Environmental management of Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*): implications of noise, light and substrate.

Rogelio Sierra-Flores

BA (Hons), MSc.

March 2014

UNIVERSITY OF STIRLING

A Thesis submitted for the degree of Doctor of Philosophy

School of Natural Sciences

Institute of Aquaculture

TABLE OF CONTENTS

List of Figures

List of tables

List of abbreviations

11KT	11-ketotesttosterone
3 β-HSD	3 β-hydroxy steroid dehydrogenation
5-HT	Serotonin
A*	asteriscus otolith of lagena
AAAD	aromatic aminoacid decarboxylase
AANAT	arylalkylamine N-acetyltransferase
ac	anterior canal
ACTH	adrenocorticotropic hormone
AgRP	Agouti-related protein
Ang II	angiotensin II
AP	anterior pit
ARA	arachidonic acid
ASP	agouti-signalling protein
AVT	arginine vasotocin
BBS	Bombesin
BPG	brain-pituitary-gonad
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CE	Cerebellum
CF	caudal fin
CI	condition index
CNS	central nervous system
CR	corticosteroid receptor
CRF	corticotropin-releasing factor
CRH	corticotrophin releasing hormone
DA	Dopamine
dB	Decibel
DHA	docosahexaenoic acid
DPH	days post hatch
DW	dry weight
E2	17β.estradiol
ED	eye diameter
EM	Electromagnetic
END	Endorphin
β-END	non-acetylated opioid beta-endorphin
EPA	eicosapentaenoic acid
ET	Ethmoid
FAA	Food anticipatory activity
FCR	Food conversion rate
FE	Food efficiency

FP	Frequency peak
Fq	Frequency
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
GAL	Galanin
GCL	ganglion cell layer
GH	Growth hormone
GLP	Glucagon-like peptide
GnRH	gonadotropin releasing hormone
GPR54	KISS-1 derived receptor
GR	glucocorticoid receptor
GRE	glucocorticoid-responsive element
GRP	gastrin-releasing peptide
GVBD	Germinal Vesicle Break-Down
hc	horizontal canal
HIOMT	hydroxyindole-O-methyltransferase
HSP	heat shock proteins
HYP	Hypothalamus
Hz	Hertz
IGF-I	Insulin-like growth factor
ILM	inner limiting membrane
INL	inner nuclear layer
IO	Infraorbital
IPL	inner plexiform layer
IR	Infrared
ir	immunoreactive
IST	isotocin
J	Joules
K	Condition factor
KISS	kisspeptin
L	lapillus otolith of utricle
L:D	Photoperiod light:dark
LED	Light emitting diode
LH	luteinising hormone
M	medulla
Ma	main trunk line anterior
MCH	melanin-concentrating hormone
MCR	melanocortin receptors
MD	mandibular
MH	Myotome height
Mp	main trunk line posterior
MR	mineralocorticoid receptor
α-MSH	alpha-melanocyte-stimulating hormone
MSH	melanocyte-stimulating hormone

NA	noradrenaline
NFL	nerve fibre layer
NPY	Neuropeptide Y
OLM	outer limiting membrane
ONL	outer nuclear layer
OPL	outer plexiform layer
OR	oral
ORN	olfactory receptor neurons
OT	otic
OX	Orexins
P450c11	deoxycortisol
P450c17	17 α-hydroxylation
P450c21	21-hydroxylation
Pa	Pascal
PACAP	pituitary adenylate cyclase-activating polypeptide
pc	posterior canal
PE	pigmented epithelium
pi	pars intermedia
PO	preopercular
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PRL	photoreceptor layer
PY	peptide Y
PYY	peptide YY
RAS	Recirculating Aquaculture Systems
Rfamides	RF amide peptides
RGB	Red, Green, Blue hue layer composition
RMS	Root mean square
rpd	rostral pars distalis
S	sagitta otolith of saccule
SE or SEM	Standard error or standard error of the mean
SG-II	secretogranin-II
SGR	specific growth rate
SL	Standard length
SO	supraorbital
SPL	Sound Pressure level
ST	supratemporal
T	testosterone
T3	triiodothyronine
T4	thyroxine
TEL	telencephalon
TGC	Thermal growth coefficient
TH	Thyroid hormones
TKs	Tachykininis

TL	Total length
TpOH	tryptophan hydroxylase
TRH	thyrotropin-releasing hormone
TSH	thyrotropin-stimulating hormone
UI	urotensin I
UV	Ultraviolet
V	Volts
VtG	Vitellogenin
W	Watts
Y	peptides Y family receptor
YS	yolk sac

Abstract

During the last decades marine aquaculture has steadily expanded and diversified to include a wider range of commercial species. Despite the intense effort towards understanding the biological requirements of farmed species, several issues remain to be addressed. Mariculture success is restricted by a number of production bottlenecks including limited seed supply, caused mainly through a combination of compromised productivity in broodstock paired with high mortalities during the early life stages. Productivity and survival success is often dependent on the successful recreation of natural environmental conditions. While in a commercial setting a concerted effort is generally made to simulate key environmental stimuli there remains a lack of understanding of the significance of many potential signals.

The overarching aim of this thesis was to investigate the effects of some of the overlooked environmental stimuli on fish performance in enclosed facilities and where possible relate this to the natural setting from which the species have been removed. The studies contained in this text are focused on the effects of anthropogenic noise, light spectral composition and substrate on the performance of broodstock and juvenile development of two valuable commercial marine species Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*).

The aim of Chapter 3 was to test if artificial sound can act as a stressor in Atlantic cod and thereafter to examine if chronic sound disturbances can compromise broodstock spawning performance in land-based facilities. Results showed that anthropogenic noises in a land-based marine farm are within the auditory thresholds of cod and other fish species. Juvenile cod exposed to 10 min of artificial noise (100-1,000

Hz) from 10 to 20 dB 1 re µPa above background sound levels presented a typical acute stress response with a 4 fold elevation of plasma cortisol levels within 20 min, with a return to basal levels after 40 min, while the intensity of the stress response (in terms of amplitude and return to normal levels) appeared to be correlated to the noise level applied. When a similar artificial noise of 35 dB 1 re µPa above background sound level was applied to a broodstock population daily on a random schedule during the spawning season, it significantly impacted on reproductive performances in comparison to a control undisturbed population with notably a reduction in fertilisation rate that correlated with increased egg cortisol contents.

Overall, these studies confirmed, for the first time, that artificial noise mimicking anthropogenic sounds generated in marine land-based facilities trigger a typical acute stress response if a similar sound exposure is then applied in a chronic manner it resulted in reduced broodstock spawning performances. Overall this work provides novel evidence on the potential of anthropogenic noise to act as stressor in fish. The possible implications for both captive and wild stock are discussed.

In chapter 4 the effects of light spectrum and tank background colour on Atlantic cod and turbot larval performance from hatch until the end of metamorphosis were investigated. In both species larvae exposed to shorter wavelengths (blue and green spectrums) showed significantly enhanced growth in terms of standard length, myotome height, eye diameter and condition factor in comparison to larvae exposed to longer wavelengths (red). Larvae performances in the colour background experiment differed between species. Atlantic cod larvae reared in a red tank background displayed the best growth and survival, while larvae in blue tank background had a significant positive effect on final survival rate. In contrast, turbot larvae survival rates were the highest in the red tank background colour with the lowest growth parameters, while larvae in the

blue tank background displayed the best growth. In both species, white tank background colour resulted in the lowest final survival rate.

These results highlight the biological relevance of light spectrum and background colour in marine larvae performance and survival, demonstrating the importance of considering the light composition of the light units used in the hatcheries for larval rearing.

Subsequently in chapter 5 the effects of light spectrum in juvenile turbot growth, appetite, stress response and skin pigmentation were investigated. Two sets of experiments were performed with post-metamorphosed (1 g) and on-growing (100 g) turbot. Results demonstrated that short wavelength treatments had a significant positive effect on growth parameters (total length and wet weight), food intake and feeding response. Light treatments caused a positive correlation between plasma glucose and cortisol levels with significant differences between the short and long wavelength treatments. Skin pigmentation was affected by the light treatments, showing a relationship between wavelength and brightness (negative) and darkness (positive). Blue light treatment resulted in brighter and lighter skin colouration, while red light had the opposite effect: darkening of the skin.

Overall these results confirm that turbot juveniles performance is enhanced by exposing them to a similar photic environment than the one from the natural ecological niche. Light spectrum intervenes in skin pigmentation and the possible mechanisms behind the variations are discussed. In general chapter 5 provides background knowledge of the possible implications of light spectrum in fish juveniles performance and possible commercial applications.

The final two experimental chapters turned focus back on the optimisation of broodstock environmental management and subsequent effects on their productivity. In

Chapter 6 the importance of crepuscular light simulation was investigated in Atlantic cod broodstock spawning performance. No significant impact could be observed in terms of egg production and quality in association with dawn/dusk simulation compared to abrupt lights on/off. This suggests, at least for Atlantic cod, that crepuscular light simulation is not a key factor affecting spawning performance during the spawning window. The possible implications of twilight on gamete quality prior ovulation are discussed. In Chapter 7 the effect of a "breeding nest" containing a substrate (i.e. sand) in turbot broodstock spawning performance was investigated. Behavioural observation recorded active occupancy of the nests with the suggestion of social structuring as specific individuals (females) occupied the nest preferentially. However no fertilised, naturally released eggs were collected from the overflow during the spawning seasons. This would suggest that the presence of a nest is not enough to induce natural spawning behaviour in turbot in itself however the elective occupancy suggests that nests and/or their substrate was a physical enrichment that was valued by the fish which should be explored further.

Overall the studies contained in this thesis highlight further the importance of considering noise and light as crucial environmental factors in marine aquaculture. Results from the different chapters offer a possible application within the enclosed facilities that might contribute to the success of the industry. Present findings contribute towards the understanding of the effects of environmental signals in fish and provide further insight to guide further lines of research on the involvement of light spectrum on fish physiology.

Acknowledgments

First of all I want to thank the National Council of Science and Technology of the Mexican Federal government (CONACyT) for the full funding of my studies in Scotland, without which this would have not been possible. I also want to thank the EU FP7 PROSPAWN project for funding the sound and light equipment required for this study. To LarvaNet for the opportunity of being part of the first LARVITA training course, which not only provided me with a deep update of the fish larvae knowledge but also allowed me to meet fantastic people.

To my supervisors, Hervé and Andrew, there are no words to thank your guidance through these turbulent times. Thank you for your advice, no matter the situation (i.e. distance, photoperiod, temperature, rainfall or endocrinal status) I always got at least one word of advice. Thank you for taking this work as your own, without your advice this thesis wouldn't exist and would be filled with (more) "mexicanisms".

For the people at Ardtoe I can only be grateful for all those years, in which you were the closest to a family, I do not have enough space to express all what I wish to say. Tim, I will always be thankful for the good and bad times, for trusting my judgment and letting me be part of it. Jim, thank you for all those laughs and words of encouragement you always had for me. Roman, my mate, thanks for all your help with the technical issues of my experiments and all those nights in the shellfish unit full of advice. Jon, you will never realise how thankful I am to you for teaching me how to keep larvae alive. Hazel, RossAmy, Kathryn, Anne, Mark, Yolanda, Miguel thanks for the long talks and help during my experiments.

Stef for being a friend, an advisor, a teacher and sometimes an extremely strict supervisor, I will never be able to thank you enough. Jul, for teaching me the wonders of fish husbandry and how to survive at the end of the world. Eric, for always being there, for your technical advice and nights out. Nicole, my lab and concert partner. Bridie, the sheep were never the same since you left the caravan. Nico, my life advisor. John for your advice and keeping the lab organised, despite our mistakes. Amy, Rania, Mairi, Elsbeth, Hazel, Antonis, Andy, Benedikt, Lucas, Gus, Sean, Cartsen, Carina, Polyana for your help, advice and company; you made this journey a pleasant one.

Silvia, Su, Gilta, Christian source of endless distractions that allowed me to survive this process, thanks for your advice, company and unconditional friendship. Pierre, for surviving and making more pleasant the last stages of the writing with me. Ben for initiating me in the most rewarding addiction a human can have, without my violin I would be lost. Lislie por esas peleas, discusiones, cenas.

A mi familia, en especial a mis padres y abuelos por su apoyo incondicional, por ser un gran ejemplo de vida, por lo que soy y lo que no soy. Si su apoyo, jamás habría llegado hasta aquí.

A.M.D.G.

Chapter 1

General Introduction

Marine aquaculture includes a diverse range of species however; ultimately the underlying methodologies, management practices employed as well as production bottlenecks have a great similarity. Key production challenges are to ensure the supply of quality juveniles and optimise the grow-out phase, addressing nutritional and environmental requirements at different stages of development and disease control through sustainable practices (Moksness *et al*., 2004). Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) are two marine species for which an extensive body of research already exits ranging from broodstock performance through larval development and juvenile growth and performance (Person-Le Ruyet *et al.*, 2002; Rosenlund and Halldórsson, 2007). Despite the significant advances achieved larvae performance remains a major constrain due to low survivals, high incidence of deformities and malpigmentation. While in a commercial context a concerted effort is generally made to simulate key environmental stimuli there remains a lack of understanding of the significance of many potential signals. The overarching aim of this thesis was to investigate the significance of some of the overlooked environmental stimuli on fish performance in enclosed facilities and where possible relate this to the natural setting from which the species originated.

1.1. OVERVIEW OF SPECIES ECOLOGY AND COMMERCIAL EXPLOITATION

1.1.1. Atlantic cod (*Gadus morhua*)

Ecology

The Atlantic cod (*Gadus morhua*, Linnaeus 1758) is an omnivorous marine fish species of the *Gadidae* family distributed across the north Atlantic Ocean (Fig. 1.1), where it is exposed to a wide range of salinities (10 to 35 ppt) and temperatures (-2° to

20°C) (Rosenlund and Halldorson, 2007). During feeding and spawning cod can be found in pelagic areas (up to 600 m), although generally Atlantic cod is considered a demersal species (150 to 200 m depth) and its optimal temperature range is from 0 to 12 °C (Rosenlund and Halldorson, 2007). Cod populations are active swimmers forming schools during the day and dispersing during the night. Although some populations are relatively stationary, it is predominantly a migratory species looking for food and adequate spawning grounds at relative long distances.

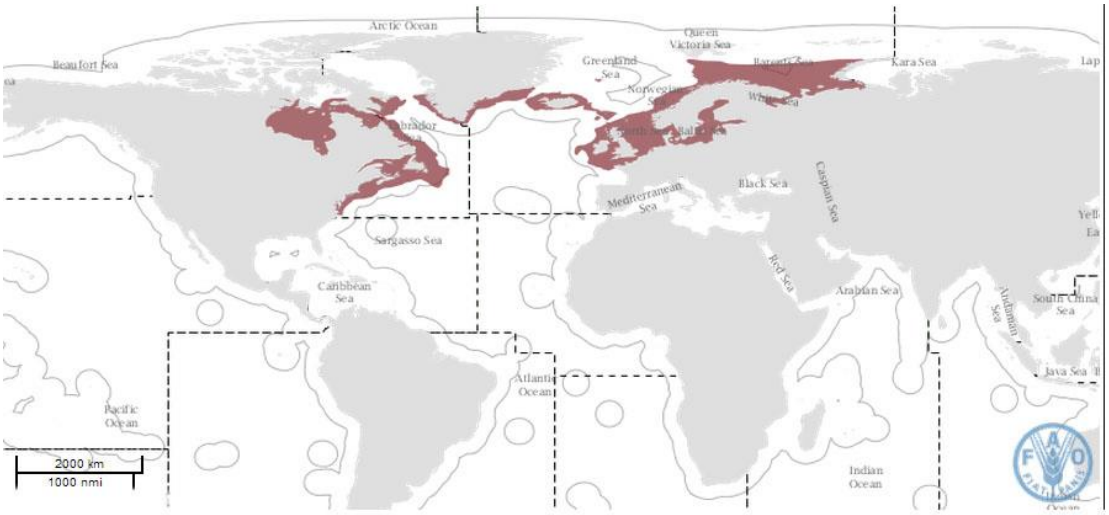

Figure 1.1. Approximate geographical distribution of Atlantic cod (*Gadus morhua*) according to FAO database (2013).

Reproduction

Atlantic cod is a batch spawner species reproducing naturally during spring, although active reproduction has been reported from November until June in some wild populations (Brander, 2005). Sexual maturity is reached at a size of ~40 cm long (2-4 years old) for the North sea populations, while northern populations are reported to take as long as 8 years (Berg and Albert, 2003). Gametes are freely released into the water column and no parental care occurs after spawning. Females have a high fecundity of 500,000 eggs per Kg of weight (Cohen *et al.*, 1990). Successful reproduction involves a complex behaviour consisting of swimming patterns and acoustic signals (Engen and

Folstad, 1999). Prior to spawning dominant males establish a territory through aggressive behaviour towards other members of the population. Courtship has been described by Engen and Folstad (1999) and it starts by the females entering the spawning grounds and settling at the bottom. Satellite males approach a single female presenting a lateral view of the flaunting display with the median fins fully erected. Those fast approaches are usually accompanied by male vocalisations that stimulate the female and prevent subordinate males from approaching. "Grunts" are believed to be the most relevant sounds produced during courtship (see section 2.1.3.). The female will then follow the selected male upwards matching their swim speeds. Males perform a dorsal mount followed by a ventral mount, releasing gametes simultaneously. If synchronisation fails the female returns to the bottom and the courtship is reinitiated. Opportunistic males surround the engaged couple disturbing the swim patterns and releasing sperm (Cohen *et al.*, 1990; Hutchings *et al.*, 1999).

Life cycle

Cod eggs are buoyant, spherical and transparent with an average diameter of 1.4 mm. Development is temperature dependant with hatching occurring between 80 to 100 °days. Cod larvae are dependent on the yolk sac reserves which are fully absorbed at 4.5 mm after which exogenous feeding is mainly based on zooplankton (Brown *et al.*, 2003). Invertebrates such as crustaceans and polychaete worms are included in the diet and within 4-5 months smaller fish become a regular part of their diet. At this stage survival rates are very low due to climatic conditions and high cannibalism (Howell, 1984; Lear, 1984; Miller and Kendall, 2009). Approximately a year after hatch cod move to deeper waters to join adult populations, where under adequate conditions (nutritional status and environmental conditions) juveniles are recruited into sexual maturation (Norberg *et al.*, 2004).

Fisheries

Atlantic cod has been one of the most valued commercial fish since the beginning of fisheries in the North seas. Cod has been an important commodity since the Vikings time that rapidly propagated to the rest of Europe in its dried and salted form. Popularity and supply increased with the colonisation of North America and the improvement of fisheries technology. Wild cod catches peaked at almost 3 million tonnes in 1968 (FAO; 2013) and then a steady decline has been registered in the fisheries (Fig. 1.2). A first major decline (~30%) occurred in the early 1970s, reaching a dramatic point that led to the total closure of the Canadian Grand Bank fisheries in 1992 (MacKenzie, 1995). Overfishing is allegedly the main reason for the wild population decline (Cook *et al.*, 1997), although temperature reduction might have contributed to the variability in recruitment, growth, mortality and ecosystem instability (prey and predators) (Lilly, 2008). Currently Atlantic cod is classified as a vulnerable species by OSPAR (2013) and FAO (2013) classified the wild populations as fully exploited to depleted.

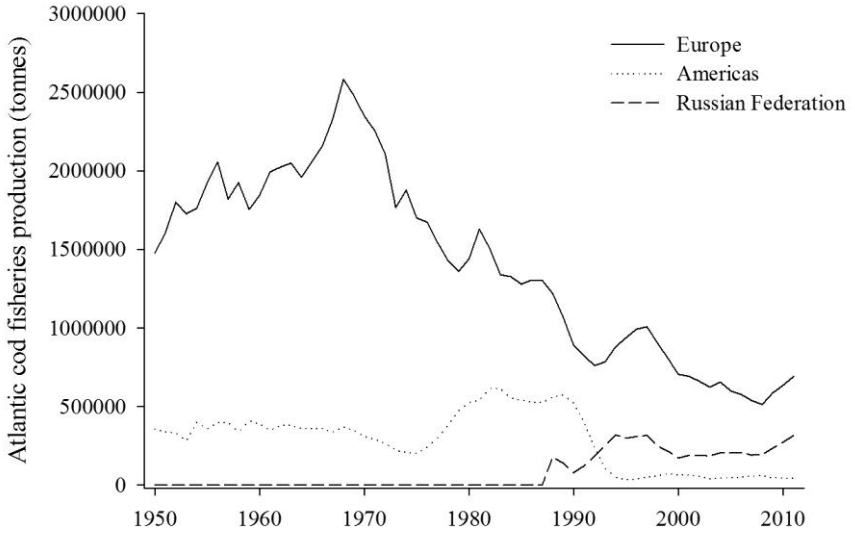

Figure 1.2. World *Gadus morhua* fisheries catches (tonnes) from 1950 until 2011 according to FAO (2013) data. Data shows total catches by region: Europe, Americas and Russia federation.

Aquaculture

The decline of fisheries and increasing commercial value made cod an excellent candidate species for Aquaculture production. First cod farming attempts were done in the late 19th century mainly as "ranching", were seed was obtained from the sea. Cod aquaculture boom from 1987 primarily pushed by Canada, Norway, Iceland and the United Kingdom (FAO; 2013). Nowadays successful production in enclosed facilities is possible; however it is still not financially viable due to the bottle neck encountered during the early stages of development (low survival, low growth rates) and ongrowing (early sexual maturation in captivity). Low survival rates have been correlated to suboptimal transition to exogenous feeds stuff during weaning. In 2010 the world cod aquaculture production (Fig. 1.3) reached a peak with almost 23,000 tonnes produced, only 13% of the targeted production according to Rosenlund and Skretting (2004). By that year the Norwegian production accounted for 94 % of the world total and there were clear signs that the cod aquaculture industry had collapsed everywhere else (Canada, Iceland, UK).

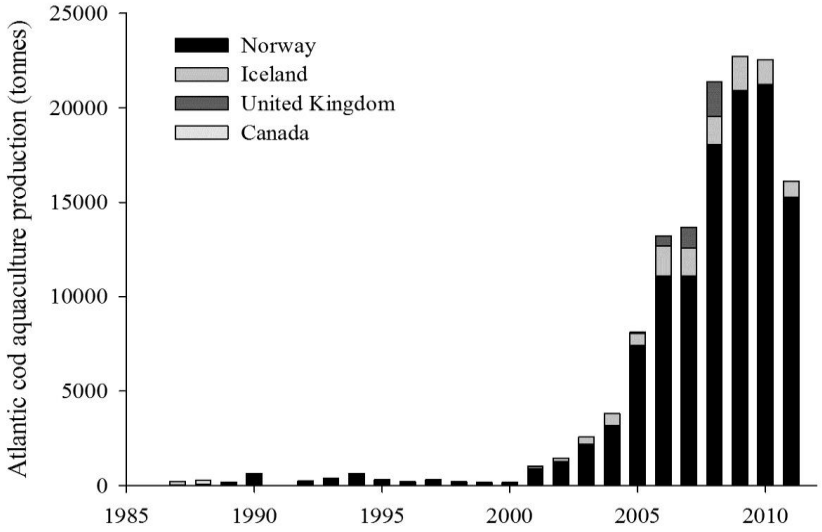

Figure 1.3. World aquaculture production of Atlantic cod from 1985 to 2011 according to FAO (2013) by major producer countries: Norway, Iceland, United Kingdom and Canada.

1.1.2. Turbot (*Scophthalamus maximus)*

Ecology

Turbot (*Scophthalamus maximus* Linnaeus, 1758; also referred as *Psetta maxima* Linnaeus 1758: taxa classification no longer valid according to the World Register of Marine Species, 2013) is a marine demersal omnivourous fish species of the order *Pleuronectiformes*, distributed across European waters from the Arctic sea to the Mediterranean and Black Sea (Fig. 1.4) (FAO, 2013). The species was introduced to Chile, New Zealand and China for commercial proposes with unknown ecological effects (Person-Le Ruyete *et al*., 2002). Turbot is very tolerant to salinity ranging from full strength sea water (34 ppt) to brackish waters (FAO, 2013). It inhabits the sea bottom on sandy, rocky or mixed soils at depths of 20 to 70 m (Støttrup and Sparrevohn, 2010). Optimum temperature ranges from 13 to 20 °C with lethal levels below 2 and above 30 °C (Burel *et al*., 1996). Theoretically turbot larvae growth is biologically arrested below 5.3 °C (Weltzien *et al.*, 1999), however some reports suggest that it might occur from 7 °C (Knudsen, 1983) and 100 % mortality was reported at 6 °C during incubation (Kuhlmann and Quantz, 1980). Low temperatures (together with salinity) reduce the overall metabolic rate: restricting growth, food efficiency but also reducing the metabolic energy cost (Imsland *et al.*, 2001). Fluctuating temperatures of 9 to 19 °C in North Europe constrict turbot's growth potential by 60 % when compared to the 14 to 18 °C Spanish range (Person Le-Ruyet *et al*., 2002). However, Burel *et al.* (1996) found that despite a significant difference in final growth (length and weight) between temperatures (8 to 22 °C), growth rates (daily percentage) and food efficiency did not differ. Final growth was correlated to feed intake/ appetite, which in turn was temperature dependant. Appetite was markedly decreased below 14 °C.

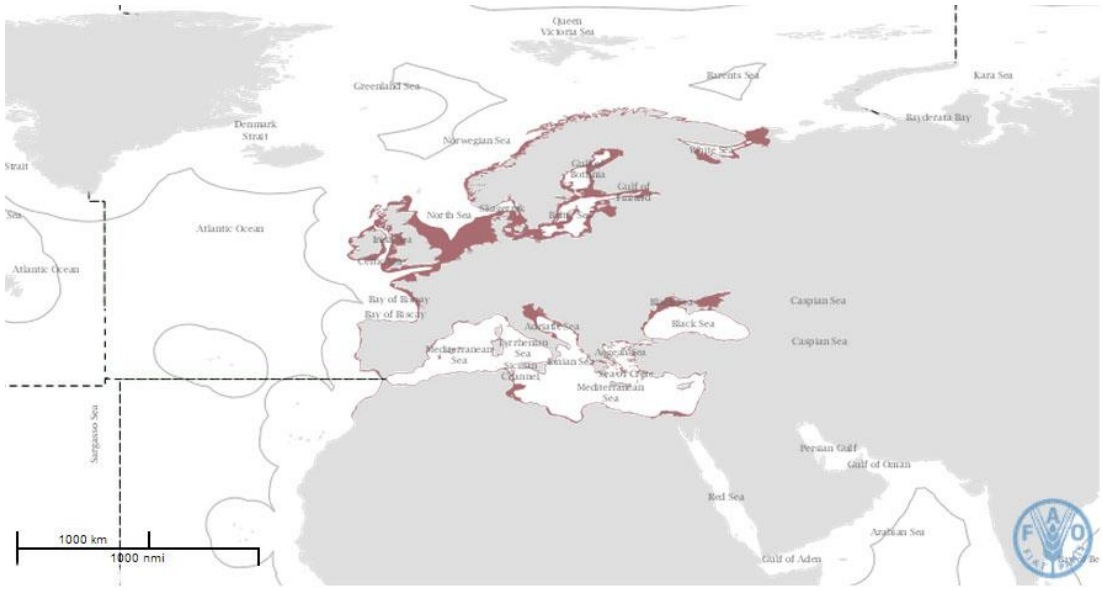

Figure 1.4. Approximate geographical distribution of turbot (*Scophthalamus maximus*) map from FAO (2013).

Reproduction

Turbot spawn between February and April for the Mediterranean stocks; and from May to July in the Atlantic sea. Turbot is a batch spawner with ovulation intervals of 2 to 4 days with an average duration of a month. There is no register of the reproductive behaviour of turbot in the wild. In captivity fish must be hand-striped to produce embryos. Stripping time determines the egg/larvae quality due to the over ripening process; eggs need to be stripped and fertilised whithin 10 h of their ovulation process (McEvoy, 1984). Synchronisation between artificial egg collection and ovulation is crucial to obtain high quality eggs and larvae (McEvoy, 1984; Howell and Scott, 1989) which can be achieved under constant temperature and lighting control. Female relative fecundity is 100,000 to 600,000 eggs per Kg of fish weight (Jones *et al.*, 1974; Aydin and Sahin, 2011) while males produce poor sperm (i.e. low motility and viscosity) compared to other teleost (Chereguini *et al.*, 1999).

Life cycle

Eggs are pelagic and spherical with a diameter of 1.1 mm and an oil globe of 0.2 mm. Incubation optimal temperature is around 13° to 15°C (Jones, 1972). Newly hatched larvae (~100° days) are symmetric measuring 2.2 to 2.8 mm length with a large yolk sac retaining the oil globule, resulting in the turbot larvae floating upside-down up to 12 hrs after hatch (Al-Maghazachi and Gibson, 1984). Turbot larvae undergo a metamorphosis (Fig. 1.5) subject to genetic control and regulated by environmental conditions (temperature, food availability and nutritional value, water quality). Active first feeding starts when the larvae are 3.1 – 3.4 mm long when the mouth and anus open (3-5 days). Between 3.5 to 4.5 mm length: the gut starts developing, swim bladder inflates and head spines appear (8-10 days). The next developmental stage (4.8 – 5.6 mm length) is characterised by the appearance of the ventral fins and a straight notochord (2-3 days). The most evident morphological changes start when larvae are more than 10 mm length. At this stage the larvae becomes asymmetrical (caudal, anal and dorsal fins) and the right eye starts migrating towards the head, the notochord bends below 45 ° (10 -15 days). Finally the spines from the lower jaw and the swim bladder are absorbed, the right eye is visible from the ocular side and the blind side loses pigmentation. Metamorphosis is considered as accomplished when the larvae reaches their adult morphology: upper eye positioned away from the margin of the head; head spines completely absorbed; swim bladder completely absorbed; dorsal fins extends to a level above the front end of the upper eye (Al-Maghazachi and Gibson, 1984).

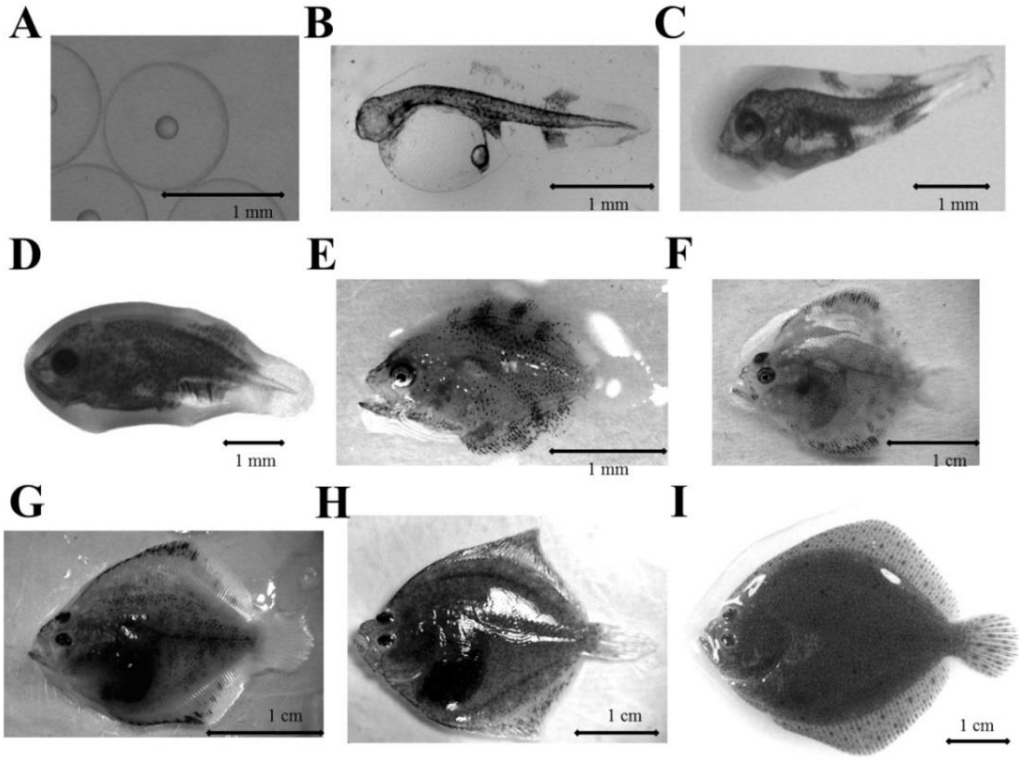

Figure 1.5. Images of turbot development from chapter 4 reared at 15°C, white light, photoperiod 24:00 L:D. Images are at the same scale: Fig A-E are shown to 1 mm, Fig. F-I scale is to 1 cm. A) turbot egg from 12th July 2011: shell diameter 1.06 mm, oil globule diameter 0.18 mm; turbot larvae B) At hatch SL 2.7 mm, oil globule diameter 0.16 mm, yolk sac diameter 0.36 mm; C) 7 DPH SL 3.5 mm; D) 14 DPH SL 4.7 mm; E) 21 DPH SL 6.6 mm; F) 35 DPH SL 18.2 mm; G) 42 DPH SL 24.2 mm; H) 56 DPH SL 35.5 mm; I) Fully metamorphosed larvae from chapter 5 ~70 DPH SL 52.2 mm.

Fisheries

Turbot is a highly valuable commercial fish, mainly in the European market and more recently in China. Turbot fisheries have remained relatively steady except in the 1980s when a dramatic decline occurred. Wild captures are mainly sourced in Europe; however catches are relatively low with a peak volume of less than 10,000 tonnes in the 1970s (Fig. 1.6).

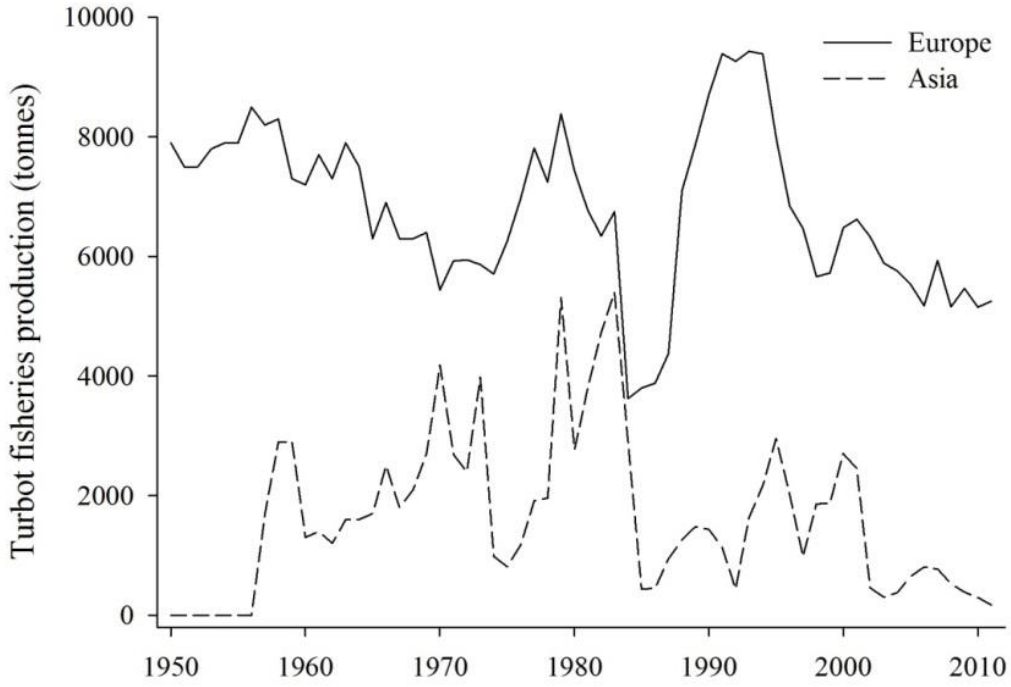

Figure 1.6. World turbot fisheries catches (tonnes) from 1950 until 2011 according to FAO (2013) data. Data shows total catches by region: Europe and Asia.

Aquaculture

Turbot farming started successfully in France and the United Kingdom in the late 1970s. Since then France has established a steady turbot industry with rather modest growth rates. By 2002 Spain and France were the top world producers of farmed turbot (Fig. 1.7). However, in 2003 China increased its turbot aquaculture production to reach over 85 % of the global volume according to FAO estimations (2013). Production levels are possible due to the relative high resistance and adaptability of turbot to temperature and salinity.

Despite the apparent success in turbot farming, there are still many problems. First the need for artificially released gametes can impact on the quality of the eggs and it is a labour intensive process that raises production costs. Beside the economical aspect, turbot aquaculture faces problems with deformities mainly in the lower jaw and

uncompleted eye migration. Malpigmentation and albinism appear to occur more frequently in aquaculture; however it is possible that the incidence is comparable to the wild (Bolker and Hill, 2000). (see section 6.)

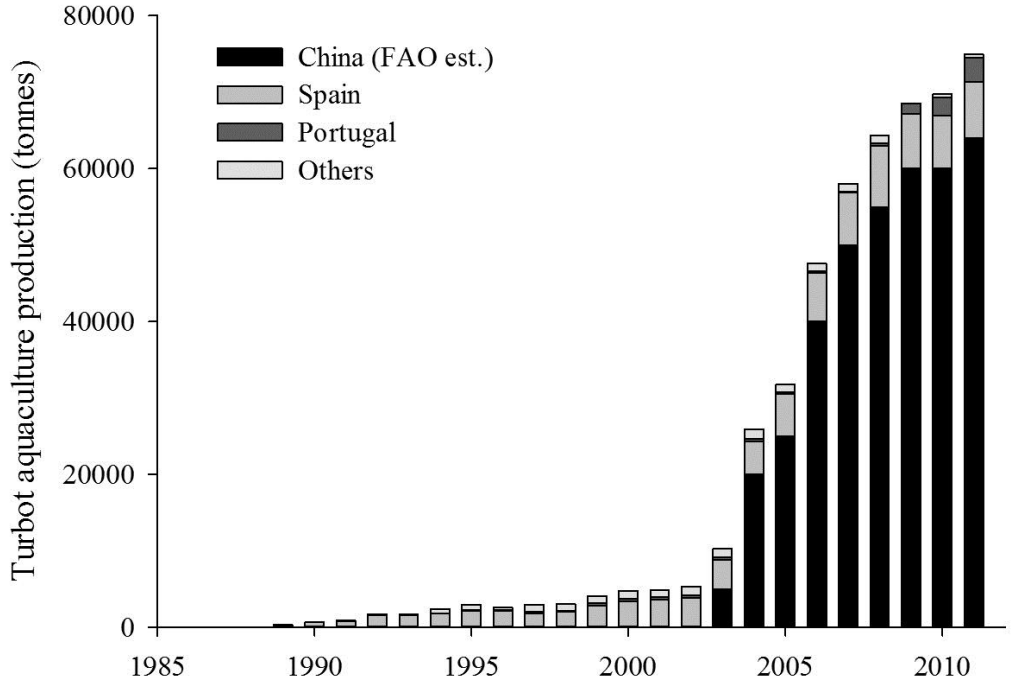

Figure 1.7. World aquaculture production of turbot from 1985 to 2011 according to FAO (2013) by major producer countries: China (FAO est), Spain, Portugal and others (e.g. France, United Kingdom, Ireland).

1.2. TELEOST SENSORY SYSTEMS

1.2.1. Acoustics

Sound propagation

Sound is energy produced by the vibration of molecules in a pattern as a mechanical wave traveling across an elastic medium (gas, liquid, solids or plasma) (Fig. 1.8) (Howard and Angus, 2009). The numbers of pressure changes over a period of time

are defined as frequency, measured in Hertz (Hz) as cycles per second. Therefore a sound with a single frequency is defined as a pure tone. Sound speed is the rate of propagation (m/s), dependant on the elasticity and density of the medium (Howard and Angus, 2009). Wavelength (ƛ) of a sound is the distance a pure tone travels during a full period (ƛ=Speed of sound / frequency) (Howard and Angus, 2009).

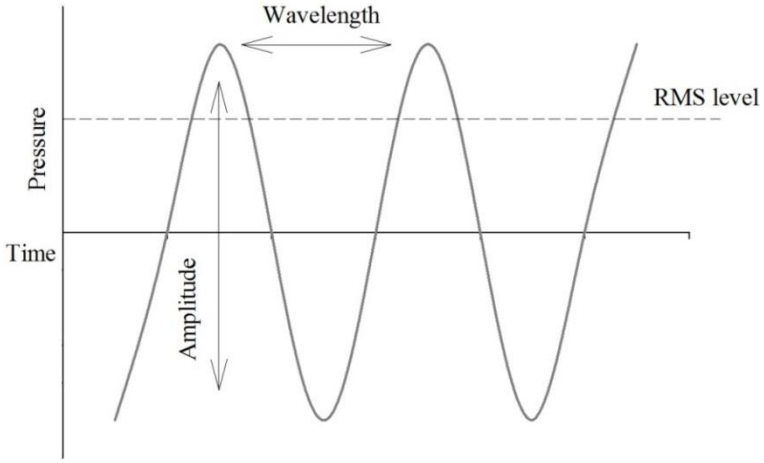

Figure 1.8. Representation of sound wave components: axis show time (sec) and pressure (Pa or dB): a) Amplitude (length of the wave from peak to peak); b) wavelength (distance between peaks across time), c) Root mean square of the total pressure of the wave. Adapted from Howard and Angus, 2009.

Sound measurements are expressed in decibels (dB), which might refer to sound amplitude or sound pressure. Sound pressure level (SPL) is the measurement of a pressure fluctuation in Pascals, while amplitude is the magnitude of the change. Because pressure variation can be large, the SPL is expressed as the pressure root mean square in a logarithmic scale representing a ratio or relative measurement in decibels (dB) (Howard and Angus, 2009). SPL is often confused with sound intensity, which refers to the flow rate of energy through a unit area (Watts m^{-2}).

Sound propagation is ruled by Newton's second law (force = mass * acceleration) and Conservation of mass (velocity is proportional to density). Furthermore sound

waves can be reflected (by encountering a denser mass), refracted (change in speed causing a direction variation) or attenuated (amplitude reduction as a function of distance) (Howard and Angus, 2009). Air and water are two completely different acoustic environments. Water is about a thousand times denser requiring a higher energy input to initiate sound propagation (acoustical impedance) resulting in greater velocity: 1,500 m sec^{-1} and 330 m sec^{-1} for water and air, respectively. In open waters sound pressure and particle motion (displacement (m), velocity (m sec^{-1}) or acceleration (m sec^{-2})) maintain a constant analytical relation, however water surface, the sea bottom and sea layers (caused by temperature differences) reflect the sound waves. As distance from the source increases sound energy is attenuated and absorbed (as a function of salinity, temperature, depth, frequency and water pH) (Howard and Angus, 2009).

Sound detection by fish: Mechanoreception

Fish use sound to detect and locate prey and predators (Sand and Karlsen, 2000). Additionally some fish species produce vocalisations to communicate with other individuals (Kasumyan, 2008). Sound detection in fish involves three interconnected systems (auditory, lateral line and equilibrium) which involve a complex network of organs and specialised cells distributed among the whole body (Sand and Enger, 1973; Hawkins and Horner, 1981).

The auditory system main organ is the inner ear, which is sensitive to mechanosensory stimuli (Fig. 1.9). The inner ear has three semi-circular canals and three otolith organs. It is divided as the pars superior (semicircular canals, 1 otolith organ and the utricle) and the pars inferior (2 otolith organs, saccule and lagena) (Platt and Popper, 1981). Sound waves produce movement at a single locus in the inner ear

making the hair cells oscillate with respect to the otolith, similar to bone conduction in terrestrial vertebrates (von Békésy, 1959).

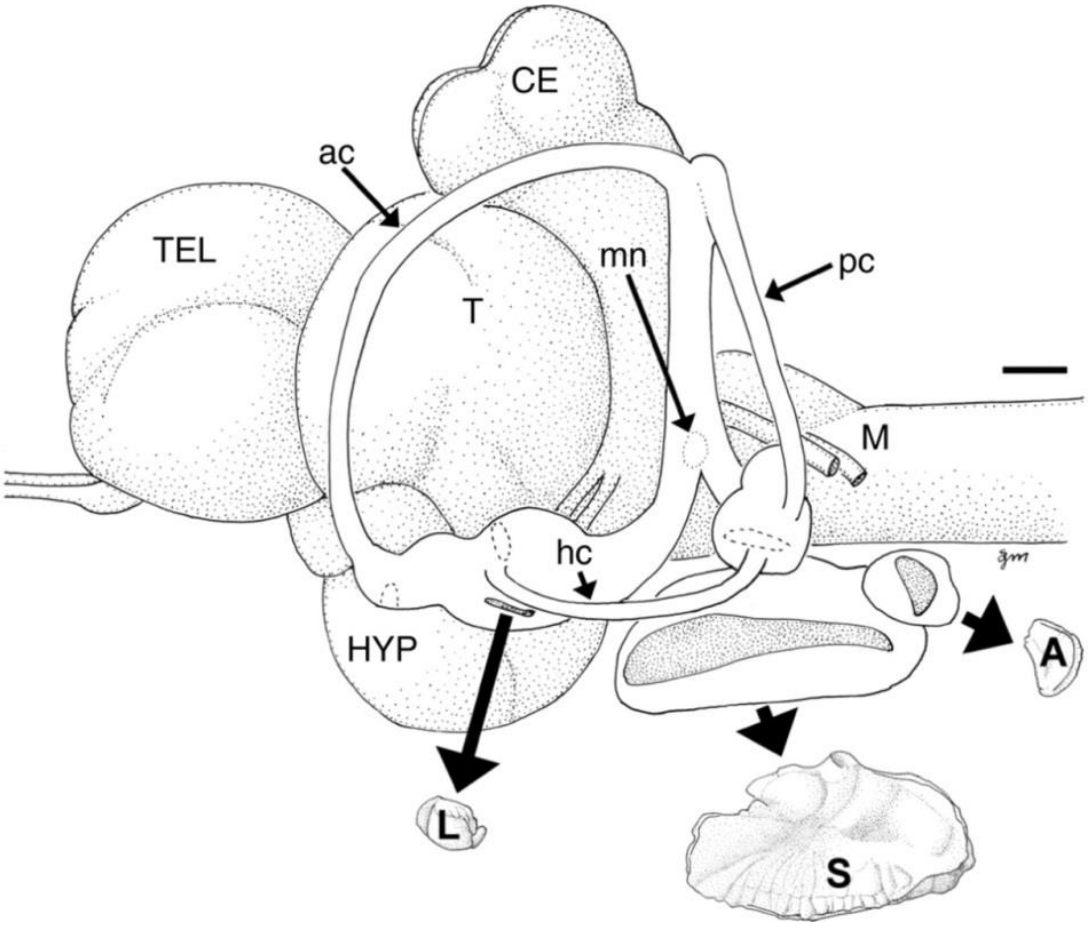

Figure 1.9. Schematic representation of the lateral view of the inner ear of a teleost. The saccule and lagena are positioned beneath the brain and oriented in the dorso-ventral plane along the presumed primary axis of particle motion during audit auditory evoked potential experiments. The position of the sensory macula is outlined and shaded within each otolithic endorgan, while its removed left otolith is illustrated below (large arrows). Dashed lines represent the location of the crista ampullaris of each semicircular canal, and the dotted line represents the position of the macula neglecta (mn). A, asteriscus otolith of lagena; ac, anterior canal; CE, cerebellum; hc, horizontal canal; HYP, hypothalamus; L, lapillus otolith of utricle; M, medulla; pc, posterior canal; S, sagitta otolith of saccule; T, tectum; TEL, telencephalon. Scale bar, 1 mm. Image taken from Maruska *et al.*, 2007.

The lateral line system (Fig. 1.10) is a mechanosensitive set of endorgans or neuromasts (Fig. 1.10b) along the entire body, composed by the sensory hair cells covered by the cupula, contributing in sound reception sensing fish motion and water movements (Hastings and Popper, 2005). The position and direction of the neuromasts allow fish to discriminate and determine the sound and its source based on the direction, distance, frequency and amplidude (Chapman and Johnstone, 1974; Hawkins and Sand, 1977; Buwalda *et al.*, 1983), the frequencies, (Enger, 1981; Hawkins and Horner, 1981) the amplitude and phase (Hawkins, 1981). The lateral line detection is limited by the distance, where the inner ear is capable of detecting sound originated at long distances.

Fish hearing bandwidths vary widely between species from below 20 Hz up to 100 kHz (Popper *et al.*, 2003). The initial studies on fish bioacoustics attempted to classify fish based on hearing range and sensitivity: hearing generalist detection thresholds are from below 50 to 1,500 Hz (optimal hearing range 100 to 400 Hz) and for hearing specialists it can go up to 3,000 Hz (optimal hearing range 300 to 1,000 Hz). However, studies on a range of species showed clearly that the auditory diversity is wider than first thought and such a classification is not possible (Popper and Fay, 2011). Fig. 1.11 compares the audiograms from seven selected species; it is evident that hearing thresholds are comparable between species of similar ecology and morphology. Nevertheless audiograms were recorded under different experimental conditions (e.g. behavioural conditioning, heart rate) therefore they cannot be taken as absolute. Moreover there is a debate over the perception of fish to particle motion and/or pressure. It appears that the less complex the hearing anatomical structure, the less sensitive fish are to sound pressure (Fay and Popper, 2012).

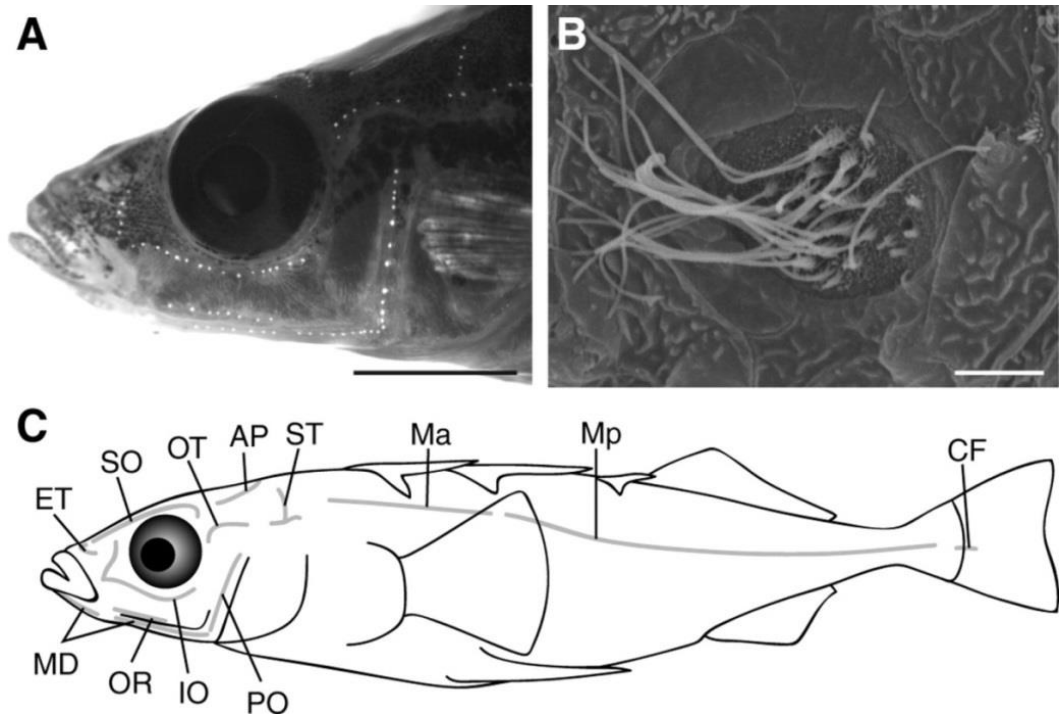

Figure 1.10. Arrangement of superficial neuromasts on the threespine stickleback. (A) View of a representative threespine stickleback head stained with DASPEI to highlight superficial neuromasts. Scale bar = 5 mm. (B) Scanning electron micrograph of a preopercular superficial neuromast on a Little Campbell marine threespine stickleback. Scale bar = 5 μm. (C) Schematic representation of neuromast arrangement. Abbreviations for line names: infraorbital (IO), oral (OR), mandibular (MD), preopercular (PO), otic (OT), supratemporal (ST), main trunk line anterior (Ma), main trunk line posterior (Mp), caudal fin (CF), ethmoid (ET), supraorbital (SO) and anterior pit (AP). Image taken from Wark and Peichel, 2010.

Recently it has been suggested that some species are capable of detecting infra- or ultra-sound. Infrasound detection in fish might be used for prey detection or navigation as seen in birds. Fish sensitive to infrasound receive the signals only in the inner ear (Sand and Karlsen, 1986; Enger *et al.*, 1993; Sand *et al.*, 2001). An experiment with spring chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) presented flight and avoidance swimming responses to an exposure of 5 seconds of frequency of 10 Hz. (Knudsen *et al.*, 1997). Sand *et al.* (2001) found that Atlantic cod detected infrasound only through the otolith organs, but did not find any physical or behavioural reaction after exposure. Similarly, despite a

behavioural response to ultrasound (> 20 kHz) in some species (Astrup and Møhl, 1998; Mann *et al.*, 1998 and 2001; Popper *et al.*, 2003), the involvement of the inner ear could not be confirmed.

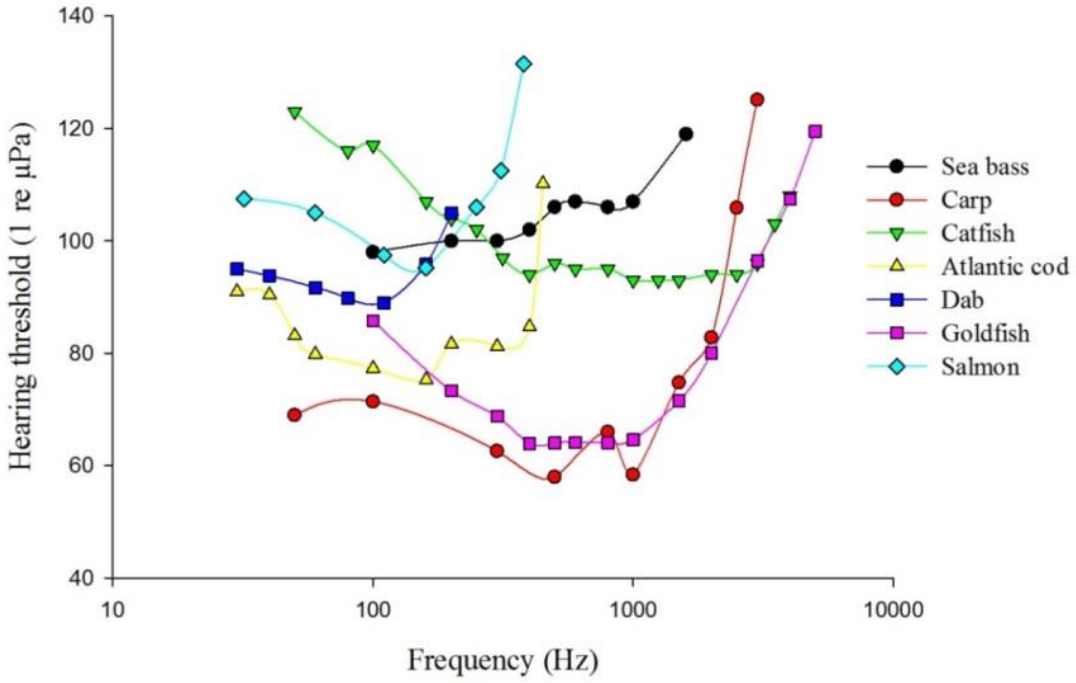

Figure 1.11. Hearing thresholds (dB re 1 µPa) of seven selected fish species. Data taken from Nedwell *et al*. (2004).

In order to improve sound perception, fish, like mammals, use an auditory filter to focus on specific tones masking background noise. This is achieved because the membrane supporting the otolith responds differently to diverse frequencies. It is believed that this discrimination capability might interfere in the communication when the background sound is higher than usual levels or is at the same or adjacent frequencies (Hawkins and Chapman, 1975). Additionally the swim bladder works has an accessory auditory function in some species (Sand and Enger, 1973; Buwalda and van der Steen, 1979). Gas contained in the swim bladder translates the particle motion into pressure, making the chamber vibrate and therefore displaying higher amplitudes (Hawkins, 1986). Yan (2001) confirmed that the swim bladder increases the sound

sensitiveness in goldfish and 3 gourami species. By deflating goldfishes' swim bladder an average of 46 dB re 1 µPa were necessary to detect the sound at the same frequencies, compared to an intact gas chamber. However, the large variability between anatomical structures (Table 1.1), added to behaviour and physiology, suggests that species detect and process sound in different ways probably as an adaptation to their environment (Popper and Fay, 1993).

Moreover fish are not only passive acoustic receptors, as daily activities produce sound in all species. Unintentional sounds are caused mainly by swimming (hydrodynamic, pneumatic), respiration and feeding (stridulation, cavitation), and in some cases, they act as signals to other individuals (Kasumyan, 2009). However under some circumstances sounds are intentionally produced under certain behavioural or specific situations.

Vocalisations are more common during reproduction or complex intraspecies relationships (Ladich, 2004; Ladich and Myrberg, 2006). Fish sounds are mainly produced at low-frequencies (below 1 kHz) consistently with their specific optimal hearing bandwidths (Ladich and Yan, 1998, Ladich, 2000). Moreover less acoustic sensitive fish compensate with louder sounds and closer proximity (Ladich and Popper, 2004). Besides the mentioned sounds (hydrodynamic, pneumatic, respiration, stridulation and cavitation), some species developed complex sound production involving the drum muscle and the swim bladder. Those sounds are produced by repeatedly contracting the drum muscle sending vibrations to the swim bladder (Rowe and Hutchings, 2004). The structure of the sound generated is variable, even between fish families. Some species possess specific canals (Table 1.1) connecting the muscle with the swim bladder; in others the presence of the sonic muscle is gender dependant,

or they compensate the absence of the drum muscle by vibrating the bones on the pectoral girdle (Kasumyan, 2008).

Table 1.1. Comparison of frequency hearing range (Hz) of selected species and type of specialised anatomical connection to the swim bladder.

Species	Common name	Family	Swim bladder connection	Hearing threshold (Hz)	Source
Alosa sapidissima	American shad	Clupeidae	Prootic auditory bullae	200- 200k	Mann *et al.*, 1997
Anguilla anguilla	European eel	Anguillidae	None	Up to 300	Jerkø *et al.*, 1989
Astyanax jordani	Mexican blind cave fish	Characidae	Weberian ossilcles	50 - 6400	Popper, 1970
Carassius auratus	Goldfish	Cyprinidae	Weberian ossilcles	100 - 5000	Yan, 2001
Clupea harengus	Herring	Clupeidae	Prootic auditory bullae	30 - 4000	Enger, 1967
Cyprinus carpio	Carp	Cyprinidae	Weberian ossilcles	50 - 3000	Popper, 1972
Dicentrarchus labrax	Sea bass	Moronidae	Extension multiple	100 - 1600	Nedwell *et al.*, 2004
Eupomacentrus dorsopunicans	Damselfish	Pomacentridae	None	100 - 1200	Myrberg and Spires, 1980
Gadus morhua L	Atlantic cod	Gadidae	None	30 - 450	Hawkins and Myrberg, 1983
Ictalurus punctatus	Catfish	Ictaluridae	Weberian ossilcles	50 - 4000	Fay and Popper, 1975
Limanda limanda	Dab	Pleuronectidae	No swimbladder	30 - 250	Chapman and Sand, 1974
Melanogrammus aeglefinus	Haddock	Gadidae	None	30 - 450	Chapman, 1973
Pimephales promelas	Fathead minnow	Cyprinidae	Weberian ossilcles	300 - 4000	Scholik and Yan, 2001
Pleuronectes platessa	Plaice	Pleuronectidae	No swimbladder	30 - 250	Chapman and Sand, 1974
Salmo salar	Atlantic salmon	Salmonidae	None	32 - 380	Hawkins and Johnstone, 1978
Sardinops melanostictus	Sardine	Clupeidae	Prootic auditory bullae	250 - 2000	Akamatsu *et al.*, 2003
Scophthalmus maximus	turbot	Scophthalmidae	No swim bladder	30 – 250	Chapman and Sand, 1974
Thalassoma bifasciatum	Blue-head wrasse	Labridae	Extension multiple	100 - 1200	Tavolga and Wodinsky, 1963
Thunnus albacares	Yellowfin tuna	Scombridae	Extension multiple	50 - 1100	Iversen, 1967
Tilapia macrocephala	African mouthbreeder	Cichlidae	Extension multiple	50 - 900	Fay and Popper, 1975

Atlantic cod bioacoustics and vocalisation

Atlantic cod has an optimal hearing bandwidth of 18 to 470 Hz (maximum response between 60 to 310 Hz), although this range should not be considered absolute (Buerkle, 1967; Chapman and Hawkins, 1973). Despite the relative narrow bandwidth gadoids appear to be among the most acoustic sensitive teleosts in terms of sound pressure/particle motion (loudness) (Popper et al, 2003; Ladich and Popper, 2004; Hastings and Popper, 2005). Cod need 16 dB above ambient noise to detect acoustic sound (Chapman and Hawkins, 1973) and above 30 dB for a significant behavioural response (Mitson, 2000).

Atlantic cod is a broadcast species that communicates at low frequencies during migration, aggression and courtship (Ladich and Myrberg, 2006). Four different vocalisations have been reported: "grunts", "clicks", "bops or knocks" and "hums" (Engen and Folstad, 1999; Midling *et al.*, 2002; Vester *et al.*, 2004, Rowe and Hutchings, 2008). It appears that captivity restricts the vocal repertoire of Atlantic cod, however grunts are the most common and the most relevant vocalisation to this work. Grunts are produced by repeatedly contracting the three pairs of drumming muscles sending vibrations to the swim bladder (Rowe and Hutchings, 2004). A grunt is the repetition of single pulses with peak sound amplitudes of 50-500 Hz with an harmonic frequency of 80 Hz, consisting in 4 to 15 pulses with a duration of 75 to 200 ms (Hawkins and Rasmussen, 1978, Finstad and Nordeide, 2004). Both genders are capable of producing grunts throughout the year, however the peak in vocalisation numbers is achieved during the spawning season (mainly after sunset) (Brawn, 1961; Langárd *et al.*, 2008). During courtship it appears that only males grunt related to mate competition (e.g. selection, territorial) (Hutchings *et al.*, 1999). The vigour of a grunt is correlated to the volume of the drum muscle mass (which enlarges during the spawning

season) and the specific immune state of the male (i.e. sick fish produce weak grunts) (Rowe and Hutchings, 2004, 2006 and 2008). The swimming sequence that precedes synchronised gamete release is accompanied by grunts throughout the courtship (Engen and Folstad, 1999). Therefore spawning success might be restricted if the vocalisations are interrupted or masked.

Anthropogenic noise

In humans, noise causes: annoyance, sleep disturbances, short-term physiological reactions or even hypertension and coronary heart disease (von Gierke and Eldred, 1993; Pearsons *et al.*, 1995; Clark and Stansfeld, 2007). This effect is very likely somehow extended to terrestrial or aquatic animals (Lonsbury-Martin *et al.*, 1987; Zelick *et al.*, 1999; Knudsen and Oen, 2003; Popper, 2003, NRC, 2005; Dooling and Popper, 2007; Baldwin and Bell, 2007; Wright *et al.*, 2007; Popper and Hastings, 2009; Wright, 2012). Anthropogenic activities are a known source of noise impacting directly on animal welfare. Fuller *et al.* (2007) found that urban European robins (*Erithacus rubecula*) switched to nocturnal singing to reduce acoustic interference. The impact of human activities in the aquatic acoustic environment have been mainly focused towards the effects on marine mammals, however there is an increased awareness of the potential negative effects on fishes also (Hatch and Wright, 2007; Popper and Hastings, 2009).

Most human activities in the aquatic environment generate noise within fish hearing thresholds (<1 kHz) (Hatch and Wright, 2007). Exposure to elevated sound levels can cause: a behavioural disturbance (flight/fight, migration patterns, reproductive strategy) (Engås *et al.*, 1996; Wardle *et al.*, 2001; Engås *et al.*, 2006), acoustic masking (changes in vocalisations) (Enger, 1973; Clark *et al.*, 2009;

Slabbekoorn *et al.*, 2010), temporary threshold shifts (Scholik and Yang, 2001 Smith *et al.*, 2004), permanent threshold shifts and tissue damage (Enger, 1981; Hastings *et al.*, 1996; McCauley *et al.*, 2003) or even mortality (Yelverton *et al.*, 1975). Fish have been reported to be attracted to sound generated by divers (Chapman *et al.*, 1974), but equally show avoidance behavioural reactions when exposed to pile-driving noise (Thomsen *et al.*, 2006, Mueller-Blenkle *et al.*, 2008), air guns used in seismic surveys (Hirst and Rodhouse, 2000; Wardle *et al.*,, 2001) vessel traffic noise (Vabø *et al.*, 2002, Handegard *et al.*, 2003) and off-shore wind farms (Wahlberg and Westerberg, 2005). Migration patterns and reproductive behaviour can be disturbed by noise as well, forcing fish to find alternative routes or prevent them from settling in their usual spawning grounds (van Opzeeland and Slabbekoorn, 2012) and impact larval settlement (Holles *et al.*, 2013). The avoidance reaction to noise sources suggests that fish have the need to escape from the unknown source, which might be triggering a physiological stress reaction in fish. Buscaino *et al.* (2010) demonstrated that sea bass *(Dicentrarchus labrax*) and sea bream (*Sparus aurata*) showed significant increases in lactate, haematocrit levels and glucose (not sea bass) in response to a sound stressor. Interestingly all these studies found an acute reaction to the sound stress that demised in the longer term. Similarly, rainbow trout (*Oncorhynchus mykiss*) exposed to sound levels comparable to those encountered in recirculating aquaculture systems (RAS) did not show any long term (chronic) stress response (Wysocki *et al.*, 2007). These studies suggest that fish have the capability to adapt or cope with chronic stressors such as sound. This topic is discussed further in experimental chapter 3.

1.2.2. Photoreception

Light definition

According to NASA (2013), electromagnetic (EM) radiation consists of travelling particles (photons) carrying discrete packets of energy (quanta) in a wave-like pattern at the speed of light. Radiation can be expressed in terms of energy content in the photons, wavelength (distance between wave peaks: metres) or frequency (cycles per second: Hertz). Table 1.2 shows the differences in terms of energy content (volts), wavelength (meters) and frequency (Hertz) between the different parts of the EM spectrum: Gamma ray, X-ray, Optical light (Ultraviolet light, visible light and Infrared light) microwaves and radio waves. EM radiation presents a "wave-particle duality" behaviour, where the higher the energy content (i.e. gamma ray), thus higher frequencies and shorter wavelengths, behaviour is mostly particle-like; while lower energy (longer wavelengths) predominantly show a wave-like behaviour. Only radio and optical waves penetrate the Earth's atmosphere up to the sea level, while the rest are absorbed.

Table 1.2. Approximate limits of the electromagnetic (EM) spectrum regions a) wavelengths (m), b) Frequency (Hz) and c) carrying energy (J). Adapted from NASA (http://imagine.gsfc.nasa.gov/docs/science/know_l1/spectrum_chart.html)

	Wavelength (m)	**Frequency (Hz)**	**Energy (J)**
Radio	$> 1 \times 10^{-1}$	$< 3 \times 10^{9}$	$< 2 \times 10^{-24}$
Microwave	1×10^{-3} to 1×10^{-1}	3×10^{9} to 3×10^{11}	2×10^{-24} to 2×10^{-22}
Infrared	7×10^{-7} to 1×10^{-3}	3×10^{11} to 4×10^{14}	2×10^{-22} to 3×10^{-19}
Optical	4×10^{-7} to 7×10^{-7}	4×10^{14} to 7.5×10^{14}	3×10^{-19} to 5×10^{-19}
UV	1×10^{-8} to 4×10^{-7}	7.5×10^{14} to 3×10^{16}	5×10^{-19} to 2×10^{-17}
X-ray	1×10^{-11} to 1×10^{-8}	3×10^{16} to 3×10^{19}	2×10^{-17} to 2×10^{-14}
Gamma-ray	$< 1 \times 10^{-11}$	$> 3 \times 10^{19}$	$> 2 \times 10^{-14}$

The Optical window of the EM spectrum includes the ultraviolet (UV) (100 – 400 nm), visible light (380 – 770 nm) and infrared (IR) (770 to $1x10^{6}$ nm). UV light

contains the highest amount of energy of the Optical window radiation forms. Based on biological effects it can be classified as: UV-A (315 – 400 nm) (e.g. “black light”, “tanning beds”), UV-B (280 – 315 nm) (e.g. damage to biological tissues, skin cancer) and UV-C (100 to 280 nm) (e.g. formation of ozone, “germicidal lamps”). Infrared light energy content is the lowest of the optical window (longer wavelengths), which often cannot be detected by a quantum detector and is invisible to the human eye. IR is commonly expressed as temperature changes caused by the energy absorption of a body. This property gives IR a practical application in imaging cooler bodies: e.g. night vision or astrophysics. The intermediate portion of the EM spectrum is referred as “Visible light”, based on the human perception of the wavelengths as colours: from red ~700 nm to ultraviolet ~380 nm. The colour appearance depends on the temperature (energy content) contained in the wave, the hotter the body the shorter the wavelength. The visibility of the energy depends on the reflection of the radiation on an object resulting in a specific spectral signature. Depending on the texture of the reflecting surface, light reflection is specular (identical angles with opposite directions between incident and reflected ray) or scattered (diffused or spread). Fig 1.12 shows an example of some Earth spectral signatures, the differences between objects reflection mirror the atoms and molecules forming it.

For instance, long wavelengths are easily absorbed by hydrogen. In the aquatic field this results in a poor penetration of the red end of the visible spectrum, while the blue component reaches depths of 100 m or more in clear water (Clarke, 1965). In addition light encounters suspended solids and dissolved materials that cause a further alteration of the spectral composition. Inland and coastal waters contain high quantities of organic matter narrowing the spectral composition to 500 to 600 nm (green/yellow). The absorbance and reflection of light does not solely affect the wavelengths but also

the direction, intensity and propagation of the light (Smith, 1974). Differences in luminance attenuation and spectral shift results in diverse underwater photic environments. Fish ecology shaped species specific functional visual features (Kusmic and Gualtieri, 2000).

