Research and development of stock management strategies to optimise growth potential in on-growing of Atlantic cod, *Gadus morhua,* and Atlantic halibut, *Hippoglossus hippoglossus*

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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

Aquaculture is an essential developing sector for world food production, however the attainment of sexual maturity during commercial on-growing is a major bottleneck to industry expansion. Sexual maturation brings a commercial loss due to reduced growth performance as well as reduced immune function. Furthermore, serious concerns exist over potential genetic interaction with native stocks through broadcast spawning or spawning interaction by escapees. In the north Atlantic region, the Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) are key aquaculture species in which industry expansion is limited by pre-harvest sexual maturation. However, through a species specific combination of modern technologies and refinement in management practices it is possible that this sexual maturation can be controlled and on-growing potential enhanced. Thus the overall aim of this thesis was to conduct novel research that will improve our understanding of the underlying mechanisms that regulate sexual maturation, whilst also advancing the optimisation of technologies for the management of maturation in cod and halibut.

In Atlantic cod, owing to the inconsistent inhibition of maturation in commercial conditions, ever increasing intensities of light and in some cases narrow spectrum technologies are being used to try to combat this problem. Firstly, this PhD project investigated the potential welfare impacts of high intensity artificial lighting which have not been studied to date (Chapter 2). The work specifically investigated the effect of traditional metal halide and novel green cathode lighting on the stress response, innate immunity, retina structure, feeding activity and light perception of Atlantic cod. Results indicated that although acute responses to light were observed, there were no clear significant long term effects of any of the lighting treatments on these parameters. Regarding light perception, interestingly even when subjected to high intensity constant

lighting (metal halide mean tank intensity: 16.6 watts m^{-2}), cod still demonstrated a day/night rhythm in melatonin release which suggests perception of the overlying ambient photoperiod. The second trial of this PhD project investigated the efficacy of shading of ambient photoperiod in addition to constant lighting to inhibit maturation of cod outdoors (Chapter 3). This aimed at improving the performance of artificial lighting regimes in the open cage system during commercial on-growing by reducing the relative difference between day/night light intensities. The trial was conducted over a one year period where a low and high shade treatment were tested in outdoor tanks. Shading increased the relative night time illumination to 6.6% and 31.3% of daytime levels respectively, compared to <2% in an unshaded set-up. Both shading treatments were effective at suppressing sexual development in cod as confirmed through measurements of gonadosomatic index, histological analysis of gonadal development, oocyte diameter measurements and sex steroid profiles as well as measurements of growth. In addition to research at the applied level in Atlantic cod, this thesis has also extended to the fundamental level and explored one of the potential mechanisms relaying photoperiod signal to the endogenous regulation of sexual maturation in cod, namely the kisspeptin system (Chapter 4). Partial sequences for the signal peptide Kiss2 and its receptor Kissr4 were isolated and described showing similarity to other teleost species such as the medaka, *Oryzias latipes* and stickleback, *Danio rerio*. Novel molecular qPCR assays were designed and developed to measure the expression of both genes in male and female cod over a maturation cycle and compared to cod under constant lighting which remained immature. Interestingly, expression patterns of *kiss2* and *kissr4* did not reveal any clear association with season or photoperiod treatment. However, pituitary expression of gonadotropins (FSH, follicle stimulating hormone; LH, luteinising hormone) did show a differential expression in relation to treatment from early winter

approximately 4-6 months after the photoperiod change. These new results are in contradiction with the hypothesis that the kisspeptin system would be involved in the initiation of gametogenesis, as shown in mammals. However, the FSH/LH data defines a window during which time kisspeptin or another GnRH stimulating mechanism must be active, this compels the need further investigation.

In Atlantic halibut farming, all-female production removes the concerns of production losses through sexual maturation. Accordingly, this thesis investigated the potential/feasibility of generating monosex populations by FACS (fluorescence activated cell sorting) semen sexing based on cellular DNA content, as proven in terrestrial agriculture. Results however did not show any clear differences between the DNA of sperm in a range of species tested (Atlantic halibut, cod, sea bass, perch) suggesting that this technique may not be applicable in such species. The project also focussed on the production of a population of sex reversed halibut broodstock (neomales) that will generate, in the long term, a basis for traditional monosex population generation in the UK. Two in feed MDHT (17α-methyldihydrotestosterone) treatments were tested with the aim to reduce the use of hormone. Results were very successful with a hormone treatment of 5ppm MDHT generating a 97% phenotypic male population thus suggesting the presence of sex-reversed halibut which can be used for future monosex production.

Overall, this work aimed to develop and/or refine potential remediation techniques for sexual maturation in two key commercially important farmed marine fish species, cod and halibut, as well as further our understanding on the regulation of puberty. The knowledge gained from this work provides a means to optimise the techniques employed in the industry and has the potential to increase production and profitability without compromising farmed animal welfare, thus ultimately promoting the sustainable expansion of the Atlantic cod and halibut aquaculture.

Keywords: Atlantic cod, *Gadus morhua*, Atlantic halibut, *Hippoglossus hippoglossus*, artificial light, kisspeptin, maturation, monosex, stress, shading, semen sexing, sex reversal, sex steroids

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LIST OF ABBREVIATIONS

AANAT	Arylalkylamine *N*-acetyltransferase
ANOVA	Analysis of variance
ARP	Acidic ribosomal protein
BCL	Biploar cell layer
BLAST	Basic local alignment search tool
BPG	Brain-pituitary-gonad
BSA	Bovine serum albumin
CA	Cortical alveoli
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CL	Cathode light
DA	Dopamine
Dio2	Type 2 iodothyronine deodinase gene
DHP	17α, 20β-dihydroxy-4-pregnen-3-one
DNA	Deoxyribonucleic acid
Dnd	Dead end
dNTP	Deoxyribonucleotide triphosphate
E_2	17β-estradiol
e.g.	For example
ESD	Environmental sex determination
EST	Expressed sequence tag
EV	Early vitellogenesis
EXP	Exponential function
FACS	Fluorescence activated cell sorting

FSH	Follicle stimulating hormone
FWHM	Full width at half maximum
GABA	Gamma aminobutyric acid
GLM	General linear model
GnRH	Gonadotropin releasing hormone
GPR54	G-coupled protein receptor 54
GSI	Gonadosomatic index
GSD	Genetic sex determination
GTH	Gonadotropin hormone
GVBD	Germinal vesicle breakdown
i.e.	That is
IgfI	Insulin-like growth factor I
IMM	Immature
IPL	Inner plexiform layer
Kiss	Kisspeptin
Kissr	Kisspeptin receptor
L	Length
LED	Light emitting diode
L_F	Fork length
LH	Luteinising hormone
LL	Continuous light
LN	Natural logarithm
LV	Late vitellogenesis
M	Moles
MDHT	17α-methyldihydrotestosterone

MERL	Machrihanish marine environmental research laboratory
MH	Metal halide
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mRNA	Messanger ribonucleic acid
n	Number
NPY	Neuropeptide Y
NVT	Nucleus ventralis tuberis
ONL	Outer nuclear layer;
OPL	Outer plexiform layer;
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Pigment epithelium
PGC	Primordial germ cell
PI	Propidium iodide
PIT	Passive integrated transponder
PNES	Photoneuroendocrine system
PR	Photopreceptor layer
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
RAD	Restriction site associated DNA
RNA	Ribonucleic acid
SARF	Scottish aquaculture research forum
SCN	Suprachiasmatic nucleus
SD	Standard deviation

SEM	Standard error of the mean
SGR	Specific growth rate
SNP	Simulated natural photoperiod
SPW	Spawning
SPT	Spent
Std	Standard
T	Testosterone
T_3	Triiodothyronine
T_4	Thyroxine
TGC	Thermal growth coefficient
UTR	Untranslated region
UV	Ultraviolet
VTG	Vitellogenesis
W	Weight
11KT	11-ketotestosterone

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

1. Overview of Atlantic cod and Atlantic halibut life history and commercial exploitation

1.1 Atlantic cod, *Gadus morhua*

The Atlantic cod belongs to the family Gadidae within the order Gadiformes. This species is distributed in both temperate and polar regions extending across the Atlantic Ocean from the North American coast in the West to the Barents Sea in the East (Cohen, 1990). Cod are considered as a demersal species and are generally found within continental shelf areas to depths of around 150-200 m although they have also been found at depths of over 600 m (Cohen *et al*., 1990). The reproductive cycle of cod is seasonal, with annual batch spawning typically occurring between January and April although this depends on the stock (Brander, 2005). Hatching of eggs is temperature dependent but usually occurs at around 80-100 degree days post fertilisation (Laurence & Rogers, 1976; Brown *et al*., 2003). Hatched larvae are pelagic and resorption of their yolk reserve takes only 6-8 days at a temperature of 6-7^{o}C (Walden, 2001) after which time they must resort to exogenous feeding, mainly zooplankton (Brown *et al*., 2003). Through the first few months after hatch larvae undergo metamorphosis and by 4-5 months they have developed into juveniles with a more demersal mode of behaviour, feeding on invertebrates including crustaceans and polychaete worms. As cod grow, fish begin to form a more regular component of their diet (Cohen *et al*., 1990). Young cod are thought to inhabit shallow nursery grounds and then move to deeper waters to join adults at feeding grounds approximately one year after hatching. Adult cod populations can exhibit annual migrations between feeding and spawning grounds (Windle & Rose, 2005). In general, wild cod reach puberty and undergo their first reproduction at three

years of age at the earliest but this is dependent on stock and location (Berg & Albert, 2003).

Atlantic cod has been fished ever since man began to exploit the seas in Europe and it is amongst the most important of all commercial fishes earning the name "beef of the sea" (Kurlansky, 1998). Throughout the last century however global capture has dramatically declined (from 1,833,877 to 764,582 tonnes, 1950-2008, FAO, 2010) and this has spurred an interest in cod aquaculture with multinational salmon producers keen to diversify into such a species. Substantial efforts have therefore been made over the last few decades to develop the farming of this species into a sustainable industry with production in Norway increasing from 200 tonnes in 1998 to over 18,000 tonnes in 2008 (Fig. 1).

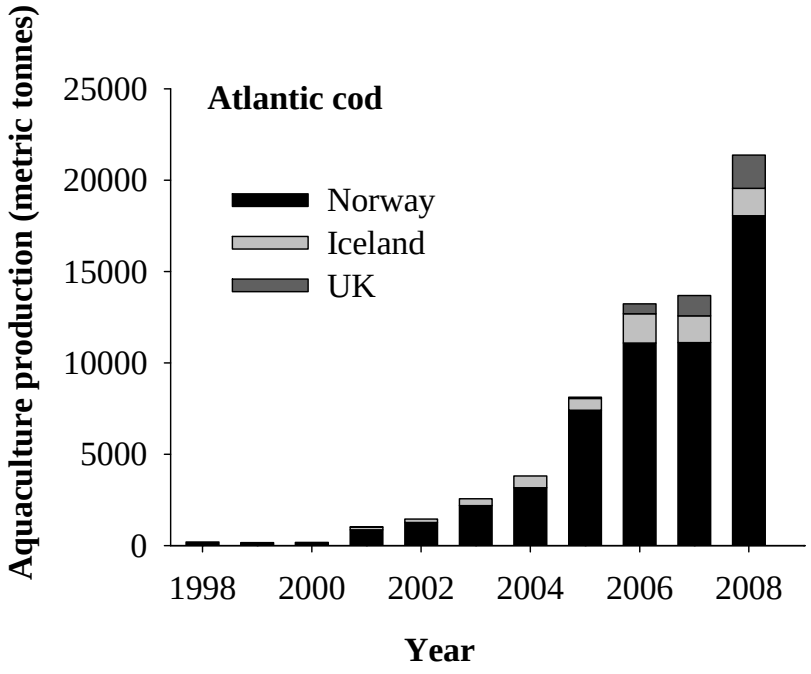

Figure 1. Total aquaculture production of Atlantic cod from 1998-2008 (FAO, 2010).

1.2. Atlantic halibut, *Hippoglossus hippoglossus*

The Atlantic halibut is a member of the family Pleuronectinae within the order Pleuronectiformes and is the largest of the right-eyed flatfish. It is widely distributed throughout the North Atlantic Ocean and parts of the Arctic Ocean and typically found at depths of 100-700 m (Haug, 1990). Like Atlantic cod, halibut are annual batch spawners and spawning takes place at discrete spawning grounds between December and April (Haug, 1990). Around 18 days (90 degree days) after fertilisation, eggs hatch giving rise to early undeveloped larvae which are approximately 7 mm long with a yolk sac and non-functional eyes and mouth, these are dependent on transportation to the photic zone where first feeding occurs. The yolk sac stage is long and can last up to 50 days (300 degree days), during this time the eyes, mouth and intestine become functional and at around 200-290 degree days, there is a transition to exogenous feeding. Following the yolk sac stage, halibut larvae metamorphose into a flatfish shape, the left eye migrates round to the right side and the fish becomes pigmented (Sæle *et al.*, 2004). At this time the larvae settles as a benthic juvenile. The diet of halibut varies depending on size/age, younger fish (~30 cm in length) feed almost exclusively on invertebrates whereas large adult fish (greater than 80 cm) feed mainly on fish (Kohler, 1967). Atlantic halibut show a distinct sex specific growth pattern and age at puberty. In the wild, males normally mature around 4-5 years of age (1.7 kg), whereas females mature around 7-8 years old (18 kg) (Jakupsstovu & Haug, 1988).

Atlantic halibut is a highly prized fish with a high market value. As with cod, global landings of Atlantic halibut have declined over the last century (from 21,644 to 5,239 tonnes, 1950-2008, FAO, 2010) and with a public demand exceeding this diminished wild catch, aquaculture of this species has also been of great interest. Accordingly commercial efforts have been undertaken in the farming of this species

particularly in Norway. However the maximum production recorded in Norway to date (2,308 tonnes in 2007) is still far below that of historical capture figures and public demands (Fig. 2).

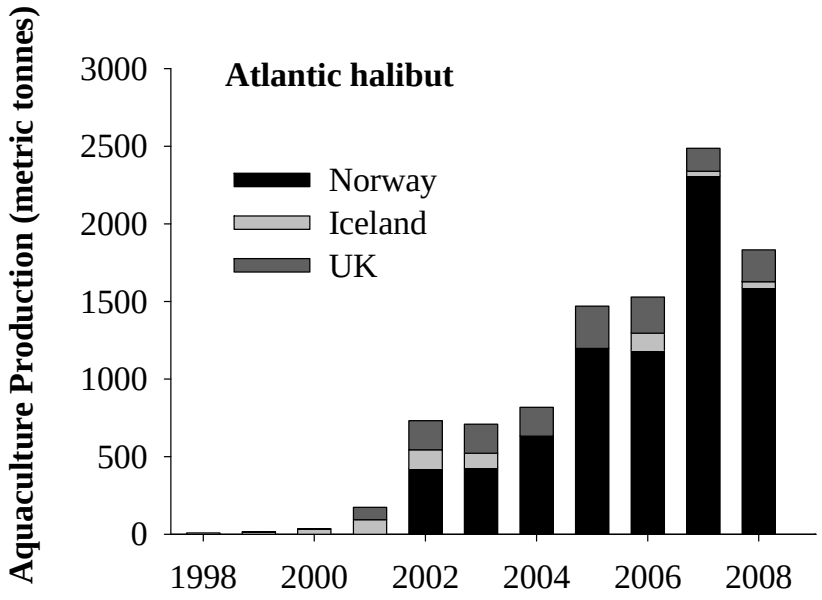

Figure 2. Total aquaculture production of Atlantic halibut from 1998-2008 (FAO, 2010).

The expansion of both the Atlantic cod and halibut industries is limited by production bottlenecks common to the culture of many finfish species. Along with restrictions in juvenile supply due to poor egg and larvae quality/survival in the hatchery as well as an increasing prevalence of disease outbreaks both in the hatchery and during on-growing, the problem of maturation prior to harvest limits both industries potential productivity. While protocols to manage maturation have been demonstrated in a range of species there are severe gaps in our understanding relating the neuroendocrine control of reproduction and the direct mechanisms by which the environment regulates this. It is essential therefore that research is directed into a better understanding of these aspects in order to improve management techniques and ultimately increase productivity.

2. Fish sex differentiation and puberty

In order for fish to reproduce they must successfully complete two key processes: sex differentiation during which individuals differentiate into the male or female phenotype followed by puberty during which individuals become reproductively functional. Both processes are under the influence of genetic and environmental drivers and are regulated by a complex neuroendocrine system. In most teleosts, gonadal differentiation progresses down a distinct developmental pathway to yield a direct and complete differentiation into a female or male phenotype which remains throughout the lifetime of the species, these individuals are named gonochorists (Nakamura *et al.*, 1998; Penman & Piferrer, 2008). Such a gender system is found in both species of interest investigated in the present thesis: Atlantic cod (Chiasson *et al*., 2008) and halibut (Luckenbach *et al.*, 2009) as well as in most other commercially important aquaculture species including salmonids and sea bass, *Dicentrarchus labrax*. However, an alternative gender system, hermaphroditism, exists in fish species such as grouper, wrasse and bream, in which individuals first differentiate into a given sex before switching to the opposite sex under the control of environmental or social cues (Devlin & Nagahama, 2002).

2.1 Sex determination and differentiation

Functional sex in fish is expressed though the differentiation of cell types and organs unique to the ovaries or testes in conjunction with interactions with neighbouring somatic cells and specialised tissues in other organs (Devlin & Nagahama, 2002). This expression is the outcome of two processes: sex determination and sex differentiation (Piferrer, 2001). Determination is used to describe the genetic and environmental influences that control sex differentiation, and differentiation is the physical realisation in terms of testicular or ovarian development in accordance with Devlin & Nagahama (2002).

There are two principle types of sex determination: (1) genotypic sex determination (GSD), where sex is determined at fertilisation by genetic differences between the sexes (e.g. salmonids) and (2) environmental sex determination (ESD), where factors of the environment can determine the sex of individuals following fertilisation (e.g. Sea bass and Atlantic silverside, *Menidia menidia*; Penman & Piferrer, 2008; Valenzuela, 2008; Luckenbach *et al*., 2009). Mechanisms of GSD vary greatly and range from minor sex factor control (polygenic) to dominant sex determining factors in conjunction with autosomal controls to highly evolved sex chromosomes with heterogametic males (XY) or heterogametic females (ZW), of which the former is more common (Devlin & Nagahama, 2002). Out of the 1700 species of fish which have been characterized cytogenetically only 10.4% contain morphologically distinct sex chromosomes, although this figure has been suggested to be higher if calculations considered only studies where sex chromosomes were specifically looked for (Devlin & Nagahama, 2002). No sex chromosomes have been detected in Atlantic cod or Atlantic halibut however they have been shown to have a clear XX/XY sex determining system with female homogameity (Tvedt *et al.,* 2006; Haugen *et al.,* 2011). Currently it is unknown where genetic sex-determination mechanisms mediate in gonadal developmental programs to instigate distinct pathways of steroid production and differentiation into the sexes. However it has been suggested that gene/allele differences that exist between the sexes will alter the biochemistry of the individual at a number of different levels including biochemical conversion, receptor sensitivity and signal transduction, and up/down regulation of gene complexes involved in the sex-determination cascade (Nakamura *et al.,* 1998; Devlin & Nagahama, 2002). In mammalian systems for example, in the primordial gonad, cell migration patterns are directed by the *Sry* gene located on the Y chromosome. This gene acts by signalling

recruitment of mesonephric cells into the gonad of males (Brennan & Capel, 2004). Thereafter, the somatic cells of the testis which are surrounding the primordial germs cells (PGCs), differentiate into seminiferous tubules and testis specific cells. With recent genomic methods it is feasible to characterise the sex determining regions of fish genomes and it would be of great interest to do this for Atlantic cod and halibut.

Regarding environmental sex determination (ESD), there are no sex specific genetic differences and thus sex is determined after conception (Valenzuela, 2008). A number of species appear to have a combination of GSD and ESD (Lagomarsino & Conover, 1993), thus it is not clear if GSD and ESD are distinct systems (Valenzuela *et al.,* 2003) or interactive (Sarre *et al.,* 2004). For example, European sea bass have a genetic basis for sex determination however they can produce variable sex ratios depending on the environmental temperature (Vandeputte *et al.*, 2007; Baroiller *et al.,* 2009). Temperature is the most common cue but factors such as population density and pH can also have an influence (Baroiller *et al.,* 2009). In cod and halibut, sex determination appears to be primarily genetic (Hughes et al., 2008; Haugen *et al.*, 2011), however to our knowledge the direct effects of environmental factors such as temperature on functional sex expression have not been comprehensively studied in these species.

Sex differentiation refers to the process by which PGCs in the gonad start to proliferate and differentiate into oogonia in females and spermatogonia in males, this generally occurs earlier in females than males (Piferrer, 2001). Development (growth and differentiation) of sex-specific gonads requires communication with non-neighbouring cells, and this is done by endocrine signalling which is distinct for the two sexes (Nagahama, 1994). This endocrine signalling works through the brain-pituitary-gonad (BPG) axis where ultimately sex steroids (i.e. 17β-estradiol, 11 ketotestosterone,

testosterone etc.) have local, direct effects on germ cell development in the gonads and also influence other cells and organs involved in sex differentiation (Bieniarz & Epler, 1992; Kime, 1993; Zohar *et al.*, 2010). Many fish are labile in their phenotypic sex and treatment with sex steroids in the time preceding sex differentiation can influence the developmental processes over-riding sex determination, this plasticity can be exploited for monosex production.

2.2 Fish puberty

Following sex differentiation, puberty refers to the process by which an immature individual develops into a reproductively functional adult (Okuzawa, 2002), this is characterised by gonadal differentiation and gametogenesis if the nutritional status and environmental conditions are suitable (Bromage *et al.*, 2001; Coward *et al.*, 2002). In culture, fish usually reach puberty at an earlier age than their wild counterparts due to the favourable farming conditions this however results in great commercial loss to the industry. The harvest goal for Atlantic cod is 3-4 kg (2.5 - 3 years from hatch) however 100% maturation is observed at 2 years of age or less in culture (Davie *et al*., 2007a). In Atlantic halibut the harvest goal is 8 kg or greater (+ 4 years from hatch) however males can reach puberty at 3 years of age (circa 20-30% of population at 3 years reaching 50% at 4 years, Norberg et al., 2001). Puberty onset has significant negative impacts on a number of important production traits such as growth, product quality and welfare. This is because sexual development requires the diversion of energy resources into gonadal growth which leads to a reduction in somatic growth and flesh quality (Porter *et al.*, 1999; Endal *et al.*, 2000; Hansen *et al.*, 200; Hemre et al., 2004) and also a reduction in appetite with Atlantic cod showing decreased feeding approximately one month prior to and during the spawning season (Fordham & Trippel, 1999). Pubertal cohorts in comparison to immature cohorts can show a weight loss of up to 60% in cod (Davie et

al., 2007a) and 17% in male halibut (Norberg *et al*., 2001). Pubertal fish have also been reported to have reduced immunocompetence (Cuesta *et al.*, 2007) as well as a greater sensitivity to environmental stressors like changes in water temperature and low oxygen levels (Makino *et al.*, 2007). Furthermore, broadcast spawning during cod on-growing leading to the release of potentially fertilized eggs into the surrounding environment creates the potential risk of genetic interaction with wild fish stocks (Taranger *et al.*, 2010). For all of these reasons, the control of puberty during on-growing of cod and halibut stocks is a priority for fish farmers to optimise fish performance, shorten the production cycle and improve overall profitability. This is a common problem to most cultured fish species and so a number of strategies have been developed to help prevent or suppress sexual maturation which will be discussed further below (see section 3.1).

2.3 Fish reproductive cycle

Once fish have sexually differentiated and are reproductively competent and environmental and/or nutritional and/or social cues are suitable, then individuals can proceed through a reproductive cycle and produce gametes.

2.3.1 Female gonadal development (oogenesis)

In females, in terms of gonad morphology and development, the mature ovaries are paired structures which contain oogonia, oocytes, follicle cells, supporting tissue, and vascular/nerve tissue (Nagahama, 1994; Coward *et al.*, 2002). There is no set classification system for the stages of development of female gametes from oogonia to mature oocytes and the number divisions along the scale of maturity varies between studies. In Atlantic cod, Tomkiewicz *et al*. (2003) classed ovarian development (based on histological analysis) into six main phases: 1) juvenile, 2) preparation, 3) ripening, 4) spawning, 5) regeneration, 6) degeneration (Table 1). This general main trend of development can also be applied to halibut and other teleost species (Coward *et al.*,

2002). During ripening, vitellogenesis is the main event responsible for oocyte growth and this involves the synthesis of vitellogenin (VTG) by the hepatic system which is released into the bloodstream, taken up by the oocytes and packaged into its yolk derivatives to form a nutrient source (Coward *et al.,* 2002). During the later stage of ripening and into the spawning phase, the process of oocyte maturation is essential prior to ovulation and involves continued VTG sequestration such that yolk globules fill the entire ooplasm, furthermore, germinal vesicle migration and germinal vesicle breakdown (GVBD) must take place (Coward *et al.,* 2002). The Atlantic cod and halibut are annual batch spawners with group-sychronous oocyte development (Wallace & Selman, 1981), female cod may release 9 to 20 pelagic egg batches at intervals of 60 to 70 hours (Trippel, 1998) throughout the spawning period, halibut females release eggs approximately every 72-80 hours over a period of 3-6 weeks (Norberg *et al.*, 1991). In cod, vitellogenesis has been found to commence during the late autumn/early winter (November/December) prior to spawning in January (Kjesbu, 1991). In halibut, vitellogenesis has been found to commence around August/September time, 5 months prior to the start of spawning (Methven *et al.*, 1992).

Table 1. Stages of ovarian development in Atlantic cod based on histological analysis (taken from Tomkiewicz *et al*. 2003). Oocyte diameters are given however these may vary between studies and fish stocks and according to shrinkage during fixation.

Phase	Stage	Histological criteria	Oocyte Diameter (µm)
Juvenile	I	Oocytes are in perinuclear stage with large circular nuclei and peripheral nucleoli in most progressed ovaries.	Up to 80 µm
Preparation	II	Oocytes with circumnuclear ring and nuclei with attached nucleoli.	90-160
Ripening	III	Oocyte recruitment: oocytes with cortical alveoli, initial chorion and detached peripheral nucleoli progressing to vitellogenesis, but yolk granules not entirely filling the cytoplasm.	170-290
	IV	Late vitellogenesis: yolk granules entirely filling the cytoplasm and expand oocytes. Nucleus central circular to slightly eccentric, irregular. Enlarged chorion.	300-530
Spawning	V	Initiation of spawning: abundant vitellogenic oocytes as in IV, but round and larger; single oocytes in final maturation, hydrating oocytes or post-ovulatory follicles exist.	500-970
	VI	Main spawning period: vitellogenic oocytes as V, oocytes in final maturation, oocytes in hydration and postovulatory follicles abundant.	510-1000
	VII	Cessation of spawning: postovulatory follicles abundant, final maturation and hydrating oocytes frequent, vitellogenic oocytes scarce or absent.	450-930
Regeneration	VIII	Spent: postovulatory follicles abundant among perinuclear or circumnuclear stage oocytes, residual eggs or atretic vitellogenic oocytes may be present.	110-140
	IX	Resting: oocytes in perinuclear or circumnuclear stage as in II, but residual eggs or atretic vitellogenic oocytes present.	140-170
Degeneration	X	Malfunction owing to high density of residual eggs encapsulated by fibroblast and macrophages among normal developing oocytes. Other abnormalities.	-

2.3.2 Male gonadal development (spermatogenesis)

In males, the testis is a paired organ consisting of two lobes. In general, these lobes are organised into branching lobules containing a germinal compartment which projects into the collective efferent duct. The germinal compartments of the lobules contain spermatocysts which comprise a clone of germ cells (i.e all at the same stage) surrounded by somatic Sertoli cells. The Sertoli cells provide physical support and factors necessary for proliferation, differentation and survival of the germ cells (Weltzien *et al.*, 2004; Schultz *et al.*, 2010). The tubules themselves are embedded in connective tissue containing fibroblast cells, blood vessels and Leydig cells, the latter of which are the site of sex steroid production for germ cell maturation and the development of secondary sexual characteristics. At the final stages of maturation, the spermatocysts release their spermatozoa into the central lumen of the lobules. These then travel through the efferent sperm duct and are released through the urogenital pore (Weltzien *et al.*, 2004). The process of spermatogenesis which refers to the development of spermatogonial stem cells into mature spermatozoa can be divided into three key stages (Nagahama, 1994; Schultz *et al.*, 2010). The first stage involves the mitotic proliferation of stem cells to produce more stem cells and differentiation into spermatogonia and at this stage the number of Sertoli cells enclosing these stem cells also increases by mitotic proliferation. Stage two involves the differentiation of spermatogonia into primary then secondary spermatids by meiosis. The third stage involves spermiogenesis whereby haploid spermatids differentiate into flagellated spermatozoa. Thereafter spermiation takes place which refers to the process whereby spermatocysts break open releasing the spermatozoa into the sperm duct (Nagahama, 1994; Almeida *et al.*, 2008). Male sexual development generally occurs earlier than oogenesis in females. This is especially clear in Atlantic halibut where males can mature

up to three years before females (Norberg *et al.*, 2001). In cod, although at a smaller scale, males have still been seen to develop up to a month or two in advance of females with meiosis and spermiogenesis being seen from October and free spermatozoa recorded in gonads in December (Almeida *et al.*, 2008).

2.4 Neuroendocrine regulation of sexual maturation

In teleosts, it is the neuroendocrine brain-pituitary gonadal (BPG) axis which acts as the endogenous master controller of puberty and adult reproductive cycles (Zohar *et al*., 2010). This axis is organised around; 1) the hypothalamus of the brain which releases neuropeptides and neurotransmitters which influence, 2) the pituitary (gonadotroph cells), which synthesises and releases gonadotropins (follicle stimulating hormone, FSH; luteinising hormone, LH) which are transferred through the bloodstream and stimulate 3) the gonads to produce sex steroids (androgens, oestrogens and progestagens) necessary for gametogenesis and positive/negative feedback regulation of reproduction (Fig. 3). All three major regulators of the BPG axis integrate with growth/energy (leptin, growth hormone, Igf1) pathways to regulate reproductive processes in synchrony with life stage and the surrounding environment to ensure spawning in favourable conditions (Migaud *et al.*, 2010).

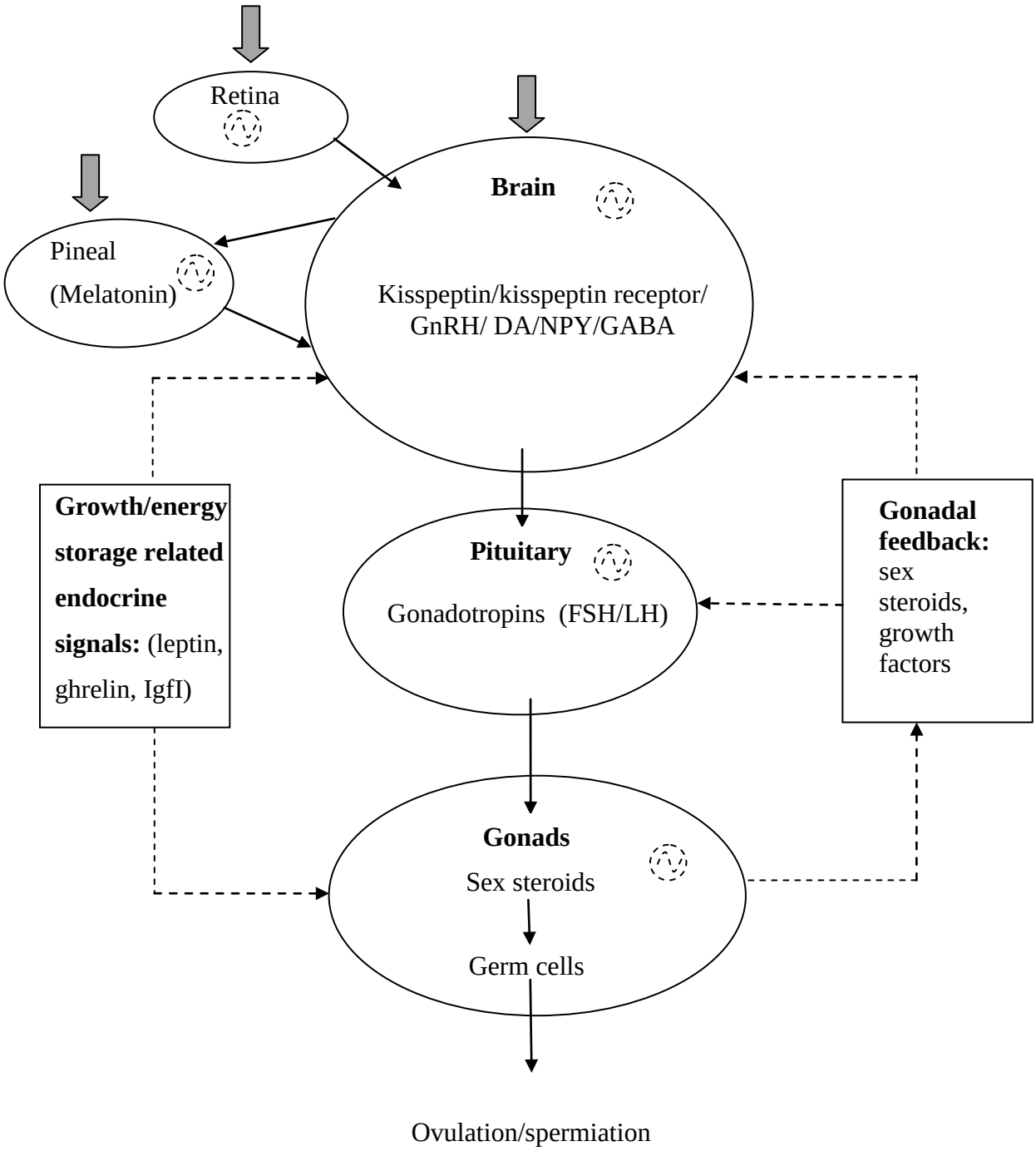

Figure 3. Schematic representation of regulatory pathways in the BPG axis during puberty in teleosts (adapted from Taranger et al., 2010; Migaud et al., 2010). Sites of light perception are indicated by grey arrows. Endogenous clocks (broken wave form) are located throughout the system although their roles and interaction with the reproductive cascade remain unclear.

2.4.1 Brain neuropeptide and neurotransmitters regulating reproduction

2.4.1.1 Gonadotropin releasing hormone (GnRH) neurons

At the level of the brain (hypothalamus), the activation of the gonadotropin releasing hormone (GnRH) neurons and subsequent release of GnRH into the pituitary has traditionally been described as the starting point of the BPG axis controlling the onset of puberty in fish. Many studies have focused on the characterisation of the GnRH system (identification, localisation, pharmacology) in a range of teleosts and it has emerged to be more complex and diverse than first thought. To date, up to 24 distinct forms of GnRH have been identified in a variety of species ranging from invertebrates to humans, and this includes eight variants isolated from fish brains (see Table 2, Kah *et al*., 2007). Based on phylogenetic analysis of sequences and associated sites of expression, variants have been segregated into three branches named type 1, type 2 and type 3 (White *et al*., 1998; Lethimonier *et al*., 2004). The first branch of GnRH forms, GnRH-1, has been suggested to be the major hypophysiotropic hormone in perciformes (Powell *et al*., 1994; Holland *et al*., 1998; González-Martínez *et al*., 2002; 2004 a, b) whereas the significance of the GnRH-2 and GnRH-3 remains unclear. GnRH-3 immunoreactive nerve fibers have been seen to innervate the pituitary of the sea bass although at a lesser extent than GnRH-1 (González-Martínez *et al*., 2002; 2004a). In terms of the physiological role of GnRHs, in the European sea bass, GnRH-3 and GnRH-1 mRNAs have been seen to increase along with the GnRH receptor and follicle stimulating hormone gene expression during sexual differentiation (Moles *et al*., 2007) indicating that these GnRHs may play a role in the BPG axis during sexual differentiation. The majority of research over the past decades has focused on sea bass, salmonids, catfish and eels which means that there is limited data available on Atlantic cod and halibut. In cod, GnRH ligand cDNAs have been isolated for all three forms with research on the mRNA expression patterns ongoing

to further understand the regulatory role of GnRH system in this species (Sandvik *et al*., 2007). In Atlantic halibut, to our knowledge, no studies on the characterisation of GnRH genes have been published yet. However studies have been performed on other pleuronectiforms such as turbot, *Scophthalmus maximus,* and barfin flounder, *Verasper moseri* in which the three different GnRH forms have been identified (Andersson *et al*., 2001; Amano *et al*., 2002a, 2002b; Weltzien *et al*., 2004).

2.4.1.2 Kisspeptins and other brain neurotransmitters

Originally it was believed that GnRH was acting as the top of the BPG axis to control the endogenous reproductive cascade however, recent research in mammals has brought to light the importance of a number of upstream signal peptides that regulate GnRH expression (kisspeptin & neurokinin B) or neurotransmitters that work in association with GnRH's actions to regulate gonadotropin synthesis e.g. dopamine, neuropeptide Y (NPY) and gamma aminobutyric acid (GABA) (Mananos *et al*., 1999; Vidal *et al*., 2004; Zohar *et al*., 2010). Research in teleosts is somewhat behind that of mammals, however, the identification of upstream regulators is as equally important and there have been a number of advances in our understanding of the kisspeptin system in particular. While only one signal peptide kisspeptin 1 (KISS1) and receptor (KISSR1, formerly referred to as G-coupled protein receptor 54 (GPR54)) are present in mammals, *in silico* analysis of the sequenced teleost genomes supported by functional analysis studies have revealed two forms of both the signal peptide (Kiss1 & Kiss2) and receptor (Kissr2 & Kissr4) in fish (Felip *et al*., 2009; Lee *et al*., 2009, Akazome *et al*., 2010). It is believed that kisspeptin performs similar roles in fish as have been reported in mammals. For example it has been associated with the onset of puberty (Filby *et al*., 2008; Martinez-Chavez *et al*., 2008), shown to have similar GnRH regulatory abilities (Elizur, 2009) as well as being susceptible to sex steroid feedback (Kanda *et al*., 2008).

Table 2. Structure of the 24 known GnRH variants taking mGnRH as the reference (Kah et al., 2007).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mammalian (mGnRH)	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH2
Chicken I (cGnRH-I)	-	-	-	-	-	-	-	Gln	-	-
Frog GnRH (frGnRH)	-	-	-	-	-	-	-	Trp	-	-
Seabream (GnRH)	-	-	-	-	-	-	-	Ser	-	-
Salmon (sGnRH)	-	-	-	-	-	-	Trp	Leu	-	-
Whitefish (whGnRH)	-	-	-	-	-	-	Met	Asn	-	-
Guinea pig (gpGnRH)	-	Tyr	-	-	-	-	Val		-	-
Medaka (mdGnRH)	-	-	-	-	Phe	-	-	Ser	-	-
Chicken II (cGnRH-II)	-	-	-	-	His	-	Trp	Tyr	-	-
Catfish (cfGnRH)	-	-	-	-	His	-	-	Asn	-	-
Herring (hgGnRH)	-	-	-	-	His	-	-	Ser	-	-
Dogfish (dfGnRH)	-	-	-	-	His	-	Trp	Leu	-	-
Lamprey I (lGnRH)	-	-	-	-	His	Asp	Phe	Lys	-	-
Lamprey III(lGnRH-III)	-	-	Tyr	-	Leu	Glu	Trp	Lys	-	-
Tunicate I (tGnRH-I)	-	-	-	-	Asp	Tyr	Phe	Lys	-	-
Tunicate II	-	-	-	-	Leu	Cys	His	Ala	-	-
Tunicate III	-	-	-	-	-	Glu	Phe	Met	-	-
Tunicate IV	-	-	-	-	Asn	Gln	-	Thr	-	-
Tunicate V	-	-	-	-	-	Glu	Tyr	Met	-	-
Tunicate VI	-	-	-	-	Lys	-	Tyr	Ser	-	-
Tunicate VII	-	-	-	-	-	Ala	-	Ser	-	-
Tunicate VIII	-	-	-	-	Leu	Ala	-	Ser	-	-
Tunicate IX	-	-	-	-	Asn	Lys	-	Ala	-	-
Octopus - Asn	Tyr	-	-	-	Asn	-	Trp	His	-	-

Studies on its temporal expression in fish species such as the female grey mullet, *Mugil cephalus* (Nocillado *et al*., 2007) have shown high levels of *kissr4* gene expression in the brain during early puberty correlated with high expression levels of the three main GnRH types. Similar results have been found in zebrafish, *Danio rerio* (van Aerle *et al*., 2008) and Nile tilapia (Parhar *et al*., 2004; Martinez-Chavez *et al*., 2008) where expression levels of *kissr4* in the brain have been found to increase during the onset of puberty. Likewise, in terms of kisspeptin expression, an increase in kisspeptin at the onset of puberty has been recorded in tilapia (Parhar *et al*., 2004), grey mullet (Nocillado *et al*., 2007), fathead minnow, *Pimephales promelas* (Filby *et al*., 2008), and zebrafish (Kitahashi *et al*., 2009). These findings have led a number of authors to suggest an important role played by the kisspeptin system in the activation of the BPG axis in fish. This research area is attracting increasing attention due to its potential application in fish farming as a tool to understand how various regulatory signals like photoperiod and/or nutritional status are integrated to entrain the BPG axis. However, despite their potential value, to the authors knowledge, no research to date has described the sequences or characterised the expression of kisspeptin system related genes in either Atlantic cod or halibut.

In addition to kisspeptin, other neurotransmitters have been demonstrated to work in association with GnRH's actions to regulate gonadotropin synthesis including dopamine, NPY and GABA. Dopamine (DA) is known to play an inhibitory role in the neuroendocrine regulation of reproduction in a number of fish species (see review by Dufour *et al*., 2010). Neuroanatomical investigations in the goldfish, *Carassius auratus* and European eel, *Anguilla anguilla* have shown that inhibitory DA neurons which originate in the brain project directly into the pituitary where gonadotroph cells are located and exert inhibitory effects on gonadotrophin production (Kah *et al*., 1987a;

Chang *et al*., 1990; Vidal *et al*., 2004). This hypophysiotropic DA activity has been shown to vary with reproductive stage (Saligaut *et al*., 1999) and is controlled by endogenous factors such as sex steroid feedback (Weltzien et al., 2006) as well as environmental cues (Sebert *et al*., 2008). This involvement of DA neurons in the neuroendocrine control of reproduction provides a further pathway for the investigation of the link between external and internal cues. Neuropeptide tyrosine (NPY) is strongly associated with feeding control in fish. Studies in the goldfish have revealed that NPY stimulates growth hormone and gonadotropin release (Kah *et al*., 1989; Breton *et al*., 1991; Peng *et al*., 1993a, 1993b) which could therefore suggest a link between feeding, growth and control of the reproductive axis (Zohar *et al*., 2010). Gama-aminobutyric acid (GABA) is an inhibitory neurotransmitter which has been shown to have stimulatory effects on gonadotropin secretion in fish. Initial research demonstrated the abundance of GABA immunoreactive fibers in the goldfish pituitary (Kah *et al*., 1987b) and this lead to the discovery of its role in the stimulation of LH secretion including GnRH release and inhibition of dopamine in the species (Kah *et al*., 1992; Sloley *et al*., 1992). It has been hypothesised that gonadotropic stimulation by GABA can be mediated through GnRH following *in vitro* work on the goldfish pituitary where GABA was seen to cause a dose-dependent in GnRH release (Kah *et al*., 1992). In the rainbow trout, GABA has been seen to stimulate both basal and GnRH-induced gonadotropin secretion from pituitary cells (Mananos *et al*., 1999). Sex, reproductive stage and ultimately sex steroid have all been shown to affect the stimulatory action of GABA on gonatropin secretion in fish (Trudeau *et al*., 1997).

