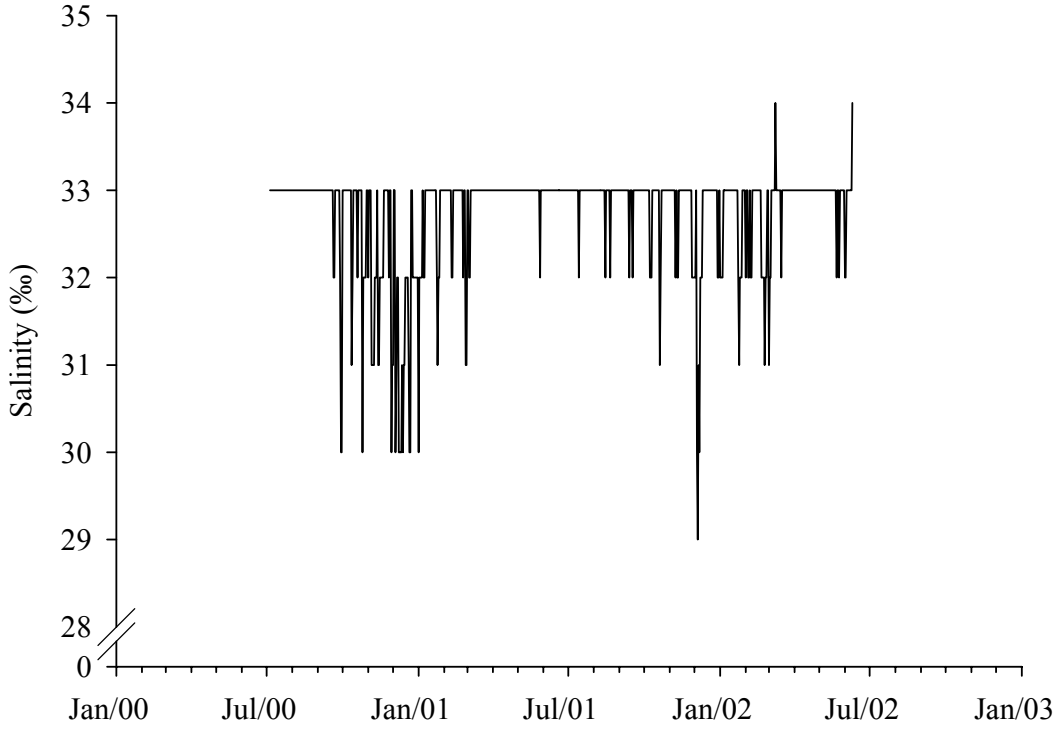


Figure 3.3: Daily salinity (‰) of seawater at Machrihanish from 1st July 2000 to 10th June 2002.

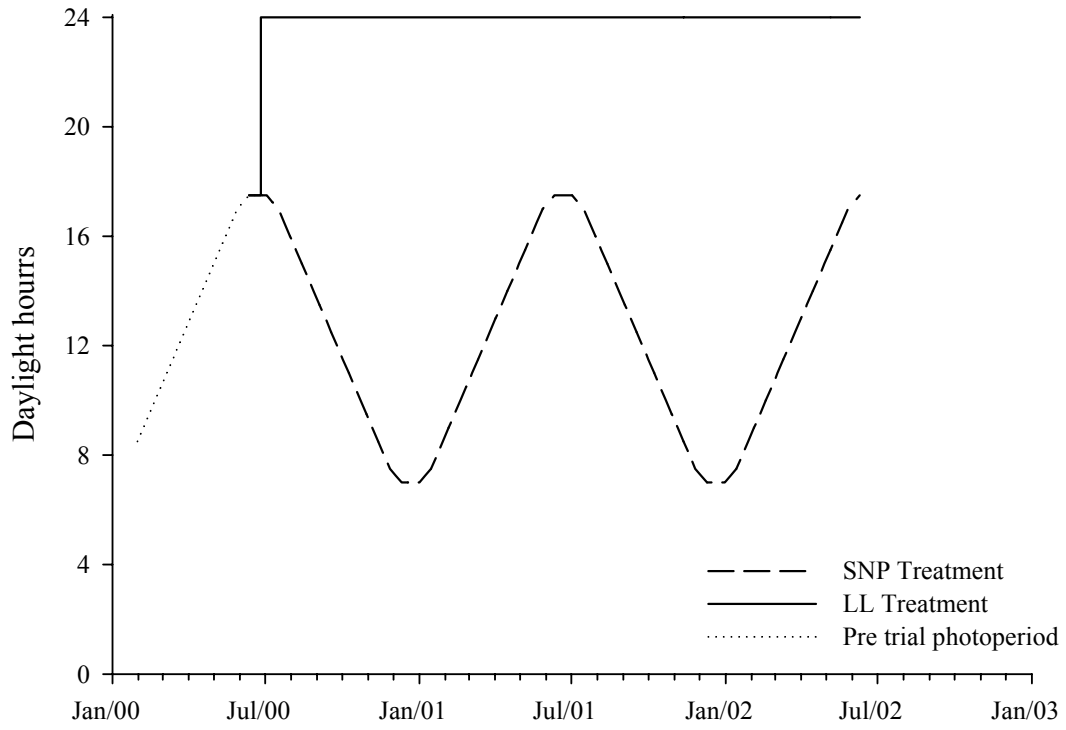


Figure 3.4: Photoperiod treatments. Simulated Natural Photoperiod, SNP (grey line) and continuous illumination, LL (black line), Pre treatment photoperiod (SNP) shown as dashed line.

and 12 am, 6 am, 10 am and 1 pm on the 16th. In the SNP treatment sunset was at 17:00 and sunrise at 07:40 on these dates. At each sample point 10 individuals per tank were anaesthetised and 1 ml of blood withdrawn into heparinised syringes. Following sampling, fish were placed in separate tanks under identical photic conditions to ensure there was no repeat sampling of individuals and all were replaced at the end of the sampling period. The plasma was separated from the blood by centrifugation and frozen over liquid nitrogen prior to storage at -70°C. Samples were assayed for melatonin content (Section 2.4.1) within 2 weeks of sampling. Samples taken at 1pm on the 16th thawed prior to assaying and hence were not analysed.

3.2.2.3 Statistical analysis

Growth parameters and plasma melatonin levels were analysed by General Linear Model (Section 2.7.6), normality and homogeneity of variance were improved where necessary by log transformations. A significance level of $p < 0.05$ was set with all significant effects being analysed by Tukey *post hoc* test. Correlations between condition factor and temperature were analysed using the Pearson's product moment method (Section 2.7.8).

3.2.3 Results

3.2.3.1 Maturation

3.2.3.1.1 Maturation: Timing and Commitment

Fish were classed as mature if, during the regular monthly sampling, gametes were released freely or after gentle abdominal massage. Figure 3.5 shows the percentage of mature individuals in either treatment over the trial length. The SNP population matured twice, once in 2001 (2 years post hatch) and again in 2002 (3 years post hatch) with spawning fish being present from February to May and February to June in the respective years. In the LL population however, no mature fish were observed during 2001, mature individuals were only seen between April and June of 2002 (approx. 3 years post hatch).

SNP photoperiod regime

The 2001 spawning season lasted for 4 months starting in February when the first males started to release milt (48% of population total) with females starting to ovulate 1 month later in March (18%). The spawning activity peaked in April with 86% of the population spawning, 57% being males and 29% females. In May, spawning activity reduced with only 54% of the population spawning (40% males and 14 % females), after which no more mature fish were observed.

In 2002 the spawning season lasted for 5 months with, as before, the first mature individuals being observed in February when both spermiating males (20%) and ovulating females (10%) were observed. In March the proportion of mature males increased to 57%. As in 2001, the peak of spawning activity was in April, when 81% of the observed population were releasing gametes (53% males to 28% females), after

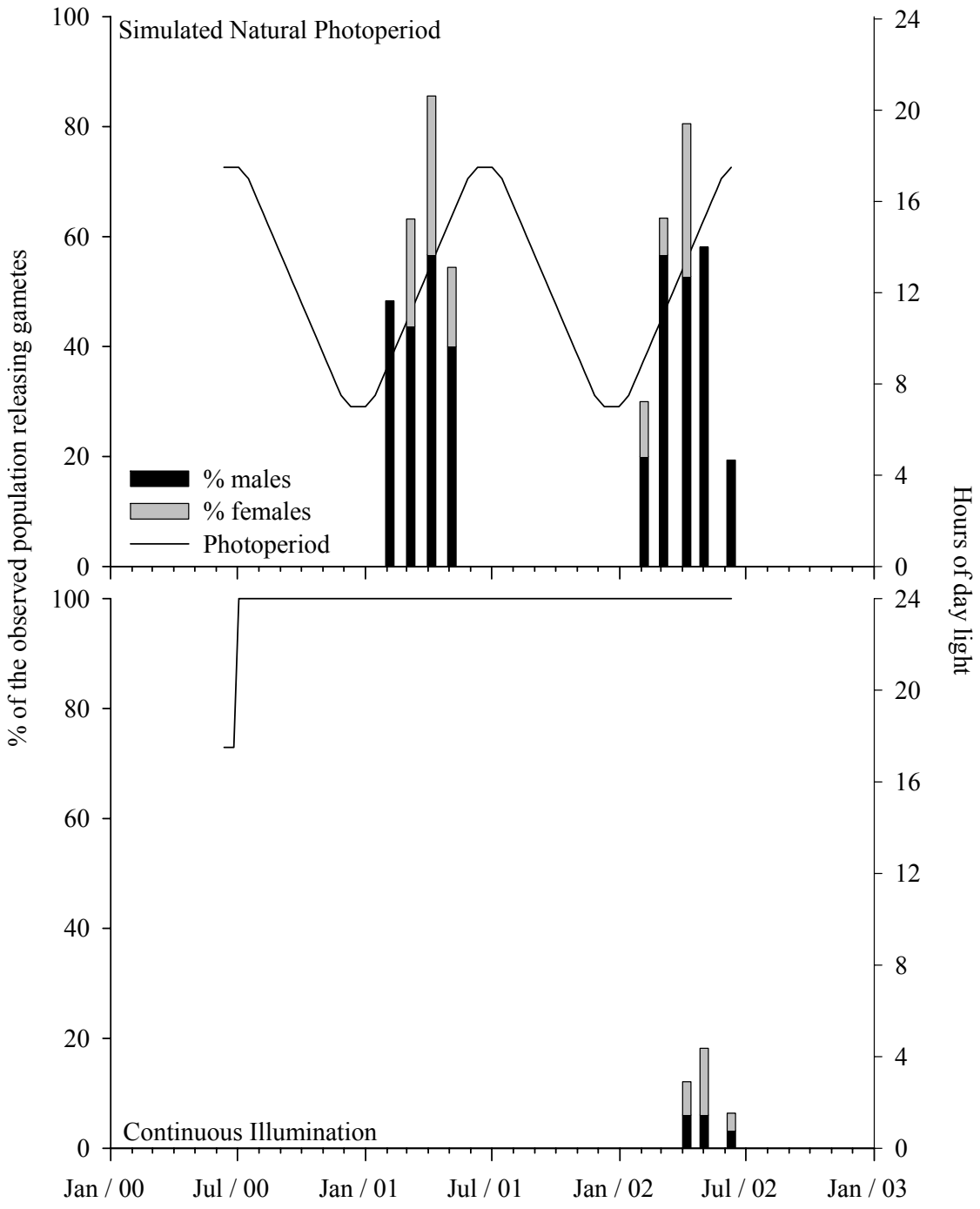


Figure 3.5: Percentage of the observed population total releasing gametes, including the relative contribution of each sex, in relation to photoperiod treatment (n= 60 in 2001 season and 30 in 2002 season)

which time no mature females were observed. Spermiating males however continued to be present in May (58%) and unlike the 2001 season were present in June (19%) as well.

LL photoperiod regime

No mature fish were observed in 2001 (Figure 3.5). In 2002 the first mature individuals were observed in April, approximately 3 years post hatch. This represented 12% of the population consisting of equal numbers of males and females (6%). The spawning activity peaked in May (18% of population total) due to the numbers of females doubling to 12% this then dropped in June to 3% for each sex.

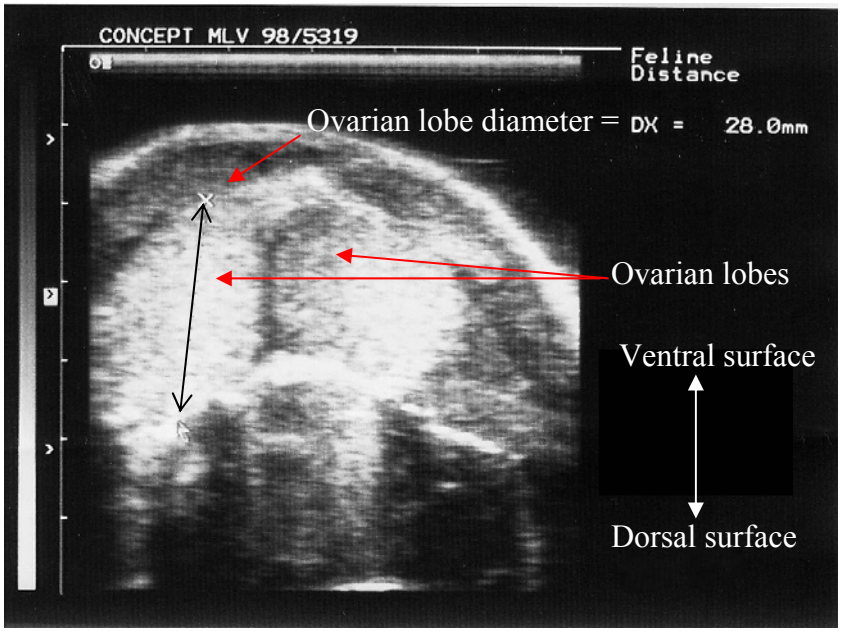
3.2.3.1.2 Gonadal Development

Ultrasound Scanning

Gonadal development (form and size) was visualised by the use of ultrasound scanning (Section 2.3.1). Although this evidence is open to individual interpretation it provides a useful non-invasive tool to estimate rates of maturation commitment and development as sacrificing of fish was not possible in this trial. To assist data interpretation, monthly observations were summarised in a three tier scale explained below:

- **X**: Gonads not detected
- **1**: Gonads small, "immature": Ovaries are generally clear with lobes being small approx < 30 mm in diameter (Plate 3.1a). Testis hard to distinguish from background: the proximal duct (see gonadal structure description in Section 4.4.1.2) is the distinguishable feature but is usually quite small (<5 mm diameter) (Plate 3.2a).

a.)



b.)

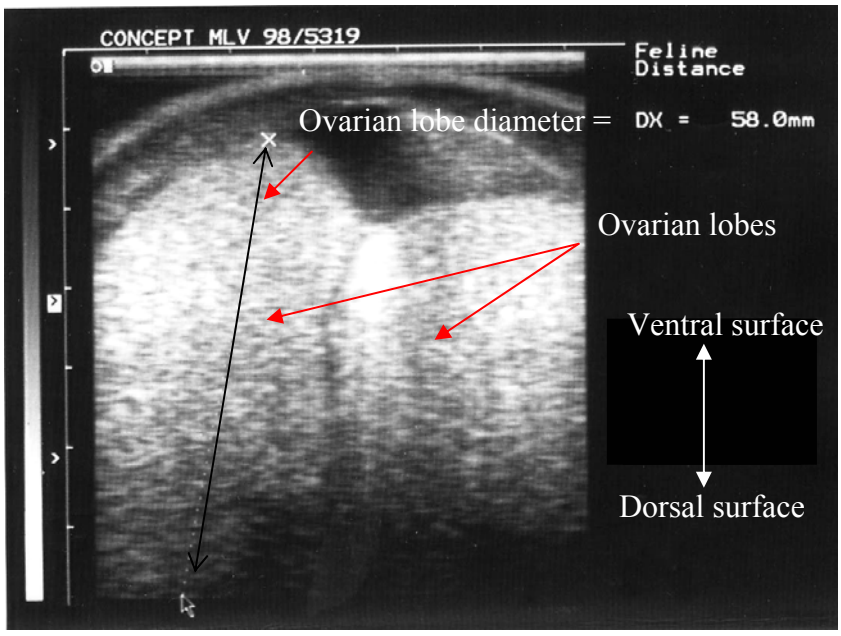
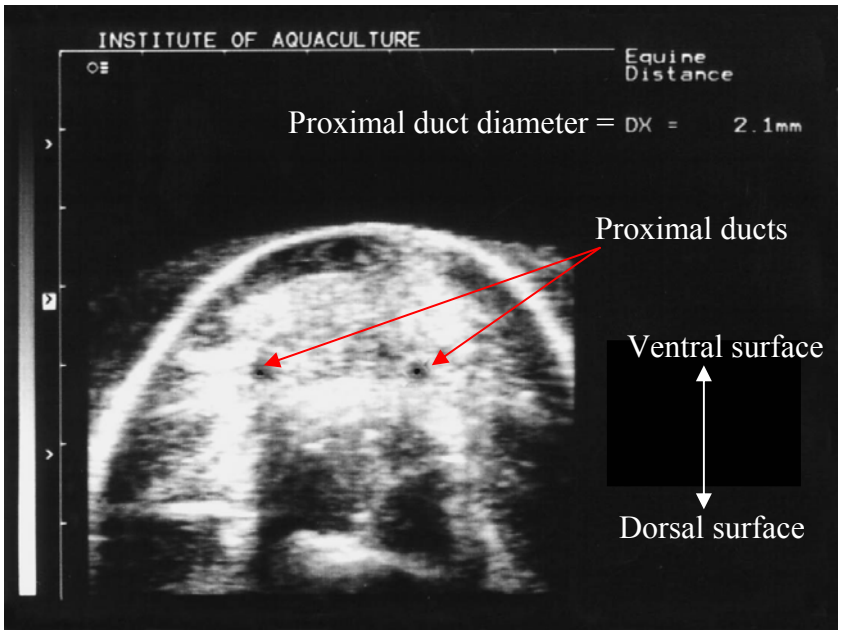


Plate 3.1: Typical ultrasound images showing ovaries of cod at different apparent stages of maturity **a.**) Female (classification scale: 1) with ovarian diameter <30 mm (Image taken 6/4/2001 LL photoperiod, x1.5 magnification), **b.**) Female (classification 2) with ovarian diameter >30 mm (Image taken 5/1/2001, SNP photoperiod, x1.5 magnification)

a.)



b.)

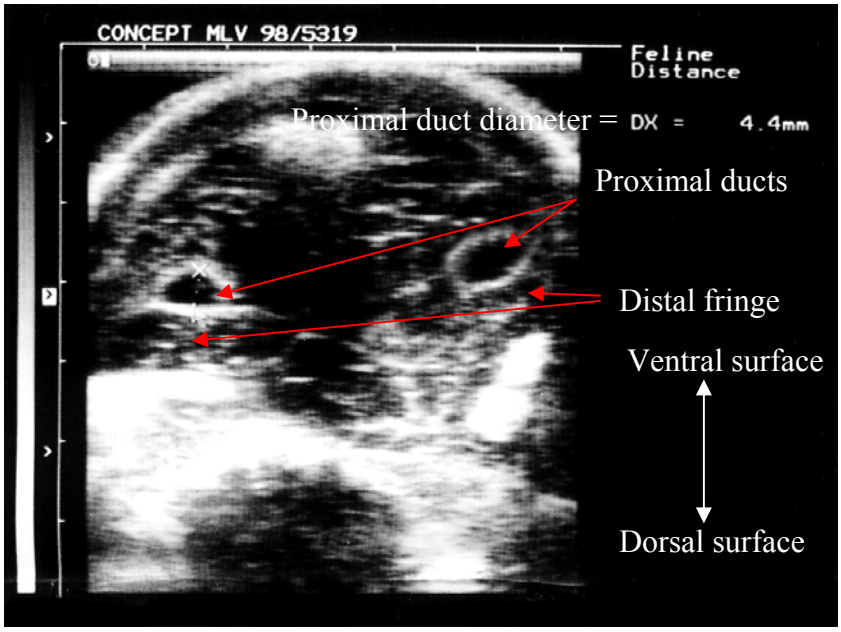
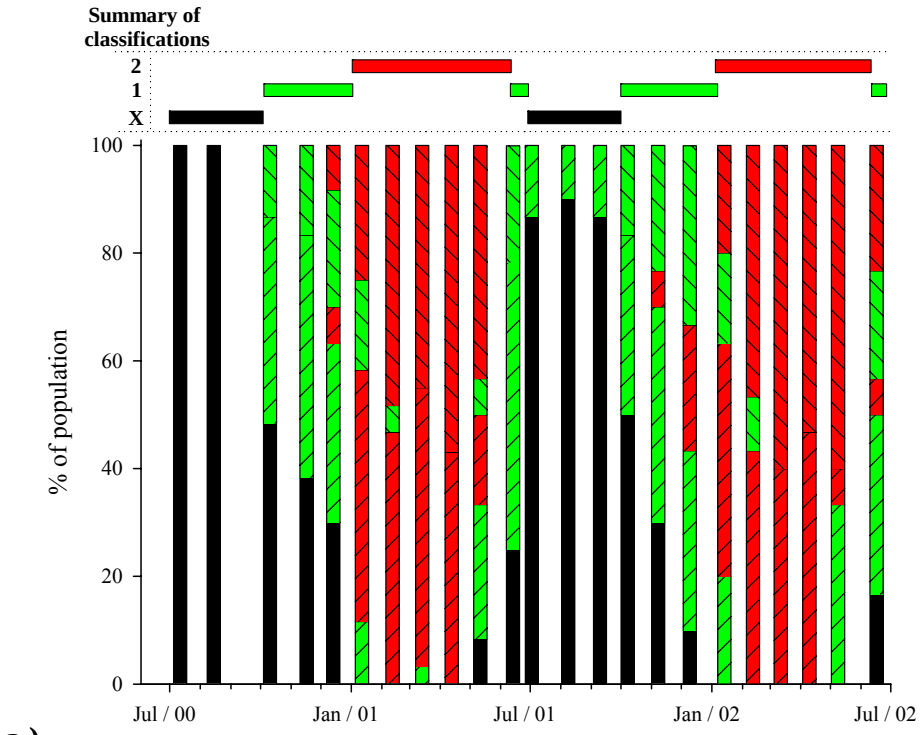


Plate 3.2: Typical ultrasound images showing testis of cod at different apparent stages of maturity **a.**) Male (classification scale: 1) with proximal duct diameter = 2 mm (Image taken 10/11/2000, SNP photoperiod, x1.5 magnification), **b.**) Male (classification scale: 2) with proximal duct diameter = 4 mm, distal fringe diameter ≈ 26 mm (Image taken 7/3/2001, SNP photoperiod, x1.5 magnification)

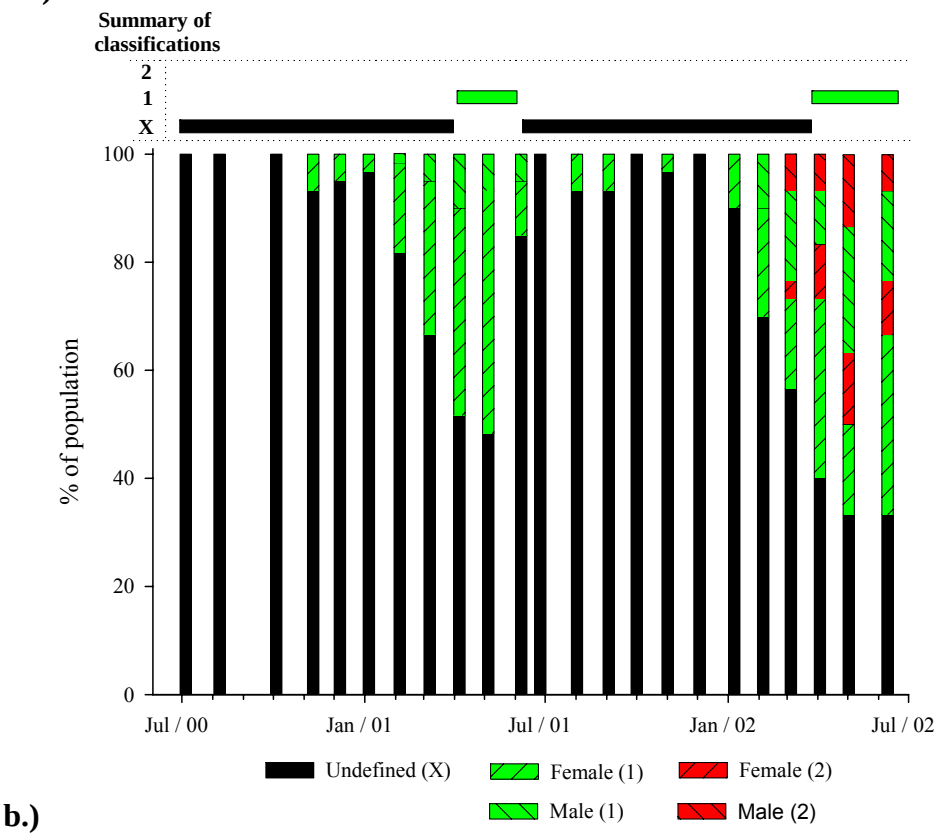
- **2**: Gonads large and well defined, "mature": Ovaries forming large dominant structure filling body cavity, each lobe > 30 mm diameter (Plate 3.1b). Testis appear with proximal duct clearly distinguishable down length of cavity with distal fringe (see gonadal structure description in Section 4.4.1.2) being visualised surrounding proximal duct in later stages of maturation (Plate 3.2b).

SNP photoperiod regime

Gonads were first detected in October 2000 (Figure 3.6). This was principally females that were distinguishable with the ovarian lobes having an initially small diameter (10-20 mm) (Plate 3.1a). Over subsequent months, the ovaries became clearer to distinguish and steadily increased in size. Males were observed in fewer numbers over these months due to the difficulty in detection. The key to successful identification was detection of the proximal ducts appearing as two dark circles (≤5 mm diameter) running in parallel, dorsally away from the uro-genital papillae (Plate 3.2a). By January 2001 gonads were distinguishable in all individuals with the majority of female ovaries appearing very large (Plate 3.1b). The male testis structure was easily identified as, during the later stages of maturation and through spawning, the distal fringe becomes more evident forming a "halo" around the proximal ducts (Plate 3.2b). For a full description of gonadal structure see section 4.4.1.2. Between March and May 2001 (i.e. the spawning season), large clearly developed gonads were observed in the majority of individuals with the females showing, in some cases, ovulated egg batches and/or areas of spent ovarian tissue which appeared as solid white striations. These "spent" regions increased in regularity as the ovaries reduced in size towards the end of the 2001 spawning season. In April 2001 ultrasound scanning was used to classify the remaining individuals that were not releasing gametes on inspection (14% of the population). All



a.)



b.)

Figure 3.6: Summary of the classification of gonad states observed under **a.**) SNP photoperiod, **b.**) LL photoperiod. Gonads visualised by ultrasound scanning and images classified using the author's three point scale explained in text. Horizontal bars denote period of predominant classification. (n = 60 up to June 2001, thereafter = 30).

individuals were females that were not releasing eggs on that day however they clearly possessed large mature ovaries. At the end of the spawning season in June both ovaries and testes were much reduced in size, thereafter from the start of July to September 2001, gonads were predominantly not detectable. From October 2001 the number of detectable females and males steadily increased. The pattern of development was similar to that observed the previous winter with size and prevalence of detectability increasing until January 2002 by which stage the sex of all individuals could clearly be identified. Testes reached maximum size in February/March while ovaries attained maximum size between March and April. Thereafter, both could be seen to reduce in size through the spawning season. Ultrasound scanning was used at the peak of spawning activity (April 2002) to classify the remainder of the population not releasing gametes (19%). These were all "mature" females that had large developed ovaries although they were not releasing eggs on the day of inspection.

LL photoperiod treatment

In the LL treatment gonads were first detected in a small number of fish from November 2000 (Figure 3.6) with only females (ovarian diameter <15 mm) being observed. From February 2001 these numbers noticeably increased and the first males were observed. Over the subsequent 2 to 3 months the ovaries increased in size reaching a maximum in May 2001 that appeared noticeably smaller than the maximum size attained by the SNP population in the same year. Plate 3.1a is a typical example of a female at the peak of development in the LL population in this year in comparison to a mature female from the SNP treatment in the same year (Plate 3.1b). Of note is that, at this stage, almost half the population still displayed no clear gonadal structure on ultrasound inspection. Thereafter, the ovaries that were detectable rapidly reduced in

size until they became undetectable in July. Gonads remained predominately undefined until February 2002 when gonadal structures in a limited number of fish of both sexes were again observed. As before, these were initially small. They increased in size, though not uniformly so that in March 2002, it became apparent that a few individuals, both male and female, were developing rapidly which was confirmed by mature ('running') individuals being recorded in April 2002. These individuals represented the exception however, with the majority of the population possessing either undetectable gonads or gonads that were present though still very small. This non-uniform spread of development in the population was present to the end of the trial.

3.2.3.2 Growth

Weight

Both populations significantly increased in weight over the length of the trial (Figure 3.7). Although the populations started at the same mean weight (561 ± 6 g), by the end of the 2 year trial the LL population was significantly heavier than the SNP population (3208 ±131 g vs. 2104 ±92 g respectively). There were two periods when the populations were significantly different from each other this was from May to July 2001 and from May to June, 2002. In both cases the LL population was significantly heavier than the SNP population.

The SNP population displayed two apparent growth cycles during the trial time frame. These cycles consisted of a period of steady weight gain (growth phase) followed by a period of growth arrest or weight loss (rest phase). The first cycle ran from June 2000 to July 2001. The growth phase was between June 2000 and March 2001 when the population displayed continued significant growth improvement from 561 ±6 g up to 1438 ±48 g. This was followed by the resting phase from March 2001 to

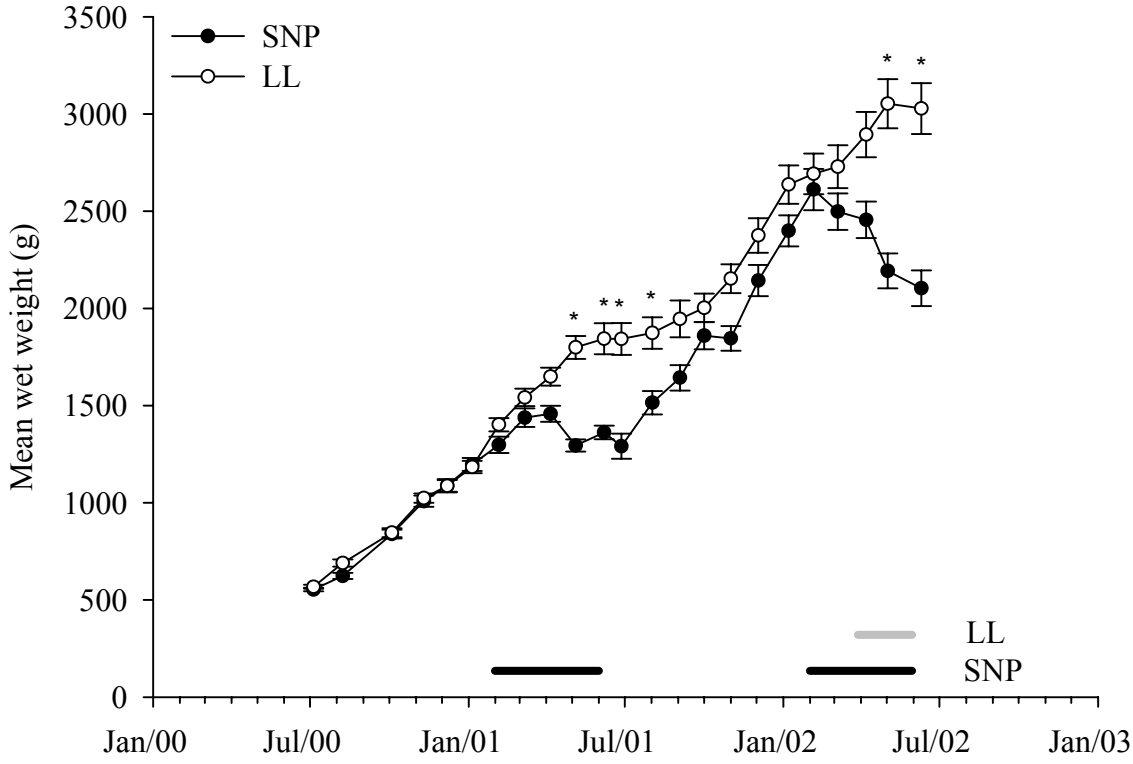


Figure 3.7: Mean individual wet weight (g) ± SEM (n=60 up to June 2001, thereafter 30), for fish maintained under SNP (●) or LL (○) from July 2000. Horizontal bars represent spawning seasons for photoperiod treatments SNP (Black), LL (Grey) respectively. * denotes a significant difference between treatments in that month ($p<0.05$) ANOVA GLM with Tukey post hoc test.

July 2001 which coincides with the first spawning season, during which time the mean weight reduced by 13%. No significant increase in weight (with relation to March 2001 mean weight) was observed until October 2001. However, the next apparent "growth phase" started in July 2001, lasting 7 months and ending in February 2002, over which period there was a significant increase in mean weight from 1291±65 g to 2611±106 g . This was then followed by the rest phase coinciding with the second spawning season from February 2002 to the trial end in June 2002. During this period the mean weight significantly decreased over the 5 month period ending the trial at 2104±92 g, a reduction of 24%.

The LL population did not exhibit the same dramatic fluctuations seen in the SNP population. The general trend was one of continuous growth. There was however an apparent period of reduced weight gain between May and October 2001 during which time weight did not significantly increase (mean weight *circa* 1800 g). At the trial end with a mean weight of 3028±131 g, the LL population was over 40% heavier than the ambient photoperiod group.

Specific Growth Rate (Weight)

As weight samples were taken from random individuals each month it was only possible to calculate mean monthly population weight specific growth rates (SGRw) (Figure 3.8). Over the first year, both populations under SNP and LL displayed a steady reduction in SGRw with the growth rate dropping below zero between May and July 2001 in the SNP population in line with the spawning season. Following this, in the second year the SNP population rapidly returned to growth rates comparable to the start of the trial (August 2000 to February 2001) which were maintained until February 2002 when, as in spring 2001, in line with the initiation of spawning within the population,

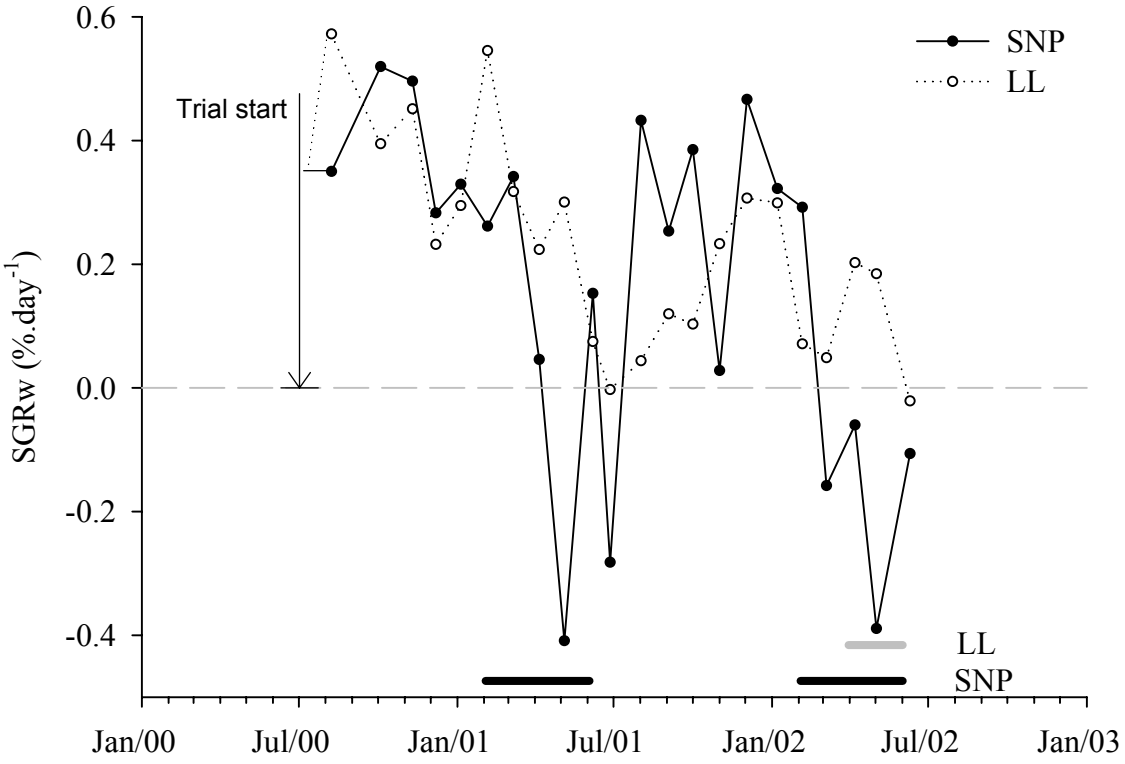


Figure 3.8: Monthly mean weight specific growth rate (SGRw) under SNP (●) or LL (○) photoperiod treatments. Horizontal bars represent spawning seasons for photoperiod treatments SNP (Black) and LL (Grey) respectively.

SGRw rapidly fell to below zero in April and remained so until the trial end. As for the LL population, SGRw rose from July 2001 and peaked in December 2002, thereafter falling to just below zero by June 2002.

Length

Both populations increased in length over the duration of the trial (Figure 3.9). Throughout the trial the LL population appeared longer though significant differences were only observed from May 2001 to August 2001. This was due to a lower length specific growth rate (SGRl) in the SNP population in the months of January, April and June of this year. Following this a period of increased SGRl (July and August 2001) reduced the divide after which the two populations displayed a comparable rate of growth.

Foulton's Condition Factor

A scatter plot of all individual weight vs. lengths recorded throughout the trial (a total of 2115 observations in the range of 365-5040 g, 280-711 mm) had a regression coefficient of 2.9 with an adjusted r-squared of 0.89 ($p<0.01$). As such, Foulton's condition factor was deemed an appropriate condition index to use. Condition factor demonstrated clear seasonal cycles in both the SNP and LL treatments (Figure 3.10).

SNP photoperiod regime

At the trial start, the SNP population had a condition factor of 1.2 ± 0.01 which rose steadily to reach a peak of 1.45 ± 0.03 in March 2001. Thereafter, it rapidly dropped back to basal levels by July 2001. This cycle was repeated in the second year with a peak of 1.67 ± 0.06 being reached in February 2002. Condition factor remained

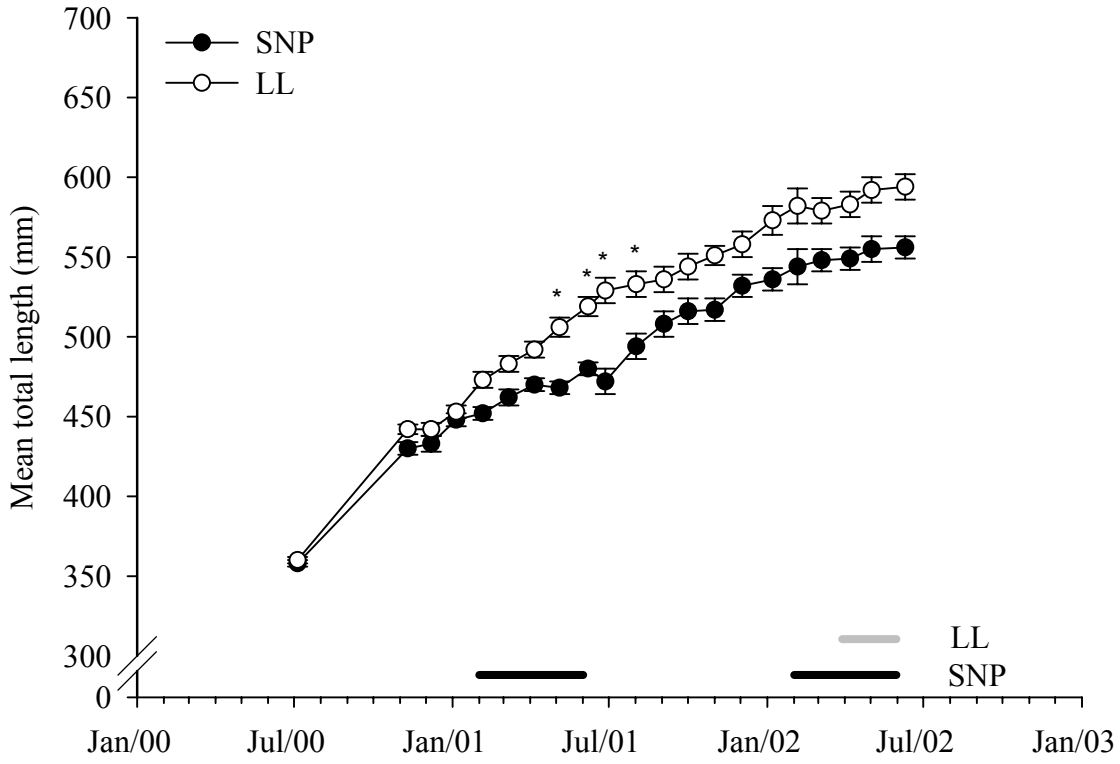


Figure 3.9: Mean individual total length (mm) ± SEM (n=60 up to June 2001, thereafter 30), for fish maintained under SNP (●) or LL (○) from July 2000. Horizontal bars represent spawning seasons for photoperiod treatments SNP (Black), LL (Grey) respectively. * denotes a significant difference between treatments in that month ($p<0.05$) ANOVA GLM with Tukey post hoc test.

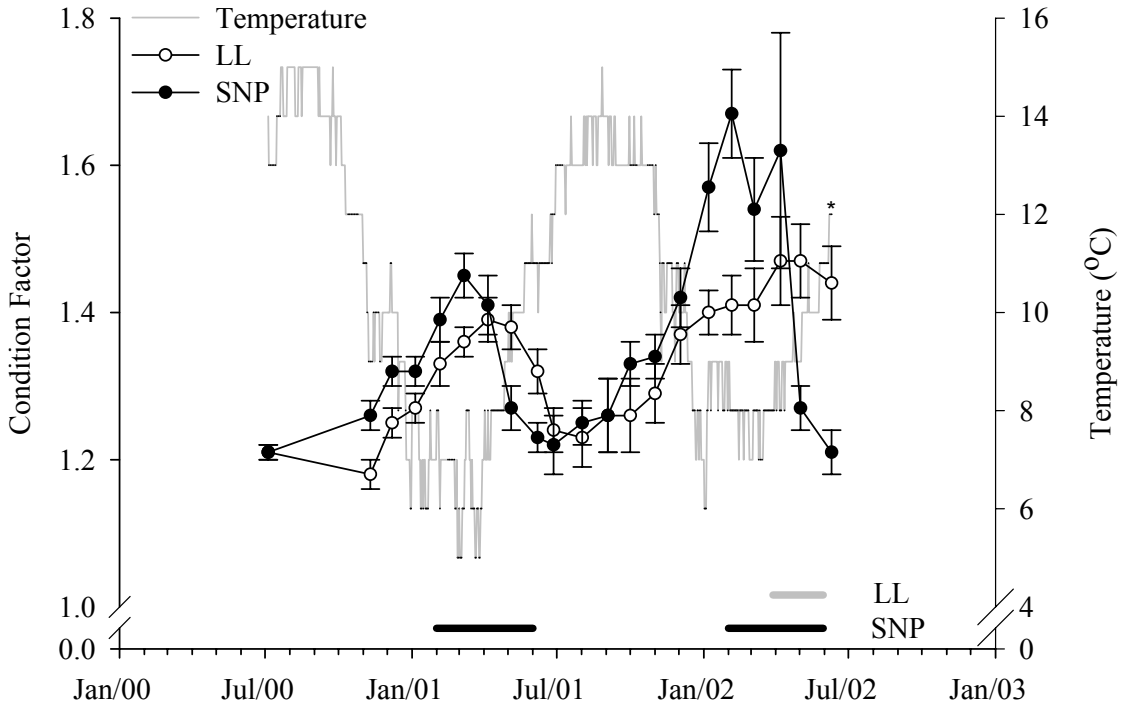


Figure 3.10: Mean individual Foulton's condition factor ± SEM for fish maintained under SNP (●) or LL (○) from July 2000, in relation to ambient water temperature (n=60 up to June 2001, thereafter 30). Horizontal bars represent spawning seasons for photoperiod treatments SNP (Black), LL (Grey) respectively. * denotes a significant difference between treatments in that month ($p<0.05$) ANOVA GLM with Tukey post hoc test.

at this elevated level until April 2002 and thereafter dropped rapidly again to basal levels for the remaining 2 months of the trial.

LL photoperiod regime

The LL population displayed a similar seasonal rhythm in condition factor though with an apparent 1 month delay in the cycle. It also appears to have reduced amplitude with regards to the peak of condition especially in the second year of the trial (January to April 2002). As with the SNP population, condition factor began at 1.21 ± 0.01 which was maintained until November 2000. Thereafter, condition steadily increased to a peak of 1.39 ± 0.03 in April 2001, dropped to 1.23 ± 0.04 in August 2001 and increased again to reach a peak of 1.47 ± 0.06 in April 2002. Thereafter the population's condition remained at this elevated level.

Foulton's condition factor correlation to temperature

In both the LL and SNP populations the cyclic patterns in condition factor appeared to be inversely related to water temperature. Both populations showed a significant negative correlation with water temperature (Table 3.1) though the r^2 is very low in both cases due to the wide spread of individuals within the limited condition factor range.

Table 3.1: Pearson's correlations between water temperature and Foulton's condition factor for the SNP and LL populations.

	Slope	Y Intercept	R^2	P value	F	n
SNP	-0.3	1.64	0.1068	<0.0001	110.8	900
LL	-0.215	1.52	0.0516	<0.0001	56.4	900

3.2.3.3 Melatonin Analysis

Melatonin levels in the SNP population displayed a clear diel pattern, rising to a peak (47 ± 5 pg.ml^{-1}) towards the end of the dark phase (Figure 3.11). The LL population levels never rose above basal levels of *circa* 18 pg.ml^{-1}during the 24 hour period.

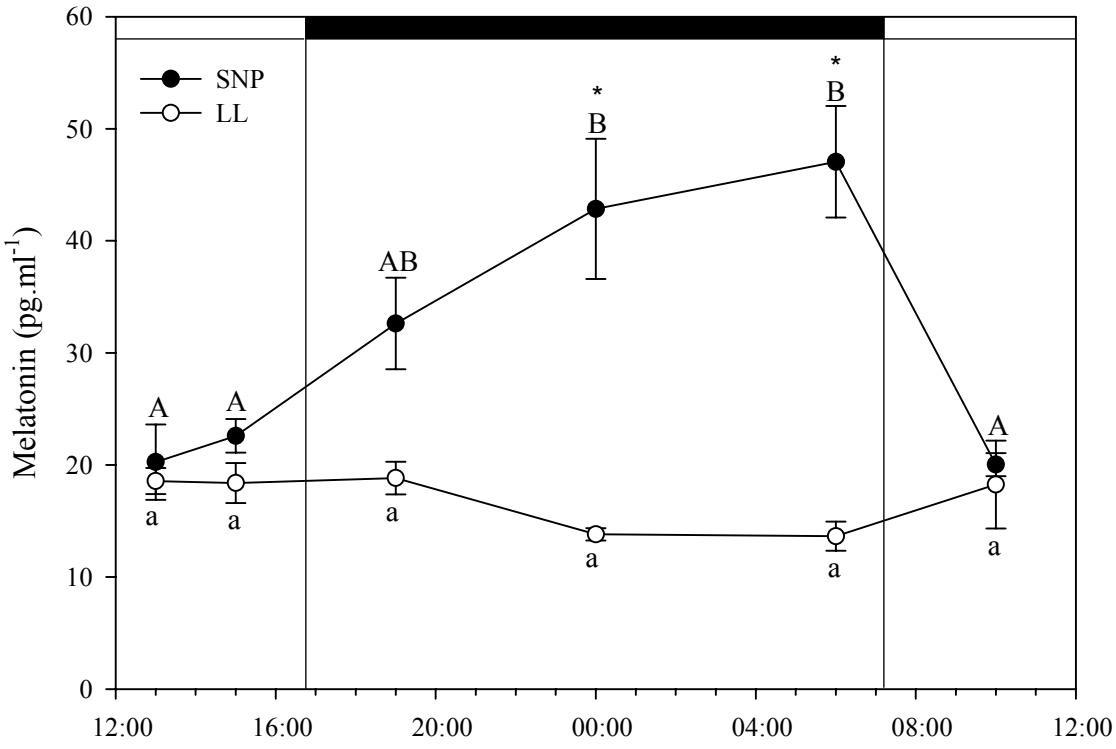


Figure 3.11: Mean plasma melatonin concentration (pg.ml^{-1}) ± SEM (n=10) measured over a light/dark cycle on the 15th-16th of February 2001 in the SNP and LL treated cod. Black bar denotes period of ambient darkness. Different letters represent significant differences within treatments while * denotes significant differences between treatments at that time point. (P<0.05) ANOVA GLM with Tukey *post hoc* test.

3.3 EXPERIMENT II. THE IMPACT OF EXPOSURE TO CONTINUOUS ILLUMINATION FROM 15 MPH ON MATURATION AND GROWTH IN AN OPEN CAGE SYSTEM.

3.3.1 Objective

The purpose of this experiment was to test the effect of the application of LL photoperiod regime from 15 MPH on maturation and growth performances in a spring spawning native strain of Atlantic cod in a commercial cage setting utilising a "high intensity" artificial lighting system.

3.3.2 Materials and Methods

3.3.2.1 Fish stocks, rearing conditions and photoperiod regimes.

Atlantic cod juveniles (20,000) were produced at Machrihanish Marine Farms, Machrihanish, UK (55:44^{o}N, 5:44^{o}W) during the spring of 2001 from an Irish Sea origin broodstock. They were maintained under the ambient conditions (photoperiod and temperature) for this location until autumn 2001 when they were transferred to a commercial scale cage site on the west coast of Scotland (56:09^{o}N, 5:32^{o}W). The population (mean weight 575 ± 9 g) was split into 2 groups of *circa* 10,000 individuals, each held in a circular cage of 19 m diameter and net depth of 10 m, creating a cage volume of 2833 m^{3}. Stocking densities ranged from 4 kg.m^{-3} to 10 kg.m^{-3} during the course of the trial. Into one cage (LL), from 3rd July 2002 until 20th August 2003, 4 submersible light units (Pisces, 400 W, Aquabeam, UK) were placed at mid cage depth (approximately 5 m) in a square arrangement to maximise light distribution. The second cage unit was unlit (Control) and held >80 m away from the illuminated cage. Both cages were completely covered with 50% shade netting. Feeding was to satiation with a commercial dry diet (EWOS, Bathgate, UK) given by a mixture of automated and hand

feeding every second day. Both populations were treated with prescribed in-feed antibiotics for outbreaks of *Vibrio anguilarium* in June 2002, September 2002 and May 2003.

3.3.2.2 Sampling

Following anaesthesia, weight to 10 g and total length to 1 mm were recorded and maturation was assessed by external examination (Section 2.3.2) in 200 individuals from each group on 3rd July 2002, 17th October 2002 and 29th March 2003. Lights were removed on 20th August 2003 and morphometric measurements were recorded finally on 9th September 2003. Gonadal development was examined using ultrasound scanning (Section 2.3.1) on a limited number of individuals (n≈20) on 17th October 2002 and on all individuals whose maturation state was not apparent on visual inspection on 29th March 2003. The observations from the March sample were collated into 3 categories for clarity of data interpretation, as explained below:

1. Spawning: Visibly swollen, releasing gametes on touch or show clear mature gonadal structures under examination with ultrasound scanning.
2. Maturing: Not releasing gametes to touch but showing clear gonadal structures which appear enlarged/developing on examination with ultrasound scanning.
3. Unknown: No visible external signs of maturation or sex or clearly detectable gonadal structures with ultrasound scanning.

3.3.2.3 Statistical Analysis

Growth parameters (weight, length and condition factor) were analysed by General Linear Model (Section 2.7.6). Data were log or reciprocal transformed to improve normality and homogeneity of variance. A significance level of $p<0.05$ was set

with all significant effects being analysed by Tukey *post hoc* test. For changes in percentage maturation rates 95% confidence limits were calculated and compared (Section2.7.7).

3.3.3 Results

3.3.3.1 Maturation

Through the use of ultrasound scanning, it was possible to non-invasively monitor gonadal development. Such scanning of a limited number of individuals in October 2002 revealed detectable ovaries in both populations with a comparable size however, male testes were not clearly identified. In April 2003, clearly mature individuals of both sexes were present in both populations. There were significantly more spawning individuals in the ambient photoperiod population than in the LL population (93.5% v 43.2%) (Figure 3.12) with a corresponding lower proportion of maturing (0% v 38.2%) or unknown individuals (6.5% v 18.6%).

3.3.3.2 Growth

Both populations showed significant increases in weight and length between each sample point over the trial period (Figure 3.13). Despite both populations originating from a common pool the LL population was significantly smaller (weight & length) at the start of the trial and it remained so until April 2003; thereafter the populations were statistically comparable.

Weight and length specific growth rates (based on population means) were higher in the LL group from July 2002 to April 2003; thereafter they were higher in the ambient photoperiod group from April to September 2003 (Table 3.2).

Condition factor significantly increased in both populations from July 2002 to October 2002 and thereafter there were no significant changes (Figure 3.14). Between the populations, condition factor was comparable until April 2003, after which point the LL population was significantly lower.

