

**Involvement of kisspeptin and Melatonin in the
seasonal entrainment of reproduction in
European sea bass (*Dicentrarchus labrax*)**

RANIA FAHMY KHEDR ISMAIL

JULY 2011



A THESIS SUBMITTED TO THE DEGREE OF DOCTOR OF PHILOSOPHY

Institute of Aquaculture

University of Stirling

Declaration

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

Candidate name:

Signature:

Date:

Supervisor name:

Signature:

Date:

Abstract

Aquaculture is an essential developing sector for world food production however one of the major bottlenecks for the sustainability of the aquaculture industry is the ability to control fish reproduction in captivity and to produce high quality seeds. European sea bass is a one of most commercially important species for the European fish farming industry. If broodstock management under captivity is well established, problems remain in hatcheries where survival can be low and deformity prevalence high as well as in on growing sites where fish reach puberty early especially with skewed sex ratio towards males. Sea bass displays strong seasonality in its physiology and is therefore an excellent candidate for the study of the photo-neuroendocrine control of reproduction and growth. The overall aims of this thesis were to better understand the molecular and endocrine drivers that control the Brain-Pituitary-Gonad axis in repeat spawner sea bass, and expand our knowledge of sea bass light and temperature regulation of melatonin production. First, this PhD project investigated the seasonal expression of kisspeptin, GnRH and gonadotropin genes in relation to the gonadal development throughout a reproductive cycle in male repeat spawning sea bass (Chapter 3). A partial sequence for the receptor *kissr4* was isolated and described showing similarity to all other teleost species sequences available to date. QPCR molecular assays were validated to measure the expression of a suite of genes along the BPG axis including kisspeptin related genes (*Kiss1* and *Kiss2* and its receptor *kissr4*) over a full reproductive cycle (12 months) in adult male European sea bass. Brain Kisspeptin mRNA expression levels (*kiss1*, *2* and *kissr4*) showed clear seasonal profiles and correlated well to other BPG markers (*GnRHs*, *fsh β* and *lh β*), supporting a possible involvement of kisspeptin genes in the seasonal control of reproduction in repeat

spawning sea bass. Moreover, clear seasonal patterns were observed for expression of the genes encoding for pituitary mRNA expression of *lhβ* and *fshβ*, with a significant correlation between expression of both subunits and GSI and steroids levels. However, no clear seasonal profiles in brain GnRHs gene expression were observed with the exception to some peaks in *GnRH1* and *GnRH2*. The second part of this PhD project investigated the potential direct effect of the two kisspeptin core peptides (*kiss1* and *kiss2*) on the pituitary gonadotropin gene expression (Chapter 4). The aim of this work was to better understand the mechanism by which kisspeptin acts on the BPG axis. This was done by testing the kisspeptin decapeptide core sequences on the *lhβ* and *fshβ* transcript expression in primary culture of sea bass pituitary cells using QPCR technique. The findings, as a whole, provided evidence that kisspeptin can act directly on the pituitary gonadotroph cells and modulate *fshβ* and *lhβ* mRNA expression in sea bass although effects were limited and not uniform. Of note, *kissr4* gene expression was also detected in the sea bass pituitary. The third part of this PhD project focused on the effects of environmental signals (photoperiod and temperature) on melatonin production (Chapter 5). Environmental manipulation is routinely used in the aquaculture industry with the purpose of enhancing growth and manipulating the timing of reproduction in seasonal fish species like sea bass. Melatonin, known as the light perception and time keeping hormone, has been suggested to play key roles in the synchronisation of most physiological functions in vertebrates, although the mechanisms by which melatonin controls reproduction, growth and behaviour are still not fully understood in fish. The studies performed aimed to determine the synergistic effects of both temperature and photoperiod on the daily phase and amplitudinal changes in melatonin production through both *in vivo* and *in vitro* trials. The results

confirmed the diel melatonin rhythm in sea bass as previously reported in many teleost species with “high at night” and “low at day” melatonin profiles. Temperature showed clear effects on the amplitude of the melatonin production under both *in vivo* and *in vitro* conditions for both long day and short day photoperiods. Furthermore, no endogenous melatonin production was found under constant darkness in both *in vivo* and *in vitro* conditions. These results suggested a lack of intrapineal (or located elsewhere such as retina and/or deep brain) oscillators in sea bass, contrasting with previous reports. These results further enhance our knowledge of light perception and circadian rhythmicity in sea bass, while the circadian system remains to be characterised in sea bass and teleosts as a whole.

Overall, this doctoral work broadened our understanding on the photoneuroendocrine control of reproduction in a seasonal fish species, sea bass. New knowledge gained and tools developed from this work should help to develop/optimize husbandry techniques for the sea bass farming industry with the view to increase production and profitability and thus promoting the sustainable expansion of the sea bass aquaculture in Europe. It has also the potential to help the fishery sector in the modelling of wild sea bass populations.

Acknowledgments

I would like to gratefully acknowledge the source of funding that made my PhD research possible. I was funded by a governmental scholarship from the Ministry of Higher Education and State for Scientific Research in Egypt.

I would like to give my sincere thanks to my principal supervisor, Dr. Herve Migaud, for his supervision, guidance, help and for the thorough revision of my thesis. Dr. Andrew Davie has been an excellent second supervisor throughout the time of the present research. He was always eager to help when I encountered difficulties. I really think this work could have not been the same without their guidance and motivation.

My sincere thanks are also due to Dr. John Taylor for his sincere help and essential lab training, statistical guidance and constructive comments. A very special 'Thank You' goes to Mrs. Fiona Muir, for her effective help and guidance during the time of the *in vitro* experiment.

The invaluable cooperation from the staff at Machrihanish Marine Environmental Research Laboratory was highly appreciated.

The hard times I faced while carrying out this research are heartily memorised and deeply appreciated, as these have added a lot to my practical experience. My stay in Scotland has been made enjoyable due to a lot of friends at the Institute of Aquaculture, University of Stirling. My warm and sincere thanks go to Eric Leclercq, Jan Heumann, Matteo Minghetti, Sean Monaghan, Sophie Fridman, and to all others with whom I shared hours in the office. A sincere and a very special 'Thank You' goes to Carlos Cristian Martinez for his effective help during the initial experimental steps of this research, to Mairi Cowan for her sincere cooperation during the sampling process and

in memory of hours on the road to and from Machrihanish, and finally to Amy Rajae for her continuous support and encouragement.

Above all, I feel a deep sense of gratitude to my parents and my sisters in Egypt. During more than four years of work my family gave me endless support and encouragement in order to attain, write and finish this research. Finally special thanks must go to my husband, Tarek, for his support and encouragement and help during the whole journey of my PhD specially the final stages of this work.

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List of Abbreviations

11-KT	11-ketotestosterone	kiss	Kisspeptin
AANAT	Arylalkylamine N-acetyltransferase	kissr	Kisspeptin receptor
ANOVA	Analysis of variance	L	Light
BPG	Brain-pituitary-gonad	LH	Luteinising hormone
cAMP	Cyclic AMP	LL	Continuous illumination
CSF	Cerebrospinal fluid	LTN	Lateral tuberal nucleus
cm	Centimetre	m	Meter
D	Dark	ml	Millilitre
DA	Dopamine	mm	Millimetre
DD	Continuous darkness	NPY	Neuropeptide Y
e.g.	For example	PACAP	Pituitary adenylate cyclase activating peptide
EFSA	European Food Safety Authority	POA	Preoptic area
FAO	Food & Agriculture organization	PNES	Photo neuro endocrine system
FSH	Follicle stimulating hormone	PNX	Pinelectomy
g	Gram	PRL	Prolactin
GABA	Gamma aminobutyric acid	PT	Pars tuberalis
GHRH	Growth hormone-releasing hormone	RHT	Retino-hypothalamic tract
GnRH	Gonadotropin-releasing hormone	RIA	Radioimmunoassay
HIOMT	Hydroxyindole-O-methyltransferase	SCN	Suprachiasmatic nuclei of the hypothalamus
hrs	Hours	TH	Tyrosine hydroxylase
IoA	Institute of Aquaculture	TPHO	Tryptophan hydroxylase
kg	Kilogramme	VTN	Ventromedial thalamic nucleus

List of Species

African catfish	<i>Clarias gariepinus</i>	Japanese flounder	<i>Paralichthys olivaceus</i>
Arctic lamprey	<i>Lampetra japonica</i>	Masu salmon	<i>Oncorhynchus masou</i>
Atlantic cod	<i>Gadus morhua</i>	Medaka	<i>Oryzias latipes</i>
Atlantic croaker	<i>Micropogonias undulatus</i>	Nile tilapia	<i>Oreochromis niloticus</i>
Atlantic halibut	<i>Hippoglossus hippoglossus</i>	orange-spotted grouper	<i>Epinephelus coioides</i>
Atlantic salmon	<i>Salmon solar</i>	Perjerrey	<i>Odontesthes bonariensis</i>
Ayu	<i>Plecoglossus altivelis</i>	Pike	<i>Esox lucius</i>
Barfin flounder	<i>Verasper moseri</i>	Pufferfish	<i>Fugu rubipe</i>
Bluefin tuna	<i>Thunnus maccoyii</i>	Rainbow trout	<i>Oncorhynchus mykiss</i>
Chub mackerel	<i>Scomber japonicus</i>	Red seabream	<i>Pagrus major</i>
Cobia	<i>Rachycentron canadum</i>	Seabream	<i>Sparus aurata</i>
Common carp	<i>Cyprinus carpio</i>	Senegalese sole	<i>Solea senegalensis</i>
Eurasian perch	<i>Perca fluviatilis</i>	Sockeye salmon	<i>Oncorhynchus nerka</i>
European eel	<i>Anguilla anguilla</i>	Spotted green pufferfish	<i>Tetraodon nigroviridis</i>
European sea bass	<i>Dicentrarchus labrax</i>	Striped bass	<i>Morone saxatilis</i>
Fathead minnow	<i>Pimephales promelas</i>	Stickleback	<i>Gasterosteus aculeatus</i>
Golden rabbitfish	<i>Siganus guttatus</i>	White sucker	<i>Catostomus commersoni</i>
Goldfish	<i>Carassius auratus</i>	Zebrafish	<i>Danio rerio</i>
Grass puffer	<i>Takifugu niphobles</i>		
Grass rockfish	<i>Sebastes rastrelliger</i>		
Grey mullet	<i>Mugil cephalus</i>		

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CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