2.4.2 Gonadotroph cells in the pituitary

In the pituitary of fish, as in other vertebrates, gonadotroph cells (FSH/LH synthesising cells) are located in the anterior lobe (pars distalis). Unlike mammals, gonadotroph and

other important cells including somatotrophs, corticotrophs and mammotrophs cells, are generally arranged together in specialised masses (Olivereau & Ball, 1964). These cells are innervated (directly or indirectly depending on the fish species) by the neurosecretory fibers (i.e. GnRH neurons, dopaminergic neurons) which release neurohormones that originate from the hypothalamus of the brain (Zohar *et al*., 2010). The distribution of GnRH immunoreactive fibers have been shown to be co-localised with gonadotroph cells in the anterior pituitary of sea bass (Gonzalez-Martinez *et al*., 2002; Zmora *et al*., 2002; Yaron *et al*., 2003). The functional role of GnRH on gonadotropin release has been demonstrated in many teleost species including the striped sea bass, *Morone saxatilis* (Hassin *et al*., 1998), the European sea bass (Mateos *et al*., 2002) and the common carp, *Cyprinus carpio* (Kandel-kfir *et al*., 2002). It is well known that pituitary gonadotropins play a key role in the regulation of sex steroid hormones produced by steroidogenic gonadal cells and the production of gametes in both male and female teleosts. The importance of gonadotropins in reproduction was first demonstrated in fish following removal of the pituitary gland (Khan *et al*., 1986, 1987) and subsequently confirmed through loss of function studies in mammals (Krishnamurthy *et al*., 2000). The measurement of circulating gonadotropins was then identified as an important tool when investigating vertebrate reproductive physiology (Weltzien *et al*., 2004) as shown by correlative analysis of the timing of gonadotropin expression levels (Mittelholzer *et al*., 2009b). There are two main forms of gonadotropins in teleosts as in most other vertebrates (Redding & Patino, 1993): follicle stimulating hormone (FSH, also referred to as GTH I) and luteinising hormone (LH, also referred to as GTH II). In salmonids, these have been shown to share a common α subunit and a hormone specific β subunit which explain their specific roles in the gonads and are therefore produced at different stages of the reproductive cycle (Prat *et al*., 1996; Planas *et al*., 2000).

Expression of pituitary gonadotropin β subunits during puberty have been investigated in a number of fish species. In females spawning single batches of eggs such as rainbow trout, gonadotropic regulation of gametogenesis is generally characterised by an elevation in FSH during early oocyte growth and vitellogenesis whereas LH is associated with final oocyte maturation and ovulation (Prat *et al*., 1996; Gomez *et al*., 1999; Hassain *et al*., 1999). Alternatively in multiple batch spawners such as Atlantic halibut, gilthead sea bream (*Sparus aurata*), goldfish, Japanese flounder *(Paralichthys olivaceus*), pituitary gonadotropin subunit expression has been seen to increase simultaneously (Sohn *et al*., 1999; Kajimura *et al*., 2001; Weltzien *et al*., 2003a, b; Meiri *et al*., 2004) as the oocytes are developing at different rates. Interestingly, expression of Atlantic cod gonadotropin mRNAs showed elements similar to both single spawner and batch spawner profiles (Mittelholzer *et al*., 2009b). *LHβ* expression increased through the reproductive cycle and peaked in February-March at spawning time, *FSHβ* expression also increased but peaked in December (2 months preceding spawning) then decreased and peaked again at spawning (Mittelholzer *et al*., 2009b). Mittelholzer *et al*. (2008b) thus speculated that the first *FSHβ* increase (September onwards) stimulates the onset of vitellogenesis whereas the second peak may be associated with ongoing vitellogenesis that runs in parallel with final oocyte maturation and ovulation during production of several batches of eggs. It has been suggested that the sequential gonadotropic activation of ovarian follicle growth and maturation in repetitive spawners is probably regulated by modulating the temporal expression of the gonadotropin receptors in the follicle (Kobayashi *et al*., 2008). In the Nile tilapia and zebrafish, the expression of the FSH receptor (*FSH-R*) and LH receptor (*LH-R*) mRNA expression are mainly associated with different phases of oocyte development with *FSH-R* mostly expressed during vitellogenesis and *LH-R* playing a role during final oocyte maturation

(Hirai *et al*., 2002; Kwok *et al*., 2005). Results on follicular expression in Atlantic cod and halibut are consistent with these. In Atlantic cod, analysis of GTH-R mRNA expression throughout a reproductive cycle showed a moderate increase in *FSH-R* during gonadal growth and a peak of expression in *LH-R* mRNA expression at spawning (Mittelholzer *et al*., 2009a). All these results therefore strongly indicate that the two receptors differ in numbers depending on the follicular stage. It has therefore been speculated that asynchronous oogenesis is regulated by different temporal profiles of the ovarian GTH-Rs that result in differential follicular responsiveness to circulating gonadotropins (Kobayashi *et al*., 2008). In male fish, FSH has been described to play a role in spermatogenesis whereas LH is associated with spermiation (Carillo *et al*., 2010). In Atlantic cod Almeida *et al*. (2009a) demonstrated high *FSH-R* mRNA expression at the start of testicular development (July) while at the more advanced stages of spermatogenesis and during spermiation (March-April), significantly high *LH* mRNA expression was recorded in parallel with elevated androgen levels (i.e. testosterone and 11KT).

2.4.3 Sex steroids and the control of gametogenesis

The BPG axis ends with the production of sex steroids at the gonadal level. There are three main types of steroids: androgens, oestrogens and progestagens. Androgens including testosterone (T) and 11-ketotestosterone (11KT), produced in the Leydig cells within the testis, are the dominant sex steroids involved in spermatogenesis in male teleosts (Kime, 1993; Borg, 1994; Weltzien *et al*., 2004). In particular, 11KT is considered as the main androgen (Borg, 1994), it can induce all stages of spermatogenesis as shown in the Japanese eel *Anguilla japonica,* through organ culture experiments (Miura *et al*., 1991a). In male Atlantic halibut, three stages in the endocrine control of spermatogenesis were characterised (Norberg *et al*., 2001; Weltzien *et al*.,

2002). In the first stage of spermatogenesis consisting of spermatogonial proliferation and meiosis with the formation of spermatocytes, T and 11KT levels remained low. In the second stage, increasing T and 11KT levels marked the presence of haploid germ cells including spermatozoa. In the third stage with regressing testis and Sertoli cells displaying signs of phagocytotic activity, T and 11KT levels returned to low levels. In Atlantic cod, plasma T levels have been found to increase from spermatogonial proliferation to meiosis whereas 11KT levels increased during spermiogenesis and spermiation (Almeida *et al*., 2009b). In females, although levels of 11KT are generally considerably lower than in males, seasonal changes can still be measured (Mayer *et al*., 1992). Females also display a marked increase in T towards the spawning season (Mayer *et al*., 1992). T is a precursor to the oestrogen 17β-estradiol (E_2) which plays an important role in the female teleost reproductive cycle (Kime, 1993). As ovarian recrudescence begins E_2 is secreted by the ovarian follicle and stimulates the hepatic synthesis and secretion of vitellogenin which is taken up by the oocyte (Nagahama, 1994). Oestrogens are considered as a 'female' hormone but are also found in male teleosts and it has been suggested that they have an important role in regulating gene expression in the testis (Schulz *et al*., 2010). Progestagens have been shown to play a major role during advanced stages of gametogenesis in both male and female teleosts. In females, progestagens such as 17α, 20β-dihydroxy-4-pregnen-3-one (also called DHP) have been found to play an important role in final oocyte maturation, namely germinal vesicle breakdown (GVBD) (Nagahama *et al*., 1987; Nagahama, 1994) which is an essential process prior to ovulation. In males, high plasma levels of DHP have been found in salmonid species during spermiation such as rainbow trout (Ueda *et al*., 1984) and it is thought to be involved in the acquisition of sperm motility (essential for fertilisation) by increasing the pH of the sperm duct thus increasing cAMP in sperm and

facilitating motility (Miura *et al*., 1991b). Furthermore precocious spermiation can be induced by an injection of DHP (Nagahama *et al.*, 1994). As well as their role in gametogenesis and secondary sexual characteristics, sex steroids are of prime importance in the feedback control of reproductive development and provide an indicator to the brain and pituitary as to the reproductive state of the animal (Zohar *et al*., 2010). Both the brain and pituitary have been found to contain high densities of estrogen and androgen receptors (Navas *et al*., 1995; Blazquez & Piferrer, 2005). Whether feedback is positive or negative depends on the physiological status of the individual and the species itself (Zohar *et al*., 2010). For example, a negative effect of estradiol on FSH synthesis was found in salmonids (Saligaut *et al*., 1998) whereas in goldfish estradiol resulted in a positive increase in FSH expression (Huggard-Nelson *et al*., 2002).

3. Control of sexual maturation in aquaculture

3.1 Strategies to suppress sexual maturation in aquaculture

Five strategies have been identified and/or commercially implemented to prevent, suppress and/or avoid sexual maturation during on-growing: 1) environmental manipulations, 2) monosex production, 3) sterility, 4) selective breeding and 5) feed management. Due to the diversity of reproductive strategies employed by fish, it is necessary to tailor the management strategy for each species as will be outlined below.

3.1.1 Photoperiodic manipulation

In temperate fish species, reproduction is generally entrained by seasonal variations in environmental conditions (namely photoperiod and water temperature) (Bromage *et al.*, 2001) with photoperiod being the principal proximate cue entraining sexual development in most temperate teleosts (Bromage *et al.*, 2001; Migaud *et al*., 2010). Thus photoperiod manipulation through the application of artificial lighting can be used to control the timing of seasonal reproductive patterns in fish and is now widely adopted in

aquaculture to alter spawning season, delay/prevent maturation during on-growing and stimulate growth (Pankhurst & Porter, 2003; Rad et al., 2006; Migaud *et al.,* 2010; Taranger *et al.,* 2010). It is now standard practice in the salmon farming industry (Taranger et al., 1999*)* and protocols have been established for cod in enclosed tank systems (Hansen *et al.,* 2001; Norberg *et al.,* 2004; Davie *et al.,* 2007a). In Atlantic halibut however, photoperiod manipulation to inhibit sexual maturation prior to harvest has not been very successful to date. Norberg *et al.* (2001) suggested that environmental factors will need to be addressed in the early stages of halibut life as this may determine when the fish enters puberty. While considered as a "less invasive" technique than some of the other potential strategies, photoperiod manipulation has a number of problems such as the power costs of running such systems and potential welfare effects of high intensity lighting (see section 3.2.4).

In temperate environments, water temperature has also been shown to factor in the regulation of fish reproduction (Peter & Yu, 1997). In female halibut for example, ovulation can be inhibited by exposing fish to temperatures of 6^0C or higher (Brown *et al.,* 2006). Rather than playing a role in directing puberty however, temperature is thought to play a more permissive ultimate role in complement to dominant control by photoperiod (Taranger *et al.,* 2010). Furthermore it cannot be easily controlled during on-growing and therefore is not used as part of the photoperiod strategy to suppress puberty.

3.1.2 Monosex production

In a number of important aquaculture species, sexual dimorphism exists in which one sex grows faster which is usually related to differences in the timing of puberty between the sexes. It is therefore desirable to culture only the sex that grows best. Effective techniques for monosex production involve the generation of monosex gametes by either

endocrine manipulation during early development or a combination of endocrine and genetic technologies (Donaldson, 1996), these are discussed in detail in section 3.3. Protocols to produce monosex stocks are already available and commercially implemented for rainbow trout, *Oncorhynchus mykiss* (all-female production, Bye & Lincoln, 1986) and tilapia, *Oreochromis niloticus* (all male production). Atlantic halibut also exhibit such a sexual dimorphism with females showing better growth with a later age at maturation which means they can be harvested well in advance of maturation (Hendry *et al.*, 2002; Hendry *et al.*, 2003; Tvedt *et al.*, 2006). Clearly the farming of all female halibut would be advantageous and infact this has already been successfully achieved in Canada with the commercial production of monosex female juveniles. This has not yet been done in Europe due to a combination of consumer perception of hormone treatments and lack of secure protocols for the generation of neomales.

3.1.3 Sterility

In species where both sexes mature before reaching harvest size, the production of sterile populations is a possible approach. The methodologies are however limited and while they focus mainly on the manipulation of ploidy there has also been research into vaccines that inhibit sexual maturation as well as transgenic approaches. Triploidy describes individuals which have three sets of chromosomes unlike normal diploids which have two (Maxime, 2008; Piferrer *et al.*, 2009). This additional chromosome set results in abnormal meiotic division during gametogenesis (Tiwary *et al.*, 2004) and thus female triploids are considered sterile as they have small undeveloped gonads and don't lose body weight as a result of maturation as seen in dipoids. Males on the other hand can develop large gonads and secondary sexual characteristics however the sperm is often recorded as aneuploid thus can not fertilise an oocyte (Tiwary *et al.*, 2004; Feindel *et al.*, 2010). The production of all-female triploid populations thus appears to be a more

effective technique. Triploidy can be artificially induced for commercial purposes by physically shocking newly fertilised eggs to suppress meiosis II to retain the second polar body (Malison *et al.,* 1996; Felip *et al*., 2001; Maxime, 2008). Physical induction includes pressure shocking, which has been successfully applied in rainbow trout (Taylor *et al.*, 2007), Atlantic salmon (Leclercq *et al*., 2010b), sea bass (Peruzzi *et al.,* 2004) and thermal (heat or cold) shock which has been successfully applied in Atlantic cod (Peruzzi *et al.,* 2007), Atlantic halibut (Holmefjord & Refstie, 1997) turbot, *Psetta maxima* (Piferrer *et al.* 2003), sea bass (Peruzzi & Chatain, 2000) and Eurasian perch, *Perca fluviatilis* (Rougeot *et al.,* 2003) among others. Triploidy is already successfully applied in the rainbow trout industry and is under extensive testing in the Atlantic salmon and sea bass industry (Peruzzi *et al.,* 2000, 2001; Zanuy *et al.,* 2001; Taylor *et al.,* in press). In Atlantic halibut, although triploidy has been sucessfully induced (Holmefjord & Refstie, 1997) to our knowledge no further investigations have been carried out in this species. In commercial conditions, it is important to consider that triploidy can only be applied following manual stripping and fertilisation. Thus in species like Atlantic cod, juvenile production methods will have to be radically changed in species where the current standard hatchery production is reliant on mass spawning in tanks.

Despite production protocols being in place there currently remains some scepticism within the industry as to the performance of triploids in comparison to diploids (Benfey, 1999). The performance of triploids has been extensively reviewed by Benfey (1999) and more recently Tiwary (2004) and Maxime (2008). In salmonids, in which triploidy has been studied more extensively than in other teleosts, a number of morphological deformities have been reported and triploid tolerance and survival under sub-optimal conditions is suggested to be poorer (Ojolick *et al.,* 1995; Hyndman *et al.,*

2003a; Atkins & Benfey, 2008), although metabolic and physiological mechanisms and pathways do not necessarily differ (Stillwell & Benfey, 1996; Sadler *et al.*, 2000; Hyndman *et al.*, 2003b; Leggat *et al.*, 2006). In terms of growth, results are variable, triploid growth has been shown as being reduced (Withler *et al.*, 1995), equal to (Galbreath & Thorgaard, 1995; McGeachy *et al.*, 1995; Taylor *et al*., 2007) and improved relative to diploids (Oppedal *et al*., 2003; Taylor *et al*., in press). It is clear that the effects of triploidy vary between species, families and strains and more studies are needed.

In addition to triploidy, other techniques such as transgenics and vaccines have also been tested for the production of sterile fish. The theory behind transgenics for the production of sterile fish is to knock out the genomic copies of a gene whose product is crucial to sexual development and gonad formation. In zebrafish, Weidinger *et al*. (2003) demonstrated that interception in the expression of the dead end (dnd) gene affects the migration of primordial germ cells leading to apoptosis and germ cell deficient gonads. In Tilapia, the GnRH and gonadotropin genes are considered as good candidates for knock-out or knock down expression (Maclean *et al*., 2002). Regarding vaccines, Riley & Secombes (1993) demonstrated that injection of rainbow trout with conjugates of gonadotropin releasing hormone (GnRH) coupled to protein carriers elicited antibody production, they suggested that such anti-GnRH formation could prevent fish maturation by neutralisation of endogenous GnRH. Currently these techniques have not yet been commercially applied in the industry.

3.1.4 Selective breeding

The technique to delay puberty in farmed fish by selective breeding has only been employed in a few farmed fish species so far (Taranger *et al*., 2010). Although selective breeding programs have been shown to effectively delay age at puberty and thereby

increase body size in Atlantic salmon for example (Gjøen & Bentsen, 1997; Gjedrem, 2000), care must be taken as improvements to feed and feeding protocols can result in rapid growth improvement which can counteract the results of breeding programs due to the strong phenotypic link between growth rate and early puberty (Taranger *et al*., 2010). To date, while the inheritance of such traits have not been examined in halibut, in Atlantic cod, positive genetic correlations between spinal deformity, body weight and sexual maturation have been observed which represents a challenge for a selective breeding program (Kolstad *et al*., 2006). So far breeding programs in Atlantic cod haven't been fully efficient however these have only been running for 2 generations (Kolstad *et al*., 2006).

3.1.5 Feed management

The potential of restricted feeding in affecting the age at puberty has been investigated in a number of fish species in the belief that a reduction in energy and adiposity will impact on sexual recruitment. For example, Shearer *et al*. (2006) demonstrated that a restricted feed ration and ultimately reduced body weight reduced the incidence of maturing male Chinook salmon, *Oncorhynchus tshawytscha*. In Atlantic cod however the various starvation regimes which have been tested have been ineffective at reducing fecundity and age at puberty (Kjesbu & Holm, 1994; Karlsen *et al*., 1995). Taranger et al. (2010) suggested that this technique may only be effective for species with more limited energy reserves. Furthermore, if this technique is to be successfully applied in the industry in species which are sensitive to dietary manipulation, impacts on the health and welfare of fish must be taken into consideration (Damsgård *et al*., 2004).

3.1.6 Drivers behind selection of a strategy

The methods used for puberty control are species specific and the technique of choice is

determined by the current understanding of the target species underlying biology (Table 3). Environmental control, namely photoperiod manipulation, requires species with a seasonal reproductive cycle whose reproduction is predominantly determined by daylength and a manageable generation time such as in Atlantic cod (Davie *et al*., 2007a). Monosex production requires species where a sex advantage is conferred such as in Atlantic halibut (Hagen *et al*., 2006). Production of single sex populations in cod would not in itself remove production loss through male sexual maturation. If however maturation were to be inhibited through photoperiod manipulation, female populations would display an improved growth performance over male cohorts of around 10% (Davie *et al*., 2007b). In this respect monosex female production would be of some value. In terms of sterility, triploidy induction is the only method that can be applied in a commercial scale currently; however there are concerns that it may compromise fish welfare and production performance (Benfey, 2001; Felip 2001; Hulata, 2001) and furthermore it can only be applied following manual stripping and fertilisation. Other maturation management strategies include breeding programs and feed management. Breeding programs usually take multiple generations to reduce the problems associated with early puberty however and they are costly. Feed management likely requires species with limited energy reserves and a small body size. For these reasons, the most viable stratagies for our target species are photoperiod manipulation in Atlantic cod and monosex production in Atlantic halibut. While these methods have already been explored and infact implemented in commercial culture in some locations there remains a number of concerns/challenges relating to their effective implementation which are explored further in the following sections.

Table 3. Drivers behind the selection of methodology for control of puberty in teleosts during on-growing. Examples of species in which protocols have already been implemented commercially (bold) or have been developed (underlined) or only tested (italic) given.

Methodology	**Underlying biological requirements**	**Species**
Environmental (photoperiod) manipulation	• Seasonal species • Short to medium generation time	**Atlantic salmon (Taranger *et al*., 1999)** **Atlantic cod (Davie *et al*., 2007a)** European sea bass (Felip *et al*., 2009)
Monosex production	• One sex exhibits more favourable characteristics for culture	**Atlantic halibut (Hendry *et al*., 2003)** **Rainbow trout (Bye & Lincoln, 1986)** **Tilapia (Phelps & Popma, 2000)** *Atlantic cod (*Haugen *et al., 2011)*
Sterility: triploidy	• Available supply of good quality eggs i.e. eggs which can easily be stripped from broodstock	**Atlantic salmon (O'Flynn *et al*., 1997; Oppedal *et al*., 2003)** **Rainbow trout (Oliva-Teles & Kaushik, 1990)** Sea bass (Peruzzi *et al*., 2000) *Atlantic halibut (Holmefjord & Refstie, 1997)* *Atlantic cod (Peruzzi et al., 2008)*
Selective Breeding	• Genetic variation is associated with important traits • Good heritability rates of desirable traits i.e. growth	**Atlantic salmon (Gjøen & Bentsen, 1997)** Rainbow trout (Kause et al., 2003) Tilapia (Ponzoni *et al*., 2011) *Atlantic cod* (Kolstad *et al*., 2006)
Feed Management	• Individuals which are sensitive to dietary manipulation i.e. with limited energy reserves	Chinook salmon (Shearer et al., 2006) *Rainbow trout (Bromage & Jones, 1991)* *Atlantic salmon (Bromage et al., 2001)*

3.2 Photoperiodic regulation of puberty

While the principle of photoperiod manipulation is conceptually a simple process, the mechanism which connects the perception of environmental signals to the entrainment of the reproductive axis is complex. However such knowledge is essential to ensure that manipulations are effective and can not be considered to harm the target animals.

3.2.1 Photoneuroendocrine system

Current knowledge on the photoneuroendocrine system (PNES) through which photoperiod rhythm regulates reproduction and physiological processes in fish has recently been reviewed by Migaud *et al*. (2010). The first step in this PNES is the circadian axis, which regulates the perception of light and transduction into a biological signal to act as a relay of 'time' to target areas and entrains biological rhythms (Falcon, 1999; Foster & Hankins, 2002). In mammals, light is perceived by the retina and this information is then relayed to the suprachiasmatic nucleus (SCN) of the brain which contains the "master" circadian clock (Simmoneux & Ribelayga, 2003). Subsequently this master clock controls mechanisms that regulate melatonin synthesis and secretion from the pineal (Schomerus & Korf, 2005). In teleosts, light perception is more complex, as well as photoreception by the eyes, the pineal gland also appears to be directly photosensitive in most teleosts studied unlike in mammals (Forsell *et al*., 2001; Falcon *et al*., 2010) and it is also believed that deep brain photoreceptors may play a role in light reception in teleosts as for other vertebrates (Menaker *et al*., 1997). The photosensitivity of each component and their relative importance differs between species, for example in tropical species such as the Nile tilapia, melatonin release has been shown to be exclusively dependent on photoreception from the eyes (Martinez-Chavez *et al*., 2009). In contrast, a combination of photic information from the eyes and the pineal are necessary for typical plasma melatonin production in Atlantic cod and sea bass while

only the pineal regulates melatonin production in salmonids (Bayarri *et al*., 2003; Migaud *et al*., 2007b). The presence of a central "master" clock in teleosts remains an open question as despite much circumstantial evidence for its presence (Holmqvist *et al*., 1992; Falcon *et al*., 2007), in-situ studies of clock gene expression in species like zebrafish have not defined any such localised expression (Whitmore *et al*., 1998, 2000). The principle output of the circadian axis is the indoleamine hormone melatonin which displays day (low) and night (high) rhythms. It is now widely known that this profile of synthesis and release is down to the degradation of the enzyme arylalkylamine *N*-acetyltransferase (AANAT) which plays a key role by catalyzing the conversion of serotonin to *N*-acetylserotonin which is ultimately methylated to produce melatonin (Klein *et al*., 1997). High levels of melatonin are produced during darkness, with the duration of this release proportional to night length, whereas lower levels are produced during daylight (Bromage *et al*., 2001; Migaud *et al*., 2010; Falcon *et al*., 2010). Thus melatonin release acts as an endocrine rhythm providing information on both the time of day and seasonal date to the fish. Measurement of melatonin is therefore a valuable tool to interpret perception of photoperiod regimes. While melatonin analysis has helped define light perception in fish, the functional link between plasma melatonin levels and the endogenous control of maturation through the BPG axis remains unclear (Migaud *et al*., 2010). In contrast, in mammals melatonin has been shown to act at various sites such as the pituitary, SCN and hypothalamus depending on the species to stimulate the reproductive axis (Goldman, 2001). In the pituitary, melatonin is thought to act either independently or through the clock gene system to entrain prolactin cycling which in turn controls a number of key functions during sexual development (Bachelot & Binart, 2007; Dardente, 2007; Dupré *et al*., 2008). In the hypothamus, it is thought that melatonin stimulates kisspeptin expression which in turn regulates GnRH function

(Revel *et al*., 2006a; 2007). It is also thought to stimulate the expression of type 2 iodothyronine deodinase (*Dio2*) which converts thyroxine (T_4) to the active form triiodothyronine (T_3) which regulates functions such as sensitivity of steroid feedback during reproduction (Yasuo *et al*., 2007; Morgan & Hazlerigg, 2008). In fish, melatonin appears to play a more detached role in reporting timing and synchronizing reproduction. However, interestingly, a recent study by Servilli *et al*., (2010) in sea bass discovered that the pineal organ receives GnRH-2 immunoreactive fibers originating from the synencephalic GnRH-2 neurons. Furthermore *in vitro* and *in vivo* experiments demonstrated stimulatory effects of GnRH-2 on nocturnal melatonin secretion by the sea bass pineal organ. This data showed, for the first time in a vertebrate species, converging evidence supporting a role of GnRH-2 in the modulation of fish pineal function. However, the lack of clear support for melatonin regulating reproduction in teleosts suggests that other driving forces might also be in place. For example, it has been suggested that the clock-gene system in fish is associated with the entrainment of reproduction from observations of the maintenance of endogenous cycles in reproduction in species such Atlantic cod (Norberg *et al*., 2004), sea bass (Carillo *et al*., 1995) and rainbow trout (Randall *et al*., 1998). In addition, in a QTL study, Leder *et al*. (2006), mapped the genes *Clock* and *Per1* to quantitative trait loci that explained up to 50% of the variance in spawning time, while other candidate genes including components of the melatonin synthesis pathway (*AANAT-1* and *AANAT-2*) were not significantly associated. It is believed that the interplay of seasonally changing environmental cues regulate the entrainment of melatonin and possibly clocks to entrain reproductive cycles although the functional link is yet to be found (Migaud *et al*., 2010). Furthermore, with the knowledge of the kisspeptin system as discussed earlier, it is believed that this too could act as a missing link between the environmental and endogenous control of

reproduction. In summary so far, while elements of the PNES have been described in teleosts, a clear description of the completed pathway remains to be presented. However, popular consensus is focusing on the interface of the PNES outputs like melatonin & clock-genes with initiators of the BPG like kisspeptin to finally complete such a description (Migaud *et al*., 2010).

3.2.2 Species specific photoperiodic regimes

Once the photoperiodic signals that entrain reproduction are defined in a species (Table 4), it is possible to design artificial regimes to manipulate reproduction. Such regimes can be used to either produce out-of-season spawning from broodstock through adjusted seasonal cycles or suppress puberty (early maturation) during on-growing through either the masking of the natural photoperiod cycle (e.g. cod, Davie et al., 2007a; Taranger *et al*., 2010) or using advancing regimes (e.g. salmon, Taranger et al., 1999; Migaud *et al*., 2010,) to skip spawning. In enclosed conditions (indoors/tanks), the management of lighting regimes is relatively easy. For example, low intensity lighting (i.e. tungsten filament or halogen bulbs) has been demonstrated to effectively manipulate photoperiod thus controlling maturation in a range of species including Atlantic cod (Hansen *et al*., 2001; Norberg *et al*., 2004; Davie *et al*., 2007a), Atlantic halibut (Norberg *et al.*, 2001), Atlantic salmon (Oppedal *et al*., 1997; Porter *et al*., 1999; Taranger *et al*., 1999), pink salmon, *Oncorhynchus gorbuscha* (Beacham *et al*., 1993), masu salmon, *Oncorhynchus masou* (Takashima *et al*., 1984), rainbow trout (Davies *et al*., 1999), turbot (Imsland *et al*., 2003) and sea bass (Zanuy *et al*., 1995). However, in open cage systems, owing to the overlying ambient photoperiod, light management is more difficult and success is dependent on species and rearing system. In Atlantic salmon, application of constant artificial lighting using metal halide technology in on-growing cages from winter solstice onwards for several months successfully prevents maturation (Oppedal *et al*., 1997;

Porter *et al*., 1999; Taranger *et al*., 1999; Peterson & Harmon, 2005; Schulz *et al*., 2006). In such a regime, the artificial lighting effectively 'masks' the ambient prevalent photoperiod, this has been indicated by permanently suppressed nocturnal and diel melatonin levels in salmon under such conditions (Porter *et al*., 1999). Photoperiod manipulation in salmon on-growing cages is therefore now standard practice in the salmon farming industry. On the contrary, in Atlantic cod, photoperiod manipulation using metal halide in cage systems has not been fully effective with at best a 4 month delay in spawning (Taranger *et al*., 2006). Due to the failure of these manipulations in open cage systems it was proposed that cod were able to perceive changes in the ambient photoperiod over and above the artificial lighting, described as relative photoreception (Taranger *et al*., 2006; Davie *et al*., 2007a; Vera *et al*., 2010). This has required extensive research into non visual light perception and the lighting technologies used.

3.2.3 Fish light sensitivity and lighting technology

When employing photoperiod manipulation for the prevention of spawning, it is important to take into account species biological differences not only in terms of seasonality (i.e. timing of regime application) but also light sensitivity, behaviour and to consider the environment in which these regimes are to be applied (i.e. seawater). In terms of light sensitivty, *in-vivo* as well as *in-vitro* pineal studies in a number of teleost species have shown a gradual suppression of melatonin in response to night-time illumination depending on the spectrum and intensity of light exposure (Ekstrom & Meissl, 1997; Migaud *et al*., 2006). For example, *in vitro* and *in vivo* experiments on salmon and sea bass have demonstrated the effectiveness of shorter wavelengths (blue-green) in reducing melatonin levels, in comparison to longer wavelengths (red) (Bayarri *et al*., 2002). Also these wavelengths have been proven to penetrate seawater more efficiently than longer wavelengths (Leclercq *et al*., in press). Regarding light intensity,

a study on pineal light sensitivity by Vera *et al*. (2010) suggested that cod are 10,000 times more sensitive to light than salmon. Furthermore they demonstrated that this sensitivity depends on preceeding light exposure. For example, suppression of night-time melatonin levels depends on the intensity of light perceived during the day this is defined as "relative photoreception". This generates the theory that if daytime lighting is reduced then the threshold light intensity required to suppress night-time melatonin levels is also reduced and could be tested in outdoor conditions using shading. Thus potentially preventing fish from perceiving a report of daylength. When considering light sensitivity in fish species it is also important to take into account the difference in photic behaviour between species. For example salmon show attraction to light whereas cod do not. This has implications for the implementation of lighting regimes in aquaculture.

At present, metal halide lighting is the predominant lighting system used in marine fish on-growing cages to manipulate photoperiod. Such systems were originally developed for salmon and they have been very successful in this respect despite being associated with high running costs. However, it is thought that their biological efficiency (i.e. reduced prevalence of maturing fish) and energy consumption could be improved using alternative technologies (e.g. light emitting diode) (Leclercq *et al.,* 2010). Metal halogen units emit a broad spectrum of light with a series of discrete peak wavelengths throughout the visible spectrum and a significant proportion of it (towards the red end of the spectrum) is absorbed within the first few meters of the water column (Migaud *et al*., 2007a). Consequently, multiple units are required in order to expose whole cage volumes to high light intensities (which are still often inadequate in the case of cod) which results in excessively high energy running costs. New lighting technologies: Cathode Lighting (CL) and Light Emitting Diodes (LED), now available, will help to address this problem as they can be tuned to emit narrow bandwidths of light specific to the photic properties

Table 4. Seasonal environmental temperate cues in temperate teleost species (taken from Migaud *et al*., 2010).

Species	Initiation Window			Completion window			References
	Prevailing Daylength	**Specific thermal requirement**	**Gametogenesis onset**	**Prevailing daylength**	**Specific thermal requirement**	**Time of spawning**	
Gadus morhua	Decrease	No	June-Dec	Increase	Yes	Feb-June	Davie *et al*. (2007a)
Hippoglossus hippoglossus	Increase	No	Jan-June	Decrease	Yes	Dec-April	Haug (1990) Norberg *et al*. (2001)
Salmo salar	Increase	No	Jan-Apr	Decrease	Yes	Oct-Dec	Taranger *et al*. (1999)
Dicentrarchus labrax	Decrease	Yes	June-Dec	Increase	Yes	Jan-June	Moretti *et al*. (1999)

of the water body and also fish light sensitivities. However, there is limited information regarding the potential 'welfare' impact of such artificial lighting technologies on fish.

3.2.4 Artificial light and welfare in fish

There is growing awareness that good welfare equates to greater production success. With the increasing number of light units of escalating power and efficiency and new narrow bandwidth technologies which are being introduced to the aquaculture industry, it is essential to understand the potential adverse impacts of artificial lighting properties on the 'welfare' of fish in order to promote health and growth whilst aiming to prevent maturation (Pickering, 1993; Ashley, 2007; Bowden, 2008). High intensity point sources of artificial light in combination with daylight, present levels of lighting beyond which a fish would be exposed in its natural environment. Furthermore, narrow bandwidth emission designed to target a species specific light sensitivity may act to increase the risk of light damage. Short wavelengths of light are considered to be much more harmful in higher vertebrates than long wavelengths (Young, 1988; Dawson *et al*., 2001; Migaud *et al*., 2007a). The general framework for evaluating welfare and suffering in both terrestrial animals and farmed fish is based on the Five Freedoms (FAWC, 1979). These include freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury or disease, freedom to express normal behaviour and freedom from fear and distress, a variety of indicators are used to assess impairment of these freedoms (Table 5). The framework recognises that animal welfare is complex, reflected in many physiological and behavioural traits, thus a combination of different indicators offers a much more useful assessment than relying on a single indicator (FSBI, 2002; Lembo & Zupa, 2009). Such indicators in fish may include measurement of the stress response (cortisol, glucose), immune function and organ damage, feed intake and swimming behaviour. In the past, the issue of welfare with regard to artificial lighting in fish has not

been investigated, recent work however has been conducted to directly address such concerns, with attention focussed more on the stress and pathological effects of high intensity lighting on fish, relating to freedom from fear and distress, freedom from discomfort and freedom from pain, injury or disease. Migaud *et al*. (2007a) studied the effect of high intensity blue LED light on post-smolt salmon and recorded an acute stress response (short-term peak in cortisol and glucose) following onset of exposure to constant high intensity blue LED light, however, there was no recorded significant effect of constant artificial lighting on the immune system specifically lysozyme activity. They also studied the visual system, extensive examination of Atlantic salmon retina following exposure to artificial light revealed no signs of light induced damage, this was thought to be due to highly efficient protective mechanisms (melanin granule migration) recorded in retina from this trial. The fish eye is known to be vulnerable to artificial light (Dawson *et al.*, 2001; Vihtelic *et al.*, 2000, 2006; Vera *et al.*, 2009) due to the absence of eyelids (palpebra) and a fixed pupil aperture (Ferguson, 2006) thus high intensity regimes could result in retinal damage potentially impacting feeding behaviour in visual feeding fish such as Atlantic salmon. Vera *et al.* (2009) demonstrated that the cod retina is much more susceptible to artificial light damage than the salmon and sea bass retina with earlier signs of retinal damage and a greater reduction in the thickness of the photoreceptor layer which parallels the previous findings on pineal sensitivity (Vera *et al*., 2010). This clearly warrants further investigation.

It's important to note that studies investigating the welfare impact of artificial lighting on fish have clearly highlighted species and environment specific light sensitivity. This emphasizes the need to determine the light sensitivity of specific species intended for photoperiod manipulation, not only to tune lighting set-ups to their perceptive range but to limit potential light-induced adverse effects on welfare.

Table 5. The five freedoms of animal welfare and the indicators used to assess impairment from these freedoms (taken from Lembo & Zupa, 2009).

Five Freedoms of Animal Welfare		**Indicators**
1	Freedom from hunger and thirst	Feed intake, growth rates, condition factor
2	Freedom from discomfort	• Physical damage: fin condition, cataracts, lesions • Immune responses (e.g. lysozyme activity, respiratory burst activity, phagocytic activity)
3	Freedom from pain, injury or disease	• Environmental monitoring: water quality monitoring (dissolved oxygen, ammonia, pH, carbon dioxide, suspended solids) • Targeted sampling of fish: gill condition and checking for parasite infestation
4	Freedom to express normal behaviour	Abnormal behaviour: swimming and feeding behaviour, distribution of the fish within a system (eg. clumping around inflows), response of fish to an approaching farmer
5	Freedom from fear and distress	Measuring primary and secondary stress responses: plasma, cortisol, glucose, lactate, muscular activity

3.3. Monosex production

In Atlantic halibut, monosex production is the technique of choice and the production of all female halibut stocks would represent a significant boost to the profitability of the industry. However this is only achievable through an advanced understanding of the mechanisms by which sex is expressed (see section 2.1). With such an understanding it is possible to then employ a number of different methodologies to influence the gender

of stocks for on-growing. While some of these methods are long established in aquaculture, gender control is a problem common to most agricultural systems which means novel technologies are always being developed which have the potential to radically change management practice if they can be effectively translated into the aquaculture environment.

3.3.1 Endocrine therapy

The manipulation of sexual phenotype can be achieved by exposing sexually undifferentiated fish during the labile period to exogenous sex steroids in order to direct the process of sex differentiation towards the desired sex (Donaldson, 1996). The labile window varies between species (See review by Piferrer, 2001), for example in Atlantic halibut phenotypic sex can be affected up to 30mm total length (Hendry *et al.,* 2002) and in Atlantic cod the window lasts up to 25mm (Haugen *et al.,* 2011). For the production of monosex populations, it is important to determine the time window of sex differentiation in order to identify the sex steroid window to alter phenotypic sex. On occasion, sex reversal by exogenous steroids has been reported to be short-lived i.e. fish revert back to their original sexual phenotype (Olito & Brock, 1991), however generally the effects are permanent. Monosex populations can be produced by either direct or indirect sex reversal. Direct sex reversal refers to the exposure of mixed sex larvae to sex steroids in order to directly generate the selected single sex population. In Nile Tilapia, treatment with synthetic testosterone (in-feed, immersion) successfully masculinises a population (Gale *et al.,* 1996; Kwon *et al.,* 2000; Wassermann *et al.,* 2003). The use of direct hormone therapies in food fish production is banned in the EU (Directorate general for health and consumers, 2003) however thus methods have been developed to control sex while not directly treating fish destined for human consumption. Indirect sex reversal involves the hormonal treatment of juvenile fish that are then used as

broodstock in order to produce progeny of the desired sex. This works on the basis that the application of exogenous steroids changes only the phenotype of the individual and not the genotype. For example in halibut, with an XY genetic sex determination system and where all-female populations are the primary production goal, masculinised female broodstock (XX genotype, referred as 'neomales') obtained by 17α-methyldihydrotestosterone (MDHT) treatment prior to sex differentiation, are crossed with normal females (XX) to naturally produce all-female progeny (Hendry *et al.,* 2003; Tvedt *et al.,* 2006). Hendry *et al.* (2003) have successfully developed a protocol to produce all-female Atlantic halibut populations using this technique. Other species in which indirect sex reversal has produced all-female stocks include the rainbow trout (Bye & Lincoln, 1986), Atlantic salmon (Johnstone & Youngson, 1984), the yellow perch, *Perca flavescens* (Malison *et al.*, 1996) and Nile tilapia (Mair *et al*., 1991). The advantage of this method is that fish intended for consumption have not been in direct contact with the synthetic hormones. However this method is time consuming as it involves more than one generation (Piferrer, 2001). Furthermore it is important to understand the genetic determination system of the species in mind in order to produce effective broodstock. For example, in Atlantic halibut, in order to produce all-female populations using masculinised females, it is necessary that the female is the homogametic sex. Monosex Atlantic halibut are already produced commercially in Canada however it has not been yet commercially performed in the UK.

3.3.2 Gynogenesis

An alternative means to produce monosex populations is by parthenogenetic (uniparental) reproduction, a process which does not require the use of exogenous hormones (Komen & Thorgaard, 2007). Androgenesis refers to the process whereby offspring inherit only paternal DNA, this can be achieved by fertilising eggs with sperm

which has been irradiated with UV radiation to destroy their nuclear content (Thorgaard, 1992; Marengoni & Onoue, 1998). Gynogenesis is a similar process where offspring inherit only maternal genetic material (Felip *et al.,* 2001; Piferrer, 2001). Gynogenesis can be induced by stimulating an egg to divide using genetically inactive (i.e. UV or gamma irradiated) spermatozoa, thus there is no genetic contribution of the sperm. The haploid embryo is then temperature or pressure shocked to prevent polar body expulsion and thus restore diploidy (Piferrer, 2001). Induced gynogenesis has been used commercially as a technique for monosex production of Silver barb, *Puntius gonionotus,* in Thailand (Pongthana *et al.,* 1999) and it has been successfully performed in Atlantic halibut (Tvedt *et al.,* 2006). However due to the significantly lower viability of gynogenetic embryos, in combination with the fact that a proportion of these females usually possess abnormal ovaries (Piferrer *et al.,* 1994) all of which is perhaps a consequence of inbreeding, means that females obtained through induced gynogenesis are not generally suitable for direct production and thus at present gynogenesis is more commonly performed on species for research purposes (Piferrer, 2001).

3.3.3 Semen sexing

Current techniques for monosex production have disadvantages such as long timeframes required to produce stocks (e.g. >5 years in Halibut) as well as public health concerns over the use of hormones. Owing to these problems, a potential new avenue for monosex production would be 'semen sexing'. In terrestrial agricultural systems, successful protocols based on identifying and isolating 'sexed' sperm cells have already been established for monosex production (Garner, 2001). The technique involves using flow cytometry to differentiate between X and Y sperm based on DNA content and is only possible in species where males are the heterogametic sex. These sperm cells can then be automatically sorted and the desired sex of spermatozoa collected and used to fertilise

egg batches (Garner, 2001). To date there has been no research on this field with teleost spermatozoa which is primarily due to the fact that success may be limited since there is a limited data suggesting that the DNA content between male and female teleost species differs (Devlin & Nagahama, 2002). However if proven to work it has the potential to radically shorten the time to generate monosex stocks fit for human consumption which warrants its consideration as a new technology to be employed.

3.3.4 Environmental sex manipulation

A final "natural" way to influence sex ratios in production stocks is by manipulation of environmental factors such as temperature, pH, stocking density and social interaction (Baroiller & D'Cotta, 2001; Penman & Piferrer, 2008). In species such as the medaka, *Oryzias latipes*, and sea bass, temperature has been shown to influence sex during the sexually labile period (Hattori *et al.,* 2007; Koumoundouros *et al.,* 2002; Saillant *et al.,* 2002) with higher temperatures yielding male skewed populations and lower temperatures favouring the formation of females in the latter species (Pavlidis *et al.,* 2000). However, rather than temperature playing a dominant role in determining the sex of fish it is thought to act as an adaptive value interacting with genetic sex determination (Baroiller & D'Cotta, 2001; Ospina-Álverez & Piferrer, 2008). In sea bass, females grow faster than males in farmed populations it is therefore of great interest to develop monosex female sea bass populations. In order to do this it is important to understand the basis of sex determination in this species considering both genetic and environmental influences. Saillant *et al.* (2002) demonstrated that genotype-temperature interactions modulated the sex-ratio of sea bass and suggested that the effects of temperature in monosex production may be eliminated by selecting non sensitive breeders (Saillant *et al.,* 2002). In halibut, the effects of rearing temperature on juvenile sex ratios were tested during the time of gonadal differentiation however there appeared to be no signifcant

influence on sex ratios (Hughes *et al*., 2008). Potential temperature effects on sex ratio earlier in development (prior to gonadal differentiation) have been recorded (van Nes & Andersen, 2006) however hormone treatments applied after this time generate successful sex reversed populations. In the rearing of putative monosex progeny (from a neomale parent) it may be considered that temperature during early development could cause a deviation from the expected 100% female outcome.

4. Aims of the thesis

Early puberty is a major problem during the on-growing stage of fish farming and in order for the industry to expand, a delay or cessation in puberty is crucial. Currently there are five management techniques which exist for the control of this problem as described above. However, there are major limitations in the commercial use of these. With the proposed expansion of the Atlantic cod and halibut industry it is of great importance to optimise management techniques for the control of puberty in these species. Photoperiod manipulation is currently the technique of choice in Atlantic cod as it exhibits a temperate reproductive cycle and has no clear sex advantage, furthermore it undergoes spontaneous mass spawning in tanks thus making timely stripping for triploidy production difficult. Photoperiod manipulation in cod works effectively in tanks however in the outdoor environment even with the application of constant artificial lighting, large differences in day and night light intensity still exist thus maturation is not effectively inhibited. For these reasons fish are being exposed to ever increasing intensities of light and narrow wavelengths are being introduced, the potential adverse impacts of this lighting on cod welfare however are unknown. At the same time the potential of shading of ambient light during the daytime is coming to the forefront however this has not been scientifically tested to date. This lack of effective control, in part, is due to our continued lack of understanding of the neuroendocrine mechanisms

which control the onset and completion of puberty. While it is known that puberty is sensitive to environmental factors, the underlying physiological mechanisms which relay this information are still to be elucidated. In this context, the role of kisspeptin has recently come to light in fishes and it is thought it may act as part of the missing link between the environmental and neuroendocrine control of reproduction which could then help scientists and farmers better understand and then control maturation during farming.

