Research And Development Of Stock Management Strategies To Optimise Growth Potential In Ongrowing Of Marine Fish

SARF027

A REPORT COMMISSIONED BY SARF
AND PREPARED BY

The Institute of Aquaculture, University of Stirling

June 2010

Published by the: Scottish Aquaculture Research Forum (SARF)

This report is available at: http://www.sarf.org.uk

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Suggested Citation
Cowan, M., Migaud, H., Davie, A. (2010) RESEARCH AND DEVELOPMENT OF STOCK MANAGEMENT STRATEGIES TO OPTIMISE GROWTH POTENTIAL IN ONGROWING OF MARINE FISH. pp 100. ISBN: 978-1-907266-35-5

Title: RESEARCH AND DEVELOPMENT OF STOCK MANAGEMENT STRATEGIES TO OPTIMISE GROWTH POTENTIAL IN ONGROWING OF MARINE FISH

ISBN: 978-1-907266-22-5

First published: June 2010

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RESEARCH AND DEVELOPMENT OF STOCK MANAGEMENT STRATEGIES TO OPTIMISE GROWTH POTENTIAL IN ONGROWING OF MARINE FISH

Project Code: SARF027

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Acknowledgements

This project was funded by the Scottish Aquaculture Research Forum (SARF) and CL systems were provided by Intravision Aqua (Norway). The authors would like to thank the staff at Machrihanish Marine Environmental Research Laboratory for their help with fish husbandry and thanks also goes to Dr Iain Berrill for training in cortisol analysis and Dr Alison Morgan for her advice regarding analysis of lysozyme activity.

Executive Summary

This project has made significant steps forward in the control of maturation in Atlantic halibut, *Hippoglossus hippoglossus,* and Atlantic cod*, Gadus morhua*, during ongrowing through a combination of cutting edge research and practical guidance. Sexual maturation is a major problem during commercial ongrowing due to vastly reduced growth rates as well as potential genetic interaction with wild stocks. Management strategies used to combat this problem are species specific, this project focussed on 1) monosex production of Atlantic halibut, and 2) photoperiod manipulation in Atlantic cod.

Firstly, for monosex production, the first UK sex reversed population of halibut broodstock (neomales) was established which will generate, in the long term, a basis for all-female population generation. Halibut juveniles were fed a diet supplemented with MDHT (synthetic testosterone) according to a previously published protocol, two in-feed hormone treatments were tested with the aim to reduce the use of hormone. Results were very successful with the more effective hormonal treatment yielding a 97% male population. Sex reversed males (neomales) within this population will be identified through progeny testing (ouside timeframe of the project). In conjunction, the feasibility of generating monosex populations using a novel semen sexing technique (flow cytometry) proven in terrestrial agriculture was investigated. 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). This study has therefore clearly demonstrated that semen sexing may not be a practical future technique for aquaculture of such species.

Importantly, this project also advanced on research by the Reproduction team at the IoA on photoperiod management of reproduction in Atlantic cod. Firstly work investigated 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. Positive findings 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. Concerning 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 suggested perception of the overlying ambient photoperiod. Leading on from this, a one year trial was

set-up to determine the potential of shading of ambient photoperiod in conjunction with constant lighting to inhibit maturation of cod outdoors. By reducing the relative difference between day/night light intensities (difference presently as high as 98.8 % in commercial cages), it was hypothesised that maturation in cod could be inhibited as fish could not perceive a daylength signal to entrain their reproductive cycle. Again, positive results from the work carried out indicated that with shade netting, relative day/night differences could be reduced to 93% (using a low shading net) and down to 69% (using a high shading net) and each were equally effective at suppressing sexual development in cod. No significant differences in weight, GSI, oocyte diameter/stage or hormone profiles were seen between these shading treatments and the indoor constant lighting control. These results are very promising for cod on growers that desperately need protocols to control maturation of their stocks.

The last deliverable of this project concerned the development and validation of a novel molecular assay to measure the expression of kisspeptin related genes (*kiss1r* and *kiss2*). This task was fully achieved with expression analyses of these genes profiled in maturing (under SNP lighting) versus immature (under constant lighting) cod. No clear pattern in *kiss1r* expression was observed however *kiss2* expression showed a significant elevation during the spawning season of mature cod. These new results, are in contradiction with the hypothesis that the kisspeptin system is involved in the initiation of gametogenesis, as shown in mammals, by stimulating the Brain-Pituitary Gonadal axis at the GnRH level (recruitment of fish into maturation).

Overall, this work aimed to develop and/or refine potential remediation techniques of sexual maturation as well as developing new tools to further our understanding on the regulation of puberty in two of the main commercially important farmed marine fish species in the UK, cod and halibut. This project has been very successful, not only has it established the first UK population of neomale halibut broodstock which will greatly increase the efficiency and profitibility of halibut farming in this country, but it has tested a new alternative technique of semen sexing for monosex production. Furthermore photoperiod manipulation in cod farming has been intensely examined: no significant welfare impacts were recorded from exposure to high intensity artificial lighting in the cod and in addition, the potential of shade netting and artificial lighting outdoors for the inhibition of maturation in cod was demonstrated. Finally, novel molecular tools for investigating the kisspeptin system in cod have been developed, and the physiological control of puberty investigated.

Ultimately the new knowledge and tools gained from this project will improve the competitiveness and sustainability of marine fish aquaculture industry within the UK.

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CHAPTER I: INTRODUCTION AND OBJECTIVES

The farming of Atlantic halibut, *Hippoglossus hippoglossus*, and Atlantic cod, *Gadus morhua,* have a number of clear differences in the strategies and production goals that is a reflection of the apparent biological differences between the two species. The key aspects can be summarised as follows:

- Atlantic halibut: harvest goal is 8 kg or greater (+ 4 years from hatch), male maturation starts at 3 years of age (circa 20-30% of population at 3 years reaching 50% at 4 years), broodstock system is reliant on stripping and artificial fertilisation.

- Atlantic cod: harvest goal is 3-4kg (2.5 - 3 years from hatch), 100% maturation is observed at 2 years or less, broodstock system is reliant on natural spawning.

A number of management strategies are available to address this problem of early puberty in farming however reproducibly successful protocols are still lacking. Monosex production is the strategy of choice in species which exhibit a favourable sex for culture such as Atlantic halibut (Piferrer, 2001). The production of all-female stocks removes any concerns of sexual maturation in production cycles as females will be harvested well in advance of sexual maturation. Furthermore female stocks have a greater growth performance (>25%) than males. 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 (circa 10%). In this respect monosex female production would be of some value. The established technique of monosex production (*n.b.* which is already proven in Atlantic halibut, Hendry *et al.*, 2003), is where sex reversed males (i.e. genotypical males which are phenotypically female) are produced by in-feed hormonal treatments, these are referred to as neomales. This is possible in halibut as the females are the homogametic sex (XX) (Hendry *et al.,* 2002)*,* sex chromosomes have not been detected in Atlantic cod yet however (Klinkdhart, 1994). Neomales are then crossed with normal females to produce a monosex population. This process is however time consuming and resource demanding (e.g. in halibut production this would be in excess of 5-6 years to produce neomales and test progeny). For this reason, terrestrial agricultural systems with similar problems e.g. dairy/beef farmers and pig producers have established alternative protocols based on semen sexing (Joerg *et al.,* 2004). In this system, male and female spermatozoa are physically identified and separated using flow cytometry based on the differences in DNA content. Thereafter eggs are fertilised with the semen “sex” of choice. It should be noted that such a technique has never

been established in a teleost species to date and as such represents a high risk of failure, however its potential benefits are all too apparent.

A second management technique, and the most popular to date, is the use of photoperiod manipulation. This is the preferred management technique for Atlantic cod, especially where organic status is targeted and has been a major focus of the Genetics & Reproduction Group over the last few years at the Institute of Aquaculture. Owing to the long production cycle of Atlantic halibut, photoperiod manipulation has limited value as to date the tested protocols have not prevented maturation, at best they reduce the male recruitment rate and delay their onset to the following year. While photoperiod protocols have already been established for cod in enclosed tank systems (Davie *et al.,* 2003, 2007; Hansen *et al.,* 2001) the lighting technology available for cage systems is poorly developed, inappropriate and not cost effective and as a result has not been successful to date. The main industry priority is therefore to refine lighting systems used, thus new narrow band-width lighting technology such as LED (light emitting diode) and CL (cathode light) units have been designed as improvements to the traditional metal halide lighting systems. There is however very limited information available at a technical level (i.e. efficiency of LED/CL units in sea water) and no information at a biological level (i.e. cod light sensitivities in terms of light perception and potential welfare impacts of high energy light on stress response or photoreceptor damage). Only when these key concerns are satisfied can such technology be successfully implemented within the cod industry. In addition to manipulation of the outdoor photoperiod through improvements in artificial lighting, it is also intended to investigate the potential of shading systems.

Not withstanding the technical constraints of the equipment employed in photoperiod manipulation there are more fundamental questions which remain unanswered concerning the endogenous regulation of maturation in species like Atlantic cod. Although seasonally-changing day-length (photoperiod) and especially decreasing photoperiod from summer to winter solstices is known as the natural time-keeping mechanism used by cod to entrain sexual maturation and other important physiological processes, the mechanisms behind the photoperiodic initiation of puberty are still unknown. Studies performed to date on sexual maturation in cod or halibut, to date, use laboratory based techniques such as sex steroid immunoassay, ultrasound scanning or histological analysis of gonadal samples. Although these techniques allow a good monitoring of the gonadal development over the six months preceding the act of spawning, they can not accurately inform on the onset of puberty as they reflect the hormonal cascade involved in the gametogenesis process or simply gonadal growth. Furthermore, no validated assays are available to date in cod to measure Gonadotropin Releasing Hormone (GnRH) which characterises the brain-pituitary stimulation

of gonadal development i.e. onset of puberty (Roa *et al*., 2008). Consequently, although we know that it is the reduction in daylength from summer to winter that initiates maturation in cod ("decision window"), it is not known exactly when during this 6 month period the decision is taken and when it becomes irreversible. This information is of prime importance as it will provide a very useful prediction model as to whether or not cod stocks are to mature. Recently, a new signal peptide was discovered in mammals (kisspeptin) and its receptor (GPR54) which have been shown to be involved in the initiation of puberty by stimulating the secretion of GnRH in higher vertebrates (Colledge *et al*., 2004; Roa *et al.,* 2008; Seminara *et al.,* 2005). A study performed in tilapia identified and characterized GPR54 for the first time in fish (Martinez-Chavez *et al.,* 2008) and IoA has been investigating and developing the tools in this species during the last few years. It is believed that kisspeptin and/or its receptor (GPR54) could provide a tool to predict cod puberty which could thereafter be used for other important marine fish species. For this reason, it is intended within this project to develop and validate a q-PCR assay for the Atlantic cod homologue of kisspeptin and characterize the expression profile across maturation cycle and specifically during decreasing daylength in order to determine precisely when the light stimuli initiates puberty.

Objectives

1. Literature review on the control of puberty in temperate marine fish species

2. Monosex Production

    a. Provide the guidance and practical help in establishing a "neomale" halibut population at a commercial hatchery which could be used in future years to produce all female halibut populations and optimisation of the technique.

    b. Determine the feasibility of semen sexing by flow cytometry in both Atlantic halibut, Atlantic cod and other commercially important UK species and develop, if successful, protocols for all female production.

3. Determine the effect of metal halide and novel green cathode lights on the stress response, innate immunity, eye structure and feeding activity of Atlantic cod.

4. Study the effect of day / night ratio, through the use of shading and constant lighting, on the maturation of Atlantic cod

5. Development of molecular tools for definitions of puberty onset in cod

a. Develop and validate molecular tools to determine the initiation of puberty in marine fish species. Determine the expresison of the kisspeptin system over a maturation cycle in cod and compare this to individuals maintained under constant lighting.

CHAPTER II: CONTROL OF PUBERTY IN TEMPERATE MARINE FISH SPECIES

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1. Puberty and aquaculture

In fish, as in other vertebrates, puberty refers to the process by which an immature individual develops into a reproductively functional adult (Okuzawa, 2002), this is characterised by subsequent gonadal maturation and gametogenesis if the nutritional status and environmental conditions are suitable (Bromage *et al.*, 2001; Coward *et al.*, 2002). Puberty is a major problem during the on-growing stage of temperate marine fish and can result in great commercial loss to the industry. This is because fish direct their energy into gonadal maturation leading to a reduction in somatic growth and flesh quality (Endal *et al.*, 2000; Hansen *et al.*, 2001; Oppedal *et al.*, 1997; Porter *et al.*, 1999) thus increasing the duration and cost of production cycles (Galbreath *et al.*, 1995). It has also been reported that pubertal fish have low immunocompetence (Cuesta *et al*., 2007) and greater sensitivity to environmental stressors like changes in water temperature and low oxygen levels (Makino *et al.,* 2007) which is accompanied by the potential risks of genetic interaction with wild fish stocks through broadcast spawning or escapees (Taranger *et al*., 2009).

This review focusses on three of the main management techniques which can be used to address the problem of puberty in on-growing of marine fish: 1) production of monosex populations, 2) production of sterile populations, 3) photoperiod manipulation. Other strategies include feed management (Shearer *et al.,* 2006) and selective breeding (Gjedrem, 2000). Prior to discussing management techniques, it is necessary to understand the biological and environmental mechanisms regulating puberty in fish. This review centres on temperate marine fish, and particularly Atlantic cod, *Gadus morhua*, and Atlantic halibut, *Hippoglossus hippoglossus*, two of the most commercially important marine fish species in the UK.

2. Expression of sex and regulation of puberty in teleosts

2.2 Expression of sex

The expression of sex in fish species is dependent on two processes: sex determination and sex differentitaion (Piferrer, 2001). A large expanse of literature is available on such processes in fish and was reviewed by Devlin & Nagahama (2002) and more recently Penman and Piferer (2009). Determination is used to describe the genetic and environmental influences that control sex differentiation, referring to the fulfilment of sex determination through testicular / ovarian development (Devlin and Nagahama, 2002). Many teleosts are gonochoristsic where individuals develop only as males or females and remain the same sex

throughout their lifespan (Devlin & Nagahama, 2002). Examples of commercially important gonochoristic species include Atlantic halibut, cod, salmon and rainbow trout. There are a number of hermaphroditic species as well which are described as either synchronous where viable eggs and sperm are released in the same spawning or successive, where the fish first differentiates as one sex and then switchess to the other. The gilthead sea bream, *Sparus aurata*, for example starts life as a male and then transforms into a female (Brusclé Sicard & Fourcault, 1997). It is believed that hermaphroditism represents cases where sex differentiation has been tuned to maximise fitness through the flexible production of gametes (Devlin & Nagahama, 2002). Regarding sex determination many teleosts have a clear primary genetic basis, this generally occurs in one of two forms (Hendry *et al*., 2002); a heterogametic male (XY) or a heterogametic female (WZ). Atlantic halibut, tilapia and rainbow trout all exhibit the former XY system whereas the black mollie, *Poecilia sphenops*, exhibits the WZ system (George & Pandian, 1995). In other species such as European sea bass, Atlantic cod and Atlantic salmon, the chromosomal basis for sex determination is not so clear and sex chromosomes have not been detected (Devlin & Nagahama, 2002; Klinkhardt, 1994; Piferrer *et al*., 2005). As well as genetics, the environment too can play a role in sex determination for example, in the European sea bass, temperature has clearly been shown to influence sex ratios, with a higher temperature yielding a greater percentage of males (Piferrer *et al*., 2005) with there being similar evidence for temperature effects on sex ratios in Turbot (Haffray *et al.* 2009) and Halibut (Hughes *et al.* 2008) as well. Also importantly, application of exogenous steroids via immersion or orally has been used to override natural steroid levels and thus reverse the phenotypic sex of treated individuals such as in Atlantic halibut (Hendry *et al*., 2003) or turbot (Haffray *et al.* 2009).

2.3 Brain-Pituitary-Gonad Axis

Puberty and the subsequent reproductive cycles are under the endogenous control of the neuroendocrine, brain-pituitary gonadal (BPG) axis (Taranger *et al.*, 2009). This axis is organised around three major regulators: 1) the hypothalamus of the brain that releases gonadotropin-releasing hormone (hypothalamic GnRH) which passes to the pituitary either through neurons or via the blood supply (Redding *et al.*, 1993; Colledge, 2004), 2) The Pituitary which is stimulated by GnRH to synthesise and release gonadotropins (FSH, LH) from the pituitary 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 (Taranger *et al*., 2009). There are two main forms of gonadotropins in teleost fish: follicle stimulating

hormone (FSH) and luteinising hormone (LH), each of which perform specific roles in the gonads and are therefore produced at different stages of the reproductive cycle. In female fish, FSH is elevated during early oocyte growth and vitellogenesis whereas LH is associated with final maturation and ovulation (Mittelholzer *et al*., 2009; Nocillado *et al.*, 2007). In male fish, FSH plays a role in spermatogenesis whereas LH is associated with spermiation (Carillo *et al*., 2010).

GnRH secretion from the hypothalamus is a critical step in the BPG axis as it triggers the hormonal cascade for gonadotropins and ultimately reproductive development (Seminara, 2005; van Aerle *et al.*, 2008). Mechanisms causing the stimulation of the GnRH neurons and hormonal release are still poorly understood (Seminara, 2005), recent research to elucidate the upstream activation of the kisspeptin system has opened up new avenues to explore (Migaud *et al*., 2010).