1.1. Overview of sea bass life history and commercial exploitation

1.1.1. Taxonomy of European sea bass

European sea bass (*Dicentrarchus labrax*) belongs to the family Moronidae within the order of the Perciformes which contains about 40 % of all bony species (Nelson, 2006). The Perciformes order includes 18 sub-orders, the largest of which is sub-order Percoidei with its three superfamilies Percoidea, Cirrhitioidea and Cepoloidea. The super family Percoidea itself is the largest amongst the three with sixty nine families including the family Moronidae (temperate basses). European sea bass represents a major fisheries and aquaculture species in the Mediterranean, the European Atlantic coasts and North Africa (Pickett & Pawson 1994; Kuhl *et al.*, 2010). The taxonomy of this species may be detailed as follow (Integrated Taxonomic Information System, www.itis.gov, 2006):

Kingdom: Animalia

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Perciformes

Suborder: Percoidei

Superfamily: Percoidea

Family: Moronidae (temperate basses)

Genus: *Dicentrarchus*

Species: *D. labrax*

1.1.2. Life cycle

Like many fish at higher latitudes, the European sea bass life cycle has a seasonal pattern, with annual batch spawning typically occurring during winter months from December to March in the Mediterranean sea, under low temperature (12-14 °C) and short day length (Barnabé 1980; Carrillo *et al.*, 1995), while spawning can be up to June in the Atlantic Ocean (Haffray *et al.*, 2007). Male spermiation lasts longer than spawning in females (Prat *et al.*, 1999). The female produces 200,000 eggs per kg body weight on average. Eggs are spherical with diameter ranging between 1.2 and 1.4 mm (Haffray *et al.*, 2007). For the aquaculture industry, the egg quality is a key factor to obtain high survival rates. Many researches have focused on the many factors which control the egg quality: nutrition, fatty acid content, egg size, buoyancy, regular shape of vitellus, regular cell divisions, hatching rates and the biochemical composition (Bromage *et al.*, 1992; Carrillo *et al.*, 1995; Bruce *et al.*, 1999). The newly hatched larva is pelagic and measures 3.3 – 4.0 mm long. The larval stage extends between day 6 and day 80 (Marino *et al.*, 1993). By day 90 a definite sea bass juvenile morphology is fulfilled. Often in the culture industry, this is the stage when the transport from hatchery tanks to on-growing facilities is possible. In the wild, bass under 30 cm spend most of the year in or near estuaries close inshore and move into deeper water as they grow (Pickett & Pawson 1994). An adult sea bass is classified as carnivorous in the wild, feeding on molluscs, shrimps, worms and fish. It may reach 1 m in length or more and 15 kg in total weight. Adult sea bass are commonly found in estuarine and costal zones in summer, while it returns back in winter to deeper warm open water where it can be found to a depth up to 100 m (Tortonese, 1984; Pickett & Pawson 1994). In most teleosts, gonadal differentiation progresses down a distinct developmental pathway to

yield a direct and complete differentiation into a female or male phenotype which remains throughout the lifetime of the species. Such species are labelled gonochoristic (Nakamura *et al.*, 1998; Penman & Piferrer, 2008). Such a gender system is found in sea bass with females growing faster and attaining larger size than males (Carrillo *et al.*, 1995; Saillant *et al.*, 2001). Under natural conditions in the Mediterranean sea, sea bass males reach sexual maturation at the age of 2 years, while females attain maturation at 3 years of age (Zanuy *et al.*, 2001).

1.1.3. Geographical distribution and Commercial exploitation of sea bass

Sea bass mainly inhabits littoral zones where food is abundant. It is usually found along rocky coasts from the North-eastern Atlantic close to Norway (60 °N) south to Western Sahara along the coasts of Senegal (30 °N) and is distributed, as well, throughout the Mediterranean and Black seas (Haffray *et al.*, 2007; EFSA, 2008). The species is eurythermal and euryhaline, able to live in a temperature ranging from 2 to 32 °C and in fresh as well as saline water up to 50 ppm (EFSA, 2008). A map showing the global distribution of wild sea bass is shown in Figure 1.1.



Figure 1.1. Global distribution of European sea bass

(http://en.wikipedia.org/wiki/File:Dicentrarchus_labrax_map.png)

From an economical point of view, sea bass is a fish with high commercial value both from capture from wild stocks and from aquaculture production. The fish has been introduced for aquaculture purpose in many countries, e.g. Iceland, Israel, Oman and the United Arab Emirates (Haffray *et al.*, 2007; EFSA, 2008). In the year 2009, the wild capture of sea bass exceeded 11,900 tonnes (FAO, 2011). Regarding aquaculture production, France and Italy were the pioneers of reliable mass production of sea bass in the late 1960s and by the late 1970s these techniques had been dispersed and developed in most Mediterranean countries (FAO, 2011). European sea bass was one of the first marine non-salmonid species to be commercially cultured in Europe and at present is a very important cultured species. The aquaculture production (mainly from cage farming), harvested in 2009 was 113,653 tonnes, valued at US\$ 673,946,000 (FAO, 2011). The most significant producers in the Mediterranean are Greece, Turkey, Italy and Spain (FAO, 2011) as shown in Figure 1.2.

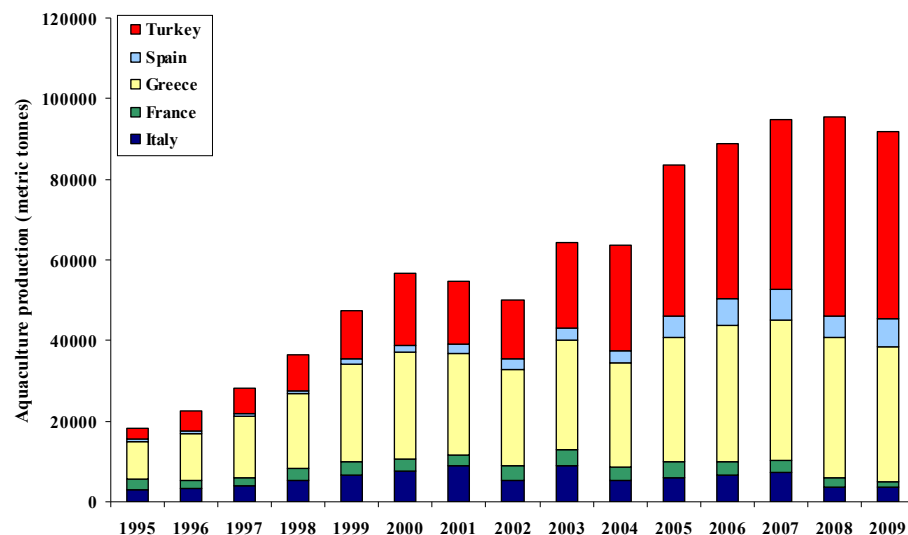


Figure 1.2. Marine Aquaculture production of the most significant producer countries European sea bass in the Mediterranean from 1995-2009 (FAO, 2011)

Accumulation of scientific knowledge on the biology, reproduction, and nutrition of sea bass in addition to the large economical investments in sea bass farming have raised the sea bass production considerably in the past two decades (Zanuy *et al.*, 2001). Despite the large industrial production of European sea bass, problems persist which negatively affect its productivity and profitability under farming conditions, which still need to be solved. In many farms located in the Mediterranean sea, the majority of larval and juvenile sea bass are reared at 19-22 °C instead of the more typical spawning temperature (~ 14 °C). This results in a male-biased sex ratio which can be as high as 100 % due to the temperature sensitive sex determination mechanism present in the species (Piferrer *et al.*, 2005). Sea bass show a clear sex-dimorphism in growth, with females growing faster and attaining larger size than males (Carrillo *et al.*, 1995; Saillant *et al.*, 2001). Females reach puberty at 3 years of age, one year after attaining marketable size, while puberty in males usually appears in the second year of life at the same time of harvesting (Zanuy *et al.*, 2001). In males, puberty is associated with growth rate delay and causes a limitation in the production as males must be harvested at smaller size or kept for longer time than females. Precocious maturation can also be observed in male sea bass, under intensive culture conditions where a considerable proportion of males mature precociously (20-30 %) around the first year of age (Carrillo *et al.*, 1999; Zanuy *et al.*, 2001). Therefore, the prevention of maturation prior to the attainment of suitable harvest size during on-growing has been identified as a principal production bottleneck. Different strategies have been taken to overcome culture problems, based on clarifying the environmental and endogenous regulation of sea bass reproduction and puberty. The use of photoperiod manipulations was successful in the prevention or delay of male first maturation (Rodriguez *et al.*, 2001;

Begtashi *et al.*, 2004; Felip *et al.*, 2008). The components and the mechanisms implicated in sex determination and differentiation in European sea bass including genetic, endocrine and environmental factors are matter of investigation to solve the culture problems (see review by Piferrer *et al.*, 2005).

1.2. Environmental control of physiology in fish

Most of the main physiological and behavioural functions in all living organisms are entrained by environmental fluctuations such as daily and seasonal changes in day length, temperature, salinity, rainfall and food availability (Gerkema, 1992). Circadian rhythms (cycling on approximately 24 hours) play a crucial role in the molecular and neuroendocrine regulation of physiology and behaviour such as seasonal reproduction, feeding, locomotor activity and migration in fish (Gerkema, 1992; Boujard & Leatherland, 1992; Foster & Kreitzman, 2005).

1.2.1. Photoperiod

There are a number of important environmental factors showing daily and annual variations like temperature, rainfall, lunar phase and food supplies that could entrain the biological rhythms of living organisms. But in temperate animals like sea bass, the seasonally changing pattern of day length (photoperiod) remains the most reliable and important signal for timing of many physiological events especially reproduction, as photoperiod represents the most predictable environmental signal being constant from year to year and reflecting seasonality (Migaud *et al.*, 2010).