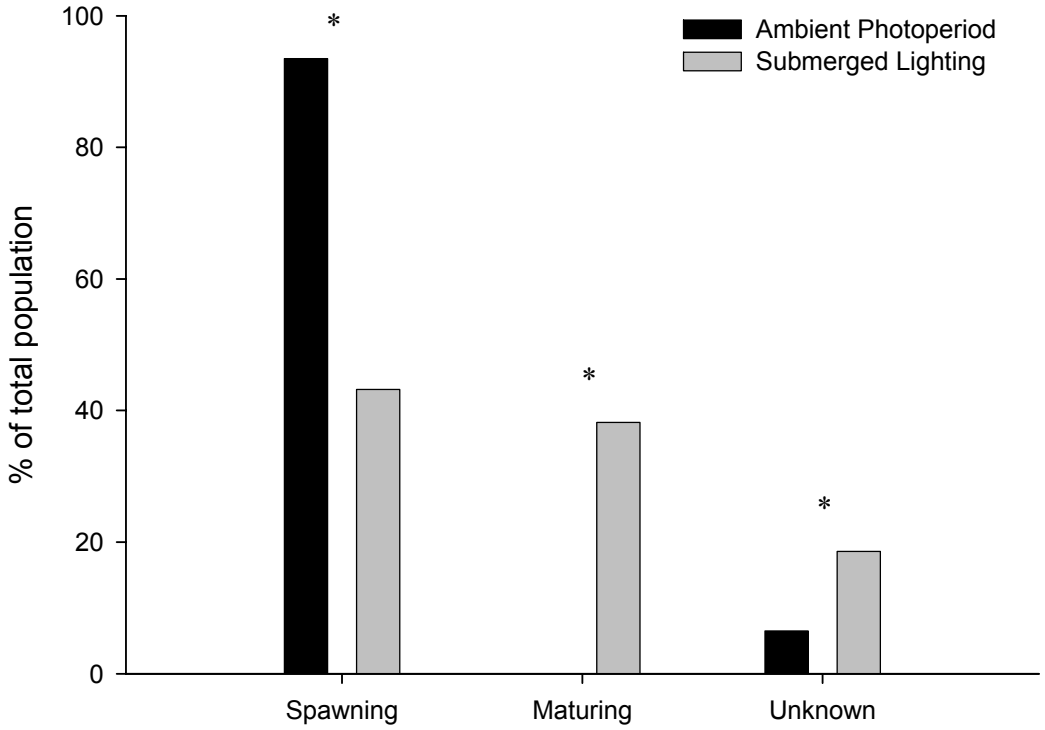
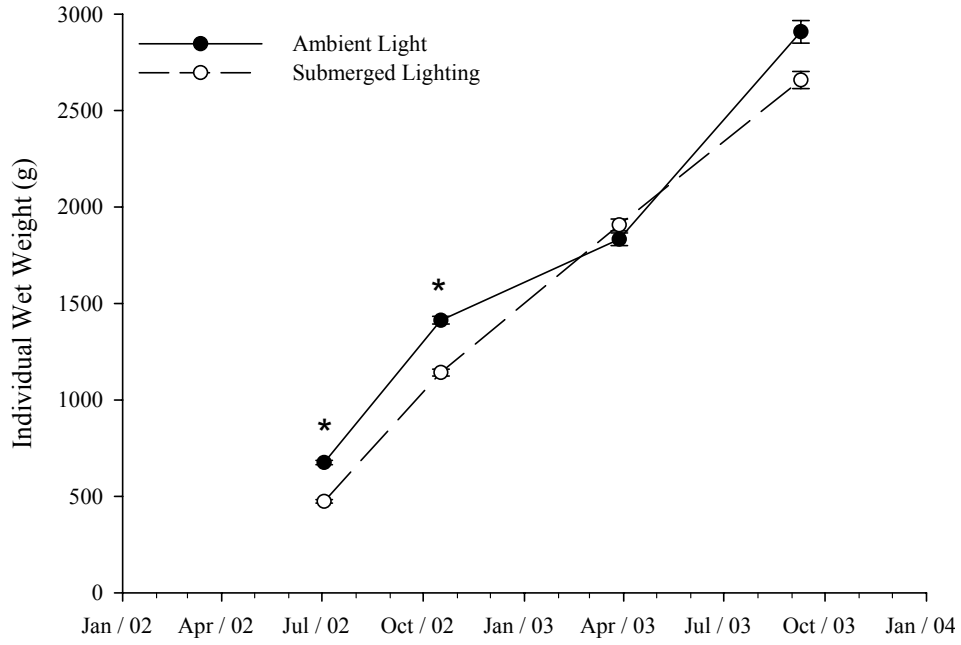


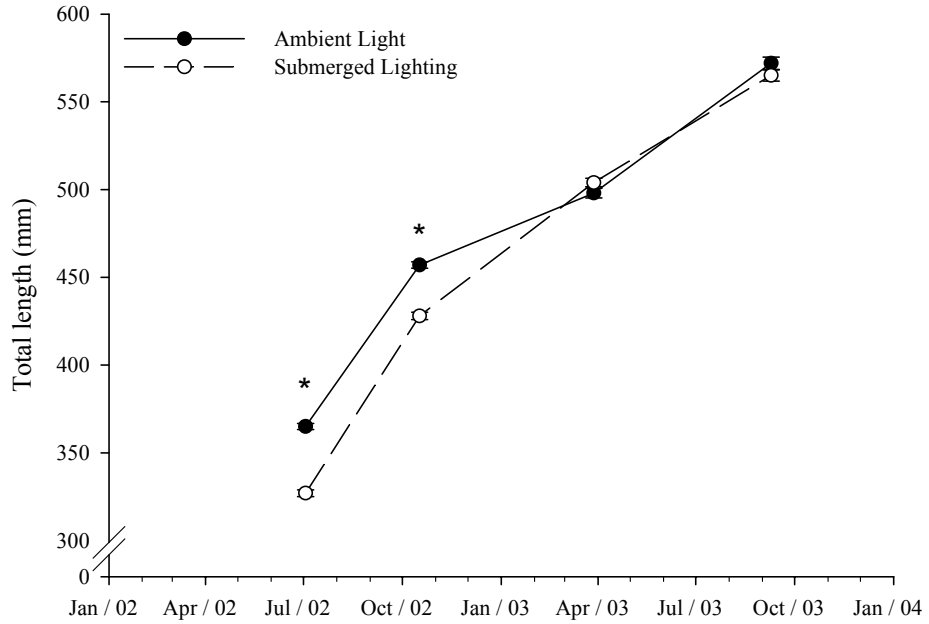
Figure 3.12: Percentage maturation rates visually assessed on 29th March 2003, in cod held under either ambient photoperiod (black bar) or ambient photoperiod with continuous artificial illumination superimposed (grey bar) from July 2002. * = significant difference ($p<0.05$) (n=200).

Table 3.2: Weight and length specific growth rates between July 2002 and September 2003 for cod held under ambient photoperiod or ambient photoperiod with continuous artificial illumination superimposed from July 2002. Data based on population mean data, with the difference in growth rates between treatments expressed as a percentage.

Growth period	Ambient light	Additional light (LL)	% difference between LL and ambient light
SGRw (%.day^{-1})			
July – October 2002	0.697	0.830	+ 19.1
October 2002 – April 2003	0.160	0.312	+ 95.0
April – September 2003	0.280	0.205	- 26.8
SGRl (%.day^{-1})			
July – October 2002	0.212	0.257	+ 21.2
October 2002 – April 2003	0.053	0.100	+ 100
April – September 2003	0.084	0.070	- 16.7



a.)



b.)

Figure 3.13: Mean individual wet weight (g) (a) and total length (mm) (b) ± SEM (n=200) for individuals held under either ambient photoperiod or ambient photoperiod with continuous artificial illumination superimposed (LL) from July 2002. * = significant difference ($P<0.05$) ANOVA GLM with Tukey *post hoc* test.

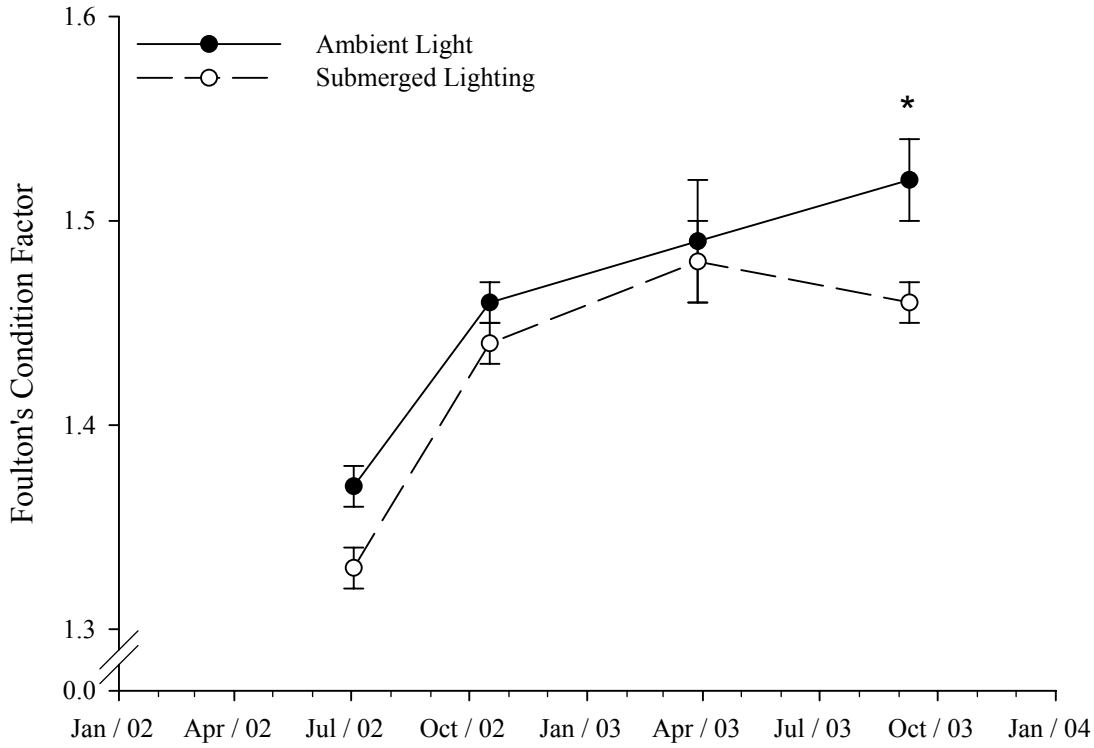


Figure 3.14: Mean Foulton's condition factor ± SEM (n=200), for individuals held under either ambient photoperiod or ambient photoperiod with continuous artificial illumination superimposed (LL) from July 2002. * = significant difference ($P<0.05$) ANOVA GLM with Tukey *post hoc* test.

3.4 EXPERIMENT III: AN INVESTIGATION INTO THE ENTRAINMENT OF REPRODUCTION UNDER CONSTANT PHOTOPERIODS

3.4.1 Objectives

The aim of this experiment was to investigate if seasonal photoperiod regimes which utilise either constant long or short days only (i.e. square wave photoperiods) can entrain maturation in Atlantic cod.

3.4.2 Materials and Methods

3.4.2.1 Fish stocks and rearing conditions

Atlantic cod juveniles were produced at Port Erin Marine Laboratory, Isle of Man, UK (54:05^{o}N, 4:45^{o}W) during the spring of 1999 from an Irish Sea origin broodstock. They were maintained under ambient conditions (photoperiod and temperature) for this location. The fish were brought to the Marine Environmental Research Laboratory (MERL), Machrihanish, Argyle, Scotland (55:44^{o}N, 5:44^{o}W) in the autumn of 1999 where they were maintained under simulated natural conditions (photoperiod and temperature) until the start of the experiment.

On the 15th December 2001, 114 individuals (mean weight 760 ± 18 g) were anaesthetised and a passive integrated transponder tag was placed subcutaneously in the dorsal flank (Section 2.1.5). Individuals were allowed to recover in clean, aerated sea water before being returned to the holding tanks. No mortalities were associated with the tagging process.

The six experimental tanks, located indoors at MERL, were circular 1.5m^3 tanks (2m radius, approximate running depth 0.5m) with light proofed lids. Artificial light was provided by a single 9W fluorescent bulb located centrally under the tank cover

approximately 0.2m above the water surface. Light intensity at the water surface was 0.186 Wm^{-2} (0.991 μmoles $m^{-2}s^{-1}$ or 84 lux) when illuminated and 0 Wm^{-2} (0 μmoles $m^{-2}s^{-1}$, 0 lux) in the dark phase. During the experimental period water temperature ranged from $5^{O}C$ to $15^{O}C$ (Figure 3.15) and the salinity ranged between 29 ‰ and 34 ‰ with an average of 33 ‰ (Figure 3.16). Fish were fed a commercial dry pellet (EWOS, Bathgate, UK) to satiation 3-5 times per day between 9 am and 5 pm, 7 days a week. Stocking densities were maintained between 10 $kg.m^{-3}$ and 20 $kg.m^{-3}$.

3.4.2.2 Photoperiod regimes

All individuals were randomly assigned to one of 6 photoperiod groups (Figure 3.17) (n= 19 to 21 per treatment) with test photoperiods being applied from the winter solstice (21st December 2000) onwards. Photoperiod 1 represented an ambient seasonal cycle while photoperiods 2 to 5 were repeating cycles with a 12 month period, consisting of a 6 month window of continuous light (24L:0D) followed by a 6 month period of continuous short days (7L :17D). Photoperiod 2 began the cycle (transferred from short days to continuous light) at the experiments start with photoperiods 3-5 being staggered thereafter with a 2 month delay respectively. Photoperiod 6 was continuous 24 hours light from the winter solstice onwards throughout the remainder of the experiment.

On the 15th July 2001 photoperiod treatment 1 was lost due to a technical problem (failure to replace a stand pipe during routine husbandry). Therefore photoperiod treatment 3 was adapted by returning it to an ambient photoperiod cycle (Figure 3.17) based on similarity of performance to that date.

The population maintained under an ambient photoperiod (*i.e.* treatment 1 or 3) was maintained in a single tank while all other populations were reared communally under either continuous illumination or short daylength as their regime dictated.

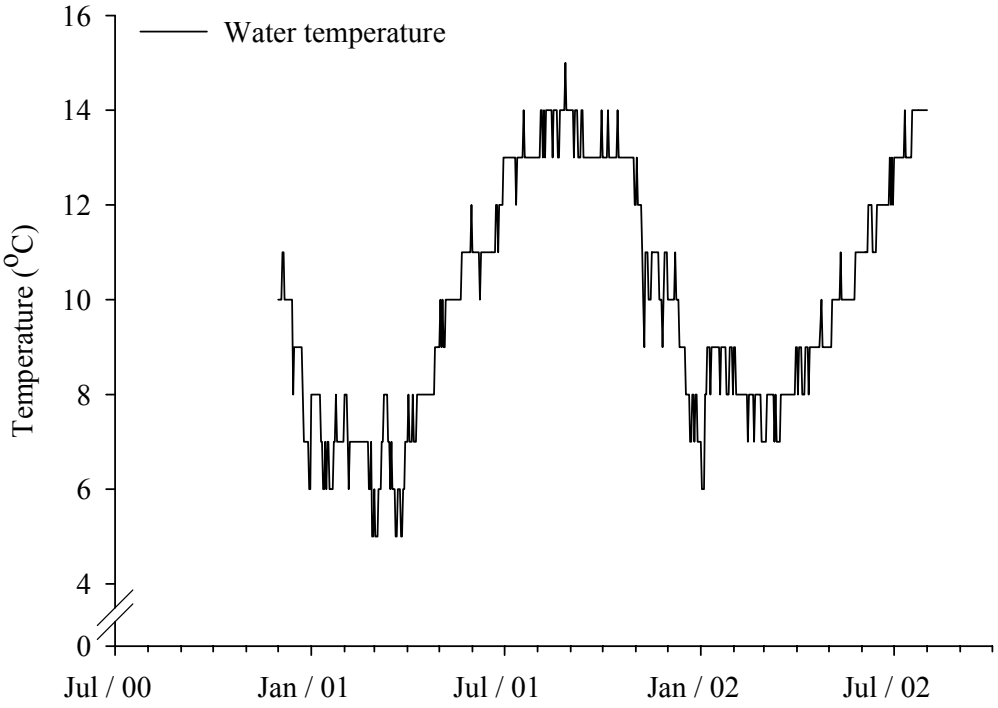


Figure 3.15: Daily temperature (°C) of seawater at Machrihanish from 1st July 2000 to 9th August 2002.

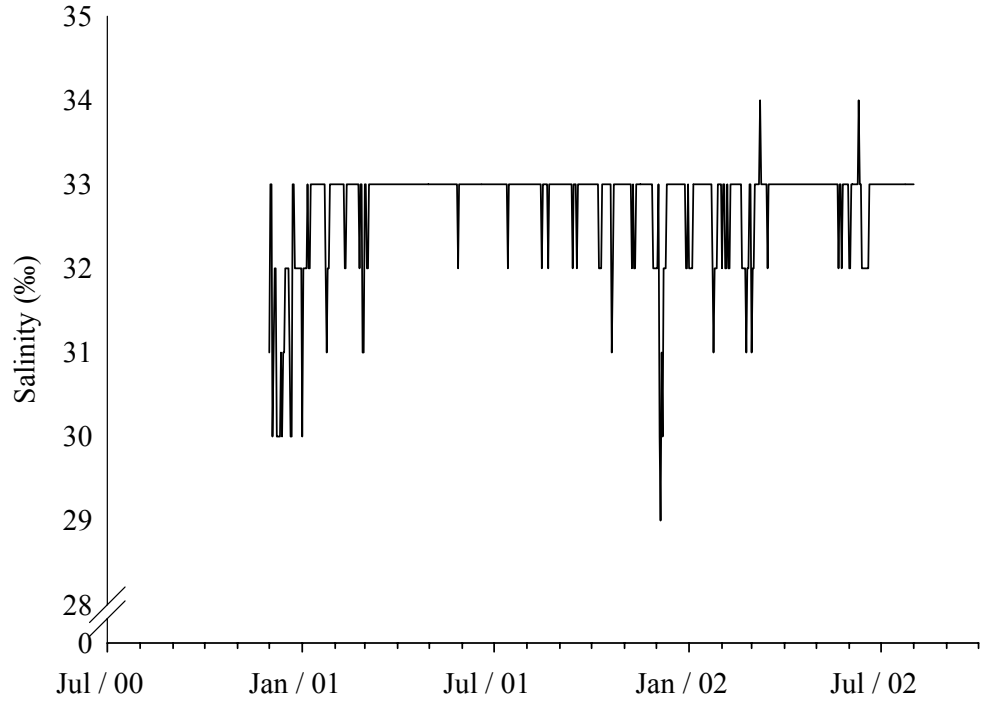


Figure 3.16: Daily salinity (‰) of seawater at Machrihanish from 1st July 2000 to 9th August 2002.

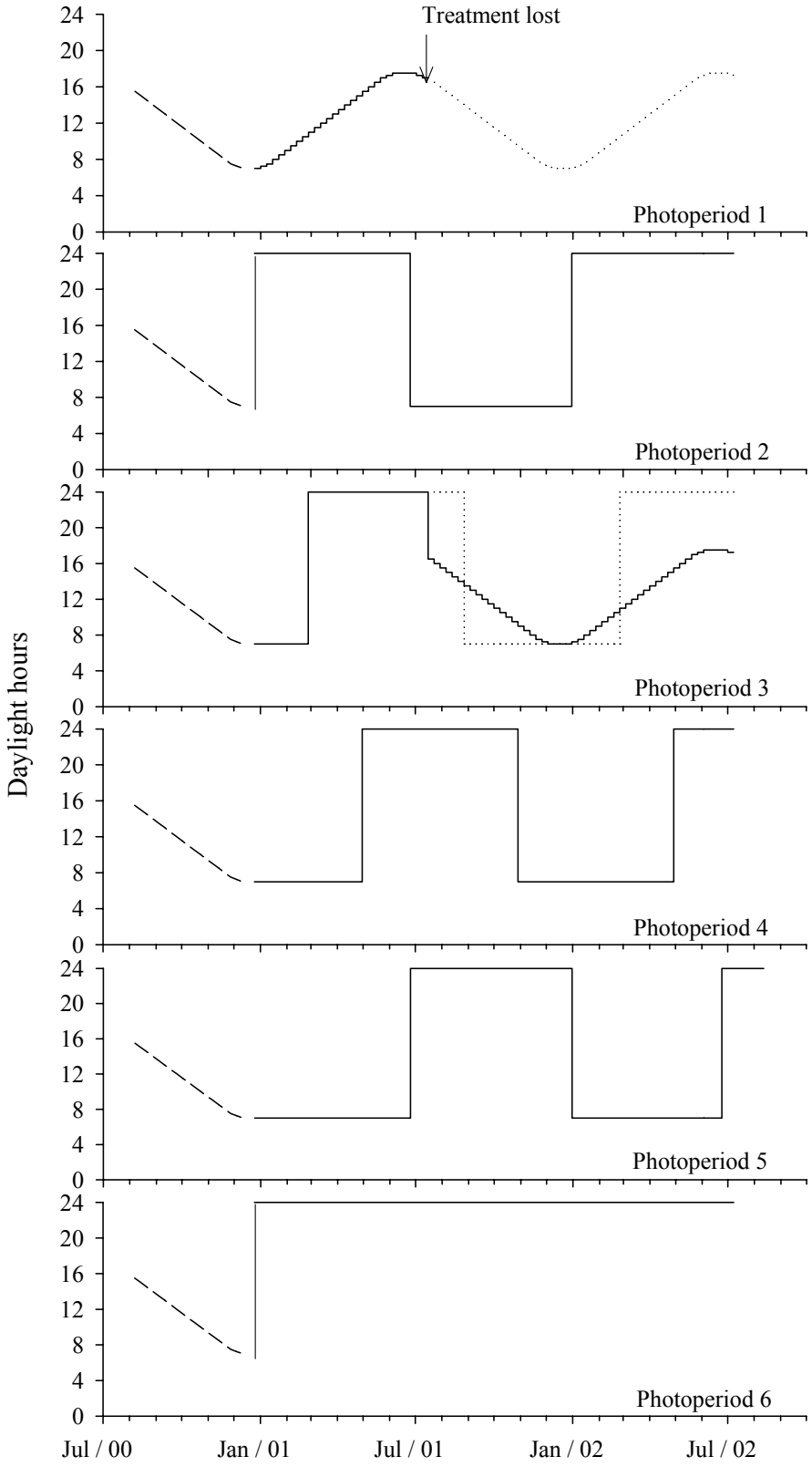


Figure 3.17: Experimental photoperiods 1 to 6 (solid line). Dotted line in photoperiods 1 and 3 represent planned treatment prior to adaptation due to mortality event. Photoperiod prior to experiment shown as dashed line.

Individuals from each population were therefore spread evenly between 2 or 3 tanks per photoperiod treatment (24:0 or 7:17, L:D). Such a strategy was necessary due to limited fish and tank availability.

3.4.2.3 Sampling regime

On the16th December 2000, 6th February 2001 and every month thereafter until the 8th of July 2002, all individuals were anaesthetised and visually inspected for maturation status (Section 2.3.2). A blood sample was removed into heparinised syringes and plasma stored at -70°C prior to later analysis for testosterone (Section 2.4.2) and calcium (Section 2.4.4). Between February and June 2001 and January and June 2002, all individuals were visually inspected for maturation status twice a month. The experiment was completed on the 6th July 2002 for all treatments except photoperiod 5 which, showing indications of pending maturation, was maintained for one further month until the 9th August 2002.

3.4.3 Results

3.4.3.1 Mortalities

Mortality rates over the trial length were high. By the trial end the cumulative mortality rates range from 47% to 60% in all treatments, except photoperiod 1, where 100% of population were lost on 15th of July 2001 due to a technical problem (Figure 3.18). No specific cause could be ascribed to these losses.

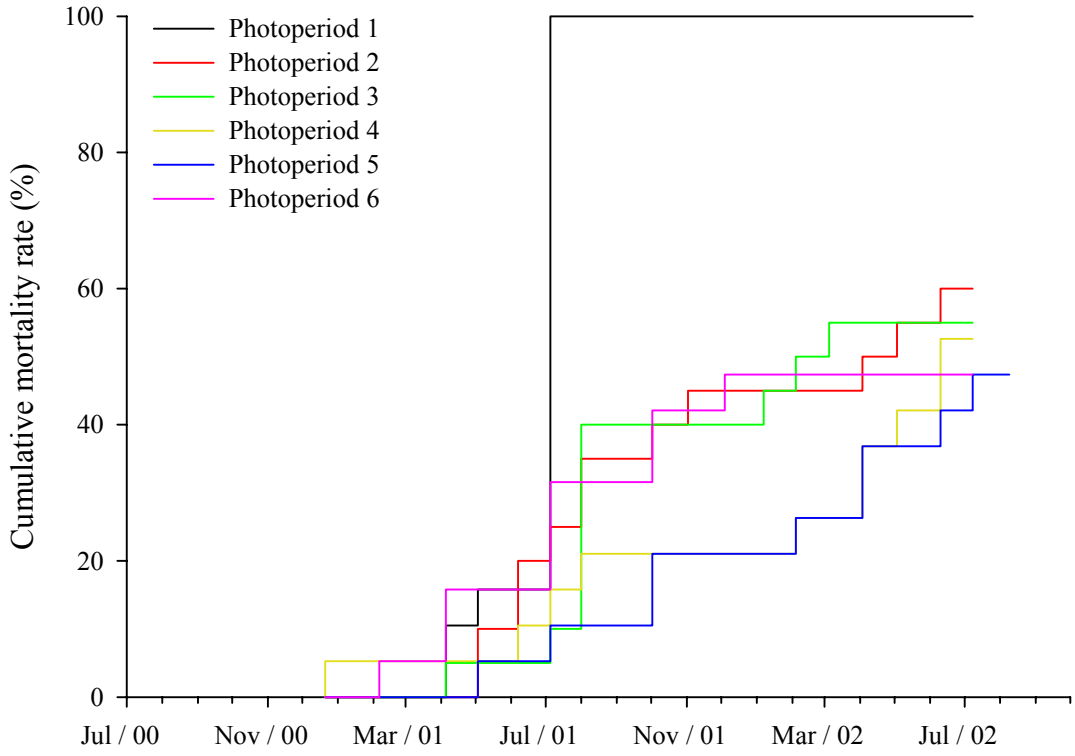


Figure 3.18: Cumulative mortality rates (% of initial population total) for photoperiod treatments 1 to 6.

3.4.3.2 Maturation: Timing and commitment.

All experimental populations, except photoperiod 1, matured twice during the experimental period (Figure 3.19). The overall sex ratio revealed through the monthly

sampling was M: F, 1: 1.34. The spawning profiles in both seasons exhibited alterations in relation to the populations' photoperiod treatments (Table 3.3) as follows:

1st Spawning Season (2001):

Ambient photoperiod (Photoperiod 1): Spermiating males were first detected at the start of February with ovulating females being detected one month later at the start of March. The spawning season lasted for 4 months ending in late May with the entire population being recorded as having spawned (M^{100}) in line with the peak in spawning activity in early April.

Transfer to LL from winter solstice (Photoperiods 2 and 6): Spermiating males and ovulating females were first detected in both treatments from the start of February, 29 days prior to ovulating females being detected in the simulated natural photoperiod. As such the date by which 100% of the population had been recorded as mature (M^{100}) was advanced in both populations by 41 days reducing the spawning season to 3 months.

Maintenance on SD from winter solstice (Photoperiods 3 to 5): The detection of first spermiating males showed no difference to the SNP population while the detection of first ovulating females was advanced by 12 days in treatments 3 and 5 but delayed by 20 days in treatment 4, compared to the SNP treatment. The maintenance on short days from the winter solstice prolonged maturation as is apparent from delays of between 15

Figure 3.19 : (Following page) Maturation profiles with percentages of male (black bars) (n=5 to 13) and female (grey bars) (n = 3 to 11) releasing gametes in relation to applied photoperiod treatments 1 to 6 (black line).

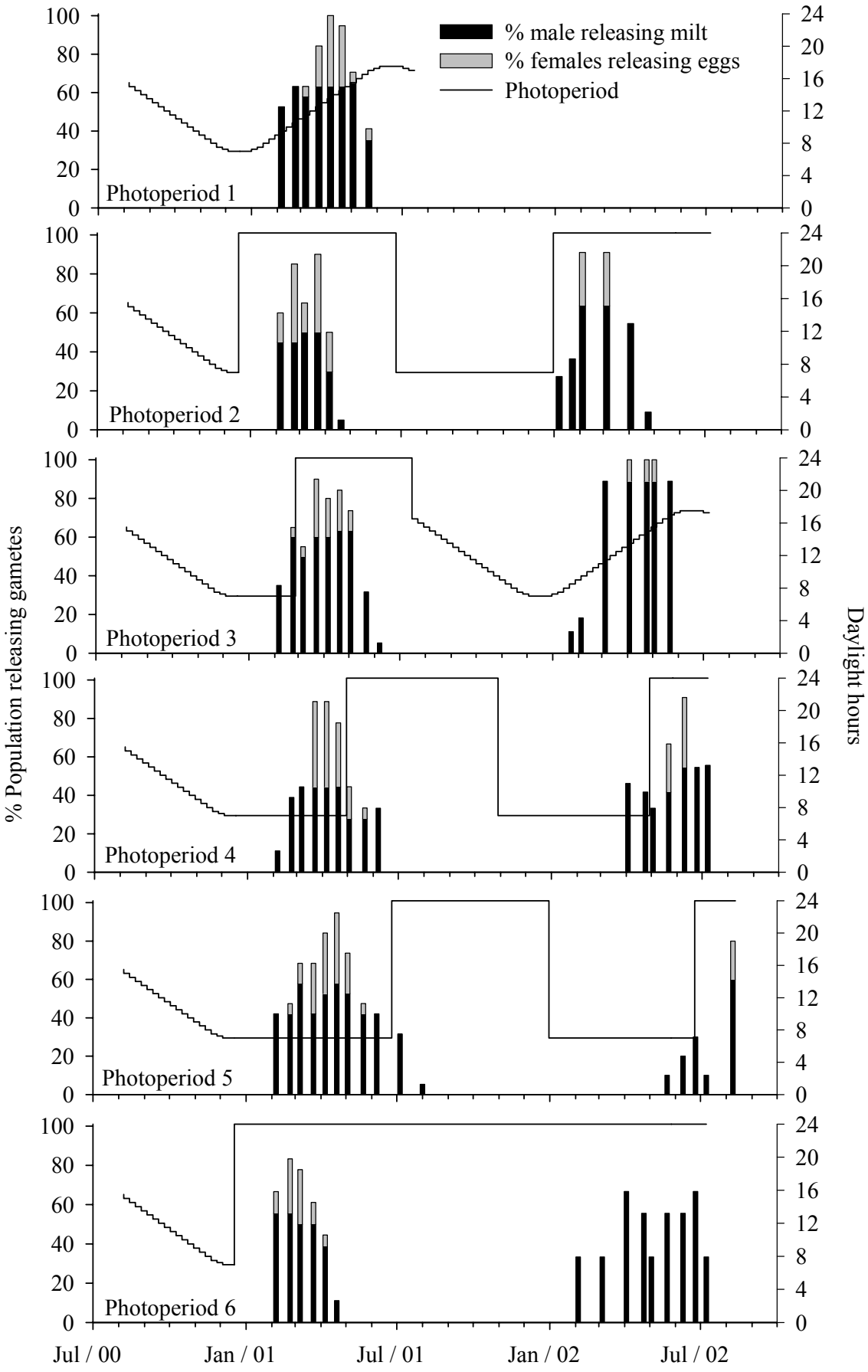

% male releasing milt
% females releasing eggs
Photoperiod
Photoperiod 1
Photoperiod 2
Photoperiod 3
Photoperiod 4
Photoperiod 5
Photoperiod 6
% Population releasing gametes
Daylight hours
Jul / 00
Jan / 01
Jul / 01
Jan / 02
Jul / 02

Table 3.3: Spawning profiles of experimental populations 1 to 6 in the first (2001) and second (2002) spawning seasons listing dates when first spermiating male (1st Male) and ovulating female (1st Female) of each population were observed along with the date when cumulative maturation rates reached 100% (M^{100}) of the population total. All are listed with respect to the timing (± no. of days) of the simulated natural photoperiod population. The length of the spawning season in months is listed along with the number of males and females in respective populations during the spawning season.

2001	**1**	**2**	**3**	**4**	**5**	**6**
1st Male	6th Feb	6th Feb	6th Feb	6th Feb	6th Feb	6th Feb
Relative to SNP	**0**	**0**	**0**	**0**	**0**	**0**
1st Female	7th Mar	6th Feb	23rd Feb	27th Mar	23rd Feb	6th Feb
Relative to SNP	**0**	**-29 days**	**-12 days**	**+20 days**	**-12 days**	**-29 days**
M^{100}	5th April	23rd Feb	20th Apr	3rd May	20th Apr	23rd Feb
Relative to SNP	**0**	**-41 days**	**+15 days**	**+28 days**	**+15 days**	**-41 days**
Spawning Season	**4 Months**	**3 Months**	**4.5 Months**	**4.5 Months**	**6 Months**	**3 Months**
Fish No. M:F	**12:7**	**10:11**	**13:7**	**9:9**	**11:8**	**11:8**

2002	**1**	**2**	**3**	**4**	**5**	**6**
1st Male		7th Jan	23rd Jan	3rd April	22nd May	4th Feb
Relative to SNP		**-16 days**	**0**	**+70 days**	**+119 days**	**+12 days**
1st Female		4th Feb	3rd April	22nd May	9th Aug	
Relative to SNP		**-58 days**	**0**	**+49 days**	**+128 days**	
M^{100}		5th Mar	3rd April	10th June	9th Aug	
Relative to SNP		**-29 days**	**0**	**+68 days**	**+128 days**	
Spawning Season		**4 Months**	**4 Months**	**3.5 Months**	**3 Months**	**5 Months**
Fish No. M:F		**7:4**	**8:4**	**5:4**	**7:3**	**7:3**

to 28 days in 100% (M^{100}) of the population maturing and the elongation of the spawning season to 4.5, 4.5 and 6 months respectively in treatments 3,4 and 5.

2nd Spawning season (2002)

Ambient photoperiod (Photoperiod 3): Because photoperiod 1 had been lost through a technical problem, photoperiod 3 was adapted to represent the SNP controls by transferring to the simulated natural photoperiod as of the 15th of July 2001. In this adapted SNP population, spermiating males were first observed on the 23rd of January 2002 with ovulating females being observed over 2 months later from the 3rd of April onwards. The spawning season lasted for 4 months until the end of May. This maturation profile mimics accurately that recorded in the SNP treatment in experiment I of this chapter (Section 3.2.3.1) which was a population of the same year class that prior to this experiment had been raised on a simulated natural photoperiod regime under comparable husbandry conditions.

Photoperiod 2, 4 and 5: The spawning seasons of treatments 2, 4 and 5 are in accordance with the timing of their respective photoperiod cycles. In all three treatments spermiating males were generally found within approximately 5 – 6 months of the population being transferred from continuous light to a short day photoperiod, generally pre-empting the subsequent photoperiod rise, while ovulating females were only detected following the reversal of photoperiod treatment. The clearest reflection of this can be expressed through the attainment of the entire population being recorded as mature (M^{100}). This was approximately 1 month advanced, 2 months delayed and 4 months delayed in photoperiods 2, 4 and 5 respectively in relation to the SNP population.

Photoperiod 6: This population had been maintained on continuous illumination from the trial start in December 2001. In the spring of 2002 the first spermiating males were observed at the start of February and remained present for 5 months until the end of the trial in July. However, at no time were the females of this population (n=3) observed to release eggs on inspection.

3.4.3.3 Plasma testosterone

Testosterone levels were significantly lower in females compared to males at all times with females exhibiting a mean range from 0.2 to 5 $ng.ml^{-1}$ (Figure 3.20) while the males exhibited a mean range from 0.5 to 20 $ng.ml^{-1}$ (Figure 3.21).

Females

In the first spawning season (2001) all treatments displayed a distinct elevation in testosterone levels ($\geq$2.5 $ng.ml^{-1}$) just prior to, or during, ovulation (Figure 3.20). In the later half of the spawning season levels fell to baseline levels of *circa* 1 $ng.ml^{-1}$. In the second spawning season (2002) only treatments 2 and 3 exhibited clear peaks in testosterone comparable to those seen in 2001, again just prior to ovulating females being observed. Treatments 4 and 5 in 2002 exhibited levels elevated to >1.5 $ng.ml^{-1}$ in the months preceding spawning, while treatment 6 displayed no such fluctuations with levels remaining $\leq$ 1 $ng.ml^{-1}$ throughout.

Males

In the first spawning season (2001) the males in all treatments showed perceptible elevations in plasma testosterone levels, generally in the middle of the window when spermiating males were observed (Figure 3.21). Levels in treatments 1

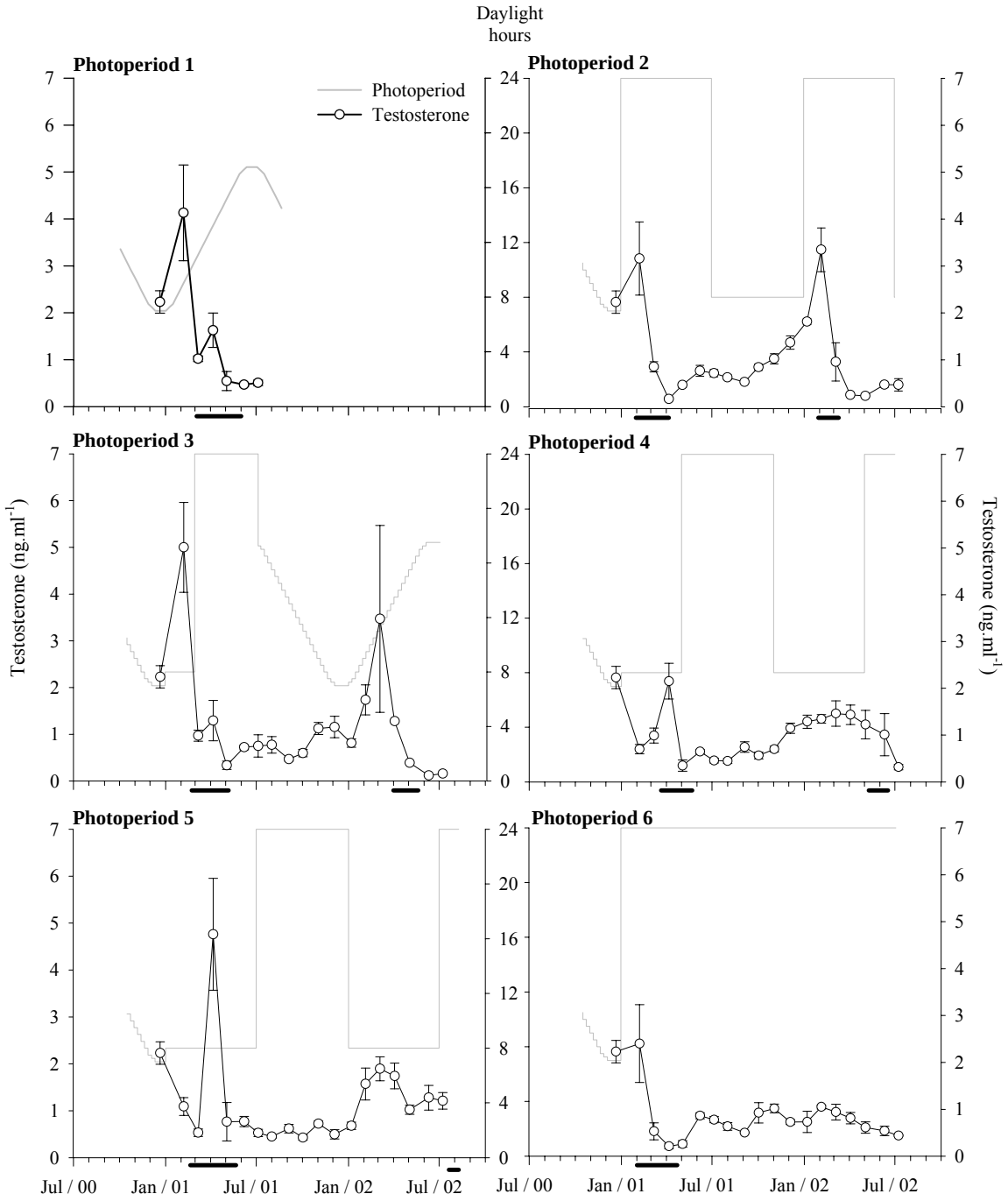


Figure 3.20: Mean female plasma testosterone concentration ($ng.ml^{-1}$) ± SEM (n= 3 to 11) in Atlantic cod maintained under photoperiod treatments 1-6 (grey line). Black horizontal bars denote period when ovulating individuals were observed.

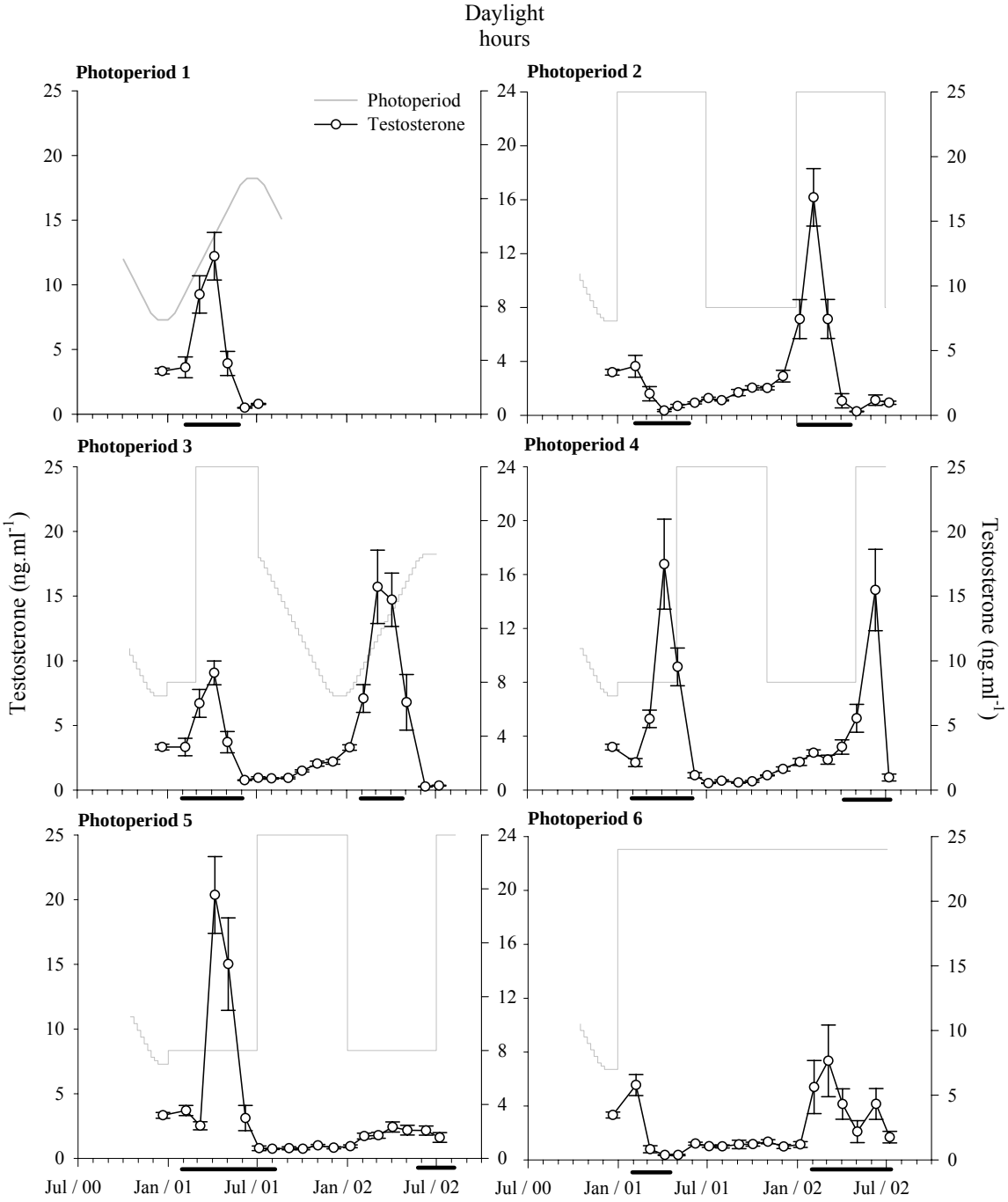


Figure 3.21: Mean male plasma testosterone concentration ($ng.ml^{-1}$) ± SEM (n= 5 to 13) in Atlantic cod maintained under photoperiod treatments 1-6 (grey line). Black horizontal bars denote period when spermiating individuals were observed.

and 3 to 5 peaked at ≥ 10 ng.ml^{-1} while treatments 2 and 6 'peaked' at ≤ 5 ng.ml^{-1}. In the second spawning season (2002) treatments 2 to 4 and 6 showed peaks in testosterone with levels ranging from 7 to 17 ng.ml^{-1}, while photoperiod 5 showed no rise above basal levels of ≤ 2.5 ng.ml^{-1} through the entire year.

As the timing of the testosterone peaks mimicked the alterations in timing of spawning activity in relation to photoperiod treatment in both sexes, a peak in testosterone in photoperiod 5 in relation to the second spawning season may have been undetected due to the completion of sampling prior to the completion of this population's maturation activity.

3.4.3.4 Plasma calcium levels

Mean plasma calcium levels in the females of all treatments ranged from approximately 150 to 300 mg.dl^{-1} (Figure 3.22). In the first spawning season (2001), levels in all treatments peaked at ≥ 200 mg.dl^{-1}, levels which were maintained broadly across the spawning season before declining to basal levels of *circa* 150 mg.dl^{-1}. In the second spawning season, in treatments 2 to 4, levels generally rose in the 3-4 months directly preceding spawning to reach a peak of ≥ 200 mg.dl^{-1} in line with the detection of first ovulating females. In treatment 5, levels displayed a rise in the months before the end of the trial to reach a peak of close to 200 mg.dl^{-1} on the last sample when first ovulating females were observed. In treatment 6 however, after the peak in 2001, plasma calcium levels remained at basal levels until the end of the trial.

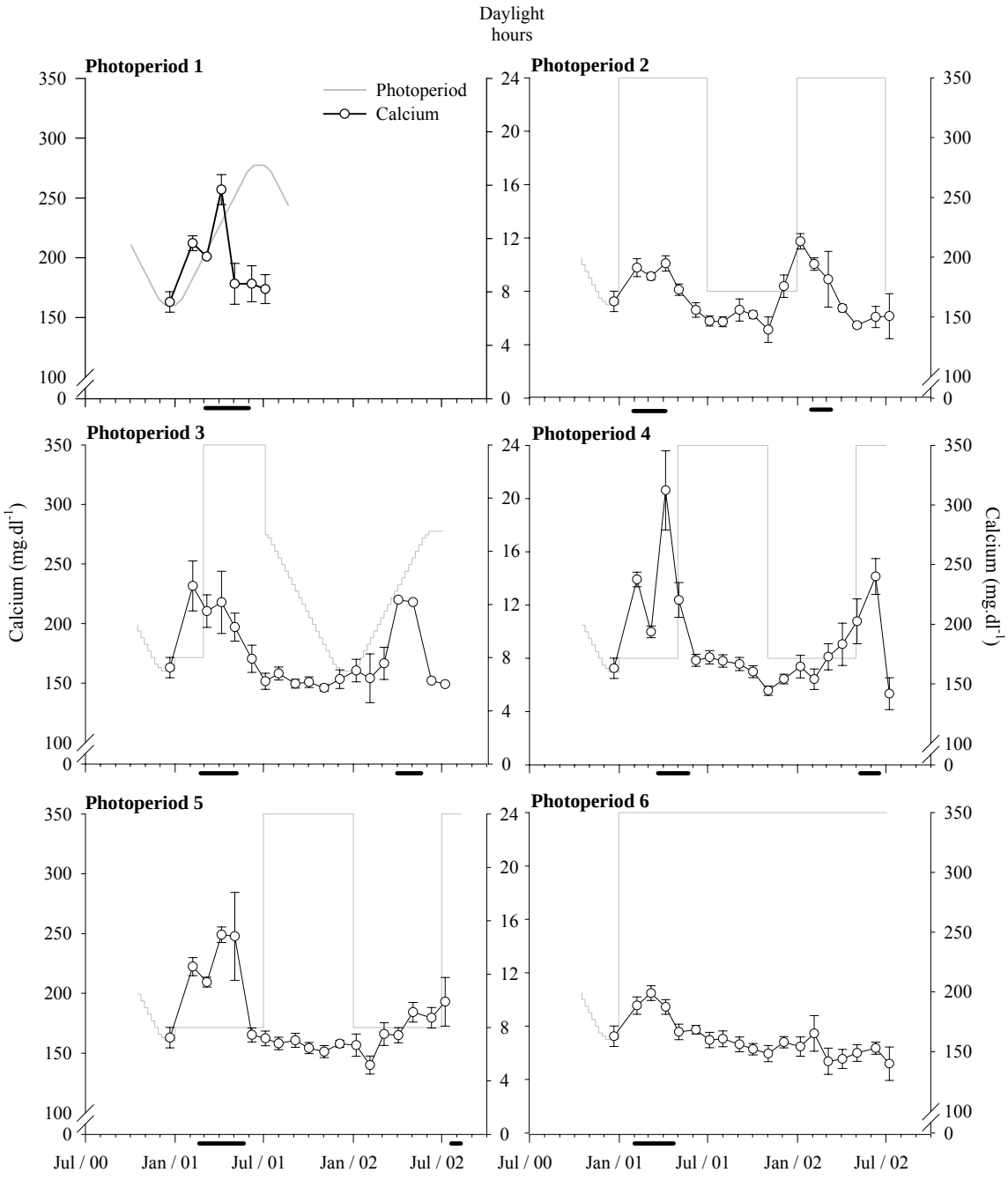


Figure 3.22: Mean female plasma calcium concentration ($mg.dl^{-1}$) ± SEM (n= 3 to 11) in Atlantic cod maintained under photoperiod treatments 1-6 (grey line). Black horizontal bars denote period when ovulating individuals were observed.

3.4.3.5 Growth performance

The principal aim of this work was to examine photoperiodic regulation of maturation however, as growth performance was measured in all individuals as part of routine husbandry practice, it was felt the presentation of limited data would be useful for later discussion of the maturation data. Due to the low number of individuals per treatment, there was increasingly wide variation in population means (Figure 3.23) hence, for the treatments 1 to 4 and 6, both sexes appeared to be of a comparable size throughout. This was in contrast to photoperiod 5 in which both sexes appeared comparable until July 2001, where after the females of this population appeared to display a greater growth rate than the males of the same population ending the trial over 60% larger.

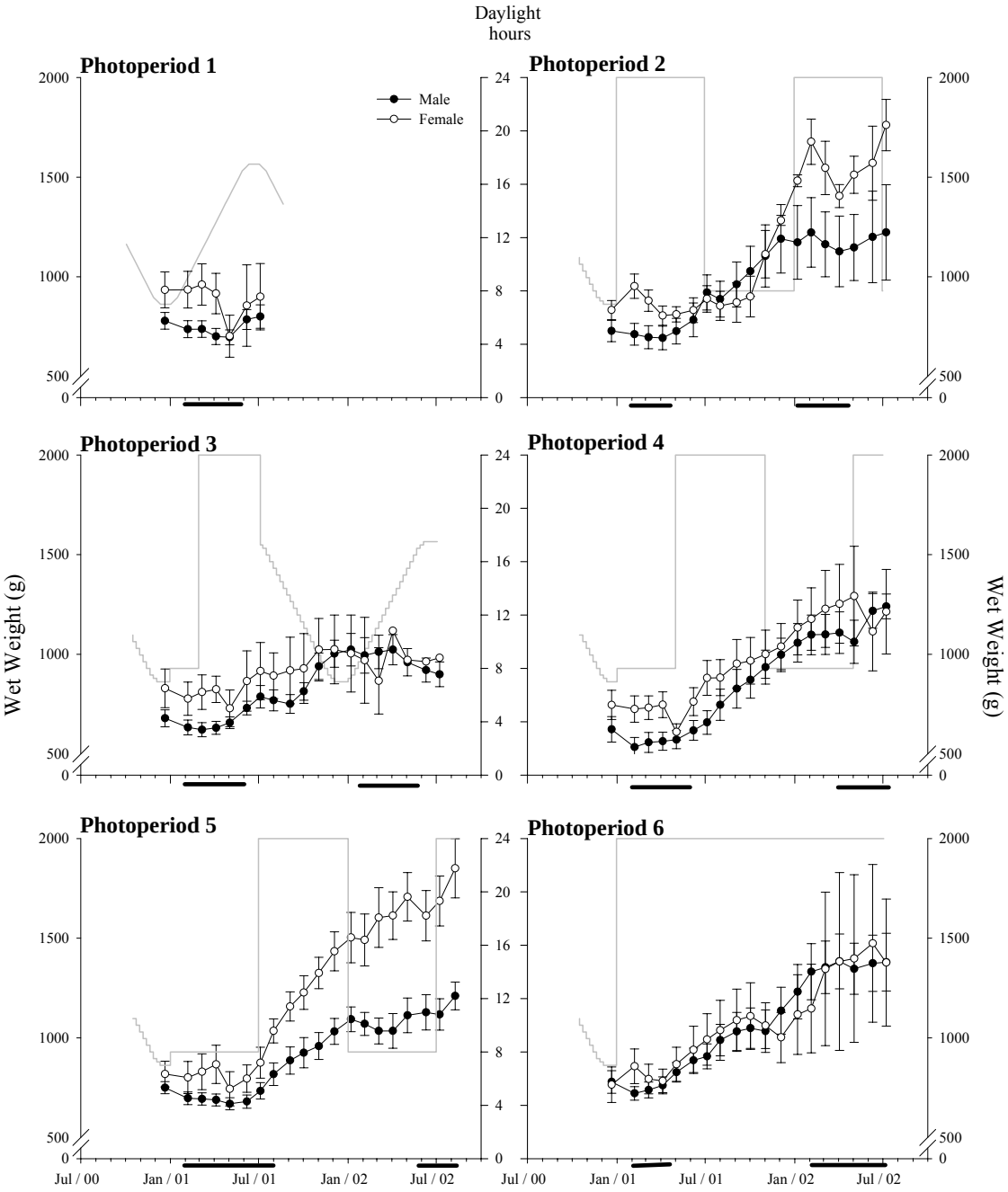


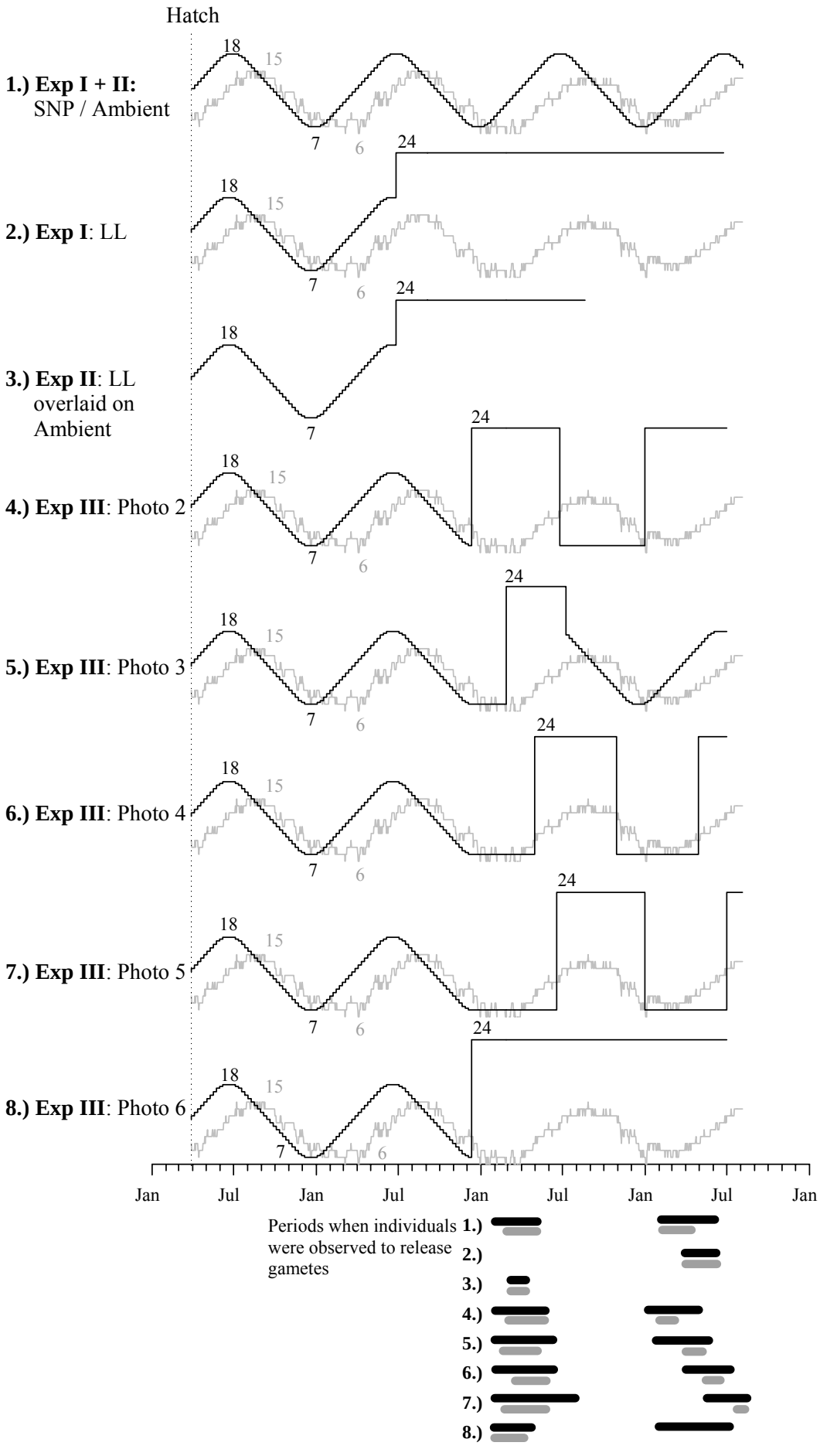
Figure 3.23: Mean individual wet weight (g) ± SEM for male (filled circles, n= 5 to 13) and female (open circles, n= 3 to 11) in Atlantic cod maintained under photoperiod treatments 1-6 (grey line). Black horizontal bars denote period when mature individuals were observed.