In halibut, the clear dimorphism in sex makes monosex production of females an obvious strategy to prevent early puberty becoming a problem. Monosex production has successfully been performed in Canada by indirect sex reversal however it has not yet been applied in Europe. The time required to monosex populations using indirect sex reversal is time consuming, so advances in methodologies like semen sexing which is standard practice in the cattle industry, potentially offers a faster approach if it can be proven to be effective in the target species.

In summary, detailed scientific knowledge is required regarding stock on-growing management techniques to improve the sustainability and profitability of the aquaculture industry and ensure a higher quality product is readily available to the customers. The overall aim of this thesis was to research and further develop management strategies to be adopted by the UK marine finfish farming sector for the control of maturation in Atlantic cod and halibut.

The specific objectives of this thesis were as follows:

1. To investigate the effect of artificial lighting on the welfare (stress response, innate immunity, eye structure and feeding activity) of Atlantic cod (**Chapter 2**).
2. To investigate the effect of combining shading and continuous lighting on the suppression of sexual maturation in outdoor reared Atlantic cod (**Chapter 3**).

3. To investigate the expression of kisspeptin and gonadotropin genes in Atlantic cod (**Chapter 4**).
4. Research the methodologies for the production of monosex Atlantic halibut in the UK (**Chapter 5**).

CHAPTER 2

RESEARCH ARTICLE

THE EFFECT OF METAL HALIDE AND NOVEL GREEN CATHODE LIGHTS ON THE STRESS RESPONSE, INNATE IMMUNITY, EYE STRUCTURE AND FEEDING ACTIVITY OF ATLANTIC COD, *GADUS MORHUA* L.

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Keywords: *Gadus morhua* L.; artificial light; green cathode; stress; lysozyme; retina, feeding activity

Abstract

High intensity constant lighting is routinely used for photoperiod manipulation in the aquaculture industry in order to prevent early maturation. The potential welfare impacts of this technology however have not been extensively studied to date, and with the implementation of more efficient narrow bandwidth lighting technologies (cathode, light emitting diodes), definitions of species specific sensitivities are becoming essential. The objective of this study was to investigate the impact of traditional metal halide and novel green cathode lighting on the welfare (stress response, innate immunity, retina structure, feeding activity) and light perception of Atlantic cod over a four week period. Results indicated that although acute responses to light were observed, there were no clear significant long term effects of any of the lighting treatments on stress levels (plasma cortisol, glucose), innate immune function (lysozyme activity), retina structure and population feeding activity (acute drop under all light treatments, most pronounced in fish exposed to higher illumination but normal feeding activity was resumed within 8 days following light onset). Regarding light perception, interestingly even when subjected to high intensity constant lighting (metal halide mean tank intensity: 16.6 watts m^{-2}), cod still demonstrated a day-night rhythm in melatonin release which suggests perception of the overlying ambient photoperiod.

1. Introduction

Sexual maturation is a major welfare concern and economic burden during the on-growing of marine finfish as energy is directed into gonadal development resulting in a loss in growth and product quality. Furthermore, during final maturation there is an increased sensitivity to disease, reduction in feeding activity, and concerns exist over potential genetic interaction with native stocks through broadcast spawning or spawning interaction by escapees (Bromage *et al*., 2001). Photoperiod manipulation is an efficient tool used to suppress early maturation in a number of commercially important marine teleosts, especially temperate species such as Atlantic cod, *Gadus morhua* L. (Hansen *et al*., 2001; Davie *et al*., 2003; Davie *et al*., 2007a), Atlantic salmon, *Salmo salar* L. (Endal *et al*., 2000) and European sea bass, *Dicentrarchus* labrax L. (Bayarri *et al*., 2003; Felip *et al*., 2009) where seasonal changes in day-length act as the principal regulator of puberty onset (Bromage *et al.*, 2001). It is believed that the indoleamine melatonin acts as the key light perception hormone and is released by the photosensitive pineal gland (Bromage *et al.*, 2001; Falcon *et al*., 2009), with high levels of melatonin produced during darkness and lower levels produced during daylight (Porter *et al*., 1999; Porter *et al*., 2000; Bromage *et al.*, 2001; Bayarri *et al.*, 2002) thus providing an entraining endocrine message. As such, plasma melatonin measurements are routinely used to assess an individual fish's perception of lighting systems (Porter *et al.*, 2000; Migaud *et al*., 2006). At present, photoperiod is manipulated in commercial, open cage systems through the use of metal halide (MH) light units. These systems however are not specifically designed for aquaculture and thus new, more cost effective technologies, (e.g. Cathode Lighting (CL) and Light Emitting Diodes (LED)) which allow the refinement of spectral content and reduce energy requirement are now being used to develop species and environment specific lighting systems. *In vitro* and *in vivo*

experiments in a number of species including sea bass and zebrafish, *Danio rerio*, have demonstrated the effectiveness of shorter wavelengths (blue-green) in reducing melatonin levels, in comparison to longer wavelengths (red) (Bayarri *et al.*, 2002; Ziv *et al*., 2007). These shorter wavelengths are also known to penetrate seawater more efficiently (Lalli & Parsons, 1993). Currently however, there is almost no published scientific information available regarding the technical performance of such systems in the marine environment. Likewise, there is limited information regarding the potential 'welfare' impact of these artificial lighting technologies on fish.

It is well known that aquaculture practices including stocking density, diet, feeding technique and management procedures may act as stressors in aquaculture and have strong effects on the health and performance of the fish (Pickering, 1993; Wendelaar Bonga, 1997; Schreck *et al*., 2001). It is essential, therefore, that work is conducted on the effects of an abrupt change in lighting conditions and continuous (LL) high intensity light on fish to avoid or mediate detrimental implications (Ashley, 2007; Bowden, 2008). Regarding welfare and the five freedoms framework (FAWC, 2009), there is a combination of possible physiological and behavioural processes that artificial illumination could influence including the stress response, the immune system, eye damage and feeding activity. To date, only Migaud *et al*. (2007) have directly considered the welfare impact of artificial blue LED lighting on Atlantic salmon. While the authors reported no chronic effects in this case, it is important to consider that species specific sensitivities to light do exist (Migaud *et al.*, 2006). Recent *in vitro* pineal studies have revealed that cod, in comparison to salmon and sea bass, have a much higher sensitivity to light (Vera *et al*., 2010). In addition, the cod retina has also been recently demonstrated to be more sensitive to light induced damage than salmon and sea bass retina (Vera & Migaud, 2009). Importantly, in cod on-growing, an increasing number of

light units of escalating power and efficiency are being used in commercial cages as photoperiodic regimes used to date (MH systems) have failed to fully suppress early maturation and at best only caused a 4 month delay (Taranger *et al*., 2006). However, so far no studies have been performed to investigate welfare indicators in cod, regarding the effects of these increasingly high intensity constant regimes.

The objective of our study was thus to investigate the potential welfare impact of two different types of artificial lighting (CL and traditional MH) currently being used to suppress maturation in commercial cod aquaculture through analysis of the stress response, innate immunity, retinal structure, feeding activity, and also to determine cod light perception of these systems.

2. Materials and Methods

2.1 Fish stock and initial rearing conditions

The trial was conducted at the Machrihanish Marine Environmental Research Laboratory (MERL, 55:44^{0}N, 5:44^{0}W) between 6th June and 16th August, 2007. Groups of 50 mixed-sex juvenile Atlantic cod produced by MERL (mean wet weight ± SEM = 142 ± 3g) previously reared in tanks under simulated natural photoperiod and ambient temperature regimes, were randomly stocked into ten white 2 m diameter, covered tanks (volume 1.6 m^3 , 0.5 m deep, approx. initial stocking density: 4.4 kg m^{-3}). Within each population, 20 individuals were selected at random and implanted with a passive integrated transponder tag (Avid Plc, Uckfield, UK). All tanks were supplied with fresh seawater, filtered to 60µm, at a flow rate of approximately 50 L min^{-1} and drained to waste. Water temperature during the trial was 14 ± 1^{0}C.

2.2 Experimental conditions

Fish were initially maintained on a 6-week acclimation period under a control simulated natural photoperiod regime (SNP, experimental light units were fitted in tanks but remained off). This control lighting was provided by two 9W fluorescent bulbs (Osram Dulux, S G23 energy saver, St Helens, UK) that were located on the underside of tank lids. Their operation was regulated by digital timers which were adjusted weekly to match the ambient photoperiod throughout the trial. Intensity measured at the water surface was 0.32 watts m^{-2} when illuminated. Intensity measurements (watts m^{-2}) were performed using a single channel light sensor set to a wavelength range of 400-740 nm (Skye Instruments Ltd., UK) and calibrated to National Physics Laboratory (UK) standards. Spectral content was recorded using a portable spectroradiometer (Model EPP 2000c, Stellarnet Inc., Tampa, USA).

Following acclimation, fish were randomly assigned to one of five light treatments (duplicated) for 4 weeks. Control lighting was provided by fluorescent bulbs (as during acclimation) and experimental lighting was provided by green cathode (CL, 40W, Intravision Aqua, Oslo, Norway) or metal halide (MH, 400W, BGB engineering, Grantham, UK) units. Experimental treatments were designed to mimic the intensities that fish would be exposed to if they were to remain in close proximity to the lighting systems in a cage environment ($\leq$ 1.5 m) and were set to a continuous light (LL) or a simulated natural photoperiod (SNP) regime, daylength for SNP treatments ranged from 16 hours at the start of the test period (19th July) to 15 hours at the end (16th August). Treatments were as follows: **1)** Control (SNP), **2)** Low CL (1 unit, LL), **3)** High CL (4 units, LL), **4)** MH-LL (1 unit), **5)** MH-SNP (1 unit). An SNP metal halide treatment was included in the trial in order to determine if there was an effect of darkness following the

highest intensity day-time lighting (Table 1, see Appendix 1 for a schematic diagram of light unit set-up).

Table 1. Mean light intensities recorded in tanks (watts m^{-2}) and the distance mimicked from the respective light unit in a cage. Data presented as mean ± SEM (n = 12).

Treatment	**Photoperiod**	**Light Intensity (watts m^{-2})**	**Position mimicked in a cage (distance from unit, m)**
Control	SNP	0.08 ± 0.03	-
Low CL	LL	0.47 ± 0.18	1.5
High CL	LL	0.82 ± 0.15	1
MH	(LL + SNP)	16.58 ± 8.77	1

Regarding wavelength, the green cathode units emitted a clear prominent green peak (546 nm) whereas metal halide units emitted a broader range of wavelength throughout the visible spectrum (Fig.1).

Fish were fed to satiation on commercial cod feed (Start/Pearl diet, Biomar, Grangemouth, UK) according to the manufacturer's guidelines via clockwork belt-feeders throughout the ambient daylight period. In order to obtain data on population feed intake, tanks were also hand-fed to satiation four times (9:30,12:00,14:30,17:00) throughout the daylight period over five days prior to light onset (baseline feeding activity) and 11 days following.

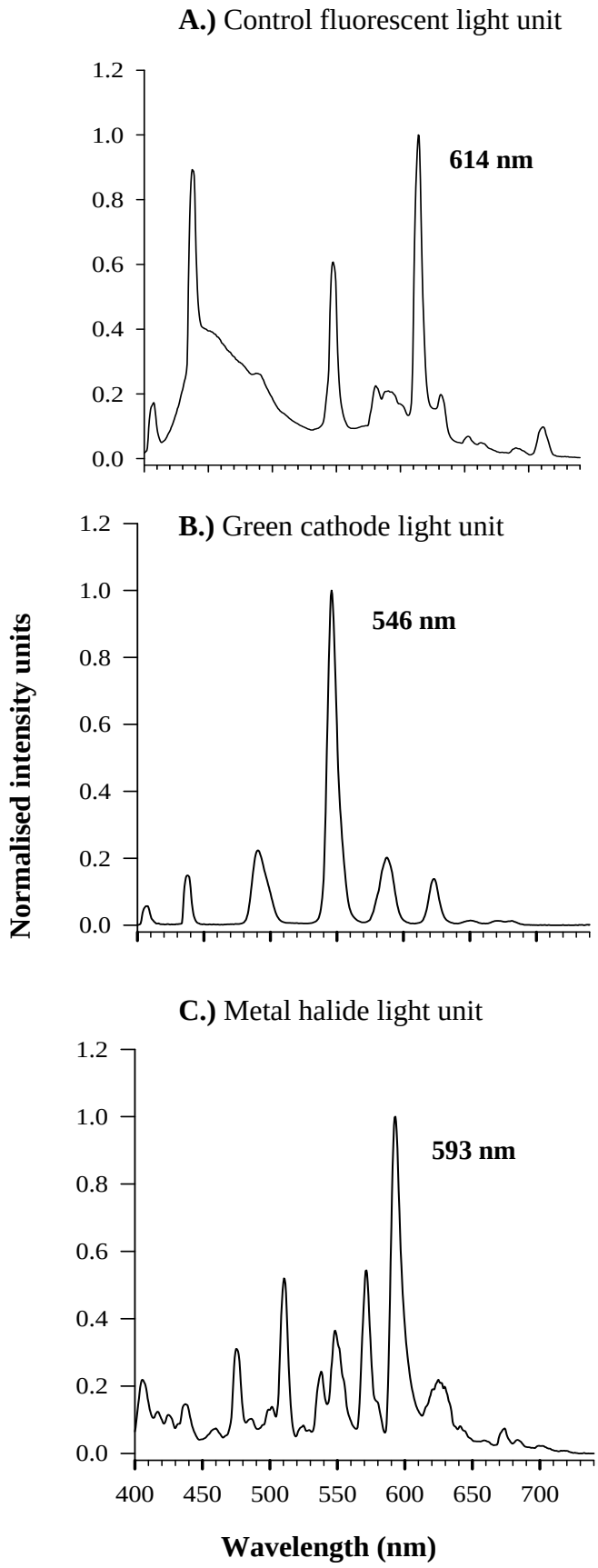

Figure 1. Normalised spectral profiles for A.) control fluorescent light, B.) cathode light and C.) metal halide light units. Wavelength of peak emission is labelled on each graph.

2.3 Sampling procedure

Five un-tagged fish per tank were sacrificed at 6 time-points during the experiment: pre-light exposure (3 and 2 weeks prior to exposure to the light treatments, during acclimation) and post-light exposure (3 hours, 1, 2 and 4 weeks). At each time-point, fish were culled by lethal anaesthesia (MS222, 80 ppm, Pharmaq, Fordingbridge, UK). Immediately after death, a heparinised syringe was used to withdraw blood from the caudal vein for cortisol and glucose analyses: fish were then measured for whole body weight (± 0.1 g) and total length (± 1 mm) then a sample of head kidney was removed and frozen at -70 ^{0}C for lysozyme analysis and both eyes were removed and fixed in bouins fixative (Bios Europe, Lancashire, UK). Blood was sampled within 5 minutes of netting, stored on ice, centrifuged at 1200 *g* for 15 min and resulting plasma was aliquoted and stored at -70 ^{0}C until analysis. At the end of the trial, 5 fish were sacrificed during night and 5 during day from all tanks: 2 ml of blood withdrawn and plasma melatonin content analysed.

2.4 Plasma analysis

Plasma cortisol levels were determined by radioimmunoassay according to North *et al*. (2006) and validated in Atlantic cod by comparing serial dilutions of pooled cod plasma to check it was immunologically comparable to purified standards (data not presented). The tritiated label (TRK407) was supplied by Amersham Pharmacia Biotech (Little Chafont, Buckinghamshire, UK) and a sheep anti-cortisol antibody from Diagnostic Scotland (Carluke, UK). Intra- and inter-coefficients of variation were 6.85% and 21.33% respectively (n = 4), with a minimum sensitivity of 0.38 ng.ml^{-1}. Glucose concentration was analysed colourimetrically using Infinity^{TM} Glucose Oxidase diagnostic kits (Alphalabs, Hampshire, UK). Melatonin was analysed by radioimmunoassay according to Porter *et al*. (2000).

2.5 Lysozyme analysis

Lysozyme activity was analysed by a modified version of the lysoplate method as described by Osserman & Lawlor (1966). The method is based on lysis of the bacterium *Micrococcus lysodeikticus* in l % agarose prepared in 0.05 M sodium phosphate buffer pH = 6.2. *Micrococcus lysodeikticus* is a gram-positive cocci particularly susceptible to the lytic action of lysozyme. The diameter of the lysed zone was visualized by lack of colour in contrast to the white unlysed area. The mean diameter (n = 2) of each zone was measured (± 0.5mm) using a ruler.

2.6 Eye histology

Once the eye was removed, a small incision was made in the sclera 90° to the right of the choroid fissure to allow fixative penetration. Eyes were fixed overnight in Bouin's fixative (less than 24 hours) and then washed and transferred twice into fresh 70 % ethanol where they remained until processing. Eyes were oriented using the location of the ventral choroid fissure and trimmed in a dorsal-ventral plane to include the optic nerve. Subsequent processing to paraffin wax was routine and sections were stained using haematoxylin and eosin.

Retinal measurements were conducted using image analysis software (Image Pro Plus, v. 4.5, Media Cybernectics Inc., Silver Spring, MD, USA) and taken at the central region of the retina ventral to the optic nerve. Two parameters were measured: 1) the thickness of the outer nuclear layer (ONL) (n = 5 measurements/ fish) and 2) the number of ONL nuclei in a 50 µm band (n = 2 counts/fish) (Fig. 2). Measurements were conducted on retina from the 2 and 4 week light exposure time-points.

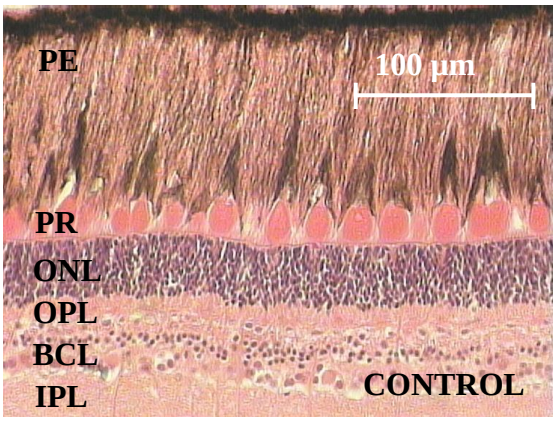

Figure 2. Histological section of Atlantic cod retina kept for 2 weeks under control conditions. Section illustrates different retinal layers: PE, pigment epithelium; PR, photopreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; BCL, biploar cell layer; IPL, inner plexiform layer.

2.7 Population feed intake

Population feed intake (% body weight / day) was determined by hand-feeding tanks to satiation and dividing total consumption by number of fish.

2.8 Specific growth rate

Specific growth rate (SGR) for all tagged individuals over the 4 week test period was calculated as follows:

SGR= ((EXP(((LN(weight end)-LN(weight start))/no. days)))-1)*100

2.9 Statistical analysis

Statistical analysis was performed with MINITAB ® version 15.0 (Minitab Ltd., Coventry, UK). All data sets were tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test, and if necessary were log transformed. All data expressed as a percentage was arcsine transformed prior to analysis. The effect of light treatment over time on all dependent variables was

compared by analysis of variance (ANOVA) manipulated using a General Linear Model that included a comparison of treatment replicates (n=2) nested within the fixed treatment effect. When no significant replicate difference was found, the model analysed treatment differences only, however for the SGR data only where a replicate difference was present, analysis was performed between replicates independently of treatment. In all cases a significance level of $p<0.05$ was set with significant interactions being analysed by Tukey *post hoc* test.

3. Results

3.1 Cortisol, glucose and lysozyme

There were no significant differences between light treatments in plasma cortisol (Fig. 3a) and glucose (Fig. 3b) levels. Although there was a significant elevation from baseline observed in cortisol (Low CL at 1 week) this deviation was transitory with a return to baseline levels 2 weeks after light onset. There were no significant differences in lysozyme activity between treatments or timepoints, mean activity (measured by clearance zone) ranged from 3.95 ± 0.25 to 5.45 ± 0.02 (mean ± SD, n = 2, 5 fish/replicate, data not shown).

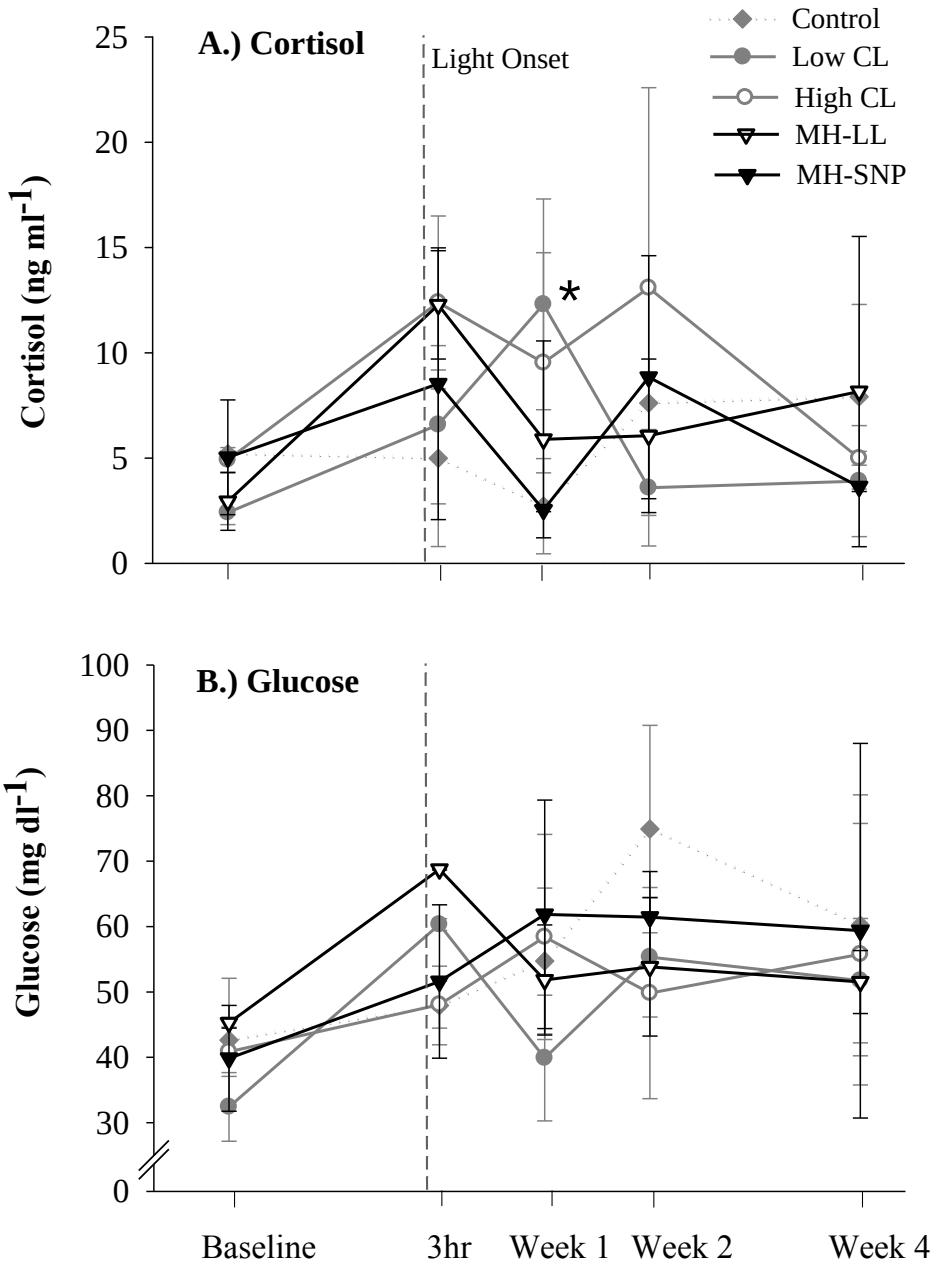

Figure 3. Plasma A.) cortisol and B.) glucose levels in Atlantic cod kept under control conditions, green cathode light (Low CL and High CL) and metal halide (MH-LL and MH-SNP) light. Data presented as mean ± SD (n = 2, 5 fish/replicate). Significant differences between baseline and post-light onset values are indicated by *. See appendices 2 and 3 for detailed tables of results.

3.2 Eye histology

No differences in ONL thickness or ONL nuclei number were observed between fish under different treatments after two or four weeks of light exposure (Table 2). ONL thickness ranged from 29.40 ± 3.75 to 37.09 ± 0.06 (mean ± SD, n = 2, 5 fish/replicate) and the number of ONL nuclei ranged from 98 ± 19.94 to 126 ± 1.48 (mean ± SD, n = 2, 5 fish/replicate).

3.3 Feeding intake

Population feed intake analyses indicated no long term effects on the feeding activity of cod in any of the light treatments (Fig. 4). There was however a significant reduction in feed intake following light onset in all experimental treatments with this being most pronounced in fish exposed to metal halide lighting. Prior to light onset cod were feeding at ≥ 0.98 % body weight per day; however following light onset, in the MH-LL treatment, this dropped to 0.2 %. By days 8 and 10 onwards however, feeding rates were no different from baseline levels. For the two CL treatments, feeding intake dropped to 0.5 % (High CL) and 0.6 % (Low CL) with fish feeding normally once more after just five days. For control fish there was no drop immediately following light onset, however feed intake was significantly reduced on day 3.

Table 2. Retinal morphometric measurements (central region) performed in Atlantic cod kept under control conditions, green cathode light (Low CL and High CL) and metal halide light (MH-SNP and MH-LL) for two and four weeks following light onset. Parameters measured include the thickness (µm) of the outer nuclear layer (ONL) and the number of ONL nuclei / 50 µm. Data is presented as the mean ± SD (n = 2, 5 fish / replicate).

Parameter	**Time (weeks)**	**Treatment**				
		Control	**Low CL**	**High CL**	**MH-LL**	**MH-SNP**
ONL Thickness	2	37.09 ± 0.06	34.12 ± 0.61	33.71 ± 0.58	30.95 ± 0.43	29.40 ± 3.75
	4	36.77 ± 1.47	36.46 ± 1.70	31.80 ± 2.09	34.14 ± 2.53	32.89 ± 0.42
ONL Nuclei	2	116 ± 15.95	111 ± 11.24	115 ± 0.00	102 ± 11.83	103 ± 18.21
	4	126 ± 1.48	107.58 ± 7.53	104 ± 4.79	106 ± 4.97	98 ± 19.94

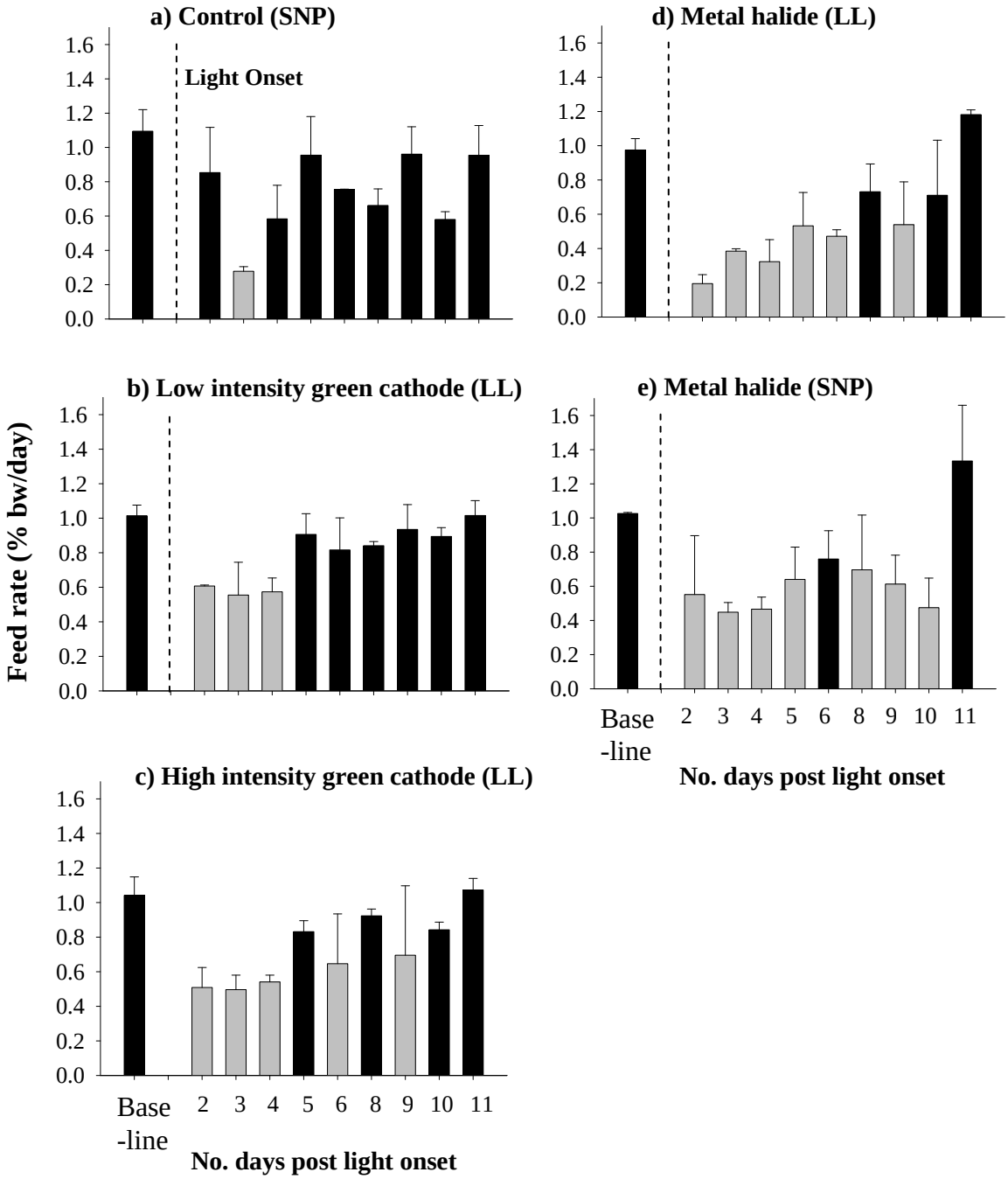

Figure 4. Population feeding behaviour in Atlantic cod kept under different lighting treatments. Data presented as mean feed rate (% body weight/day) per tank (n = 2) ± SD. Dark bars indicate baseline feeding levels, light bars indicate a significant reduction from baseline levels.

3.4 Melatonin

While no significant differences between treatments were observed in melatonin levels during the daytime, there was a significant night-time elevation in plasma melatonin levels in fish under all treatments except Low CL (Fig. 5). Day-time and night-time Low CL levels of melatonin did not significantly differ from other treatments at those timepoints however.

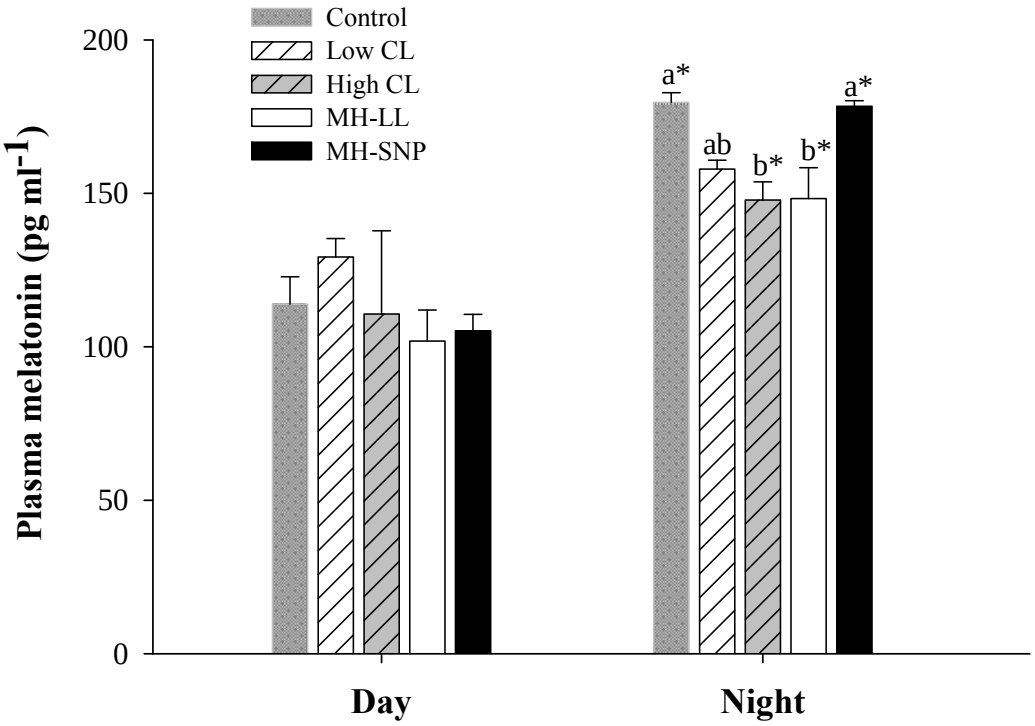

Figure 5. Plasma melatonin levels sampled at day and night in Atlantic cod under control conditions, green cathode light (Low CL and High CL) and metal halide (MH-LL and MH-SNP) light. Data presented as mean ± SD (n = 2, 5 fish/replicate). Significant differences between day-time and night-time values are indicated by *. Significant differences between light treatments at a given time-point are indicated by different superscripts.

3.5 Survival and SGR

Survival rate over the trial period was 100%. No significant differences were found between treatments for SGR (Table 3), mean SGR ranged between -0.05 ± 0.1 (MH-LL) and 0.58 ± 0.14 % day $^{-1}$ (Control).

Table 3. Specific growth rate (% body weight / day) of Atlantic cod kept under different lighting treatments for four weeks. Data presented as treatment replicate mean ± SE (n = 20). Treatments which exhibit a significant replicate difference are indicated by *.

Treatment	**SGR**	
	Replicate 1	**Replicate 2**
Control	0.33 ± 0.06	0.58 ± 0.14
Low CL	0.38 ± 0.06	0.16 ± 0.12
High CL	0.30 ± 0.11	0.21 ± 0.10
MH-LL	-0.05 ± 0.10	0.27 ± 0.09
MH-SNP*	0.37 ± 0.12	0.16 ± 0.06

4. Discussion

Photoperiod manipulation is used extensively throughout the aquaculture industry to induce out of season spawning, to control the timing and completion of smoltification in salmonids and suppress early maturation (Endal *et al*., 2000; Bromage *et al*., 2001; Davie *et al*., 2007a; Norberg *et al*., 2004). However, whilst considered to be less invasive than other techniques used to control puberty such as hormonal sex reversal for monosex production (Piferrer, 2001; Hendry *et al*., 2003; Taranger *et al*., 2010) or chromosome manipulation for sterility induction (Benfey, 2001; Tiwary *et al*., 2004; Maxime, 2008), studies investigating the potential 'welfare' impacts of such technology on fish are lacking. This is especially important in Atlantic cod which are being exposed to increasingly higher light intensities during on-growing in open cage systems owing to the relatively unsuccessful outcomes of photoperiodic manipulations (Taranger *et al.*, 2006) in comparison to tank based studies where 100% suppression has been demonstrated (Davie *et al*., 2007a).

Lighting treatments tested in the current study were designed to recreate the light intensities within a 1.5 m distance from a light source in a cage environment. MH lights delivered a much brighter light intensity (x 20) across a broad range of wavelengths in comparison to the CL technology. Surprisingly, no significant differences between treatments were observed in the stress response (cortisol and glucose) following light onset however cortisol did increase significantly with respect to basal levels in the CL treatment (at 1 week). Although treatment differences were not apparent, it must be recognised that the large variability observed between individuals in conjunction with the limited sampling size may have prevented the detection of further differences. When the current levels of cortisol are compared to other studies however, it could be concluded that their range is not indicative of stress. The maximum mean cortisol value

recorded was 12.3 ng ml^{-1} (Low CL treatment) which when compared to studies by King & Berlinsky (2006a), King *et al*. (2006b) and Perez-Casanova *et al*. (2008a) is far below the level representative of stress in cod. Although there are no studies specifically addressing the effect of light on stress levels of cod of a similar size, studies of stressors such as netting, transport and grading on smaller cod (~40 g) have been found to elicit a peak in plasma cortisol concentration of over 60 ng ml^{-1} after 30-60 minutes, with a return to basal levels after 24 hours (King, 2006a). Also a temperature rise of up to 16^0C resulted in cortisol levels of over 50 ng ml^{-1} (Perez-Casanova *et al*, 2008b). These results are similar to cortisol levels reported in other teleosts subjected to similar stressors (Barton & Iwama., 1991). Migaud *et al.* (2007) observed cortisol levels in Atlantic salmon following light onset reached a peak value > 100 ng ml^{-1}. In haddock, *Melanogrammus aeglefinus* L., a 30 second net stressor resulted in a peak of 86 $ng.ml^{-1}$ plasma cortisol after 6 hours (King *et al*., 2006b). This said however, in our study it must be considered that since measurements were performed at 3 hours following light onset, a temporary elevation within this window could have been missed (King *et al*., 2006b). It would be of interest to Glucose levels also showed large variability. According to Perez-Casanova *et al*. (2008a), the maximum mean value recorded in our present study (74.91 mg dL^{-1}, control) was within the basal range (60-100 mg dL-1) for cod maintained under their control conditions. The relevance of glucose as a reliable indicator of stress in gadoids has been questioned however (Perez-Casanova *et al*., 2008b).

Light treatments tested in this current study also appeared to have no significant effects on the innate immune response, studied through lysozyme activity. In fish, lysozyme activity is usually measured by the turbidity assay adapted from Lygren *et al.* (1999) however due to difficulties encountered with this methodology when used with cod, an agar plate (lyso-plate) method was developed and refined from Osserman &

Lawlor (1966). Very few studies have been performed so far specifically looking at the immune response in this species, and it is therefore difficult to interpret results when no baseline levels have been published (Bowden, 2008). Regarding literature relating to the effects of stressors on lysozyme activity, results are very variable. For example, Migaud *et al.* (2007) demonstrated that constant high light intensity had no effect on lysozyme activity in Atlantic salmon. In contrast however, Demers & Bayne (1997) found that an elevation in plasma lysozyme was the typical immediate response of rainbow trout, *Oncorhynchus mykiss* (Walbaum), to acute handling stress. Also, Taylor *et al*. (2007) demonstrated elevated plasma lysozyme activity in rainbow trout following seawater transfer. Clearly the type and duration of an environmental change/stressor and the fish species involved, will determine if there is a consequent change in lysozyme activity. Future studies of interest may involve performing direct measurements of the immune system following bacterial challenge in order to assess welfare issues such as reduced immune function as a consequence of lighting treatments (Dautremepuits *et al*., 2006).

In terms of retinal morphology, there were no significant differences in outer nuclear layer (ONL) thickness or ONL nuclei in any of the treatments, a reduction in ONL thickness or number of nuclei could be considered a sign of retinal damage (Allen & Hallows, 1997; Vihtelic & Hyde, 2000; Dawson *et al*., 2001) however this was not apparent in these fish following light exposure.

Regarding feeding activity, acute effects of the light treatments on population feeding response were characterised by a transient reduction in feeding, in all treatments, though normal feeding resumed within a few days (approximately 8 days in fish exposed to MH light). Interestingly, the time needed to return to normal feeding behaviour appeared to be related to the light intensity of the treatments taking 5, 6 and 8 days under the CL treatments (0.5, 0.8 watts m^{-2}), MH-LL (16.6 watts m^{-2}) and MH-SNP treatments,

respectively. It must be noted that although feeding activity remained steady immediately following light onset in control fish (SNP treatment), a reduction at day 3 was observed that can not be explained and might simply reflect natural patterns of variation in feed intake (Kadri *et al*., 1996; Lokkeborg, 1998).

Interestingly, with respect to the perception of the light by the cod populations, under the metal halide and High CL treatments, a day/night rhythm of plasma melatonin levels was still maintained, probably resulting from increased light intensities at day due to ambient light pollution entering the tanks through the feeding hatch. These results confirm previously obtained *in vitro* pineal results on the effects of day/night ratio on melatonin production (Vera *et al.*, 2010). The potential entrainment of melatonin rhythm by internal clocks was ruled out as when Atlantic cod were subjected to constant lighting in fully light-proofed tanks on the same site: melatonin levels remained constant (Davie, 2005).

As a whole, results from this study indicate that the light treatments tested, which mimicked cod light exposure at night time in an open cage system when maintained within 1.5 m of the light unit, did not have any clear chronic effects on the stress response, immune function, retinal structure and feeding activity of cod. These are important physiological parameters described in the five freedoms of welfare (FAWC, 2009) and have relevant implications for cod culture where increasing light intensities are being used in an attempt to make the response to photoperiod management more consistent. Further studies should be carried out to determine if there are light intensity thresholds above which the welfare of fish could be compromised as well as testing the effects of various spectral profiles.

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CHAPTER 3

RESEARCH ARTICLE

THE EFFECT OF COMBINING SHADING AND CONTINUOUS LIGHTING ON THE SUPPRESSION OF SEXUAL MATURATION IN OUTDOOR-REARED ATLANTIC COD, *GADUS MORHUA*.

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Keywords: Atlantic cod, maturation, light, shading, sex steroids, growth

Abstract

Sexual maturation of Atlantic cod, *Gadus morhua*, is a major problem during commercial on-growing as fish divert energy away from growth into gonadal development. While the photoperiod regimes that inhibit maturation are well described, when manipulations are applied in a commercial cage setting, using standard lighting technology, maturation is not completely inhibited. It has been hypothesised that the enhanced light sensitivity of cod allows it to perceive ambient illumination over the artificial lighting. Therefore, a 13 month trial was conducted to determine the effectiveness of net shading ambient photoperiod in addition to constant lighting to suppress maturation of cod in outdoor conditions. By reducing the relative difference between day and night light intensities, it was hypothesised that maturation in cod could be inhibited as fish could not perceive, and thus use the 'ambient daylength' signal to entrain their reproductive cycle. Two outdoor tanks were covered in either a low density (70% reduction in ambient illumination) or high density (90% reduction in ambient illumination) shade netting and then illuminated continuously by cathode lighting. These were compared to two indoor tanks in which ambient light was excluded and instead were illuminated by similar lighting running either under a simulated natural photoperiod or continuous illumination. The study demonstrated that shade netting could improve the relative performance of artificial lighting outdoors from <2% in a non shaded system to 6.6% (low shade treatment) and 31.3% (high shade treatment) of the day light intensity. Importantly, both shading treatments in combination with constant light were effective at suppressing sexual development as confirmed through histology and reduced gonadosomatic index (female mean ≤ 2.18%, male mean ≤ 1.39%), oocyte diameter (mean <400µm) and sex steroid profiles (female 17β-estradiol mean ≤ 0.24 $ng.ml^{-1}$, male 11-ketotestosterone mean ≤3.35 $ng.ml^{-1}$) as well as enhanced growth.

These results are a promising demonstration of the potential value of shading systems to enhance the efficacy of photoperiod control.

1. Introduction

Sexual maturation during on-growing is a major problem in Atlantic cod farming as fish allocate considerable amounts of energy into gonadal development which results in reduced flesh quality, increased mortality and severely impeded growth (Hansen *et al*., 2001; Karlsen *et al*., 2006; Davie et al., 2007b) as seen in other temperate species like Atlantic salmon, *Salmo salar* (Oppedal *et al.*, 1997; Endal *et al.*, 2000). Furthermore, broadcast spawning of on-growing cod in cages results in the release of gametes into the surrounding environment that could lead to genetic interaction with native stocks (Jorstad *et al*., 2008). Prevention of sexual maturation is thus crucial to the profitability and sustainability of cod farming.