Photoperiod manipulations are commonly used in the culture of temperate fish species to enhance growth performances, suppress or delay puberty and manipulate spawning windows. Both the daily and annual rhythms of key hormones seem to be of

critical importance to attain normal spawning at the appropriate time of the year for the optimal survival of the progeny (Migaud *et al.*, 2010; Taranger *et al.*, 2010). In Atlantic salmon (*Salmon salar*), photoperiodic regimes are used throughout the production cycle to alter the timing of spawning (Bromage *et al.*, 2001; Pankhurst & Porter, 2003) or smoltification (Duston & Saunders, 1992; Berrill *et al.*, 2003), or to suppress early maturation (Taranger *et al.*, 1998; Endal *et al.*, 2000). Importantly, the manipulation of these physiological events is restricted to 'windows of opportunity' during which photoperiod is acting as the main environmental cue (Bromage *et al.*, 2001; Migaud *et al.*, 2010). As such, the transition from a short (winter) to long (summer) day photoperiod is the signal used to entrain sexual development in Atlantic salmon while a long to short photoperiod shift is the main driver of smoltification. In other salmonid species such as masu salmon (*Oncorhynchus masou*) male testicular maturation can be accelerated by short days (8 Light (L): 16 Dark (D)) and delayed by long days (16L: 8D) (Amano *et al.*, 2000). These regulatory signals differ between temperate species, reflecting their different life cycle strategies. In Atlantic cod (*Gadus morhua*), it is the decreasing daylength that initiates reproduction and therefore the shift from long to short photoperiod is used to entrain sexual maturation (Davie *et al.*, 2007a). These authors proposed that the window of opportunity is opened by decreasing photoperiod whereas the use of continuous illumination (LL) closes that window.

In European sea bass, as reproduction is in winter and decreasing photoperiod is the environmental signal to recruit fish into sexual maturation (Rodriguez *et al.*, 2001), using extended photoperiod (application of simulated natural photoperiod of complete one year for 18 months instead of 12 months) can advance the onset of puberty in male sea bass (Rodriguez *et al.*, 2001) while constant long photoperiod (Rodriguez *et al.*,

2001) or LL (Begtashi *et al.*, 2004, Felip *et al.*, 2008) reduce or fully arrest maturation (Bayarri *et al.*, 2009). Applications of long photoperiod (18L: 6D) for two years on immature male sea bass delay timing of first spermiation and enhance growth (Carrillo *et al.*, 2010). Also under LL the gene expression of the of pituitary gonadotropin subunits is altered by the light regime during the sexual maturation of male sea bass (Rodriguez *et al.*, 2005; Felip *et al.*, 2008). In mature female sea bass, short days (9L: 15D) advanced the spawning time while long days (15L: 9D) delayed maturation and spawning time without any adverse effects on the brood stock fecundity or egg and fry quality and survival (Carrillo *et al.*, 1989; Zanuy *et al.*, 1995 ; Prat *et al.*, 1999). Falcon *et al.* (2010) summarised the effects of photoperiod on entraining the existing endogenous rhythms of reproductive hormones that affect spawning time and gonadal development in sea bass. The critical photo-sensitive period at which decreasing photoperiod occurs (photolabile period) is from August to October (Carrillo *et al.*, 2009). The specific change on the direction of the photoperiod is critical for entraining the existing endogenous rhythms of reproduction (Carrillo *et al.*, 1993; 1995); it also affects the daily rhythms of plasma melatonin (Bayarri *et al.*, 2010) and of LH and 11-Ketotestosterone (11-KT) (Bayarri *et al.*, 2009). The annual rhythms of gonadotropin expression and plasma levels of sex steroids were also affected by the photoperiod (Rodriguez *et al.*, 2005). However, LL superimposition over the natural autumnal decreasing photoperiod is very effective in suppressing the daily melatonin rhythms (Bayarri *et al.*, 2010), altering the phase of LH daily plasma levels pattern (Bayarri *et al.*, 2009) and provoking a significant depletion of mRNA levels of the gonadotropin sub-units and a significant reduction of plasma levels of 11-KT during the spawning time (Rodriguez *et al.*, 2005) (Fig. 1.3). The presence of daily or annual hormonal

rhythms thus seems critical to develop and maintain the reproductive function in fish (Falcon *et al.*, 2010). These studies clearly demonstrate that photoperiod plays important roles in the entrainment and control of maturation and growth performance in temperate species. Although the amplitude of seasonal variations can be weak in tropical and subtropical latitudes, photoperiod remains a key signal.

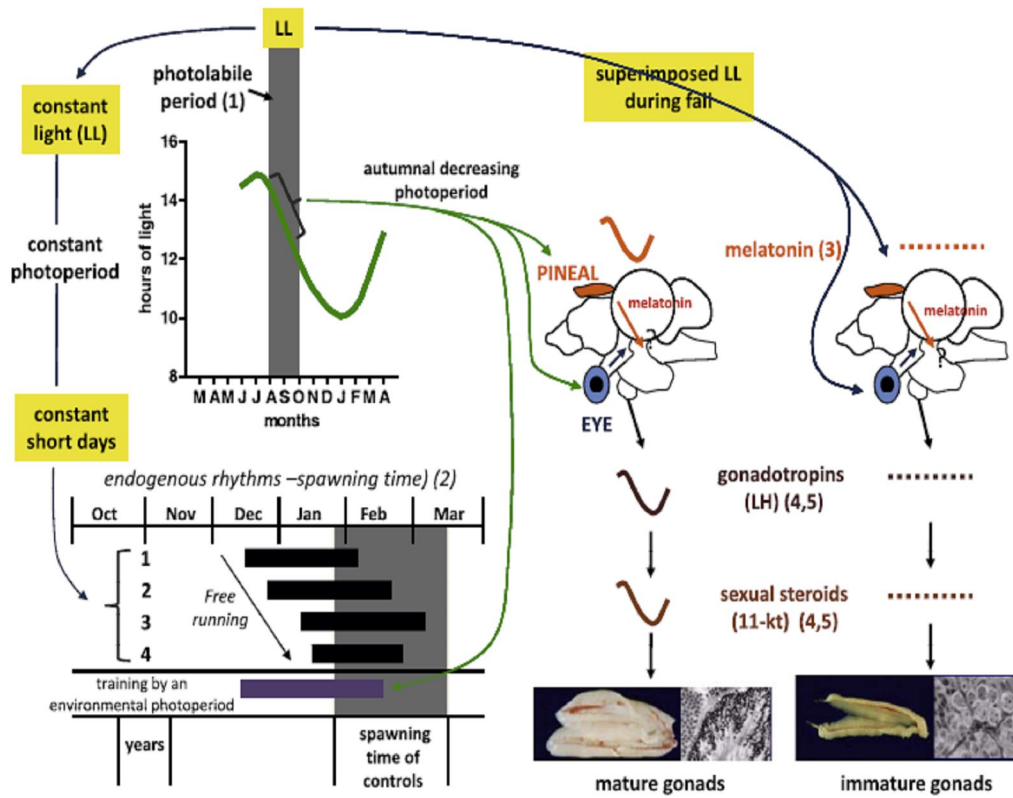


Figure 1.3. Effects of constant photoperiods on the rhythms of reproductive hormones that affect spawning time and gonadal development in sea bass. Numbers refer to the references from which the present figure is constructed, (1, Carrillo *et al.*, 2009),(2, Carrillo *et al.*, 1993; 1995), (3, Bayarri *et al.*, 2010), (4, Bayarri *et al.*, 2009),(5, Rodriguez *et al.*, 2005). Discontinuous line indicates the absence or the alteration of the daily rhythms (From Falcon *et al.*, 2010)

1.2.2 Temperature

In temperate regions, temperature is also an important cue in regulating reproductive cycles in many springs spawning species (Migaud *et al.*, 2010). In sea bass (Zanuy *et al.*, 1986; Prat *et al.*, 1999), striped bass *Morone saxatilis* (Clark *et al.*, 2005) and Eurasian perch *Perca fluviatilis* (Migaud *et al.*, 2004) the combined reduction in day-length associated with decreasing water temperature are suspected to be required for maturation initiation and successful spawning. In tropical and sub-tropical species temperature has a proximate role in gonadal development, whereas thermal manipulations can be used in controlling spawning time (Bromage *et al.*, 2001). Maturation and spawning of carps and other cyprinids, and many other tropical and sub-tropical species, are cued by temperature (Bromage *et al.*, 2001). In a study performed in Nile tilapia, *Oreochromis niloticus*, temperatures above 20 °C were required for reproduction stimulation, while high temperature (above 30 °C) affected spawning activity, egg quality and hatching success (Rana, 1988). In salmonids, temperature plays only a modulating role by affecting the metabolic processes of gametogenesis (e.g. cellular metabolism, gene expression and protein activity) (Bromage *et al.*, 2001; Wang *et al.*, 2010), while photoperiod is the only proximate factor to drive the reproductive cycle (Wang *et al.*, 2010).

1.2.3. Food availability

Food availability is one of the important environmental factors that affect the organism's survival and ability to successfully initiate a reproductive cycle following the correct environmental signal within the right time. As in mammals, thresholds for factors like growth rate and energy storage must be surpassed to permit the development of sexual maturation as has been observed in salmonids (Thorpe *et al.*,

1998; Taranger *et al.*, 1999), whereas individuals assess themselves on the basis of whole body lipid. Feeding larger rations or high energy diets is known to increase fecundity (Shearer & Swanson, 2000), while feed deprivation reduce both fecundity and maturation rates (Bromage *et al.*, 1992). Different animals developed several systems to adapt feeding time with different physiological variables and behavioural activities (Boulos & Terman, 1980). Furthermore, endocrine signals like growth hormone, insulin-like growth factor, leptin and thyroid hormone have significant roles in conveying growth and energetic information to the Brain-Pituitary-Gonadal axis (BPG) to interact with gonadotropin regulation (Migaud *et al.*, 2010), as gonadotropins are central to the BPG axis (Zohar *et al.*, 2010).

1.2.4. Other factors

Other environmental factors, such as salinity, lunar/tidal cycles, water quality, rainfall, current and stress, that do not act as proximate cues can nonetheless contribute and impact on growth and reproductive performance in fish (Ross, 2000; Pankhurst & Porter, 2003). In seasonal species these factors can be involved in the timing and synchronizing of the final stage of reproductive development and spawning time (Pankhurst & Porter, 2003).

1.3. The circadian axis: How light entrains physiology?

The daily and annual response of the organism to the environmental variations can be just passive in some cases, while in most cases these rhythms are driven through internal clocks entrained to a 24 hours (circadian rhythms) or annual (cicannual rhythms) cycle. A circadian light system consists of all the different components (structures and pathways) by which light enters the organism and is transformed into a

biological time signal (Foster & Hankins, 2002). Melatonin is one of the most important output signals of vertebrate circadian system which is mainly produced by the pineal organ (Falcon *et al.*, 2007). The daily pattern of pineal melatonin secretion is conserved across a range of vertebrates, in which the melatonin is produced and released into the blood circulation and cerebrospinal fluid (CSF) at night (Tricoire *et al.*, 2002).