Studies in mammals and subsequently fish have demonstrated the critical role of GPR54 (G-protein-coupled receptor 54) and its ligands, the kisspeptins (peptide products of the KISS1 gene), in stimulating GnRH release (Colledge, 2004; Parhar *et al.*, 2004; Seminara, 2005; Nocillado *et al.*, 2007; van Aerle *et al.*, 2008). A number of studies for example, have shown that mammals with an absence or mutations in the gene encoding GPR54, exhibit impaired secretion of FSH and LH resulting in complete or partial failure of pubertal development (Parhar *et al.*, 2004; Seminara, 2005; van Aerle *et al.*, 2008). Furthermore, studies on its temporal expression in fish species such as the female grey mullet, *Mugil cephalus* (Nocillado *et al*., 2007) have recorded high levels of GPR54 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 Tilapia, *Oreochromis niloticus* (Parhar *et al.*, 2004; Martinez-Chavez *et al.,* 2008) where expression levels of GPR54 in the brain have been found to increase during the onset of puberty. These findings have lead a number of authors to suggest the important role of GPR54 in the activation of the BPG axis.

2.4 Environmental regulation of puberty and integration with the BPG axis

In temperate marine teleosts, the seasonal environment plays a primary role in controlling puberty and entraining reproductive cycles (Bromage *et al.*, 2001; Migaud *et* al., 2010). Proximate environmental factors cue the start of maturation (gonadal and gamete development can take over six months) and principally co-ordinate reproduction (Bromage *et*

al., 2001; Migaud *et al.,* 2010) such that fish spawn and larvae hatch in favourable 'ultimate' environmental conditions,

More specifically, the seasonally changing pattern of daylength has been described as the key environmental determinant of maturation in gadoids, salmonids, bass, the breams, flatfish etc. (Bromage *et al.*, 2001; Migaud *et* al., 2010). For example in cod, it is the reduction in daylength after the summer solstice (Davie *et al.*, 2007a), which acts as the 'proximate' cue for individuals to enter into maturation mode. This is followed by spawning in 'ultimate' conditions between February and May depending on location (Cohen *et al.*, 1990).

A number of authors have suggested that a 'window of opportunity' directs the age of puberty in seasonal spawning fish (i.e. salmonids, gadoids) (Taranger *et al.*, 1999; Bromage *et al.*, 2001). This window represents the period of 'proximate' environmental cue and, if the physiological (developmental/energetic) state of a fish is sufficient, it will proceed to puberty. In the case of Atlantic cod, farmed stocks usually reach sexual maturity at 2 years of age (Hansen *et al.*, 2001; Norberg *et al.*, 2004) prior to attainment of harvest weight (Davie *et al.*, 2007a) while in wild stocks this can range from 2 to 6 years depending on location. It has been demonstrated that in tanks, the application of constant light (LL) at 15 months post hatch (July), prevented cod from spawning during the natural spawning season between April-June at two years old (Hansen *et al.* 2001, Davie *et al.* 2007a). From these results, authors have proposed that the 'proximate' cue accountable for recruiting individuals to into sexual maturation is the decreasing photophase in Autumn (Davie 2005, Davie *et al* 2007a).

While the environmental signals that entrain reproduction are now clear the mechanisms by which they perceive this information and thus regulate the BPG axis remain uncertain. It is believed that operating at the core of every vertebrate is the 'circadian axis' which comprises the retina, the pineal complex and the suprachiasmatic nucleus (SCN) or SCN like structure of the hypothalamus (Menaker *et al.*, 1997). Studies on non-mammalian vertebrates, such as fish, have identified circadian oscillators within these components, capable of sustaining rhythmicity in vitro but sensitive to entraining signals from photoreceptors (Menaker *et al.*, 1997). The photosensitivity of each component and their relative employment differs between species, however as a whole, they constitute the 'circadian axis' and ultimately generate photoperiod entrained rhythms (Migaud *et al.,* 2006, 2010).

The subsequent output mechanism of the axis is essential in relaying 'time' to target areas and controlling biological rhythms (Falcon, 1999). At present, the two photoreceptive organs in teleosts, believed to be most important in interpreting photoperiod are the eyes

(retina) and the pineal gland (Falcon, 2010; Forsell *et al.,* 2001) that together regulate the main output signal which is melatonin. In salmonids, it is the pineal gland which has been proposed to dominate while in Atlantic cod and Seabass it appears both the retina and pineal are involved in regulating melatonin signalling (Migaud et al. 2007). 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). Thus melatonin release acts as a hormonal rhythm providing information on both the time of day and seasonal date to the fish.

The question comes however as to the link between this environmental control and the endogenous control of maturation through the BPG? It is thought that the GPR54 / kisspeptin system may play a part in this link however, more work needs to be carried out to elucidate its expression over a maturation cycle in various fish species and its correlation to environmental conditions (i.e. photoperiod) (Migaud et al 2010).

3. Stock management strategies to suppress early maturation

Management strategies employed to alleviate problems of puberty can be directed towards its avoidance: 1) monosex production, or towards its control: 2) sterility (prevention) 3) environmental control (inhibition).

3.1 Monosex production

The production of monosex populations can be applied in sexually dimorphic species where one sex reaches harvest size before maturation. For example, all-female populations of Atlantic halibut would be greatly advantageous as they reach market size and can be harvested well in advance of maturation (Hendry *et al.*, 2002; Hendry *et al.*, 2003; Tvedt *et al.*, 2006). In Nile Tilapia on the other hand, it is the males which grow faster and are more economically profitable for production.

3.1.1 Hormonal Therapy

Monosex populations can be produced by either direct or indirect sex reversal. Direct sex reversal refers to the exposure of mixed sex larvae (prior to sexual differentiation) to a hormone in order to directly generate the selected single sex population. In Nile Tilapia, treatment with synthetic testosterone (in-feed, immersion) successfully masculinises a population (Fitzpatrick *et al.*, 1998; Fitzpatrick *et al.*, 1999; 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 and India.

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. 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 MDHT treatment prior to sex differentiation, are crossed with normal females (XX) to produce all-female progeny (Piferrer, 2001). 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, *Salmo gairdneri* R. (Bye *et al.*, 1986) and the yellow perch, *Perca flavescens* (Malison *et al.*, 1996). 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).

3.1.2 Gynogenesis

Monosex populations can also be generated by gynogenesis. This is a process of asexual reproduction where offspring inherit only maternal genetic material (Piferrer, 2001). Gynogenesis can be induced by stimulating an egg to divide using genetically inactive (i.e. UV or gamma irradiated) spermatozoa. The haploid embryo is then temperature or pressure shocked to 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) however at present, it is more commonly performed on species for research purposes (Piferrer, 2001).

3.1.3 Semen Sexing

Current techniques for monosex production have disadvantages such as long term processing and public concern over the use of hormones. Owing to these problems, a potential new avenue for mono sex production would be 'semen sexing'. In 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. 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 lack of data suggesting that the DNA content between male and female teleost species differs.

3.2 Sterility in farmed stocks

In species where both sexes mature before reaching harvest size, the production of sterile populations is a possible approach. The methodologies are limited and focus mainly on the manipulation of ploidy

Triploidy describes individuals which have three sets of chromosomes in their somatic cells (Benfey, 2001), unlike normal diploids which have two, this results in abnormal meiotic division of chromosomes during gametogenesis (Benfey, 1999; Tiwary *et al.*, 2004). Triploid fish are considered sterile as the females have small undeveloped gonads, while the males still develop large gonads and secondary sexual characteristics however the sperm is aneuploid thus can not fertilise an oocyte (Taranger, 2010). Thus to boost production further it is therefore believed that production of monosex all-female (monosex) stocks is more favourable than mixed triploid stocks for culture. This technique is already commercially applied in the rainbow trout industry, is under testing in Atlantic salmon and has been investigated in sea bass (Zanuy *et al.*, 2001).

3.2.1 Techniques

Triploidy can be artificially induced for commercial purposes by shocking newly fertilised eggs (zygote cells) to inhibit the second meiotic division (Malison *et al.*, 1996; Maxime, 2008). This prevents exclusion of the second polar body from the egg thus three chromosome sets are retained (Benfey, 1999; Maxime, 2008; Tiwary *et al.*, 2004). Induction may be physical or chemical (Maxime, 2008). Physical induction is the preferred method as chemical shocking (i.e. colchicine, anaesthetics) has been found to lead to mosaicism (Maxime, 2008). Physical induction includes pressure shocking, which has been successfully applied in Rainbow trout, (Taylor *et al.*, 2007) and thermal (heat or cold) shock which has been successfully applied in turbot (Piferrer et al. 2003) as well as Eurasian perch, *Perca fluviatilis* (Rougeot *et al.*, 2003) and Blue tilapia, *Oreochromis aureus* (Byamungu *et al.*, 2001). In comparison of success, although more costly than thermal shock, pressure shocking has been found to be more consistent (Maxime, 2008) and produce higher triploid yields (McGeachy *et al.*, 1995). Also it has been described as causing less harm than thermal shocking (Maxime, 2008; Peruzzi *et al.*, 2000), however this said, Maxime (2008) states triploidy induction overall rarely results in damage. In order to detect triploidy under circumstances of commercial production, indirect techniques such as checks of nuclear or cell size by flow cytometry, are commonly employed. In fact, erythrocyte size is frequently used as the sole indicator for triploidy in fish (Benfey, 1999).

3.2.2 Impact on performance and welfare

Regarding commercial production of triploids, scepticism exists as to their performance in comparison to diploids as triploids tend to have larger but fewer cells (Benfey, 1999). The performance of triploids has been extensively reviewed by Benfey (1999) and more recently Tiwary (2004) and Maxime (2008) in species such as Atlantic salmon, brook trout, rainbow trout, Nile tilapia and grass carp. Firstly, in terms of growth, preceding the 'normal' diploid timing of first sexual maturation, numerous studies have demonstrated comparable growth rates (and survival) between diploids and triploids. For example, in Coho salmon, Johnson *et al*. (1986) found no significant differences in growth between ploidies reared separately in both freshwater and seawater. Similar results have been observed in Rainbow trout (Taylor *et al.*, 2007) and Turbot (Cal *et al.*, 2006). In the post-maturation stage however, the sterility advantage of triploids has been demonstrated with a 10-30 % faster growth rate than diploids in Atlantic salmon (Taranger *et al.,* 2009). In terms of morphological deformities, a number of deformities have been reported in triploid salmonids, and this has negatively affected farmer decisions to employ triploidy. It is believed however that these deformities may be more due to the family strains and egg quality, eggs are generally selected from end of the season batches to prevent risk of loss of good quality eggs. Recent studies show that diploid and triploid fish, under optimal rearing conditions, can have similar anaerobic capacities (i.e. brown trout in 9 ^{o}C water, Mercier *et al*., 2002). When subjected to sub-optimal conditions (chronic stressors) such as higher water temperature (oxygen solubility is reduced and oxygen demand is increased) however, the survival of triploids relative to diploids varies. For example, Ojolick *et al*. (1995) and Hyndman (2003b) studied the effect of chronic high temperature on rainbow trout and brook trout respectively and found that mortality rates were increased in triploids relative to diploids. It has been suggested that under these conditions, triploids have greater difficulty in using anaerobic energy stores and recovering(Hyndman *et al.*, 2003a). On the contrary, experiments on brook char (Benfey, 1997), brook trout and rainbow trout (Galbreath, 2006) have shown that triploid and diploid fish exhibit identical thermal tolerance. It is clear that the effects of triploidy vary between species, families and strains and more studies needed.

3.3 Photoperiod control of early maturation

Photoperiod manipulation can be applied in seasonal temperate species, where a sex advantage is not conferred and both sexes reach maturation before harvest. Essentially, it acts to control the timing of seasonal reproductive patterns in fish and is now widely used in aquaculture to alter spawning season, delay/prevent maturation during ongrowing and

stimulate growth (Migaud *et al.,* 2010; Pankhurst & Porter, 2003; Rad *et al.*, 2006). It may be described as a less invasive technique than hormonal sex reversal or chromosome manipulation. Although not feasible for some species such as Atlantic halibut which have a long production cycle (Norberg *et al.,* 2004), photoperiod manipulation for the prevention of maturation is standard practice in the salmon farming industry (Taranger *et al.,* 1999) and protocols have already been established for cod in enclosed tank systems (Davie *et al.*, 2007a;Hansen *et al.,* 2001; Norberg *et al.,* 2004).

3.3.1 Lighting systems and fish light sensitivity

At present, metal halide lighting is the predominant routine lighting used in marine fish on-growing cages to manipulate daylength and thus control maturation, the potential of this lighting in terms biological and economic success is limited however since it's not specifically designed for aquaculture. Units emit a broad spectrum of light, the majority of which is absorbed by the water column (Migaud *et al.*, 2007). 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) and this results in excessively high energy running costs.

New lighting technologies: Cold Cathode Lighting (CCL) and Light Emitting Diodes (LED) are being developed to combat this problem. These units can be specified to emit a narrow band-width of light specific to the photic properties of the water body and also a fish species light sensitivity. 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 (Migaud *et al.*, 2007). In addition to species specificity, LED and CCL units have lower energy costs and a longer life-span. Current research is aimed at investigating the technical performance of these units in the marine environment and their impact on the 'welfare' of fish species.

3.3.2 Application in aquaculture

Management of lighting regimes in enclosed conditions (indoors/tanks) 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 salmon (Oppedal *et al.*, 1997; Porter *et al.*, 1999; Taranger *et al.*,

1999), pink salmon (Beacham *et al.*, 1993), masu salmon (Takashima *et al.*, 1984), rainbow trout (Davies *et al.*, 1999), Atlantic cod (Davie *et al.*, 2007a; Hansen *et al.,* 2001*;* Norberg *et al.,* 2004), Atlantic halibut (Norberg *et al.,* 2001), turbot (Imsland *et al.*, 2003) and sea bass (Zanuy *et al.*, 1995).

In open cage systems however, owing to the overlying ambient photoperiod, light management is more difficult and success is dependent on species. In Atlantic salmon, application of constant metal halide artificial lighting in ongrowing cages successfully prevents maturation (Oppedal *et al.*, 1997; Porter *et al.*, 1999), it is hypothesised that this is due to the artificial lighting effectively 'masking' the ambient photoperiod. In a study by Porter et al. (1999), permanently suppressed nocturnal and diel melatonin levels were recorded in salmon under such conditions. Accordingly, application of constant lighting (LL) in industry ongrowing cages from January onwards has been found to prevent maturation and thus inhibit spawning in salmon in the following autumn (Taranger *et al.*, 1999). Photoperiod manipulation in salmon ongrowing cages is now standard practice in the salmon farming industry.

On the contrary, in Atlantic cod, metal halide cage lighting has not been effective and only acted to delay maturation by four months at best (Taranger *et al.,* .2006) thus having no signficant influence on fish growth. Recent studies at the Institute of Aquaculture on pineal light sensitivity suggest that cod are 10,000 times more sensitive to light (i.e. the relative difference between day and night) than salmon (Vera *et al.* 2009). As such, it is proposed that cod are better able to perceive changes in ambient photoperiod even when exposed to artificial lighting intensities comparable to that for salmon. Furthermore, these species contrast in their behaviour such that cod, unlike salmon are not attracted to light (*pers. obs.*). The failure of photoperiod manipulation of cod in outdoor cages is not thought to be due to the specific timing of the lighting regime as the timing has been verified in enclosed conditions but rather the non-specificity of the metal halide lighting technology.

In order to design artificial lighting for successful photoperiod manipulation in a broader range of species, future research needs to concentrate on the specific light (chromatic and luminescent) sensitivities of each. This may be explored through ex vivo pineal and retina cultures and localisation and characterisation of opsin structures. Furthermore a precise understanding of the timing of recruitment and the size at which certain species can enter reproduction would greatly help in determining the timing of lighting regimes. This could be investigated through analysis of the kisspeptin system expression.

3.3.3 Additional effects (welfare concerns)

It's important 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). High intensity point sources of artificial light in combination with daylight, present levels of lighting beyond that 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 (Dawson *et al.*, 2001; Migaud *et al.*, 2007; Young, 1988).

In the past, this issue of welfare with regard to artificial lighting has seldom been a focus, recent work at the Institute of Aquaculture however has been conducted to directly address such concerns with investigations into effects on stress, immune function and the visual system (Migaud *et al.*, 2007). Migaud *et al.* (2007) recorded an acute stress response (short-term peak in cortisol and glucose) (Wendelaar Bonga, 1997) in Atlantic salmon 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.

It has been demonstrated that the fish eye is vulnerable to artificial light (Dawson *et al.*, 2001; Vera *et al.,* 2009; Vihtelic *et al.*, 2000; Vihtelic *et al.*, 2006) and high intensity regimes could result in retina damage thus impacting feeding behaviour in visual feeding fish such as Atlantic salmon. In their study, extensive examination of retina 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. However recent work at the Institute of Aquaculture has revealed that the cod retina is much more susceptible to artificial light damage than the salmon retina (Vera *et al.,* 2009*)* which parallels the previous findings on pineal sensitivity (Vera et al 2010).

To conclude, these 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

4. Conclusions

The UK marine aquaculture industry has identified that a delay or cessation in maturation during on-growing is crucial for the profitable farming of marine fish species owing to the otherwise loss in growth and condition. At present, three main management strategies (species specific) exist for controlling this problem; monosex production, triploid production and photoperiod manipulation. Techniques relating to these strategies however, demand further research and refinement. Firstly, regarding monosex production, techniques such as direct/indirect sex reversal and gynogenesis exist however the novel technique of semen sexing offers huge benefits (practical/economical) over these if shown to be feasible. Secondly, results on triploid fish indicate that optimal husbandry conditions need to be defined for specific species in order to improve performance and survival of individuals. Thirdly, in terms of photoperiod manipulation: 1) species specific light sensitivities and behaviour must be determined, 2) physiological parameters that regulate recruitment to maturation need to be defined, and 3) welfare concerns of artificial lighting resolved for planned lighting set-ups and species. Once these photoperiod manipulation issues have been addressed, species specific artificial lighting (wavelength, intensity) can be designed and lighting regimes refined thus providing farmers with reproducible successful protocols.