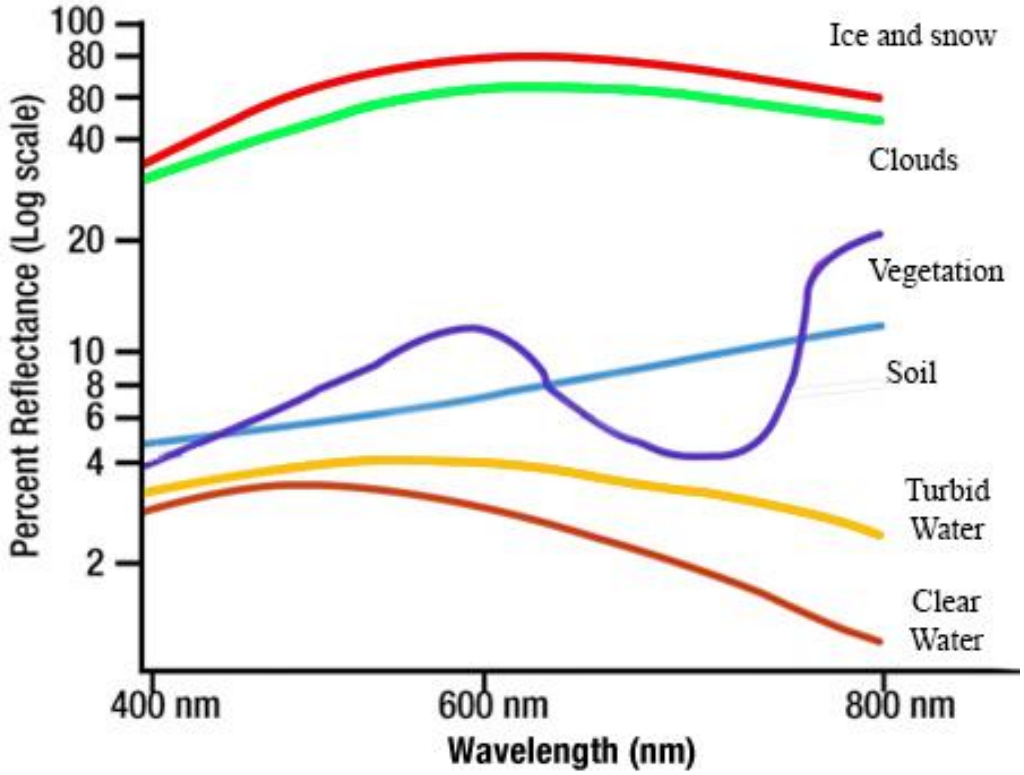

Figure 1.12. Reflectance of light of spectral signatures of Earth features. Arranged from NASA http://missionscience.nasa.gov/ems/09_visiblelight.html Credited within to: Jeannie Allen.

Fish light perception

Light is perceived through the photoreceptors contained in the eye (retina), the pineal complex and possibly in the deep brain (Migaud *et al.*, 2010) where it is transformed into electrical neuro-signals.

Eye

The basic anatomy of the fish eye is conserved across vertebrates (Bowmaker, 1998), where light is focused by the cornea and lens onto the retina containing the photoreceptor cells and transformed into neural impulses (Dowling 1987). Adult fish possess a duplex retina (Fig. 1.13) composed of: pigmented epithelium (PE), outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) (nerve fibres connected to the brain). The main photoreceptor cells in the fish retina are cones and rods (distinguishable by their shape) contained in the ONL. Retina development starts with the appearance of cones (Blaxter and Staines, 1970), responsible for colour and central vision; while rods (light intensity and peripheral vision) develop further during the larval stages (Table 1.3). Light photon absorption occurs in the photopigments (opsin (protein membranes) and chromophore bound) by ratio-changes in vitamin A_1-A_2 (Beatty, 1975) or changes in the amino-acid sequence (Cowing *et al.*, 2002).

Table 1.3. Main differences between retinal photoreceptors: rods and cones. Adapted from Evans and Browman (2004) and Jobling *et al*. (2012).

	Rods	**Cones**
Photopigment	High concentration	Low concentration
Photoreceptor	Achromatic	Chromatic
	Intensity	Colour vision
Sensitivity	High (at least one time more)	Low
Temporal integration time	Slow	Fast
Spatial acuity	Low	High
Saturation	Low light intensity	High light intensity
Detection	Scotopic, low intensity	Photopic, high intensity

Pineal complex

The pineal complex in teleosts compromises two intracranial components: the pineal gland and parapineal gland (likely derivate from one bipartite pineal complex in early vertebrates). The parapineal gland is a rudimentary body located above the epithalamus (Ekström and Meissl, 1997). The pineal gland, in most fish species, is a vesicle (distal pineal) attached to the diencephalon roof below a skull window connected by a slim stalk. The end-vesicle can be differentiated in terms of their predominant cell types in: distal, intermediate and proximal regions (Ekström and Meissl, 2003). The pineal epithelium is formed by photoreceptor cells, neurons and ependymal interstitial cells. Contrasting the more complex retinal structure, the pineal epithelium contains only cone-like photoreceptor cells in contact with the ganglion cells. Therefore the pineal organ acts as a day-length and luminance detector, with a basic spectral differentiation (Vera *et al.*, 2010). See section 1.3.1. for the pineal involvement in photic signalling. (see section 1.3.1. for Melatonin secretion)

1.2.3. Thermoreception

The ectothermic nature of fish requires that fish detect variations in temperature to find suitable thermal conditions (Evans and Clairborne, 2006). Temperature preference varies between species and developmental stages and plays an important role on most physiological aspects: from cellular processes to physiological functions (Coutant, 1977). Thermoreceptors, mainly located in the skin and hypothalamus, detect water temperature and integrate the signals through the central nervous system, triggering a behavioural response (e.g. swim, movements in the water column) (Jobling *et al.*, 2012). Receptors in the skin appear to act as mechanoreceptors as well, while the brain receptors respond to chemical stimuli (Willmer *et al.*, 2005).

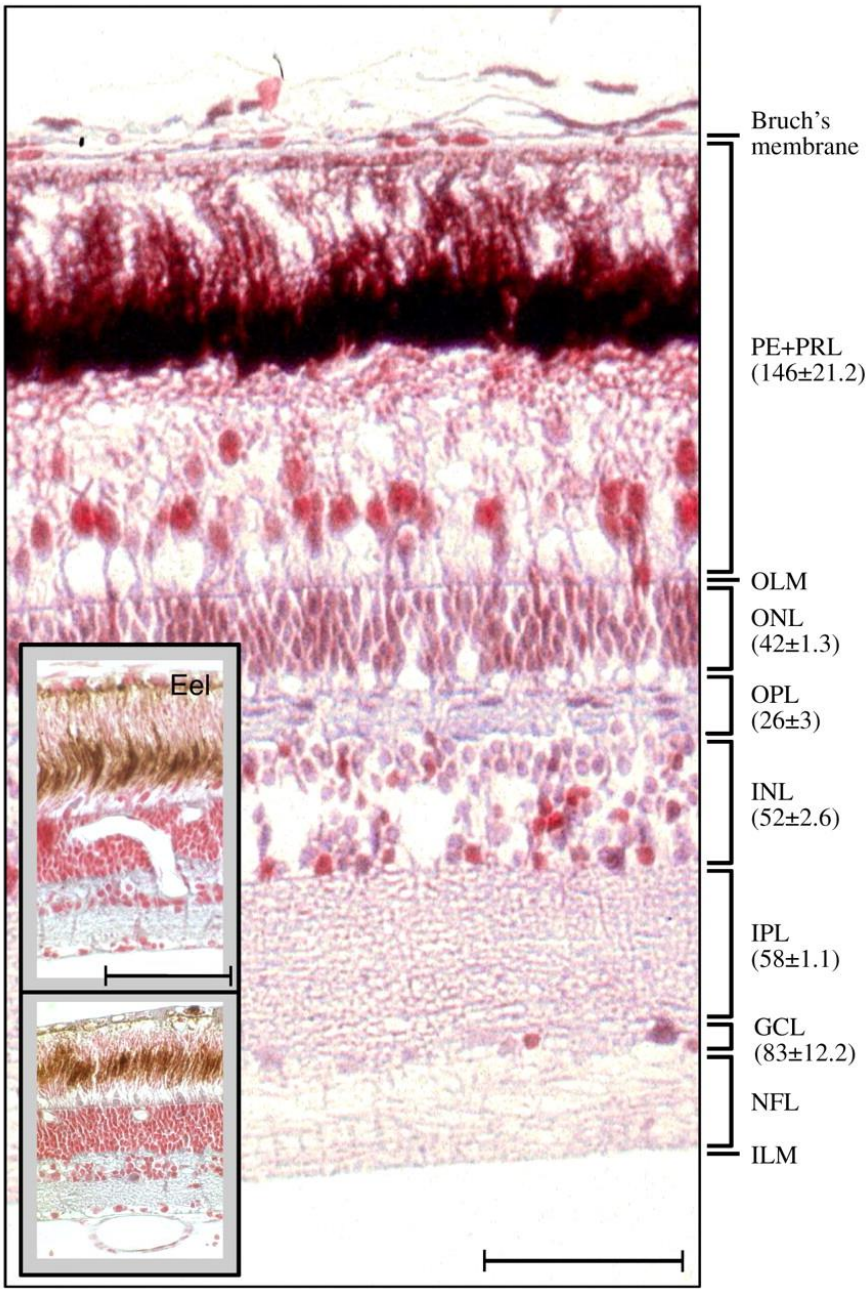

Figure 1.13. Microphotograph of trout retina section (PE+PRL, pigment epithelium and photoreceptor layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fibre layer; ILM, inner limiting membrane). The trout retina is completely devoid of blood vessels. Numbers denote thickness ± s.d.(N=3) in µm of the respective layers. For GCL and NFL the combined thickness of the two layers is given. Insert: Microphotographs of eel retina as an example of a teleost retina with intraretinal vascularization. Large blood vessels visible on ILM reach into the retina to OLM. Bars, 100 µm, 50 µm. Image taken from Waser and Heisler, 2005.

1.2.4. Chemoreception

Fish detect dissolved chemical substances in water through sensory cells mediating processes (e.g. food intake, predator avoidance, migration) as a behavioural response. Chemoreception involves two systems: gustation and olfaction. The gustatory system input is originated from cranial nerves in the epithelial cells located on the "taste bud" organ. They are more abundant in the oral cave and less prominent in the facial nerves, the lips, barbels or in some species in the entire body (Michel, 2006). Until now literature suggest that the use of gustation is food related only and no other apparent application has been reported. Similarly to humans, taste detection in fish allows discernment between beneficial (food) and potential harmful (toxic) molecules. Integration between gustation and the gastrointestinal tract receptors are believed to regulate, to some extent, appetite and feeding response though the central nervous system (Sorensen and Caprio, 1998). In contrast to the gustation receptors, olfactory cells possess axons projecting directly from the olfactory epithelium to the brain. Paired olfactory organs are located on the dorsal side of the snout connected to the olfactory nerve (formed by the olfactory receptor neurons (ORN) axons). The ORN interact with odorants (amino acids, sex steroids, prostaglandins and bile acids) (Hara, 1994) in relation to prey detection, predator risk or reproductive behaviour. However, the olfaction neural pathways in fish differ from the mammalian models and between fish species in ways that are not yet understood (Laberge and Hara, 2001).

The sensory systems are, to some extent, shared across living organisms. They are adaptive responses to the evolutionary pressure to allow fish to perceive and pro-actively react to the surrounding environment. Perception of environmental conditions allows fish to foresee changes that ultimately are involved in the entrainment of the physiology and behaviour.

1.3. CHRONOBIOLOGICAL CONTROL OF FISH PHYSIOLOGY

All living organisms were subjected to evolutionary pressure to develop biological mechanisms capable of anticipating the Earth rhythmicity. As such, cyclic environmental events such as day/night, lunar cycles, rainfall, temperature variations, seasonal fluctuations entrain biological rhythms in most organisms. Synchronization with the cyclic events coordinates fish physiology and behaviour, maximising chances of survival and optimising energy cost (larval development, locomotor activity, skin pigmentation, thermoregulation, food intake, social organisation, reproduction). For instance, feeding activity is harmonised with the daily time periods in which food is more abundant and environmental conditions are optimal (Blanco-Vives *et al.*, 2009); while reproduction is synchronised with the time (clearly seasonal for temperate species) when progeny have the greatest chances of survival (e.g. food availability, temperature, salinity) (Migaud *et al.*, 2010). Biological rhythms appear to be dictated by endogenous clock mechanisms (biological clocks or pacemakers); however environmental cues (e.g. temperature, salinity/rainfall, tidal movements, photoperiod and light composition) act as "zeitgebers" entraining the intrinsic clock (López-Olmeda *et al.*, 2006b). The latter is more evident in the plasticity of circadian activity rhythms in fish (Reebs, 2002) when a temporal adjustment can take place.

1.3.1. Photic signals and melatonin

Light works as a "noise free" signal giving accurate information of diel and seasonal conditions. Photoperiod, light intensity and spectral composition vary between day/night and seasons, caused by the Earth's rotation (dawn/dusk, latitude or season), influencing the fish behaviour and physiology (Boeuf and Le Bail, 1999; Boeuf and Falcón, 2001; Bromage *et al.*, 2001). Photic perception varies widely between species

(as discussed below) however, in general terms, photic signals are transduced into nervous and endocrine signals in both the retina and pineal gland. The retina decodes the visual information (shapes and colours) by processing the information in the inner nuclear layer inter-neurons. In the pineal, information is sent directly though the ganglion cells creating a light dose-dependent electrical discharge (Migaud *et al.*, 2010). Despite high variability between species photosensitivity, both retinal and pineal ganglion cells appear to target similar brain areas (Ekström and Meissl, 1997); suggesting an integrative function of environmental cues (Falcón *et al.*, 2009).

Melatonin: the light perception hormone

Melatonin is synthesized during the scotophase 1) from tryptophan in the pineal cells, 2) catalysed by tryptophan hydroxylase (TpOH) into tryptophan hydroxylation, 3) decarboxylased by the aromatic aminoacid decarboxylase (AAAD) into serotonin. Two further enzymatic steps transform it into melatonin: 4) the arylalkylamine N-acetyltransferase (AANAT) catalyses into N-acetylserotonin and; 5) hydroxyindole-O-methyltransferase (HIOMT) into melatonin (Falcón *et al.*, 2010). Melatonin is a highly lipophilic molecule that penetrates cell membranes, reaching the blood stream. Melatonin concentration is an accurate reflection of the duration of the day length (photoperiod). Across vertebrates the highest concentration of circulating melatonin occurs during the scotophase and returns to basal levels during the photophase, while serotonin levels are inversed (Falcón, 1999). Nocturnal melatonin production responds to an increase in AANAT activity. Unlike other vertebrates, fish possess at least three homologous forms of AANAT: AANAT-1a and 1b (localised between the retina and brain) and AANAT-2 (in the pineal gland) (Falcón *et al.*, 2010). However the presence of subtypes is species specific and appears to dictate the degree in which fish can foresee photic changes. Furthermore those subtype variations appear to regulate the

photic entrainment of the circadian rhythms differently. The latter is translated into three melatonin production patterns: A-type, production starts after the initiation of the dark phase, reaching its peak towards the end; B-type, discrete peak in the middle of the scotophase; and, C-type (the most common) melatonin production rapidly increases following the onset of the scotophase and rapidly decreases with the onset of the photophase (Migaud *et al.*, 2010). Retinal melatonin is usually produced in parallel with the pineal; however some species show a reversed shift (e.g. highest during photophase). There is still uncertainty surrounding the exact role of melatonin in fish, driven by inconsistency in experimental settings: gender, reproductive status, time, season, methods of melatonin administration and role of pineal neural output (Ekström and Meissl, 1997). However retinal melatonin appears to be involved in homeostasis of the retina and act as local antioxidants (Falcón *et al.*, 2003), while circulating melatonin has been associated with many rhythmic patterns: locomotor activity, sedation (sleep), thermal preference, vertical migration, shoaling, skin pigmentation, osmoregulation, metabolism, smoltification, growth and reproduction (Ekström and Meissl, 1997; Sánchez-Vazquez *et al.*, 2000; Pinillos *et al.*, 2001; Tricoire *et al.*, 2003; López-Olmeda *et al.*, 2006a; Herrero *et al.*, 2007; Falcón *et al.*, 2007; Azpeleta *et al.*, 2010).

Diel rhythmicity of melatonin production relies on the photic signals; not only on photoperiod, but also on the perception of day/night caused light intensity thresholds and spectral composition (Bromage *et al.*, 2001; Bayarri *et al.*, 2002; Migaud *et al.*, 2006 and 2007; López-Olmeda *et al.*, 2009; Migaud *et al.*, 2010; Oliveira *et al.*, 2011). For instance, Oliveira *et al.* (2007) reported that in sole (*Solea senegalensis*) melatonin production was significantly reduced under LL photoperiod, with a strong correlation between the light intensity and the inhibition effect (1 hr light pulses during the dark cycle) and inhibited by violet (368 nm) but not red (>600 nm) light. The authors

suggested that moon light (dim light) may play a role in the photic entrainment of the biological rhythms. Similarly in Atlantic cod red light was less effective in melatonin secretion suppression that the shorter wavelengths being 100 to 1,000 times more sensitive to light that other species (Vera *et al.*, 2010). Furthermore several other hormones present cyclic patterns likely regulated by daily/seasonal changes although they are still not fully understood and are not solely correlated to light variations: e.g. Insulin-like growth factor (IGF-I) (Davie *et al.*, 2007b, Taranger *et al.*, 2010), growth hormone (GH) (Falcón *et al.*, 2003) and thyroid hormones (TH) (Miwa et al, 1992; Power *et al.*, 2001).

1.3.2. Temperature

Thermoreception was briefly mentioned in section 1.2.3. Body temperature regulation, by means of locomotor activity or physical relocation (i.e. displacements in the water column) is crucial for fish survival (e.g. respiration, metabolism). Recent studies in zebrafish suggests that temperature regulates directly the internal clock even in the absence of light (Lahiri *et al.*, 2005), driven through its effects on the metabolic processes (e.g. cellular metabolism, gene expression or protein activity; including melatonin synthesis) (Bromage *et al.*, 2001; Pankhurst and Porter, 2003; Falcón *et al.*, 2006). Therefore, temperature appears to modulate the efficiency of the physiological responses (e.g. gonadal development, spawning time).

1.3.3. Salinity

The effects of salinity/rainfall on fish biological rhythms and reproduction are scarce. It was mentioned before that photoperiod and temperature play a crucial role in fish reproduction. However in the tropics, both photoperiod and temperature remain relatively steady throughout the year. Some tropical species are seasonal breeders;

however it is possible that neither photoperiod nor temperature are the ultimate signals regulating reproduction, but rather salinity/rainfall might be a seasonal indicator. Literature suggests that salinity, influences reproductive success but do not regulate it directly (Lee *et al.*, 1992; Haddy and Pankhurst, 2000).

1.3.4. Lunar cycles / Tides

Some marine species, more evident in the tropical species, spawn in synchronisation with the lunar cycles (Rahman *et al.*, 2004). For instance, seagrass rabbitfish (*Siganus canaliculatus*), a tropical species from the Pacific Ocean with a circannual reproductive cycle, showed the maximum reproductive activity (e.g. gonadosomatic index) in coordination with the moon cycles. Lunar cycles and diel tidal changes appear to synchronise the oocyte development, although the perception and regulation of the periodicity remains to be defined (Tamekmura et al., 2010).

1.3.5. Feeding entrainment

Feeding is a key physiological function that is regulated by environmental signals through clock rhythms. Food anticipatory activity (FAA) involves molecular, physiological and behavioural responses (Sánchez-Vázquez and Madrid, 2001; Vera *et al.*, 2007; Montoya *et al.*, 2010; López-Olmeda *et al.*, 2012) directly correlated to the fish predominant activity phase (diurnal, nocturnal or crepuscular) (Sánchez-Vázquez *et al.* 1998). FAA may improve food acquisition and nutrient utilisation (Comperatore and Stephan, 1987) therefore the seasonal shift between phases (dualism) found in many species: e.g. Arctic charr (*Salvelinus aplinus*) (Jørgensen and Jobling, 1990) and European sea bass (*Dicentrarchus labrax*) (Sánchez-Vázquez *et al.* 1998). However fish activity patterns are plastic and vary widely between species, stages and even within individuals (Reebs, 2002). Although feeding itself can entrain the circadian

rhythms (Spieler, 1992), other environmental factors are believed to be the proximate signals synchronising the internal clocks (Bromage *et al.*, 2001). However feed- and light- entrainable oscillators appear to be loosely coupled (Kohbara *et al.*, 2000; Vera *et al.*, 2006; Noble *et al.*, 2007), allowing a multioscillator system recognising different "zeitgebers" simultaneously (Boujard and Leatherland, 1992). Feeding can act as a biological rhythms entrainer; however it also involves a series of physiological and behavioural mechanisms that are discussed in section 8.

1.4. FISH REPRODUCTIVE PHYSIOLOGY AND NEUROENDOCRINE CONTROL

1.4.1. Brain-pituitary-gonadal axis

The cyclic production of melatonin (section 3.3.1) as a response to the day length is recognised as the timekeeper element of the biological rhythms. Melatonin receptors are found throughout the fish anatomy, especially in the brain-pituitary-gonad axis (BPG) (Falcón *et al.*, 2009). This suggests that melatonin acts as intermediary in the reproductive cascade translating the photic signals.

Research on the exact role played by melatonin on the BGP axis is still in its infancy (Fig. 1.14). It is clear now that kisspeptin (KISS) acts directly on the gonadotropin releasing hormone (GnRH) neurons though GPR54 membrane receptors. Recent studies suggest that melatonin may initiate the reproductive neurocascade by stimulating, directly or indirectly, KISS (Taranger *et al.*, 2010). Revel *et al.* (2006) suggested that photoperiod regulates KISS-1 expression through melatonin in Syrian hamsters (*Mesocricetus auratus*), a seasonal breeder. Similarly KISS-1 expression in sheep (*Ovis aries*) is down regulated outside the reproductive period (Clarke *et al.*, 2009). Recent studies have confirmed kisspeptin involvement in puberty onset and on

the gonadotropin cascade in teleosts: e.g. Nile Tilapia (*Oreochromis niloticus*) (Martínez-Chávez *et al.*, 2008), Atlantic cod (Cowan *et al.*, 2012; Karlsen *et al.*, 2014), sea bass (*Dicentrarchus labrax*) (Migaud *et al.*, 2012, Alvarado *et al.*, 2013), club mackerel (*Scomber 38ranulose*) (Selvaraj *et al.*, 2012); Senegalese sole (*Solea senegalensis*) (Mechaly *et al.*, 2012). Those studies suggested that the intermediate function of kisspeptin on the BPG axis through photoperiod (i.e. melatonin) has been highly preserved across vertebrates. However placental mammals have a single gene for the ligand (KISS) and for the receptor (KISSR) while fish have up to three KISS genes and up to four KISSR genes due to genome duplication events (Mechaly *et al.*, 2013).

KISS/GPR54 activates the GnRH cells in the hypothalamus and neurotransmitters that stimulate the gonadotroph cells in the pituitary gland. Secretion of GnRH into the hypophysial portal system stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary, which are transferred though the bloodstream to the gonads (Zohar *et al*., 2010). Gonadotropins act directly on the ovaries and testis stimulating the steroidogenic cells for steroid secretion: oestrogens, progesterone and androgens (i.e. 11-ketotesttosterone (11KT) (Migaud *et al*., 2010). In females FSH initiates vitellogenesis by recruiting vitellogenin into the ovaries and regulates egg growth, while LH secretion is delayed in respect to FSH, because it is involved in the final maturation and ovulation of the oocyte. In males, both LH and FSH, stimulate 11KT production during gametogenesis (Zohar *et al.*, 2010). The BPG axis regulators are integrated with the growth and energy pathways: e.g. growth hormone (GH), insulin-like growth factor (IGF-1), leptin, ghrelin, cortisol (Migaud *et al*., 2013).

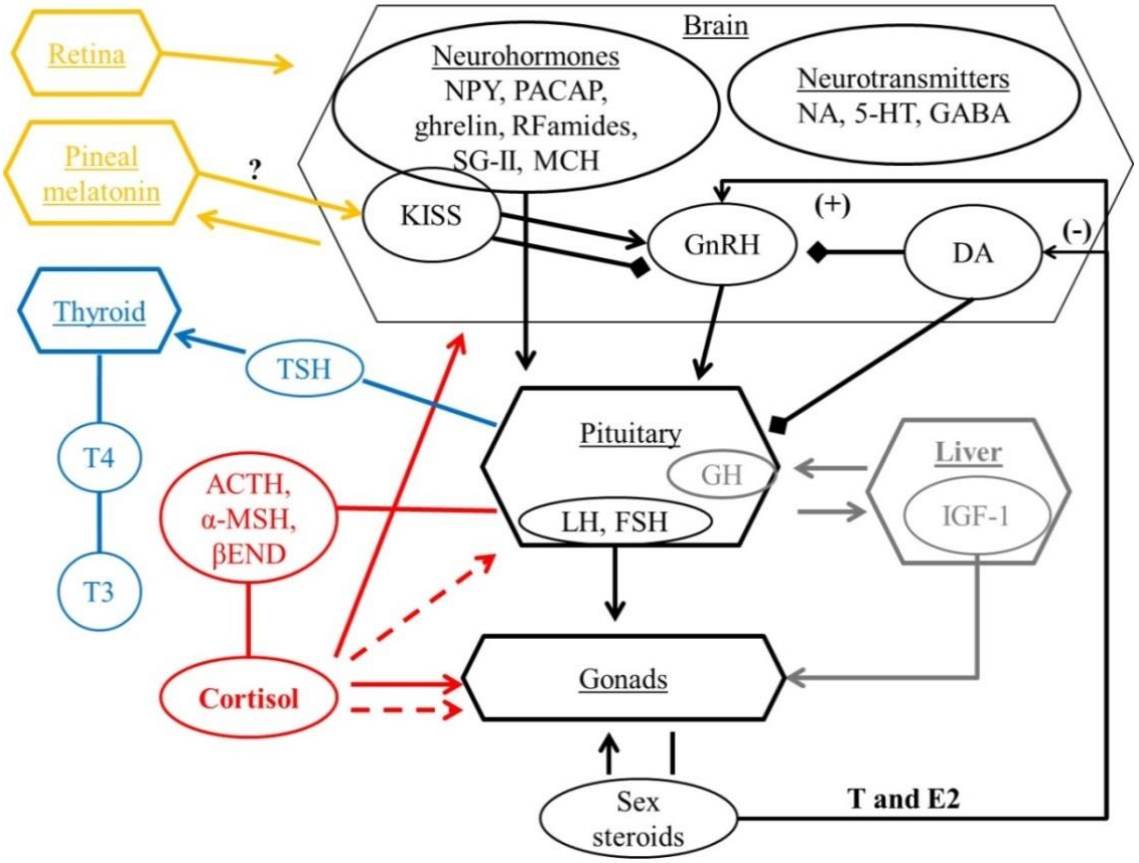

Figure 1.14. Schematic representation of the major factors involved in the control of the reproductive axis (black), growth and energy balance (grey), photic pathway (yellow), thyroxine secretion (blue) and 39ranulose39phin axis (red) on teleost. Solid arrows represent stimulation; diamond-end indicates inhibition and dashed arrow line indicated negative feedback. 5-HT; serotonin; α-MSH; alpha-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; DA, dopamine; E2, 17β.estradiol; FSH, follicle-stimulating hormone; GABA, γ-aminobutyric acid; GH, growth hormone; GnRH, gonadotropin-releasing hormone; IGF-I, insulin-like growth factor; KISS, kisspeptines; LH, luteinising hormone; MCH, melanin-concentrating hormone; NA, noradrenaline; β-END; non-acetylated opioid beta-endorphin; NPY, neuropeptide; PACAP, pituitary adenylate 39ranulo-activating polypeptide; Rfamides, RF amide peptides; SG-II, secretogranin-II; T, testosterone; T3, triiodothyronine; T4, thyroxine; TSH, thyrotropin-stimulating hormone. Adapted from Bernier *et al.*, 2009 ; Milla *et al.*, 2009 ; Migaud *et al.*, 2010 ; Taranger *et al.*, 2010 and Zohar *et al.*, 2010.

1.4.2. Gametogenesis

Oogenesis

Most fish are gonochoristic that release gametes annually: once (iteroparous) or several times (semelparous) during the life cycle; with some exceptions where sex changes depending on the population (i.e. hermaphrodites)

Lubzens et al. (2010) describe in detail the process of oogenesis through which a primordial germ cell develops into a mature egg. The basic stages of development are: a) previtellogenesis (oogonia multiply by mitotic division and become protoplasmic oocytes); b) endogenous vitellogenesis (Differentiation of follicular cells (theca and granulosa), nucleolus formation, yolk vesicle and lipid accumulation); c) exogenous vitellogenesis (gonadotropins stimulate the true yolk formation and accumulation into the eggs); d) final oocyte maturation (oocyte hydratation, germinal vesicle migration e.g. mGV and break down e.g. GVBD); and e) ovulation (expulsion into the ovary). Mature eggs are released into the water column and fertilised by the sperm which corresponds to the start of the embryogenesis (Lubzens *et al.*, 2010).

Some physiological and/or environmental factors can prevent the egg release which is then reabsorbed by the ovary. In both captivity as well as the natural environment, atresia occurs if reproduction is prevented when conditions are suboptimal: e.g. stress, food deprevation, interruption of mate selection etc. (Mañanós *et al.*, 2008). In captivity some fish species will mature and undergo final oocyte maturation and ovulation however they will not spawn in captivity, in these cases manual stripping and artificial fertilisation is required; this is the case of most flatfish species (Gibson, 2008). In this situation synchronisation between ovulation and stripping time is required to ensure that eggs are not going through the overipening

process, the impact of ovulation cycles on gamete quality is discussed further below (section 1.4.3).

Spermatogenesis

Spermatogenesis can be categorised in three stages: a) spermatogonial (germ cells multiply by mitotic division and are differentiated into spermatogonia); b) spermatocytary (spermatogonia are differentiated into primary/secondary spermatids by meiosis), and, c) spermiogenic (haploid spermatids differentiate into flagellated spermatozoa). Spermatozoa are released into the sperm duct by the break of spermatocysts (spermiation) (Schulz *et al.*, 2010). In the sperm duct it is mixed with the seminal fluid, both seminal fluid and spermatozoa form the milt.

1.4.3. Gamete quality

The success of a reproductive cycle is dependent on the quality of the gamete. Gamete quality is loosely defined as the ability for fertilisation and subsequent embryo development to occur (Bobe and Labbe, 2010). Gamete quality is key for the future of the progeny from successful fertilisation, embryonic development, hatching success, larval development and survival. In practical terms quality is assessed initially through simple parameters (which individually are not necessarily reliable indicators of quality): batch size, egg diameter, buoyancy, spermatozoa concentration and motility, fertilisation rate and development of the first blastomere divisions (Cabrita *et al.*, 2008, Bobe and Labbé, 2010; Migaud *et al.*, 2013). A symmetrical cleavage pattern with regular sized and shaped blastomeres are associated with higher survival (Shields *et al.*, 1997). Egg quality assessment gives an accurate forecast of future larval viability, as seen in several other marine species such as sea bream (*Sparus aurata*) (Lahnsteiner

and Patarnello, 2004), turbot (*Scophthalmus maximus*) (Kjørsvik *et al.*, 2002) and red porgy (*Pagrus pagrus*) (Aristizibal *et al.*, 2008).

Several physiological and environmental factors (Fig. 1.15) influence gamete quality including temperature, photoperiod, water quality, nutritional status, immune condition, age and size of the broodfish, hormonal disturbance, handling and stress (Bobe and Labbé, 2010, Migaud *et al.*, 2013).

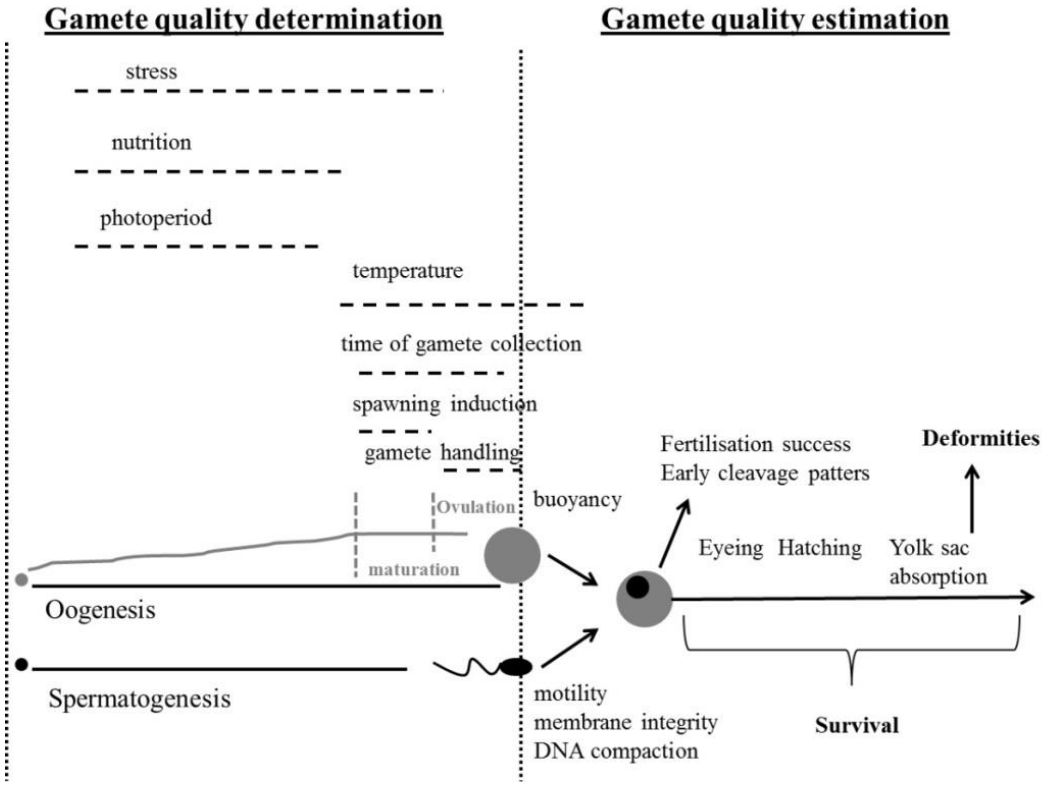

Figure 1.15. Main factors determining gamete quality in fish and practical estimation of gamete, embryo and larvae quality. Dotted lines represent time windows of influence during gametogenesis. Adapted from Bobbe and Labbé, 2010.

1.5. STRESS RESPONSE IN FISH

The definition of stress has been debated in the literature for many decades. Understanding stress requires the differentiation between a stress stimuli and the physiological stress response it triggers. A stress response was defined as any non-specific reaction threatening homeostasis (physiological equilibrium) (Chrousos, 2009). However, common physiological indicators of stress (e.g. blood pressure, blood glucose or intracellular osmolality) also respond to many biological processes and are therefore not always reliable stress indicators (Romero *et al*., 2009). The broad definition of stress as anything affecting homeostasis overlooks the actual implication on fish welfare caused by external signals (e.g. anthropogenic sources). Koolhaas *et al.* (2011) proposed that the definition of stress should be limited to the conditions in which environmental demand surpasses the intrinsic regulatory capacity of the animal, showing a physiological unpredictability (no anticipatory reaction) or limited recovery (uncontrollable). Furthermore, they propose that under this definition, stress effects should be analysed in terms of adaptive nature (homeostasis recovery: acute response) or maladaptive (compromise physiological performance: chronic). Taking into account the divergence of cause-effect between different stress stimuli and the system involved, might explain the variability between stress research results between species and within individuals (Barton, 2002). It is likely that a stress stimuli caused by excess heat will not be identical to light-induced stress. The stress response is triggered by a rapid input from the sensory system, triggering a behavioural, endocrine and metabolic responses in all levels of organisation (from the cell, individual organism, to the population structure).

1.5.1. Behavioural response to stress

Behavioural responses are widely used as welfare indicators in fish, more evident in enclosed systems. Behavioural changes (e.g. swimming activity of an individual or group), foraging behaviour, feed intake, food anticipatory activity, ventilator activity, aggression) are commonly used by the "well trained eye" in fish farms as indicators of fish welfare affections. Abnormal behaviour is a non-invasive indicator of a stress reaction in fish, however specific responses depend on the type and intensity of the stressor and the coping strategy of the individual (Koolhaas *et al.*, 1999; Martins *et al.*, 2012). The appearance of a behavioural response (or lack of it) is not a clear indicator of a stress reaction in fish. A true stress coping strategy involves a behavioural and physiological (biochemical and molecular) reaction. Coping strategies can be: proactive (fight-flight; low corticosteroid production) and reactive (conservation withdrawal; high corticosteroid production).These coping strategies are defined based on the immediate response of the fish to unexpected stress stimuli. Long term corticosteroid levels are influenced by the social hierarchy nonetheless they won't be discussed in this text.

1.5.2. Physiological stress reaction: Corticotropic axis

Stress exposure triggers a cascade of biochemical and physiological responses to cope with the stress stimuli and return to homeostasis. The stress response in teleost is broadly categorised into primary (e.g. stress hormone release), secondary (e.g. increased plasma glucose levels) and tertiary responses corresponding to the physiological effects (decreased productivity due to energy allocation: e.g. gonad reabsorption, immune suppression, restricted growth) (Pankhurst, 2011). The main endocrine outcome of a stress in fish is through the corticosteroid axis leading to the production and release of cortisol into the bloodstream (Fig. 1.16).

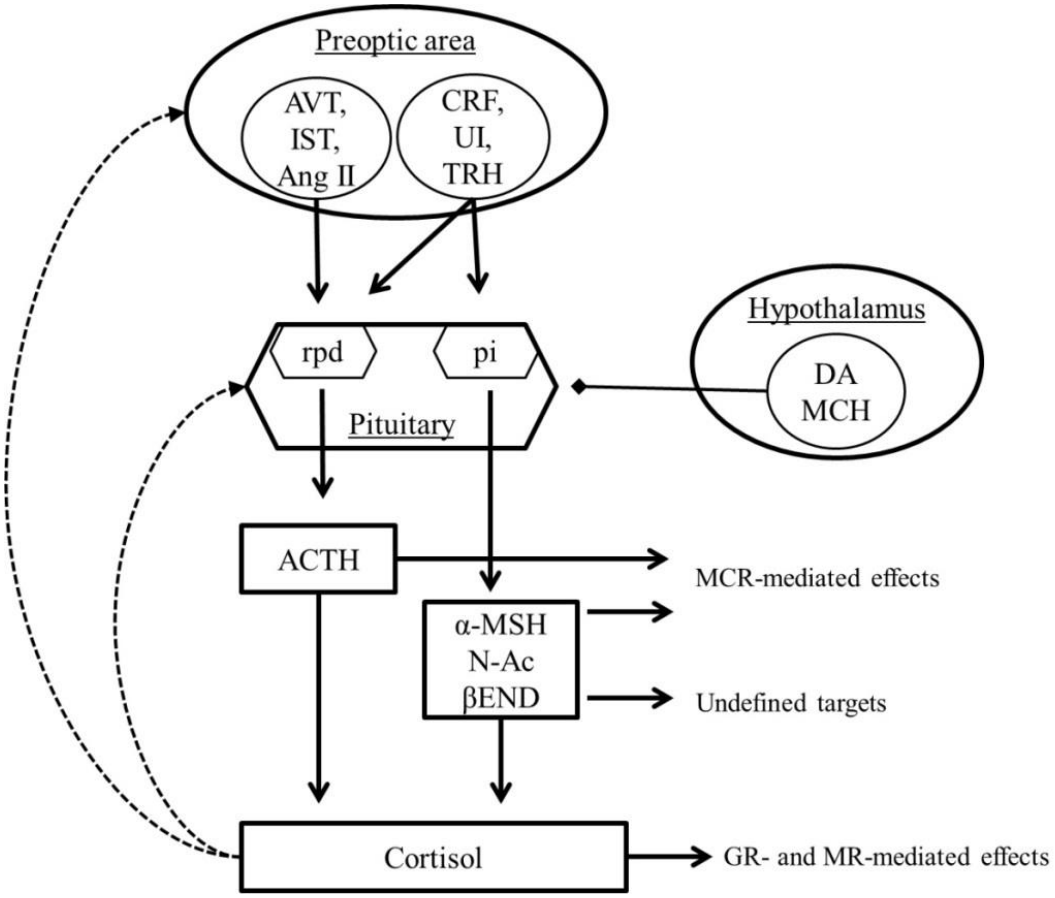

Figure 1.16. Schematic representation of the major factors that affect the activity of the corticotropic axis and melanotropic axis in teleosts. Solid arrows represent stimulation; diamond-end indicates inhibition and dashed arrow line indicated negative feedback. . α-MSH; alpha-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; Ang II, angiotensin II; AVT, arginine vasotocin; CRF, corticotropin-releasing factor; DA, dopamine; GR, glucocorticoid receptor; IST, isotocin; MCH, melanin-concentrating hormone; MCR, melanocortin receptors; MR, mineralocorticoid receptors; pi; pars intermedia; rpd, rostral pars distalis; N-Ac β-END; non-acetylated opioid beta-endorphin; TRH, thyrotropin-releasing hormone; UI, urotensin I. Adapted from Bernier *et al.*, 2009.

The cortisotropic pathway starts with the production of corticotrophin releasing hormone (CRH) in the hypothalamic nuclei that stimulates cells in the pars distal of the pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the vascular system. Cortisol is catalysed from cholesterol by enzymes of the cytochrome P450

family, similarly to the mammalian models in the intrarenal cells distributed in the head-kidney region (Sampath-Kumar *et al.*, 1996). Cholesterol is catalysed: 1) by cytochrome P450scc into pregnenolone; 2) which is catalysed by 17 α-hydroxylation (P450c17) into 17-OH-pregnenolone; 3) 3 β-hydroxy steroid dehydrogenation (3 β-HSD) synthetises 17-OH-progesterone; 4) further into 11-deoxycortisol by 21-hydroxylation (P450c21); 5) and deoxycortisol (P450c11) into cortisol (Lehoux *et al.*, 1972). The corticosteroid receptor (CR) consists of two classes (based on molecular sequencing): glucocorticoid receptors (GR) and mineralocorticoid receptor (MR) (Colombe *et al.*, 2000; Bury *et al.*, 2003). GR are present in different tissues: liver, kidney, gill, intestine, muscle and brain (Mommsen *et al.*, 1999). The glucocorticoid-responsive element (GRE) acts as a transcription factor regulating the expression of genes related to metabolic regulation, osmatic regulation and immune system (Flik and Wendelaar Bonga, 2001; Milla *et al.*, 2009).

Cortisol can also interfere with other hormone signalling as GRE lacks specificity and therefore can bind with other steroid receptors, particularly sex steroids involved in the control of gametogenesis (Mommsen *et al.*, 1999, Milla *et al.*, 2009, Schreck, 2010). The stress hormones mediate a secondary stress response resulting in the activation of several biochemical and physiological pathways (e.g. blood glucose, lactate or lactic acid, chloride, sodium, potassium, glycogen and Heat Shock Proteins) (Barton, 2002; Hori *et al.*, 2010). Stress itself is a high-energy demand process. The production of glucose provides a source of energy to respond efficiently to the stress stimuli, explaining why blood glucose levels are widely used as a stress indicator in fish. However, the secondary stress response varies widely between individuals and depends on the stress source. For instance, sea bass and sea bream exposed to noise as a stressor showed significant differences in haematocrits and lactate levels, while glucose

levels were only impacted in sea bream (Buscaino *et al*., 2010). Furthermore, it appears that the stress response in gadoids differs from the general teleost response. Cortisol increase remains the main indicator of physiological stress, while no glucose-stress response or hsp70 expression was found (Herbert and Steffensen, 2005; Afonso *et al.*, 2008, Pérez-Casanova *et al.*, 2008). As mentioned before the secondary, and therefore the tertiary stress response is species specific, likely linked to the metabolic rate of the species.

1.5.3. Corticosteroids and fish reproduction

In the previous section the discussion was focused on the general negative effects of cortisol in fish performance. In terms of reproductive performance chronic levels of cortisol can results in arrestment of gametogenesis and gonad atresia, failure of gonadal maturation, low gonad mass, reduced oocyte size, delayed ovulation, decreased plasma gonadotropins, steroids and vitellogenin (VtG) that all impact on the reproductive performances (Schreck *et al*., 2001; Leatherland and Barakataki, 2010). The neuroendocrine activation of the stress axis (Fig. 13) occurs as a response to a stimuli perceived as a threat, therefore it can overlap and interfere with other biological processes. Corticosteroids play a role in fish reproduction (gonads may be able to produce them). Some species exhibit an increase in plasma cortisol concentrations during the pre-spawning and spawning period (Noaksson *et al*., 2005, Westring *et al*. 2008).

However a physiological stress reaction would release high concentrations of cortisol that can affect the hormonal balance of the endocrine system. Furthermore, the stress behavioural reaction could interfere or prevent mate selection. Atlantic cod broodstock engaged in fewer and altered courtship sequences (Morgan *et al.*, 1999) and

abandoned spawning grounds (Engås *et al*., 1996) have been reported in relation to stress.

High levels of circulating cortisol in the female can be transferred to the eggs, impacting its' quality in terms of fertilisation ability (Mileva *et al.*, 2011). Schreck *et al.* (2001) suggested that females have a protection mechanism that would shield the eggs from over accumulating cortisol. Although its effectiveness will depend on the nature, severity and duration of the stressor, as well as the stage of gonadal development. However, high concentrations of cortisol in spawning cod females differentiated the expression of genes in eggs and embryos linked mainly to cytogenesis and mesodermal fate (Kleppe *et al.*, 2013) and resulted in a higher prevalence of deformities in larvae (Morgan *et al*., 1999). Cortisol is involved in fish embryonic development and metamorphosis (de Jesus *et al*., 1991; Hwang *et al*., 1992; Brown and Kim, 1995). Endogenous cortisol production starts after hatching, therefore initial levels are linked to maternal hormonal transfer. By any means an excess in cortisol transfer could result in severe malformations and have an impact on larvae performance.

In males, moderate doses of cortisol (<100 ng mL^{-1}) was shown to enhance DNA replication and mitosis in spermatogonia in Japaneese eel (Ozaki *et al*., 2006), however doses >100 ng mL^{-1} had a negative effect on spermatogonia proliferation. Excess cortisol levels inhibit the reproductive physiology of the males, delaying testicular development, spermatogenesis and result in lower sperm counts (Carragher *et al*., 1989; Campbell *et al*, 1992; Consten *et al*., 2001).

1.6. PIGMENTATION: MELANOTROPIC AXIS

Skin colouration is one of the most striking features of the teleost phenotypes. Fish pigmentation is a plastic feature that can be adjusted according to fish needs: social interactions (Grosenick *et al.*, 2007), camouflage (predator avoidance) (Doolan *et al.*, 2009) or mating selection (Grether *et al.*, 2005). Pigmentation (colour and pattern) is determined by the amount, type and distribution of the pigment cells (chromatophores), which vary according to the genotype, species, gender and developmental stage. Changes in skin colouration can be morphological (changes in the intracellular pigment material) or physiological (pigment motility within chromatophores). The latter can be: primary (caused by environmental factors: e.g. light) or secondary (nervous and endocrinal translocations: e.g. ACTH and alpha-melanocyte-stimulating hormone, α-MSH). Physiological colour changes are virtually instantaneous (visible within minutes or hours) caused by movements of pigment vesicles or reflective structures within their respective cell (Leclercq *et al.*, 2010). Morphological changes are slower and have a long lasting effect on the colouration. They are driven by changes in the skin pigment density and distribution (expand or contract) due to environmental stimuli (proximate) or as part of the phenotype transition between developmental stages (ultimate) (Leclercq *et al.*, 2010) (Fig. 1.17).

1.6.1. Chromatophores

Chromatophores are specialised cells that synthesise and store pigment structures that can absorb or reflect light. Based on their light sensitivity, chromatophores are categorised in: melanophores (black), erythrophores (red), xanthophores (yellow), iridophores (silver) and leucophores (white). In flatfish only three types of chromotophores are present: melanophores, xanthophores and iridophores (Bolker and

Hill, 2000). They originate during early embryo ontogeny from neural crest cells (Bolker *et al.*, 2005). During the embryonic and early larval stages, stem cells migrate symmetrically on both sides. During metamorphosis, cells undergo a second differentiation producing additional melanophores and complement pigment cells, which results in a blind side and the ocular side pigmentation pattern (Seikai *et al.*, 1987). The melanophores and xanthophores are dendritic cells that translocate light-absorbing pigment granules and store melanins and pteridines, respectively. Iridophores are not dendritic, but rather stacked refractosomes (transparent reflecting purine) with a non-fixed structure and organisation (colour reflectance depends on the angle of observation) (Menter *el al*. 1979).

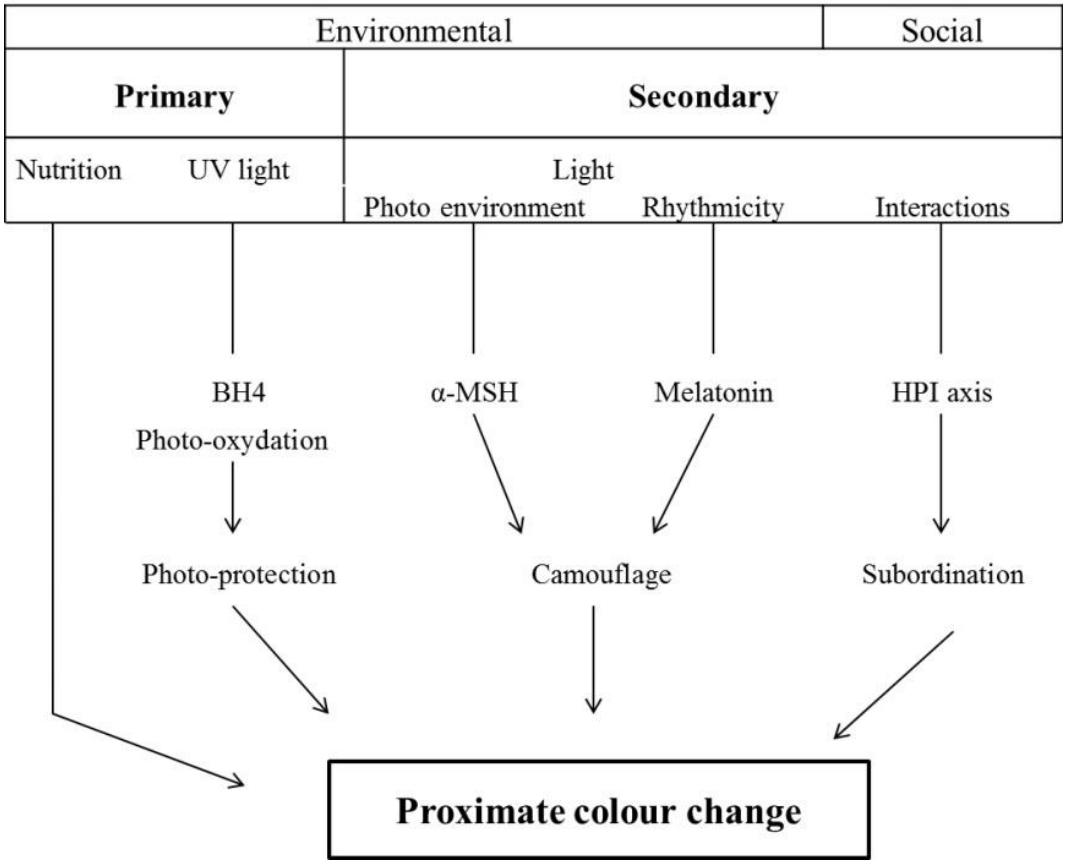

Figure 1.17. Simplified schematic representation of the types of proximate morphological changes by proximate environmental factors. Adapted from Leclercq *et al.*, 2010.

Flatfish pigmentation patterns respond easily to proximate stimuli, for example the rapid colour match from the upper side to that of the background (Healey, 1999). Light perception plays a crucial role allowing the dispersion or concentration of melanophores to match the photic environment. Furthermore, melanin synthesis in the chromatophores is initiated in flatfish before metamorphosis by the regulation of Melanocyte Stimulating Hormone form the pituitary (Suzuki *et al.*, 1998). Light intensity (and possibly spectral composition) play a crucial role in the initiation of the pigment synthesis and remain so during fish adulthood (Leclercq *et al.*, 2010). Incidence of abnormal pigmentation (albinism, malpigmentation or bicolouration) in flatfish larvae are increased by light intensity (darkness or high intensity) during rearing (Venizelos and Benetti, 1999). However, partially albino colouration can be corrected (prior the end of metamorphosis) when light intensity is adjusted closer to the optimal (Denson and Smith, 1997).

1.6.2. Involvement of the visual system

Since the early studies in the 19th century it became clear that vision plays a crucial role in the regulation of pigmentation in fish. Despite not being able to gain access to the original publications, Henslow (1872) highlighted in a letter to Nature that the work of Pouchet (1872) demonstrated that skin colouration mimicking background in prawns was prevented by eye ablation. Afterwards Pouchet (1876) further demonstrated that turbot skin was darkened by sectioning the peripheral nerves and whitened (pale colouration) by electrical stimulation of the spinal nerves (cited in Scott, 1965). More recently Kanazawa (1993) found that abnormally pigmented Japanese flounder (*Paralichthys olivaceus*) had no preference between light (exposed) and dark (covered) tank compartments, while normally pigmented fish would gather in the dark section. The author suggested that the differences in background preference were due to

an impaired visual system in the malpigmented fish. Supplementation of vitamin A and fatty acids (particularly: docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) in the diets improved pigmentation, likely driven by retinal rhodopsin formation that ultimately leads to melanin production. Vitamin A is a group of morphogenetic nutrients that fish are not able to synthetize and need to be absorbed though the diet. The active retinal form is used as the chromatophore of rhodopsins (Peppe, 1999), while other forms are involved in a range of functions: e.g. skeletal tissues development (Fernández and Gisbert, 2011).

1.6.3. Involvement of nutrition

Essential fatty acids are also involved in a whole range of functions, being critical for the nervous system development during the larval stages (Hamre *et al.*, 2013). Nutritional deficiencies in the artificial diets are linked to a higher incidence of abnormal pigmentation in aquaculture (Bolker and Hill, 2000). Turbot fry fed artificial diets rich in DHA achieved the highest pigmentation levels (Dhert *et al.*, 1993), however it has been suggested that the ratio between DHA to EPA is the key factor, rather than absolute fatty acid levels (Dhert *et al.*, 1993; Sargent *et al.*, 1997; Hamre *et al.*, 2013). Furthermore, turbot larvae fed live-prey enriched with high ratios of arachidonic acid (ARA) to EPA resulted in a higher incidence of abnormal pigmentation (Estevez *et al.*, 1999). The variance between results suggests that nutrition plays a crucial role in determining pigmentation success. However it is clear that pigmentation does not depend solely on the proximate factors but rather on their neuroendocre effects.

1.6.4. Endocrinology of pigmentation

The two major peptides associated with physiological pigmentation changes are melanocyte-stimulating hormone (MSH) and melanin-concentrating hormone (MCH) (Fig. 16). MSH is produced in the neurointermediate lobe of the pituitary gland derived from proopiomelanocortin (POMC) that stimulates pigment dispersion in the chromatophores (skin darkening). MCH is produced in the hypothalamus and secreted by the hypothalamus-pituitary complex through neural fibres and induces pigment aggregation (skin lighten) (Balm and Gröneveld, 1998). The opposing functions between MSH and MCH in pigment migration are clearly evident *in vitro* (Baker, 1993; Burton and Vokey, 2000). However *in vivo* MSH does not necessarily disperse pigments, because interrelation between them is masked by the dominant neural effects (Mizusawa *et al.*, 2013). Five melanocortin receptors (MCR) subtypes (MCR1 – MCR5) have been cloned in tetrapods, highly homologous in lower vertebrates. The number of receptors diverges between fish species (Metz *et al.*, 2006; Kobayashi *et al.*, 2008; Sánchez *et al.*, 2009 a, b and 2010; Kobayashi *et al.*, 2010): e.g. zebrafish possess six (two copies of MCR5) and pufferfish lacks MCR3.

1.7. Interactions beteween Corticotropic, Melanotropic and Thyrotropic axis

MCR regulate melanogenic activity through high affinity to α-MSH but also to ACTH (Selz *et al.*, 2007). MSH (α-, β, γ-MSH) shares POMC as the common precursor with the ACTH (Metz *et al.*, 2006). Fig. 16 integrates the major factors affecting the melanotropic and corticotopic axis in fish. It is important to mention the contribution of the melanotropic axis on the endocrine stress response is species specific and depends on the nature and intensity of the stress stimuli. For instance, in sea bream (*Sparus*

aurata) air exposure (3 min) resulted in increased plasma levels of cortisol, α-MSH, glucose and lactate; but ACTH and βEND were maintained. Stress by confinement (70 Kg m^{-3}) triggered a rapid increase (1 h) of cortisol, ACTH and α-MSH (Arends *et al.*, 1999). Plasma α-MSH differed based on background adaptation (highest in the white background), but plasma cortisol levels were similar between treatments (Arends *et al.*, 2000). Similarly, white background increases aggressive behaviour and skin darkening in Arctic charr (*Salvelinus alpinus*) (Höglund *et al.*, 2002). The skin darkening was firstly correlated to a stress-induced reaction due to the higher plasma levels of α-MSH, ACTH and cortisol (Höglund *et al.*, 2000). It appears that multiple interactions interconnect the corticotropic and melanotropic axis. However, available information is scarce or contradictory, mainly due to the different experimental approaches, species analysed and the still undefined targets and functions of these hormones in fish. For instance, skin darkening in sole was correlated to cortisol levels but not α-MSH (Ruane *et al.*, 2005).

Pigmentation is a complex mechanism that can be adapted based on the needs of the fish but also reflects the physiological status of the animal. The narrow connection with other systems (i.e. food intake, growth and stress) are not fully understand and further research is required to clarify the mechanisms behind skin pigmentation and its interaction with other mechanisms, mainly stress and nutritional status.

1.8. ENDOCRINE REGULATION OF FEED INTAKE

Food intake regulation is a complex process that involves central and peripheral factors, stimulated by environmental signals, energy reserves, metabolic rate, developmental stage and reproductive status. The indeterminate growth of fish results in a continuous resource allocation between reproduction and growth. The sole role of

food intake regulation is to maintain the energy homeostasis through orexigenic (appetite stimulation) and anorexic (appetite inhibition) factors (Volkoff *et al*., 2005). The brain (mainly the hypothalamus) and peripheral hormones regulate food intake, through signalling energy status of different systems. This added to the interaction between hormones and apparent shared functions of several orexigenic and anorexic factors, results in a complex neuroendocrine process that is still not fully understood. Neuropeptides interact closely with each other and in parallel. The orexigenic and anorexic factors are briefly described, followed by the current knowledge of interaction between different neuroendocrine systems relevant for this thesis.

1.8.1. Orexigenic factors

Neuropeptide Y (NPY) family consists on NPY, peptide YY (PYY), pancreatic polypeptide (PP) and peptide Y (PY). They regulate food intake in teleost modulating other appetite regulators. So far five receptors have been cloned: Y1, Y2, Y4, Y5 and Y6 (Larhammar *et al*., 2001) widely distributed in the CNS, pituitary and gut (Volkoff *et al*., 2009). NPY is a strong orexigenic factor and levels undergo periprandial variations in several species: e.g. Atlantic cod (Kehoe and Volkoff, 2007). Orexins (OX-A and OX-B) are peptides produced in the brain including the hypothalamus, in pituitary and in peripheral tissues (i.e. gut) (Xu and Volkoff, 2007). Galanin (GAL) is produced also in the brain, pituitary and perfierial tissues (i.e. olfactory bulb in the goldfish) (Unniappan *et al*., 2004). Agouti-related protein (AgRP) physiological role in fish is unknown (Volkoff *et al*., 2005), however in mammals AgRP is involved in the control of energy homeostasis and feeding. Furthermore the agouti-signalling protein (ASP) is a competitive antagonist of the melanocortin receptors: MCR1 and MCR4. Ghrelin is predominately secreted in by the stomach and into less extent in the brain. In

mammals, ghrelin stimulates appetite (by activation/inhibition of AgRP, NPY and POMC neurons) and GH secretion (Schmid *et al*., 2005). The orexigenic function of ghrelin has been confirmed in some fish species (e.g. Miura *et al*., 2009) and its interaction with other appetite peptides (NPY and OX) (Miura *et al*., 2006 and 2007).

1.8.2. Anorexic factors

Cocaine- and amphetamine-regulated transcript (CART I and CART II) peptides are present in the fish brain, pituitary and peripherial tissues (i.e. gonads and kidney) (Singru *et al*., 2007). Human CART injection inhibit feeding in goldfish (Volkoff and Peter, 2000), however orexigenic actions have also been reported in rats (Abbot *et al*., 2001). CART inhibits both NYP and OX-A (Volkoff and Peter, 2000) and interacts with leptin (Volkoff *et al*., 2003). Cholecystokinin (CCK) is structurally related to gastrin. CCK mRNA is detected in the brain, pituitary and intestine (Peyon *et al*., 1999). It is released when food is present in the intestine, influencing digestion, feeding processes and inhibiting appetite (Volkoff *et al*., 2003). Bombesin and gastrin-releasing peptide (BBS/GRP) regulate gut motility in several fish species (Bjenning *et al*., 1991) and is expressed in the gastrointestinal tract, cardiovascular system and CNS (Bjenning *et al*., 1991; Himick *et al*., 1995). Glucagon-like peptide (GLP) is produced in the pancreas and intestine. GLP induces anorexia and causes gastric emptying (Plisetskaya and Mommsen, 1996). Leptin is mainly expressed in the fish liver, brain, pituitary and peripheral tissues (e.g. ovary of pufferfish) (Kurokawa *et al*., 2005; Wong *et al*., 2007). In most fish species, leptin decreases appetite and increases fat metabolism (Londraville and Duvall, 2002). Tachykininis (TKs) is expressed in the CNS, it inhibits appetite and is involved in feeding and digestion processes (Peyon *et al*., 2000).

1.8.3. Interaction with the Melanocortin axis

In fish and mammals it appears that the MCR4 play a key role in the regulation of food intake and energy balance (Metz *et al*., 2006). In zebrafish larvae, α-MSH and AgRP cells in the hypothalamus are more pronounced coinciding with the appearance of active feeding behaviour (Forlando and Cone, 2007). In goldfish feeding was inhibited by central injections of NDP α-MSH (MCR4 agonist) or MT II (MSH agonist), but does not modify mRNA levels of POMC in the brain (Cerdá-Reverter *et al*., 2003; Matsuda *et al*., 2008). MCH involvement in food intake regulation remains controversial; however in mammals it is involved in energy homeostasis and feeding. In goldfish MCH reduces food intake and fasting decreases MCH-like immunoreactive (ir) neurons (Matsuda *et al*., 2007). The process seems to be regulated through NPY and MSH but not CRF, PACAP or CCK (Shimakura *et al*., 2008; Matsuda *et al*., 2009). Controversially barfin flounder reared in a white background presented enhanced body growth and greater expression of the MCH gene and number of MCH neurons (Takahashi *et al*., 2004).

1.8.4. Interaction with the Corticotropin axis

One of the additional involvements of the CRF system appears to be the regulation of food intake and energy balance, linked to some extent to the stress response (Richard *et al*., 2002). CRF-related peptides play a role regulating appetite in fish through increased CRF mRNA in the forebrain (Bernier and Peter, 2001; Doyon *et al*., 2003). Based on mammalian models it appears that CRF modulates gastrointestinal motility and gastric emptying (Wang *et al*., 2001; Tache and Perdue, 2004), however the CRF-related peptides interactions knowledge with food intake regulation in fish are

still in its infancy (Bernier *et al*., 2004). Moderate chronic administration of cortisol stimulates food intake, by increasing NPY and decreasing CRF brain expression, while high doses does not impact food intake or NPY expression (Bernier *et al*., 2004).

1.8.5. Influence of light on feed intake

Feeding activity is regulated by photoperiod likely by increasing food intake and/or muscle mass by exercise (Boeuf and Le Bail, 1999). Melatonin administration reduces food intake and body weight in goldfish, but does not affect plasma leptin, ghrelin or brain NPY levels (Pinillos *et al*., 2001, De Pedro *et al*., 2008). Studies on the effects of light intensity in growth are normally focused on fish growth in term of the visibility of the prey or food-stuffs and a direct implication in appetite stimulation has not been investigated (Bouf and Le Bail, 1999). In terms of the light spectrum, the information is rather scarce. Nile Tilapia (*Oreochromis niloticus*) when subjected to a photoperiod of 12L:12D, presented a reduced weight gain under red light as opposed to blue, violet, yellow and green light (Luchiari and Freire, 2009). Controversially, Volpato *et al*., (2013) reported that tilapias appetite was stimulated by red light without a visible effect in growth rates. Furthermore, tilapia exposed to blue light increased their reproduction success (i.e. fertility) (Volpato *et al*,. 2004) and reduced stress related plasma cortisol levels (Volpato and Barreto, 2001). In other fresh water species results vary. Light spectrum did not have any influence in yellow perch (*Perca flavescens*) growth parameters or plasma cortisol (Head and Malison, 2000). Growth was enhanced by green and yellow light in Crucian carp (*Carassius carassius*); green and blue light in Chinese sleeper (*Perccottus glenii)* and blue light in guppy (*Poecilia reticulata*), while red light impacted negatively all of them (Ruchin, 2004). Red light increased only brain dopaminergic activity in gilthead sea bream, with no other effect (Karakatsouli *et al.*,

2007). Growth and blood parameters did not differ in European sea bass (*Dicentrachus labrax*) between blue and white light treatments, however stress (1 h confinement by reducing water level) increased haematocrits and plasma cortisol (but not glucose) in fish exposed to blue light (Karakatsouli *et al.*, 2012).

1.9. EXPERIMENTAL AIMS

The current global status of marine aquaculture is below the projections and estimations from different international organisations as reported by FAO (2013). Industry and research have directed their efforts together to improve fish welfare and satisfy the biological requirements of cultured species, which at the same time would make the marine aquaculture industry profitable. Marine farming faces several bottlenecks throughout the production cycle, from low survivals and malformations, to slow grow-out phases and early maturation in captivity which limit its expansion. Fish are the most diverse group of vertebrates, occupying a vast range of ecological niches and environmental conditions and therefore optimised conditions in captivity must be defined. Irrespective of the evolutionary divergence, fish share, to some extent, common features including: locomotion and buoyancy, senses, metabolism and digestive extraction; and water balance. Many of those requirements have been addressed in the few scientifically studied species up to date. Environmental stimuli regulate or modulate different biological processes: from basic functions (e.g. vision, communication) to the regulation of complex neuroendocrine systems. The experimental aim of this work was to address some of the overlooked environmental conditions to which fish are exposed in captivity (e.g. stress through sound and light).

The studies of this thesis were focused on Atlantic cod and turbot, two commercially important species. Cod is a very valuable species in Europe that appears

to be very sensitive to light and noise, allowing the results of those studies to act as a baseline for other species in Chapters 3 and 6. Turbot is a well-known farmed species in Europe and is rapidly expanding to the Americas, Asia and Africa. Despite the apparent success, several limitations need to be addressed (i.e. malpigmentation, natural spawning and larval rearing/survival). Furthermore, chapter 4 allowed a direct comparison between the two marine species with fundamental physical differences.

The thesis is divided into five experimental chapters addressing the specific aims listed below:

Chapter 3. Stress response to anthropogenic noise in Atlantic cod (*Gadus morhua L.)*

a) To map out the sound levels produced within an aquaculture facility, with emphasis on sound disturbances created by anthropogenic activities.

b) To investigate the short term effects of sound levels similar to common husbandry activities in Atlantic cod juveniles and study sound stress physiological reaction.

c) To investigate the effects of chronic noise exposure on Atlantic cod broodstock reproductive performance

Chapter 4. Effects of light spectrum and tank background colour on Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) larvae performance.

a) To investigate the effects of light spectral composition on larval performance and survival of Atlantic cod and turbot.

b) To investigate the effects of tank background colour on larval performance and survival of Atlantic cod and turbot.

Chapter 5. Effect of light wavelengths on growth, appetite, skin pigmentation and stress response in turbot (*Scophthalmus maximus*) juveniles.

a) To investigate the effect of light spectral composition on turbot juvenile appetite and growth.

b) To investigate the effects of light spectral composition on short and long term skin pigmentation of turbot juveniles.

c) To investigate the effects of the light spectral composition on physiological stress response in turbot.

Chapter 6. Effects of twilight illumination on Atlantic cod (*Gadus morhua*) spawning performance

a) To investigate the effects of light dimming systems recreating crepuscular conditions (Dawn/Dusk) conditions on Atlantic cod broodstock reproductive performance.

Chapter 7. Effects of breeding nest on turbot (*Scophthalmus maximus*) spawning performance

a) To investigate the effects of breeding nests (sand substrate) on turbot broodstock reproductive performance.

Chapter 2

General Materials & Methods

The following section is a general description of the materials and methods utilised during the experimental procedures sample and data analysis for the studies contained in this thesis. Specific chapter material and methods are described independently in its corresponding chapter of this thesis.

2.1.Location

All the experiments of the present work were carried on at the facilities of Viking Fish Farms Ltd, Ardtoe Marie Laboratory, Ardtoe, Acharacle, Scotland (N 56°46' W 05°53'). All the experiments were carried out in compliance with the Animals (Scientific Procedures) Act of 1986, United Kingdom and following approval from the Institute of Aquaculture, University of Stirling, local ethical review committee.

2.2.Animal husbandry

The seawater supply was from a pumped shallow water intake 500 m off-shore and passed through mechanical filtration systems (sand filters followed by 5 µm cartridges filters) before UV sterilisation. Mean annual water temperature was 10.5 ± 3.0 °C ± SD (ranging from 5.5 to 15.4 °C) with a salinity of 34.4 ± 0.4 ppt ± SD (ranging from 32.0 to 35.5 ppt). All fish used in the experiments were from hatchery reared sources as described in the specific chapters. In all cases fish were held in enclosed tank facilities where they were fed manually *ad libitum* using commercially available food. Larvae were reared under continuous photoperiod (24:00 LL:DD), juveniles under an artificial (12:12 LL:DD) and broodstock under ambient photoperiod corresponding to the laboratory geographical location as explained in the specific experimental chapters.

2.3.Sampling procedures

2.3.1. Anaesthesia and euthanasia

All experimental procedures were preceded by anaesthetic baths freshly made at regular intervals. In all cases fish showed signs of equilibrium loss within 3 minutes. Full recovery (within 5 min) took place in a well aerated tank filled with clean sea water. Atlantic cod juveniles from Chapter 3 were anaesthetised in a tricaine methanesulponate (50 mg L^{-1}, Pharmaq, Fordingbridge, UK) bath. turbot juveniles for Chapter 5 were anaesthetised in a bath containing 2 ml of 2-phenoxyl ethanol (Sigma-Aldrich, UK) per litre of sea water. No accidental mortalities were associated with this procedure. Fish larvae (Chapter 4) and juveniles (Chapter 5) were sedated in a bath containing a lethal dose of 2-phenoxyl ethanol (8 mL L^{-1}) prior to decapitation.

2.3.2. Blood sampling

Blood was collected from anaesthetised or sacrificed fish from the caudal vein into 2 mL sterile syringes with a 25G hypodermic needle (Terumo N.V, Belgium) and transferred to 1.5 mL eppendorfs. Prior to sampling syringes were fitted with a needle and flushed with a 4 mg mL^{-1} solution of ammonium heparin salt (Sigma-Aldrich Ltd., UK). Within 10 min of the blood collection, samples were centrifuged at 750 g for 15 min and plasma transferred in 0.5 mL eppendorfs then stored at -20°C until further analysis.

2.3.3. Egg sampling

In chapter 3, 4, 6 and 7 freely spawned eggs were collected from egg net collectors located externally to the spawning tanks. The collector consisted of a conical mesh bag (5 µm mesh size) floating on the surface of a 2 m^3 external tank. A surface skimming outflow pipe drawing 10 cm of surface water fed into the centre of the

collection net with released eggs being retained in the net while excess water flowed to waste. Nets were inspected daily at 15:00 hrs with any released eggs being transferred to a clean 19 L white plastic bucket and then the net was rinsed thoroughly with a pressure hose supplying fresh water before returning to the external tank.