It is important to consider that the organization of the circadian system has evolved across vertebrates. Mammals have the most complex and evolved form of organisation, where the photic information is perceived through the eye and transmitted through a retino-hypothalamic tract (RHT) to the suprachiasmatic nuclei of the hypothalamus (SCN) in the brain where the mammalian master clock is present (Simonneaux & Ribelayga, 2003). In response to the light/ dark signals perceived by the eyes, the master clock in the SCN stimulates and entrains the appropriate mechanisms and peripheral oscillators that will regulate melatonin synthesis from the pineal (Jin *et al.*, 1999; Taghert, 2001; Foster & Hankins, 2002; Schomerus & Korf, 2005). In the mammalian model the pineal gland lost its direct photosensitivity and is enslaved to the master oscillator. The circulating melatonin can both act as a feedback mechanism to the SCN as well as act on the pars tuberalis of the pituitary and other brain areas to modulate seasonal neuroendocrine functions (Falcon *et al.*, 2007). Figure 1.4 a (i) presents a simple schematic diagram of the mammalian photoperiodic and circadian control of neuroendocrine functions.

In teleosts, the structure and function of the circadian system is less clear with there being extensive evidence highlighting the complexity of the light perception system which includes a range of potential photoreceptive structures (e.g. retina, pineal gland and deep brain photoreceptors), which convey information both centrally to the

brain and peripherally around the animals physiology by a range of neuroendocrine signals. The circadian axis in fish has been described as being decentralized, as all studied fish species to date have shown independent (directly photosensitive) light perception components (retina, pineal gland), while in mammals both pineal gland and retina form part of a centralized light entrainment organization (Falcon *et al.*, 1989; Falcon *et al.*, 1992; Falcon, 1999; Falcon *et al.*, 2007). There is a strong indication that the control of the pineal activity has changed dramatically during phylogeny, probably as a response to 500 million years of evolution to the diverse environments occupied by vertebrates during that time (Mayer *et al.* 1997; Falcon, 1999). Different specialized structures (e.g. pineal complex, retina, parietal eye, deep brain) and pathways would have thus evolved in vertebrates, although the main basic components e.g. the non-visual photoreceptors are likely to be conserved (Menaker *et al.* 1997; Foster & Hankins 2002; Klein, 2004) (Fig. 1.4 a-ii). The photoneuroendocrine regulation in fish is described in Falcon *et al.* (2007) as light impacts on photoreceptor cells of both pineal organ and retina, enabling synchronisation of their internal molecular clocks. Furthermore, light might also impact on other possible photosensitive and circadian structures in the ventral diencephalon (POA and hypothalamic area; and peripheral organs). In response to the photoperiodic information, the retina and the pineal organ produce two types of rhythmic information: a) The neural information from the retina and pineal organ reach the ventral diencephalon through the RHT and the pineal tracts: this information provides an indication of day length, as well as of subtle variations in ambient illumination. b) The hormonal information is relayed by melatonin, the production of which reflects day length and season. In the retina, melatonin is an autocrine and/or paracrine factor, which is metabolised locally. Pineal melatonin is

released into the cerebrospinal fluid (CSF) and blood, and acts on specific targets through melatonin receptors. In the hypothalamus, melatonin might contribute to synchronising the activities of circadian oscillatory units [SCN and others] (Falcon *et al.*, 2007) (Fig. 1.4 b).

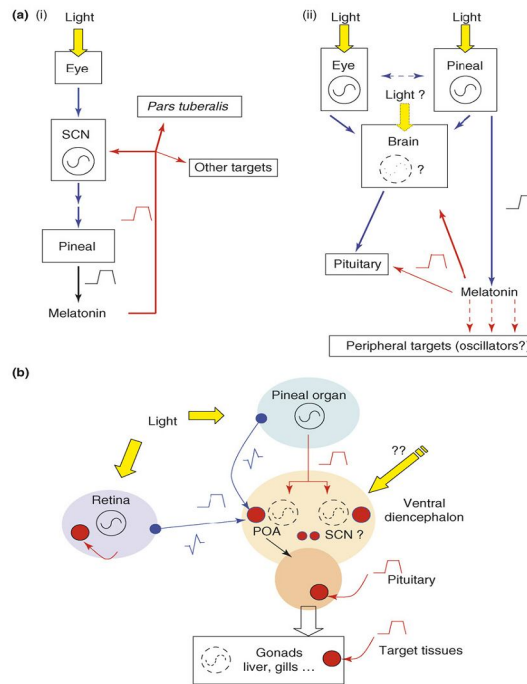


Figure 1.4. Photoperiodic and circadian control of neuroendocrine functions.

(a) Fish versus mammals. In mammals (i) a linear flow leads to the rhythmic production of melatonin. Nonvisual information from the retina reaches the SCN of the hypothalamus through the retinohypothalamic tract (blue arrow). The periodic signals enable synchronizing of the circadian activity of the SCN clocks, which, in turn, impact on the pineal gland through a multisynaptic pathway (blue arrows), thus controlling cyclical melatonin secretion. Melatonin feeds back to the SCN and acts on the pars tuberalis of the pituitary and other brain areas to modulate seasonal neuroendocrine functions. The situation is more complex in fish (ii): the photoneuroendocrine system seems to be organized as a network of independent and interconnected light-sensitive oscillatory units in the retina, the pineal and, perhaps, in the brain. The dashed blue arrow indicates a hypothetical connection. ‘?’ in the brain indicates the hypothetical presence of brain circadian oscillators. (b) Photoneuroendocrine regulation in fish. Light (yellow arrows) impacts on photoreceptor cells of the pineal organ and retina, enabling synchronization of their internal molecular clocks. Light might also impact on other possible photosensitive and circadian structures in the ventral diencephalon (POA and hypothalamic area; yellow arrow with ‘??’) and peripheral organs. In response to the photoperiodic information, the retina and the pineal organ elaborate two types of rhythmic information. The neural information (blue arrows) from the retina and pineal organ reach the ventral diencephalon through the retinohypothalamic and the pineal tracts, respectively. This information provides an indication of day length, as well as of subtle variations in ambient illumination. The hormonal information is relayed by melatonin (red arrows), the production of which reflects day length and season. In the retina, melatonin is an autocrine and/or paracrine factor, which is metabolized locally. Pineal melatonin is released into the cerebrospinal fluid and blood, and acts on specific targets through melatonin receptors (red filled circles). In the hypothalamus, melatonin might contribute to synchronizing the activities of circadian oscillatory units [SCN and others (depicted by ‘?’)] and modulating the production of pituitary gland releasing factors. Melatonin receptors have been identified in areas that impact on pituitary function, including the POA, which also receives nervous input from both the pineal organ and the retina. Melatonin impacts on the pituitary gland itself to modulate the production of hormones. Taken from Falcon et al. (2007)

1.3.1. Melatonin synthesis and regulation

Melatonin was found in the earliest life forms and is present in all organisms studied to date, ranging from bacteria to humans (Conti *et al.*, 2002; Tan *et al.*, 2003). Melatonin was found to be produced mainly by the pineal gland and retina (described below) but also by many other organs and tissues including the gastrointestinal tract (Bubenik & Pang, 1997), skin (Slominski *et al.*, 2005), lymphocytes (Carrillo-Vico *et al.*, 2004) and bone marrow (Conti *et al.*, 2000), suggesting that melatonin is involved in a range of physiological processes. In mammals and humans, melatonin acts as a sleep regulator and was reported to have a role in sleep initiation (Shochat *et al.*, 1997; Zisapel, 2007; Pandi-Perumal *et al.*, 2008). Melatonin has been described to have other roles: it can act as a dopamine release inhibitor from hypothalamus and retina (Zisapel, 2001), it can be involved in the aging process (Reiter *et al.*, 1998), and it can regulate blood pressure (Cavallo *et al.*, 2004; Grossman *et al.*, 2006) and immune response (Carrillo-Vico *et al.*, 2006) among other roles.

Melatonin secretion reflects the length of the day throughout the seasons; hence it is described as a “zeitgeber” or the biological time keeping hormone which entrains circadian (daily) and circannual (seasonal) rhythms in vertebrates (Menaker *et al.*, 1997; Falcon *et al.*, 2006) although direct evidence is scarce in fish. Melatonin represents the main hormonal output of the pineal organ in response to photoperiodic changes and it is involved in the control of daily and seasonal biological rhythms. Daily rhythms include locomotor activity, rest, food intake, vertical migration and shoaling, skin pigmentation, osmoregulation and metabolism, whereas seasonal processes include growth, reproduction and smoltification for migrating salmonids (Falcon *et al.*, 2007).

1.3.1.1. The pineal gland

The pineal complex in teleosts is part of the central nervous system that is formed as an invagination of the diencephalon. In fish, it consists of the pineal and parapineal organs; the parapineal organ remains rudimentary, while the pineal organ grows to form a large rounded body located dorsally to the forebrain below the “pineal window” of the skull where light can penetrate (Ekstrom & Meissl, 1997; 2003). The pineal organ is often differentiated into proximal slender pineal stalk attached to the diencephalon and a distal expanded end-vesicle (Ekstrom & Meissl, 1997). The wall of the pineal organ is formed by unistratified epithelium cells. The pineal epithelium of teleost fish is made of photoreceptor cells, neurons, and ependymal interstitial cells; the photoreceptors produce melatonin that is released into the CSF and blood circulation (Ekstrom & Meissl, 1997; Falcon, 1999). The photoreceptor cells establish synaptic contacts with neurons (ganglion cells) that send their axons to the brain. Therefore, the signals that are transmitted to the brain reflect the response of the photoreceptor cells (Ekstrom & Meissl 1997; Falcon *et al.*, 2010). In most vertebrates, the melatonin synthesised by the pineal organ is regulated by light intensity and reaches its highest levels in complete darkness. Pinealectomy studies have confirmed that the pineal gland is the main source of circulating melatonin found at night in the blood although it has been suggested that melatonin produced in other tissues or organs such as the eyes or even the gut (Bubenik & Pang, 1997) could also enter the blood stream (Porter *et al.* 1996; Ekstrom & Meissl, 1997, 2003; Migaud *et al.*, 2006; Falcon *et al.*, 2010).

1.3.1.2. The retina

The teleost eye has evolved and specialised according to the surrounding environments (Kusmic & Gualtieri, 2000). The fish eyes have visual and non-visual photoreceptors with the ability of circadian entrainment and production of melatonin (Falcon *et al.*, 2003; Foster & Bellingham, 2004). In most vertebrates retinal melatonin is produced in parallel with that of the pineal mainly at night following the cell depolarization (Falcon *et al.*, 1999; Falcon *et al.*, 2007). However, teleost fish show different patterns of retinal melatonin production with zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) displaying a nocturnal melatonin pattern (Cahill, 1996; Iigo *et al.*, 1997a), while other fish species either do not show any rhythm or show peaks at different times of the L/D cycle (Iigo *et al.*, 1997b; Besseau *et al.*, 2006), as in the case of European sea bass, in which the phase of retinal melatonin rhythm changed between different seasons (Garcia-Allegue *et al.*, 2001).