In conclusion, detailed scientific knowledge is required regarding stock on-growing management techniques in order to refine and improve their efficiency and better guarantee their success. This is currently the focus of the Genetics and Reproduction Group at the Institute of Aquaculture with the ultimate aim to improve the sustainability and profitability of the aquaculture industry and ensure a higher quality product is readily available to the customer.

CHAPTER III: MONOSEX PRODUCTION OF ATLANTIC HALIBUT

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 (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 (Hendry *et al*., 2002, 2003; Tvedt *et al*., 2006). Maturation is a major problem during ongrowing as energy is shifted into sexual development resulting in a loss in somatic growth and flesh quality and increased susceptibility to disease (Taranger *et al*., 2009). Thus monosex production of female Atlantic halibut clearly potentially holds great commercial and economic benefit for the industry. To date research in this field has been focused outside the EU so that currently there is no means to generate all-female halibut populations in the UK.

As with a number of commercial fish and also mammalian species, genetic sex in Atlantic halibut is determined at fertilisation by the gamete of the heterogametic male (X or Y) (Hendry *et al*., 2002). This is followed by gonadal sex differentiation and expression of the genetic sex via the appropriate phenotype (Hendry *et al*., 2002). In order to generate a monosex population, hormonal sex reversal (exposure of individuals to exogenous steroids aimed to over-ride natural endocrine signalling) is commonly applied during the intermediary period following genetic sex determination but before sexual differentiation, when gonads are still in an undifferentiated state which will result single phenotypic sex population. This therapy is described as direct sex reversal and has commonly been used in Tilapia farming (Fitzpatrick *et al.*, 1998; Fitzpatrick *et al.*, 1999; Gale *et al.*, 1996; Kwon *et al.*, 2000; Wassermann *et al.*, 2003). Indirect sex reversal however is more widely favoured as the fish intended for consumption have not been in direct contact with the additive hormones. In this situation a potential broodstock population is directly sex reversed and then at maturity the appropriate "neo-parents" are identified and crossed with a normal parent in order to produce the monosex population. Indirect sex reversal has been successfully demonstrated in halibut in a study by Hendry *et al.* (2003) where masculinised female broodstock (XX neomales) fed with methyldihydrotestosterone (MDHT, synthetic testosterone) prior to sex differentiation, were crossed with normal females (XX) to produce all-female progeny (Piferrer, 2001). Atlantic halibut lend themselves well to sex reversal as they start to differentiate after first feeding has started thus hormones can be administered in the feed (Hendry *et al*., 2003). Results from Hendry *et al.* (2002) indicate that the sexually 'indifferent' period lasts from

prior to hatch to 38 mm fork length (L_F). This establishes therefore that sex steroids should be administered before individuals reach 38 mm. In the recent hormonal sex reversal experiment, Hendry *et* al (2003) successfully sex reversed halibut at 5 ppm in-feed MDHT for 6 weeks with halibut starting at a mean length of 30 mm L_F.

These indirect techniques for the production of single sex stocks in fish are time-consuming taking a minimum of two generations confirm the techniques success (Piferrer, 2001), in the case of Halibut this would represent a minimum of 4-5 years. Thus the identification of any 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 sperm according to DNA differences using flow cytometery (Joerg *et al*., 2004). In all mammals investigated so far the total length of the X-chromosome-bearing spermatozoa has been found to be in the region of 4 % greater than that of Y-chromosome bearing spermatozoa (Seidel, 2002). 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. Populations can then be frozen, 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. It must be considered however that although sex-related DNA differences have been found in the ninespine stickleback (Ocalewacz *et al.*, 2008a), measurable differences have not been reported in commercially important species such as sea bass and a recent investigation into the chromosome morphology of halibut revealed no clear sex related differences (Ocalewicz *et al.,* 2008b). This said, to our knowledge, semen sexing has never been scientifically tested, or at least reported in teleosts.

The objective of this study was 1) to establish the first UK population of broodstock neomales for the production of monosex (all-female) halibut populations, based on the published protocol by Hendry *et al*. (2003) and 2) to test the feasibility of semen sexing in a variety teleost fish species compared to a mammalian control.

2. Materials and Methods

2.1 Sex reversal experiment

Fish stock and initial rearing conditions

Weaned mixed sex halibut larvae (mean L_F ± SD = 40.12 ± 3.23 mm, mean wet weight ± SD = 0.51 ± 0.11 g) were obtained from a commercial halibut hatchery and transferred to Machrihanish Marine Environmental Research Laboratory (MERL, 55:44^{0}N, 5:44^{0}W) for hormone treatment. Six tanks were prepared, each with 230 halibut. Tanks were part of a seawater flow-through system with water running at at a flow rate of approximately 50 L min^{-1} at ambient temperatures and filtered to 60 µm.

Experimental Conditions

In-feed hormone treatments started on the 16th August 2007 (one day following transfer) for a total of 43 days.

Three treatments were tested in duplicate: 1) 6 weeks steroid free diet (control conditions), 2) 6 weeks MDHT in-feed (5 ppm), 3) 3 weeks MDHT in-Feed (10 ppm) followed by 3 weeks steroid-free diet. Feeders 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 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 steroid contamination. The same process was conducted with the control diets but with steroid free ethanol.

Sampling regime

Five sampling time-points were conducted throughout the 6 week trial period, these included: baseline sample (pre-treatment), 12, 22, 33 and 43 day post treatment onset. At each sampling point, for each tank, water samples (2 x 500 ml) were collected at approximately 12:30 pm and frozen at -20^{0}C, 10 fish were then sacrificed by lethal anaesthesia, weight/length taken and frozen at -20^{0}C. Water and fish samples will later be analysed for MDHT content. In addition, a length measurement of 50 fish and 3 batch weights (20 fish/batch) were taken for

each tank. Halibut were transported back to the commercial fish farm on the 17th of October (3 weeks later) for ongrowing and to await sex determination.

Sex determination

Once halibut had reached a weight of 80 g (6th December 2007), 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. Once it was confirmed that sex could be determined at this stage, a further 60 individuals / treatment were sacrificed and sexed. Thus a total of 80 individuals per treatment were sampled for sex deternination, due to a loss during processing or difficulty in sex identification however, 3-4 samples per treatment could not be assessed (see table 1 for exact numbers of individuals per treatment).

Ongrowing of potential broodstock.

On the 15th of May 2008, 60 control fish (30 / replicate) and 150 5ppm fish (75 / replicate) were tagged with a passive integrated transponder tag (Fish Eagle Co., Lechlade, UK) to be retained until they reach maturity.

Statistical analysis

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 ($p < 0.05$). Growth data (length and weight) were analysed using MINITAB ® version 15.0 (Minitab Ltd., Coventry, UK) statistical software. Initially data were tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test. Growth throughout the hormone treatment period was 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. Growth following the hormone treatment period was compared between treatments (with replicates nested) at each time point using a GLM.

2.2 Semen sexing feasibility study

Sperm Collection and Fixation

Semen samples were collected from cod and sea bass (Machrihanish Marine Environmental Research Laboratory, Machrihanish, UK), halibut (Ardtoe Marine Laboratory, Acharacle, UK) and perch (Niall Bromage Freshwater Research Laboratory, Stirling, UK). Ninespine stickleback testes samples were kindly donated by Konrad Ocalewicz (Poland) and mammalian 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 by pipetting and then stored at 4^0C until ready for 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 peleted cell debris being prepared as above.

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 0.45 µm mesh using 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 for purity and the concentration of sperm confirmed. Volumes of buffers used during the staining preparation of 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, the ratio of sperm suspension:lysing buffer and PI stain remained consistent however.

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, CA, 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 by subjective review of the 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.

3. Results

3.1 Sex reversal experiment

Sex Ratios

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

Growth

Throughout the hormone treatment period, there were no significant differences in length and weight of halibut between tank treatments over time (Figure 1). In the retained potential broodstock growth has been excellent and by September 2009 control fish were found to be significantly larger than the 5 ppm treated fish, 1847 ± 203 g and 1548 ± 127 g (mean ± standard deviation) respectively (Figure 2).

3.2 Semen sexing feasibility study

Total cell fluorescence measurements of bull sperm produced fluorescence frequency distributions with two peaks (Fig 3a) indicating a bimodal distribution of DNA content, and thus confirming 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 3. 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.

4. Discussion

Indirect sex reversal is a standard and accepted method for the production of monosex populations in aquaculture (Pandian & Kirankumar, 2003; Piferrer, 2001; Taranger *et al.*, 2009). 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 a UK neomale population which can be used as future broodstock to generate monosex female populations. Interestingly, the 5 ppm MDHT treatment for 6 weeks was more effective for masculinisation than the higher concentration treatment (10 ppm MDHT) for 3 weeks thus suggesting that a hormonal treatment for a duration of at least three weeks is necessary to masculinise a juvenile population of halibut, this is probably a reflection of the length of time that differentiation takes within a heterogeneous population. In terms of growth, no differences in weight and length were found between treatments during the in-feed hormone period. At the latest growth monitoring time point however (26 months following hormonal treatment), control fish were found to be significantly larger than 5 ppm fish (1847 g compared to 1548 g respectively). It is thought that this is the sexual dimorphism of growth becoming apparent (Imsland & Jonassen, 2005), where the 50% phenotypic females in the control population are growing faster than the males and thus raising the population mean size over the MDHT treated population where at least 97% are pheotypically male. This is further supported by the standard deviation of the mean within these populations, the control group has a high standard deviation (± 203 g) suggesting a mixed sex population whereas the MDHT group has a lower standard deviation (± 127 g) suggesting a more uniform, male population.

Samples of feed, water and halibut were also collected in this study, throughout the period of the hormonal treatment, and these will be analysed for hormone content by high performance liquid chromatography (HPLC) thus building up a picture of hormone leaching. This could help identify the potential impacts that in-feed hormone treatments may have on the water and rearing environment of the halibut.

In conclusion of the sex-reversal of halibut, it has been established that an in-feed treatment of 5 ppm MDHT for 6 weeks is a robust and effective method for masculinising a juvenile population of halibut and producing a potential population of neomales for the future generation of monosex female populations. Once these individuals have reached maturity (expected: March 2010), crosses between normal females and phenotypic males will be performed and compared. Quantification of offspring sex ratios will identify the presence of neomales in the population. Thereafter these neomales can be used in routine juvenile production generating 100% female stocks for ongrowing.

Semen sexing by flow cytometery 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, when tested at this resolution. It is possible that there was not a measureable difference between the spermatozoa DNA content of the commercial fish species tested, for example recent work published during the course of this study which has investigated chromosome morphology of flat fish has suggested that there are no visible differences between sexes (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 detected 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 ranging from cattle to humans to chinchillas which do display sex–related DNA content, the latter demonstrating a difference of up to 7.5 % (Johnson *et al.,* 1987), differences in fish sperm may not be so widely common. 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. Recent 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).

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 by flow cytometry is limited and is not a viable approach for producing monosex populations for the aquaculture industry

Table 1: 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 ratio)

	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	

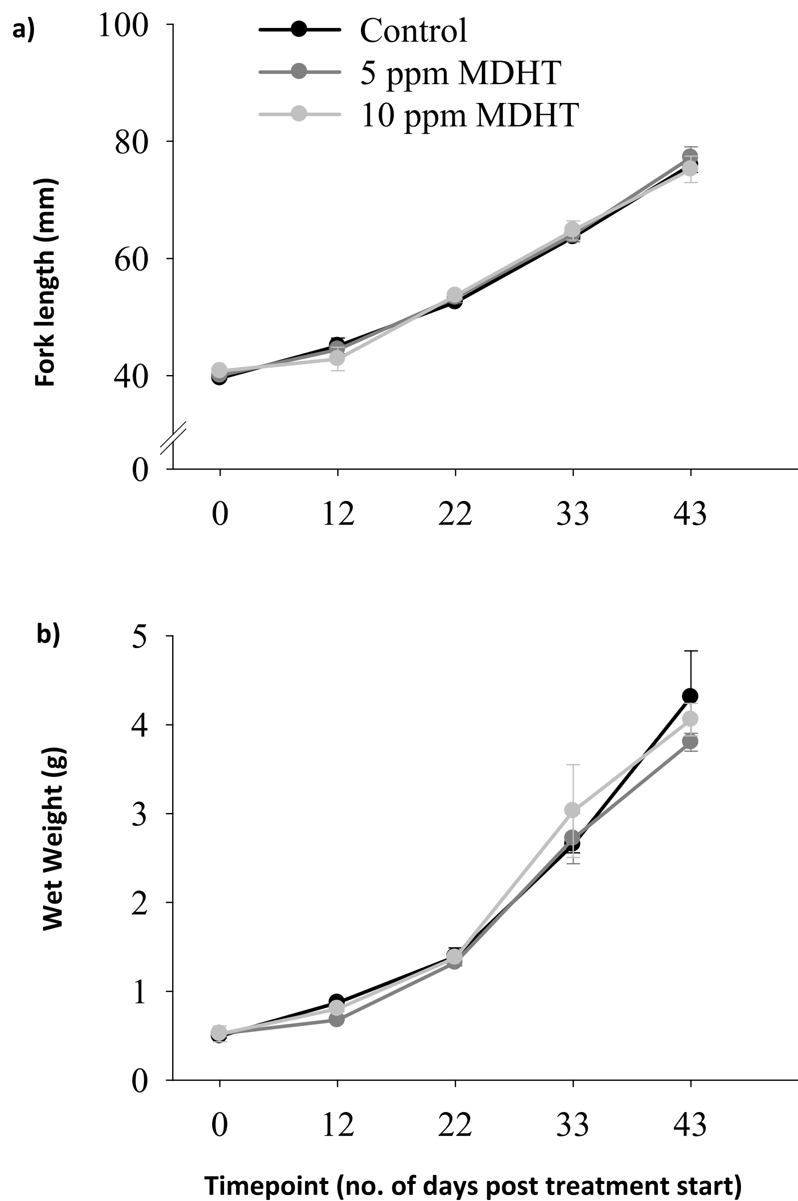


Figure 1: length and weight of halibut throughout the period of hormone treatment. Length data (a) presented as mean ± SD (*n* = 2, 60 individuals / replicate), weight data (b) presented as mean ± SD (*n* = 2, 13 individuals / replicate).

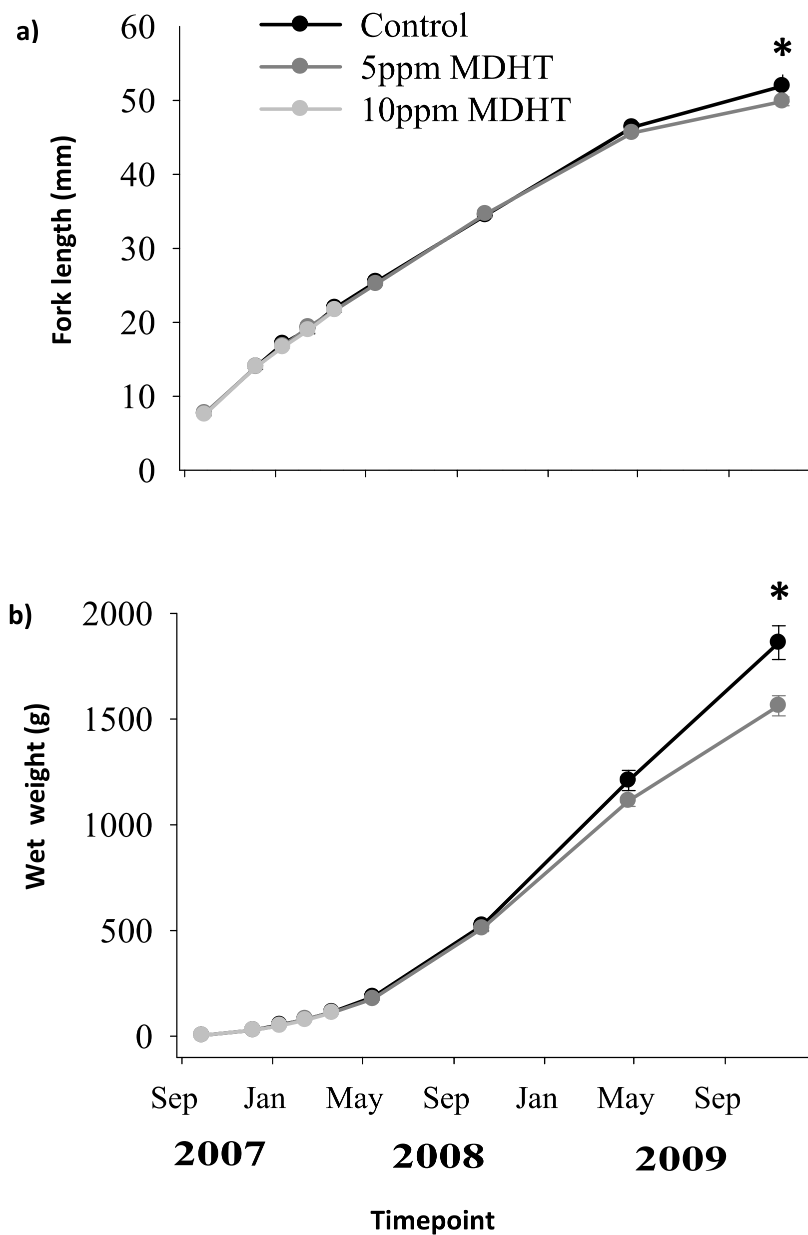


Figure 2: length and weight of halibut following the period of hormone treatment. Length data (a) presented as mean ± SD (*n* = 2, 9-31), weight data (b) presented as mean ± SD (*n* = 2, 9-30). Significant differences between treatments at each timepoint are indicated by *.