Wild cod stocks have been found to mature from two to four years of age or even later depending on location (Cook *et al.*, 1999; Berg & Albert, 2003) while under the favourable conditions experienced in culture (i.e. high food abundance) cod are capable of maturing at the end of their first year (Davie *et al.*, 2007a). Photoperiod manipulation appears to be the only commercially viable strategy to combat this culture-related "precocious" maturation (Davie *et al.*, 2007a; Taranger *et al.*, 2006, 2010) although sterility through triploidisation is being explored as an alternative solution (Trippel *et al*., 2008). In temperate latitudes, the seasonally changing pattern of daylength is generally accepted to be the key environmental regulator of puberty in gadoids, salmonids, bass, breams and flatfish (Bromage *et al.*, 2001; Carrillo *et al.*, 2009; Migaud *et al*., 2010). In cod, it is the decreasing daylength from summer solstice (Davie *et al*., 2007a) which acts as the 'proximate' cue for individuals to enter into puberty through stimulation of the brain pituitary gonadal (BPG) axis which in turn regulates gonadal development

(Migaud *et al*., 2010). Research has shown that exposure of fish to continuous light from the summer solstice prior to likely maturation, effectively masking the daylength decrease, leads to full suppression of puberty in cod in enclosed tanks (Hansen *et al*., 2001; Davie *et al.,* 2007a). However, such treatments are far less efficient when applied outdoors using standard submersible lighting systems and maturation may at best be delayed by four to five months (Taranger *et al.,* 2006). In contrast, when such photoperiod manipulations are applied to farmed Atlantic salmon (Endal *et al*., 2000; Hansen *et al*., 2008), they have been consistently shown to arrest maturation and are thus routinely applied in the industry. Similarly, photoperiod regimes have been designed for European sea bass, *Dicentrarchus labrax* (Begtashi *et al*., 2004; Rodríguez *et al*., 2005) and Senegalese sole, *Solea senegalensis* (García López *et al*., 2006). This differential response between species led to the hypothesis that cod might have an enhanced sensitivity to light (Davie *et al.,* 2007a) as compared to other commercially important species. This was confirmed by Vera *et al.* (2010) who studied the response of the cod pineal gland to light *ex vivo* and showed that cod would be 100 times more "sensitive" to light in comparison to European sea bass and 10,000 times more "sensitive" than Atlantic salmon. However, more importantly, the same authors demonstrated that this light sensitivity in cod is not a definable single intensity, rather it appears that the minimum detection of light is dependent on the intensity of light experienced during the day in a process the authors referred to as "relative photoreception". If daylight intensity is reduced, so the threshold of night is also reduced which opens an interesting possibility when this relationship is translated into the cage farming context. If ambient daylight intensity is reduced, for example by shading, so the efficacy of artificial light may be improved at night as the difference between the two intensities, *i.e.* day vs. artificial light, is reduced.

Therefore, the aim of this study was to investigate the efficacy of two different levels of net shading to suppress daylight in combination with constant artificial lighting in an outdoor culture system on the prevention of sexual maturation in two-year old Atlantic cod. The effects of shading were assessed through measurement of a series of parameters including growth performance, gonadal development and plasma sex steroid levels.

2. Materials and Methods

2.1 Fish Stock and Initial Rearing Conditions

The trial was conducted at the Machrihanish Marine Environmental Research Laboratory (MERL, Scotland, 55:44^{0}N, 5:44^{0}W) between the 18th June 2008 and the 24th August 2009. Groups of immature (before 1st maturation) mixed sex Atlantic cod, produced by MERL (411 ± 5.59 g, mean wet weight ± SEM) previously reared in tanks under simulated natural photoperiod (SNP) and natural temperature regimes, were randomly stocked into two indoor and two outdoor circular tanks (10.6 m^3, 9.4m circumference, water depth of 1.5 m). 187 cod were stocked into each of the two indoor tanks and 97 cod were stocked into each of the two outdoor tanks (18th June 2008). Owing to fish availability, it was not possible to stock as many fish in the outdoor tanks, and it was thus decided that high resolution sampling was more important in the indoor tanks to provide a clear benchmark of maturing and immature populations. Within each of the four populations, 20 individuals were selected at random and implanted with a passive integrated transponder tag (Avid Plc, Uckfield, UK). Tanks were supplied with fresh seawater, filtered to 60µm, at a flow rate of approximately 50 L min^{-1}. Surface skimming egg collectors as described in Thorsen *et al.* (2003) were fitted at the outflow of all four tanks from 28th January until 30th June 09 and were inspected daily.

2.2 Experimental Conditions

Fish were initially acclimated for 5 weeks under an SNP regime, at this time indoor and outdoor tanks were fitted with lids and lighting was provided by two 9W fluorescent bulbs (Osram Dulux, S G23 energy saver, UK) located on the underside of the tank lids. Their operation was regulated by digital timers which were adjusted weekly to match the ambient sunrise/sunset times through the course of the trial. Intensity measured at the water surface was 0.32 watts m^{-2} when illuminated. Experimental light units were positioned in tanks across the centre on the water surface and secured at their ends however these remained off during acclimation.

Experimental treatments commenced on the 24th July and were maintained for 13 months. The four treatments tested were: 1) Indoor simulated natural photoperiod (indoor SNP), 2) Indoor constant light (indoor LL), 3) Outdoor, LL and 70 % shade (outdoor low shade LL) and 4) Outdoor, LL and 94 % shade (outdoor high shade LL). Lids remained on the indoor tanks to light-proof them from any extraneous ambient illumination while the lids were removed from the outdoor tanks and shade netting was stretched over their open surface and secured around the edge (see Appendix 4 for a schematic diagram of shading and light unit set-up). In both cases the shade netting was a knitted polyethylene monofilament mesh, however the measurement of shading effect differed from the manufacturers specifications with the low shade netting being provided by LBS Garden Houseware, Colne, UK (marketed as 40% shading) while the high shade netting was provided by Aaask, Glasgow, UK, (marketed as 90% shading). Lighting in all cases was provided by one green cathode light unit per tank (232W, Intravision Aqua, Oslo, Norway). The wavelength of peak emission was 546 nm and the full width at half maximum (FWHM: description of the range in light wavelength with an intensity half that of the peak wavelength) was 8.5nm.

Light intensity in the tanks was measured during the daytime at solar noon and at midnight on the 24th July 2008. Down-welling intensity measurements (1.5 m below the water surface) were taken beneath the light source, mid way between the light and the tank edge (1.5 m from centre) and at the tank edge (3 m from centre). Light intensity (watts·m^{-2}) was measured using a single channel light sensor with a non biased spectral range of 400-740 nm (Skye Instruments Ltd., Powys, UK) and calibrated to National Physics Laboratory (UK) standards. The proportion of night-time illumination relative to day in each treatment was calculated as follows: Night-time illumination = (mean night intensity / mean day intensity) *100. In the outdoor low shade LL tank, night-time illumination (1.55 ± 1.14 watts m^{-2}) represented 6.6% that of daytime light levels (23.5 ± 9.56 watts m^{-2}) whereas in the outdoor high shade tank LL, night levels (1.49 ± 1.11 watts m^{-2}) were equal to 31.3 % of the day levels (4.77 ± 1.70 watts m^{-2}). In the absence of shading, night levels (1.55± 0.66 watts m^{-2}) were equal to only 2% of the day levels (78.93 ± 2.02 watts m^{-2}). In the indoor tanks illumination was at 0.54 ± 0.32 watts m^{-2} during the day in both the LL and SNP tanks and 0.57 ± 0.38 watts m^{-2} or 0 ± 0 watts m^{-2} at night in the LL and SNP tanks respectively.

Fish were fed to satiation on commercial cod diet (Biomar, Grangemouth, UK) according to the manufacturer's guidelines via clockwork belt-feeders throughout the ambient daylight period. Owing to an outbreak of furunculosus in the indoor treatment tanks at the start of the trial an oral antibiotic treatment was prescribed by a veterinarian (oxytetracyline, 100 mg/kg fish weight) and administered for 5 days on, 5 days off, 5 days on, starting on the 27th August 2008. All procedures were performed in accordance with the Animals (Scientific Procedures) Act, UK, 1986 under the approval of the local ethical review board.

2.3 Sampling Procedure

A basal (pre-treatment) sample was taken on the 24th July 2008 where 20 fish were sacrificed at random (7♀, 13♂). Blood was withdrawn from the blood vessels in the caudal peduncle using a 2 ml syringe and 23G sterile hypodermic needle, round weight (± 0.1g) was measured and gonads were removed and fixed for histological assessment. The data from these fish was applied to all treatments. Then in August and every two months thereafter PIT-tagged fish were anaesthetised (1:10,000 concentration of 2-phenoxyethanol, Sigma-Aldrich Co Ltd, Poole, UK) prior to blood being sampled and round weight and total length being measured. PIT-tagged fish which died during the trial were removed from the dataset (data from 4 to 8 individuals/sex/treatment were analysed, Table 1). TGC_w was calculated for tagged fish every two months from August 2008 until the following August 2009 according to the equation: $((\sqrt[3]{weight_{end}} - \sqrt[3]{weight_{start}})*1000)/degree\ days$. Furthermore at these time-points, 10-12 fish (un-tagged) were sacrificed at random from each indoor and outdoor tank. On the intervening months, an additional 12 fish were sampled from the two indoor tanks. Due to the lack of clear external sexual dimorphism it was not always possible to get a balanced sex ratio (Table 1). Sacrificed fish were blood sampled and measured for whole body weight (± 0.1 g) and total length (± 1 mm) before having their gonads dissected and weighed and a sample taken and fixed in 10 % neutral buffered formalin. In all cases the chilled blood samples were later centrifuged at 1200 *g* for 15 min and the resulting plasma was aliquoted and stored at -70 ^{0}C for subsequent steroid analyses. In addition, 10-18 fish were blood sampled in August 2009 in each experimental tank at day (between 10:00-14:00 pm) and night (between 01:00 – 03:00 am) for melatonin analysis. Night samples in the indoor SNP population were performed under the illumination of dim red light.

2.4 Analyses

Plasma levels of testosterone and 17β-estradiol (E_2) were measured by radioimmunoassay (RIA) according to Duston & Bromage (1987). Plasma levels of 11-ketotestosterone (11-KT) were measured by RIA according to Fostier *et al.* (1982), following a parallelism test to confirm that serial dilutions of cod plasma extracts and hormone standards were immunologically comparable. In order to assess how fish perceived the light regimes and potential differences between day and night light intensities, melatonin was analysed by radioimmunoassay adapted from Fraser *et al.* (1983) and validated for use in cod by Porter *et al*. (2000). Intra- and inter-assay coefficients of variation were 3.73, 3.97, 3.13 and 17.02%, and 17.25, 5.08, 10.88 and 23.33% for T, E_2, 11-KT and melatonin respectively (n=2 to 8 assays for each hormone).

The diameter of dissociated, fixed oocytes was measured by image analysis using a protocol adapted from Thorsen & Kjesbu (2001). Measurements (mean of 2/oocyte) were done in a random sample of *circa* 50 oocytes/fish gonad using a digital image processing software (Image Pro Plus™, Media Cybernectics, Silver Spring, Maryland, USA). From this sample the mean ± SEM of the 10 largest oocytes was calculated and designated as the leading cohort or G1 oocytes (Thorsen & Kjesbu 2001). It was not possible to perform measurements of oocyte diameter before September 2008 or after June 2009 as oocytes were too small to be processed accurately (< 152µm). Sections of the same ovarian tissue sample, following fixation in 10 % neutral buffered formalin, were also dehydrated and embedded in paraffin wax, 5 µm sections were then cut and stained using haemotoxylin and eosin. Sections were examined under a light microscope classified into one of seven stages (see Table 2) with reference to Dahle *et al*. (2003) and Tomkiewicz *et al*. (2003). Male development was not considered due to the complicated

gradient of spermatogonial development in the species preventing easy classification of a specific developmental stage (Almeida *et al*., 2008).

2.5 Data and statistical analyses

Statistical analysis was performed with MINITAB ® version 15.0 (Minitab Ltd., Coventry, UK). All data sets were tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test, and if necessary were log or arcsine-transformed when normality and/or homogeneity of variance was violated. Two sets of data were analysed differently: data obtained from PIT-tagged fish (weight and sex steroid data) were analysed using a General Linear Model using individuals as a covariate followed by Tukey post hoc test to analyse time by treatment interaction; data from sacrificed fish (not tagged, weight, GSI, sex steroid and melatonin) were analysed by one way ANOVA to test for treatment effects. In all cases a significance level of $p<0.05$ was set. Baseline data (collected in July 2008) were pooled according to sex for weight, histology and sex steroid profiles. Data are presented as mean ± SE of the mean.

Table 1. Number of female and male cod sampled at each timepoint over the trial period, both the numbers of sacrificed fish and tagged fish are displayed. Data for fish sacrificed in July was pooled according to sex.

	Indoor SNP				**Indoor LL**				**Outdoor Low Shade LL**				**Outdoor High Shade LL**			
	Sacrificed		Tagged		Sacrificed		Tagged		Sacrificed		Tagged		Sacrificed		Tagged	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Jul	20 fish sacrificed (7 females, 13 males)															
Aug	6	6	6	6	6	6	6	5	3	7	7	7	3	7	3	8
Sep	6	6			6	6										
Oct	6	6	6	6	8	6	6	4	5	5	6	7	5	5	3	8
Nov	6	6			6	6										
Dec	6	6	6	6	6	7	6	5	6	4	6	7	5	5	3	8
Jan	6	6			6	6										
Feb	6	6	6	6	10	4	6	5	5	5	6	7	6	4	2	8
Mar	7	6			6	6										
Apr	2	9	6	6	7	3	6	5	4	6	7	7	5	5	3	8
May	1	8			8	1										
Jun	2	8	6	5	3	7	5	5	2	8	7	7	6	4	3	7
Aug	6	6	5	6	6	5	6	5	11	10	7	7	3	8	3	8

Table 2. Classification stages of female ovarian development with reference to Dahle *et al*. (2003) and Tomkiewicz *et al*. (2003).

Stage		Description
1	Immature	Oocytes are small, transparent and pre-vitellogenic with a round circumnuclear ring (cr) and attached peripheral nucleoli.
2	Cortical alveoli (CA)	Cortical alveoli present, cr has moved to the outer part of the cell and is gradually disintegrating and nucleoli are becoming detached.
3	Early vitellogenesis	Yolk granules are present but not filling the cytoplasm entirely. Nucleus becoming irregular in shape.
4	Late vitellogenesis	Cytoplasm is entirely filled by yolk granules. Nucleus is migrating to the animal pole. Thickening of chorion.
5	Spawning	Hydrated oocytes, postovulatory follicles (POFs) and late vitellogenic oocytes present.
6	Spent	Residual (atretic) oocytes are being reabsorbed. Abundance of POFs. Small pre-vitellogenic oocytes present.
7	Regressing	Atresia of developing oocytes.

3. Results

3.1 Growth performance and mortality

There were no treatment differences in TGC prior to February 2008 (Fig. 1). In the period from February to April 2009, individuals under indoor SNP showed significantly lower TGC_w than the indoor LL treatment and outdoor high shade treatments. From April to June 2009, the TGC_w of individuals under SNP increased and was significantly higher from that of low and high shade treatments however in the last three months of the trial all treatments showed comparable TGC_w.

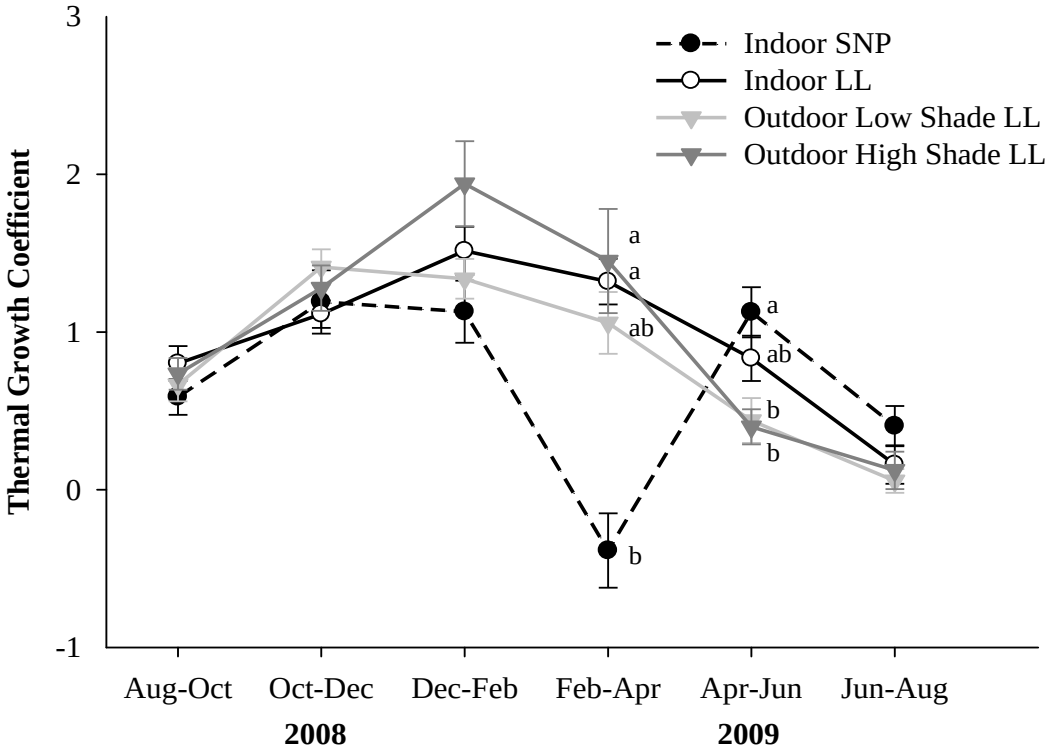

Figure 1. Mean thermal growth coefficient (TGC_w) ± SEM for tagged individuals (gender pooled, 10-19 individuals/time point/treatment/sex) maintained under indoor simulated natural photoperiod (Indoor SNP), indoor constant lighting (Indoor LL), low shade with constant lighting (Outdoor Low shade LL) and high shade with constant lighting (Outdoor High shade LL). Data presented for every two month interval. Differences between treatments at a given timepoint are indicated by superscripts.

With regard to female weight (Fig. 2), all treatments were comparable at the start of the trial however the weights of females under the indoor SNP treatment were significantly lower than the high shade treatment in April and the indoor LL treatment in May (sacrificed fish). By the end of the trial, weights of females under the indoor SNP and outdoor high shade treatment appeared to be higher than the SNP treatment (tagged fish, sacrificed fish) (Fig. 2 A and B). The weights of males under all treatments were comparable at the start of the trial however in males under the indoor SNP treatment, weights were significantly lower in comparison to the indoor LL treatment in December, March and May and the outdoor low shade treatment in December and April (sacrificed fish) (Fig. 2 C and D). By the end of the trial both shading treatments and the idoor SNP treatment appeared to reach a higher mean weight than the SNP treatment (tagged fish, sacrificed fish) (Fig. 2 C and D).

At the start of the trial (July – August 2008) an outbreak of *furunculosis* struck the high shade and indoor SNP tanks which led to 16% and 14% mortality losses respectively in this period. An in-feed antibiotic treatment prevented further mortality or spread to other treatments and growth (TGC_w and weight) remained comparable when compared to indoor LL and the low shade treatments at this time. Losses were not replaced as photoperiod treatments had already begun. Over the remaining course of the trial mortality rates ranged between 5% (high shade) and 13% (low shade).

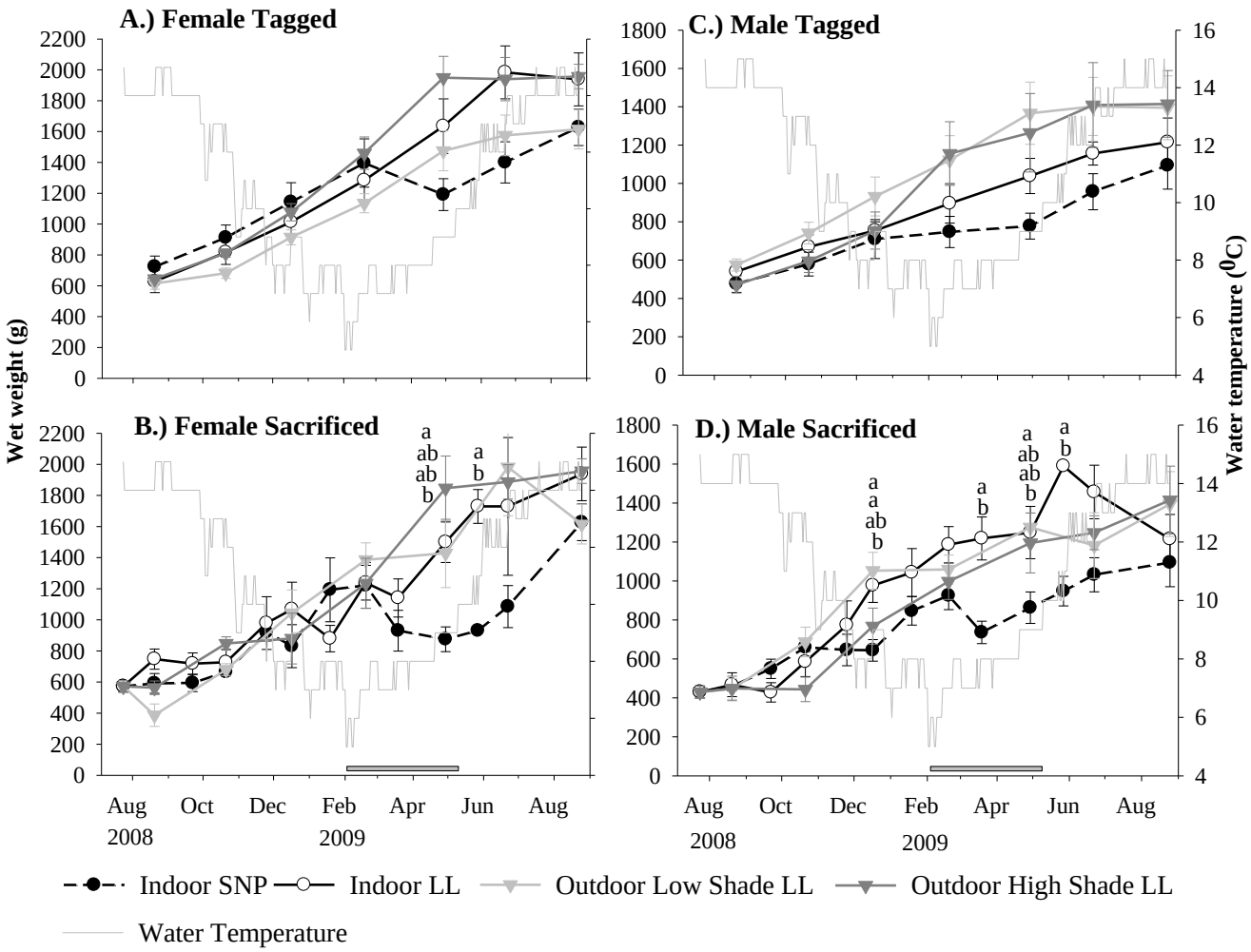

Figure 2. Wet weight of female (A-tagged, B-sacrificed) and male (C-tagged, D-sacrificed) fish maintained under indoor (SNP or LL) or outdoor (low/high shade) lighting treatments. Data are presented as mean ± SEM for every two month interval. On the intervening months, only sacrificed fish from the two indoor treatments are displayed (see Table 1 for number of fish sampled). Differences between treatments at each time point are indicated by superscripts (GLM and one way ANOVA for tagged and sacrificed fish respectively). Grey bar indicates period of spawning in the indoor SNP treatment. See appendices 5-8 for detailed tables of results.

3.2 Gonadal Development

Gamete release was observed in the indoor SNP treatment with eggs being collected from February 5th to May 11th and peak activity recorded during March and April. No spawned eggs were collected from the indoor LL treatment or the two outdoor shading treatments however two spermiating males were observed in February in the indoor LL treatment.

Females under indoor SNP showed a significant increase in GSI in November (with respect to indoor LL) with a maximum mean value reached in March 2009 (21.49 ± 6.29 %) (Fig. 3A). Male GSI increased in December with the highest mean reached in February (6.9 ± 2.4 %)(Fig. 3B). Male and female GSI remained low in the indoor LL and outdoor low shade and high shade treatments (<1.35 %, <2.14 %, respectively).

G1 oocyte diameters were comparable in all treatments up to October 2008, thereafter diameter in individuals under the indoor SNP treatment increased from November 2008 onwards with all individuals having vitellogenic oocytes >350µm from December 2008 onwards (Fig. 4). In March 2009, all indoor SNP individuals contained oocytes with a large diameter, indicating hydration and spawning. An indoor SNP individual with large oocyte diameter (1636 µm) was also recorded in May 2009, however histology and GSI measurements confirmed that this individual was mainly spent with only some remaining large oocytes. Limited numbers of individuals from the high shade and low shade treatments had early vitellogenic oocytes from December to June 2009 (mean G1 diameters ≤ 383µm). A number of indoor LL individuals also had early vitellogenic oocytes in January and April 2009 (G1 diameters ≤ 426 µm). There was however no clear full commitment to maturation in fish under the shading and indoor LL treatments, and no significant differences in oocyte diameter were recorded between these three treatments.

Histological examination of ovarian development supported the G1 oocyte diameter analysis (Figure 5). Individuals under the indoor SNP treatment displayed a typical reproductive cycle with the majority of individuals spawning in March. Spent gonads were then observed from April onwards. There were indications of early stages of vitellogenesis in the other treatments (indoor LL, low shade, high shade) and even one female with hydrated oocyte in the indoor LL in April and one female with late vitellogenic oocytes in the high shade treatment in February. However there was also evidence of regression (atresia) from December onwards in the LL and low shade treatments and in February in the high shade treatment.

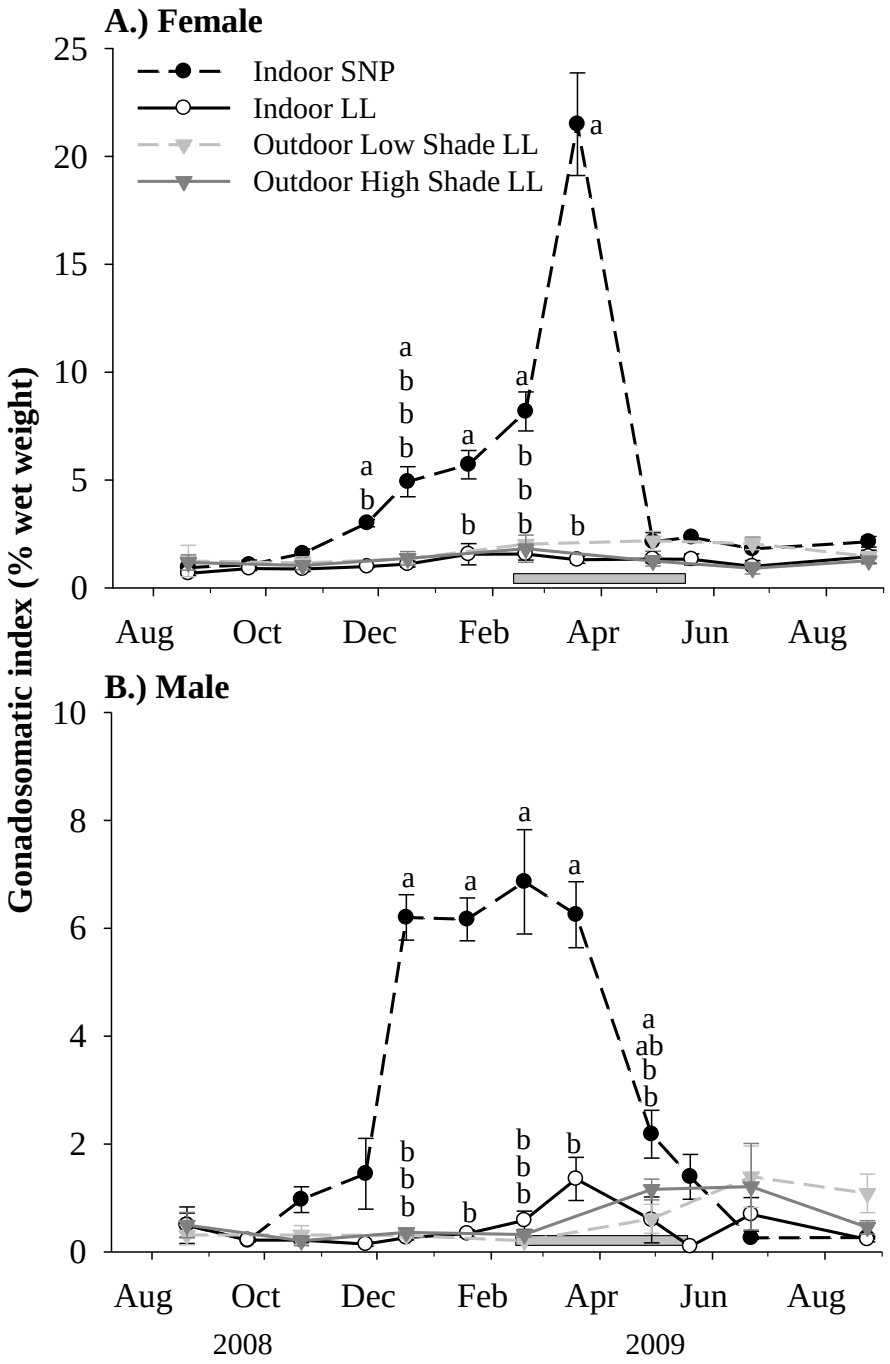

Figure 3. Female (A) and male (B) mean individual GSI ± SEM for sacrificed individuals maintained under one of four lighting treatments (see Table 1 for number of fish sampled). Differences between treatments at a given timepoint are indicated by superscripts. Grey bar indicates period of spawning in the indoor SNP treatment. See appendices 9 and 10 for detailed tables of results.

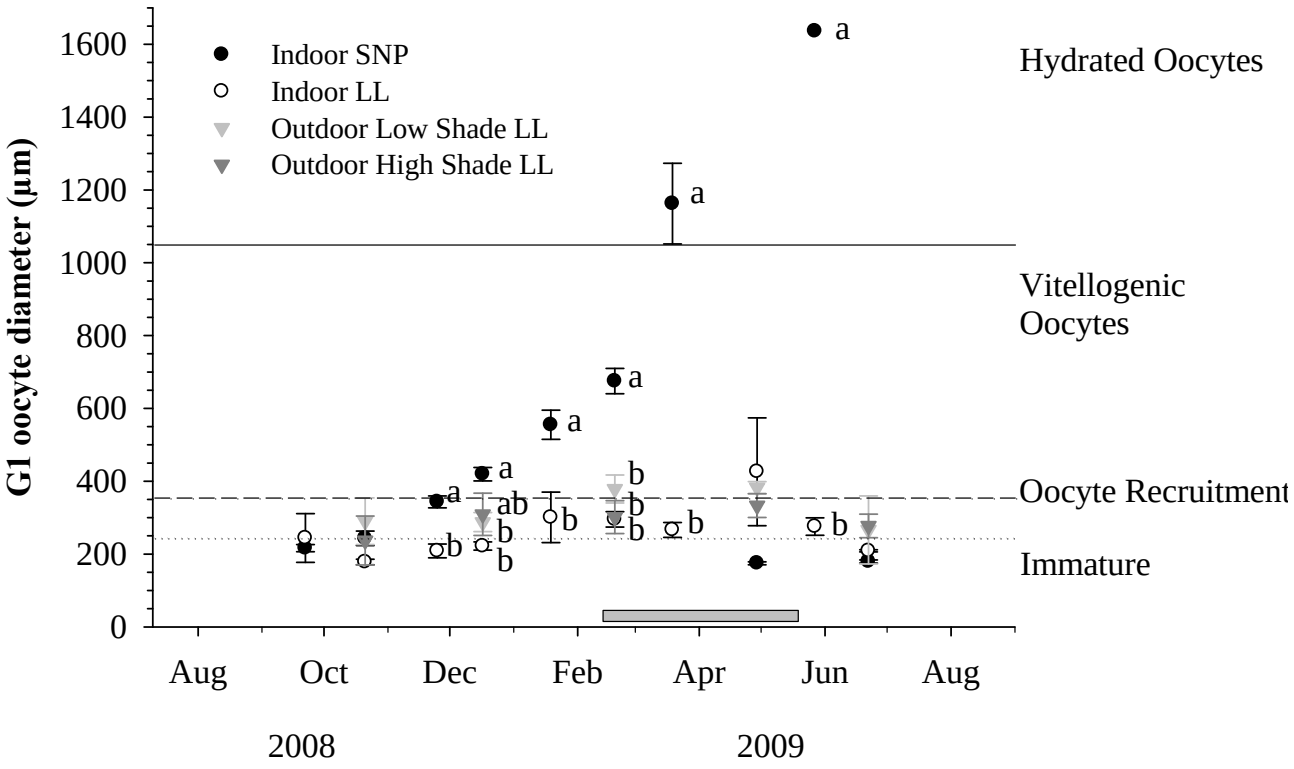

Figure 4. Mean oocyte diameter of leading cohort ± SE (see Table 1 for number of fish sacrificed). Grey indicates period of spawning in the indoor SNP treatment. Development size classifications are in reference with the definitions of Kjesbu (1991). See appendix 11 for a detailed tables of results.

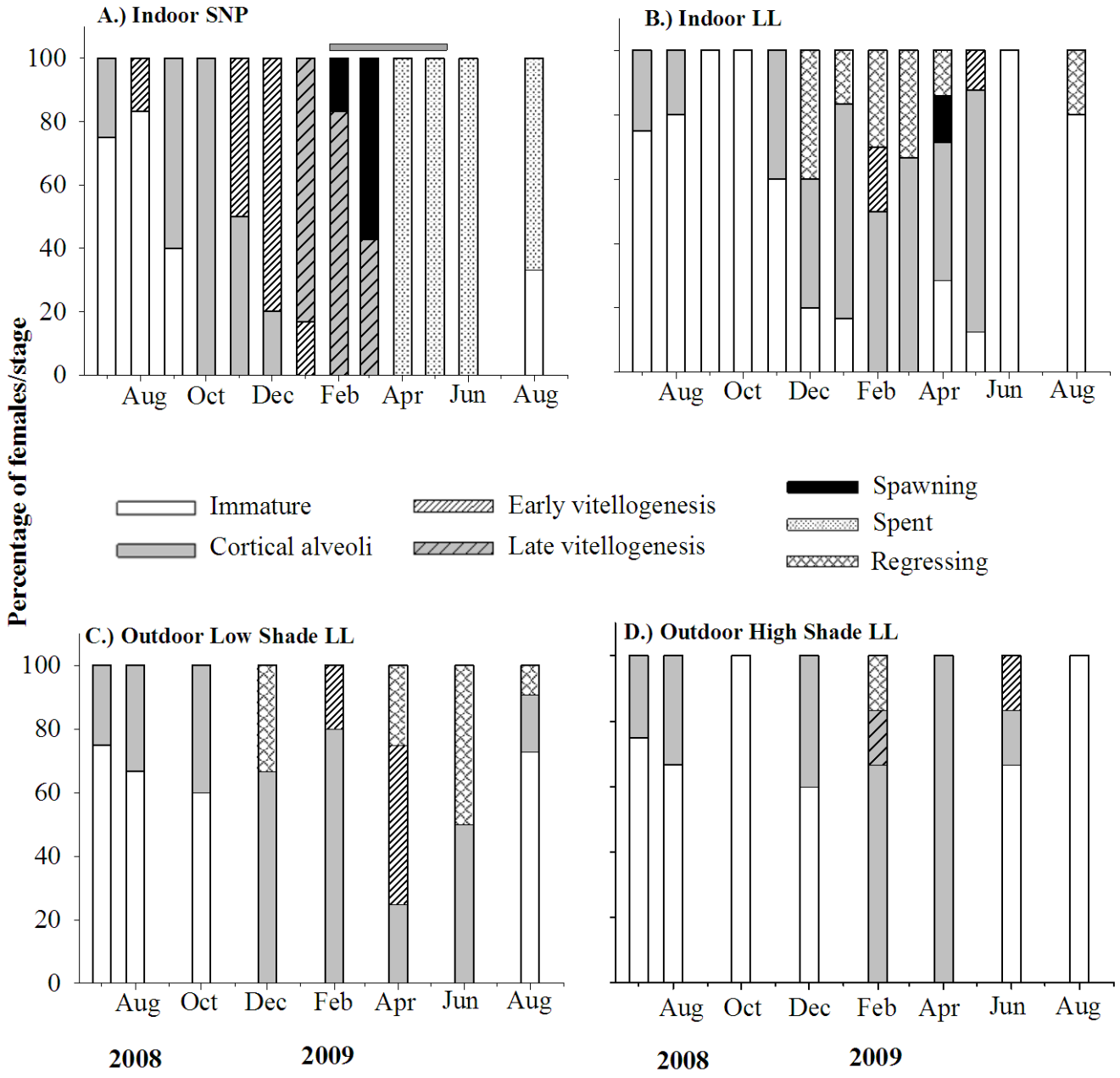

Figure 5. Gonadal staging of sacrificed female individuals maintained under all lighting treatments (A-D) (see Table 1 for number of fish analysed). Grey bar indicates period of spawning in the indoor SNP treatment.

3.3 Sex steroid profiles

In indoor SNP females, plasma testosterone (T) levels began to rise in September 2008 and significantly peaked in December (tagged fish, circa 1.4 $ng.ml^{-1}$) or from November to March (sacrificed fish, circa 0.8-1 $ng.ml^{-1}$) with respect to the LL treatment (Fig. 6A and B). T levels in SNP fish then decreased by the end of trial. Significant differences were also observed between indoor SNP and outdoor shading treatments in tagged fish (high shade treatment in October) and in sacrificed fish (from October to March except in December). Plasma 17β-estradiol (E_2) levels showed a significant elevation in both tagged and sacrificed SNP females with peak levels of circa 1.2-2.3 $ng.ml^{-1}$ in February and from January to March respectively (Fig. 6C and D). E_2 levels in indoor LL fish remained <0.25 $ng.ml^{-1}$ and there were no significant differences between these and the outdoor shading treatments throughout the period of indoor SNP spawning. However, a significant rise was observed in June in the outdoor low shade treatment relative to indoor SNP and LL treatments. In indoor SNP males, plasma T levels started to increase in October and reached peak levels in February (tagged fish, circa 2.2 $ng.ml^{-1}$) and January to March (sacrificed fish, circa 2-3 $ng.ml^{-1}$) before returning to basal levels from May onwards (Fig. 7A and B). In tagged fish, T levels in the indoor SNP treatment were significantly higher than the outdoor high shade treatment in October and both shade treatments in February. In sacrificed fish, T levels in SNP fish remained significantly higher than all other treatments from September to March. There was no difference in plasma 11-ketotestosterone (11-KT) concentrations between any treatments until February (tagged fish, circa 7 $ng.ml^{-1}$) and January to March (sacrificed fish, circa 4-6 $ng.ml^{-1}$) when levels in the indoor SNP population significantly peaked compared to outdoor shading treatments (Fig. 7C and D).

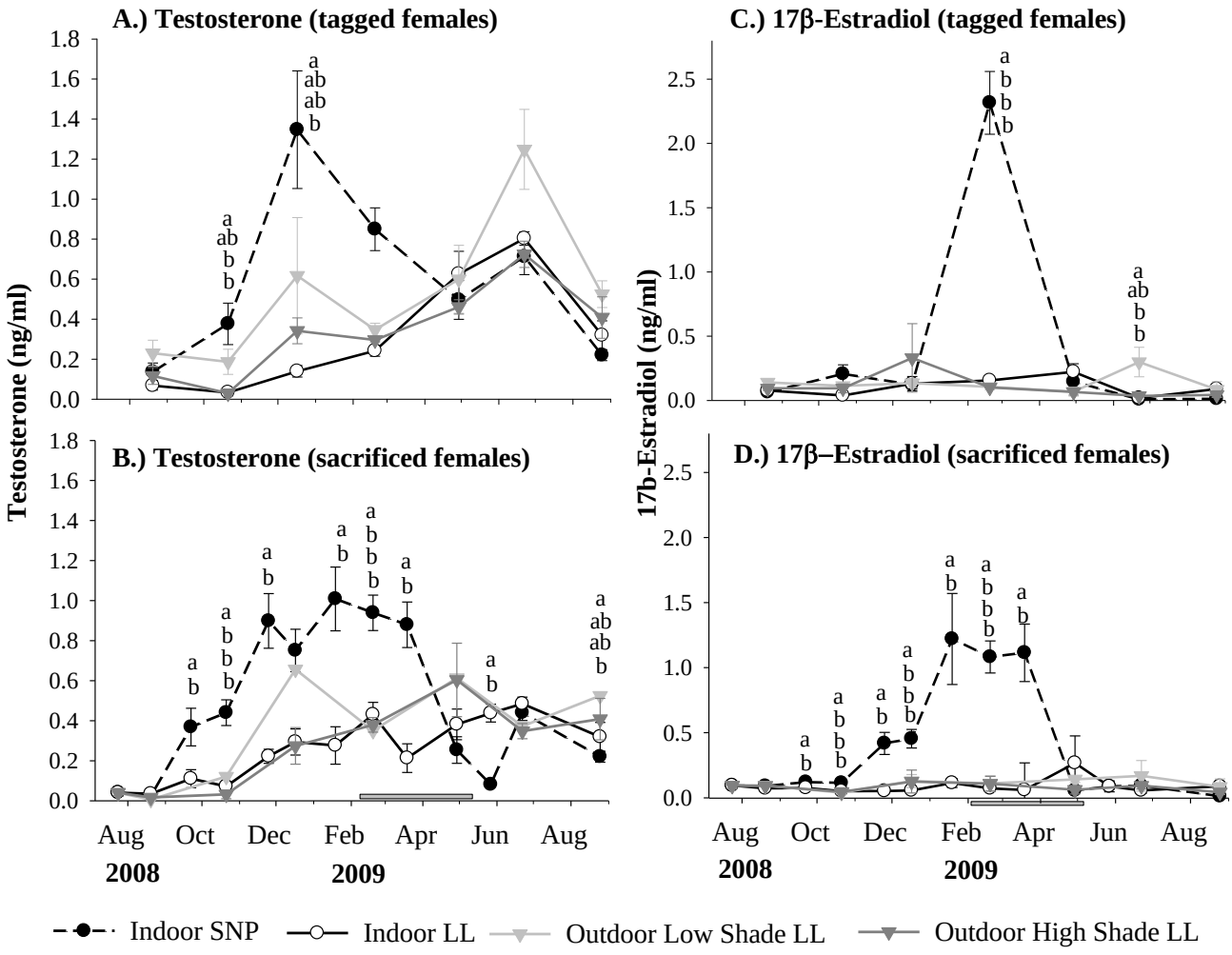

Figure 6. Plasma testosterone (A-tagged, B-sacrificed) and 17β-estradiol (C-tagged, D-sacrificed) levels in female fish maintained under indoor (SNP or LL) or outdoor (low/high shade) lighting treatments. Data are presented as mean ± SEM for every two month interval. On the intervening months, only sacrificed fish from the two indoor treatments were measured (see Table 1 for number of fish sampled). Significant differences between treatments at each time point are indicated by superscripts (GLM and one way ANOVA for tagged and sacrificed fish respectively). Grey bar indicates period of spawning in the indoor SNP treatment. See appendices 12-15 for detailed tables of results.