3.5 Summary of results from experiment I to III

3.5.1 Experiment I

- Application of continuous light from 15 MPH prevented maturation at 2 years and significantly reduced maturation at 3 years of age (Figure 3.24).
- The LL population ended the trial being significantly heavier (40%) than their SNP reared siblings.
- There was an apparent cycle in gonadal development at 2 years observed in the LL population via ultrasound scanning though no "mature" individuals were observed. Then, in the third year, gonadal development appeared non synchronous in the LL population with some fish maturing, however the majority remained "immature".
- Condition factor shows a clear seasonal cycle in both the SNP and LL treatments showing an inverse correlation to the ambient temperature cycle.
- Plasma melatonin measured in February 2001 (19 months post light treatment) showed a diel rhythm in the SNP population while the LL population levels did not rise above basal levels over the diel cycle.

Figure 3.24: (Following page) Illustrative summary of experimental photoperiod treatments (black line) tested in Chapter 3 in relation to the control populations. Temperature profiles (grey line) are shown where recorded. Horizontal bars represent periods of observed mature males (black) and females (grey) for the respective treatments. Range of daylight hours administered in photoperiod treatments along with ambient water temperatures are shown on the respective diagrams.


Hatch
1.) Exp I + II: SNP / Ambient
2.) Exp I: LL
3.) Exp II: LL overlaid on Ambient
4.) Exp III: Photo 2
5.) Exp III: Photo 3
6.) Exp III: Photo 4
7.) Exp III: Photo 5
8.) Exp III: Photo 6
18
15
7
6
24
Jan
Jul
Jan
Jul
Jan
Jul
Jan
Jul
Jan
Periods when individuals were observed to release gametes
1.)
2.)
3.)
4.)
5.)
6.)
7.)
8.)

3.5.2 Experiment II

- Exposure to submerged lighting from approximately 15 MPH in an open cage structure significantly altered maturation rates (Figure 3.24).
- Both the LL and ambient photoperiod groups significantly increased in size between each sample and ended the trial a comparable size despite the LL group starting significantly smaller; this was due primarily to higher growth rates over the winter period.

3.5.3 Experiment III

- Out of season "square wave" photoperiods significantly altered timing of maturation in repeat spawning Atlantic cod (Figure 3.24).
- In "mature" individuals, which had previously experienced a simulated natural photoperiod up to the winter solstice at approximately 21 MPH, the transition to LL compressed spawning while maintenance on short days extended spawning at 2 years of age.
- Continuous LL exposure from the winter solstice prior to first maturation (21 MPH) failed to prevent male maturation at 2 and 3 years of age, however it did appear to prevent female maturation at 3 years.

3.6 Discussion

The aim of this chapter was to investigate photoperiod regulation of maturation in an Irish Sea strain of Atlantic cod reared in its native thermal conditions. Therefore, the photoperiod regime reported to most successfully inhibit maturation in Atlantic cod in coastal Norway was examined. Physical maturation and growth parameters were quantified both in an enclosed tank system, and in a commercial open cage system. Then, "square wave" photoperiods, which utilised simplified seasonal transfers between long and short day lengths were tested to further investigate how the timing of maturation, in terms of both physiological and endocrinological parameters, can be altered. As is discussed below, this dataset provides a valuable insight into the natural rhythms of maturation under both entrained (seasonally varying photoperiod regimes) and un-entrained (prolonged exposure to continuous illumination) photic conditions.

3.6.1 Maturation

Maturation under ambient, simulated natural photoperiod conditions

In all three experiments, the fish held in ambient and simulated natural photoperiod treatments matured between February and May, which is in close accordance with that reported for the ambient spawning season for wild cod in the eastern Atlantic Ocean (Hislop, 1984; Bye, 1990; Kjesbu, 1994; Cardinale and Modin, 1999) as well as in the captive studies of Hansen *et al.* (1995)[1], Norberg *et al.* (1995) [2],

[1] The work described in the introduction by Hansen *et al.* (1995) was, during the course of this work, published in fuller form, Hansen *et al.* (2001), which will be referred to from here on.

[2] The work described in the introduction by Norberg *et al.* (1995) was, during the course of this work, published in fuller form, Norberg *et al.* (2004), which will be referred to from here on.

Kjesbu *et al.* (1996b), Cyr *et al.* (1998), Karlsen *et al.* (2000) and Dahle *et al.* (2000,2003). Furthermore, in both the SNP photoperiod treatments of experiments I and III, the entire population was observed to be mature which is in agreement with studies of captive populations by Karlsen *et al.* (2000), Dahle *et al.* (2000) and Hansen *et al.* (2001).

The SNP (photoperiod 1) and adapted SNP (photoperiod 3) photoperiod regimes in experiment III demonstrated the concomitant seasonal fluctuations in plasma testosterone and calcium levels that occur in relation to maturation under natural conditions. The mean levels of plasma testosterone reported in this study (Females: 0.2 to 5 $ng.ml^{-1}$, Males: 0.5 to 20 $ng.ml^{-1}$) are comparable to those previously documented in Atlantic cod, as was the significant difference in levels between males and females (Kjesbu *et al.*, 1996a; Cyr *et al.*, 1998; Dahle *et al.*, 2003; Norberg *et al.*, 2004). Both sexes displayed a peak in plasma testosterone levels in the early stages of spawning activity (Females: February 2001, March 2002, Males: April 2001, March 2002) as was observed by Norberg *et al.* (2004). In females, testosterone is the precursor to estradiol which Norberg *et al.* (2004) reports as fluctuating in a similar manner to testosterone in cod. Estradiol passes through the vascular system to stimulate the hepatic tissue which releases vitellogenin (Nagahama, 2000). As explained earlier (Section 1.5), plasma calcium levels have been shown to act as an indirect measurement of vitellogenesis in teleosts (Elliot *et* al., 1984; Norberg *et al.*, 1989; Norberg *et al.*, 2004). Therefore, in the present study, plasma calcium levels indicated that vitellogenic activity increased in the months preceding spawning activity (3 months approximately) to attain a maximum (April 2001/2002) in the initial months of spawning activity in females. The maintenance of elevated calcium levels throughout the spawning period is a reflection of the batch spawning nature of cod as the successive batches of eggs continue to

sequester vitellogenin through the spawning season until they enter final oocyte maturation and are released (Kjesbu, 1989; Kjesbu *et al.*, 1996b).

Maturation under non-entraining photic conditions

The application and subsequent maintenance on LL in all three experiments demonstrated different results which can be ascribed to differences in the rearing system and timing of LL application. In experiment I, although no fish under the LL regime were observed to have released gametes during 2001 (2 years post hatch) a period of reduced weight gain between May and October 2001 was observed. Over this period weight did not significantly increase while prior to and following this the population had displayed consistent, significant weight gain. During this period, water temperature rose from 10^{o}C to 14^{o}C in August and September which represents the optimal thermal range for cod growth performance (Jobling 1988; Pedersen and Jobling, 1989) and food was readily available. In relation to this period, some limited gonadal activity (development followed by resorbtion) was observed via ultrasound imaging. It could be possible that this represented what has been referred to as adolescence (Dennison and Bukley, 1972) or a "dummy run" (Trout, 1957; Woodhead, 1959; Eliassen and Vahl, 1982a; Hassin *et al.*, 1997). Teleosts have been shown to exhibit significant alterations in behavioural, physiological and endocrinological parameters of the maturation process in the years prior to first final maturation. Fluctuations in GSI in immature fish in line with the natural spawning season have been recorded in a number of species including two members of the order Gadiformes, cod (Eliassen and Vahl, 1982a) and Atlantic hake (Hickling, 1935). A seasonal cycle of vitellogenesis that does not lead to spawning has been recorded in black bullheads (Dennison and Bukley, 1972) while Elliot *et al.* (1984) recorded small, though significant, rises in sex steroids, vitellogenin and calcium

one year prior to maturation in rainbow trout. Weltzien *et al.* (2003) noted detectable changes in androgens one year before first maturation in Atlantic halibut. Interestingly Trout (1957), Woodhead (1959) and Rose (1993) have all recorded immature cod joining in spawning migrations in the years prior to maturation and increased thyroid activity in the immature cod partaking in the migrations with respect to younger non-migrating immature individuals. These "dummy runs" are in part believed to be a survival strategy to allow immature fish to "learn" migration routes and even courtship behaviour prior to first maturation (Rose, 1993; Arnold and Metcalfe, 1995). With these different responses being recorded as dummy runs, it is not unreasonable to assume that in the LL population of experiment I, fish which were of a physiological size capable of maturing were however "inhibited" by photoperiod manipulation but still exhibited some form of limited maturational development. Gonadal development and subsequent resorbtion may have been partially responsible for the associated period of depressed growth. Hansen *et al.* (2001), examining an identical treatment in cod, did not observe a similar period of weight stasis. This may be due to a combination of reduced resolution of sampling times and the completion of recording in July causing their study not to witness the monthly weight fluctuations recorded in this present study. However, these authors did report that in July (approximately 27 MPH), one male out of the 11 fish inspected (8 male: 3 female) (9% of the population sample) recorded a GSI of 6%. Up to this stage, all other fish in the LL treatment had displayed GSI levels below 1%. With reference to the recent work by Dahle *et al.* (2003), it could be suggested that this fish was in the early to mid stages of maturation so not discounting the possibility that there may be some form of limited maturation exhibited in the first year by the LL population under this photoperiod treatment. In the second year of this present work (Experiment I, 2002, 3 years post hatch), a small percentage of the fish matured (18%), starting 93

weeks after the rise in photoperiod from SNP to LL. Interestingly, however, the fish spawned in a comparative timeframe with the natural spawning season by which stage the photoperiod had been unchanged for almost 2 years. The role of temperature as an environmental zeitgeber which could entrain this maturation rhythm should not be ignored. After all, the observed spawning did coincide with the seasonal low in ambient water temperatures (Figure 3.24). However, it is commonly believed that, certainly in animals from the higher latitudes including Atlantic cod, temperature itself plays a more permissive role in regulating events like reproduction (Norberg *et al.,* 2004). As temperature cannot provide the same rigid seasonal timing signal that photoperiod provides, animals have evolved to use this environmental signal as more of an "ultimate" factor which is responsible for subtle alteration in reproductive activity during the spawning season e.g. determination of the timing of initiation and/or inhibition of spawning, as local conditions dictate (Sims *et al.,* 2004; Okuzawa *et al.,* 2003; Webb and Mclay, 1996). As such, this present data suggests that there could be an endogenous component to maturation regulation that may entrain sexual maturation in the absence of entraining photoperiod signals.

In experiment II, the LL treated population matured in a similar timeframe to the ambient photoperiod population. The significantly higher proportion of "maturing" (38.2%) and "unknown" (18.6%) individuals at the time of inspection (29th of March), suggests that the mean spawning date of this population was delayed by approximately 1 to 2 months, however it appeared that close to 100% of the population would mature. Such delayed responses in cage culture have been mentioned by Dahle *et al.* (2000) and Karlsen *et al.* (2000). These authors suggested that differences in illumination levels and/or spectral content between the ambient daylight and the artificial illumination

system applied over the scotophase in open cage systems, may still have allowed the fish to perceive the ambient photoperiod signal. This will be discussed further later.

In experiment III, LL was applied in an enclosed tank system from the winter solstice prior to maturation (i.e. 6 months later than experiments I and II). In the first instance, application of LL at this stage, in close proximity to first maturation, apparently advanced maturation and shortened the spawning season by 1 month in the first year (realised in both treatments 2 and 6). This was achieved primarily through the advancement of spawning in the females. It is possible that spermiation was also advanced in the males, however the sampling regime (first sampling on the 6th of February 2001) precluded detection of such an occurrence. What is evident in both treatments 2 and 6 is a 2 month phase advance in elevated testosterone levels in the males (Peak = February 2001) with respect to all other treatments (Peak = April 2001). Furthermore, the reduced levels of these peaks compared to the levels attained in treatments 1, 3-5 suggest that the LL application could have phase advanced the testosterone peak further still to earlier in January between the December and February sampling. Hansen *et al.* (2001) reported a comparable 28 day advancement in median spawning date and a reduction in the length of the spawning season by 26 days in recruit spawning cod experiencing LL from the winter solstice prior to maturation. In the present work, exposure to continuous illumination was then maintained in treatment 6 through to their third year (2002) when spermiating males were observed from February onwards (as under the control photoperiod 3) until the trial end (5 months) however, no mature females were witnessed in this population. This physiological response was confirmed at the endocrinological level with a subdued peak in testosterone in the males being evident in March while no fluctuations in plasma testosterone or calcium levels up to the trial end were recorded in the females. To date,

there are no comparable works in this species. As with experiment I these data allude to an endogenous rhythm that regulates maturation however, this appears to be sex specific.

Endogenous regulation of maturation.

The results of experiment I and III suggested the presence of an endogenous "clock" component that could regulate maturation activity in the absence of photoperiodic signals. The possibility of such an endogenous rhythm regulating maturation in cod has recently been proposed by Norberg *et al.* (2004) who reported the failure to significantly alter maturation in the short term with altered seasonal photoperiod cycles as evidence for this. Gwinner (1981, 1986) first outlined the characteristics of an endogenous rhythm: 1) the rhythms should be observed for at least 2 full cycles to establish that it is self-sustaining; 2) it should free-run with a periodicity that approximates to, but is significantly different from, 12 months; 3) it should be entrainable by an environmental *zeitgeber*; and 4) it should be temperature compensated. In the present work, several lines of evidence exist to support the hypothesis that maturation is under an endogenous control; the presence of a "dummy run" maturation cycle followed by a limited number of mature individuals in the spring of 2002 in experiment I, along with the presence of spermiating males in the second year under experimental photoperiod 6 of experiment III, demonstrate that there is some form of entraining mechanism that can sustain and co-ordinate maturation, certainly in the first case, over two complete seasonal cycles. Furthermore, the fact that individuals were observed in varied gonadal development states via ultrasound scanning at the end of experiment I and that the males of photoperiod 6 in experiment III were observed to be spermiating for a period of 5 months with some individuals showing no indication of

stopping at the trial's end suggest a "free-running behaviour". Of great interest in this respect is the fact that no mature females were observed in the second spawning season of photoperiod 6 in experiment III. The absence of fluctuations above base line levels in plasma testosterone or calcium indicate that maturation from certainly the onset of vitellogenesis was completely inhibited. If there was an endogenous clock mechanism regulating maturation in the males in the absence of entraining photoperiod signals, these results may indicate that this regulating mechanism in not operational in females. The reason why such a sex related difference would exist is unknown. Obviously, the low numbers involved at this stage (n=3) require these results to be further validated before any firm conclusion can be drawn. A possible explanation for skipped maturation in individuals could be through reduced nutritional/energetic status causing individuals to skip spawning (Rideout and Burton, 2000; Rideout *et al.*, 2000). However, with a mean weight of 1400 ± 300 g and condition factor of 1.2 ± 0.2 (data not presented), these individuals were not different from the males of this treatment or from the mature females of treatments 2-5 in this spawning season, suggesting this was not a point of concern.

With evidence for self-sustaining and free-running behaviour, the remaining criteria to be validated are those of entrainability to an environmental *zeitgeber* and evidence for temperature compensation. While the experiments described herein were not designed to explore the later, results from experiment III provide an insight into the former. It is apparent that the continuation of short days (7:17 L:D) from the winter solstice prior to maturation (treatments 3-5) extended the spawning season up to 6 months (photoperiod 5), while LL treatment from a similar date reduced the spawning season to 3 months and advanced maturation in the population by over 1 month. In the second spawning season the timing of maturation in treatments 2, 4 and 5 paid close

adherence to that of the experimental photoperiod. As discussed by Bromage *et al.* (2001), in teleosts, when natural maturation cycles are compressed or extended there is a concomitant delay or advancement in spawning time with respect to the photoperiod signal. Within experiment III such phase shifts are most clearly evident in the male spawning profiles in treatments 4 and 5 when compared to photoperiod 2. In these treatments an increasing proportion of the mature males were observed prior to the photoperiod change from short to long days that marks the end of the "winter" period as the maturation date is delayed. In these treatments plasma testosterone and calcium levels exhibit similar adjustment with the maturation cycles due to the photoperiod manipulation. This, along with the results of Norberg *et al.* (2004), demonstrate that maturation is entrainable to altered photoperiod cycles and the present work further demonstrates that such a signal can be broken down to its simplest components of transitions between long and short day and still act as an entraining mechanism. Therefore, it could be stated with some confidence that maturation in Atlantic cod is under the control of an endogenous "clock" mechanism that is entrainable to photoperiod signals which does have the ability to self-regulate under constant photic conditions. This is in accordance with results from other species such as European seabass (Zanuy *et al.*, 1991), rainbow trout (Randall *et al.*, 2000), Atlantic salmon (Eriksson and Lundqvist, 1982), Indian catfish, stickleback and barbel (Bromage *et al.*, 2001) and adds further credence to the hypothesis that "…endogenous mechanisms underlie the timing of reproduction in fish…" (Bromage *et al.*, 2001).

Photoperiod inhibition of maturation and its commercial significance

As explained earlier (section 1.3.3), the prevention of maturation during on-growing to realise maximum growth potential is an important concern for profitable

production. Since commercial cod culture will be performed in open systems where ambient photoperiods will be perceived, the only possible light manipulation will be the exposure to continuous illumination at set points in the production cycle. Hence work in this chapter and that published before it (Karlsen *et al.*, 2000; Dahle *et al.*, 2000; Hansen *et al.*, 2001) has focused on such manipulations. As was explained earlier (Section 1.2), in cod the commitment to spawn is believed to be initiated one year prior to actual completion. Mean weight post-spawning or at the equivalent time as a one year old for virgin spawners (i.e. April-June) has been correlated to the initial number of oocytes entering "maturation commitment" (Waiwood, 1982; Kjesbu and Holm, 1994). If body size at such a time is a regulating factor of maturation, this would imply that it is the rising photoperiod during the spring that could be responsible for initiating maturation commitment. Although the application of LL from summer solstice in experiment I was successful in preventing final maturation at 2 years of age and significantly reducing it at 3, evidence suggests that there was still an underlying maturation cycle. Such behaviour is also possibly alluded to, by the presence of a maturing male at the same time period in the results of Hansen *et al.* (2001). The same authors concluded that "…a reduction in day length is a vital environmental signal regulating the maturation and spawning of cod, and that oocyte development may be arrested or considerably delayed in its absence", thereby suggesting that maturation was not necessarily prevented but instead possibly delayed. As a whole, this data suggests that the application of continuous light from the summer solstice at one year of age may in fact be too late to prevent maturation commitment and rather the subsequent LL photoperiod regime merely prevents the completion of final maturation. With this provisional evidence the role of an endogenous maturation rhythm synchronised prior to the summer solstice cannot be ruled out, which demands that further investigations are

carried out into the significance of LL application prior to this date. Furthermore, in experiment II when such a summer solstice application was applied in an open cage system, maturation was delayed rather than inhibited. As explained above this is in agreement with the limited published work available, which draws the ultimate conclusion that "...the intensity and/or spectral composition of light may be the important difference between the situation in tanks and cages exposed to LL..." (Karlsen *et al.*, 2000). While access was not possible during experiment II to the cage site at night to record intensity levels and spectral profiles in the illuminated cage, data are available from comparable systems. Figure 3.25 shows the spectral profiles recorded of daylight after passing through 2 and 10 m of seawater, a 400 W submerged lighting unit (Aquabeam, UK) and test lighting in the tank system utilised in all experiments, the latter two through 2 m of seawater. The figures show clearly that through the cage during the day the majority of light is in the 450 to 600 nm range. This will of course fluctuate with seasonal parameters such as sun elevation and suspended solids, plankton, etc. (Open University 1989; Loew and McFarland, 1990). However, when considering the artificial submerged light unit, a single peak at 538 nm dominated the light output. With regards to the light intensity, the light system used (4 x 400 W) is significantly more powerful than would normally be applied in such a cage unit containing Atlantic salmon (Blair C., *pers comm.*). Similar lighting arrangements as those used within this present work can at best be expected to produce in the region of <2% of the ambient daylight intensity (Davie *et al,.* unpublished). Whether the fish could differentiate the clearly different spectral profiles or light intensity levels would be dependent on the chromatic and luminescent sensitivities of the species. Some limited work into light perception has been performed to date in cod. Firstly, with regards to the chromatic response, this is best quantified through studies of the

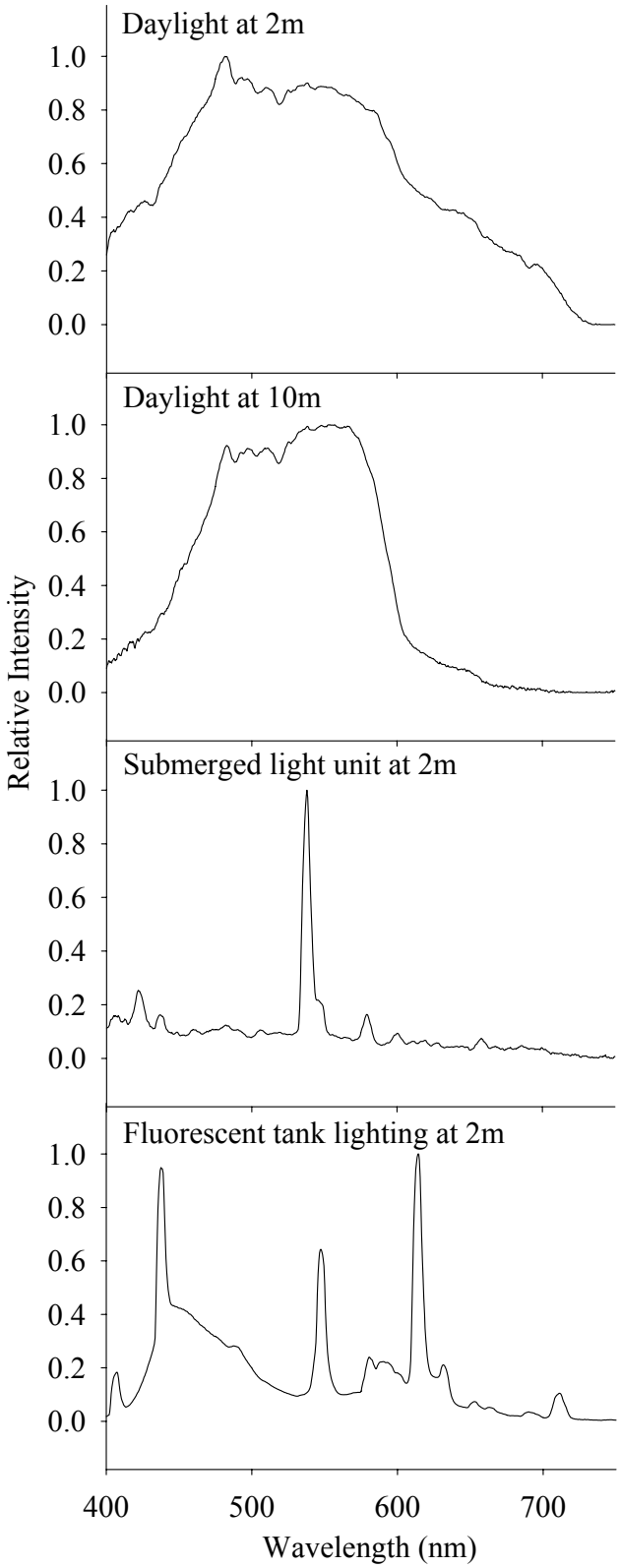


Figure 3.25: Normalised spectral profiles of ambient daylight through 2 and 10m of seawater and a submerged 400W light unit and above surface fluorescent tank lighting through 2m of seawater. (Adapted from Migaud *et al.* 2004a)

photoreceptors. To date, in cod, four different "non visual" opsins have been identified. Drivenes *et al.* (2003) isolated two subtypes of melanopsin genes expressed in the inner retina and deep brain of Atlantic cod. The expression in the deep brain was localised to the supraoptic/suprachiasmatic nucleus (SOC) and the habenula. Importantly, the SOC has been proposed to act as one of the principal sites to regulate photoperiod entrained circadian/circannual physiological activity in teleosts (Holmqvist *et al.*, 1994; Drivenes *et al.*, 2003). Interestingly, the expression of the two opsin genes was not detectableabove baseline levels in the pineal gland (Helvik J.V., *pers comm.*). Forsell *et al.* (2001) reported localisation of a "teleost green cone opsin" in the retina and pineal gland of cod and haddock. However, much work remains to be performed in cod as numerous "non-visual" opsin structures have been identified in vertebrates to date. Those identified in teleosts include cone like opsins (Forsell *et al.*, 2001), parapineal opsin (Blackshaw and Snyder, 1997), teleost multiple tissue opsin (Moutsaki *et al.*, 2003), melanopsin (Bellingham *et al.*, 2002; Drivenes *et al.*, 2003), vertebrate ancient opsin (Philp *et al.*, 2000), rhodopsin (Mano *et al.*, 1999) and cryptochrome (Whitmore *et al.*, 2000). Identification and localisation of "non-visual" opsin structures would demonstrate light reactive centres, while sequencing, reconstruction and spectrophotometric studies would then help define the absorption spectra of these proteins so, definitively outlining the chromatic sensitivities of the species. Of note, however, is that the spectral responsiveness described in a number of species so far could not be described as being highly specific (Kavaliers, 1980; Meissl and Ekstrom, 1988; Kusmic and Gualtieri, 2000; Bozzano *et al.*, 2001). While the focus (λmax) is generally at the "blue end" (450-550 nm) of the spectrum, the actual range spreads over a significant portion of the lower visible wavelength range 400-600nm (Kavaliers, 1980; Meissl and Ekstrom, 1988; Kusmic and Gualtieri, 2000; Bozzano *et al.*, 2001). In

this sense it appears while there may be an optimum sensitive wavelength (λmax), it is perhaps not in the fishes' interest to refine sensitivities due to the dynamic nature of the photic environment in which they live. The potential presence of so many different opsin structures may be a reflection of a more cautionary environmental adaptation where detection of light no matter where an individual is located is paramount. Hence, the spectral content of lights utilised may be less biologically important but more physically and/or economically important to ensure that energy is not wasted in producing light at wavelengths that do not readily transmit through the water column.

With regards to luminescent sensitivities in cod, Kristoffersen *et al.* (2004) has reported that additional artificial illumination applied to cod in open tank systems at two intensity levels (900 and 300 Lux) had significant impacts on maturation in male cod with "…arrest in GSI development being more apparent in the higher intensity treatment". Unfortunately, no information was provided about ambient intensities so relative illumination levels (artificial scotophase vs. ambient photophase) cannot be compared. Equally, Taranger *et al.* (2004a) and Taranger *et al.* (2004b) have reported this and related work which states that intensity levels in the range of 1:1 to 1:0.001 (relative photophase intensity : relative scotophase intensity) were equally effective in suppressing melatonin levels. However, this experiment was not maintained to record impacts on maturation levels.

As in the trials cited above, plasma melatonin levels were measured in experiment I of this present work as an indirect indicator of light perception in the species. The inability to gain access to the cage site at night in experiment II prevented gathering the same information, as did the limited number of individuals in experiment III. The results showed a clear diel pattern being exhibited under SNP, with melatonin rising to a peak of 47 $pg.ml^{-1}$ at the end of the scotophase before returning to basal

levels of approximately 20 $pg.ml^{-1}$. There have been limited published data with regards to melatonin in Atlantic cod. The work presented here confirms the "A profile" first reported by Porter *et al.* (2000b), which along with haddock (Davie *et al.,* unpublished) appears to be the only fish to display such a rhythm of melatonin secretion. Such profiles have been described in a number of nocturnal rodents (Reiter, 1988) however, the significance of this is not fully understood. The levels recorded in this experiment are far lower than those reported by Porter *et al.* (2000b). This cannot easily be ascribed to differences in temperature or age of fish which have been shown to influence melatonin profiles in cod (Porter *et al.*, 2000b). However, Reiter (1988) states that with regard to the nocturnal rise in melatonin, "by convention, it is assumed that a doubling of basal daytime levels constitute a physiological relevant change that will induce a physiological response at the target organ level." So, it can be concluded that the diel variations described within this experiment by the SNP population are physiologically significant while those of the LL population are not.

After 19 months of continuous light exposure there was no diel rhythm in plasma melatonin levels in this study. However, Hall (2000) reported the maintenance of a rhythm of melatonin secretion after 5 months of LL exposure in juvenile cod and Bolliet *et al.* (1996) reported the maintenance of rhythmic secretion of melatonin in isolated cod pineal glands under conditions of continuous darkness over a period of 4 days. Both these authors suggested the role of endogenous rhythms and/or clocks which maintain this cyclical endocrine signal in the lack of entraining light stimuli. If this were to be the case then the present data suggest that between 5 and 19 months of LL exposure, the endogenous rhythm of melatonin secretion becomes uncoupled from its regulating mechanism and ceases to operate. Further work is required to confirm the existence of such a rhythm in cod, identify how this mechanism is regulated under

constant lighting conditions, and reveal the physiological significance of this endogenous rhythmicity.

3.6.2 Growth

It had previously been documented that, one year of continuous light exposure from the summer solstice in 1+ cod resulted in an apparent 60% to 80% increase in wet weight (Dahle *et al.*, 2000). In the first experiment outlined within this chapter, after one years' exposure to LL, a 43% improvement in wet weight over the SNP population was recorded. This is primarily explainable through the apparent weight loss over each spawning season (11 and 19%) observed in the ambient photoperiod regime. These losses are slightly lower than those presented by Braaten (1984), who reported that mean weight loss was around 21% with females recording a significantly greater weight loss than males (27.4% vs. 15.9%). The limitations of the present trial obviously did not allow such a differentiation. Although experiment III allowed the monthly monitoring of individual growth in relation to maturation these results are not felt to be representative and hence, were not further analysed in this manner, as the relatively high mortality rates combined with the regular blood sampling of all individuals may have compromised growth rates.

What the previously published literature regarding growth improvement in maturation inhibited populations has not shown to date is subsequent rapid compensatory growth in the SNP population from the end of their first spawning season in July to return to statistically comparable weights with the LL population within a month. Within 3 months both populations displayed comparable weight and growth rates until the onset of spawning in the 2nd spawning season of the SNP population,

which caused the second divergence in mean weight and caused a 44% difference between populations at the end of the trial.

Compensatory growth is a well documented physiological response in which individuals exhibit a period of rapid growth in response to periods of suboptimal growth. This is usually in response to food deprivation (Jobling *et al.*, 1994; Saether and Jobling, 1999) although it has been associated with restrictive environmental conditions like low temperatures (Purchase and Brown, 2001), hypoxic conditions (Foss and Imsland, 2002), or even following hydrogen peroxide treatments of disease in rainbow trout (Speare and Arsenault, 1997). In their review of compensatory growth, Ali *et al.* (2003) stated "…the consequence of a compensatory growth response is the attainment of a size status relative to the size achieved by an organism that has not experienced any phase of growth impairment." When looking at the compensatory growth response in cod in relation to nutritional history, Jobling *et al.* (1994) concluded that cod exhibit a rapid and complete recovery of body weight when provided with adequate food following a period of undernutrition. In agreement with this, Pedersen and Jobling (1989), studying the growth rates of "large sexually mature" cod, reported compensatory growth displayed in the few months post-spawning. It could therefore be proposed that the spawning season during spring at 2 years post hatch represents the period of "suboptimal growth" following which the SNP population showed a rapid weight gain principally between July and September to attain a weight comparable to a population that has not experienced any growth impairment (i.e. the LL population). This would imply that the LL population did not exhibit any additional somatic growth benefit from the LL treatment.

However, in experiment II the LL population in the first growth period between July and October showed a higher growth rate. Such a growth period is theoretically

prior to maturation activity like gametogenesis (Burton *et al.*, 1997) or even elevation in steroid levels (Experiment III of this work and Norberg *et al.*, 2004) and hence is more likely to be a reflection of somatic growth. It must however be acknowledged that, at the start of the trial, the LL population had a smaller mean size than the ambient photoperiod group which may have influenced the outcome, as smaller individuals generally exhibit higher specific growth rates than larger counterparts.

Similar confusion abounds in the published literature. Hansen *et al.* (2001) suggested that LL did not stimulate somatic growth when they compared the growth performance in the first 6 months of their LL and LL/SNP treatments to the SNP and SNP/LL treatments. The data presented here in experiment I concurs with their findings by not demonstrating a difference in mean weight or total length until 10 months post light treatment application i.e. during the spawning season. Equally, Hall (1988) failed to show any difference of growth rate or feeding activity in wild sourced juvenile cod maintained under divergent photoperiods (fish were placed under either a reducing "oncoming winter" or increasing "oncoming summer" photoperiod regime) initiated at either the spring or autumn equinox. However, conflictingly, Folkvord and Ottera (1993) reported an increase in SGR in juveniles held under continuous light and Hall (2000) reported significantly heavier juvenile cod after 5 months of continuous light treatment in juveniles between 10 and 15 months post hatch, which is in agreement with the findings in experiment II.

Boeuf and Le Bail (1999) and Boeuf and Falcon (2001) both extensively reviewed light regulation of growth in fish. From this review it appears that cod is not the only species which has an uncertain growth response to light. While it is well documented that salmonids display enhanced growth in response to long photoperiods (Stefansson *et al.*, 1991; Hansen *et al.*, 1992) and as such this tool is used in commercial

culture to improve growth potential (Forsberg, 1995), other species from similar environmental niches so far studied display a varied response. In cod's close relative the haddock, Trippel and Neil (2003) reported improved growth in juveniles under continuous illumination while in Atlantic halibut, both Simensen *et al.* (2000) and Norberg *et al.* (2001) reported improved growth under continuous light. However, Hallaraker *et al.* (1995) failed to show any effect of extended day length on juvenile Atlantic halibut growth rate. In turbot, Imsland *et al.* (1995) and Imsland *et al.* (1997b) reported improved growth in response to 24 hours light, although they suggested that marine fish exhibit a reduced growth-promoting effect of photoperiod compared to freshwater species. A greater understanding of the photoperiod interactions with the somatic growth axis in cod is required before firm conclusions can be drawn about environmental regulation. The principal problem is that the trials in this chapter as well as those of Karlsen *et al.* (2000), Dahle *et al.* (2000) and Hansen *et al.* (2001) included individuals that passed through sexual maturity and only round wet weight was presented. As such, the concomitant fluctuations in associated tissues i.e. the gonads and liver during maturation (Eliassen and Vahl, 1982a, 1982b; Schwalme and Chouinard, 1999; Yaragina and Marshall, 2000) are not taken into account in the assessment of "growth". Equally neither is the possible anabolic effect of elevated steroid levels (Hunt *et al.*, 1982). It is, therefore, clearly necessary to examine growth in response to photoperiod with these variable components being removed and/or accounted for to quantify the true underlying somatic growth response.

Related to the above discussion, is the seasonal variation of condition factor exhibited by both populations in experiment I. Foulton's condition factor is an accepted condition index used in Atlantic cod as well as in other species to describe energetic status of an individual or population (Love 1970; Bolger and Connoly, 1989; Lambert

and Dutil, 1997). The condition factor range recorded in this experiment is comparable with other studies performed in cod under farmed conditions with *ab libitum* feeding [experiment II of this work, Svasand *et al.* (1996) and Morais *et al.* (2001)]. Within this experiment, both populations displayed peaks in condition factor between February and April, which interestingly is in conflict with the discussion of Love (1970) who, after reviewing the available literature, concluded condition factor peaks in cod to be from September to December. All studies discussed by these authors were based on wild caught, spring spawning, fish. This highlights the difference in growth responses between cultured fish that have feed available all year round in comparison to wild origin fish that have exogenous restrictions like seasonal food availability. Consequently, any comparisons of farmed and wild origin cod should be interpreted carefully. Interestingly, the LL population of experiment I exhibited a seasonal cycle in condition which is significantly correlated to the seasonal fluctuations in water temperature, as did the SNP population. It is not proposed that temperature is the cause of these fluctuations. It is felt that this is a coincidental relationship. In the first year, the drop of condition observed between April and August 2001 was due to the cessation in weight gain related to maturation over this period. However, the exact reasons for this change in condition cannot be deciphered from the data as whole wet weight was used in the calculation. Hence, the fluctuations could be due to changes in liver weight, gonad weight or flesh weight or any combination of these (Schwalme and Chouinard, 1999). As was discussed above, with regards to deciphering the somatic growth response, future work should try to disassociate these components to understand the underlying processes (somatic growth vs energy reserve and gonadal tissue fluctuations) and their relationship with the principal environmental signals of photoperiod and temperature.

3.7 Conclusions

The three experiments described in this chapter have demonstrated that with regards to photoperiod regulation of growth and maturation, an Irish Sea strain of Atlantic cod performs in a very similar manner to that reported in Norwegian strains. As such, it is concluded that the statements made here should be true not only for an Irish Sea stock but Atlantic cod in general, at least from the eastern Atlantic Ocean. This work provides evidence for the hypothesis that cod possess an endogenous "clock" rhythm regulating maturation that is entrained by seasonal photoperiod signals. Thus artificial photoperiod manipulation offers the potential to be an invaluable tool for stock management during the commercial culture of the species, on the one hand allowing the production of out of season juveniles in a confident and predictable manner while also allowing commercial ongrowers to realise maximum growth potential through the timely inhibition of maturation in culture. The refinement of such a management strategy is the principal focus of this work and has so far confirmed that long term exposure to continuous light in enclosed conditions from the summer solstice in 1+ cod significantly suppresses maturation at both 2 and 3 years of age. However, an underlying maturation rhythm is still present. A similar application in an open cage system delays maturation rather than inhibits it. Further studies are therefore required to better understand how photoperiod regimes regulate maturation and growth in Atlantic cod, as outlined below:

- The timing of LL application must be further investigated to quantify the importance of photoperiod signals prior to the summer solstice at approximately 15MPH. Such applications may be able to eradicate the apparent underlying maturation "dummy runs" witnessed.

- The potential for somatic growth enhancement requires further investigation as work to date has not been able to clearly quantify this phenomenon due to the possibility that physiological changes associated with the endogenous maturation cycles have not been fully taken into account. Future work must consider not only changes in whole wet weight but also changes in liver and gonad weight to decouple somatic growth from maturation associated growth.
- With regards to the failure of application in open cage commercial systems, this may first be addressed by the above-mentioned refinement of photoperiod timing. Failing this, extensive work remains to be done in the quantification of spectral and luminescent sensitivities of the species, an understanding of which would aid the development of biologically "in tune" lighting systems that could be applied in commercial settings.

CHAPTER 4: EFFECTS OF PHOTOPERIOD MANIPULATION ON GROWTH AND REPRODUCTION IN ATLANTIC COD: THE IMPORTANCE OF TIMING OF LL EXPOSURE ON THE INHIBITION OF MATURATION

4.1 Introduction

Evidence of an endogenous "dummy run" of maturation in experiment I (Section 3.2) and the theoretical delay witnessed in experiment II (Section 3.3) suggest that both the timing of application of LL and the method of application in open systems required further investigation. The former was examined further in the present chapter in an attempt to define the photoperiod signals that recruit individuals into the sexual maturation cycle while also examining the interaction of photoperiod manipulation and somatic growth. The clear commercial benefit of such knowledge would be the optimisation of industry guidelines for maturation control and subsequently stock performance.

To date, all long term photoperiod designs to regulate maturation in Atlantic cod have focused on applying LL on, or after, the summer solstice prior to maturation (experiment I and II of this work; Karlsen *et al.*, 2000; Dahle *et al*., 2000; Hansen *et al.*, 2001). While all tank trials reported the prevention of maturation at 2 years of age, evidence for underlying maturation rhythms exist (experiment I of this work and Hansen *et al.*, 2001) that are in line with the maturation cycle exhibited by cod maintained under a comparable photoperiod regime in open cage systems (experiment II of this work and Dahle *et al.*, 2000; Karlsen *et al.*, 2000). As was explained previously (Section 1.2), it has been postulated that the recruitment of individuals into the reproductive cycle begins in the late spring/early summer. If true, the application of LL from the summer solstice may, in fact, be too late as puberty has already been initiated.

This could then explain the limited apparent physiological fluctuations observed in the previous work. Therefore, it was felt the significance of applying LL at earlier life stages should be further explored.

Norberg *et al.* (2001) investigated the use of LL treatments applied up to 2 years prior to first maturity to regulate growth and maturation in male Atlantic halibut. Interestingly, the authors found that prolonged exposure to LL enhanced both growth and maturation, recruiting 100% of the male population compared to <60% in the control populations. These authors found that the lowest numbers of mature individuals were observed following a combination of exposure to LL early in the on-growing production cycle before returning the population to an ambient photoperiod at the summer solstice prior to spawning. Such an approach has not been applied to Atlantic cod and may provide a useful insight into photoperiod regulation of maturation. Furthermore, it would be interesting to have a direct comparison between both species to further examine the apparent species-specific nature of photoperiodic regulation in teleosts (Imsland *et al.*, 2003), especially as both these species inhabit comparable regions and spawn in comparable time frames (Jakupsstovu and Haug, 1988).

As has been previously demonstrated (experiment I of this work and Karlsen *et al.*, 2000; Dahle *et al*., 2000; Hansen *et al.*, 2001), the inhibition of maturation through the timely application of photoperiod manipulation realises a significant growth benefit (over 40% in the present work). However, at present, the potential for direct promotion of somatic growth through photoperiod manipulation is some what unclear. This question is by no means limited to Atlantic cod, with much work having been performed in numerous other teleosts which both directly and indirectly examine this phenomenon (see reviews of Boeuf and Le Bail, 1999; Boeuf and Falcon, 2001). The most compelling argument for light stimulating somatic growth is the demonstration of

improved feed conversion efficiencies (Boeuf and Le Bail, 1999), which is only achievable through direct stimulation of the somatic growth axis.

Somatic Growth Axis

Sumpter (1992) states "many hormone systems are involved in the extracellular control of growth and there is a considerable degree of interaction between these systems". Figure 4.1 is an overall schematic representation of the somatic growth axis. The exact mode of operation of this system has recently been brought into contention (Dickhoff 2004). This new, alternative view however, does not contend the network's components but rather, the hierarchy in which they operate. Therefore, what will be presented here is a brief summary of the principal components in order to identify key points of interest for the identification of photoperiod stimulation of the somatic growth axis.

Growth Hormone

Somatotropin (growth hormone, GH) originating from the anterior pituitary gland typically has 188 amino acids with between 4 and 5 cysteine residues (Peter and Marchant, 1995) and acts as one of the key regulators of the growth axis (Boeuf and Le Bail, 1999). Expression of GH itself is enslaved to a range of neuroendocrine regulators which have been listed as including somatostatin, serotonin, norepinephrine, dopamine, gonadotropin–releasing hormone, neuropeptide Y, GH-releasing factor, thyrotropin releasing hormone and cholecystokinin (Peter and Marchant, 1995). The hierarchy and interaction of these regulating factors is not clear as yet, though the possibility that their functional significance changes with different developmental states has been suggested (Peter and Marchant, 1995). The growth mediating effects of GH have been

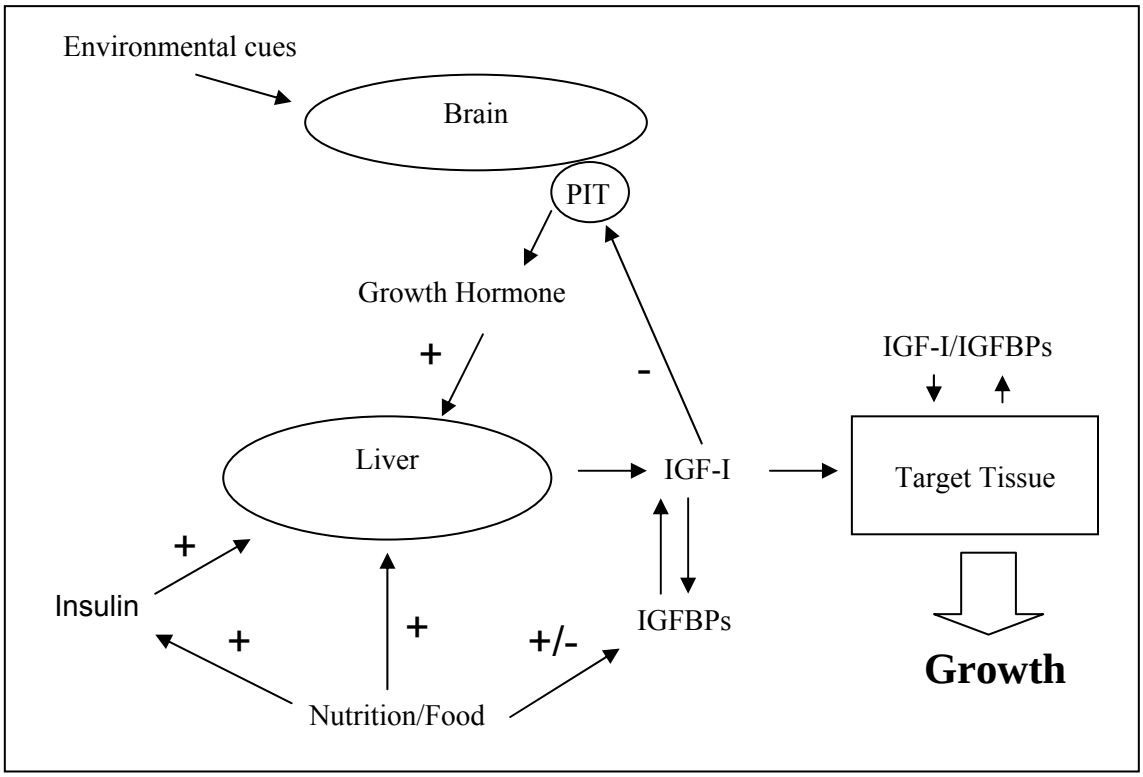


Figure 4.1: Diagrammatic representation of the somatic growth axis in fish (from Duan, 1997) (PIT = pituitary, IGF-I = Insulin like growth factor-I, IGFBPs = IGF-I binding proteins)

demonstrated most consistently through exogenous administration in salmonids to the degree that it is possible to predict the magnitude of the growth promoting effect on the basis of GH source and dose, rearing conditions and fish strain (Le Bail *et al.*, 1993; Perez-Sanchez and Bail, 1999). The 2-3 fold improvement in growth rate recorded following administration has been demonstrated to be achieved through increased appetite and improved feed conversion efficiency (Donaldson *et al.*, 1979). The obvious value of GH in somatic growth promotion is reflected through the controversial development and commercial application of transgenic fish with GH gene additions to produce individuals that perform up to 3-fold better than non-transgenic cohorts (Cook *et al.*, 2000). As well as its role in growth, it appears that GH may represent an

important and multifunctional hormone in Atlantic salmon acting as a central mediator of seasonal changes in physiology and behaviour (Björnsson, 1997). Rhythms in GH secretion have been associated with seasonal fluctuations in day length (Pérez-Sánchez *et al.*, 1994) as well as diel cycles in light intensity (Bates *et al.*, 1989; Boujard and Leatherland, 1992), yet the link between GH and light perception remains unclear (Boeuf and Le Bail, 1999). With regards to the growth promoting effects of GH, with the exception of direct stimulation of developing tissues (usually limited to early developmental stages: Pérez-Sánchez and Bail, 1999), the principal target is the liver (Boeuf *et al.*, 1999), where direct administration of GH has been shown to significantly increase hepatic insulin-like growth factor-I (IGF-I) mRNA (Duan, 1997). IGF-I, another significant component in the somatotropic axis, is described as having a synergistic existence with GH, providing a negative feedback mechanism which inhibits GH release (Pérez-Sánchez *et al.*, 1993).

IGF-I

IGF-I is a 70 amino acid basic peptide containing 3 intra-chain disulfide bridges (Duan, 1997). The IGF system is unique among peptide hormone systems in that IGFs, in the extracellular environment, are bound to members of a group of high affinity binding proteins (IGFBPs) (Duan, 1997) which will be discussed later. IGF-I plays a key role in the somatomedin hypothesis by regulating the majority of GH actions (Leroith *et al.*, 2001). The principal action of IGF-I is to increase growth by stimulating nutrient uptake, protein synthesis and eventually cell division (Phillips *et al.*, 1990; McCormick *et al.*, 1992; Negatu and Meier, 1995; Oksbjerg *et al.*, 2004). As such, positive relationships between feeding level, IGF-I level and growth have been found in a range of teleosts (McCormick *et al.*, 1998; Beckman *et al.*, 1998; Beckman and

Dickhoff, 1998; Silverstein *et al.*, 2000; Larsen *et al.*, 2001; Pierce *et al.*, 2001; Beckman *et al.*, 2001, 2004; Mingarro *et al.*, 2002; Dyer *et al.*, 2004a). Apart from growth, IGF-I has been associated with normal development (Duan *et al.*, 1995), smoltification in salmonids (Beckman and Dickhoff, 1998), osmoregulation (McCormick *et al.*, 1991; Mancera and McCormick, 1998; Riley *et al.*, 2003) and finally reproduction where its actions include regulation of steroidogenesis prior to, and the induction of, germinal vesicle breakdown during final oocyte maturation (Maestro *et al.*, 1997; Kagawa *et al.*, 1994), as well as germ cell proliferation in the testis (Le Gac and Loir, 1993). In circulation IGFs have a functional half life of approximately 10 minutes, however over 90% of IGF circulates in conjunction with one of 4 IGFBP's identified in fish (6 IGFBP's have been identified in higher vertebrates) that extend this half life to between 12 and 15 hours (Duan, 1997; Shimizu *et al.*, 1999, 2004). IGFBPs appear to provide a regulatory link controlling availability of IGF-I as they themselves are regulated by a variety of physiological factors, for example the prevalence of the <31 kDa IGFBP in fish has been linked to cortisol release, suggesting that under stressed conditions the rapid elevation of cortisol provides a concomitant rise in this specific IGFBP that binds to available IGF-I to restrict its availability (Kelley *et al.*, 2001). With regards to growth promotion in teleosts, it is thought that the 40-50 kDa fish IGFBP, which is suggested to be analogous to mammalian IGFBP 3 (Kelley *et al.*, 2001), controls the availability of IGF-I to two different IGF receptors (Duan, 1997). The dominant one is IGF-IR, which has been found to be expressed in a wide range of tissues, which correlates with the recorded sites of action of IGF-I, i.e. ovary, heart, brain and skeletal muscle (Duan, 1997).

Due to the negative feedback interaction of IGF-I and GH, the optimal growth performance in fish could be characterized by low GH levels and high circulating levels

of IGF-I in plasma (Pérez-Sánchez and Bail, 1999). The close association of IGF-I to growth behavior has meant that it has become a popular component in recent research. With the development and validation of a commercially available radioimmunoassay kit (Dyer *et al.*, 2004b) it is possible to rapidly assess total plasma IGF-I in, it appears, most widely studied teleost species (Dyer *et al.*, 2004b). As such, the measurement of circulating IGF-I has been offered as a valuable tool to the aquaculture industry to, for example, evaluate diet related growth potential (Dyer *et al.*, 2004a) or husbandry stressors (Dyer *et al.*, 2004b). These are assessed through the corresponding up or down regulation of IGF-I levels that mediate growth which would be characterised as the typical phenotypic response. Clearly, this offers a valuable opportunity to examine the potential for direct light stimulation of the somatic growth axis by the use of artificial photoperiod manipulation. Enhancement of IGF-I and somatic growth by long day photoperiods has been demonstrated in red deer (Webster *et al.*, 1999) and due to the highly conserved nature of the vertebrate IGF system throughout evolution (Duan, 1997), it is quite possible that if there is a link between photoperiod and somatic growth then the IGF system in teleosts may provide evidence of this. Hence, the measurement of plasma IGF-I levels was employed in such a manner in the experiment described within this chapter.

4.2 Objectives

The aim of this experiment was to investigate the impact of transfer of populations from a simulated natural photoperiod to constant illumination at three monthly intervals from 6 MPH onwards on both the gonadal and somatic axes at both the physiological and endocrinological levels up to 2 years of age.

4.3 Materials and Methods

4.3.1 Fish stocks and rearing conditions

Atlantic cod juveniles were produced at Machrihanish Marine Farms, Machrihanish, Argyle, Scotland (55:44^{o}N, 5:44^{o}W) during the spring of 2001 from an Irish Sea origin broodstock maintained on an ambient photo-thermal regime for this location. The juveniles were maintained on ambient conditions until the start of the experiment.

On 26th September 2001, 830 individuals (mean weight 33 ± 0.3 g) were anaesthetised and a Passive Integrated Transponder tag was placed subcutaneously in the dorsal flank (Section 2.1.5). Fish were allowed to recover in clean, aerated water before being returned to holding tanks. There were no mortalities associated with the tagging process. Following recovery, the fish were transferred to circular 7 m^3 tanks (3 m diameter, running depth approximately 1m) with light proofed lids located indoors at Machrihanish Marine Environmental Research Laboratory. Artificial light was provided by two 9W fluorescent bulbs located centrally under the tank cover, approximately 0.5 m above the water surface. Light intensity at the water surface was 0.32 Wm^{-2} (1.705 µmoles $m^{-2}s^{-1}$ or 105 lux) when illuminated and 0 Wm^{-2} (0 µmoles $m^{-2}s^{-1}$ or 0 lux) in the dark phase. Throughout the experimental period, water temperature ranged from 5^{O}C to 15^{O}C (Figure 4.2) and the salinity ranged between 29 ‰ and 34 ‰ with an average of 33 ‰ (Figure 4.3). During the experimental period the fish were held within a stocking density range of 10 to 30 $kg.m^{-3}$. Every 4-6 weeks a small number of individuals from each experimental tank (approximate n = 10 to 20) were weighed to update farm husbandry records and to estimate stocking density fluctuations. Every 3 months, tank biomass was recalculated following the complete population sampling (Section 4.3.3) and, subsequently, tank depth was adjusted by standpipe height in order to balance

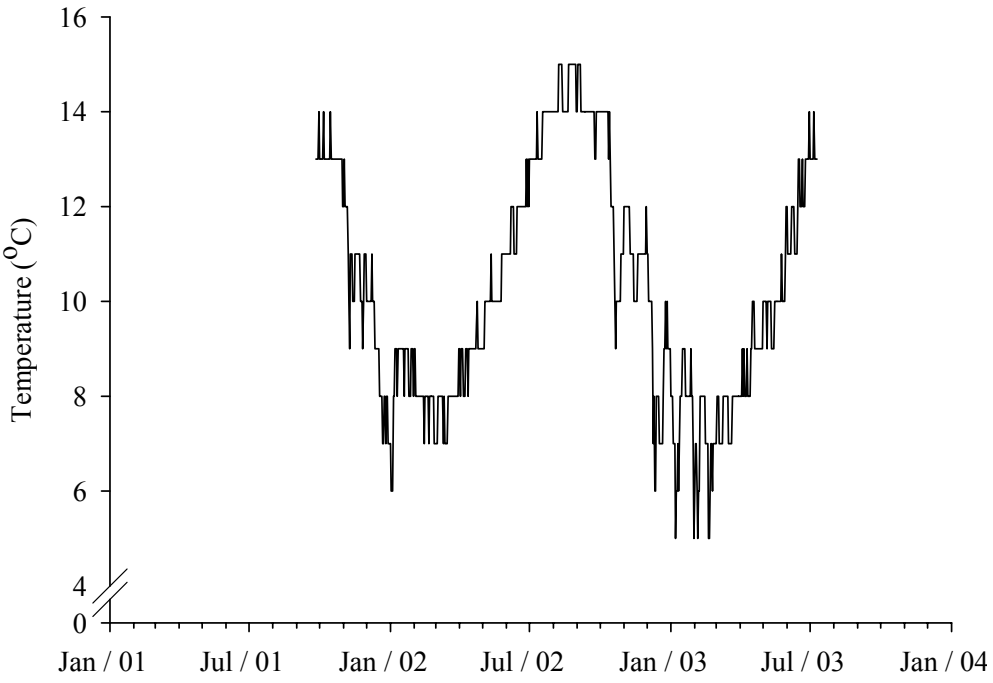


Figure 4.2: Daily temperature (°C) of seawater at Machrihanish from 26th September 2001 to 8th July 2003.