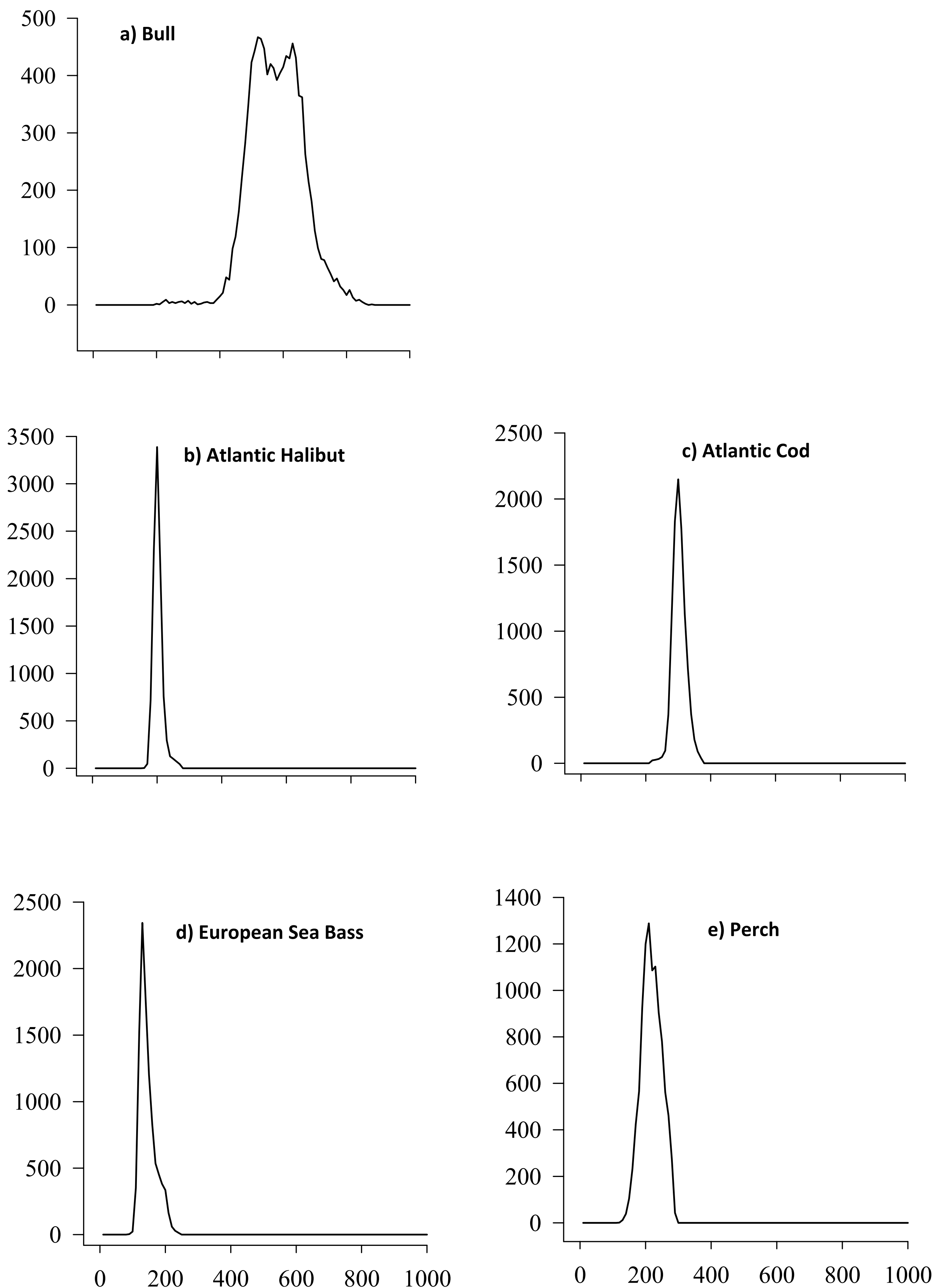


Figure 3. Flow cytometric DNA analysis of mammalian (bull) and a variety of fish species sperm nuclei, stained with propidium iodide. Graphs present a moving average of the number of sperm / fluorescence bin (total cell fluorescence / sperm has been grouped into bins of 10, number of sperm = 10,000).

CHAPTER IV: THE EFFECT OF METAL HALIDE AND NOVEL GREEN CATHODE LIGHTS ON THE STRESS RESPONSE, INNATE IMMUNITY, EYE STRUCTURE AND FEEDING ACTIVITY OF ATLANTIC COD

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*., 2007), Atlantic salmon, *Salmo salar* L. (Endal *et al*., 2000) and European sea bass, *Dicentrarchus* labrax L. (Bayarri *et al*., 2003; Felip *et al.,* 2008) 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 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, 1995). 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). There are a number 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 and Migaud, 2009). 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 ongrowing, an increasing number of light units of escalating power and efficiency are being used in commercial cages as photoperiod 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, no documented studies have so far been performed on welfare of 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

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 ± 1^0C.

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, 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., 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 (≤ 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. 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.

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.

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 Biotech (UK) and a sheep anti-cortisol antibody from Diagnostic Scotland (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™ Glucose Oxidase diagnostic kits (Alphalabs, Hampshire, UK).

Melatonin was analysed by radioimmunoassay according to Porter *et al*. (2000).

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 1 % 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.

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 (H&E).

Retinal measurements were conducted using image analysis software (Image Pro Plus, v. 4.5, Media Cybernectics, Inc. 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. 1). Measurements were conducted on retina from the 2 and 4 week light exposure time-points.

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.

Specific Growth Rate (SGR)

Specific growth rate 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

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

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).

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).

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 days 3.

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.

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).

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*., 2007; 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*., *in press*) 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*., 2007).

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-Cassanova *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^{0}C resulted in cortisol levels of over 50 ng ml^{-1} (Perez-Cassanova *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). Glucose levels also showed large variability. According to Perez-Cassanova *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-Cassanova *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 (Migaud *et al*., 2007) 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.

In terms of retinal morphology, there were no significant differences in 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 results 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.

Table 1: Mean light intensities recorded in tanks (watts m^{-2}). Data presented as mean ± SD ($n = 12$).

Treatment	Photoperiod	Light Intensity
Control	SNP	0.08 ± 0.03
Low CL	LL	0.47 ± 0.18
High CL	LL	0.82 ± 0.15
MH	(LL + SNP)	16.58 ± 8.77

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

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**

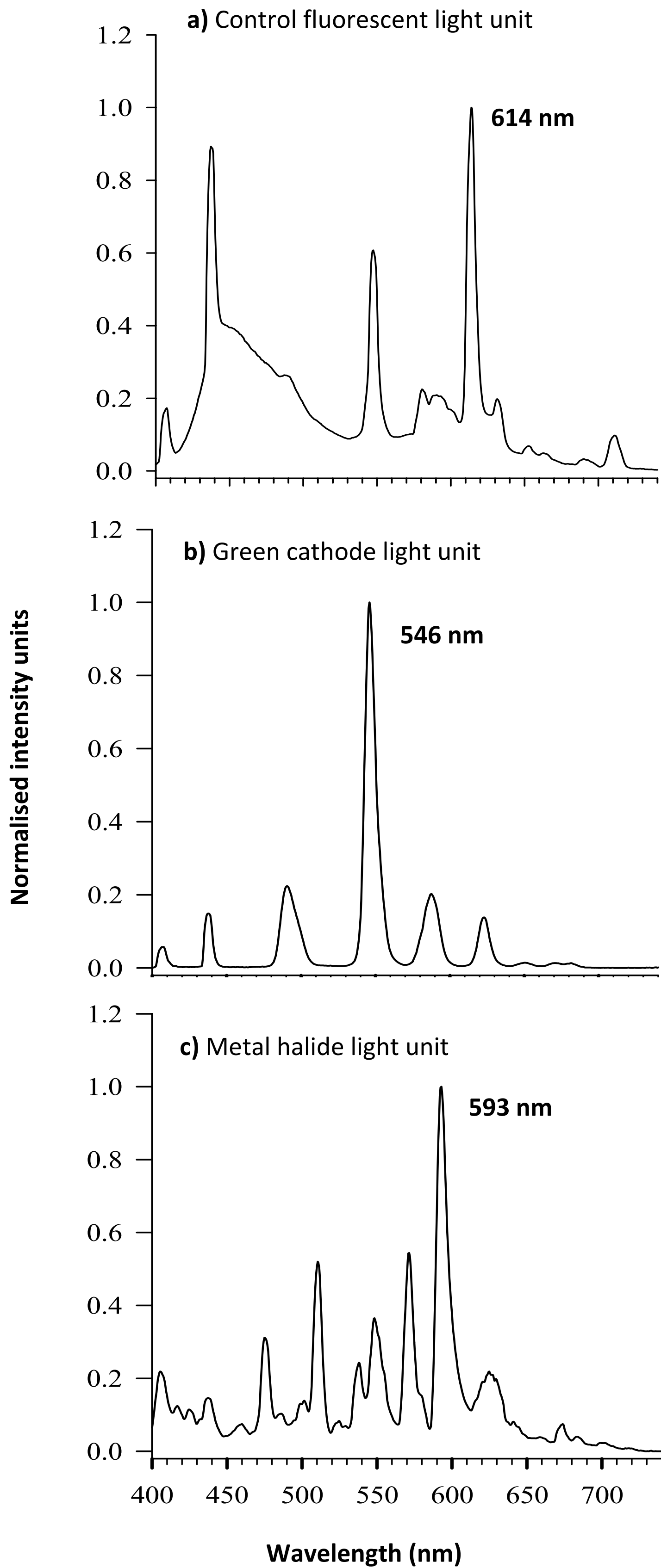


Figure 1. Normalised spectral profiles for a) control fluorescent light, b) cathode light and c) metal halide light units.

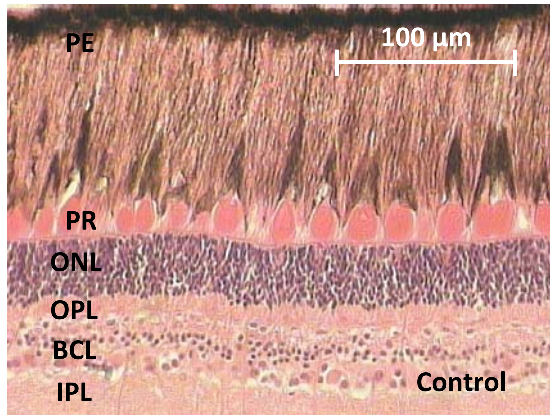


Figure 2. Histological section of Atlantic cod retina kept for 2 weeks under control conditions, illustrating the different layers of the retina (PE, pigment epithelium; PR, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; BCL, bipolar cell layer; IPL, inner plexiform layer).

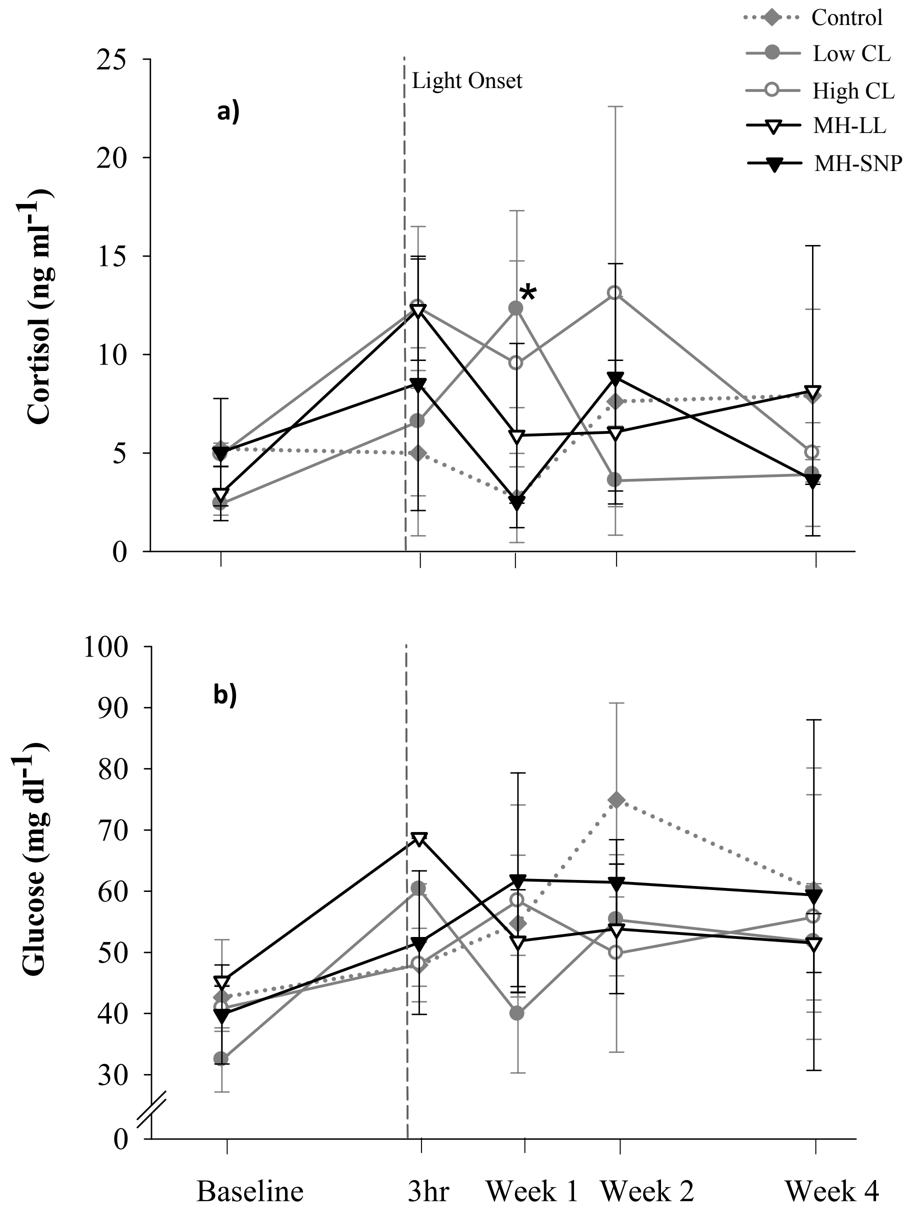


Figure 3. Plasma a) cortisol and b) glucose levels in Atlantic cod kept under control conditions, green cathode light (Low CL and High CL) and metal halide (MH-LL and MH-SNP) light. Data presented as mean ± SD (n = 2, 5 fish/replicate). Significant differences between baseline and post-light onset values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

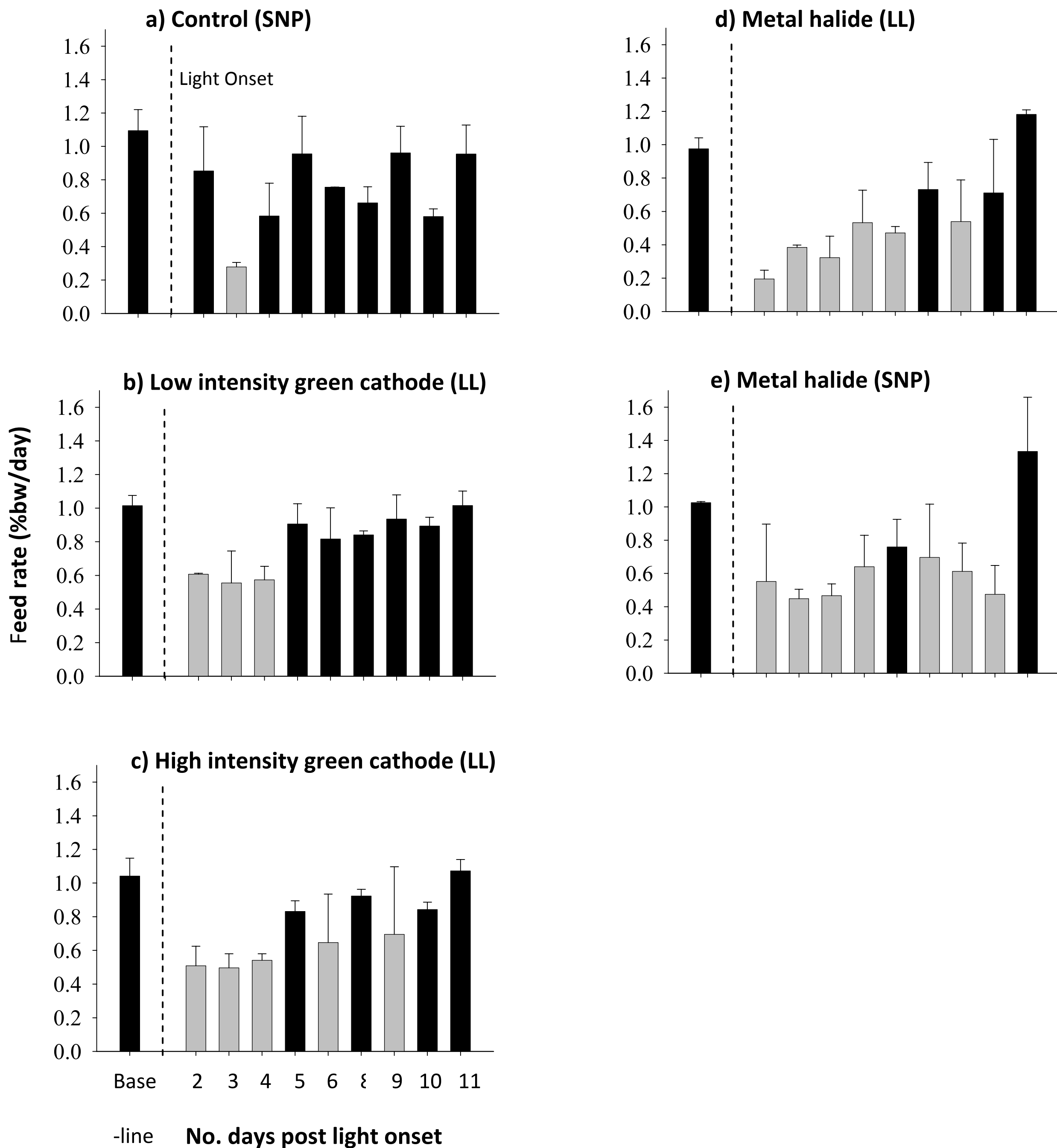


Figure 4. Population feeding behaviour in Atlantic cod kept under different lighting treatments. Data presented as mean feed rate (% body weight/day) per tank (n = 2) ± SD. Dark bars indicate baseline feeding levels, light bars indicate a significant reduction from baseline levels.