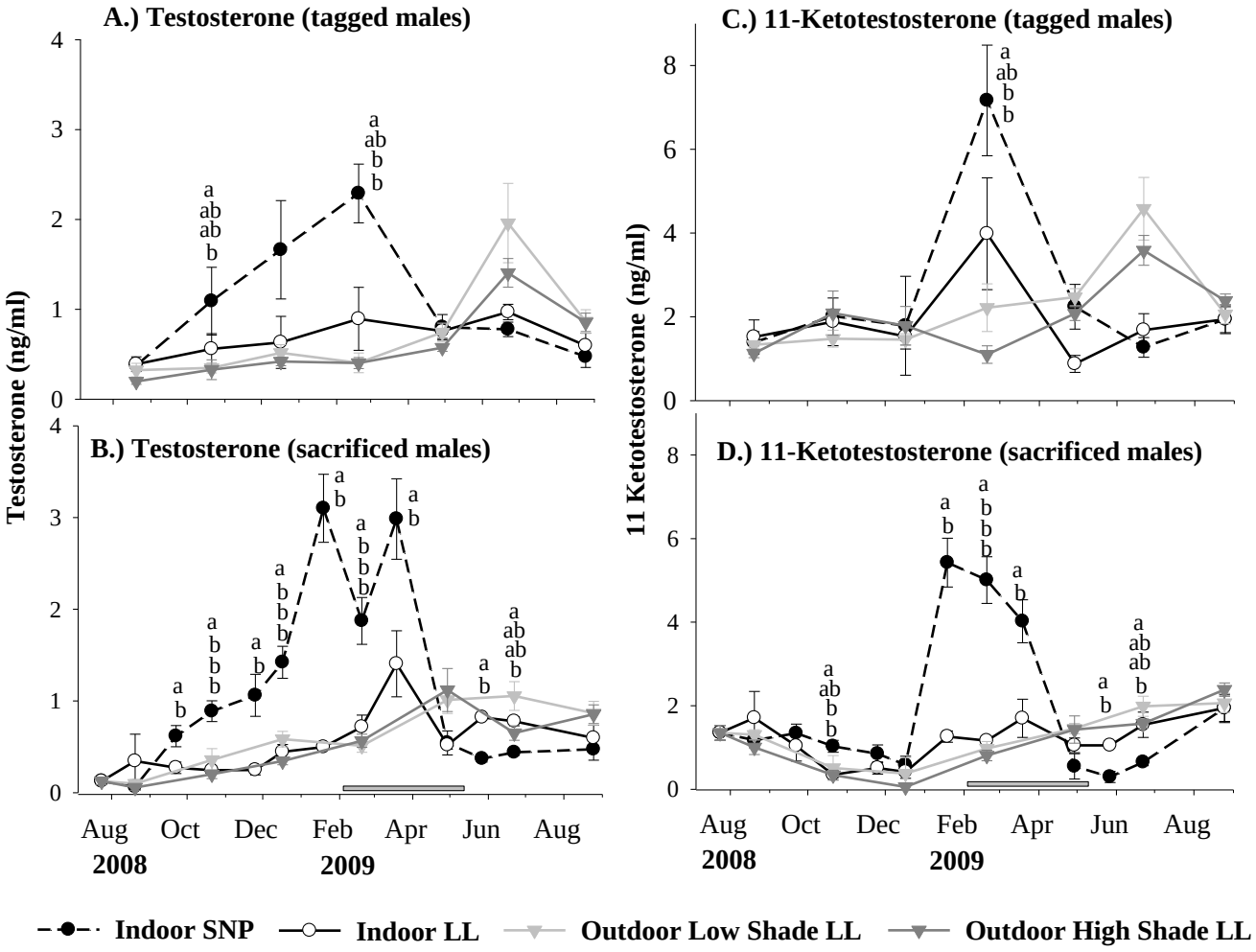

Figure 7. Plasma testosterone (A-tagged, B-sacrificed) and 11-ketotestosterone (C-tagged, D-sacrificed) levels in male fish maintained under indoor (SNP or LL) or outdoor (low/high shade) lighting treatment. Data are presented as mean ± SEM for every two month interval. On the intervening months, only sacrificed fish from the two indoor treatments were measured (see Table 1 for number of fish sampled). Significant differences between treatments at each time point are indicated by superscripts (GLM and one way ANOVA for tagged and sacrificed fish respectively). Grey bar indicates period of spawning in the indoor SNP treatment. See appendices 16-19 for detailed table of results.

3.4 Melatonin

Indoor SNP fish showed a significant night-time elevation in plasma melatonin (40.67 ± 4.91 and 69.22 ± 11.09 pg·ml^{-1} for day and night respectively)(Fig. 8). There were no significant differences between day or night plasma concentrations in melatonin observed in fish maintained under the indoor LL or either outdoor shading treatments (mean melatonin/treatments ≤ 43.86 pg.ml^{-1}).

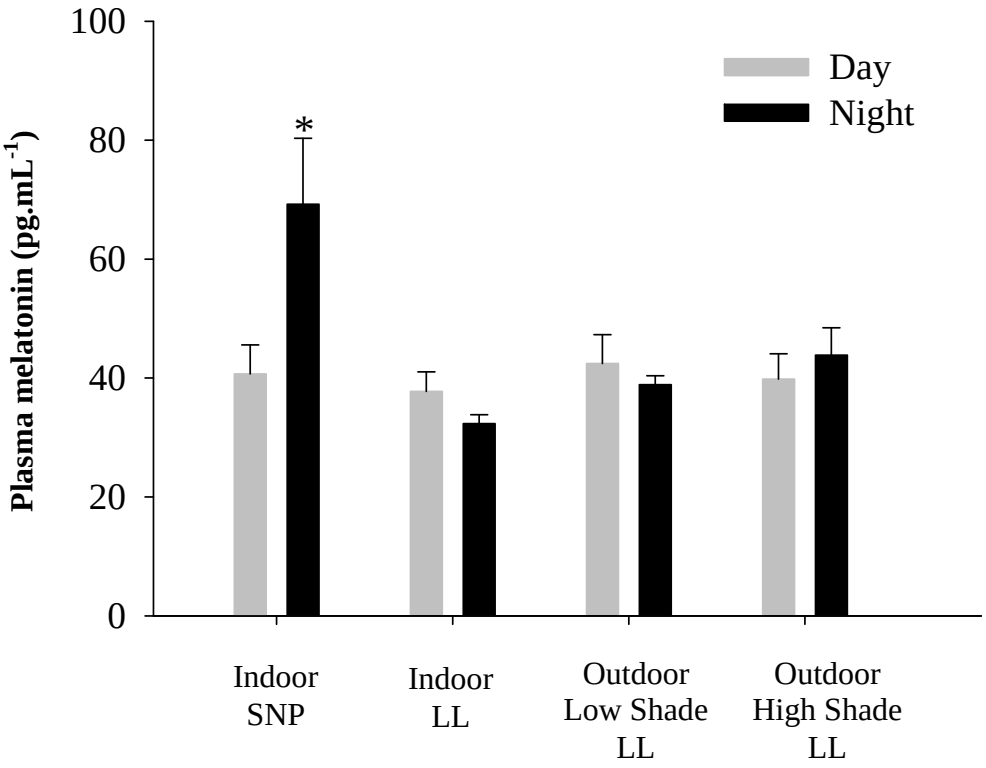

Figure 8. Plasma melatonin levels (mean ± SE, *n* = 10-18 for day and night) in fish maintained under four different lighting treatments. Significant night-time elevations relative to day are indicated by an asterix.

4. Discussion

This current study is the first to demonstrate that shading in combination with continuous artificial lighting in outdoor conditions can successfully suppress sexual maturation in 2 year old Atlantic cod. Both the outdoor high and low shading treatments tested were shown to be effective at suppressing sexual maturation when compared to the two indoor populations which either fully matured (SNP) or remained immature (LL) during the course of the experiment.

As expected, all fish exposed to the indoor SNP photoperiod regime matured and spawned as demonstrated by fertilised eggs collected in the tank outflow from February to May 2009. Such a spawning season is comparable to that reported for wild cod (Vitale *et al.*, 2005) and past studies of captive cod reared in sea cages (Taranger *et al*., 2006) and tanks (Kjesbu, 1989; Norberg *et al*., 2004; Davie *et al.*, 2007a). The temporal increase in GSI observed in our study is in accordance with previous reported data although our absolute female GSI values were higher (~12 % in Davie *et al.*, 2007a) which could be a reflection of sampling female ovaries that contain hydrated oocytes. With the exception of two spermiating males and one female with hydrated oocytes in the indoor LL treatment, no spawning activity was observed in individuals under any of the indoor or outdoor LL treatments. Accordingly female mean GSI values remained below a mean of 2.2 %, male values remained below a mean of 1.4 %. These values are significantly lower than those recorded in spawning siblings and are in keeping with levels reported for immature cod in previous light manipulation studies (Hansen *et al*., 2001; Karlsen *et al*., 2006; Davie *et al.*, 2007a).

Histological analysis of ovarian development (both measurement of leading cohort oocyte diameters and histology) was used to classify the reproductive development within each of the treatment populations. In the indoor SNP females,

measurements of G1 oocyte diameter showed a steady increase in vitellogenic oocytes from December onwards, peaking in March, which correlated with the spawning activity recorded. Conversely mean oocyte diameter did not exceed 400 µm in the indoor LL and outdoor shading treatments. It appears from the literature that the threshold for vitellogenic oocytes lies somewhere between 350 and 400 µm (Kjesbu 1991; Taranger *et al*., 2006) thus oogenesis would not have been initiated in all LL treatments. It must be acknowledged that in the present study, oocyte measurements were not corrected for the possible shrinkage owing to fixation (Kjesbu, 1994; Dahle *et al*., 2003). However, as this shrinkage is usually in the region of 5%, the current figures will still give an accurate comparison of oocyte development between females. Histological examination of the ovarian development confirmed normal development in indoor SNP females following the basic teleost pattern (Coward *et al*., 2002). Vitellogenesis (early and late) corresponding to the critical period of oocyte growth, was apparent in females in November and continued through to March after which all females sampled displayed spent ovaries containing oocytes and post-ovulatory follicles in resorption accompanied by primary growth oocytes (Kjesbu & Kryvi 1989). In contrast, the majority of females exposed to LL treatments did not progress beyond the cortical alveoli phase and from December 2008 onwards, a number of females under the LL conditions had ovaries dominated by atretic oocytes characterised by a hypertrophy of the follicular cells and irregular shape. This indicates that early phases of oogenesis may have been initiated but were then arrested with developing oocytes undergoing subsequent resorption (Miranda *et al*., 1999; Rideout *et al*., 2000).

A cycle in testosterone (♂ + ♀), 17β-estradiol (♀) and 11-ketotestosterone (♂) levels was observed under indoor SNP conditions with levels increasing up to the spawning window and decreasing to reach basal values thereafter. Treatment differences

were more apparent in fish which were sacrificed and had not been handled at previous timepoints. The repeated handling of tagged fish involving anaesthesia, weight/length and blood sampling throughout the trial may have had an effect on sex steroid levels. Furthermore sampling of tagged individuals from indoor SNP and LL fish was at lower resolution (every two months) than those sacrificed from these treatments (every month) thus peaks in sex steroid levels may have been missed. Sex steroids are known to play a key role in the control of gametogenesis in fish including the synthesis of vitellogenin by the liver which then accumulates in the oocytes (Silversand *et al*., 1993), and the multiplication of spermatogonia at the onset of spermatogenesis (11-KT, Schulz et al., 2010). In addition, T is known to serve as a precursor for estrogens in female teleosts (Tanaka *et al*., 1992, Schulz & Miura, 2002) and potentially other androgens such as 11-KT in male teleosts (Young *et al*., 2005). The T and 11-KT results (amplitudinal changes, peak levels and overall profiling) match previous sex steroid data however, absolute E_2 levels measured in the current study were significantly lower than those previously reported for cod (Davie *et al*., 2007a; Norberg *et al*., 2004). This said, the relative change (*circa* 10 fold change from basal to peak levels) and profile of change was consistent with previous observations (Dahle *et al*., 2003; Norberg *et al*., 2004; Almeida *et al*., 2009b). The lower absolute E_2 levels could be a reflection of the analytical methodology, age, size and/or origin of the stocks studied. In contrast to the indoor SNP population, sex steroid levels in fish reared under LL treatments (indoor LL and outdoor low & high shade LL) in general remained low. There was one notable difference however in June 09 where E_2 (♀) in the low shade population showed a significant elevation with respect to indoor (both SNP and LL) levels. This hormonal change did not appear to correlate with GSI values measured as no significant increase was observed. In fact the large proportion of fish that showed atretic oocytes under the

LL conditions confirmed that some gonadal development had been initiated but then arrested which would probably be due to the lack of suitable hormonal stimulation. A number of authors have shown that the incidence of atresia decreases as sex steroid levels start to rise both in mammals (Woolveridge *et al*., 1999) and teleosts (Almeida *et al*., 2009b), thus in our study the lack of sex steroid surge could explain the restricted gonadal recruitment in the LL fish followed by apoptosis and regression.

The efficacy of the LL treatments was inferred by the lack of observed spawning in any of the three LL populations. However, the E_2 fluctuation under the low shade treatment paralleled by the higher proportion of "regressing" ovarian samples suggests that perhaps the relative difference within this setup was closer to a theoretical threshold of sensitivity than in the high shade setup. At this stage, it must be acknowledged that no outdoor control LL treatment (without shade netting) could be tested due to limitations in facility availability. Thus the effects of shading on the efficacy of the LL regime in an open system could not be directly confirmed especially under the increased night-time illumination achieved in this study (1.5 watts m^{-2}) as compared to previous commercial trials performed in UK standard cage set up (0.13 watts m^{-2} in 25x25x15 m square pen when using 15x400 W metal halogen units, Migaud, per. com.). This said, the actual night levels relative to daylight in a control outdoor LL tank (without shade) would have been similar to a commercial set up previously tested depending on the cod swimming depth (2 % vs. 0.05-3.4 % respectively). Based on previous tank and cage studies as well as industry reports, it is therefore to be expected that fish exposed to LL in an outdoor tank without shade would have spawned with a 3-4 month delay as seen previously (Taranger et al., 2006).

Ultimately a key outcome of such photoperiod manipulations is an improvement of growth performance during culture. Whilst it must be acknowledged that the

experimental design led to differences in stocking densities between treatments, the maximum densities (10 kg/m^3 indoor *vs*. 7 kg/m^3 outdoor) did not exceed levels reported as compromising growth performance in the species (Lambert & Dutil 2001) which allows for a valid comparison of performance. The improved growth, observed in the present study, in fish exposed to the indoor LL and outdoor high shade regimes is believed to be principally due to the suppression of maturation rather than direct photostimulation of the somatic growth axis as was proposed by Davie *et al*. (2007b). Interestingly, females from the low shade LL treatment did not show any weight enhancement as compared to SNP. This is surprising as although some females showed signs of gonadal development and sex steroid increases especially in June, no fish fully matured. One possible explanation could be that sexual maturation under the low shade LL treatment could have been delayed as described in previous cage on-growing studies (Taranger *et al.* 2006). However, while the trial could not be continued past August, due to limitations in stock availability, the histological analysis of females in this treatment at the trials end recorded a G1 oocyte diameter <300µm and showed no evidence of vitellogenesis that means no likelihood of spawning for at least the next 3-4 months (Kjesbu 1994). As for males, no significant differences in weight were observed between the high shade treatment and the SNP treatment however both the high shade treatment and the low shade treatment appeared to reach a higher wet weight than the SNP fish by the end of the trial. Growth rates differed between sexes in all treatments, and expecially in the indoor LL and outdoor high shade LL treatments with females from treatments being in the region of 40% bigger than males. This is in accordance with results from Davie *et al*. (2007b) in immature cod and has also been reported in turbot, *Scopthalmus maximus* (Imsland *et al*., 1997). Such sex differences in growth are not fully understood but may involve different sex specific windows of muscle fibre recruitment (Johnston *et*

al., 2003). In light of these results, the production of monosex female populations together with light and shading control of early maturation could become a valuable strategy for farmers to realise maximum growth potential of their stock, however, robust maturation control would be essential.

Plasma melatonin levels measured in this study provided as expected a day night rhythm present only in the indoor SNP population. There was no night-time elevation observed in the indoor LL or shading treatments and this is in accordance with past *in vitro* and *in vivo* pineal studies on cod which have shown that LL effectively suppresses melatonin rhythms (Porter *et al*., 2000; Vera *et al.* 2010).

In summary, this study demonstrated that by reducing the ambient daylight intensity using shade netting in combination with constant artificial light, the maturation of cod can be fully suppressed compared to controls reared under SNP. Both outdoor shade LL treatments were equally effective at suppressing gonadal development as shown by the GSI data. This suggests that such a difference in light intensity between daylight and artificial light during the subjective night was too weak a signal for the recruitment of cod into a reproductive cycle. However, data also suggest the low shade treatment (6.6%) to be closer to the threshold as shown by rise in steroid towards the end of the trial. Therefore, a cautious approach, prior further confirmation of the current data, would be to recommend using night-time illumination ≥31% of daylight (high shade treatment) to suppress cod maturation in outdoor set ups. This preliminary benchmark must be tested in a commercial setting and further refined to accurately define a cut-off threshold to be applied within the industry. However, as field data reveals that current submersible lighting technology can only achieve levels of 1-2% then the current findings provide a compelling argument for the development and testing of robust commercial scale shading to make such thresholds achievable. Another key advantage of

using shade netting to reduce ambient light would also be a potential reduction in the amount of artificial light needed to suppress maturation. This is especially important as increasing intensities are being used during cod on-growing in cages through higher number of more powerful lighting systems in attempts to reduce the rate of fish maturing.

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CHAPTER 4

RESEARCH ARTICLE

PHOTOPERIOD EFFECTS ON THE EXPRESSION OF KISSPEPTIN AND GONADOTROPIN GENES IN ATLANTIC COD, *GADUS MORHUA* DURING FIRST MATURATION

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In Preparation

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Keywords: Atlantic cod, kisspeptin, brain, maturation, light

Abstract

The kisspeptin system has recently been identified as a key regulator of sexual maturation in mammals and it is believed that this system is conserved in fish. In order to investigate the potential role of the kisspeptin system in the entrainment of reproduction in Atlantic cod, *Gadus morhua*, qPCR assays were developed and validated for the Atlantic cod homologue of kisspeptin (Kiss2) and its receptor (Kissr4). Expression was characterised in the brain over 13 months (July 2008-August 2009) in 2 populations of males and females: 1) a maturing population (exposed to simulated natural photoperiod, SNP) and 2) a maturation inhibited population (exposed to constant light, LL). In the initial 8 months of the trial corresponding to the onset of puberty, pituitary expression of gonadotropin subunit mRNA (*fshβ* and *lhβ*) was also measured. Results from this study indicated no clear pattern in expression of *kiss2* or *kissr4* mRNAs in either population of cod, acute elevations did occur in maturing individuals however, namely an elevation in *kiss2* in females (January) and an elevation *kissr4* in males (November). Gonadotropin mRNA expression displayed strong amplitudinal changes over time, as expected, with *fshβ* and *lhβ* mRNA expression increasing towards spawning in maturing individuals while no significant elevations were recorded in immature individuals. These results do not clearly support the hypothesis that the kisspeptin system would play a key role in the initiation of gametogenesis, as shown in mammals and suggested in other fish species, by stimulating the brain pituitary gonadal axis at the gonadotropin releasing hormone (GnRH) level which would result in recruitment of fish into maturation. This work therefore opens up interesting new avenues to unravel the role of kisspeptins in teleost reproductive physiology.

1. Introduction

In teleosts, it is well known that puberty and subsequent gonadal development are under the neuroendocrine control of the brain pituitary gonadal (BPG) axis (Weltzien *et al.,* 2004; Zohar *et al.,* 2010). While the BPG cascade is well described in vertebrates, the initiation and subsequent regulation of reproduction by external factors such as environmental changes or adiposity remains to be defined. However, in recent years genetic analysis of idiopathic hypogonadotropic hypogonadism in humans has identified two new candidate neuropeptides, Kisspeptin and Neurokinin B, which have both been shown to regulate GnRH neuron activity and ultimately sexual maturation (Oakley *et al.,* 2009; Wakabayashi *et al.,* 2010). Kisspeptin, in particular, has seen extensive investigation across several disciplines and while it appears its regulatory role in puberty is conserved across most of the vertebrate classes, a clear description of its mode of action remains to be presented (Akazome *et al.,* 2010; Lehman *et al.,* 2010).

Kisspeptin (Kiss) and its receptor (Kissr), formally called G-coupled protein receptor-54 (GPR54), have been subjected to duplication events in the early vertebrate lineage with at least two isoforms of both being present in teleosts and amphibians as well as the platypus, a monotreme mammal (Um *et al.,* 2010), which complicates the functional characterisation of this system. The two paralogous genes of kisspeptin (*kiss1* and *kiss2*) have been reported in a number of teleost species including Zebrafish (*Danio rerio*), Medaka, (*Oryzias Latipes*), Fugu (*Takifugu rubripes*), Goldfish (*Carassius auratus*) and European Seabass (*Dicentrarchus labrax*) (Felip *et al.,* 2009; Lee *et al.,* 2009; Akazome *et al.,* 2010). However, it appears that some species, like the three spined Stickleback (*Gasterosteus aculeatus*), have lost one isoform (*kiss1*) from their genome. While 4 different isoforms of Kissr (1-4) have been described in vertebrates only Kissr2 & 4 have been reported in teleosts with Kissr4 being the most prevalent

(Akazome *et al.,* 2010). In order to discuss expression patterns and physiological functions of these kisspeptin forms from studies on different animal and fish species, systematic nomenclature in accordance with Akazome *et al.* (2010) has been adopted throughout this paper.

Functional studies of Kisspeptin in zebrafish have revealed that the peptide-receptor relationship appears to be rather promiscuous with both Kiss1 and Kiss2 shown to activate both types of kisspeptin receptors with different potencies (Lee *et al.,* 2009). However, Felip *et al.* (2009) demonstrated that Kiss2 was a more potent inducer of gonadotopin expression than Kiss1 in European seabass, which has led to the suggestion that Kiss2 is functionally more important in fish. With regards to the receptors, studies performed to date have focussed on the Kissr4 with Kissr2 only found in a few species, leading to the suggestion that the Kissr4 form would be functionally more important . Parhar *et al.* (2004) were the first to report co-localization of *kissr4* (cited as *kiss1r*) and *GnRH* expression in the Nile Tilapia, *Oreochromis niloticus*. Later studies have all supported this finding with significant positive correlatation between *kissr4* and *GnRH* gene expression as documented in tropical species such as the zebrafish (Kitashi *et al.,* 2009) and cobia, *Rachycentron canadum* (Mohammed *et al.,* 2007) and temperate species including grey mullet, *Mugil cephalus* (Nocillado *et al.,* 2007), fathead minnow, *Pimephales promelas* (Filby *et al.,* 2008) and chub mackerel, *Scomber japonicus* (Selvaraj *et al.,* 2010).

Interestingly, a number of studies performed in seasonal mammals have shown correlations between kisspeptin expression, sexual development and photoperiod (Revel *et al.,* 2006a; Grieves *et al.,* 2007; Mason *et al.,* 2007). For example, in the Syrian hamster, a spring breeder, transfer from a long day to short day photoperiod inhibits sexual development and results in down regulation of *Kiss1* expression (Revel *et al.,*

2006a; 2006b, Grieves *et al.*, 2007). Similar results were obtained in Soay sheep, an Autumn breeder, when transferred from a short to long day photoperiod (Wagner *et al.*, 2007). As in mammals, puberty and subsequent reproduction of temperate teleosts is typically in tune with the seasonal environmental changes and more specifically photoperiod (Bromage *et al.*, 2001). In Atlantic cod it has been demonstrated that the decreasing daylength after the summer solstice acts as the proximate cue to recruit individuals into a reproductive cycle (Davie *et al.*, 2007a). However the cascade of events from seasonal perception to stimulation of the BPG has not been clearly described yet. Differential gene expression of the gonadotropin, follicle stimulating hormone (*fshβ*), was shown from September onwards in male cod exposed to either a continuous (LL) or simulated natural photoperiod (SNP) regime (Almeida *et al.*, 2009a). This would suggest that between the switch of photoperiod at the summer solstice and the *fshβ* gene expression surge seen in September the GnRH neurons would have been stimulated by a signal peptide which is as yet unidentified, potentially kisspeptin?

This study aimed to investigate the expression profiles of kisspeptin genes (*kiss2* and *kissr4*) in the brain in correlation with gonadotropin genes (*fshβ, lhβ*) in the pituitary in reproductively active (exposed to SNP) or suppressed (exposed to LL) Atlantic cod populations. The objective being to test the hypothesis that the kisspeptin system could act as a marker for the decision to commit to sexual maturation and thus differential expression patterns will be evident between the SNP and LL treatments prior to the onset of gametogenesis.

2. Materials and Methods

2.1 Fish and sample collection

The trial was conducted at the Machrihanish Marine Environmental Research Laboratory (MERL, Scotland, 55:44^{0}N, 5:44^{0}W). Prepubescent mixed sex Atlantic cod (411.0 ± 5.6 g, mean wet weight ± SEM) were randomly allocated into two indoor tanks (10.6 m^3, 9.4 m circumference, 1.5 m running depth, 187 fish/tank) which were fully covered with light proof lids. These fish had been previously reared on site under simulated natural photoperiod (SNP) and ambient temperature regimes. Fish were acclimatised to their experimental tanks during 5 weeks (18th June – 24th July). On the 24th July 2009, a baseline sample of 13 fish (6 males, 7 females) was taken before the populations were subjected to their experimental treatments for 13 months. Two treatments were set up: 1) SNP (stimulating reproduction) and 2) LL (inhibiting reproduction) with lighting in all cases being provided by one green cathode light unit (232 W, peak wavelength: 546 nm, Intravision Aqua, Oslo, Norway) per tank located across the centre of the tank and suspended on the water surface. Downwelling light intensity (at 1.5m, maximum tank depth) measured in July 2008, was 0.54 ± 0.32 watts m^{-2} (n= 3 measurements/tank) during the solar noon for both tanks and 0 watts m^{-2} and 0.57 ± 0.38 watts m^{-2} for the SNP and LL tank respectively, at midnight. Light intensity (watts.m^{-2}) was measured using a single channel light sensor with a non biased wavelength range of 400-740 nm (Skye Instruments Ltd., Powys, UK).

Every month from 24th July 08 to the 24th August 09, up to 12 fish were sacrificed from each treatment (individuals were chosen at random when sex could not be externally identified, Table 1). No samples were taken in July 2009, instead fish were maintained for an additional month and sampled in August 2009. At each time-point, fish were culled by lethal anaesthesia (MS222, 80ppm, Pharmaq, Fordingbridge, UK),

blood was withdrawn from the blood vessels in the caudal peduncle using a 2 ml syringe and 23G sterile hypodermic needle and stored on ice for later processing (see below). Cod whole brains and pituitaries were dissected and then frozen separately, within 1 minute of dissection, over liquid nitrogen vapour before transferring to a -70°C freezer. Gonads were then dissected from sacrificed fish, weighed and a sample taken and fixed in 10 % neutral buffered formalin for histological examination.

2.2 RNA extraction and cDNA synthesis

RNA was extracted from whole brain samples for analysis of *kiss2* and *kissr4* expression and from pituitary samples for analysis of *fshβ* and *lhβ* expression. Extraction consisted in thawing brain and pituitary samples in 1 ml TRIzol® Reagent (Invitrogen, UK) per 100 mg of tissue before being homogenized over ice. RNA was extracted in accordance with guidelines (Invitrogen, UK) with RNA pellets being reconstituted in 50 µl of MilliQ water. RNA quality checks were performed with a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). cDNA was synthesized from 1 µg of DNase treated (DNA-Free, Ambion, UK) total RNA using a 2 µl blend of random primers, 0.8 µl of 100 mM dNTPs, 1 µl of multiscribe^{TM} reverse transcriptase (50 U/µl) with provided buffers and nuclease free H_20 in a final volume of 20 µl (High-Capacity cDNA Archive Kit - Applied Biosystems, UK). Thermal cycling conditions consisted of 10 minutes at 25^0C, 120 minutes at 37^0C and 5 minutes at 85^0C.

2.3 Molecular cloning of Atlantic cod *kiss2, kissr4, fshβ & lhβ*

Partial cDNA sequences for each target gene were generated by designing primers (Table 2) using one of the following strategies: designed on an expressed sequence tag clone identified by BLAST analysis of published sequences (*kiss2*); designed on highly conserved regions of sequences from other teleost species already published (*kissr4*);

designed on previously published sequences for Atlantic cod (*fshβ*, *lhβ & ARP*). Partial cDNA sequences were generated by PCR using 0.5 µM of primers (Eurofins MWG Operon, Edersberg, Germany) (Table 2) one fortieth of the original cDNA synthesis reaction, Klear Taq polymerase with supplied buffer (Kbiosciences, UK), and 1 mM MgCl2 in a final volume of 20 µl using a routine PCR strategy: 15 min 95 °C followed by 30 cycles of 95 °C 20 s, X °C 20 s, 72 °C 1 min. The annealing temperature is denoted as X °C in the description as it varied with the different primer pairs (see Table 2). All primer pairs generated a single PCR product and those products which were to be used for qPCR standards were cloned into a pGEM-T Easy vector (Promega, UK) and sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA). The identities of the cloned PCR products were then verified (100% overlapping) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequencing was performed using a Beckman 8800 autosequencer. Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA sequences. ClustalW (Thompson et al., 2000) was used to generate multiple alignments of deduced protein sequences. MEGA version 4 was used (Tamura et al., 2007) to deduce and bootstrap phylogenetic trees using the neighbor joining method (Saitou & Nei, 1987).

2.4 Quantitative PCR

Expression of the genes of interest was measured by absolute quantification with all samples being normalised with Acidic ribosomal protein (ARP) mRNA expression as this was previously shown to be the most stable candidate reference gene in Atlantic cod brain cDNA (Olsvik et al. 2008). All cDNA for qPCR were synthesised as described previously and qPCR primers (Table 2) were used at 0.5 µM, with one twentieth of the total cDNA synthesis reaction and SYBR-green qPCR mix which consists of Thermo-Start^{TM} DNA polymerase, a propietary reaction buffer, dNTP's and SYBR Green I with

Mg^{++} at a concentration of 3 mM in the final 1X reaction (ABsolute™ QPCR SYBR Green Mix, ABgene, UK), the total reaction volume was 20 µl. The thermal cycling protocol run in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) consisted of 15 min at 95 °C followed by 45 cycles of 95 °C for 15 s, X °C for 15 s and 72 °C for 30 s followed by a temperature ramp from 70 to 90 °C for melt-curve analysis. The annealing temperature (X) was changed in accordance with the primer pair (Table 2). Melt-curve analysis verified the primer sets for each qPCR assay generated one single product and no primer-dimer artefacts. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing partial cod cDNA sequences generated as described above. All samples were run in duplicate together with non-template controls.

2.5 Validation procedures

All sample extraction and qPCR assays were conducted where practically possible according to MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al*., 2009). In order to validate the qPCR assays, a serial dilution of linearised plasmid for each gene was tested and details on assay validation including linear dynamic range, PCR efficiency and repeatability are presented in table 3. It was not possible to perform RNA contamination assessment as there was no access to an agilent bioanalyser, the A_{260}/A_{280} ratio (range: 1.82-2.13) provided an indication of RNA purity however.

2.5 Analysis of gonadal stage and sex steroids

Histology was performed on the ovaries only. Sections of ovarian tissue, following fixation in 10 % neutral buffered formalin, were dehydrated and embedded in paraffin

wax, 5 µm sections were then cut and stained using haemotoxylin and eosin. Sections were examined under a light microscope and classified into one of five stages of development (according to Tomkiewicz *et al.*, 2003, see Chapter 3 for a description of histological stages). Testes were not analysed due to the differential stages of development within each lobe (Almeida et al., 2008).

2.6 Data analysis

qPCR data were analysed by Techne Quansoft version 1.1.21. using a fit points approach with the number of fit points set at 2 and standard deviations above the average of readings set in the range 3-10 for *fsh, lh* and *ARP* and 3-14 for *kiss2* and *kissr4*.

Statistical analysis of data sets was performed with MINITAB ® version 15.0 (Minitab Ltd., Coventry, UK). All data sets were tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test, and if necessary were log or arcsine-transformed prior to analysis. Gene expression data were analysed by analysis of variance (ANOVA) manipulated using a general linear model. In all cases a significance level of $p<0.05$ was set with significant interactions being analysed by Tukey *post hoc* test. Baseline data (collected in July 2008) was pooled according to sex.

Table 1. Number of fish sampled at each month over the trial period from the simulated natural photoperiod (SNP) and constant light (LL) treatments.

	SNP		**LL**	
	Female	**Male**	**Female**	**Male**
July	7	5	7	5
August	6	6	6	6
September	6	6	6	6
October	6	4	8	6
November	6	6	6	6
December	6	6	6	5
January	6	6	6	6
February	6	6	5	4
March	7	6	6	6
April	2	9	7	3
May	1	8	8	1
June	2	8	3	7
August	6	5	6	6

Table 2. Primer name, procedure for which the primers were used, sequence, predicted amplicon size, annealing temperature and accession number for the different genes studied.

Name	Procedure	Sequence	Product size	Annealing temperature (X°C)	Accession Number
*kiss2*F	Cloning & qPCR std	5'-CTGAGAGGGAACGACGAG-3'	317 bp	59°C	FG321938
*kiss2*R		5'-CAAGATTGTAAAAGATGGGATAG-3'			
*kiss2*qPCRF	qPCR	5'-TGAGAGGGAACGACGAGCAG-3'	78 bp	59°C	
*kiss2*qPCRR		5'-GGAGCCCGAACGGATTGTAG-3'			
*kissr4*F	Cloning & qPCR std	5'-TATGAGTGGAGACCGCTGTTACG-3'	556 bp	59°C	To be registered
*kissr4*R		5'-CTATGGGGTTGACAGAGGAGTTG-3'			
*kissr4*qPCRF	qPCR	5'-CATCAGCATACGGAGCAAGGTGTC-3'	123 bp	62°C	
*kissr4*qPCRR		5'-TTGGGCTGGTACTGGGGATAGAAG-3'			
fshF	Cloning, qPCR std & qPCR	5'-CGCCCGACCCCGACCACCATT-3'	236 bp	68°C	DQ402373
fshR		5'-TTGCTGCTCTGACACAGGGAACAC-3'			
*lh*F	Cloning & qPCR std	5'-CTCCGTGGAGAAGAAGGGCTGTC-3'	300 bp	68°C	DQ402374
*lh*R		5'-GCTGAGTGCGGCGGGGTAGTGGAC-3'			
lhqPCRF	qPCR	5'-CCCGCGGCTCAGCAAGGTGGTG-3'	114 bp	62°C	
lhqPCRR		5'-GCTGAGTGCGGCGGGGTAGTGGAC-3'			
*ARP*F	Cloning & qPCR std	5'- AGGTGCTCGGTTCTTCATCTG-3'	400 bp	59°C	EX741373
*ARP*R		5'- TCGTCCTTCTTCTCCTCTTTCTTC-3'			
*ARP*qPCRF	qPCR	5'-TAGCACCGTTAAATTTAGGCATCC-3'	90 bp	59°C	
*ARP*qPCRR		5'-AGCAGAGTAAATACAAGCGAGTTC-3'			

Table 3. Details on validation assays for qPCR of *fsh*, *lh*, *kiss2*, *kissr4* and *ARP*. Details include the quantification cycle (Cq) number of no template controls (NTC), specifics of the standard curve including slope and Y intercept, qPCR efficiency calculated from the slope and r^2 value and the linear dynamic range including Cq variation at the lower limit.

Assay	**Cq NTC (mean ± standard deviation)**	**Slope**	**Y intercept**	**Efficiency (%)**	**r^2**	**Linear range**	**Lower limit Cq variation (coefficient of variation, %)**
fsh	29.59 ± 0.34	-3.433	35.87	96	0.99	10-10^8	1.2
lh	28.81 ± 0.19	-3.465	38.33	94	1	10^4-10^8	0.4
kiss2	35.12 ± 0.76	-3.358	36.01	98.5	1	10-10^8	0.6
kissr4	34.21 ± 0.34	-3.329	35.66	99.7	0.99	10-10^8	0.5
ARP	35.28 ± 1.32	-3.099	34.85	94.9	0.99	10-10^8	0.1

3. Results

3.1 Atlantic cod *kiss2* and *kissr4* partial cDNA sequences and phylogenetic analyses

BLAST analysis of Atlantic cod EST clones registered in public databases identified a single sequence (Accession no: FG321938) with high homology to other teleost *kiss2* sequences. This 479 bp partial sequence consists of a 329 bp coding sequence (cds) and 150 bp 3'untranslated region (UTR), importantly the cds contains the decapeptide epitope *kiss-10* sequence that defines the gene (Fig. 1). The deduced amino acid (aa) sequence for this epitope is "FNYNPFGLRF" which has 100 % identity with the kiss2 epitope in zebrafish, medaka and goldfish though is one amino acid different, Tyrosine (Y) in place of Phenylalanine (F), from European seabass and Orange spotted Grouper, *Epinephelus coioides* (Fig. 1). Phylogenetic analysis of the deduced aa sequence in relation to other teleost kisspeptins shows the fragment grouped within the Kiss2 cluster (Fig. 2) having the greatest identity with European seabass Kiss2 (60 %) and in the order of 50-60 % identity with all other teleost Kiss2 sequences.

Primer pair *kissr4* F/R generated a 556 bp product from cod brain cDNA samples (Fig. 3). This fragment covers 50 % of the target gene cds which spans five of the receptor's seven trans-membrane domains. Phylogenetic analysis of the deduced aa sequence for Kissr4 in relation to other teleost kisspeptins shows the fragment grouped within the Kissr4 cluster having >80 % identity with all other teleost *kissr4* sequences (Fig. 4).

A.)

```
2     TCG AGC TCT GCG GAG TGG CCC CTC GCG ACA GGT TGC GGA CCG ACC   46
1      S   S   S   A   E   W   P   L   A   T   G   C   G   P   T    15

47    AGT TTC TCA GAG CTG ACG GTG GCC AAG AGG ACG GAT TTA ACG GAC   91
16     S   F   S   E   L   T   V   A   K   R   T   D   L   T   D    30

92    ATC TTA CCG GAG AAC CAC AAC CCG TGC ATC TCC CTG AGA GGG AAC   136
31     I   L   P   E   N   H   N   P   C   I   S   L   R   G   N    45

137   GAC GAG CAG CGT CAA CTG CTC TGC AAC GAC CGG CGG AGT CCG TTC   181
46     D   E   Q   R   Q   L   L   C   N   D   R   R   S   P   F    60

182   AAC TAC AAT CCG TTC GGG CTC CGC TTC GGG AAA CGG TTT CTG CCC   226
61     N   Y   N   P   F   G   L   R   F   G   K   R   F   L   P    75

227   GTG AAA ACC GAC CGC TTG TCT CAA GGA AGT CTC GCG AGA CCG AGG   271
76     V   K   T   D   R   L   S   Q   G   S   L   A   R   P   R    90

272   ACG ATA ACT TTT TTA CCC GTT TTC CTC AAC CCG CGA GAT TTG GAA   316
91     T   I   T   F   L   P   V   F   L   N   P   R   D   L   E   105

317   ATC ACG ACC TAA TGA AGT AGC CGT GTC CGC CTG TCC AGC AGT TTT   361
106    I   T   T   *                                             109

362   CGC GTT GCA AGT CAA ATA TTT TAA ATT AAT GTC GTT AAC TTC ACG   406
407   ATT TGT GAT ATT CCT ATC CCA TCT TTT ACA ATC TTG TGT TAT TTT   451
452   TTC CCA TGA ATA AAC CTT TTG TAC CGT                         478
```

B.)

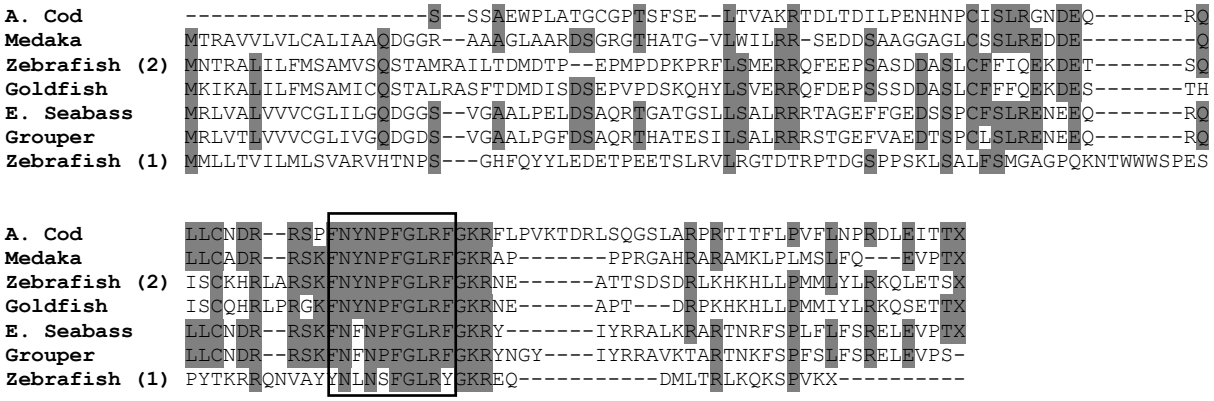

Figure 1. A.) Partial nucleotide and deduced amino acid sequence of Atlantic cod *kiss2* (FG321938). The predicted kisspeptin-10 eptitope is underlined. * marks the stop codon. B.) Alignment of the deduced protein sequence for Atlantic cod, Medaka, zebrafish, Goldfish, European Seabass and Orange spot grouper kiss2 in comparison with zebrafish kiss1. The conserved amino acid residues are shaded. The kisspeptin-10 epitope is boxed.

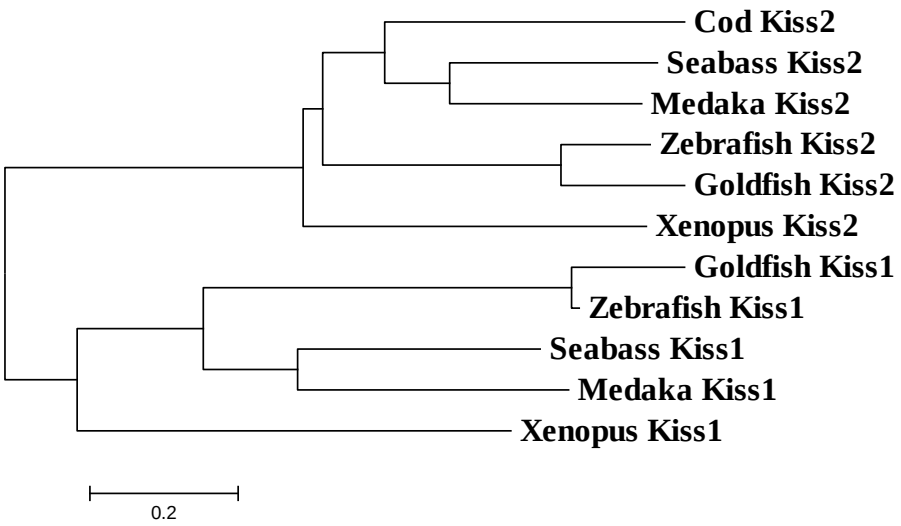

Figure 2. Phylogenetic tree analysis of teleost *kiss2* genes. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.

A.)

```
2     ATG AGT GGA GAC CGC TGT TAC GTC ACC GTG TAC CCG CTC AAG TCG   46
1      M   S   G   D   R   C   Y   V   T   V   Y   P   L   K   S   15
            TM3
47    CTC CGT CAC CGC ACG CCG AGG GTG GCC ATG ATC GTC AGC GTC TGC   91
16     L   R   H   R   T   P   R   V   A   M   I   V   S   V   C   30
                                    TM4
92    ATT TGG ATA GGT TCC TTC ATC CTT TCC ATT CCC ATC TTC CTG TAC   136
31     I   W   I   G   S   F   I   L   S   I   P   I   F   L   Y   45

137   CAA CAC ATC GAG GAG GGC TAC TGG TAC GGT CCC AGG CAG TAC TGC   181
46     Q   H   I   E   E   G   Y   W   Y   G   P   R   Q   Y   C   60

182   GTG GAC AGG TTC CCC AGC AAG ACG CAC GAG AGG GCC TAC ATC CTC   226
61     V   D   R   F   P   S   K   T   H   E   R   A   Y   I   L   75
                                                TM5
227   TAC CAG TTC ATA GCC GCC TAC CTG CTC CCC GTC CTC ACC ATC TCC   271
76     Y   Q   F   I   A   A   Y   L   L   P   V   L   T   I   S   90

272   TTC TGC TAC ACG CTG ATG GTG AAG AGG GTA GGG CGG CCC ACC GTG   316
91     F   C   Y   T   L   M   V   K   R   V   G   R   P   T   V   105

317   GAG CCA GTA GAC AAC AAC TAC CAG GTC AAC CTG CTG TCG GAG AGG   361
106    E   P   V   D   N   N   Y   Q   V   N   L   L   S   E   R   120

362   ACC ATC AGC ATA CGG AGC AAG GTG TCC AGG ATG GTG GTG GTG ATC   406
121    T   I   S   I   R   S   K   V   S   R   M   V   V   V   I   135
                                            TM6
407   GTC CTT CTG TTC ACC GTG TGC TGG GGT CCC ATC CAG TTC TTC GTC   451
136    V   L   L   F   T   V   C   W   G   P   I   Q   F   F   V   150

452   CTC TTT CAG TCC TTC TAT CCC CAG TAC CAG CCC AAC TAC GCC ACA   496
151    L   F   Q   S   F   Y   P   Q   Y   Q   P   N   Y   A   T   165

497   TAC AAG ATC AAG ACG TGG GCC AAC TGC ATG TCC TAC GCC AAC TCC   541
166    Y   K   I   K   T   W   A   N   C   M   S   Y   A   N   S   180
                TM7
542   TCT GTC AAC CCC ATA   556
181    S   V   N   P   I
```

B.)