In chapters 4 and 7 as well as collecting freely released eggs, artificial fertilisation was also performed which required manual stripping of turbot gametes. Tanks were drained down to a 50 cm water level at least 2 hours prior stripping. Fish were uplifted by hand into a stripping table inside the tank and eyes covered with a wet towel and the gonadal area dried with paper towel. Eggs were collected into disinfected dry 2 L plastic jugs, egg batches from different females were usually mixed in the same jug. Once the collection jug contained at least 1 Kg of eggs, males were manually selected. Milt was collected with a 2 mL sterile syringe by applying pressure to the testis. Milt from 2 or 3 males was poured into the collection jug containing the eggs and left in darkness for 10 min. After which the mixture was transferred into the laboratory where the 2 L jug was filled with clean sea water and left in darkness for 30 min. After that, the mixture was gently poured into a 12 L transparent plastic aquarium and filled to 10 L and left in darkness for 2 more hours. The floating fraction was poured into a fine mesh (5 µm) and weighed; both floating and sinking fractions were recorded. The subsequent incubation process is defined in the material and methods section of each chapter.

Egg quality parameters were evaluated (total volume, floating fraction, sinking fraction, fertilisation rate, egg diameter, and embryonic developmental stage) for each batch. Floating and sinking fractions were measured independently by gently pouring the eggs into a fine mesh (5 µm) and later into a plastic bowl containing water, total egg volume was the sum of both floating and sinking fractions. A sample from the floating fraction (n = ~50) was taken to estimate: fertilisation rate, egg diameter and embryonic

developmental stage under the microscope. In addition in chapter 3 eggs from both, control and noise tanks, were treated as described above and triplicate samples (0.2 g) were taken to calculate the number of eggs containing in one gram of wet weight. Triplicated samples were taken from the floating and sinking portions in a 1.5 mL eppendorf (~1.5 g of eggs each) and kept at -20°C for cortisol content analysis as measured by radioimmunoassay as described below.

2.3.4. Morphological and weight measurements.

Morphological measurements (individual fish total length and wet weight) were recorded on anaesthetised or recently euthanised fish greater than 1 g, with length being measured to the nearest 1 millimetre and weight to the nearest 0.01 g. Larvae morphological measurements (Fig. 2.1) were determined through the use of digital image analysis to calculate individual standard length, myotome height, eye diameter and yolk sac diameter.

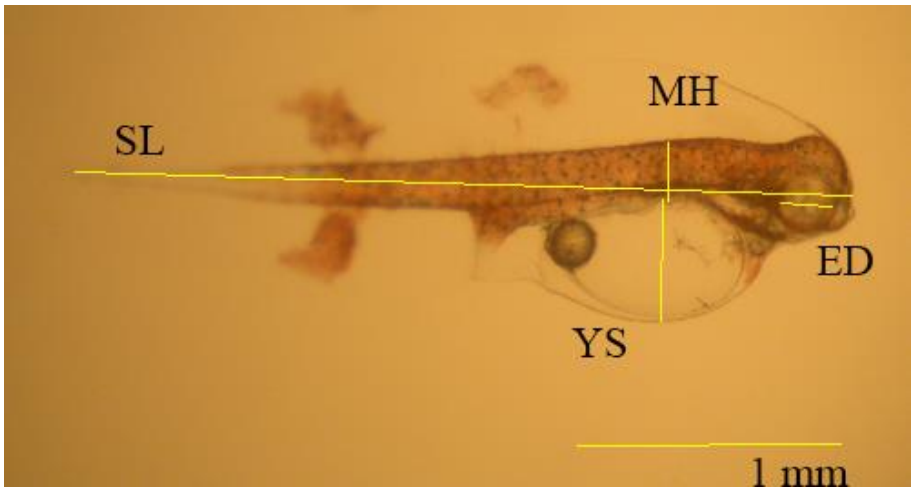

Figure 2.1. Turbot larvae morphological measurements through image analysis: SL, Standard lenght, MH, myotome height, ED, eye diameter and YS, yolk sac diameter. Scale 1 mm.

Image analysis was carried on in all experiments using freeware software based on java Image J from the National Institute of Health (NIH, USA http://rsbweb.nih.gov/ij/). The image analysis methodology was adapted from Rajaee

(2011). For the pigmentation analysis an additional RGB analysis software plug-in was required, available in the same website (http://rsb.info.nih.gov/ij/plugins/rgb-measure.html). The image analysis methodology was tested and validated for turbot juveniles pigmentation by comparing the RGB measurements from 144 individuals performed using the "wand tool" *v.s.* the manual selection tool (variation between results was below 2% between methods).

Photographs taken with a digital camera (either SZH-ILLD, Olympus Optical Company, Japan. or VMS-001 200x USB Digital Microscope, Veho, UK) were then processed using the Image J package, all dimensions were measured to the nearest 1 µm. Whole body wet weight was determine in situ (SK-WP scale A&D weighting, UK OR ICETAC PRO Mini pocket scale 200g/0.01 g). In Chapter 4 larval dry weight was used instead of wet weight due to the small size of the animals being studied. In these situations, triplicated pre weighted (AFA-210LC, Asten Instruments Ltd., UK) 0.5 mL Eppedorfs (weight recorded to the nearest 0.01 g) containing 10 larvae from each treatment replicate were washed with fresh water three times. Water was removed using a disposable plastic pipette and then eppendorfs were frozen at -20°C for at least 72 hrs prior to freeze drying over 24 hrs (Edwards Freeze Drier Micro Modulvo). Individual dry weight was calculated as follows:

$DW = [(W_1 – W_0)/ n];$

where W was the weight of the eppendorf at the given time; and n, number of larvae in the eppendorf (n = 10).

2.3.5. Egg and larvae image collection and analysis

Individual larvae photographs were taken with a digital camera (either SZH-ILLD, Olympus Optical Company, Japan. or VMS-001 200x USB Digital Microscope, Veho, UK) depending of the fish size. Each batch of photographs was preceded by a

picture of a scale/ruler (to the nearest 1 µm/ 1 mm, respectively) to determine the image scale during digital analysis. The image analysis was performed as follows:

Step 1: The replicate group of Images were imported as RGB (8 bit) image files (original files were created as .jpg). Using the "line selection" tool a distance in the ruler was selected and in \Analyse\Set scale…\ the actual scale of the image determines (Fig. 2.2). This process was repeated with each batch of photographs. By doing this the software transforms the distance (sample resolution 1 µm) in pixels selected into the known actual size established through the ruler.

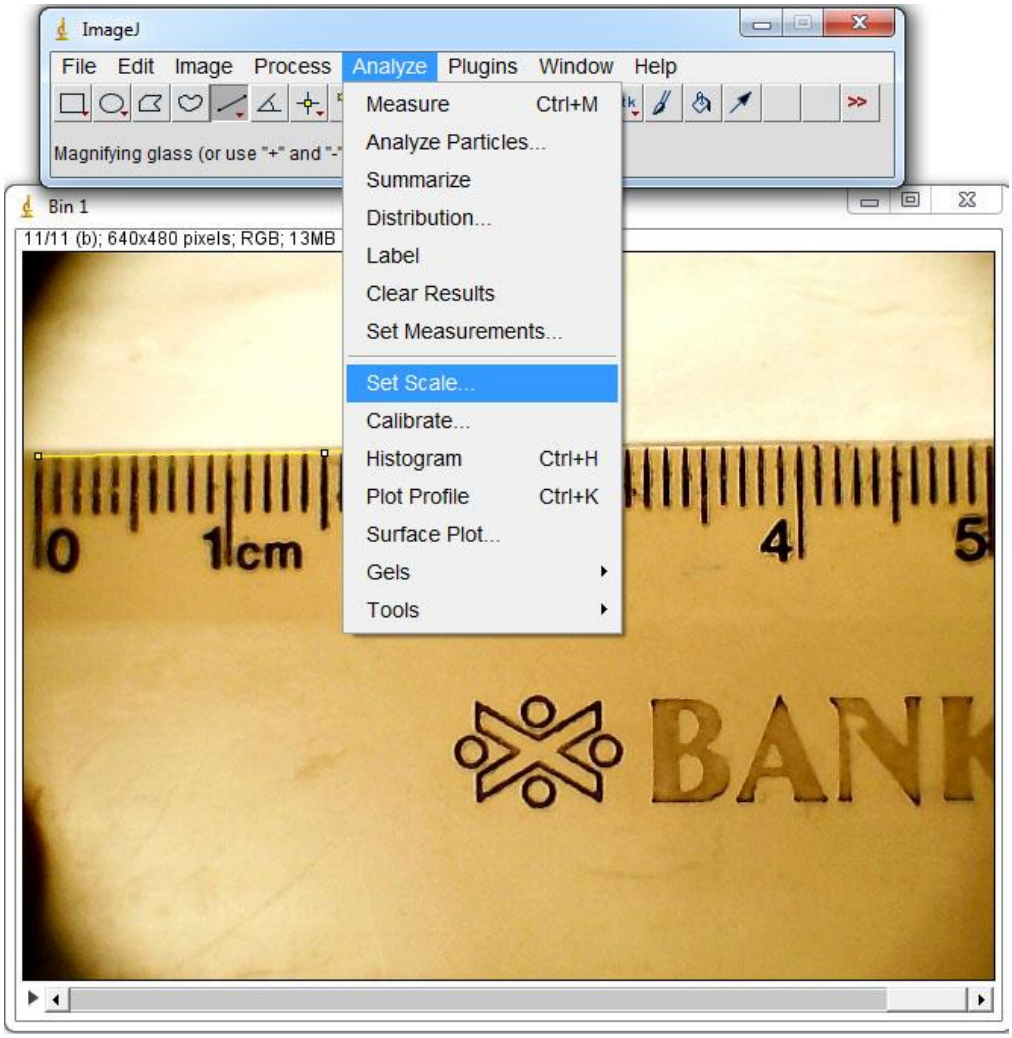

Figure 2.2. View of the software ImageJ to set scale of the imaging for further analysis.

Step 2: Using the "line selection" tool larvae morphological measurements (Fig. 2.3) (standard length, myotome height, eye diameter and yolk sac diameter when appropiate) or egg diameter were measured. The image was zoomed in when needed, mainly to measure the eye diameter accurately. Measurements were obtained through \Analyse\Measure…\ and a second table with the values appeared. Once all the images were processed, the table was copied into Office Excel software as raw data.

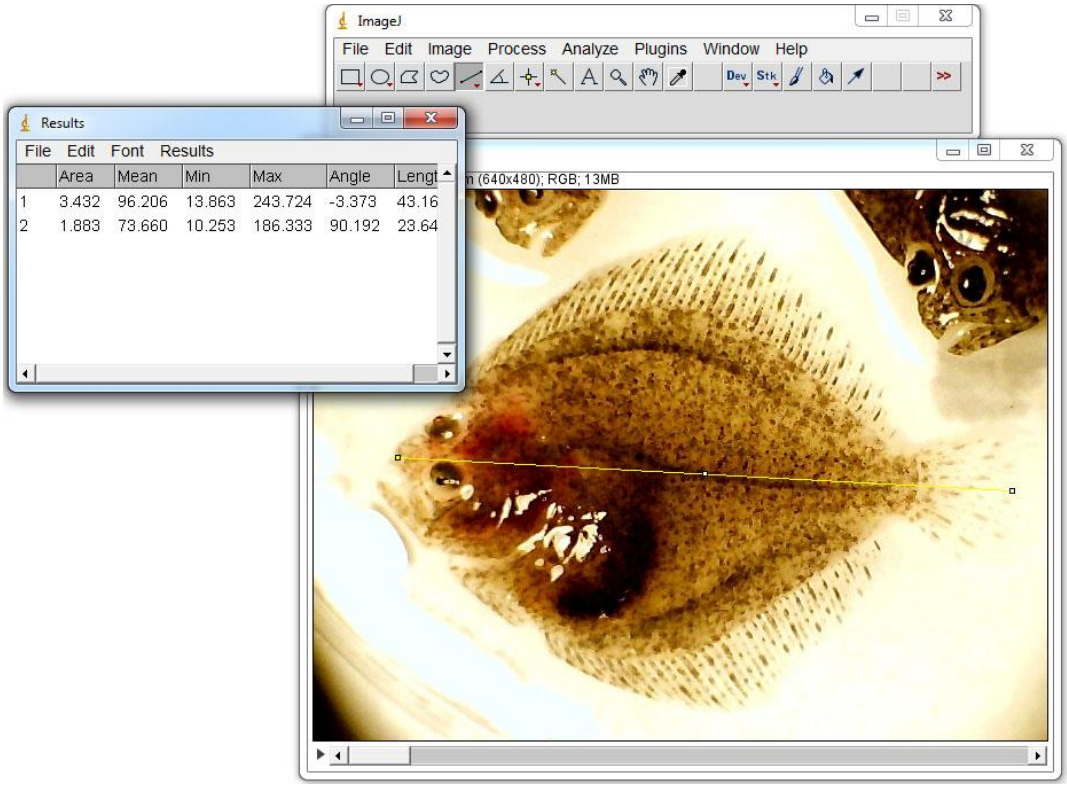

Figure 2.3. View of an example of the measurement of the standard length of a turbot larva.

2.4.Sound trials

In chapter 3 we investigated the effects of anthropogenic noise as a stressor in Atlantic cod. First we determined the common anthropogenic sound levels and cod vocalisations within the enclosed facilities. The experiment consisted in exposing fish to previously established sound levels (noise) in juveniles for an acute response and in broodstock for a chronic effect. Different specialised audio equipment and software

were required to ensure accurate measurements and controlled noise exposure. A description of the equipment and experimental settings used are described below.

2.4.1. Sound recording equipment

Sound data was collected using an omnidirectional hydrophone (RESON TC4034, usable Frequency range: 1 Hz to 470 kHz +3,-10 dB; receiving sensitivity -218 dB ±3dB (at 250Hz) (Avisoft Bioacoustics, Germany) with a 10 m cable (DSS-2 Mil C-915 Twisted pair + Shield). Amplifier UltrasSoundGate Charge with Hi-Pass filter (Avisoft Bioacoustics, Germany) with cut-off frequencies below 25 Hz for facilities sound mapping, 100 Hz cut-off for noise exposure trials and cut off of 0 Hz for fish vocalization recordings. Differences in the cut-off frequency were determined based on the nature of the sound. Cut-off frequency for the facilities sound mapping was set at 25 Hz to reduce the recording pollution generated by the power installation of the facilities (e.g. bulbs, transformers) and to standardise between tank sizes and locations. Noise exposure experiments were set at 100 Hz to accurately measure the sound levels created by the audio generator (underwater speakers min exposure frequency is 100 Hz), therefore lower frequencies were reflection of the noise exposure. Cod vocalisation monitoring required to record the lowest frequencies possible, to do so no cut-off frequency was used and a holding tank away from the electrical installation monitored.

Sound data was digitalised though a USB 2.0 audio interface pre-amplifier (E-MU tracker pre 24-bit/192 kHz, E-MU, USA) and Avisoft SASLab Pro software (Avisofts Bioacoustics, Germany) to a computer hard disk (Toshiba Satellite L635-S3011, Japan) at a sampling rate of 48 kHz with 16-bit resolution (Fig. 2.4).

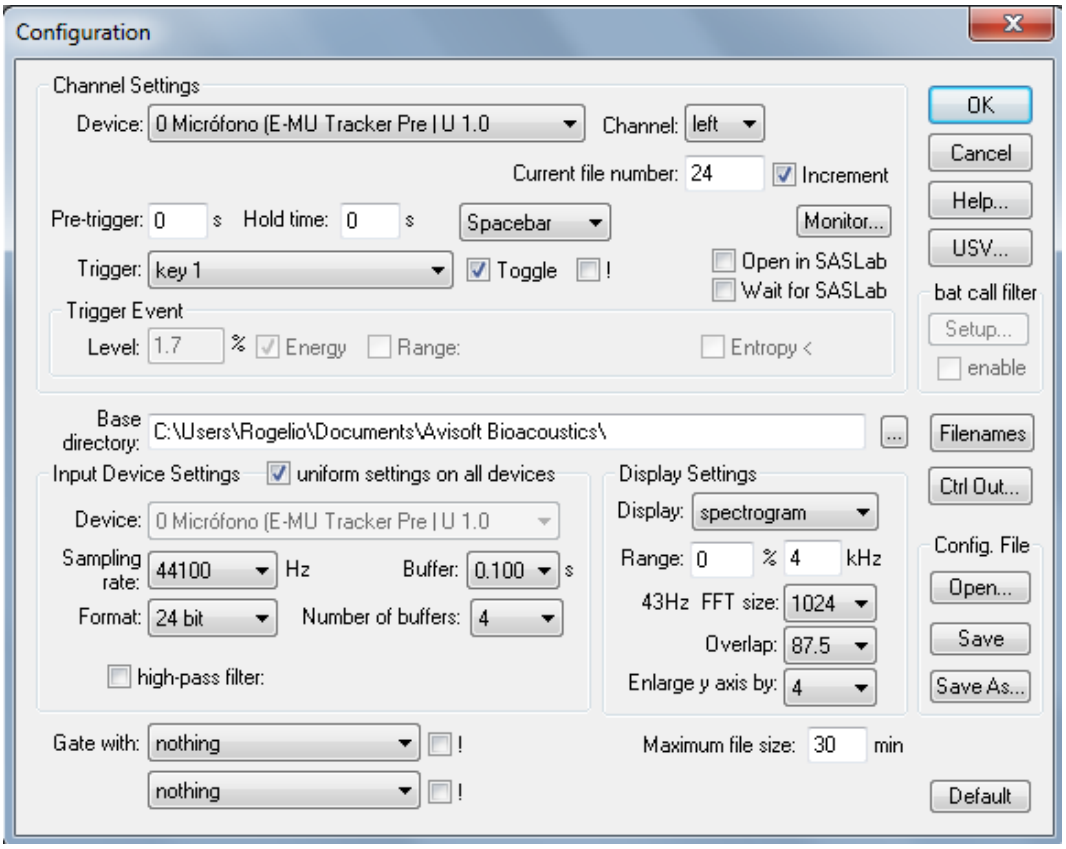

Figure 2.4. View of the configuration settings of the Avisoft recorder software used during the sound data collection. Sampling rate 44,100 Hz, format 24 bit

2.4.2. Sound exposure

The experimental noise exposure for Chapter 3 Experiment 3.1 was created using an audio function generator (PM 5131 0.1 Hz – 2MHz, Philips, Germany) transmitted into the tanks through suspended omnidirectional underwater loudspeakers (EV UW30, Frequency response 100-10,000 Hz; Impedance 8 ohms) connected to a commercial amplifier (RX-4105 2 channel stereo, Sherwood). The noise consisted in a linear sweep of 10 sec with frequencies from 100 – 1,000 Hz (Fig. 2.5) repeated during 10 min simultaneously in every experimental tank. Sound levels were determined based of the energy input of the system to mimic those sound levels encountered during the anthropogenic perturbation sound mapping. The energy input was 20, 15 and 10 V,

which corresponded to a RMS sound pressure level (SPL) of 20.9, 18.7 and 10.2 dB 1 re µPa above background noise, respectively.

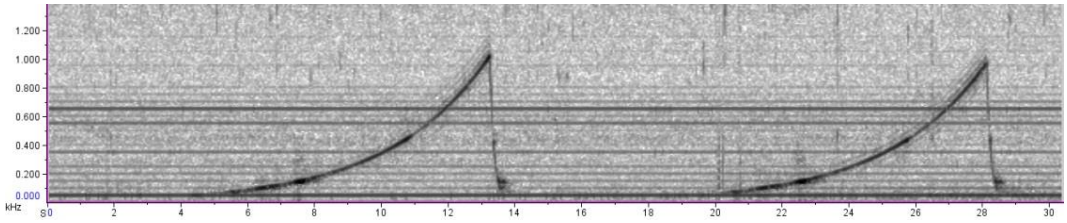

Figure 2.5. View of the 10 sec linear sweep (100-1,000 Hz) used for Chapter 3 Experiment 3.1 view of Raven Lite software.

For experiment 2 in Chapter 3 a comparable 10 s linear sweep (Fq. 100 to 1,000 Hz) (Fig. 2.6) was created using a tone generator software (NCH Software, Australia). An audio file (.wav, 16 bits) containing 60 min of the 10 sec linear sweep repetition was created using Audacity software.

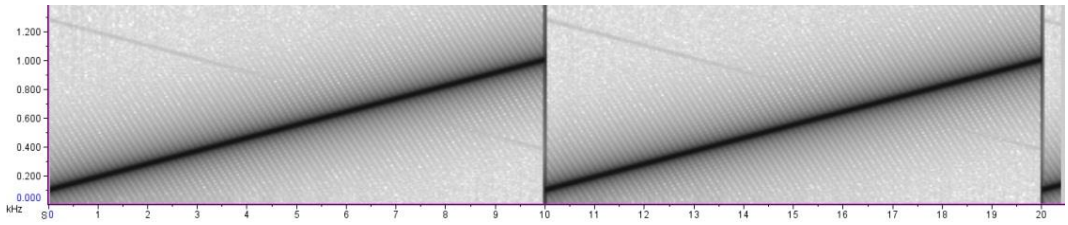

Figure 2.6. View of the 10 sec linear sweep (100 – 1,000 Hz) used for Chapter 3 Experiment 3.2 created with the tone generator software. View of Raven Lite software.

The noise exposure consisted in repeating those 60 min of noise at 6 random times every day. To do so, a list of 24 elements was created corresponding to 6 hours of noise and 18 hours of silence. The list was randomised ten times using the website www.random.org, which states: *"The randomness comes from atmospheric noise, which for many purposes is better than the pseudo-random number algorithms typically used in computer programs."* Those 10 randomised lists were used to create the 24 h audio files that would be used for the experiment. The ten 24 hr digital audio files (Table 2.1) were saved in an external USB hard disk (250 Gb Maxton, China) and played using a HD Media Player (Digi-Fun, UK) connected to the same stereo and

speakers as described above. The initial exposure started 9:00 hrs on the 20th February 2012 with File 1 (Table 2.1). Due to unsettled weather conditions the main power supply was interrupted twice. The power cut set the media player into a standby mode, despite the emergency power generator in place. On 10th March 2012 the power cut occurred around 2:00 hrs and the list was restarted on File 1 (Fig. 2.7 and Table 2.1) at 19:00 hrs once the main power supply was restored. On 16th March the electricity power supply was stopped around 7:00 hrs and the noise play list restarted on File 3 (Table 1) at 11:30 hrs. However those interruptions did not affect the noise exposure but rather increased the randomisation of the list. The noise exposure ended on 13th May 2012 when there were clear signs that the spawning season had come to an end due to the absence of eggs in the egg collector after 5 consecutive days.

2.4.3. Sound analysis

Sound levels were determined by analysing the digital audio file created using audio analysis software Audacity (http://audacity.sourceforge.net/). Sound levels were measured in dB re 1 µPa. Sound profiles were determined by analysing the sound spectrum of the digital audio file. The spectrum was plotted from 0 Hz to 22 kHz. The audio analysis procedure is described step by step as follows:

Table 2.1. Summary of the noise treatment exposure in Chapter 3 experiment 3.2. Ten audio files of 24 hours containing 6 randomised hours of noise (10 sec linear sweep 100 -1,000 Hz). Dark squares correspond to the 60 min repetition of the 10 sec linear sweep.

	File number									
Time										
1		■	■	■		■		■		
2										
3			■	■		■				
4	■									
5				■						■
6										■
7				■						
8										
9	■	■			■			■		■
10	■								■	
11		■	■					■		
12					■		■			
13									■	
14						■	■		■	
15			■		■	■				
16		■								■
17					■			■		
18	■			■			■	■		■
19	■		■		■		■		■	
20						■			■	
21	■	■	■			■	■			
22		■						■		■
23							■			
24				■	■				■	

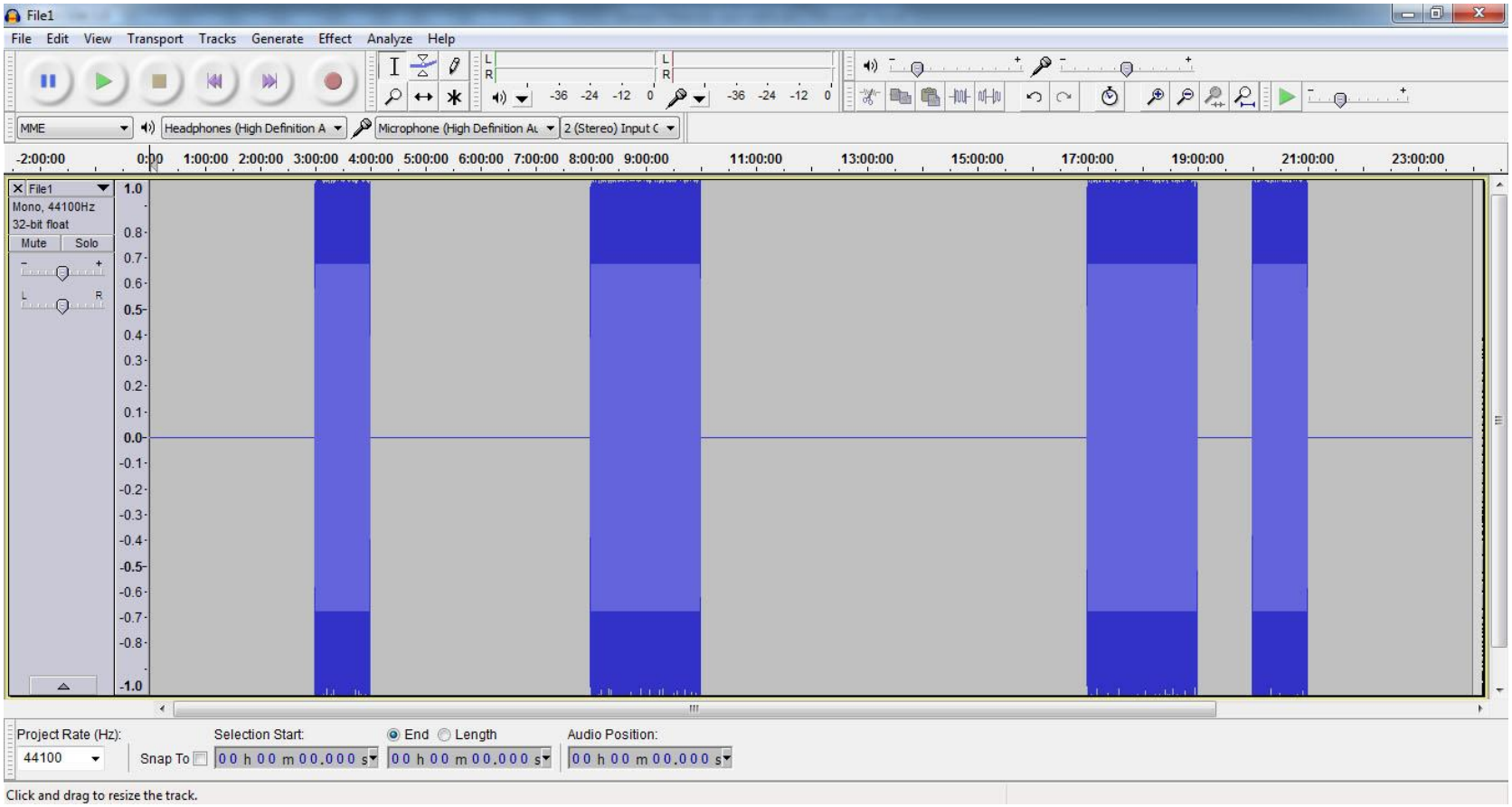

Figure 2.7. View of Audacity software of File 1 noise exposure treatment containing the 6 hours of sound exposure and 18 hours of silence as recorded in .wav form

Step 1: The audio file was opened with the software. Fig. 2.8 shows an example of the sound analysis of an experimental tank "knocks disturbance. The waveform shows first the background noise recorded followed by the induced perturbations.

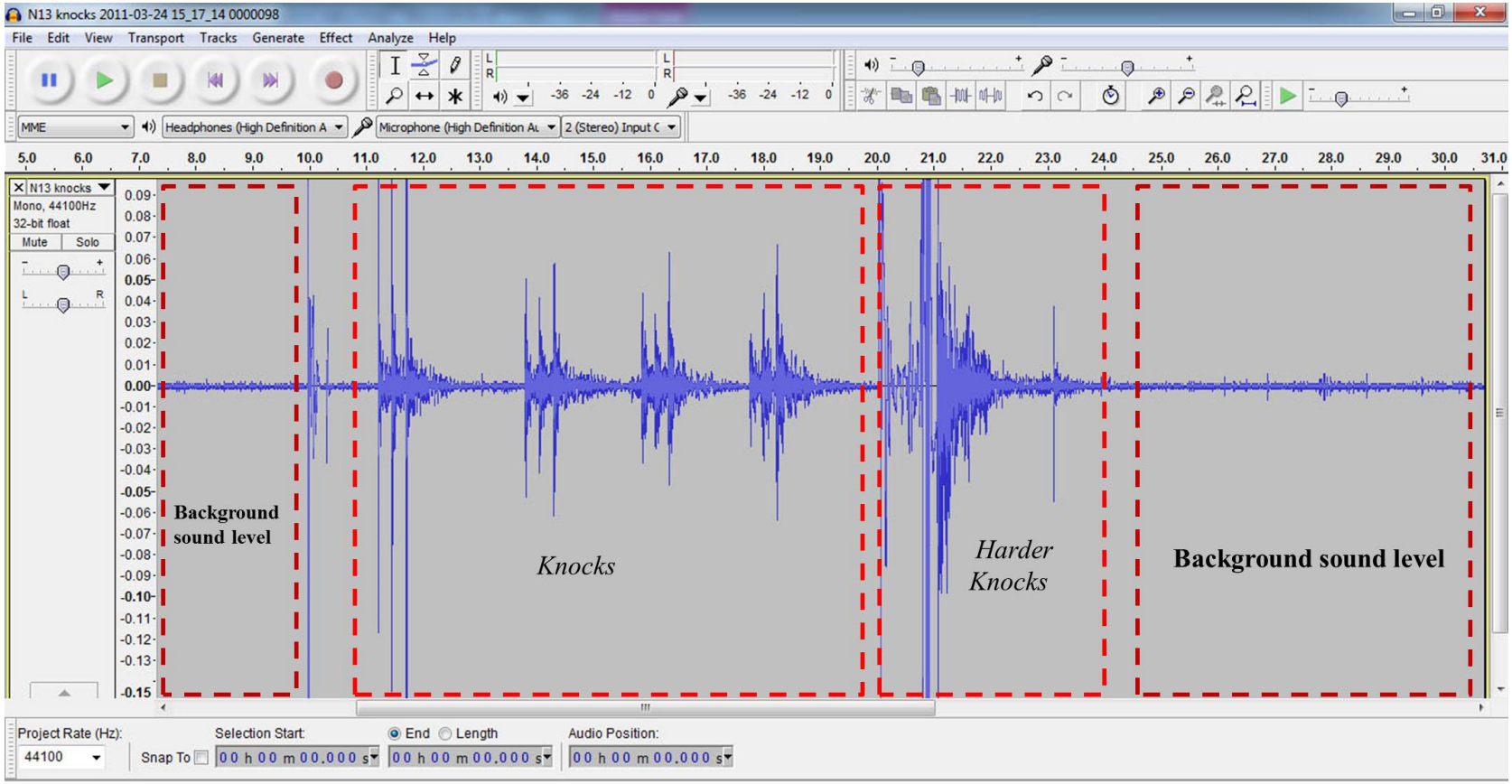

Figure 2.8. View of the audio file recorded in a 1 m tank creating sound perturbations (*knocks* and *harder knock*) in the Audacity software.

Step 2: A section of the audio file was selected to analyse the sound levels. First a section of the background sound levels recorded in the tank were selected to determine the baseline noise level of the tank (Fig. 2.9). The selection was analysed by "Plot Spectrum". This process was repeated for each sound or noise of interest.

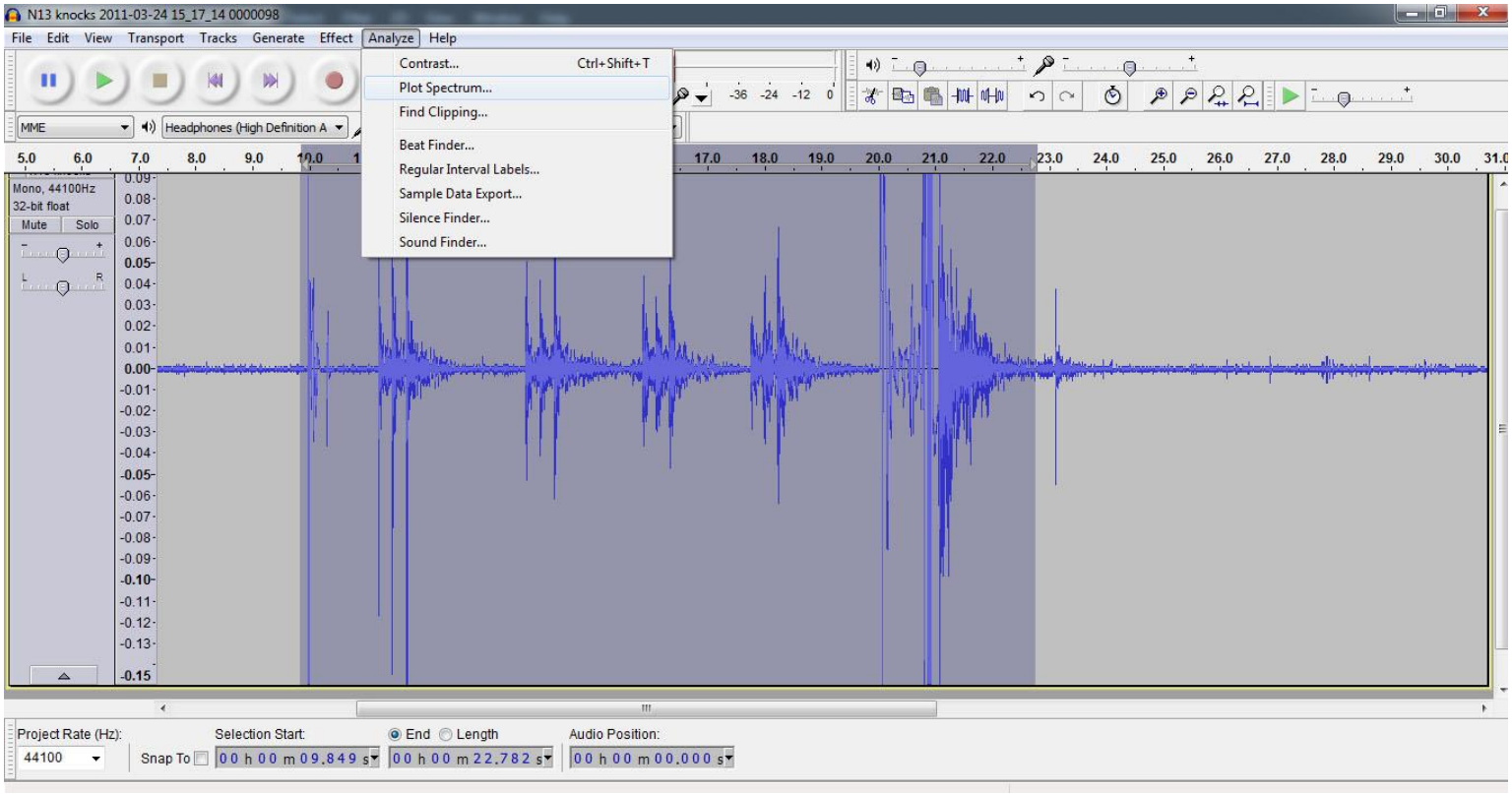

Figure 2.9. View of the audio spectrum analysis of induced noise in the tanks using Audacity software.

Step 3: The software created the spectrum plot. All audio files were analysed with a sample size of 8192 audio samples (resulting in a time resolution of 185 milliseconds and frequency resolution of 5 Hz) with a Rectangular Function with a Log frequency Axis (Fig. 2.10). The data resulting from this analysis was exported as a .txt file.

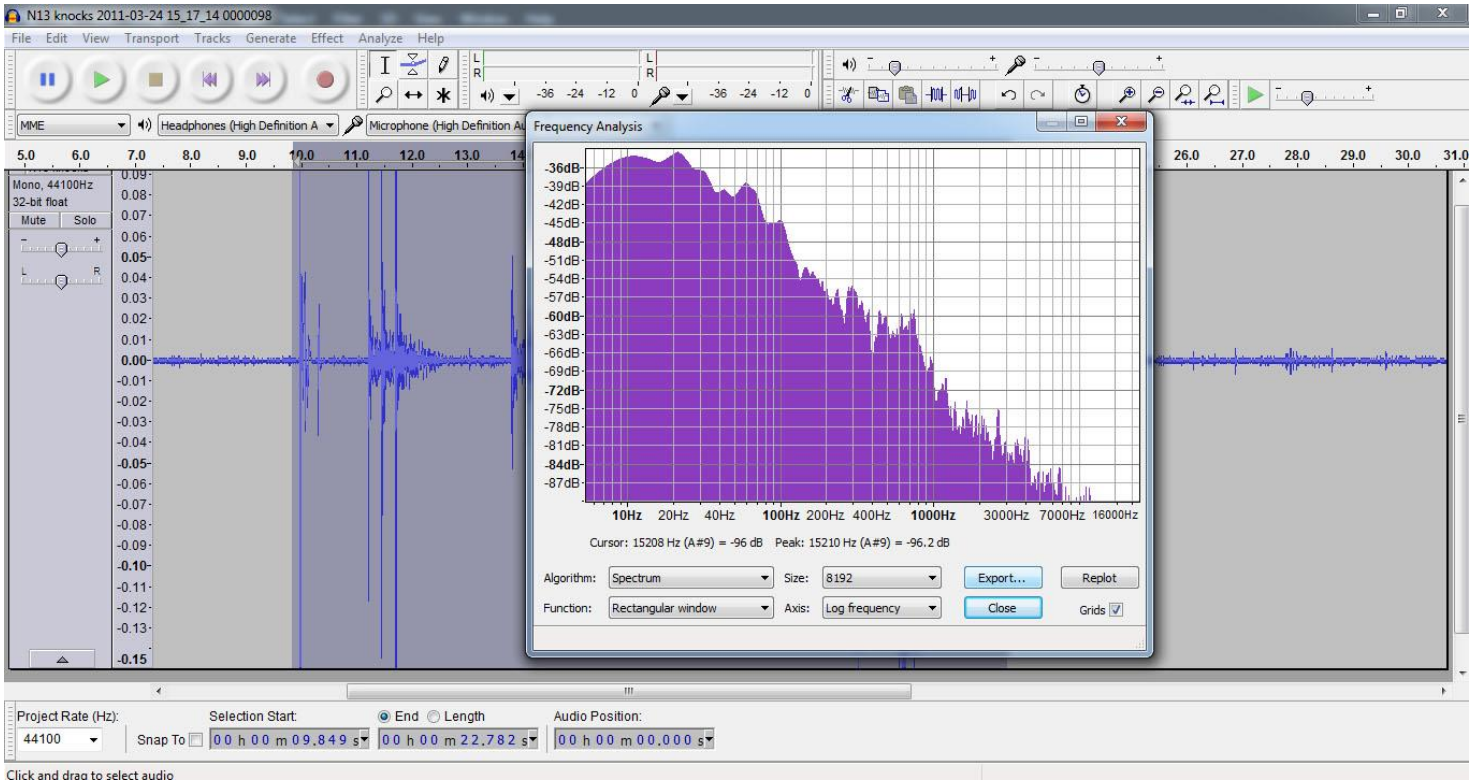

Figure 2.10. View of the software Audacity with the audio spectrum plot created to analyse sound levels.

Step 4: The previously saved .txt file was then opened using Windows Notepad and the data was selected and copied in Office Excel.

Step 5: The audio analysis from Audacity software resulted in 2 columns of data including: Frequency and "volume". The root mean square (RMS) of the SPL increase was calculated (see below) and expressed in dB 1re µPa. The frequency range selected for the audio analysis was from 100 to 1,000 Hz.

$$RMS = \sqrt[2]{(\sum x1\text{^}2 + x2\text{^}2 + \cdots xn\text{^}2)/(n)}$$

Where, x is the SPL at a given frequency (e.g. x1 = 1 Hz = 120 dB, x2 = 2 Hz = 121 dB) and n is the number of sampled frequencies (100 – 1,000 Hz).

2.4.4. Atlantic cod vocalisation sound analysis

Atlantic cod vocalisations ("grunts") were monitored for Chapter 3 by taking 5 min audio samples every hour during a 24 hours sampling period with the sound recordings then being analysed as described above. "Grunts" were first localised within the audio file (Fig. 2.11). When a "grunt" was found, it was selected (within the audio file) and "copy and paste" into a new Audacity file to facilitate its' individual analysis. Individual sound spectrum plots were processed for each grunt and sound analysis (SPL, Frequency peaks, Maximum levels) performed as with the induced noise perturbations. Grunt repetition frequency was determined as the sum of the number of grunts in the 5 m audio sample. The harmonics of the grunt were determined by manually adjusting the spectrogram sharpness to ~4,300 samples.

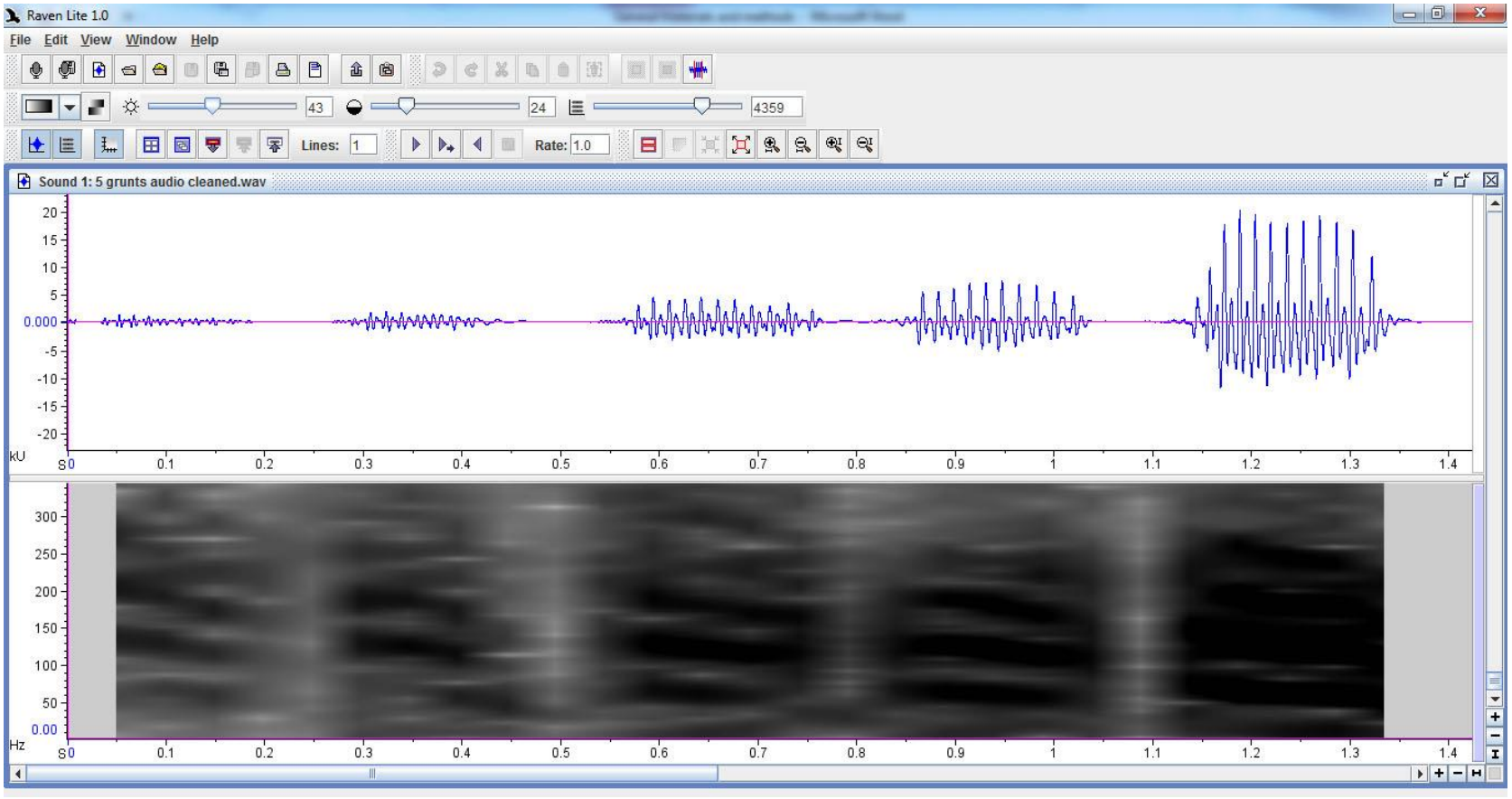

Figure 2.11. View of 5 Atlantic cod "grunts" in Raven Lite software. The upper window shows the waveform and the lower window the spectrogram of the corresponding grunts harmonics.

2.5.Image pigmentation analysis

2.5.1. Image collection turbot juveniles

Turbot pigmentation (Chapter 5) photographs were obtained using a waterproof digital camera (Lumix DMC-FT25, 16.1 MP, Panasonic). The camera settings were adjusted at every sample to balance the ISO manually at every sample point. A white background with a scale was used to set up the fish with the digital camera 20 cm above the fish. Extra lighting was added by four (40 W) fixed Osram energy saving white bulbs. Two professional portable photography card: 18 % grey and pure white card (GC-2 Pocket size Grey and white card, Kaavie, UK); were included in each image for later brightness digital whites balance. Images were saved as .jpg files (18 bit resolution) by the digital camera.

2.5.2. Pigmentation image analysis

Pigmentation image analysis was carried on using ImageJ and additional RGB plug-ins as described above. The procedure was followed was:

Step 1: Using Photoshop CS6 software (Adobe, USA) the "whites" were balanced for each image based on the grey and white photography cards to standardise the further colour analysis. In \Image\Adjustments\Levels…\ a new window "pops out" from which a grey point and a white point was establish which adjusted the light levels of the image (Fig. 2.12). This procedure balanced automatically the colours to adjust the image to the true colour intensity. The image was saved as a new file in .jpg format. Each photograph was balanced individually, based on its own grey and white photography cards.

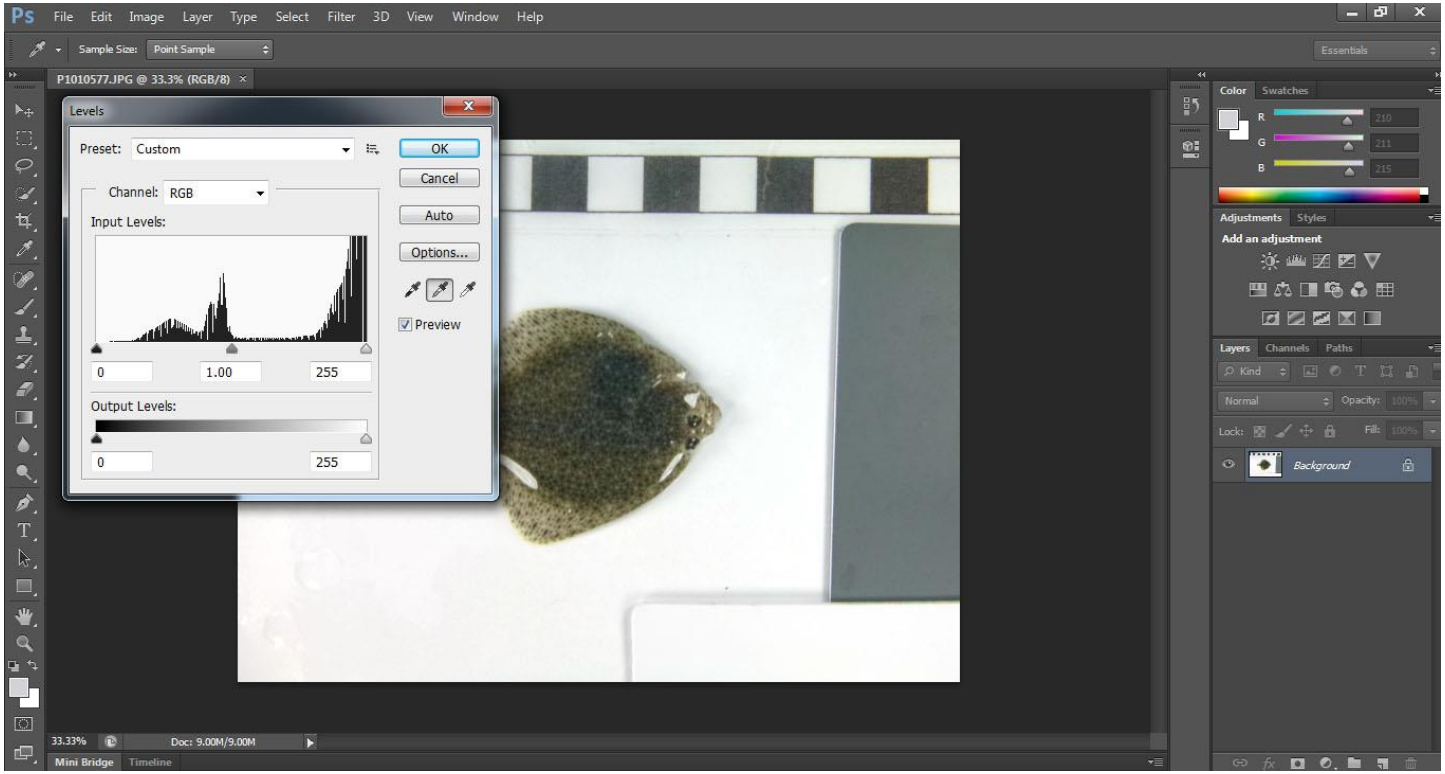

Figure 2.12. View of Photoshop suite to adjust light and colour balance using a grey and black photography cards

Step 2: The new balanced image was further transformed into black and white format: (\Image\Adjustments\Black&White) and saved in B&W but as a Red, Green and Blue hue composition (RGB) image for blackness analysis (Described further below). This allowed the RGB values to be determined within the image.

Step 3: The balanced image was opened using Image J software. The image sequence of each replicate were imported and the size scale determined as for morphometric measurements.

Step 4: The fish skin area was selected using the "wand tool" (Fig. 2.13). The settings of the "wand tool" were adjusted to "4-connected pixels" with a sensitivity/tolerance of 75.0 pixels. These parameters were homogenous throughout the pigmentation image analysis.

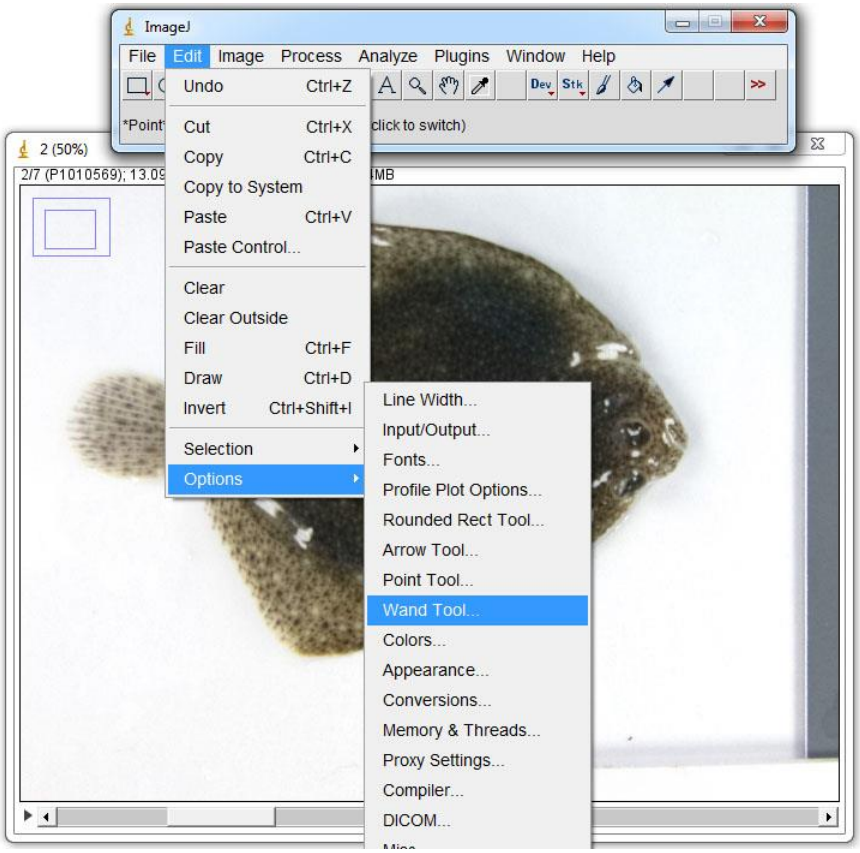

Figure 2.13. View of the ImageJ software to set the configuration on the "wand tool" used to measure the RGB composition of the turbot skin pigmentation

Step 5: The wand tool was used to select the coloured area. A "click" with the computer mouse in the middle of the fish skin automatically selected the pigmented area of the fish skin avoiding the tail and part of the fins (Fig. 2.14). Several tests were carried on to determine the accuracy of the wand tool. Manual selection (as in Rajjai 2011) vs. wand tool showed minimal variations in the RGB analysis; automation (i.e. wand tool) of the measurements reduced significantly the time required for the image analysis and any possible human subjective error. Furthermore, automation supressed the need on removing reflection areas, fins and eyes.

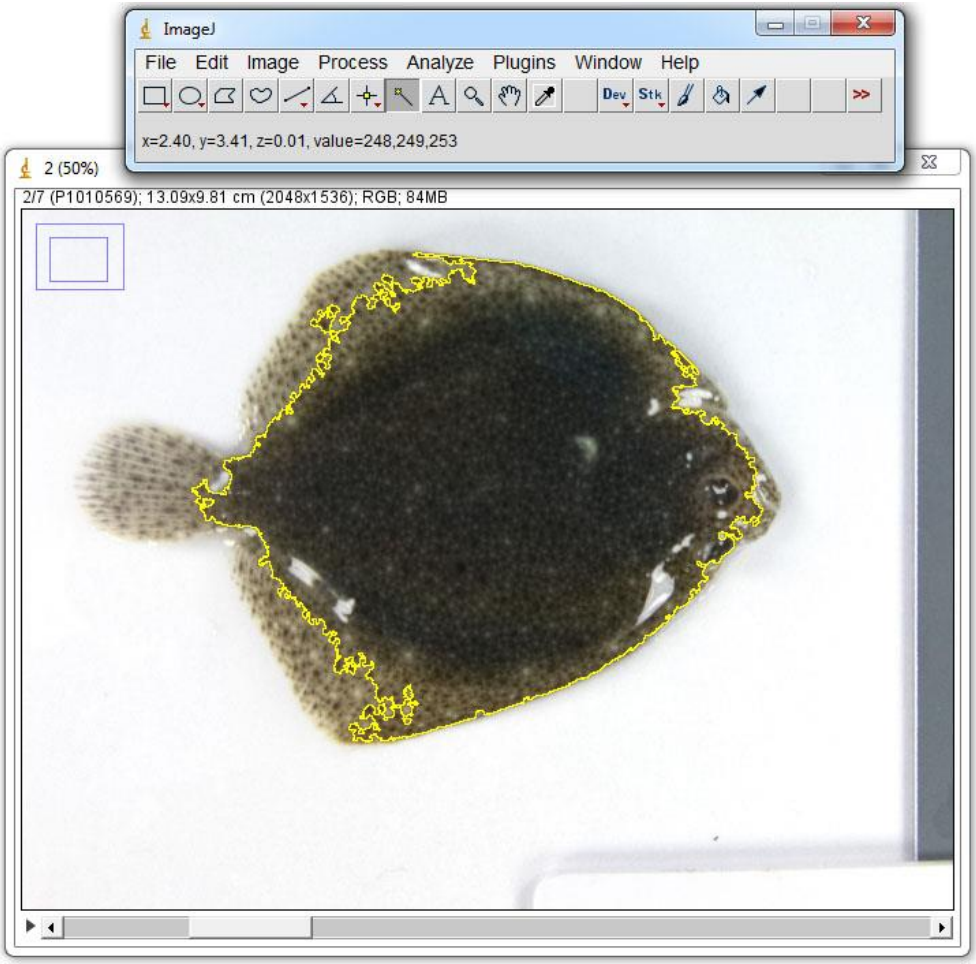

Figure 2.14. View of the skin surface selected using the "wand tool" with ImageJ to measure the RGB composition.

Step 6: The RGB composition of the selected area was measured using the "Measure RGB" plug in (Fig. 2.15). The software measures the mean number of pixels

of each colour layer and displays the output in a new window. The results window includes individual mean values for RGB and two intensity brightness equations: one expressing the image saturation (R+G+B /3) and the other is a weighted equation of brightness in terms of human perception (0.299R + 0.587G + 0.114B). Those results were copied into an excel sheet.

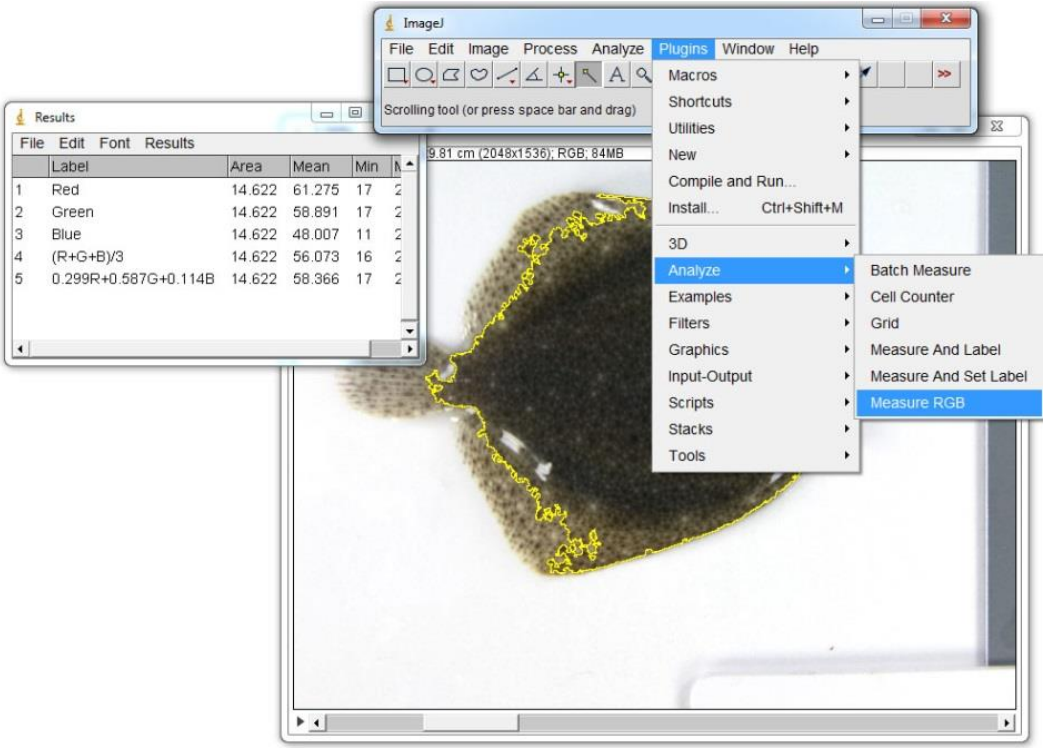

Figure 2.15. View of the Plug in to measure the RGB composition in ImageJ software.

Step 7: Steps 1 to 6 were repeated for each individual using the previously transformed Black and White image. The RGB analysis gave the same value for all three RGB layers.

Step 8: The gathered data was used to determine the colour composition and perception using the following calculations: [example: Fish from green light treatment Day 30. (RGB = 65, 63, 50] [Blackness = RBG (58, 58, 58)]

Red hue % = R / (R+G+B) [R% = 65 / (65+63+50) = 0.3651 * 100 = 36.51 %)]

Green hue % = G / (R+G+B) [G% = 63 / (65+63+50) = 0.3539 * 100 = 35.39 %)]

Blue hue % = B / (R+G+B) [B% = 50 / (65+63+50) = 0.2808 * 100 = 28.08 %)]

Saturation = Maximum (RGB) – Minimum (RGB) [Saturation = (65 – 50) = 15]

Brightness = (0.299R + 0.587G + 0.114B) [Brightness = (0.299* 65) + (0.587 * 63) + (0.114 * 50) = 62.11]

Blackness % = (1- (RGB)/255) e.g. [1 – (58 / 255) = 0.7725 * 100 = 77.25 %]

Where, RGB value is the red, green and blue hue from the analysis in pixels. In the grey scale photos the RGB values were identical to each other (R=G=B). The top limit of the RGB scale is 255 pixels corresponding to absolute blackness. In the blackness % equation, result (%) describe the percentage of closeness to absolute black (RBG= 0,0,0).

2.6.Blood parameters analysis

2.6.1. Plasma glucose

Plasma glucose levels were measured in serum using a portable glucose meter (USB Contour Bayern, Germany) with a minimum detection level of 0.6 nmol L^{-1} previously validated for fish (Fanouraki *et al*., 2011). Test strips are designed to absorb 6 µL of sample and calculate the glucose levels. The portable glucose meter was tested using glucose standard provided by the supplier with the glucose meter at the beginning and every 50 samples; no error in the readings was found (standard measurement = 7.0 nmol L^{-1} every time). Further 30 random samples were tested by triplicate to ensure accuracy of the equipment and values were always identical to the first measurement (Standard deviation of the measurement between samples was < 0.01 nmol L^{-1}).

2.6.2. Plasma cortisol

Cortisol levels were determined using an indirect competitive radioimmuno assay (RIA) adapted from North *et al.* (2006) and previously validated for Atlantic cod by Cowan *et al*. (2011). Plasma cortisol for turbot was validated as described below prior to analysis.

Plasma cortisol extraction

Plasma cortisol was extracted in ethyl acetate by suspension of the plasma organic phase. Plasma samples were defrosted at room temperature. In a 4.5 mL polypropylene assay tube (12 x 75 mm polypropylene round bottom, Alpha labs Ltd, UK) 200 µL of plasma and 1 mL of ethyl acetate added (BDH Chemicals Ltd, Pool, UK). Assay tubes with stoppers were vigorously vortexed and then spun on a rotary mixer for 1 hr at room temperature (room air conditioning was set at 16°C at least 60 min before any work). The mixture was centrifuged at 430 G for 10 min at 4°C and kept at 4°C until analysis.

Atlantic cod whole egg cortisol extraction

Whole egg cortisol content analysis protocol was adapted from McCormick (1998) and Wunderink *et al*. (2011). 0.5 g of frozen egg sample was added to a 4.5 mL LPL assay tubes together with 350 µL of 0.01M PBS (Sigma-Aldrich Ltd, UK) and homogenised. Sample tubes were centrifuged (1,200 G, 5 min, 4°C) to condense foam formed. Homogenised sample was transferred to 1.5 mL eppedorfs and centrifuged (10,000 G, 1 min, 4°C) to form a pellet. In a clean LPL 4.5 mL assay tube with stopper, 400 µL of homogenised mixture and 1 mL of ethyl acetate were mixed, vigorously vortexed for 1 min and spun for 1.5 hr on a rotary mixer. The rotary mixer was stopped

and tubes additionally vortexed every 20 min. The mixture was centrifuged (430 G, 10 min, 4°C) and then stored in a cold room (4°C) until analysis.

2.7.Cortisol radioimmunoassay (RIA)

Cortisol extractions were analysed by RIA using the same protocol. However the volume of extraction used varied between species and whether it was plasma or eggs.

Working and charcoal buffers

Fresh working buffer was prepared for each assay on the same day and allowed to cool down (4°C) before the analysis. Charcoal buffer was prepared the night before and chilled down (4°C) overnight (Activate charcoal and Dextran were added and stirred 30 min before being used) (Table 2.2).

Table 2.2. Radioimmuno assay buffers for cortisol content analysis**.**

Working buffer	**Concentration**
NaH2PO4	1.48 g
2NaHPO4	5.76 g
Bovine serum albumin (BSA)	2 g
NaCl	8 g
Ethylenediaminetetraacetic acid	0.3 g
NaN3	0.1 g
Charcoal buffer	
NaH2PO4	1.48 g
2NaHPO4	5.76 g
Gelatine	1 g
Activated charcoal	5 g
Dextran (H(C6H10O5)xOH)	1 g

Cortisol antibody

Cortisol antibody for the assay was used in a dilution of 1:10,000 (binding target of 35%). The aliquots were originally obtained from diluting neat anti-serum (Guildhay Antisera Ltd, UK) in working buffer (as detailed before) and kept frozen at -20°C. On

the day of the assay the required amount of aliquots (dilution 1:1000) were defrosted at room temperature (16°C) and diluted in working buffer in 5 mL glass vials.

Cortisol ^{3}H- radio label

Cortisol ^{3}H radiolabel was prepared from a concentrated stock of 9.25 MBq in 0.25 mL toluene-ethanol (Perkin-Elmer Ltd., UK). The "hot stock" was diluted in absolute ethanol to prepare "stock A" and kept at -20°C. Working radiolabel was prepared fresh for every assay by diluting "stock A" in working buffer. The required volume was determined every time by reading the activity of 100 µL of working radiolabel solution in 4 mL of scintillation fluid. Working radiolabel solution concentration was adjusted to reach an activity of ~5,000 disintegrations per minute (DPM) / 100 µL of working radiolabel solution.

Cortisol standards, standard lines and controls

Working cortisol standard was prepared by a series of stock dilutions. Stock 1 was prepared using 0.01g Hydrocortisone powder (Sigma-Aldrich Ltd., UK) in 20 mL of absolute ethanol (500µg mL^{-1}). For stock 2, 100 µL of stock 1 were further diluted in 10 mL of absolute ethanol (5µg mL^{-1}). Then 100 µL of stock 2 diluted again in 10 mL of absolute ethanol (50 ng mL^{-1}) and stored at -20°C. For each assay 400 µL of stock 3 were diluted in 4.6 mL of ethyl acetate to give a working concentration of 4 ng mL^{-1}. This solution was used to prepare the cortisol standard curve (Fig. 2.16). Standard curve consisted of none specific binding (binding not related to the cortisol Ab), binding zero (cortisol binding without sample or cortisol standard) and 8 cortisol concentrations (800, 400, 200, 100, 50, 25, 12.5 and 6.25 pg of cortisol) prepared from 200 µL of working standard and further diluted and vortexed in 200 µL of ethyl acetate. Cortisol

standard used for quality control (n = 4) consisted of a solution containing 1:16 dilution of cortisol standard in ethyl acetate to give 50 pg mL^{-1}. This was stored at -20°C and used between assays to assess reproducibility.

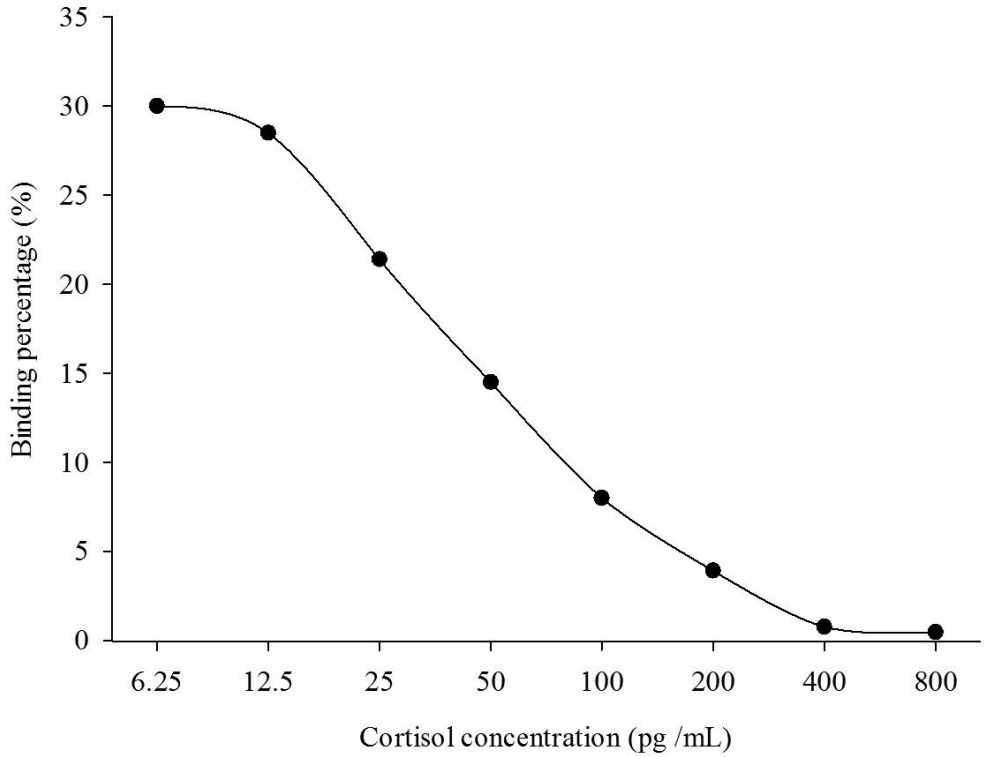

Figure 2.16. Cortisol RIA typical standard curve with a 30% binding.

Assay protocol

The cortisol RIA protocol is described in Table 2.3 and takes two days to complete. Day 1 included Steps 1 to 16 and Day 2 from Step 17. On day 2 the room air conditioning was started at least 60 min before the charcoal was added to the buffer to ensure a constant room temperature of ~16°C throughout the whole assay. Sample extraction volumes for assay were: 200 µL for cod plasma, 50 µL for turbot plasma and 400 µL for cod egg homogenisation.

Table 2.3. Protocol of the cortisol radioimmunoassay performed.

Cortisol Radioimmunoassay protocol	
Day 1	
1.	Prepare working buffer and cool down to 4°C
2.	Prepare cortisol standard solution, cortisol antibody solution and
3.	Add 100 µL of working ^{3}H radiolabel and 4 mL of scintillation
4.	Adjust the ^{3}H radiolabel concentration as needed.
5.	Prepare the assay standard curve dilutions
6.	Prepare the quality controls
7.	Add sample extraction to assay tubes
8.	Dry down sample tubes in vacuum rotary evaporator (30°C for
9.	Cool down sample tube to 4°C (~20 min)
10.	Add 100 µL of working buffer to each tube (200 µL to NSB
11.	Add 100 µL of working Antibody to each tube (except NSB
12.	Add 100 µL of working ^{3}H radiolabel to each tube
13.	Centrifuge tubes at 430 G for 10 sec (to "wash down" solutions
14.	Vortex tubes
15.	Incubate tubes at 4°C for 18 hrs
16.	Prepare charcoal buffer (charcoal and dextran are added 30 min
Day 2	
17.	In an Ice box add the charcoal and dextran and stir for 30 min
18.	Add 1mL of charcoal buffer to each tube on ice box
19.	Vortrex each tube
20.	Incubate tubes at 4°C for 30 min
21.	Centrifuge tubes at 1270 G for 12 min at 4°C
22.	Transfer 1 mL of supernatant to scintillation vials
23.	Add 4mL of scintillation fluid to each vial
24.	Add cap to vial and vortrex until pellet is mixed
25.	Arrange scintillation racks

Calculations

Results were given by Riasmart software (Canberra Packard, UK) as a percentage of ^{3}H radiolabel binding from the disintegrations per minute (DPM) considering the no specific binding (NSB) and binding zero (B0). Readings given by the software express the transformation of DPM proportions into cortisol content in each tube (pg of cortisol). Those values were adjusted to express the cortisol concentration in the original sample (ng mL^{-1} for plasma and pg egg^{-1} for whole eggs). To do so a simple equation was applied using Microsoft Excel.

$$Cortisol\ in\ tube = \frac{Cortisol\ reading * Supernatant\ volume}{Volume\ of\ extract\ used}$$

$$Cortisol\ in\ sample\ = \frac{Cortisol\ in\ tube * Volume\ of\ extraction}{Sample\ volume\ for\ extraction}$$

Where cortisol reading is the value from Riasmart; Supernatant volume is 1.3 mL; volume of extract used corresponds to 200 µL for cod plasma, 50 µL for turbot plasma and 400 µL for cod eggs. Volume of extraction is 1200 µL (volume of sample plus 1 mL of ethyl acetate). Sample volume for extraction is the amount in µL of sample used for the extraction before the ethyl acetate addition. For whole egg cortisol content, the result of cortisol concentration in sample was divided by the number of eggs in the sample.