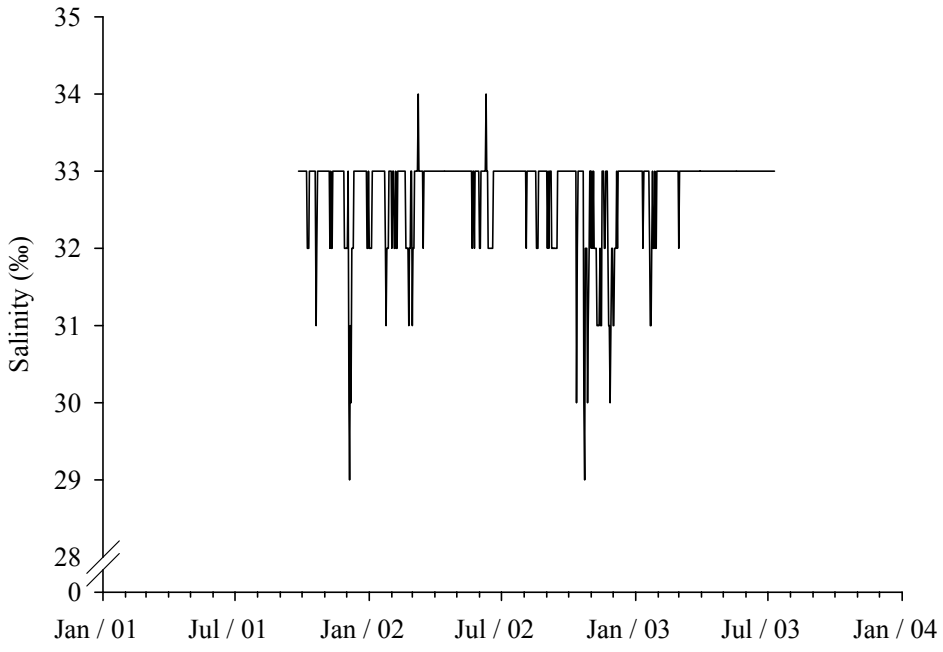


Figure 4.3: Daily salinity (‰) of seawater at Machrihanish from 26th September 2001 to 8th July 2003.

stocking densities and return them to a common basal level in accordance with the predetermined range. Fish were fed a commercial dry pellet (EWOS, Bathgate, UK) to satiation 3-5 times per day between 9 am and 5 pm, 7 days a week.

4.3.2 Photoperiod regimes.

All fish were randomly assigned to one of 8 photoperiod regimes (Figure 4.4, Table 4.1). Unequal numbers were assigned to populations in order to optimise the experimental setup due to the restricted resources available. Population size varied as a reflection of the number of terminal samples (Section 4.3.3) scheduled for that treatment. In treatments 1-4, 50 additional individuals were included to investigate plasma melatonin response (Section 4.3.4); these individuals were removed from the experimental setup on the 1st of July 2002. Photoperiod treatments in tanks were held constant (SNP or LL) with experimental populations being transferred between tanks as the experimental photoperiod regime dictated. The number of treatment tanks used (Figure 4.5) was regulated by fish number and size with the stocking density ranges being the limiting factors. Where more than one treatment tank (i.e. SNP or LL) was required, individuals for each photoperiod group were evenly assigned between the tanks.

4.3.3. Sampling regime.

Monitoring of the population for growth and reproduction: From 1st October 2001 onwards, a 3 monthly sampling schedule was undertaken. At every sample time, following anaesthesia, all individuals were measured (weight to 0.1 g and total length to 1 mm) and their maturation status visually assessed (Section 2.3.2). Individuals were designated mature when gametes were released on inspection. In the spring of 2003 additional checks for maturation status only of all populations were also made on 24th

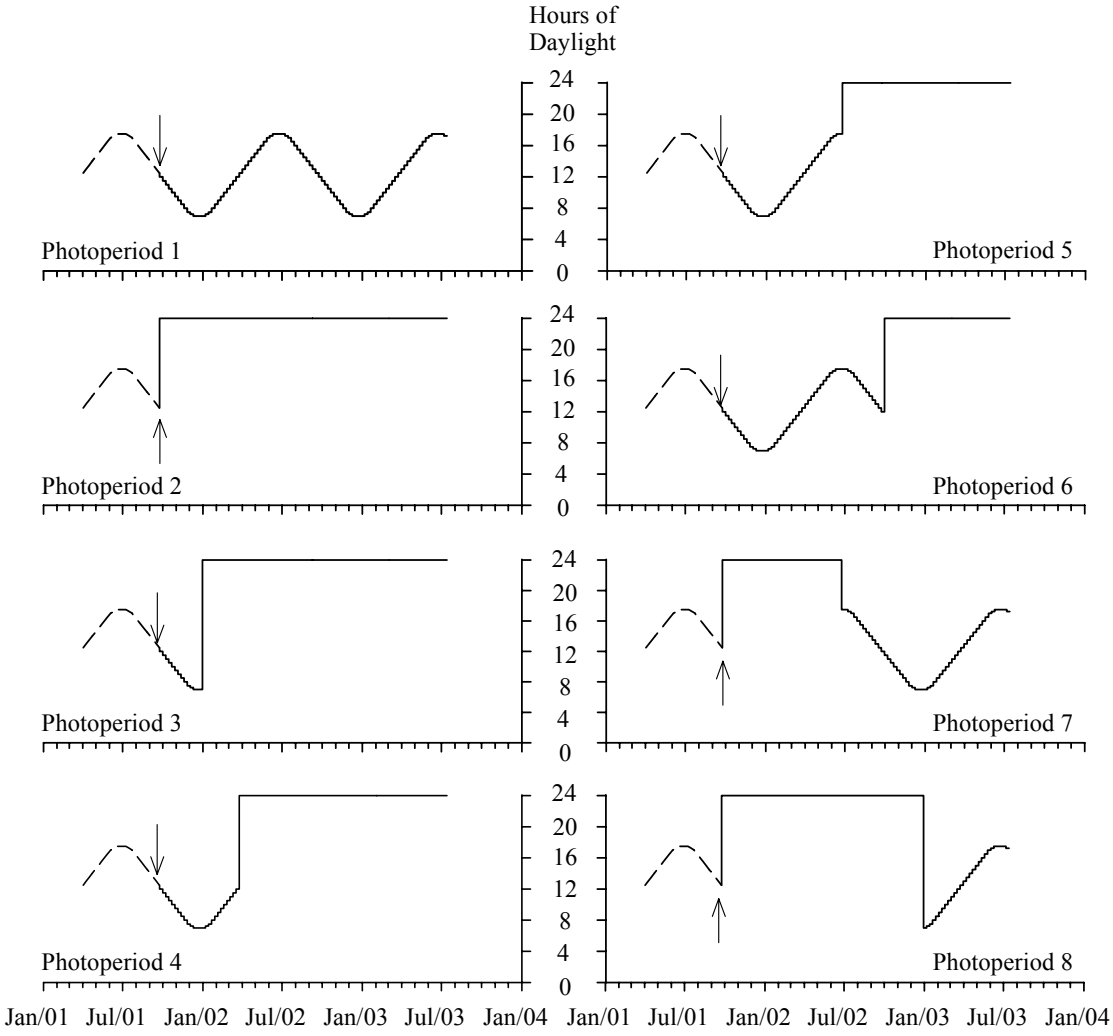


Figure 4.4: Photoperiod treatments 1-8. Arrow indicates start of experimental regime. Photoperiod prior to experimental period shown as broken line.

Table 4.1: Experimental groups, listing numbers of individuals and a description of the respective photoperiod treatment.

Photoperiod Treatment	No. of Individuals	Description
Photoperiod 1	160	Simulated Natural Photoperiod (SNP)
Photoperiod 2	150	SNP until 1st October 2001 (6 months post hatch (MPH)) then continuous illumination (LL)
Photoperiod 3	140	SNP until 1st January 2002 (9 MPH) then LL
Photoperiod 4	130	SNP until 1 April 2002 (12 MPH) then LL
Photoperiod 5	70	SNP until 1 July 2002 (15 MPH) then LL
Photoperiod 6	60	SNP until 1 October 2002 (18 MPH) then LL
Photoperiod 7	70	SNP until 1st October 2001 (6 MPH) then LL until 1st July 2002 (15 MPH) then SNP
Photoperiod 8	50	SNP until 1st October 2001 (6 MPH) then LL until 1st January 2003 (21 MPH) then SNP

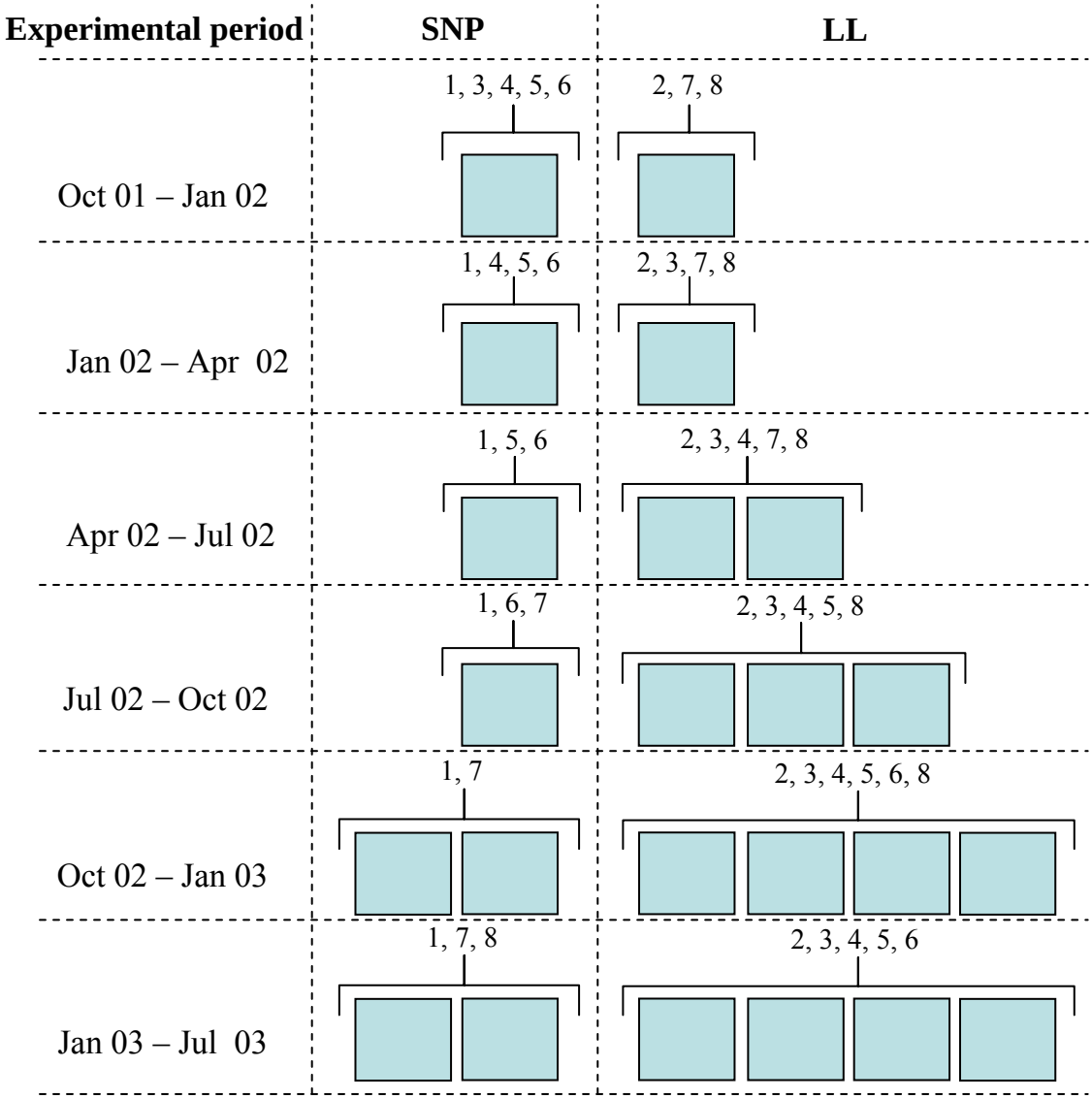


Figure 4.5: Schematic diagram outlining the number of tanks used and their allocation to the photoperiod treatments (SNP and LL) in association with the presence of experimental populations throughout the trial period.

February and 28th of May.

Terminal sampling: As part of the 3 monthly sampling schedule, from each different photoperiod treatment, 10 individuals were pre-selected at random. Once identified [sampling error and tag failure caused this number to vary on occasions between 4 and 11 individuals (Table 4.2)] these individuals were sacrificed. Following morphometric measurements, a blood sample was withdrawn into heparinised syringes and the plasma, separated by centrifugation, was stored at -70°C prior to later analysis for testosterone (Section 2..4.2), calcium (Section 2.4.4) and IGF-I (Section 2.4.3). The abdominal cavity was scanned with a 7.5 MHz horizontal probe ultrasound scanner (Section 2.3.1) and images of the gonads recorded. Following this, the liver and gonads were removed and weighed to 0.01 g in order to calculate both HSI and GSI values, and gonad

Table 4.2: Numbers of individuals terminally sampled at each sample for each photoperiod treatment (1 to 8).

	January 2002		April 2002		July 2002		October 2002		January 2003		April 2003		July 2003	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Photo 1	5	4	6	4	7	3	3	6	3	1	2	7	5	5
Photo 2	6	4	4	6	7	3	5	5	6	4	5	5	3	5
Photo 3	5	4	5	4	5	5	5	5	7	1	6	4	4	6
Photo 4	5	4	6	4	3	5	4	6	4	5	6	3	6	4
Photo 5	5	4	6	4	7	3	5	5	3	7	6	4	6	4
Photo 6	5	4	6	4	7	3	3	6	1	6	8	2	5	5
Photo 7	6	4	4	6	7	3	5	5	6	4	3	7	5	3
Photo 8	6	4	4	6	7	3	5	5	6	4	4	6	8	3

developmental stage was assessed on a 5 point visual scale taken from Morrison (1990), a summary of which is shown in Table 2.1. Thereafter, the liver and carcass were stored at -20^{o}C prior to proximate compositional analysis (Section 2.5) and gonads preserved in 10% buffered formalin for histological analysis (Section 2.3.4). At times when populations experienced common photoperiod histories, terminal samples were taken from one population and the subsequent data assigned to all relevant treatments (e.g. data collected in April 2002 sample from photoperiod 1, was also assigned to photoperiods 4 to 6). This was necessary to optimise resources.

At the end of the experiment in July 2003 there remained a core group of 32-43 individuals per photoperiod treatment for which a complete growth history had been recorded. These were sacrificed and dissected to record the sex of the individuals.

The overall population sex ratio (males to females) was 1:1.19 the range within the treatment groups was 1:0.94 to 1:1.58 (Table 4.3). Throughout the trial period the overall mortality rate was 5%. The range within the photoperiod groups was 1.4 to 8.3% (Table 4.3) however, there was no significant difference between treatments. Mortalities could not be ascribed to any particular symptom or event. There was also no significant effect of sex.

Table 4.3: Sex ratio (M:F) and percentage mortality for each photoperiod treatment.

	Photo 1	Photo 2	Photo 3	Photo 4	Photo 5	Photo 6	Photo 7	Photo 8
Sex ratio (M:F)	0.94	0.95	1.58	1.06	0.97	1.33	1.09	0.94
Mortality rate (%)	7.1	3.0	2.2	4.2	8.3	6.3	1.4	6.0

4.3.4. Melatonin Study

On 1st July 2002, 50 tagged individuals from photoperiod treatments 1 to 4 were removed from the communal experimental tank setup and placed into 4 additional 7 m^3 tanks (1 treatment population per tank) under identical photic conditions. On the $10^{th}/11^{th}$ of July a diel sampling regime was performed for melatonin analysis. There were 5 sample points in total taken at 20:00 and 23:00 on the 10^{th} and 01:30, 04:00, 07:00 on the 11^{th}, in the SNP treatment sunset was at 22:00 and sunrise at 05:00 on these dates. At each sample point 10 individuals were anaesthetised and 1ml of blood withdrawn into heparinised syringes. Following sampling, fish were placed in stock tanks to ensure there was no repeat sampling and were thereafter not returned to the experimental setup. The plasma was separated by centrifugation and frozen over liquid nitrogen prior to storage at -70^{o}C. Samples were assayed for melatonin content within 1 month of sampling by direct radioimmunoassay (Section 2.4.1).

4.3.5 Histological analysis

A limited number of gonad samples (n = 6, 3M:3F) from the July 2003 sample (end of trial) from each photoperiod treatment were processed for histological analysis (Section 2.3.4). Histological staging was based on scales presented in Kjesbu and Kryvi (1989), Morrison (1990), Burton *et al*. (1997), Saborido-Rey and Junquera (1998), Tomkiewicz *et al.* (2003) and Dahle *et al*., (2003). In the ovarian samples, the diameters of oocytes (longest diameter through nucleus) (n = 100 per individual) were measured only in oocytes which were sectioned through the nucleus using image processing software.

4.3.6. Analytical calculations

The potential interaction of seasonally fluctuating gonad and liver weight on the calculation of relative weight indexes GSI (Section 2.3.3) and HSI (Section 2.6.2) was investigated prior to statistical analysis as follows:

GSI: The standard GSI calculation (Section 2.3.3) was correlated against a GSI calculation based on "carcass" weight (i.e. whole wet weight – liver weight) to quantify the importance of this potential variance in the calculation. A regression of:

$$\frac{Gonad\quad weight}{Whole\quad wet\quad weight} \; vs. \; \frac{Gonad\quad weight}{(Whole\quad wet\quad weight - liver\quad weight)}$$

using all data points (n = 368), had an $r^2 = 0.9997$ and $p<0.0001$ with the slope of the regression line being 1.05. As such, liver weight was not deemed to provide a significant influence over GSI calculation and this correction was not included in the subsequent analysis.

HSI: The standard HSI calculation (Section 2.6.2) was correlated against a HSI calculation based on "carcass" weight (i.e. round weight – liver weight) to quantify the importance of this potential variance in the calculation. A regression of:

$$\frac{Liver\quad weight}{Whole\quad wet\quad weight} \; vs. \; \frac{Liver\quad weight}{(Whole\quad wet\quad weight - gonad\quad weight)}$$

using all data points (n = 378) had an $r^2 = 0.99$ and $p<0.0001$ with the slope of the regression line being 0.98. As such, gonad weight was not deemed to provide a

significant influence over HSI calculation and this correction was not included in the subsequent analysis.

4.3.7. Statistical analysis

Growth parameters, GSI, HSI, oocyte diameters, hormonal levels and flesh and liver proximate compositions were analysed using a General Linear Model (Section 2.7.6). Normality and homogeneity of variance were improved where necessary by either log or reciprocal transformations. Parameters expressed as a proportion or percentage were first subjected to arcsine transformation. A significance level of $p<0.05$ was set with all significant interactions being analysed by Tukey *post hoc* test. Correlations between IGF-I vs. growth rates, HSI and temperature and amongst flesh and liver proximate components were analysed using the Pearson's product moment method (Section 2.7.8). For changes in % maturation and mortality rates, 95% confidence limits were calculated and compared (Section 2.7.7).

4.4 Results

4.4.1 Maturation

4.4.1.1 Maturation: Timing and commitment

2002

There were two individuals (males) which sexually matured in 2002. The first, in April (12 MPH), was a male from photoperiod 3 which was found to be releasing milt following gentle abdominal pressure. This individual, part of the terminal sample group at this time, had a GSI of 1.9%. No other individuals were found to be releasing gametes at this time.

The second incidence of maturation was discovered on 10th June 2002 (14 MPH). Following a routine monthly inspection to update husbandry records a single male from photoperiod 4 was found to be releasing milt. As this was not a scheduled sampling the entire population could not be inspected. The next complete sample performed one month later on the 9th of July 2002 revealed no individuals releasing gametes in any treatment.

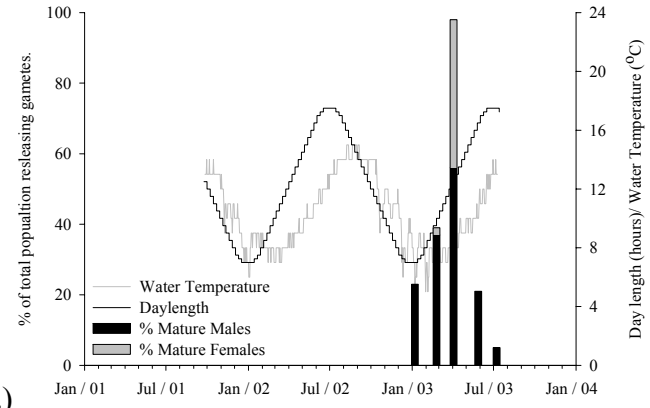
2003

In the second year of the trial, mature individuals were detected in 4 of the photoperiod treatments. These were groups 1, 6, 7 and 8 (Figure 4.6, Figure 4.7).

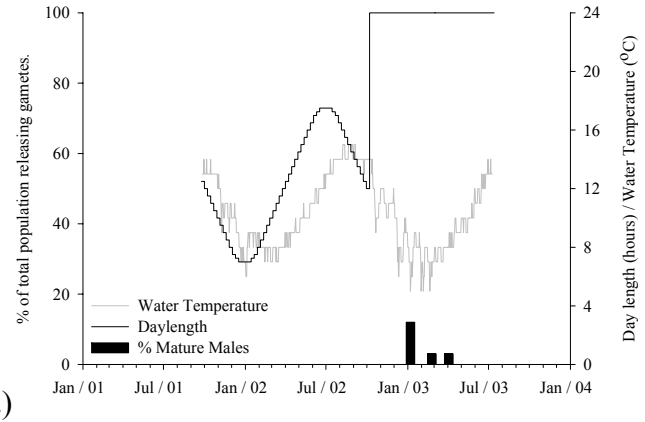
Photoperiod 1 and 7

For both photoperiod 1 and 7 the spawning season lasted from January through

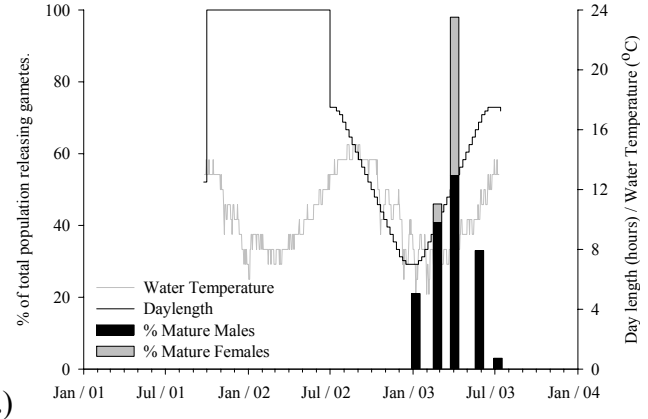
Figure 4.6: (Following page) Percentage of the total population releasing gametes in relation to photoperiod treatment and water temperature for photoperiods 1 (a.), 6 (b.), 7 (c.) and 8 (d.) (n=33 to 43)


% of total population resleasing gametes.
Day length (hours)/ Water Temperature (°C)
Water Temperature
Daylength
% Mature Males
% Mature Females
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

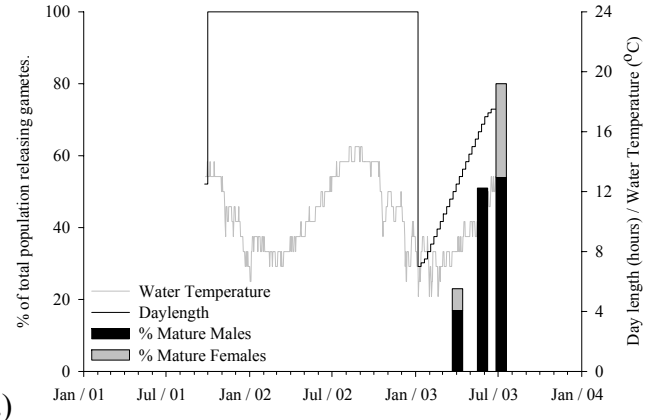
a.)


% of total population releasing gametes.
Day length (hours) / Water Temperature (°C)
Water Temperature
Daylength
% Mature Males
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

b.)


% of total population releasing gametes.
Day length (hours) / Water Temperature (°C)
Water Temperature
Daylength
% Mature Males
% Mature Females
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

c.)


% of total population releasing gametes.
Day length (hours) / Water Temperature (°C)
Water Temperature
Daylength
% Mature Males
% Mature Females
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

d.)

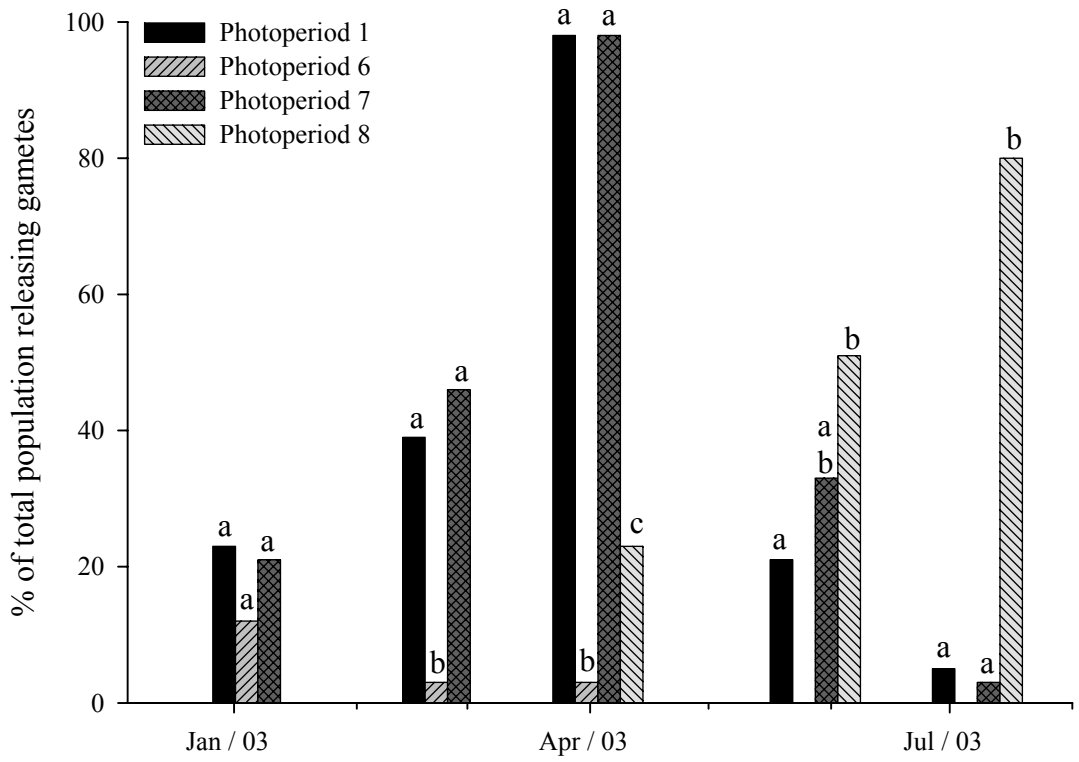


Figure 4.7: Percentage of total population releasing gametes for individuals maintained under photoperiods 1, 6,7 and 8 in 2003 spawning season (n =33 to 43). Different letters denote significant differences between treatments. Analysis of proportions (P<0.05).

to July 2003 (21 to 27 MPH). While all individuals in photoperiod 7 (n = 39) were recorded as releasing gametes during the trial, in photoperiod 1 (n = 43), a single female (2% of population) was not. In both treatments males were spermiating from January to July 2003, however females were only ovulating between the end of February (22 MPH) through to April (24 MPH). In both treatments the peak of spawning activity was in April with 98% of both populations releasing gametes on inspection.

Photoperiod 6

The spawning season lasted from January through to April 2003 (21 to 24 MPH). Only five spermiating males, representing 13% of the population (n = 40), were recorded as releasing gametes through the trial. The peak of activity was in January (21

MPH), thereafter one male (3% of population) continued to release milt until April (24 MPH). Maturation commitment (% of population releasing gametes) in January was comparable with that of photoperiods 1 and 7; thereafter it was significantly reduced.

Photoperiod 8

The spawning season lasted from April to July 2003 (24 to 27 MPH). 97% of the total population (population n = 35) were recorded as releasing gametes by the end of the trial while a single female (3%) was not. Mature individuals of both sexes were identified in April 2003. The number of mature individuals increased steadily to reach a peak at the trial end in July 2003 when 80% of the population were releasing gametes on inspection. Maturation commitment in April was higher than in Photoperiod 6 though lower than 1 and 7. In May (25 MPH) commitment was comparable to photoperiod 7 though higher than 1, while in July it was significantly higher than in all other treatments. Interestingly, when the maturation "profile" (spawning commitment over time) was aligned to start on the same theoretical date as photoperiods 1 and 7 (i.e. advanced by 3 months) then there was no significant difference in the maturation commitment (% mature individuals) between treatments 1, 7 and 8 over the comparable relative time frame.

4.4.1.2 Gonadal development

Ultrasound Scanning

Gonadal development (form and size) was visualised by ultrasound scanning (Section 2.3.1) in the sacrificed fish of each photoperiod treatment prior to dissection. This data was included to allow direct comparison with the later recorded physical size (GSI) and morphology of the gonads as well as histological staging.

The observed data was summarised, as before (Experiment I), using the 3 tier scale outlined in section 3.2.3.1. In brief, gonadal tissues were not detectable in any population until October 2002 (18 MPH) (Table 4.4, Figure 4.8). At this stage, in the majority of populations, ovaries were identifiable though small (*circa* 10-20 mm diameter) and testis were not consistently evident. After which, the division between mature and immature populations became apparent.

Mature Populations

Photoperiods 1 and 7: Developmental rates mimicked that reported for the SNP treatment in experiment I (Section 3.2.3.1). In January 2003 (21 MPH) both sexes were clearly identifiable with ovarian diameters of *circa* 20 to 50 mm, while faint echoes of the distal frills (see below) were recorded in the male testes, the efferent ducts (see

Table 4.4: Summary of the classification of gonad states for all photoperiod treatments. Gonads were visualised by ultrasound scanning and images were classified using the three point scale outlined in Section 3.2.3.1.

	Oct 01	Jan 02	Apr 02	Jul 02	Oct 02	Jan 03	Apr 03	Jul 03
Photoperiod 1	X	X	X	X	1	2	2	1
Photoperiod 2	X	X	X	X	1	1	1	1
Photoperiod 3	X	X	X	X	1	1	1	1
Photoperiod 4	X	X	X	X	1	1	1	1
Photoperiod 5	X	X	X	X	1	1	1	1
Photoperiod 6	X	X	X	X	1	1	1	1
Photoperiod 7	X	X	X	X	1	2	2	1
Photoperiod 8	X	X	X	X	1	1	2	2

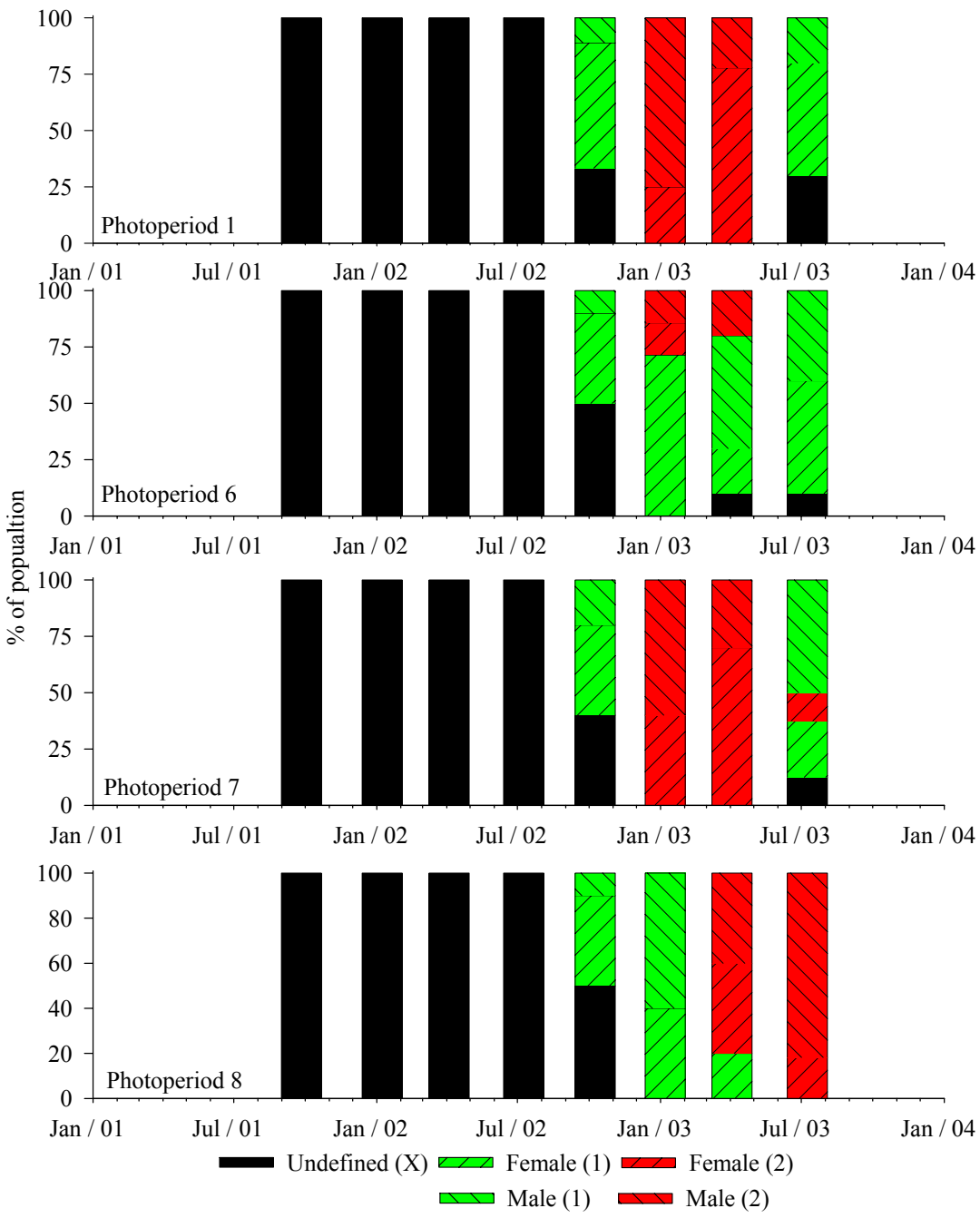


Figure 4.8: Graphical summary of the classification of gonad states recorded for individuals maintained under photoperiod treatments 1, 6, 7 and 8 which displayed spawning activity in the 2003 spawning season. Gonads were visualised by ultrasound scanning and images classified using the three point scale outlined in Section 3.2.3.1. (n = 4 to 11).

below) were the clearest structures with a diameter of *circa* 2-5 mm. By April 2003 (24 MPH) gonadal dimensions reached their peak in line with the spawning activity with ovarian diameter extending to 70 mm while testis structures increased to 30 mm (including both distal tissue and efferent ducts). In July 2003 (27 MPH) ovarian diameters had reduced to <20 mm while the testes were small and difficult to differentiate from the background noise.

Photoperiod 6: In January 2003 (21 MPH) it was possible to differentiate both sexes. While female ovarian diameters were predominately small (*circa* 10-30 mm), a single female was observed with enlarged ovaries (≈45 mm), while the only observed male displayed developed proximal ducts (diameter >7 mm). After this stage no change was observed in ovarian diameter in the females above basal levels to the trial end. With regards to the males, in April 2003 (24 MPH), a single male was again observed with clearly developed proximal ducts, however this appeared to be the exception with all other males inspected to the end of the trial displaying testis which remained small and difficult to differentiate from the background signal.

Photoperiod 8: There was no apparent change in gonad size until April 2003 (24 MPH) when the gonads of both sexes showed marked increase in line with sizes observed in January 2003 (21 MPH) in treatments 1 and 7, then by July 2003 (27 MPH) gonad size had reached its peak in line with the spawning activity.

Immature populations

In treatments 2-5 from January 2003 (21 MPH) onwards both sexes were definable. They were however clearly far smaller in size than treatments 1, 6, 7 and 8.

Male proximal duct diameters were *circa* 1-3 mm, detectable most consistently using moving images to visualise the parallel running channels. Ovarian diameters were *circa* 10-30 mm.

Gross gonadal description.

A gross morphological scale taken from Morrison (1990) as described previously (Table 2.1) was used to visually "stage" the gonads once they were removed from the sacrificed individuals (Table 4.5, Figure 4.9). Gonad structures were not consistently identified in October 2002 (6 MPH) so gross morphology was not recorded on this date. It was difficult to differentiate any apparent difference between classifications 1 and 2 hence, individuals were classified as 1/2 in these situations.

Table 4.5: Summary of gonad gross morphology staging of males (M) and females (F) maintained under photoperiod treatments 1 to 8 over the trail period, using a visual scale from Morrison (1990) as outlined in Table 2.1. Where individuals fell into more than one classification, all are listed.

	January 2002		April 2002		July 2002		October 2002		January 2003		April 2003		July 2003	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Photo 1	1	1	1/2	1/2	1/2	1/2	1/2	1/2	3,4	3	4	4	5	5
Photo 2	1	1	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
Photo 3	1	1	1/2,4	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
Photo 4	1	1	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
Photo 5	1	1	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
Photo 6	1	1	1/2	1/2	1/2	1/2	1/2	1/2	4	1/2,3	1/2,5	1/2	1/2	1/2
Photo 7	1	1	1/2	1/2	1/2	1/2	1/2	1/2	3,4	3	4	4	5	5
Photo 8	1	1	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	3,4	3,4	4	4

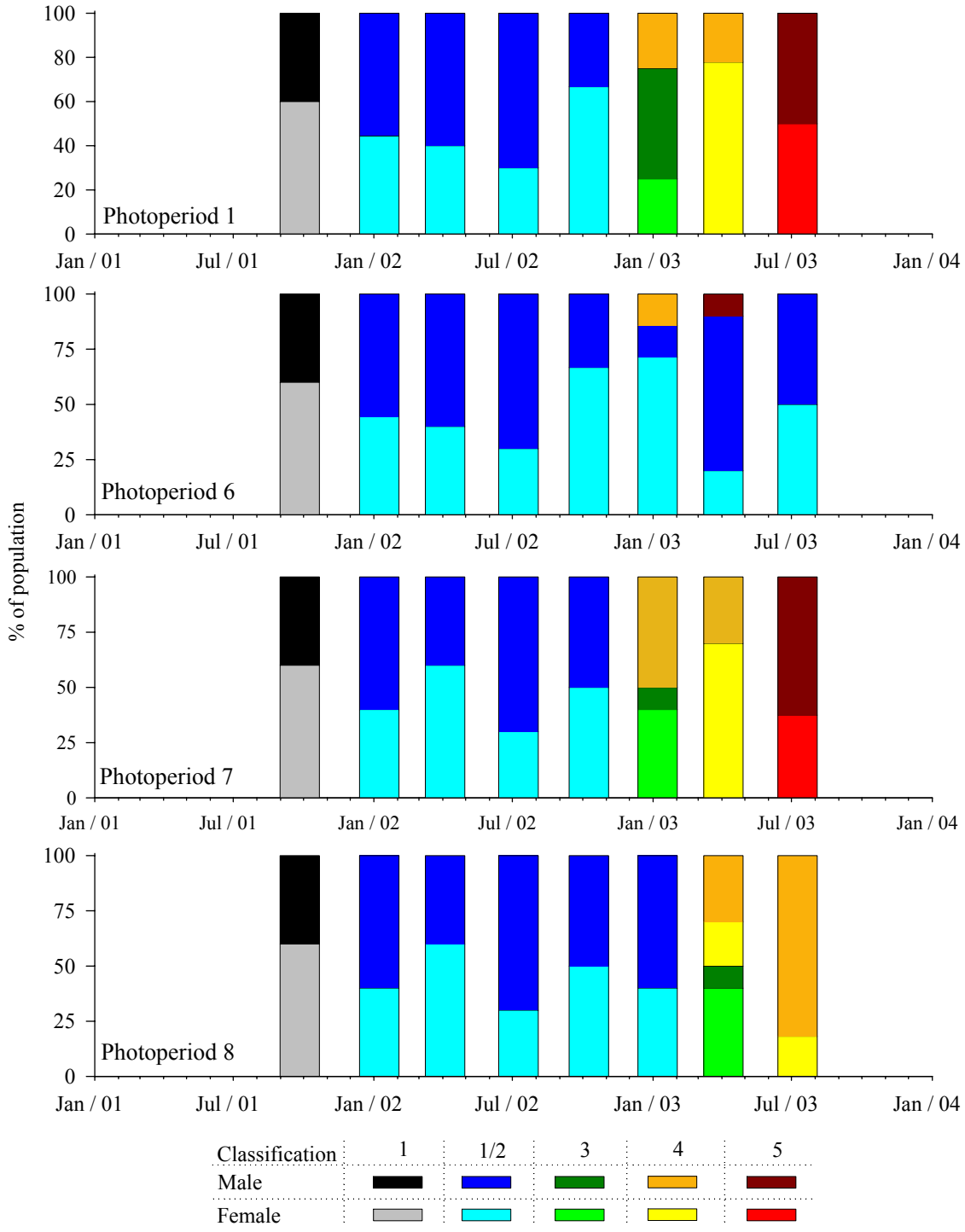


Figure 4.9: Graphical summary of the classification of gonad gross morphological states recorded for individuals maintained under photoperiod treatments 1, 6, 7 and 8 which displayed spawning activity in the 2003 spawning season. Gonads classified using a 5 point visual scale from Morrison (1990) (Table 2.1) (n = 4 to 11).

Immature

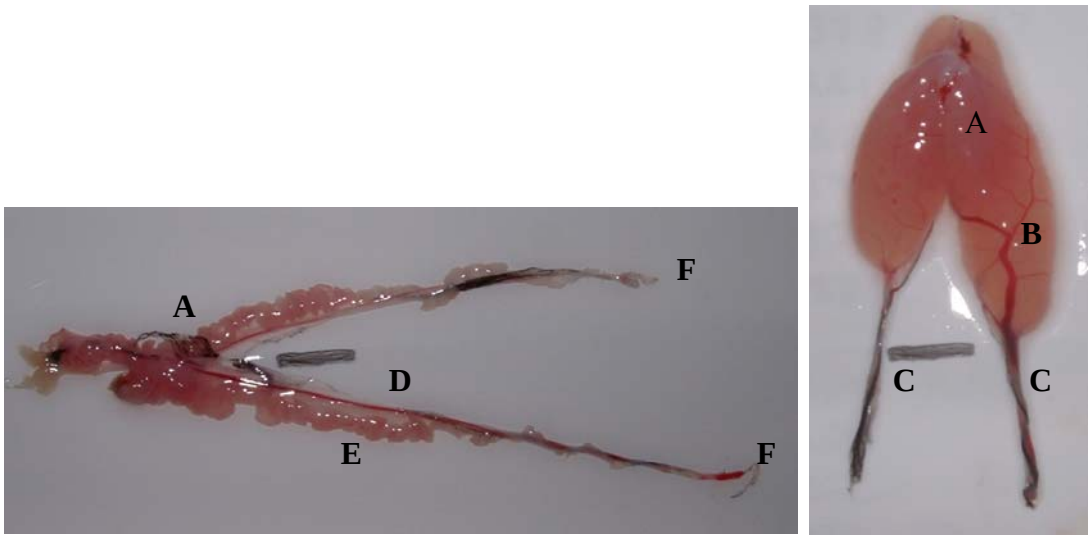
For the majority of inspections, gonads were banded between the 1 and 2 classification (immature and/or early ripening). The different sexes could be characterised as follows:

Males: (Plates 4.1a, 4.2a, 4.3a) Testes, initially joined posteriorly to form the sperm duct just above the genital pore ran anteriorly as two separate arms attached to the dorsal wall of the body cavity by the mesorchium. The organs were thin and insignificant, the most obvious structures were two blood vessels that ran along the entire length of the attachment along with a "proximal duct" structure. It was this duct structure that was detectable in the latter stages (January 2003, 21 MPH, onwards) by the ultrasound scanning. Extending to approximately ¾ the length of the testes was a distal "frill". Both this and the proximal duct structure were a translucent pink/red colour at all times.

Females: (Plates 4.1b, 4.2b, 4.4a) In females, the two ovarian lobes joined dorsally above the genital pore to form the common oviduct. Anteriorly the lobes separated and extended along the roof of the body cavity attached by the mesovaria. The ovaries generally extended to just under $^{1}/_{3}$ of the body cavity length. However, the mesovaria and blood vessels extended to the anterior limit of the cavity. The lobes themselves had a pale pink translucent appearance and always exhibited extensive vasculature.

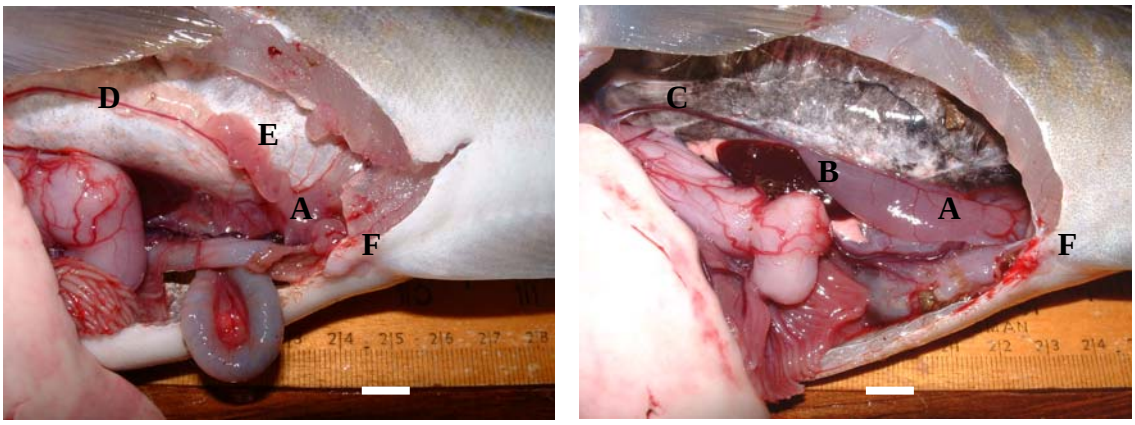
Mature

Gross morphology changed rapidly from the previously described "immature" state to late ripening and/or ripe/spawning individuals (classification 3 and 4) consistently within a 3 month sample period. Such classified individuals were observed



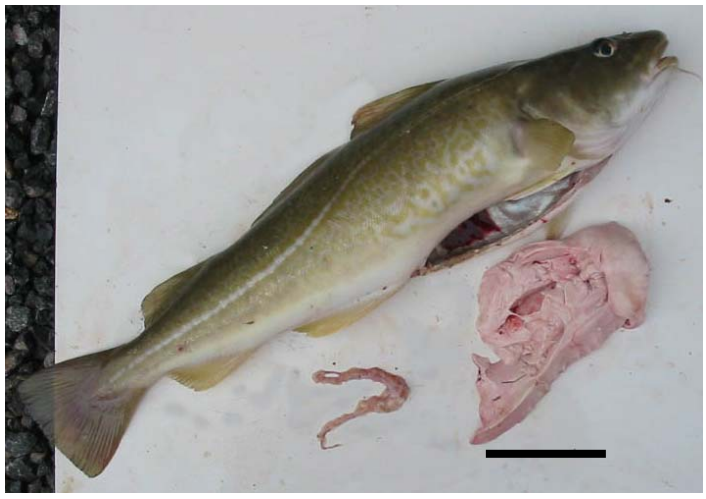
a.) **b.)**

Plate 4.1: Immature gonads of a.) male and b.) female showing **A.** Common caudal region connected to genital pore. **B.** Rostral end of ovary showing vascalature **C.** Blood vessel and mesovaria. **D.** Proximal channel and blood vessel of testes. **E.** Distal frill of testes. **F.** Mesochrium and blood vessels. (Taken April 2002, 12 MPH) Scale bar = 1 cm.



a.) **b.)**

Plate 4.2: Immature gonads *in situ*, of a.) male and b.) female showing **A.** Common caudal region connected to genital pore. **B.** Rostral end of ovary **C.** Blood vessel and mesovaria. **D.** Proximal channel and blood vessel of testes. **E.** Distal frill of testes. **F.** Anus and urogenital papillae. (Taken October 2002, 18 MPH) Scale bar = 1 cm .



a.) Weight: 2023 g
Length: 535 mm
GSI: 0.20 %
HSI: 8.35 %

b.) Weight: 2261 g
Length: 542 mm
GSI: 7.61 %
HSI: 3.98 %

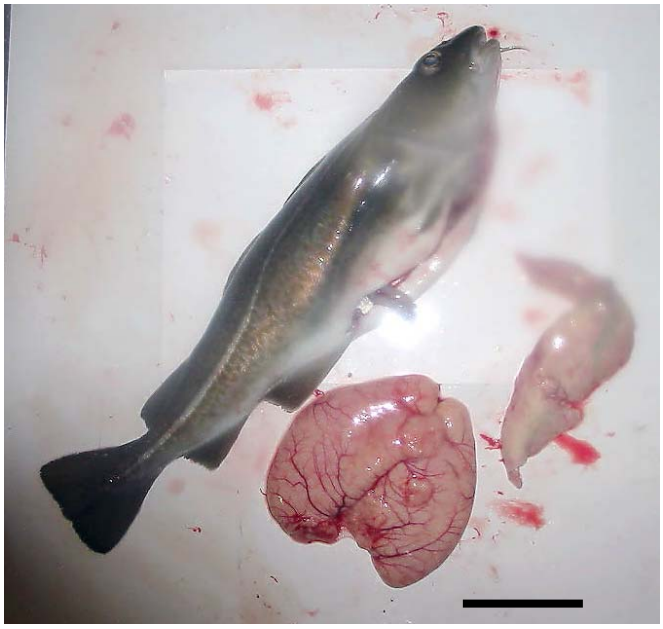
c.) Weight: 983 g
Length: 422 mm
GSI: 0.34 %
HSI: 2.04 %

Plate 4.3: Male cod showing liver along side a.) immature (photoperiod 5), b.) ripe (photoperiod 8) and c.) spent testes (photoperiod 1). (Taken July 2003, 24 MPH) Scale bar = 10 cm.

a.)

Weight: 2405 g
Length: 560 mm
GSI: 0.81 %
HSI: 8.36 %

b.)



Weight: 1580 g
Length: 480 mm
GSI: 18.42 %
HSI: 5.63 %

c.)

Weight: 1819 g
Length: 497 mm
GSI: 0.82 %
HSI: 5.11 %

Plate 4.4: Female cod showing liver along side a.) immature (photoperiod 5) b.) ripe (photoperiod 8) and c.) spent ovaries (photoperiod 1). (Taken July 2003, 24 MPH) Scale bar = 10 cm.

in photoperiod 3 in April 2002 (24 MPH) (1 male out of 9 individuals, 4F:5M) and photoperiod 6 in January 2003 (21 MPH) (1male and 1 female out of 7 individuals, 1M:6F) while in photoperiods 1,7 and 8 all individuals changed to mature in January 2003 (1 and 7) or April 2003 (8). Ripe individuals were characterised as follows:

Males: (Plate 4.3b) In the testes, both the proximal duct and distal frill enlarged dramatically, especially the distal frill which developed into a very complex over folding arrangement which became the dominant structure of the testes. Filling the entire length of the body cavity, the structure was an off-white colour (spermiating males) with thin vasculature evident over the distal frills. It was noted that, in non-spermiating males examined at the start of the spawning season, there was a translucent edge to the distal frill and the frill did not appear as well developed as was observed in spermiating males.

Females: (Plate 4.4b) In the females, both ovarian lobes had enlarged dramatically to fill over ¾ of the body cavity length. They were firmer and denser than before and had changed to an opaque, off white/pink colour with extensive vasculature spread across the ovarian wall. Dissection revealed densely packed internal folds in which individual oocytes were clearly visible. At later stages hydrated oocytes were visible through the ovarian wall.

Spent

In all of the inspected individuals of treatments 1 and 7 in July 2003 (27 MPH) and a single male out of 10 individuals (2F:8M) in treatment 6 in April 2003 (24 MPH),

the gonadal morphology progressed from mature to the classification of spent (classification 5).These fish were characterised as follows:

Males: (Plate 4.3c) While the testes had maintained their opaque off-white colour they were much reduced in size, extending to no more than ½ of the length of the body cavity. The distal frills had shrunk significantly leaving the proximal tubules to become the dominant structure.

Females: (Plate 4.4c) The ovaries had also reduced significantly in size. While taking on a dark red/purple colour. They had become soft, "loose" structures that often contained waxy deposits within the central lumen of the ovaries.

Gonado-somatic index (GSI)

Gonad structures were not consistently identified in October 2001 so GSI was not recorded on this date. There was no significant change in GSI in either sex up to October 2002 (18 MPH) (Figure 4.10). Thereafter fluctuations were observed as follows:

Photoperiod 1 and 7

GSI levels in both populations rose from basal levels in October 2002 (19 MPH) to between 4 and 7 % in both sexes in January 2003 (21 MPH) and 10 to 12 % by April 2003 (24 MPH). Then in July 2003 (27 MPH) the GSI of both populations returned to a size that was statistically comparable to pre-January 03 level.

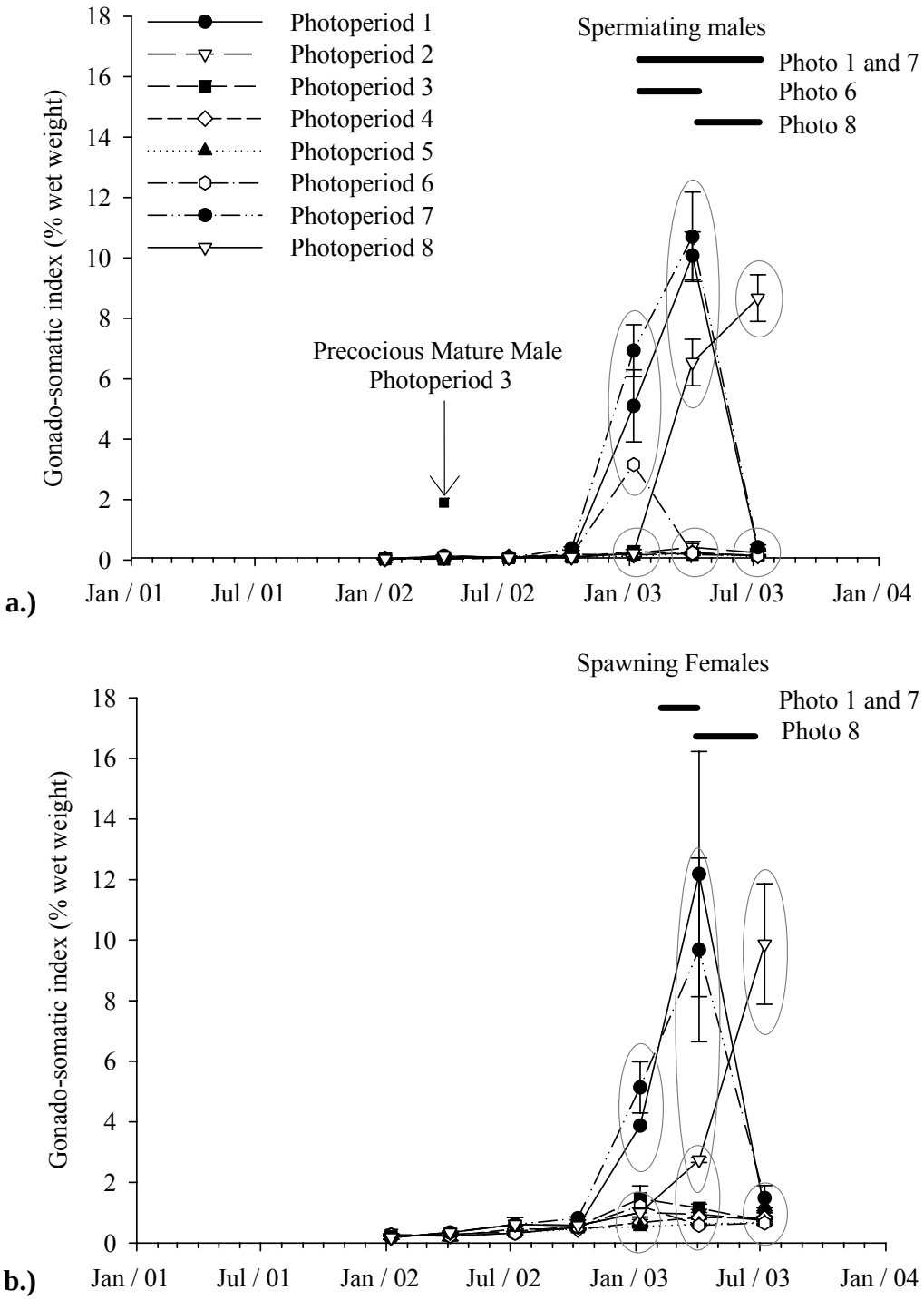


Figure 4.10: Mean gonado-somatic index (GSI) ± SEM for a.) Males (n = 1 to 9) and b.) Females (n = 1 to 7) for individuals maintained under photoperiod treatments 1 to 8. Horizontal bars denote periods when spermiating males or spawning females were present. (Where significant differences existed within a given date, homogeneous subsets are enclosed within an ellipse, $p<0.05$. Where no ellipse is present there were no significant differences within that time point. ANOVA GLM with Tukey *post hoc* test).