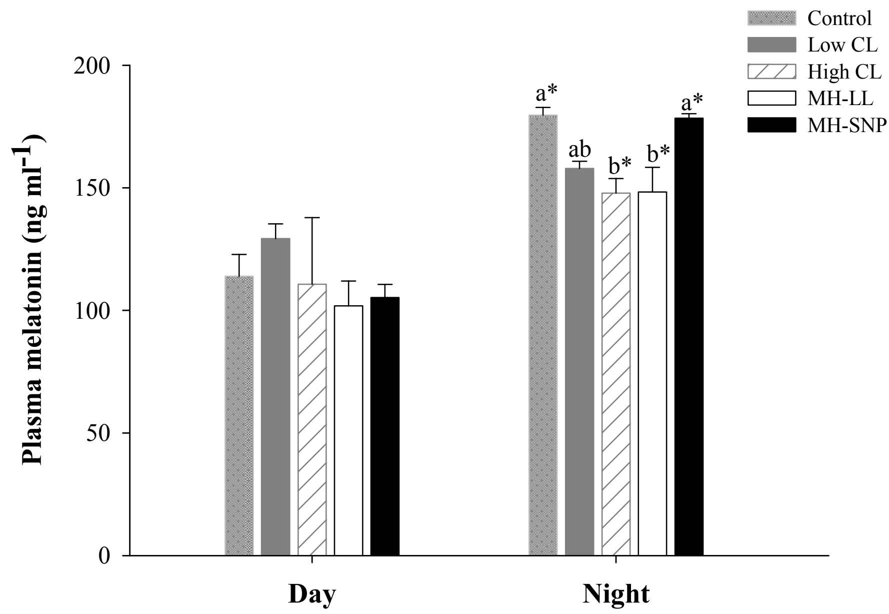


Figure 5. Plasma melatonin levels sampled at day and night in Atlantic cod under control conditions, green cathode light (Low CL and High CL) and metal halide (MH-LL and MH-SNP) light. Data presented as mean ± SD (n = 2, 5 fish/replicate). Significant differences between day-time and night-time values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

CHAPTER V: THE EFFECT OF DAY/NIGHT LIGHT RATIO ON THE MATURATION OF ATLANTIC COD

1. Introduction

Photoperiod manipulation is currently the only commercially viable strategy to manage early maturation during ongrowing of Atlantic cod (Davie *et al.,* 2007). In the open cage setting however the efficacy of the lighting treatments has been brought into question. When the most efficacious light treatment (*i.e.* continuous illumination from summer solstice prior to likely spawning) is applied in an open cage using submersible lighting, maturation is not inhibited as is seen in the enclosed tank environment but rather delayed by only four months at best (Taranger *et al.,* .2006).

The objective of photoperiod manipulation is to subject the cod to constant lighting conditions in which they cannot perceive the difference between day and night light intensities thus the decreasing daylength cue triggering maturation is masked. Recent work looking at the perception of light by the cod pineal gland (*ex vivo)* has demonstrated that cod are 100 times more sensitive to light than the European seabass and 10,000 times more sensitive than Atlantic salmon (Vera et al 2010). Furthermore this same work demonstrated that the definition of what a cod considered to be night time was relative and dependent on the intensity of light perceived during the day. It is highly plausible therefore that cod's acute sensitivity of the relative difference between day and night light intensities enables them to perceive the daylength cue over and above the artificial illumination in the cage environment. This demands further optimisation of the lighting fields to which the cod are exposed and in response, new lighting systems tuned to species sensitivity and optimised for the marine photic environment have been developed however large step changes in the lighting fields are not being achieved. There remains however another opportunity to improve the efficacy of light manipulation by using the cods natural relative perception of day:night ratios. If the day light intensity is concomitantly reduced by shading then the animals theoretical threshold of night will be reduced and thus the artificial lighting should become more potent.

The aim of this study was to investigate the effectiveness of shading (suppression of daylight) in addition to constant lighting for the prevention of maturation in cod reared outdoors.

2. Materials and Methods

Fish Stock and Initial Rearing Conditions

The trial was conducted in Scotland at the Machrihanish Marine Environmental Research Laboratory (MERL, 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 and ambient temperature regimes, were randomly stocked into two indoor and two outdoor tanks (7 m^3). 187 cod were stocked into each of the two indoor tanks and 97 cod were stocked into each of the two outdoor tanks. Within each population, 25 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}. Egg collectors were fitted on all four tanks from 28th January – 28th May 09, collectors were then removed from the indoor tanks but remained on outdoor tanks until 30th June 09.

Experimental Conditions

Fish were initially maintained on a 5 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, 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.

Following acclimation, a baseline sample of 20 fish was taken (24th July), experimental light units and shading were then set-up and fish were assigned to light treatments for 13 months. Four treatments were tested, indoor treatments consisted of: 1) simulated natural photoperiod control (control SNP), 2) constant light control (control LL), outdoor treatments consisted of 3) LL and 40% shade (low shade LL) and 4) LL and 90% shade (high shade LL). Lighting was provided by one green cathode light unit / tank (40 W, Intravision Aqua, Oslo, Norway) and shading was provided by either low density (40 %, LBS Garden Houseware) or higher density (90%, aaask) netting. Tank light intensity measurements and day:night ratios are presented in table one.

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.

Light Scan Measurements

Light intensity measurements were conducted in July 2008. Down-welling intensity measurements (submerged at 1.5 m) were taken at the light source, the tank mid point (1.5 m) and at the tank edge (3 m). 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. Measurements were performed at day and night in all tanks with the day:night relative difference in intensity (table 1) being calculated as follows:

Day / night ratio = 100 – (mean night intensity / mean day intensity)*100

Sampling Procedure

Following baseline sampling and treatment onset in July 08, samples were taken in August and every two months thereafter where all tagged fish were weighed and total length recorded and a further 6 males and 6 females were sacrificed where possible. It was not always possible to accurately select 6 males and 6 females however due to an absence of external sexual morphological features, furthermore towards the end of the trial there were fewer remaining fish due to mortalities and slightly skewed sex ratios resulted in a lack of females under SNP and a lack of males under LL (see Table 2 for exact numbers of fish sacrificed). At each time-point, fish were culled by lethal anaesthesia (MS222, 80ppm, Pharmaq, Fordingbridge, UK). Immediately after death a heparinised syringe was used to withdraw blood from the caudal vein for steroid hormone analyses (testosterone, 17β-estradiol and 11 keto-testosterone). Fish were then measured for whole body weight (± 0.1g) and total length (± 1mm), gonads were dissected and weighed and a representative sample of the tissue was fixed in 10 % neutral buffered formalin. SGR was calculated for fish from the onset of lighting in August 2008 until the following August 2009 according to the equation:

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

Blood was sampled within 5 minutes of netting the fish, stored on ice, centrifuged at 1200 *g* for 15 min and resulting plasma was aliquoted and stored at -70 ^{0}C for later anlaysis. In

conjunction, samples from the indoor tanks were also taken in the intervening months for measurements of the gPR54 / kisspeptin system gene expression (see Chapter VI).

Analysis

Plasma levels of testosterone and 17β-estradiol were measured using a direct radioimmunoassay according to Duston and Bromage (1987). Melatonin was analysed by radioimmunoassay according to Porter *et al*. (2000).

Regarding oocyte diameter, the diameters of 10-30 of the largest oocytes were measured from a random sample of 30-50 oocytes / female (mean of 2 measurements / oocyte) using digital image processing software (Image Pro Plus^{TM}, Media Cybernectics, Silver Spring, Maryland). 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 staged according to Tomkiewicz *et al*. (2003).

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. Weight, GSI and hormone data (sampled every two months) 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. For oocyte diameter and histology parameters, data is presented for consecutive months (obtained from individuals sampled for gene expression data, under SNP and LL). It was not possible to perform measurements of oocyte diameter before September 2008 as oocytes were too small for the software to accurately define.

3. Results

Light Scan Measurements

The low shade outdoor treatment tank had a high day:night relative difference (93.4 %) whereas the high shade treatment tank had a lower day:night relative difference (68.7 %).

Growth performance

Growth was as expected for cod under farmed conditions with all treatments showing comparable growth until spring 2009. Individuals under control SNP showed significantly lower weight in April 2009 than the high shade treatment (females) and the low shade treatment (males), it is thought that this is due to weight loss of individuals following spawning in the SNP treatment (Fig. 1). Females and males showed increasing weight gain throughout the trial period under the control LL, low shade and high shade treatments, with no significant differences found between each. Furthermore, individuals under SNP showed a significantly lower specific growth rate compared to those under the high shade treatment (Table 3).

Gonadal Development

Regarding spawning and egg collection, no eggs were collected under any of the LL treatments whereas under the SNP lighting, eggs were collected from 5th February through to the 11th May.

Females under control SNP showed an increase in GSI in December with a maximum mean value reached in February 2009 (8.2 ± 2.2 %, mean ± SD) (Fig. 2). Males also showed a significant increase in December with highest values reached in February (6.9 ± 2.4 %). Male and female GSI remained low with no significant increases or treatment differences between individuals under control LL, low shade and high shade.

Oocyte diameter of individuals under SNP increased from September 2008 onwards with all individuals having vitellogenic oocytes from November. Oocyte diameter peaked in March 2009 which contained hyaline oocytes (indicating spawning individuals, Fig. 3). There was no clear full commitment to maturation in fish under LL, low shade and high shade treatments. There was a limited proportion of individuals with early vitellogenic oocytes between December and March however thereafter the oocytes reverted back to immature sizes.

The histological examination of ovarian development supported the G1 oocyte diameter analysis by showing a typical cycle of maturation in the SNP treatment with

spawning in February / March time followed by spent gonads observed in April / May (Fig. 4). While there was indications of early stages of ovarian maturation in the other treatments (LL, low shade, high shade), there was also clear evidence of atresia from December onwards which was not evident in the SNP treatment and thus marks the onset of regression in the constant light populations.

Hormone Profiles

Testosterone levels in control SNP and low shade females were significantly higher than those under control LL, high shade treatments in December, in February, control SNP levels were significantly higher (0.9 ± 0.3 ng.ml^{-1}, mean ± SD) than all three treatments, between which there were no differences (Fig. 5 a, b). SNP levels dropped back down in April, following spawning, with no more significant differences found.

In males, testosterone levels were significantly higher in those under control SNP in December and February, with a drop back down in April.

Female estradiol levels were significantly higher in control SNP fish than control LL fish in December and higher than all treatments in February, no significant differences were found between treatments thereafter (Fig. 5 c).

4. Discussion

This study demonstrated that maturation in Atlantic cod can be inhibited outdoors through the use of shade netting in conjunction with constant artificial lighting. Results indicated that relative day:night differences of 69% (high shade treatment) and up to 93% (low shade treatment) effectively inhibited development in the cod and no significant differences between weight, GSI or hormone profiles were seen between these shading treatments and the indoor constant lighting control. Furthermore, oocyte diameter measurements and histological characterisation of oocyte stage indicated that the majority of individuals under shading treatments possessed ovaries which remained at immature stages throughout the period of a normal maturation cycle in cod. Although there was still a large percentage difference between day and night light intensities (69-93 %) outdoors, shading still acted to modify the cage lighting field such that cod were unable to percieve a daylength cue to enter maturation. In industry cages the day:night ratio has been recorded at around 98.8%, this suggests that there could be a threshold cut-off in the region of 93-98.8% at which cod start to perceive

ambient daylength. Thus more studies could be conducted to further define this cut-off threshold.

This trial has confirmed that shading along with constant artificial lighting can effectively inhibit maturation in cod reared outdoors. The next step therefore is the application of purpose-built shading technology in the industry. There are a number of important factors which must be considered however when designing these shading systems. Firstly, systems must meet biological requirements thus the day:night ratio must be reduced below the threshold at which cod can perceive differences, throughout the whole cage. Secondly, systems must meet the physical demands and absorb the physical/environmental impacts subjected to cages in the marine environment. In the past, pilot application has had poor results with nets becoming blown away in storms or such rigid shade netting structures that cages become distorted. Thirdly, systems must be user-friendly so not to impede routine husbandry. Thus although shading could prove to be an effective component in photoperiod manipulation, the engineering of shading systems needs to be improved.

Table 1: Mean light intensities (watts m^{-2}) recorded in indoor and outdoor tanks at day and night. Data presented as mean ± SD (n = 3). The day:night ratio (presented as relative difference, %) in the outdoor tanks is also shown.

	Mean light intensity		**Relative difference (%)**
	Day	Night	
Control SNP	0.54 ± 0.32	0 ± 0	n/a
Control LL	0.54 ± 0.32	0.57 ± 0.38	-5.6
Low Shade LL	23.5 ± 9.56	1.55 ± 1.14	93.4
High shade LL	4.77 ± 1.70	1.49 ± 1.11	68.7

Table 2: Number of females and males sacrificed at each timepoint over the trial period. The number of fish sampled on the intervening months which were included in the oocyte diameter and gonad classification results are also shown (in italics). Months with less than 5 fish are highlighted in grey.

	Control SNP		**Control LL**		**Low Shade LL**		**High Shade LL**	
	Female	Male	Female	Male	Female	Male	Female	Male
Jun	20 fish (7 females, 13 males)							
Aug	**8**	**6**	**6**	**6**	**3**	**7**	**3**	**7**
Sep	*6*	*6*	*7*	*6*				
Oct	**6**	**6**	**10**	**6**	**5**	**5**	**5**	**5**
Nov	*6*	*6*	*6*	*6*				
Dec	**6**	**6**	**6**	**7**	**6**	**4**	**5**	**5**
Jan	*6*	*6*	*6*	*6*				
Feb	**6**	**6**	**10**	**4**	**5**	**5**	**6**	**4**
Mar	*7*	*6*	*6*	*6*				
Apr	**2**	**9**	**7**	**3**	**4**	**6**	**5**	**5**
May	*1*	*8*	*8*	*1*				
Jun	**2**	**8**	**3**	**7**	**2**	**8**	**6**	**4**
Aug	**6**	**6**	**6**	**5**	**11**	**10**	**3**	**8**

Table 3. Specific growth rate (% body weight / day) of tagged Atlantic cod kept under different lighting treatments for one year. Data presented as treatment mean ± SD (n = 11-15). Treatments which exhibit a significant replicate difference are indicated by superscripts.