Assay quality control and validation

The sensitivity of the assay i.e. the minimum amount of cortisol statistically distinguishable from zero was 0.05 pg.tube^{-1} this corresponded to 0.86 ng mL^{-1} cod plasma; 0.24 pg egg^{-1} for cod eggs; and, 0.56 ng mL^{-1} for turbot plasma; depending on the extraction protocol and amount of extract analysed as explained in the specific chapters. Cortisol standard, stored in absolute ethanol at -20°C at a concentration of 50 pg mL^{-1}, was used to confirm the inter- and intra-assay variation with results being listed in the corresponding chapter.

The assay had previously been validated for cod plasma (Cowan *et al*., 2011) however, in addition to this, serial dilutions of pooled cod egg extracts and turbot plasma were used to obtain an inhibition curve (Figure 2.17, 2.18). When plotted against the standard curve, it was observed that both curves were parallel to the standard curve and no statistical difference in the gradient was found (ANCOVA), indicating that the samples were immunologically similar to the standards.

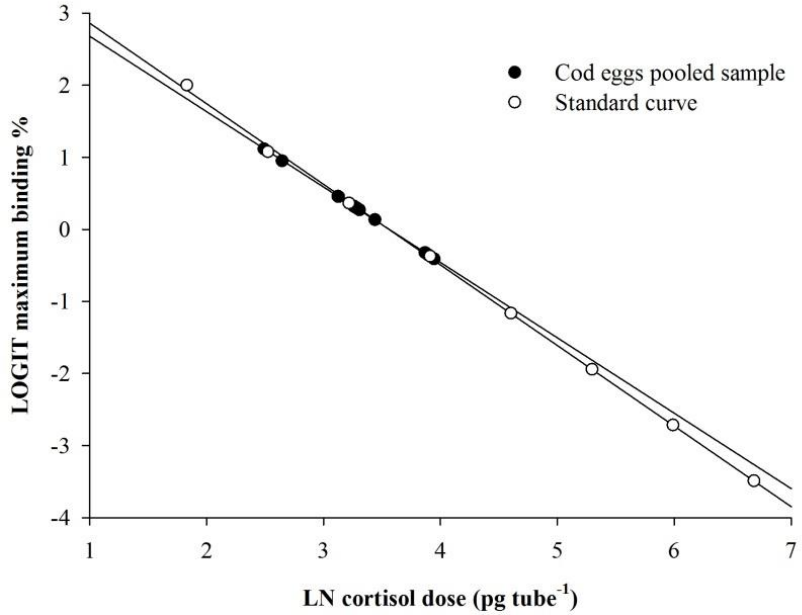

Figure 2.17. Logarithmic transformation of cortisol content of a pooled sample of cod eggs extractions and cortisol standard curve. The parallelism between regression lines shows the direct correlation and validation of the assay for Atlantic cod egg cortisol extraction.

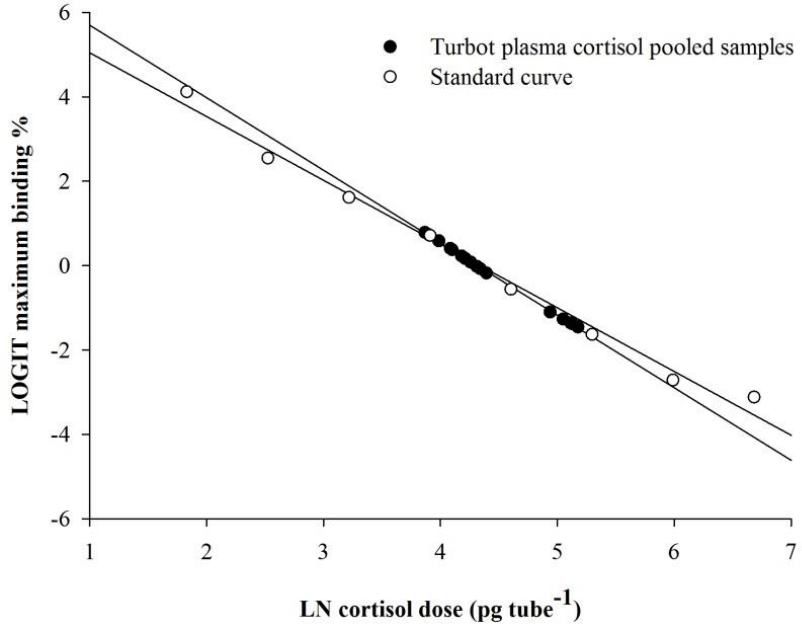

Figure 2.18. Logarithmic transformation of cortisol content of a pooled sample of turbot plasma extractions and cortisol standard curve. The parallelism between regression lines shows the direct correlation and validation of the assay for turbot plasma cortisol extraction.

Cortisol recovery

Waring *et al*. (1996) reported that turbot plasma contained high volumes of lipid that interfered with cortisol RIA measurements. To test this statement prior to the sample RIA analysis cortisol recovery for turbot plasma samples was calculated based on 8 turbot plasma cortisol extractions. Each extraction was performed adding 200 µL of plasma into 1 mL of ethyl acetate following the standard procedure described above. The RIA samples were analysed by adding 25 µL of extraction sampled in quadruplicate. A second set of replicates also contained 25 µL of cortisol standard equivalent to 158 pg of cortisol. Recovery was calculated by transforming the RIA result into the volume contained in the original sample (RIA reading * 1.3 mL), plus the cortisol standard added (158 pg). The RIA result from the addition was divided by the expected dose and multiplied by 100. Total recovery was estimated as 101.0 ± 14.4 % (Table 2.4). Our results showed that cortisol recovery was effective and therefore freely lipids did not interfere with the analysis.

Table 2.4. Summary of cortisol recovery % from turbot plasma cortisol samples (n=8)

Sample	**Dose**	**Expected dose**	**Actual dose**	**Recovery %**
		Dose + 158 pg		
1	94.4 ± 2.4	252.5 ± 2.4	253.5 ± 10.9	100.3 ± 3.3
2	112.0 ± 2.1	270.0± 2.0	234.1 ± 8.3	86.7 ± 2.4
3	26.6 ± 6.9	184.6 ± 6.8	231.7 ± 18.8	125.7 ± 14.8
4	54.8 ± 8.8	212.7 ± 8.7	254.1 ± 5.4	119.5 ± 7.4
5	71.5 ± 0.7	229.5 ± 0.7	219.7 ± 1.0	95.7 ± 0.7
6	66.2 ± 5.6	224.2 ± 5.5	192.4 ± 15.0	85.9 ± 8.8
7	70.9 ± 9.3	228.8 ± 9.2	230.4 ± 0.6	100.7 ± 3.8
8	74.3 ± 6.1	232.3 ± 6.1	217.7 ± 1.7	93.72 ± 3.2
			average	**101.0 ± 14.4**

2.8.Statistical analysis

The statistical analysis for this work were performed using MiniTab statistical software (Version16.1, MiniTab Inc., USA).The statistic methods used in this work were based on Zar (1999). Each chapter describes the specific statistical method used however they can be briefly summarised as:

Data and statistical analysis

Results are presented as the arithmetic average of the replicate values ± the Mean Standard Error (SEM). All data sets were first tested for normality using the Anderson-Darling test. When data were not normally distributed they were transformed as required using a range of potential manipulations (log, inverse (1/x), sin, cos, tan). Percentages or ratios were arcsine square root transformed before analysis. Replicate sample data were analysed using the analysis of variance method (ANOVA) to test variance of treatment effect in all chapters (e.g. wet weight *vs.* treatment). Variance was analysed with a General Linear Model (GLM) to compare the effect of treatments, time and replicates at each experimental chapter. In all cases a p value < 0.05 was considered to be statistically significant in which cases a Tukey *post hoc* test was performed to define the significant relationship.

Linear regression was used to analyse the correlation between two variables in Chapters 3, 4 and 5. Linear correlation was calculated with Office Excel data analysis add by using the Pearson product moment correlation coefficient (r). Run's test was used to confirm linearity and all linear relationships presented had a slope different from zero.

The correlation between weight and length was analysed for Chapter 5 based on the mathematical function: $W = a L^b$; where W is total body weight (g), L total length (cm) and a and b are coefficients from the functional regression between W and L. The

coefficient a expresses the body shape, while coefficient b expresses the growth: if b = 3, growth is isometric. If b ≠ from 3, growth is allometric (if b < 3, growth is towards length; if b > 3, growth towards weight.). The values of a and b constants were estimated from the natural logarithm transformed values of length and weight through least square linear regression: Ln W = Ln a + b Ln L . The degree of association between the W and L is expressed as the r^2 coefficient of the linear regression and the significance of the b coefficient is expressed as its p value.

Chapter 3

STRESS RESPONSE TO ANTHROPOGENIC NOISE IN ATLANTIC COD *GADUS MORHUA L*

Research article

Submitted to: The Canadian Journal of Fisheries and Aquatic Science

Authors: ***Sierra-Flores, R****; Davie, A. Atack, T and Migaud, H.*

3.1. Introduction

The acoustic environment plays an important role throughout the life cycle of most animals however, anthropogenic noise can interfere acting as a stressor and impacting negatively on behaviour and physiology. In the aquatic environment there is clear evidence that anthropogenic sound can disturb aquatic animals and impact on their welfare (Popper 2003; NRC 2005; Popper and Hastings 2009). While most studies on the impact of noise in the aquatic environment have been performed in marine mammals (Thomsen *et al.*. 2006; NRC 2005), there is an increasing awareness of the potential negative effects on other marine organisms including invertebrates (Aguilar de Soto *et al.*. 2013) and fish (Hatch 2007; Holles *et al.*. 2013; Popper and Hastings 2009). Fish have been reported to be attracted to sound but equally show avoidance reactions depending on the source (Chapman *et al.*. 1974; Mueller-Blenkle *et al.*. 2008, 2010; Løkkeborg *et al.*. 2012; Reyff 2012; Spiga *et al.*. 2012). It has also been suggested that migration patterns and reproductive behaviour may be disturbed by noise as well, forcing fish to find alternative routes or prevent them from settling in their usual spawning grounds (van Opzeeland and Slabbekoorn 2012) and thus possibly impacting on larval settlement (Stanley *et al.*. 2012). The acoustic field in enclosed aquaculture systems is not exempt from sound pollution but its impact on fish stocks has been widely overlooked to date despite the drive towards increasing land-based facilities.

Most human activities in the aquatic environment generate noise in the frequency range below 1 kHz (Hatch and Wright, 2007) which corresponds broadly to the optimal auditory range of most fish species (Hall *et al.*. 1981; Popper *et al.*. 2003). Fish hearing bandwidths for most species, formerly referred to as “hearing generalists”, range between <50 to 500-1500 Hz (Chapman and Hawkins 1973), however a few species have specialised anatomical structures connecting the swim bladder to the inner ear that

allows them to detect sounds over 3kHz and possibly up to 100 kHz (Mann *et al.*. 2001; Popper *et al.*. 2003).

When exposed to sound pollution, fish audition can be altered with temporary threshold shifts (Scholik and Yang 2001; Smith *et al*., 2004), auditory tissue damage (McCauley *et al*., 2003) and physical injuries can be induced that can reduce hearing capability (Hastings, 1995). Importantly, noise perturbations can also restrict or mask communication when it covers similar frequencies to the vocalizations (Hawkings and Chapman 1975). Physiological response to stress varies widely between species (Barton, 2002) however it has been observed that noise can cause behavioural changes in fish (Kasumyan, 2008) as well as affect typical stress biomarkers including glucose, lactate and haematocrit (Buscaino *et al.*, 2010).

The Atlantic cod (*Gadus morhua*) is a species of great commercial fisheries and aquaculture interest. To optimise fisheries technology design, the auditory ability of cod has been well studied during which it was established that it has an optimal hearing bandwidth ranging from 18 to 470 Hz, although this range should not be considered absolute (Buerkle, 1967; Chapman and Hawkins, 1973). Importantly, Atlantic cod is a vocal species that communicates at low frequencies for migration, aggression, escaping, but mainly during courtship (Ladich and Myrberg, 2006). One of the species main vocalisations, "grunts", are produced by repeatedly contracting the drum muscle sending vibrations to the swim bladder (Hawkins and Rasmussen, 1978) generating repeated single pulses with frequencies ranging from 30 to 250 Hz and duration of 60 to 200 ms. Grunts are an important part of the mating courtship in Atlantic cod (Engen and Folstad, 1999). The volume of the drum muscle mass is correlated to the vigour and number of grunts and therefore to mating success (Rowe and Hutchings 2006, 2008).

Very few studies have focused on the negative effects of anthropogenic noise on Atlantic cod behaviour and physiology.

Behavioural responses have been reported such as avoidance to wind turbine noise, freeze reactions to pile-driving noise (Mueller-Blenkle *et al.,* 2010) and attraction to divers breathing air release (Chapman *et al.*, 1974). However, the possible negative physiological effects caused by noise have not been reported yet in Atlantic cod in either a wild "fisheries" or captive "aquaculture" context.

The aims of the present study were to 1) investigate whether sound can elicit an acute stress response in Atlantic cod using plasma cortisol as a stress biomarker, and 2) study the potential chronic effects of sound disturbances on spawning performance over a spawning season.

3.2. Materials and Methods

3.2.1. Facilities

All the experiments were carried out at the facilities of Viking Fish Farms Ltd., Ardtoe Marine Laboratory, Acharacle, Scotland (N 56°46' W 05°53'). All working procedures complied with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the ethics committee of the University of Stirling

Sound recording system

Sound recordings were performed using an omnidirectional hydrophone (RESON TC4034, usable Frequency range: 1 Hz to 470 kHz +3,-10 dB; receiving sensitivity -218 dB ±3dB (at 250Hz) (Avisoft Bioacoustics, Germany) connected to an UltrasSoundGate Charge Amplifier with Hi-Pass filter (Avisoft Bioacoustics, Germany) to reduce low frequency pollution (cut-off frequencies of 25 Hz for facilities mapping,

100 Hz cut-off for noise exposure trials and 0 Hz for fish vocalization recordings). Sound data was recorded through a pre-amplifier (E-MU tracker pre 24-bit/192kHz) and Avisoft SASLab Pro software (Avisofts Bioacoustics, Germany) to a computer hard disk at a sampling rate of 48 kHz with 16-bit resolution. Sound equipment was powered with the internal battery of the laptop to avoid low frequency noise recording pollution from the AC power supply.

3.2.2. Sound mapping and vocalizations

Sound mapping analysis consisted in determining background sound levels for a range of tank systems (indoors and outdoors 1 to 5m diameter), followed by testing the impact of husbandry activities (hand feeding), disturbances (talking, walking, netting, knocks against the tank walls) and equipment (aerator, water inflow, oxygenator) on the acoustic environment of the tank (Sound pressure level (SPL) and frequencies). Sound recordings were repeated on several days and times of the day to obtain a representative sound profile. In addition, vocalisations generated by Atlantic cod broodstock in captivity were recorded during the spawning season. To do so, sound recordings were made during a 24 hour period taking a 5 minute sample at the start of every hour to record number of vocalisations and then perform further audio analysis (frequency, duration, SPL).

3.2.3. Experiment 3.1: Sound as an acute stressor in Atlantic cod.

Prior to the experiments Atlantic cod (total length 40.4 ± 2.8 cm, body weight 806.8 ±173.5 g) were maintained in 2 m^3 black circular fibreglass tanks under a constant 12L:12D artificial photoperiod with illumination provided by fluorescent lights distributed evenly across the experimental room. Tanks were on a flow through system with ambient water temperature of 6.9 ± 2.3 °C and constant salinity of 34.4 ± 0.4 ‰

over the course of the experiment (24th February to 23rd March 2011). Fish were fed *ad libitum* everyday using commercial 4.5 mm marine dry pellets (Classic marine, Biomar Norway).

Naïve cod were randomly selected from the stock tanks and randomly allocated to seven identical experimental tanks (volume 2 m^3) (6 fish per tank) where they were acclimated for a week prior to the noise exposure. Each tank was equipped with a suspended omnidirectional underwater loudspeaker (UW30, Frequency response 100-10,000 Hz; Impedance 8 ohms, EV, USA). The experimental noise exposure consisted in a linear sweep of 10 seconds (Frequency range 100-1000 Hz) using a sweep function generator (FS502, Feedback Instruments Ltd., UK) and a commercial amplifier (RX-4105 2 channel stereo, Sherwood, UK). The linear sweep was repeated for ten minutes simultaneously in all tanks. The energy input used for the noise generation was 10, 15 and 20 volts, that resulted in a final root mean square (RMS) SPL of 10, 18 and 20 dB re 1 µPa above background noise within the 100-1,000 Hz frequency range analysed (Fig. 3.1). Noise levels tested were intended to reflect the range in SPL levels recorded during the sound mapping of the facilities (from feeding: 8 dB re 1 µPa to hitting the tank wall: 26 dB 1 re µPa; above background noise levels). Noise treatments applied reached the peak SPL at around 150 Hz with maximum recorded values of 121, 127 and 128 dB re 1 µPa for each increasing RMS respectively. For each sound level tested, the seven experimental tanks corresponded to a different sample point with the first tank being sampled prior to the noise onset to determine basal levels, followed by the 10 min. sound exposure. The remaining 6 tanks were sampled at 10, 20, 30, 40, 60 and 120 min. post sound exposure. At each sampling point, 6 fish per tank were netted, anaesthetised using MS-222 (50 mg L-1, Pharmaq, Fordingbridge, UK) and blood sampled from the caudal vein using pre-heparinised syringes. Sampling, from netting

the fish to blood withdrawal of 6 individuals was performed in less than 5 minutes to minimise potential handling effects on cortisol release. Blood was kept on ice and haematocrit levels were determined within 10 min. Blood samples were then centrifuged at 1200G for 10 minutes and plasma samples stored at -20°C until further analysis.

3.2.4. Experiment 3.2: Effects of chronic noise exposure on spawning performance

Atlantic cod broodstock (total length 59.9 ± 5.6 cm, body weight 3.4 ± 1.0 Kg mean ± SEM) were maintained in a circular holding tank (5.3 m diameter, 88 m^3) under ambient photoperiod with an automatic dimming system to recreate dawn-dusk (SunMatch, AquaBioLab, Canada) using 40 W incandescent tungsten halogen bulbs. 20 females and 12 males were randomly split into two identical tanks (10 females and 6 males in each tank) (same as source stock tank) two weeks prior to the initiation of first egg release.

Both tanks were equipped with the same underwater loudspeaker as in exp. 1. One of the tanks was exposed daily to six noise events lasting for 1 hour, played at random times throughout the day while the other tank remained silent. The sound recording was a repetition of the same 10 sec linear sweep (Frequency 100-1,000 Hz) described above, however it was played for 60 min.

Ten different audio files with a duration of 24 hrs (6 randomised clusters of noise and 18 of silence, 1 hr each) were created and randomly played throughout the experimental period to ensure fish could not acclimate to the sound playback. The SPL was 34 dB re 1µPa above background noise levels, corresponding to the same energy input as tested at the highest exposure in experiment 3.1 (20 Volts). Fish were fed three

times a week using commercial marine fish diets (Classic marine, Biomar Norway). Cod eggs were collected daily from an external overflow egg collector at 15:00 hrs each day and egg quality parameters monitored. The total egg volume was measured volumetrically recording both the floating and sinking fraction volumes separately following standardisation of salinity to 35‰. Fertilisation rate (%) and egg diameter was measured from a sample (n=50 eggs) of the floating fraction only, as was the egg wet weight (triplicate measurement of the number of eggs in one gram). Viability percetange was calculated as follows: floating portion (%) * fertilisation rate (%). Furthermore, triplicate egg samples (~1.5 grams) were taken from both the floating and sinking fractions from each batch in 1.5 ml eppendorfs and stored at -20 °C for later cortisol analysis.

3.2.5. Cortisol analyses

Plasma cortisol levels were determined by radioimmunoassay (RIA) in duplicate according to North *et al.* (2006) and previously validated for Atlantic cod plasma by Cowan *et al.* (2011), similarly egg extracts were confirmed to be immunologically comparable to purified standards by comparing serial dilutions before analysis was performed (Data not presented). For egg cortisol analysis, an extraction, modified from McCormick (1998), was first carried out where egg samples were homogenised, in triplicate (0.5 g each), using 350 µl of PBS (0.01 M Tablets, Sigma-Aldrich, UK). Tubes were centrifuged for 5 minutes at 1200 G at 4 °C. Supernatants were then transferred to clean eppendorfs and centrifuged 1 min at 10,000 G. 400 µl of supernatant were then taken and added to 1ml of ethyl acetate. Tubes were vortexed and mixed in a rotary mixer for 1.5 hrs, then centrifuged at 720 G at 4 °C for 10 min. Extraction samples were kept at 4 °C until assayed as described above. 400 µl of sample extract were used in duplicate. Intra- and inter-coefficients of variation were 8.5

and 8.9 %, respectively (n=11), with a minimum sensitivity of 0.05 ng mL-1 which equates to 0.24 pg.egg^{-1}.

3.2.6. Data analysis

Sound data is expressed as sound pressure level (SPL) in the decibel scale. The impact of artificially created perturbations on noise levels is presented as the root mean square (RMS) of the SPL. Statistical analysis was performed using MiniTab version 16.2.2. All data sets were first tested for normality using the Anderson-Darling test. Cortisol concentrations and haematocrit percentages across experiments and sample points were analysed by one-way ANOVA. Plasma cortisol concentrations were normalised by $\log_{10}$ transformation. Egg cortisol concentrations were normalised by its' inverse value. Haematocrit levels, fertilisation rates and floating fraction % were arcsine, square root transformed. Significance levels ($p<0.05$) were determined by Tukeys post hoc test. The degree of linear relationship between egg cortisol content and fertilisation rate was analysed using the Pearson correlation coefficient (r). If the calculated r value was greater than the tabulated r value, at the 5% level, the correlation between variables was considered to be significant, with Run's test used to check for departure from linearity (INSTAT version 3.0). All data are presented as mean ± SEM.

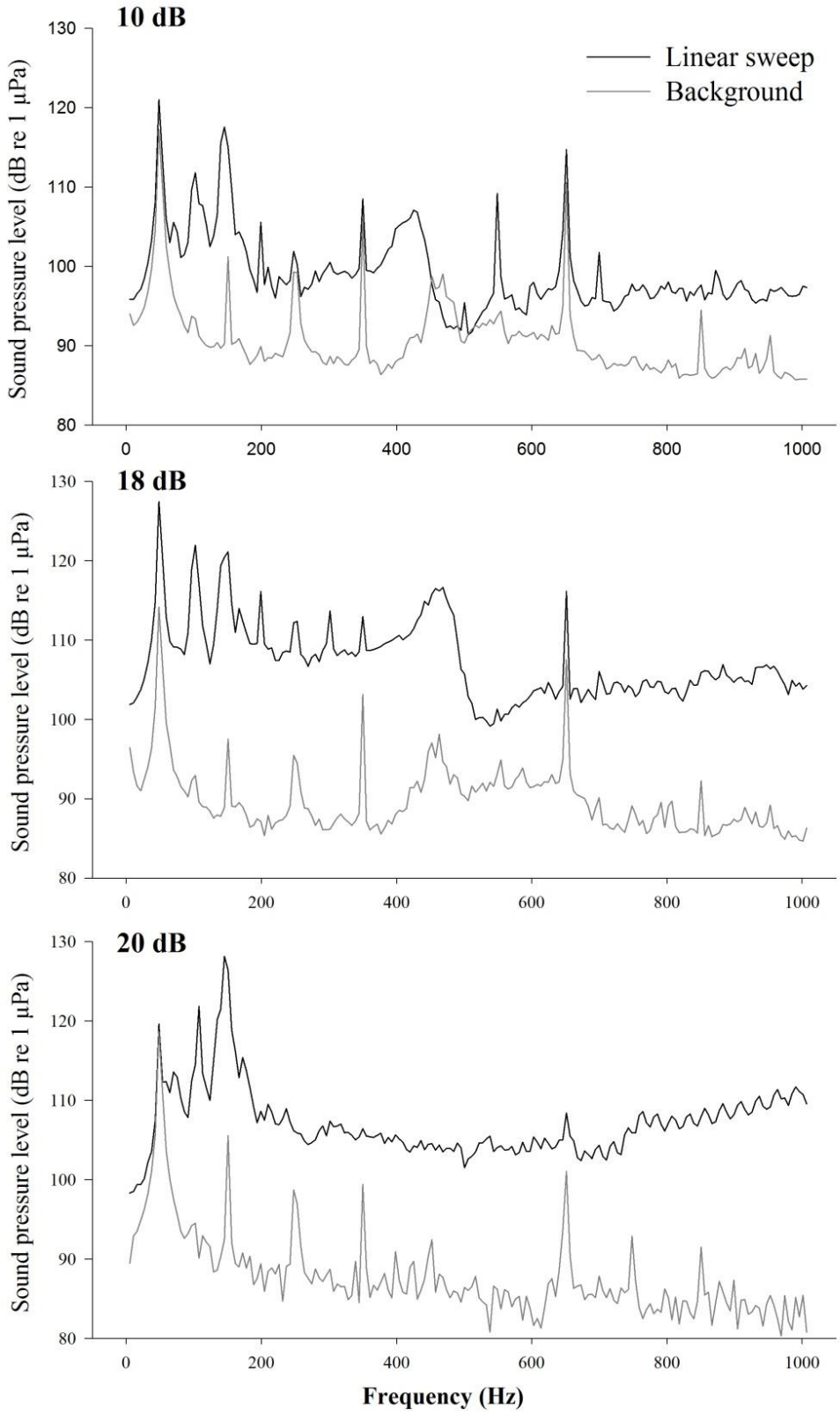

Figure 3.1. Sound levels of the background sound levels and linear sweep tested in experiment 3.1 for the stress reaction in Atlantic cod juveniles (100-1,000 Hz). At 10, 18 and 20 dB re 1 µPa above background noise, with energy input of 10, 15 and 20 Volts, respectively.

3.3. Results

3.3.1. Sound mapping and Broodstock vocalisations.

Background sound levels ranged from 89 to 111 dB re 1 µPa across the different tanks studied with a peak frequency ranging from 50 to 200 Hz (Table 3.1, Fig. 3.2). Different perturbations mimicking husbandry activities induced sound perturbations from 11 – 39 dB re 1 µPa above background levels (Table 3.1, Fig. 3.2). A survey of recorded vocalisation activity during the spawning season revealed a distinct nocturnal focus of activity (Fig. 3.3a) with the frequency of calls ranging from 36 -132 vocalisations per hour when extrapolated from the 5 mins. recordings. Analysis of recorded grunts (n=59) revealed that they typically had an average duration of 192 ± 21 ms (range from 144 to 215 ms) (Fig. 3.3b), with a frequency range from 28 to 252 Hz with peak frequencies around 48 Hz (Fig. 3.3 c&d). Sound levels ranged from 129 to 141 dB 1re µPa with a RMS of 132 ± 3 dB re 1 µPa.

Table 3.1. Analysis of common anthropogenic perturbations in the experimental tanks. Sound levels are expressed as the RMS SPL (dB re 1 µPa) ± S.E.M. in the frequency range 100-1000 Hz. Background sound levels are the mean RMS (n = 4). Sound perturbations are the RMS sound level gain over the background sound level (n = 6). Peak levels are the maximum sound pressure level registered. Frequency Peak (FP) range expresses the frequency range where the highest sound levels were registered.

Sound source	**Mean level ± SEM** (dB re 1 µPa)	**Peak level** (dB re 1 µPa)	**FP range** (Hz)
Background	**89.5 ± 0.5**	**109.1**	**48-210**
feeding	11.6 ± 0.4	27.1	Na
netting	22.9 ± 0.3	48.1	Na
soft knocks	21.5 ± 0.4	34.1	Na
knocks	26.2 ± 0.3	40.9	Na
hard knocks	39.4 ± 0.3	55.8	Na

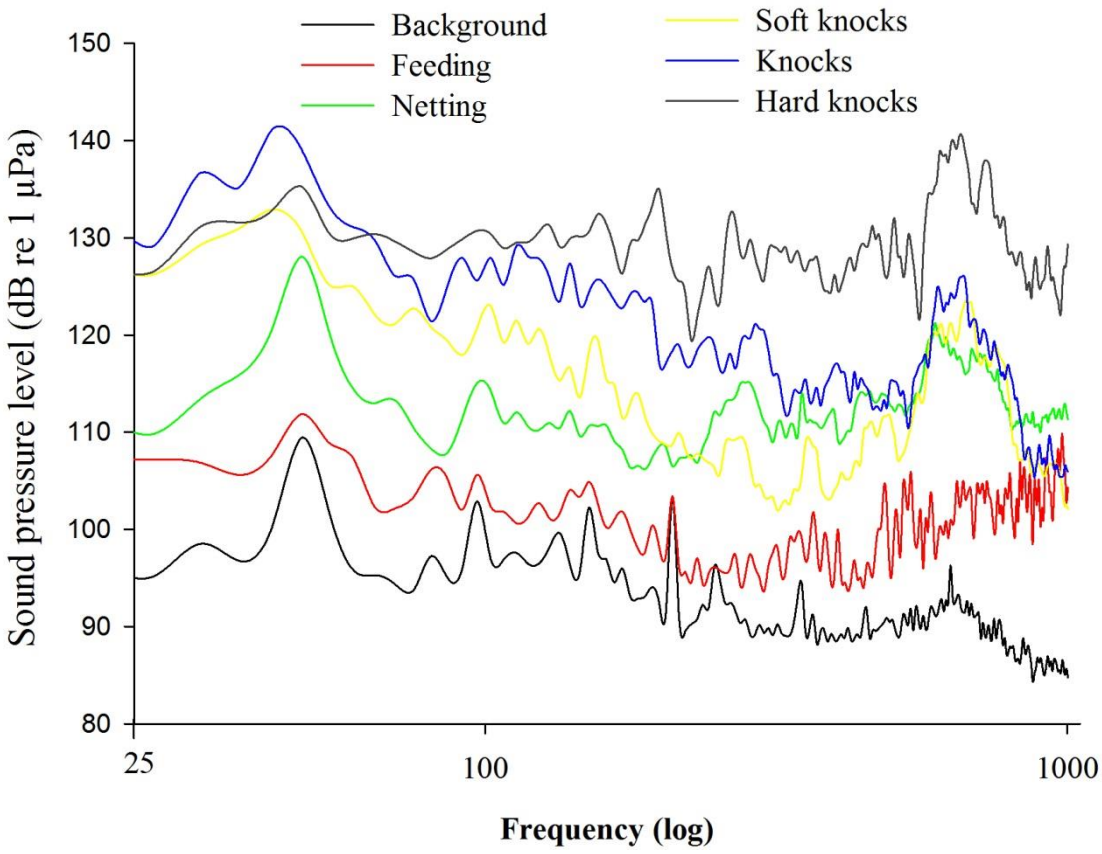

Figure 3.2. Background and husbandry activities sound levels dB re 1 µPa recorded during the facilities sound mapping (25-1,000 Hz). SPL levels corrected by a calculated factor of 183 dB: 2 m diameter indoor tank as used in Experiment 3.1.

3.3.2. Experiment 3.1: Sound as an acute stressor in Atlantic cod.

All noise levels tested showed a significant increase in plasma cortisol concentrations (Fig. 3.4). SPL of 20 and 18 dB above background noise resulted in a significant peak in cortisol concentration 20 minutes after the noise exposure (26.4 ± 9.9 and 29.3 ± 3.5 ng mL^{-1}, respectively) while in the 10 dB noise exposure, cortisol peak level was reached within 10 min. following the noise onset (22.2 ± 3.5 ng mL^{-1}). Plasma cortisol returned to basal levels 40 and 20 minutes post exposure for the 20/18 and 10 dB treatments respectively. Haematocrit levels (Total experimental mean 22.9 ±

1.2 %) did not show significant differences between sound exposures (data not shown). Casual observation of the behavioural response of the fish revealed that they usually displayed a "freeze" reaction immediately after the sound started with typical swimming resuming only after the noise exposure finished (data not shown).

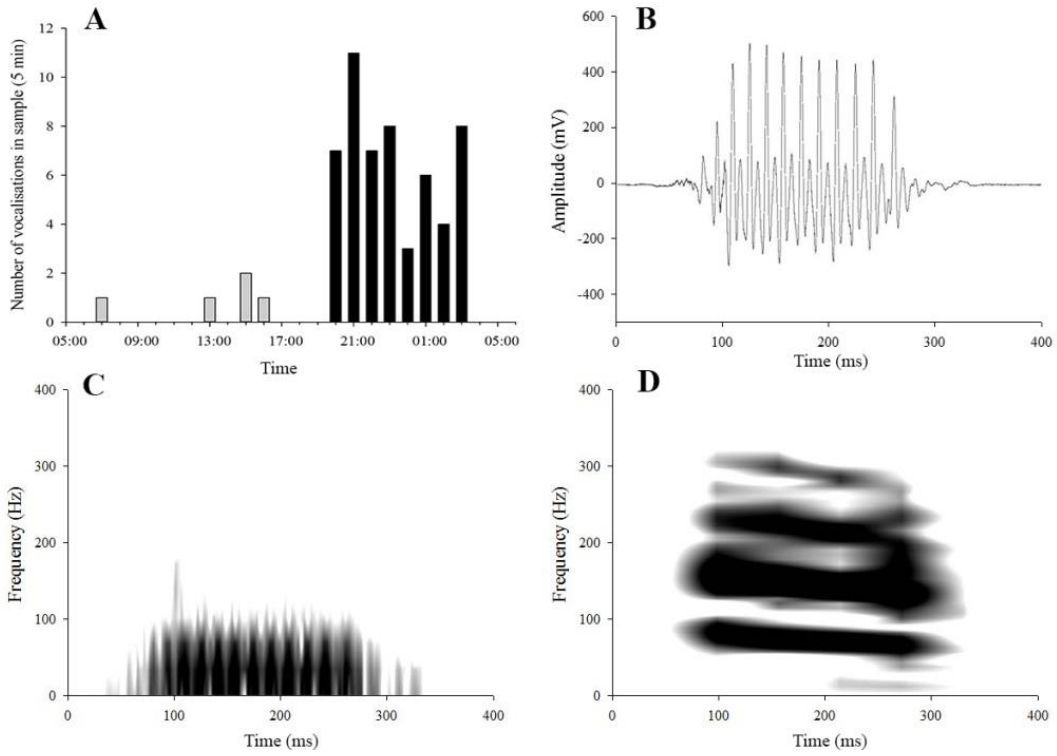

Figure 3.3. (A) Histogram of the number of Atlantic cod grunts recorded in a 5 min sample every hour during a 24 hr period (Grey bars correspond to day time and black bars to darkness). Typical waveform (B), spectrogram (C) and harmonised spectrogram (D) of a single grunt recorded during the scotophase (21:00 hrs) from one broodfish.

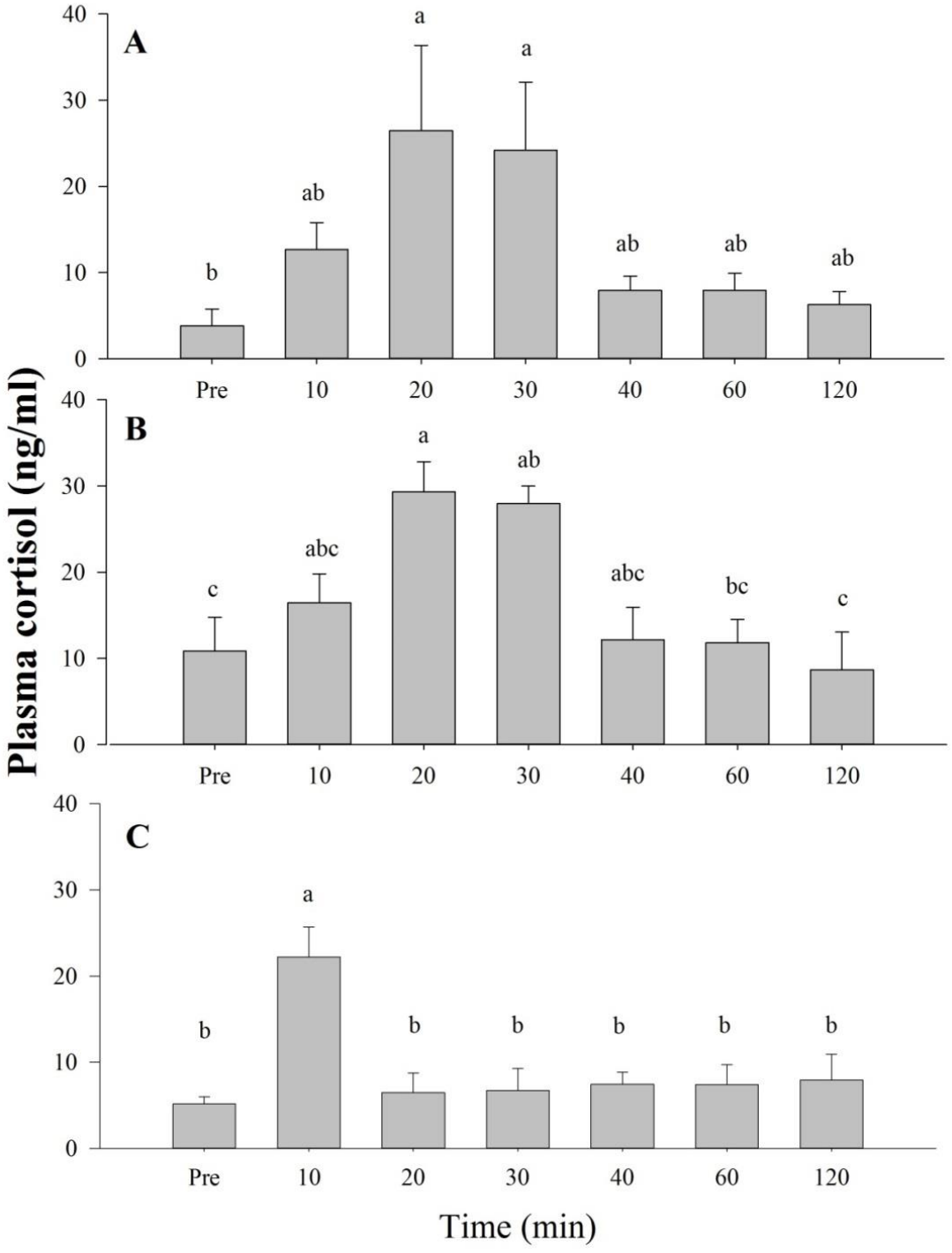

Figure 3.4. Plasma cortisol concentration (ng mL^{-1}) of Atlantic cod juveniles exposed to noise with RMS SPL above background noise of (a) 20 dB re 1 µPa, (b) 18 dB re 1 µPa; and (c) 10 dB re 1 µPa. Data are presented as mean ± SEM. Different superscripts denote significant differences (One-Way ANOVA $p < 0.05$)

3.3.3. Experiment 3.2: Effects of chronic noise exposure on spawning performance

Spawning in the control population started on March 4th and ended on May 8th, 2012 (65 days) (Fig. 3.5a). The spawning period in the sound exposed population was 23 days shorter starting on March 6th and ending on April 17th (Fig. 3.5b). Total egg volume produced during the spawning period was comparable with 29.79 and 25.50 kg (relative fecundity of 0.75-0.88 kg/female) for control and treated populations, respectively (Table 3.2). Larger egg batches were collected from the noise exposed population in the first four weeks of the experiment (Fig. 3.5b and 3.6a) however no eggs were released in the last two weeks of the study from the sound exposed population.

Table 3.2. Mean egg quality parameters over the spawning season ± S.E.M.. Total volume is the sum of the daily egg collection. Eggs per gram measured from 0.2 gram samples (n=3) per day. Egg diameter (n=50). Mean floating percentage over the spawning season. Mean of daily fertilisation rates. Mean viability percentage. * shows significant differences (One-Way ANOVA $p < 0.05$)

	Control	**Noise**
Total volume (Kg)	29.7	25.5
Eggs per gram	580 ± 12	579 ± 12
Egg diameter (mm)	1.28 ± 0.06	1.31 ± 0.07
Floating fraction (%)	70.4 ± 2.9	71.2 ± 4.5
Fertilisation rate (%) *	58.4 ± 3.8	37.12 ± 4.2
Viability (%) *	47.7 ± 3.7	33.22 ± 3.6

The proportion of floating fraction was comparable between treatments with the exception of weeks 3, 4, 5 and 7 (Fig. 3.6b) where it was higher in the control population. Overall mean batch fertilisation rate was significantly lower in the treated population (37.1 ± 4.2 %) compared to the control population (58.5 ± 3.8 %) (Table

3.2). When compared by weekly performance it was clear that the fertilisation rate within the floating fraction was significantly higher in the control population in most weeks from week three of the study onwards (Fig. 3.6c) with a similar trend apparent in the calculated viable egg volume from week 6 onwards (Fig. 3.6d). For both fertilisation rate and calculated viable egg volume there was a tendency towards reduced fertilisation rate/viable volume during the spawning period in both treatments. Egg diameter was statistically comparable between treatments throughout the study (Fig. 3.6e).

Cortisol levels in eggs batches ranged from 0.33 ± 0.04 to 14.83 ± 1.76 pg egg^{-1} across the study period (n=6). There was a wide variability between batches within treatments, when the data was collated by weekly average concentration within treatments there was no significant time by treatment interaction (P=0.069) but there was a significant difference in treatment overall mean cortisol levels with the sound exposed mean batch cortisol level being significantly higher (2.09 ± 0.43 pg egg^{-1}; P = 0.036) compared to the population mean level of the control population (1.56 ± 0.21 pg egg^{-1}). Cortisol levels were also monitored in the sinking fraction of each egg batch. These levels were in general about half of those recorded in the floating fraction, 0.83 ± 0.09 *vs.* 1.01 ± 0.17 pg egg^{-1} for control and sound exposed overall treatment means respectively. Regression analysis of weekly mean egg cortisol content and fertilisation rate revealed a significant negative relationship in the sound exposed population that explained almost 70% of the data variability while no such significant relationship was present in the control population (Fig. 3.7).

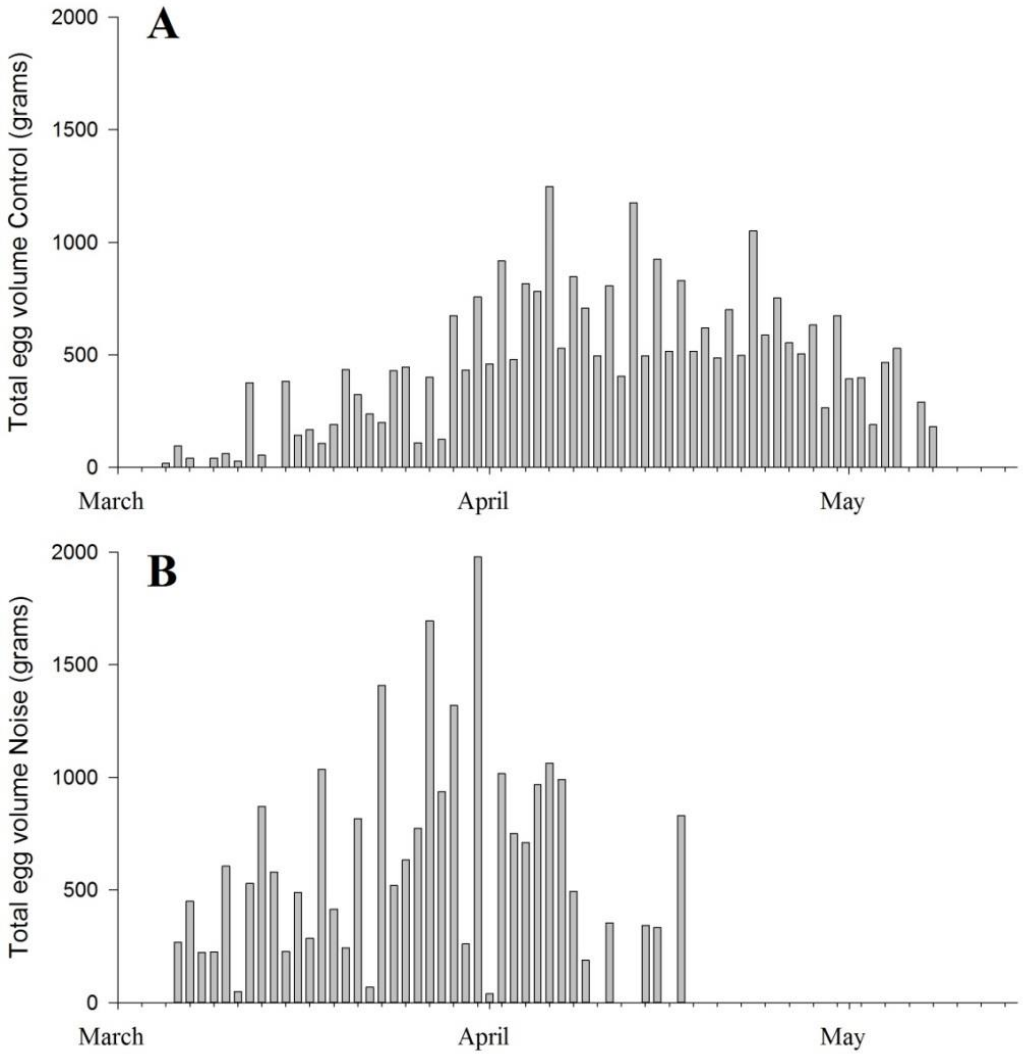

Figure 3.5. Daily total volume of eggs (Floating and sinking fraction combined) collected for each tank (control (A) and noise exposed (B) tanks) during the spawning season (March-May 2012).

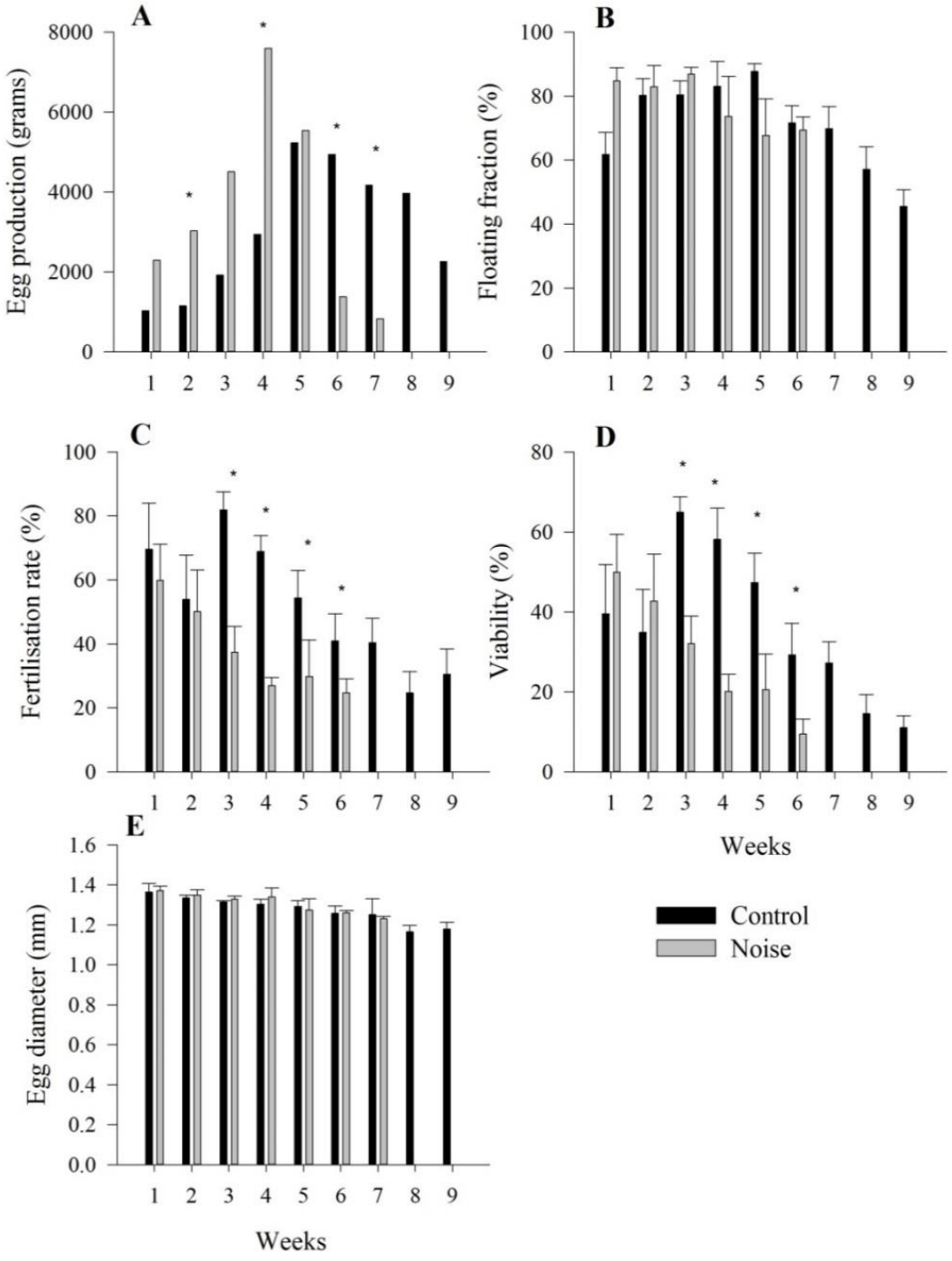

Figure 3.6. Egg quality parameters presented in mean values per week (n = 9). A) Total egg volume collected during the week (including floating and sink fractions); B) Floating fraction from each treatment C) Mean fertilisation rates (n = 100 per batch) from the floating fraction. D) Mean proportion of viable eggs. E) Mean egg diameter. * denotes statistical significant differences Tukey *post hoc* test following One-Way ANOVA.

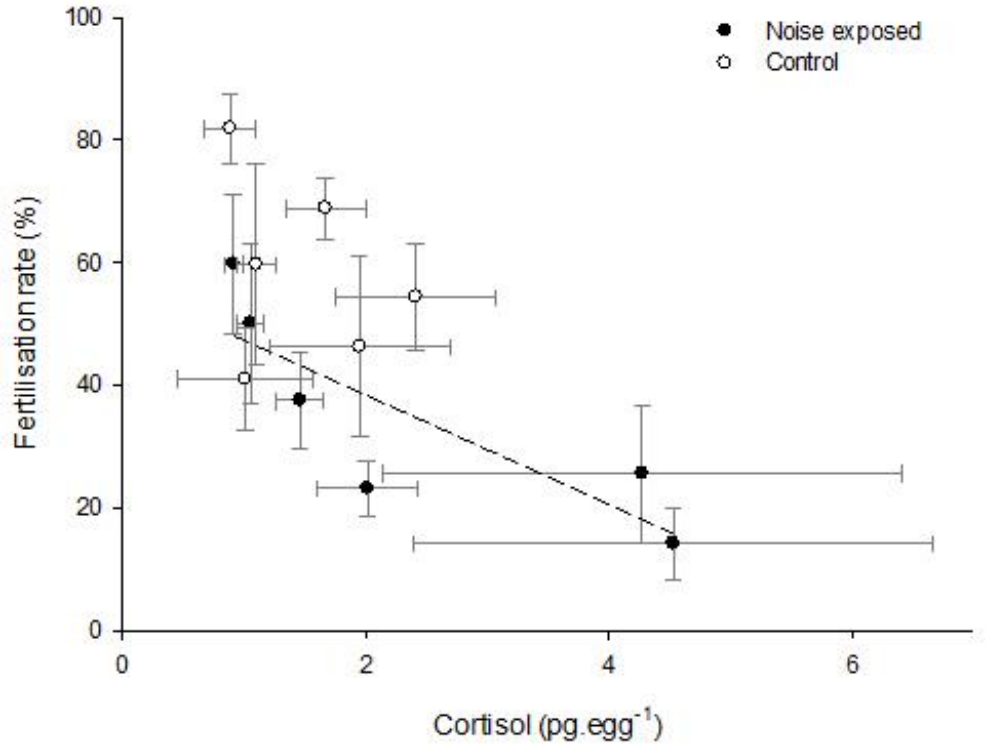

Figure 3.7. Scatter plot and linear regressions representing the relationship between weekly mean egg cortisol content and average fertilisation rate from the buoyant fraction. Bidirectional error bars represent the SEM for each axis. Dotted line is the linear regression for sound exposed population: *y* (Fertilisation rate) = 56.24 – 8.9*x* (egg cortisol level) r^2=0.69, P =0.04.

3.4. Discussion

The aim of this study was to investigate the effects of anthropogenic noise on both the acute stress response and chronic effects on spawning performance. Results showed, for the first time, that artificial noise mimicking anthropogenic sound levels generated in land-based aquaculture facilities can be perceived by cod and trigger a transitory acute stress response. Similar sound stressors applied over a longer time frame also appeared to negatively impact on spawning performance in terms of spawned embryo viability. These results have clear implications for both captive aquaculture stock management as well as the planning and management of offshore engineering works in relation to wild stocks.

During the initial sound mapping it was evident that background acoustic levels varied between tanks, as reported previously (Bart *et al.*, 2001), which could depend on size, material and tank position within the facilities. In general the background sound levels observed were lower than those previously reported in shallow open waters or in recirculating aquaculture systems (Wysocki *et al.*, 2007; Mueller-Blenkle *et al.*, 2010). During the initial empirical monitoring of the facilities, we catalogued a series of common husbandry activities and perturbations that caused a behavioural reaction in the fish (i.e. flight, freeze or attraction) such as hand feeding, dropping hand nets, knocks on the tank walls or people talking and walking. Each tested sound perturbation had an impact on the sound pressure levels increasing the overall SPL. The frequency range of such disturbances was in general at low frequencies (20-1,000 Hz) which are within the hearing thresholds of most fish species including Atlantic cod (Chapman and Hawkins 1973, Popper *et al.*, 2003). Many of the perturbations recorded in the present study are very common but easily avoidable. As Davidson *et al.*, (2007) demonstrated it is important to consider noise perturbations when designing land based aquaculture facilities and simple engineering solutions (i.e. suspended, insulated pipeline or physical distance) can be applied to efficiently minimise the transmission of extraneous noise.

In the acute stress response study total sound pressure levels tested were in the range to those recorded in common husbandry activities (10, 18 and 20 dB above background noise). In all cases noise triggered a physiological stress response showing a significant increase in cortisol plasma levels within 10-20 minutes after the noise exposure. The effects of cortisol in fish have been widely reviewed (Mommsen *et al.*, 1999, Milla *et al.*, 2009; Ellis *et al.*, 2012) and reported to affect growth, food conversion, immune response, flesh quality and reproduction. Cortisol peak levels have

been reported to vary from ~27 to 127 ng mL^{-1} in Atlantic cod when exposed to a capture/confinement stress (Morgan *et al.*, 1999), ~115 ng mL^{-1} when exposed to progressive hypoxia (Herbert and Steffensen, 2005) and > 400 ng mL^{-1} when kept at 24 °C (Pérez-Casanova *et al.*, 2008). In the present study, the peak cortisol concentration (up to 30 ng mL^{-1}) and the fast return to basal levels (within 20-40 mins) in the current study are typical of an acute stress reaction in Atlantic cod. Results also suggest that cortisol increase is sound pressure dependant (26 to 29 ng mL^{-1} for 20 and 18 dB and 22 ng mL^{-1} for 10 dB). Cortisol levels recorded in the present study are much lower than reported in previous stress trials in Atlantic cod, indicating that noise perturbation could be considered as a mild stressor compared to more conventional stressors tested (e.g. confinement, handling).

It is envisaged that such a temporary mild increase in blood corticosteroid level would not have a major impact on fish performances (Mommsen *et al.*, 1999) as fish are likely to acclimatise over a short period although this may vary between individuals as a factor of age, reproductive stage, nutritional status, social rank or immune condition and species (Martins *et al.*, 2012). The physiological reaction to a given stressor will depend on the perception or correlation that the animal associates with it i.e. threat or reward. During a light-feed conditioning study, Nilsson *et al.*, (2008) found that cod needed repeated light exposure to "switch" from a flight response to the positive food call. Similarly trout exposed to constant noise showed no significant difference in growth parameters after 9 months (Wysocki *et al.*, 2007). However, the ability of a fish population or individual to adapt to a chronic sound perturbation appears to be dependent upon the predictability of the perturbation (Mueller-Blenkle *et al.*, 2010). Most common anthropogenic perturbations found in a land based facilities (knocks, walking, oxygenator switching on) can be considered to be random and

therefore it can be hypothesised that fish would take longer to adapt however further studies are clearly needed.

Haematocrit levels did not show any significant variation between fish exposed to noise and controls during the sampling period. In a similar experiment with sea bass and sea bream, haematocrit and lactate levels were altered following noise exposure (Buscaino *et al.*, 2010). Interestingly, in the same study glucose levels differed in sea bream but not in sea bass. These species specific differences are most likely reflective of the general variability in physiological responses to stress widely reported between species (Barton, 2002).

In the chronic sound exposure study our results showed that noise had a significant negative effect on Atlantic cod broodstock performance. While the spawning window in the treated population was much shorter than the control population, the total egg volume was not significantly different between populations due to higher egg volumes being released during the early phases of the spawning period. This is in agreement with previous reports showing irregular spawning cycles caused by confinement stress with cod broodstock engaged in fewer courtships and skipping the initial steps of the mating sequence (Morgan *et al.*, 1999). Most egg quality parameters (total egg volume, floating fraction, egg diameter and egg weight) appeared to not be negatively affected by the noise treatment. However, fertilisation rates were reduced by 40 % reducing viable egg productivity by over 50 %. Overall, 18 kg of viable eggs were collected from the control population as compared to 8 Kg from the sound exposed population. Based on the egg counts during the trial this reduction in 10 Kg of viable eggs equates to approximately 5,800,000 less viable embryos from a comparably sized spawning population. In a hatchery environment this would translate to a loss of

approximately 300,000 weaned juvenile cod (assuming a survival rate of 5 % from egg to a 20 g weaned juvenile) which would have a farm gate value in excess of £500,000.

Analysis of cortisol content in freely released eggs showed great batch variability across the spawning season with mean levels ranging from 0.3 – 14.8 $pg.egg^{-1}$ which is comparable to levels measured in a range of other teleost species oocytes (Hwang *et al.*. 1992). The overall mean cortisol levels were 34% higher in the sound exposed population than in the control population. While it appeared that there was a differential shift in cortisol levels between treatments with levels increasing over time, this trend fell short of significance (P= 0.069). There was however a clear negative relationship between cortisol level and fertilisation rate in the sound exposed population that did not exist in the control population. Kleppe *et al.*, (2013) reported that cortisol concentration in cod eggs and embryos was positively correlated to the plasma concentration of the female. Furthermore high concentrations of plasma cortisol in spawning cod females ~30 ng mL^{-1} resulted in a higher frequency of deformities in larvae (Morgan *et al.*, 1999) and ~150 ng mL^{-1} caused differential gene expression in eggs and embryos in pathways linked mainly to cytogenesis and mesodermal fate (Kleppe *et al.*, 2013). By association we can propose that in the current study the sound stressor provided to the broodstock, which we had previously been shown to induce an acute mild cortisol response in cod, has acted as a chronic stressor and the subsequent maternal transfer of elevated cortisol is associated with compromised spawning performance. While the long term survivability of embryos was not recorded, the work of Kleppe *et al.*, (2013) would suggest that those which did survive were possibly compromised further as they could exhibit an elevated proportion of abnormal development, as has also been recently shown in marine invertebrates (Aguilar de Soto *et al.*, 2013).

While convention has focused on the maternal influence on embryo viability it must be recognised that the male broodfish were equally subjected to the noise stressor with a presumed concomitant increase in plasma cortisol concentration. Moderate doses of cortisol (<100 ng mL^{-1}) enhance DNA replication and mitosis in spermatogonia in Japaneese eel (Ozaki *et al.*, 2006), however doses >100 ng mL^{-1} had a negative effect on spermatogonia proliferation. Excess cortisol levels inhibit the reproductive physiology of the males, delaying testicular development, spermatogenesis and lower sperm counts (Carragher *et al.*, 1989; Campbell *et al.*, 1992; Consten *et al.*, 2001). It is therefore possible that lower sperm quality could have also contributed to the reduction in fertilisation success observed in the current study though this requires confirmation. In addition to the physiological responses to stress observed, noise can mask natural communication (Hawkins and Chapman, 1975) or damage the auditory system (Hall *et al.*, 1981; Hastings *et al.*, 1996; McCauley *et al.*, 2003). Luczkovich *et al.*, (2012) found that vocal fish of the family Sciaenidae produced fewer vocalizations when ambient noise increased due to vessel and ferry traffic, although this reduction happened only outside the reproductive season. In cod it is known that captivity does not inhibit reproduction while it may restrict the vocalisation repertoire (Midling *et al.*, 2002; Finstad and Nordeide, 2004). As grunts have been correlated to mating success (Rowe and Hutchings, 2006, 2008), even if the randomised noise in the current study did not always conflict with the vocalisation climax, hearing thresholds might have been adjusted to block the unwanted noise throughout the experiment, reducing the communication success and mate choice process even in the absence of the noise exposure at the exact time of the grunts (Scholik and Yang 2001; Smith *et al.*, 2004).

This study focused on the effects of noise in enclosed aquaculture facilities where activities are limited to the specific husbandry needs. The implications are clear and

support previous work proposing simple engineering fixes to minimise the presence and vigour of anthropogenic sound sources in aquaculture facilities to maximise productivity (Davidson *et al.,* 2007). What is perhaps more overlooked are the implications for wild fisheries management. Initial interest in sound perception in fish was to minimise the flight reaction to ship trawling noise (e.g. Buerkle, 1977), more recently attention has been directed towards the potential negative effect of the rapid increasing human activities in the oceans, mainly related to maritime traffic and offshore engineering (Slabbekoorn *et al.*, 2010). Hildebrand (2009) reports that shipping activity globally has increased by a factor of 6 from 1955 to 2005 and thus has also manifested an increase in low frequency ambient noise of at least 20dB in the same time frame. In addition, the need to move towards "green energy" production has heralded the expansion of off-shore wind farm developments. During construction the noise caused by the pile-driving into the sea bed creates sounds up to 250 dB re 1 µPa causing a known flight reaction in Atlantic cod and Dover sole (Mueller-Blenkle *et al.*, 2010). During the off-shore wind farms construction an "air bubble wall" is installed to minimise the noise pollution expansion, however this also means that fish are restricted to approach those grounds while the construction lasts. Post construction there is already evidence that sound generated by operational wind farms affects cod residency patterns (Wahlberg and Westerberg, 2005). Offshore turbines' rotors noise levels were estimated to be 150 dB 1 re µPa at a frequency of 60 and 180 Hz within 1m, this model estimates that cod is capable of detecting the rotor noise as far as 2 km considering a 10 dB detection threshold above background noise (Wahlberg and Westerberg, 2005). Importantly, this same threshold, "10 dB above background noise", proved to trigger a physiological stress reaction in the current study. Traditionally sound mapping datasets have focused on areas of intense shipping activity, given the

current results; the authors would advocate more detailed work around offshore engineering construction. This is particularly pertinent in the European context due to the expansion of offshore wind farm projects in the North Sea that overlay established cod spawning grounds (Fox *et al.*, 2008; FourC Offshore, 2013). In this context setting a provisional threshold of concern at >30 dB re 1 µPa above background noise (reflective of the levels tested in Exp 3.2) would help identify areas where spawning performance could potentially be compromised.

Overall this work is the first demonstration that sound can act as a stressor in Atlantic cod and that furthermore a chronic sound exposure can significantly compromise cod spawning performance. There is a suggestion that maternal cortisol transfer could be associated with this reduction in spawning performance however other mechanisms could be at work which warrants further investigation. The implications for farm management of the species are clear, where every effort should be made to minimise the propagation of anthropogenic sound within culture facilities. What is perhaps of greater concern is the potential significance of these findings for wild fisheries management. Further work is required to model the expansion of anthropogenic sound sources associated with offshore engineering and confirm if sound pollution is passing levels that have here-in been demonstrated to severely impact of cod spawning performance.

Chapter 4

EFFECTS OF LIGHT SPECTRUM AND TANK BACKGROUND COLOUR ON ATLANTIC COD (*GADUS MORHUA*) AND TURBOT (*SCOPHTHALMUS MAXIMUS*) LARVAE PERFORMANCE

Research article

Submitted to: Aquaculture

Authors: ***Sierra-Flores, R****; Davie, A.; Grant, B.; Carboni, S.; Atack, T and Migaud, H.*

4.1. Introduction

Protocols for the optimal rearing of marine fish larvae have seen significant improvements in the last decade through the definition of the most appropriate rearing environmental conditions in culture (rearing systems, temperature, live feeds, weaning.) (Planas and Cunha, 1999; Hamre *et al*., 2013). However, of all the abiotic and biotic parameters that can influence larval performance and survival, the impact of light has received comparatively little attention (Koven, 2003; Villamizar *et al*., 2011; Pittman *et al*., 2013). Fish feeding and foraging activity involves different sensory mechanisms e.g. vision, mechanoreception, chemoreception, electroreception (Guthrie, 1986; Finn and Kapoor, 2008). However, at the early stages of larval development, none of those systems are fully functional. Eye development is one of the first changes in larvae morphology with the appearance of a duplex retina (rods and cones), that enlarges as the fish grows (Evans and Browman, 2004). Retina development starts by the appearance of cone photoreceptors (Blaxter and Staines, 1970), responsible for colour detection, while rods (intensity reception) develop further during metamorphosis. The basic structure of photoreceptors (retinal and non-retinal) are well conserved across vertebrates, although specific morphological and functional (i.e. sensitiveness) features vary widely across species and within developmental stages (Blaxter, 1969; Britt *et al*., 2001). Fish living in different underwater photo-environments therefore appear to have adapted their maximum photopigment sensitivity according to the species ecological niche (Kusmic and Gualtieri, 2000). In addition water selectively absorbs light wavelengths (< 390 nm, > 600 nm) with blue wavelengths (~450 nm) being the most efficient at penetrating the sea water column (>100 metres depth depending on water turbidity) (Utne-Palm, 2002). This explains the increased sensitivity to short

wavelengths e.g. blue/green displayed by most marine fish species (Lythgoe, 1979; Villamizar *et al*., 2011).

Photoreception plays a crucial role throughout the whole teleosts' life cycle regulating (directly or indirectly) physiological and behavioural (feeding activity, swimming, schooling, migration and reproduction) responses (Boeuf and Le Bail, 1999; Bromage *et al*., 2001; Migaud *et al*., 2010). In an aquaculture context incident light, in terms of intensity and spectral content, have been shown to influence larval growth, performance and survival in a number of marine teleosts however response appears to be species specific as reviewed by Villamizar *et al*. (2011). Tank colour, which is less well studied, has also been shown to impact on growth and/or development in a limited number of species including haddock (*Melanogrammus aeglefinus*) (Downing and Litvak, 1999), spotted sea bass (*Paralabrax maculatofasciatus*) (Pena *et al*., 2005), striped trumpeter (*Latris lineta*) (Cobcroft and Battaglene, 2009) and orange spotted grouper (*Epinephelus suillus*) (Duray *et al*., 1996). The drivers behind these physiological effects remain to be clearly defined however visual acuity and thus perception of prey may be one of the main influences (Utne-Palm, 2002).

The aim of this study was to test the effects of light spectrum and tank background colour on larvae development and survival of two commercially important fish species inhabiting different, but overlapping, ecological niches (benthopelagic Atlantic cod, *Gadus morhua*, *vs.* benthic turbot, *Scophthalamus maximus*) as there remains a lack of information regarding the impact of these two parameters on their larval rearing performance.

4.2.Materials and methods

4.2.1. Broodstock and egg production

All the experiments were carried out at the facilities of Viking Fish Farms Ltd., Ardtoe Marine Laboratory, Acharacle, Scotland (N 56°46' W 05°53') and conducted in accordance with the Animals Scientific Procedures Act of 1986, UK following independent ethical review. Hatchery reared Atlantic cod broodstock were kept in an indoor circular tank (4.6 m diameter, depth 1.9 m) with a flow through system at ambient temperature (7-8 °C spawning season), salinity (~34.5 ppt) and photoperiod (except for experiment 4.2a where eggs were obtained from cod broodstock exposed to a 6 week advanced photoperiod). Fish were fed three times a week using commercial dry feed (Classic Marine, Biomar). Fish gender ratio was kept at 1:1 male:female. Atlantic cod broodstock spawned naturally in the tanks and eggs were collected daily at 15:00 hrs through a 100 µm mesh egg collector.

Turbot broodstock were kept in an outdoor tank (5.3 m diameter, depth 1.9 m) under natural ambient light conditions, temperature (12-14 °C during spawning season) and salinity (~34.5 ppt). Fish were fed three times a week using commercial dry feed (EFICO Sigma 570, Biomar) and sex ratio was kept at 1:2 male:female. As turbot do not spawn spontaneously in captivity under these stocking conditions, fish were stripped every third day by gentle abdominal massage according to hatchery procedure. Eggs from several females (3-5) were stripped and pooled into a clean plastic 2 L jug. Milt from at least two males was collected into individual sterile 2.5 mL syringes. Artificial fertilisation was done by gently mixing milt and eggs and adding filtered sea water (5 µm). After 5 mins, fertilised eggs were transferred into a 10 L plastic container and 8 L of filtered seawater were added and eggs were left in darkness undisturbed for 30 min.

Eggs used in the present experiment for both cod and turbot were first checked for quality (floating fraction, development through blastomere scoring, fertilisation rate and total egg production). Cod egg floating fractions were disinfected for 45 sec (Kickstart, RS Hygene, UK, concentration of 1:250) and then rinsed in filtered sea water for 30 sec, weighed and transferred to 80 L conical black incubators. Eggs were first incubated in darkness at ambient temperature (7-8 °C and 12-14 °C for cod and turbot eggs, respectively) at a density of 6 g of eggs L^{-1} and provided with continuous water flow at 400 mL min^{-1}. Water flow and aeration was turned off daily for 30 minutes to allow dead eggs to sink to the bottom and be gently siphoned out. Embryo development was assessed daily by visual inspection under the microscope of a small egg sample taken from of the floating fraction. Cod eggs were transferred to the experimental system at 75-80 °C days (prior to hatching) while turbot eggs were left to hatch in the incubator and transferred at 100 °C days as larvae in the experimental system. Numbers were assessed volumetrically from the floating fraction in the incubators prior to transfer.