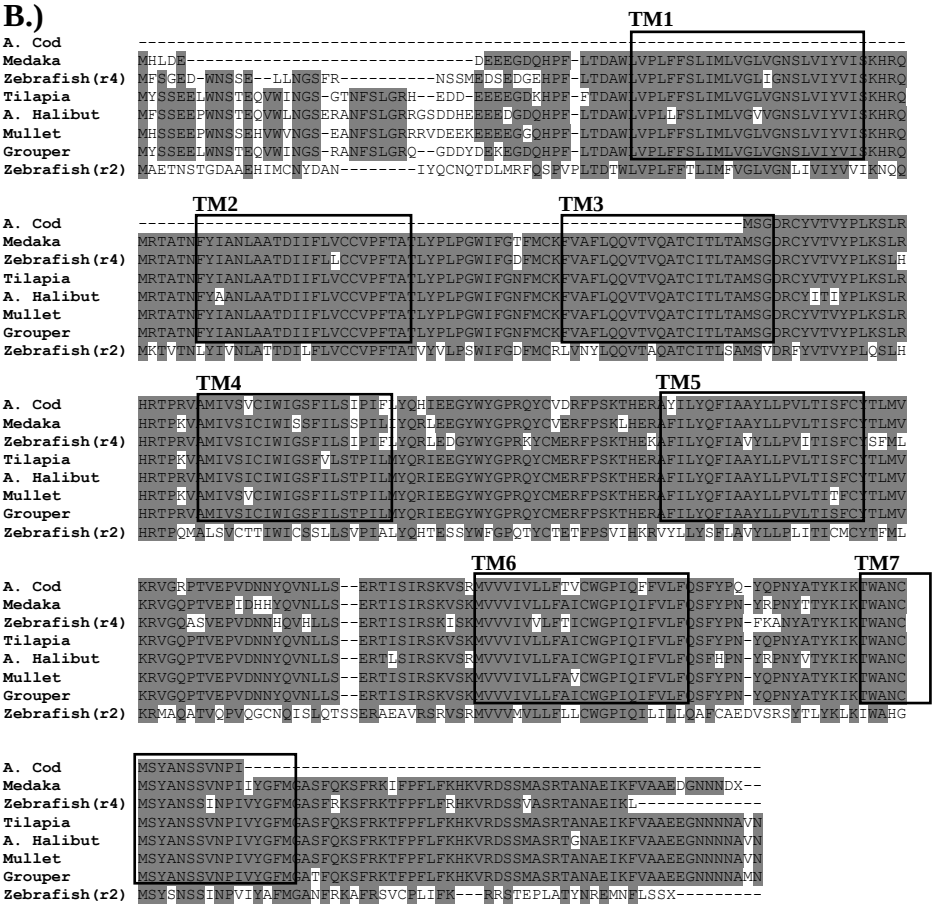

Figure 3. A.) Nucleotide and deduced amino acid sequence of Atlantic cod *kissr4* partial cDNA fragment. Predicted transmembrane domains as defined in Tilapia by Parhar *et al.* (2004) are underlined. B.) Alignment of the deduced protein sequence for Atlantic cod, Medaka, zebrafish, Nile tilapia, Atlantic halibut, Flathead mullet and Orange spot grouper kissr4 in comparison with zebrafish kissr2. The conserved amino acid residues are shaded. Predicted transmembrane domains as defined by Parhar *et al.* (2004) are boxed.

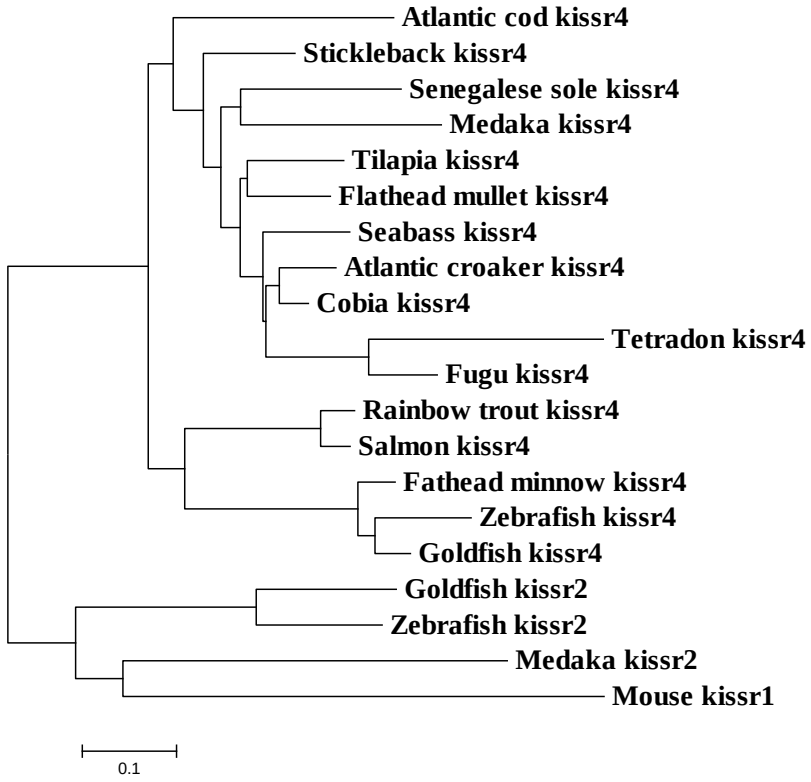

Figure 4. Phylogenetic tree analysis of teleost *kissr4* genes. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.

3.2 Sexual maturation

A fuller description of the effects of light treatment on sexual maturation including detailed descriptions of gonad morphology/histology and plasma sex steroid levels has previously been described in Chapter 3. In brief, females and males under SNP (simulated natural photoperiod) conditions matured whereas those under LL (constnat light) conditions remained immature as shown by gonadosomatic index (GSI) measurements (Fig. 5a, b). In SNP females, GSI showed an increase in November and peaked in March corresponding to spawning of individuals with fertilised eggs being collected in the outflow from February 5th to May 11th. In males under SNP conditions, GSI increased in December with the highest mean reached in February.

3.3 Pituitary expression of *fshβ* and *lhβ*

Levels of *fshβ* mRNA in females under SNP increased significantly over the first 8 month period of the trial with a 5 to 6-fold elevation from September 2008 to January 2009, these levels were significantly higher than those in fish under LL which showed no temporal variation (Fig. 5a). Maximum mean *fshβ* mRNA expression levels in SNP females were 2 to 3-fold greater than LL males. In males under SNP, there was no significant temporal variation in *fshβ* mRNA expression or significant elevation relative to fish under LL due to large individual variability however SNP males still showed a circa 2 fold increase in expression from October to December 2008 (Fig. 5b). *lhβ* mRNA expression in females under SNP showed a 10.5 fold increase from November 2008 to February 2009 (Fig. 5c), with levels significantly higher than that of LL individuals who showed no significant temporal variation. In SNP males, temporal variation in *lhβ* mRNA was not significant and there were no differences between these and LL males. Again maximum mean *lhβ* mRNA expression levels in SNP females were greater than in SNP males (~3-fold).

According to stage of gonadal development, both *fshβ* and *lhβ* gene expression increased during gametogenesis in SNP females (Fig. 7a, b). Expression levels started to rise significantly in females at the early vitellogenic stage and peaked during late vitellogenesisa. The only spawning individual showed reduced *fshβ* expression and steady high *lhβ* expression.

3.4 Brain expression of *kiss2* and *kissr4*

There were no significant differences in kiss2 or kissr4 expression in either male or females over the course of the trial nor was there any significant effect of treatments (Figure 6). In general there was no greater than a 2 fold variation in expression level across the course of the experiment. Furthermore there was no clear correlation between *kiss2* or *kissr4* mRNA levels and stage of gonadal development in SNP females (Fig. 7c, d).

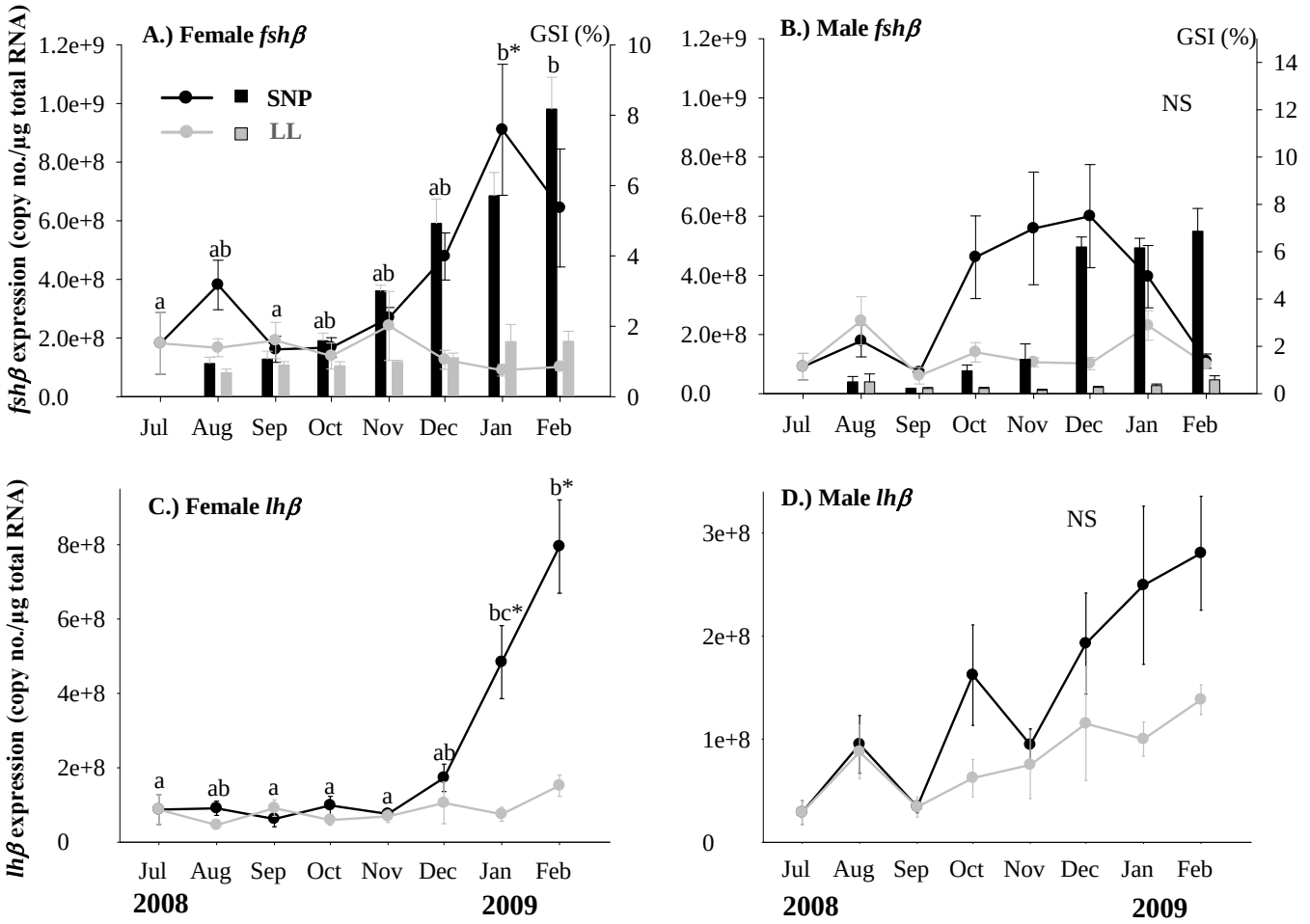

Figure 5. Absolute mRNA expression levels and gonadosomatic index (GSI) from July 2008 to February 2009 in Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). A.) GSI (bars) and *fshβ* (line) expression in the pituitary of females, B.) GSI (bars) and *fshβ* (line) expression in the pituitary of males, C.) *lhβ* expression in the pituitary of females, D.) *lhβ* expression in the pituitary of males. Data is normalised to ARP and presented as treatment mean ± SE. Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *. Graphs are labelled as non-significant (NS) where no significant time or treatment differences in expression were apparent. See appendices 20-23 for detailed tables of results.

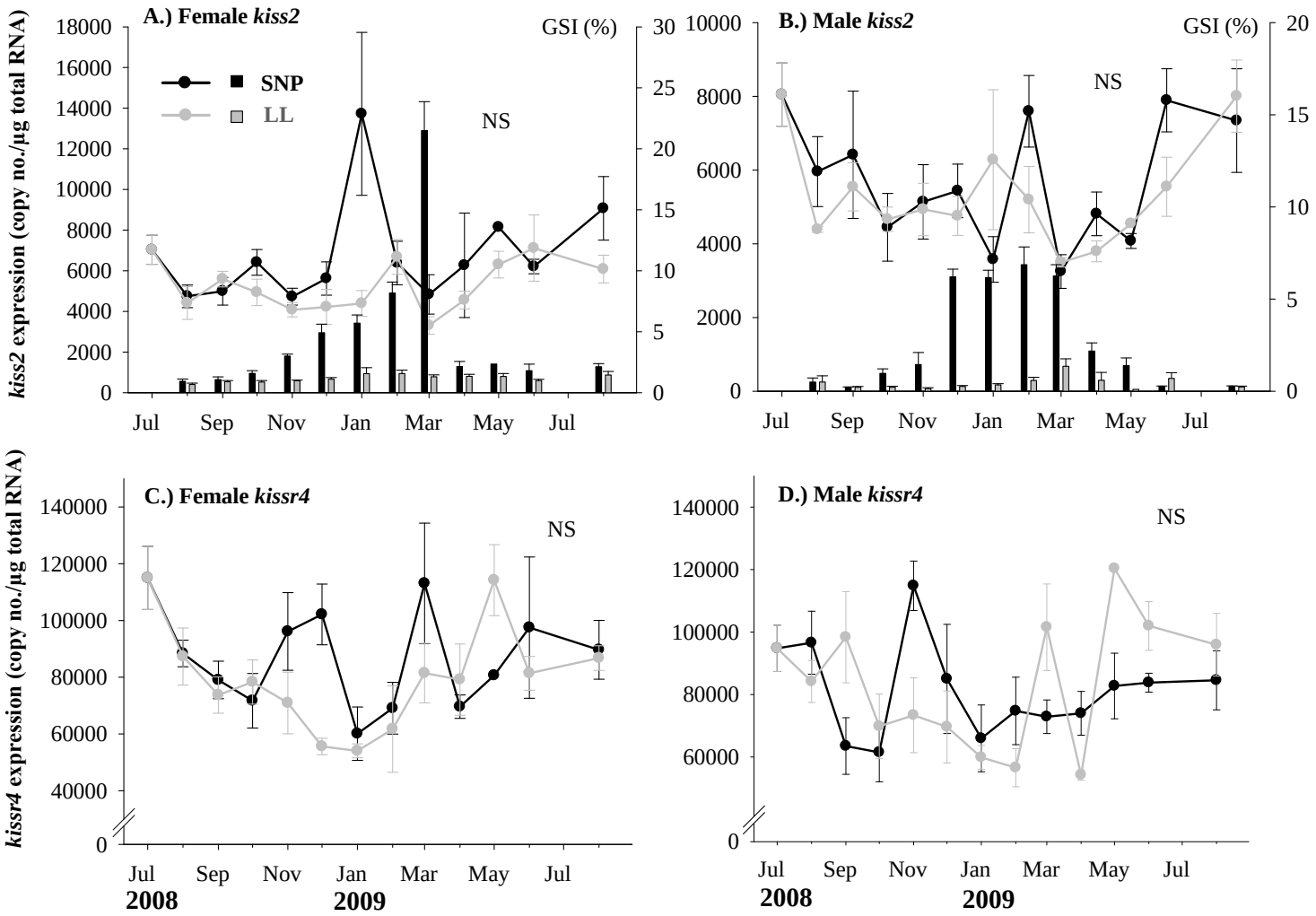

Figure 6. Absolute mRNA expression levels and gonadosomatic index (GSI) from July 2008 to September 2009 in Atlantic cod exposed to simulated natural photoperiod (SNP) and constant light (LL). A.) GSI (bars) and *kiss2* (line) expression in the pituitary of females, B.) GSI (bars) and *kiss2* (line) expression in the pituitary of males, C.) *kissr4* expression in the pituitary of females, D.) *kissr4* expression in the pituitary of males. Data is normalised to ARP and presented as treatment mean ± SE. Graphs are labelled as non-significant (NS) where no significant time or treatment differences in expression were apparent. See appendices 24-27 for detailed tables of results.

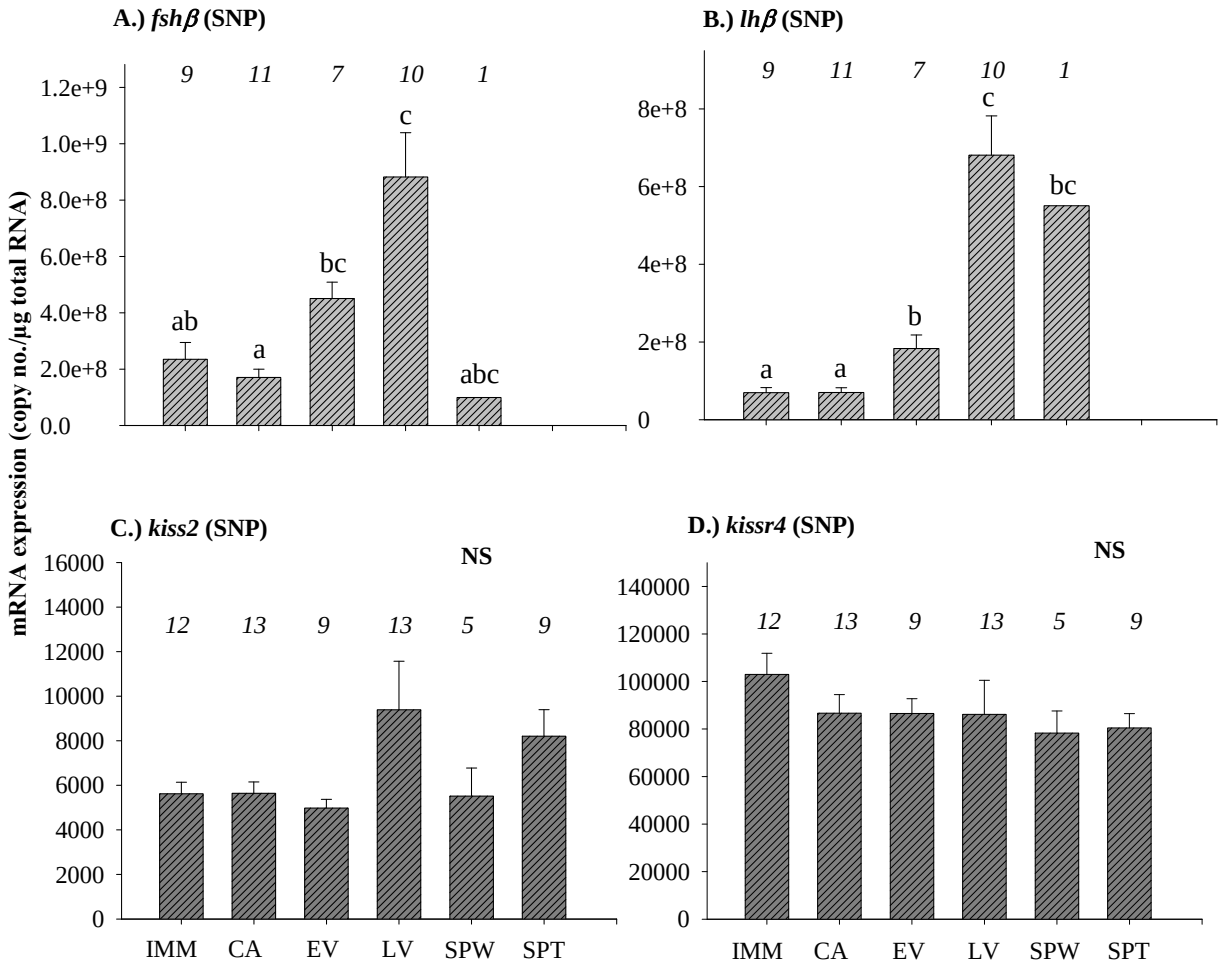

Figure 7. Relative *fshβ* (A.), *lhβ* (B.), *kiss2* (C.) and *kissr4* (D.) expression in females under the SNP lighting treatment, classed according to stage of maturity (IMM: immature, CA: cortical alveoli, EV: early vitellogenesis, LV: late vitellogenesis, SPW: spawning, SPT: spent). Data presented as mean ± SE. Numbers above the bars indicate the number of fish corresponding to each stage.

4. Discussion

To the authors knowledge this is the first description of partial cDNA sequences coding for kisspeptin and its receptor in Atlantic cod. Expression analysis indicated that there was no clear seasonality in expression of *kiss2* or *kissr4* mRNA transcripts in relation to puberty in Atlantic cod and no significant differences between these and individuals exposed to a constant lighting regime, while gonadotropin expression did show a time and treatment effect. Morphological (GSI, oocyte diameter, histological staging) and endocrine (sex steroid profiles) assessment showed that individuals exposed to simulated natural photoperiod (SNP) underwent a typical seasonal reproductive cycle with spawning from February to May (Vitale *et al.*, 2005; Taranger *et al*., 2006; Davie et al., 2007a) whereas individuals exposed to LL remained immature. In the present study, SNP female *fshβ* and *lhβ* expression showed an increase towards spawning in line with previous studies of seasonal expression on gonadotropin and gonadotropin receptor expression in Atlantic cod (Almeida et al., 2009a; Mittelholzer et al., 2009a, 2009b). Levels of expression of male *fshβ* and *lhβ* also showed a seasonal pattern in expression however with a larger variability between individuals. Classification of SNP female gonadotropin expression with stage of gonadal development demonstrated that *fshβ* and *lhβ* expression are most closely associated with vitellogenesis and spawning.

Analysis of the deduced amino acid sequence of the *kiss2* gene in Atlantic cod and in particular the kisspeptin-10 epitope with other fish species indicated high sequence homology, this suggests the gene has been conserved during evolution which may be a reflection of its important functional role in cod (Eipper et al., 1992; Kitahashi et al., 2009). The reduced identity (circa 50-60 %) out with the eptitope with other species is a common feature of kisspeptins as it is proposed that due to post translational modifications of the propeptide these regions are cleaved off to leave the highly

conserved signal peptide (Oakley et al. 2009). Analysis of the predicted amino acid sequence of the Atlantic cod *kissr4* revealed high structural similarity with other teleost Kissr4s such as the medaka, stickleback and tilapia. Clearly full length descriptions of both partial fragments should be completed to formally describe both genes, which at the same time may help further our understanding of the regulation of their expression through examination of their untranslated regions. However, of greater priority will be localisation studies to provide some spatial definitions of expression that will help focus future works.

Significant seasonal patterns in *kiss2* mRNA were not evident, however in the female dataset, the treatment by time interaction was close to significance ($p = 0.087$). The restricted sample size in conjunction with the apparent limited range in expression will have contributed to this perceived lack of effect, however one point of interest is the *kiss2* expression increase in females sampled in January. Under the SNP conditions, mean expression was approximately 3 fold higher than the LL treatment. Examination of the data at this time reveals that 3 individuals had expression levels ranging from 4 – 8000 copies per µg total RNA, while three individuals had expression levels ranging from 16 – 28000 copies per µg total RNA which is approximately 3-5 fold above basal levels. This heterogeneous spread could be a reflection of pulsatile release of kisspeptin as has been described in mammals (Keen et al. 2008). These authors demonstrated in pubertal monkeys, that hypothalamus KISS1 protein release pulsed with a spike approximately every hour which matched closely similar pulses in GnRH release. If such a signal mechanism was in place in cod and repeated at the mRNA level then the current sampling regime could easily have sampled individuals at different states of signalling resulting in such a heterogeneous sample. Thus a more focused study of expression in the late stages of maturation may be warranted. Increases in *kiss2* expression late in the

reproduction cycle have already been reported in the grass puffer, *Takifugu niphobles* (Shahjahan et al., 2010) and also in male European sea bass, a repeat spawner, together with high levels of gonadotropins and sex steroids (Cowan *et al*., 2010).

When looking at the kisspeptin receptor, the present study showed no clear seasonal profile in brain *kissr4* expression over time in SNP females or males. However males did show an elevation in expression in November prior to GSI and sex steroid peaks (refer to Chapter 3 for assessment of maturation). Interestingly, no such receptor expression increase was seen in LL males at this time. It is possible that this variation may be related to the later stages of spermatogenesis in accordance with the suggestions of Nocillado *et al.* (2007) who studied the temporal expression of *kissr4* in grey mullet and found that it significantly increased from the intermediate stage of puberty rather than the early stages. Equally however it could be that the receptor does not show significant variations in expression and that the only notable increase is that associated with the development of reproductive competence during early development (Martinez-Chavez et al. 2008)

Since there was no clear seasonal expression evident in our maturing male and female populations, it was difficult to unravel the effect of the LL regime. Revel *et al.* (2006a) have shown that pineal ablation in the Syrian hamster prevented the expected down regulation of *Kiss1* expression following transfer to short day photoperiod. This appeared to be melatonin dependent knowing that the pineal is the sole source of circulating melatonin in mammals, however, it remains unclear whether melatonin acts directly on Kiss1 neurons (Revel et al. 2006a). Localisation studies of kisspeptin genes in Atlantic cod may help to elucidate their possible sensitivity to photoperiod. It is important to note that our study only investigated gene expression in whole brain samples, although this is still believed to reveal a pattern if one exists, as seen in

Martinez-Chavez *et al. (*2008) and Cowan *et al.* (2010). Recent studies on the anatomical distribution of kisspeptin neurons have shown interesting localisation patterns in sea bass (Aguirre et al., 2010) and zebrafish (Servili et al., 2010). In sea bass, cells expressing *kiss1* and *kiss2* were found in the mediobasal hypothalamus suggesting their involvement in GnRH signalling however *kiss1* genes alone were also expressed in the habenular region (Aguirre et al., 2010). In zebrafish, localisation showed two separated neuronal systems with *kiss2* expressed in cells mostly in the mediobasal hypothalamus and kiss1 neurons localised in the habenular region (Servili et al., 2010). These findings strongly suggest that kiss1 neurons may be linked to light perception because the habenula receives pineal and parapineal projections (Kitahashi et al., 2009). In addition, in medaka, photoperiod was shown to have an effect on the number of nucleus ventralis tuberis (NVT) Kiss1 neurons, with increased *kiss1* expression under long day conditions (necessary for reproductive development) (Kanda *et al*., 2008).

While there was no clear association between kisspeptin and photoperiod observed in the present study, clear effects on gonadotropin signalling were found. In males and females in particular, both *fshβ* and *lhβ* gene expression increased under SNP but not the LL treatment from December and arguably as early as October which reflects previous findings of gonadotropin signalling in photoperiod treated male cod (Almedia 2009). It therefore appears that there must be some, as yet, unknown signalling pathway that integrates photoperiod signals to stimulate *fsh*/*lh* expression in early winter. The original hypothesis driving this work was that kisspeptin genes (*kiss2, kissr4*) may be playing a role at the onset of maturation during the 'window of decision' between July and October. Results did not however support this role in the cod even though it has been suggested in several other fish species including tilapia (Parhar *et al.*, 2004; Martinez-Chavez *et al.*, 2008), fathead minnow (Filby *et al.*, 2008) and zebrafish

(Kitahashi *et al.*, 2009). Considering the presence of two forms of both kisspeptin (Kiss1 & Kiss2) and its receptor (Kissr4 & Kissr2) in fish (Lee *et al.,* 2009; Akazome *et al.*, 2010), it is possible that one subtype may be more potent in the control of reproduction in cod than the other. Akazome *et al.* (2010) have highlighted the distinct expression patterns and activities of multiple forms of kisspeptins suggesting that the KISS1/KISSR1 system has functionally diverged, this can lead to differences in function and potency between the sexes and species. Functional studies for example have shown that in goldfish, administration with kiss1 but not kiss2 stimulates LH secretion (Li *et al.,* 2009). In contrast, in zebrafish, Kiss2 but not Kiss1 significantly increased the expression of *fshβ* and *lhβ* transcripts in the pituitary inferring that Kiss2 is the more potent activator for gonadotropins in the female zebrafish (Kitahashi *et al.,* 2009). Likewise in pre-pubertal sea bass, *kiss2* administration was more potent in stimulating FSH and LH secretion in comparison to kiss1 (Felip *et al.,* 2009). The absence of any clear seasonal expression pattern of *kiss2* in the present study suggests that another subtype of kisspeptin ie. *kiss1* may play a more functional role in puberty/gonadal maturation in Atlantic cod. Likewise, it is possible that Kissr2 may play a more functional role than Kissr4 in cod although, to date, most studies performed in fish focused on Kissr4 (Oakley *et al.,* 2009). It is important to note that the sampling resolution (once a month) and number of individuals sampled (6 fish/sex/time point) combined with clear intra population variability, might have not allowed to detect potential acute peaks. Alternatively it is possible that kisspeptin role in the regulation of cod reproduction might not be as important as originally believed. Interestingly, in avian species nether the *kiss1 or kiss2* have been found, their absence is thought to be due to gene loss during evolution (Um et al., 2010). Thus alternative signalling pathway(s) must be operating in avian species which could well be present in fish.

In conclusion, the present study did not reveal any strong correlations between puberty and the kisspeptin system in contrast with previous findings obtained in other teleost species. It is difficult at this stage therefore to conclude on the functional role of these genes at the onset of puberty. Localisation studies of kisspeptin neurons and pharmacological studies of the kisspeptin receptor in Atlantic cod should help to shed light on the importance of this system in cod reproductive physiology

Acknowledgements

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CHAPTER 5

RESEARCH ARTICLE

RESEARCH ON METHODOLOGIES FOR THE PRODUCTION OF MONOSEX ATLANTIC HALIBUT, *HIPPOGLOSSUS HIPPOGLOSSUS* IN THE UK

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In Preparation: due for publication once progeny testing data has been obtained (May-June, 2011)

Contributions: The present manuscript was written and compiled in full by the author of this thesis. Sampling, lab and statistical analysis was carried out by the candidate with the support of thesis supervisors (Drs. Herve Migaud, Andrew Davie, David Penman) who also proof read the manuscript.

Keywords: Atlantic halibut; sex reversal; monosex; 17α-methyldihydrotestosterone; semen sexing

Abstract

The attainment of sexual maturity during commercial on-growing of marine species brings a commercial loss due to reduced growth potential and poor flesh quality. Female Atlantic halibut, *Hippoglossus hippoglossus*, mature later and grow faster than males and can be harvested before maturation becomes a problem, monosex female production is therefore an important strategy to increase the profitability of the UK halibut farming industry. A pilot study was conducted to examine the feasibility of generating a monosex population using semen sexing through based on cellular DNA content by flow cytometry that is routinely used in terrestrial agriculture. Semen from a range of teleost species including Atlantic halibut, Atlantic cod, *Gadus morhua*, sea bass, *Dicentrarchus labrax*, and perch, *Perca fluviatilis* were collected and tested. Results did not show any clear sex related size differences in the DNA of sperm analysed in any of the species and therefore demonstrated that semen sexing based on total DNA content may not be an applicable technique for monosex production of such species. The second part of this study involved the development of a population of sex reversed halibut broodstock (neomales) that will generate, in the long term, a basis for traditional monosex female population generation. To do so, halibut juveniles were fed a diet supplemented with 17α-methyldihydrotestosterone (MDHT) according to a previously published protocol. Two in-feed treatments were tested (5ppm for 6 weeks versus 10ppm for 3 weeks) with the aim to reduce the window of exposure to the hormone. Results were very successful with the 6 week treatment yielding a 97% male population (based on the sex ratio of a sub-sample of fish). The growth of halibut thereafter was monitored up until they reached an age of 36 months, at the time of first male maturation.

1. Introduction

Monosex production is an effective management technique used to address the problem of early maturation in a number of commercially important aquaculture species which exhibit sexual dimorphism in growth and age at first maturation (Pandian & Kirankumar, 2003; Piferrer, 2001). In Atlantic halibut, *Hippoglossus hippoglossus*, it is the females which grow faster and larger than males and reach market size before maturation (Bjornsson, 1995; Imsland & Jonassen, 2005). Bjornsson (1995) demonstrated that female halibut reared in tanks under natural photoperiod matured at a mean weight of 12.7 kg whereas males matured at just 3.2 kg. Furthermore females showed a higher growth rate during the period of male maturation, 3.2 versus 1.4 kg/year for females and males respectively. Maturation is a major problem during on-growing as energy is shifted into sexual development resulting in a loss in somatic growth and flesh quality and increased susceptibility to disease. Thus monosex production of female Atlantic halibut clearly holds great commercial and economic benefit for the industry. However, to date, little research has been done into the implementation of a monosex strategy in the European halibut industry as opposed to Canada where research led to the publication of a protocol for farmers to produce monosex commercially (Hendry *et al.*, 2003).

Traditional techniques for the production of single sex stocks such as indirect sex reversal in fish are time-consuming taking a minimum of two generations to confirm the success of the technique (Piferrer, 2001), in the case of halibut this represents a minimum of 4-5 years. Thus the identification of an alternative and faster approach would help realise the commercial benefit far sooner. In the terrestrial livestock industry, semen sexing is used for the production of monosex populations in order to increase efficiency in producing meat or milk (Joerg *et al.*, 2004). Semen sexing is based on

sorting of X and Y-bearing spermatozoa according to differences in DNA content using flow cytometry (Joerg *et al.,* 2004). Sex related differences of 4.2% have been recorded in cattle (Johnson, 1992) and differences of as much as 7.5% have been recorded in chinchillas (Johnson *et al.,* 1987). Fluorescence activated cell sorting (FACs) is then used to apply a charge to the droplets containing the desired cells and these are subsequently sorted into male and female populations. The sexed semen stocks can then be cryopreserved, stored and used when necessary for artificial insemination (Seidel, 2009). This technique of semen sexing could be of great advantage to the aquaculture industry giving major benefits in the production of monosex populations. However, although sex related differences have been found in some fish species such as the ninespine stickleback, *Pungitis pungitis,* where chromosome number 1 is found to be longer in males, no percentage difference was reported (Ocalewicz *et al.,* 2008a). Furthermore, sex chromosomes have not been detected (by karyotype analysis) in most commercially important species including Atlantic halibut (Ocalewicz *et al.,* 2008b) and cod, *Gadus morhua* (Klinkhardt, 1994) (see review by Devlin and Nagahama, 2002). This said, to our knowledge, semen sexing has never been scientifically tested, or at least reported in teleosts.

The current most common way to produce all female fish populations is by indirect sex reversal and the use of 'neomales' (masculinised females) (Devlin & Nagahama, 2002). This technique requires that females are the homogametic sex. This is true in the case of Atlantic halibut where the gamete of the heterogametic male determines the sex of offspring at fertilisation although no specific sex chromosomes have been found yet (Hendry *et al.,* 2002; Tvedt *et al.,* 2009). Indirect sex reversal for the production of all-female populations is a two step process. The first consists of hormone treating juveniles with testosterone during the labile period which is the

window following genetic sex determination but before phenotypic differentiation, when gonads are still in an undifferentiated state. Exposure of individuals to exogenous steroids is aimed to over-ride natural endocrine signalling. Hormone treatment results in a predominantly male phenotypic sex population containing both normal males and masculinised females called 'neomales' which are genetically female fish but with testes and sperm, the sperm being carriers of only the female genotype. The second step of the process involves crossing these neomales (carrying the female genotype) with normal broodstock females to produce progeny consisting only of females. Indirect sex reversal has been successfully demonstrated in halibut in a study by Hendry *et al.* (2003), halibut lend themselves well to this as they start to differentiate after weaning at the time of first feeding, thus hormones can be administered in the feed (Hendry *et al.*, 2003). Results from Hendry *et al.* (2002) indicated that halibut are sexually 'labile' from prior to hatch and the first signs of ovarian cavity formation appear at 38 mm fork length (L_F). In their study, male development was much later than females and there was still no differentiation of testis by 43.5 mm L_F, by 74 mm L_F spermatogonia started to appear along with interstitial tissue. Hendry et al. (2003) have demonstrated that treatment with in feed 17α-methylhydrotestosterone (MDHT) for six weeks at a mean size of 30 mm L_F successfully masculinised halibut. Monosex production by indirect sex reversal has already been successfully implemented in the commercial production of rainbow trout, *Oncorhynchus mykiss* (Kuzminski & Dobosz, 2010) and tilapia, *Oreochromis niloticus* (McAndrew, 1993).

The objective of this study was 1) to test the feasibility of semen sexing in a variety of teleost fish species compared to a mammalian control semen (bull) and 2) to establish the first UK population of broodstock neomales for the production of monosex (all-female) halibut populations by indirect sex reversal based on the published protocol

by Hendry et al. (2003) and to test the efficacy of a higher hormonal treatment for a shorter period of time with the aim to reduce handling time of the hormone.

2. Materials and Methods

2.1 Semen sexing feasibility study

2.1.1. Sperm collection and fixation

Semen samples were collected by stripping male broodstock, samples from Atlantic cod, *Gadus morhua* and sea bass, *Dicentrarchus labrax* (Machrihanish Marine Environmental Research Laboratory, MERL, Machrihanish, UK), Atlantic halibut (Ardtoe Marine Laboratory, Acharacle, UK) and perch, Perca *fluviatilis* (Niall Bromage Freshwater Research Laboratory, Stirling, UK). Ninespine stickleback, *Pungitis pungitis* testes samples previously fixed in 80% ethanol were kindly donated by Konrad Ocalewicz (Poland) and cryopreserved bull semen samples were donated by the Sustainable Livestock System group at the Scottish Agricultural College (Edinburgh, UK).

For fixation, fish and bull semen samples were divided into 500 µl aliquots, 1000 µl PBS (filtered to 0.45 µm) was then added and the contents of the tube centrifuged for 10 minutes at 430 *g*. Supernatant was then removed and 1000 µl of 80 % ethanol (filtered to 0.45 µm) added to the pelleted spermatozoa, contents were mixed gently and then stored at 4^0C until staining (Johnson *et al.*, 1987). The stickleback whole testes were gently minced in 1000 µl PBS, filtered through 74 µm mesh to remove large particulate matter and then centrifuged for 10 minutes at 430 *g*, with the pelleted cell debris being prepared as above.

2.1.2. Staining

Sperm were recovered from fixation by centrifugation for 5 minutes at 400 *g* (4^0C). Ethanol was then removed and 1400 µl PBS/1 % BSA buffer (filtered to 0.45 µm, BSA included to help prevent coagulation) added, contents were gently mixed by pipetting and the sample was left on ice for 5 minutes. Sample was then centrifuged (5 minutes at 400 *g*), supernatant removed and the PBS/1 % BSA wash repeated. Following the wash, 900 µl of PBS/ 1 % BSA was added, and samples gently mixed by pipetting to obtain a homogenous suspension of spermatozoa. 500 µl of this suspension was then added to a fresh aliquot of 500 µl PBS/1 % BSA and filtered twice through 30 µm mesh attached to a syringe and ejected through a 23G needle.

Following filtration, 300 µl of filtrate was added to 600 µl lysing buffer (0.1 % triton X100 and 0.1 % sodium citrate) along with 6 µl of 10 mg/ml Propidium Iodide (PI, Sigma Aldrich, UK), samples were left at 4^0C for one hour before flow cytometry. During the one hour staining period, subsamples from the different species were examined by microscopy to confirm purity and the concentration of the sperm. Volume of buffers used during the staining preparation of the sperm samples were slightly modified if necessary depending on species in order to yield an optimal final concentration of ~1 x 10^6 spermatozoa / ml for flow cytometry, however the ratio of sperm suspension:lysing buffer and PI stain remained consistent.

2.1.3. Flow cytometry

The fluorescence of the stained sperm (a total of 10,000 cells / sample) was measured using a standard fluorescence activated cell analyser (FACsCalibur™, Becton Dickinson, BD Biosciences, California, USA) and analysed using CellQuest 3.3 software. Fluorescent light emitted by individual spermatozoa nuclei was recorded through a combination of forward scattered and right angle scattered laser light

(Chilmonczyk & Monge, 1999). Populations of cells were gated according to the pulse width of cells versus the total cell fluorescence in order to remove aggregates and debris (neither of which was present in abundance), and the DNA content of these gated cells was then analysed through frequency distributions of total cell fluorescence. The frequency distributions in this paper have been displayed as a moving average in order to highlight the data trend of DNA content and indicate if there is unimodal or bimodal pattern of distribution.

2.2 Sex reversal experiment

2.2.1. Fish stock and initial rearing conditions

Weaned mixed sex halibut larvae (mean total length ± SE of 40.1 ± 0.2 mm, mean wet weight ± SE of 0.5 ± 0.01 g) were obtained from a commercial halibut hatchery and transferred to MERL (55:44^{0}N, 5:44^{0}W) for hormonal treatment. Six tanks were prepared, each with 230 halibut juveniles. Tanks were part of a seawater flow-through system with water running at a flow rate of approximately 50 L min^{-1} at ambient temperatures and filtered to 60 µm.

2.2.2. Experimental conditions

In-feed hormone treatments started on the 16th August 2007 (one day following transfer) and continued for a maximum of 6 weeks, until the 28th September 2007. Three treatments were tested in duplicate: 1) 6 weeks steroid free diet (control conditions), 2) 6 weeks MDHT in-feed (5 ppm) and 3) 3 weeks MDHT in-Feed (10 ppm) followed by 3 weeks steroid-free diet. Food was provided by automated feeders which were programmed to shake pellets into the tanks every 12 minutes throughout the 24 hour cycle to ensure that fish could feed to satiation.

To incorporate steroids into the manufactured feed, two stock solutions of 17α-methyldihydrotestosterone (MDHT: Sigma–Aldritch Co Ltd, Poole, UK) dissolved in 100% ethanol) were made up at 5ppm (based on published protocol) and 10ppm (experimental protocol) (Hendry et al., 2003). Trays containing manufactured feed (Low Energy Marine Larval diet, EWOS, West Lothian, UK) were prepared and covered in the appropriate MDHT solution (2.5 ml solution/g feed). These were then left in a fume extraction cupboard overnight to facilitate evaporation of the ethanol. Dividers were placed between the trays to prevent any potential steroid contamination. The same process was conducted with the control diets but with steroid free ethanol.

2.2.3. Sampling regime

Five sampling time-points were conducted throughout the 6 week hormonal treatment period, these included: baseline sample (pre-treatment, day 0), 12, 22, 33 and 43 day post treatment onset. At each sampling point, for each tank, 10 fish were culled by lethal anaesthesia and weight/length taken. Length was then taken for another 50 halibut/tank which were anaesthetised (1:10,000 concentration of 2-phenoxyethanol, Sigma-Aldrich Co Ltd, Poole, UK) and then returned back to respective tanks. Water, feed and fish samples were also taken for analysis of MDHT content by high performance liquid chromatography (HPLC) however the sensitivity of the analytical technique employed did not have a high enough senstivity to allow detection of MDHT in the samples. Halibut were transported to a commercial fish farm on the 17th of October (3 weeks later) for on-growing and sex determination.

2.2.4 On-growing of MDHT treated fish and sex determination

Halibut have been maintained in commercial on-growing facilities (Otterferry Seafish Ltd., Argyll, UK) and monitored for growth performance. Fish were initially maintained

in separate tanks according to their respective treatment replicates however once they had reached a mean weight of 28.4 ± 0.4 g (mean ± SE) they were marked with panjet dye and replicates were pooled according to treatment (3 tanks in total, approximately 300 fish/tank, 6th December, 2007) due to limitation in on farm facilities. The panjet mark was re-applied approximately every 1.5 months to retain the identity of fish treatments. Once halibut had reached a weight of 79 ± 2.95 g (mean ± SE) (14th February 2008), 20 individuals from each treatment were sampled for histological determination of sex to ensure that gonadal differentiation had occurred. The posterior gut cavity (containing the region of gonadal development) was dissected from individuals, fixed in 10% neutral buffered formalin, processed by histology and stained using haemotoxylin and eosin. Following confirmation that sex could be determined at this stage, a further 60 individuals / treatment were sacrificed and sexed (21st March 2008). Thus a mean total of 80 individuals per treatment were sampled for sex determination however, due to a loss during processing or difficulty in sex identification owing to the small size of gonads, 3-4 samples per treatment could not be assessed (see table 2 for exact numbers of individuals per treatment).