Photoperiod 6

In January 2003 (21 MPH), female GSI doubled from basal level of *circa* 0.6% to 1.2% due to a single female with a GSI of 4.6%. The remaining individuals had a mean GSI of 0.6% (n = 8). Male GSI levels rose to 3%, however, it should be noted that this calculation was based on one male sacrificed at this point. The remaining individuals sacrificed from this treatment were females. Thereafter, GSI returned to basal levels.

Photoperiod 8

No significant change was noted until April 2003 (24 MPH) when the GSI in photoperiod 8 rose to around 3% in the females and 8% in the males. This increase continued in July 2003 (27 MPH) when levels reached of 9% and 10% for males and females respectively.

Photoperiods 2-5

GSI in treatments 2 to 5 stayed below 0.5% and 1.5% for males and females respectively throughout the trial period, with the exception of a single spermiating male in photoperiod 3 in April 2002 (12 MPH) with a GSI of 1.9%. There was an apparent trend for the GSI to increase over time in both sexes. This was clearest in the females with the July 2003 (27 MPH) levels being significantly larger than their corresponding sample in January 2002 (9 MPH).

4.4.1.3 Histological analysis

A limited number of fixed gonad samples taken at the trial end in July 2003 (27 MPH) were processed for histological analysis (n = 6 per treatment, 3 males and 3 females) to define the developmental state of the populations at this time. Interestingly

this revealed apparent developmental differences between the sexes in some photoperiod treatments.

Photoperiod 1 and 7

The males testes in both photoperiods 1 (Plate 4.5a) and 7 (Plate 4.5g) predominately contained large areas of spermatozoa with no earlier germ cell stages apparent. The females in both photoperiods 1 and 7 possessed a clear mix of primary growth oocytes in between postovulatory follicles and atretic oocytes (Plates 4.6 a, b). Mean oocyte diameter was 128 ± 3 μm and 109 ± 4 μm in treatments 1 and 7 respectively (Figure 4.11).

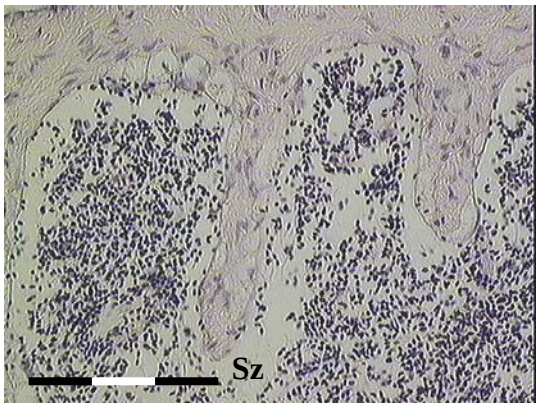
Photoperiod 6

The males in photoperiod 6 (Plate 4.5f) possessed germ cells at most stages of development. The tissues were generally dominated by the earlier developmental stages of spermatogonia and spermatocytes, however apparent pockets of spermatozoa were observed. The ovarian sections revealed only primary growth oocytes (Plate 4.6c,d) with mean diameter of 112 ± 2 μm (Figure 4.11).

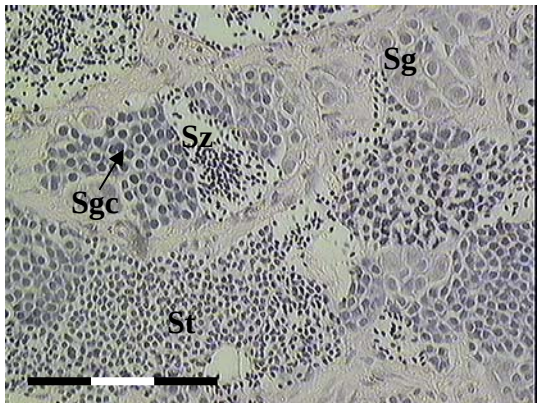
Photoperiod 8

The male testes in photoperiod 8, like those of photoperiods 1 and 7, were

Plate 4.5: (Following page) Photomicrographs of sections (5 μm thick) of Atlantic cod testes taken in July 2003 (27 MPH, trial end) from individuals maintained under **A.**) Photoperiod 1, **B.**) Photoperiod 2, **C.**) Photoperiod 3, **D.**) Photoperiod 4, **E.**) Photoperiod 5, **F.**) Photoperiod 6, **G.**) Photoperiod 7 and **H.**) Photoperiod 8. Showing: Sz, Spermatozoa, Sg, Spermatogonia, Sgc late spermatogonia or early spermatocyte, St, Spermatid, H, Spermatozoa head, F, Spermatozoa flagellum. Sections were stained with Mayer's haematoxylin and eosin Y. Scale bar divisions = 25 μm.



A.



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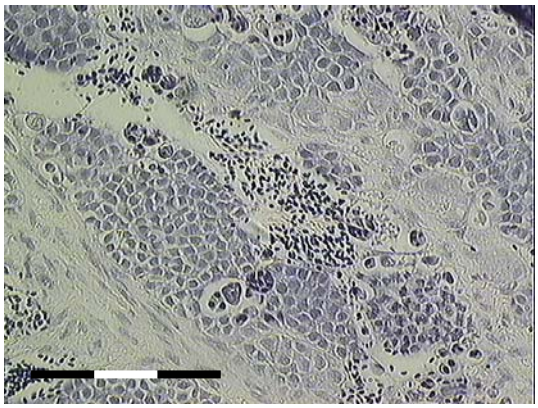

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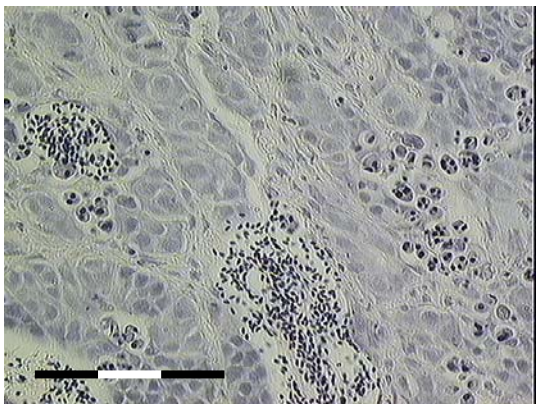

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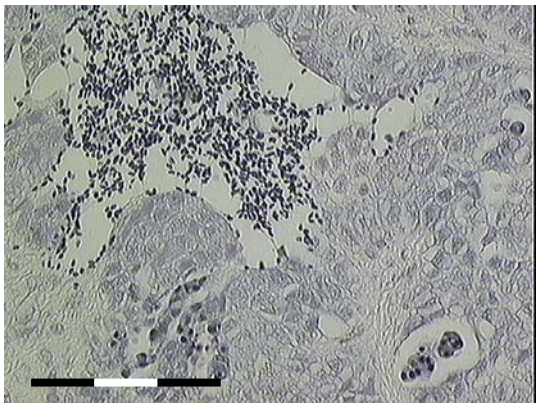

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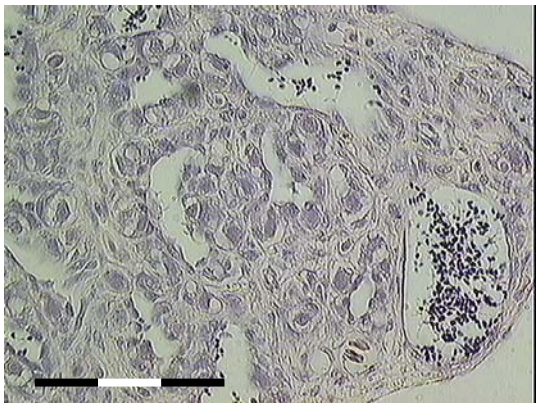
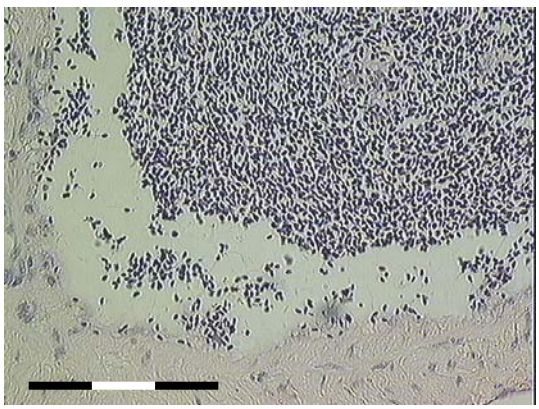
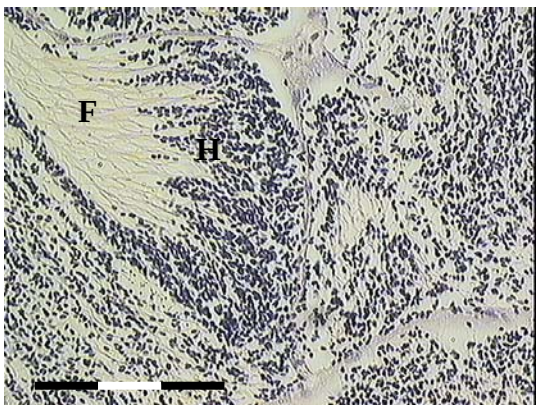


F.



G.



H.

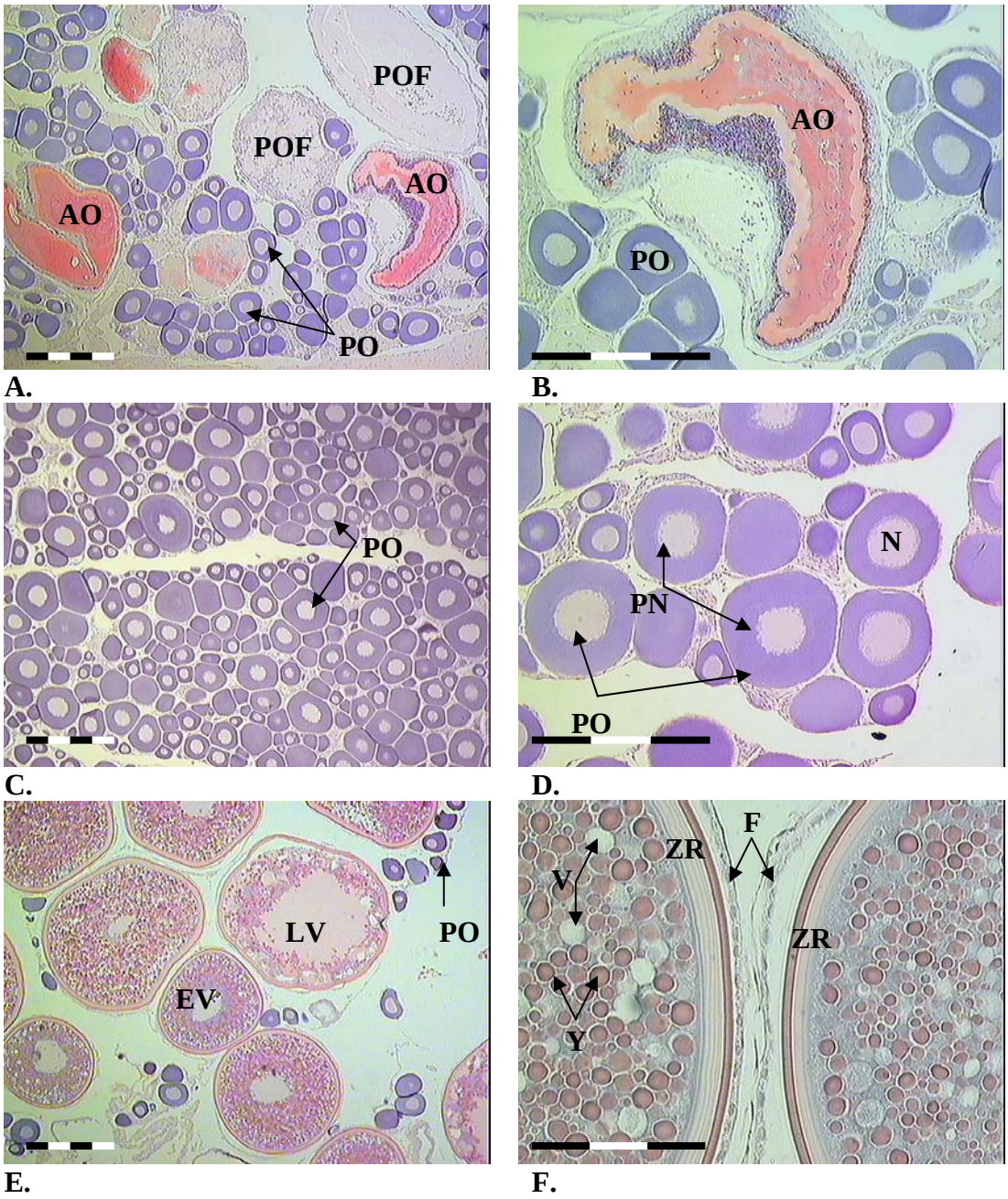


Plate 4.6: Photomicrographs of sections (5 µm thick) of Atlantic cod ovaries taken in July 2003 (27MPH, trial end), showing females at different developmental stages. **A.** and **B.**) Female from photoperiod 1 (spent) showing primary oocytes, postovulatory follicles and atretic oocytes. **C.** and **D.**) Female from photoperiod 5 (immature, typical of photoperiods 2 to 6) showing primary growth oocytes. **E.** and **F.**) Female from photoperiod 8 (spawning) showing oocytes in varying stages of vitellogenesis. PO, Primary growth oocyte, AO, Atretic oocyte, POF, Postovulatory follicle, PN, peripheral nucleolus, N, Nucleus, EV, early vitellogenic oocyte, LV, Late vitellogenic oocyte, Y, Yolk droplets, V, Vesicle, ZR, Zona radiata, F, Follicular cell layer. Sections were stained with Mayer's haematoxylin and eosin Y. Scale bar divisions = 100 µm in **A.** to **E.** and 25 µm in **F.**

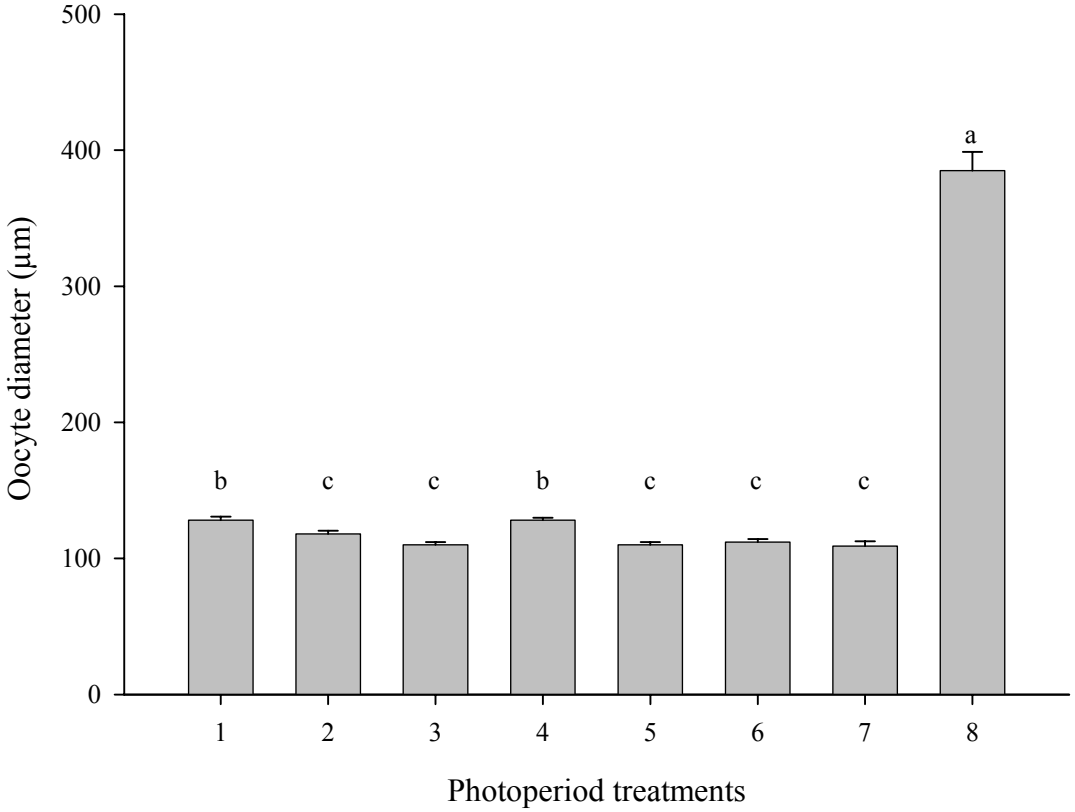


Figure 4.11: Mean oocyte diameter (µm) ± SEM, measured as longest axis through the nucleus (n = 300; 100 randomly selected oocytes from three individuals) from individuals maintained under photoperiod treatments 1 to 8 in July 2003 (27 MPH). Ovary samples were previously fixed in 10% buffered formalin prior to dehydration, embeded in paraffin wax and sectioned at 5 µm thickness. Oocyte diameter was calculated by image processing software. Different letters denote significant differences between treatments, $p<0.05$, ANOVA.

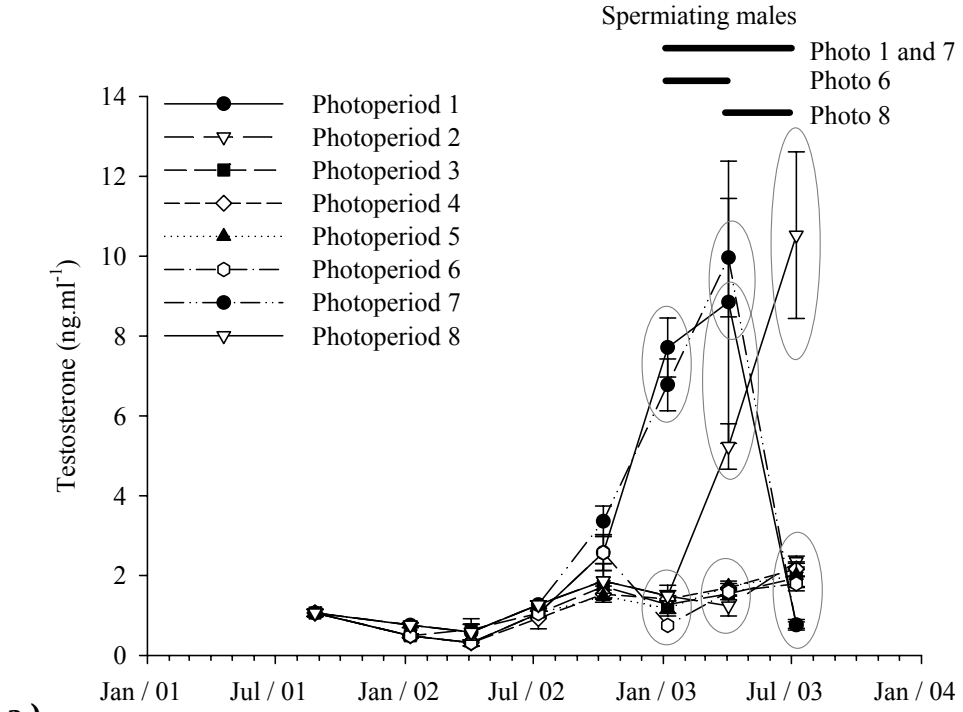
dominated by spermatozoa (Plate 4.5h). However, they appeared to be arranged into more structurally organised groups with clusters orientated in a common direction and tightly packed areas of spermatozoa heads and spermatozoa flagellum being apparent. The females possessed oocytes in various stages of vitellogenesis including up to those about to enter final oocyte maturation (FOM) (Plate 4.6e). These were interspersed in places with occasional small primary growth oocytes. The vitellogenic oocytes contained large yolk droplets (Plate 4.6f) that, in the later stages of development, were clearly coalescing to form a single homogenised yolk (Plate 4.6e) in preparation for FOM. Mean oocyte diameter was significantly larger than all other treatments at 385 ± 14 μm (Figure 4.11).

Photoperiod 2 to 5

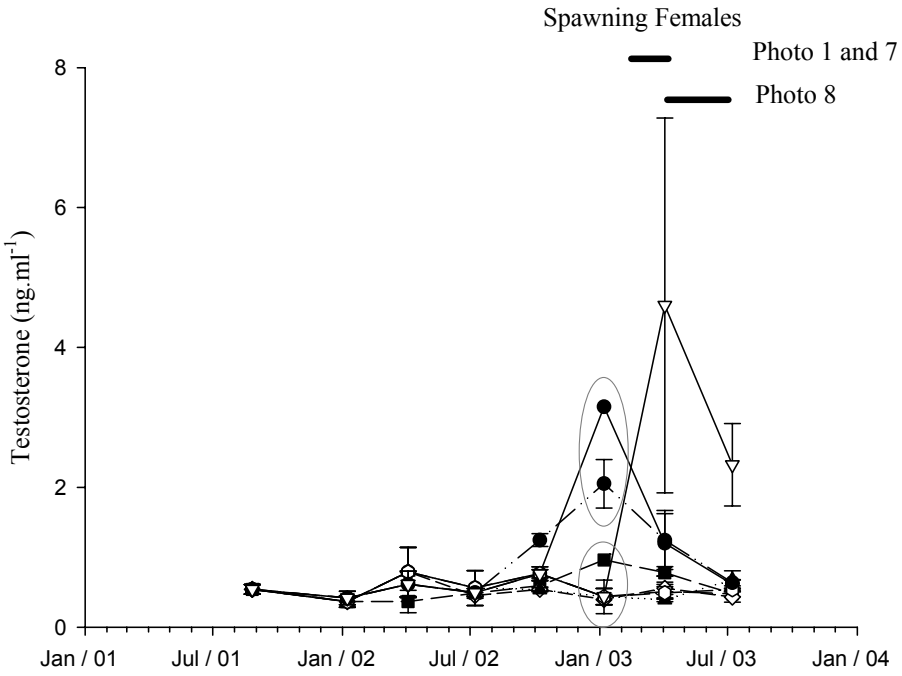
The males in photoperiods 2 to 5 (Plates 4.5, b to f) were very similar to those observed in photoperiod 6. While the testes were dominated by the earlier developmental stages of spermatogonia and spermatocytes in all samples examined, there were apparent pockets of spermatozoa observed. In the females of treatments 2 to 5, only primary growth oocytes were observed in the ovaries (Plate 4.6c) with the peripheral nucleoli being clear in the larger oocytes (Plate 4.6d). Mean oocyte diameter ranged between 110 and 128 μm (Figure 4.11).

4.4.1.4 Plasma testosterone

Maximum mean plasma testosterone levels were significantly higher in the males (mean range 0.31 to 10.6 $ng.ml^{-1}$) than in the females (mean range 0.36 to 4.6 $ng.ml^{-1}$) (Figure 4.12). However, both sexes displayed perceptible differences in plasma levels in relation to the photoperiod treatments.



a.)



b.)

Figure 4.12: Mean plasma testosterone concentration ($ng.ml^{-1}$) ± SEM for **a.)** Males (n = 1-9) and **b.)** Females (n = 1-7) maintained under photoperiod treatments 1 to 8. Horizontal bars denote period when spermiating or spawning individuals were present. (Where significant differences existed on a given date, homogeneous subsets are enclosed within an ellipse, $p<0.05$. Where no ellipse is present there were no significant differences at that time point: $p> 0.05$. ANOVA GLM with Tukey *post hoc* test).

Photoperiod 1 and 7

Up to July 2002 (15 MPH), testosterone levels in males remained at basal levels of <2 $ng.ml^{-1}$ after which time levels started to rise and by January 2003 (21 MPH) they were significantly higher than in all other treatments. They reached a peak of between 9 and 10 $ng.ml^{-1}$ in April 2003 (24 MPH) which was significantly higher than all other treatments apart from the levels in photoperiod 8. Following this, the levels descended to basal levels at the trial end in July 2003 (27 MPH). Testosterone levels in the females predominately stayed at basal levels of <1 $ng.ml^{-1}$, however they rose significantly in January 2003 to reach a peak of 2 to 3 $ng.ml^{-1}$ before returning to basal levels in April 2003.

Photoperiod 6

The males of photoperiod 6 showed an apparent rise in testosterone comparable to photoperiod 1 and 7 in October 2003 (15 MPH) (not significant compared to other treatments) and thereafter, returned to basal levels, while the females displayed no evident fluctuations.

Photoperiod 8

Males showed no significant fluctuations in testosterone until after January 2003 (21 MPH) when levels rapidly rose to attain a peak of 10.6 $ng.ml^{-1}$ by July 2003 (27 MPH) that was significantly higher than all other treatments. In the females, levels raised from basal to *circa* 5 $ng.ml^{-1}$ in April 2003 (24 MPH). However, because of large individual variation, this was not significantly higher than the other treatments. In July (27 MPH), these levels fell back to approximately 2 $ng.ml^{-1}$.

Photoperiods 2 to 5

In photoperiods 2 to 5, testosterone levels remained below 2 ng.ml-1 in the males and 1 $ng.ml^{-1}$ in the females, furthermore, there were no significant fluctuations over time.

4.4.1.5 Plasma calcium

Calcium concentrations in the plasma of females were used as an indirect assessment of mobilisation of vitellogenin. In photoperiods 2 to 6, a constant basal level of *circa* 140-170 $mg.dl^{-1}$ was maintained throughout the trial (Figure 4.13). Levels in photoperiods 1 and 7 rose significantly in January 2003 (21 MPH) to ≥200 $mg.dl^{-1}$ and thereafter declined to reach basal levels in July 2003 (27 MPH). As for photoperiod 8, calcium levels rose above the basal levels after January 2003 (21 MPH) to reach a peak of >200 $mg.dl^{-1}$ in April (24 MPH) which was maintained to the trial end.

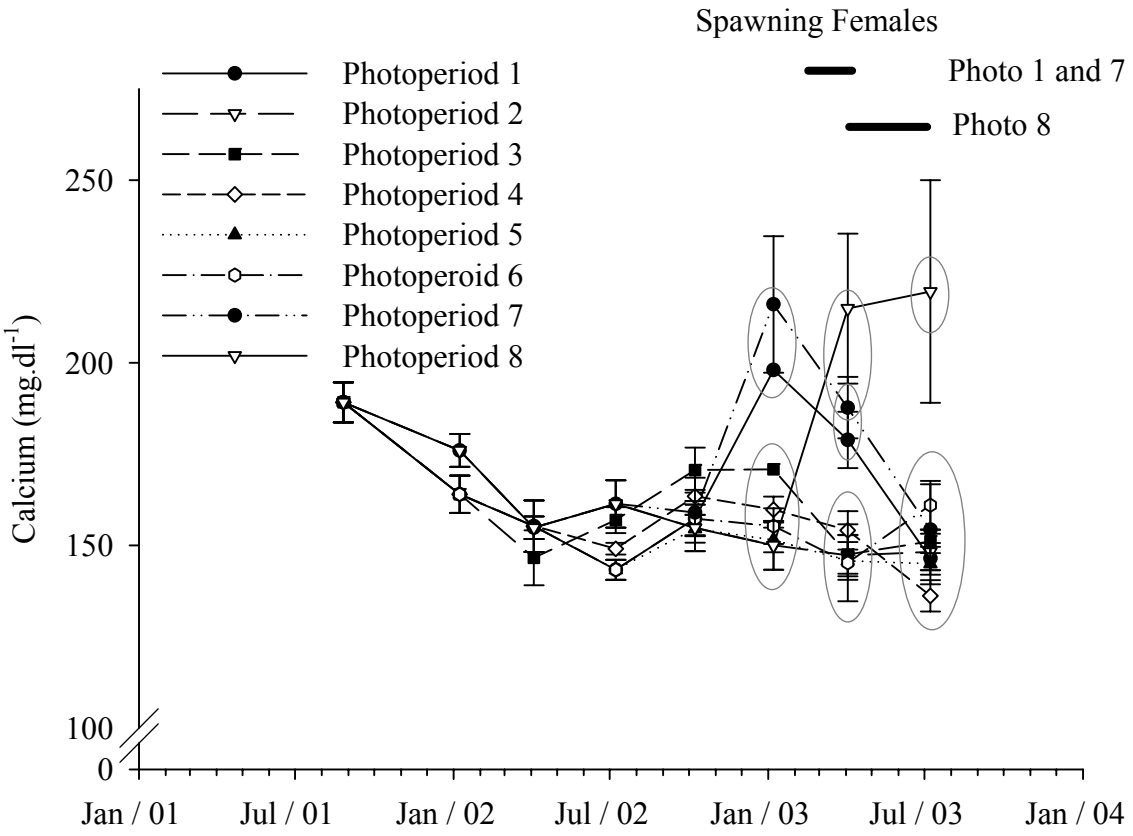


Figure 4.13: Mean plasma calcium concentration ($mg.dl^{-1}$) ± SEM (n = 1-7) for females maintained under photoperiod treatments 1 to 8. Horizontal bars denote the periods when spawning females were present. (Where significant differences existed within a given date, homogeneous subsets are enclosed within an ellipse: $p<0.05$. Where no ellipse is present there were no significant differences within that time point: $p>0.05$, ANOVA GLM with Tukey *post hoc* test).

4.4.2. Growth

4.4.2.1. Weight

There was no significant effect of sex within the treatments, hence the data presented are those of the mixed sex population mean. All populations significantly increased in weight over the length of the trial (Figure 4.14). Photoperiods 2-6 all significantly increased in weight between each sample point throughout the trial length, while photoperiods 1 and 7 significantly increased in weight except between January and April 2003 (21 to 24 MPH) when weight stayed consistent at approximately 1200 g and 1400 g respectively. Equally, photoperiod 8 did not significantly increase in weight after April 2003 (24 MPH).

Impact of transfer to LL on weight

Fish in photoperiods 3 to 5, 7 and 8 were significantly heavier than photoperiod 1 fish (SNP control) when measured 3 months post transfer to LL from SNP (Figure 4.15). Fish in photoperiods 2 and 6 however were not significantly heavier than those in photoperiod 1 until 6 months following transfer to LL. Once heavier than the "SNP" population, photoperiods 3 to 6 and 8 all remained significantly heavier for the remainder of the trial. Photoperiod 7 fish were significantly heavier at all times except on the last sampling in July 2003 (27 MPH) when there was no significant difference compared to the SNP population. Photoperiod 2 fish were not significantly different from the SNP population between July 2002 and January 2003 (15 to 21 MPH) (dates inclusive). However, following this date, the population was again significantly heavier. Between the treatments there was a general trend for those populations on continuous light to have heavier weights compared to those that were not on LL at that time (Figure 4.14).

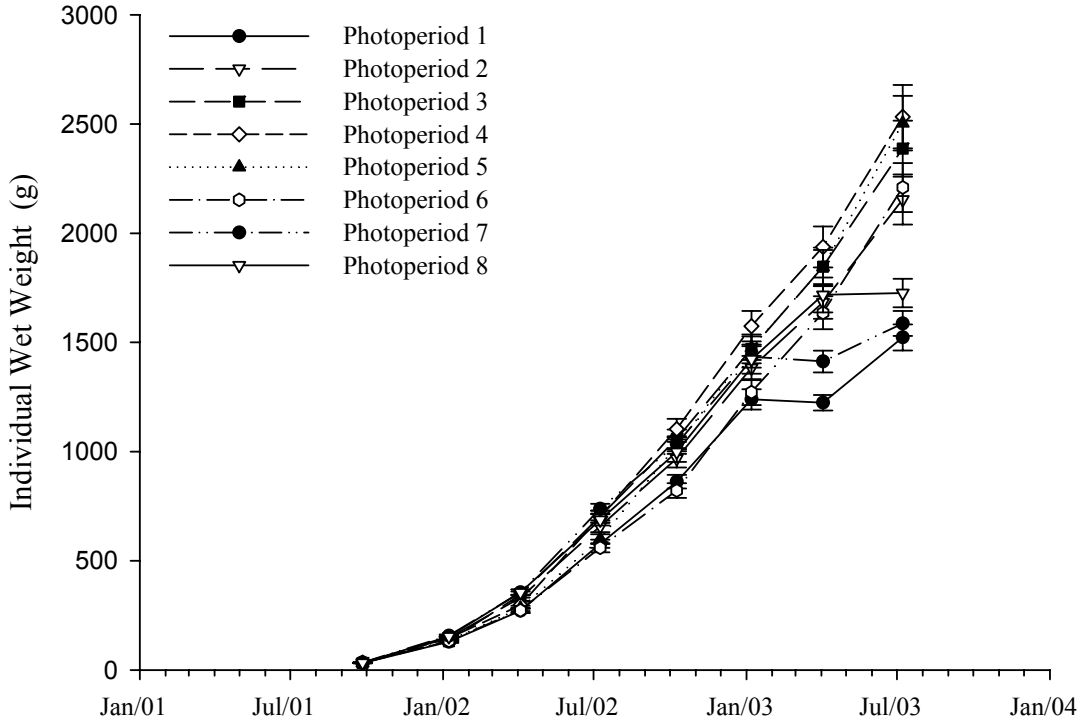
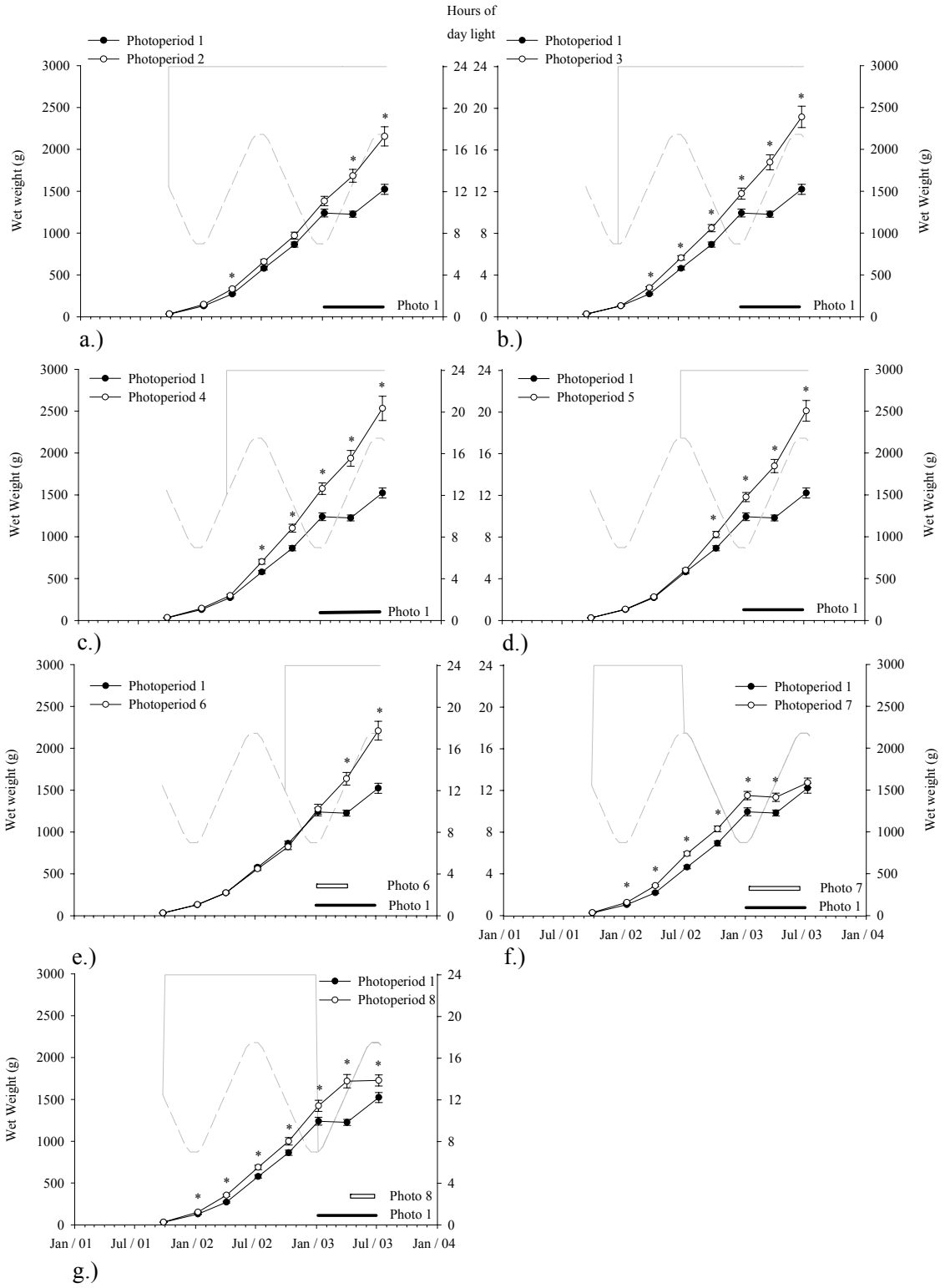


Figure 4.14: Mean wet weight (g) ± SEM of individuals (n = 32 to 43) maintained under photoperiod treatments 1 to 8.

Figure 4.15: (Following page) Mean wet weight (g) ± SEM for individuals (n = 32-43) maintained under Photoperiod 1 vs. **a.)** Photoperiod 2 **b.)** Photoperiod 3 **c.)** Photoperiod 4 **d.)** Photoperiod 5 **e.)** Photoperiod 6 **f.)** Photoperiod 7 **g.)** Photoperiod 8. Photoperiod treatments are shown as grey lines, Photoperiod 1 is broken line, photoperiods 2-8 are solid line. Horizontal bar denotes spawning season for respective photoperiods. * denotes significant differences between treatments ($p<0.05$) ANOVA GLM with Tukey post hoc test.


Hours of day light
Photoperiod 1
Photoperiod 2
Photoperiod 3
Photoperiod 4
Photoperiod 5
Photoperiod 6
Photoperiod 7
Photoperiod 8
Wet weight (g)
Wet Weight (g)
Photo 1
Photo 6
Photo 7
Photo 8
a.)
b.)
c.)
d.)
e.)
f.)
g.)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

Weight at trial end (27 MPH)

At the end of the trial, fish under photoperiods 3, 4, 5 and 6 were the largest (2387±128 g, 2533±145 g, 2504±125 g and 2209±112 g respectively) with no statistical difference between these populations (Figure 4.16). Photoperiod 2 at 2154±115 g was significantly smaller than photoperiods 4 and 5 though not 3 and 6. All of these were heavier than photoperiod 8 at 1726±65 g, which though heavier than the SNP population (1522±60 g) was not different from photoperiod 7 (1587±57 g) which ended the trial at a comparable weight to the SNP controls. Population weight structure was broken into 500 g cohorts to provide a clearer indication of the population spread (Table 4.6). In the populations which matured during the trial (treatments 1, 7 and 8) the greatest proportion of the populations (77 to 87%) were between 1000 and 2000 g with no individuals over 2500 g, while the populations exposed to photoperiods 2 to 6 (i.e. the populations that contained predominately immature individuals) were widely spread from 1000 to 4000 g with all treatments possessing between 11 and 30% of the population with a wet weight greater than 3000 g.

Table 4.6: Population structure at trial end, July 2003 (27 MPH) for individuals maintained under photoperiods 1 to 8, described as % of population divided in 500 g weight bands between <1000 g and 4499 g.

	Photo 1	Photo 2	Photo 3	Photo 4	Photo 5	Photo 6	Photo 7	Photo 8
n	43	37	41	32	34	39	39	35
<1000g	12%					3%	3%	
1000 – 1499g	40%	19%	22%	16%	9%	13%	41%	31%
1500 – 1999g	40%	22%	12%	13%	15%	26%	46%	46%
2000 – 2499g	9%	32%	17%	19%	26%	33%	10%	23%
2500 – 2999g		16%	29%	25%	21%	10%		
3000 – 3499g		3%	10%	13%	24%	10%		
3500 – 3999g		8%	5%	13%	6%	5%		
4000 – 4499g			5%	3%				

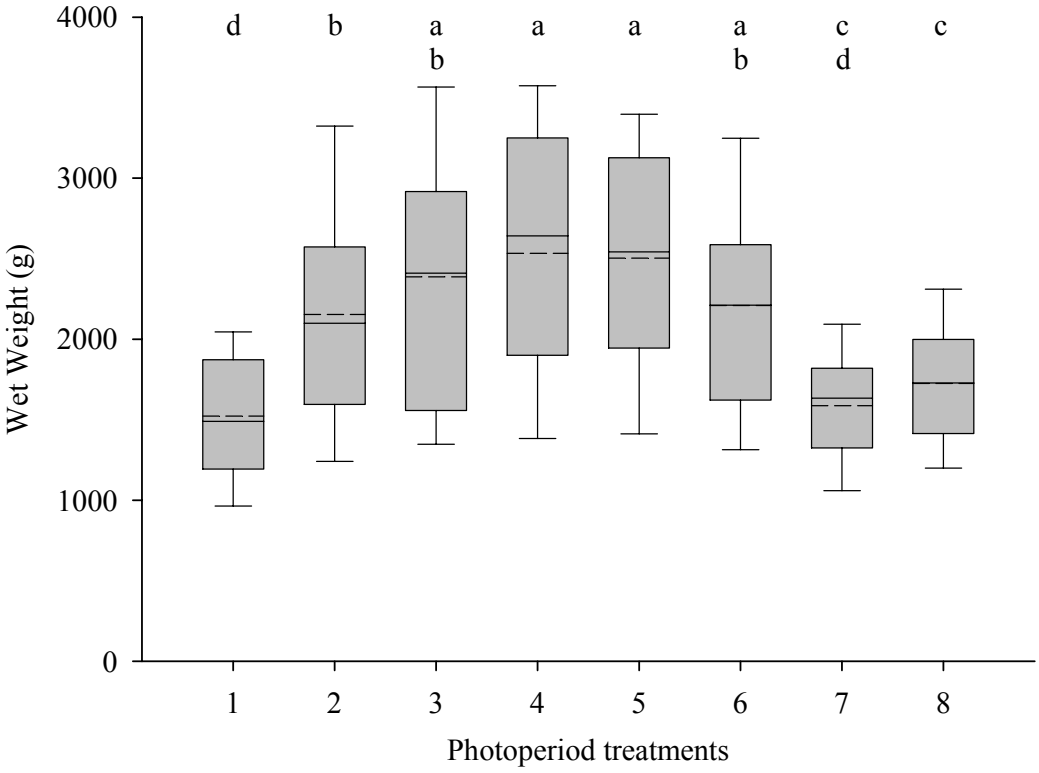
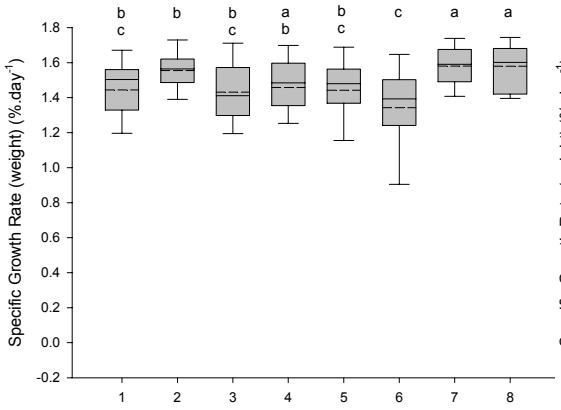


Figure 4.16: Box plot showing population weight (g) distribution in individuals maintained under photoperiod treatments 1 to 8 (n = 32-43) at the trial end in July 2003 (27 MPH). Median and mean of population weights are shown as solid and broken lines respectively where different. Box denotes 25th and 75th percentile and whiskers denotes 5th and 95th percentiles. Different letters denote significant differences between treatments ($p<0.05$: ANOVA GLM with Tukey *post hoc* test).

4.4.2.2. Specific Growth Rate (weight)

Both sexes performed comparably at all times except between October 2002 and January 2003 when a difference was observed within certain treatments. Hence, outwith this time period, data are presented as the mean of the mixed sex population. The weight specific growth rate (SGRw) displayed a clear decline over the trial period in all populations (Figure 4.17, a-g). In the period from January to April 2003 (21 to 24 MPH), SGRw in photoperiods 1 and 7 was close to 0 with at least 50% of the populations recording negative growth rates, while all other treatments displayed significantly higher growth rates of around 0.2-0.3 % per day. In the last 3 months of the trial from April to July 2003 (24 to 27 MPH), photoperiod 1 growth rates recovered to be comparable to treatments 2 to 4 although still significantly lower than treatments 5 and 6. Photoperiod 7 however, was still significantly lower than treatments 1 to 6. The SGRw of photoperiod 8, like 1 and 7 in the previous 3 month period, had decreased to around 0 and was significantly lower than all other treatments. Over the whole trial period, SGRw could be divided into two groups of photoperiod treatment: treatments 2 to 6 were significantly higher than 1,7 and 8 (Figure 4.17h).

Figure 4.17: (Following page) Box plots of specific growth rate of weight (SGRw) for photoperiods 1 to 8, between **a.)** October 2001 and January 2002. **b.)** January and April 2002. **c.)** April and July 2002. **d.)** July and October 2002. **e.)** October 2002 and January 2003. **f.)** January and April 2003. **g.)** April and July 2003. **h.)** Over trial length October 2001-July 2003. Box represents 25^{th} and 75^{th} percentile and whiskers denote 5^{th} and 95^{th} percentile range. Median and mean are shown as solid and dashed line respectively where different. (n = 32-43) except e.) where (n = 13-26). Different letters denote significant differences between treatments ($p<0.05$: ANOVA GLM with Tukey *post hoc* test).



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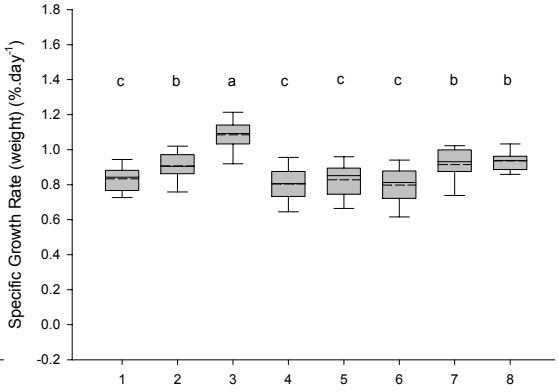
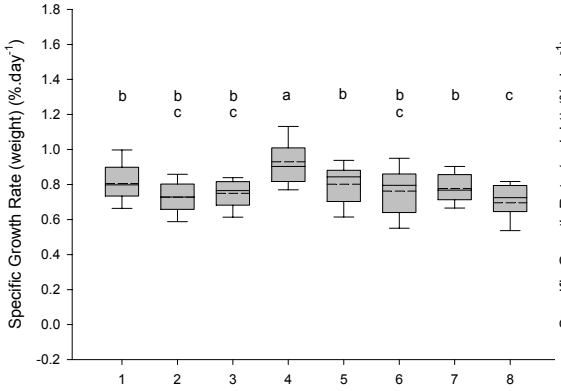
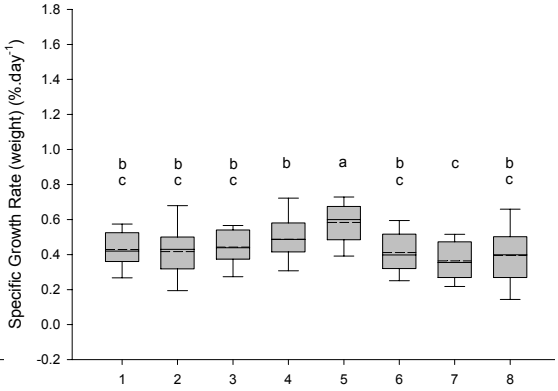


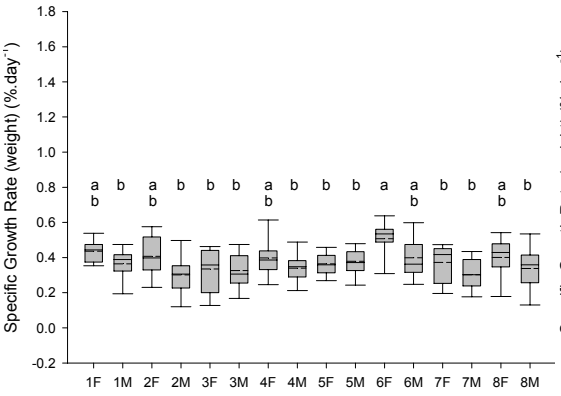
b.) Jan – April 02



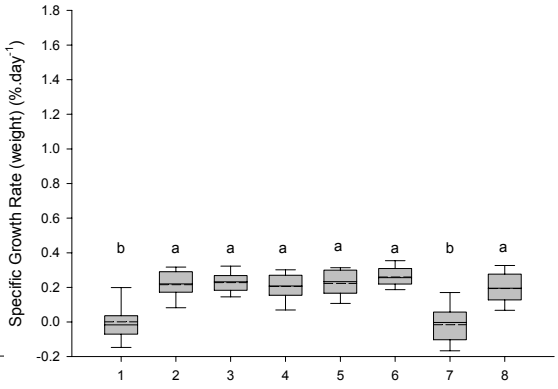
c.)April – July 02



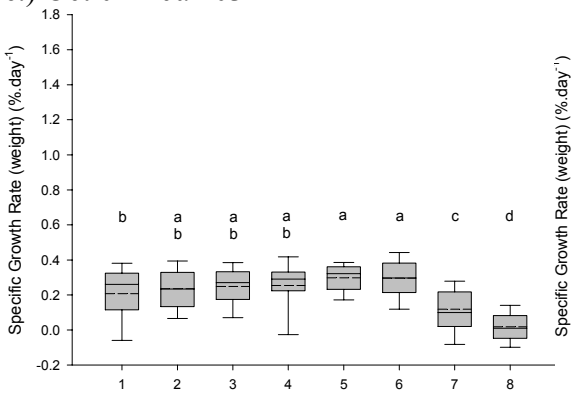
d.) July – Oct 02



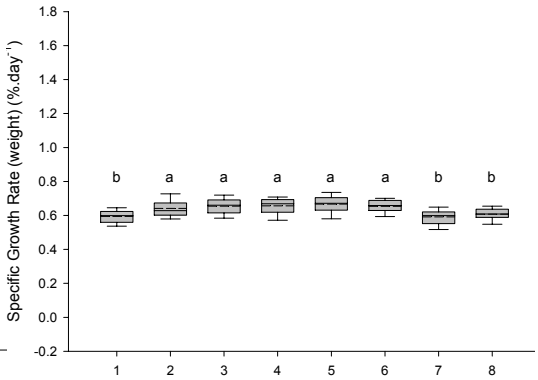
e.) Oct 02 – Jan 03



f.) Jan – April 03



g.) April – July 03



h.) Trial mean (Oct 01 – July 03)

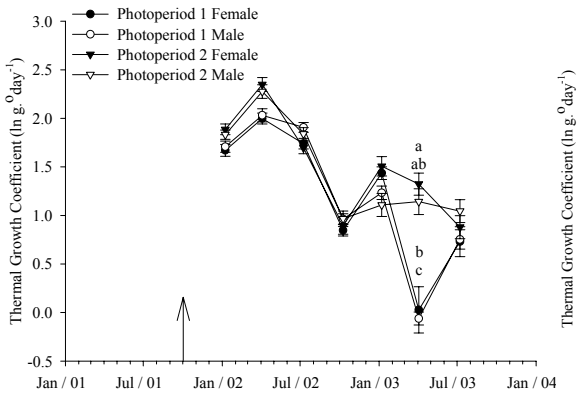
Impact of LL transfer on SGRw

It is evident that up to July 2002 (15 MPH) transferring populations from the ambient photoperiod to continuous light caused a transient elevation in SGRw with respect to the ambient photoperiod populations during the subsequent growth period. This was a significant elevation for all transfers with the exception of photoperiod 2 between October 2001 and January 2002 (6 and 9 MPH). After this initial "growth boost" in the subsequent 3 month window, SGRw returned to a rate comparable with the SNP treatment. When photoperiod 6 was transferred to LL in October 2002 (18 MPH) the SGRw in the following 3 months in the males was unaffected compared to all other treatments. However, the females displayed a significantly higher SGRw than the males in all other treatments as well as females in treatments 3, 5 and 7.

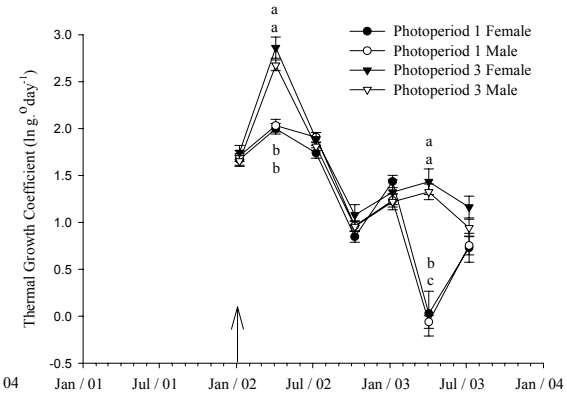
4.4.2.3. Thermal Growth Coefficient (TGCw)

Thermal growth coefficient was calculated in order to compare growth rates over the trial period. The limitations of SGR calculations do not allow such a comparison. Sex had a significant overall interaction within the treatments. Hence, the data presented are those of individual sex means within populations. There was a general trend for TGCw to decline over the trial period (Figure 4.18) with all treatments showing a significant fall in TGCw from July 2002 to October 2002 (15 to 18 MPH)

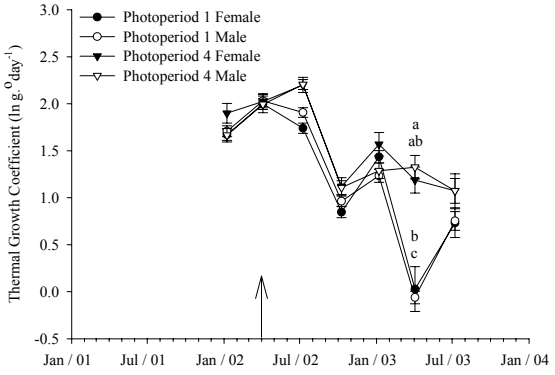
Figure 4.18: (Following page) Mean thermal growth coefficient ± SEM for males (n = 13-26) and females (n = 13-23) maintained under Photoperiod 1 vs. **a.)** Photoperiod 2 **b.)** Photoperiod 3 **c.)** Photoperiod 4 **d.)** Photoperiod 5 **e.)** Photoperiod 6 **f.)** Photoperiod 7 **g.)** Photoperiod 8. Arrows on Y axis denote transfer to or from LL photoperiod in experimental regime (i.e. photoperiods 2 to 8). Different letters denote significant differences between treatments ($p<0.05$: ANOVA GLM with Tukey *post hoc* test).


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 2 Female
Photoperiod 2 Male
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

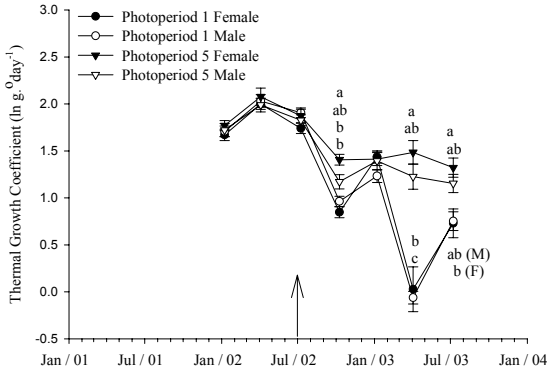
a.)


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 3 Female
Photoperiod 3 Male
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

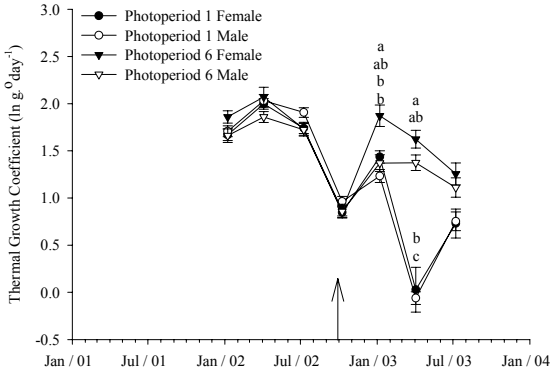
b.)