4.2.2. Experimental set up and treatments

Two sets of experiments were performed in both species: effects of narrow bandwidth light on larvae performances (Exp. 4.1 a and b for cod and turbot, respectively) and effects of tank background colour on larvae performances (Exp. 4.2 a and b for cod and turbot, respectively). All trials were performed in the same experimental setup that consisted of 12 flow through tanks (triplicated design with four treatments) under a continuous illumination (24L:0D). Temperature was controlled for turbot (Exp. 4.1b and 4.2b) and kept at 18.5 ± 0.9 °C. Seawater was passed through mechanical filtration systems (sand filters and cartridges; 5 µm particles size) and UV sterilised. All experimental tanks received gentle aeration via a glass tube (0.5 mm) and

water was exchanged at 0.2 L min^{-1} with temperature and dissolved oxygen being monitored daily.

Light spectrum experiments (Exp. 4.1a and 4.1b) were performed in 150 L black, flat bottom circular tanks running at a 100 L capacity. Light was provided by white (peaks: 460 and 560 nm), green (530 nm), blue (455 nm) and red (640 nm) dimmable Light-Emitting Diode (LED) lamps (Intravision Aqua AS, Norway) suspended 40 cm above the water surface. In Experiment 4.1b (turbot) the same LED lights were used except for the white spectrum which was created using 60 watts dimmable incandescent tungsten bulbs suspended 40 cm above the water surface. Spectral profiles are shown in Figure 4.1. Light intensity in the experimental tanks was balanced to 1.3 ± 0.01 W m^{-2} at the surface and 0.4 ± 0.01 W m^{-2} at the bottom for all light treatments.

Tank background colour experiments (Exp. 2a and 2b) were performed in 100 L polyethylene coloured (white, blue, red and black) flat bottom circular tanks (Mailbox mouldings, UK) filled to 75 L. Light was delivered by 60 watts dimmable incandescent tungsten bulbs (same as Exp. 4.1b). Light intensity in the experimental tanks was balanced to 1.25 ± 0.04 W m^{-2} at the surface, and 0.18 ± 0.01 W m^{-2} at the bottom.

Light irradiance (Watts m^{-2}) for all treatments was measured with a calibrated single channel light sensor (Skye Instruments Ltd., Powoys, UK) and balanced for the surface and bottom of the tanks. Spectral composition was measured using a portable spectroradiometer with an umbilical fibre optic based sensor head (Stellarnet Inc, USA).

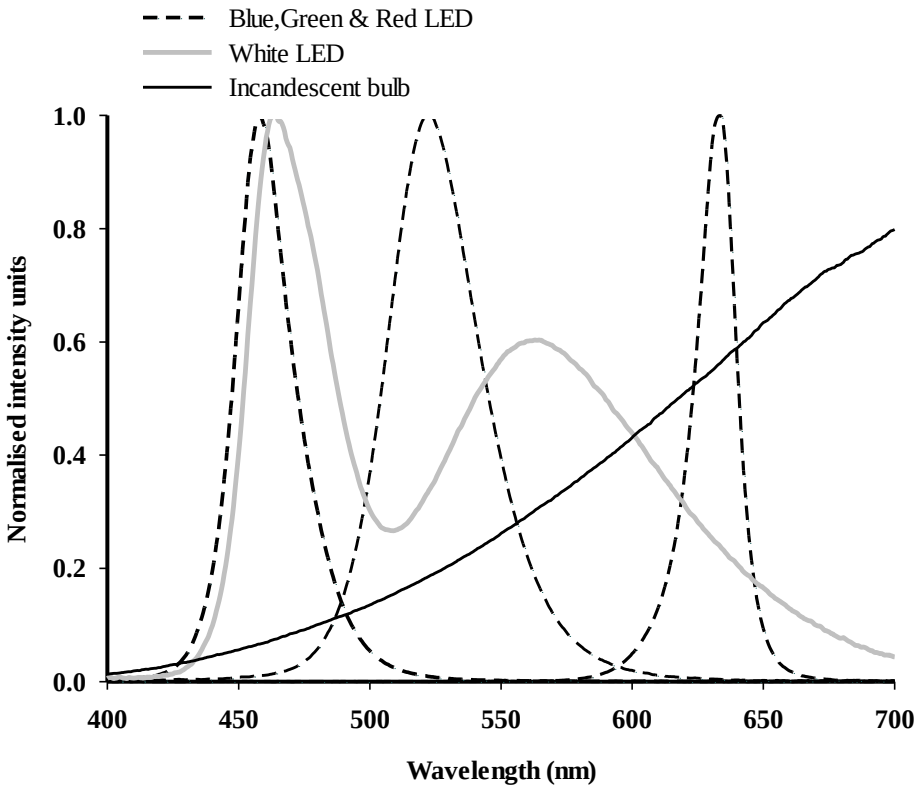

Figure 4.1. Normalised spectral profiles data from the lighting used in light exposure treatments and tank background treatments. LED light provided by Intravision Aqua AS, Norway. Peak wavelengths: blue (455 nm), green (550 nm), red (640 nm) and white (460 and 560 nm). White light spectral profile for light used in experiment 4.2b (Incandescent tungsten bulb 60 W) is also provided.

4.2.3. Larval rearing

At 100 °C days Atlantic cod eggs hatching rates were determined (Exp. 1a: 96.6 % and 2a: 96.0 %) and light treatments as well as gentle aeration and water inflow started (0.1L min^{-1}). Larvae (50 larvae L^{-1} for Exp. 1a and 75 larvae L^{-1} for Exp. 2a) were reared in "green water" using 50:50 mixture of *Nannochloropsis atomus* and *Isochrysis galbana* (20 x 10^6 and 13 x 10^6 cells mL^{-1} respectively). Microalgae were added every day. On the 3rd day post hatch (DPH), rotifers enriched with *Pavlova lutheri* (5 individuals mL^{-1}) were introduced and water inflow increased (0.2 L min^{-1}). From 5 DPH, rotifer residuals were counted and more were added to maintain a density

of 5 mL^{-1}. At 30 DPH (240°C days) *Artemia salina* nauplii (6 hours enriched with AlgaeMac-2000) was first introduced (0.3 individuals mL^{-1}). By 36 DPH rotifers and microalgae were completely substituted by 0.5 *Artemia naupii* mL^{-1} (supplied twice a day from 42 DPH to 53 DPH, after 6 hours of enrichment enriched). Dry artificial diet (AgloNorse, EWOS Ltd., UK) was first introduced on 54 DPH for Exp. 4.1a.

In turbot Exp. 4.1b and 4.2b, all tanks were stocked with 50 larvae L^{-1} (100 °D at 100 % hatch). The same larviculture protocol than for cod experiments was used except that *Artemia* were introduced in small quantities (0.2 individuals mL^{-1}) from 12 DPH (200 °C days) and by 30 DPH dry feed was first introduced (Otohime Hirame, Japan) and pellet size gradually increased (Larviva ProStart, Biomar, France) according to the farms established turbot protocols.

4.2.4. Sampling

In order to monitor Atlantic cod larvae performance (Exp. 1a and 2a) samples were taken at hatch (15 larvae/tank) and every 10 days afterwards (10 larvae/tank). Pictures of individual larvae were taken to measure standard length (SL), myotome height (MH) and eye diameter (ED) by image analysis (ImageJ, USA).

Condition index (CI) of larvae was calculated using

CI = (myotome height (mm) / standard length (mm)) x100.

In addition, 10 larvae were stored in pre-weighed eppendorfs for dry weight (DW) analysis (3 replicates per tank).

Weight specific growth rates (SGR, % d^{-1}) was calculated using

$SGR = (e^{g}-1)\times 100$ were $g=(\ln X_2-\ln X_1)/t_2-t_1$

where X_2 and X_1 are weights at times t_2 and t_1 respectively.

Survival rates, estimated as percentage of stocked eggs, were determined at the end of the trial (50 and 60 DPH for Exp. 4.1a and 4.2a, respectively) by counting the remaining larvae in all experimental tanks. Samples to monitor turbot larvae performance (Exp. 4.1b and 4.2b) were taken at hatch and every 7 days afterwards (15 larvae/tank). Pictures of individual larvae were taken to measure standard length (SL), myotome height (MH) and eye diameter (ED) by image analysis (ImageJ, USA). 10 larvae were stored in pre-weighted eppendorfs for dry weight analysis (3 replicates per tank). Survival rates, estimated as a percentage of stocked larvae, were determined at the end of the trial (56 DPH) by counting the remaining larvae in all experimental tanks. From 32 DPH no more samples were taken from the tank background colour trial (Exp. 4.2b) due to the limited number of fish left, however fish were weaned and survivals at 56 DPH determined by comparison with initial number of larvae stocked into the tanks.

4.2.5. Statistical analysis

Statistical analysis was performed using Minitab 16 (Minitab Ltd., UK). All datasets were first tested for normality and homogeneity using the Anderson-Darling test. Growth parameters (SL, MH, ED and DW) were analysed using a General Linear Model which accommodated for the replication effects within treatments. Condition index was arcsine square root transformed. All data are presented as the mean value ± standard error (SEM) (n=30 per treatment). Survival rates were arcsine square root transformed and variance tested by One-Way ANOVA. Significance level was set at $p < 0.05$ and significant interactions evaluated by Tukey *post hoc* test.

4.3. Results

4.3.1. Effects of light spectrum on Atlantic cod (Exp. 4.1a) and turbot (Exp. 4.1b) larvae performance

Cod larvae reared under a blue spectrum showed a significantly higher dry weight (DW) than those reared in the red treatment from 40 DPH and from 50 DPH, to the end of the trial, the red treatment yielded larvae with significantly lower dry weights than all other treatments (Fig. 4.2). There was however no statistical differences in treatment mean weight SGR within isolated sample periods or over the complete experimental period. A similar trend to the DW data was apparent in the other morphometric measurements with standard length (SL), myotome height (MH) and eye diameter (ED) being significantly smaller under red light than all other treatments from 30 DPH, equally condition index (CI) was significantly lower from 30 DPH under red light however at the end of the trial all treatments had comparable condition.

In turbot, larvae under green light had significantly heavier DW than all other treatments from 21 DPH and, with the exception of 42 DPH, remained so for the remainder of the trial (Fig. 4.3). There was however no statistical differences in treatment mean weight SGR within isolated sample periods or over the complete experimental period. For both SL and MH the green treatment was significantly higher than all others from 14 DPH and remained so to the end of the trial while ED was significantly larger under green from 14 DPH by the end of the trial. Larvae under green, blue and white treatments were comparable and significantly larger than under red light. As for CI at 14 and 21 DPH there was a transitory separation in condition with larvae under green light having a higher CI however thereafter all treatments were comparable.

Due to wide replicate variation there was no statistical difference in treatment mean survival for either cod or turbot. The highest survival rate for cod larvae was found in the red light treatment (6.3 %) followed by the blue and white treatments (3.6 %), with the lowest survival observed in the green light (2.8 %) (Table 4.1). In turbot, the best survival rates were observed in the blue, white and red treatments (2.2 to 2.7 %) while survival in larvae under the green light appeared to be much lower (0.8 %).

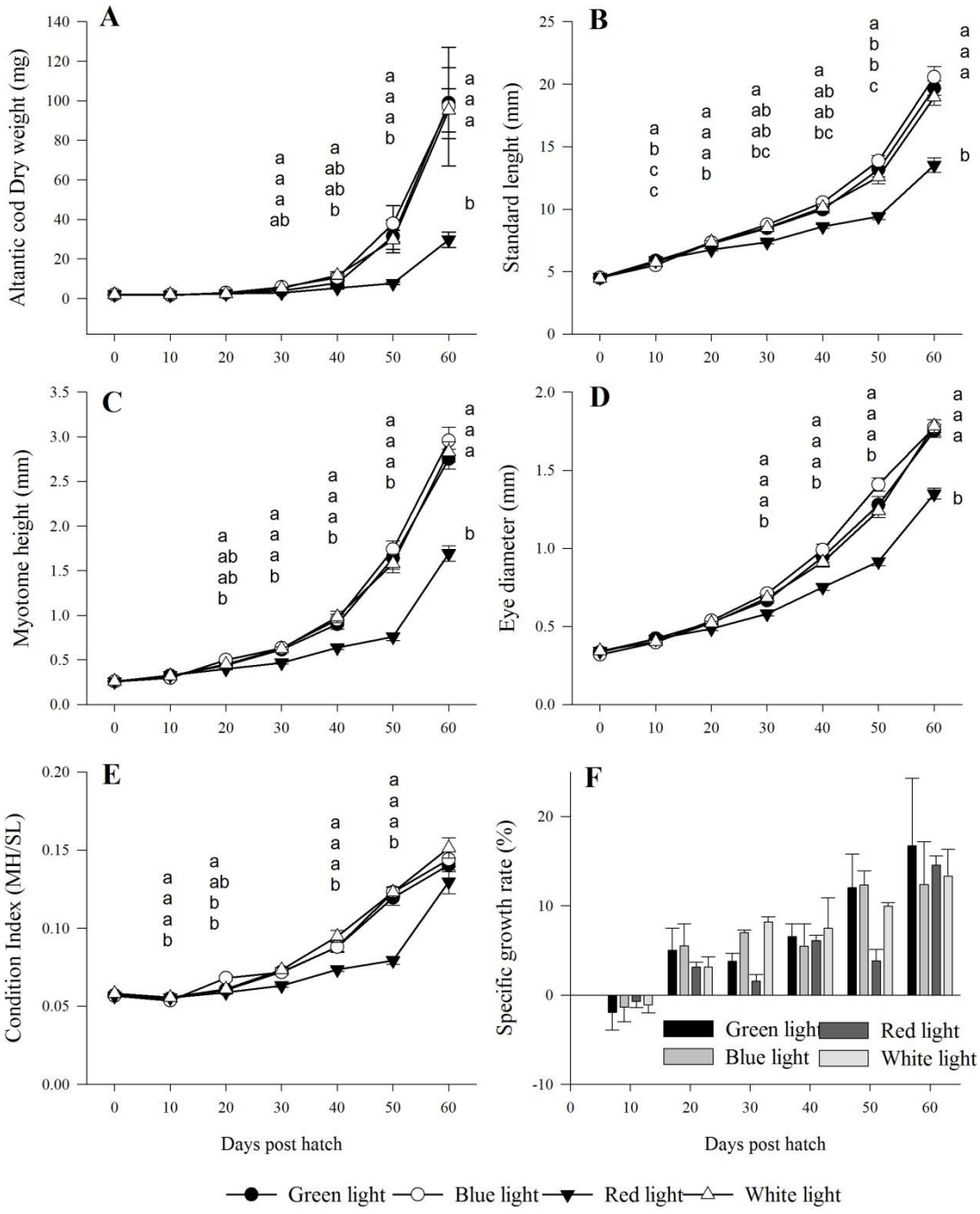

Figure 4.2. Atlantic cod larvae growth parameters exposed to the light treatments: Green light, Blue light, Red light and White light. Dry weight (A), Standard length (B), Myotome height (C), Eye diameter (D), Condition index (E) and Specific growth rate (F). Growth parameters are expressed as mean ± SEM (n = 30, 10 larvae/replicate). Superscripts denote statistical significant differences at a given time point.

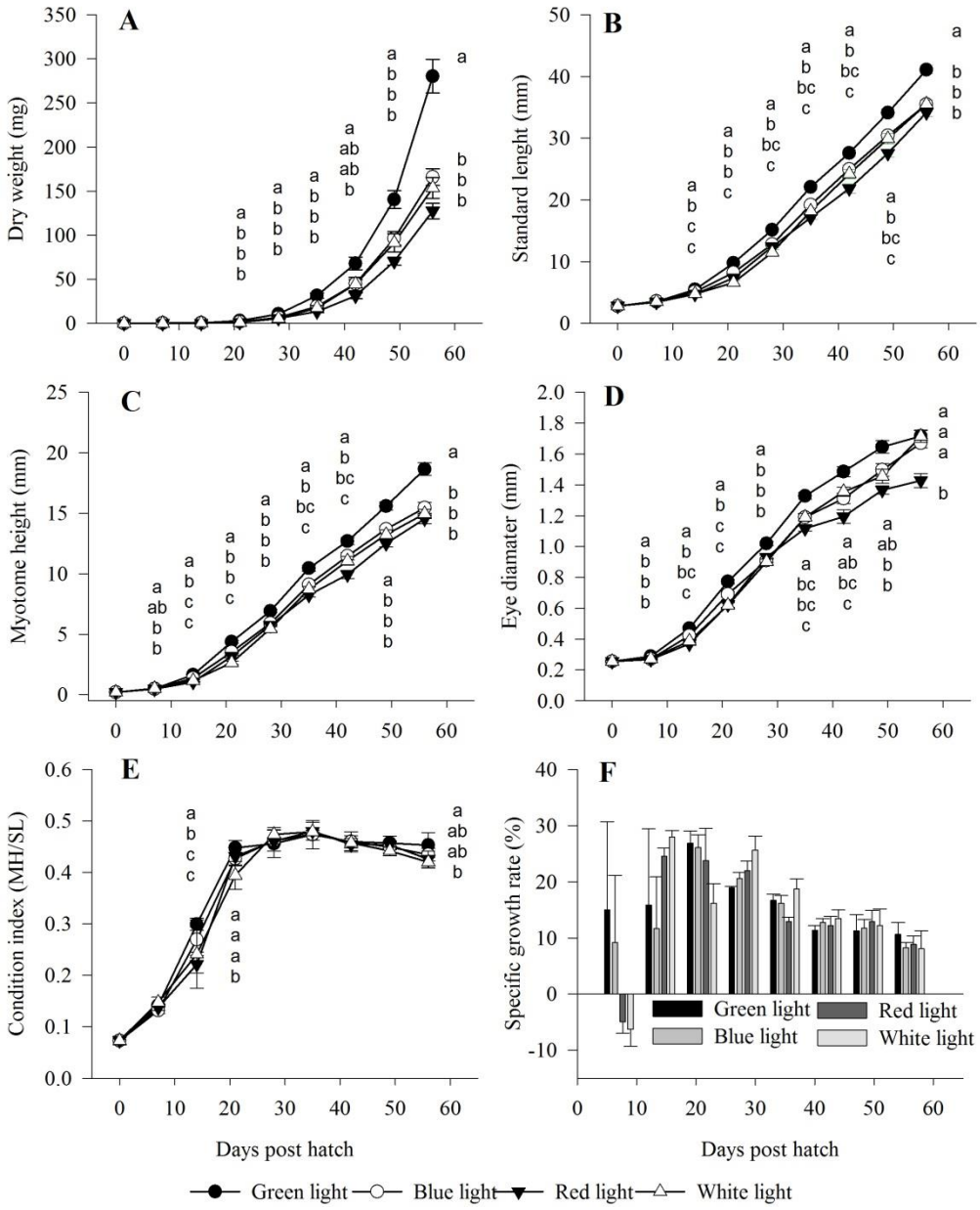

Figure 4.3. Turbot larvae growth parameters exposed to the light treatments: Green light, Blue light, Red light and White light. Dry weight (A), Standard length (B), Myotome height (C), Eye diameter (D), Condition index (E) and Specific growth rate (F). Growth parameters are expressed as mean ± SEM (n = 45, 15 larvae/replicate). Superscripts denote statistical significant differences at a given time point.

4.3.2. Effects of tank background colour on Atlantic cod (Exp. 4.2a) and turbot (Exp. 4.2b) larvae performance

Significant differences in cod DW were only observed between treatments at 50 DPH with larvae reared in blue tanks being lighter than all other treatments (Fig. 4.4). There was however no statistical differences in treatment mean weight SGR within isolated sample periods or over the complete experimental period. The same treatment effect on DW was apparent from 40DPH for SL, MH, ED and CI.

Turbot larvae DW showed significant differences at 15 and 22 DPH with white and red treatments significantly lighter than the blue and black treatments (Fig. 4.5). However, thereafter from 28 DPH until the end of the trial there were no significant differences between any treatments. There were no statistical differences in treatment mean weight SGR within isolated sample periods or over the complete experimental period. turbot SL, ED, MH and CI showed a comparable trend as DW.

Cod population mean survival was significantly higher in blue tanks (12.6%) compared to all other tank colours (4.7-5.1%) (Table 4.1) however due to wide replicate variation there was no statistical difference in treatment mean survival for turbot where the highest survival rates were observed in the red tank (2.1 %) followed by the blue, black and white tank treatments (0.7 - 1.4 %).

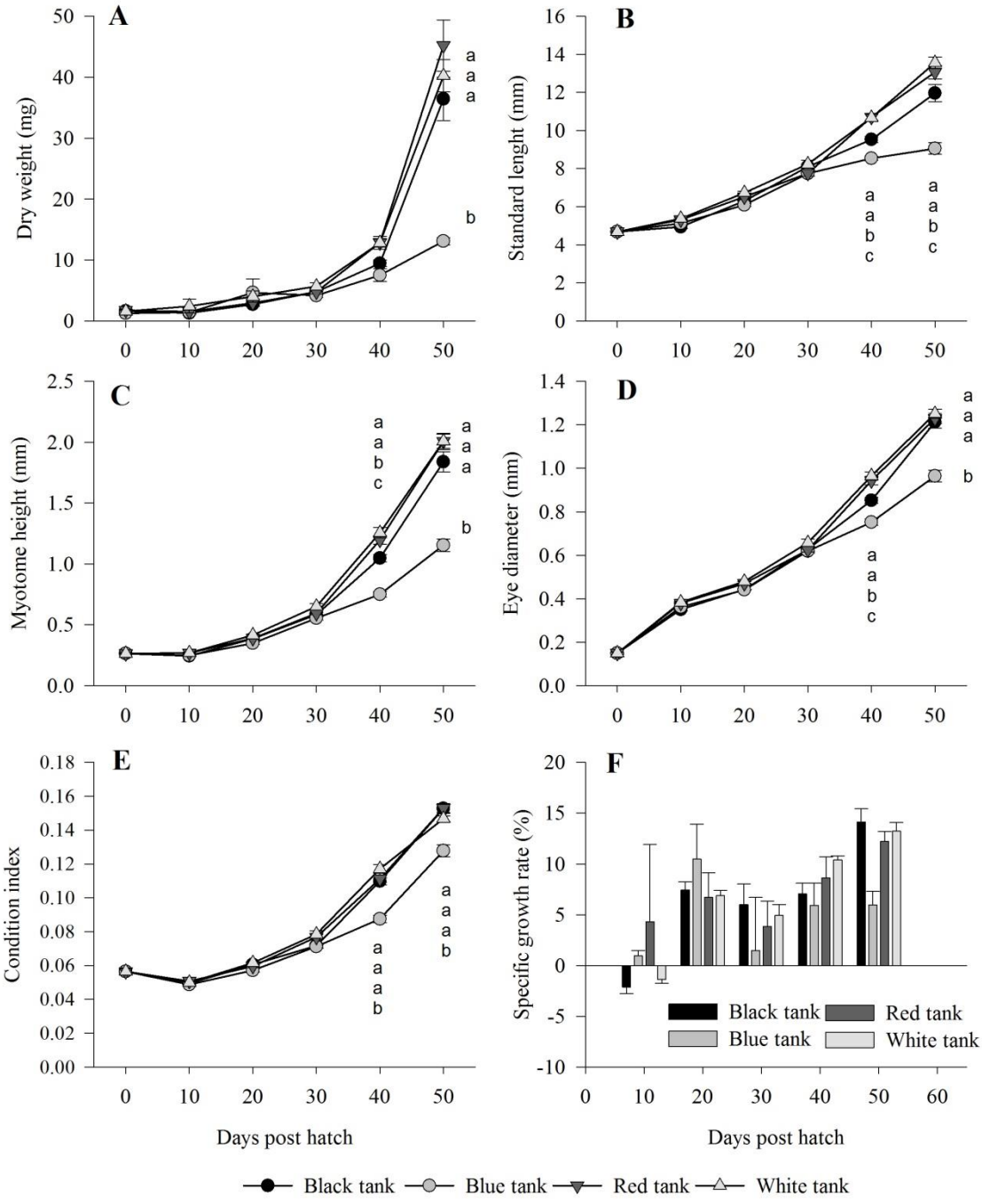

Figure 4.4. Atlantic cod larvae growth parameters exposed to tank background colour treatments: black tank, blue tank, red tank and white tank. Dry weight (A), Standard length (B), Myotome height (C), Eye diameter (D), Condition index (E) and Specific growth rate (F). Growth parameters are expressed as mean ± SEM (n = 30, 10 larvae/replicate). Superscripts denote statistical significant differences at a given time point.

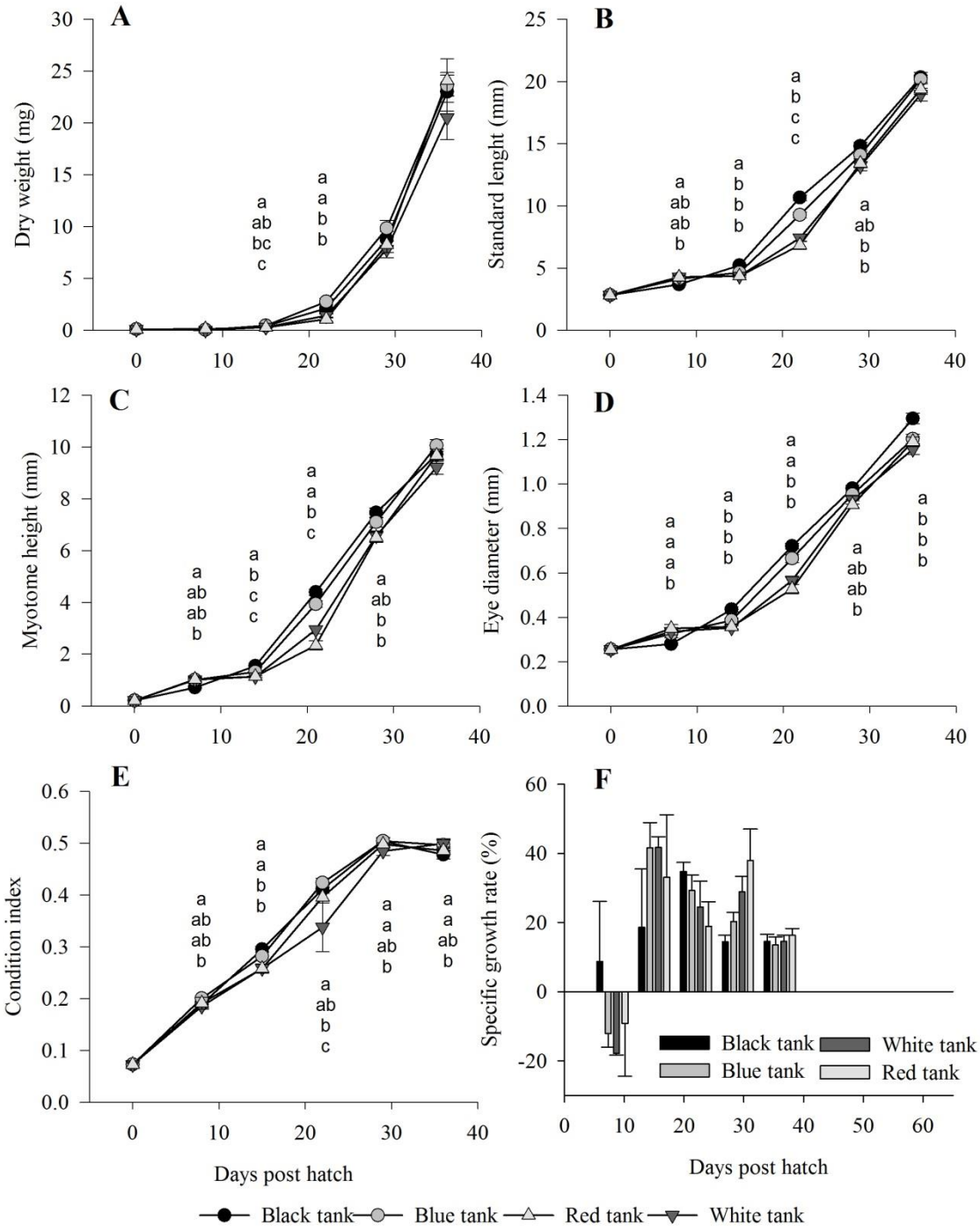

Figure 4.5. Turbot larvae growth parameters exposed to tank background colour treatments: black tank, blue tank, red tank and white tank. Dry weight (A), Standard length (B), Myotome height (C), Eye diameter (D), Condition index (E) and Specific growth rate (F). Growth parameters are expressed as mean ± SEM (n = 45, 15 larvae/replicate). Superscripts denote statistical significant differences at a given time point.

Table 4.1. Survival rate (%) per treatment (n = 3) at the end of the trial for light spectrum treatments in Atlantic cod (60 DPH) and turbot (56 DPH); and tank background colour treatments for Atlantic cod (Day 50 DPH) and turbot (56 DPH). Mean survival ± S.E.M.

		Atlantic cod	*turbot*
Light spectrum	***Green***	2.8 ± 0.7	0.8 ± 0.2
	Blue	3.6 ± 1.3	2.7 ± 0.7
	Red	6.3 ± 0.6	2.2 ± 0.5
	White	3.6 ± 0.5	2.4 ± 0.7
Tank background	***Black***	5.1 ± 0.5^{b}	1.1 ± 0.4
	Blue	12.6 ± 0.9^{a}	1.4 ± 0.7
	Red	5.9 ± 1.5^{b}	2.1 ± 0.3
	White	4.7 ± 0.6^{b}	0.7 ± 0.1

4.4.Discussion

First feeding is considered to be an extremely important part of marine larviculture and high mortality rates are common during the early life history stages, mainly due to starvation and/or cannibalism. Successful growth and survival is dependent on the ability of larvae to detect prey and any adaptations set forth to increase the probability of larvae encountering prey may increase larval rearing success (Britt *et al*., 2001). As most marine larvae are visual feeders it is important to provide a rearing environment that will provide an adequate background contrast for larvae to detect and forage on live or inert preys. Light environment and its interaction with visual photoreceptors have therefore the potential to directly affect larval growth and survival. Light can be manipulated in many ways, including light source, distance, tank depth, and tank colour (Downing and Litvak, 1999). Factors such as light intensity (Downing and Litvak, 1999), light spectrum (Villamizar *et al*., 2011), photoperiod (Puvanendran and Brown, 2002; Monk *et al*., 2003) and tank colour (Pena *et al*., 2005)

were shown to impact on larval growth and survival. However, to date, work reporting effects of light conditions on marine fish larvae performances are scarce and results appear to be species specific. The aim of the present study was to compare fish larvae performances from both pelagic Atlantic cod and benthic turbot exposed to different light spectrum conditions through the use of either narrow bandwidth light or tank background colours.

Fish larvae have visual pigments present within their retina that respond best to specific wavelengths allowing for object detection (Moksness *et al.*, 2004). During early lifecycle stages, marine larvae mainly have cone photoreceptor cells within their retina, which are used for colour detection while rod cells that are involved in light intensity perception develop later (Blaxter and Staines, 1970; Kusmic and Gualtieri, 2000). Visual pigments in marine fish larvae generally appear to be more sensitive to shorter wavelengths e.g. green and blue (Britt *et al.*, 2001; Villamizar *et al.*, 2011) which may in part explain the enhanced growth performances under short wavelengths observed in the current series of trials. Among the considered growth indicators, dry weight showed the most significant differences with cod larvae reaching 5 times higher DW in the short wavelengths treatments (blue and green) than red spectrum at 60 DPH. The comparable results observed under white light to both the blue and green treatments could be explained by the spectral profile of the white LEDs which contains a high proportion of transmitted light in the blue and green wavelengths (54% of the total output <550 nm) although longer wavelengths were also present. In turbot, similar effects were observed however larvae performed significantly better when exposed to green light (up to 300 % enhanced growth) as opposed to blue, white and red. Enhancing growth effects of short wavelengths are consistent with other studies performed in marine species such as gilthead sea bream (Karakatsouli *et al.*, 2007), sea

bass (*Dicentrarchus labrax*), and Senegale sole (*Solea senegalensis*) (Villamizar *et al*., 2009; Blanco-Vives *et al*., 2010) as well as freshwater species (Ruchin, 2004; Karakatsouli *et al*., 2007, 2008). As live feed density in the present study was delivered in excess in all experimental treatments, it can be concluded that larval performance was not restricted by food availability and therefore differences were associated with the sensitiveness of each species, which are adapted to their chosen natural ecological niche. While it is possible that light can influence growth and development via direct stimulation of the growth axis, there remains a lack of clear evidence of the pathway by which this may occur (Boeuf, and Le Bail, 1999). The more common pathway thought to influence growth relates to visual perception of prey/feed for foraging activity (Browman and Marcotte, 1987; Volpato *et al*., 2013). Interestingly, the current results showed differences in most growth parameters from the time corresponding to the introduction of *Artemia nauplii* in both species.

As incident light can influence prey detection, so background colour should be optimised to provide the maximum contrast against which larvae can see and capture food, this is particularly important during the switch from endogenous to exogenous feeding. Therefore, tank wall colour may influence the rate of prey capture by either increasing or decreasing prey-background contrast (Downing and Litvak, 1999). Results from the current study showed that in cod the larval growth (DW, SL, MH, ED) in blue background tanks was reduced so that at the trial end larvae were 3-5 times smaller than the other treatments. This is supported by approximately 50% reduction in SGR between 40 and 50 DPH. As with the light trial, larvae in all treatments were transferred from a rotifer based diet onto an *Artemia* diet from 30 DPH. Again it is possible that suboptimal background contrast resulted in larvae not being able to forage on enough prey to sustain the growth rates observed in other treatments. While much

smaller effects were observed in turbot larvae these also occurred during *Artemia* feeding. These findings suggest that tank colours that promote the best larval growth rates vary between species and possibly between feeding periods depending on prey items. Previous studies have shown different background tank colour effects on growth, also depending on species. In general, it has been suggested that black tanks are best for marine larval rearing as they provide a background colour and light regime that best represent natural conditions (Duray *et al*., 1996; Monk *et al*., 2008). Pena *et al*. (2005) found that tank colour significantly affected feeding intensity in spotted sand bass (*Paralabrax maculatofasciatus*) larvae with the highest feeding intensity recorded in black rearing tanks, intermediate in grey tanks, and lowest in white tanks. Conversely, Duray *et al*. (1996) concluded that grouper (*Epinephelus suillus*) larvae were significantly longer after 14 DPH when reared in tan tanks as opposed to black tanks and that larvae in tan tanks ingested more rotifers. Duray *et al.* (1996) suggested that the way light scatters in black coloured tanks gives prey a clearer visual contrast, and that light coloured tanks could cause excess reflection interfering with larval vision and therefore prey detection. However, a study conducted by Monk *et al*. (2008) tested the effects of different tank bottom colours and showed no significant difference in growth of Atlantic cod larvae when reared in black tanks with either black or tan bottoms. In addition, haddock larvae appeared to perform better (SL and survival) when reared in white background tanks (Downing and Litvak, 1999). In agreement with this later study, our results suggest that, for larval Atlantic cod, white as well as red and black tanks may provide a better background contrast to promote growth. While the main focus in this study and the majority of the published work has been on growth and feeding responses, recent work in southern flounder (*Paralichthys lethostigma*) has shown that tank colour can influence stress response in terms of whole body cortisol

levels which in turn was shown to influence sex determination (Mankiewicz *et al*. 2013). All of the current trials ran through the sex differentiation window for both species however phenotypic sex ratios were not measured but in light of the results of Mankiewicz *et al*. (2013) it is a parameter that perhaps should receive greater attention as it could also influence growth performance.

Beside the assessment of treatment effects on growth parameters, this study also considered differences in survival which commercially is perhaps of greater importance for marine hatcheries. In the light studies, treatment mean survival ranged from 2.8 to 4.3 % in cod and 0.8 to 2.7 % in turbot without significant differences between narrow bandwidth treatments although lowest survival were seen in green treatments for both species. In the tank background trials, best survivals were found in cod larvae reared in blue tanks (12.6%) and turbot larvae reared in red tanks (2.1%). Low survival from hatch to metamorphosis is commonly reported within cod (1-10%) and turbot (0-40%) larval rearing (Howell, 1984, Person-Le Ruyet *et al*., 2002) however comparisons are not straightforward due to a lack of standardisation in the manner that survival has been calculated. In larval rearing, the main causes of mortalities are a lack of and/or late active exogenous feeding, poor acceptance/palatability of exogenous feed as well as cannibalism. Several studies including Howell (1984) and Brown *et al*. (2003) reported that cannibalism is an important component of cod larvae mortality and it is particularly severe on the onset of metamorphosis (12-15 mm) (Brown *et al*., 2003) when difference in size between siblings is more evident. In the current trial, cannibalism could not be assessed. However, the faster growth showed under shorter wavelengths may also increase size variability within the populations, increasing therefore the likelihood of cannibalism which could partially explain the reduced survival (e.g. green light treatment in turbot resulting in enhanced growth but also reduced survival). Ultimately

this confirms the complexity of marine fish larviculture where growth must be balanced against potential cannibalism and thus reduced survival.

While behaviour was not quantified or directly monitored throughout the experiments, casual observation suggested that larvae distribution and swimming behaviour was influenced. Both cod and turbot larvae exposed to red light were widespread in the water column from stocking, while fish in all other treatments remained close to the surface until approximately 10 DPH. Furthermore turbot (from circa. 40 DPH) exposed to short wavelengths appeared to swim more actively, while red light larvae swam slower and white light larvae remained for longer periods at the bottom of the tank. In the tank colour experiments there was increased "walling behaviour" observed in both species in the white and red tank treatments. Walling is a behaviour associated with larvae vigorously swimming into the tank walls that can be potentially damaging to larval health and survival (Cobcroft and Battaglene 2009). Cobcroft and Battaglene (2009) reported increased walling behavior in striped trumpeter (*Latris lineta*) reared in red, green, white, and blue tanks compared to black or marble coloured tanks. They found a direct link of walling behavior to jaw malformations and survival rate and concluded that growth and survival was highest in black tanks and lowest in red tanks. These authors stressed that hard surface interactions can have effects on growth and survival of cultured marine fish and that identifying the specific tank colour preference for a given species can both increase survival rates and reduce malformation prevalence seen in cultured fish. Increased jaw malformations was also reported in Senegal sole larvae exposed to a blue spectrum associated to a low survival (Blanco-Vives *et al*., 2010).

Overall this present study confirms that for both cod and turbot larvae performance is affected both by the incident light wavelength as well as the background

colour. The influence is potentially related to the environmental impact on foraging activity due to visual contrast of prey items however this needs more specific investigations. In general, shorter wavelengths improved growth performance although this gain can be offset by reduced survival. In terms of tank background it appears that the industry standard black tanks perform well in both species however care should be taken with cod if adjusting from this as they showed a clear differential impact.

Chapter 5

EFFECT OF LIGHT WAVELENGTHS ON GROWTH, APPETITE, SKIN PIGMENTATION AND STRESS RESPONSE IN TURBOT (*SCOPHTHALMUS MAXIMUS*) JUVENILES.

5.1. Introduction

Light is a key environmental signal regulating (directly or indirectly) physiological and behavioural (feeding activity, schooling, migration and reproduction) responses throughout the teleosts' whole life cycle, although the exact pathways are not yet fully described (Boeuf and Le Bail, 1999; Bromage *et al.*, 2001, Marchesan *et al.*, 2005; Falcón et al., 2010; Migaud *et al.*, 2010).

Recently, most of the studies investigating the effects of light wavelength in fish have focused on the larval stages. In an aquaculture context it has been shown that larvae growth, performance and survival are influenced by light including photoperiod, intensity and spectral composition (Downing and Litvak, 1999; Monk *et al.*, 2003; Blanco-Vives *et al.*, 2010; Villamizar *et al.*, 2011). The response appears to be species specific (Vera *et al.*, 2010) and to some extent congrouns in species that share similar ecological niches. Literature suggest that larvae exposed to a photic environment (light spectrum or background colour) similar to the one encountered in their natural habitat perform better in terms of growth and survival. This is likely to be due to a better visibility that facilitates prey detection and foraging activity. However, the underlying mechanisms along the hypothalamus-pituitary axis mediating such effects remain to be understood. For instance, metamorphosis in halibut (*Hippoglossus hippoglossus*) larvae is clearly affected by photoperiod. Larvae exposed to continuous light showed a lower ratio between standard length and eye migration than those reared under a 12L:12D artificial photoperiod, while overall growth was not affected (Solbakken and Pittman, 2004). The authors suggested that the underlying mechanisms might be related to the regulation of melatonin secretion through photoperiod and an interaction with the thyroid hormones, one of the main endocrine driver of larvae metamorphosis (Power *et*

al., 2001). Despite the relatively abundant literature on the effects of light on fish, focus has mainly been on larvae with far fewer studies performed on juvenile stages.

In the juvenile and adult stages, research has mainly looked at the effects of photoperiod on precocious sexual maturation. Suppression of puberty is a key milestone to prevent energy diversion towards gonadal development, which results in enhanced somatic growth. Rainbow trout (*Oncorhynchus mykiss*) growth rates and feeding efficiency were enhanced by extended photoperiods from autumn to spring, likely through the up-regulation of IGF- I secretion (Taylor *et al.*, 2005; 2006). Similarly, an exposure of 3 months under continuous lighting enhanced growth in Atlantic cod juveniles (*Gadus morhua*), the effect persisted for almost 3 years after fish were transferred to sea cages and exposed to ambient photoperiod (Imsland *et al.*, 2007). In the red sea bream (*Pagrus major*) increasing photoperiods (24L:0D or 16L:8D) positively affected weight gain, growth rates, food intake and food efficiency conversion (Biswas *et al.*, 2005). In gilthead sea bream (*Sparus aurata*) continuous light exposure increased food intake, however food conversion was more enhanced under a 16L:8D photoperiod, resulting in a higher weight gain (Ginés *et al.*, 2004). In flatfish, exposure to extended photoperiod does not seem to enhance growth as much as in round fish species. Growth rates and survivals were not affected by photoperiod (24L:0D, 18L:6D and 12L:12D) in yellow tail flounder (*Pleuronectes ferrugineus*) juveniles (Purchase *et al.*, 2000). Long photoperiods (24L:0D and 20L:4D) resulted in higher growth rates and lower mortality rates in halibut juveniles, however the positive effects were not immediate and required a period of at least 3 weeks of exposure (Simensen *et al.*, 2000; Imsland *et al.*, 2006). Furthermore, Jonassen *et al*. (2000) found a positive correlation between growth rates and low temperatures under continuous light in this species. Food intake was temperature dependant while food conversion efficiency

appears to dependant on photoperiod treatment. In turbot, growth is enhanced by continuous light with a maximum condition index during winter (Imsland *et al.*, 1995). All of these examples confirm the importance of photoperiod on feeding activity and growth.

Light spectrum appears to be involved in the juveniles' somatic growth as well, although data is very scarce and effects species specific. Nile Tilapia (*Oreochromis niloticus*) (intial weight 60 g) exposed for 30 days (12L:12D, at 150 lux) to red light had a lower weight gain than fish grown in blue, violet, yellow or green light (Luchiari and Freire, 2009). In freshwater species, red light usually negatively affected growth rates which were enhanced by green and yellow in Crucian carp (*Carassius carassius*) (initial weight 3 g), green and blue in Chinese sleeper (*Perccottus glenii)*(initial weight 1 g) and blue in guppy (*Poecilia reticulata*) (initial weight 50 mg) (Ruchin, 2004). In gilthead sea bream 11 weeks of red light exposure increased brain dopaminergic activity, but had no effect on growth, carcass composition, blood parameters or other brain neurotransmitters compared to white and blue light treatments (Karakatsouli *et al.,* 2007). Growth and blood parameters did not differ in European sea bass (*Dicentrachus labrax*) (initial weight 145 g)between blue and white light treatments, however stress (1 h confinement by reducing water level) increased haematocrits and plasma cortisol (but not glucose) in fish exposed to blue light (Karakatsouli *et al.*, 2012). If the effects of the light spectrum are preserved from the larval into the juvenile stages, it is possible that marine fish will perform best under the short wavelengths as seen in sea bass, Atlantic cod, Senegal sole and turbot larvae (Villamizar *et al*., 2011; Chapter 4) .

Light intensity (and possibly spectral composition) appears to influence the development of pigmentation during the early stages of development in flatfish (Venizelos and Benetti, 1999). Post-metamorphosis flatfish typically present a well-

defined coloured ocular side and a blind side. Pigmentation is a plastic feature in fish that can be modified by social factors (Grosenick *et al.,* 2007) or as a predator avoidance tactic (Doolan *et al.,* 2009). Colour changes can be a rapid reaction (physiological) or a long term effect (morphological) (Leclercq *et al.,* 2010). Changes in pigmentation are driven by expanding or contracting the chromatophores (pigment storage/reflecting cells) in the skin layers. turbot possess three types of chromatophores: melanophores (black), xanthophores (yellow) and iridophores (silver/reflecting) which contain pigments (melanin, pteridine and purine, respectively) (Bolker and Hill, 2000). Several proximate factors intervene on the distribution and concentration of the pigment cells including light. Light stimuli can act directly on the colouration mainly through stimulating the brain-pituitary axis (alpha melanocyte-stimulating hormone (α-MSH) (pigment dispersion) and/or its antagonist (*in vitro*) melanin concentrating hormone (MCH) (pigment aggregation)) (Burton and Vokey, 2000).

The pigmentation axis acts in parallel with other physiological functions and vision plays a crucial role for the expression of pigmentation. It has been suggested that abnormally pigmented flatfish have an impaired visual system that interferes with the skin colouration (Kanazawa, 1993). Supplementation of vitamin A and fatty acids (crucial for retinal rhodopsin formation) in the diet was shown to improve pigmentation in flatfish (Pepe, 1999; Bolker and Hill, 2000). Stress can also affect fish external pigmentation. Skin darkening is correlated to elevated plasma cortisol levels (Ruane *et al.,* 2005) and in some cases elevated levels of α-MSH and ACTH (Höglund *et al.,* 2000). The exact pathway by which pigmentation is regulated remains unclear. α-MSH appears to be one of the main pigmentation driving hormones, however it is also related to stress response (cortisol secretion) (Metz *et al.,* 2006) and has an apparent anorexic effect (Matsuda *et al.,* 2009). This suggests that growth, pigmentation, metabolism,

stress and also maturation are highly interconnected which is not surprising given the co-localisation of most hormones within the pituitary (growth hormone, gonadotropins, ACTH, MSH among others).

Published reports on the effects of light on fish larvae and juveniles are highly variable probably due to species plasticity and differential sensitivities to light. However, these contrasting results may also come from the complexity to study light impact on fish physiology including several variables (photoperiod, intensity, spectrum) with daily and seasonal changes and the difficulty to test light treatments due to lighting technologies with very different light outputs, water absorbance and light pollution.

The aim of this study was to investigate the effects of light spectrum (white, blue, green and red) on turbot growth, appetite, blood parameters (plasma glucose and cortisol) and skin colouration and determine possible applications of light spectrum manipulation in enclosed facilities to enhance juvenile performances.

5.2. Materials and methods

All the experiments were carried out at the facilities of Viking Fish Farms Ltd., Ardtoe Marine Laboratory, Acharacle, Scotland (N 56°46' W 05°53') and conducted in accordance with the Animals Scientific Procedures Act of 1986, UK following independent ethical review.

5.2.1. Experiment 5.1: Spectrum effects on turbot growth and stress

On 14th October 2011 turbot juveniles (length: 16.9 ± 0.7 cm, weight: 72.4 ± 9.8 g) were stocked in eight black flat-bottomed circular tanks (45 fish/tank) of 1 m^{-3}. With a flow through system (5 L min^{-1}) and ambient water temperature (ranging from 9.0 to 13.4 °C; mean 11.6 ± 0.9 °C). Fish were fed to satiation twice a day (10:00 and 17:00 hrs.) using commercial dry feed (EFICO Sigma, Biomar, Spain).

Fish were exposed to continuous lighting regimes (24:00 L:D) of different wavelengths: white, blue (476 nm), green (526 nm) and red (643 nm) in duplicate. White light was produced by a dimmable incandescent flood bulb (80 W, angle 30°, 2,900 K, GE). Coloured lights were produced using dimmable Light-Emitting Diode (LED) green, blue and red LED units with controllers (Intravision Aqua AS, Norway) suspended 1 m above the water surface. The spectral composition was measured prior to the experiment with a portable spectoradiometer (Model EPP 2000c, Stellamet Inc, USA) (Fig. 5.1). The intensity of the light units was measured at nine points in the tank (1 on the surface directly under the light bulb and on four sides both surface and bottom) using a single sensor light channel watts meter set to a wavelength range of 400-740 nm (Skye Instruments Ltd., UK) and calibrated to National Physics Laboratory standards. Light intensity was balanced between treatments (surface: 4.8 ± 1.7 W m^{-2}, bottom: 2.7 ± 0.6 W m^{-2}). Experimental tanks were exposed to coloured lights for 30 days, then switched to ambient room white light using fluorescent tubes (70 W, 3,500 K, Osram) distributed across the experimental room for ten days (up to day 40) and finally returned to the coloured LEDs for a further 10 days (up to day 50).

At 30, 40 and 50 days post light exposure 10 individuals per tank were hand netted and culled using a lethal dose of 2-phenoxyl ethanol (4 ml L^{-1}). Growth parameters were measured: total length (min 1 mm) and weight (min. 0.1 g). Blood samples were taken from the caudal vein in pre-heparinised syringes and kept on ice. Blood was centrifuged at 250 g for 15 min and plasma stored at -20 °C until analysed.

Glucose levels were measured in serum using a portable glucose meter (USB Contour Bayern, Germany) with a minimum detection level of 0.6 nmol L^{-1} previously validated for fish (Fanouraki *et al.*, 2011). Plasma cortisol levels were determined by radioimmunoassay (RIA) in duplicate according to North *et al*. (2006). A set of serial

dilutions of turbot plasma were assayed in comparison to a comparable dilution of purified cortisol standards to demonstrate the plasma was immunologically comparable prior to the assay being used. Intra-assay and inter-assay coefficients of variation were 8.32% and 14.98%, respectively (n = 5), with a minimum sensitivity of 0.56 ng mL^{-1}. Cortisol recovery was 97.81 %. For further details of assay conditions and validation refer to section 2.7.

5.2.2. Experiment 5.2. Spectrum effect on turbot feed intake, growth and pigmentation

Fully metamorphosed hatchery reared turbot (length: 4.2 ± 0.4 cm; weight: 1.2 ± 0.4 g) were randomly selected from a commercial production tank previously reared under constant white light (incandescent dimmable flood bulb, GE 80 W, angle 30°, 2,900 K) with a light intensity at the tank water surface directly under the light source of 4.6 Watts m^{-2} and acclimatised to 15 °C three weeks prior the start of the experiment. On 11th January 2013, 100 fish were stocked in each of twelve 100 litre black flat-bottomed (30 cm diameter) round tanks with a flow through system (0.5 L min^{-1}), salinity (34.5 ppt) with controlled water temperature of 13.9 ± 0.05 °C (mean all treatment replicates ± SEM.) (Range 11.2 – 15.9 °C).

Fish were exposed to continuous lighting regimes (24:00 L:D) of different wavelengths. Coloured dimmable LED bulbs (3x3 W spotlight, EPISTAR, Taiwan) were used for the specific colour treatment while daylight halogen bulbs were used for the white light treatment (35 Watts, 3,500K, SoLux, US). The spectral composition was measured prior the experiment as above (Fig. 5.1): white, blue (peak 458 nm), green (peak 516 nm) and red (peak 632 nm). Light intensity was measured as described above and balanced at the surface: 4.9 ± 0.3 W m^{-2} which equated to a tank bottom intensity of 0.6 ± 0.2 W m^{-2}. Fish were fed to satiation twice a day (8:00 and 17:00 hrs) using

commercial dry food (INICIO Plus, Biomar, Spain). The bottom of the tank was siphoned 2 hours after feeding every day.

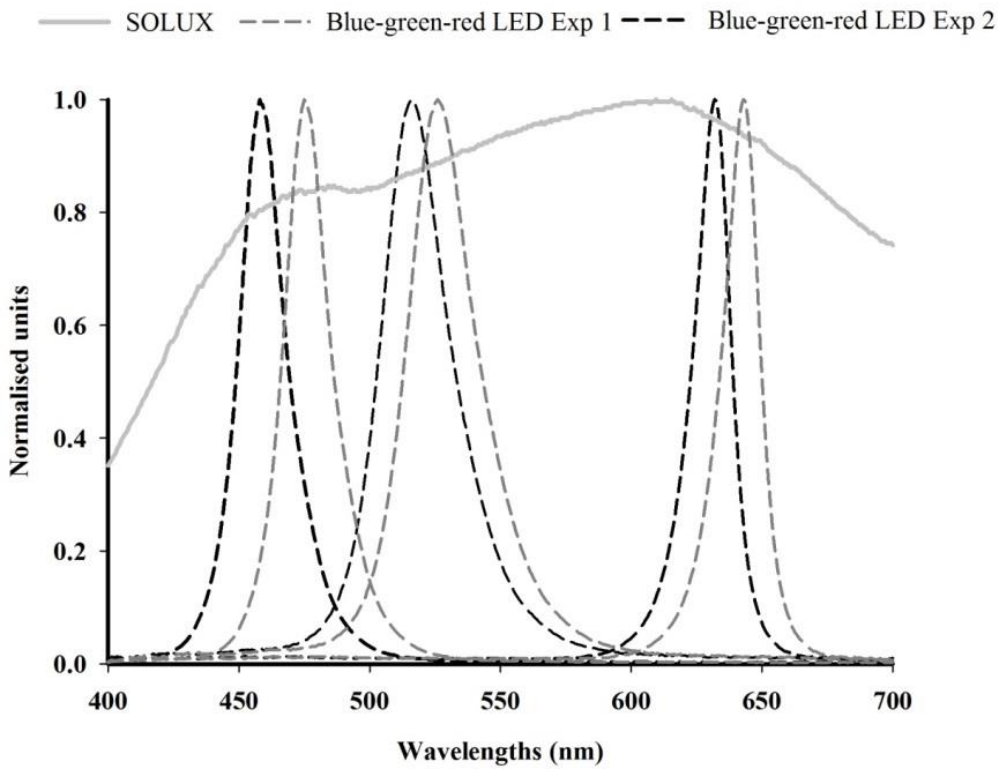

Figure 5.1. Normalised spectral profiles from the lighting used in experiment 5.1 and 5.2.

Growth and food intake were monitored once a week. Fish (n = 20) were anaesthetised (2 ml L^{-1}, 2-phenoxyl ethanol, Sigma-Aldrich, UK) and total length (min 1 mm) and weight recorded (Mini pocket Scale 200g/0.01g, ICETAC PRO, US). Fish were returned to the experimental tank once fully recovered (within 5 min). No mortalities were associated with this procedure. Growth parameters were calculated as follows.

Fultons condition factor (K) was calculated by K = 100 x (weight (g)) / (total length (cm))^3;

Weight gain = $W_1 - W_0$, where W mean weight (g).

Food intake by biomass was calculated as Weekly food / biomass, where weekly food was the sum of food weight used (g) and biomass the mean fish weight x number of fish in the tank.

Food conversion efficiency (FCR) was calculated by:

Mean feed amount per individual (total feed (g) / number of fish) / mean individual weight gain ((Mean weight $_{T1}$- mean weight $_{T0}$) / number of fish) where T is sample time point.

Weight specific growth rates (SGR, % d^{-1}) were calculated using

SGR = $(e^{g}-1)\times 100$ where $g=(\ln X_2-\ln X_1)/t_2-t_1$ and X_2 and X_1 are weights (g) at times t_2 and t_1 respectively.

Thermal growth coefficient (TGC) was calculated: $TGC = [(\sqrt[3]{W_1} - \sqrt[3]{W_0}) /(T^\circ \times t)] \times 1000$; where W is weight (g), T° is mean temperature (°C) and t is time (days).

Additionally individual photos were taken for pigmentation image analysis (days 2, 4, 6, 8, 20 and 30). The methodology used for image collection and analysis is detailed in Chapter 2: General Materials and Methods. Briefly, photographs were taken using a commercial digital camera (Lumix 16.1 MP, Panasonic) on a white background with a dimension scale and an 18% digital grey and white photography balance card (GC2 Pocket size 3-1 cards, Kaavie, UK) included in shot for later calibration. Image light intensity was balanced *post hoc* using the grey and white photography cards using Photoshop CS5 software (Adobe, US) for colour analysis and a second file in grey scale created for darkness analysis. Determination of the skin coloration was adapted from Rajaee (2011) using image analysis software (ImageJ, NIH, US) with additional RGB measurement plug-in. RGB values (range 0 to 255) were used to determine: colour composition/hue as percentages (e.g. R / R+G+B), brightness/intensity of the colours (0.299R + 0.587G + 0.114B) and saturation of the colours (Max RGB value – Min RGB

value). Using the grey scale picture the RGB analysis was repeated to determine the darkness/blackness of the fish presented as a percentage (1- (RGB/255)*100) of closeness to the absolute black (i.e. RGB 0,0,0) .

5.2.3. Statistical analysis

The correlation between weight and length was analysed based on the mathematical function: $W = a L^b$; where W is total body weight (g), L total length (cm) and a and b are coefficients from the functional regression between W and L. The coefficient a expresses the body shape, while coefficient b expresses the growth: if b = 3 growth is isometric. If b ≠ from 3 growth is allometric (if b < 3, growth is towards length; if b > 3 growth is towards weight.). The values of a and b constants were estimated from the natural logarithm transformed values of length and weight through least square linear regression: Ln W = Ln a + b Ln L . The degree of association between the W and L is expressed as the r^2 coefficient of the linear regression and the significance of the b coefficient is expressed as its p value.

Statistical analysis was performed using MiniTab version 16.2.2. All data sets were first tested for normality using the Anderson-Darling test. All data for growth parameters (total length, weight and condition factor), pigmentation (red hue, green hue, blue hue, brightness/intensity, saturation and darkness) and blood parameters (glucose and cortisol) variance was analysed using One-Way ANOVA model at given time points. Colouration saturation and plasma cortisol data was first natural log transformed to improve homogeneity of variances. Significance levels ($p<0.05$) were determined by Tukeys *post hoc* test. All data are expressed as mean ± SEM (n=20 and 30 for experiment 5.1 and 5.2, respectively).

5.3.Results

5.3.1. Experiment 1: Spectrum effects on turbot growth and stress

Growth parameters

After 30 days of coloured light exposure fish from the red treatment were significantly smaller than the green treatment (Fig. 5.2a). Fish from the green and blue treatments were significantly heavier than red and white treatments (Fig. 5.2b). Condition factor was significantly higher for the green and blue treatments than the other treatments (Fig. 5.2c). Similarly, specific growth rates (SGR) and thermal growth coefficient (TGC) were significantly higher for the green and blue treatments (Table 5.1). Weight gain in fish under the green treatment was significantly higher than all the other treatments, followed by the blue treatment, while red and white treatments were not different.

Table 5.1. Turbot growth rates (Experiment 5.1) exposed to the light treatments: Overall (Day 0 to 50), Day 30 (post 30 days of coloured light exposure), Day 40 (after 10 days of white light exposure) and Day 50 (after subsequent 10 days of coloured light exposure). Mean temperature, light colour exposure and experimental treatments relate to the given time window. Specific growth rate (SGR), Thermal growth coefficient (TGC), mean weight gain (g day^{-1}) and mean daily food intake (g day^{-1}). Data expressed as the mean (n = 20) ± SEM; 10 fish per replicate. Scripts denote statistical differences (One-Way ANOVA) at given time windows.

Time window	Temp. (°C)	Lights	Treatment	SGR (%)	TGC	Weight gain (g day^{-1})	Mean food intake (g day^{-1})	FCR
Overall	11.6 ± 0.9	Green	Green	0.86 ± 0.08 (a)	1.10 ± 0.12 (a)	0.78 ± 0.13 (a)	N/A	N/A
		Blue	Blue	0.81 ± 0.03 (ab)	1.02 ± 0.04 (ab)	0.72 ± 0.04 (ab)		
		Red	Red	0.20 ± 0.03 (c)	0.24 ± 0.04 (c)	0.15 ± 0.04 (c)		
		White	White	0.48 ± 0.07 (bc)	0.59 ± 0.09 (bc)	0.39 ± 0.09 (bc)		
Day 0-30	12.1 ± 0.7	Green	Green	1.31 ± 0.03 (a)	1.60 ± 0.04 (a)	1.16 ± 0.04 (a)	N/A	N/A
		Blue	Blue	1.12 ± 0.03 (a)	1.35 ± 0.04 (a)	0.96 ± 0.04 (b)		
		Red	Red	0.57 ± 0.05 (b)	0.67 ± 0.07 (b)	0.45 ± 0.07(c)		
		White	White	0.39 ± 0.00 (b)	0.46 ± 0.01 (b)	0.30 ± 0.01 (c)		
Day 30-40	11.4 ± 0.2	**White**	Green	0.00 ± 0.23	0.00 ± 0.32	0.00 ± 0.36	8.35 ± 0.21 (c)	0.00 ± 0.93
		White	Blue	0.00 ± 0.43	0.00 ± 0.58	0.01 ± 0.62	7.95 ± 0.21 (c)	0.00 ± 0.51
		White	Red	-0.33 ± 0.09	-0.42 ± 0.12	-0.28 ± 0.12	9.0 ± 0.42 (b)	-0.98 ± 0.26
		White	White	1.07 ± 0.14	1.38 ± 0.18	0.93 ± 0.18	13.85 ± 0.35 (a)	0.43 ± 0.05
Day 40-50	10.56 ± 0.84	Green	Green	0.38 ± 0.28	0.57 ± 0.43	0.42 ± 0.45	14.35 ± 1.62 (a)	3.05 ± 2.22
		Blue	Blue	0.67 ± 0.18	1.00 ± 0.26	0.71 ± 0.24	17.40 ± 2.41 (a)	1.07 ± 0.36
		Red	Red	-0.36 ± 0.26	-0.49 ± 0.36	-0.29 ± 0.30	3.95 ± 0.91 (b)	-1.28 ± 1.02
		White	White	0.15 ± 0.49	0.22 ± 0.70	0.14 ± 0.64	6.55 ± 0.07 (b)	-0.21 ± 0.64

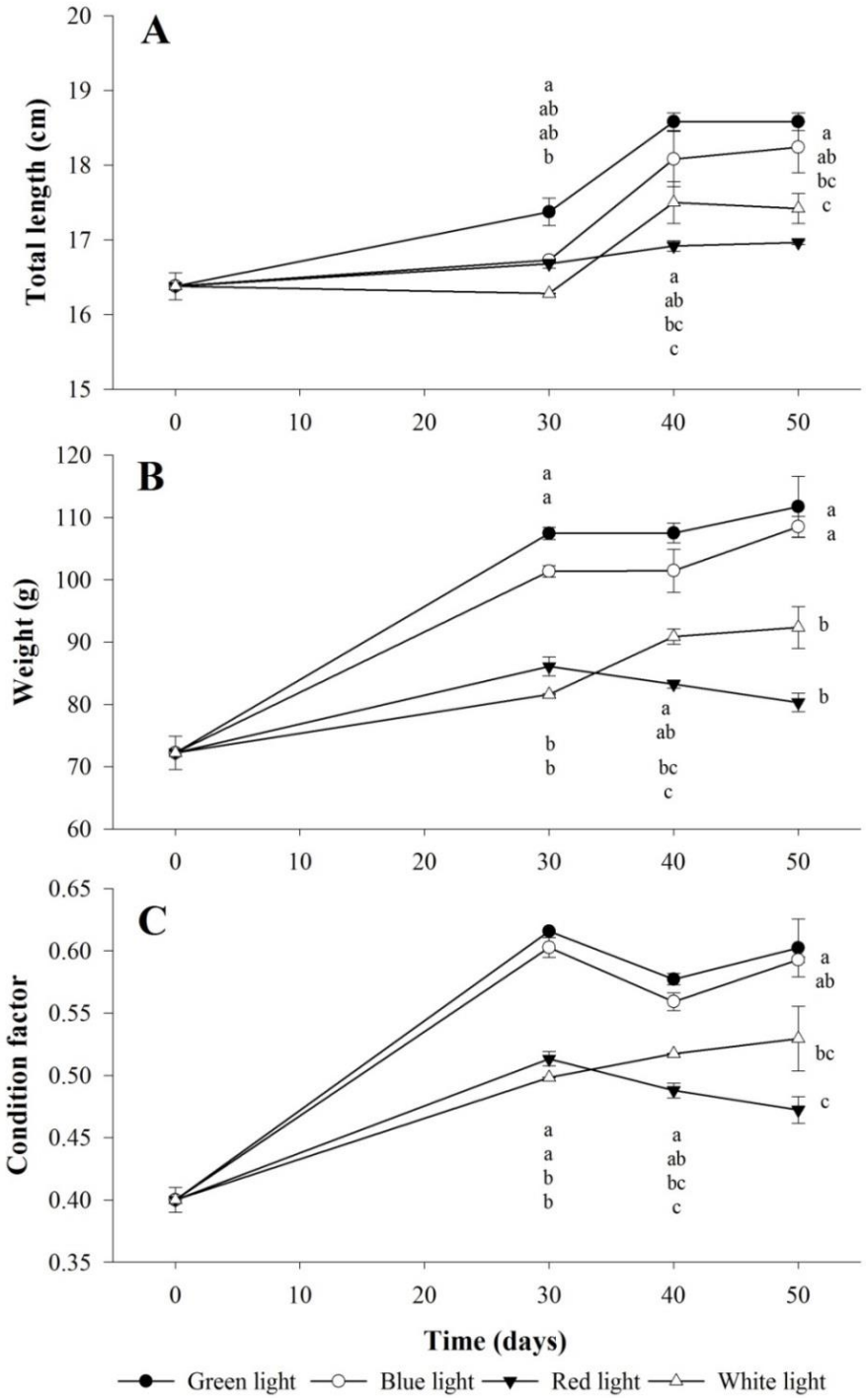

Figure 5.2. Turbot growth parameters (Experiment 5.1) exposed to the light treatments days post light exposure: green, blue, red and white light. Total length (A), weight (B) and condition factor (C). Growth parameters expressed as the mean (n = 20) ± SEM; 10 fish/replicate. Superscripts denote statistical significant differences (One-Way ANOVA) at given time point between treatments.

On day 40 (after 10 days of intermediate white light exposure) fish from the previous green and blue treatments were significantly larger than fish from the red treatment (Fig. 5.2a). Fish from the previous white treatment were significantly smaller than the green treatment. Similarly, fish from the green and blue treatment were significantly heavier than the red light treatment (Fig. 5.2b). Fish from the white light treatment were significantly lighter than the green treatment. Condition factor (K) was reduced in all treatments except the previous white treatment. K was significantly higher in the green treatment than red (Fig. 5.2c). SGR, TGC and weight gain (Table 5.1) were not different between treatments, however, the previous white light treatment was the only one with a mean positive SGR, TGC and weight gain. Mean total daily food intake was significantly higher in the previous white treatment, followed by the previous red treatment. Total food intake was the significantly lower in the previous green and blue treatments (Table 5.1). FCR was not different between treatments.

On day 50 (after 10 days of subsequent coloured light exposure) fish from the green treatment were significantly larger than the white and red treatments (Fig. 5.2a). Blue treatment length was only different from the red treatment. Wet weight was significantly higher in the green and blue treatments than the white and red treatments (Fig. 5.2b). K was significantly higher in the green treatment than white and red, but not blue (Fig. 5.2c). SGR, TGC and weight gain were not different between treatments. Mean food intake was significantly higher in the green and blue treatments than white and red treatments (Table 5.1). FCR was not different between treatments.

The overall final growth rates from the onset of the light treatments until day 50 showed that SGR, TGC and weight gain in the green light treatment was significantly higher than the other treatments except for the blue light treatment. SGR, TGC and

weight gain were significantly lower in the red light treatment except for the white light treatment.

The relationship between length and weight ($W = a L^b$) showed a b value of < 3 for the green and blue treatments while b > 3 for the red and white treatments on day 30. On day 40 all b values were similar and < 2. On day 50 all treatments showed a b value of < 3, but the relationship in terms of r^2 value for the green treatment was not significant (Table 5.2).

Blood parameters

Plasma glucose levels at day 30 in the green treatment were significantly higher than blue treatment, but not red nor white (Fig. 5.3a). On day 40 glucose was significantly higher under green compared to white treatments. On day 50 glucose levels in the green and blue treatments were significantly higher than red and white treatments.

Plasma cortisol concentrations showed significant differences after 30 days of exposure between treatments, with the blue treatment being significantly lower than the rest of the treatments (Fig. 5.3b). On day 40 cortisol levels in white treatment was significantly lower than the other treatments, except for the red treatment. On day 50 plasma cortisol levels in the blue treatment were significantly higher than all other treatments, except white treatment. Cortisol levels in the green treatment were significantly lower, except for the red treatment.

Light treatments had an effect on plasma glucose and cortisol levels correlation throughout the whole experiment (Fig. 5.4). On day 30 and 40 the correlation was strong with an r^2 of 0.87 and 0.83, respectively. On day 50 the correlation was maintained but reduced ($r^2 = 0.22$). Plasma glucose levels were correlated to the food

intake (g fish^{-1}) (Fig. 5.5). The correlation was negative on day 40 (r^2 = 0.42) and positive on day 50 (r^2 = 0.77).

Table 5.2. Turbot weight and length correlation (Experiment 5.1) exposed to the light treatments ($W = a\ L^b$). Day 30 (post 30 days of coloured light exposure), Day 40 (after 10 days of white light exposure) and Day 50 (after subsequent 10 days of coloured light exposure). Light colour exposure and experimental treatments during the given time window. Mean total length (cm) (MTL), L_{min}-L_{max} range of length (cm), mean weight (g), W_{min}-W_{max} range of weight (g). Data expressed as the mean (n = 20) ± SEM; 10 fish per replicate. Coefficients of the linear regression from natural logarithm *a* (body shape) and *b* (growth relationship), SE of *b* coefficient regression and r^2 and p value of the regression equation.