In fish and other non-mammalian vertebrates, it has been suggested that retinal melatonin is not released into the blood circulation due to strong melatonin deacetylase activity (Grace *et al.*, 1991). Retinal melatonin has been suggested to serve local functions, including modulation of neurotransmitter release, retinomotor movement, neuronal electrical activity and act as a local antioxidant (Besseau *et al.*, 2006; Ping *et al.*, 2008). The relationship between pineal and retinal melatonin, as part of the circadian system in different fish species, has been studied by Migaud *et al.* (2007). They proposed at least three different types of organisation: 1) salmonids with a “decentralized” system in which the pineal gland responds directly to light, independently of the eyes; 2) sea bass and cod in which both the eyes and the pineal gland are required to sustain full night-time melatonin production; and 3) tilapia and

catfish in which the pineal gland would not be light sensitive (or only slightly) and the eyes are required to perceive light and inhibit melatonin synthesis during the day.

1.3.2. Melatonin biosynthesis and diel profile

Melatonin is synthesised by photoreceptor cells in the pineal by taking up tryptophan and converting it to serotonin (5-hydroxytryptamine) via two enzymatic steps (Fig. 1.5): the first step is hydroxylation of tryptophan by means of tryptophan hydroxylase (TPHO) producing 5-hydroxytryptophan, which is then decarboxylated by means of the aromatic amino-acid decarboxylase forming serotonin. Melatonin is then synthesized from serotonin through another two steps; first, serotonin is N-acetylated by arylalkylamine N-acetyltransferase (AANAT) into N-acetylserotonin which is then converted into melatonin by the action of hydroxyindole-O-methyltransferase (HIOMT) (Falcon *et al.*, 1999; Falcon *et al.*, 2007; Falcon *et al.*, 2010). The HIOMT and AANAT enzymes have been studied in a range of vertebrates as both enzymes are controlling melatonin synthesis. AANAT is found in all vertebrates and is known to be the melatonin rate-limiting enzyme (Klein *et al.*, 1997; 2002). In mammals, birds and anurans, only one type of AANAT has been found, while for all studied teleosts two AANAT genes are present, probably attributed to genome duplication in early evolution of the teleost lineage (Falcon *et al.*, 2007; Coon & Klein, 2006). The AANAT activity increases at night with elevation in melatonin production, while the daylight produces a proteasomal degradation of the enzyme reflecting termination of melatonin production (Ekstrom & Meissl 1997; Falcon *et al.*, 2010) (Fig. 1.5). On the other hand HIOMT enzyme activity does not show any rhythmic changes and remains steady throughout the LD cycle (Klein *et al.*, 2002). The HIOMT was suggested to be involved in seasonal rather than daily rhythmic oscillations in melatonin production (Ribelayga *et al.*, 2002).

As explained above, the AANAT enzyme in fish has two forms (AANAT-1 and AANAT-2). These forms display tissue specific distribution: AANAT-1 is more expressed in retina and brain, while AANAT-2 is more specific to the pineal in fish (Falcon *et al.*, 2003; Coon & Klein, 2006). Recent findings in teleosts suggested the presence of two AANAT-1 forms (1a and 1b) (Coon *et al.*, 1999; Coon & Klein, 2006, Falcon *et al.*, 2010). At night, photoreceptor depolarization allows accumulation of cyclic AMP (cAMP) and Ca^{2+} entry. Both actions regulate AANAT-2 amount and activity at the cellular level through AANAT-2 protein phosphorylation (Falcon *et al.*, 1999 & 2010) and lead to rising melatonin levels. This mechanism is reversed by light, as the light triggers hyperpolarization of photoreceptors and causes proteasomal proteolysis, leading to AANAT-2 degradation and less melatonin secretion (Falcon *et al.*, 2001&2010), while a minor part of the pineal AANAT-2 protein pool is photo-stable (Falcon *et al.*, 2010). Contrasting to AANAT-2 protein and AANAT-2 enzyme activity, *Aanat2* mRNA is not light sensitive (Coon *et al.*, 1999), and *Aanat2* gene expression is controlled by the clock machinery (Appelbaum *et al.*, 2006).

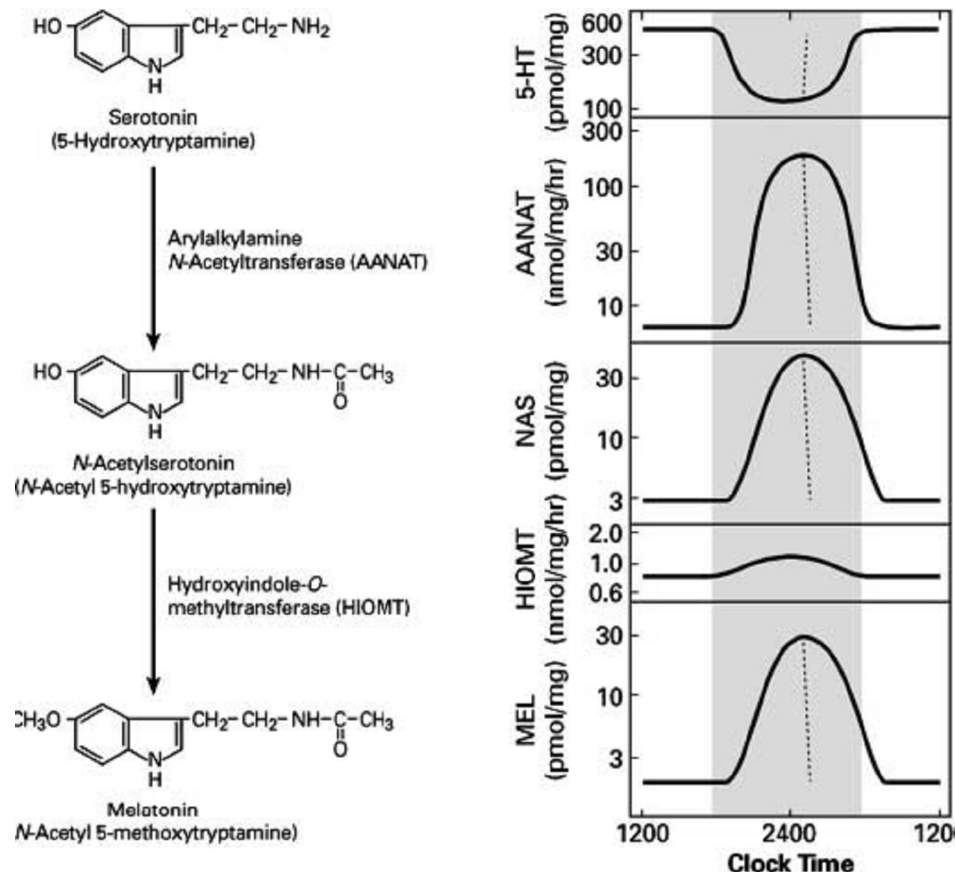


Figure 1.5. Melatonin pathway is shown on the left; changes in pineal levels of the compounds and enzymes are shown on the right (From Klein *et al.*, 2002)

Although a melatonin production rhythm is conserved across all vertebrates (high at night and low during the day), three variant profiles have been identified (Fig. 1.6). The A-type is characterised by a delay of melatonin rise after the start of the dark phase, showing the peak towards the end of the dark phase (Reiter, 1988). This peak type was found in mouse and Syrian hamster as well as in gadoid species (Atlantic cod and haddock) (Reiter, 1988; Porter *et al.*, 2001; Davie *et al.*, 2007b). The B-type profile is characterised by a distinct peak in the middle of the dark phase as found in human and tilapia (Reiter, 1988; Nikaido *et al.*, 2009). The third profile is the C-type; it is the

most common profile in vertebrates, where melatonin rises immediately following the onset of the dark phase to the maximum, which remains high and then falls rapidly to the basal level once the light phase starts. This profile is found in salmonids as well as other vertebrates (Randall *et al.*, 1995; Reiter, 1988).

Although pineal melatonin production is regulated by light intensity in teleosts, the spectral content of the light can also have an impact by modulating the amount of light reaching the pineal photoreceptors depending on the light transmittance properties of the cranial bones (Falcon *et al.*, 2010). The spectral quantity (light intensity) and quality (spectral content) that penetrate the pineal window varies from one species to another (Migaud *et al.*, 2006). Both light intensity and spectral content are subjected to daily variations depending on the time of the day, weather conditions and moon phase (Falcon *et al.*, 2010). Some other internal factors can play a role in the control of melatonin production (Falcon *et al.*, 2007). Temperature can also modulate melatonin production, as temperature acts directly on the pineal organ and regulates AANAT-2 activity as shown in previous studies (Coon *et al.*, 1999; Falcon *et al.*, 1999; Benyassi *et al.*, 2000; Zachmann *et al.*, 1992). Rainbow trout, *Oncorhynchus mykiss*, showed an optimal temperature of 12 °C for pineal AANAT-2 physiological activity (Benyassi *et al.*, 2000), whereas AANAT-2 recorded its highest activity at 20 °C in pike, *Esox lucius* (Coon *et al.*, 1999).