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 4 Female
Photoperiod 4 Male
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

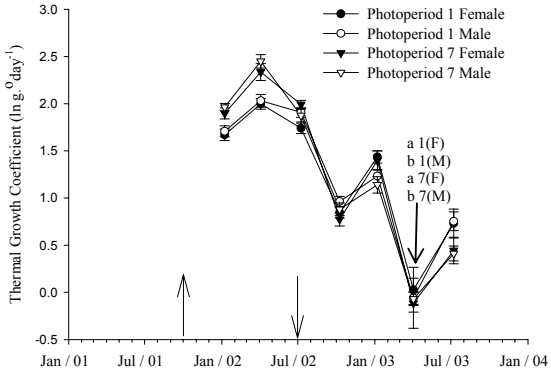
c.)


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 5 Female
Photoperiod 5 Male
ab (M)
b (F)
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

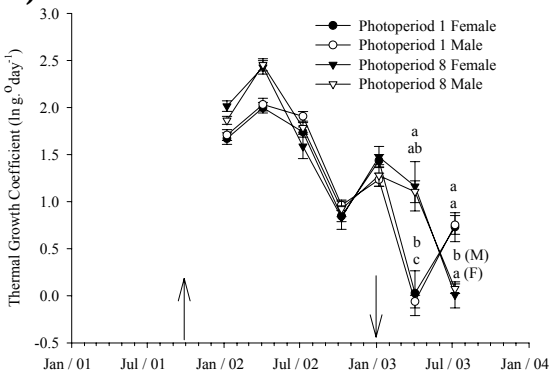
d.)


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 6 Female
Photoperiod 6 Male
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

e.)


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 7 Female
Photoperiod 7 Male
a 1(F)
b 1(M)
a 7(F)
b 7(M)
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

f.)


Photoperiod 1 Female
Photoperiod 1 Male
Photoperiod 8 Female
Photoperiod 8 Male
b (M)
a (F)
Thermal Growth Coefficient (ln g. °day-1)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

g.)

with subsequent recovery to comparable levels prior to this time over the following three months (October 2002 to January 2003, 18 to 21 MPH). Following this, groups 1 and 7 exhibited a further rapid fall in TGCw between January and April 2003 (21 and 24 MPH) with levels falling to close to 0 with, in both cases, males being significantly lower than females. This difference appears to be due to the greater variation in TGCw in the females with some exhibiting growth rates comparable until January 2003 (21 MPH) while others demonstrated dramatic weight loss. By the trial end (27 MPH) TGCw in treatments 1 and 7 had returned to rates comparable to all other treatments except group 8 which in the same period had exhibited a significant decline in TGCw to close to 0, as groups 1 and 7 had done 3 months earlier. However, at this time females had a significantly lower TGCw than the males.

Impact of LL transfer on TGCw

It is apparent that as with SGRw the transfer of groups from SNP to LL caused a transient elevation in TGCw. This rise was not always statistically significant and with the exception of photoperiods 2, 7 and 8 this elevation returned to comparable levels with the ambient treatment during the following growth period. The females in both treatments 5 and 6 showed a greater increase in TGCw following transfer to LL from SNP than males under the same treatment. This difference was not apparent in earlier transfers.

4.4.2.4. Length

There was no significant effect of sex within the treatments hence the data presented are those of the mixed sex population mean. All populations significantly increased in length over the period of the trial (Figures 4.19, 4.20). The results reported

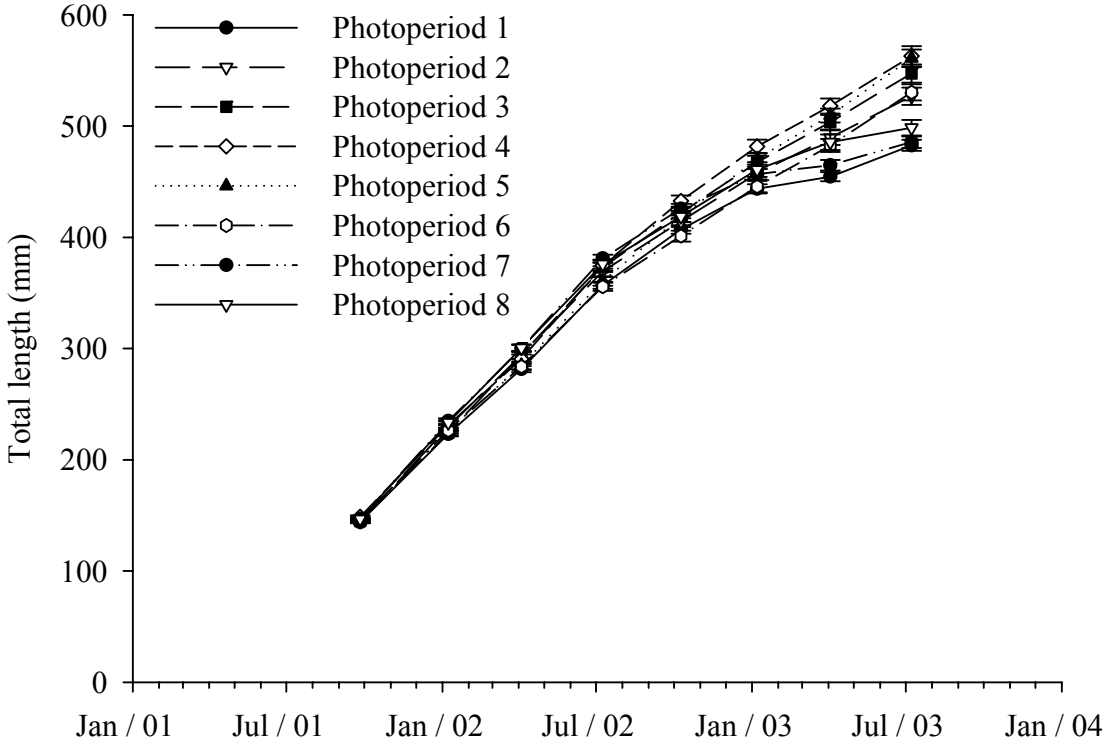
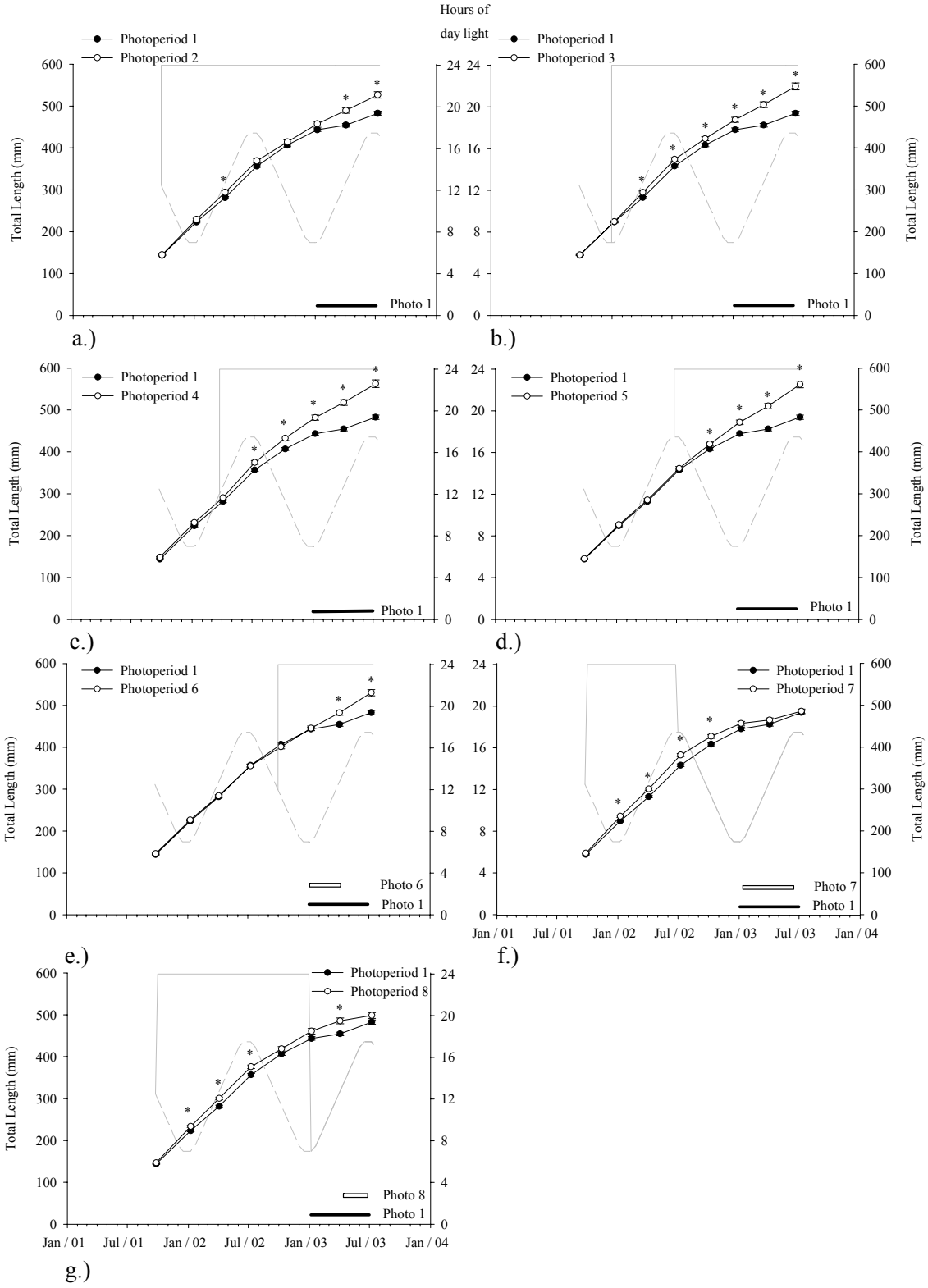


Figure 4.19: Mean total length (mm) ± SEM of individuals (n = 32 to 43) maintained under photoperiod treatments 1-8.

Figure 4.20: (Following page) Mean total length of individuals (mm) ± SEM (n = 32-43) maintained under Photoperiod 1 vs. **a.)** Photoperiod 2 **b.)** Photoperiod 3 **c.)** Photoperiod 4 **d.)** Photoperiod 5 **e.)** Photoperiod 6 **f.)** Photoperiod 7 **g.)** Photoperiod 8. Photoperiod treatments shown as grey lines, Photoperiod 1 is broken line, photoperiods 2-8 are solid line. Horizontal bar denotes spawning season for respective photoperiods. * denotes significant differences between treatments ($p<0.05$: ANOVA GLM with Tukey *post hoc* test).


Hours of day light
Photoperiod 1
Photoperiod 2
Photoperiod 3
Photoperiod 4
Photoperiod 5
Photoperiod 6
Photoperiod 7
Photoperiod 8
Total Length (mm)
Photo 1
Photo 6
Photo 7
Photo 8
a.)
b.)
c.)
d.)
e.)
f.)
g.)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

for weight were generally repeated in the length growth response. Photoperiod 1 and 7 significantly increased in length between each sample except between January 2003 and April 2003 (21 and 24 MPH), equally photoperiod 8 did not significantly increase in length after April 2003 (24 MPH). Photoperiods 2-6 all significantly increased in length between each sample point throughout the trial.

Impact of LL transfer on length

Photoperiods 3 to 5, 7 and 8 were significantly longer than the SNP controls (photoperiod 1) following transfer from SNP to LL when next measured, 3 months post-transfer (Figure 4.20). Photoperiods 2 and 6 however were not significantly longer than photoperiod 1 until 6 months following transfer to LL. Once longer than photoperiod 1, photoperiods 3 to 6 all remained significantly larger for the remainder of the trial. Photoperiod 7 was significantly longer until October 2002 (18 MPH) where after it fell to a comparable size as photoperiod 1. With regard to photoperiod 2, throughout the trial duration, it was significantly longer only in April 02 (12 MPH) and April to July 03 (24 to 27 MPH). After July 2002 (15 MPH) photoperiod 8 was only significantly longer than the SNP population in April 2003 (24 MPH). At all other times it was comparable with photoperiod 1.

Length at trial end (27MPH)

At the trial end, the fish in photoperiods 3 to 5 were the largest (547±8 mm, 563±9 mm and 561±8 mm respectively) with no statistical difference between these populations (Figure 4.21). Photoperiod 6 at 530±7 mm was significantly smaller than photoperiods 4 and 5 though not photoperiod 3 or 2 which at 527±8 mm was significantly smaller than 3 to 5. All of these were longer than photoperiod 1, 7 and 8 at

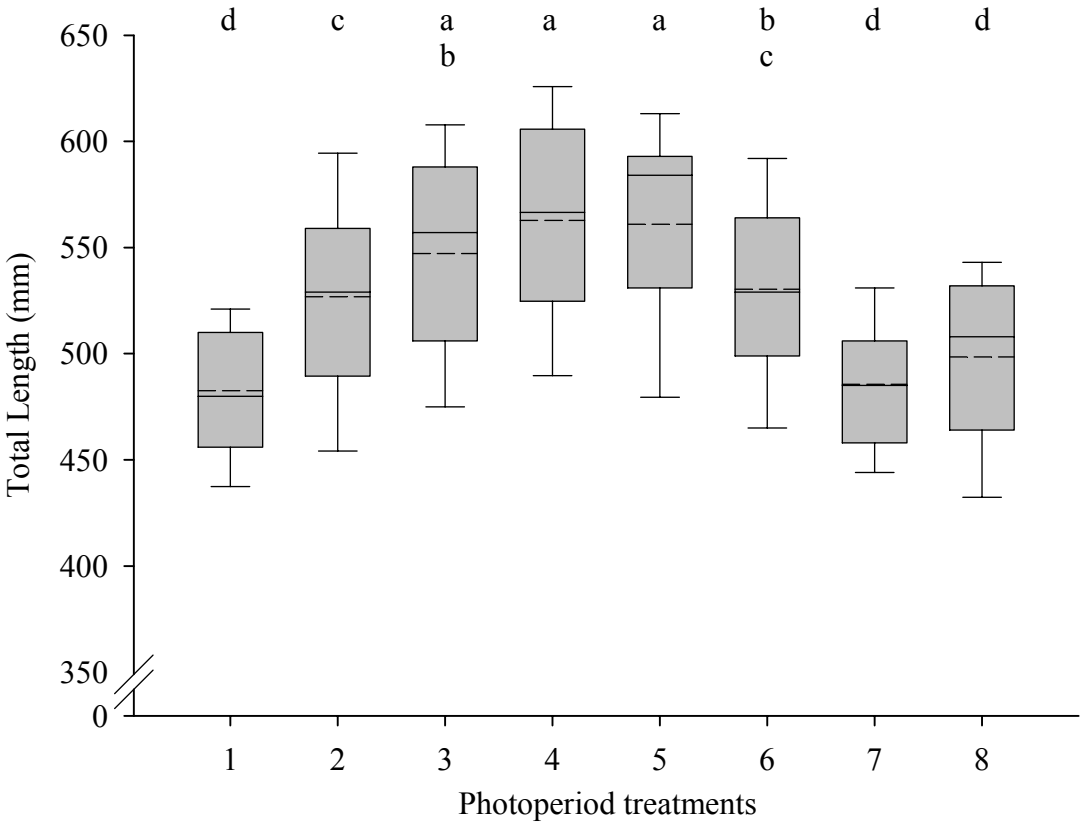


Figure 4.21: Box plot showing population length (mm) distribution at the trial end in July 2003 (27 MPH) for individuals maintained under photoperiods 1 to 8. Box denotes 25th and 75th percentile and whiskers denotes 5th and 95th percentiles, median and mean are shown as solid and broken line respectively where different (n = 32-43). Different letters denote significant differences between treatments ($p<0.05$: ANOVA GLM with Tukey *post hoc* test).

483±5 mm, 486±5 mm and 498±7 mm respectively.

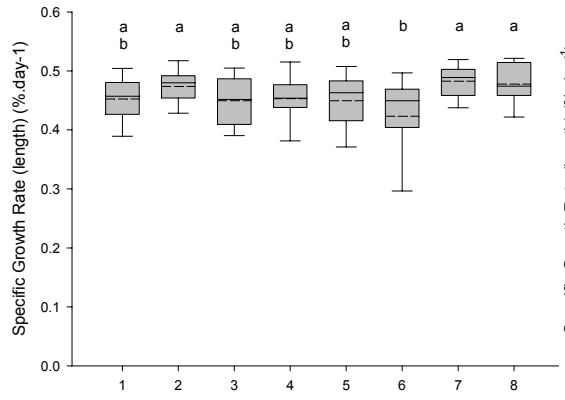
4.4.2.5. Specific Growth Rate (length)

There was no significant effect of sex within treatments hence, the data presented are those of the mixed sex population. Over the trial period the length specific growth rate (SGRl) displayed a clear decline in all populations (Figure 4.22, a-g). In the January to April 2003 growth period both photoperiods 1 and 7 exhibited a significant decline in SGRl compared to treatments 2-6 to reach growth rates close to 0. Photoperiod 8 exhibited a similar decline in growth rate, though three months delayed, to reach a low between April and July 2003. SGRl over the whole trial period could be divided into two groups of photoperiod treatment, treatments 2-6 which were significantly higher than 1,7 and 8 (Figure 4.22h).

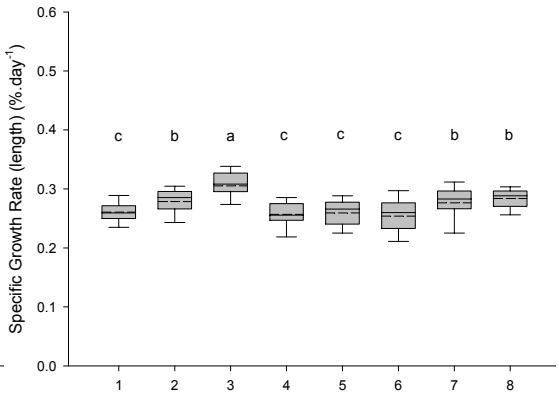
Impact of LL transfer on SGRl

Changes in SGRl were not as distinct as was the case with SGRw. It could be suggested that transfer from SNP to LL enhanced SGRl though this was not always clear and predominantly not statistically significant (Figure 4.22, a-e).

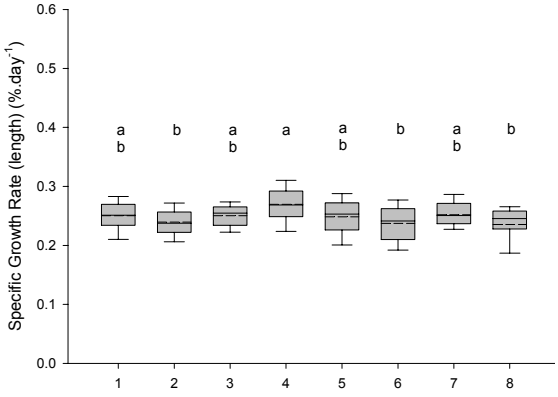
Figure 4.22: (Following page) Box plots of specific growth rate of length (SGRl) for individuals maintained under photoperiods 1-8 between a.) October 2001 and January 2002. b.) January and April 2002. c.) April and July 2002. d.) July and October 2002. e.) October 2002 and January 2003. f.) January and April 2003. g.) April and July 2003. h.) Over trial length October 2001-July 2003. Box represents 25^{th} and 75^{th} percentile and whiskers denote 5^{th} and 95^{th} percentile range, median and mean are shown as solid and dashed lines respectively where different (n = 32-43). Different letters denote significant differences between treatments ($p<0.05$: ANOVA GLM with Tukey *post hoc* test).



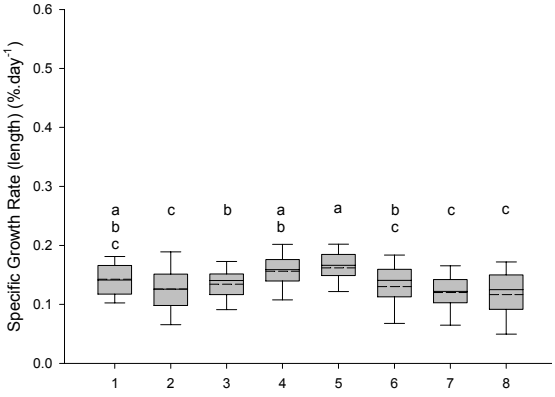
a.) Oct 01 – Jan 02



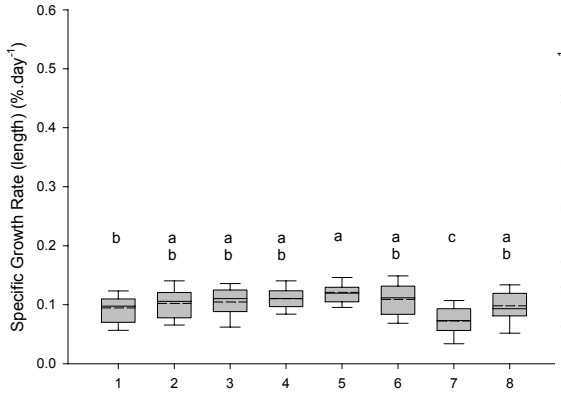
b.) Jan – April 02



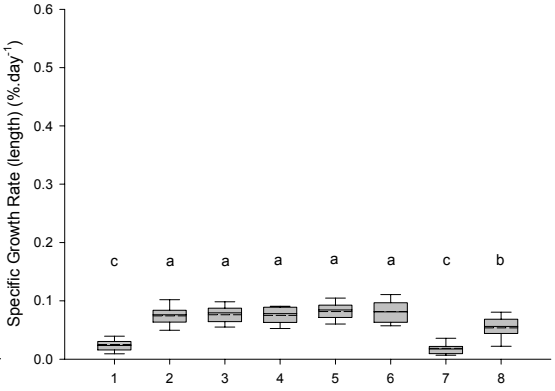
c.) April – July 02



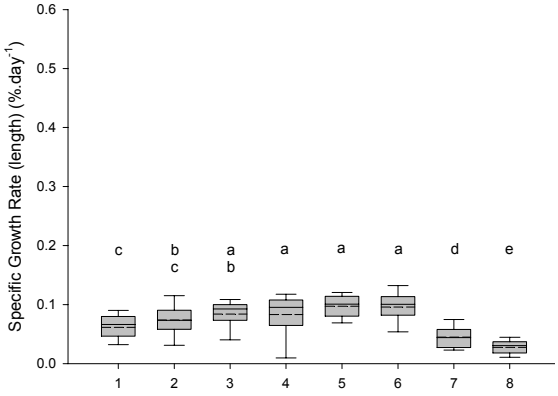
d.) July – Oct 02



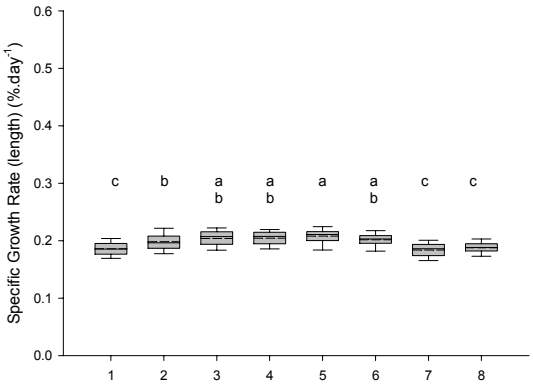
e.) Oct 02 – Jan 03



f.) Jan – April 03



g.) April – July 03



h.) Trial Mean (October 2001 – July 2003)

4.4.2.6. Condition Factor

A scatter plot of all individual weight vs. lengths for the total trial (a total of 2401 observations in the range of 16.5-4239 g, 115-642 mm) had a regression coefficient of 3.2 with an adjusted r-squared of 0.99 ($p<0.01$). Therefore, Foulton's condition factor was deemed an appropriate condition index to use.

There was no significant effect of sex within treatments, hence the data presented are those of the mixed sex population mean (Figure 4.23, 4.24). Photoperiod 1 steadily increased in condition over the first 9 months of the trial from October 2001 to July 2002 (6 to 15 MPH), following which condition remained comparable except for a transient peak in January 2003 (21 MPH). In comparison photoperiods 3 to 6 all showed a significant rise in condition following transfer to LL (Figure 4.24, b-e); this level was then maintained for the remainder of the trial. Photoperiod 2 (Figure 4.24a), like groups 3-6, showed an increase in condition following transfer to LL. However, it was not as distinct as the previous groups and continued to rise as the trial progressed. Group 7's change in condition throughout the trial mirrored that of group 1 including the transitory peak in January 2003 (21 MPH). However, at all times it was significantly higher except on the first and last samples. Photoperiod 8 displayed a general trend for increased condition from the trial start to April 2003 (6 to 21 MPH). However, in July 2003 (21 MPH), photoperiod 8 showed a significant decline in condition to end the trial comparable with photoperiod 1.

4.4.2.7. Sexual Dimorphism

Although size (weight or length) was not shown to be statistically different between sexes, the data were examined for evidence of sexual dimorphism (Figure 4.25). In treatments 2 to 6 there was an apparent trend that females exhibit greater

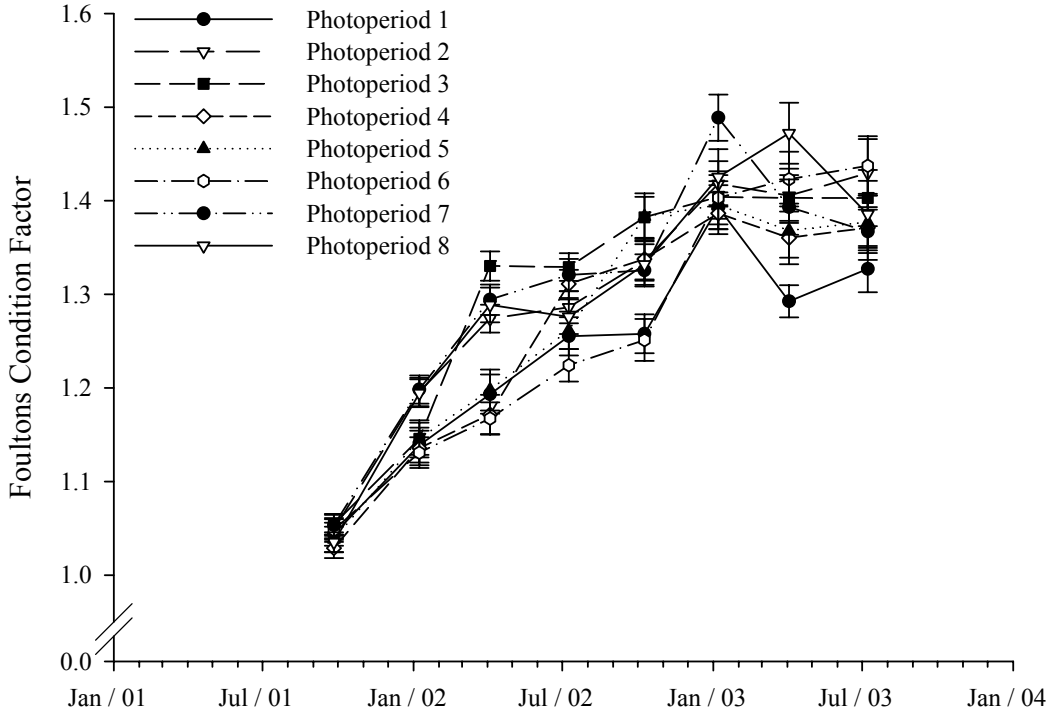
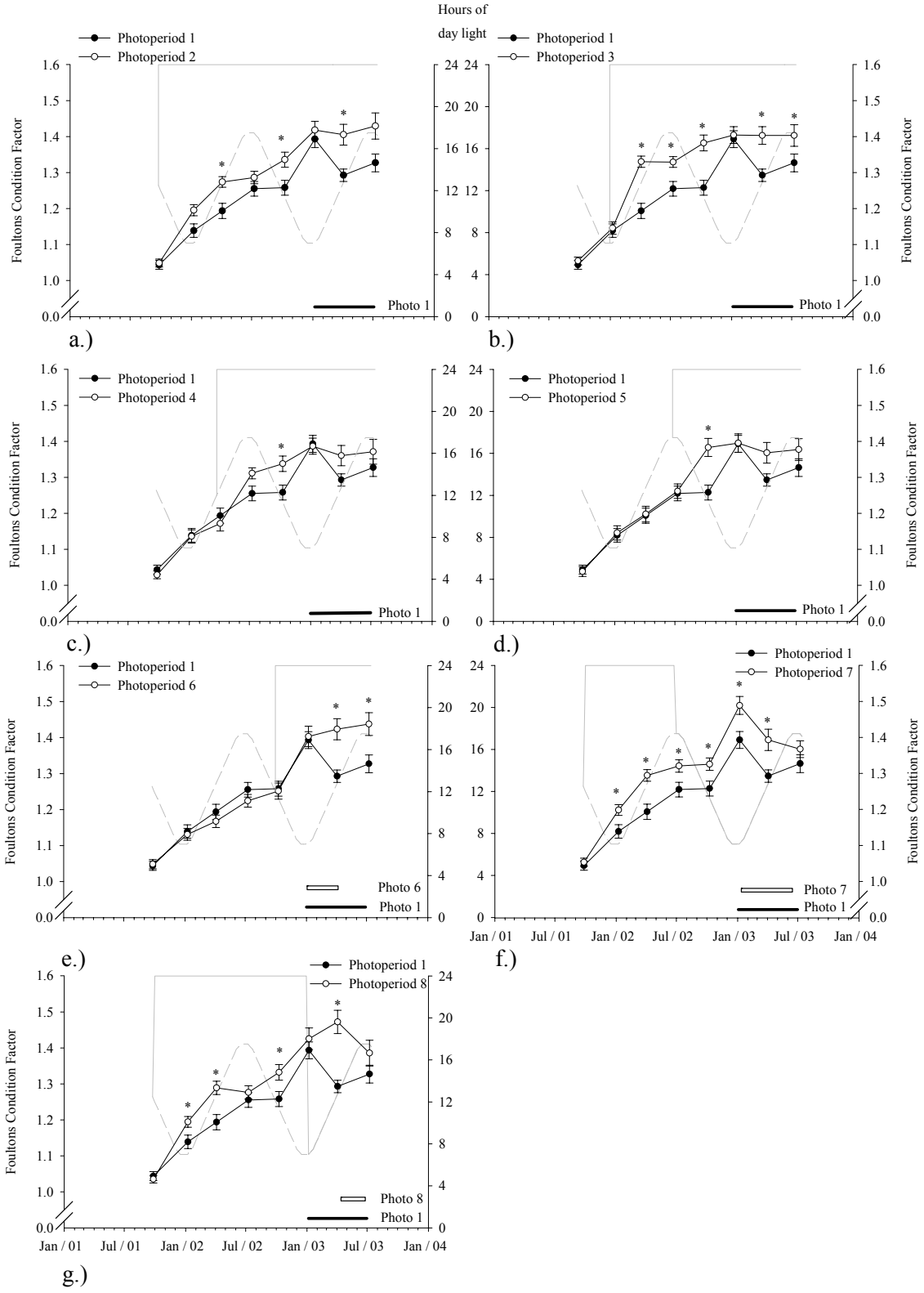


Figure 4.23: Mean population Foulton's condition factor ± SEM (n = 32-43) for individuals maintained under photoperiods 1 to 8.

Figure 4.24: (Following page) Mean Foulton's condition factor ± SEM of individuals (n = 32-43) maintained under Photoperiod 1 vs. **a.)** Photoperiod 2 **b.)** Photoperiod 3 **c.)** Photoperiod 4 **d.)** Photoperiod 5 **e.)** Photoperiod 6 **f.)** Photoperiod 7 **g.)** Photoperiod 8. Photoperiod treatments are shown as grey lines. Photoperiod 1 is a broken line and photoperiods 2-8 are solid lines. Horizontal bar denotes spawning season for respective photoperiods. * denotes significant differences between treatments ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.


Hours of day light
Photoperiod 1
Photoperiod 2
Photoperiod 3
Photoperiod 4
Photoperiod 5
Photoperiod 6
Photoperiod 7
Photoperiod 8
Foultons Condition Factor
Photo 1
Photo 6
Photo 7
Photo 8
a.)
b.)
c.)
d.)
e.)
f.)
g.)
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04

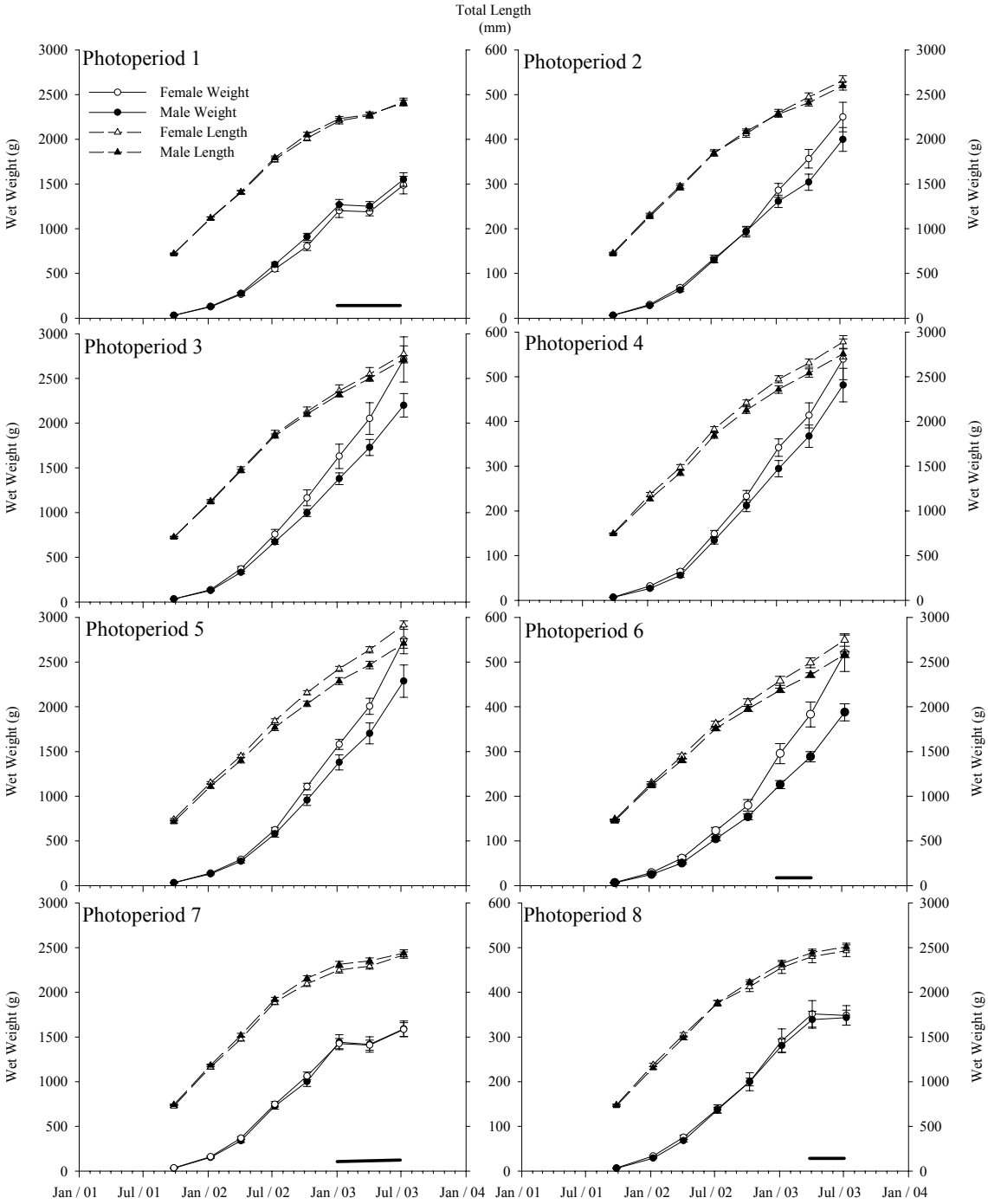


Figure 4.25: Mean weight (g) and total length (mm) (± SEM) for males and females maintained under Photoperiods; 1 (n = 19 M : 23 F); 2 (n = 23 M : 14 F); 3 (n = 15 M : 26 F); 4 (n = 14 M : 18 F); 5 (n = 16 M : 18 F); 6 (n = 16 M : 23 F); 7 (n = 18 M : 21 F) and 8 (n = 13 M : 22 F). Horizontal black bar denotes period when mature individuals were present in treatments 1, 6, 7 and 8. No statistical differences exist within treatments.

growth under conditions of continuous light than males. Such dimorphism was not evident in treatments 1, 7 and 8. When the trial ended, males in treatments 2 to 6 were 11 to 25% smaller than their female counterparts while males in treatments 1, 7 and 8 are comparable to the females (± 4% of female size).

4.4.2.8 Growth and Maturation

While the statistical modelling demonstrated the clear performance differences between "mature" (Photoperiods 1,7 and 8) and "immature" (Photoperiods 2-6) populations, it was unable to isolate within-population performance differences relating to the precocious males detected in 2002 and the low incidence of maturation in 2003 in photoperiod 6. The characterisation of the growth performance of these individuals in comparison to their siblings was felt to be important hence, these data were further examined in isolation.

Maturation in 2002

Photoperiod 3: The mature male identified in April 2002 (12 MPH) had a wet weight of 200 g, total length of 253 mm (Figure 4.26) with a condition factor of 1.24. This individual was at all times in the bottom 10% of the population and its growth rates were in the lower 30%.

Photoperiod 4: The mature male identified in June 2002 (14 MPH) had a wet weight 433 g (Figure 4.27). When next inspected with the entire population in July 2002 (15 MPH) the individual in question weighed 559 g, had a total length of 351 mm and a condition factor of 1.29. Prior to April 02 (12 MPH) this individual was part of the upper 50% of the population. However, after April 02, prior to releasing milt the

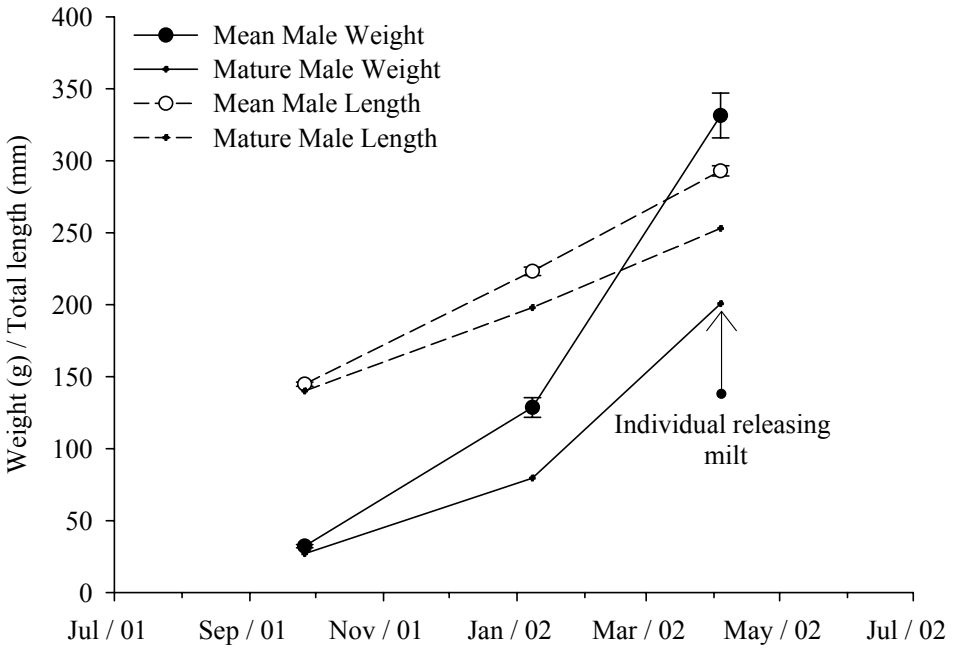


Figure 4.26: Mean weight (g) and total length (mm) ± SEM (n=26) from October 2001 to April 2002 for males maintained under photoperiod 3 with mature male growth performance plotted as single line.

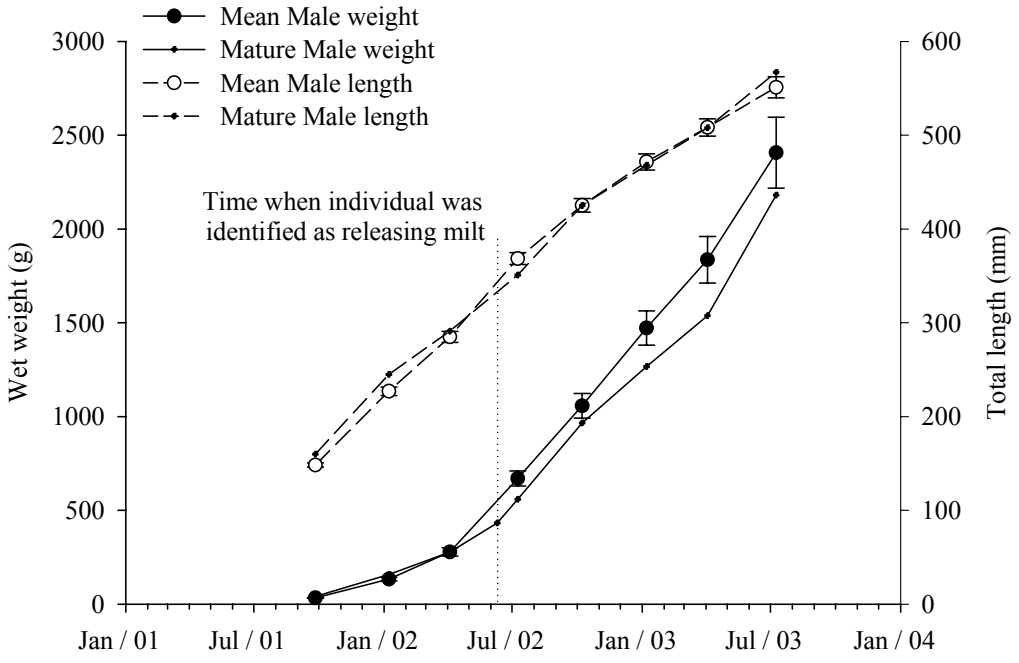


Figure 4.27: Mean weight (g) and total length (mm) ± SEM (n=18) from October 2001 to July 2003 for males maintained under photoperiod group 4 with mature male growth performance plotted as single line.

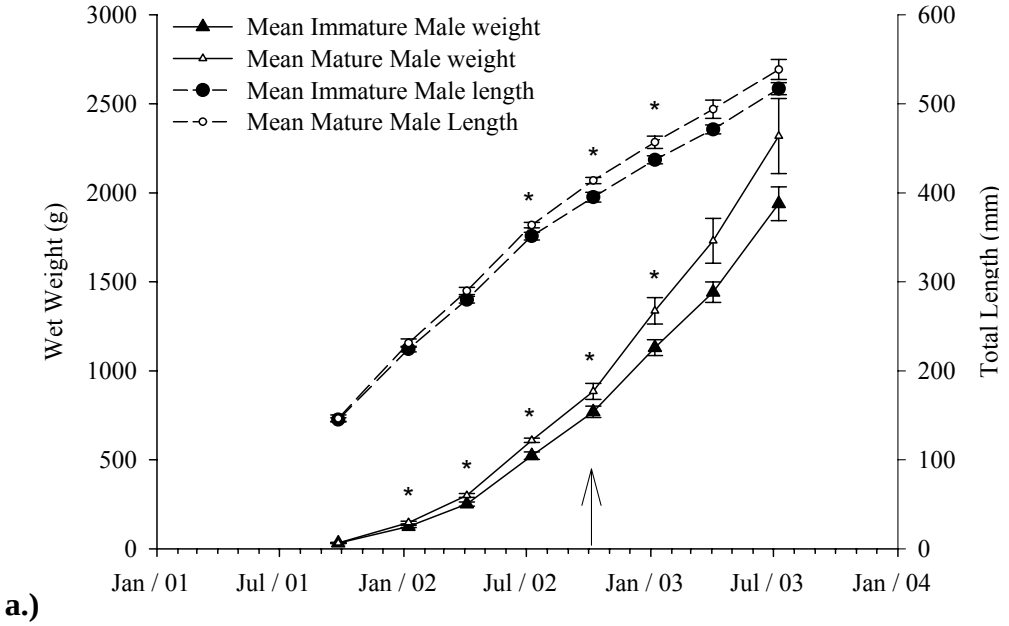
individual displayed a much reduced growth rate. In the period April to July 2002 this individual had the lowest growth rate (SGRw, SGRl and TGC) of the population. This individual was part of the core group of fish maintained to the trial end so its long term growth performance could be compared to its "non-precocious" maturing cohorts. At the trials end in July 2003 (27 MPH) the individual at 2181 g was 9% smaller than the population mean. This was the result of a 6% reduction in SGRw over the trial length.

Maturation in 2003

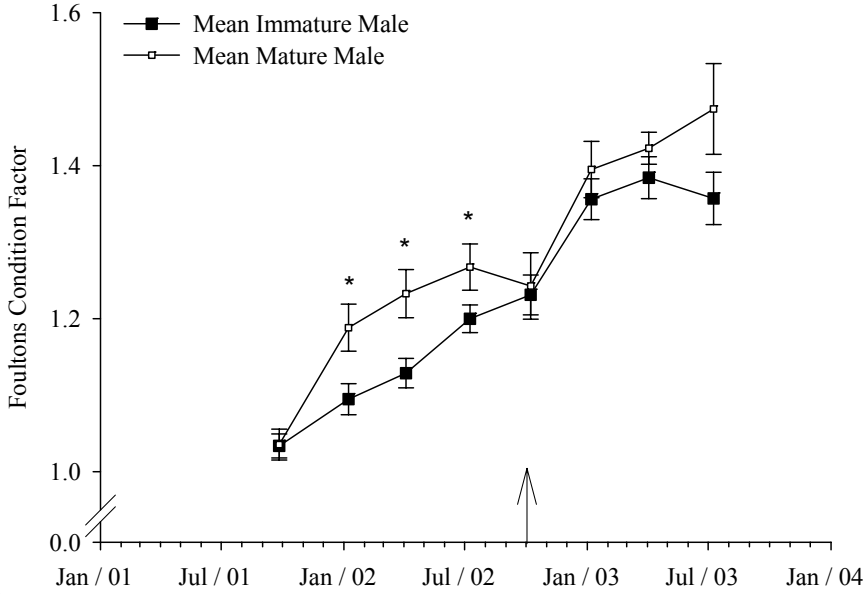
Photoperiod 6: Only five mature males (13%) were detected within this population. In comparison to the immature fish, maturing individuals were heavier between January 2002 and January 2003 (9 to 21 MPH) and longer from June 2002 to January 2003 (15 to 21 MPH). Thereafter, they were comparable to the non-maturing males (Figure 4.28a). These growth paterns culminated in a higher condition factor from January 2002 to July 2002 (9 to 15 MPH) (Figure 4.28b).

4.4.2.9. Insulin like Growth Factor-I (IGF-I)

There was no significant effect of sex within treatments hence, the data presented are those of the mixed sex population. The total plasma IGF-I was in the range of 5 to 32 $ng.ml^{-1}$ (Figure 4.29). There were only significant differences between photoperiod treatments in the months of April and July 2003 (24 and 27 MPH) (Figures 4.29 and 4.30). Prior to this date however, there was a suggestion that in photoperiod treatments 2, 3, 4, 7 and 8, IGF-I levels were elevated, though not significantly, compared to the simulated natural photoperiod following transfer to LL in 2002.



a.)



b.)

Figure 4.28: Mean a.) weight (g), total length (mm) and b.) condition factor all (± SEM) for mature (open triangle, circle and square respectively, n = 5) and immature (closed triangle, circle and square respectively, n = 18) males maintained under photoperiod 6 (arrow denotes transfer from SNP to LL). (* denotes significant difference: $p<0.05$, ANOVA GLM of log transformed data with Tukey *post hoc* test)

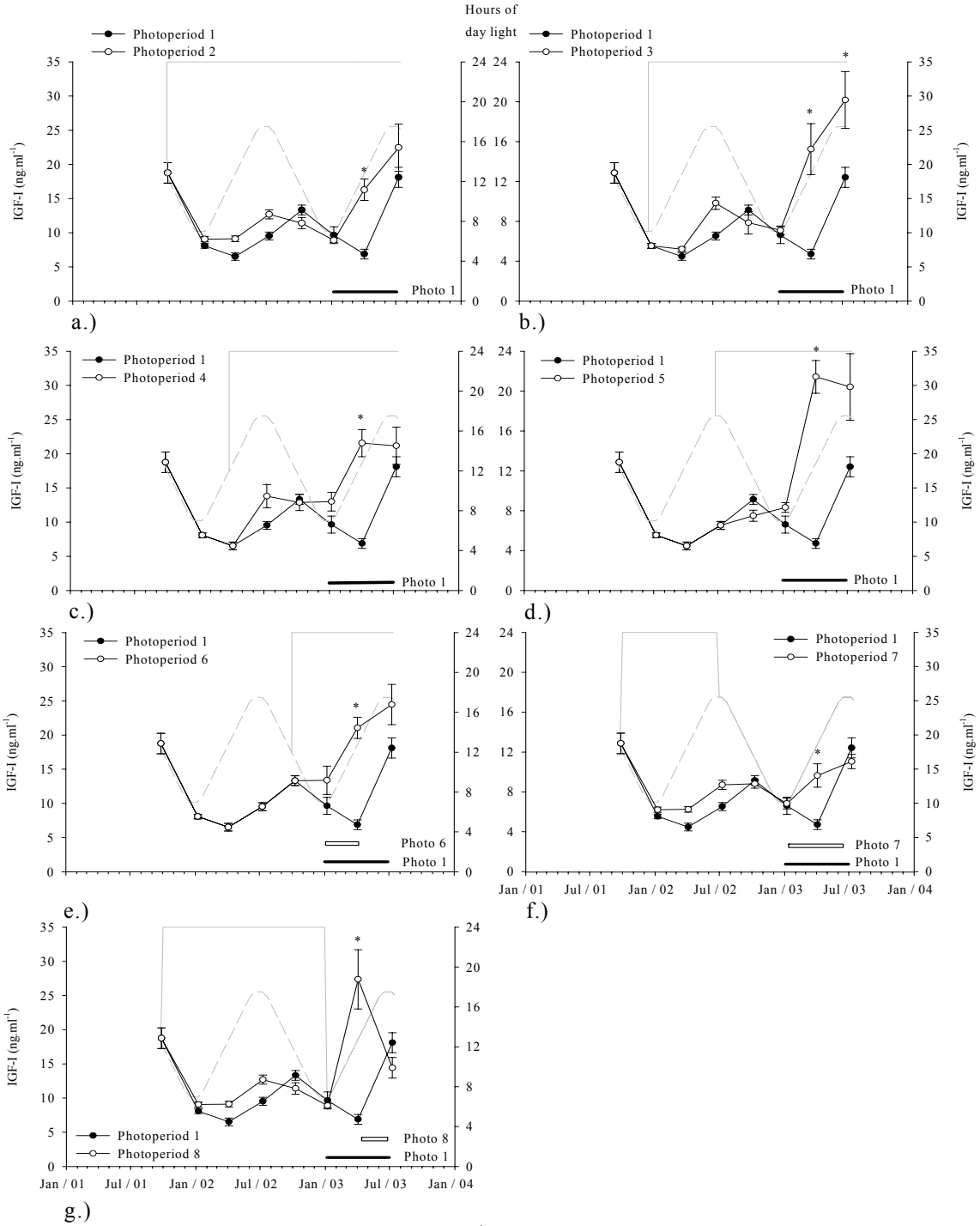
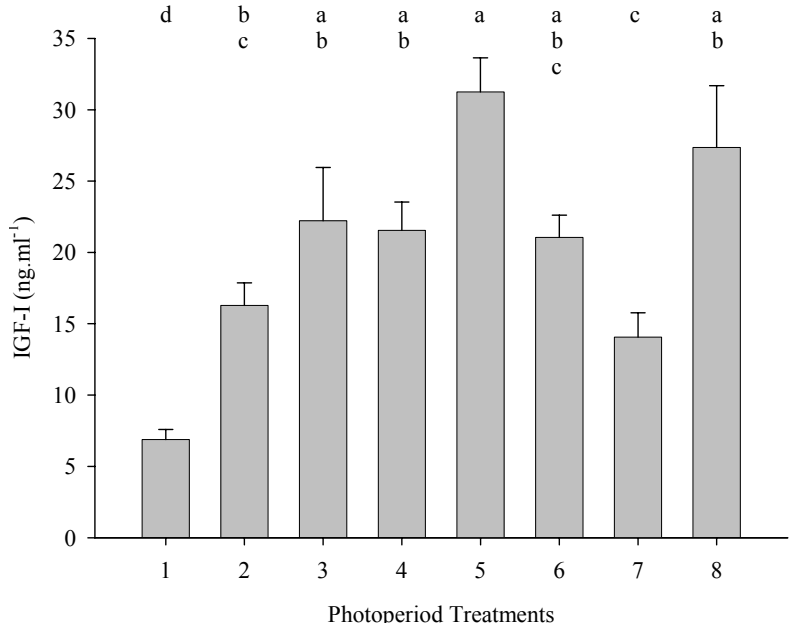
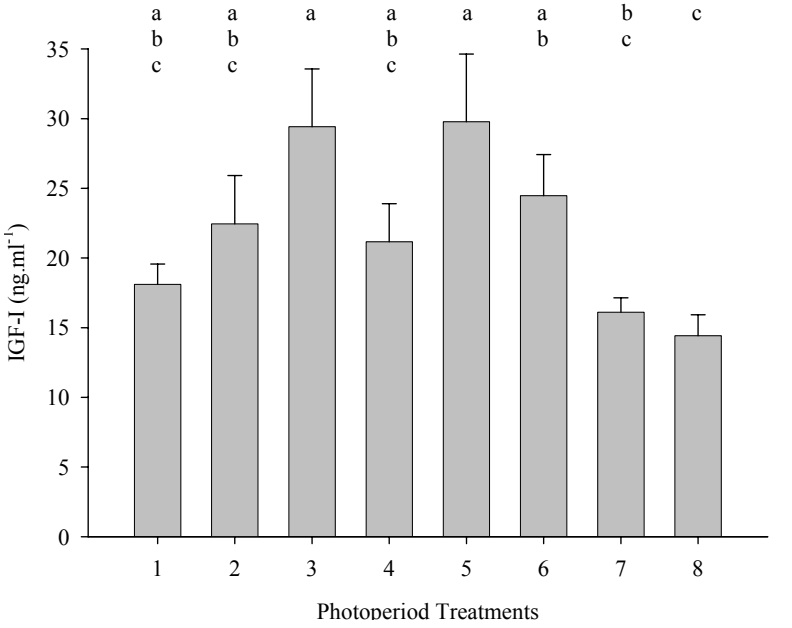


Figure 4.29: Mean plasma IGF-I ($ng.ml^{-1}$) ± SEM for individuals maintained under Photoperiod 1 vs **a.)** Photoperiod 2, **b.)** Photoperiod 3, **c.)** Photoperiod 4, **d.)** Photoperiod 5, **e.)** Photoperiod 6, **f.)** Photoperiod 7, **g.)** Photoperiod 8, (n = 4 to 10). Photoperiod treatments shown as grey lines. Photoperiod 1 is broken line and photoperiods 2-8 are solid line. * denotes significant differences between treatments ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.



a.)



b.)

Figure 4.30: Mean plasma IGF-I (ng.ml^{-1}) ± SEM for individuals maintained under photoperiods 1 to 8 at a.) 24 MPH (April 2003) (n=9-10) and b.) 27 MPH (July 2003) (n = 8-11) Different letters denote significant differences between treatments ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.

In April 2003 (24 MPH), photoperiod 1 had significantly lower levels than all other treatments, while photoperiod 7, although higher than 1, was still significantly lower than all other treatments apart from photoperiod 2 and 6 (Figure 4.30). The remaining treatments were statistically comparable except group 2 which was significantly lower than group 5. In July 2003 (27 MPH), while IGF-I levels in treatment 1 showed a significant rise to attain a level that was statistically comparable to all other treatments, photoperiod 7 was still significantly lower than treatments 3 and 5. At the same time, photoperiod 8 IGF-I levels had significantly fallen from the April (24 MPH) sample to be statistically comparable with the lower level treatments 1, 2, 4 and 7.