Time	Lights	Treatment	MTL	L_{min}-L_{max}	MW	W_{min}-W_{max}	a	b	SE (b)	r^2	p
Day 0 - 30	Green	Green	17.3 ± 0.2	14.1 - 18.5	107.4 ± 3.5	70 - 137	0.08	2.49	0.24	0.84	0.000
	Blue	Blue	16.7 ± 0.2	14.7 - 18.5	101.3 ± 4.1	56 - 126	0.01	3.42	0.41	0.79	0.000
	Red	Red	16.6 ± 0.2	15 - 18.9	86.1 ± 3.4	63 - 130	0.02	3.04	0.25	0.88	0.000
	Blue	White	16.4 ± 0.2	15.1 - 17.9	83.6 ± 2.9	61 - 103	0.03	2.76	0.41	0.72	0.000
Day 30 - 40	**White**	Green	18.5 ± 0.2	17.3 - 20.1	107.5 ± 3.0	85 - 136	0.11	2.33	0.39	0.65	0.000
	White	Blue	18.1 ± 0.2	16.5 - 19.7	101.5 ± 3.2	78 - 124	0.03	2.81	0.33	0.79	0.000
	White	Red	16.9 ± 0.2	14.8 - 19.6	83.2 ± 4.1	53 - 123	0.02	2.87	0.33	0.8	0.000
	White	White	17.5 ± 0.2	14.6 - 19.2	92.3 ± 3.6	71 - 126	0.25	2.05	0.35	0.64	0.000
Day 40 - 50	Green	Green	18.5 ± 0.2	17.3 - 20.1	111.7 ± 3.5	88 - 150	71.8	0.14	0.72	0.00	0.840
	Blue	Blue	18.3 ± 0.2	16 - 19.7	108.5 ± 3.4	80 - 134	0.18	2.19	0.33	0.71	0.000
	Red	Red	16.9 ± 0.3	15 - 19.6	80.3 ± 3.2	61 - 121	1.46	1.41	0.47	0.32	0.008
	White	White	17.5 ± 0.3	14.6 - 19.2	90.9 ± 3.5	72 - 132	2.07	1.32	0.55	0.25	0.029

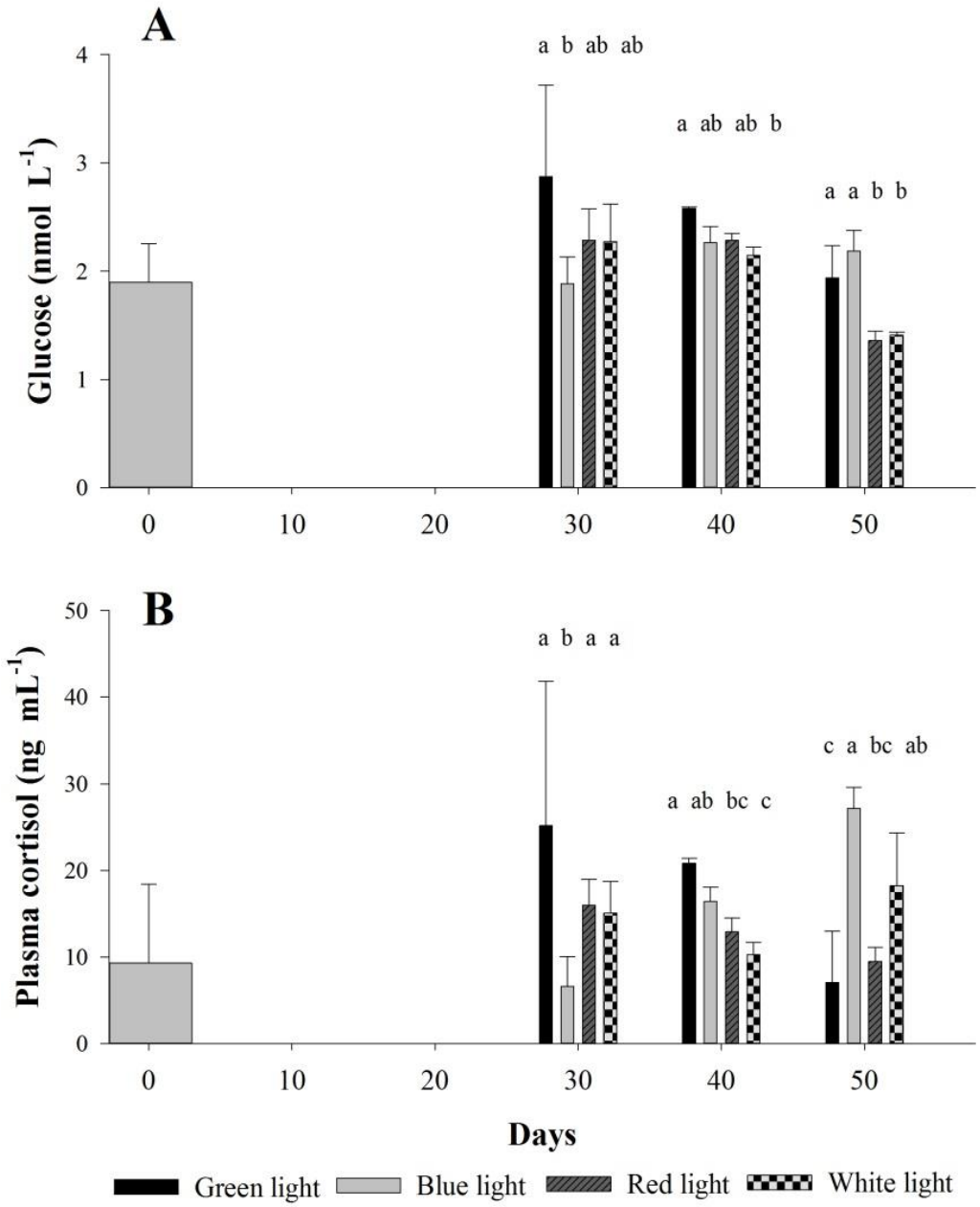

Figure 5.3. Plasma glucose (A) and cortisol (B) in turbot juveniles exposed to light treatments at given time point in experiment 5.1: Day 30 (coloured light treatments); Day 40 (white fluorescent light) and Day 50 (coloured light treatments). Data expressed as the mean (n = 20) ± SEM (10 individuals per replicate). Superscripts denote significant differences (One-Way ANOVA) at a given time point between treatments.

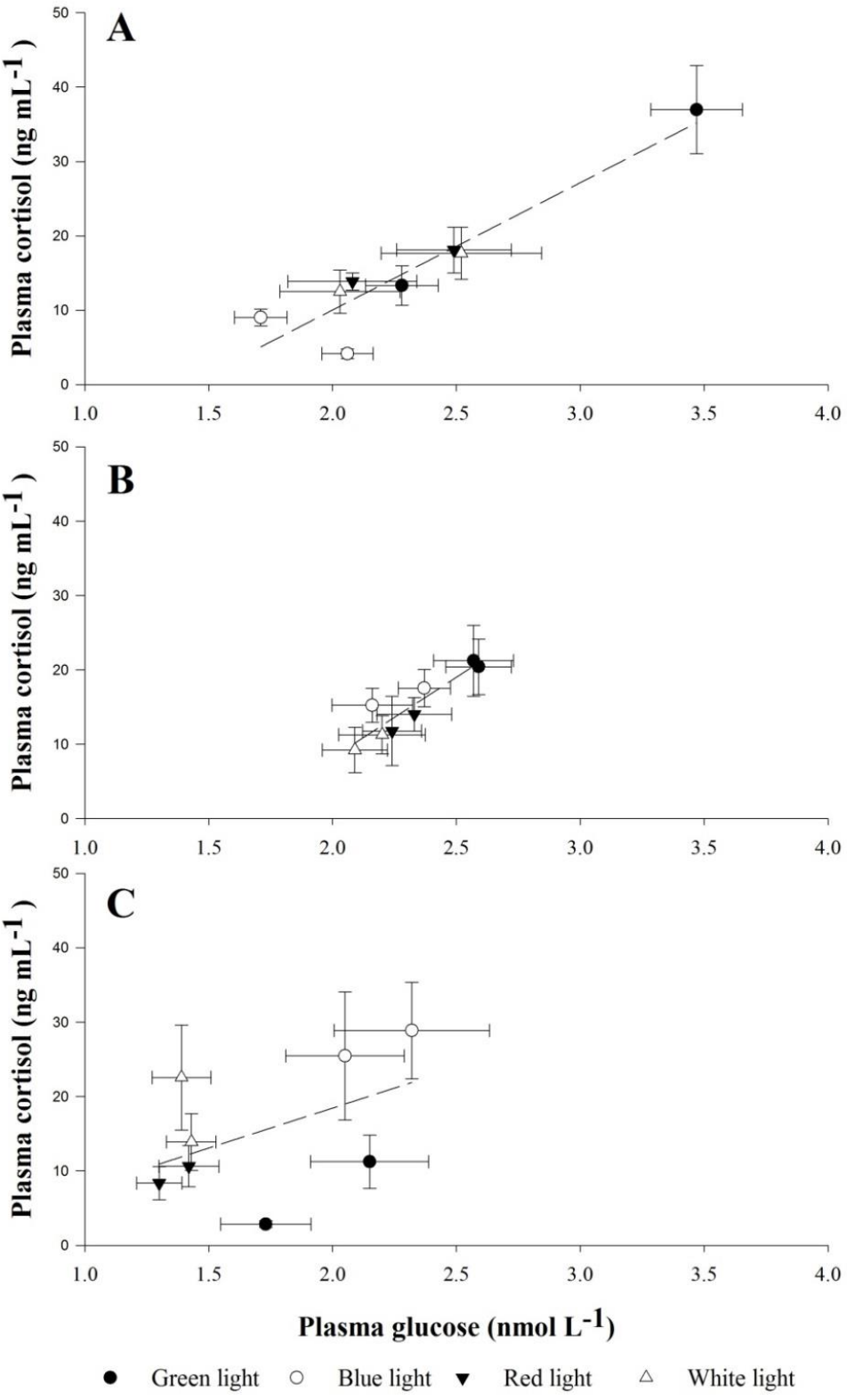

Figure 5.4. Correlations between plasma glucose and cortisol levels in turbot juveniles exposed to coloured light (A, day 30), white light (B, day 40) and coloured light (C, day 50) in Experiment 5.1. (A) $r^2 = 0.87$ (coloured light treatments); (B) $r^2 = 0.83$ (white light fluorescent tubes) and (C) $r^2 = 0.22$ (coloured light treatments). Data expressed as the mean (n = 20) ± SEM (10 individuals per replicate).

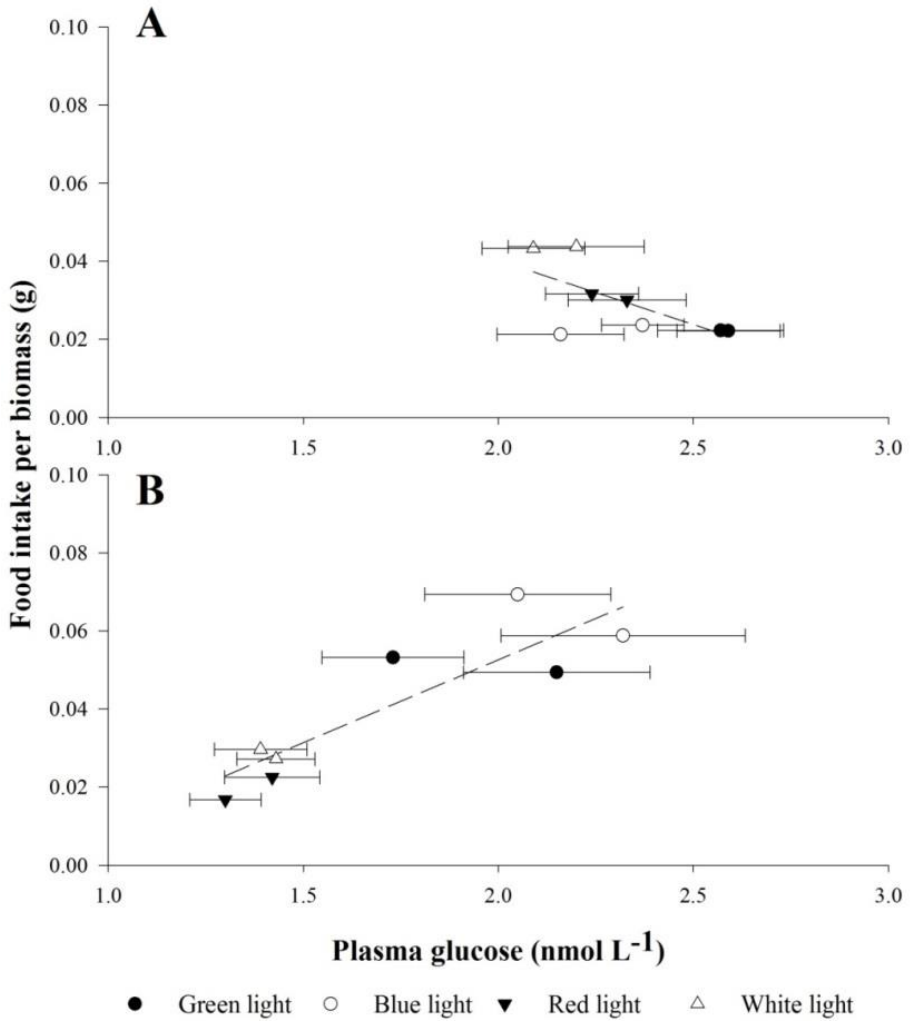

Figure 5.5. Correlations between plasma glucose and food intake (g $fish^{-1}$) levels in turbot juveniles exposed to coloured light (A) day 40), and (B) day 50 in Experiment 5.1. (A) r^2 = 0.42; y = -0.0329x + 0.1061 (coloured light treatments); (B) r^2 = 0.77; y = 0.0424x - 0.0322 (white light fluorescent). Data expressed as the mean (n = 20) ± SEM. Glucose 10 individuals per replicate, food intake food amount divided by number of fish.

5.3.2. Experiment 5.2.: Spectrum effect on turbot feed intake, growth and pigmentation

Growth parameters

Total length was significantly influenced by the light treatment following the first week of light treatment (Fig. 5.6a). Fish exposed to the red light were significantly smaller throughout the experiment, while blue, green and white treatments were not significantly different between each other. Turbot exposed to the red light were significantly lighter than the blue treatment after one week of exposure and, from 3 weeks onwards, turbot under red light were significantly lighter than all other treatments (Fig. 5.6b). Across the length of the trial, condition factor was highest under the blue treatment and lowest in the red. By the end of the trial K was significantly higher for blue treatment with respect to the red and green treatments, but it was not different from white (Fig. 5.6c).

Food intake divided by the biomass (Fig. 5.7a) was significantly higher for the blue and green treatments on the first week post light exposure onset. During the second week, consumption remained significantly higher under the blue light compared to the red and white; and thereafter there was no difference in feed intake between treatments. Weekly food conversion rate (FCR) was not significantly different between treatments throughout the trial (Fig. 5.7b). Weekly specific growth rate (SGR) (Fig. 5.7c) and thermal growth coefficient (TGC) (Fig. 5.7d) were significantly higher in the blue treatment compared to the red treatment during the first week. From week two there were no significant differences in SGR or TGC observed between treatments.

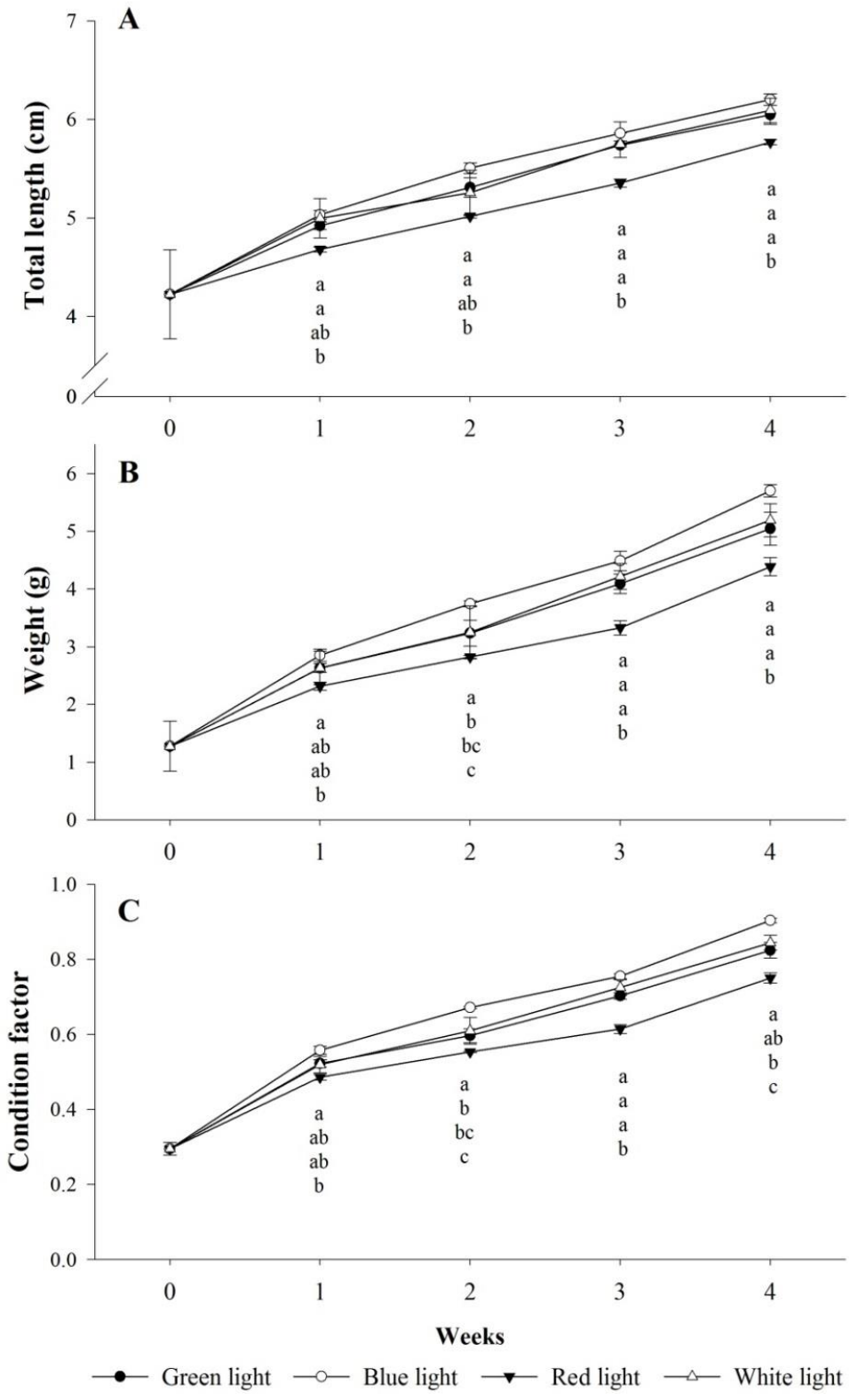

Figure 5.6. Growth parameters in turbot juveniles sampled weekly (Experiment 5.2) exposed to the light treatments: Green, Blue, Red and "white light". Standard length (A), weight (B) and condition factor (C). Growth parameters expressed as the mean (n = 60) ± SEM; 20 individuals per replicate. Superscripts denote significant differences (One-Way ANOVA) at a given time point between treatments.

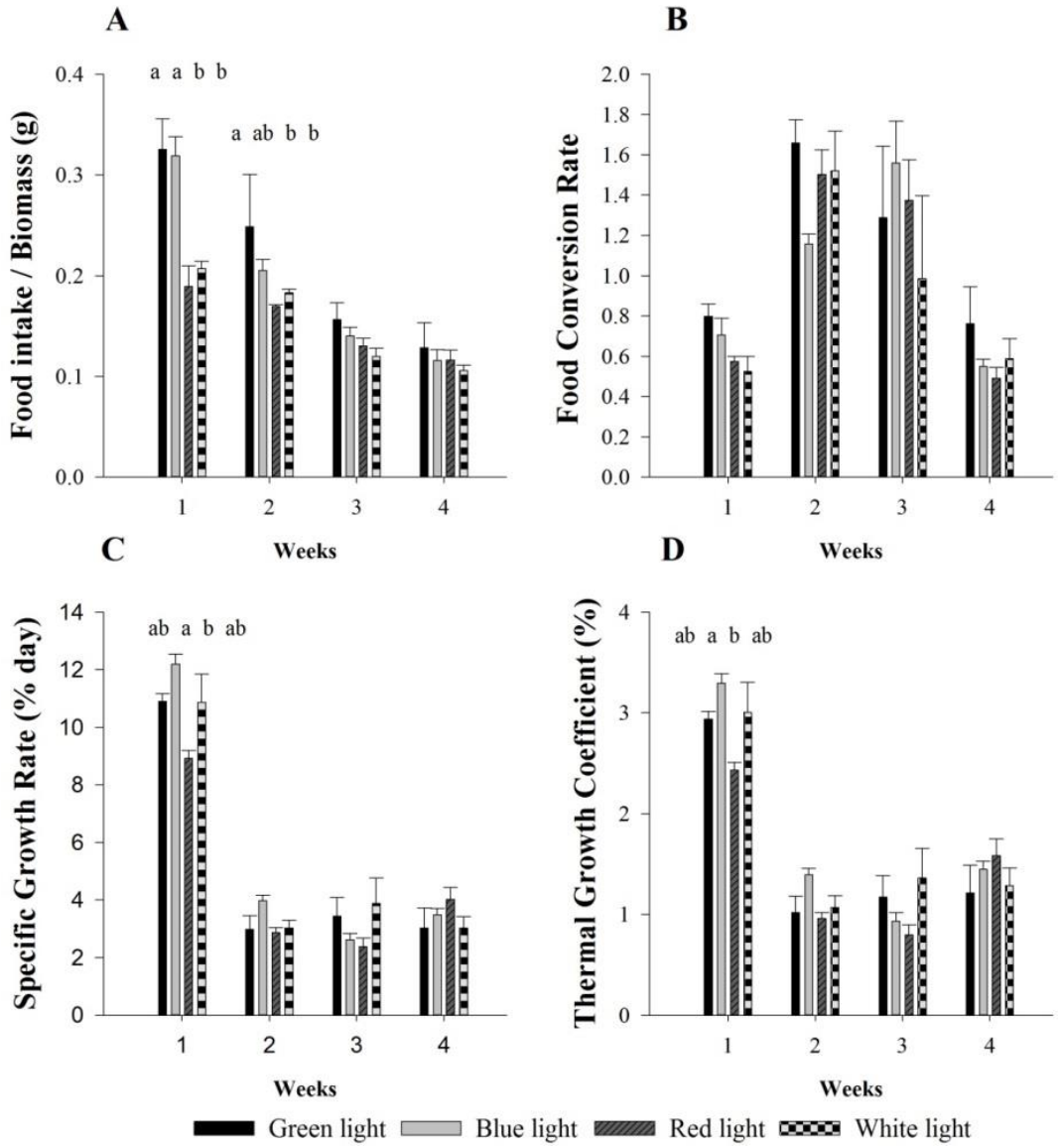

Figure 5.7. Growth rates in turbot juveniles sampled weekly (Experiment 5.2) exposed to light treatments: green, blue, red and white light. (A) Food intake (amount of food (g) / biomass), (B) Food conversion rate (FCR), (C) specific growth rate (SGR) and (D) thermal growth coefficient (TGC). Data expressed as the mean (n = 3) ± SEM. Superscripts denote significant differences (One-Way ANOVA) at a given time point between treatments.

Growth parameters analysed overall (from day 0 to 30) show differences between treatments (Table 5.3). Weight gain in the blue light treatment was higher than the green and red light treatments, but not white. Total food intake was higher in the blue and green treatments, followed by the white treatment and the lowest in the red treatment.

The food intake divided by the biomass was significantly higher in the blue and green treatments than red and white. Food efficiency was the highest in the white treatment and different from the blue and green. FE in the red treatment was different from the green treatment. FCR was not different between treatments after 30 days of light exposure. SGR in the red treatment were lower than the rest of the treatments. SGR in the white treatment was only different from the red treatment, while blue was higher than green. TGC was similar in all treatments except for the red treatment, which was comparable to the green light.

Relationship between length and weight ($W = a L^b$) showed similar b values for all treatments at all sample points, always $b < 3$, which were significantly representative of the growth parameters in terms of their r^2 and individual p values (Table 5.4).

Table 5.3. Turbot mean growth parameters and food intake and efficiency (Experiment 5.2) exposed for 30 days to the light treatments: A) Mean weight gain (g), B) Mean food intake (g day^{-1}), C) Mean food intake divided by the tank biomass (g); D) Food efficiency; E) Food conversion rate; F) Specific growth rate (SGR); G) Thermal growth coefficient (TGC). Growth parameters data expressed as the mean (n = 60) ± SEM; 20 fish per replicate. Scripts denote statistical differences (One-Way ANOVA) at given time point.

	Weight gain (g)	**Food intake (g day^{-1})**	**Food intake $biomass^{-1}$ (g)**	**FE**	**FCR**	**SGR**	**TGC**
Green light	3.8 ± 0.1 (b)	7.3 ± 0.2 (a)	0.21 ± 0.0 (a)	1.04 ± 0.0 (c)	1.32 ± 0.1	4.91 ± 0.1 (b)	1.58 ± 0.0 (ab)
Blue light	4.4 ± 0.0 (a)	7.4 ± 0.2 (a)	0.19 ± 0.0 (a)	1.21 ± 0.0 (bc)	0.99 ± 0.0	5.35 ± 0.0 (a)	1.77 ± 0.0 (a)
Red light	3.1 ± 0.1 (c)	4.6 ± 0.1 (c)	0.15 ± 0.0 (b)	1.28 ± 0.0 (ab)	1.13 ± 0.1	4.41 ± 0.0 (c)	1.44 ± 0.0 (b)
White light	3.9 ± 0.2 (ab)	5.4 ± 0.1 (b)	0.15 ± 0.0 (b)	1.46 ± 0.0 (a)	0.90 ± 0.0	5.01 ± 0.1 (ab)	1.68 ± 0.0 (a)

Table 5.4. Turbot weight and length correlation (experiment 2) exposed to the light treatments ($W = a L^b$). Mean total length (cm) (MTL), L_{min}-L_{max} range of length (cm), mean weight (g), W_{min}-W_{max} range of weight (g). Data expressed as the mean (n = 60) ± SEM; 20 fish per replicate. Coefficients of the linear regression from natural logarithm *a* (body shape) and *b* (growth relationship), SE of *b* coefficient regression and r^2 and p value of the regression equation.

Treatment	Week	MTL	L_{min}-L_{max}	MW	W_{min}-W_{max}	a	B	SE (b)	r^2	p
Green	1	4.9 ± 0.1	3.6 - 6.2	2.6 ± 0.1	1.3 - 4.7	0.03	2.65	0.12	0.88	0.000
	2	5.3 ± 0.1	4.2 - 6.5	3.2 ± 0.1	1.6 - 5.5	0.03	2.78	0.09	0.93	0.000
	3	5.8 ± 0.1	4.5 - 6.8	4.1 ± 0.1	2.1 - 6.6	0.08	2.26	0.1	0.88	0.000
	4	6.1 ± 0.1	5.1 - 7.2	5.0 ± 0.1	3.1 - 7.9	0.05	2.58	0.11	0.9	0.000
Blue	1	5.0 ± 0.0	3.8 - 6.0	2.8 ± 0.1	1.3 - 4.6	0.05	2.44	0.11	0.89	0.000
	2	5.4 ± 0.1	4.1 - 6.4	3.7 ± 0.1	1.6 - 5.3	0.03	2.68	0.13	0.87	0.000
	3	5.8 ± 0.1	4.4 - 7.1	4.4 ± 0.2	2.0 - 7.2	0.04	2.57	0.08	0.93	0.000
	4	6.2 ± 0.1	4.5 - 7.3	5.6 ± 0.2	2.6 - 9.0	0.05	2.61	0.08	0.94	0.000
Red	1	4.6 ± 0.1	3.6 - 5.7	2.3 ± 0.1	1.1 - 3.7	0.04	2.63	0.14	0.84	0.000
	2	5.0 ± 0.1	3.8 - 6.1	2.8 ± 0.1	1.2 - 4.7	0.03	2.7	0.09	0.94	0.000
	3	5.3 ± 0.1	4.1 - 6.5	3.3 ± 0.1	1.8 - 5.0	0.06	2.39	0.16	0.78	0.000
	4	5.7 ± 0.1	4.6 - 6.8	4.3 ± 0.1	2.3 - 6.5	0.04	2.63	0.09	0.92	0.000
White	1	4.9 ± 0.1	3.6 - 5.7	2.6 ± 0.1	1.2 - 4.5	0.04	2.54	0.13	0.86	0.000
	2	5.2 ± 0.1	4.0 - 6.1	3.2 ± 0.2	1.4 - 5.4	0.03	2.67	0.12	0.89	0.000
	3	5.7 ± 0.1	4.5 - 7.6	4.2 ± 0.1	2.3 - 7.1	0.06	2.39	0.11	0.88	0.000
	4	6.1 ± 0.1	4.9 - 7.0	5.2 ± 0.1	3.0 - 8.4	0.05	2.53	0.12	0.88	0.000

Skin colouration

Red hue (%) (Fig. 5.8a) in fish was different at day 2 post light onset between blue and green treatments and not the other treatments. From day 4 to day 6 fish from the blue treatment were significantly different only to those from the red treatment, while at day 8 green treatment fish were different from those in the white treatment. At day 20 blue treatment fish were different from those in the white and red treatments, but not green. Green hue (%) (Fig. 5.8b) was different at day 4 when this parameter was higher in fish from the blue treatment than those from the red and white treatments. At day 6 blue treatment fish were different only from those in the red treatment, while at day 8 red treatment fish were different from those in the blue and green treatments. At day 20, blue treatment fish showed lower percentage green hue than those in the white and red but not green treatments. By the end of the trial fish in the red treatment were different from other treatments, except the green treatment fish. Blue hue (%) (Fig. 5.8c) at day 4 was lower in the blue treatment fish except the fish in the green treatment. On day 6 fish in the red treatment B% was higher than those in the blue treatment. On day 8 green treatment fish B% was lower than the rest, except for the fish in the blue treatment. On day 20 fish in the blue was lower than the rest except for the fish in the green treatment.

Brightness showed significant differences from day 4 post light exposure (Fig. 5.9a) On day 4 brrighntess in the fish in the red treatment was lower than those in the blue treatment. On days 8 and 20 the blue treatment was brighter than the white and red treatments. On day 30 this parameter was lower in the fish in the red treatment than those in the rest of the treatments. Colour saturation (Fig. 5.9b) was higher in fish from the blue treatment than those in the red treatment. Blackness (Fig. 5.9c) was higher (darker) in fish from the red treatment than those in the green and blue treatments,

except on day 8 when fish from the white and red treatments were similar. On day 20 red treatment was darker than blue and green treatments. On day 30 red light treatment fish were different from all the other treatments.

On day 30 brightness and darkness were correlated to the wavelength exposed showing a positive correlation between the length of the wavelength and the darkness of the skin. Fig. 5.10 shows an example of each light treatment at the end of the trail as it was perceptible to the human eye.

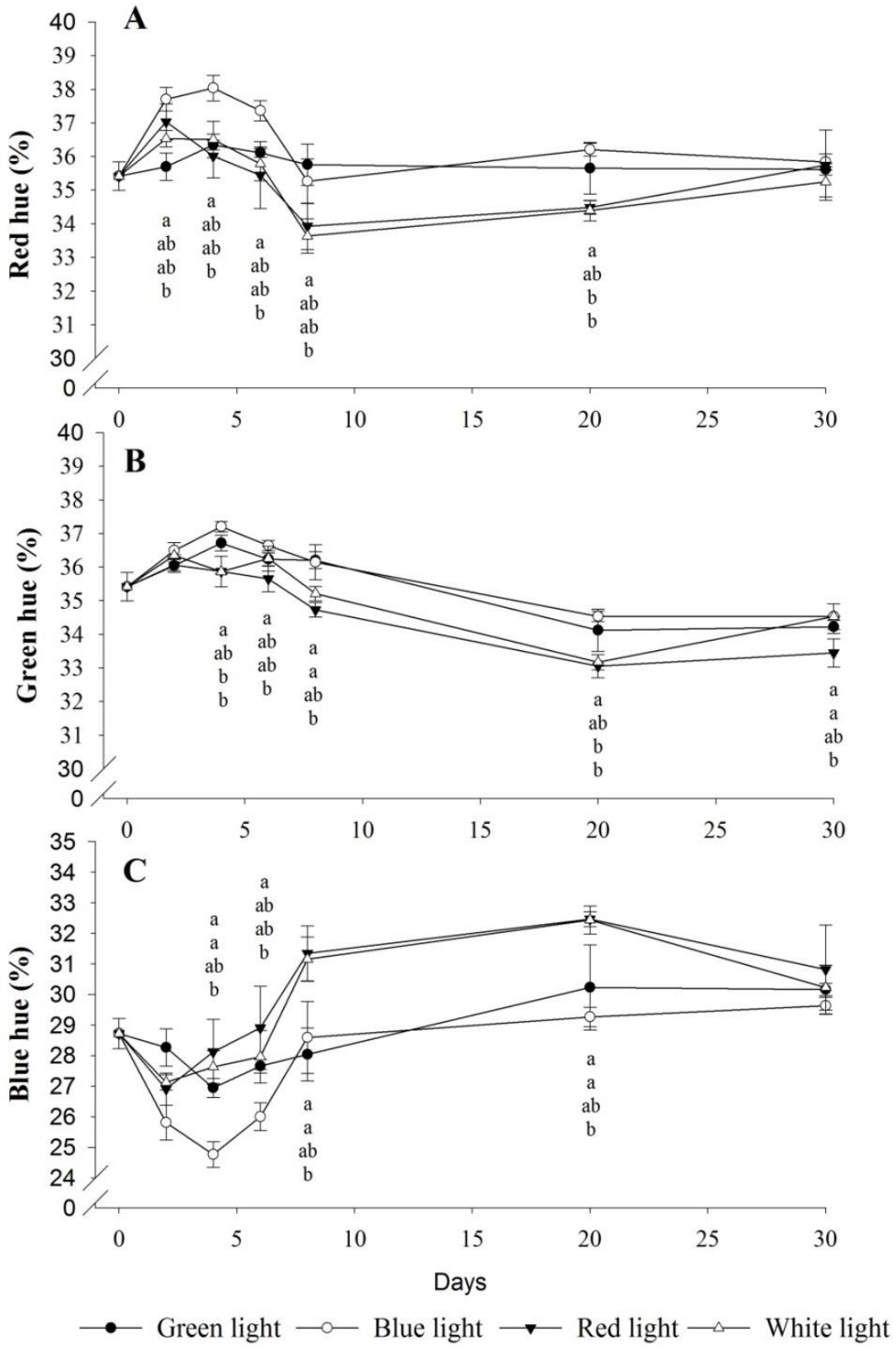

Figure 5.8. Colour composition (RGB) by Image analysis expressed as a percentage (%) at a given time point. (A) Red, (B) Green and (C) Blue hue expressed as the mean (n = 18) ± SEM. 6 individuals/replicate. Superscripts denote significant differences (One-Way ANOVA) at a given time point between treatments.

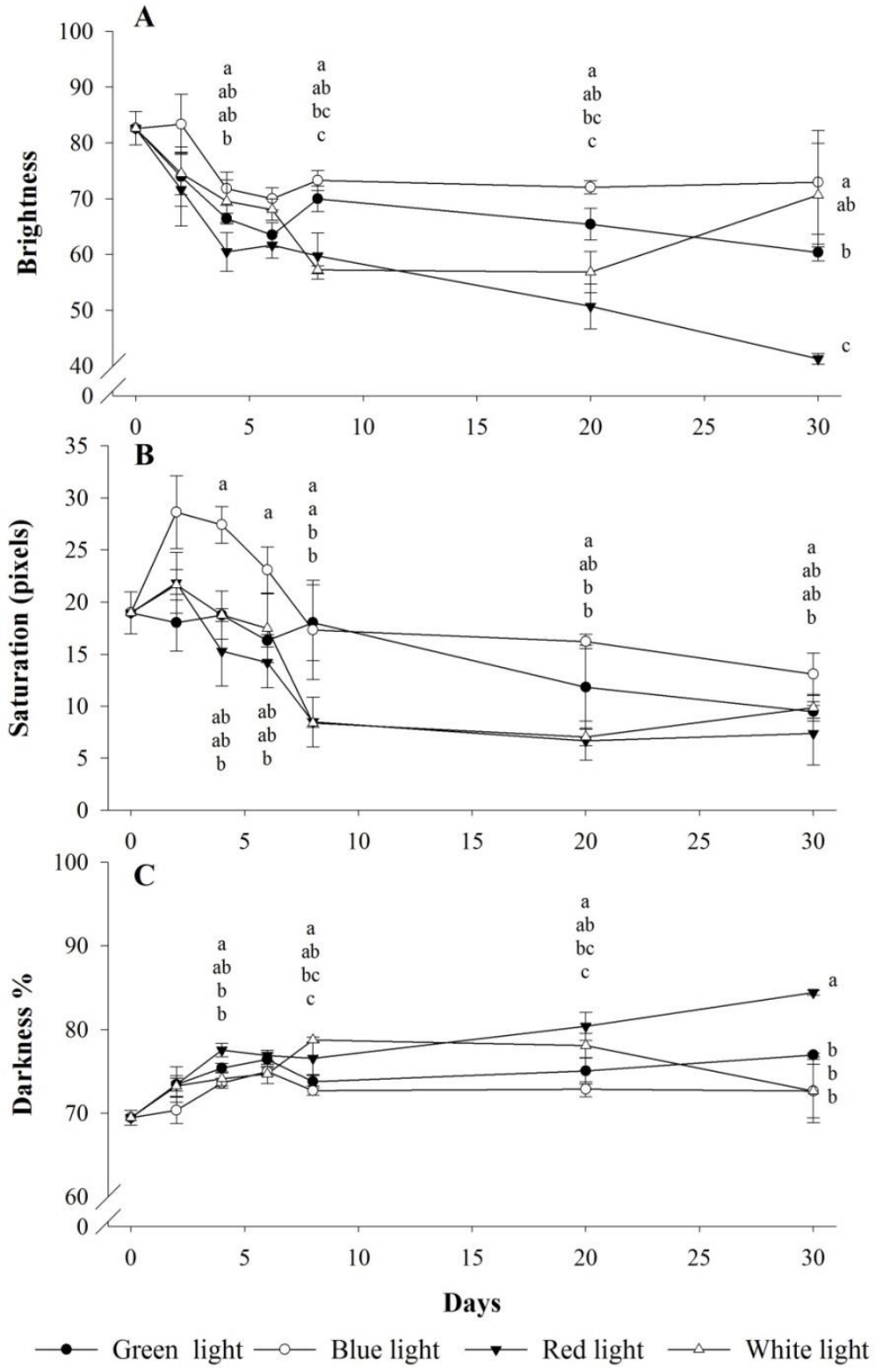

Figure 5.9. Colouration analysis by Image analysis at a given time point. (A) Intensity/brightness, (B) colour saturation and (C) darkness/blackness expressed as the mean (n = 18) ± SEM. 6 individuals/replicate. Superscripts denote significant differences (One-Way ANOVA) at a given time point between treatments.

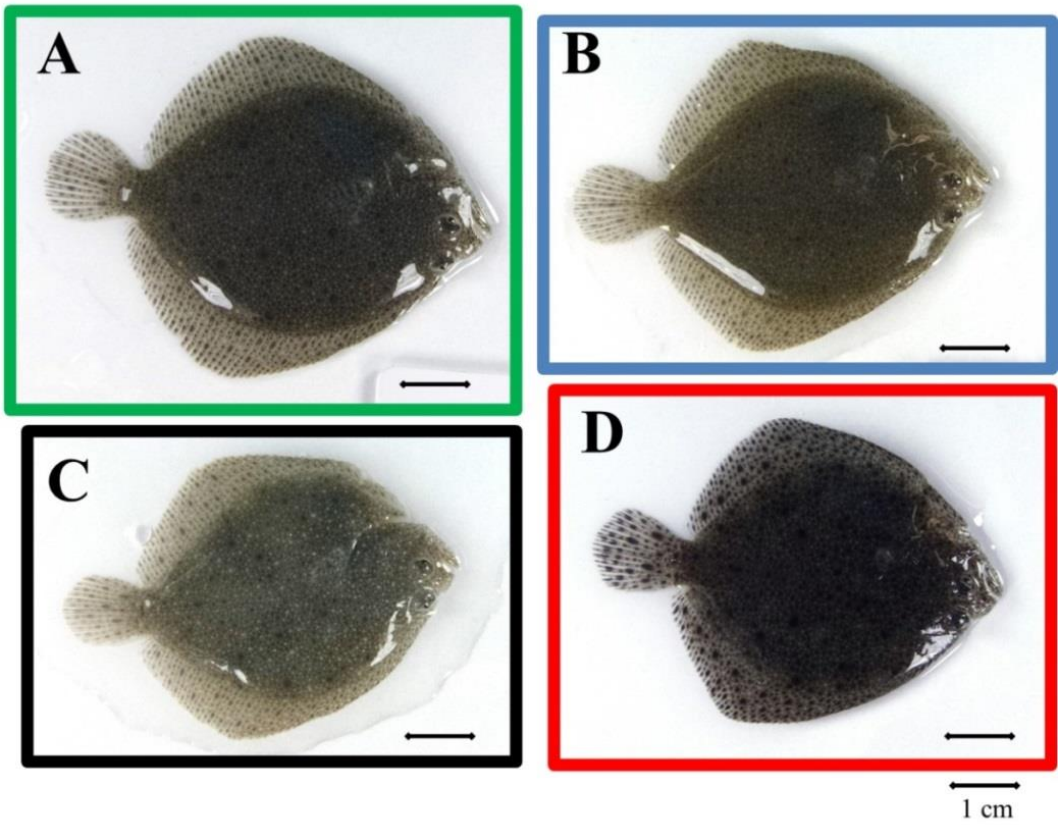

Figure 5.10. Turbot sample of colouration at day 30 from the (A) green, (B) blue, (C) white and (D) red treatments. Scale represents 1 cm.

5.4. Discussion

Results from the present study are the first to show that light spectrum has a direct effect on turbot juveniles' physiology and morphology. In both experiments, growth was enhanced by the short wavelength treatments and supressed by the red light exposure. Light spectrum treatment affected in a similar way growth rates, food intake, food efficiency and had an impact on plasma glucose and cortisol levels and skin colouration. It is very likely that turbot is predisposed to perform better under short wavelengths given its natural habitat at depths where blue/green wavelengths are predominant (Jerlov, 1968).

In Experiment 5.1, fish exposed to the green light had the highest total length, weight and condition factor throughout the experiment. However, fish exposed to blue light were not different other treatment fish at any sampling points. On the other hand, red light negatively affected turbot growth with reduced total length, weight and condition factor. In Experiment 5.2 turbot exposed to blue and green light showed the highest total length, weight and condition factor. These results, together with the larvae trials presented in chapter 4, confirm that turbot growth is enhanced by short wavelengths at the larval and juvenile stages. However, fish exposed to white light (daylight bulb) in Experiment 5.2 were not different from the blue/green treatments. Similar results were found in sea bass (Karakatsouli *et al.,* 2012) and sea bream (Karakatsouli *et al.,* 2007) where growth was not different between blue and white spectra using coloured filters. This can be explained by the spectral profile of the Solux bulb which covered wavelengths from the violet up to the red colours. It appears that red light has a negative effect on juvenile growth in most of the studied species: tilapia, crucian carp, Chinese sleeper, guppies, sea bass, and Senegale sole (Ruchin, 2004; Luchiari and Freire, 2009; Blanco-Vives *et al.,* 2010; Karakatsouli *et al.,* 2012).

However, in sea bream, red light did not have a negative effect on growth parameters after 11 weeks of exposure but an increase in dopaminergic activity was observed (Karakatsouli *et al.*, 2007) suggesting a stress condition. A recent study (Volpato *et al.* 2013) reported that tilapia appetite is enhanced by red light however without a positive effect on growth. It is possible that food was not absorbed efficiently, or most likely that the red light increased the energy cost by stimulating swimming activity or triggering a stress reaction. However the experimental settings (i.e. light intensity) might have caused the differences between their light treatments (white, blue, green, yellow and red).

Experimentation with light is not an easy task as spectral content and intensity perceived by fish must be controlled and these will be impacted by the light technology used, the volume and quality of the water that will absorb wavelengths differentially and system set up with potential light pollution. A key criticism with most studies performed to date is the use of lux as light intensity unit.

Photometric units (i.e. lux) are quantitative relative measurements of luminance created to represent the spectrum perceived by the human eye from 400 nm to 700 nm (peak at 555 nm) and therefore lux is not suitable for fish studies (Murray, 1993; Boeuf and Le Bail, 1999; Migaud et al., 2010, Villamizar *et al*., 2011). From the literature it appears that the effects of light on fish growth are mainly mediated by feeding activity and food intake (Boeuf and Falcón, 2001). As food intake has been reported to be enhanced in fish exposed to increased light intensity (Boeuf and Le Bail, 1999; Noble *et al.*, 2005; Strand *et al*., 2007; Yoseda *et al.*, 2008), if this is true then the effects of the light spectrum in those trials might have been masked by differences in light intensity between spectral treatments.

Results from Experiment 5.1 at day 40, after the intermediate white light exposure, confirmed that the enhanced growth was induced by the blue and green light treatments. During the 10 days of white light exposure, fish in all treatments except red increased in total length. However, only fish that had previously been exposed to white light for 30 days gained weight, while the blue and green treatments weight remained steady and the fish initially under the red light lost weight. Condition factor in all light treatments was reduced at the end of the intermediate white light window. When the fish were returned to their original light treatments from day 40, fish under white light continued to gain weight; however, weight gain was not as pronounced as in the blue and green treatments. The relationship between length and weight regression analysis (in terms of the b value from $W = a L^b$) showed that growth in fish from Experiment 5.1 was allometric mainly towards length, rather than weight, except at day 30 in the blue and red treatments. In Experiment 5.2 growth was allometric towards length as well and no differences were found between treatments, despite the growth and weight gain differences. It is possible that turbot's metabolic rate responds to instrinsic autoregulators rather than external stimuli (e.g. light, temperature, food availability).

turbot growth depends on the genetic background, feeding regime and temperature (Person Le-Ruyet, 2002). Appetite is regulated by temperature, with a marked decrease when it drops < 14 °C, however the metabolic rate does not seem to be affected by it (Burel *et al.*, 1996). In both experiments presented here temperature remained relatively constant (Experiment 5.1: 11.6 ± 0.9 °C and Experiment 5.2: 13.9 ± 0.05 °C) but food intake was different between treatments, which can be directly associated with the light treatments. The greater food intake in the blue and green treatments was translated into a higher SGR and TGC, but FCR was similar between treatments. Feed waste could not be monitored in the present experiments due to the

small pellet size and system used with no waste feed collectors and therefore true biological FCR could not be calculated. FCR values are calculated from the total amount of food given over the course of a day to reach satiation. It is likely that feed given to the fish does not accurately represent feed intake especially in blue and green treatments where feeding activity appeared to be increased. Furthermore, through casual observations it appeared that the initial feeding reaction was faster in the blue and green treatments and lasted longer than in the white and red light although no quantitative assessment was done.

Several neuroendocrine systems are involved in the regulation of metabolism, to maintain the energy homeostasis through orexigenic and anorexic factors (Volkoff *et al.,* 2005). The theoretical sustained growth of fish throughout their life requires a constant resource allocation between biological processes. In mammals glucose is a key energy source, but its exact functions in fish are still unclear (Enes *et al.,* 2009). Ultimately glucose is catalysed into energy used to maintain the endocrine balance (Hemre *et al.,* 2002). Stress stimuli require a rapid response to allow the fish to cope with the threat and maintain its homeostatic state, which is achieved through the increase of plasma corticosteroids (Barton, 2002). Together plasma cortisol and glucose levels are widely used as indicators of a physiological stress reaction in fish. In Experiment 5.1, on day 30, plasma glucose and cortisol levels were higher in fish under green light, while the lowest were in the blue treatment. At that time point there was a strong positive correlation between cortisol and glucose levels. However, the data cannot explain such differences between green and blue treatments. In the green treatment both parameters peaked similarly which might suggest a physiological stress reaction. However, on day 50, plasma cortisol levels in fish exposed to blue light peaked, while glucose levels remained unchanged. The glucose stress response appears

to be dependent on temperature: the lower the temperature, the weaker the glucose response in turbot (van Ham *et al.,* 2003). Furthermore, the cortisol response in sea bass after 1 hour confinement was higher in fish reared under blue light (Karakatsouli *et al.*, 2012), which could explain the difference on day 50 between the blue and the other treatments.

Karakatsouli *et al*. (2008) reported that plasma glucose, cortisol and dopaminergic activity are negatively affected by light spectrum in rainbow trout (blue light) and sea bream (red light). However, unstressed rainbow trout showed no significant differences between light treatments (white, blue, red and yellow), while stressed trout (1-hour stress by reducing water level) under yellow light showed significantly lower cortisol levels than their stressed counterparts (white, blue and red) (Heydarnejad *et al.*,, 2013). The involvement of cortisol in metabolism regulation, osmoregulation, stress response and its interaction with other hormones is complicated and requires further investigation (Ellis *et al.,* 2012).

Apart from the cortisol peaks (green at day 30 and blue at day 50), the rest of the blood levels at the other time points for both glucose and cortisol, appear to be within the basal levels for the species (Waring *et al.,* 1996, 1997; van Ham *et al.,* 2003). Therefore differences in glucose and cortisol between treatments might be a reflection of the metabolic state of the fish at the sample point, rather than stress indicators. Blood glucose levels are known to be correlated to the food intake levels. Food deprivation reduces circulating blood glucose levels and its receptors expression in the brain (Barton *et al*., 1988; Blasco *et al*., 1996; Power *et* al., 2000; Soengas *et al*., 2006; Viegas *et al*., 2013). The food intake and glucose levels at day 50 show a strong correlation based on the r^2 value of 0.77. Both red and white treatments had a lower food intake and lower glucose levels which could be associated with the metabolism

(i.e. food intake) (Polakof *et al.*, 2012). On day 40 the correlation slope was inverted, however the r^2 value was 0.42 which suggests that the correlation does not explain the ultimate cause.

Light treatments had also a clear effect on turbot skin pigmentation. The image analysis of the hue composition showed that the light treatments had short term effects (resumed within 8 days) on the RBG composition of the skin pigmentation. This is a very interesting outcome as pigmentation in flatfish, especially turbot, is a commercial problem that leads to product downgrading and economic losses. Blue light treatment triggered an increase in the percentage of the red and green hue, and a reduction of the blue hue. By the end of the trial 2, on day 30, there were no significant differences between treatments in any of the RGB hues. However, the RGB scale cannot truly represent the visual pigmentation of turbot. Species like tilapia and salmon, where the red hue (%) is the predominant colour; it can then be used as a functional tool. The RGB scale goes from pure black (RGB = 0,0,0) to pure white (RGB = 255,255,255). For example, one turbot exposed to green light for 30 days had a light brown colouration, which in RBG scale is defined as a RGB of 65,63,50. In fish with erythrophores (red pigment cells) like salmon, tilapia or goldfish, the analysis clearly reflects the skin colouration showing redness percentage. turbot chromatophores in the skin are mainly composed of melanophores, xanthophores and iridophores which contains black, yellow and silver/reflecting pigments, respectively (Bolker and Hill, 2000). Unfortunately, due to the small size of the fish used in the study, pigment analyses could not be performed. However, such analyses may have been more informative to compare skin pigmentation between treatments.

In visual terms, the hue composition showed that fish under red light appeared black/dark, fish under green light were dark brown and fish under blue light were light

brown while fish under white light were light grey but less homogenous than the other treatments. This is clearly reflected by the brightness, saturation and darkness data. For instance, darkness in the red treatment was the highest, followed by the green and blue/white. Consistently blue light exposure in red porgy (*Pagrus pagrus*) increased skin lightness (Pavlidis *et al.*, 2008). Interestingly in the present study, there appears to be a correlation between the wavelength and the skin colour of the fish, the longer the wavelengths, the darker the fish are (Fig. 5.10). Skin darkening is usually associated to a stress due to higher plasma levels of cortisol, ACTH and α-MSH (Höglund *et al.*, 2000). Melanocyte Stimulating Hormone, as its name states, stimulates the dispersion of the pigments darkening the skin; however its role on pigmentation can be modulated due to interaction with other hormones (Mizusawa *et al.*, 2013). MCH has an opposite function from MSH, both *in vivo* and *in vitro* (Kawauchi and Baker, 2004). In barfin flounder (*Verasper moseri*) MCH brain and plasma levels were increased in fish reared in a white background tank, while the α-MSH levels were increased in a black tank and also in the white tank after 7 days (Amiya *et al.*, 2008; Mizusawa *et al.*, 2013). It is possible that different spectral profile of the light impacted on α-MSH and MCH explaining the lighting or darkening of the skin. However, red porgy exposed to blue light under a 12L:12D photoperiod, became gradually paler but no differences was found in melanin content, hue, glucose, thyroid hormones, or MSH levels (Szisch *et al.*, 2002; Pavlidis *et al.*, 2008). α-MSH shares proopiomelanocortin (POMC) as a common precursor with ACTH and both can stimulate cortisol synthesis. The pathways by which MSH is involved in pigmentation, nutrition and stress reaction need to be studied further. Differences in pigmentation found in the present study could have been caused by melanin dispersion due to a direct effect of the light spectrum, differences in food intake, stress reaction or the interaction between all.

In conclusion, we confirmed for the first time that light spectrum affects turbot growth, food intake, plasma glucose and cortisol, and pigmentation. However, all these biological processes interact with each other at the level of pituitary and form a complex network. Further research is needed, first to understand the direct effects of the light spectrum on the neuroendocrine control of appetite and second better characterise the pathways at work and their interactions between the different systems involved (food intake, stress, pigmentation). The results of the present study suggest that turbot performance can be enhanced by blue and green light. Light spectrum can easily be manipulated in enclosed facilities and potentially could benefit the farmers. Further studies should investigate further the nature of the pigmentation change (stress, light spectrum or metabolic) and the potential implementation of light as a tool to improve turbot farming.

Chapter 6

EFFECTS OF TWILIGHT ILLUMINATION ON ATLANTIC COD (*GADUS MORHUA* L) SPAWNING PERFORMANCE

6.1. Introduction

Marine aquaculture is confronted by several restrictions that prevent the industry from becoming financially profitable. The cod industry faces low survival rates of 1- 30 % (Brown *et al.*, 2003) during the larval stages, limiting the seed supply and creating a production bottleneck. Poor gamete quality and weaning are likely causes of the low survival. Gamete quality is generally defined as the ability to successfully fertilise the oocyte (Bobe and Labbe, 2010), however gamete quality appears to determine the viability of later stages of the animal as well.

Several interrelated physiological and environmental factors directly influence the egg quality. In captivity these factors need to be controlled to ensure that the optimal conditions for fish welfare are given. Broodstock management is relatively simple as cod is a mass spawner that freely releases gametes in the holding tanks (Kjørsvik and Holmefjord, 1995). However the technical simplicity of the aquaculture systems might overlook some of those factors contributing negatively to cod spawning success. The genesis of egg quality is still unclear; however it seems to be influenced by: genetics, broodstock nutrition, environmental conditions and endocrinal status of the broodfish (Migaud *et al.*, 2013). For instance, if those factors do not cover some minimal or optimal conditions, spawning can be completely skipped (Jørgensen *et al.*, 2006). However the recruitment threshold for maturation is more complicated, as it appears to depend on energy resources (based of age, size, food availability, population mortality) prior or at early stages of vittelogenesis due to allocation towards somatic growth or reproduction (Jørgensen and Fiksen, 2006; Skjæraasen *et al.*, 2012).

Light is a key factor entraining reproductive activity in temperate fish (Migaud *et al.*, 2010). The underlying mechanisms behind seasonal entrainment by light remain unclear, although there is wide evidence of the direct implications of photoperiod in the

reproductive axis (Migaud *et al*., 2010). Photoperiod regulates the timing of spawning and hormonal levels in mature fish (Norberg *et al*., 2004) and is responsible for recruiting individuals into sexual maturation (Davie *et al*., 2007a). The decreasing autumnal day length is perceived by sexually immature cod as the signal to initiate gonadal development, around 5 months before spawning occurs. The advanced reaction is essential to coordinate mature gamete release with optimal ecological and environmental conditions (e.g. temperature, food availability) to maximise the offspring survival.

Female cod spawn between 9 to 20 egg batches per season with intervals of 60 -70 hours (Kjesbu, 1989 and 1990). The duration of the spawning period, and ovulation *per se*, is related to the physiological (hormonal) and morphological (e.g. size and age) status of the fish and regulated by temperature (Kjesbu, 1994). Since temperature remains relatively constant in higher latitudes throughout the spawning period, other signals (e.g. light regime, pheromones) might act as ultimate factors determining the actual egg batch release. As mentioned in previous chapters, it is believed that cod spawn mainly during the scotophase, which can be corroborated by the embryonic stage of development and the nocturnal peaks in vocalisations (Fudge and Rose, 2009). Therefore, daily light variations are likely signals influencing the ovulatory rhythms, possibly by interacting with other diel variations.

Day/night perception might play a crucial role in starting the actual spawn (from mate selection to fertilisation), through hormonal signalling and/or behavioural changes. During dawn/dusk light composition changes in intensity and spectral composition. At dusk the decreasing light intensity is accompanied by a reduction of the short wavelengths, resulting in a predominantly long wavelength photo environment, while the inverse is true during dawn (Jerlov, 1968). The photoreceptors (possibly

through melatonin regulation) might be involved in the control of the ovulatory rhythms. For instance, Vera *et al.* (2010) found that red light does not efficiently inhibit melatonin secretion, depending on light intensity. Therefore it is possible that the daily reduction in short wavelengths act as a signal activating the melatonin secretion, although secretion patterns vary between species (Migaud *et al*., 2010). Furthermore, reproductive light entrainment is not solely related to day length. In Senegal sole the lunar cycle, through light perception, regulates melatonin, vittelogenin and sex steroids (Oliveira *et al*., 2010). In enclosed systems such environmental signals (twilight, moon light) are overlooked and might be contributing to the low egg quality found in captivity. Simulating similar lighting conditions in stocking tanks as in the wild, could possibly improve egg quality and enhance the commercial seed supply of marine fish.

The aim of this study was to investigate the effects of different lighting systems (on/off timer switch vs. a dimming system simulating the twilight light intensity) in terms of egg production and quality.

6.2.Materials and methods

The experiments were carried out at the facilities of Viking Fish Farms Ltd., Ardtoe Marine Laboratory, Acharacle, Scotland (N 56°46' W 05°53') and conducted in accordance with the Animals Scientific Procedures Act of 1986, UK following independent ethical review.

Atlantic cod broodstock (total length 50.5 ± 5.3 cm, body weight 2.0 ± 0.5 Kg) were maintained (48 females and 10 males) in a circular holding tank (5.3 m diameter, 88 m^3) at ambient temperature (8.4 ± 0.1 °C: range 6.7 – 10.2 °C) and salinity (34.5 ± 0.6 ppt: range 32 – 35 ppt) under ambient photoperiod using 40 W incandescent tungsten halogen bulbs with an automatic dimming system to recreate dawn-dusk (SunMatch, AquaBioLab, Canada) for 2 years prior the start of the experiment. On 26th February

2011, 24 females and 5 males were randomly allocated into two identical tanks (5.3 m diameter, 88 m^3). No treatment replication could be performed due to limitations in fish and tank availability. Photoperiod was controlled in one experimental tank with a timer switch, while the other was supplied with an automatic dimming system to recreate dawn-dusk (SunMatch, AquaBioLab, Canada). Fish were fed to satiation three times a week using commercial marine fish diets (Classic Marine, Biomar, Norway). From 28th February to 10th May 2011 cod eggs were collected daily from an external overflow egg collector as 15:00 hrs each day and egg quality parameters monitored. Total egg volume was measured volumetrically recording both floating and sinking fractions separately. Fertilisation rate (%) and embryonic stage of development was determine from a sample (n = 50 per tank) of the floating fraction.

Statistical analysis was performed using Minitab 16 (Minitab Ltd., UK). All datasets were first tested for normality and homogeneity using the Anderson-Darling test. Fertilisation rates, floating fraction and egg viability percentage were arcsine square root transformed and variance tested by One-Way ANOVA. Significance level was set at $p < 0.05$ and significant interactions evaluated by Tukey *post hoc* test.

6.3.Results

The spawning period for both tanks lasted from February 28th to May 10th, 2011 (Fig. 6.1). Total egg volume produced during the spawning period was comparable with 45.1 and 43.7 kg (relative fecundity of 0.75 kg/female) for the on/off timer and dawn/dusk dimmer treatments, respectively (Table 6.1). Overall egg parameters showed no differences between treatments.

Table 6.1. Total spawning production and batch mean ± S.E.M. egg quality parameters over the spawning season.

	On/Off	**Dawn/Dusk**
Total egg volume (Kg)	45.1	43.7
Floating fraction (%)	66.8 ± 2.5	62.8 ± 2.7
Fertilisation rate (%)	64.8 ± 3.5	65.8 ± 3.9
Viability (%)	46.1 ± 2.9	46.2 ± 3.6

Weekly egg parameters: total weekly egg production sum (Fig. 6.2a); weekly mean fertilisation rate (Fig. 6.2c), mean weekly floating fraction (Fig. 6.2d) and weekly mean viability rate (Fig. 6.2e) (Floating fraction x fertilisation rate) over the spawning period were similar between treatments. Mean daily egg production (Fig. 6.2b) was different between treatments at week 1, 2, 3 and 5.

No differences were found in embryonic stage of development. Fertilised eggs from both tanks were consistently at the blastula: blastodisc and sphere stage of development (Hall *et al.*, 2004) (Fig. 6.3), which corresponds to > 128 cells – gastrula development. Based on the blastula development equation ($D° = a\ (T + 2)^{b}$) (Geffen *et al.*, 2006*)*, fertilisation in the experimental tanks occurred between 17 and 20 hours prior to collection at the ambient temperature of 8°C. Recorded developmental stage suggests that fertilisation consistently occurred during the scotophase, between 22:00 and 02:00 hrs.

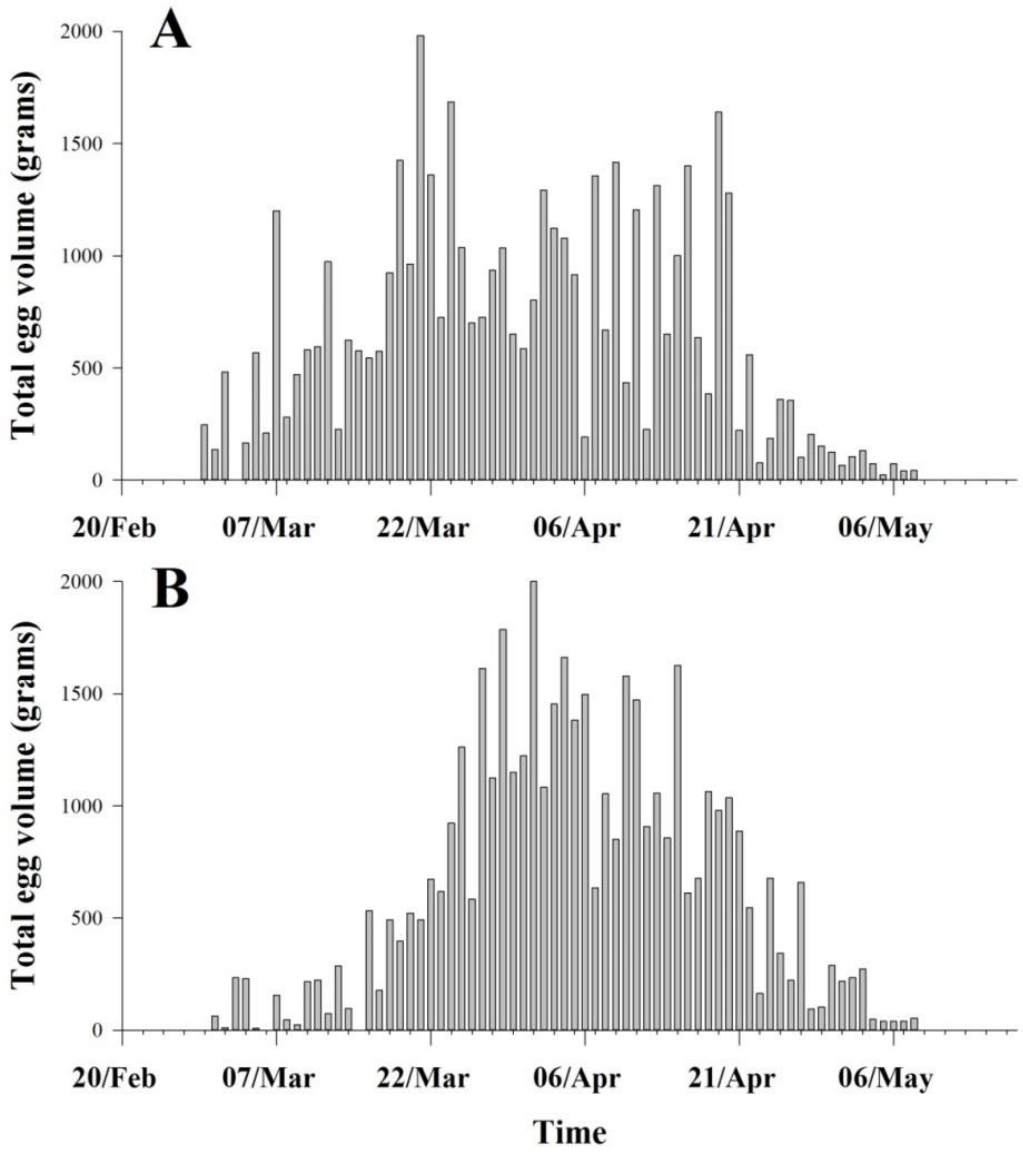

Figure 6.1. Daily total volume of eggs (floating and sinking fraction combined) collected from each tank (On/Off timer (A) and Dawn/Dusk dimmer system (B) tanks) during the spawning season (February-May 2011).

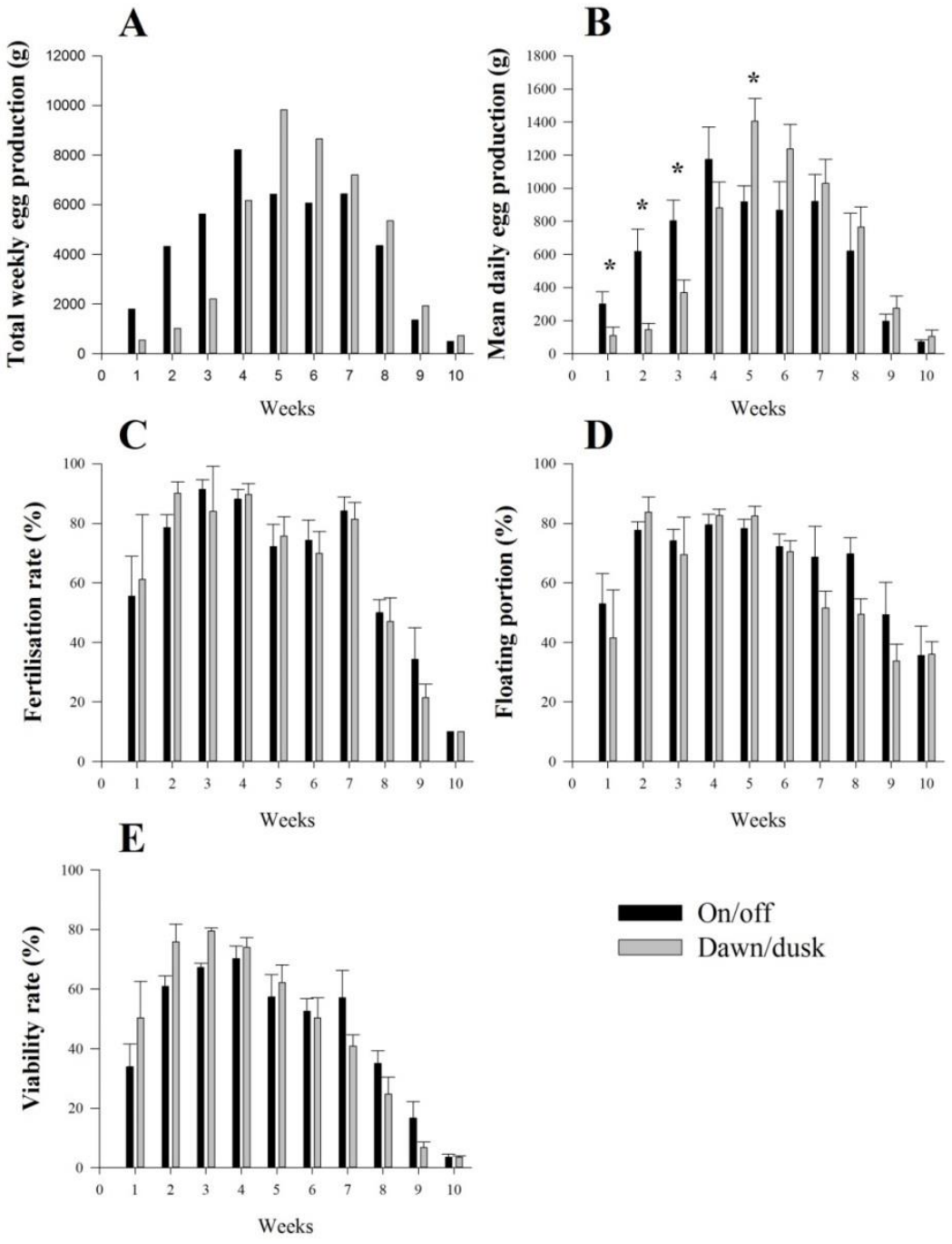

Figure 6.2. Egg quality parameters presented in mean values per week ± SEM. A) Total weekly egg production; B) Week mean egg production; C) Weekly mean fertilisation rate (n = 50 per treatment); D) Weekly mean floating fraction from each treatment; E) Weekly mean fraction of viable eggs (Viability (%) = Floating fraction * fertilisation rate). * denotes significant differences (One-Way ANOVA).

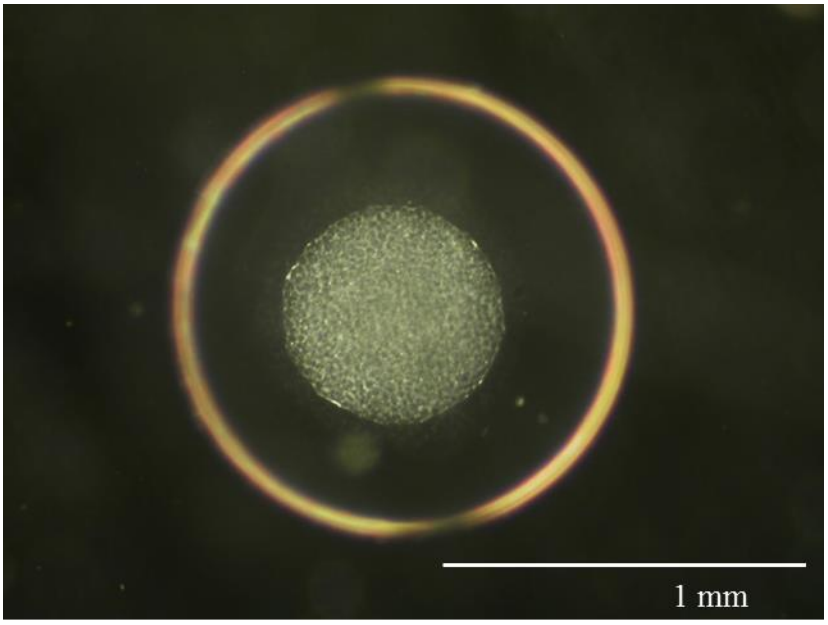

Figure 6.3. Image of Atlantic cod egg at collection. Stage of development: "blastodisc". Approximate equivalence of 17-20 hours post fertilisation at 8°C (fertilisation time: 22:00 and 2:00 hrs). Scale represents 1 mm

6.4. Discussion

Results show that the lighting system used during the spawning season does not have a significant impact on egg production (total egg volume and spawning window) and quality (fertilisation rate, floating fraction and viability percentage). Mean daily egg production was different at weeks 1, 2, 3 and 5, showing that higher volumes of eggs were released earlier in the spawning season under the on/off treatment compared to the dawn/dusk simulation treatment. However the difference in egg volumes collected between tanks are between 200 and 400 grams. In practical terms it means that the difference between tanks was created by one or two extra females spawning (Cohen *et al*., 1990) and does not necessarily show a relevant difference between treatments.