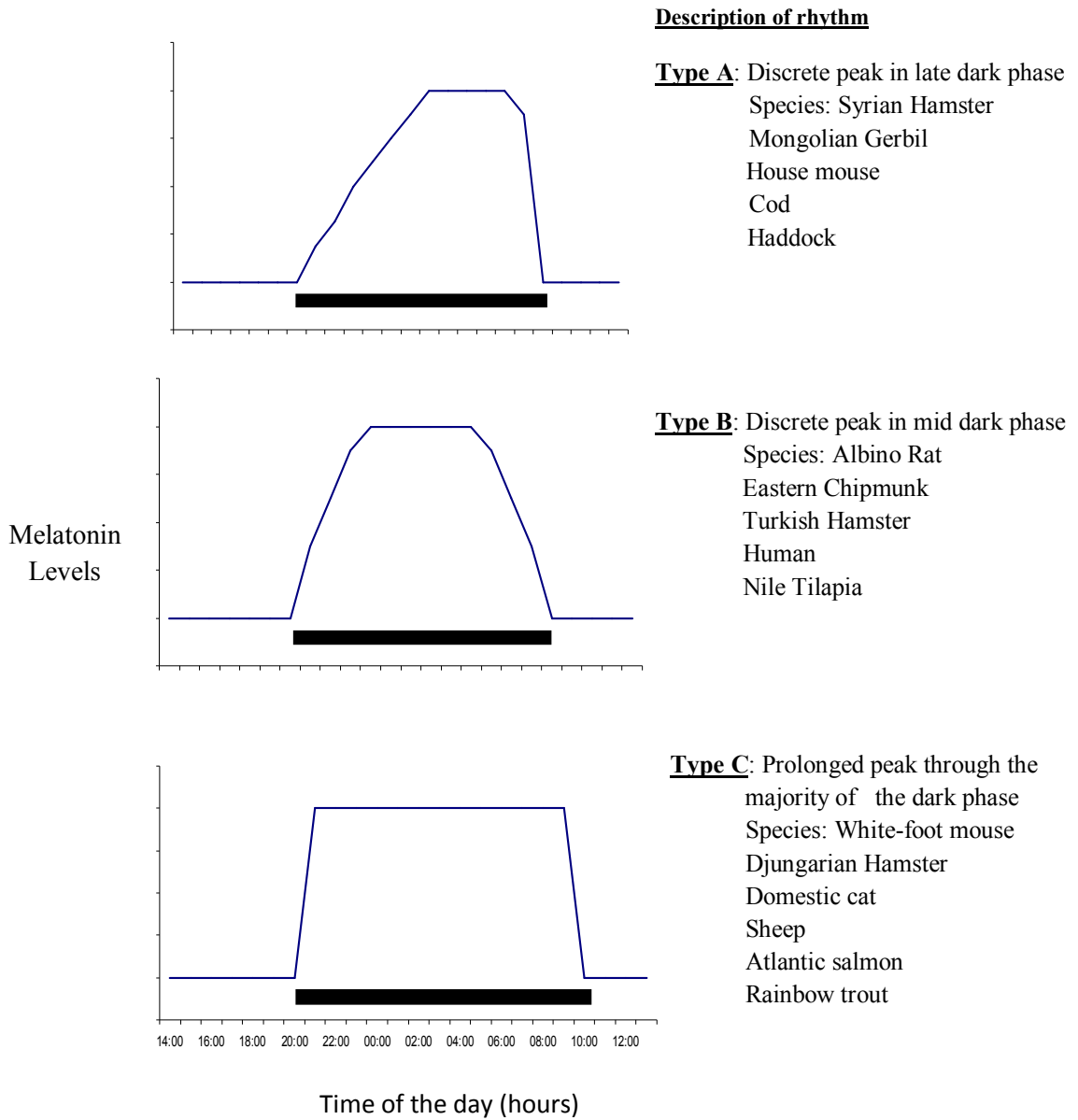


Figure 1.6. Schematic representation of the different melatonin profiles recorded in vertebrates. Examples of species which express such pattern of plasma melatonin for each profile are listed. Horizontal black bar denotes subjective dark period (adapted from Reiter, 1988; Falcon *et al.*, 2010)

The enzymatic activity showed the same response to the temperature when measured from either cultured pineal organ homogenates or the recombinant AANAT-2 (Benyassi *et al.*, 2000). It is therefore thought that the control of melatonin synthesis by the pineal in fish is more complex than first thought and will depend on a multitude of variables including environmental factors, of which light intensity and temperature are the best known, and physiological factors (age, size, reproductive status, stress ...).

Circadian melatonin production in sea bass showed the classical pattern observed in teleost fish: under natural photoperiod, melatonin level was high during night and low during the day (Sanchez-Vasquez *et al.*, 1997; Gracia-Allegue *et al.*, 2001; Bayarri *et al.*, 2010), with type A profile in which the melatonin peak occurred in late dark phase (Migaud *et al.*, 2007). The annual rhythms of melatonin profiles appear to be with large differences in nocturnal melatonin levels (plasma levels from 144 pg/ml in summer to 23 pg/ml in autumn) (Garcia-Allegue *et al.*, 2001). Circadian melatonin rhythm in sea bass has been shown to persist *in vitro* under constant darkness (Bayarri *et al.*, 2004a), while under continuous light, plasma melatonin showed no elevation during the subjective night (Bayarri *et al.*, 2010). In contrast, no persistency of the intrapineal oscillators was found *in vivo* under continuous darkness (DD) conditions; melatonin was high without any significant variation after two days of DD application (Iigo *et al.*, 1997b). Confirmation of the presence or absence of intrapineal oscillators in such seasonal species like sea bass has become important for better understanding the regulatory mechanisms of melatonin production

1.3.3. Melatonin receptors

The first melatonin receptor was cloned from *Xenopus* dermal melanophores (Ebisawa *et al.*, 1994). Thereafter many studies have been conducted and led to the

identification of many receptor subtypes in vertebrates by using both cloning and radioligand techniques. Two mammalian subtypes of G protein coupled melatonin receptors (MT1 and MT2) have been identified (Reppert *et al.*, 1994; Dubocovich & Markowska, 2005). The MT2 receptor has lower affinity for ^{125}I -melatonin while the MT1 receptor shows high affinity for ^{125}I -melatonin. A third low affinity mammalian melatonin receptor (MT3) has been characterized as the enzyme quinone reductase 2, which belongs to a group of reductases involved in the protection against oxidative stress (Barrett *et al.*, 2003; Mailliet *et al.*, 2005). The Mel1c subtype is another high affinity melatonin receptor, which has been identified only in non-mammalian vertebrates (amphibians, birds and fish) (Sugden *et al.*, 1997; Dubocovich *et al.*, 1998; Barrett *et al.*, 2003; Sauzet *et al.*, 2008). Few studies have been conducted on fish melatonin receptors; full length sequences of melatonin receptor have been reported in rainbow trout (MT1), pike (MT2) (Gaildrat & Falcon, 2000), and golden rabbitfish (*Siganus guttatus*) (MT1 and Mel1c) (Park *et al.*, 2007 a, b). In European sea bass three melatonin receptors have been cloned belonging to the MT1, MT2 and Mel1c subtypes (Sauzet *et al.*, 2008). Melatonin receptors display a wide distribution in fish and are associated with areas that receive or integrate information from sensory organs (olfactive bulbs, telencephalon, diencephalon, optic tectum and cerebellum) and are mostly incorporated in areas receiving input from the retina and pineal (Ekstrom & Meissl, 1997; Falcon *et al.*, 2010). Melatonin receptors are also present in the preoptic area (POA) and pituitary gland of pike, rainbow trout and sea bass (Gaildrat *et al.*, 2002; Falcon *et al.*, 2003; Sauzet *et al.*, 2008). Furthermore, melatonin receptors and ^{125}I binding sites have been detected in different peripheral tissues (kidney, gills,

intestine and gonads) (Kulczykowska *et al.*, 2006; Park *et al.*, 2006; Sauzet *et al.*, 2008).

1.3.4. Melatonin and hypothalamic interaction

In mammals, melatonin receptors show a high density in both the supra chiasmatic nucleus (SCN) and the pars tuberalis (PT) of the adenohypophysis in the pituitary, and interact with the molecular clocks (Korf & von Gall, 2006). In fish, photoperiodic and circadian systems are less clear and no SCN-like structure has been identified as yet in the brain (Migaud *et al.*, 2010). Melatonin function in the central nervous system is still to be found in teleosts (Falcon *et al.*, 2007). It has been suggested that melatonin action is mediated through the hypothalamic-pituitary axis in fish (Falcon *et al.*, 2007, 2010). Melatonin hormonal signal would also act on the diencephalic areas, the rostral POA, lateral tuberal nucleus (LTN) and ventromedial thalamic nucleus (VTN) as shown through receptor expression studies (Ekstrom & Vanecek, 1992; Sebert *et al.*, 2008). The POA receives nervous information from both the retina and pineal organ (Ekstrom & Meissl, 1997). The POA and hypothalamic neurons convey dopamine and releasing factors that control pituitary cell activity including pituitary adenylate cyclase activating peptide (PACAP), growth hormone-releasing hormone (GHRH), neuropeptide Y (NPY), and gonadotropin-releasing hormones (GnRHs) (Batten *et al.*, 1999; Montero *et al.*, 2000; Gonzalez-Martinez *et al.*, 2002 a&b). It is also suggested that melatonin might regulate photoneuroendocrine functions by targeting the pituitary gland itself, as the photic information can reach the pituitary directly via melatonin hormone signal and pituitary melatonin receptors (Falcon *et al.*, 2010). The actions of melatonin at the brain and pituitary levels remain to

be found in fish and can be considered as the missing link between light perception regulating melatonin production and circadian / circannual control of physiology.

1.3.5. Evidence for melatonin effects on physiology

The effects of photoperiod on the timing of reproduction are well investigated and provide clear evidence that melatonin is involved in this process (Bromage *et al.*, 2001). The clear definition for melatonin role in reproductive axis was studied by two main approaches; the first is the pinealectomy (PNX); for female rainbow trout pineal removal at the summer solstice resulted in a spawning delay comparing to the controls (Randall *et al.*, 1995) suggesting the importance of pineal for the entrainment of final stage of reproduction, via the decreasing day length and increased daily duration of melatonin secretion (Bromage *et al.*, 2001). While PNX failed to influence the timing of early maturation in male Atlantic salmon (Mayer, 2000), pinealectomized and ophthalectomized ayu (*Plecoglossus altivelis*) were sexually mature under short day photoperiod, while those under long day did not show maturity (Masuda *et al.*, 2005).