On the 15th of May 2008, at a mean size of 180.8 ± 3.1 g (mean ± SE), 60 control fish (30 / replicate) and 150 5ppm fish (75 / replicate) were tagged with a passive integrated transponder tag (Fish Eagle Co., Lechlade, UK) and only these individuals were monitored for weight/length thereafter. All the remaining fish from these treatments and those from the 10 ppm treatment were culled. At the subsequent sampling points, tagged individuals were selected at random, anaesthetised and their weight and length taken, a minimum of 20 individuals/treatment were sampled. At the final sampling point on 8th April 2010 all tagged fish were measured and owing to the presence of maturing males, individuals were classified into maturing or immature

cohorts (Table 1). The specific growth rate (SGR) of immature individuals has been calculated retrospectively for tagged fish in the months leading up to 8th April 2010. SGR was calculated according to the equation: SGR = (e^g – 1) x 100, where g = [LN($weight_{end}$)- LN($weight_{start}$)] x number of days.

2.2.5. Statistical analysis and data presentation

For sex ratio determination, divergence from the expected 1:1 sex ratio were evaluated statistically using a Chi-square (χ^2) formula, with an χ^2 value of 3.84 ($p < 0.05$). All data were analysed using MINITAB ® version 15.0 (Minitab Ltd., Coventry, UK) statistical software. Length and weight (L/W) data were initially tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test. L/W data throughout the hormone treatment period were compared by analysis of variance (ANOVA) manipulated using a General Linear Model (GLM) that included a comparison of treatment replicates (n=2) nested within the fixed treatment effect. L/W during on-growing was compared between treatments (with replicates pooled as there were no significant differences between each) at each time point using a GLM.

Table 1. Number of tagged control and 5 ppm fish sampled at each time point. Fish have been classed into a maturing or immature group according to their maturation status in April 2010.

		Immature		**Mature**	
Year	**Date**	**Control**	**5 ppm**	**Control**	**5 ppm**
2008	15th May	56	47	4	12
	9th October	18	34	2	8
2009	24th April	21	40	0	13
	12th November	18	31	2	10
2010	8th April	53	105	4	33

3. Results

3.1 Semen sexing feasibility study

Total cell fluorescence measurements of bull sperm produced fluorescence frequency distributions with two peaks (Fig. 1a) indicating a bimodal distribution of DNA content, which according to the literature is due to the presence of X and Y chromosome bearing sperm cells. This validated that the methodology and instrumentation used in this study was sensitive to the sex difference in DNA content of bull (approx 4% difference). Analysis of all fish samples tested did not however show distinct peaks (Fig. 1 b-e), with fluorescence data showing a unimodal population distribution, thus suggesting no measurable differences of the DNA content between the two sexes of these fish sperm tested. Unfortunately we were unable to test or demonstrate this difference in the Ninespine stickleback due to difficulty in recovering sperm from the testes. The tissue samples were too small to recover a suitable volume of a good quality suspension of sperm cells preventing a robust analysis from being completed.

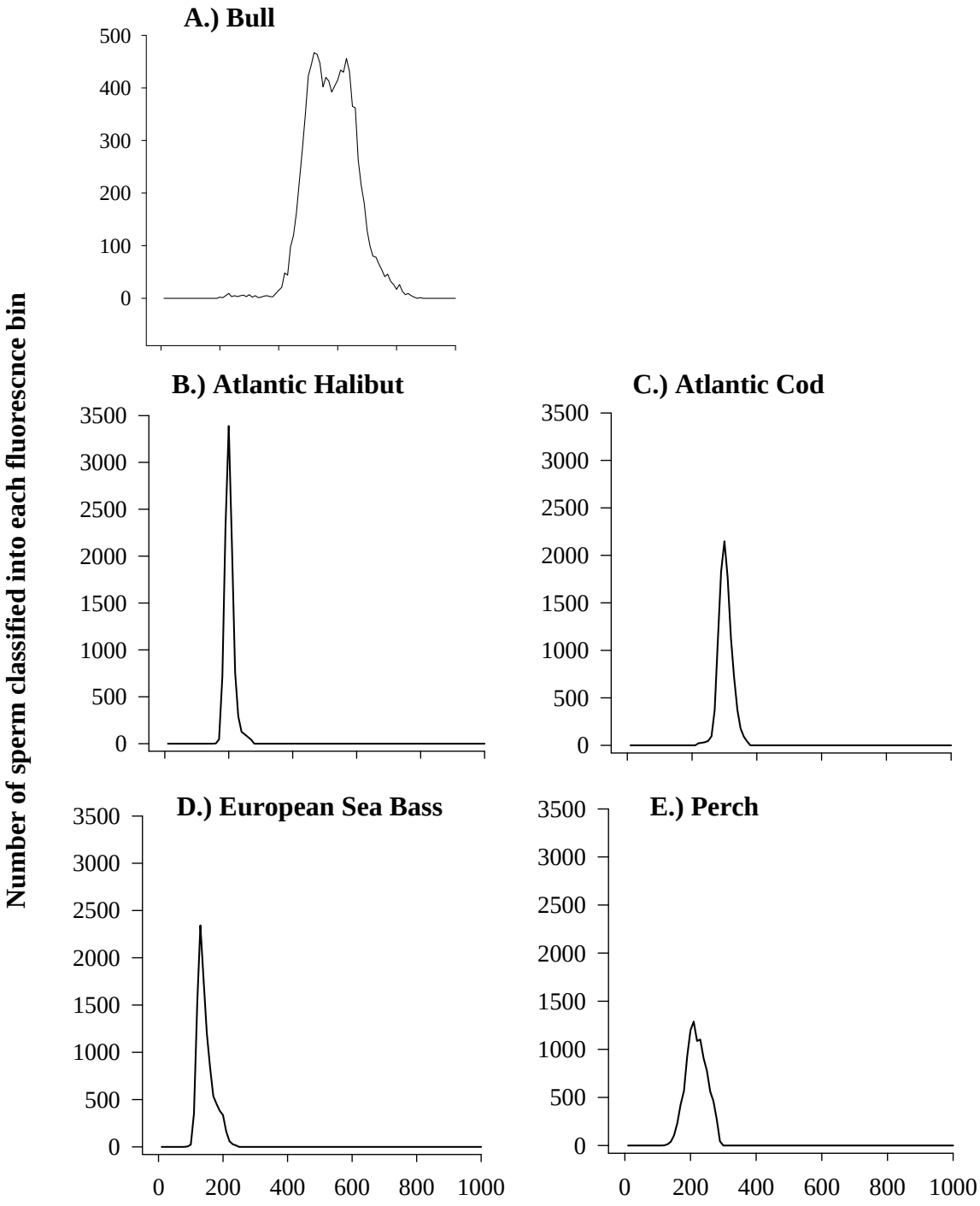

Figure 1. Flow cytometric DNA analysis of mammalian (A. bull) and several fish species (B. Atlantic halibut, C. Atlantic cod, D. European sea bass and E. Eurasian perch) sperm nuclei, stained with propidium iodide. Graphs present a moving average of the number of sperm/fluorescence bin (total cell fluorescence/sperm has been grouped into bins of 10, number of sperm = 10,000).

3.2 Sex reversal experiment

3.2.1. Sex ratios

The control population exhibited the expected 1:1 phenotypic male to female sex ratio (52% male: 48 % female) whereas 97 % of the 5 ppm population and 70 % of the 10 ppm populations were confirmed as phenotypic males (Table 2). Chi square analysis confirmed that both in feed treatments significantly altered the natural sex ratio in favour of the male phenotype.

Table 2. Results of chi-squared analyses comparing control group sex ratios with MDHT (5 ppm and 10 ppm) treatments (Obs., observed sex ratio; Exp., expected sex).

	Control		**5 ppm**		**10 ppm**	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
N	77	77	77	77	76	76
Male	40	38.5	75	38.5	53	38
Female	37	38.5	2	38.5	23	38
χ^2	0.12		69.2		11.8	
P	> 0.05		< 0.001		< 0.001	
df	1		1		1	
Sex Ratio	52 % male		97 % male		70 % male	

3.2.2. Growth performance and maturation

Throughout the hormone treatment period, there were no significant differences in length and weight of halibut between tank treatments over time (Fig. 2). Following the hormone treatment period, the growth of halibut remained comparable up until November 2009 at which point individuals from the 5 ppm treatment showed a reduced length and weight in comparison to control individuals (Fig. 3). By April 2010 the 5ppm fish were 84% of the mean weight of control fish (1968.26 ± 47 g and 2331.44 ± 81 g respectively, mean ± SE). Also at this timepoint, a number of maturing males were observed in the control (7%, 4 out of 60 individuals) and 5 ppm populations (21%, 32 out 150 individuals). Retrospective analysis of growth performance of the mature versus immature cohorts showed growth was comparable until April 2010, at which point their weight and length were significantly lower (Fig. 4). This was more pronounced in those under the control treatment which were just 66% of the mean weight of immature fish (1586 ± 277 g versus 2388 ± 80 g mature and immature mean weights ± SE respectively), in the 5 ppm treatment the mature individuals were equivalent to 71% of the immature mean weight (1508 ± 59g versus 2113 ± 51g). There were no significant differences in the SGR of tagged individuals that remained immature throughout the period measured, from May 2008 - April 2010 (Table 3).

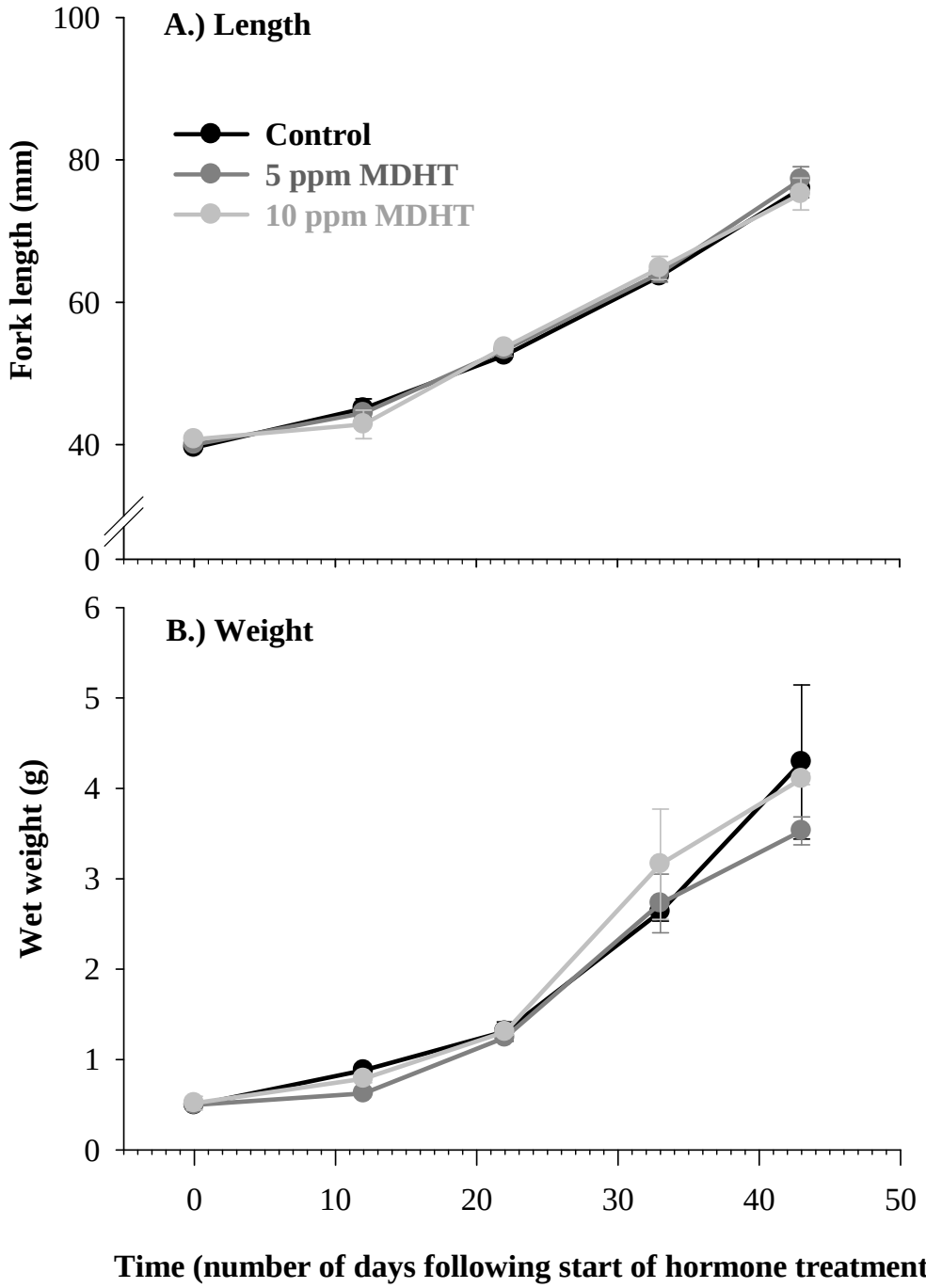

Figure 2. Length (a) and weight (b) of halibut treated (5 or 10 ppm) or not (control) with MDHT throughout the period of hormone treatment. Length and weight data presented as mean ± SD (*n* = 2, 60 or 10 individuals / replicate respectively). No significant differences were recorded between treatments.

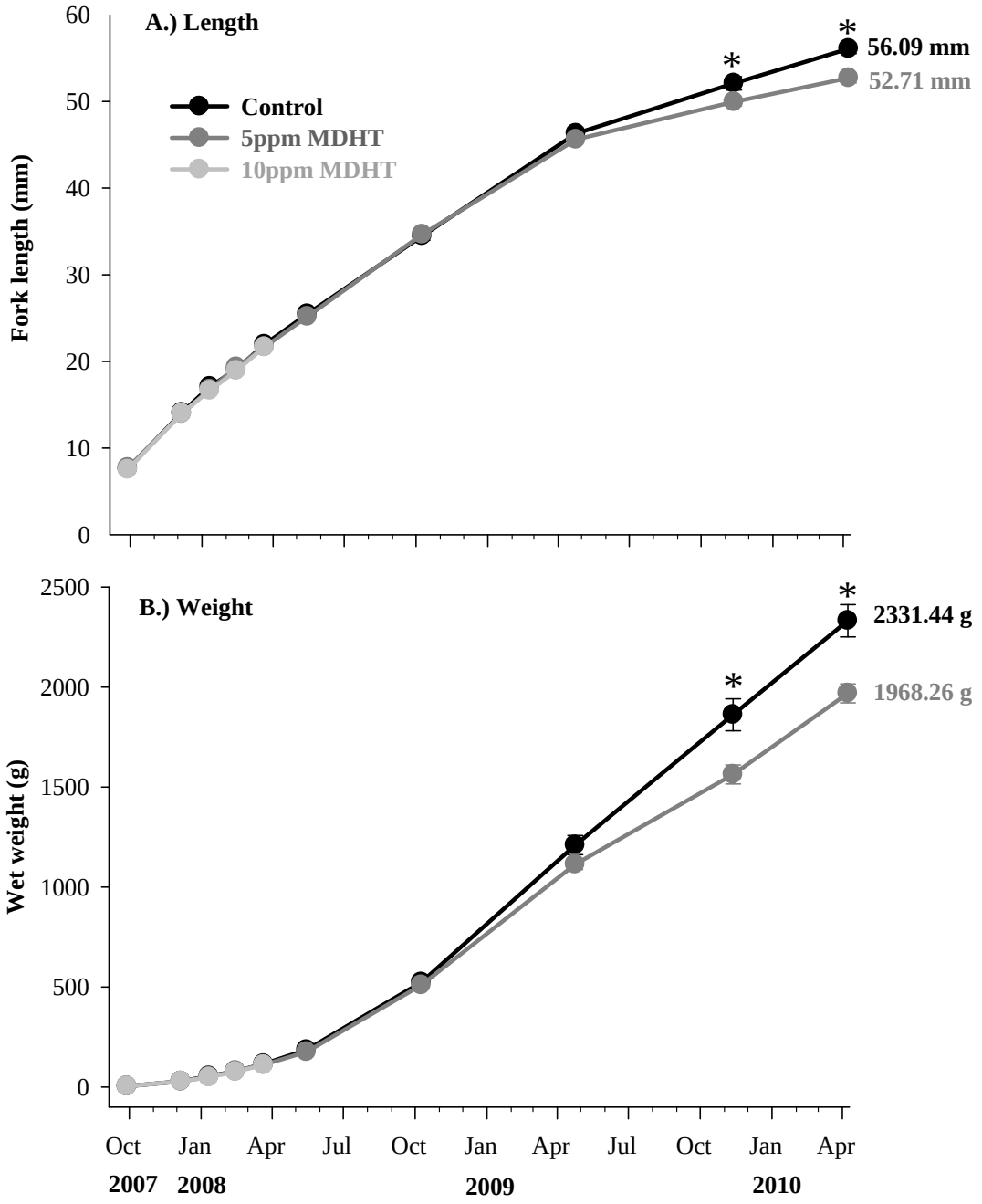

Figure 3. Length (a) and weight (b) of halibut treated (5 or 10 ppm) or not (control) with MDHT during ongrowing (data displayed from the last hormone treatment sampling on 28th Sep 2007 to the latest PIT-tag sample on 8th April 2010). Data for replicates is pooled and presented as treatment mean ± SE (10 ppm *n* = 20-100, 5 ppm *n* = 20-138, control ppm *n* = 20-83 individuals / treatment). Significant differences between treatments at each timepoint is indicated by an asterix.

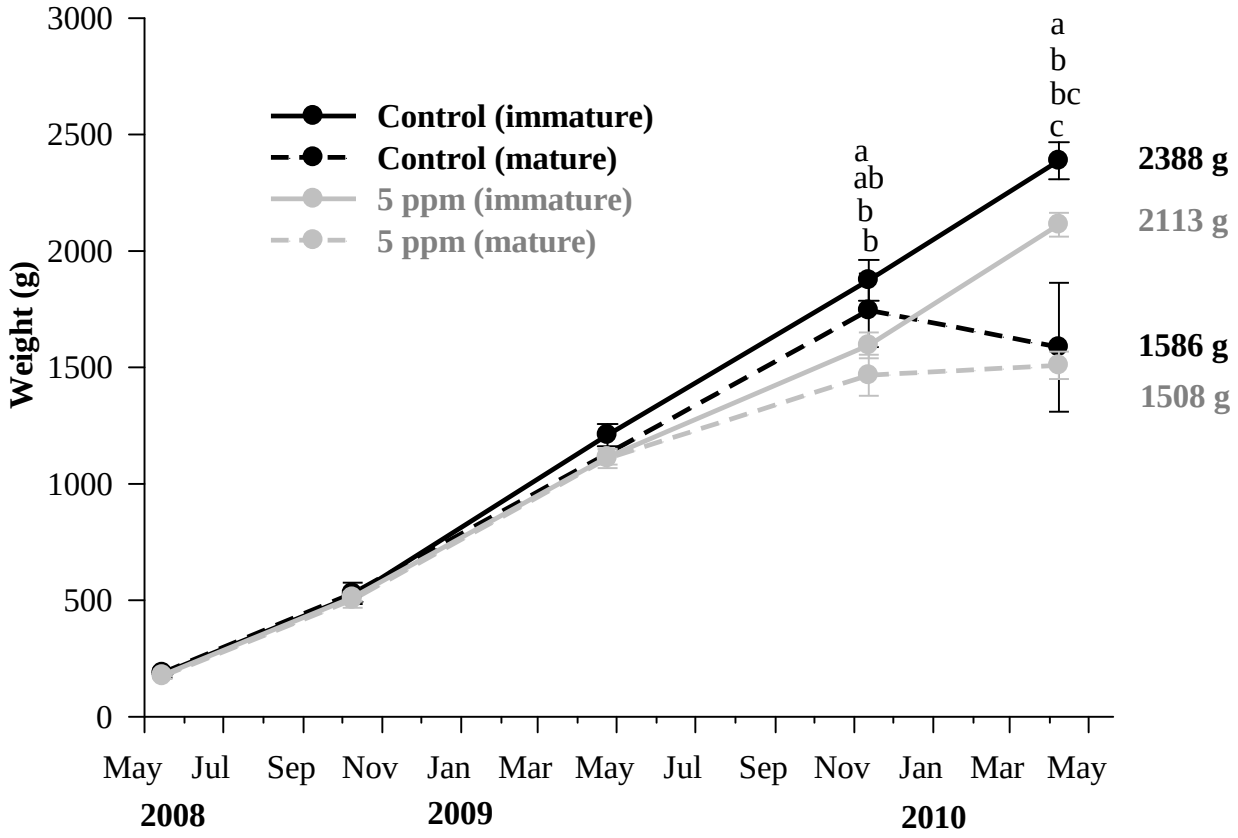

Figure 4. Weight of immature versus maturing halibut according to hormone treatment (control or 5 ppm). Data is presented as treatment mean ± SE (number of fish/timepoint is presented in table 1). Significant differences between treatments and maturation status are indicated by superscripts.

Table 3. Specific growth rate of tagged halibut which have remained immature throughout the sampling points so far. Data is presented as treatment mean ± SE (n = 6-31).

Date	**Treatment**	
	Control	**5 ppm**
May 08-Oct 08	0.69 ± 0.04	0.73 ± 0.02
Oct 08-Apr 09	0.38 ± 0.03	0.37 ± 0.02
Apr 09-Nov 09	0.25 ± 0.01	0.20 ± 0.02
Nov 09-Apr 10	0.16 ± 0.03	0.14 ± 0.02

4. Discussion

Semen sexing by flow cytometry is standard practice in the cattle industry (Joerg *et al.*, 2004) and is based on the difference in DNA content of X- and Y-bearing spermatozoa (Siedel *et al.*, 2002). Results from the current trial have shown that the flow cytometry instrumentation and technique employed was sensitive enough to identify a bimodal distribution in DNA content of bovine semen based on their sex-specific chromosome differences. Regarding the commercial fish species tested however, no such bimodal distribution was recorded suggesting that semen sexing based on DNA content is not feasible for these species either because a difference doesn't exist or because the detection threshold is not at a high enough resolution. It is possible that there was not a measureable sex difference between the spermatozoa DNA content of the commercial fish species tested as explained for the halibut (Azevedo *et al.*, 2007; Ocalewicz *et al.*, 2008b). Although recent work on chromosome morphology has demonstrated sex-related differences in chromosome size in sperm cells of the ninespine stickleback (which could not be tested in this current study), based on a sex-related length heteromorphism of chromosome one (Ocalewicz *et al.*, 2008a), this difference appears to be species specific. Unlike a large variety of mammalian species which do display sex–related DNA differences, ranging from 2.8% in humans, 4.2 % in cattle (Johnson, 1992; Johnson & Welch, 1999; O'Brein *et al.*, 2002) to 7.5 % in the chinchilla (Johnson *et al.*, 1987), differences in fish sperm may not be so widely common (Martínez *et al.*, 2009). It could also be argued that the instrumentation employed for this trial may not have been sensitive enough to pick up potentially finer differences between the fish sperm cells. Research in the livestock industry has focussed on the design of flow cytometry nozzles in order to maintain proper orientation of cells (sperm head to laser excitation) as they pass through the laser beam (Rens *et al.*, 1999). Since sperm heads are flat in shape,

fluorescence can be affected by their orientation through the flow cytometer i.e. high emission of light from the narrow edge due to a high index of refraction (Dean *et al.*, 1978). Thus only sperm which are oriented correctly can be accurately analysed. Rens *et al.* (1999) described that novel nozzel designs compared to the original bevelled needle system can increase correct orientation rates within bull/boar sperm samples from 20-30% to over 60%. Thus the precision of our instrumentation used may have been hindered partly by misorietation of a proportion of the cells thereby preventing the detection of more subtle differences in DNA, below the level of that found in mammals. It can be concluded from this trial that there was no detectable sex specific DNA differences between the sperm cells of the fish species tested. While it is possible that the methodology could be refined to improve the sensitivity there is no evidence to date to suggest that a morphological difference in sex chromosomes exist in any commercially important fish species that could be differentiated by flow cytometery. Thus it appears that the potential for semen sexing based on DNA content by flow cytometry is limited and is not a viable approach for producing monosex populations for the aquaculture industry. However, this system could potentially be used if sex specific markers were identified and then non-destructive fluorescent labels developed against these.

Indirect sex reversal is a standard and accepted method for the production of monosex populations in aquaculture (Pandian & Kirankumar, 2003; Piferrer, 2001). Based on a published protocol by Hendry *et al.* (2003), this study has demonstrated the effective direct masculinisation of Atlantic halibut for the production of the first UK neomale population which can be used as future broodstock to generate monosex female populations. This will have significant benefit to the industry, removing the problem of male maturation prior to harvest. Our results suggested that the 5ppm treatment for 6 weeks was the most effective and this supports the study by Hendry *et al.* (2003)

confirming that hormone treatment can be conducted when the halibut move from weaning onto the first feeding diet (Pandian & Sheela, 1995). In our study, hormone treatments commenced with halibut at a size of 40 mm, which was later than planned due to difficulties with facility access, according to Hendry *et al.* (2002) ovarian cavity formation has begun by 38 mm. However, this did not appear to affect our results as we effectively generated a male skewed population under both hormone treatments, this suggests that the opportunity for hormonal sex reversal may extend to a later period than previously thought. It is clearly not necessary to treat the halibut immediately from first feeding and this is true for other species with research being focussed on identifying the most sensitive period for steroid action prior to morphological sex differentiation (Kavumpurath & Pandian, 1993). Interestingly, the 10 ppm treatment was less effective, this may suggest that a longer period of treatment is required to induce masculinisation. Alternatively it may just be a reflection of the intra-population variability in developmental stage. It is unlikely that all individuals within the population will be at a uniform stage of development therefore it is understandable that a minimum period of hormone treatment is required to effectively sex reverse the majority of target individuals.

In terms of growth, no differences in length and weight were found between treatments during the in-feed hormone period. By April 2010, maturing males were evident in both treatments and these clearly showed a lower mean weight in comparison to immature individuals. This was expected since as males enter maturation, resources are diverted into reproduction. Hagen *et al.* (2006) reported a cessation in muscle fibre recruitment from November to April in male halibut undergoing maturation. Interestingly in November and April 2010, immature individuals in the 5 ppm treatment consistently showed lower weight than the control individuals. This can not be attributed

to maturation and is more likely due to body size dimorphism with a higher female proportion in the control population (circa 50 % female) as compared to the 5 ppm population (97% phenotypic male). Studies with 1-2 kg halibut have shown that females have a 1.9 fold higher fast myotomal muscle fiber number than males (Hagen *et al.*, 2006, 2008) reflecting the larger ultimate body size in females. The coefficient of variation however was not different between treatments suggesting that there didn't appear to be distinct population differences between the 5 ppm and control population at this stage. Furthermore although the muscle fiber recruitment rates of males and females appear to differ, the sexual dimorphism in weight between gender generally only became apparent at the start of male maturation (Hagen *et al.*, 2006). The precise physiological mechanisms regulating differences in muscle fiber recruitment between male and female fish showing a sexual dimorphism in body size are unknown (Hagen *et al.*, 2008), thus weight differences cannot be attributed to genetics or phenotype alone. The SGR of tagged immature fish did not vary between treatments, as expected. Halibut growth is generally calculated on the basis of yearly weight gain due to the seasonal changes associated with sexual maturation (Imsland & Jonassen, 2005), furthermore since SGR reduces as weight increases, it makes the evaluation of growth difference difficult (Bjornsson, 1995), yearly weight gain will be calculated in the future after the halibut have been monitored for greater then 3 years

In order to determine neomales within our 5 ppm treatment, as a pilot study, fertilisation crosses have been performed with four of the early maturing 5 ppm putative neomales (spring 2010) and sex determination (identification of monosex female progeny) will be conducted on the progeny once they have obtained a suitable size (~80 g) (Spring 2011). Large scale fertilisation crosses will take place when halibut have reached four years of age (Spring 2011) at which point a greater percentage of males

should be maturing (Jákupsstovu & Haug, 1998) and from this, broodstock for future monosex production will be selected. In order to promote the chances of selecting a neomale, as many individuals as possible should be tested and the ability to pool progeny from different putative neomales would greatly help in this respect. Work is currently being directed into investigating genetic marker (microsatellite) variation between potential male broodstock (taking into account the females to which they are crossed) with the aim to identify their offspring within pooled progeny. Moreover, the ability to determine the genetic sex of halibut would greatly facilitate and fast-track the process of monosex production i.e. neomales could be identified at maturation.

Acknowledgements

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CHAPTER 6

SUMMARY OF FINDINGS

CHAPTER 6: MAIN FINDINGS

In this section, the main findings of each research chapter are summarised:

Chapter 2. ***The effect of metal halide and novel green cathode lights on the stress response, innate immunity, eye structure and feeding activity of Atlantic cod, Gadus morhua L.***

- There were no clear significant long term effects of any of the lighting treatments on stress levels (plasma cortisol, glucose), innate immune function (lysozyme activity), retina structure and population feeding activity.
- A transient reduction in feeding occurred following light onset, most pronounced in fish exposed to higher illumination however this resumed to normal activity after 8 days.
- Even under high intensity constant lighting, cod still demonstrated a day-night rhythm in melatonin release suggesting perception of the overlying ambient photoperiod.

Chapter 3. ***The impact of combining shading and continuous lighting on the suppression of sexual maturation in outdoor reared Atlantic cod, Gadus morhua.***

- Shading treatments in conjunction with continuous lighting were effective at suppressing sexual development in male and female Atlantic cod, confirmed through histology, reduced GSI, oocyte diameter and sex steroid profiles.
- No significant differences in day/night melatonin levels were observed in fish under the shading treatments and continuous light regime.

Chapter 4. ***Photoperiod effects on the expression of kisspeptin and gonadtropin genes in Atlantic cod, Gadus morhua, during first maturation.***

- Expression patterns of *kiss2* and *kissr4* did not reveal any clear association with season or photoperiod treatment.
- Pituitary expression of FSH and LH showed a differential expression in relation to photoperiod treatment from December onwards.

Chapter 5. ***Research on methodologies for the production of monosex Atlantic halibut, Hippoglossus hippoglossus, in the UK.***

- Flow cytometry of fish species sperm cells did not reveal any clear sex related size difference in DNA of species tested thus demonstrating that semen sexing may not be an applicable technique for monosex production of such species.
- Indirect sex reversal was successfully performed in Atlantic halibut yielding a male-skewed population and thus potential neomales for all-female production.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7: GENERAL DISCUSSION

The overall objective of this thesis was to improve the management of sexual maturation in Atlantic cod and halibut with the aim to promote the sustainability and expansion of their commercial culture. A targeted approach was adopted whereby a specific management technique for the control of maturation in each species was investigated: 1) photoperiod manipulation during cod on-growing in cages and 2) monosex production in juvenile halibut. Research was conducted at both the applied and fundamental levels to provide valuable information for the optimisation and implementation of these techniques within the cod and halibut industry. This general discussion will expand on the wider implications of the main findings from the thesis, discuss the limitations of the experimental studies performed and identify current research priorities for future development in these fields of investigation.

1. Photoperiodic control of puberty in Atlantic cod

Photoperiod manipulation has been demonstrated to be fully effective at suppressing puberty in indoor tanks but not in outdoor commercial rearing systems. In an experiment by Taranger *et al*. (2006) only a four month delay at best was recorded using four 150W metal halide units. Recent commercial trials using as many as twenty 1000W metal halide units in a single cage showed improved results with signs of partial inhibition however maturation was still not inhibited in 100% of the population (CodLight–Tech, 6th EU Framework Programme project). This latter set-up was considered as "over-kill" and serious concerns were raised regarding the potential impacts of such high light wattage on the general health and welfare of the fish. Importantly, an increase in the number of light units in a cage does not appear as a viable option for the future due to technical and economical limitations. This PhD project therefore focussed on two key

aspects of the use of high intensity lighting regimes in commercial on-growing of cod: 1) potential welfare impacts (Chapter 2) and 2) the potential of shade netting systems to improve the efficiency of systems (Chapter 3). Furthermore, this project also investigated the interface between the perception of light signals and the endogenous regulation of the brain-pituitary-gonadal axis by firstly developing molecular tools for key targets of the kisspeptin system and then comparing their seasonal profile of expression in ambient vs. photoperiod manipulated cod populations (Chapter 4).

1.1 Welfare impacts of artificial lighting

Research into the potential adverse impacts of artificial lighting regimes in aquaculture is seriously lacking despite the clear importance of these regimes in the industry especially in the case of cod farming where fish are exposed to extremely high light intensities in comparison to other commercially important species (e.g. salmon). Findings from our present work are promising and suggest that high light intensity regimes do not compromise cod juveniles in the long run in terms of the parameters measured including stress indicators (cortisol, glucose), feeding behaviour and aspects of the non specific immune system such as lysozyme activity. It is acknowledged that our experiment was conducted in indoor tanks and therefore ambient conditions especially the additive effect of natural daylight were not encompassed. In particular the UV component of the daylight spectrum has been shown to be damaging to the teleost eye when applied at high intensity (Cullen & Monteith-McMaster, 1993; Chen *et al*., 1999). Furthermore our study was only performed on a small scale (1.6 m^3 volume of water) and for a short duration (up to 4 weeks). This said, the objective of this trial was to mimick the 'worst-case' scenario of artificial lighting conditions that fish would be exposed to in a commercial cage, based on the different lighting systems which are employed by the industry. Our findings came as a surprise considering the levels of light tested (a mean of

16.6 watts m^{-2} in the metal halide treatment), the proximity of fish to the light units (fish were within 1m of the light unit) and the light units themselves (metal halide and narrow bandwidth green lighting, peak emission of 546 nm). This demonstrates the ability of cod to cope and adapt to acute changes in their photic environment. Of particular interest was the lack of observed morphological eye damage following the sudden onset of the constant high intensity lighting. Since the teleost eye does not possess eyelids and has a fixed pupil aperture (Ferguson, 2006) it may be conceived that they are more susceptible to light-induced damage than other vertebrate species. However, mechanisms to protect the retinal photoreceptors from high illumination are present in fish and include the migration of melanosomes to enshroud the photoreceptors and photoreceptor motility. These appear to be highly effective in cod. In a previous study, Vera et al. (2009) showed that morphological damage of the retinal layers does occur above a certain light intensity although the threshold remains to be determined. Retinal damage indicated by a reduced photoreceptor layer and a reduction in the number of outer nulear layer nuclei, was shown in cod, salmon and sea bass after 3 days of exposure to illuminations of 51-380 watts m^{-2}. What is remarkable is the ability of the teleost retina to regenerate thereafter, this is a known feature of fish retina (Cameron & Easter, 1995) which was confirmed by Vera et al (2009). In contrast, retinal regeneration does not occur in higher vertebrates and humans and death of photoreceptors can result in permanent loss in vision. With this in mind and in light of our results, clearly the teleost retina is a very interesting model for understanding mechanisms of retinal cell apoptosis and regeneration in relation to light-induced damage. This work could prove very interesting to study ocular disease and develop therapies for the human eye (Easter & Hitchcock, 2000). Further work in teleosts should be conducted to test the effect of narrow bandwith light especially high energy wavelengths (blue light, 400-500 nm) on the retina. Indeed,

these shorter wavelengths are known to be harmful to the eyes of mammals (Young, 1988; Dawson et al., 2001) and are considered as the most efficient at penetrating the seawater column thus having an increasing potential in the aquaculture industry as compared to metal halide broad spectrum technologies (Lalli & Parsons, 1995). In addition, fish have been shown to be more sensitive to short rather than longer wavelengths (Vera et al., 2010). Blue LED lighting (peak wavelength of 424 nm) has been tested in Atlantic salmon and had no clear effects (Migaud et al., 2007) but it remains to be tested in cod, a more light sensitive species (Vera *et al*., 2009; 2010).

Although there were no clear long term effects of lighting in our study, a transient reduction in feeding was observed. This has also been recorded in Atlantic salmon on many commercial sites following the onset of lighting in on-growing cages and is referred to as the "growth dip". It is thus possible that lighting is having an effect at the physiological level which in turn affects feeding activity. It must also be acknowledged that the lighting regimes may have also had an effect on other parameters not measured in our study. For example, it would be interesting to study the effects of artificial lighting on skin pigmentation, organisation and mucus production. Although UV radiation is not a component of the artificial lighting used, it is known that it can have damaging effects on the skin of teleosts such as a reduction in epidermial goblet and mucous cells, alteration in mucous production, epidermis sloughing (Blazer *et* al., 1997; Leclercq *et al*., 2010a) it would therefore also be of interest to investigate the effects of high intensity narrow bandwidth light. Light-induced skin effects could not only be an indicator of poor health but also affect the marketability of fish. To date, it appears that no studies have been performed to answer these important questions. Furthermore it would be of interest to study the effect of lighting on swimming behaviour and if this results in increased allocation of energy. Atlantic salmon for

example are known to be attracted to light, this said, cod did not show such photo attraction however neither did they show an avoidance. It is also important to note that light sensitivity may vary depending on the age of the fish, for example it is known that the wavelength sensitivity of fish species such as the Atlantic salmon changes throughout its life history (Kusmic *et al.,* 2003), it is therefore possible that in cod, the repertoire of photoreceptors in the retina may change throughout its life history thus also affecting its susceptibility to light.

In summary, this study provides very interesting preliminary information on the effect of high intensity light on the welfare of cod. It is important to acknowledge however that animal welfare is complex, and this may be reflected in many physiological and behavioural traits, thus although we have assessed a number of different indicators to provide a broad assessment with respect to the five freedoms of welfare (FAWC, 2009) a variety of other parameters including swimming behaviour, could be measured to provide an even more comprehensive study of potential welfare impacts. Welfare assessment is of particular importance considering the prevalence of lighting intervention and associated concerns in commercial cod farming, and the desire of regulators to steer best practice. Currently welfare standards are only available for Atlantic salmon and these are very simplistic with respect to light (RSPCA, 2010). The research conducted in this study and other studies by Vera *et al*. (2009; 2010) suggest that these standards should be reconsidered.

1.2 Efficacy of shading - day/night light ratio perception

Research into the non visual light perception in cod *in-vitro* brought to the forefront the concept of "relative photoreception" (Vera *et al*., 2010) which suggests that by reducing daylight intensities, the impact of night time illumination may be improved such that fish cannot perceive daylength seasonal changes. The study in chapter 3 exploited this

concept by investigating the impact of shading of ambient daylight in addition to constant lighting to suppress maturation in cod on-growing. Positive results from the study revealed that the shading treatments in combination with constant light, effectively suppressed maturation and this was shown through a comprehensive analysis of growth, gonadal development and sex steroid data. It is suggested therefore that the efficacy of artificial lighting systems employed to control sexual maturation can be improved through the use of shade netting. Unfortunately no outdoor control with constant lighting and no shading could be tested in this study due to limitations in facility/fish availability. However it must be remembered that the important concept in this study is that a reduction in daylight ratio can effectively suppress maturation in outdoor tanks and this reduction was achieved by combining shading with constant artificial light. It is acknowledged that these results are preliminary and require further confirmation in commercial cage systems before implementation can be done across the industry. Unfortunately, validation of these results at a commercial scale, originally planned as part of the project, could not be performed during the time course of the PhD project due to the collapse of the UK cod industry in 2009. However, such implementation is not straightforward and two key areas must be considered for future research. Firstly, the definition of the night-time illumination threshold needs to be set. The concept of "relative photoreception" recently proposed in fish (Vera *et al.*, 2010) was based on *in vitro* pineal studies and melatonin analyses alone. Although results were extrapolated to *in vivo* conditions by considering the skull light transmittance of cod, it is important to to take into account the circadian organisation in the species with respect to the control of melatonin. Indeed, the pineal gland is only one of the photosensory structures that form the light perception network which also include the retina and deep brain photoreceptors (Migaud *et al*., 2010). Future experiments should therefore try to validate further the

concept of "relative photoreception" *in vivo*. Furthermore, although Vera *et al.* (2010) tested a number of different wavelengths of light (blue, green and red) other components of the daylight such as ultraviolet wavelengths may have an important influence. For example, juvenile salmonids when reared in freshwater present UV cones which suggests that they may play an important role in this fish species at this age (Kusmic & Gualtieri, 2000). Thus definitions based on isolated wavelengths and intensities alone may not be an accurate benchmark for commercial implementation. It also remains to be determined how the photic information is relayed to the BPG axis. Although melatonin release is known to reflect daylength and theoretically light perception in fish species, no direct link to the BPG axis has been demonstrated yet (Migaud *et al*., 2010). To help elucidate this, the expression of key genes involved in the initiation of the BPG axis and their response to photoperiod need to be determined such as those encoding for kisspeptin (Chapter 4) and GnRH subtypes. Furthermore localisation studies of these genes will help identify their potential sensitivity to photic stimuli, for example are they co-localised with key light perception centres centrally and peripherally?

The second area that needs to be investigated prior to any implementation of shading systems in the industry is the technical challenges associated with their development. Shade netting might not sufficiently reduce the light intensity throughout the entirety of the cage thus importantly, relative night time illumination intensities might be lower than levels achieved in the experimental tanks. All of these might interact and impact on the end result. Shading systems must also meet the physical demands and absorb the physical/environmental impacts subjected to cages in the marine environment. In the past, pilot application gave poor results with nets blown away during winter storms when not robust enough or compromising the cage structure when rigid shade netting was used. Furthermore, systems must be user-friendly so not impede on

routine husbandry (e.g. feeding, monitoring,..). Thus although shading could prove to be an effective component in photoperiod manipulation, the engineering of shading systems must be researched. If confirmed commercially, this would have very significant implications for the cod on-growing industry that desperately needs to control puberty to improve profitability.

Another concept to reduce the day/night ratio is the use of submersible cages, this would immediately decrease the amount of ambient daylight to which fish are exposed creating a more constant environment for the fish. However this said, cod naturally inhabit deepwater thus they must still be receiving a cue such as daylength which drives their seasonality. Although offshore submersible cages are not commercially practiced at a large scale presently in cod aquaculture due to the need for development of supporting technologies for autonomous operation of tasks such as feeding (Langan, R., 2010), preliminary results have indicated that cod may be well suited to this rearing system (Chambers & Howell, 2006).

1.3. Photoperiodic effects on the expression of kisspeptin and gonadotropin genes

Despite photoperiod effects having been extensively studied in fish, the pathways by which photoperiod control the neuroendocrine systems and regulate puberty still remain unknown. The kisspeptin system in mammals is known to play a crucial role in the activation of the BPG axis however little work has been carried out on the importance of the kisspeptin system on fish reproduction and its potential link to photoperiod. In Chapter 4 we reported on the identification of cod specific transcripts for the signal peptide *Kiss2* and its receptor *kissr4* which lead to the development of quantitative assays to measure their expression for the first time in Atlantic cod. Interestingly, results indicated that there was no clear seasonality in the selected kisspeptin genes measured in cod that underwent a typical reproductive cycle. This contrasted with our original

hypothesis that kisspeptin expression would display a clear association with sexual development as already suggested in other fish species and mammals (Oakley *et al*., 2009; Filby *et al*., 2008). It is acknowledged that in our study, expression was only measured once a month therefore a higher resolution profile may have captured a peak in expression**.** However, when expression was presented according to gonad developmental stages, there was no evidence of clear patterns of expression. The data obtained conflicts with the previously suggested mode of action of the kisspeptin system and requires more work before any firm conclusions can be made. There are however a number of interesting questions emerging from this work. First of all, perhaps other subtypes of kisspeptin (e.g. Kiss1) and receptor (Kissr2) if proven to exist in cod could play a more important role in the control of reproduction of this species (Akazome et al., 2010). However, irrespective of signal peptide/receptor subtype, in all cases, there is a need for co-localisation studies such as those conducted in zebrafish and sea bass (Aguirre et al., 2010; Servili et al., 2010). In such studies, the presence of Kiss1 neurons in the habenula region suggest that they might be linked to photoreception, whereas the presence of kiss2 in the hypothalamus suggest that it is directly involved in the BPG axis. Similar studies performed in cod will help elucidate the sites of action of the kisspeptin peptide and identify candidate targets for further expression studies relating to the light perception and BPG axis regulation. Regarding regulation of the BPG axis, the role of GnRH in cod also needs to be elucidated. Although the coding sequences of GnRH genes have been published (Accession numbers: GU332296, GnRH1; GU332294, GnRH2; GU332295, GnRH3), to our knowledge no localisation or expression studies have been performed to date. Again it is of importance to determine the localisation of these subtypes as has been done in the sea bass (Kah *et al.*, 2007) to help identify which subtypes appear to play a role in the regulation of the BPG axis and provide neuroanatomical evidence between

kisspeptin and GnRH neurons (Tena-Sempere *et al*., 2010). Results obtained so far in fish clearly indicate species specific adaptations with variation in the subtypes of kisspeptin and GnRHs which play an active role, this emphasises the need for comparative studies to be performed.