Individual growth rate (measured as thermal growth coefficient, TGC) correlated with individuals' plasma IGF-I, levels in all growth periods except between July and October 2002 (15 and 18 MPH) (Table 4.7). Only from October 2002 (18 MPH) onwards did all growth rate parameters (SGRw, SGRl and TGC) correlate significantly with plasma IGF-I however in no case did the r^2 value exceed 0.29. When individual TGCw was compared to individual plasma IGF-I levels within the individual photoperiod treatments over the complete trial length no significant correlations were observed. There were significant correlations with HSI (Section 4.4.3.1) in the months of July 2002 (15 MPH) and January to July 2003 (21 to 27 MPH). When group mean plasma IGF-I was examined in relation to water temperature only photoperiod 1 had a significant correlation ($r^2 = 0.55$; $p = 0.04$).

Table 4.7: Correlations of individual plasma IGF-I levels with individual growth rate assessments and HSI.

	Slope	**Y intercept**	**r^2**	**P**	**F**	**df**
Oct 01 - Jan 02						
SGRw	2.26	5.17	0.22	0.0493	4.526	17
SGRl				ns		
TGC	1.66	5.54	0.29	0.02	6.39	17
Jan 02 - April 02						
SGRw				ns		
SGRl				ns		
TGC	1.59	4.16	0.15	0.036	4.9	28
April 02 - July 02						
SGRw				ns		
SGRl				ns		
TGC	4.36	4.25	0.19	0.0066	8.3	37
July 02 – Oct 02						
SGRw				ns		
SGRl				ns		
TGC				ns		
Oct 02 - Jan 03						
SGRw	11.76	6.76	0.14	0.0047	8.7	57
SGRl	36.13	7.92	0.08	0.03	4.9	57
TGC	3.83	5.85	0.21	0.0004	14.04	57
Jan 03 - April 03						
SGRw	28.75	16.04	0.21	<0.0001	19.93	77
SGRl	161.15	10.73	0.25	<0.0001	24.82	77
TGC	4.97	15.81	0.22	<0.0001	21.99	77
April 03 - July 03						
SGRw	24.67	16.38	0.14	0.001	11.82	76
SGRl	135	11.97	0.20	<0.0001	18.43	76
TGC	6.34	16.36	0.15	0.0005	13.26	76
HSI						
Oct 01				ns		
Jan 02				ns		
April 02				ns		
July 02	0.94	4.48	0.21	0.0037	9.64	37
Oct 02				ns		
Jan 03	0.71	5.51	0.20	0.0006	13.33	56
April 03	1.72	10.50	0.19	<0.0001	17.01	75
July 03	2.22	4.28	0.25	<0.0001	24.97	77

4.4.3. Somatic tissues: size and composition.

4.4.3.1. Hepatosomatic index (HSI)

Livers were removed and weighed from the sacrificed fish (n = 4 to 11 per treatment per time point) to calculate the hepatosomatic index (HSI) (Section 2.6.2). There was no significant effect of sex within treatments hence the data presented are those of the mixed sex population. Population means ranged from 3 to 10% (Figure 4.31). Significant differences were only recorded in HSI in the months of April and July 2003 (24 and 27 MPH). In April the HSI of treatments 1 and 7 fell to below 5% which although lower than the remaining treatments was only significantly so compared to treatments 3, 5, 6 and 8. In July 2003 photoperiod 8 showed a significant decline in HSI to 3.4% which was significantly smaller than treatments 3 to 6. Meanwhile photoperiods 1 and 7 exhibited a moderate increase in HSI to be statistically comparable with treatments 2-6.

4.4.3.2. Liver proximate composition.

The impact of maturation and photoperiod treatments on liver composition was analysed in selected samples. The basis for selection of livers to be analysed was as follows. Three pools were chosen to represent theoretical extremes of condition. The first sample was photoperiod group 1 in January 2002 (9 MPH) which represented individuals prior to signs of maturation (i.e. low GSI, testosterone and calcium levels) with a "high" HSI score (mean HSI 8.4%). The second sample was photoperiod group 1 in April 2003 (24 MPH) which represented mature individuals (i.e. releasing gametes, high GSI, testosterone and calcium levels) with reduced liver size (mean HSI of 4.4%) and the final sample analysed was that of photoperiod 3 in April 2003 (24 MPH): these individuals while of a comparable age to sample 2, were classified as immature and had a similar HSI status (mean HSI of 9.3%) to the first samples.

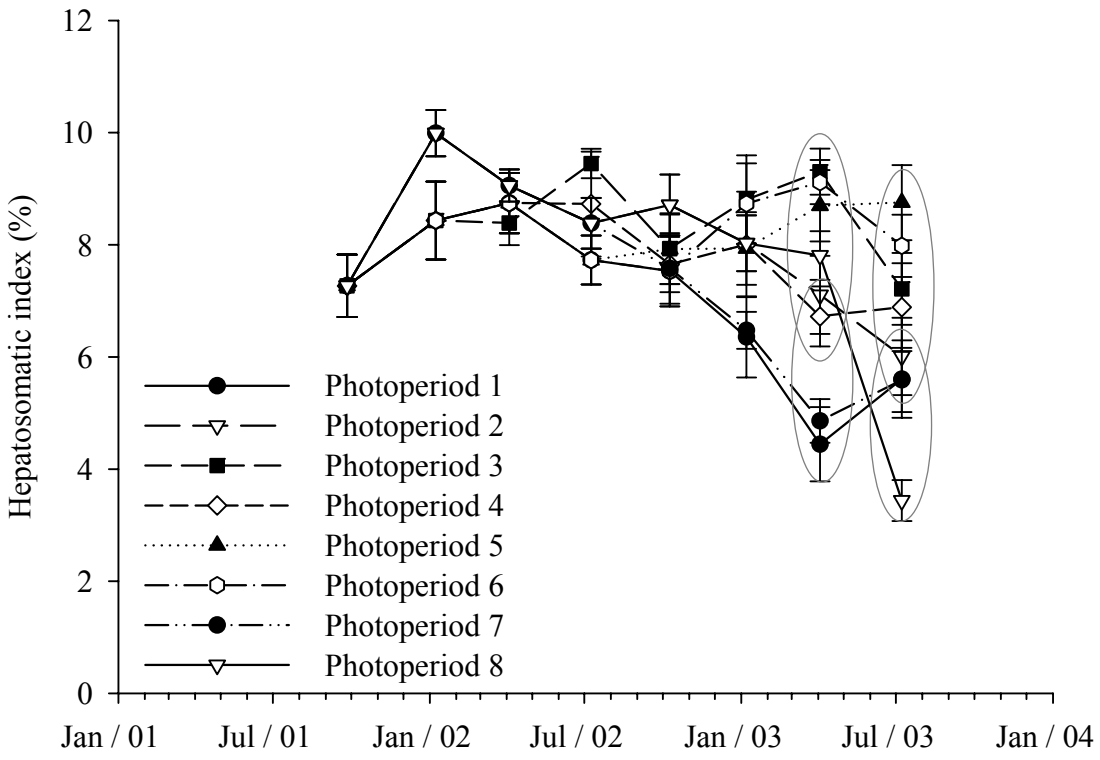


Figure 4.31: Group mean hepato-somatic index (HSI) ± SEM (n = 4-11) for individuals maintained under photoperiod treatments 1 to 8. Where significant differences existed within a given date, homogeneous subsets are enclosed within an ellipse. Where no ellipse is present there were no significant differences within that time point ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.

Both the "immature" groups (sample groups 1 and 3) exhibited moisture levels around 30% with lipid levels around 65% while the mature population (sample group 2) possessed significantly higher moisture levels (45%) and correspondingly significantly lower lipid levels (40%) (Figure 4.32). A regression of % lipid content vs. % moisture content returned a significant negative correlation between the two:

$$\%lipid = -1.1536(\%moisture) + 95.775$$

$$(r^2 = 0.98, p < 0.0001, n = 22)$$

The subsequent aim was to investigate whether the lipid composition could be related to the condition index of relative liver weight (HSI). A regression of % lipid content vs. HSI showed a significant positive correlation between the two ($r^2 = 0.41$, $p < 0.0014$, $n = 22$). Although this relationship was significant it was felt that its accuracy could be improved upon. Therefore the actual lipid content of the liver was calculated for each individual which was then expressed as a % of the whole body weight in a similar manner to the HSI calculation this was termed the hepato-lipid-somatic index ($H^L SI$).

$$H^L SI = \frac{\left(\frac{\%\,liver\;\;lipid\;\;content}{100}\right) \times liver\;\;weight}{Body\;\;weight} \times 100$$

A regression of $H^L SI$ vs. HSI showed a significant positive correlation between the two ($r^2 = 0.76$, $p < 0.0001$, $n = 22$). Hence it can be stated that within the confines of this dataset HSI accurately reflects liver lipid content.

4.4.3.3. Muscle proximate composition

The proximate composition (total protein, lipid and moisture content) of the flesh was measured in the individuals taken from the July 2003 sample (27 MPH). The muscle of cod was very lean (mean composition: 77% moisture, 20.9% protein and

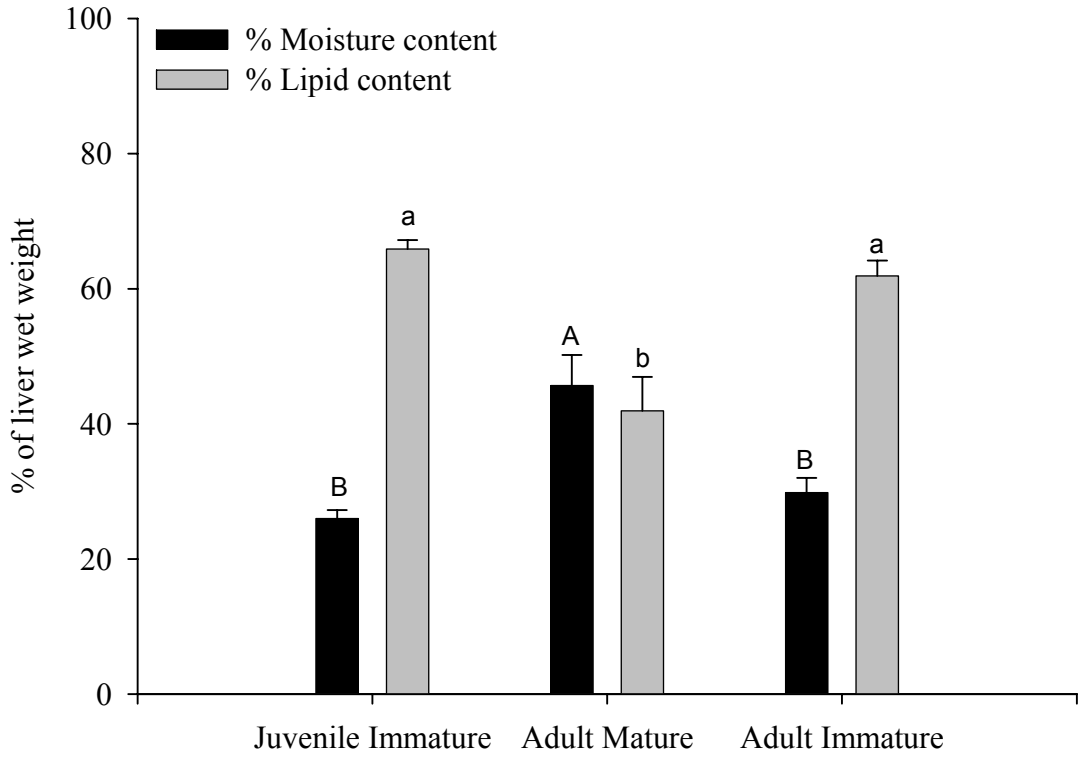


Figure 4.32: Proximate composition (% moisture and lipid content) of livers sampled from juvenile (immature, n = 7) and adult (immature, n = 10 and mature, n = 5) Atlantic cod. Different letters denote significant differences between populations for each parameter ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.

0.16% lipid) (Table 4.8). Protein accounted for over 90% of the dry matter within the muscle and as such exhibited a significant correlation with moisture content ($r^2 = 0.958$: $p<0.0001$, $n = 74$) when lipid content was added to the relationship it was shown to also play a significant role in describing the variation in moisture content ($p = 0.0034$) with the final model being:

$$\text{Moisture content} = 101.45 - 1.135*[\text{protein content}] - 2.287*[\text{lipid content}]$$

$$(r^2 = 0.963: p<0.0001, n = 74)$$

There was no significant effect of sex within treatments hence the data presented are those of the mixed sex population mean. However, of note was the fact that males as a whole had significantly higher lipid levels than females (0.158 ± 0.007 g per 100 g vs. 0.141 ± 0.008 g per 100 g (n = 41 males: 33 females); p = 0.009). The differences in proximate composition between populations were evident in all three parameters, however they were only significant with regards to moisture (as well as its reciprocal

Table 4.8: Proximate composition of muscle, mean (g per 100 g) ± SEM, of individuals (n= 8 to 10) maintained under photoperiods 1 to 8 in July 2003 (27 MPH), (Superscript text denotes significant difference between photoperiods, ANOVA GLM)

Treatment	n	Moisture (g per 100g)	Dry matter (g per 100g)	Protein (g per 100g)	Lipid (g per 100g)
Photo 1	9	78.2 ± 0.4^{ab}	21.8 ± 0.4^{cd}	20.1 ± 0.3^{cd}	0.15 ± 0.02
Photo 2	8	77.4 ± 0.2^{abcd}	22.6 ± 0.2^{abcd}	21.0 ± 0.2^{abc}	0.15 ± 0.02
Photo 3	9	76.7 ± 0.4^{bcd}	23.3 ± 0.4^{abc}	21.5 ± 0.3^{ab}	0.17 ± 0.01
Photo 4	10	77.3 ± 0.4^{bcd}	22.7 ± 0.4^{abc}	21.0 ± 0.3^{abc}	0.17 ± 0.01
Photo 5	10	76.4 ± 0.3^{cd}	23.6 ± 0.3^{ab}	21.7 ± 0.2^{ab}	0.17 ± 0.01
Photo 6	10	76.1 ± 0.3^{d}	23.9 ± 0.3^{a}	22.0 ± 0.2^{a}	0.17 ± 0.01
Photo 7	8	77.9 ± 0.5^{abc}	22.1 ± 0.5^{bcd}	20.5 ± 0.4^{bcd}	0.14 ± 0.02
Photo 8	10	78.9 ± 0.4^{a}	21.1 ± 0.4^{d}	19.6 ± 0.4^{d}	0.12 ± 0.02

measurement of dry matter) and protein content (Table 4.7). Photoperiod 8 had significantly higher moisture levels than treatments 3 to 6, while photoperiod 1 had significantly higher moisture content than photoperiods 5 and 6 and photoperiod 7 was significantly higher than photoperiod 6 only. Hence, the reverse was true with regards protein content with photoperiod 8 having significantly lower levels of protein than treatments 2 – 6, while photoperiod 1 had significantly less protein than photoperiods 3, 5 and 6 and photoperiod 7 had significantly lower levels than photoperiod 6 only.

4.4.3.4. Carcass weight

Since both HSI and GSI have been shown to fluctuate with season, the gross changes in relative carcass weight over the season were quantified. Liver and gonad weights were subtracted from the whole wet weight of the sacrificed individuals in each population and this was then expressed as a percentage of the whole wet weight (Figure 4.33). There was no significant change in carcass weight between treatments 2 to 6. Over the length of the trial it remained in the range of 90-94% of the whole wet weight. Treatments 1, 7 and 8 exhibited a significant drop in carcass weight in April 2003 (24 MPH) compared to their respective previous time points. With regards to the other treatments at this time, this was only significantly lower than treatments 2 and 4. Then in July 2003 (27 MPH), while photoperiods 1 and 7 exhibited a significant rise in carcass weight to return to comparable proportions to treatments 2 to 6, photoperiod 8 maintained the significantly lower levels.

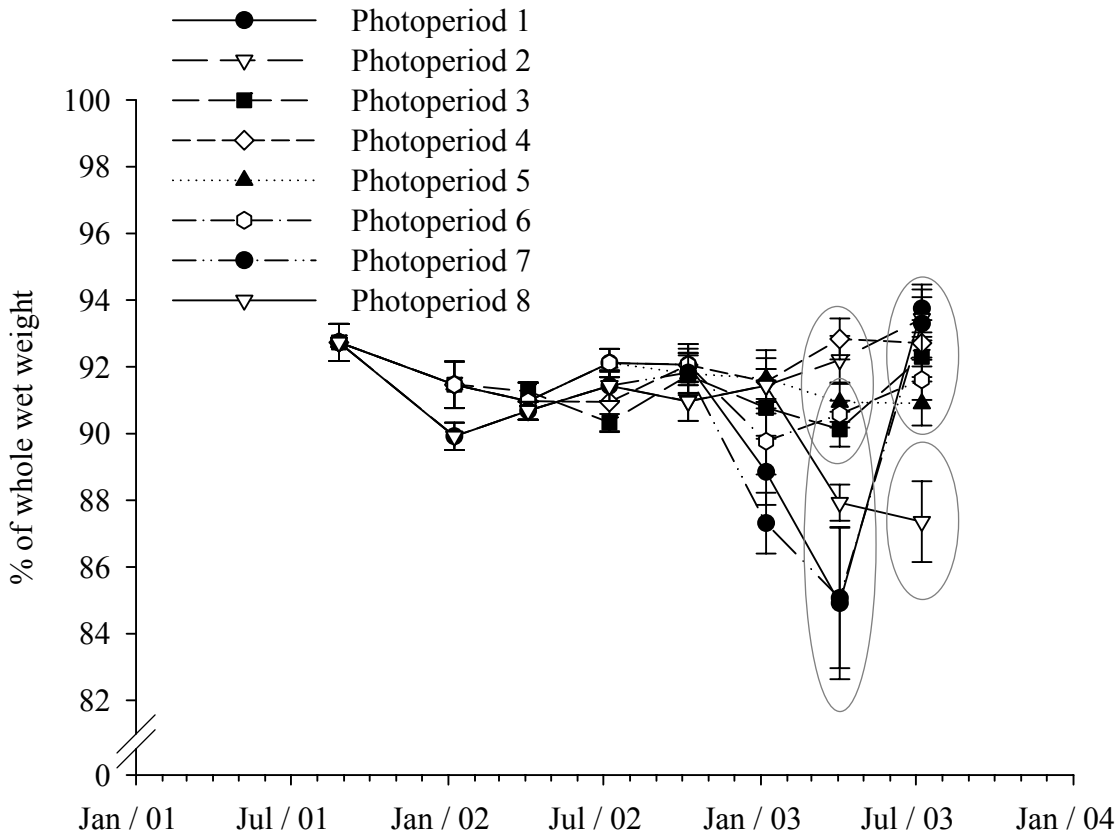


Figure 4.33: Mean carcass weight (% of round fish weight) ± SEM (n = 4-11) for individuals maintained under photoperiod treatments 1 to 8. Where significant differences existed within a given date, homogeneous subsets are enclosed within an ellipse. Where no ellipse is present there were no significant differences within that time point ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.

4.4.4 Melatonin profiles

Plasma melatonin in the SNP population displayed a clear diel pattern with dark phase levels rising significantly compared to daylight levels, attaining a peak of 55±4 $pg.ml^{-1}$ towards the end of the dark phase (Figure 4.34). All of the LL treated populations maintained mean levels between 20 and 25 $pg.ml^{-1}$ throughout the subjective diel cycle, a level which was comparable to the light phase levels of the SNP treatment.

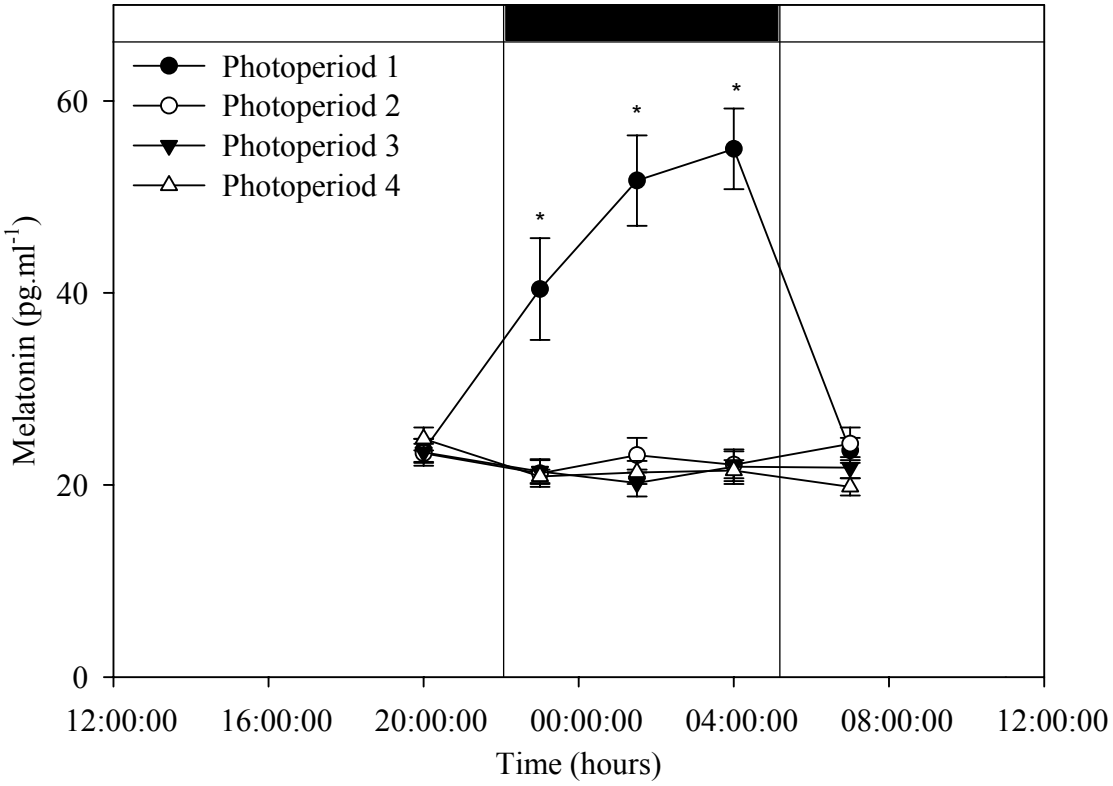


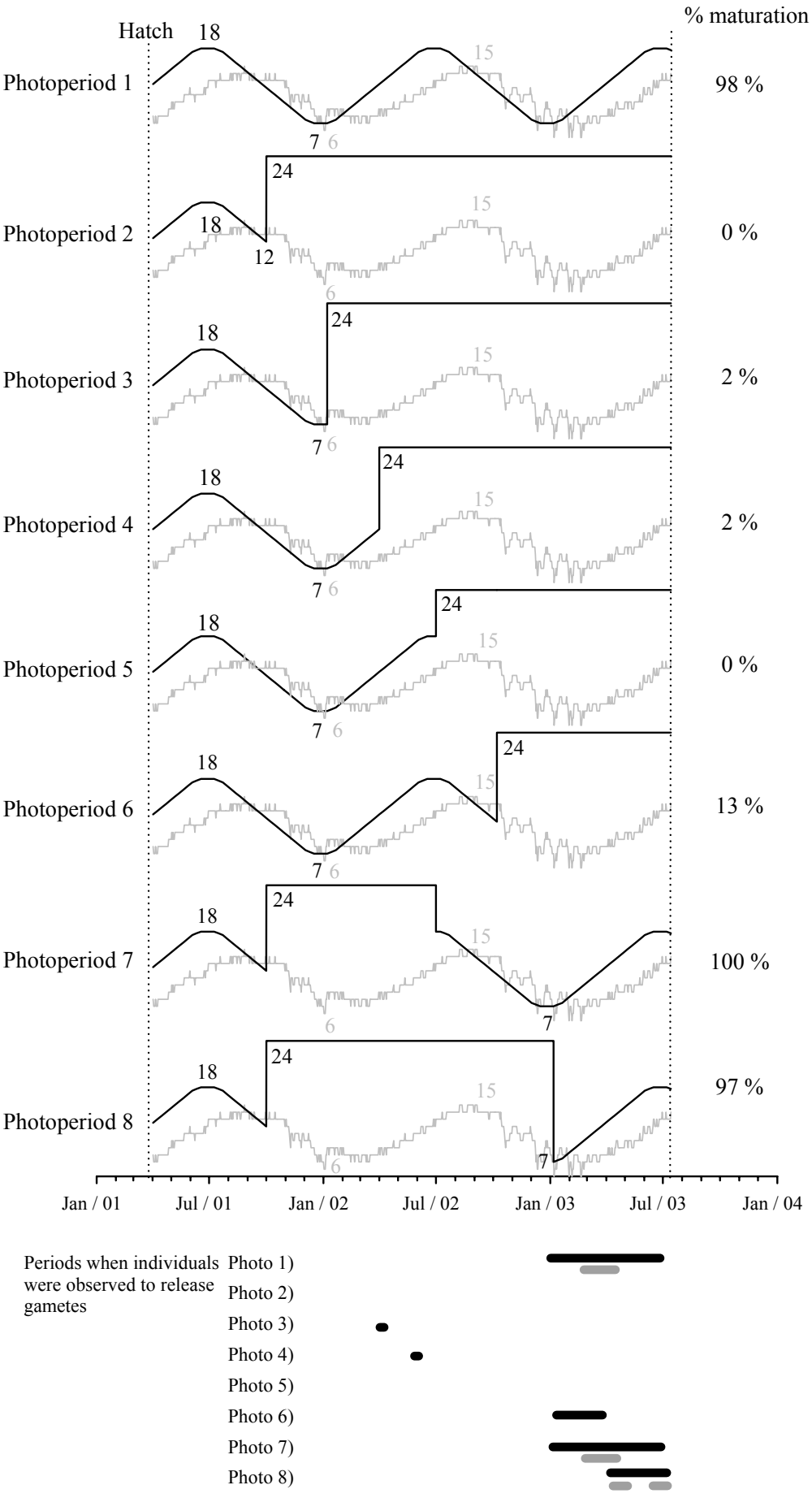
Figure 4.34: Mean plasma melatonin concentration ($pg.ml^{-1}$) ± SEM (n = 10) measured from 20:00hrs to 07:00 hours on the 10th and 11th of July 2002 (15 MPH) for individuals maintained under photoperiod 1 (SNP), photoperiod 2 (9 months exposure to LL), photoperiod 3 (6 months exposure to LL) or photoperiod 4 (3 months exposure to LL). Filled bar represents period of natural darkness for photoperiod 1 (SNP). * denotes significant difference ($p<0.05$) ANOVA GLM with Tukey *post hoc* test.

4.5 Summary of results from experiment IV

- The populations maintained under photoperiods 1, 7 and 8 matured within 27 months post hatch, while those under photoperiod 6 showed limited male maturation between 21 and 24 MPH (Figure 4.35).
- Plasma testosterone levels only rose above baseline levels in the males and females of photoperiods 1, 7 and 8. Levels rose prior to spawning activity in both sexes, with the levels being maintained throughout the spawning season in the males.
- Calcium levels in the females of photoperiods 1, 7 and 8 were elevated just prior to and during spawning activity but did not fluctuate in any other treatment or in any males.
- Histological analysis of gonads at 27 MPH revealed evidence for spermatogenesis in the males raised under photoperiods 2 to 5 while the females under the same treatments showed no advances in gametogenesis beyond immature primary growth oocytes.
- Groups 2 to 6 were all significantly larger than groups 1, 7 and 8 at the trial end.
- Growth rates (SGRw, SGRl) declined over the trial period. Transfer to LL from SNP in the short term elevated growth rates. Periods of reduced and/or negative growth in treatments 1, 7 and 8 coincided with spawning activity.
- There is evidence that populations under treatments 2 to 6 exhibited sexually dimorphic growth patterns in the later stages of the trial, with males ending the trial 11-25% smaller than the females.
- Condition factor increased over the trial period in all treatments. Transfer to LL elevated condition factor and those groups that matured in the second year had a transient elevation in condition factor just prior to spawning activity.

- Plasma IGF-I correlated with growth rates in most growth periods.
- HSI correlated to liver lipid content and both significantly dropped during spawning activity.
- Flesh proximate composition showed limited fluctuations in relation to the photoperiod treatments at the trial end.
- Exposure to LL for 3 to 9 months prevented the diel expression of plasma melatonin.

Figure 4.35: (Following page) Illustrative summary of maturation performance of individuals maintained under photoperiod treatments 1 to 8 (black line) tested in Chapter 4 in relation to ambient temperature (grey line). Horizontal bars represent periods of observed mature males (black) and females (grey) releasing gametes for the respective treatments. Percentage of the total population being recorded as spawning (% maturation) is listed. Range of daylight hours administered in photoperiod treatments (black text) and ambient water temperatures (grey text) are shown on the respective diagrams.


% maturation
Hatch
Photoperiod 1
98 %
Photoperiod 2
0 %
Photoperiod 3
2 %
Photoperiod 4
2 %
Photoperiod 5
0 %
Photoperiod 6
13 %
Photoperiod 7
100 %
Photoperiod 8
97 %
18
15
7
6
24
12
Jan / 01
Jul / 01
Jan / 02
Jul / 02
Jan / 03
Jul / 03
Jan / 04
Periods when individuals were observed to release gametes
Photo 1)
Photo 2)
Photo 3)
Photo 4)
Photo 5)
Photo 6)
Photo 7)
Photo 8)

4.6 Discussion

The experiment detailed in this chapter aimed to further investigate the photic regulation of both the gonadotropic and somatropic axes in Atlantic cod. This was achieved through the transfer of populations from a simulated natural photoperiod to continuous light at different life stages from 6 to 18 months post hatch. The results demonstrated that maturation at approximately two years of age was initiated only in populations that experienced a descending photoperiod at or after the age of 15 MPH. Maturation was associated with clear production losses: gonadal investment is at the cost of somatic growth and arguably flesh composition as well as a reduction in liver size and its corresponding lipid content. All of these could be prevented, resulting in significantly enhanced somatic growth, through the timely application of artificial photoperiod manipulation. Thus, this work has improved the present understanding of the photoperiodic regulation of maturation and growth in Atlantic cod which has informed the applied aim of this work, to refine industry guidelines on this topic.

4.6.1. Maturation

The principal focus of this work was to determine the impact of the timing of LL photoperiod regimes on the recruitment of individuals into the maturation cycle. While a limited number of mature individuals were observed within the first year of the trial (discussed further below), the main spawning event occurred in the second year, between January and July 2003 (i.e. 21 to 27 MPH), when mature individuals were observed in treatments 1, 6, 7 and 8 only. While spawning in these treatments occurred within a relatively compact timeframe there were apparent differences in a number of aspects of the "spawning profiles" of the four populations. With the exception of photoperiod 6 in which only 13% of the total population were observed to mature and

spawn, all other photoperiod treatments recorded close to 100% maturation commitment (Photoperiod 1, 98%; Photoperiod 7, 100%; and Photoperiod 8, 97%). Spawning activity was first recorded in January 2003 (21 MPH) for treatments 1, 6 and 7 while it was delayed until April 2003 (24 MPH) in those individuals exposed to photoperiod 8. After this time, in treatments 1, 7 and 8, spawning activity rose to reach the above listed peaks in activity over the subsequent 3 months (i.e. the peak in spawning activity was observed in April 2003 [24 MPH] for treatments 1 and 7 and July 2003 [27 MPH] for treatment 8). Spawning activity in photoperiod 6 reduced over the subsequent 3 months so that by April 2003 (24 MPH), no further mature individuals were observed. Of these spawning profiles, only the populations reared under photoperiods 1 and 7 were comparable (timing and rate of commitment) with the natural spawning season for cod in the eastern Atlantic Ocean (Hislop, 1984; Bye, 1990; Kjesbu, 1994; Cardinale and Modin, 1999), with the timing of the ambient photoperiod populations reported in experiments I, II and III of this work, and that of the ambient photoperiod reared populations in numerous studies (Hansen *et al.*, 1995, 2001; Kjesbu *et al.*, 1996b; Cyr *et al.*, 1998; Karlsen *et al.*, 2000; Dahle *et al.*, 2000, 2003; Norberg *et al.*, 2004). With regards to photoperiod 8, despite the initial three month delay, the relative time frame of the spawning profile was comparable to the natural populations. However, despite the fact that the spawning of those individuals exposed to photoperiod 6 appeared to begin at a comparable time and with a similar degree of commitment (% of mature individuals) to the "natural" populations (Photoperiod 1 and 7). Thereafter, spawning activity was significantly reduced and stopped prematurely by the onset of the LL regime.

For a more complete description of the maturation activity in all populations a range of associated physiological and endocrinological parameters were also assessed.

These included techniques like ultrasound scanning of gonads *in situ*, visual staging of gonad morphology, the calculation of the relative contribution of gonad weight to the whole animals mass (GSI), histological staging as well as the measurement of plasma testosterone and calcium levels.

As in experiment I and II, ultrasound imaging allowed non-destructive visualisation of gonadal structures. Previous criticism of such a technique has been that it is open to individual interpretation. However in the present case the subsequent sacrificing of individuals allowed direct comparison of this ultrasound imagery with the physical gonadal morphology. The technique did have limitations in not being able to differentiate gonadal structures in the first year of this trial despite their obvious presence. This may be addressed through the use of smaller sized, higher frequency scan heads where technology and budgets would allow. More significantly, however, it was clear that when mean weight ≈1000 g or above, ultrasound imagery was capable of both differentiating the sexes, and detecting the transition of individuals from immature status to ripening and spawning as was recorded in both the gross morphological observations and the GSI calculations. Thus, these data further define the effective range that ultrasound imagery can work within as a rapid, non-destructive tool for sexing and maturation staging as originally outlined in cod by Karlsen and Holm (1994).

Following dissection, gonads were scored on a 5 point scale according to Morrison (1990). This scale was selected due to its inclusion of both sexes and its simplicity. The lack of clearer separation between “immature” and “ripening stage 1” meant that most sexes at most time-points were banded as crossing over both of these categories. The principle reason for this in females was Morrison’s (1990) inclusion of visible blood vessels in the ripening stage. At all times, vascularisation was clearly seen

to cover the ovary structures (Plate 4.1, 4.2, 4.4). Equally with males, Morrison's (1990) definition catalogues the development of a pink colour as a ripening characteristic. However, in this study males testes always had a pink translucent appearance (Plate 4.1, 4.2, 4.3). This highlights the weakness of such visual macroscopic scales. The differentiation between mature, spent and immature is clear however, subtle differentiation between intermediate states can not easily be assessed.

Following gross morphological staging, the temporal fluctuations GSI were recorded. As liver weights in cod are known to fluctuate with season and/or maturation (Schwalme and Chouinard, 1999; Yaragina and Marshall, 2000) the potential impact of such fluctuations on GSI values was investigated but shown to have no significant influence on the results. Thus, the simplest form of calculation was adopted because of its widespread use and the fact that such a calculation is a true reflection of gonadic contribution to animal weight.

The GSI data reflected what was seen in the spawning activity and in the later stages of the trial was accurately recorded using the ultrasound scanning technique, as explained above. In the immature stages the gonads represent <1% of the round weight of an individual (<0.5% in males). However, as an individual enters maturation the GSI increases to reach a peak of *circa* 12%, at which point it is the largest paired organ in the individual. These levels are comparable to those reported by Hansen *et al.* (2001) and Kristoffersen *et al.* (2004). The relative size of the gonads has previously been correlated to maturation condition in either both sexes (Dahle *et al.* 2003), or just females (Tomkiewicz *et al.,* 2003). Unfortunately, the GSI calculations in both these papers are different from each other and also from that which is used in the present work. The calculation utilised by Dahle *et al* (2003) compared gonad weight against body weight with both gonad and liver weight removed. The calculation utilised by

Tomkiewicz *et al* (2003) was based on gonad weight against gutted weight (i.e. with gonad, liver, stomach and viscera weights removed). In both cases, these actions result in an elevation of the GSI values, so definitive comparison between these studies and this work is difficult. As demonstrated earlier, the removal of liver weight had no significant influence on GSI variations. Further removal of gonad weight from the body weight used within the calculation in accordance with Dahle *et al.* (2003), should not have any major further consequences in the majority of situations where gonad size is small. With this condition accepted, it is possible to state that the individuals in treatments 2 to 5 should be classified as "immature" at all stages. In photoperiod 6 the single male at 21 MPH by these standards most probably falls in the maturing classification as did one female observed at that time. Otherwise, all other samples would be classed as immature. While all individuals in photoperiods 1, 7 and 8, up to 18 MPH (1 and 7) and 21 MPH (8) appeared to fall within the "immature" classification, after this date, when gonads started to increase in relative weight, comparison with Dahle *et al.* (2003) and Tomkiewicz *et al.* (2003) becomes difficult. However, what is clear is that all three populations entered the "mature" classification.

While the above techniques are popular rapid assessments of the stage of reproductive activity, the clearest definition can only be provided through histological analysis of the gonadal tissue to characterise the cellular activity of the germ cells. Unfortunately because of time constraints, histological analysis could not be performed in all samples. Hence, only a limited number of individuals (n = 3) of both sexes from each photoperiod taken in July 2003 (27 MPH) were analysed to define the developmental stage the populations were in at the trials' completion. The females from photoperiods 1 and 7 displayed clear evidence of recently completed spawning, evident from the postovulatory follicles and atretic oocytes in conjunction with predominately

primary growth oocytes which would be recruited in later maturation cycles. For this reason, mean oocyte diameters were low (128 ± 3 μm and 109 ± 4 μm). Whilst it is acknowledged that oocyte measurements were not corrected for the possible impact of the fixing and sectioning process (i.e. oocyte shrinkage, see Kjesbu, 1994; Svasand *et al.,* 1996; Dahle *et al.,* 2003), the measurements still provided a further valuable indication of oocyte development. The males of both treatments evidently still contained spermatozoa within clear empty lobular regions with little evidence, in the sections screened, for early germ cell stages. Such characteristics have been referred to by Dahle *et al.* (2003) as being indicative of "stage IV", spent testes. As for photoperiod 8, ovarian sections were dominated by vitellogenic oocytes at various stages of development. This was reflected in the mean oocyte diameter (385 ± 14 μm) which was significantly higher than those in all other treatments. With the individuals in question being recorded as releasing eggs on inspection, the lack of any significant presence of postovulatory follicles suggests that they were in the early stages of spawning (Tomkiewicz *et al.*, 2003). The males, which were spermiating, clearly showed dense clusters of spermatozoa throughout the sections with little evidence for the presence of any earlier stages of germ cells. As for photoperiods 2 to 6, there were apparent developmental differences between the sexes in all treatments. While all ovarian sections revealed only primary growth oocytes with small oocyte diameters (treatments mean, ranged between 110 and 128 μm) which suggested no gonadal development, the testes samples consistently demonstrated germ cells at varying stages of development. Although cells in the early stages of spermatogenesis (spermatogonia and spermatocytes) dominated the testes structures, clusters of later stages including spermatozoa were apparent. This is interesting as, with the exception of the males of photoperiod 6, in all other treatments, all reproductive parameters so far investigated,

suggested no gonadal activity. There is, at present, limited published work concerning the histological observations of Atlantic cod following photoperiod manipulations. Interestingly, Kristoffersen *et al.* (2004), also noted evidence of limited spermatogenesis (as was observed in treatments 2 to 6 of this work) in male cod maintained on LL photoperiods, comparable to photoperiod 5 in this present work, which had been held in either enclosed systems, or tank systems that also allowed a degree of ambient illumination. The same authors report this was in association with "an increase in germ cell death (apoptosis)". Hansen *et al.* (2001) noted that females held under LL treatments, comparable to photoperiod 5 of this work, possessed oocytes in pre-vitellogenic "immature" states up to approximately 24 MPH but did not report the examination of any males. Clearly the information from the present study provides a useful insight to the developmental stages of the populations at the end of the trial. The remaining histological analysis will be competed at a later date to catalogue the temporal alterations in histological characteristics in all treatments. In particular, the apparent developmental difference between sexes under photoperiods 2 to 6 requires further investigation to determine whether the apparent limited male gametogenesis is a significant phenomenon. The demonstration that it appears in all "immature" photoperiod treatments (photoperiods 2 to 5) is at least suggestive that it is not linked to any specifically timed photoperiodic event.

This physical assessment of maturity status was further supported by the measurement of plasma testosterone and calcium levels. The levels of plasma testosterone reported in this study (males, 0.3 to 10.6 ng.ml-1; females, 0.4 to 4.6 ng.ml^{-1}) are comparable to those previously documented in Atlantic cod, as was the significant difference in levels between males and females (Experiment III of this work: Cyr *et al.*, 1998; Dahle *et al.*, 2003; Norberg *et al.*, 2004). Testosterone levels in males on

photoperiods 1, 7 and 8 rose from basal levels (<0.5 $ng.ml^{-1}$) at the onset of maturation, at the same time as the rise in GSI and corresponding transition of morphological characteristics from immature to the late ripening stage. It reached a peak prior to spawning (≥ 7 $ng.ml^{-1}$) which was maintained during the spawning period before falling rapidly to basal levels at the end of the spawning season, when GSI had shown an equally dramatic reduction and the gonads themselves were spent. This apparent link with maturity status is in line with that reported by Dahle *et al.* (2003), although these authors reported testosterone levels [Stage I (Immature, GSI <0.6%) ≈ 0.5 $ng.ml^{-1}$, Stage II (Showing evidence of active spermatogenesis, GSI ≈ 4%) ≈ 1.5 $ng.ml^{-1}$, Stage III (Spermiating males, GSI = 12%) ≈3.4 $ng.ml^{-1}$) and Stage IV (Spent, GSI <1.4%) <0.2 $ng.ml^{-1}$] for fish in the later stages of maturation that appear to be somewhat lower than those recorded here. A spike in testosterone levels in males in photoperiod 6 was noted at 21 MPH when a limited number of spermiating males were discovered, however at all other times in this, and all remaining photoperiod treatments (i.e. 2 to 5), the lack of detectable fluctuations suggests a lack of significant testicular development. Testosterone is said to play a dominant role in the early stages of spermatogenesis (Scott *et al.*, 1980; Borg, 1994). However, this is in conflict with the histological data discussed above, which showed evidence for active spermatogenesis in individuals raised under treatments 2 to 5 when testosterone was low. While it is possible that the three monthly sampling schedule was unable to detect a related testosterone fluctuation if indeed it did exist, the completion of further histological analysis is required to better explain this apparent conflict in results.

As with the males, in the females on photoperiod 1, 7 and 8 plasma testosterone levels showed a brief elevation (rising from <0.5 to between 2 and 4 $ng.ml^{-1}$) that coincided with the "late ripening stages" of ovarian development when GSI shows its

first significant rise. Dahle *et al.* (2003) did not record a significant difference in testosterone levels from immature, through ripening to spawning stages in females. They did however, record a significantly higher level of testosterone between all these stages (mean levels ranging between 0.5 and 0.8 $ng.ml^{-1}$) and spent females (<0.3 $ng.ml^{-1}$). Norberg *et al.* (2004a) however, did record seasonal fluctuations in plasma testosterone in female cod which were described as "largely following the pattern of circulating estradiol -17β". Together, these hormones were shown to peak in the females just prior to/at the start of the spawning activity, as was the case in the present trial. The authors' maximum measured values (varying between 3.5 and 4.4 $ng.ml^{-1}$) are in close accordance with those recorded here (4.6 ± 2.6 $ng.ml^{-1}$). In females, testosterone is the precursor to estradiol which, passed through the vascular system, stimulates the hepatic tissue to release vitellogenin (Nagahama, 2000). Plasma calcium levels are used as an indirect measurement of vitellogenin (Norberg *et al.*, 2004). In the current study elevation in calcium coincided with the detected peaks of testosterone in the same females, confirming the onset of vitellogenesis. Levels were elevated (*circa* 200 $mg.dl^{-1}$) from just prior to the onset of vitellogenesis through to the peak in spawning activity, thereafter returning to basal levels (*circa* 150 $mg.dl^{-1}$) once spawning activity had ended in photoperiods 1 and 7. Vitellogenesis is a continuous process in females during the spawning season as cod is a serial batch spawner which explains this prolonged elevation (Kjesbu *et al.*, 1996a). Again, the lack in fluctuation of calcium or testosterone in females in treatments 2 to 6 indicates a lack of any significant ovarian development.

The impact of maturation was also assessed on a number of other traits in relation to the key somatic tissues. The liver is the principle energy reserve in cod (Holdway and Beamish, 1984) and, as such, HSI is a valuable tool to describe

fluctuations in this energy resource (Lambert and Dutil, 1997). As with GSI, the impact of the fluctuating weight of the gonads on HSI was assessed and with the data showing no significant effect, the simplest formula was thus adopted. No difference in HSI levels between the sexes were recorded, as Lloret and Ratz (2000) noted in stocks off Greenland. However, Karlsen *et al.* (1995) did note temporal differences in HSI between the sexes which led them to conclude that male gadoids prepare for spawning earlier than females. The lack of such detection in the present trial may be a reflection of the restricted sample time-frame and/or numbers analysed. Furthermore, the fact that the majority of individuals included in the study of Karlsen *et al.* (1995) had experienced a period of starvation may have increased their dependence on endogenous energy reserves compared with the present trial where feeding was unrestricted. Within the present work, the only significant difference noted was the temporary decline in HSI in populations 1, 7 and 8 which coincided with the peak in spawning activity in these treatments, as Eliassen and Vahl (1982b) and Hansen *et al.* (2001) noted in their spawning populations. The lack of detectable fluctuations in the other populations confirms, once again, the lack of maturation commitment under these photoperiod treatments. Furthermore, HSI was shown to directly reflect total lipid content, to demonstrate that during maturation there was not only a decline in liver size but also in relative lipid content. This relationship between HSI and lipid content is indirectly confirmed by the studies of Lambert and Dutil (1997), who firstly demonstrated that changes in liver energy levels are primarily associated with fluctuations in lipid content in cod and secondly, that the relationship between HSI and liver energy content was not linear, but exponential. Hence, through association of the results it could be concluded that the decline in HSI is associated with a reduction in liver lipid content.

Fluctuations in muscle proximate composition were also examined at the trial end which represented a theoretical harvest sample. Due to the significant inverse relationship between water content versus protein and lipid content in cod muscle, the mature treatments 1, 7 and 8 exhibited a tendency towards higher water content and consequently, lower protein and lipid content than the immature treatments 2 to 6. The range recorded in this present trial (78.9% to 76.1% moisture content vs. 19.6% to 22% protein content; from Photoperiod 8 [mature] vs Photoperiod 6 [immature] respectively; these values represent the maximum observed difference) is comparable with that noted in other studies examining fluctuations in muscle proximate composition over seasonal cycles in cod [Dambergs, 1964 (1% variation in moisture content); Love 1970 (3% variation in moisture content); Eliassen and Vahl, 1982a (1% variation in moisture content); Hemre *et al.,* 2004 (1 – 2% variation in moisture content)]. While it is recognised that these variations are low, the present conclusion that this differentiation is associated with reproduction is in accordance with Love (1970) and Hemre *et al.* (*2*004). In further support, Hemre *et al.* (2002), who studied whole body proximate content instead of muscle alone, observed a significant decline in whole body protein content and a reciprocal rise in moisture content in relation to spawning in cod, which the authors believed was a reflection of the requirement for protein in oocyte development. While it is apparent this protein loss in the muscle can be prevented through the inhibition of maturation, such variations should not be considered to be commercially significant, although the results can say nothing about their impact on other flesh quality parameters which were not investigated in this trial.

Another concern that this work addressed in relation to maturation under farmed conditions rose from anecdotal reports from industry representatives that mortality increases during spawning seasons (Robertson D. *pers comm.*). In the present study,

there was no significant impact of any treatment on mortality rates, nor was there any increase in mortality rates in association with maturation in the populations that did pass through this process. It is acknowledged that increased mortality during spawning has been previously reported in wild stocks (Dutil and Lambert, 2000; Dutil *et al.*, 2003). These occurrences are linked to lack of energy reserves and feed availability over the winter period immediately prior to maturation. The lack of such specific mortalities in the present work is most likely a reflection of the *ab libitum* feeding conditions and good husbandry practices, ensuring energy levels never descended to life-threatening levels.

Although the majority of data discussed so far relate to maturation in the second year of this study, it must be recognised that there were isolated incidences of maturation recorded in the first year. At both 12 MPH and 14 MPH, mature, spermiating males (one in each case) were observed in photoperiod treatments 2 and 4 respectively. At these times, both these precocious males had within the past three months been placed onto LL following a simulated winter photoperiod. Maturation at such an age in cod has been reported in farming trials in Norway (Norberg B., Karlsen O. *pers comm*.). However, no such occurrences had been reported by the farms in the UK prior to this. Cook *et al.* (1999) did however, report a limited proportion of mature fish of such a size and age in North Sea surveys. Such "precocious" maturation (within 1 year following hatch) has been reported in a limited number of commercially significant teleost species including masu salmon (Aida and Amano, 1995; Amano *et al.*, 1999, 2000), Atlantic salmon (Jones and King, 1952; Saunders *et al.*, 1982; Fleming, 1996), chinook salmon (Unwin and Glova, 1997; Shearer and Swanson, 2000) and sea bass (Carrillo *et al.*, 1995; Zanuy *et al.*, 2001; Rodriguez *et al.*, 2001). Precocious maturation has become a financial liability within the salmon farming

industry in particular due to the precocious individuals exhibiting reduced growth rates (Berglund *et al.*, 1992), low immunotolerance (Murphy, 1980) and poor seawater survivability (Saunders *et al.*, 1994). It remains to be seen whether precocious maturation will become an issue within cod farming. Although it must be acknowledged that the 3 monthly sampling schedule may have precluded the detection of some precocious individuals, the levels observed within this study (≈2%) should not represent a commercial significance and it is further heartening to see that the male in photoperiod 4 continued to end the trial in close accordance to the population mean size suggesting that such precocious maturation did not significantly affect the individuals' growth performance.

The elemental causation of maturation (be it precocious or not) is believed to be linked to the attainment of a suitable energetic status in association with appropriate environmental signals (Godo and Haug, 1999; Bromage *et al.*, 2001). The fact that it is predominately males that undergo precocious maturation (Jones 1959; Saunders *et al.*, 1982, 1994; Berrill, 2004) has been ascribed to the lower energetic cost of male maturation over female maturation, allowing males to mature at a smaller size (Jonsson *et al.*, 1991). The results in this present experiment further support this viewpoint in cod as no mature females were discovered in the first year; equally the reports of precocious maturation in farmed cod in Norway consisted entirely of males. While some fisheries studies have reported that males mature at a younger age and smaller size than females (Hislop, 1984; Cardinale and Modin, 1999), other studies did not note any significant difference between size and age at first maturation (O'Brien, 1999). In salmon, precocious parr have been shown to be present in the "upper modal" group of the population, exhibiting greater growth rates and hence being of a greater size prior to maturation completion (Saunders *et al.*, 1982; Bohlin *et al.*, 1994). Equally, size has

been described as the dominant determining factor in maturation commitment in haddock, as size also reflects overall growth conditions (Korsbrekke, 1999). Saborido-Rey and Junquera (1999) demonstrated in cod stocks of the Flemish Cap that faster growing individuals mature sooner and at a smaller size than similarly aged, slower growing fish. While the male reared under photoperiod 4 appeared to have superior size and growth prior to maturation, no such enhanced growth was apparent in the male from treatment 3. However, it is possible that the entraining growth pattern that committed this individual to mature may have occurred prior to this experiment's inception.

4.6.2. Photoperiod regulation of maturation

As a whole, this dataset clearly demonstrates that populations under photoperiod 1 and 7 matured and spawned between 21 MPH (January 2003) and 27 MPH (July 2003), with a peak of activity at 24 MPH (April 2003) while individuals held under photoperiod 8 matured with a spawning season starting at 24 MPH (April 2003) and reached a peak at 27 MPH (July 2003). Of the remaining photoperiod groups only a limited number of males in photoperiod 6 matured at 21MPH. However, all evidence of energetic investment in maturation had been eliminated by 24 MPH. No evidence existed for any significant reproductive development in any other treatment (photoperiods 2 to 5). As Norberg *et al.* (2004) stated "photoperiod acts as an important *zeitgeiber* for the timing of spawning in Atlantic cod". This experiment was designed to further investigate the photoperiod signals that initiate and regulate maturation. These results demonstrate that transition to LL at or before 15 MPH (or in other words the summer solstice prior to first maturation) held individuals in an "immature" state. Hence, it can be concluded that a descending photoperiod on or after this date (15 MPH) will recruit individuals to begin the maturation cycle. Exposure to LL from 18

MPH (Photoperiod 6, i.e. following a reduction in daylength from 17.5 to 11.5 hours over three months), led to the recruitment of only a limited percentage of male individuals (13%) to mature. Interestingly, both experiment III of this work and Hansen *et al.* (2001) have shown that application of LL three months later still, at approximately 21 MPH, in line with the winter solstice, actually advances maturation by *circa* 1 month. Hence, it can be suggested that with regards to reproduction, within this window of three months, the role of "long day" photoperiods reverses from an inhibitory to a stimulatory photoperiodic signal. This is indicative of a phase response curve for an endogenous clock controlled rhythm in maturation (Randall *et al.,* 2000; Bromage *et al.,* 2001). Randall *et al.* (2000) demonstrated for the first time in a single stock experiment, complete phase response curves utilising light pulses of both long and short day lengths applied throughout the natural seasonal photoperiod cycle to both advance and delay spawning in rainbow trout. Long day exposure up to September was shown to delay maturation while exposure after this date resulted in an advancement of maturation. A similar mechanism is most likely at work in the present experiments.

As has been discussed previously, the endogenous control of reproduction is reliant on a gating theory in which "windows of opportunity" (Bromage *et al.*, 2001) regulate when an individual can initiate a physiological process like maturation. This window corresponds to a specific entraining photoperiod signal in conjunction with a permissive physiological state. In this present case, to maintain the analogy, the window would be opened by a descending photoperiod (autumnal period in ambient conditions) and in the case of the population maintained under photoperiod 6, the application of LL from October 2002 closed this window before most of the individuals had attained a suitable physiological status or had perceived the window was open in the first place. Of interest is the fact that these mature individuals under photoperiod 6 were exclusively

male, which prior to the opening of the window could be characterised as having a larger size and better condition than those non-maturing fish. This is in accordance with the theories that elevated "physiological condition" allows earlier commitment to maturation (Bromage *et al.*, 2001) and that males frequently mature earlier than females (Karlsen *et al.*, 1995; Imsland *et al.*, 1997a).

Further insights into the definition of this, photoperiod regulated, window of opportunity are highlighted when comparing populations exposed to photoperiods 7 and 8. Although both populations showed 100% maturation, fish under photoperiods 7 and 8 spawned 6 months and 3 months after the reduction from LL to SNP respectively. As no evidence exists to demonstrate a physiological difference between both groups prior to the photoperiod transfer, it can only be suggested that, either the photoperiod signal was perceived differently and/or temperature plays a regulatory role in maturation initiated by photoperiod signalling (Bromage *et al.,* 2001). It is possible that the decrease from 24 to 17.5 daylight hours (reduction of 6.5 hours), as in photoperiod 7, was still considered by the fish to be a long day with the natural seasonal decline thereafter acting as the entraining signal to coordinate reproduction, as was the case in the SNP treatment (Photoperiod 1), explaining the comparable timing of spawning between these two populations. In contrast the descent from 24 to 7 daylight hours (reduction of 17 hours) as in photoperiod 8 was considered to be a long to short day switch, hence recruiting individuals to mature at the fastest possible pace which would explain the observations of spawning within 3 months. However, the role of temperature on maturation should not be ignored. It has been reported that high temperatures can delay maturation in a number of species (Bromage *et al.*, 2001). Nevertheless, Norberg *et al.* (2004) concluded that photoperiod is dominant over temperature in regulating maturation in cod, and the results of experiment III of this work demonstrate that

reproduction entrains to photoperiods out of phase of natural temperature cycles, suggesting the differentiation of day length to be the main regulating mechanism at work.