The second approach to define melatonin's role in reproduction is the direct effect of melatonin administration. Intraperitoneal injection elicited significant elevations in plasma LH levels during the late-light phase of the day-night in Atlantic croaker, *Micropogonias undulates* with fully developed gonads. In this case, the stimulatory effect of melatonin was dose-dependent over a range of 5–500 ng g⁻¹ body mass (Khan & Thomas, 1996). In addition, low concentrations (0.2 ng ml⁻¹) of melatonin stimulated *in vitro* LH release from pituitary fragments of fish with fully developed gonads, suggesting that melatonin can also stimulate gonadotropin secretion directly at the pituitary level; melatonin injection (1 or 10 ng/g body weight) into the third ventricle in the preoptic anterior hypothalamic area of croaker with fully

developed gonads resulted in an elevation in plasma LH concentrations. In addition, low concentrations (0.2 ng ml^{-1}) of melatonin stimulated *in vitro* LH release from pituitary cells of fish with fully developed gonads, suggesting that melatonin can also act both at POA/hypothalamus and pituitary levels (Khan & Thomas, 1996). This was recently confirmed in the European eel, *Anguilla anguilla*, where melatonin implants induced a decrease of gonadotropin (*lh β* and *fsh β*) mRNA gene expression in the pituitary as well as a reduction of plasma sex steroid levels (11-KT) whereas the treatment increased brain tyrosine hydroxylase (TH) which is the rate limiting enzyme of dopamine production, leading then to a stimulation of the dopaminergic system in the POA (Sebert *et al.*, 2008). The same authors have proposed that one route for melatonin's action is thought to be the dopaminergic system. Interestingly, a similar pathway (i.e. melatonin's action *via* the dopaminergic system) has been proposed in mammals where it was suggested that melatonin can stimulate dopaminergic neurons that then inhibit GnRH and kisspeptin signalling *via* RFRP (RFamide-Related Peptide) expression (Smith *et al.*, 2008a). However, in other species such as mature female common carp (*Cyprinus carpio*), melatonin administration reduced the dopamine levels in the hypothalamus and led to an increase in LH secretion (Popek *et al.*, 2005; 2006). In an *in vitro* study Ribelayga *et al.* (2004) demonstrated that an isolated goldfish retina cultured in continuous darkness, clearly exhibited a circadian rhythm of endogenous dopamine with high values during the subjective day, while this circadian rhythm was abolished by continuous presence of melatonin (with low values equal to the night-time value) or the melatonin antagonist luzindole (but the values were high and equal to the daytime values). The effects of melatonin on other physiological processes in fish were also investigated. As such, fish growth rate was shown to be affected by melatonin

levels in circulation as for the control of food intake and other behavioural rhythms (Falcon *et al.*, 2010). However, again, *in vivo* melatonin studies have given contradictory results with respect to feeding and growth (Falcon *et al.*, 2010). *In vitro* melatonin administration appeared to stimulate growth hormone (GH) production and inhibit prolactin (PRL) release from trout cultured pituitary cells (Falcon *et al.*, 2003). Melatonin has been found to have different effects on behavioural responses. Melatonin administration was shown to have a different action on locomotor activity depending on the lighting conditions and species life cycle (Lopez-Olmeda *et al.*, 2006). Melatonin decreased the locomotor activity in sea bass (Herrero *et al.*, 2007) and zebrafish (Lopez-Olmeda *et al.*, 2006). Melatonin was also found to promote a sleep-like state and influence aging in zebrafish (Zhdanova *et al.*, 2001; 2008).

Overall, all these results point towards one main conclusion: there is no strong case to argue that the photoneuroendocrine system (PNES) in fish is as melatonin-dependent as is the case in mammals. Rather than being a direct driving force for the Brain-Pituitary-Gonad (BPG) axis, melatonin appears to play a more detached regulatory role integrating timing messages with numerous discrete processes. As such, other driving processes must be at work.

1.4. Neuroendocrine control of reproduction

In teleosts, it is the neuroendocrine BPG axis that acts as the master regulator of puberty and adult reproductive cycles (Zohar *et al.*, 2010). This axis is organised around a series of hormones that are produced, released into the blood circulation and act on target tissues within the brain, pituitary, liver and gonads to initiate and control sexual development (Zohar *et al.*, 2010). It is organised around: 1) the hypothalamus of the brain which releases neuropeptides and neurotransmitters which innervate and influence directly 2) the pituitary (gonadotroph cells), which synthesises and releases gonadotropins (follicle stimulating hormone, FSH; luteinising hormone, LH) which are transferred through the bloodstream and stimulate 3) the gonads steroidogenic cells (sertoli cells in testes and follicular cells in ovary) to produce sex steroids (androgens, oestrogens and progestagens) necessary for gametogenesis and positive/negative feedback regulation of reproduction (Fig.1.7). All three major regulators of the BPG axis integrate with growth/energy pathways (e.g. leptin, growth hormone, Igf1) to regulate reproductive processes in synchrony with life stage and the surrounding environment to ensure spawning in favourable conditions (Migaud *et al.*, 2010).

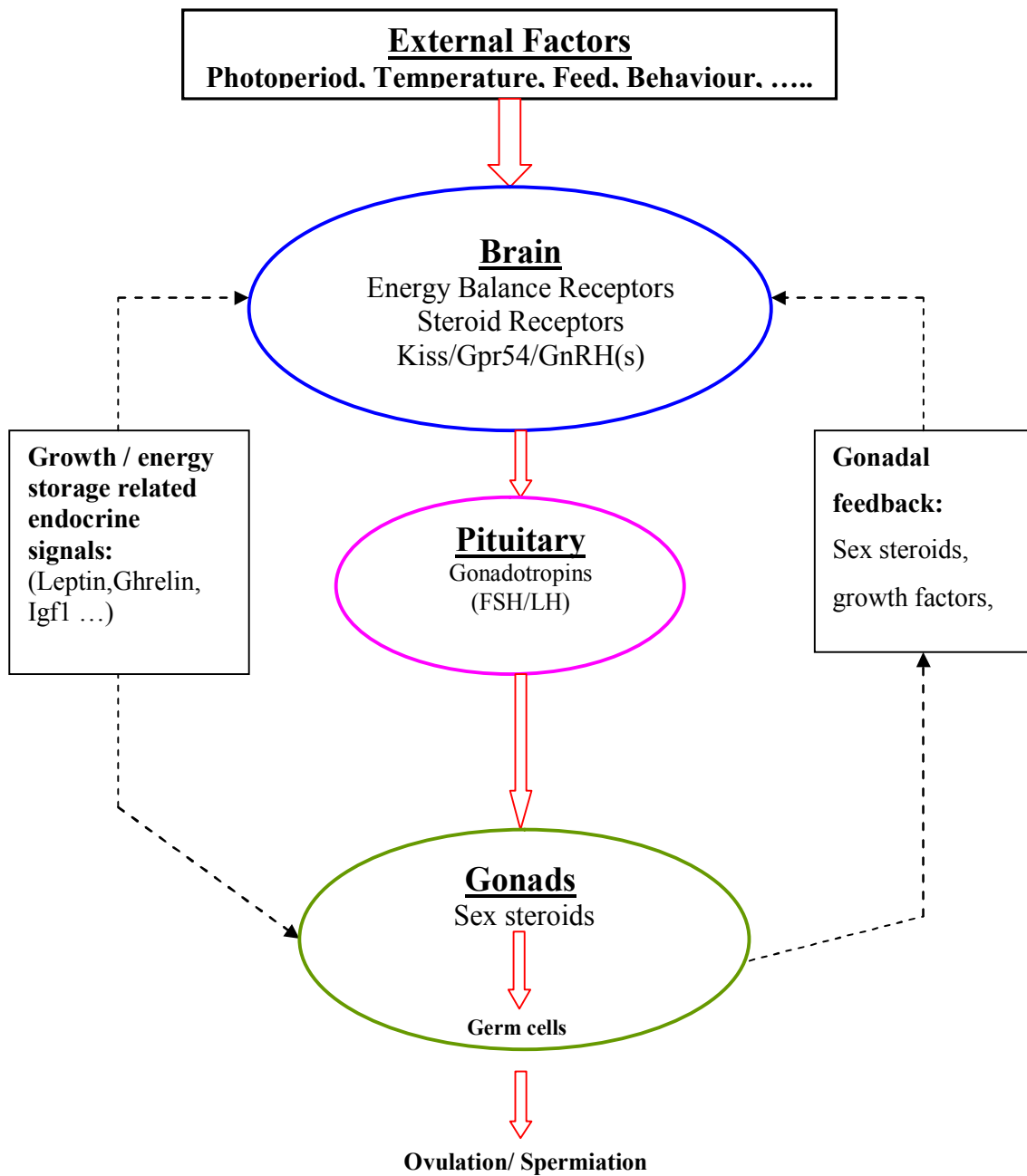


Figure 1.7. Schematic representation of regulatory pathways in the BPG axis during puberty in teleosts (adapted from Migaud *et al.*, 2010; Taranger *et al.*, 2010)

1.4.1. Gonadotropin Releasing Hormone (GnRH) Neuropeptide

The Gonadotropin releasing hormone (GnRH) is a neuropeptide produced in the brain (hypothalamus), which was discovered more than 30 years ago and is known to regulate the reproductive activity by stimulating pituitary gonadotropin secretion in vertebrates (Zohar *et al.*, 2010). GnRH is expressed and produced very early in development in olfactory neurons and migrates to three or four brain regions: the preoptic area (POA), the midbrain and the terminal nerve as well as some other areas in the forebrain (Yamamoto, 2003; Sherwood & Adams, 2005; Cariboni *et al.*, 2007). Many studies have investigated the characterisation of the teleost GnRH system (identification, localisation, pharmacology) and it has emerged to be more complex and diverse than first thought. To date, 24 forms of GnRH have been discovered in vertebrates, protochordates and invertebrates, with eight GnRH forms being found in teleost brains (Lethimonier *et al.*, 2004; Kah *et al.*, 2007; Van Der Kraak, 2009) (see Tables 1.1 and 1.2). The GnRH forms are usually named after the species from which they were first isolated. Fish share two forms of GnRH with other vertebrates; mammalian GnRH (GnRH I) and chicken GnRH (GnRH 2) (Pawson & McNeilly, 2005; Sherwood & Adams, 2005). Based on phylogenetic analysis of sequences and associated sites of expression, teleost GnRHs variants have been segregated into three clades named GnRH1, GnRH2 and GnRH3 (White & Fernald, 1998; Lethimonier *et al.*, 2004; Kim *et al.*, 2011). Teleost brains express at least two GnRH forms; the existence of three GnRH forms was confirmed in perciformes (Gothilf *et al.*, 1996, Okuzawa *et al.*, 1997, Weber *et al.*, 1997; Holland *et al.*, 2001, Zamora *et al.*, 2002; Zohar *et al.*, 2010) and there is now evidence on the existent of the three different GnRH forms in many teleost orders (Zohar *et al.*, 2010).The diversity of GnRH forms in teleosts is

suggested to be due to at least two genomic duplication events (Sherwood & Adams, 2005). In the European sea bass, these three forms are also identified as sea bream GnRH, sbGnRH (GnRH1); chicken GnRH-II, cGnRH-II (GnRH2) and salmon GnRH, sGnRH (GnRH3) (Zmora *et al.*, 2002; Gonzalez-Martinez *et al.*, 2001; 2002 a&b). The first branch of GnRH forms, GnRH1, has been suggested to be the major hypophysiotropic hormone in perciformes (Powell *et al.*, 1994; Holland *et al.*, 1998; Gonzalez-Martinez *et al.*, 2002a; 2004 a, b) whereas the significance of the GnRH2 and GnRH3 remains unclear. The three GnRH forms have differential expression patterns with GnRH1 expressed mainly in the POA cells, GnRH2 in the olfactory bulbs and GnRH3 in the mid brain tegmentum (White *et al.*, 1995 ; Senthilkumaran *et al.*, 1999; Amano *et al.*, 2002; Andersson *et al.*, 2001; Gothilf *et al.*, 1996 ; Gonzalez-Martinez *et al.*, 2002a; 2004a).