Treatment	SGR (%)
Control SNP	0.21 ± 0.03^{a}
Control LL	0.27 ± 0.07^{ab}
Low Shade	0.25 ± 0.05^{ab}
High Shade	0.29 ± 0.05^{b}

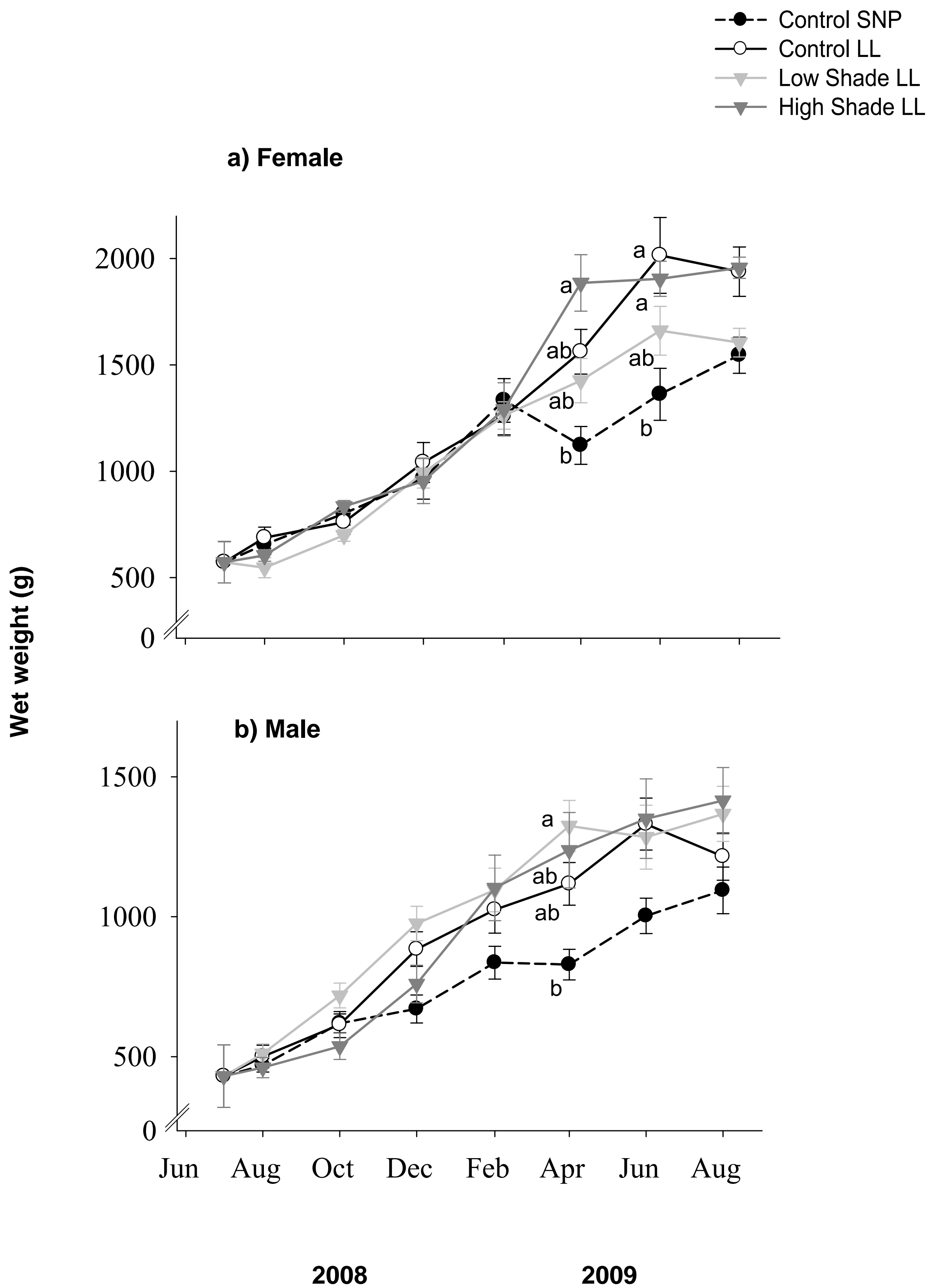


Figure 1. Female (a) and male (b) mean individual wet weight ± SD of the mean (females n = 7-19, males n = 8-17) for sacrificed and tagged individuals maintained under one of four lighting treatments. Differences between treatments at timepoints indicated by superscripts.

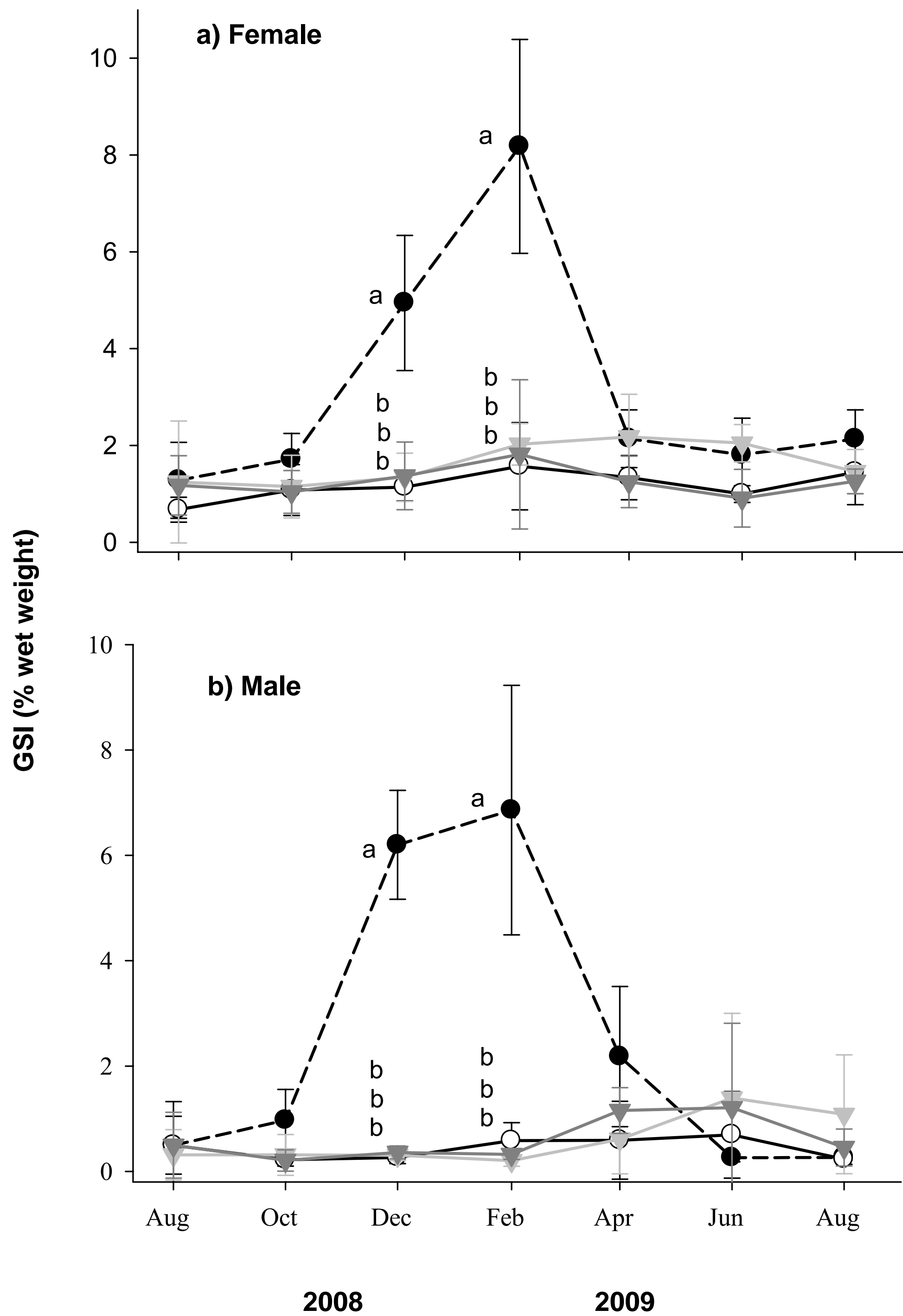


Figure 3. Female (a) and male (b) mean individual GSI ± SD of the mean for individuals (females n = 2-10, males n = 3-9) maintained under one of four lighting treatments. Differences between treatments at timepoints indicated by superscripts.

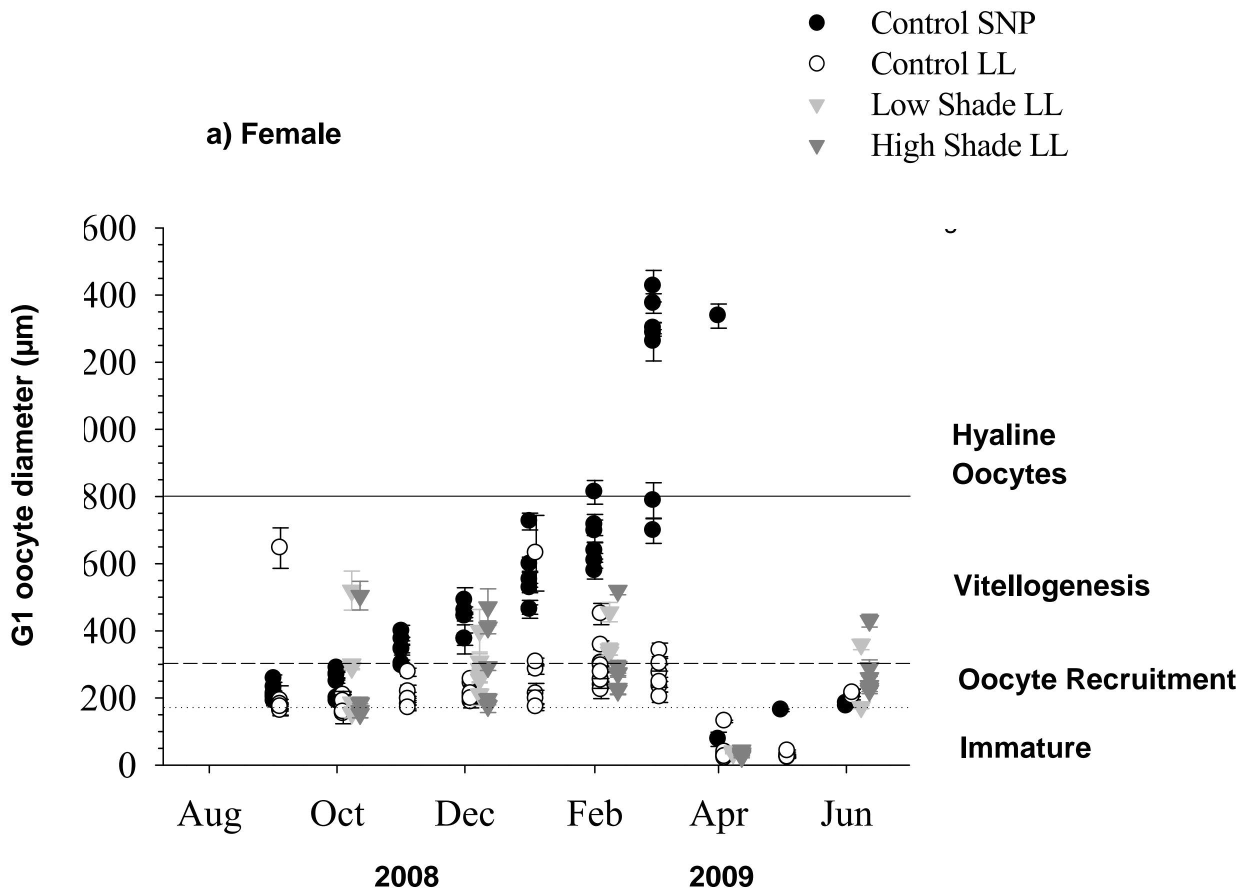
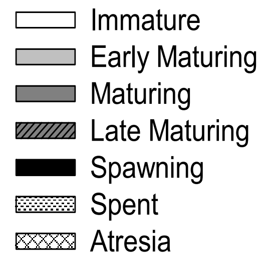
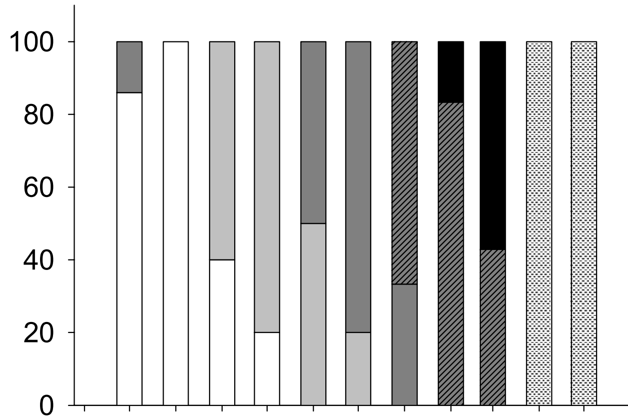
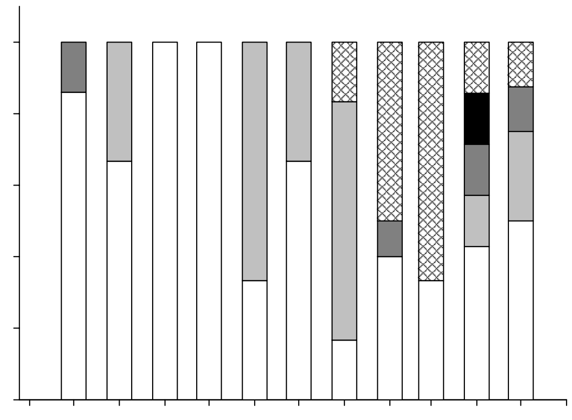
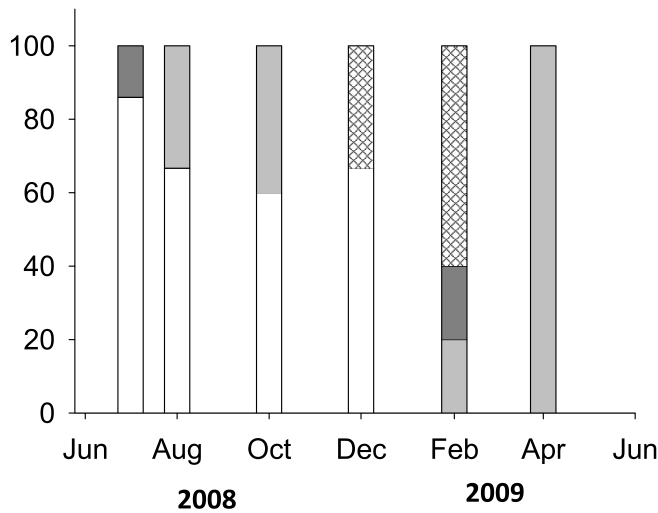


Figure 4. Mean oocyte diameter of leading cohort per individual ± SD (*n* = 10 measurements/individual, 2-10 individuals/treatment).









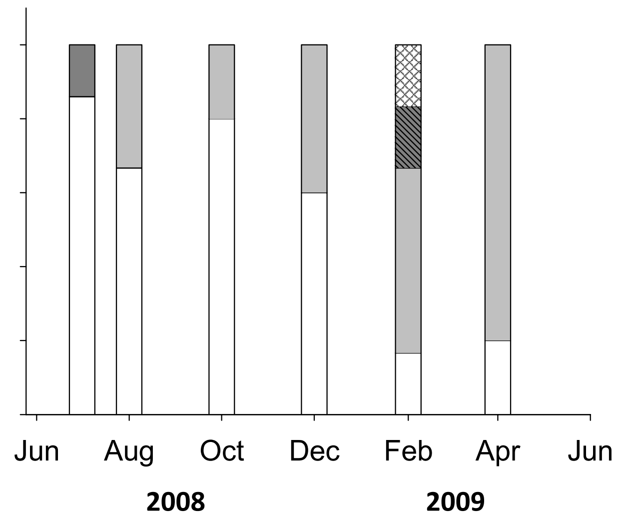


Figure 4. Graphical summary of classification of gonad stage from individuals maintained under all lighting treatments (a-d, *n* = 1 - 8 individuals / bar).

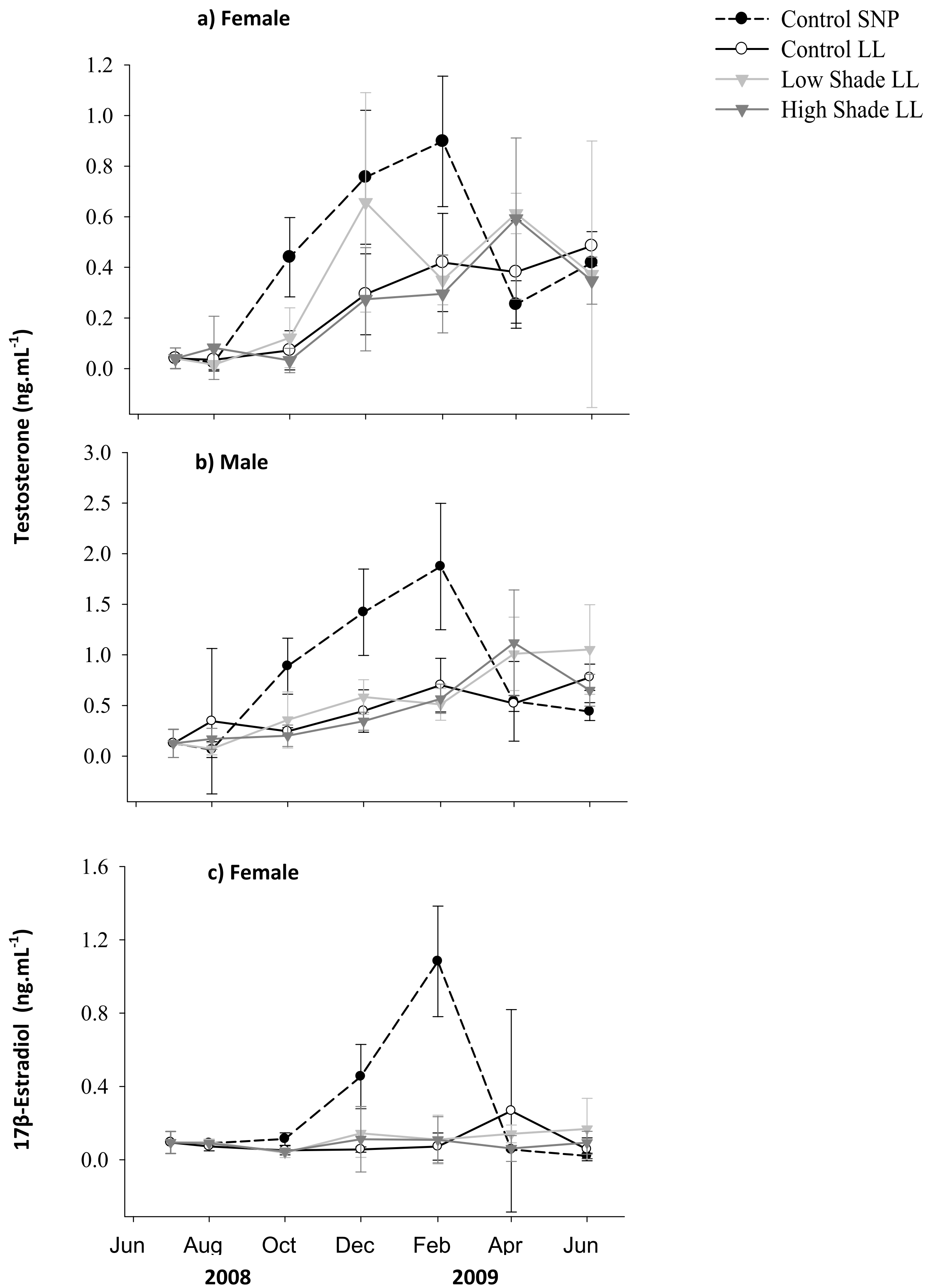


Figure 5. Female and male testosterone (a, b) and female 17β-estradiol (c) levels in fish maintained under different lighting treatments. Significant differences between treatments at each timepoint are indicated by superscripts (n = 6).