Similarities between treatments suggest that the use of a simulated dawn/dusk system does not impact Atlantic cod broodstock in terms of egg production and fertilisation success. However reproduction is a long lasting process that starts months

before the release of the mature gametes in the tank (Norberg *et al*., 2004; Migaud *et al*., 2013). Prior to this experiment all fish shared a tank equipped with a dawn/dusk light simulator under ambient photoperiod. Egg quality might have been dictated months before the fish were split into two tanks. Furthermore, egg quality does not solely compromise fertilisation success but rather has an impact during further development. Embryonic and larval development was not monitored during this trial and we cannot determine whether the lighting system had an impact on larval quality parameters (i.e. hatching success, survival, yolk sac absorption, deformity incidence). For that reason we cannot completely discard a possible beneficial effect of dimmed light on fish welfare.

It is evident that gadoids reacted negatively to the sudden presence of light. At the facilities of the marine laboratory the clearest example was the flight behavioural reaction of cod and haddock to the presence of any light during the night. Fish would desperately swim immediately after the light appearance, hitting the tank walls and jumping out. Light can trigger a physiological stress reaction, for instance constant high light intensity works as a chronic stressor in Atlantic cod (Vera and Migaud, 2009). Our results did not show any signs that could suggest a chronic physiological stress reaction caused by the lighting treatments in the short term. However weekly mean egg production showed that the on/off treatment released higher volumes of eggs during the first 3 weeks compared to the dawn/dusk, which could be related to acute stress as seen in the noise experiments (Chapter 3). If the latter is true, then the stress reaction would have been acute and the ovaries would have naturally prevented an excess cortisol accumulation in the oocytes (Schreck *et al*., 2001).

Based on the embryonic stage of development there was also no difference on the spawning timing. In all cases embryos were at the same level of development

(predominately blastodiscs as in Fig. 5.3), showing that fertilisation occurred almost in parallel in both tanks between 22:00 and 2:00 hrs. Similar results were found in Senegalense sole where 81% of the eggs were released between 21:00 and 1:00 hrs (under ambient photoperiod) with a collection peak between 21:00 and 22:00 hrs (Oliveira *et al.*, 2009a). Sole daily spawning rhythms (egg release, oestradiol and testosterone) appear to be consistently synchronised with the sunset and lunar cycle, regardless of the photoperiod/season (Oliveira *et al.*, 2009 a and b, 2010). However sole exposed to continuous light appear to maintain an endogenous spawning rhythm, suggesting that twilight perception might act as a diel zeitgeber.

Effective fertilisation in cod occurs after a successful courtship that involves swim/chase and vocalisations sequence (Engen and Folstad, 1999). "Grunts" occur more frequently during the dark period in which fertilisation happens (Rowe and Hutchings, 2006), specifically soon after sunset (Fudge and Rose, 2009; Chapter 3). A sudden change in light (on/off) could affect the reproductive behaviour of cod. Morgan *et al*. (1999) showed that stressed cod broodstock (capture/confinement stress) engaged in fewer or altered sequence of courtships, however there was no significant difference in egg quality parameters.

The present study showed that lighting system (timer or dimmer) does not significantly affect broodstock performance during the spawning window. However further research is needed to investigate if the dawn/dusk lighting simulation systems have a positive impact during vitellogenesis.

Chapter 7

EFFECTS OF SUBSTRATE REFUGES ON TURBOT (*SCOPHTHALMUS MAXIMUS*) SPAWNING PERFORMANCE

7.1. Introduction

Turbot (*Scophthalamus maximus)* is an omnivorous demersal flatfish which is widely distributed from the North Sea to the Mediterranean Sea. turbot farming became popular in the late 1970's in Europe; however in 2003 it was introduced in China which nowadays represents over 85% of the global aquaculture production (FAO est., 2013). Despite the apparent aquaculture success of turbot, the industry still faces many limitations. As in many marine fish, poor larval survival creates production bottlenecks. Survival varies widely (0 to 40 %) between farms and countries likely linked to poor egg quality (Person-Le Ruyete *et al*., 2002) caused by poor nutrition, environmental conditions and furthermore artificial gamete collection.

The ecological conditions in enclosed aquaculture systems differ widely from the wild. Turbot broodstock spawn in the wild on rocky or sandy soils at depths from 10 to 40 m (Støttrup and Sparrevohn, 2010), far deeper than the standard broodstock tanks used in farming facilities. The sea bed at those depths maintains relatively constant environmental conditions (temperature, salinity, light) that are difficult to recreate. For instance, sudden changes in temperature are more evident in shallow tanks than at 40 m deep, especially in small outdoor tanks like the ones used for this study. Maturation and reproduction are successfully entrained under controlled light regimes and temperature (Imsland *et al*., 1997; Imsland and Jonnasen, 2003), however spontaneous spawning does not normally occur in captivity.

Turbot farming requires hand stripping of the gametes every 2-3 days, a time consuming procedure that affects egg quality (McEvoy, 1984). Hand stripping requires applying pressure directly on the gonads creating a stressful situation and does not differentiate fertile mature eggs from ripened eggs that have been retained within the fish too long after ovulation (*circa*. 10 hrs in turbot) (McEvoy, 1984), resulting in high

variability in fertilisation and hatching rates (Jones, 1974; Downing, 1980). Additionally, excessive pressure can cause damage to the gonads (both, ovaries or testis) and skin of the broodfish, which in extreme cases results in bleeding from the duct or even mortality (*Pers. Observ.*). Natural spawning would therefore result in higher gamete quality through synchronised egg batches collection, reduced stress and damage to the broodfish and requiring less manpower.

Natural spawning has been observed in turbot in ordinary tanks, though with half the production of eggs as compared with stripping (Devauchelle *et al*., 1988). A better simulation of the ecological niche in enclosed systems might enhance broodstock performance, allowing the "natural instinct" to be realised. Most flatfish species held in 'bare' tanks (i.e. without sand) form dense clusters, perhaps using fellow co-habitants as a sand "substitute". The resulting congregation of males and females may interfere with the normal progress of mating behaviour, which in some species is initiated by close 'inspection' of individual females by males, presumably to identify those in which ovulation and subsequent spawning is imminent (Gibson, 2008). The situation in communal populations is further complicated by evidence of the establishment of hierarchies, though it is not known whether these are mediated by physical (e.g. size) or physiological (e.g. pheromones) factors (Stacey and Sorensen, 2005; Fleming and Huntingford, 2012).

Most flatfish species present a preference for small particle substrates which appears to be species specific and not correlated to the size of the fish (Moles and Norcross, 1995). In the wild, higher catches of flatfish are reported in the sea areas containing predominantly sandy bottoms (Amezcua and Nash, 2001). The sediment selection is correlated to food availability (Neuman and Able, 1998) and the ability of the flatfish to bury itself (Stoner and Ottmar, 2003). Burying is used as a predator

avoidance tactic and as means of energy conservation by reducing the metabolic rate and sheltering from strong currents (Gibson and Robb, 2000).

"Substrate refuges" (filled with sand and river gravel) were introduced for the first time in wild captured halibut broodstock (holding tank: 20 m diameter, 3 m depth) in the 90`s with the purpose of providing each female with a personalized site from where female fish could be subject to undisturbed courtship from numerous males (sex ratio of 1:2.5 female:male). High quality fertilised eggs were collected from 1990 to 1993 (3 years after capture) (Vikinstad, *Pers. Comm.*). Results in turbot would be expected to be similar. The aim of this study was to test if the presence of nests could lead to spontaneous spawning in a standard aquaculture broodstock tank.

7.2. Materials and methods

The experiments were carried out at the facilities of Viking Fish Farms Ltd., Ardtoe Marine Laboratory, Acharacle, Scotland (N 56°46' W 05°53') and conducted in accordance with the Animals Scientific Procedures Act of 1986, UK following independent ethical review.

Turbot broodstock (total length 49.3 ± 1.5 cm) were maintained in an outdoor circular tank (5.3 m diameter, depth 1.9 m, 42 m^3) at ambient temperature (spawning season 13.6 ± 0.6 °C: range 12.3 to 15.1 °C) and salinity (34.5 ± 0.6 ppt: range 32 – 35 ppt) under natural ambient light conditions. On 8th April 2010 fish from both tanks were measured and the sex identified using portable ultrasound equipment (7.5 MHz, Dynamic Imaging, UK). Three females (length 58.7 ± 4.7 cm) and 6 males (length 51.0 ± 5.0 cm) were moved into an experimental tank (3.6 m diameter, depth 2 m, 20 m^3). During the 2010 spawning season a wooden box (2 m length x 1 m wide x 20 cm height) filled with clean sand was submerged at the bottom of the tank. During the 2011

spawning season the nest box was replaced by 3 plastic trays (1.5 m length x 1 m wide x 20 cm height) attached to iron pipes to keep them static at the bottom of the tank and filled with clean sand. Another tank (5.3 m diameter, depth 1.5 m) was used as control containing the rest of the broodfish: 14 females (length 52.1 ± 7.7 cm) and 15 males (length 44.2 ± 10.7 cm). Fish were fed three times a week using commercial dry feed (EFICO Sigma 570, Biomar Spain).

From 26th June to 18th August 2011 turbot eggs were collected daily from an external overflow egg collector at 15:00 hrs each day and egg quality parameters monitored. Additionally all fish were stripped every third day by gentle abdominal massage according to hatchery procedure. Eggs from several females (3-5) were stripped and pooled into a clean plastic 2 L jug. Milt from at least two males was collected into individual sterile 2.5 mL syringes. Artificial fertilisation was done by gently mixing milt and eggs and adding filtered sea water (5 µm). After 5 mins, fertilised eggs were transferred into a 10 L plastic container and 8 L of filtered seawater were added and eggs were left in darkness undisturbed for 30 min.

Total egg volume was measured volumetrically recording both floating and sinking fractions separately. Eggs were first incubated in darkness at ambient temperature (12-14 °C) at a density of 6 g of eggs L^{-1} and provided with continuous water flow at 400 mL min^{-1}. Water flow and aeration was turned off daily for 30 minutes to allow dead eggs to sink to the bottom and be gently siphoned out. Embryos development was assessed daily by visual inspection under the microscope of a small egg sample taken from the floating fraction. Hatching rates were estimated at 100° days as the portion of hatched larvae from initial egg volume stocked.

. Fertilisation rate was estimated 2 hours after fertilisation from the remaining floating fraction from overall egg volume collected by stripping. Viability percentages

was calculated from floating fraction * fertilisation rate. Hatching rates were estimated as the percentage of successfully hatched larvae at 100° days from the initial egg stocking volume (Remaining egg volume in incubation tank after 100°D / Initial floating fraction for incubation).

Statistical analysis was performed using Minitab 16 (Minitab Ltd., UK). All datasets were first tested for normality and homogeneity using the Anderson-Darling test. Fertilisation rates, floating fraction, egg viability % and hatching rates were arcsine square root transformed and variance tested by One-Way ANOVA. Significance level was set at $p < 0.05$ and significant interactions evaluated by Tukey *post hoc* test.

7.3. Results

Results show no significant difference in the duration of the spawning period between tanks (Fig. 7.1). Differences in tank size and stocking density prevented a direct comparison in total egg volume production. Relative egg volume (Fig. 7.2) (total egg volume / number of females) was higher in the nest tank. No freely released fertilised eggs were collected during any of the two spawning seasons analysed.

Egg quality parameters (Table 7.1.) during the experiment showed no significant differences between tanks in terms of the floating fraction as percentage. Mean fertilisation rate and viability percentages were significantly different between treatments. Hatching rate showed no significant differences between treatments.

During the spawning season of 2010 the only nest was occupied most of the days. One specific female had a preference to occupy the single nest. On 3 separate occasions the nest was occupied by two females at the same time. On 4 occasions one or two males occupied the nest; however there is no record of both sexes nesting at the same time. During 2011, when the tank was supplied with 3 nests, most of the monitored

times at least 2 nests were used by different females. On some occasions observations showed males to be completely buried in the sand box; this situation became more frequent towards the end of the season. Once, a female and a male occupied the nest together for a brief period of time.

Table 7.1. Mean egg quality parameters over the spawning season ± S.E.M.. Total volume is the sum of the daily egg collection: Total egg volume (net collection + strip), Net collection and strip collection. [Relative per female] (Total volume / 13 (Control) and 3 (Nest). Mean floating portion over the spawning season. Mean fertilisation rates (artificial dry fertilisation). Mean viability percentage (floating fraction * fertilisation rate). * indicates significant differences (One-Way ANOVA. $p < 0.05$)

	Control	**Nest**
Egg volume [relative per female]		
Total (Kg)	21.2 [1.6]	7.9 [2.6]
From net (Kg)	8.4 [0.6]	3.7 [1.2]
From stripping (Kg)	12.8 [0.9]	4.2 [1.4]
Egg quality parameters		
Floating fraction (%)	24.3 ± 2.8	31.3 ± 3.9
Fertilisation rate (%) *	44.5 ± 3.8	63.6 ± 3.8
Viability (%) *	21.4 ± 3.3	41.4 ± 4.3
Hatching rate (%)	32.2 ± 5.3	35.7 ± 7.8

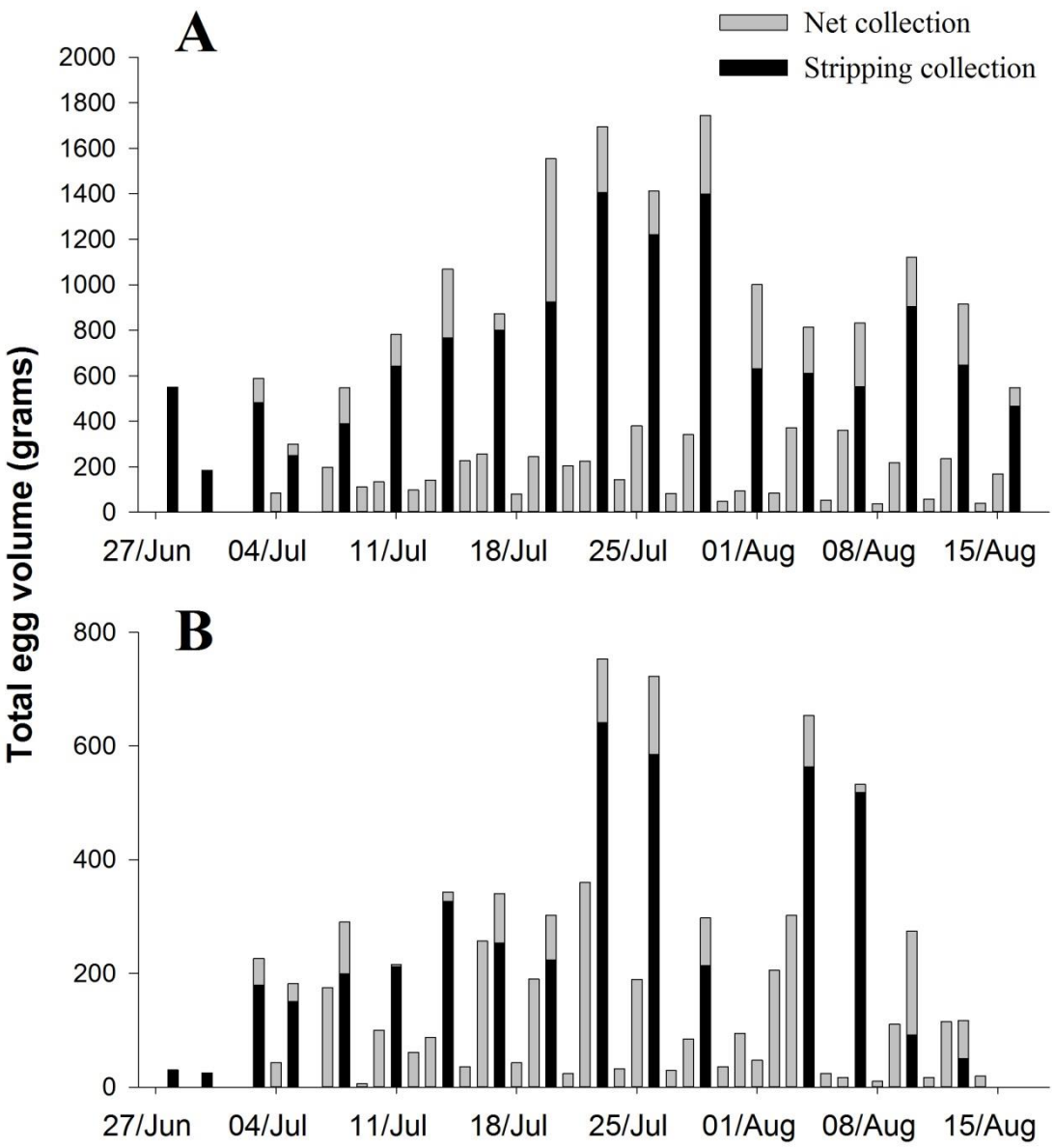

Figure 7.1. Daily volume of eggs collected for each tank (control (A) and nest (B) tanks) during the spawning season (June-August 2011). Total volume includes floating and sunken portions of eggs collected from the overflow egg collector and by abdominal massage.

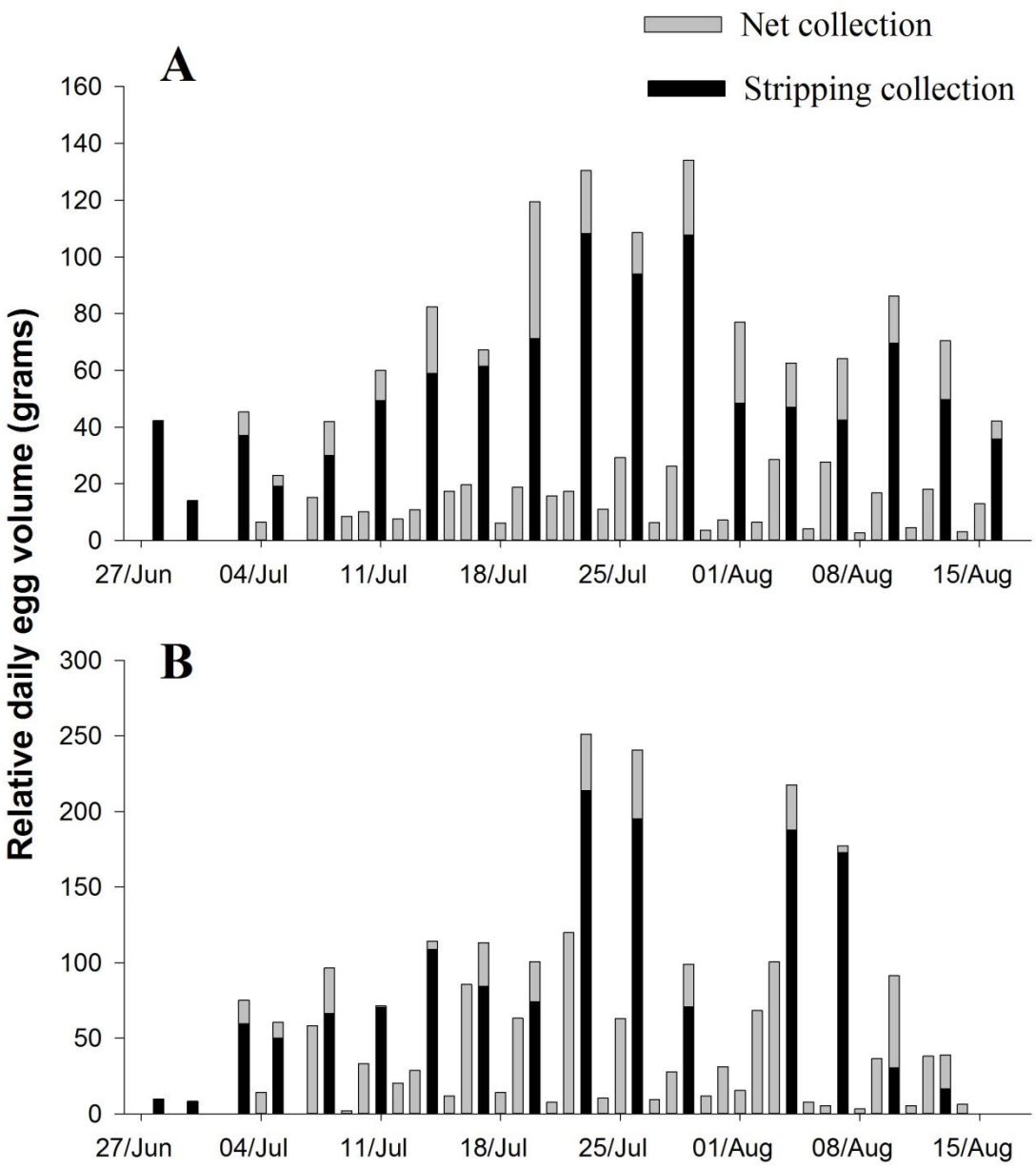

Figure 7.2. Relative daily volume of eggs collected for each tank (control (A) (Total volume / 13 females) and nest (B) (Total volume / 3 females) tanks) during the spawning season (June-August 2011). Relative total volume includes floating and sink portions of eggs collected from the overflow egg collector and by abdominal massage.

7.4. Discussion

Results of the present study do not show a clear effect of breeding nests in turbot broodstock performance. Egg batches appeared to be naturally released in the holding tank, however no fertilised eggs were collected during the trial suggesting that reproductive ecology and/or behaviour remains restricted.

The constant occupancy of the nest suggests that turbot broodstock actively looked for the nest. It is not possible to determine if the attractiveness of the nest was caused by the box itself or the substrate within and whether it impacted on reproductive activity. The sand allowed turbot to bury themselves presumably following their natural instinct as means of energy conservation or camouflage (Burrows and Gibson, 1995).

Differences in total egg volume between tanks are evident as the control tank contained ten more females than the nest tank. Relative egg production appeared to show differences between tanks. However differences might be explained by individual reproductive status of the females (size, age, gonadal development state). Turbot females have a relative fecundity of 1×10^6 eggs per Kg weight (Jones *et al.*, 1974) corresponding to 100,000 released eggs per spawn (Bromley *et al.*,, 1986). Roughly this means that female turbot release around 100 g of eggs per batch on average, which suggests that females were actively releasing eggs into the water column throughout the experiment, regardless of the presence of a breeding nest. Nevertheless, no fertilised eggs were collected during any of the monitored spawning seasons (2010 and 2011) in any tank.

The incapability of turbot to naturally reproduce in these captive conditions might be caused by the holding tanks characteristics, which probably interfere with the natural reproductive behaviour. Wild turbot spawn at depths between 10 to 40 metres with no apparent adult congregation (Støttrup and Sparrevohn, 2010). Flatfish's courtship, as in

many teleosts, follows specific swimming sequences that eventually would lead to a synchronised spawn. Turbot females swim to the surface pursued by one single male. Female then swims in circles followed or in unison with the male, accompanied by synchronous undulations of the musculature. At the end of the sequence the female returns to the bottom and the male settles near or over the female. The swimming sequence does not always involved gamete release (Bromley *et al.*, 1986; Gibson, 2008).

Bromley *et al*. (1986) reported successful natural turbot reproduction in enclosed systems. However several differences between our experimental tanks (and common hatchery procedures) exist. First, the tank size and volume are different. turbot spontaneously reproduced in a 40 m^3 tank (5.1 m diameter, 2.7 m depth), twice as big as the tank supplied with the nests. Space limitation could interfere with the courtship sequence, preventing the natural spawn. Interestingly the study from Bromley *et al*. (1986) reported that the swimming sequence was performed at variable depths. Furthermore despite collecting fertilised eggs, the authors never registered a gamete release following the swimming sequence. Furthermore stocking density was different. They stocked 8 fish (4:4 female:male) while our control consisted of 29 fish (14:15 female:males). High stocking density could impede the courtship behaviour. Moreover it is possible that, apart from the reduced space, the nest tank sex ratio (1:2 female:male) interfered with the mate selection.

The apparent required solitude of turbot couples to successfully spawn observed in wild populations of flatfish (Félix *et al*., 2011) contrasts strongly with the juvenile behaviour in captivity, where low densities have a negative effect in turbot performance. This highlights that the ecological/behavioural requirements of turbot vary widely and are poorly understood (Able and Grothues, 2007; Florin and Höglund,

2007, van der Hammen *et al*., 2013). For instance, turbot juveniles can be found at variable depths (Sparrevohn and Støttrup, 2008) and adults perform a diel migration to depths below 4 m during the dark phase (Støttrup *et al*., 2002), both presumably to feed. Furthermore, daily activity rhythms are regulated by light intensity and are accompanied by movements between sediment types, depending on food foraging activity (Burrows and Gibson, 1995).

Apart from the ecological limitations in captivity, other environmental signals remain crucial in turbot reproduction (Migaud *et al*., 2013). turbot, as other benthic fish, appear to be more susceptible to changes in temperature and lighting regimes. Environmental variations (temperature, salinity, light) are less pronounced at the sea bottom than in shallow enclosed systems (Volckaert, 2012). Turbot gamete supply can be extended all year long by exposing fish to artificial photoperiods (Forés *et al*., 1990, Imsland *et al*., 1997), however artificial photoperiods can impact negatively on the broodstock performance if temperature is not rigorously controlled.. A study from Devauchelle *et al*. (1988) confirmed that spontaneous spawning can be achieved without hormonal implants, nevertheless stocking density was very low (1 Kg m^{-3}), sex ratio (0.7 male:female), lower egg volumes (50% compared to stripping) and fertilisation rates were low (< 20%). When exposed to identical conditions but under an artificial photoperiod (advanced or delayed), turbot did not spontaneously spawn, and required hand stripping. Authors concluded that the despite the effective photo control of reproduction; turbot spawning is more susceptible to temperature changes than in other species (Smith *et al*., 1999; Weltzien *et al*., 1999; Herrera *et al*., 2011).

Inducing natural spawning in captivity could have many positive implications for turbot farming. First, it would reduce the labour required for the gamete collection and cost associated with it. For example at the marine laboratory, stripping required that the

holding tanks were drained at least 2 hours prior the strip, followed by 2-3 hours of fish selection and gamete collection; and lastly, the fertilisation and incubation process. Second, the ovulatory rhythms monitoring is time consuming and impractical in the given holding facilities. This results in a mixed egg quality. By applying pressure on the ovaries, eggs are collected regardless of their developmental stage (mature or over ripped). Natural spawning would release the synchronised gametes following the optimal window post ovulation, increasing egg quality and likely fertilisation success.

The presence of a nest in the holding tank did not trigger the required spawning behaviour. The occupancy of the breeding nest suggests that turbot broodstock might benefit by the presence of a substrate in the holding tanks. Further research is needed to investigate the ecological (e.g. tank depth, size, stocking density, sex ratio) and environmental (e.g. twilight perception, lunar cycles) cues that affect behaviour and physiology preventing natural spawning in turbot.

Chapter 8
General discussion

The general aim of this research project was to investigate the effects of different environmental signals/stressors (sound, light and substrate) on marine finfish performance throughout their life cycle (from egg to broodstock). The work performed is both fundamental to better understand how marine fish respond to environmental conditions (stress response, spawning performances, larvae development, juvenile growth and pigmentation) and applied to refine commercial protocols and overall improve fish welfare and performances. The two species selected (Atlantic cod and turbot) are commercially important and represent the two main types of marine fish cultured in temperate conditions (round and flatfish). The results have therefore strong commercial relevance to help the industry tackle some of the limiting factors that restrict the industry aspiration for expansion in Europe (e.g. reliable supply of high numbers of good quality eggs and optimal larvae/juvenile growth, survival and quality (pigmentation of flatfish).

The global challenge addressed by this doctoral work is the difficulty to recreate species specific ambient natural conditions in enclosed facilities that are optimal for spawning and growth performances of marine fish species. It must be acknowledged that within the time frame of a doctoral project, only few targeted environmental factors (e.g. sound, light and substrate) contributing to the suboptimal performance of marine fish stock in captivity could be studied.

Sound perception and stress

Studies on the effect of anthropogenic noise in fish are scarce and have been focused mainly on behavioural and welfare consequences in wild stocks. In the aquaculture context, the biological relevance of fish acoustics has been widely overlooked and started gaining attention recently. Results from Chapter 3 showed that

noise can negatively impact on fish performance kept in captivity by triggering a physiological stress reaction.

Noise pollution is likely acting as a stressor in other species as well. Hearing bandwidths and thresholds vary widely between species, although sensitivity to the lower frequencies appears to be to some extent conserved (Nedwell *et al.*, 2004). Differences in hearing sensitivity between fish species may result in different thresholds above which a physiological stress is triggered. Auditory detection involves two components: sound pressure (dB re 1 μPa) and particle motion (dB re 1 m s^{-1}). Particle motion is detected by the inner ear and neuromasts of the lateral line in the "near field", while pressure appears to be perceived only by fish with a swim bladder. The swim bladder converts the sound pressure into a displacement stimulus, enhancing hearing sensitivity. The contribution of sound pressure and particle motion in auditory detection is difficult to separate and up to date there is no technology available that can measure both components simultaneously (Fay and Popper, 2012). The present study did not test whether cod responded to sound pressure or particle motion, still our results remain valid as particle motion sensitivity appears to be relatively constant between teleosts, while sound pressure detection varies depending on the specialised hearing structures (Radford *et al.*, 2012). Furthermore, fish audiograms have traditionally been measured under sound pressure responses (Nedwell *et al.*, 2004). In shallow tanks fish are exposed indiscriminately to both auditory components (pressure and particle motion) and regardless of which contributes the most, noise pollution is triggering a physiological stress reaction.

Noise pollution in aquaculture facilities can impact on broodstock performance and fertilisation success as shown in Chapter 3. The relationship between stress stimuli, plasma cortisol and reproductive dysfunction is complex, as females appear to have an

adaptation/protection mechanism to prevent over-accumulation of cortisol in the oocytes (Schreck *et al*., 2001) and the magnitude of the stress response varies widely (i.e. time of the reproductive cycle, reproductive strategy, nutritional state, genetic variation). In addition, the effects of cortisol concentrations on oocyte quality, embryo and larvae performance vary widely between species and the administration method. Atlantic salmon broodstock with a cortisol implant resulted in smaller embryos, increased mortality, reduced yolk sac volume and increased malformations (Eriksen *et al*., 2006). In the longer term, growth (length and body mass) was not different between the previously stressed and control stocks (Eriksen *et al.*, 2007), however salmon juveniles from stressed parents showed a more aggressive behaviour and swimming reaction to a stressor was shorter (Eriksen *et al.,* 2011). Kleppe *et al.* (2013) found no differences in fertilisation or hatching rates in Atlantic cod from cortisol implanted females. However egg/embryonic transcriptome was affected, mainly in genes related to cytogenesis, which could have implications in the later stages of development.

Some studies suggest that fish are capable of coping with stressors through an adaptation mechanism (Pickering and Pottinger, 1985, Waring *et al.*, 1997), defined as the absence of a physiological stress-related reaction to a stimuli. By definition a true acclimation could only occur under repeated exposures that the animal associate as non-life threatening (Wright *et al.*, 2007). However, most chronic stressors in aquaculture facilities would fall under this definition and yet trigger a physiological stress reaction. Physiological and behavioural adaptations are believed to be caused by the neural plasticity and cognition of the fish; therefore will vary widely between species, individuals, age and history (Sørensen *et al.*, 2013; Vindas *et al.*, 2014). This explains the differences commonly reported between captive and wild fish stress reactions (i.e. threat perception). Furthermore, progeny can inherit epigenetic tags providing

resistance to specific stimuli experienced by the parents (Ho and Burggren, 2010). For example, juvenile F1 sticklebacks (*Gasterosteus aculeatus*) formed a tighter schooling (predator risk strategy) when their parents had been subjected to a chronic predator simulation (Giesing *et al.*, 2011). On the other hand rats injected with dexamethasone prenatally or during pregnancy, resulted in progeny with lower weight at birth and an altered glucose metabolism (associated with cardiovascular diseases) that persisted for 3 generations (Drake *et al.,* 2005). This implies that noise pollution might have an intergenerational effect in addition to the negative effects seen in Chapter 3. Further research is needed to elucidate whether progeny are inheriting a noise resistance mechanism that would minimise the adverse effects of anthropogenic noise and/or the negative effects of high cortisol concentrations.

Broodstock environmental management

Similarly other environmental signals influence broodstock performance and progeny quality. The aquaculture industry makes every effort to optimise the holding facilities and recreate key environmental signals involved in the regulation of reproduction. Atlantic cod spawn freely in the holding tanks and all-year long seed supply can be assured through artificial photoperiod and temperature control. Despite this, the egg quality and subsequent performance from captive broodstock is lower than in wild stocks (Lanes *et al.*, 2012). Nutrition plays a key role in egg quality, though cod nutritional requirements have been studied for several years to optimise the artificial diets used in captivity. Thus it is possible that other overlooked environmental conditions (e.g. light spectral composition, twilight, lunar cycles, substrate, space: size and depth) are impacting fish behaviour and physiology that could explain differences in offspring quality between farmed and wild stocks.

The application of dimmed light (Chapter 6) simulating twilight during the sunrise and sunset during the actual spawning period or inclusion of a substrate refuge in the holding tank (Chapter 7) did not show any significant differences in terms of broodstock performance. There are, however, three considerations to take into account. First, the broodstock trials were not replicated due to limited fish stocks and broodstock tanks availability. Second, only a few parameters could be monitored to avoid disturbing the stocks (e.g. by blood sampling). Third, in all cases the environmental factor investigated (dimmed light, breeding nest) was applied during or soon before the spawning season.

The inclusion of twilight prior to ovulation might have a clearer impact on broodstock performance, synchronising the ovulatory rhythms similarly to the lunar cycles (Oliveira *et al.* 2009 a, b). In the case of the breeding nest, it is possible that flatfish courtship requires building a nest prior ovulation or the prescence of a refuge. Nesting behaviour might be imprinted through observation in the wild and would be lost in hatchery reared fish. Successful fertilisation in the tanks would improve turbot seed quality ensuring the synchronization of the egg release and ovulatory rhythm and reducing the man power required by stripping the broodfish. Further studies are required to better understand the environmental requirement and conditioning factors required for wild and farmed marine fish to perform optimally.

Light spectrum and fish performance

The results of the present study consistently showed that exposure to short wavelengths have a positive impact on marine larvae performance (Chapter 4) and juveniles (Chapter 5), while the opposite is true for longer wavelengths of light. This confirms that species are predisposed to perform better under similar photic

environments to those encountered in their ecological niche (Ruchin, 2004; Villamizar *et al.*, 2011).

It is widely recognised that light plays a crucial role during the early stages of development as dicussed in previous chapters. All three components of light (photoperiod, intensity and spectrum) influence larval growth, development, survival and pigmentation. The effects vary between species and most likely during developmental stages. Within those variations, it appears that extended photoperiods enhance larval growth and survival, although it is unclear whether long days enhance food intake or food conversion efficiency. In terms of light quantity (intensity, irradiance) the optimal thresholds vary between species, but most fish larvae appear to perform better under high or intermediate intensities (within their specific thresholds). Intensities below or above the optimal thresholds commonly result in negative effects on survival, deformities and malpigmentation. Moreover high light intensities can induce temporal retinal damage in juveniles although further studies are required to determine species specific thresholds of light intensity exposure (Vera and Migaud, 2009).

Less is known about the effects of light spectrum in larval performance, our results suggest that growth is enhanced by exposing them to the wavelengths naturally encountered in their ecological niche. However, in apparent contradiction, survival appears to be negatively influenced by the short wavelengths. Within the context of this thesis (and survival variability between replicates), it appears that short wavelengths would produce fewer stronger fish, while the long wavelengths would results in many smaller fish. Here is an interesting dilemma. In marine hatcheries the main target is to produce as many weaned fish as possible and growth can be enhanced during the on-growing phase. Such an analogy between experimental results and commercial scale

needs to be carefully considered. Commercial large scale larval rearing settings differ from experimental small scale conditions, despite a comparable stocking density and food availability, the size of the tanks and lighting setting are usually different. Those differences result in a different photic environment (e.g. light intensity is uneven), which might impact fish differently.

Experimental settings differ also between larval studies which make any comparison between results of different studies difficult. There is no standardisation in light intensity units used in the scientific literature and photometric units (lumen or lux) are still commonly used despite being a biased light intensity measurement unit for aquaculture studies. Lux reflects intensity in terms of human perception, overlooking the spectral composition of the light unit used. This is relevant not only when coloured lights are used, but also because the commercially available bulbs do not emit a true solar light spectrum, but rather have peaks at different wavelengths which might impact fish performance. For example, a white incandescent bulb is dominated by the red spectrum and very reduced at the violet/blue end of the spectrum. Those factors need to be accounted for when selecting the light equipment commercially and experimentally.

The exact mechanism behind the effect of light on larval performance remains unclear. Marine larvae morphology changes drastically through metamorphosis and most likely the light requirements vary based on the stage of development. For instance, the retina develops during the early stages, which might suggest that optimal lighting conditions might vary within a short period of time. Further research is needed to clarify the basic underlying effects of light in larvae from visual perception to its physiological effects at the different stages of the photoreceptors (retinal and non-retinal) development. Furthermore, light intensity and spectrum might be perceived as a single stimulus because in nature fish are exposed simultaneously to diel variations in

light intensity and spectral composition (i.e. twilight). Recently Pauers *et al.* (2012) reported that light colour, rather than intensity, acts as the primary synchroniser of the circadian rhythms in two cichlid species. Investigating how intensity and spectral composition interact together and impact fish larvae growth and overall performance would help refining larviculture lighting conditions. .

Skin colouration

Differences registered in food intake might have contributed to the skin pigmentation variations observed between light treatments. Fish exposed to red light had a lower food intake, lower weight gain, reduced total length and a darker skin colouration. The opposite was true for the blue light, showing a strong correlation between the light wavelength and the brightness/darkness of the skin. Darker skin is commonly a morphological indicator of a physiological stress reaction (i.e. cortisol, α-MSH and ACTH) in flatfish (Ruane *et al.*, 2005). The red light might act as a stress stimuli due to the poor visibility (Owen *et al.* 2010) and food deprivation (Wunderink *et al*., 2012). On the other hand the blue light exposure resulted in a "light *crème*" skin colouration which corresponds to the preferences of the European consumer according to the Sea Food Guide (Seafish, UK). Differences in brightness/darkness were evident after 8 days of coloured light exposure. Farmers could improve the market value of the fish by exposing turbot to blue light one week before harvest. Furthermore, Ruff *et al.* (2002) found that turbot skin and flesh have a long shelf life after slaughter in terms of coloration and texture preservation. Nevertheless, the intrinsic limitations of the study prevented an analysis of the underlying mechanism behind, which could have contributed to explain the nature of the pigmentation changes. An analysis of the pigment cells variation (dispersion and type) together with an analysis of the expression

of the pigment related hormones receptors (e.g. MCH or α-MSH), would have contributed to better understand if the colouration was driven directly by the light, or through nutrition or as a stress response.

Conclusions

The present research project endorses the importance of environmental management in enclosed aquaculture facilities. It proves for the first time to our knowledge that noise levels generated by anthropogenic activities can act as an acute stressor in farmed Atlantic cod and impairs broodstock performance. The implementation of noise mitigating measures might contribute to enhance fish performance in captivity and reduce the variability commonly seen between tanks in most fish rearing facilities (experimental and commercial). This doctoral work also provides supporting evidence of the potential detrimental effects that anthropogenic activities might have on wild stocks. This project also provides an insight on the potential commercial application of coloured lights during the larval and on-growing stages to enhance productivity in marine farms. Present findings provide a preliminary insight on the effects of light spectrum on appetite and pigmentation in flatfish, pointing towards the need for further research into the effects of light as a whole on fish physiology, an environmental cue critical to most living organisms but largely overlooked in aquaculture.

References

Abbott, C. R., Rossi, M., Wren, A. M., Murphy, K. G., Kennedy, A. R., Stanley, S. A., Zollner, A.N., Morgan, D.G.A., Morgan, I., Ghatei, M.A., Small, C.J. & Bloom, S. R. (2001). Evidence of an orexigenic role for cocaine-and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. Endocrinology, 142(8), 3457-3463.

Able, K. W., & Grothues, T. M. (2007). An approach to understanding habitat dynamics of flatfishes: Advantages of biotelemetry. Journal of Sea Research, 58(1), 1-7.

Afonso, L.O.B., Hosoya, S., Osborne, J., Gamperl, A.K. & Johnson, S. (2008) Lack of glucose and hsp70 responses in haddock *Melanogrammus aeglefinus* (L.) subjected to handling and heat shock. Journal of Fish Biology 72, 157-167.

Aguilar de Soto, N. Delorme, N., Atkins, J., Howard, S., Williams, J., & Johnson, M. (2013). Anthropogenic noise causes body malformations and delays development in marine larvae. Scientific Reports, 3.

Akamatsu, T., Nanami, A., & Yan, H. Y. (2003). Spotlined sardine *Sardinops melanostictus* listens to 1-kHz sound by using its gas bladder. Fisheries Science, 69(2), 348-354.

Al-Maghazachi, S. J., & Gibson, R. (1984). The developmental stages of larval turbot, *Scophthalmus maximus* (L.). Journal of experimental marine biology and ecology, 82(1), 35-51.

Alvarado, M. V., Carrillo, M., & Felip, A. (2013). Expression of Kisspeptins and their Receptors, gnrh-1 gnrhr-II-1a and Gonadotropin Genes in the Brain of Adult Male and Female European Sea Bass During Different Gonadal Stages. General and comparative endocrinology. 187. 104-116

Amezcua, F., & Nash, R. D. M. (2001). Distribution of the order Pleuronectiformes in relation to the sediment type in the North Irish Sea.Journal of Sea Research, 45(3), 293-301.

Amiya, N., Amano, M., Iigo, M., Yamanome, T., Takahashi, A., & Yamamori, K. (2008). Interaction of orexin/hypocretin-like immunoreactive neurons with melanin-concentrating hormone and α-melanocyte-stimulating hormone neurons in brain of a pleuronectiform fish, barfin flounder. Fisheries Science, 74(5), 1040-1046.

Arends, R. J., Mancera, J. M., Munoz, J. L., Bonga, S. W., & Flik, G. (1999). The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. Journal of Endocrinology, 163(1), 149-157.

Arends, R. J., Rotllant, J., Mancera, J. M., Bonga, S. W., & Flik, G. (2000). alpha-MSH acetylation in the pituitary gland of the sea bream (*Sparus aurata* L.) in response to different backgrounds, confinement and air exposure. Journal of endocrinology, 166(2), 427-435.

Aristizabal, E., Suárez, J., Vega, A., & Bargas, R. (2008) Egg and larval quality assessment in the Argentinean red porgy (*Pagrus pagrus*). Aquaculture 287,329–334.

Astrup, J & B. Møhl. (1998). Discrimination between high and low repetition rates of ultrasonic pulses by the cod. Journal of Fish Biology. 52:205-208.

Aydin, I., & Sahin, T. (2011). Reproductive performance of turbot (*Psetta maxima*) in the southeastern Black Sea. Turkish Journal of Zoology, 35, 109-113.

Azpeleta, C., Martínez-Álvarez, R. M., Delgado, M. J., Isorna, E., & De Pedro, N. (2010). Melatonin reduces locomotor activity and circulating cortisol in goldfish. Hormones and behavior, 57(3), 323-329.

Baker, B. I. (1993). The Role of Melanin-Concentrating Hormone in Color Change. Annals of the New York Academy of Sciences, 680(1), 279-289.

Baldwin, A. L., & Bell, I. R. (2007). Effect of noise on microvascular integrity in laboratory rats. Journal of the American Association for Laboratory Animal Science, 46(1), 58-65.

Balm, P. H., & Gröneveld, D. (1998). The Melanin-Concentrating Hormone System in Fish. Annals of the New York Academy of Sciences, 839(1), 205-209.

Bart, A. N., Clark, J., Young, J., & Zohar, Y. (2001). Underwater ambient noise measurements in aquaculture systems: a survey. Aquacultural engineering 25, 99-110.

Barton, B. A., Schreck, C. B., & Fowler, L. G. (1988). Fasting and diet content affect stress-induced changes in plasma glucose and cortisol in juvenile chinook salmon. The Progressive Fish-Culturist, 50(1), 16-22

Barton, B. (2002) Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids Intgrated and Comparative Biology. 42, 517-525

Bayarri, M. J., Madrid, J. A., & Sánchez-Vázquez, F. J. (2002). Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. Journal of pineal research, 32(1), 34-40.

Beatty, D. D. (1975). Rhodopsin—Porphyropsin Changes in Paired-Pigment Fishes. In Vision in Fishes (pp. 635-644). Springer US.

Berg, E., & Albert, O. T. (2003). Cod in fjords and coastal waters of North Norway: distribution and variation in length and maturity at age. ICES Journal of Marine Science: Journal du Conseil, 60(4), 787-797.

Bernier, N. J., & Peter, R. E. (2001). Appetite-suppressing effects of urotensin I and corticotropin-releasing hormone in goldfish (*Carassius auratus*).Neuroendocrinology, 73(4), 248-260.

Bernier, N. J., Bedard, N., & Peter, R. E. (2004). Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. General and comparative endocrinology, 135(2), 230-240.

Bernier, N. J., Flik, G., & Klaren, P. H. (2009). Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes. Fish Physiology, 28, 235-311.

Biswas, A. K., Seoka, M., Inoue, Y., Takii, K., & Kumai, H. (2005). Photoperiod influences the growth, food intake, feed efficiency and digestibility of red sea bream (*Pagrus major*). Aquaculture, 250(3), 666-673.

Bjenning, C., Farrell, A. P., & Holmgren, S. (1991). Bombesin-like immunoreactivity in skates and the in vitro effect of bombesin on coronary vessels from the longnose skate, *Raja rhina*. Regulatory peptides, 35(3), 207-219.

Blanco-Vives, B., & Sanchez-Vazquez, F. J. (2009). Synchronisation to light and feeding time of circadian rhythms of spawning and locomotor activity in zebrafish. Physiology & behavior, 98(3), 268-275.

Blanco-Vives, B., Villamizar, N., Ramos, J., Bayarri, M.J., Chereguini, O., & Sánchez-Vázquez, F.J., (2010). Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture 306, 137–145.

Blasco, J., Fernandez-Borras, J., Marimon, I., & Requena, A. (1996). Plasma glucose kinetics and tissue uptake in brown trout in vivo: effect of an intravascular glucose load. Journal of Comparative Physiology B, 165(7), 534-541.

Blaxter, J. H. S., (1969). Visual thresholds and spectral sensitivity of flatfish larvae. Journal of Experimental Biology 51, 221-230.

Blaxter J.H.S. & Staines M., (1970). Pure-cone retinae and retinomotor responses in larval teleosts. Journal of Marine Biology Association U. K. 50, 449-460.

Bobe, J., & Labbé, C. (2010). Egg and sperm quality in fish. General and Comparative Endocrinology, 165(3), 535-548.

Boeuf, G., & Le Bail, P. (1999). Does light have an influence on fish growth?. Aquaculture 177, 129–152

Boeuf, G., & Falcón, J. (2001). Photoperiod and growth in fish. Vie et milieu,51(4), 247-266.

Bolker, J. A., & Hill, C. R. (2000). Pigmentation development in hatchery-reared flatfishes. Journal of Fish Biology, 56(5), 1029-1052.

Bolker, J. A., Hakala, T. F., & Quist, J. E. (2005). Pigmentation development, defects, and patterning in summer flounder (*Paralichthys dentatus*). Zoology, 108(3), 183-193.

Boujard, T., & Leatherland, J. F. (1992). Circadian rhythms and feeding time in fishes. Environmental Biology of Fishes, 35(2), 109-131.

Bowmaker, J. K. (1998). Evolution of colour vision in vertebrates. Eye, 12, 541-547.

Brander, K. M. (2005). Cod recruitment is strongly affected by climate when stock biomass is low. ICES Journal of Marine Science: Journal du Conseil, 62(3), 339-343.

Brawn, V. M. (1961). Reproductive behaviour of the cod (*Gadus callarias* L.). Behaviour, 177-198.

Britt, L., Loew, E., & McFarland, W. (2001). Visual pigments in the early life stages of Pacific Northwest marine fishes. The Journal of Experimental Biology 204, 2581-2587.

Bromage, N., Porter, M., & Randall, C. (2001). "The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin". Aquaculture, vol. 197, pp. 63-98.

Bromley, P. J., Sykes, P. A., & Howell, B. R. (1986). Egg production of turbot (*Scophthalmus maximus* L.) spawning in tank conditions. Aquaculture, 53(3), 287-293.

Browman, H. I., & Marcotte, B. M. (1987). Effects of prey color and background color on feeding by Atlantic salmon alevins. The Progressive Fish-Culturist, 49(2), 140-143.

Brown, C. L., & Kim, B. G. (1995). Combined application of cortisol and triiodothyronine in the culture of larval marine finfish. Aquaculture, 135(1), 79-86.

Brown, J., Minkoff, G., & Puvanendran, V. (2003). "Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols, and problems", Aquaculture, vol. 227, pp. 357-372.

Buerkle, U. (1967). An audiogram of the Atlantic cod, *Gadus morhua* L. Journal of the Fisheries Research. Board of Canada . 24, 2309-2319.

Buerkle, U. (1977). Detection of trawling noise by Atlantic cod (Gadus morhua L.). Marine & Freshwater Behaviour & Physiology, 4(4), 233-242.

Burel, C., Ruyet, P. L., Gaumet, F., Le Roux, A., Severe, A., & Boeuf, G. (1996). Effects of temperature on growth and metabolism in juvenile turbot. Journal of Fish Biology, 49(4), 678-692.

Burrows, M. T., & Gibson, R. N. (1995). The effects of food, predation risk and endogenous rhythmicity on the behaviour of juvenile plaice, *Pleuronectes platessa* L. Animal Behaviour, 50(1), 41-52.

Burton, D., & Vokey, J. E. (2000). The relative in vitro responsiveness of melanophores of winter flounder to α-MSH and MCH. Journal of Fish Biology, 56(5), 1192-1200.

Bury, N. R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Robinson-Rechavi, M., Laudet, V. Rafestin-Oblin, M.E. & Prunet, P. (2003). Evidence for two distinct functional glucocorticoid receptors in teleost fish. Journal of Molecular Endocrinology, 31(1), 141-156.

Buscaino, G., Filiciotto, F., Buffa, G., Bellante, A., Di Stefano, V., Assenza, A., Fazio, F., Caola, G. & Mazzola, S. (2010). Impact of an acoustic stimulus on the motility and blood parameters of European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). Marine environmental Research 69, 136-142.

Buwalda, R. J. A., & Van der Steen, J. (1979). The sensitivity of the cod sacculus to directional and non-directional sound stimuli. Comparative Biochemistry and Physiology Part A: Physiology 64, 467-471.

Buwalda, R. J. A., Schuijf, A., & Hawkins, A. D. (1983). Discrimination by the cod of sounds from opposing directions. Journal of comparative physiology 150, 175-184.

Cabrita E,; Robles V, & Herráez MP (2008). Sperm quality assessment. In: Cabrita E, Robles V, Herráez MP (*Eds*) Methods in Reproductive Aquaculture: Marine and Freshwater Species, pp. 93–147. CRC Press, Boca Raton.

Campbell, P. M., Pottinger, T. G., & Sumpter, J. P. (1992). Stress reduces the quality of gametes produced by rainbow trout. Biology of Reproduction 47, 1140-1150

Carragher, J. F., Sumpter, J. P., Pottinger, T. G., & Pickering, A. D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout*, Salmo trutta* L. and *Salmo gairdneri* Richardson. General and comparative endocrinology, 76(2), 310-321.

Cerdá-Reverter, J. M., Ringholm, A., Schiöth, H. B., & Peter, R. E. (2003). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. Endocrinology, 144(6), 2336-2349.

Chapman, C. J. (1973). Field studies of hearing in teleost fish. Helgoländer wissenschaftliche Meeresuntersuchungen, 24(1-4), 371-390.

Chapman, C. and A. Hawkins (1973). "A field study of hearing in the cod, *Gadus morhua* L." Journal of Comparative Pathology 85, 147-167.

Chapman, C. J., & Johnstone, A. D. F. (1974). Some auditory discrimination experiments on marine fish. Journal of Experimental Biology, 61(2), 521-528.

Chapman, C. J., Johnstone, A. D. F., Dunn, J. R., & Creasey, D. J. (1974). Reactions of fish to sound generated by divers' open-circuit underwater breathing apparatus. Marine Biology, 27(4), 357-366.

Chapman, C.J. & Sand, O. (1974) “Field studies of hearing in two species of flat fish” Comparative Biochemistry Physiology. 47A, 371-385

Chereguini, O., La Banda, D., García, I., Rasines, I., & Fernandez, A. (1999). Artificial fertilization in turbot, *Scophthalmus maximus* (L.): different methods and determination of the optimal sperm–egg ratio. Aquaculture Research, 30(5), 319-324.

Chrousos, G. P. (2009). Stress and disorders of the stress system. Nature Reviews Endocrinology, 5(7), 374-381.

Clark, C., & Stansfeld, S. A. (2007). The effect of transportation noise on health and cognitive development: A review of recent evidence. International Journal of Comparative Psychology, 20(2).

Clark, C. W., Ellison, W. T., Southall, B. L., Hatch, L., Van, S., Parijs, A. F., & Ponirakis, D. (2009). Acoustic masking in marine ecosystems as a function of anthropogenic sound sources. Report to the International Whaling Commission. SC-61 E, 10.

Clarke, G.L. (1965). Light. In: Elements of Ecology. Wiley, New York, pp. 185–242

Clarke, I. J., Smith, J. T., Caraty, A., Goodman, R. L., & Lehman, M. N. (2009). Kisspeptin and seasonality in sheep. Peptides, 30(1), 154-163.

Cobcroft, J.M., & Battaglene, S.C. (2009). Jaw malformation in striped trumpeter (*Latris lineta*) larvae linked to walling behavior and tank colour. Aquaculture 289, 274-282

Cohen, D.M., Inada, T., Iwamoto, T., & Scialabba, N., (1990), FAO species catalogue: Gadiform fishes of the world (Order *Gadiformes*). An annotated and illustrated catalogue of cods, hakes, grenadiers and other gadiform fishes known to date, Rome. No. 125, vol. 10, pp. 1-442.

Colombe, L., Fostier, A., Bury, N., Pakdel, F., & Guiguen, Y. (2000). A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. Steroids,65(6), 319-328.

Comperatore, C. A., & Stephan, F. K. (1987). Entrainment of duodenal activity to periodic feeding. Journal of biological rhythms, 2(3), 227-242.

Consten, D., Bogerd, J., Komen, J., Lambert, J. G. D., & Goos, H. T. (2001). Long-term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L. Biology of reproduction, 64(4), 1063-1071.

Cook, R. M., Sinclair, A., & Stefansson, G. (1997). Potential collapse of North Sea cod stocks. Nature, 385(6616), 521-522.

Coutant, C. C. (1977). Compilation of temperature preference data. Journal of the Fisheries Board of Canada, 34(5), 739-745.

Cowan, M., Davie, A., & Migaud, H. (2011). The effect of metal halide and novel green cathode lights on the stress response, innate immunity, eye structure and feeding activity of Atlantic cod, *Gadus morhua* L. Aquaculture Research 42, 115-124.

Cowan, M., Davie, A., & Migaud, H. (2012). Photoperiod effects on the expression of kisspeptin and gonadotropin genes in Atlantic cod, *Gadus morhua*, during first maturation. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 163(1), 82-94.

Cowing, J., Poopalasundaram, S., Wilkie, S., Robinson, P., Bowmaker, J., & Hunt, D. (2002). The molecular mechanism for the spectral shifts between vertebrate ultraviolet-and violet-sensitive cone visual pigments. Biochemistry. J, 367, 129-135.

Davidson, J., Frankel, A. S., Ellison, W. T., Summerfelt, S., Popper, A. N., Mazik, P., & Bebak, J. (2007). Minimizing noise in fiberglass aquaculture tanks: noise reduction potential of various retrofits. Aquacultural engineering, 37(2), 125-131.

Davie, A., Porter, M. J., Bromage, N. R., & Migaud, H. (2007a). The role of seasonally altering photoperiod in regulating physiology in Atlantic cod (*Gadus morhua*). Part I. Sexual maturation. Canadian Journal of Fisheries and Aquatic Sciences, 64(1), 84-97.

Davie, A., Porter, M. J., Bromage, N. R., & Migaud, H. (2007b). The role of seasonally altering photoperiod in regulating physiology in Atlantic cod (*Gadus morhua*). Part II. Somatic growth. Canadian Journal of Fisheries and Aquatic Sciences, 64(1), 98-112.

De Jesus, EG, Hirano, T & Inui, Y (1991). Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. General and Comparative Endocrinology 82, 369-276

De Pedro, N., Martínez-Álvarez, R. M., & Delgado, M. J. (2008). Melatonin reduces body weight in goldfish (*Carassius auratus*): effects on metabolic resources and some feeding regulators. Journal of pineal research, 45(1), 32-39.

Denson, M.R., & Smith, T.I.J., (1997). Diet and light intensity effects on survival, growth and pigmentation of southern flounder *Paralichthys lethostigma*. Journal of World Aquaculture Society. 28 (4)., 366–373.

Devauchelle, N., Alexandre, J. C., Le Corre, N., & Letty, Y. (1988). Spawning of turbot (*Scophthalmus maximus*) in captivity. Aquaculture, 69(1), 159-184.

Dhert, P., Lavens, P., Dehasque, M., & Sorgeloos, P. (1993). Improvements in the larviculture of turbot*, Scophthalmus maximus*: zootechnical and nutritional aspects, possibility for disease control. In turbot Culture: Problems and Prospects, Proceedings of the Satellite Workshop of World Aquaculture 93, 25-27.

Doolan, B. J., Booth, M. A., Allan, G. L., & Jones, P. L. (2009). Changes in skin colour and cortisol response of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801) to different background colours. Aquaculture research, 40(5), 542-550.

Dooling, R. J., & Popper, A. N. (2007). The effects of highway noise on birds. See http://www.dot.ca.gov/hq/env/bio/files/caltrans_birds_10-7-2007b.pdf.

Downing, N., (1980). Maintenance of turbot broodstock. The production of turbot eggs and larvae, 1979/1980. Field Report No. 893, White Fish Authority, Hunterston, Great Britain, 22 pp.

Dowling, J. E. (1987). The retina: an approachable part of the brain. Harvard University Press.

Downing, G., & Litvak, M.K.. (1999). The effect of photoperiod, tank colour and light intensity on growth of larval haddock. Aquaculture International 7, 369-382.

Doyon, C., Gilmour, K. M., Trudeau, V. L., & Moon, T. W. (2003). Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic

area of socially subordinate rainbow trout. General and comparative endocrinology, 133(2), 260-271.

Drake, A. J., Walker, B. R., & Seckl, J. R. (2005). Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 288(1), R34-R38.

Duray, M., Estudillo, C., & Alpasan, L., (1996). "The effect of background colour and rotifer density on rotifer intake, growth, and survival of the grouper (*Epinephelus suillus*) larvae", Aquaculture, vol. 146, pp. 217-224.

Ekström, P., & Meissl, H. (2003). Evolution of photosensory pineal organs in new light: the fate of neuroendocrine photoreceptors. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences,358(1438), 1679-1700.

Ekström, P., & Meissl, H. (1997). The pineal organ of teleost fishes. Reviews in Fish Biology and Fisheries, 7(2), 199-284.

Ellis, T., Yildiz, H. Y., López-Olmeda, J., Spedicato, M. T., Tort, L., Øverli, Ø., & Martins, C. I. (2012). Cortisol and finfish welfare. Fish physiology and biochemistry, 38(1), 163-188.

Enes, P., Panserat, S., Kaushik, S., & Oliva-Teles, A. (2009). Nutritional regulation of hepatic glucose metabolism in fish. Fish physiology and biochemistry, 35(3), 519-539.

Engås, A, S Løkkeborg, E Ona, & AV Soldal. (1996) Effects of seismic shooting on local abundance and catch rates of cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*). Canadian. Journal of Fisheries and Aquatic Sciences 53:2238-2249.

Engås, A. Eli Kyrkjebø Haugland & Jan Tore Øvredal (2006) Reactions of cod (*Gadus morhua* L.) in the pre-vessel zone to an approaching trawler under different light conditions Hydrobiologia 371/372: 199–206

Engen, F., & Folstad, I. (1999). Cod courtship song: a song at the expense of dance?. Canadian Journal of Zoology 77, 542-550.

Enger, P. S. (1967). Hearing in herring. Comparative Biochemistry and Physiology, 22(2), 527-538.

Enger, P. S. (1973). Masking of auditory responses in the medulla oblongata of goldfish. Journal of Experimental Biology, 59(2), 415-424.

Enger, PS. (1981). Frequency discrimination in teleosts central or peripheral? In: Hearing and Sound Communication in Fishes, W.N. Tavolga et al., eds. Springer-Verlag, New York, pp. 243-255.

Enger, P. S., Karlsen, H. E., Knudsen, F. R., & Sand, O. (1993). Detection and reaction of fish to infrasound. In ICES mar. Scientific Symposiums 196: 108-112.

Eriksen, M.S., Bakken, M., Espmark, Å., Braastad, B.O.,& Salte, R., (2006). Prespawning stress in farmed Atlantic salmon *Salmo salar*: maternal cortisol

exposure and hyperthermia during embryonic development affect offspring survival, growth and incidence of malformations. Journal of Fish Biology. 69, 114–129. 801

Eriksen, M.S., Espmark, Å., Braastad, B.O., Salte, R., & Bakken, M., (2007). Long-term effect of maternal cortisol exposure and mild hyperthermia during embryogeny on survival, growth and morphological anomalies in farmed Atlantic salmon *Salmo salar* offspring. Journal of Fish Biology. 70, 462–473.

Eriksen, M. S., Færevik, G., Kittilsen, S., McCormick, M. I., Damsgård, B., Braithwaite, V. A., Braastad, B.O. & Bakken, M. (2011). Stressed mothers–troubled offspring: a study of behavioural maternal effects in farmed *Salmo salar*. Journal of fish biology, 79(3), 575-586.

Estevez, A., McEvoy, L. A., Bell, J. G., & Sargent, J. R. (1999). Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in Arachidonic and Eicosapentaenoic acids. Aquaculture, 180(3), 321-343.

Evans, B.I., & Browman, H.I.. (2004). Variation in the the development of the fish retina. Pp. 145-166, In, Govoni, J.J. (Ed) Development of form and function in fishes and the question of larval adaptation. American Fisheries Society Symposium, vol. 40

Evans, B.I. & Clariborne, J.B. (2006) The Physiology of Fishes. Third edition. CRC Marine Biology Series. Taylor and Francis.

Falcón, J. (1999). Cellular circadian clocks in the pineal. Progress in neurobiology, 58(2), 121-162.

Falcon, J., Gothilf, Y., Coon, S. L., Boeuf, G., & Klein, D. C. (2003). Genetic, temporal and developmental differences between melatonin rhythm generating systems in the teleost fish pineal organ and retina. Journal of neuroendocrinology, 15(4), 378-382.

Falcón, J., Besseau, L., & Boeuf, G. (2006). Molecular and cellular regulation of pineal organ responses. Fish Physiology, 25, 243-306.

Falcón, J., Besseau, L., Sauzet, S., & Boeuf, G. (2007). Melatonin effects on the hypothalamo–pituitary axis in fish. Trends in Endocrinology & Metabolism,18(2), 81-88.

Falcón, J., Besseau, L., Fuentès, M., Sauzet, S., Magnanou, E., & Boeuf, G. (2009). Structural and functional evolution of the pineal melatonin system in vertebrates. Annals of the New York Academy of Sciences, 1163(1), 101-111.

Falcon, J., Migaud, H., Munoz-Cueto, J. A., & Carrillo, M. (2010). Current knowledge on the melatonin system in teleost fish. General and comparative endocrinology, 165(3), 469-482.

Fanouraki, E., Mylonas, C. C., Papandroulakis, N., & Pavlidis, M. (2011). Species specificity in the magnitude and duration of the acute stress response in

Mediterranean marine fish in culture. General and comparative endocrinology,173(2), 313-322.

FAO (2013) Food and Agriculture Organisation of the United Nations. Fisheries and Aquatic Department http://www.fao.org/fishery/statistics/global-aquaculture-production/en revised in October 2013.

Fay, R. R., & Popper, A. N. (1975). Modes of stimulation of the teleost ear. Journal of Experimental Biology 62, 379-387.

Fay, R. R., & Popper, A. N. (2012). Fish hearing: new perspectives from two 'senior' bioacousticians. Brain, Behavior and Evolution, 79(4), 215-217.

Félix, P. M., Vinagre, C., & Cabral, H. N. (2011). Life-history traits of flatfish in the Northeast Atlantic and Mediterranean Sea. Journal of Applied Ichthyology, 27(1), 100-111.

Fernández, I., & Gisbert, E. (2011). The effect of vitamin A on flatfish development and skeletogenesis: a review. Aquaculture, 315(1), 34-48.

Finn, R.N. & Kapoor, B.G.. (Ed.) (2008) Fish larval physiology. Science Publishers. Part 4 pp. 395-424

Finstad, J. L., & Nordeide, J. T. (2004). Acoustic repertoire of spawning cod, *Gadus morhua*. Environmental Biology of Fishes, 70(4), 427-433.

Fleming, I. A. & Huntingford, (2012). Reproductive Behaviour.In Huntingford, F., Jobling, M., & Kadri, S. (*Eds*.). Aquaculture and Behavior, 286-321.

Flik, G. & Wendelaar Bonga, S.E. (2001) "Stress in very young and adult fish". Vie et milieu 51, 229-236

Florin, A. B., & Höglund, J. (2007). Absence of population structure of turbot (*Psetta maxima*) in the Baltic Sea. Molecular Ecology, 16(1), 115-126.

Forés, R., Iglesias, J., Olmedo, M., Sánchez, F. J., & Peleteiro, J. B. (1990). Induction of spawning in turbot (*Scophthalmus maximus* L.) by a sudden change in the photoperiod. Aquacultural engineering, 9(5), 357-366.

Forlano, P. M., & Cone, R. D. (2007). Conserved neurochemical pathways involved in hypothalamic control of energy homeostasis. Journal of Comparative Neurology, 505(3), 235-248.

FourC Offshore (2013). Global Offshore Wind Farms Database [online]. Available from http://www.4coffshore.com/offshorewind/ [accessed 29 October 2013].

Fox, C. J., Taylor, M., Dickey-Collas, M., Fossum, P., Kraus, G., Rohlf, N., Munk, P. & Wright, P. J. (2008). Mapping the spawning grounds of North Sea cod (Gadus morhua) by direct and indirect means. Proceedings of the Royal Society B: Biological Sciences, 275(1642), 1543-1548.

Fudge, S. B., & Rose, G. A. (2009). Passive-and active-acoustic properties of a spawning Atlantic cod (*Gadus morhua*) aggregation. ICES Journal of Marine Science: Journal du Conseil, 66(6), 1259-1263.

Fuller, R. A., Warren, P. H., & Gaston, K. J. (2007). Daytime noise predicts nocturnal singing in urban robins. Biology letters, 3(4), 368-370.

Gibson, R. N., & Robb, L. (2000). Sediment selection in juvenile plaice and its behavioural basis. Journal of Fish Biology, 56(5), 1258-1275.

Gibson, R. N. (Ed.). (2008). Flatfishes: biology and exploitation. Blackwell Science

Giesing, E. R., Suski, C. D., Warner, R. E., & Bell, A. M. (2011). Female sticklebacks transfer information via eggs: effects of maternal experience with predators on offspring. Proceedings of the Royal Society B: Biological Sciences, 278(1712), 1753-1759.

Ginés, R., Afonso, J. M., Argüello, A., Zamorano, M. J., & López, J. L. (2004). The effects of long-day photoperiod on growth, body composition and skin colour in immature gilthead sea bream (*Sparus aurata* L.). Aquaculture Research, 35(13), 1207-1212.

Grether, G. F., Kolluru, G. R., Rodd, F. H., De la Cerda, J., & Shimazaki, K. (2005). Carotenoid availability affects the development of a colour-based mate preference and the sensory bias to which it is genetically linked. Proceedings of the Royal Society B: Biological Sciences, 272(1577), 2181-2188.

Grosenick, L., Clement, T. S., & Fernald, R. D. (2007). Fish can infer social rank by observation alone. Nature, 445(7126), 429-432.

Guthrie, D. M. (1986). Role of vision in fish behaviour. In The behaviour of Teleost fishes, pp. 75-113. Springer US

Haddy, J.A., & Pankhurst, N.W., (2000). The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri*. Aquaculture 188, 115–131.

Hall L, Patricoski M, & Fay RR (1981) Neurophysiological mechanisms of intensity discrimination in the goldfish. In: Tavolga WN, Popper AN, Fay RR (*Eds*) Hearing and sound communication in fishes. Springer, New York, pp 243–255.

Hall, T. E., Smith, P., & Johnston, I. A. (2004). Stages of embryonic development in the Atlantic cod Gadus morhua. Journal of morphology, 259(3), 255-270.

Hamre, K., Yúfera, M., Rønnestad, I. Boglione, C. Conceição,L.E.C. & Izquierdo, M. (2013) Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Reviews in Aquaculture 5: S26-S58.

Handegard, N. O., Michalsen, K., & Tjøstheim, D. (2003). Avoidance behaviour in cod (*Gadus morhua*) to a bottom-trawling vessel. Aquatic Living Resources, 16(3), 265-270.

Hara, T. J. (1994). Olfaction and gustation in fish: an overview. Acta physiologica scandinavica, 152(2), 207-217.

Hastings, M. C. (1995). Physical effects of noise on fishes. Proceedings of INTER-NOISE 95, The 1995 International Congress on Noise Control Engineering II 979–984.

Hastings, MC, Popper, A.N., Finneran, J.J. & Lanford, P.J. (1996) Effects of low-frequency underwater sound on hair cells of the inner ear and lateral line of the teleost fish *Astronotus ocellatus*. Journal of Acoustic Society of America. 99, 1759-1766.

Hastings, M. C. & Popper, A. N. (2005). Effects of sound on fish (p. 82). California Department of Transportation.

Hatch, L.T. & Wright, AJ. (2007) A brief review of anthropogenic sound in the oceans. International Journal of Comparative Phycology 20: 121-133

Hawkins, A.D. & Chapman, C.J. (1975) Masked auditory thresholds in the cod, *Gadus morhua* L. Journal Comparative Physiology A 103: 209-226.

Hawkins, A. D. & Sand, O. (1977). "Directional hearing in the median vertical plane by the cod." Journal of Comparative Physiology. 122(1): 1-8.

Hawkins, A. D., & Johnstone, A. D. F. (1978). The hearing of the Atlantic salmon, *Salmo salar*. Journal of Fish Biology, 13(6), 655-673.

Hawkins, A. D. & Rasmussen, K.J. (1978). "The calls of gadoid fish." Journal of Marine Biology Association. 58(4): 891-911.

Hawkins, A. D. (1981). The hearing abilities of fish. In Hearing and sound communication in fishes (pp. 109-137). Springer New York.