Therefore, from the results of the present study, it can be concluded that in Atlantic cod, long day photoperiods serve to inhibit maturation with a descending photoperiod acting as the initiating cue, opening the window that recruits individuals into the maturation cycle. More specifically, in an ambient photo-thermal maintained population, it is the decrease in day length from October onwards that appears to play the main role in entraining commitment to the reproductive cycle. As shown in experiment III and in previous studies (Hansen *et al.,* 2001), once maturation has been initiated, long day lengths result in accelerating maturation and consequently advancing spawning. Such a mechanism could explain the presence of the two precocious males in experiment IV within 3 months following transfer to LL on a rising winter photoperiod while no mature individuals were observed in the ambient population.

Such findings in cod are of great interest as they are not in accordance with the photoperiod regulation of maturation in other northern latitude aquaculture species such as Atlantic salmon and Atlantic halibut. In the Atlantic halibut, another marine batch spawner found in comparable latitudes as Atlantic cod (Jakupsstovu and Haug, 1988; Neilson *et al.*, 1993), it has been demonstrated that long days enhanced growth but also recruitment of maturing males (Norberg *et al.*, 2001). The authors reported that, following exposure to LL, the return to an ambient photoperiod at the summer solstice prior to maturation, resulted in the maximum reduction in maturation commitment. These results suggest that in halibut, an increasing daylength or long day photoperiod is the window opening signal, as proposed for flatfish in general by Bromage *et al.* (2001), while a descending photoperiod is the window closing. This is the reverse of what is

seen in cod and very interestingly highlights the difference between two species inhabiting comparable habitats that reproduce in a similar time frame but appear to have adopted different responses to environmental signals to coordinate their reproductive cycles. This may be a reflection of a shorter term maturation cycle in cod compared to halibut and hence, in halibut, a longer maturation cycle will require a more distant entraining "proximate" photoperiod signal. As with Atlantic halibut, in Atlantic salmon, a rising photoperiod or application of long day lengths recruits individuals into and advances the time course of reproduction (Hansen *et al.*, 1992; Duston and Saunders, 1992; Endal *et al.*, 2000). Therefore, to improve productivity in the salmonid aquaculture industry it is common practice to apply LL during early winter, which opens the window earlier when fewer individuals have suitable energetic status to commit to maturation, so the majority of the population skip maturation that year and instead redirect resources to somatic growth. Combined, these data (summarised in Figure 4.36) highlights how different cod are in this respect: the management strategy being formulated for cod in this work is based on holding this fish in an immature state by not opening the maturation recruitment window through the maintenance of populations on LL from the end of summer. A similar technique, for example in salmonids, would be holding populations on short days which clearly is not feasible in an open commercial system. Overall, these findings add further credence to the theory that the "gate open" position can vary between species (Imsland *et al.*, 2003).

The definition of the photoperiod window that recruits and subsequently regulates reproduction is the first step in complete stock management. The second vital component to this gating theory however is a classification of the physiological status that can define whether or not an individual can mature. The presence of precociously maturing individuals in this present trial highlights an important concern. Atlantic cod

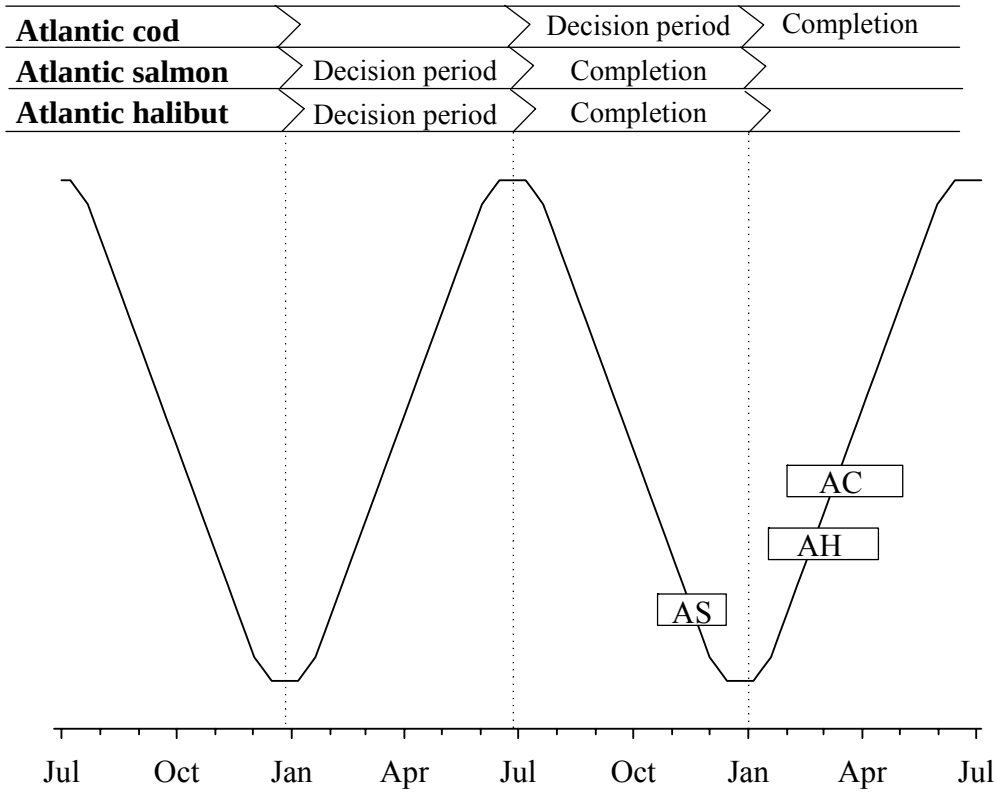


Figure 4.36: Model indicating the proposed role of seasonally changing photoperiod in recruiting individuals into sexual maturation (decision period) and subsequently regulating the final stages of the process (completion) in relation to natural spawning seasons (open boxes) in Atlantic cod (AC), Atlantic salmon (AS) and Atlantic halibut (AH). Figure adapted from Duston and Saunders (1992).

are capable of maturing far sooner than the two year age limit that this work was designed to investigate. At present, there is much research on age and size at first maturity in cod within the fisheries management field. Size and age at maturation in wild populations is becoming a contentious issue as it is argued that the data sets available are now demonstrating clear evidence for "fisheries induced evolution of maturation patterns" (Olsen *et al.*, 2004). Godo and Haug (1999) postulate "… the onset of maturation is initiated when the fish accumulate enough energy per unit of time and/or have enough reserves of energy in the body tissue to be able to fulfil maturation

and spawning in the coming season, i.e. a satisfactory maturation condition". A more simplified summary is that size with respect to age is believed to be the ultimate determinant of maturation. In this sense, as age increases, so does the minimum size at maturation (Godo and Haug, 1999). It should be stressed that the only available data for size and age at first maturation in cod comes from wild catch surveys where, during the natural spawning season, individuals either caught in the field or surveyed in commercial landings are measured (length is the standard measurement) and maturation status scored based on a gross morphometric analysis of gonads, as was used in this present trial (O'Brien, 1999; Saborido-Rey and Junquera, 1999; Cardinale and Modin, 1999). O'Brien (1999) reported a median size at maturity of 45 cm in the Georges Bank and 42 cm in the Gulf of Maine (mixed sex populations) however, the range reported was 31.3 to 57 cm and 27.9 to 71.7 cm respectively with a tendency towards maturity at smaller sizes in the more recent years of the survey. Saborido-Rey and Junquera (1999) reported minimum female size at maturity in the 1997 year class on the Flemish cap ranging from 39 to 41 cm. Cardinale and Modin (1999) reported median size at maturity in Baltic sea cod stocks ranging from 27 to 41 cm in males and 33 to 49 cm in females, while Cook *et al.* (1999) and Hislop (1984) reported mature individuals between 5-700 g or 38-43 cm in North Sea surveys. The western Atlantic stocks are reported as being 2 years of age at maturation while the North Sea and Baltic Sea survey reports mature individuals as 1 and 2 years old (ageing by otolith zonation). Together, this heterogeneous dataset has one common fact in that mature individuals are seen once they reach approximately 40 cm total length. Growth performance in this present experiment demonstrated such sizes can be easily attained in culture in *circa* 12 - 16 months. Hence, back projection of growth data by approximately 9 months (to reach the summer solstice, the start of the hypothesised decision period see discussion above) will

suggest an ultimate determinant size in the region of 20 cm (based on present trial growth rates). This is purely conjectural at this stage and requires further extensive investigation, however, of note is data that have come to light, reporting that cod produced from out of season broodstocks (October spawning) and reared under standard commercial culture conditions, matured (estimated at 100%, males and females) in spring 2004, 16 MPH, with a mean weight of *circa* 500 g and total length of ≈ 30 cm (size at summer solstice was *circa* 78 g, 20 cm, H Richards *pers comm.*). As production moves to year round supply, such events will become more prevalent. This report suggests that the threshold differences between sexes may not be widely different, but alarmingly for the industry, far lower than minimum harvest weights. This clearly highlights the now pressing necessity for the definition of the physiological parameters that regulate recruitment to maturation.

4.6.3. Melatonin

In an attempt to directly assess how the light manipulations were perceived by the cod, melatonin levels were assessed at 15MPH in subsets of photoperiods 1 to 4. These populations were specifically selected to examine the impact of 3, 6 and 9 months of LL exposure in comparison to those reared on SNP to that date. As was seen in experiment I of this work, LL applications again abolished the diel rhythm in melatonin release. These results are in direct conflict with Hall (2000), who surprisingly recorded the maintenance of a melatonin rhythm after 5 months exposure to LL in juvenile cod. Further recent work has demonstrated suppression of diel melatonin profiles in cod exposed to as little as 2 weeks of LL (Taranger *et al.*, 2004a, b) hence demonstrating the overriding effect of light on any endogenous cycling of melatonin release. All of these results contradict those of Hall (2000) and may indicate a light pollution problem

entraining the rhythmic release of melatonin in that study. As Porter *et al.* (2000b) and Taranger *et al.* (2004a) have both shown, in certain circumstances melatonin secretion maintains a diel rhythm when artificial LL is overlaid on an ambient photoperiod regime.

4.6.4. Photoperiod regulation of somatic growth

To date the relationship between photoperiod and somatic growth in cod had not been extensively explored and results to date remain ambiguous (Chapter III of this work; Hall, 1988; Folkvord and Ottera, 1993; Hall, 2000; Hansen *et al.*, 2001). The experiment outlined in this chapter examined for the first time the impact of long-term exposure to LL on somatic growth in non-maturing cohorts. What is apparent from this study is that in the immature populations i.e. photoperiods 2 to 5, the transfer from SNP to LL resulted in an elevation in growth rate, lasting between 3 and 6 months. These findings are in agreement with Hall (1988), Folkvord and Ottera (1993) and Hall (2000), all of whom investigated the effect of photoperiod treatment in immature cod over a short term period. However, the present results further demonstrated that such a growth enhancement was not maintained thereafter and that outwith this window, growth rates were comparable in all treatments, including LL vs SNP treatments. The outcome of this was that by the trial end, groups 3 to 5 were a comparable size while, under photoperiod treatment 2 (longest exposure to LL), fish were significantly smaller than those under photoperiod regimes 4 and 5 (shortest exposure to LL). Endal *et al.* (2000) tested comparable photoperiod treatments in Atlantic salmon and reported both larger mean weights and increased maturation rates (8-37%) with increasing exposure to LL. However, population mean growth data were presented without any distinction between mature and immature cohorts. Equally, Norberg *et al.* (2001), reported elevated

growth rates in male Atlantic halibut following transfer from SNP to LL at approximately 14, 20 and 26 MPH, all of which ended the trial at approximately 40 MPH with comparable final weights as well as resulting in 100% maturation. However, females under the same treatments which remained immature throughout, displayed significantly improved growth in those populations reared under LL photoperiods (Norberg B. *Pers comm.*).

The growth benefit following continuous light exposure is commonly ascribed to the shifting of "endogenous growth rhythms" (Oppedal *et al.*, 1999; Duncan *et al.*, 1999; Endal *et al.*, 2000). Within this present work however, it appears that the transfer to LL from SNP instead caused a temporary direct stimulation of somatic growth. Following this enhancement, growth rates were maintained at a similar rate compared to other LL and SNP treatments. Furthermore, the transfer of photoperiod treatment 7 to SNP at 15 MPH, following 9 months of LL exposure, did not suppress growth rate in the subsequent 3 months, instead it remained comparable to both photoperiod 2 and the ambient photoperiod control. It has been reported that reduction in photoperiods is a stronger *zeitgeiber* of endogenous circadian rhythms (Bromage *et al.*, 1993; Endal *et al.*, 2000). The lack of influence on growth in the present study further implies that no such "endogenous" growth rhythms exist in cod. For a rhythm and/or system to be described as endogenous, there are a number of criteria which have been proposed (Gwinner, 1981, 1986) (See Section 3.6.1. for further details). Very few studies to date have attempted to address these appropriately. The biggest criticism must be the lack of studies under truly "free running" conditions (i.e. no fluctuations in all potential *zeitgeibers* e.g. photoperiod, temperature, food availability): this present work is no exception. This has been attempted with salmonids, which have been shown to demonstrate clear endogenous circannual cycles in principal physiological activities

such as smoltification and maturation (Eriksson and Lundqvist, 1982; Randall *et al.*, 2000). However the apparent mistake is to assume that the associated growth patterns are endogenously regulated rhythms in themselves. As will be discussed below it is suggested instead that at least in the case of Atlantic cod, light is stimulating the somatic growth axis directly. This is made evident from the fact that in all cases transfer to LL caused a significant rise in condition factor and once elevated it was generally then unchanging in the immature treatments. Increased condition implies an increase in weight with respect to length. At the same time, GSI and HSI, the two principle seasonally fluctuating organs in cod (Eliassen and Vahl, 1982b; Schwalme and Chouinard, 1999) did not increase in response to LL treatment suggesting that the relative carcass weight for a given length or ultimately muscle mass has increased in response to LL exposure.

Johnston *et al.* (2003, 2004) have recently demonstrated that the growth enhancement under LL treatment over the winter in postsmolt salmon is due to increased muscle fibre recruitment. Over a 20-fold increase in daily recruitment rates weas recorded during the first 40 days post light application, however, thereafter both populations exhibited comparable recruitment rates. Hence, the subsequent hypertropic growth of the increased muscle fibre number (22.9% higher in the LL population) caused the mass increase recorded over the following sampling interval. The authors hypothesised that muscle fibre recruitment in both treatments ceased at the same time in association with the winter solstice in the ambient photoperiod, suggesting this was a natural cut-off time as fibre recruitment is believed to stop in early adult age [*n.b.* muscle fibre recruitment ceases once an individual reaches approximately 44% of the species ultimate size (Weatherley *et al.*, 1988)]. However with this signal not being present in the LL treatment, it was suggested it may be under an “endogenous” control

(Johnston, 1999). The same authors then questioned whether an earlier or later LL application would have caused a respectively greater or lesser recruitment increase with respect to the studied LL treatment. If muscle fibre recruitment is causing the somatic growth enhancement in this present experiment then the results suggest that extended exposure to LL does not recruit more muscle fibres. It can be hypothesised that within a 6 month window, following transfer to LL, muscle fibre recruitment may be enhanced to attain a new, higher "FN_{max}" (Fibre number maxima) and these fibres, newly recruited through hypertophic growth, cause the recorded mass and condition increase. This increase in FN_{max} is permanent in later adult stages (Johnston, 1999; Johnston *et al.*, 2003, 2004) hence also offering an explanation for the lack of growth depression and maintenance of elevated condition in treatment 7 with respect to photoperiod 1 following return to SNP at 15 MPH. Unfortunately, samples of the muscle blocks of any individuals that could allow investigation into muscle fibres and their recruitment in this present work were not preserved so this theory, though plausible, remains unconfirmed.

The direct action by which light stimulates growth is not yet known in teleosts. It is believed that photic signals are transduced to the brain - pituitary axis which subsequently entrains the somatotrophic axis and growth as outlined above (Section 4.1). Hence IGF-I was chosen as a target hormone to monitor the growth axis throughout this trial. To date and to the author's knowledge, no published data of plasma IGF-I in Atlantic cod is available; the levels herein reported (5-35 $ng.ml^{-1}$) are comparable to those quoted for a wide range of other teleosts (Table 4.9). Mean plasma IGF-I levels under ambient photo-thermal conditions in this present work exhibited a circannual pattern that correlates with the ambient water temperature. Weltzien *et al.* (2003) recorded no such seasonal association in halibut while McCormick *et al.* (2000) and Taylor *et al.* (*in review*) recorded a significant effect of temperature on IGF-I levels

Table 4.9: Typical ranges of plasma IGF-I measured in a range of teleosts.

Common name	Reported range of plasma IGF-I **Concentrations (ng.ml^{-1})**	**Reference**
Atlantic halibut	10 – 30	Weltzein *et al.* (2003)
Atlantic salmon	10 – 50	Dyer *et al.* (2004b)
rainbow trout	1 – 25	Taylor *et al.* (2003)
chinook salmon	5 – 35	Beckman *et al.* (2001)
coho salmon	4 – 16	Larsen *et al*. (2001)
Southern bluefin tuna	25 – 50	Dyer *et al.* (2004b)
black bream	25 – 50	Dyer *et al.* (2004b)
barramundi	20 – 30	Nankervis *et al.* (2000)
silver perch	20 – 40	Dyer *et al.* (2004b)
channel catfish	4 – 12	Silverstein *et al.* (2000)
Atlantic cod	5 – 35	**Present study**

in salmonids. The subsequent application of artificial photoperiods influenced IGF-I levels to erase this seasonal rhythm however, in all cases, IGF-I correlated with growth rate except between 15 and 18 MPH (July to October 2002). Such a correlation between IGF-I and growth has been reported in many previous studies (McCormick *et al*., 1998; Beckman *et al.*, 1998; Beckman and Dickhoff, 1998; Silverstein *et al.*, 2000; Larsen *et al.*, 2001; Pierce *et al.*, 2001; Beckman *et al.*, 2001; Mingarro *et al.*, 2002; Beckman *et al.*, 2004; Dyer *et al.*, 2004a) and in fact has provided the basis for a theory that the monitoring of IGF-I can act as a rapid assessment of growth response under various experimental conditions e.g. nutritional level or stress response (Dyer *et al.*, 2004a,b). Growth rate correlations in this present study, although significant, were generally weak because of a wide variation within the data (r^2 range 0.08-0.25). The growth rate assessment used (average rate over previous 3 months) was probably not the most appropriate, as it has been proposed that plasma IGF-I levels reflect more recent, short term, growth activity (Dyer *et al.*, 2004a).

No clear physiological trends can be directly linked with the plasma IGF-I levels recorded. As described above, IGF-I levels weakly reflect growth rate during all periods except between 15 and 18 MPH. Interestingly, in this period all groups showed a significant fall in thermal growth coefficient (TGC) which subsequently recovered. This period from July to October 2002 corresponds to the period of highest water temperature (14 to 15^{o}C) observed during the trial. Such temperatures are above the thresholds reported as being suboptimal for growth in cod of this size (Pedersen and Jobling, 1989). Furthermore, Jobling (1988) reported that in temperatures above 14^{o}C, cod display reduced appetite, metabolic rates and gastric evacuation rates. Hence, the disassociation of IGF-I and growth rates may reflect a down regulation of IGF-I due to such a chronic stressor (Dyer *et al.*, 2004b). Outwith this period, when groups were transferred to LL there was an apparent, though not significant, elevation of IGF-I levels with respect to the ambient treatment. Amongst its range of actions, IGF-I has been shown to stimulate both the rate of proliferation and differentiation of muscle cells (Oksbjerg *et al.*, 2004; Castillo *et al.*, 2004), potentially adding further credence to the suggestion that LL was directly stimulating muscle growth in immature fish. Only at 24 and 27 MPH (April and July 2003) were IGF-I levels significantly different between groups. These variations reflect those seen in growth rate as maturing populations go through periods of significantly reduced growth which are reflected in the fall in IGF-I levels. Another association may be with liver size. In July 2002 as well as January to July 2003, HSI was significantly correlated with IGF-I levels, at a level comparable to those seen with growth rates during this period. The liver is the principle source of circulating IGF-I (Duan, 1997) and its fluctuating size could impact on IGF-I expression though no previous works have specifically examined, or recorded, such a link. Interestingly, IGF-I levels fell in photoperiods 1 and 8 during the spawning season

despite reported links with reproductive development having been shown (Le Gac and Loir, 1993; Kagawa *et al.*, 1994; Maestro *et al.*, 1997). It is important to note that over 99% of circulating IGF-I is bound to one of at least 4 known IGFBP's that have been identified in teleosts to date (Shimizu *et al.*, 1999, 2004) and it is the IGFBP's that extend the functional life span of IGF-I and along with IGF-I receptors mediate the biological actions of IGF-I (Duan, 1997, 2004; Oksbjerg *et al.*, 2004). Common scientific opinion is now moving away from linking physiological significance to plasma IGF-I levels and is instead turning towards understanding the expression, prevalence and variation in the IGFBP's and IGF-IR's to get a greater grasp on the IGF system and its actions (Duan, 2004; Beckman *et al.*, 2004; Shimizu *et al.*, 2004). However, this should not detract from the fact that this present work has demonstrated that plasma IGF-I measurement does reflect growth activity in another commercially important species and as such represents a multifunctional tool (Dyer *et al.,* 2000a,b).

As the trial progressed, it became apparent that growth in the immature populations was showing sexually dimorphic tendencies with, in all cases, females being larger than males at the end of the trial. Male weight as a percentage of the female size was in the range of 75 – 89% which is comparable with the summary data presented by Imsland *et al.* (1997b) (Table 4.10). Calvo (1989), while studying Argentine hake, reported that 4 other members of the family *Merlucciidae* exhibit a sexual growth dimorphism. The dimorphism in the *Merlucciidae*, a member of the Gadiformes, was linked to females recruiting muscle fibres for a longer period than males, thus allowing females to attain a greater muscle mass and hence, larger size through subsequent hypertrophic growth (Calvo, 1989). These authors demonstrated that in both sexes fibre recruitment falls significantly in the size range of 32-33.9 cm and recruitment stops in males above 44.5cm but continues in females up to 53.9 cm.

Table 4.10: Typical examples of relative weight of males to females in a range of marine teleosts. Origin of fish W = Wild, HR = hatchery. Adapted from Imsland *et al.* (1997b).

Common name	Male size (% of female)	Age (years)	Origin of Fish	Reference
Brill	**72**	3.0	W	(Robert and Vianet, 1988)
Turbot	**86**	5.0	W	(Deniel, 1990)
	96	2.5	W	(Deniel, 1990)
	67	2.5	HR	(Devauchelle *et al.*, 1988)
	74	2.0	HR	(Imsland *et al.*, 1997b)
Atlantic Halibut	**73**	6.0	W	(Jakupsstovu and Haug, 1988)
	62	6.0	W/HR	(Bjornsson, 1995)
	38	30.0	W	(Devold, 1938)
Dab	**94**	2.0	W	(Lozan, 1992)
Smooth flounder	**88**	3.0	W	(Armstrong and Starr, 1993)
Flounder	**93**	3.0	W	(Berner and Sager, 1985)
Plaice	**88**	2.0	W	(Rijnsdorp and Ibelings, 1989)
Winter Flounder	**88**	3.0	W	(Vaillancourt *et al.*, 1985)
Queensland halibut	**95**	3.0	W	(Druzhinin and Petrova, 1980)
Sole	**62**	8.0	W	(De Veen, 1976)
	49	8.0	HR	(Houghton *et al.*, 1985)
Argentine Hake	**78**	Unknown	W	(Calvo, 1989)

While cataloguing the development of skeletal muscle fibres in cod, Greer-Walker (1970) did not consider the potential for any sex related differences, although he did note a significant reduction in fibre recruitment at around 35-40 cm total length. Because there is sexual dimorphism in muscle fibre recruitment within the Gadiformes and this same recruitment has been shown to be promoted by LL application (Johnston *et al.*, 2003), and/or the prevention of maturation causes the prevention of the associated cessation of recruitment (Greer-Walker, 1970), then there is a firm body of evidence to support the conclusion that such LL treatment could induce dimorphic growth patterns as recorded in this present trial. Such evidence could support the argument for the creation of all female populations in preference to mixed sex. This, in conjunction with appropriate maturation inhibition techniques like photoperiod manipulation, could further improve the growth performance of farmed stocks. However, extensive research is still required into the potential creation of such populations before it could be realistically commercially exploited.

Ultimately however, the greatest divergence in growth is associated with maturation. Until 21 MPH the difference between "immature" and "mature" populations was <25% however, in the subsequent 6 months to the end of the trial, there was a significant drop in gutted weight and HSI in the mature populations and a corresponding increase in gonadal development which was then released through the spawning seasons. Hence, at the trial end there was an improvement of over 60% in round and gutted weight in the immature populations in comparison with the ambient mature population. Therefore, it must be concluded that the most important factor in growth improvement in farmed cod is the prevention of maturation.

4.7 Conclusions

The experiment described within this chapter aimed to further characterise the environmental regulation of both maturation and somatic growth in Atlantic cod, through careful examination of both physiological and endocrinological parameters. With regards to sexual maturation, this work has allowed for the first time the definition of the photoperiod signal that initiates maturation. It appears that, unlike most other studied higher latitude teleost species of commercial aquaculture interest, in cod a descending photoperiod is the initiating cue or window that recruits individuals to begin maturation and, in fact, in an ambient photo-thermal maintained population, it is the descending photoperiod signal from October onwards that appears to play the more important role. Furthermore, a potential benchmark for the threshold size for individuals to be recruited into sexual maturation has also been proposed, which along with the definition of the recruiting environmental signals will aid future research specifically aimed at defining such limits.

With regards to somatic growth in cod, this work has demonstrated the measurement of plasma IGF-I levels in Atlantic cod, a key endocrinological component of the somatic growth axis. In so doing it also correlated IGF-1 levels to growth rates, demonstrating the potential of the technique as a tool to indirectly assess growth rates as proposed in recent literature (Dyer *et al.* 2004a). As a whole, the work described in this chapter has provided the first evidence for direct light stimulation of somatic growth in cod. This theory, based around the stimulation of various aspects of muscle fibre recruitment by LL photoperiods, though backed by compelling evidence, will require confirmation and should be a priority for future work.

CHAPTER 5: GENERAL DISCUSSION AND SUGGESTIONS FOR FUTURE WORK.

The principal aim of this research was to elucidate further the photoperiod mechanisms that regulate the key physiological processes of both sexual maturation and somatic growth in Atlantic cod. This information would then be used to formulate industry guidelines for "on-farm management" of reproduction which would principally be employed by the aquaculture industry to realise maximum growth potential in cultured stocks.

Because of the widely reported variation in growth potential and age at first maturation in wild stocks of Atlantic cod, no initial assumptions were made as to the reproductive and growth characteristics of a native British strain, subjected to artificial photoperiod manipulation when reared within its otherwise natural environment conditions. From the outset however, it was acknowledged that under commercial cage aquaculture conditions the only light manipulation that can be applied consistently is that of continuous illumination and hence, this focused the design of most experiments. Therefore, in chapter 3 a number of experimental conditions were investigated during which both reproductive as well as growth performances of an Irish Sea strain of cod were assessed. In experiments I and II, populations were subjected to continuous illumination from the summer solstice in their second year (15 MPH), in enclosed tank and open cage systems respectively, to investigate their response to the most influential photoperiod manipulation previously reported to inhibit maturation in a Norwegian coastal cod strain (Dahle *et al.,* 2000; Karlsen *et al.,* 2000; Hansen *et al.,* 2001). Furthermore, in experiment III, populations were maintained under a number of out of phase, "square wave" photoperiods which, when previously applied to salmonids (Bromage and Duston 1986; Randall *et al.,* 1991, 1998; Randall 1992) had proven to be most successful in demonstrating the entrainment of reproductive processes. This work

demonstrated that reproduction in Atlantic cod is apparently under the regulatory influence of an endogenous clock mechanism that is itself entrained by photoperiod. Furthermore, this mechanism can be entrained using simple transfers between long and short day lengths (Experiment III). This technique when applied in commercial conditions is far simpler and arguably safer to apply than the daily or weekly adjustment of day length more commonly applied by the industry (Bromage and Duston 1986; Bromage *et al*., 2001). It is acknowledged that under the present conditions (Experiment III), growth and survival performances were poor in such treated populations and further work should address these issues to ascertain whether these poor performance results were atypical. Furthermore, the effects of such photoperiod treatments on gamete quality should be investigated prior to the technique being offered to the industry. However, the demonstration that such simplified photoperiod techniques can be used to regulate reproduction in cod offers the opportunity to adopt more transparent experimental conditions to be applied in future studies of reproduction. The application of constant photoperiods allows both the clear definition of the seasonal state that an individual is held in (i.e. long days = summer, short days = winter) as well as providing clear reference points of seasonal change (i.e. date of transfer from a long to a short day length and *vice versa*) which would allow a more analytical description of the timing of reproductive activity.

Most importantly, the experiments described in Chapter 3 clearly demonstrated that continuous illumination applied from the summer solstice prior to maturation, i.e. 15 MPH, can inhibit reproductive development but, that this is dependent on the rearing system in which it is applied, as was suggested by Dahle *et al.* (2000) and Karlsen *et al.* (2000). In an enclosed tank environment (Experiment I) such an application significantly inhibited sexual maturation at both 2 and 3 years of age,

though in both cases evidence for limited reproductive activity was shown (fluctuations in gonadal morphology observed by ultrasound scanning along with a cessation of growth at approximately 2 years; spawning individuals at 3 years of age). The commercial benefit of this inhibition was the significant improvement in somatic growth (over 40% over 2 years). However, when the same photoperiod was applied in an open cage environment (Experiment II), maturation at 2 years of age was not inhibited but the peak in spawning activity was delayed by an estimated 1 to 2 months with subsequently limited impact on somatic growth. These results led to two conclusions. Firstly, in conjunction with evidence proposing that reproduction in cod was initiated in the late spring almost 1 year prior to a given spawning event (Waiwood 1982; Kjesbu *et al.*, 1991) these data suggested photoperiod signals prior to the summer solstice may entrain the recruitment of individuals into the maturation cycle. Secondly, differences in the photic environment (light intensity or spectral content) between the two rearing conditions, specifically due to the presence of an overlying ambient photoperiod signal in the open cage system, might have been responsible for entraining maturation in experiment II.

The experiment described in chapter 4 was designed to be a comprehensive study of photoperiod regulated reproduction and somatic growth activity in Atlantic cod. This was achieved through the transfer of populations from a natural photoperiod cycle to continuous illumination at various stages from 6 to 18MPH in an enclosed tank environment. This made it possible, for the first time in Atlantic cod, to define the photoperiodic "window of opportunity" that recruits individuals into the reproductive cycle and provide valuable information into the intrinsic links of gonadal and somatic growth. The results showed that in Atlantic cod it is the descending photoperiod after the summer solstice, and more precisely after October, which is responsible for

initiating reproductive development. This is apparently unique to cod as the reverse is true for most other commercially important teleost species studied to date such as Atlantic salmon (Duston and Saunders, 1992; Hansen *et al.,* 1992; Endal *et al.,* 2000) and Atlantic halibut (Norberg *et al.,* 2001). Now that the timing of this photoperiodic window of opportunity has been determined, it may help elucidate the physiological condition such as threshold size or energetic status that must be met before an individual can enter puberty, which are, as yet, undefined in Atlantic cod. Interestingly, the extrapolation of data from available literature (Hislop, 1984; Cardinale and Modin, 1999; Cook *et al.,* 1999; O'Brien, 1999; Saborido-Rey and Junquera, 1999) and field reports suggest this may in fact be quite low (approximately 20 cm total length or ≈ 100g). It would be possible to define such a limit by maintaining fish under conditions favourable to growth, i.e. constant long day photoperiods (maturation inhibition) with cohorts being "dropped" to short daylength photoperiods (maturation recruitment) at progressively increasing physical sizes and subsequent rates of maturation being monitored to determine critical size limits. The incorporation of variation in nutritional status, regulated through the inclusion of varying degrees of restrictive feeding in such studies, could be used to investigate the plasticity of this threshold level (Berrill, 2004). Such work should focus on what role the liver plays in defining this limit or whether only physical size and/or condition are critical.

The determination of such a threshold would be of use to the fisheries sector in its continued efforts to optimise the management and in fact preservation of the now endangered wild population (Olsen *et al.,* 2004), while in the aquaculture sector such a definition would allow the careful planning of production schedules. By knowing when stocks enter this permissive state the application of photoperiod regimes can be timed appropriately. This is of greatest concern with the move towards year round juvenile

supply, producing stocks that exhibit altered growth profiles to those studied in this and previous similar works (Dahle *et al.,* 2000; Karlsen *et al.,* 2000; Hansen *et al.,* 2001).

Another benefit of the work described in Chapter 4 was the ability to study the long term somatic growth response in immature cohorts under photoperiod manipulation. Along with demonstrating the measurement of plasma IGF-I, this work was also able to confirm the potential of this technique as a tool to assess somatic growth, as has been proposed in a number of other teleosts (Dyer *et al.,* 2004a,b). However, if used in future research a more intensive sampling schedule (weekly to monthly) should be employed to get the greatest benefit from the technique. More importantly, the examination of the somatic growth response did appear to support the hypothesis that constant light or long day lengths directly stimulates somatic growth. Based on the evidence of temporary elevation of growth rate and the permanent elevation of condition in conjunction with recent published research in salmonids (Johnston *et al.,* 2003, 2004), it is proposed that this growth promotion may occur through the stimulation of muscle fibre recruitment. However, this is unsubstantiated at present. Short term trials monitoring growth rates, condition and fibre recruitment in populations recently transferred to continuous light would succinctly clarify this theory. Such experiments should also contemplate monitoring the IGF-I system (plasma levels, binding proteins and even receptor expression) as it is known to be associated with muscle proliferation (Oksbjerg *et al.*, 2004; Castillo *et al.*, 2004). While scientifically interesting, the potential benefit of direct LL stimulation of somatic growth (<25% gain) was far outweighed by the benefit realised through the timely prevention of maturation, which, in the case of this trial, realised an improvement of up to a 60% in one year in all "inhibited" treatments.

Through an improved understanding of the environmental regulation of maturation and growth, this work aimed to optimise industrial guidelines for photoperiod inhibition of maturation during ongrowing. In the commercial application of photoperiod management there must be a balance between cost and benefit wherein the simplest sense cost can be measured as time of light application while benefit is marked as firstly preventing maturation and secondly through further enhancing somatic growth. The present work shows that to prevent maturation, continuous illumination should be applied from the summer solstice prior to likely maturation (15 MPH in the present case) which serves to mask the opening of the above described window of opportunity. Earlier application of LL photoperiods realised no further significant commercial benefit with regards to somatic growth which, therefore, defines the summer solstice photoperiod as the optimum timing of application to prevent maturation and realise maximum growth potential in farmed Atlantic cod. Thereafter, if maturation is to remain inhibited in the majority of the population, the treatment should be maintained until a suitable harvest weight is achieved as any subsequent reduction in photoperiod daylength will serve to initiate the maturation process.

The failure to inhibit maturation when such a light regime was applied in a commercial cage system in experiment II of this work, as also reported by Dahle *et al.* (2000) and Karlsen *et al*. (2000), suggests that the fish did not perceive the artificial illumination at night as a continuation of the ambient day length. The lighting system used in the present experiment (4 x 400 W light units) was far brighter than would normally be applied in such a cage system to successfully manipulate Atlantic salmon for example (Johnson *et al.,* 2003, 2004), suggesting that light perception is species-specific. Recent, industry based trials managed to inhibit maturation using the summer solstice application of LL in open cages which were restricted in size (5x5x5 m square

pen) and illuminated with two submersible 400 W lighting units (Richards, H. *pers. comm.*), however these are not commercially viable conditions. Research is therefore required to define both the chromatic and luminescent sensitivities of Atlantic cod as it is only then that species-specific lighting systems can be designed. Sensitivities could be explored through studies of melatonin production by *ex vivo* cultures of isolated pineal glands and retinas in response to variable light qualities (intensity and spectral content) applied through the subjective night (Bolliet *et al.,* 1996; Migaud *et al.,* 2004b). However, the localisation and characterisation of opsin structures (Forsell *et al.*, 2001) may prove to be a more informative approach. Such a study would in the first case, demonstrate the location of the light sensitive tissues which are not necessarily restricted to the pineal or retina (Philip *et al.,* 2000; Forsell *et al.,* 2001; Drivenes *et al.,* 2003), while subsequent research into the structure of the identified proteins and hence, spectral sensitivity along with quantification of relative abundance would categorically define the chromatic and luminescent sensitivities of cod. Such knowledge could then be applied to the design of bespoke lighting systems which, dependent on the above definitions, would aim to produce greater general illumination or illumination at specific stimulatory wavelengths.

These findings highlight the need for a greater understanding of the mechanism responsible for light entrainment of physiological functions in teleosts as a whole. While this work and that of other authors clearly demonstrate the functional entrainment of the reproductive mechanism by photoperiod signals, the specific neuroendocrine link between environment and the HPG axis remains unclear. As a reflection of extensive work in higher vertebrates (Goldman, 1999; Gerlach and Aurich, 2000) the working model is that light perception via the pineal gland is transferred to an endocrine signal (melatonin) that acts in discrete regions of the hypothalamus to entrain the HPG axis. In

fact melatonin levels are commonly used as an indirect measurement of "light perception" in teleosts (Porter *et al.*, 1999; Porter *et al.*, 2000a) and due to an apparent "dose response" to light intensity (Futter *et al.*, 2000; Porter *et al.*, 2000a; Migaud *et al.,* 2004b) have been proposed to act as a long term predictor of physiological response to artificial light manipulations (Porter *et al.*, 2000a). However recent work (Taranger *et al.*, 2004a) has questioned this association between diel melatonin responses to light and the corresponding maturation response to photoperiod manipulations in cod, which agrees with other reports that fail to functionally link melatonin and reproduction directly in any lower vertebrate (see reviews of Ekstrom and Meissl, 1997; Mayer *et al.*, 1997). Clearly, some form of tool to predict long term physiological response to artificial photoperiod manipulations would be of value. Whether melatonin is such a tool remains to be seen.

To conclude, the work presented in this thesis represents novel research which explores the interactions of photoperiod, reproduction and growth in Atlantic cod. It provides useful information that can be exploited by the fisheries sciences community but, more significantly, by the aquaculture industry. In so doing however, it has also demonstrated that substantial further research remains to be performed to more clearly define the mechanisms that regulate maturation and growth in one of the most commercially significant marine teleosts of the northern hemisphere.

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APPENDIX I: LIST OF SUPPLIERS OF EQUIPMENT AND REAGENTS USED

EQUIPMENT LIST

Equipment description	Supplier
Assayzap software	Elsevier Biosoft, USA.
Atomic absorption spectrophotometer (Model 2280 AAS)	PerkinElmer, Boston, USA.
Binocular microscope (Model BH-2)	Olympus Optical Co., London, UK.
Borosilicate glass tubes (3ml)	Fisher Scientific, Manchester, UK.
Centrifuge (Model CT422) (Model 1-15)	Jouan Ltd, Herts, UK. Philip Harris, Sigma-Aldritch Co. Ltd. UK.
Digital Timers (Model ETU11)	Smiths, UK.
Dim red light (670-800nm)	Eastman Kodak Company, Rochester, USA, Philip Harris Education, UK.
Drying oven	Gallenkamp, Loughborough, UK.
Eppendorf tubes	BDH, VWR International Ltd, Lutterworth, UK.
Extended range reader for PIT tag (Model Power Tracker III)	AVID, Norco, USA.
Extraction thimble	Whatman International Ltd, Maidstone, UK
9W Fluorescent bulbs	Osram Dulux S G23 energy saver, UK
Gamma Counter (Model Wallac Wizard 3", 1480 Automatic Gamma Counter)	PerkinElmer Boston, USA.
High performance glass vials (20ml)	Packard BioScience Company: Little Chalfort, UK.
Image capture/processing software	Image ProPlus, Data Cell Ltd, UK.
Hypodermic needles (Sterile) 25G, 23G and 21G	Terumo Europe N.V., Belgium
Light intensity meters	Skye Instruments Ltd, Powys, UK.
Passive integrated transponder tag	AVID, Norco, USA.
Polyethylene scintillation vials (6ml)	Packard BioScience Company, Groningen, The Netherlands.
Polypropylene tubes (3ml)	LP3P, Thermo Life Sciences, Basingstoke, UK.
Polystyrene tubes (3ml)	LP3, Thermo Life Sciences, Basingstoke, UK.

Riasmart software	Canberra Packard Ltd, Pangbourne, UK
Scales 0.1-10g (Model QC7DCE-S) 0.01g (Model: BFS-242-020C) 0.0001g (Model AE100)	Sartorius, UK. Sartorius, UK. Mettler Toledo, UK.
Scintillation counter (Model 1900TR LSA)	Canberra Packard Ltd, Pangbourne, UK.
Soxtec HT 6 extraction unit	Tecator AB, Höganäs, Sweden.
Supercut automatic retracting microtone	Reichart-Jang, Cambridge Instrument Gnbh, Germany.
Syringes (1ml or 2ml)	Terumo Europe N.V., Belgium.
Ultrasound scanner (7.5MHz)	Dynamic Imaging, UK.
Zoom lens (Model 18-108/2.5	Olympus Optical Co., London, UK.

REAGENT LIST

Reagent	Supplier
Calcium chloride (1000mg.l^{-1} stock standard)	Sigma–Aldritch Co Ltd, Poole, UK.
Chloroform	Fisher Scientific UK, Manchester, UK.
Cicatrin antibiotic	The Wellcome Foundation Ltd, Middlesex, UK.
Dextran coated charcoal	Sigma–Aldritch Co Ltd, Poole, UK.
Disodium hydrogen phosphate	BDH, VWR International Ltd, Lutterworth, UK.
Eosin (aq)	BDH, VWR International Ltd, Lutterworth, UK.
Ethanol (Absolute)	Fisher Scientific UK, Manchester, UK.
Ethyl acetate	BDH, VWR International Ltd, Lutterworth, UK.
Formalin (40% formaldehyde)	BDH, VWR International Ltd, Lutterworth, UK.
Freeze dried rabbit anti-testosterone antiserum	Biogenesis Ltd, Poole, UK.
Freeze dried sheep anti-melatonin antiserum	Stockgrand Ltd, Surrey, UK
Gelatine	BDH, VWR International Ltd, Lutterworth, UK.
Glass beads	BDH, VWR International Ltd, Lutterworth, UK.
Haematoxylin	BDH, VWR International Ltd, Lutterworth, UK.
HCL	Fisher Scientific UK, Manchester, UK.
Heparin ammonium salt	Sigma–Aldritch Co Ltd, Poole, UK.
IGF-I RIA kit	GroPep, Thebarton, Australia.
Lanthanum chloride	Sigma–Aldritch Co Ltd, Poole, UK.
Melatonin, N-acetyl-5-methoxytryptamine,	Sigma–Aldritch Co Ltd, Poole, UK.
Mercury Kjeltabs	Fisher Scientific UK, Manchester, UK.
Methylated spirit	Fisher Scientific UK, Manchester, UK.
Nitric acid	BDH, VWR International Ltd, Lutterworth, UK.
Orahesive powder	Squibb and Sons Ltd, Hounslow, UK.

Pertex mountant	CellPath Plc, Herts., UK.
Petroleum ether	Fisher Scientific UK, Manchester, UK.
2-Phenoxyethanol	Sigma–Aldritch Co Ltd, Poole, UK.
Putts's Eosin	BDH, VWR International Ltd, Lutterworth, UK.
Scintillation fluid (Ultima Gold)	Packard BioScience Company, Goningen, The Netherlands.
Scotts tap water substitute	BDH, VWR International Ltd, Lutterworth, UK.
Sodium chloride	BDH, VWR International Ltd, Lutterworth, UK.
Sodium chloride	BDH, VWR International Ltd, Lutterworth, UK.
Sodium dihydrogen phosphate	BDH, VWR International Ltd, Lutterworth, UK.
Sodium thiosulphate	Fisher Scientific UK, Manchester, UK.
Sulphuric acid	Fisher Scientific UK, Manchester, UK.
Testosterone	Sigma–Aldritch Co Ltd, Poole, UK.
Tricine [N-tris(hydroxymethyl)methylglycine]	BDH, VWR International Ltd, Lutterworth, UK.
Tris(hydroxymethyl)methylamine (Tris)	Fisher Scientific UK, Manchester, UK.
Tritiated melatonin, [*O-methyl-*^{3}H]melatonin	Amersham Pharmacia Biotech UK Ltd, Little Chalfort, UK
Tritiated testosterone [1,2,6,7-^{3}H]testosterone	Amersham Pharmacia Biotech UK Ltd, Little Chalton, UK.
Urea standard tablet (50mg)	Fisher Scientific UK, Manchester, UK.
Wax	BDH, VWR International Ltd, Lutterworth, UK.
Xylene	Fisher Scientific UK, Manchester, UK.

APPENDIX II: MANUFACTURER'S PROTOCOL FOR FISH IGF-I RIA KITS

Determining fish IGF-I levels using the GroPep Fish IGF-I RIA Kit

28 Dalgleish Street
Thebarton SA 5031
PO Box 10065 BC
Adelaide SA AUSTRALIA
Ph (+61 8) 8354 7700
Fax (+61 8) 8354 7788
Email: bioreagents@gropep.com.au
Website: http://www.gropep.com.au

Intended Use

This radioimmunoassay (RIA) kit provides materials required for the quantitative *in vitro* measurement of serum IGF-I in various fish species. This is the only intended use for the kit. Using the kit, serum IGF-I parallelism has been demonstrated for barramundi, bream, salmon, tilapia, trout and tuna. Other species are under investigation.

Summary and Explanation

- Insulin-like growth factor I (IGF-I) is a single chain polypeptide that stimulates growth and differentiation in many cell types.
- The measurement of serum IGF-I is useful in a variety of different applications. For example, IGF-I mediates many of the properties of growth hormone and is therefore a useful indicator of some growth hormone actions.
- IGF-binding proteins present in the serum actively bind IGF-I and may interfere with any assay for IGF-I by sequestering the IGF-I present in the reaction mixture.
- IGF-binding proteins MUST therefore be removed from the serum prior to assay (e.g. using an acid ethanol extraction protocol).

Principle of the Test

- Radioimmunoassays rely on the specific interaction between antigen and antibody.
- A limited amount of radioactive antigen, together with a known amount of corresponding antibody, is combined with the assay sample. The radiolabelled and unlabelled antigens compete for the same antibody binding sites.
- A second antiserum precipitates the antibody-bound antigen, separating it from the unbound antigen which remains in solution. After careful removal of the supernatant, the precipitate is counted in a gamma-counter.
- The amount of radiolabel present in the precipitate is directly related to the amount of unlabelled antigen present in the original sample.
- The IGF-I concentration in the samples is calculated from a calibration curve generated using the standards provided.

Reagents Supplied:

- Store kits at 2-8°C. Lyophilized components, with the exception of the tracer, are stable for at least 3 months at this temperature.
- Sufficient reagents are supplied to assay 100 serum samples in triplicate. For reliable data, each sample should be assayed in triplicate.
- Sufficient IGF-I standard is supplied for three standard curves, so three assay runs can be performed.

a. Standard (Lyophilized): 3 vials of fish IGF-I (105 ng/vial).
See Reagent Preparation for details.
b. 125I-Fish IGF-I tracer (Lyophilized). See Certificate of Analysis for details of radiolabel and activity date.
c. Antiserum 1 (Rabbit) (Lyophilized): 1 vial.
d. Antiserum 2 (Sheep) (1.1 ml): 1 vial.
e. IgG for precipitation: (Rabbit) 1 vial.
f. QC Serum (Lyophilized): 3 vials.
g. RIA buffer (50 ml) 10 X concentrated, pH 7.5
h. PEG solution (100 ml) 5 X concentrated.

Materials needed but not provided

- Gamma counter
- Vortex mixer
- Centrifuge refrigerated capable of 4,000 g
- Microfuge capable of 10,000 g
- Glass / plastic disposable test tubes (12 X 75 mm)
- Sterile bottles (500 ml)
- Sterile distilled or Milli-Q water
- Micropipettes 20, 100, 500 µl and 1.0 ml
- Test tube racks
- Acid ethanol solution, 0.855 M Tris and acid ethanol blank solution.

Specimen collection

1. Blood collected in tube and left overnight at 4°C.
2. Centrifuge at 10,000 g in a microfuge (13,000 rpm or maximum speed) for 15 minutes at 4°C.
3. Remove serum and store at -20°C.

Method & Protocol

Reagent Preparation

1. Standards. Three vials of lyophilized IGF-I in carrier protein are supplied for 3 standard curves.
   For each assay a series of ten standards is prepared from one of the lyophilized vials. Add 1.5 ml of RIA buffer to one vial, mix well and leave overnight at 4°C to reconstitute. This will give an IGF-I concentration of 70 ng/ml. Nine serial 1/3 dilutions are then made from the starting dilution, i.e. 350 µl of standard plus 700 µl of RIA buffer to give a final volume of 1.05 ml. Mix well. Each of these dilutions is one standard. Reconstitute a new vial of lyophilized IGF-I standard for each new assay.

2. ^{125}I-Fish IGF-I tracer. Reconstitute in 80 µl RIA buffer. Store this stock solution at -20°C. Immediately before use, calculate the dilution in RIA buffer required to give a working solution of ~20,000 cpm/50 µl. Add 50 µl to ALL tubes including 'Totals', Blanks, Reference, Standards, QC and unknown Samples. Make up fresh working solution for each new assay.
3. Antiserum 1. Add 500 µl of RIA buffer to one vial to reconstitute. This will produce a stock solution equivalent to a 1/50 dilution. Store stock solution at -20°C.
   Prepare sufficient fresh working solution immediately prior to assay and discard after use. For the working solution, the stock must be diluted 1/85.7 in RIA buffer giving a final dilution in the assay tube of 1/30,000. 50 µl is required for each tube as shown in Table 1.
4. Antiserum 2. Store stock solution (1.1 ml) at 2-8°C. Prepare sufficient fresh working solution immediately prior to assay and discard after use. The working solution is a 1/20 dilution of the stock solution in RIA buffer. 50 µl is required for each tube as shown in Table 1.
5. IgG. Add 225 µl of RIA buffer to the vial to reconstitute. Store stock solution at 2-8°C.
   Prepare sufficient fresh working solution immediately prior to assay and discard after use. The working solution is a 1/20 dilution in RIA buffer. 10 µl is required for each tube as listed in Table 1.
6. RIA Buffer. Pour the 10 X concentrate into a sterile 500 ml bottle. Wash the concentrate from the bottle with sterile distilled or Milli-Q water and make up to a final volume of 500 ml with sterile distilled or Milli-Q water. Check pH of solution and adjust to pH 7.5 if necessary with 5 M HCl. Store at 4°C.
7. PEG solution. Pour the 5 X concentrate into a sterile 500 ml bottle. Wash the concentrate from the bottle with sterile distilled or Milli-Q water and make up to a final volume of 500 ml with sterile distilled or Milli-Q water. Store at 4°C.
8. Acid/Ethanol Extraction Mix.
   Carefully add 62.5 ml of 2 M HCl to 437.5 ml of 100 % Ethanol. Mix gently and when cool transfer to a sterile 500 ml bottle and store at -20°C.
9. Acid/Ethanol Blank Solution. Combine:
   1 ml RIA buffer
   4 ml Acid/Ethanol Extraction Mix
   2 ml 0.855 M Tris
   Make up fresh for each standard curve.
10. QC Serum. Three vials of fish serum (lyophilized) are provided. Reconstitute overnight at 4°C in 40 µl RIA buffer. Vortex vigorously prior to the acid ethanol extraction step. Use a new vial for each acid/ethanol extraction and assay. This QC sample contains approximately 30-50 ng/ml of IGF-I.
11. 0.855 M Tris. Dissolve 51.8 g of Tris base (MWt 121.14) in 350 ml sterile distilled or Milli-Q water in a sterile 500 ml bottle. Make up to a final volume of 500 ml with sterile distilled or Milli-Q water. Store at 4°C.

Acid Ethanol Extraction of Serum Samples

1. Extract the QC serum samples provided in parallel with the unknown samples to be assayed.
2. To 40 µl of plasma/serum (and QC samples), add 160 µl of acid-ethanol (a/e) extraction mix.
3. Vortex and incubate at room temperature for 30 minutes.
4. Add 80 µl of 0.855 M Tris and vortex.
5. Spin in a microfuge at 10,000 g (~13,000 rpm or maximum speed) for 10 minutes at 4°C.
6. Collect supernatant and assay 50 µl (in triplicate).

Radioimmunoassay Procedure

For optimal results ensure that assay is set up on ice.

1. Set up and label required number of tubes, in triplicate, for the "Totals", Blanks, reference, Standards, QC and Samples to be assayed. Tube contents are detailed in Table 1.
2. Add 50 µl of acid ethanol (A/e) extracted sample to the sample tubes, 50 µl of A/e blank solution to standard, reference and blank tubes, and 50 µl of a/e extracted QC serum to QC tubes.
3. Add 200 µl of RIA buffer to the sample, reference and QC tubes.
4. Add 250 µl of RIA Buffer to the blank tubes.
5. Add 200 µl of the appropriate standard to the standard tubes.
6. Add 50 µl of diluted antiserum-1 to all tubes except the Blanks and "Totals" (tubes to determine the total amount of radioactivity added to the assay).
7. Add 50 µl of diluted tracer to all tubes.
8. Cap tubes for "Totals" to prevent adding any further solution.
9. Vortex all tubes and incubate overnight at 4°C.

Table 1: Assay tube contents. (All volumes in µl.)

	'Totals'	Blank	Ref	Standard	QC or Sample
RIA buffer	-	250	200	-	200
A/e Extract	-	-	-	-	50
Standard	-	-	-	200	-
A/e blank	-	50	50	50	-
Antiserum1	-	-	50	50	50
Tracer	50	50	50	50	50
Total Volume	50	350	350	350	350

NEXT DAY

For optimal results ensure that all steps are performed on ice.

1. Add 50 µl of diluted antiserum-2 to all tubes except 'Totals'.
2. Add 10 µl of diluted IgG to all tubes except 'Totals'.
3. Vortex all tubes and incubate for 30 minutes at 4°C.
4. Add 1 ml of cold PEG solution (4°C) to all tubes except 'Totals'.
5. Vortex all tubes.
6. Centrifuge all tubes (except 'Totals') at 4000 rpm for 20 minutes at 4°C.
7. Carefully remove supernatant and count all tubes.
8. Process data using RIA software packages or manually using the appropriate methods.

Serum Parallelism

- Supplied standards have been tested across a variety of different fish sera for parallelism of assay.
- If you are using this kit for a species other than those described in 'intended use' you are strongly advised to test for parallelism before adopting this methodology.

Performance Characteristics:

Sensitivity

The limit of detection for this assay is typically 0.15 ng/ml IGF-I.

Precision and reproducibility

- The intra-assay precision was determined from the mean of nine triplicates from fish serum samples.
- Overall coefficient of variation was 3.0%.
- The inter-assay precision was determined from the mean of three triplicates of fish serum samples.
- Overall coefficient of variation was 16.0%.

Recovery

Four different fish serum samples were spiked with known amounts of IGF-I and extracted and assayed. Recoveries of spiked IGF-I are as shown in Table 2.

Table 2: IGF-I recovery in various fish species

	Sample Added (ng/ml)	% Recovery
Barramundi	2	115
	5	102
	10	107
Salmon	2	94.2
	5	96
	10	92
Tuna	2	98.7
	5	92.6
	10	90
Tilapia	2	82
	5	86.6
	10	92.1

Performance Characteristics:
Specificity (cross-reactivity)

Recombinant human IGF-I < 0.5%, Recombinant human IGF-II < 1%, Recombinant salmon insulin ND

APPENDIX III: RESEARCH PUBLICATIONS

PEER REVIEWED JOURNALS

Davie A., Porter, M.J.R. and Bromage N.R. (2003) Photoperiod manipulation of maturation and growth in Atlantic cod (*Gadus morhua*). *Fish Physiology and Biochemistry* **28,** 399-401

PEER REVIEWED ORAL PRESENTATIONS

Davie A., Porter, M.J.R. and Bromage N.R. (2003) Photoperiod manipulation of maturation and growth in Atlantic cod (*Gadus morhua*): The effect of timing period and system on successful application. In: *7th International Symposium on Reproductive Physiology of Fish*. May 18th -23rd, 2003, Mie, Japan

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