Also in light of our results, there is the possibility that the kisspeptin system may not play such an important role in the entrainment of sexual maturation in cod as was first hypothesised. Interestingly, neither kisspeptin or its receptor have been isolated in the avian lineage (Um et al., 2010) which suggests that another signalling pathway must be operating in avian species which could very well be present in fish. What our work did show was that a differential response in gonadotropin signalling was present from December onwards between the maturing population under SNP and the immature population under LL thus an undescribed pathway is at work during this time. With high throughput transcriptomic/proteomic methodologies becoming available and the cod genome soon to be fully sequenced, target tissues such as the hypothalamus of the brain and the pituitary could be screened to identify candidate genes involved in the photic regulation of puberty. For example, microarray and/or based gene expression profiling could be used to identify genes whose expression changes in response to photoperiod treatment and maturity status.

An understanding of the underlying control of reproduction is of key importance in order to fully exploit the seasonal control of puberty. The development of molecular assays to measure genes indicating maturity status will be of great use in helping to design and validate the efficacy of lighting regimes. In a wider context, such tools could be used to look at maturation in wild fish stocks and potentially assess population reproduction status and dynamics of population growth.

2. Methodologies for the production of monosex Atlantic halibut

Monosex production is a key remediation technique to the problem of early maturation in Atlantic halibut as the production of all-female populations prevents the problem of early maturation as females reach a suitable harvest size before undergoing maturation.

Based on an established form of monosex production in the cattle industry the potential of semen sexing by flow cytometry in fish species was explored (Chapter 5). This technique however was not able to differentiate cells into discrete populations based on total DNA content. It is possible that the sensitivity of the instrumentation employed was not at a high enough resolution to detect differences in the DNA content however it is more likely that there were no detectable differences in the DNA supported by the fact that there remains a lack of reported morphological differentiation between male and female sex chromosomes in most teleost species (Devlin & Nagahama, 2002; Tvedt *et al.*, 2006). However advances in DNA analysis techniques have facilitated the search for sex linked and sex specific markers (Penman and Piferrer, 2008) even in the absence of apparent sex chromosomes. Should robust markers be identified in halibut then the fluorescent based cell sorting could be re-employed with non destructive fluorescent labels developed against these targets.

A second methodology for the production of monosex populations is the approach of indirect sex reversal. In this study, the initial steps of the traditional method of indirect sex reversal were successfully completed with the production of potential neomales for the first UK female monosex halibut population. Importantly, a shorter duration of hormone treatment was tested with the intention to reduce overall handling time of the hormone however this was not as effective as the longer treatment. This highlights the importance of setting a minimum treatment time for the commercial production of juveniles. This may be a reflection of the amount of time an individual

must receive hormones to over-ride its natural steroid signalling profile. Alternatively it may be an indication of the heterogeneous development rates present in a batch of juveniles for treatment which ultimately means a blanket period of treatment is necessary to capture all individuals during the labile period prior to sexual differentiation. The drawback to indirect sex reversal is the time taken to firstly generate neomales which is then further compounded by the additional time required to validate the broodstocks genotype by progeny testing. In our study, crosses between potential neomales and normal females have been conducted (Spring 2010) however it will take one year following these crosses (Spring 2011) before progeny have reached a size in which they can be sexed through histology by their phenotype. Only then can the neomales with an XX genotype be identified. For the future sustainability of this indirect sex reversal for monosex production, it is important that tools are developed that will help identify genotype by other means than progeny testing. To this end, as with the semen sexing, the identification and validation of sex related/specific markers are a priority goal. Recent advances in DNA technologies such as linkage mapping or Restriction site Associated DNA marker tagging (so called "RAD" tagging) could be used to identify such markers. This means that juveniles could be sexed at a much earlier age and thus hormone treatments for the production of neo-broodstock confirmed as successful or not (based on population sex ratios). Furthermore neoparents could be identified as if their genotype is known then all that remains, is to determine their sexual phenotype. Furthermore, hatcheries selling juveniles marketed as monosex populations could benefit from the technology by screening and confirming population sex prior to sale.

3. Conclusions

This thesis presents novel research investigating the management of sexual maturation in Atlantic cod and halibut. In cod, not only has it assessed serious concerns regarding the

welfare impacts of artificial lighting but it is the first to experimentally document and positively support the concept of shading to improve the efficiency of photoperiod manipulations in outdoor conditions. Furthermore it has ventured into the mechanisms behind the photoperiodic modulation of the BPG axis stimulation and is the first to describe expression of kisspeptin genes in Atlantic cod. In halibut, this project tested the alternative practice of producing monosex populations by semen sexing and has produced putative neomale halibut for the first UK production of monosex female halibut. These latter studies have emphasised the need for the development of sex specific/linked markers in such a species. Importantly the valuable information generated from this PhD project can be used in a species targeted approach to optimise and enhance the control of maturation in each and promote sustainable expansion. It may also be considered in a broader sense for the control of maturation in other fish species. As a whole, sustainable expansion of the aquaculture industry is vital in a world where wild fish stocks are diminishing yet food demand is accelerating rapidly.

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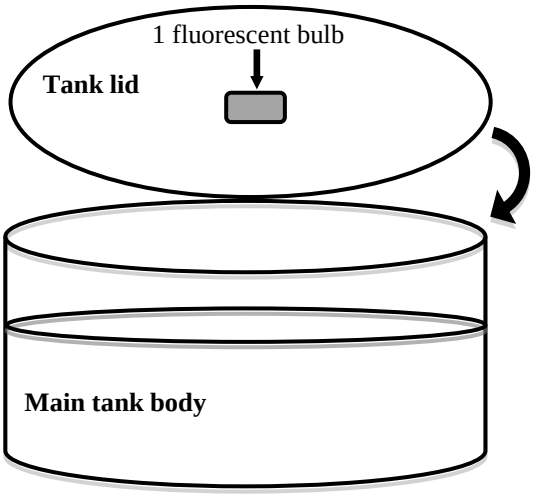
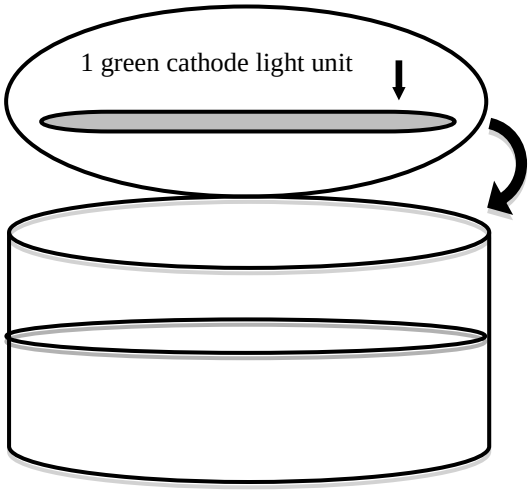
APPENDIX

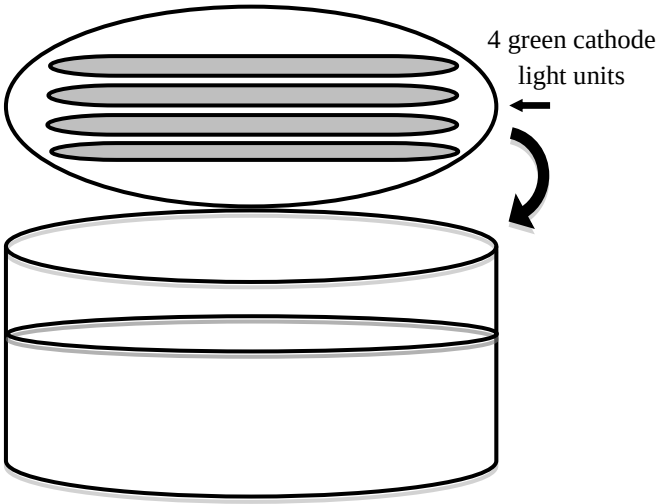
A.) Control (SNP)



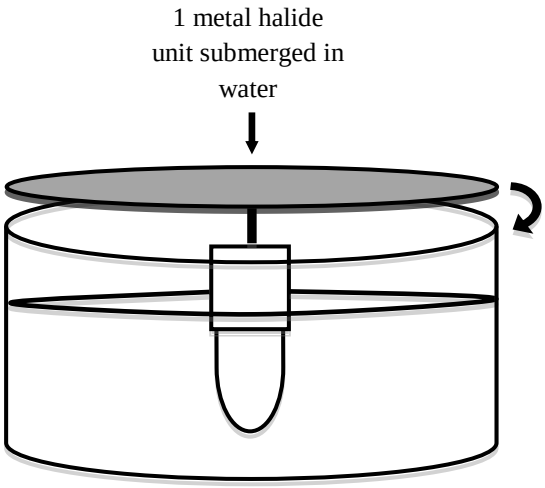
B.) Low intensity green cathode light (LL)



C.) High intensity green cathode light (LL)



D.) Metal halide (LL and SNP treatments)



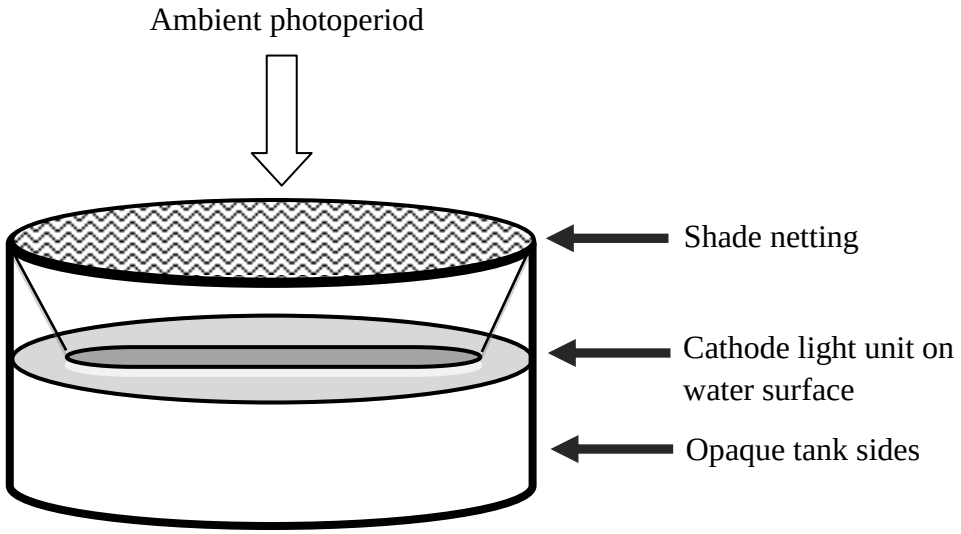
Appendix I. Schematic representation of the light unit set-up in tanks in Chapter 2. Lights were either programmed to a continous light regime (LL) or a simulated natural photoperiod (SNP). Set-ups are displayed as follows: a) control (SNP), b) low intensity green cathode light (1 unit, LL), c) high intensity green cathode light (4 units, LL), and d) metal halide (1 unit, SNP and LL treatments). Cathode light units were secured to tank lids and remained clear of the water surface. Metal halide units were secured to the tank lid and submerged into the water. Tanks were opaque and lids remained closed throughout the trial.

Appendix 2. Plasma cortisol levels (ng ml^{-1}) in Atlantic cod kept under control conditions, green cathode light (Low CL and High CL) and metal halide (MH-LL and MH-SNP) light. Mean and standard deviation (SD) are presented (n=2, 5 fish per replicate). Significant differences between baseline and post-light onset values are indicated by *.

Treatment		Baseline	3 Hour	1 Week	2 Week	4 Week
Control	**Mean**	5.21	4.99	2.72	7.61	7.91
	SD	0.14	4.19	2.26	5.33	4.38
Low CL	**Mean**	2.41	6.58	12.30*	3.59	3.90
	SD	0.57	3.75	5.00	2.76	2.63
High CL	**Mean**	4.92	12.39	9.52	13.08	4.99
	SD	0.58	4.11	5.23	9.51	0.33
MH-LL	**Mean**	2.94	12.28	5.89	6.06	8.16
	SD	1.37	2.57	4.67	3.64	7.37
MH-SNP	**Mean**	5.04	8.53	2.54	8.84	3.64
	SD	2.72	6.45	0.08	5.77	0.23

Appendix 3. Plasma glucose levels (mg dl^{-1}) in Atlantic cod kept under control conditions, green cathode light (Low CL and High CL) and metal halide (MH-LL and MH-SNP) light. Mean and standard deviation (SD) are presented (n=2, 5 fish per replicate).

Treatment		Baseline	3 Hour	1 Week	2 Week	4 Week
Control	**Mean**	42.63	47.92	54.72	74.91	60.19
	SD	9.46	6.01	11.16	15.87	19.95
Low CL	**Mean**	32.42	60.31	39.89	55.33	51.73
	SD	5.24	0.90	9.62	9.16	9.50
High CL	**Mean**	40.85	48.06	58.41	49.83	55.77
	SD	3.76	3.59	15.68	16.14	20.00
MH-LL	**Mean**	45.27	68.76	51.84	53.83	51.52
	SD	0.81	0.13	8.42	10.58	4.81
MH-SNP	**Mean**	39.85	51.60	61.87	61.44	59.36
	SD	8.09	11.73	17.48	6.96	28.65



Appendix 4. Schematic representation of the shading and light unit set-up in Chapter 3. Shaded tanks were opaque and shade netting was stretched across their open surface. The cathode light unit was positioned to float on the water surface and secured to the tank by its ends.

Appendix 5. Weights (g) of tagged female fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval.

		Aug'08	**Oct**	**Dec**	**Feb'09**	**Apr**	**Jun**	**Aug**
Indoor SNP	Mean	723.67	911.67	1142.50	1395.83	1190.83	1400.00	1627.00
	SEM	69.07	82.53	126.12	156.71	103.38	133.70	117.53
	n	6	6	6	6	6	6	5
Indoor LL	Mean	628.33	816.67	1014.17	1283.33	1635.00	1984.00	1938.33
	SEM	72.92	78.06	91.13	135.45	177.54	171.12	172.33
	n	6	6	6	6	6	5	6
Outdoor low shade LL	Mean	615.14	681.67	914.17	1135.00	1475.00	1575.00	1615.00
	SEM	36.95	33.13	48.28	61.98	128.80	132.00	127.37
	n	7	6	6	6	7	7	7
Outdoor high shade LL	Mean	645.00	811.67	1076.67	1460.00	1950.00	1940.00	1956.67
	SEM	13.23	25.87	56.00	105.00	137.69	140.21	79.29
	n	3	3	3	2	3	3	3

Appendix 6. Weights (g) of sacrificed female fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: one-way ANOVA).

		Jul'08	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
Indoor SNP	Mean	572.14	591.25	594.17	665.00	920.00	830.83	1193.33	1221.67	930.00	875.00[b]	930.00[b]	1085.00	1627.00
	SEM	36.72	63.33	56.18	25.50	55.53	138.21	205.61	147.13	130.92	80.00	-	135.00	117.53
	n	7	6	6	6	6	6	6	6	7	2	1	2	6
Indoor LL	Mean	572.14	747.50	717.86	727.50	979.17	1068.33	879.17	1238.64	1140.83	1499.29[ab]	1729.38[a]	1729.38	1938.33
	SEM	36.72	64.61	69.53	81.93	169.25	173.62	84.95	111.12	122.30	130.55	108.89	443.14	172.33
	n	7	6	6	8	6	6	6	10	6	7	8	3	6
Outdoor low shade LL	Mean	572.14	386.67	-	680.00	-	1044.17	-	1386.00	-	1428.75[ab]	-	1982.50	1615.00
	SEM	36.72	72.19	-	38.08	-	147.49	-	108.72	-	221.54	-	312.50	127.37
	n	7	3	-	5	-	6	-	5	-	4	-	2	11
Outdoor high shade LL	Mean	572.14	563.33	-	848.00	-	880.00	-	1235.00	-	1847.00[a]	-	1887.50	1956.67
	SEM	36.72	43.33	-	42.80	-	163.52	-	160.90	-	206.24	-	110.64	79.29
	n	7	3	-	5	-	5	-	6	-	5	-	6	3

Appendix 7. Weights (g) of tagged male fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval.

		Aug'08	**Oct**	**Dec**	**Feb'09**	**Apr**	**Jun**	**Aug**
Indoor SNP	Mean	476.67	579.17	710.00	746.67	777.50	957.00	1094.17
	SEM	44.72	62.11	101.55	81.53	67.41	94.20	123.47
	n	6	6	6	6	6	5	6
Indoor LL	Mean	540.00	668.75	754.00	896.00	1039.00	1156.00	1215.00
	SEM	52.44	63.72	45.56	102.82	91.93	60.22	126.39
	n	5	4	5	5	5	5	5
Outdoor low shade LL	Mean	574.57	740.71	931.71	1122.14	1366.43	1401.71	1395.00
	SEM	31.69	56.55	101.55	127.46	162.42	151.47	166.52
	n	7	7	7	7	7	7	7
Outdoor high shade LL	Mean	471.88	595.00	754.38	1155.63	1264.38	1409.29	1415.00
	SEM	43.60	59.40	97.19	165.93	204.75	221.50	173.88
	n	8	8	8	8	8	7	8

Appendix 8. Weights (g) of sacrificed male fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: one-way ANOVA).

		Jul'08	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
Indoor SNP	Mean	430.00	460.33	549.17	659.17	645.83	644.17[b]	846.67	925.00	735.83[b]	862.78[b]	948.13[b]	1031.88	1094.17
	SEM	30.94	22.23	49.49	20.14	80.63	55.31	72.72	72.26	57.47	80.71	75.62	87.45	123.47
	n	13	6	6	6	6	6	6	6	6	9	8	8	6
Indoor LL	Mean	430.00	467.50	428.33	585.00	773.33	977.14[a]	1043.33	1186.25	1218.33[a]	1248.33[ab]	1590.00[a]	1456.43	1215.00
	SEM	30.94	60.87	49.49	75.40	124.82	86.91	122.69	93.15	110.23	134.24	-	137.59	126.39
	n	13	6	6	6	6	7	6	4	6	3	1	7	5
Outdoor low shade LL	Mean	430.00	446.43	-	687.00	-	1052.50[a]	-	1059.00	-	1275.83[a]	-	1181.88	1395.00
	SEM	30.94	53.23	-	75.92	-	94.48	-	74.10	-	73.57	-	168.92	166.52
	n	13	7	-	5	-	4	-	5	-	6	-	8	10
Outdoor high shade LL	Mean	430.00	448.57	-	444.00	-	768.00[ab]	-	997.50	-	1195.00[ab]	-	1247.50	1415.00
	SEM	30.94	62.75	-	62.76	-	92.41	-	92.41	-	153.60	-	87.14	173.88
	n	13	7	-	5	-	5	-	4	-	5	-	4	8

Appendix 9. Female mean individual GSI for sacrificed individuals maintained under one of four lighting treatments. Mean, standard error of the mean (SEM) and number of fish are presented. Differences between treatments at a given timepoint are indicated by superscripts.

		Aug'08	Sep	Oct	Nov	Dec	Jan'09	Feb	Mar	Apr	May	Jun	Aug
Indoor SNP	Mean	0.94	1.07	1.59	3.00[a]	4.92[a]	5.71[a]	8.18[a]	21.49[a]	2.14	2.35	1.80	2.13
	SEM	0.17	0.22	0.22	0.17	0.70	0.66	0.90	2.38	0.42	-	0.54	0.25
	n	6	6	6	6	6	6	6	7	2	1	2	6
Indoor LL	Mean	0.67	0.90	0.87	0.99[b]	1.10[b]	1.56[b]	1.57[b]	1.30[b]	1.34	1.32	1.00	1.45
	SEM	0.11	0.10	0.12	0.04	0.14	0.49	0.29	0.16	0.17	0.25	0.10	0.30
	n	6	6	8	6	6	6	10	6	7	8	3	6
Outdoor low shade LL	Mean	1.25	-	1.15	-	1.34[b]	-	2.03[b]	-	2.18	-	2.05	1.46
	SEM	0.73	-	0.29	-	0.20	-	0.19	-	0.44	-	0.27	0.14
	n	3	-	5	-	6	-	5	-	4	-	2	11
Outdoor high shade LL	Mean	1.18	-	1.04	-	1.37[b]	-	1.82[b]	-	1.25	-	0.91	1.26
	SEM	0.35	-	0.20	-	0.31	-	0.63	-	0.24	-	0.27	0.15
	n	3	-	5	-	5	-	6	-	5	-	6	3

Appendix 10. Male mean individual GSI for sacrificed individuals maintained under one of four lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented. Differences between treatments at a given timepoint are indicated by superscripts.

		Aug'08	Sep	Oct	Nov	Dec	Jan'09	Feb	Mar	Apr	May	Jun	Aug
Indoor SNP	Mean	0.50	0.21	0.97	1.45	6.20[a]	6.16[a]	6.86[a]	6.25[a]	2.18[a]	1.39	0.26	0.27
	SEM	0.22	0.02	0.24	0.65	0.42	0.40	0.97	0.61	0.44	0.42	0.03	0.02
	n	6	6	6	6	6	6	6	6	9	8	8	6
Indoor LL	Mean	0.49	0.22	0.22	0.15	0.26[b]	0.34[b]	0.58[b]	1.35[b]	0.59[b]	0.10	0.70	0.23
	SEM	0.34	0.04	0.05	0.04	0.04	0.07	0.17	0.40	0.43	-	0.31	0.04
	n	6	6	6	6	7	6	4	6	3	1	7	5
Outdoor low shade LL	Mean	0.31	-	0.31	-	0.30[b]	-	0.20[b]	-	0.61[b]	-	1.39	1.08
	SEM	0.18	-	0.17	-	0.05	-	0.05	-	0.27	-	0.57	0.36
	n	7	-	5	-	4	-	5	-	6	-	8	10
Outdoor high shade LL	Mean	0.50	-	0.21	-	0.36[b]	-	0.32[b]	-	1.16[ab]	-	1.21	0.45
	SEM	0.24	-	0.09	-	0.06	-	0.05	-	0.19	-	0.80	0.12
	n	7	-	5	-	5	-	4	-	5	-	4	8

Appendix 11. Oocyte diameter of leading cohort of sacrificed females maintained under one of four lighting treatments. Mean, standard error of the mean (SEM) and number of fish are presented. Development size classifications are with reference to the definitions by Kjesbu (1991). Differences between treatments at a given timepoint are indicated by superscripts.

		Sep'08	Oct	Nov	Dec	Jan'09	Feb	Mar	Apr	May	Jun
Indoor SNP	Mean	216.32	243.22	343.31[a]	419.37[a]	555.05[a]	675.06[a]	1162.39[a]	174.70	1635.71[a]	179.62
	SEM	9.92	20.02	16.44	18.38	40.16	34.72	110.82	4.12	-	4.44
	n	6	6	6	6	6	6	7	2	1	2
Indoor LL	Mean	244.20	178.16	208.59[b]	222.13[b]	300.73[b]	295.24[b]	266.47[b]	426.20	275.73[b]	208.90
	SEM	67.09	7.82	18.92	11.39	69.38	21.14	20.36	147.93	24.10	3.03
	n	6	8	6	6	6	10	6	7	8	3
Outdoor low shade LL	Mean	-	290.36	-	287.78[b]	-	378.93[b]	-	382.54	-	266.42
	SEM	-	64.04	-	26.17	-	38.44	-	16.64	-	93.39
	n	-	5	-	6	-	5	-	4	-	2
Outdoor high shade LL	Mean	-	237.24	-	309.22[ab]	-	301.37[b]	-	333.12	-	277.37
	SEM	-	67.18	-	57.92	-	45.09	-	32.59	-	32.21
	n	-	5	-	5	-	6	-	5	-	6

Appendix 12. Plasma testosterone (ng/ml) in tagged female fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval. Significant differences between treatments at each timepoint are indicated by superscipts (Statistical analysis: GLM).

Treatment		**Aug '08**	**Oct**	**Dec**	**Feb'09**	**Apr**	**Jun**	**Aug**
Indoor SNP	Mean	0.14	0.38[a]	1.35[a]	0.85	0.50	0.71	0.22
	SEM	0.04	0.10	0.29	0.11	0.10	0.09	0.03
	n	6	6	6	6	6	6	5
Indoor LL	Mean	0.07	0.03[b]	0.14[b]	0.24	0.63	0.80	0.32
	SEM	0.02	0.01	0.03	0.03	0.11	0.03	0.07
	n	6	6	6	6	6	5	6
Outdoor low shade LL	Mean	0.23	0.19[ab]	0.62[ab]	0.35	0.60	1.25	0.44
	SEM	0.06	0.06	0.29	0.03	0.17	0.20	0.09
	n	7	6	6	6	7	7	7
Outdoor high shade LL	Mean	0.12	0.03[b]	0.34[ab]	0.30	0.46	0.72	0.41
	SEM	0.04	0.02	0.06	0.01	0.04	0.07	0.10
	n	3	3	3	2	3	3	3

Appendix 13. Plasma testosterone (ng/ml) in sacrificed female fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: one way ANOVA).

Treatment		**Jul '08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan '09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
Indoor SNP	Mean	0.04	0.02	0.37[a]	0.44[a]	0.90[a]	0.75	1.01[a]	0.94[a]	0.88[a]	0.25	0.08[a]	0.44	0.22[b]
	SEM	0.02	0.01	0.09	0.06	0.14	0.11	0.16	0.09	0.11	0.07	-	0.04	0.03
	n	7	6	6	6	6	6	6	6	7	2	1	2	6
Indoor LL	Mean	0.04	0.03	0.11[b]	0.07[b]	0.22[b]	0.29	0.28[b]	0.43[b]	0.21[b]	0.38	0.44[b]	0.48	0.32[ab]
	SEM	0.02	0.02	0.05	0.02	0.03	0.07	0.09	0.06	0.07	0.08	0.04	0.03	0.07
	n	7	6	6	8	6	6	6	10	6	7	8	3	6
Outdoor low shade LL	Mean	0.04	0.01	-	0.12[b]	-	0.66	-	0.35[b]	-	0.61	-	0.37	0.53[a]
	SEM	0.02	0.00	-	0.05	-	0.18	-	0.04	-	0.04	-	0.37	0.07
	n	7	3	-	5	-	6	-	5	-	4	-	2	11
Outdoor high shade LL	Mean	0.04	0.02	-	0.03[b]	-	0.27	-	0.38[b]	-	0.60	-	0.35	0.41[ab]
	SEM	0.02	0.01	-	0.02	-	0.09	-	0.04	-	0.18	-	0.04	0.10
	n	7	3	-	5	-	5	-	6	-	5	-	6	3

Appendix 14. Plasma 17β-estradiol (ng/ml) in tagged female fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: GLM).

Treatment		**Aug '08**	**Oct**	**Dec**	**Feb '09**	**Apr**	**Jun**	**Aug**
Indoor SNP	Mean	0.06	0.21	0.12	2.32[a]	0.15	0.01[b]	0.01
	SEM	0.02	0.07	0.03	0.24	0.05	0.00	0.00
	n	6	6	6	6	6	6	5
Indoor LL	Mean	0.08	0.04	0.13	0.16[b]	0.22	0.02[b]	0.09
	SEM	0.01	0.00	0.06	0.03	0.06	0.00	0.03
	n	6	6	6	6	6	5	6
Outdoor low shade LL	Mean	0.14	0.11	0.13	0.11[b]	0.07	0.30[a]	0.09
	SEM	0.03	0.02	0.04	0.02	0.03	0.11	0.06
	n	7	6	6	6	7	7	7
Outdoor high shade LL	Mean	0.09	0.09	0.33	0.10[b]	0.07	0.04[ab]	0.04
	SEM	0.02	0.03	0.27	0.01	0.02	0.01	0.02
	n	3	3	3	2	3	3	3

Appendix 15. Plasma 17β-estradiol (ng/ml) in sacrificed female fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: one-way ANOVA).

Treatment		**Jul '08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan '09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
Indoor SNP	Mean	0.09	0.09	0.12[a]	0.11[a]	0.42[a]	0.45[a]	1.22[a]	1.08[a]	1.11[a]	0.06	0.09	0.09	0.01
	SEM	0.02	0.01	0.02	0.01	0.08	0.07	0.35	0.12	0.22	0.00	-	0.01	3.43E-03
	n	7	6	6	6	6	6	6	6	7	2	1	2	6
Indoor LL	Mean	0.09	0.07	0.08[b]	0.05[b]	0.05[b]	0.06[b]	0.11[b]	0.07[b]	0.06[b]	0.27	0.09	0.06	0.10
	SEM	0.02	0.01	0.00	0.01	0.01	0.01	0.03	0.02	0.21	0.21	0.04	0.02	0.03
	n	7	6	6	8	6	6	6	10	6	7	8	3	6
Outdoor low shade LL	Mean	0.09	0.09	-	0.04[b]	-	0.13[b]	-	0.11[b]	-	0.14	-	0.17	0.09
	SEM	0.02	0.00	-	0.01	-	0.05	-	0.06	-	0.02	-	0.12	0.06
	n	7	3	-	5	-	6	-	5	-	4	-	2	11
Outdoor high shade LL	Mean	0.09	0.09	-	0.05[b]	-	0.12[b]	-	0.11[b]	-	0.06	-	0.09	0.04
	SEM	0.02	0.00	-	0.00	-	0.09	-	0.06	-	0.03	-	0.03	0.02
	n	7	3	-	5	-	5	-	6	-	5	-	6	3

Appendix 16. Plasma testosterone (ng/ml) in tagged male fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: GLM).

Treatment		**Aug'08**	**Oct**	**Dec**	**Feb'09**	**Apr**	**Jun**	**Aug**
Indoor SNP	Mean	0.38	1.09[a]	1.66	2.29[a]	0.80	0.78	0.52
	SEM	0.03	0.38	0.55	0.33	0.14	0.08	0.13
	n	6	6	6	6	6	5	6
Indoor LL	Mean	0.39	0.56[ab]	0.63	0.89[ab]	0.76	0.97	0.60
	SEM	0.08	0.17	0.29	0.35	0.08	0.09	0.14
	n	5	4	5	5	5	5	5
Outdoor low shade LL	Mean	0.32	0.35[ab]	0.52	0.40[b]	0.74	1.96	1.19
	SEM	0.08	0.06	0.09	0.11	0.10	0.44	0.16
	n	7	7	7	7	7	7	7
Outdoor high shade LL	Mean	0.20	0.33[b]	0.42	0.40[b]	0.57	1.41	0.85
	SEM	0.03	0.11	0.05	0.06	0.04	0.16	0.12
	n	8	8	8	8	8	7	8

Appendix 17. Plasma testosterone (ng/ml) in sacrificed male fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: one way ANOVA).

Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
Indoor SNP	Mean	0.13	0.06	0.62[a]	0.89[a]	1.06[a]	1.42[a]	3.10[a]	1.87[a]	2.98[a]	0.54	0.37[a]	0.44[b]	0.47
	SEM	0.04	0.04	0.12	0.11	0.23	0.17	0.37	0.26	0.44	0.13	0.04	0.03	0.12
	n	13	6	6	6	6	6	6	6	6	9	8	8	6
Indoor LL	Mean	0.13	0.34	0.27[b]	0.24[b]	0.25[b]	0.45[b]	0.50[b]	0.72[b]	1.41[b]	0.52	0.82[b]	0.78[ab]	0.60
	SEM	0.04	0.29	0.06	0.01	0.05	0.08	0.03	0.13	0.36	0.05	-	0.05	0.14
	n	13	6	6	6	6	7	6	4	6	3	1	7	5
Outdoor low shade LL	Mean	0.13	0.10	-	0.36[b]	-	0.58[b]	-	0.51[b]	-	1.01	-	1.05[a]	0.87
	SEM	0.04	0.05	-	0.12	-	0.09	-	0.07	-	0.15	-	0.16	0.13
	n	13	7	-	5	-	4	-	5	-	6	-	8	10
Outdoor high shade LL	Mean	0.13	0.06	-	0.20[b]	-	0.34[b]	-	0.57[b]	-	1.12	-	0.65[ab]	0.86
	SEM	0.04	0.03	-	0.05	-	0.04	-	0.07	-	0.23	-	0.08	0.10
	n	13	7	-	5	-	5	-	4	-	5	-	4	8

Appendix 18. Plasma 11-ketotestosterone (ng/ml) in tagged male fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented for every two month interval. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: GLM).

Treatment		**Aug'08**	**Oct**	**Dec**	**Feb'09**	**Apr**	**Jun**	**Aug**
Indoor SNP	Mean	1.39	2.02	1.79	7.16[a]	2.24	1.27	1.94
	SEM	0.26	0.16	1.18	1.32	0.53	0.23	0.32
	n	6	6	6	6	6	5	6
Indoor LL	Mean	1.52	1.88	1.53	3.98[ab]	0.88	1.68	1.94
	SEM	0.41	0.57	0.30	1.33	0.20	0.39	0.35
	n	5	4	5	5	5	5	5
Outdoor low shade LL	Mean	1.33	1.48	1.46	2.22[b]	2.47	4.58	1.62
	SEM	0.05	0.20	0.14	0.57	0.20	0.75	0.19
	n	7	7	7	7	7	7	7
Outdoor high shade LL	Mean	1.13	2.09	1.79	1.10[b]	2.08	3.59	2.38
	SEM	0.10	0.53	0.46	0.21	0.16	0.36	0.16
	n	8	8	8	8	8	7	8

Appendix 19. Plasma 11-ketotestosterone (ng/ml) in tagged male fish maintained under indoor (SNP or LL) or outdoor (low or high shade) lighting treatments. Mean, standard error of the mean (SEM) and number (n) of fish are presented. Significant differences between treatments at each timepoint are indicated by superscipts (statistical analysis: one way ANOVA).

Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
Indoor SNP	Mean	1.35	1.17	1.34	1.03[a]	0.85	0.58	5.42[a]	5.01[a]	4.02[a]	0.55	0.29[a]	0.65[b]	1.94
	SEM	0.18	0.18	0.22	0.14	0.21	0.21	0.58	0.56	0.51	0.30	0.13	0.08	0.32
	n	13	6	6	6	6	6	6	6	6	9	8	8	6
Indoor LL	Mean	1.35	1.71	1.03	0.35[b]	0.51	0.41	1.26[b]	1.16[b]	1.70[b]	1.05	1.05[b]	1.54[ab]	1.94
	SEM	0.18	0.64	0.36	0.07	0.15	0.15	0.14	0.05	0.46	0.18	-	0.30	0.35
	n	13	6	6	6	6	7	6	4	6	3	1	7	5
Outdoor low shade LL	Mean	1.35	1.30	-	0.51[ab]	-	0.38	-	0.98[b]	-	1.45	-	1.99[a]	2.06
	SEM	0.18	0.12	-	0.30	-	0.38	-	0.17	-	0.12	-	0.24	0.23
	n	13	7	-	5	-	4	-	5	-	6	-	8	10
Outdoor high shade LL	Mean	1.35	1.00	-	0.34[b]	-	0.06	-	0.81[b]	-	1.43	-	1.57[ab]	2.38
	SEM	0.18	0.17	-	0.12	-	0.06	-	0.13	-	0.33	-	0.11	0.16
	n	13	7	-	5	-	5	-	4	-	5	-	4	8

Appendix 20. Absolute *fshβ* mRNA expression levels (copy no./µg total RNA) and gonadosomatic index (GSI, %) from July 2008 to February 2009 in female Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

	Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**
fshβ	**SNP (In)**	Mean	1.82E+08^{a}	3.81E+08^{ab}	1.61E+08^{a}	1.67E+08^{ab}	2.69E+08^{ab}	4.78E+08^{ab}	9.10E+08^{b*}	6.43E+08^{b}
		SEM	1.06E+08	8.44E+07	4.46E+07	3.38E+07	3.54E+07	8.04E+07	2.24E+08	2.01E+08
		n	7	6	6	6	6	6	6	6
	LL (In)	Mean	1.82E+08	1.67E+08	1.92E+08	1.39E+08	2.41E+08	1.26E+08	9.00E+07	1.01E+08
		SEM	1.06E+08	3.08E+07	6.17E+07	4.43E+07	1.18E+08	3.26E+07	1.96E+07	1.24E+07
		n	7	6	6	8	6	6	6	5
GSI	**SNP (In)**	Mean	-	0.94	1.07	1.59	3	4.92	5.71	8.18
		SEM	-	0.17	0.22	0.22	0.17	0.7	0.66	0.9
		n	-	6	6	6	6	6	6	6
	LL (In)	Mean	-	0.67	0.9	0.87	0.99	1.1	1.56	1.57
		SEM	-	0.11	0.1	0.12	0.04	0.14	0.49	0.29
		n	-	6	6	8	6	6	6	5

Appendix 21. Absolute *fshβ* mRNA expression levels (copy no./µg total RNA) and gonadosomatic index (GSI, %) from July 2008 to February 2009 in male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering.

	Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**
fshβ	**SNP (In)**	Mean	9.12E+07	1.78E+08	7.22E+07	4.61E+08	5.59E+08	6.00E+08	3.95E+08	1.10E+08
		SEM	4.47E+07	5.40E+07	7.15E+06	1.40E+08	1.90E+08	1.74E+08	1.05E+08	2.40E+07
		n	6	6	6	4	6	6	6	6
	LL (In)	Mean	9.12E+07	2.46E+08	6.03E+07	1.40E+08	1.06E+08	1.02E+08	2.30E+08	9.86E+07
		SEM	4.47E+07	8.10E+07	2.78E+07	3.31E+ 7	1.53E+07	2.09E+07	5.0E+07	4.96E+06
		n	6	6	6	6	6	5	6	4
GSI	**SNP (In)**	Mean	-	0.50	0.21	0.97	1.45	6.20	6.16	6.86
		SEM	-	0.22	0.02	0.24	0.65	0.42	0.40	0.97
		n	-	6	6	4	6	6	6	6
	LL (In)	Mean	-	0.49	0.22	0.22	0.15	0.26	0.34	0.58
		SEM	-	0.34	0.04	0.05	0.04	0.04	0.07	0.17
		n	-	6	6	6	6	5	6	4

Appendix 22. Absolute *lhβ* mRNA expression levels (copy no./µg total RNA) and gonadosomatic index (GSI, %) from July 2008 to February 2009 in female Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**
SNP	Mean	8.77E+07^{a}	9.12E+07^{ab}	6.23E+07^{a}	9.87E+07^{a}	7.58E+07^{a}	1.73E+08^{ab}	4.84E+08^{bc}*	7.95E+08^{b}*
	SEM	4.02E+07	1.94E+07	2.09E+07	2.46E+07	9.27E+06	3.68E+07	9.83E+07	1.25E+08
	n	7	6	6	6	6	6	6	6
LL	Mean	8.77E+07	4.59E+07	9.16E+07	5.95E+07	6.94E+07	1.05E+08	7.50E+07	1.52E+08
	SEM	4.02E+07	9.59E+06	2.21E+07	1.40E+07	1.64E+07	5.62E+07	1.88E+07	2.85E+07
	n	7	6	6	8	6	6	6	5

Appendix 23. Absolute *lhβ* mRNA expression levels (copy no./µg total RNA) and gonadosomatic index (GSI, %) from July 2008 to February 2009 in male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**
SNP	Mean	2.88E+07	9.50E+07	3.44E+07	1.62E+08	9.45E+07	1.93E+08	2.49E+08	2.80E+08
	SEM	1.15E+07	2.80E+07	5.90E+06	4.87E+07	1.54E+07	4.90E+07	7.67E+07	5.53E+07
	n	6	6	6	4	6	6	6	6
LL	Mean	2.88E+07	8.77E+07	3.42E+07	6.23E+07	7.51E+07	1.15E+08	1.00E+08	1.38E+08
	SEM	1.15E+07	2.60E+07	9.85E+06	1.81E+07	3.27E+07	5.52E+07	1.67E+07	1.46E+07
	n	6	6	6	6	6	5	6	4

Appendix 24. Absolute *kiss2* mRNA expression levels (copy no./µg total RNA) and gonadosomatic index (GSI, %) from July 2008 to February 2009 in female Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

			Jul'08	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
kiss2	**SNP**	Mean	7030.65	4742.63	4990.72	6412.76	4724.83	5618.40	13722.35	6383.34	4834.74	6268.56	8146.10	6208.34	9068.36
		SEM	713.74	557.32	679.87	633.14	411.65	822.51	4006.46	1068.26	968.83	2570.11	0.00	356.48	1564.10
		n	7	6	6	6	6	6	6	6	7	2	1	2	6
	LL	Mean	7030.65	4412.49	5589.62	4936.43	4079.93	4221.06	4394.68	6682.01	3308.67	4559.11	6305.32	7115.55	6081.26
		SEM	713.74	805.16	382.43	652.19	350.22	865.99	633.78	856.66	430.77	433.33	656.64	1628.34	682.82
		n	7	6	6	8	6	6	6	5	6	7	8	3	6
GSI	**SNP**	Mean	-	0.94	1.07	1.59	3.00	4.92	5.71	8.18	21.49	2.14	2.35	1.80	2.13
		SEM	-	0.17	0.22	0.22	0.17	0.70	0.66	0.90	2.38	0.42	-	0.54	0.25
		n	-	6	6	6	6	6	6	6	7	2	1	2	6
	LL	Mean	-	0.67	0.90	0.87	0.99	1.10	1.56	1.57	1.30	1.34	1.32	1.00	1.45
		SEM	-	0.11	0.10	0.12	0.04	0.14	0.49	0.29	0.16	0.17	0.25	0.10	0.30
		n	-	6	6	8	6	6	6	5	6	7	8	3	6

Appendix 25. Absolute *kiss2* mRNA expression levels (copy no./µg total RNA) and gonadosomatic index (GSI, %) from July 2008 to February 2009 in male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

			Jul'08	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
kiss2	**SNP**	Mean	8047.12	5958.90	6413.29	4446.44	5136.65	5435.13	3574.78	7594.26	3246.80	4811.66	4075.83	7891.10	7341.46
		SEM	856.23	948.56	1727.70	920.37	1009.88	726.10	616.52	969.26	457.72	592.68	203.49	855.78	1406.96
		n	6	6	6	4	6	6	6	6	6	9	8	8	6
	LL	Mean	8047.12	4389.03	5543.24	4664.46	4931.28	4756.42	6279.38	5196.18	3493.88	3794.02	4544.85	5545.26	8004.10
		SEM	856.23	81.01	652.68	328.90	710.73	529.33	1897.19	900.20	124.62	280.81	0.00	801.96	978.40
		n	6	6	6	6	6	5	6	4	6	3	1	7	5
GSI	**SNP**	Mean	-	0.50	0.21	0.97	1.45	6.20	6.16	6.86	6.25	2.18	1.39	0.26	0.27
		SEM	-	0.22	0.02	0.24	0.65	0.42	0.40	0.97	0.61	0.44	0.42	0.03	0.02
		n	-	6	6	4	6	6	6	6	6	9	8	8	6
	LL	Mean	-	0.49	0.22	0.22	0.15	0.26	0.34	0.58	1.35	0.59	0.10	0.70	0.23
		SEM	-	0.34	0.04	0.05	0.04	0.04	0.07	0.17	0.40	0.43	0.00	0.31	0.04
		n	-	6	6	6	6	5	6	4	6	3	1	7	5

Appendix 26. Absolute *kissr4* mRNA expression levels (copy no./µg total RNA) from July 2008 to February 2009 in female Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
SNP	Mean	115000	88400	79000	71700	96100	102000	60100	69100	113000	69600	80700	97500	89700
	SEM	11200	4670	6620	9590	13700	10700	9400	9130	21200	4130	-	24900	10400
	n	7	6	6	6	6	6	6	6	7	2	1	2	6
LL	Mean	115000	87300	73700	78300	70900	55600	54100	61700	81500	79100	114000	81400	86800
	SEM	11200	10100	6400	7820	10900	2900	2460	15300	10500	12600	12500	5930	4360
	n	7	6	6	8	6	6	6	5	6	7	8	3	6

Appendix 27. Absolute *kissr4* mRNA expression levels (copy no./µg total RNA) from July 2008 to February 2009 in male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL). Significant differences in mRNA expression between months for the SNP treatment are denoted by lowercase lettering. Significant treatment differences at each month are indicated by *.

Treatment		**Jul'08**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan'09**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Aug**
SNP	Mean	94800	96600	63500	61500	115000	85000	66000	74800	72900	74000	82700	83800	84600
	SE	7420	10100	9050	9430	7920	17500	10700	10800	5380	7010	10500	3000	9450
	n	6	6	6	4	6	6	6	6	6	9	8	8	6
LL	Mean	94800	84200	98400	69800	73400	69600	59900	56600	102000	54300	120000	102000	96000
	SE	7420	6740	14600	10300	12000	11500	3830	6120	13900	1650	-	7800	10000
	n	6	6	6	6	6	5	6	4	6	3	1	7	5