CHAPTER VI: THE EXPRESSION OF *KISS1R* AND *KISS2* OVER A MATURATION CYCLE IN ATLANTIC COD

1. Introduction

The kisspeptin system has recently been identified as a potential key regulator in the initiation of puberty. Original work in mammals identified only one signal peptide (Kiss1) and receptor (kiss1r, formally called GPR54) however *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 its receptor (Kiss1ra & Kiss1rb) in fish (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 *et al.* 2008b), shown to have similar GnRH regulatory abilities (Elizur, 2008) as well as being susceptible to sex steroid feedback (Kanda *et al.* 2009). Yet there remains no data describing this signal peptide and its role in the entrainment of reproduction in Atlantic cod. Considering its potential as a regulator of GnRH expression however, which is the start of the BPG cascade, it clearly represents a key landmark signal peptide which should be investigated. This project therefore aimed to develop and validate a qPCR assay for the Atlantic cod homologue of kisspeptin and characterize the expression profile in the months before sexual maturation. Once established such assays will be used to further our understanding of puberty in the species and specifically the environmental entrainment of the system. Such knowledge and tools will be of great relevance to the aquaculture industry as well as fisheries scientists.

2. Materials and Methods

Animals and experimental procedures

The indoor treatment tanks (SNP and LL) set up in Chapter V, provided a maturing and an immature population of fish which were sampled for kisspeptin system expression.

Samples were taken every month (extending from 24th July 08 – 24th August 09) where approximately 6 males and 6 females were sacrificed for every tank, towards the end of the trial there were fewer remaining fish however and slightly skewed sex ratios however (see Tables 1 and 2 for fish numbers). No samples were taken in August 2008 as it was decided to maintain the fish for another month to determine any effects over this extra time. At each time-point, fish were culled by lethal anaesthesia (MS222, 80ppm, Pharmaq, Fordingbridge, UK). Immediately after death a heparinised syringe was used to withdraw blood from the

caudal vein for steroid hormone analyses (testosterone, 17β-estradiol and 11 keto-testosterone). Fish were then measured for whole body weight (± 0.1g) and total length (± 1mm) and gonad weight and a sample of the gonad was fixed in 10 % neutral buffered formalin. Cod whole brains, pituitaries and sections of gonad were dissected and then frozen rapidly over liquid nitrogen vapour before transferring to a -70°C freezer.

RNA extraction and cDNA synthesis

Samples were thawed 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). RNA was cleaned with a DNase treatment (DNA-Free, Ambion, UK) to remove any genomic DNA contamination and then cDNA was synthesized from 1 µg of total RNA using random primers provided by the supplier (High-Capacity cDNA Archive Kit - Applied Biosystems, UK).

Synthesis of Kiss1r and Kiss2 cDNA

Partial cDNA sequences for each target were generated by PCR using 0.5 µM of primers (Table 3), 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, 59°C 20 s, 72°C 1 min where annealing temperature 59°C is listed in table 3. All primer pairs generated a single PCR product that was 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/).

Sequence Analysis

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).

Quantitative PCR

All cDNA for qPCR was synthesised as described previously and qPCR primers (Table 1) were used at 0.5 µM, with one fortieth of the total cDNA synthesis reaction and SYBR-green qPCR mix (ABgene, UK) in a total volume of 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, 59°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 (59) were changed in accordance with the primer pair (Table 3). 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, linearized plasmid containing partial cod cDNA sequences generated as described above. All samples were run in triplicate together with non-template controls, standards, and internal controls to correct expression levels between plates. Normalisation was performed using Acidic ribosomal protein (ARP) as it was previously shown to be the most stable candidate reference gene in Atlantic cod brain cDNA (Olsvik et al. 2008).

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. The effect of time and light treatment on all dependent variables was compared 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.

3. Results

Kiss1r

Following an *in-silico* analysis of teleost *Kiss1r* sequences to identify conserved domains in the sequence structure, we designed primer pair Kiss1rF/R which on testing generated a 556 bp product from cod brain cDNA samples (Table 3). This fragment covers 50% of the target genes coding sequence (cds) within which are four of the receptor's seven trans-membrane domains. This fragment has >80% identity with all other teleost *Kiss1r(a)* sequences (Figure

1) which is the functional isoform of this receptor in teleosts. Comparable sized amplified products were generated when the same primers were tested on Haddock and Halibut cDNA.

Kiss2

Primer pairs *Kiss2aF/R* and *Kiss2bF/R* were designed on an Atlantic cod expressed sequence tag (Accession number: FG321938.1) which together generated a 409bp partial fragment from cod brain cDNA. The fragment consists of a 292bp cds and 117bp 3'untranslated region but importantly the cds contains the decapeptide epitope Kiss-10 sequence that defines the gene. The deduced amino acid (aa) sequence for the epitope is "FNYNPFGLRF" which has 100% identity with Kiss2 epitope in zebrafish, Medaka and goldfish though is one aa different from European seabass and Orange spotted Grouper. Overall the translated nucleotide sequence has greatest identity with European seabasss Kiss2 (60%) and in the order of 50-60% identity with all other teleost *Kiss2* sequences (Figure 2). Comparable sized amplified products were generated when the same primers were tested on Haddock and Halibut cDNA.

Seasonal expression profile of Kiss1r and Kiss2 mRNA in the brains of Atlantic cod held under simulated natural photoperiod in comparison to continuous illumination.

As previously described (Chapter V) cod under SNP exhibited a typical cycle of maturation with spawning in February / March 2009 (Fig. 3). In contrast, maturation in cod maintained under constant light was inhibited. Regarding gene expression, there were no significant elevations in *Kiss1r* expression throughout a natural maturation cycle in female or male cod and there were no differences between individuals maintained under the SNP and those kept under LL which remained immature (Fig. 4). There was however a significant elevation in *Kiss2* expression in SNP females (Fig. 5) immediately before the spawning season (January) and this expression was significantly higher than the mean expression in the LL treated fish (over a 2.5-fold difference, 12302 ± 9715 versus 4395 ± 1552 copy numbers / µg total RNA respectively, mean ± SD). Males did not show such an elevation.

When expression levels were corrected for stage of gonadal development (staging data was only available for females) there was no significant differences in *Kiss1r* expression over the maturation cycle or between SNP and constant lighting treatments (Fig. 6). There was also no clear significant elevation in *Kiss2* expression over the maturation cycle and between lighting treatments.

4. Discussion

There appeared to be no seasonal patterns in *Kiss1r* expression nor was there any difference in expression between sexually mature or immature siblings. However sexually mature female cod did show an elevation in *Kiss2* expression immediately prior to spawning that was not evident in immature fish. A clear relationship between *Kiss2* expression levels and maturation stage was not clear perhaps because when the gonad samples are classified there are no clear cut boundaries between the consecutive categories of development. It is possible that the elevation immediately prior to spawning may be related to final maturation however this is not the presumed principle role for this signal peptide. In support of this potential secondary role Nocilado *et al.* (2007) studied the temporal expression of *Kiss1r* and *GnRH* in the grey mullet, *Mugil cephalus* and reported an increase in GnRH1 in the brain at the advanced stage of puberty (Nocillado *et al*., 2007). An alternative hypothesis is that the elevation may be linked to the onset of maturity in the subsequent year which is more fitting with the genes proposed role.

The hypothesis being tested was that the kisspeptin system could act as a marker for the decision to commit to sexual maturation and thus differential expression patterns would be evident between the SNP and LL treatments prior to the onset of gametogenesis. This has clearly not been the case so alternative regulatory pathways must be investigated. It has recently been reported that in cod maintained under a similar experimental paradigm differential expression of the gonadotropin follicle stimulating hormone (FSH) is evident from September (Almedia *et al*. 2009). This would suggest that between the change in light treatment at the end of June and the FSH surge in September the appropriate GnRH signalling mechanism will have been stimulated by an as yet unidentified signal peptide. It is important to consider however that other subtypes of kisspeptin (*Kiss1*) and the alternative receptor isoform (*Kiss1rb*) may have more potent effects than the genes measured in this study, thus it would be of interest to go on and study the expression of these genes too.

In conclusion, it was hypothesised that the kisspeptin system would play a significant role at the start of puberty however no such role was evident during the maturity ‘decision window’ (July -October) in this study. This suggests that measurement of these genes may therefore not be a useful indicator of the onset of maturation in Atlantic cod.

Table 1: Number of fish sampled at each month over the trial period. Months with less than 5 fish are highlighted in grey.

	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: Number of female fish classed into each maturation category over the duration of the trial, for SNP and LL treatments.

	SNP	**LL**
Immature	12	**41**
Early Maturing	11	**15**
Maturing	9	**3**
Late Maturing	12	**-**
Spawning	4	**1**
Spent	4	**-**
Atresia	**-**	**9**

Table 3: Primer name, sequence, predicted amplicon size, and annealing temperature for the different genes studied.

Name	Sequence	Product size	Annealing temperature
Kiss1rF	5’-TATGAGTGGAGACCGCTGTTACG-3’	556 bp	59°C
Kiss1rR	5’-CTATGGGGTTGACAGAGGAGTTG-3’		
Kiss1rqPCRF	5’-CATCAGCATACGGAGCAAGGTGTC-3’	123 bp	62°C
Kiss1rqPCRR	5’-TTGGGCTGGTACTGGGGATAGAAG-3’		
Kiss2aF	5’-GTTGCGGACCGACCAGTTTCTCAG-3’	319 bp	59°C
Kiss2aR	5’-GACAGGCGGACACGGCTACTTCAT-3’		
Kiss2bF	5’-CTGAGAGGGAACGACGAG-3’	317 bp	59°C
Kiss2bR	5’-CAAGATTGTAAAAGATGGGATAG-3’		
Kiss2qPCRF	5’-TGAGAGGGAACGACGAGCAG-3’	78 bp	59°C
Kiss2qPCRR	5’-GGAGCCCGAACGGATTGTAG-3’		

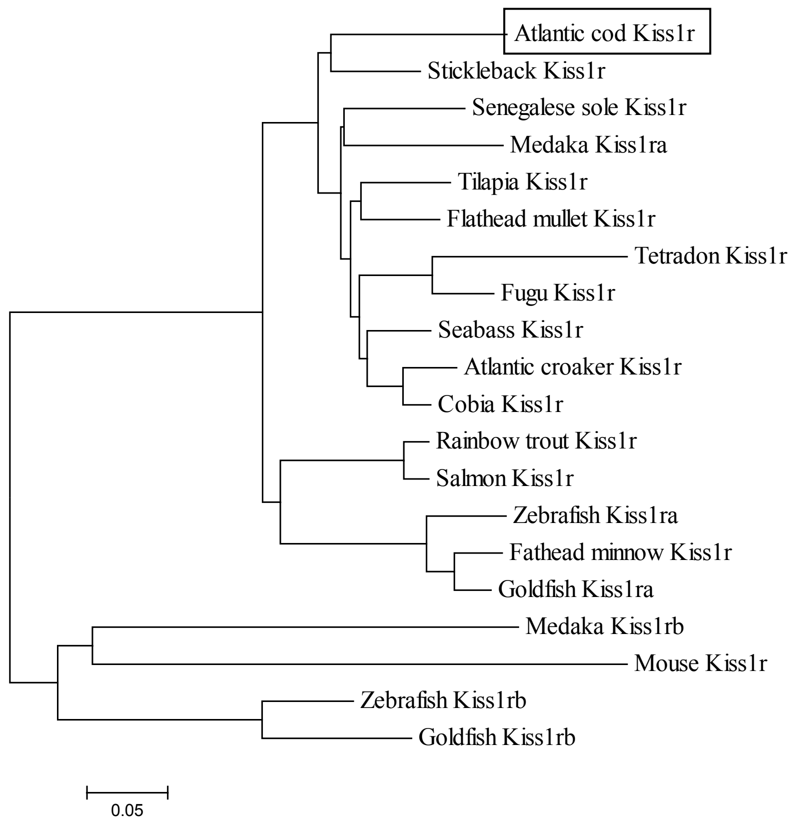


Figure 1: Phylogenetic tree analysis of teleost *Kiss1r* genes. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.

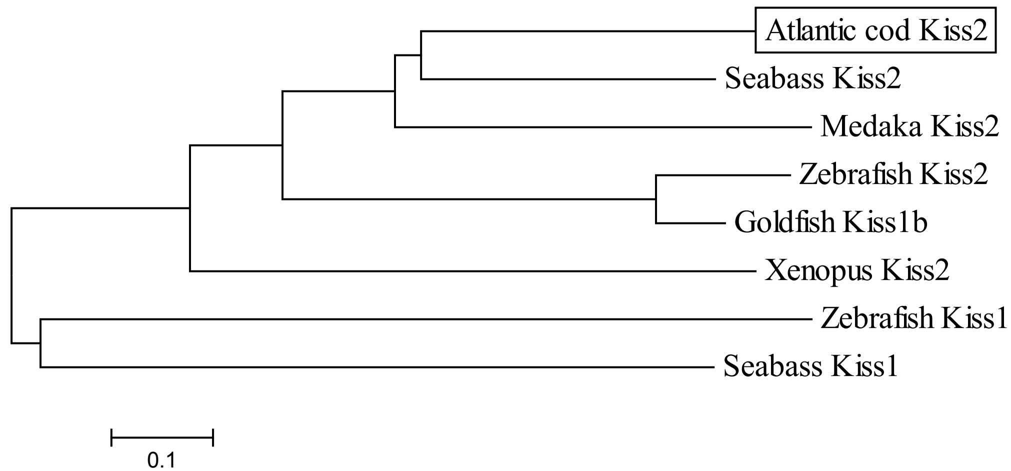


Figure 2: Phylogenetic tree analysis of teleost *Kiss2* genes. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.

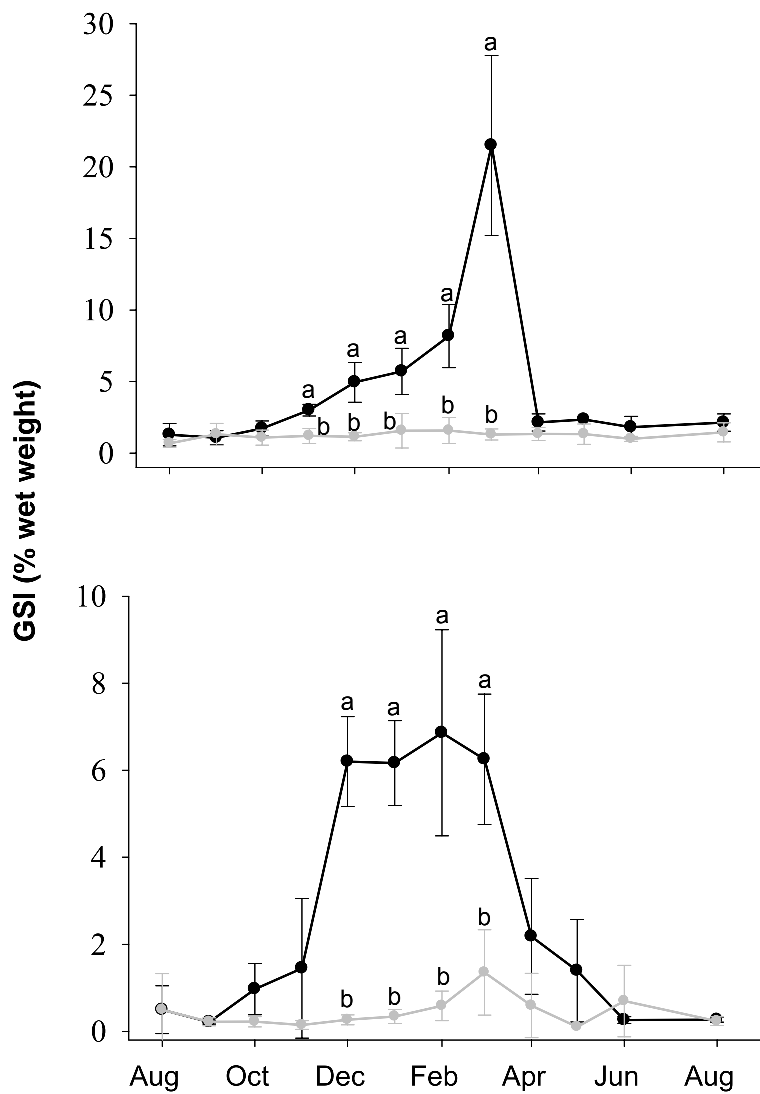


Figure 3. Female (a) and male (b) mean individual GSI ± SD of the mean for individuals (*n* = 6) maintained under SNP and LL. Differences between treatments at timepoints indicated by superscripts.