Hawkins, A. D. & Horner, K. (1981). The acoustico lateralis system of aquatic vertebrates. The Sense Organs, Blackie, Glasgow, 220-254.

Hawkins, A. D. & Myrberg Jr, A. A. (1983). Hearing and sound communication under water. Bioacoustics, a comparative approach. Academic Press, London, 347-405.

Hawkins, A. D. (1986). Underwater sound and fish behaviour. In The behaviour of teleost fishes (pp. 114-151). Springer US.

Head, A. B., & Malison, J. A. (2000). Effects of lighting spectrum and disturbance level on the growth and stress responses of yellow perch *Perca flavescens*. Journal of the World Aquaculture Society, 31(1), 73-80.

Healey, E. G. (1999). The skin pattern of young plaice and its rapid modification in response to graded changes in background tint and pattern. Journal of fish biology, 55(5), 937-971.

Hemre, G. I., Mommsen, T. P., & Krogdahl, Å. (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquaculture Nutrition, 8(3), 175-194.

Henslow, G. (1872). Conscious Mimicry. Nature, 5, 480.

Herbert, N. A., & Steffensen, J. F. (2005). The response of Atlantic cod, *Gadus morhua*, to progressive hypoxia: fish swimming speed and physiological stress. Marine Biology, 147(6), 1403-1412.

Herrera, M., Hachero-Cruzado, I., García, S., Mancera, J. M., & Navas, J. I. (2011). Reproduction of the wedge sole (*Dicologoglossa cuneata* Moreau) in captivity: spawning parameters and influence of the natural temperature. Reviews in Fish Biology and Fisheries, 21(3), 517-526.

Herrero, M. J., Martinez, F. J., Miguez, J. M., & Madrid, J. A. (2007). Response of plasma and gastrointestinal melatonin, plasma cortisol and activity rhythms of European sea bass (*Dicentrarchus labrax*) to dietary supplementation with tryptophan and melatonin. Journal of Comparative Physiology B, 177(3), 319-326.

Heydarnejad, M. S., Parto, M., & Pilevarian, A. A. (2013). Influence of light colours on growth and stress response of rainbow trout (*Oncorhynchus mykiss*) under laboratory conditions. Journal of Animal Physiology and Animal Nutrition,97(1), 67-71.

Hildebrand, J. A. (2009). Anthropogenic and natural sources of ambient noise in the ocean. Marine Ecology Progress Series, 395(5).

Himick, B. A., Vigna, S. R., & Peter, R. E. (1995). Characterization and distribution of bombesin binding sites in the goldfish hypothalamic feeding center and pituitary. Regulatory peptides, 60(2), 167-176.

Hirst, A.G. & Rodhouse, P.G. (2000) Impacts of geophysical seismic surveying on fishing success. Reviews in Fish Biology and Fisheries 10, 113–118

Ho, D. H., & Burggren, W. W. (2010). Epigenetics and transgenerational transfer: a physiological perspective. The Journal of experimental biology, 213(1), 3-16.

Höglund, E., Balm, P. H., & Winberg, S. (2000). Skin darkening, a potential social signal in subordinate Arctic charr (*Salvelinus alpinus*): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. Journal of Experimental Biology, 203(11), 1711-1721.

Höglund, E., Balm, P. H., & Winberg, S. (2002). Behavioural and neuroendocrine effects of environmental background colour and social interaction in Arctic charr (*Salvelinus alpinus*). Journal of Experimental Biology,205(16), 2535-2543.

Holles, S., Simpson, S. D., Radford, A. N., Berten, L., & Lecchini, D. (2013). Boat noise disrupts orientation behaviour in a coral reef fish. MEPS, 485, 295-300.

Hori, T., Gamperl, A. K., Afonso, L., Johnson, S., Hubert, S., Kimball, J., Bowmann, S. & Rise, M. (2010). Heat-shock responsive genes identified and validated in Atlantic cod (*Gadus morhua*) liver, head kidney and skeletal muscle using genomic techniques. BMC genomics, 11(1), 72.

Howard, D. M., & Angus, J. (2009). Acoustics and psychoacoustics. Taylor & Francis.

Howell, B.R., (1984). The intensive rearing of juvenile cod, *Gadus morhua* L. In: Dahl, E., Danielssen, D.S., Moksness, E., Solemdal, P. (Eds.), The Propagation of Cod *Gadus morhua* L. Flodevigen Rapportser. vol. 1, 657–675.

Howell, B. R., & Scott, A. P. (1989). Ovulation cycles and post-ovulatory deterioration of eggs of the turbot (*Scophthalmus maximus* L.). Rapports et Procès-Verbaux des Réunions Conseil International pour l'Exploration de la Mer, 191, 21-26.

Hutchings, J. A., Bishop, T. D., & McGregor-Shaw, C. R. (1999). Spawning behaviour of Atlantic cod, *Gadus morhua*: evidence of mate competition and mate choice in a broadcast spawner. Canadian Journal of Fisheries and Aquatic Sciences, 56(1), 97-104.

Hwang, P. P., Wu, S. M., Lin, J. H., & Wu, L. S. (1992). Cortisol content of eggs and larvae of teleosts. General and comparative endocrinology, 86(2), 189-196.

Imsland, A. K., Folkvord, A., & Stefansson, S. O. (1995). Growth, oxygen consumption and activity of juvenile turbot (*Scophthalmus maximus* L.) reared under different temperatures and photoperiods. Netherlands Journal of Sea Research, 34(1), 149-159.

Imsland, A. K., Folkvord, A., Jónsdóttir, Ó. D., & Stefansson, S. O. (1997). Effects of exposure to extended photoperiods during the first winter on long-term growth and age at first maturity in turbot (*Scophthalmus maximus*). Aquaculture, 159(1), 125-141.

Imsland, A. K., Foss, A., Gunnarsson, S., Berntssen, M. H., FitzGerald, R., Bonga, S. W., Naevdal, G. & Stefansson, S. O. (2001). The interaction of temperature and salinity on growth and food conversion in juvenile turbot (*Scophthalmus maximus*). Aquaculture, 198(3), 353-367.

Imsland, A. K., Foss, A., Stefansson, S. O., Mayer, I., Norberg, B., Roth, B., & Jenssen, M. D. (2006). Growth, feed conversion efficiency and growth heterogeneity in Atlantic halibut (*Hippoglossus hippoglossus*) reared at three different photoperiods. Aquaculture Research, 37(11), 1099-1106.

Imsland, A. K., Foss, A., Koedijk, R., Folkvord, A., Stefansson, S. O., & Jonassen, T. M. (2007). Persistent growth effects of temperature and photoperiod in Atlantic cod *Gadus morhua*. Journal of Fish Biology, 71(5), 1371-1382.

Iversen, R. T. (1967). Response of yellowfin tuna (*Thunnus albacares*) to underwater sound.

Jerkø, H., Turunen-Rise, I., Enger, P. S., & Sand, O. (1989). Hearing in the eel (*Anguilla anguilla*). Journal of Comparative Physiology A, 165(4), 455-459.

Jerlov, N.G. (1968). Optical Oceanography. Elsevier, Amsterdam

Jobling, M. (2012). Huntingford, F., Jobling, M., & Kadri, S. (Eds.). Aquaculture and behaviour. John Wiley & Sons

Jobling, M., Huntingford, F., & Kadri, S. (2012). Fish. In Aquaculture Environments. In Aquaculture and Behavior, 36-64. Kadri, S., & Huntingford, F. (Eds.). Blackwell.

Jonassen, T. M., Imsland, A. K., Kadowaki, S., & Stefansson, S. O. (2000). Interaction of temperature and photoperiod on growth of Atlantic halibut *Hippoglossus hippoglossus* L. Aquaculture Research, 31(2), 219-227.

Jones, A. (1972). Studies on egg development and larval rearing of turbot, *Scophthalmus maximus* L., and brill, *Scophthalmus rhombus* L., in the laboratory. Journal of Marine Biology Association. UK, 52, 965-986.

Jones, A. (1974). Sexual maturity, fecundity and growth of the turbot *Scophthalmus maximus* L. Journal of the Marine Biological Association of the United Kingdom, 54(01), 109-125.

Jones, A., Alderson, R. & Howell, B.R., (1974). Progress towards the development of a successful rearing technique for larvae of the turbot (*Scophthalmus mawimus* L.) . In: J.H.S. Blaxter (Ed.), The Early Life History of Fish. Proc. Int. Symp. Dunstaffnage. Springer-Verlag, pp. 731-737.

Jørgensen, E. H., & Jobling, M. (1990). Feeding modes in Arctic charr, *Salvelinus alpinus* L.: The importance of bottom feeding for the maintenance of growth. Aquaculture, 86(4), 379-385.

Jørgensen, C., & Fiksen, Ø. (2006). State-dependent energy allocation in cod (*Gadus morhua*). Canadian journal of fisheries and aquatic sciences, 63(1), 186-199.

Jørgensen, C., Ernande, B., Fiksen, Ø., & Dieckmann, U. (2006). The logic of skipped spawning in fish. Canadian Journal of Fisheries and Aquatic Sciences,63(1), 200-211.

Kanazawa, A. (1993). Nutritional mechanisms involved in the occurrence of abnormal pigmentation in hatchery-reared flatfish. Journal of the World Aquaculture Society, 24(2), 162-166.

Karakatsouli, N., Katsakoulis, P., Leondaritis, G., Kalogiannis, D., Papoutsoglou, S. E., Chadio, S., & Sakellaridis, N. (2012). Acute stress response of European sea bass *Dicentrarchus labrax* under blue and white light. Aquaculture. 364. 48-52.

Karakatsouli, N., Papoutsoglou, S.E., Pizzonia, G., Tsatsos, G., Tsopelakos, A., Chadio, S., Kalogiannis, D., Dalla, C., Polissidis, A. & Papadopoulou-Daifoti Z.. (2007). Effects of light spectrum on growth and physiological status of gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) reared under recirculating system conditions. Aquacultural Engineering 36, 302-309.

Karakatsouli, N., Papoutsoglou, S.E., Panopoulos, G., Papoutsoglou, E.S., Chadio, S. & Kalogiannis, D. (2008). Effects of light spectrum on growth and stress response of rainbow trout (*Oncorhynchus mykiss*) reared under recirculating system conditions. Aquacultural Engineering 38, 36-42.

Karlsen, Ø., Mittelholzer, C., Andersson, E., Norberg, B., & Taranger, G. L. (2014). Continuous light affects onset of puberty and associated changes in pituitary gonadotropin subunits transcript levels, and plasma estradiol-17β and testosterone levels in Atlantic cod (*Gadus morhua* L.) females. Aquaculture. doi. 10.1016/j.aquaculture.2013.12.029

Kasumyan, A. O. (2008). Sounds and sound production in fishes. Journal of Ichthyology, 48(11), 981-1030.

Kasumyan, A. O. (2009). Acoustic signaling in fish. Journal of Ichthyology,49(11), 963-1020.

Kawauchi, H., & Baker, B. I. (2004). Melanin-concentrating hormone signalling systems in fish. Peptides, 25(10), 1577-1584.

Kehoe, A. S., & Volkoff, H. (2007). Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 146(3), 451-461.

Kjesbu, O. S. (1989). The spawning activity of cod, *Gadus morhua* L. Journal of Fish Biology, 34(2), 195-206.

Kjesbu, O. S., Witthames, P. R., Solemdal, P., & Walker, M. G. (1990). Ovulatory rhythm and a method to determine the stage of spawning in Atlantic cod (*Gadus morhua*). Canadian Journal of Fisheries and Aquatic Sciences,47(6), 1185-1193.

Kjesbu, O. S. (1994). Time of start of spawning in Atlantic cod (*Gadus morhua)* females in relation to vitellogenic oocyte diameter, temperature, fish length and condition. Journal of fish biology, 45(5), 719-735.

Kjørsvik, E. & Holmefjord, I. (1995). Atlantic Halibut, *Hippoglossus hippoglossus*, and Cod, *Gadus morhua*. In Broodstock management and egg and larval quality. (Eds.) Bromage, N. & Roberts R.J. Blackwell Science Ltd.

Kjørsvik E., Hoehne-Reitan, K. & Reitan, KI (2002) Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.) Elsevier Science Aquaculture 227 9 –20

Kleppe, L., Karlsen, Ø., Edvardsen, R. B., Norberg, B., Andersson, E., Taranger, G. L., & Wargelius, A. (2013). Cortisol treatment of prespawning female cod affects cytogenesis related factors in eggs and embryos. General and Comparative Endocrinology. 189, 84-95.

Knudsen, E. (Ed.), 1983. The Effect of Temperature and Salinity on Growth and Oxygen Consumption of the turbot, *Scophthalmus maximus* L., Odense University, Denmark, p. 19, Special report.

Knudsen, F. R., Schreck, C. B., Knapp, S. M., Enger, P. S., & Sand, O. (1997). Infrasound produces flight and avoidance responses in Pacific juvenile salmonids. Journal of Fish Biology, 51(4), 824-829.

Knudsen, S. K., & Øen, E. O. (2003). Blast-induced neurotrauma in whales. Neuroscience research, 46(3), 377-386.

Kohbara, J., Hidaka, I., Kuriyama, I., Yamashita, M., Ichikawa, M., Furukawa, K., Aida, K., Sánchez-Vázquez, F.J. & Tabata, M. (2000). Nocturnal/diurnal demand-feeding pattern of yellowtail *Seriola quinqueradiata* under different keeping conditions. Fisheries Science, 66(5), 955-962.

Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C. G., Hopster, H., I.C. De Jong, Ruis, M.A.W. & Blokhuis, H. J. (1999). Coping styles

in animals: current status in behavior and stress-physiology. Neuroscience & Biobehavioral Reviews, 23(7), 925-935.

Koolhaas, J. M., Bartolomucci, A., Buwalda, B., De Boer, S. F., Flügge, G., Korte, S. M., Meerlo, P.; Murison, R.; Olivier, B., Palanza, P.; Richter-Levin, G., Sgoifo, A., Steimer, T., Stiedl, O.; van Dirk, G., Wöhr, M. & Fuchs, E. (2011). Stress revisited: a critical evaluation of the stress concept. Neuroscience & Biobehavioral Reviews, 35(5), 1291-1301.

Kobayashi, Y., Chiba, H., Amiya, N., Yamanome, T., Mizusawa, K., Amano, M., & Takahashi, A. (2008). Transcription elements and functional expression of proopiomelanocortin genes in the pituitary gland of the barfin flounder. General and comparative endocrinology, 158(3), 259-267.

Kobayashi, Y., Tsuchiya, K., Yamanome, T., Schiöth, H. B., & Takahashi, A. (2010). Differential expressions of melanocortin receptor subtypes in melanophores and xanthophores of barfin flounder. General and comparative endocrinology, 168(1), 133-142.

Koven, W. (2003). Key factors influencing juvenile quality in mariculture: a review. Israeli Journal of Aquaculture-Bamidgeh 55, 283-297.

Kuhlmann, D., & Quantz, G., (1980). Some effects of temperature and salinity on the embryonic development and incubation time of the turbot *Scophthalmus maximus* L., from the Baltic Sea. Meeresforschung 28, 172–178.

Kurokawa, T., Uji, S., & Suzuki, T. (2005). Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. Peptides, 26(5), 745-750.

Kusmic, C. & Gualtieri, P. (2000). Morphology and spectral sensitivines of retinal and extraretinal photoreceptors in freshwater teleosts. Micron 31. 183-200

Laberge, F., & Hara, T. J. (2001). Neurobiology of fish olfaction: a review. Brain research reviews, 36(1), 46-59.

Ladich, F. (2000). Acoustic communication and the evolution of hearing in fishes. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 355(1401), 1285-1288.

Ladich, F. (2004). Sound production and acoustic communication. In The Senses of Fish (pp. 210-230). Springer Netherlands.

Ladich F, & Popper A.N. (2004) Parallel evolution in fish hearing organs. In: Manley G, Popper AN, Fay RR (*Eds*) Evolution of the vertebrate auditory system. Springer, New York, pp 95–127

Ladich, F., & Myrberg, A. A. (2006). Agonistic behavior and acoustic communication. Communication in fishes 1, 121-148.

Ladich, F., & Yan, H. Y. (1998). Correlation between auditory sensitivity and vocalization in anabantoid fishes. Journal of Comparative Physiology A, 182(6), 737-746.

Lahiri, K., Vallone, D., Gondi, S. B., Santoriello, C., Dickmeis, T., & Foulkes, N. S. (2005). Temperature regulates transcription in the zebrafish circadian clock. PLoS biology, 3(11), e351.

Lahnsteiner, F. & Patarnello, P. (2004) Egg quality determination in the gilthead seabream, *Sparus aurata*, with biochemical parameters. Elsevier Science. Aquaculture 237 (2004) 443–459.

Lanes, C. F. C., Bizuayehu, T. T., Bolla, S., Martins, C., de Oliveira Fernandes, J. M., Bianchini, A., Kiron, V. & Babiak, I. (2012). Biochemical composition and performance of Atlantic cod (*Gadus morhua* L.) eggs and larvae obtained from farmed and wild broodstocks. Aquaculture, 324, 267-275.

Langård, L., Øvredal, J. T., Johannessen, A., Nøttestad, L., Skaret, G., Fernö, A. & Wahlberg, M. (2008). Sound production in pre-spawning herring, cod and haddock in a naturally enclosed ecosystem. Bioacoustics, 17(1-3), 38-40.

Larhammar, D., Wraith, A., Berglund, M. M., Holmberg, S. K., & Lundell, I. (2001). Origins of the many NPY-family receptors in mammals. Peptides, 22(3), 295-307.

Lear, W.H. (1984), Underwater World: Atlantic Cod, Department of Fisheries and Oceans, Ontario, Canada, pp. 1-8.

Leatherland, J.F.; Li, M & Barkataki, S. (2010) "Stressors, glucocorticoids and ovarian function in teleost." Journal of Fish Biology 76, 86-111.

Leclercq, E., Taylor, J. F., & Migaud, H. (2010). Morphological skin colour changes in teleosts. Fish and Fisheries, 11(2), 159-193.

Lee, C.-S., Tamaru, C.S., Kelley, C.D., Moriwake, A., & Miyamoto, G.T. (1992). The effect of salinity on the induction of spawning and fertilization in the striped mullet, *Mugil cephalus*. Aquaculture 102, 289–296.

Lehoux, J.G., Fazekas, A.G., Leblanc, H., Chapdelaine, A. & Sandor, T. (1972) Pteridins and steroid hydroxylation – I. The effect of folic acid and aminopterine on the steriod 11_- and 21- hydroxylation by domestic duck (*Anas platyrhynchos*). Journal of Steroid Biochemistry. 3, 773–779.

Lilly, G. R. (2008). The decline, recovery, and collapse of Atlantic cod (*Gadus morhua*) off Labrador and eastern Newfoundland.

Løkkeborg, S., Ona, E., Vold, A., & Salthaug, A. (2012). Sounds from seismic air guns: gear-and species-specific effects on catch rates and fish distribution.Canadian Journal of Fisheries and Aquatic Sciences, 69(8), 1278-1291.

Londraville, R. L., & Duvall, C. S. (2002). Murine leptin injections increase intracellular fatty acid-binding protein in green sunfish (*Lepomis cyanellus*). General and comparative endocrinology, 129(1), 56-62.

Lonsbury-Martin, B. L., Martin, G. K., Probst, R., & Coats, A. C. (1987). Acoustic distortion products in rabbit ear canal. I. Basic features and physiological vulnerability. Hearing research, 28(2), 173-189.

López-Olmeda, J.F., Madrid, J.A., & Sánchez-Vázquez, F.J. (2006a). Melatonin effects on food intake and activity rhythms in two fish species with different activity patterns: diurnal (goldfish) and nocturnal (tench). Comparative Biochemistry and Physiology A 144, 180–187.

López-Olmeda, J. F., Madrid, J. A., & Sanchez-Vazquez, F. J. (2006b). Light and temperature cycles as zeitgebers of zebrafish (*Danio rerio*) circadian activity rhythms. Chronobiology international, 23(3), 537-550.

López-Olmeda, J. F., Montoya, A., Oliveira, C., & Sánchez-Vázquez, F. J. (2009). Synchronization to light and restricted-feeding schedules of behavioral and humoral daily rhythms in gilthead sea bream (*Sparus aurata*).Chronobiology international, 26(7), 1389-1408.

López-Olmeda, J. F., Noble, C., & Sánchez-Vázquez, F. J. (2012). Does feeding time affect fish welfare?. Fish physiology and biochemistry, 38(1), 143-152.

Lubzens, E., Young, G., Bobe, J., & Cerdà, J. (2010). Oogenesis in teleosts: how fish eggs are formed. General and comparative endocrinology, 165(3), 367-389.

Luchiari, A. C., & Freire, F. A. M. (2009). Effects of environmental colour on growth of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), maintained individually or in groups. Journal of Applied Ichthyology, 25(2), 162-167.

Luczkovich, JJ, Krahforst, CS & Sprague M W (2012) Dies vessel noise change the calling rate and intesnsity of Soniferous Fishes? Advances in Experimental Medicine and Biology. 730 pp 375-378

Lythgoe, J.N., (1979). The Ecology of Vision. Clarendon Press, Oxford.

MacKenzie, D. (1995). The cod that disappeared. New Scientist, 147(1995), 24-9.

Mankiewicz, J. L., Godwin, J., Holler, B. L., Turner, P. M., Murashige, R., Shamey, R., Daniels, H.V. & Borski, R. J. (2013). Masculinizing effect of background color and cortisol in a flatfish with environmental sex-determination. Integrative and comparative biology, 53(4), 755-765.

Mann, D. A., Lu, Z., & Popper, A. N. (1997). A clupeid fish can detect ultrasound. Nature, 389, 341.

Mann, D. A., Lu, Z., Hastings, M. C., & Popper, A. N. (1998). Detection of ultrasonic tones and simulated dolphin echolocation clicks by a teleost fish, the American shad (*Alosa sapidissima)*. The Journal of the Acoustical Society of America, 104, 562.

Mann, D. A., Higgs, D. M., Tavolga, W. N., Souza, M. J., & Popper, A. N. (2001). Ultrasound detection by clupeiform fishes. The Journal of the Acoustical Society of America, 109, 3048.

Mañanós, E., Duncan, N., & Mylonas, C. C. (2008). Reproduction and control of ovulation, spermiation and spawning in cultured fish. Cabrita, E., Robles, V., & Herráez, P. (Eds.). In Methods in Reproductive Aquaculture: Marine and Freshwater Species, CRC Press, Taylor and Francis Group, Boca Raton, 3-80.

Marchesan, M., Spoto, M., Verginella, L., & Ferrero, E. A. (2005). Behavioural effects of artificial light on fish species of commercial interest. Fisheries research, 73(1), 171-185.

Martinez-Chavez, C. C., Minghetti, M., & Migaud, H. (2008). GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. General and comparative endocrinology, 156(2), 224-233.

Martins, C.I.M.; Galhardo, L.; Noble, C.; Damsgard, B.; Spedicato, M.T.; Zupa, W.; Beauchaud, M.; Kulczykowska, E.; Massabuau, J.C.; Carter, T.; Planellas, S.R. & Kristiansen, T. (2012) Behavioural indicators of welfare in farmed fish. Fish physiology Biochemistry 38: 17-41.

Maruska, K. P., Boyle, K. S., Dewan, L. R., & Tricas, T. C. (2007). Sound production and spectral hearing sensitivity in the Hawaiian sergeant damselfish, *Abudefduf abdominalis*. Journal of Experimental Biology, 210(22), 3990-4004.

Matsuda, K., Shimakura, S. I., Miura, T., Maruyama, K., Uchiyama, M., Kawauchi, H., Shioda, S.& Takahashi, A. (2007). Feeding-induced changes of melanin-concentrating hormone (MCH)-like immunoreactivity in goldfish brain. Cell and tissue research, 328(2), 375-382.

Matsuda, K., Kojima, K., Shimakura, S. I., Wada, K., Maruyama, K., Uchiyama, M., Kikuyama, S. & Shioda, S. (2008). Corticotropin-releasing hormone mediates α-melanocyte-stimulating hormone-induced anorexigenic action in goldfish. Peptides, 29(11), 1930-1936.

Matsuda, K., Kojima, K., Shimakura, S. I., Miura, T., Uchiyama, M., Shioda, S., Ando, H. & Takahashi, A. (2009). Relationship between melanin-concentrating hormone-and neuropeptide Y-containing neurons in the goldfish hypothalamus. Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology, 153(1), 3-7.

McCauley, RD, Fewtrell, J. & Popper, A.N.. (2003). High intensity anthropogenic sound damages fish ears. Journal of Acoustic Society of America 113(1):638-642.

McCormick, M. I. (1998). Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology, 79(6), 1873-1883.

McEvoy, L. A. (1984). Ovulatory rhythms and over-ripening of eggs in cultivated turbot, *Scophthalmus maximus* L. Journal of Fish Biology, 24(4), 437-448.

Mechaly, A. S., Viñas, J., & Piferrer, F. (2012). Sex-specific changes in the expression of kisspeptin, kisspeptin receptor, gonadotropins and gonadotropin receptors in the Senegalese sole (*Solea senegalensis*) during a full reproductive cycle. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 162(4), 364-371.

Mechaly, A. S., Viñas, J., & Piferrer, F. (2013). The kisspeptin system genes in teleost fish, their structure and regulation, with particular attention to the situation in *Pleuronectiformes.* General and comparative endocrinology.188. 258-268

Menter, D.G., Obika, M., Tchen, T.T. and Taylor, J.D. (1979) Leucophores and iridophores of *Fundulus heteroclitus*: biophysical and ultrastructural properties. Journal of Morphology 160, 103–119.

Metz, J. R., Peters, J. J., & Flik, G. (2006). Molecular biology and physiology of the melanocortin system in fish: a review. General and comparative endocrinology, 148(2), 150 162.

Michel, W. C. (2006). Chemoreception. In The physiology of fishes. CRC Press, Boca Raton, 471-497. (Eds. Evans, D. H., and Claiborne, J.B.)

Midling, K., Soldal, A. V., Fosseidengen, J. E., & Øvredal, J. T. (2002). Calls of the Atlantic cod: Does captivity restrict their vocal repertoire?. Bioacoustics, 12(2-3), 233-235.

Migaud, H., Taylor, J. F., Taranger, G. L., Davie, A., Cerdá-Reverter, J. M., Carrillo, M., Hansen, T. & Bromage, N. R. (2006). A comparative ex vivo and in vivo study of day and night perception in teleosts species using the melatonin rhythm. Journal of pineal research, 41(1), 42-52.

Migaud, H., Davie, A., Martinez Chavez, C. C., & Al-Khamees, S. (2007). Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. Journal of pineal research, 43(4), 327-335.

Migaud, H., Davie, A., & Taylor, J.F.T., (2010). Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. Journal of Fish Biology 76, 27-68.

Migaud, H., Ismail, R., Cowan, M., & Davie, A. (2012). Kisspeptin and seasonal control of reproduction in male european sea bass (*Dicentrarchus labrax*). General and Comparative Endocrinology. 179. 384-399

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez M.P. & Carrillo, M. (2013). Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture, 5(s1), S194-S223.

Mileva, V.R.; Gilmour, K.M. and Balshine, S. (2011) Effects of maternal stress on egg characteristics in a cooperatively breeding fish. Comparative Biochemistry and Physiology Part A 158: 22-29.

Milla, S., Wang, N., Mandiki, S. N. M., & Kestemont, P. (2009). Corticosteroids: Friends or foes of teleost fish reproduction?. Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology,153(3), 242-251.

Miller, B. S., & Kendall, A. W. (2009). Early life history of marine fishes (Vol. 36, No. 4). Berkeley: University of California Press.

Mitson RB (2000) ICES Cooperative Research Report 209: Five Years On. - ICES Fisheries Acoustic

Miura, T., Maruyama, K., Shimakura, S. I., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S. & Matsuda, K. (2006). Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. Neuroscience letters, 407(3), 279-283.

Miura, T., Maruyama, K., Shimakura, S. I., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S. & Matsuda, K. (2007). Regulation of food intake in the goldfish by interaction between ghrelin and orexin. Peptides, 28(6), 1207-1213.

Miura, T., Maruyama, K., Kaiya, H., Miyazato, M., Kangawa, K., Uchiyama, M., Shioda, S. & Matsuda, K. (2009). Purification and properties of ghrelin from the intestine of the goldfish, *Carassius auratus*. Peptides, 30(4), 758-765.

Miwa, S., Yamano, K., & Inui, Y. (1992). Thyroid hormone stimulates gastric development in flounder larvae during metamorphosis. Journal of Experimental Zoology, 261(4), 424-430.

Mizusawa, K., Kobayashi, Y., Yamanome, T., Saito, Y., & Takahashi, A. (2013). Interrelation between melanocyte-stimulating hormone and melanin-concentrating hormone in physiological body color change: roles emerging from barfin flounder *Verasper moseri*. General and comparative endocrinology. 181, 229-234

Moksness, E., Kjorsvid, E., & Olsen, Y. (Ed) (2004). Culture of cold-water marine fish. Blackwell Publishing Ltd., Oxford. 528 pp.

Moles, A., & Norcross, B. L. (1995). Sediment preference in juvenile Pacific flatfishes. Netherlands Journal of Sea Research, 34(1), 177-182.

Mommsen, T.; Vijayan, M.M. & Moon, T. (1999). "Cortisol in teleost: dynamics, mechanisms of action, and metabolic regulation." Fish Biology and Fisheries 9: 211-268.

Monk J., Puvanendran V. & Brown J. A., (2003). Do different light regimes affect the foraging behavior, growth and survival of larval cod (*Gadus morhua*)? Aquaculture. 257, 287-293.

Monk J., Puvanendran V. & Brown J.A. (2008). Does different tank colour affect the growth, survival and foraiging behaviour of Atlantic cod (*Gadus morhua*) larvae?. Aquaculture. 277, 197-202.

Montoya A, López-Olmeda JF, Yúfera M, Sánchez-Muros MJ, & Sánchez-Vázquez FJ (2010) Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (*Sparus aurata*). Aquaculture 306:315–321

Morgan, M.J.; Wilson, C.E. & Crim, L.W. (1999) The effects of stress on reproduction in Atlantic cod. Journal of Fish Biology 54: 477-488.

Mueller-Blenkle, C., Jones, E., Reid, D., Lüdeman, K., Kaffeman, R. & Elepfandt, A. (2008) Reactions of cod *Gadus morhua* to low-frequency sound resembling offshore wind turbine noise emissions. Bioacoustics 17, 207-209.

Mueller-Blenkle, C., Gill, A.B., McGregor, P.K. Metcalfe, J., Bendall, V., Wood, D., Andersson, M.H., Sigray, P. & Thomsen, F. (2010) "Behavioral reactions of cod and sole to playback of pile driving sound." The Journal of the Acoustical Society of America 128 (2010): 2331.

Murray, K.R. (1993) Editorial comment on light intensity measurements. Aquaculture Engeneering. 13, 242-243

Myrberg Jr, A. A., & Spires, J. Y. (1980). Hearing in damselfishes: an analysis of signal detection among closely related species. Journal of comparative physiology, 140(2), 135-144.

NASA (http://imagine.gsfc.nasa.gov/docs/science/know_l1/spectrum_chart.html) revised October 2013

Nedwell, J. R., Edwards, B., Turnpenny, A. W. H., & Gordon, J. (2004). Fish and Marine Mammal Audiograms: A summary of available information. Subacoustech Report, (534R0214).

Neuman, M. J., & Able, K. W. (1998). Experimental evidence of sediment preference by early life history stages of windowpane (*Scophthalmus aquosus*). Journal of Sea Research, 40(1), 33-41.

Nilsson, J.; Kristiansen, T.S.; Fosseidengen, J.E.; Ferno, A. and van den Boss, R. (2008) Learning in cod (*Gadus morhua*): long trace interval retention. Animal Cognition 11: 215-222.

Noaksson, E., Linderoth, M., Gustavsson, B., Zebühr, Y., & Balk, L. (2005). Reproductive status in female perch (*Perca fluviatilis*) outside a sewage treatment plant processing leachate from a refuse dump. Science of the total environment, 340(1), 97-112.

Noble, C., Mizusawa, K., & Tabata, M. (2005). Does light intensity affect self-feeding and food wastage in group-held rainbow trout and white-spotted charr?. Journal of fish biology, 66(5), 1387-1399.

Noble, C., Kadri, S., Mitchell, D.F. & Huntingford, F.A. (2007). The impact of environmental variables on the feeding rhythms and daily feed intake of cage-held 1+ Atlantic salmon parr *(Salmo salar* L.). Aquaculture 269, 290-298.

Norberg, B., Brown, C. L., Halldorsson, O., Stensland, K., & Björnsson, B. T. (2004). Photoperiod regulates the timing of sexual maturation, spawning, sex steroid and thyroid hormone profiles in the Atlantic cod (*Gadus morhua*). Aquaculture, 229(1), 451-467.

North, B. P., Turnbull, J. F., Ellis, T., Porter, M. J., Migaud, H., Bron, J., & Bromage, N. R. (2006). The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *255*(1), 466-479.

NRC/National Research Council (2005). Marine Mammal Populations and Ocean Noise. Determining When Noise Causes Biologically Significant Effects. The National Press, Washington DC. 126 pp Academies.

Oliveira, C., Ortega, A., Lopez-Olmeda, J. F., Vera, L. M., & Sanchez-Vazquez, F. J. (2007). Influence of constant light and darkness, light intensity, and light spectrum on plasma melatonin rhythms in Senegal sole. Chronobiology International, 24(4), 615-627.

Oliveira, C., Dinis, M. T., Soares, F., Cabrita, E., Pousão-Ferreira, P., & Sánchez-Vázquez, F. J. (2009a). Lunar and daily spawning rhythms of Senegal sole *Solea senegalensis*. Journal of fish biology, 75(1), 61-74.

Oliveira, C., Vera, L. M., López-Olmeda, J. F., Guzmán, J. M., Mañanós, E., Ramos, J., & Sánchez-Vázquez, F. J. (2009b). Monthly day/night changes and seasonal daily rhythms of sexual steroids in Senegal sole (*Solea senegalensis*) under natural fluctuating or controlled environmental conditions. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 152(2), 168-175.

Oliveira, C., Duncan, N. J., Pousão-Ferreira, P., Mañanós, E., & Sánchez-Vázquez, F. J. (2010). Influence of the lunar cycle on plasma melatonin, vitellogenin and sex steroids rhythms in Senegal sole, *Solea senegalensis*. Aquaculture, 306(1), 343-347.

Oliveira, C., Mañanós, E., Ramos, J., & Sánchez-Vázquez, F. J. (2011). Impact of photoperiod manipulation on day/night changes in melatonin, sex steroids and vitellogenin plasma levels and spawning rhythms in Senegal sole, *Solea senegalensis*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 159(3), 291-295.

OSPAR (Oslo/Paris Convention for the protection of the marine environment of the north-east Atlantic) http://qsr2010.ospar.org/media/assessments/Species/P00482_Cod.pdf accesed November 2013.

Owen, M. A., Davies, S. J., & Sloman, K. A. (2010). Light colour influences the behaviour and stress physiology of captive tench (*Tinca tinca*). Reviews in fish biology and fisheries, 20(3), 375-380.

Ozaki, Y., Miura, C., & Miura, T. (2006). Molecular cloning and gene expression of Spo11 during spermatogenesis in the Japanese eel, *Anguilla japonica*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 143(3), 309-314.

Pankhurst, N.W. & Porter, M. (2003). Cold and dark or warm and light: variations on the theme of environmental control of reproduction. Fish Physiology and Biochemistry 28, 385-389.

Pankhurst, N.W. (2011) The endocrinology of stress in fish: An environmental perspective. General and comparative Endocrinology. 170: 265-275.

Pauers, M.J., Kuchenbecker, J.A.; Neitz, M. & Neitz, J. (2012). Changes in the colour of light cue circadian activity. Animal behaviour, 83, 1443-1151.

Pavlidis, M., Karkana, M., Fanouraki, E., & Papandroulakis, N. (2008). Environmental control of skin colour in the red porgy, *Pagrus pagrus*.Aquaculture research, 39(8), 837-849.

Pearsons, K. S., Barber, D. S., Tabachnick, B. G., & Fidell, S. (1995). Predicting noise-induced sleep disturbance. The Journal of the Acoustical Society of America, 97, 331.

Pena, R., Dumas, S., Trasvina, A., Garcia, G., & Pliego-Cortez, H. (2005). Effects of tank colour and prey density on first feeding of the spotted sand bass *Paralabrax maculatofasciatus* (Steindachner) larvae". Aquaculture Research 36, 1239-1242

Pepe, I.M. (1999). Rhodopsin and phototransduction. Journal of Photochemistry and Photobiology B: Biology. 48, 1–10

Pérez-Casanova, J.C.; Afonso, L.O.B.; Johnson, S.C.; Currie, S. & Gamperl, A.K. (2008) The stress and metabolic response of Atlantic cod *Gadus morhua* L. to an acute thermal challenge. Journal of Fish Biology 72: 899-916.

Person-Le Ruyet, J., Baudin-Laurenc, F., Devauchelle, N., Metailler, R., Nicolas, J. L., Robin, J., & Guillaume, J. (2002). Culture of turbot (*Scophthalmus maximus*). IFREMER, France http://archimer.ifremer.fr/doc/00048/15930/13356.pdf

Peyon, P., Saied, H., Lin, X., & Peter, R. E. (1999). Postprandial, seasonal and sexual variations in cholecystokinin gene expression in goldfish brain. Molecular brain research, 74(1), 190-196.

Peyon, P., Saied, H., Lin, X., & Peter, R. E. (2000). Preprotachykinin gene expression in goldfish brain: Sexual, seasonal, and postprandial variations. Peptides, 21(2), 225-231.

Pickering, A. D., & Pottinger, T. G. (1985). Acclimation of the brown trout, *Salmo trutta* L., to the stress of daily exposure to malachite green. Aquaculture, 44(2), 145-152.

Pinillos, M. L., De Pedro, N., Alonso-Gómez, A. L., Alonso-Bedate, M., & Delgado, M. J. (2001). Food intake inhibition by melatonin in goldfish (*Carassius auratus*). Physiology & behavior, 72(5), 629-634.

Pittman, K., Yúfera, M., Pavlidis, M., Geffen, A. J., Koven, W., Ribeiro, L., Zambonino-Infante, J.L. & Tandler, A. (2013). Fantastically plastic: fish larvae equipped for a new world. Reviews in Aquaculture, 5(s1), S224-S267.

Planas, M., & Cunha, I. (1999). Larviculture of marine fish: problems and perspectives. Aquaculture, 177(1), 171-190.

Platt, C., & Popper, A. N. (1981). Fine structure and function of the ear. In Hearing and sound communication in fishes (pp. 3-38). Springer New York.

Plisetskaya, E. M., & Mommsen, T. P. (1996). Glucagon and glucagon-like peptides in fishes. International review of cytology, 168, 187-257.

Polakof, S., Panserat, S., Soengas, J. L., & Moon, T. W. (2012). Glucose metabolism in fish: a review. Journal of Comparative Physiology B, 182(8), 1015-1045.

Popper, A. N. (1970). Auditory capacities of the Mexican blind cave fish (*Astyanax jordani*) and its eyed ancestor (*Astyanax mexicanus*).Animal behaviour, 18, 552-562.

Popper, A. N. (1972). Auditory threshold in the goldfish (*Carassius auratus*) as a function of signal duration. The Journal of the Acoustical Society of America,52, 596.

Popper, A. N., & Fay, R. R. (1993). Sound Detection and Processing by Fish: Critical Review and Major Research Questions (Part 1 of 2). Brain, behavior and evolution, 41(1), 14-25.

Popper, A.N. (2003) Effects of anthropogenic noise in fishes. Fisheries research 28:10 24-31.

Popper, A.N., Fay, R.R., Platt, C. & Sand, O. (2003) "Sound detection mechanisms and capabilities of teleost fishes." In Sensory processing in aquatic environments, pp. 3-38. Springer New York

Popper, A.N & Hastings, M.C (2009). The effects of human-generated sound on fish. Integrative Zoology 4: 43-52

Popper, A. N., & Fay, R. R. (2011). Rethinking sound detection by fishes. Hearing research, 273(1), 25-36.

Pouchet, G. (1872). On the mechanism of the change of colour in fishes (and crustacea). British Association Reproduction, 42.

Pouchet, G. (1876). Color changes in crustaceans and fishes. Journal of Anatomical Physiology ,12, 1-90.

Power, D. M., Melo, J., & Santos, C. R. A. (2000). The effect of food deprivation and refeeding on the liver, thyroid hormones and transthyretin in sea bream. Journal of fish biology, 56(2), 374-387.

Power, D. M., Llewellyn, L., Faustino, M., Nowell, M. A., Björnsson, B. T., Einarsdottir, I. E., Canario, A.V.M. & Sweeney, G. E. (2001). Thyroid hormones in growth and development of fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 130(4), 447-459.

Purchase, C. F., Boyce, D. L., & Brown, J. A. (2000). Growth and survival of juvenile yellowtail flounder *Pleuronectes ferrugineus* (Storer) under different photoperiods. Aquaculture Research, 31(6), 547-552.

Puvanendran, V. & Brown J.A. (2002). Foraging, growth and survival of Atlantic cod larvae reared in different light intensities and photoperiods. Aquaculture 214, 131-151.

Radford, C. A., Montgomery, J. C., Caiger, P., & Higgs, D. M. (2012). Pressure and particle motion detection thresholds in fish: a re-examination of salient auditory cues in teleosts. The Journal of experimental biology, 215(19), 3429-3435.

Rahman, M., Kim, B. H., Takemura, A., Park, C. B., & Lee, Y. D. (2004). Influence of light–dark and lunar cycles on the ocular melatonin rhythms in the seagrass rabbitfish, a lunar-synchronized spawner. Journal of pineal research,37(2), 122-128.

Rajaee, A. H. (2011). Genetic Approaches to the Analysis of Body Colouration in Nile Tilapia (*Oreochromis niloticus*) (Doctoral dissertation, University of Stirling).

Reebs, S.G. (2002). Plasticity of diel and circadian activity rhythms in fishes. Reviews in Fish Biology and Fisheries 12, 349-371.

Revel, F. G., Saboureau, M., Masson-Pévet, M., Pévet, P., Mikkelsen, J. D., & Simonneaux, V. (2006). Kisspeptin mediates the photoperiodic control of reproduction in hamsters. Current Biology, 16(17), 1730-1735.

Reyff, J. (2012). Underwater sounds from unattenuated and attenuated marine pile driving. In The Effects of Noise on Aquatic Life (pp. 439-444). Springer New York.

Richard, D., Lin, Q., & Timofeeva, E. (2002). The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. European journal of pharmacology, 440(2), 189-197.

Romero, L.M., Dickens, M.J., & Cyr, N.E., (2009). The reactive scope model: a new model integrating homeostasis, allostasis, and stress. Hormonal Behavior. 55, 375–389.

Rosenlund, G., & Skretting, M. (2004). Worldwide status and perspective on gadoid culture. ICES Journal of Marine Science: Journal du Conseil, 63(2), 194-197.

Rosenlund, G., & Halldorsson, O., (2007), "Cod juvenile production: Research and commercial developments", Aquaculture, vol. 268, pp. 188-194.

Rowe, S., & Hutchings, J. A. (2004). The function of sound production by Atlantic cod as inferred from patterns of variation in drumming muscle mass.Canadian Journal of Zoology, 82(9), 1391-1398.

Rowe, S & Hutchings, J.A. (2006) Sound production by Atlantic Cod during Spawning. Transactions of the American Fisheries Society, 135: 529-538.

Rowe, S & Hutchings, J.A (2008) A link between sound producing musculature and mating success in Atlantic cod. Journal of Fish Biology 72: 500.511

Ruane, N. M., Makridis, P., Balm, P. H. M., & Dinis, M. T. (2005). Skin darkness is related to cortisol, but not MSH, content in post-larval *Solea senegalensis*. Journal of Fish Biology, 67(2), 577-581.

Ruchin, A. B. (2004). Influence of colored light on growth rate of juveniles of fish. Fish Physiology and Biochemistry, 30(2), 175-178.

Ruff, N., Fitzgerald, R. D., Cross, T. F., & Kerry, J. P. (2002). Comparative composition and shelf-life of fillets of wild and cultured turbot (*Scophthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture International, 10(3), 241-256.

Sampath-Kumar, R., Lee, S. T. L., Tan, C. H., Munro, A. D., & Lam, T. J. (1997). Biosynthesis in vivo and excretion of cortisol by fish larvae. Journal of Experimental Zoology, 277(4), 337-344.

Sánchez, E., Rubio, V. C., & Cerdá-Reverter, J. M. (2009b). Characterization of the sea bass melanocortin 5 receptor: a putative role in hepatic lipid metabolism. Journal of Experimental Biology, 212(23), 3901-3910.

Sánchez, E., Rubio, V. C., & Cerdá-Reverter, J. M. (2010). Molecular and pharmacological characterization of the melanocortin type 1 receptor in the sea bass. General and comparative endocrinology, 165(1), 163-169.

Sánchez, E., Rubio, V. C., Thompson, D., Metz, J., Flik, G., Millhauser, G. L. & Cerdá-Reverter, J. M. (2009a). Phosphodiesterase inhibitor-dependent inverse agonism

of agouti related protein on melanocortin 4 receptor in sea bass (*Dicentrarchus labrax*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 296(5), R1293 R1306.

Sánchez-Vasquez, F.J., Azzaydi, M., Martinez, F.J., Zamora, S. & Madrid, J.A. (1998). Annual rhythms of demand-feeding activity in seabass: evidence of a seasonal phase inversion of the diel feeding pattern. Chronobiology International 15, 607-622.

Sánchez–Vázquez, F. J., Iigo, M., Madrid, J. A., & Tabata, M. (2000). Pinealectomy does not affect the entrainment to light nor the generation of the circadian demand-feeding rhythms of rainbow trout. Physiology & behavior,69(4), 455-461.

Sánchez-Vázquez FJ, & Madrid JA (2001) Feeding anticipatory activity in fish. In: Houlihan DF, Boujard T, Jobling M (eds) Food intake in fish. Blackwell Science, Oxford, pp 216–232

Sand, O. & P. S. Enger (1973). "Evidence for an Auditory Function of the Swimbladder in the Cod." J Exp Biol 59(2): 405-414.

Sand, O. & Karlsen, H. E. (1986). Detection of infrasound by the Atlantic cod. Journal of Experimental Biology, 125(1), 197-204.

Sand, O., & Karlsen, H. E. (2000). Detection of infrasound and linear acceleration in fishes. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 355(1401), 1295-1298.

Sand, O., Enger, P. S., Karlsen, H. E., & Knudsen, F. R. (2001). Detection of infrasound in fish and behavioral responses to intense infrasound in juvenile salmonids and European silver eels: a minireview. In American Fisheries Society Symposium (pp. 183-193). American Fisheries Society.

Sargent, J. R., McEvoy, L. A., & Bell, J. G. (1997). Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155(1), 117-127.

Schmid, D. A., Held, K., Ising, M., Uhr, M., Weikel, J. C., & Steiger, A. (2005). Ghrelin stimulates appetite, imagination of food, GH, ACTH, and cortisol, but does not affect leptin in normal controls. Neuropsychopharmacology, 30(6), 1187-1192.

Scholik AR, & Yan HY (2001) Effects of underwater noise on auditory sensitivity of a cyprinid fish. Hearing Research. 152:17–24

Schreck, C.B.; Contreras-Sánchez, W. & Fitzpatrick, M.S. (2001) Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197: 3-24.

Schreck, C. B. (2010). Stress and fish reproduction: the roles of allostasis and hormesis. General and comparative endocrinology, 165(3), 549-556.

Schulz, R. W., de França, L. R., Lareyre, J. J., LeGac, F., Chiarini-Garcia, H., Nobrega, R. H., & Miura, T. (2010). Spermatogenesis in fish. General and comparative endocrinology, 165(3), 390-411.

Scott, G. T. (1965). Physiology and pharmacology of color change in the sand flounder *Scopthalamus aquosus*. Limnology and Oceanography, 230-246.

Seafish, UK. (2014) The Sea Food guide. Available at: http://www.seafoodacademy.org/Documents/Seafood%20Guide.pdf Revised February 2014.

Seikai, T., Matsumoto, J., Shimozaki, M., Oikawa, A. & Akiyama, T. (1987). An association of melanophores appearing at metamorphosis as vehicles of asymmetric skin color formation with pigment anomalies developed under hatchery conditions in the Japanese flounder, *Paralichthys olivaceus*. Pigment Cell Research 1, 143–151.

Selvaraj, S., Kitano, H., Amano, M., Ohga, H., Yoneda, M., Yamaguchi, A., Shimizu, A. & Matsuyama, M. (2012). Increased expression of kisspeptin and GnRH forms in the brain of scombroid fish during final ovarian maturation and ovulation. Reproductive Biology and Endocrinology, 10, 64.

Selz, Y., Braasch, I., Hoffmann, C., Schmidt, C., Schultheis, C., Schartl, M., & Volff, J. N. (2007). Evolution of melanocortin receptors in teleost fish: the melanocortin type 1 receptor. Gene, 401(1), 114-122.

Shields R.J., Brown N.P., & Bromage, N.R. (1997) Blastomere morphology as a predictive measure of fish egg viability. Aquaculture 155 (1997) I-12

Shimakura, S. I., Kojima, K., Nakamachi, T., Kageyama, H., Uchiyama, M., Shioda, S., Takahashi, A. & Matsuda, K. (2008). Neuronal interaction between melanin-concentrating hormone-and α-melanocyte-stimulating hormone-containing neurons in the goldfish hypothalamus. Peptides, 29(8), 1432-1440.

Simensen, L. M., Jonassen, T. M., Imsland, A. K., & Stefansson, S. O. (2000). Photoperiod regulation of growth of juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture, 190(1), 119-128.

Singru, P. S., Mazumdar, M., Sakharkar, A. J., Lechan, R. M., Thim, L., Clausen, J. T., & Subhedar, N. K. (2007). Immunohistochemical localization of cocaine-and amphetamine-regulated transcript peptide in the brain of the catfish, *Clarias batrachus* (Linn.). Journal of Comparative Neurology, 502(2), 215-235.

Skjæraasen, J. E., Nash, R. D., Korsbrekke, K., Fonn, M., Nilsen, T., Kennedy, J., Nedreaas, K.H., Thorsen, A., Witthames, P.R, Geffen, A.J., Høie, H. & Kjesbu, O. S. (2012). Frequent skipped spawning in the world's largest cod population. Proceedings of the National Academy of Sciences, 109(23), 8995-8999.

Slabbekoorn, H., Bouton, N., van Opzeeland, I., Coers, A., ten Cate, C., & Popper, A. N. (2010). A noisy spring: the impact of globally rising underwater sound levels on fish. Trends in Ecology & Evolution, 25(7), 419-427.

Smith, R.C., (1974). Structure of solar radiation in the upper layers of the sea. In: Gerlov, N.G., Stieman Nielsen, E. (*Eds*.), Optical Aspects of Oceanography. Academic Press, London, pp. 95–119.

Smith, T. I., McVey, D. C., Jenkins, W. E., Denson, M. R., Heyward, L. D., Sullivan, C. V., & Berlinsky, D. L. (1999). Broodstock management and spawning of southern flounder, *Paralichthys lethostigma*. Aquaculture, 176 (1), 87-99.

Smith, M. E., Kane, A. S., & Popper, A. N. (2004). Noise-induced stress response and hearing loss in goldfish (*Carassius auratus*). Journal of Experimental Biology, 207(3), 427-435.

Soengas, J. L., Polakof, S., Chen, X., Sangiao-Alvarellos, S., & Moon, T. W. (2006). Glucokinase and hexokinase expression and activities in rainbow trout tissues: changes with food deprivation and refeeding. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 291(3), R810-R821.

Solbakken, J. S., & Pittman, K. (2004). Photoperiodic modulation of metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus* L.).Aquaculture, 232(1), 613-625.

Sørensen, P.W. & Caprio, J. (1998) Chemoreception. In The Physiology of Fishes, Evans, D.H. (ed.) CRC Press, Boca Raton, Florida 1998, 375-405.

Sørensen, C., Johansen, I. B., & Øverli, Ø. (2013). Neural plasticity and stress coping in teleost fishes. General and comparative endocrinology, 181, 25-34.

Sparrevohn, C. R., & Støttrup, J. G. (2008). Diet, abundance, and distribution as indices of turbot (*Psetta maxima* L.) release habitat suitability. Reviews in Fisheries Science, 16(1-3), 338-347.

Spieler, R.E. (1992). Feeding-entrained circadian rhythms in fishes. In: Rhythms in fishes, Ed. Ali, M.A., Plenum Press. New York, USA. pp.137-147.

Spiga, I., Fox, J., & Benson, R. (2012). Effects of Short-and Long-Term Exposure to Boat Noise on Cortisol Levels in Juvenile Fish. In The Effects of Noise on Aquatic Life (pp. 251-253). Springer New York.

Stacey, N., & Sorensen, P. (2005). Reproductive pheromones. In Sloman, K.A., Wilson, R.W. & Balshine, S. (Eds.) Fish physiology,24, 359-412.

Stanley, J. A., Radford, C. A., & Jeffs, A. G. (2012). Effects of Underwater Noise on Larval settlement. In The Effects of Noise on Aquatic Life (pp. 371-374). Springer New York.

Stoner, A. W., & Ottmar, M. L. (2003). Relationships between size-specific sediment preferences and burial capabilities in juveniles of two Alaska flatfishes. Journal of experimental marine biology and ecology, 282(1), 85-101.

Støttrup, J. G., Sparrevohn, C. R., Modin, J., & Lehmann, K. (2002). The use of releases of reared fish to enhance natural populations: a case study on turbot *Psetta maxima* (Linne, 1758). Fisheries Research, 59(1), 161-180.

Støttrup, J. G., & Sparrevohn, C. R. (2010). Stock enhancement Europe: turbot *Psetta maxima*. In. Practical Flatfish Culture and Stock Enhancement, (Eds.) Daniels, H.V. and Watanabe W.O 219-233.

Strand, Å., Alanärä, A., Staffan, F., & Magnhagen, C. (2007). Effects of tank colour and light intensity on feed intake, growth rate and energy expenditure of juvenile Eurasian perch, *Perca fluviatilis* L. Aquaculture, 272(1), 312-318.

Suzuki, T., Oohara, I. & Kurokawa, T. (1998). Hoxd-4 expression during pharyngealarch development in flounder (*Paralichthys olivaceus*) embryos and effects of retinoic acid on expression. Zoological Science 15, 57–67.

Szisch, V., Van der Salm, A. L., Bonga, S. W., & Pavlidis, M. (2002). Physiological colour changes in the red porgy, *Pagrus pagrus*, following adaptation to blue lighting spectrum. Fish Physiology and Biochemistry, 27(1-2), 1-8.

Tache, Y., & Perdue, M. H. (2004). Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. Neurogastroenterology & Motility, 16(s1), 137-142.

Takahashi, A., Tsuchiya, K., Yamanome, T., Amano, M., Yasuda, A., Yamamori, K., & Kawauchi, H. (2004). Possible involvement of melanin-concentrating hormone in food intake in a teleost fish, barfin flounder. Peptides,25(10), 1613-1622.

Takemura, A., Rahman, M. S., & Park, Y. J. (2010). External and internal controls of lunar-related reproductive rhythms in fishes. Journal of fish biology,76(1), 7-26

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.A. & Hansen, T. (2010). Control of puberty in farmed fish. General and Comparative Endocrinology, 165(3), 483-515.

Tavolga, W. N., & Wodinsky, J. (1963). Auditory capacities in fishes: pure tone thresholds in nine species of marine teleosts. Bulletin of the AMNH; v. 126, article 2.

Taylor, J. F., Migaud, H., Porter, M. J. R., & Bromage, N. R. (2005). Photoperiod influences growth rate and plasma insulin-like growth factor-I levels in juvenile rainbow trout, *Oncorhynchus mykiss*. General and comparative endocrinology, 142(1), 169-185.

Taylor, J.F., North, B.P., Porter, M.J.R., Bromage, N.R. & Migaud, H. (2006). Photoperiod can be used to enhance growth and improve feeding efficiency in farmed rainbow trout, *Oncorhynchus mykiss*. Aquaculture 256, 216-234.

Thomsen, F., Lüdemann, K., Kafemann, R., & Piper, W. (2006). Effects of offshore wind farm noise on marine mammals and fish. Biola, Hamburg, Germany on behalf of COWRIE Ltd.

Tricoire, H., Møller, M., Chemineau, P., Malpaux, B., Campbell, B. K., Webb, R.,Dobson, H. & Doberska, C. (2003). Origin of cerebrospinal fluid melatonin and possible function in the integration of photoperiod. In Reproduction in domestic ruminants V. Proceedings of the Sixth International Symposium on Reproduction in Domestic Ruminants held in Crieff, Scotland, UK, August 2002. (pp. 311-321). Society for Reproduction and Fertility.

Unniappan, S., Cerdá-Reverter, J. M., & Peter, R. E. (2004). In situ localization of preprogalanin mRNA in the goldfish brain and changes in its expression during feeding and starvation. General and comparative endocrinology, 136(2), 200-207.

Utne-Palm, A.C. (2002). Visual feeding of fish in a turbid environment: physical and behavioural aspects. Marine and Freshwater Behaviour and Physiology,35(1-2), 111-128.

Vabø, R., Olsen, K. & Huse, I.. (2002) The effect of vessel avoidance of wintering Norwegian spring-spawning herring. Fisheries Research. 58, 59–77

van der Hammen, T., Poos, J. J., van Overzee, H. M., Heessen, H. J., Magnusson, A., & Rijnsdorp, A. D. (2013). Population ecology of turbot and brill: What can we learn from two rare flatfish species?. Journal of Sea Research, 84, 96-108.

Van Ham, E. H., Berntssen, M. H., Imsland, A. K., Parpoura, A. C., Wendelaar Bonga, S. E., & Stefansson, S. O. (2003). The influence of temperature and ration on growth, feed conversion, body composition and nutrient retention of juvenile turbot (*Scophthalmus maximus*). Aquaculture, 217(1), 547-558.

van Opzeeland, I., & Slabbekoorn, H. (2012). Importance of Underwater Sounds for Migration of Fish and Aquatic Mammals. In The Effects of Noise on Aquatic Life (pp. 357-359). Springer New York.

Venizelos, A., & Benetti, D. D. (1999). Pigment abnormalities in flatfish. Aquaculture, 176(1), 181-188.

Vera, L.M., Madrid, J.A. & Sanchez-Vazquez, F.J. (2006). Locomotor, feeding and melatonin daily rhythms in sharpsnout seabream (*Diplodus puntazzo*). Physiology and Behavior 88, 167-172.

Vera, L.M., De Pedro, N., Gomez-Milan, E., Delgado, M.J., Sanchez-Muros, M.J., Madrid, J.A. & Sanchez-Vazquez, F.J. (2007). Feeding entrainment of locomotor activity rhythms, digestive enzymes and neuroendocrine factors in goldfish. Physiology and Behavior 90, 518-524.

Vera, L.M., & Migaud, H. (2009) Continuous high light intensity can induce retinal degeneration in Atlantic salmon, Atlantic cod and European sea bass. Aquaculture 296: 150-158

Vera, L. M., A. Davie, J. F. Taylor, & H. Migaud. (2010). "Differential light intensity and spectral sensitivities of Atlantic salmon, European sea bass and Atlantic cod pineal glands *ex vivo*." General and comparative endocrinology 165, no. 1: 25-33.

Vester, H. I., Folkow, L. P., & Blix, A. S. (2004). Click sounds produced by cod (*Gadus morhua*). The Journal of the Acoustical Society of America, 115, 914.

Viegas, I., Rito, J., González, J. D., Jarak, I., Carvalho, R. A., Metón, I., Pardal, M.A.., Baanante, I.V. & Jones, J. G. (2013). Effects of food-deprivation and refeeding on the regulation and sources of blood glucose appearance in European seabass (*Dicentrarchus labrax* L.). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 166(3), 399-405.

Villamizar, N., García-Alcazar, A., & Sánchez-Vázquez, F.J. (2009). Effect of light spectrum and photoperiod on the growth, development and survival of European sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 292, 80-86.

Villamizar, N., Blanco-Vives, B., Migaud, H., Davie, A., Carboni, S., & Sánchez-Vázquez, F. J. (2011). Effects of light during early larval development of some aquacultured teleosts: A review. Aquaculture 315, 86-94.

Vindas, M. A., Sørensen, C., Johansen, I. B., Folkedal, O., Höglund, E., Khan, U. W., Khan, U.W., Stien, L.H., Kristiansen, T.S., Braastad, B.O. & Øverli, Ø. (2014). Coping with Unpredictability: Dopaminergic and Neurotrophic Responses to Omission of Expected Reward in Atlantic Salmon (*Salmo salar* L.). PloS one, 9(1), e85543.

Volckaert, F. A. (2012). (Flat) fish stocks in an ecosystem and evolutionary perspective. Journal of Sea Research. (75) 19-32

Volkoff, H., & Peter, R. E. (2000). Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, *Carassius auratus*: actions on neuropeptide Y-and orexin A-induced feeding. Brain research, 887(1), 125-133.

Volkoff, H., Joy Eykelbosh, A., & Ector Peter, R. (2003). Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. Brain research, 972(1), 90-109.

Volkoff, H., Canosa, L. F., Unniappan, S., Cerda-Reverter, J. M., Bernier, N. J., Kelly, S. P., & Peter, R. E. (2005). Neuropeptides and the control of food intake in fish. General and comparative endocrinology, 142(1), 3-19.

Volkoff, H., Unniappan, S., & Kelly, S. P. (2009). The endocrine regulation of food intake. In Bernier, N. J., Van Der Kraak, G., Farrell, A. P., & Brauner, C. J. (*Eds*.). Fish Physiology: Fish Neuroendocrinology. 28, 421-465.

Volpato, G. L., & Barreto, R. E. (2001). Environmental blue light prevents stress in the fish Nile tilapia. Brazilian Journal of Medical and Biological Research, 34(8), 1041-1045.

Volpato, G. L., Duarte, C. R. A., & Luchiari, A. C. (2004). Environmental color affects Nile tilapia reproduction. Brazilian journal of medical and biological research, 37(4), 479-483.

Volpato, G. L., Bovi, T. S., de Freitas, R. H., da Silva, D. F., Delicio, H. C., Giaquinto, P. C., & Barreto, R. E. (2013). Red Light Stimulates Feeding Motivation in Fish but Does Not Improve Growth. PloS one, 8(3), e59134.

Von Békésy, G. (1959). Neural funneling along the skin and between the inner and outer hair cells of the cochlea. The Journal of the Acoustical Society of America, 31, 1236.

Von Gierke, H. E., & Eldred, K. M. (1993). Effects of noise on people. Noise/News International, 1(2), 67-89.

Wahlberg, M., & Westerberg, H. (2005). Hearing in fish and their reactions to sounds from offshore wind farms. Marine Ecology Progress Series, 288, 295-309.

Wang, L., Martínez, V., Rivier, J. E., & Taché, Y. (2001). Peripheral urocortin inhibits gastric emptying and food intake in mice: differential role of CRF receptor 2. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 281(5), R1401-R1410.

Wardle, C.S.; Carter, T.J.; Urquhart, G.G.; Johnstone, A.D.F.; Ziolkowski, A.M.; Hampson, G. & Mackie, D. (2001) Effects of seismic air guns on marine fish. Continental shelf research 21: 1005-1027.

Waring, C. P., Stagg, R. M., & Poxton, M. G. (1996). Physiological responses to handling in the turbot. Journal of Fish Biology, 48(2), 161-173.

Waring, C. P., Poxton, M. G., & Stagg, R. M. (1997). The physiological response of the turbot to multiple net confinements. Aquaculture International, 5(1), 1-12.

Wark, A. R., & Peichel, C. L. (2010). Lateral line diversity among ecologically divergent threespine stickleback populations. The Journal of experimental biology, 213(1), 108-117.

Waser, W., & Heisler, N. (2005). Oxygen delivery to the fish eye: Root effect as crucial factor for elevated retinal PO2. Journal of experimental biology, 208(21), 4035-4047.

Weltzien, F. A., Planas, M., & Fyhn, H. J. (1999). Temperature dependency of early growth of turbot (*Scophthalmus maximus* L.) and its implications for developmental progress. Journal of experimental marine biology and ecology, 242(2), 201-210

Westring, C. G., Ando, H., Kitahashi, T., Bhandari, R. K., Ueda, H., Urano, A., Dores, R.M., Sher, A.A.& Danielson, P. B. (2008). Seasonal changes in CRF-I and urotensin I transcript levels in masu salmon: correlation with cortisol secretion during spawning. General and comparative endocrinology, 155(1), 126-140.

Willmer, P., Stone, G., & Johnston, I. (2005). Environmental physiology of animals. 2nd edition. John Wiley & Sons.

Wong, M. M., Yu, R. M., Ng, P. K., Law, S. H., Tsang, A. K., & Kong, R. Y. (2007). Characterization of a hypoxia-responsive leptin receptor (omLepR(L)) cDNA from the marine medaka (*Oryzias melastigma*). Marine pollution bulletin, 54(6), 797-803.

World Register of marine species (http://www.marinespecies.org/aphia.php?p=taxdetails&id=127149) accesed January 2014

Wright, A.J., Aguilar Soto, N., Baldwin, A.L., Bateson, M., Beale, C.M., Clark, C., Deak, T., Edwards, E.F., Fernández, A., Godinho, A., Hatch, L.T., Kakuschke, A., Lusseau, D., Martineau, D., Romero, M.L., Weilgart, L.S., Wintle, B.A., Notarbartolo-di-Sciara, G. & Vidal, M. (2007) "Anthropogenic noise as a stressor

in animals: a multidisciplinary perspective." International Journal of Comparative Psychology 20, no. 2.

Wright, A. J. (2012). Noise-Related Stress and Cumulative Impact Assessment. In The Effects of Noise on Aquatic Life (pp. 541-543). Springer New York.

Wunderink, Y. S., Engels, S., Halm, S., Yúfera, M., Martínez-Rodríguez, G., Flik, G., Klaren, P.H.M. & Mancera, J. M. (2011). Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): The involvement of cortisol, CRH and CRH-BP. *General and comparative endocrinology*, *171*(2), 203-210.

Wunderink, Y. S., Martínez-Rodríguez, G., Yúfera, M., Martín Montero, I., Flik, G., Mancera, J. M., & Klaren, P. H. (2012). Food deprivation induces chronic stress and affects thyroid hormone metabolism in Senegalese sole (*Solea senegalensis*) post-larvae. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 162(4), 317-322

Wysocki, L.E; Davidson III, J.W.; Smith, M.E; Frankel, A.; Ellison, W.T; Mazik, P.M.; Popper, A.N. & Bebak, J. (2007) Effects of aquaculture production noise on hearing, growth, and disease resistance of rainbow trout *Oncorhynchus mykiss*. Aquaculture 272: 687-697

Xu, M., & Volkoff, H. (2007). Molecular characterization of prepro-orexin in Atlantic cod (*Gadus morhua*): Cloning, localization, developmental profile and role in food intake regulation. Molecular and cellular endocrinology, 271(1), 28-37.

Yan, H. Y. (2001). A non-invasive electrophysiological study on the enhancement of hearing ability in fishes. Proceedings-Institute of Acoustics, 23(4), 15-26.

Yelverton, JT, DR Richmond, W Hicks, Sanders, & E R Fletcher. (1975). The relationship between fish size and their response to underwater blast. Topical Report DNA 3677T. Defense Nuclear Agency, Department of Defense, Washington, DC.

Yoseda, K., Yamamoto, K., Asami, K., Chimura, M., Hashimoto, K., & Kosaka, S. (2008). Influence of light intensity on feeding, growth, and early survival of leopard coral grouper (*Plectropomus leopardus*) larvae under mass-scale rearing conditions. Aquaculture, 279(1), 55-62.

Zar, H. J. (1999). Biostatistical analysis. Biostatistical analysis. Prentice Hall

Zelick, R., Mann, D. A., & Popper, A. N. (1999). Acoustic communication in fishes and frogs. In Comparative hearing: fish and amphibians (pp. 363-411). Springer New York

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., & Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. General and comparative endocrinology, 165(3), 438-455.

Publications, Conferences and Awards

Publications

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. Noise as a source of stress for farmed fish. 2012 July-August International AquaFeed Magazine pp. 28-30

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. Rudio como fuente de estrés en peces de cultivo. 2012 Julio-Agosto International Aquafeed Magazine (Spanish edition) pp. 27-30

Noise pollution in the aquatic environment. 2012 July-August Fish Farmer Magazine p.41 (*Article about my experimental work*)

Novartis winner visits PEI. 2012 September/October Fish Farmer Magazine pp. 36-37 (*Interview article*)

Peer reviewed publications

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. (2013) Stress response to anthropogenic noise in Atlantic cod *Gadus morhua* L. (Submitted: *The Canadian Journal of Fisheries and Aquatic Science*)

Sierra-Flores, R., Grant, B., Carboni, S., Davie, A., Atack, T & Migaud, H. (2013) Effects of light wavelength and tank background colour in Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) larvae development. (Submitted *Aquaculture*)

Sierra-Flores, R. Davie, A. & Migaud, H. Effect of light wavelength on growth, appetite, skin pigmentation and stress response in turbot (*Scophthalmus maximus*) juveniles. (*In preparation*)

Conferences and courses attended

Sierra-Flores, R. (November 2013). Seminar "Light beyond on/off: There is something about a coloured light bulb!". Institute of Aquaculture, University of Stirling. (*Seminar oral presentation*)

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. (2012) Noise as a stressor in Atlantic cod *Gadus morhua*. PhD conference, Institute of Aquaculture, University of Stirling. (*Oral presentation*)

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. (2012) Effects of light wavelength and tank colour on turbot (*Scophthalmus maximus*) larvae performance. PhD conference, Institute of Aquaculture, University of Stirling. (*Poster presentation*)

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. (2012) Noise as a stressor in Atlantic cod *Gadus morhua*. UK Aquaculture Conference, Aviemore, Scotland. (*Oral presentation*)

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. (2012) Noise as a stressor in Atlantic cod *Gadus morhua* 2012. Atlantic Veterinary College, Prince Edward Island University, PEI, Canada. (*Invited Informal Oral presentation*)

Sierra-Flores, R., Davie, A., Atack, T. & Migaud, H. (2012) Noise as a stressor in Atlantic cod *Gadus morhua*. Novartis Animal Health Research facilities, Victoria, PEI, Canada (*Invited Oral presentation*)

Sierra-Flores, R., Davie, A. & Migaud, H. (2011). Noise as an acute stressor in Atlantic cod juveniles. European Aquaculture Society Conference, Rhodes, Greece. (*Oral presentation*)

LARVITA Fish larvae training school. (2010) LARVANET EU-COST. Gambelas/Faro, Algarve, Portugal. (*Training course)*

Awards

Second place: Best oral presentation. Institute of Aquaculture, University of Stirling PhD Conference 24th October 2012. Stirling, Scotland.

"**Novartis Young Scientist Award 2012**", 23rd – 24th May 2012. Aviemore, Scotland. (Invitation to the opening of the new research facilities at Victoria, PEI, Canada; incl. all travel expenses).

EU E-COST LarvaNet: LARVITA Fish larvae training course 22nd – 26th November 2010. (All travel expenses).