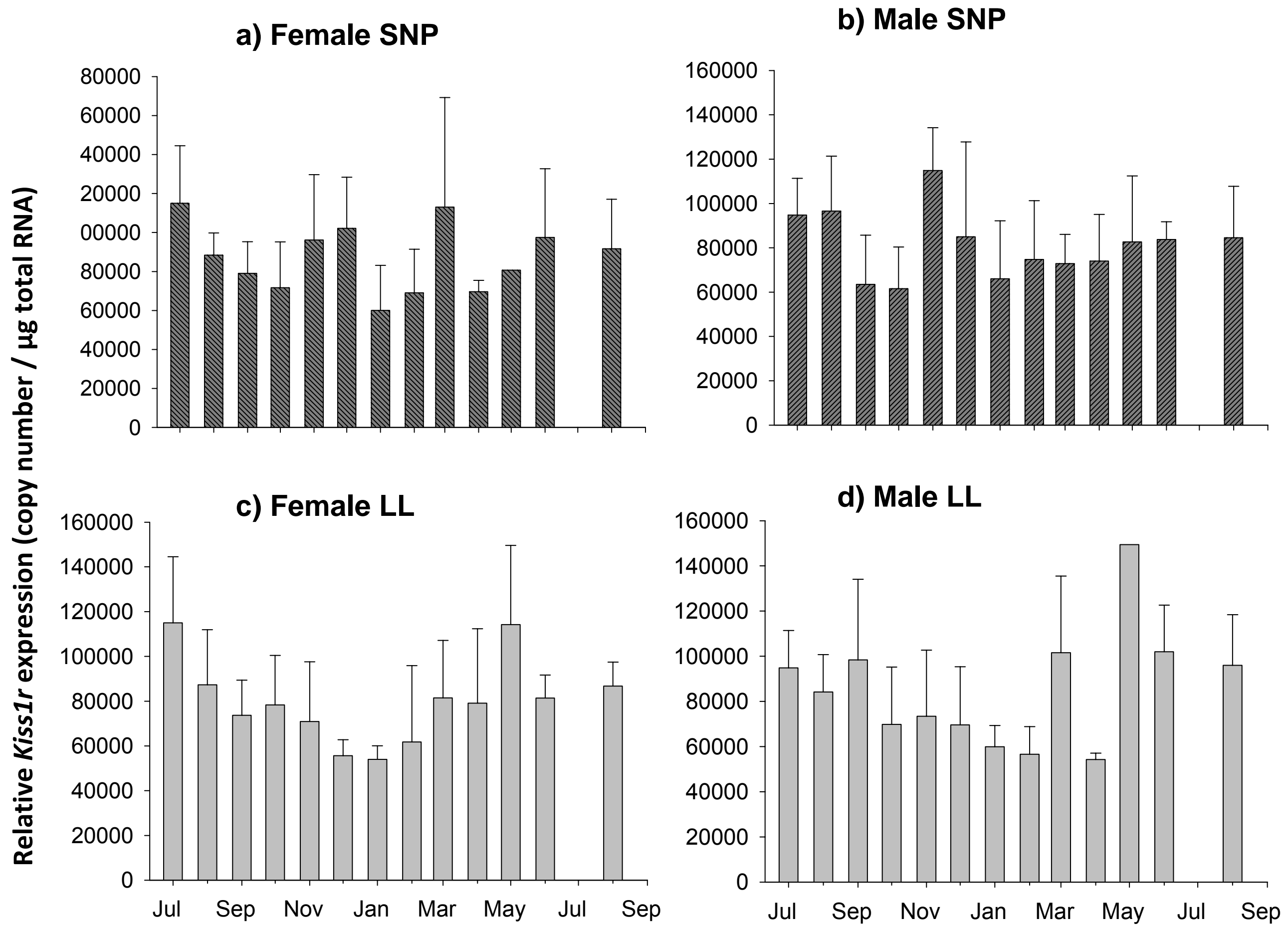


Figure 4. Relative *Kiss1r* expression in the brain of females and males under SNP (a,b) and LL (c,d) lighting treatments. Data presented as mean ± SD (*n* = 1-6). Significant differences in expression between months are indicated by *.

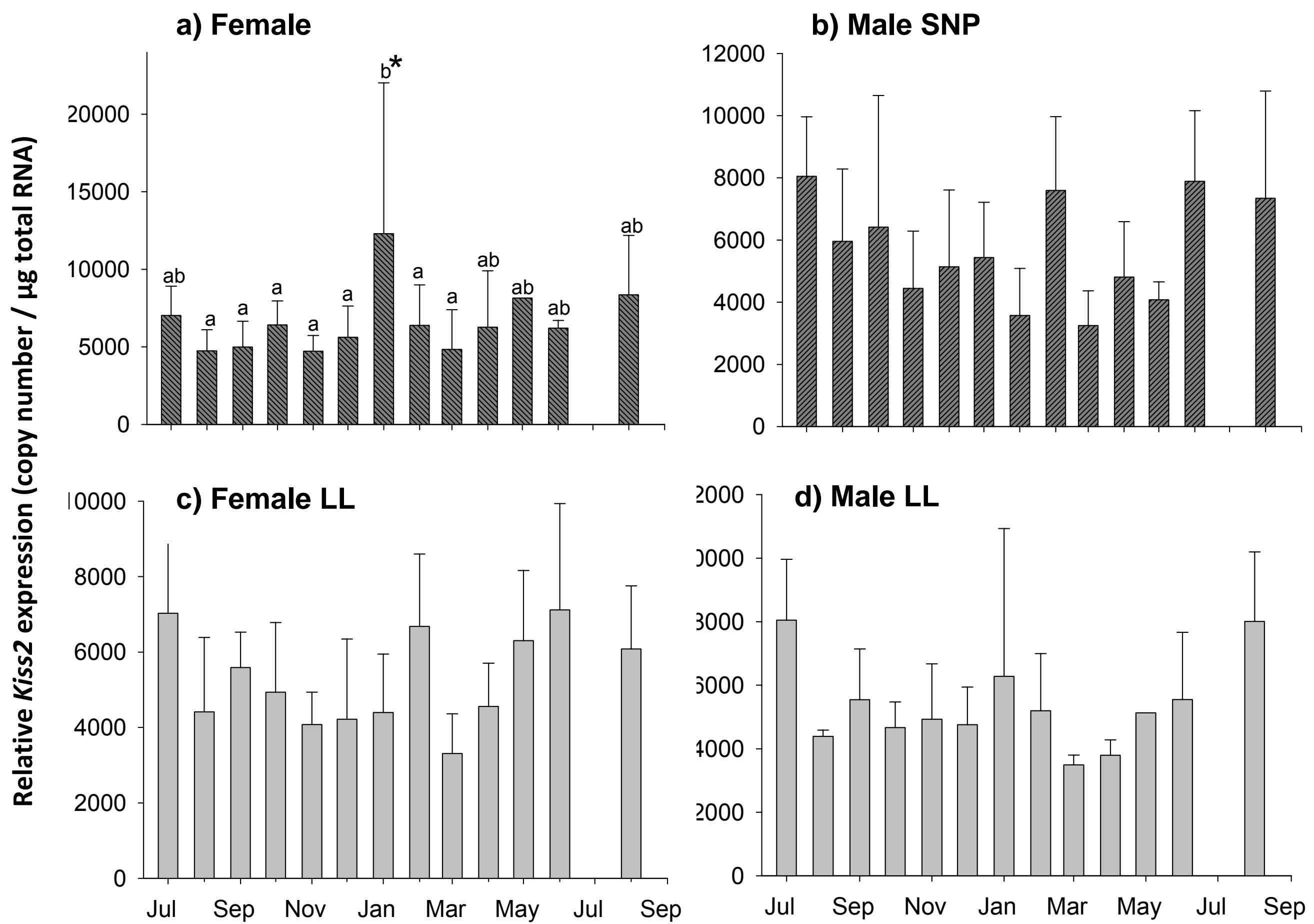


Figure 5. Relative *Kiss2* expression in the brain of females and males under SNP (a,b) and LL (c,d) lighting treatments. Data presented as mean ± SD (n = 1-6). Significant differences in expression between months are indicated by superscripts and differences between treatments (within a month) are indicated by *.

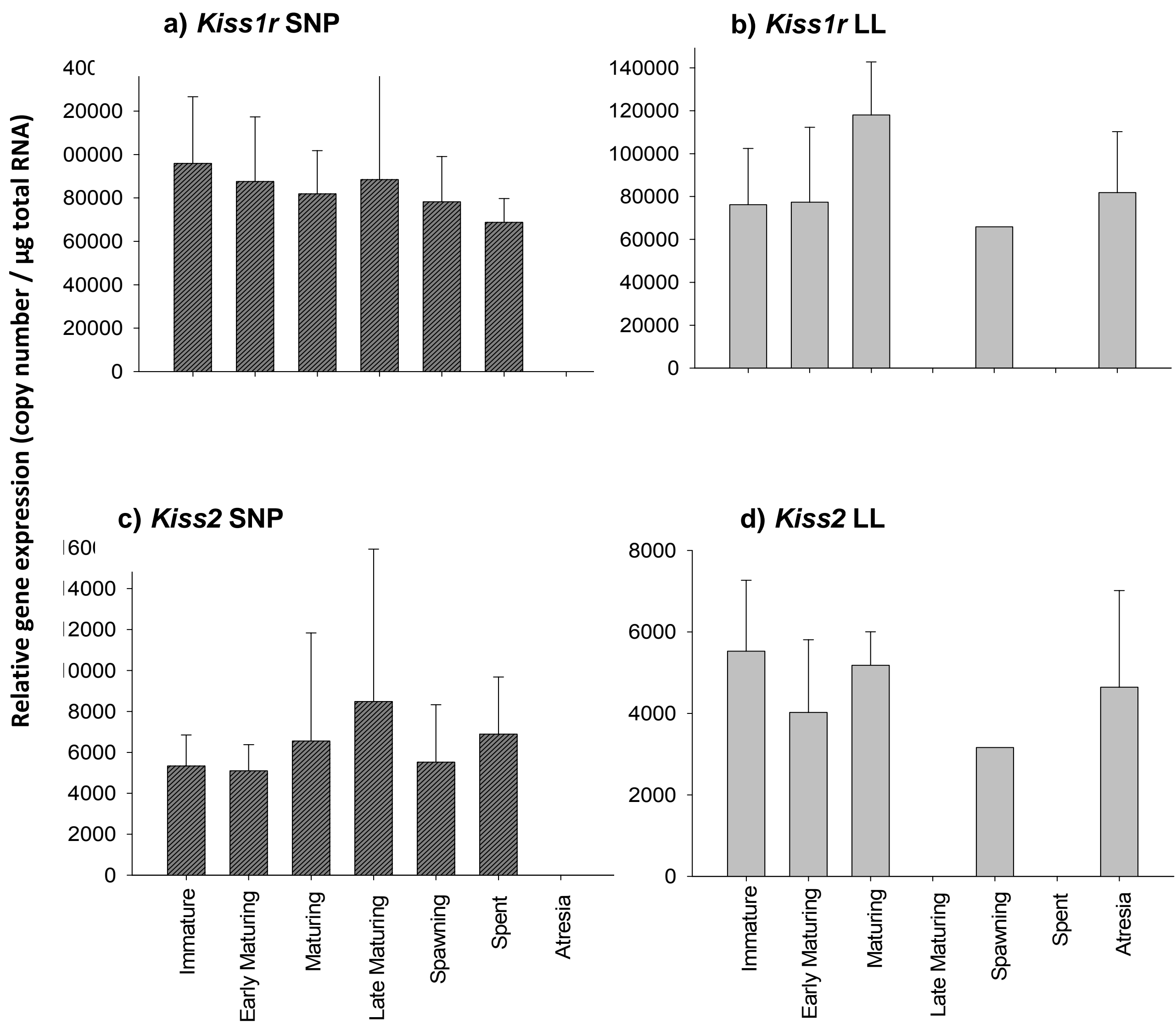


Figure 6. Relative *Kiss1r* (a,b) and *Kiss2* (c,d) expression in the brain of females under SNP and LL lighting treatments, classed into different stages of maturity. Data presented as mean ± SD (*n* = 1-6). Significant differences in expression between maturity stages are indicated by *.

CHAPTER VII: CONCLUSIONS

The British marine aquaculture industry has identified that a delay or cessation of maturation during on-growing is crucial for profitable farming as maturation in marine fish species usually results in a loss of somatic growth rate, reduction in condition, change in flesh composition and up to at least a 25% loss in wet weight in cod for example. To date, there are no protocols that can be reproducibly employed by the industry to overcome this problem in halibut or cod fish farming. As such the management of sexual maturation on farm remains unpredictable and limits profitability. This project was thus designed to provide significant steps forward in the refinement of potential reproduction management strategies and development of new tools to further our understanding of puberty in the two most commercially important marine fish species within the UK, Atlantic halibut and cod.

Firstly the project focused on the development of monosex production in Atlantic halibut, with the attainment of all-female stocks as the primary goal. A population of sex reversed halibut broodstock (neomales) was established, and this was most successful through a 6 week MDHT in-feed treatment at 5 ppm. The neomales will, in the long term, be used as a basis for traditional monosex population generation with the first progeny testing begining in spring 2010. Furthermore, for the first time in fish, the potential for generating monosex populations using a novel semen sexing technique (flow cytometry) proven in terrestrial agriculture was investigated. Results however indicated that in the species tested (Atlantic halibut, cod, sea bass, perch) there was no detectable sexual dimorphism in DNA content of individual sperm which suggests that this technique may not be practical for mono sex production of farmed fish species.

This project also expanded on the work already completed by IOA on photoperiod management of Atlantic cod by looking into the welfare concerns of utilising new lighting technology (e.g. cathode light (CL). A study was set-up 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. 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). These results suggest the lighting at the intensities tested (which reflect those used in commercial culture) have no severe adverse welfare effects on the cod which is promising for the implementation

of such lighting in the industry. 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. Leading on from this, a one year trial was set-up to determine the effectiveness of shading of ambient photoperiod in addition to constant lighting to inhibit maturation of cod reared outdoors. By reducing the relative difference between day / night light intensities (difference presently as high as 98.8 % in commercial cages), it was hypothesised that maturation in cod could be inhibited. Positive results indicated that with shade netting, relative day / night differences of 69% (high shade treatment) and up to 93% (low shade treatment) effectively inhibited sexual development in the cod and no significant differences between weight, GSI or hormone profiles were seen between these shading treatments and the indoor constant lighting control, oocyte diameter measurements and histological characterisation of oocyte stage confirmed this inhibition of maturation.

During the course of this work we have successfully isolated and characterised partial cDNA sequences for two key components of the kisspeptin system (*Kiss1r* and *Kiss2*) which have been previously reported as playing regulator roles in teleost puberty. However an investigation of their expression in relation to the onset of puberty in cod has demonstrated no clear association between this signal peptide and receptor and the recruitment into sexual reproduction. This suggests that other messenger will be responsible in this process which could include the other subtypes of kisspeptin and its receptor (i.e. *Kiss1* and *Kiss1rb*) which should be investigated prior to dismissal of kisspeptin as a key regulator in this species. However in the context of this study, it must be concluded that the measurement of *Kiss1r* and *Kiss2* does not accurately predict the timing of the onset of maturation in Atlantic cod.

Overall, this project has provided valuable information and detailed scientific knowledge for the refinement of maturation management techniques in the aquaculture of Atlantic cod and halibut. As well as establishing the first UK population of neomale halibut for long-term traditional monosex production, it has investigated semen sexing in a number of species and identified that such a technique may not be practical in their respective culture. Progeny testing is now the last step before monosex female population can be produced by the UK halibut industry. This will be done in spring 2010 with confirmed support from SARF. With regards to cod, welfare studies on the effects of high intensity green CL and metal halide lighting reported no severe adverse impacts, furthermore, it was demonstrated that the use of shading could significantly improve the potency of artificial photoperiod manipulation in the cage environment. This could lead to the implementation of new lighting management regimes for cod on growers. In addition new molecular tools were developed to measure the expression of components of the kisspeptin system however no clear role for

either *Kiss2* or *Kiss1r* could be identified during the onset of maturation. These results bring into question the role of the Kisspeptin system in Atlantic cod which conflicts findings in other vertebrates which requires further investigation to be performed.

Chapter VIII: Project Outputs

1. Conferences

2008

- Cowan M., Migaud H., Davie A., & Penman D., 2008. Research and Development of Stock Management Strategies to Optimise Growth Potential in Ongrowing of Marine Fish. Institute of Aquaculture PhD Research Conference, 29th October 2008.

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- Migaud H., Davie A., Cowan M. and Taylor J.F., (2009) Optimisation of on growing of marine finfish- a welfare perspective. Scottish Aquaculture - A Sustainable Future, The Royal Society of Edinburgh and Scottish Agriculture Research Forum, 21st - 22nd April 2009.

- Cowan M., Davie A. and Migaud H., (2009) Effects of shading in conjunction with constant green cathode lighting on sexual maturation of Atlantic cod, *Gadus morhua*. EAS conference, Trondheim Aug. 2009

2010

- Marine Biological association Postgraduate Conference, Glasgow, May (poster)
- Institute of Aquaculture Conference, June (poster)

2. Publications

- Cowan M., Davie A., Migaud H (2010) The effect of metal halide and novel green cathode light on the stress response, innate immunity, eye structure and feeding activity of Atlantic cod, *Gadus morhua* L. *Aquaculture Research In Review*
- (in progress, Aquaculture Research)
- Additional publications are planned

Chapter IX: References

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