Table 1.1. Structure of the 24 known GnRH variants taking mGnRH as the reference (Kah *et al.*, 2007).

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

Table 1.2. Summary of the eight variants of GnRH identified in teleosts (Van Der Kraak, 2009).

Position	1	2	3	4	5	6	7	8	9	10
GnRH 1 clade										
Mammalian GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-H2
Pejerrey GnRH	—	—	—	—	Phe	—	—	Ser	—	—
Sea bream GnRH	—	—	—	—	—	—	—	Ser	—	—
Catfish GnRH	—	—	—	—	His	—	—	Asn	—	—
Herring GnRH	—	—	—	—	His	—	—	Ser	—	—
Whitefish GnRH	—	—	—	—	—	—	Met	Asn	—	—
GnRH 2 clade										
Chicken GnRH-II	—	—	—	—	His	—	Trp	Tyr	—	—
GnRH 3 clade										
Salmon GnRH	—	—	—	—	—	—	Trp	Leu	—	—

Immunohistochemistry and *in situ* hybridization studies have been performed to investigate the spatial distribution of the three GnRH variants in the European sea bass brain; only one form (GnRH1) appears to be expressed in neurons located in the POA and projecting to the pituitary, thus it must be involved in the regulation of gonadotropin secretion in the pituitary and represents the main hypophysiotrophic hormone (Gonzalez-Martinez *et al.*, 2001; 2002a; Zmora *et al.*, 2002). GnRH2 immunoreactivity was only detected in large synencephalic cells which are the motor and sensorymotor brain areas and did not project to the pituitary, while GnRH3 fibers were detected in forebrain, optic tectum, and mid brain tagmentum (Gonzalez-Martinez *et al.*, 2002). GnRH3 immunoreactive nerve fibres have been seen to innervate the pituitary of the sea bass although at a lesser extent than GnRH1 (Gonzalez-Martinez *et al.*, 2002a; 2004a). The organization of the GnRH system in sea bass is presented in Figure 1.8. While GnRH1 is clearly involved in the gonadotropin secretion (Kah *et al.*,

2007) GnRH2 and GnRH3 functions are largely unknown. GnRH2 has been associated with sexual behaviour (Kauffmann *et al.*, 2005; Millar, 2005) and it has more recently been specifically linked to pineal-specific melatonin signalling in Sea bass (Servili *et al.*, 2010). Both GnRH2 and GnRH3 influence reproductive and probably other behavioural functions through neuromodulatory actions in the central nervous system (Hofmann, 2006; Oka, 2009). Possible sex specific roles of both GnRH2 and GnRH3 are proposed by Hofmann, 2006, in which GnRH2 influences reproductive behaviour and food intake in female mammals, while GnRH3, as it is present only in teleosts, could be involved in teleost male reproductive behaviour and possibility of acting as neuromodulator (Fig. 1.9). Clearly further studies are required to get clearer definitions of the GnRH system in fish.

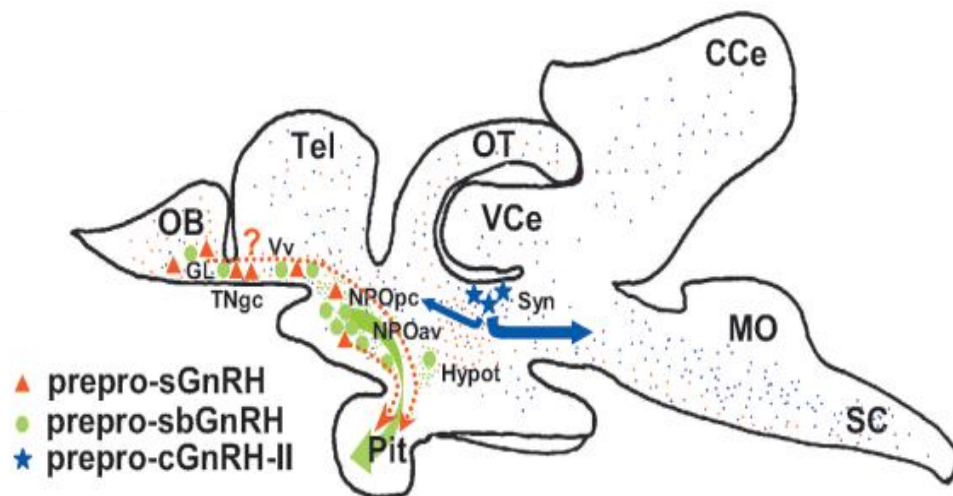


Figure 1.8. Organization of the GnRH system in the sea bass brain: sagittal drawing of sea bass brain summarizing the distribution of GnRH1 (sbGnRH) (green circles), GnRH2 (cGnRH-II) (blue stars) and GnRH3 (sGnRH) (red triangles). Hypot, Hypothalamus; MO, Medulla oblongata; OB, olfactory bulbs; Pit, pituitary; POA, preoptic area; SC, spinal cord; Syn, synencephalon; Tel, telencephalon (From Gonzalez-Martinez *et al.*, 2002a).

In addition to the brain, *GnRH* mRNA was found to be expressed in the gonads of many fish species including goldfish (Lin & Peter 1996), sockeye salmon (*Oncorhynchus nerka*) (Von Schalburg & Sherwood, 1999), seabream (*Sparus aurata*) (Nabissi *et al.*, 2000), African catfish, *Clarias gariepinus* (Bogerd *et al.*, 2002), rainbow trout (Uzbekova *et al.*, 2001&2002), spotted green pufferfish, *Tetraodon nigroviridis* (Ikemoto & Park 2005) and Perjerrey, *Odontesthes bonariensis* (Guilgur *et al.*, 2009). GnRH has also been localised in other regions including retina, trigeminal ganglion and trunk (Abraham *et al.*, 2008; Zohar *et al.*, 2010). The functional significance of this peripheral expression is yet to be investigated.

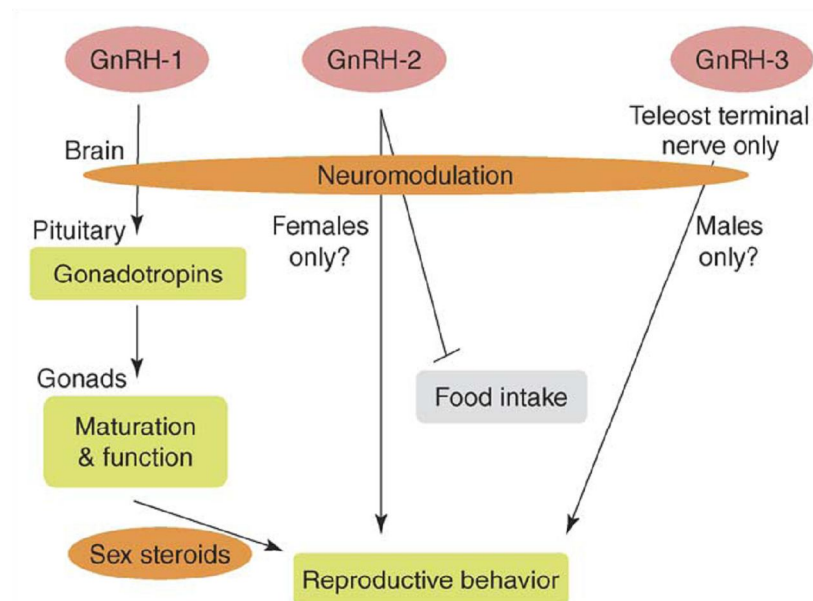


Figure 1.9. Three GnRH subtypes influence reproductive behaviour through hormonal and neuromodulatory pathways. GnRH1, which is thought to mainly control gonadal maturation through gonadotropin release from the pituitary, probably also, has neuromodulatory functions throughout the brain. Both GnRH2 and GnRH3 influence reproductive and probably other behavioural functions through neuromodulatory actions in the central nervous system (From Hofmann, 2006).

1.4.1.1 GnRH gene structure and regulation

The isolation, cloning and sequencing of the genes encoding for the various GnRHs from a variety of vertebrates has shown their conserved organisation among the different forms (Okubo & Nagahama 2008). The GnRH encoding genes consist of three introns and four exons (Okubo & Nagahama 2008; Zohar *et al.*, 2010). The second, third and parts of the fourth exons represent the encoding region of the GnRH and are called prepro-GnRH, which consists of a signal peptide of 21-23 amino acids that allows the protein to be transferred to the endoplasmic reticulum, a core sequence of GnRH (10 amino acids), a proteolytic cleavage site (Gly-Lys-Arg) and a GnRH associated peptide (GAP, 40-60 amino acids) (Okubo & Nagahama 2008). The signal peptide, GnRH decapeptide, proteolytic cleavage site and N-terminal region of GAP are encoded in exon 2, the main sequence and C-terminal parts of GAP are encoded in exon 3 and 4, while the 5' and 3' UTR are encoded in exon 1 and 4, respectively (Okubo & Nagahama, 2008; Zohar *et al.*, 2010) (Fig.1.10). The second GnRH exon shows the most conserved sequence; while the other exons show higher variability, the signal peptides and the GnRH decapeptide are well conserved, whereas the GAPs show less homology (Zohar *et al.*, 2010). In sea bass the GAP sequence of the of the three GnRHs precursors showed only 42 % identity with each other but 88-90 % identity with the corresponding sequences in other fish (Zmora *et al.*, 2002). The regulation of GnRH gene expression through the promoter region has been studied in many animal models (Kitahashi *et al.*, 2005; Sherwood & Adams, 2005). GnRH expression has been found to be regulated at the post-transcriptional level as the mRNA expression level has not been correlated to pre-pubertal transcriptional levels. The surge in GnRH transcript level and its receptors are commonly observed at puberty and used as a way to study the

mechanisms regulating the release of gonadotropin and onset of puberty in many vertebrates including fish (Gore, 2002; Nocillado *et al.*, 2007; Mohamed *et al.*, 2007).

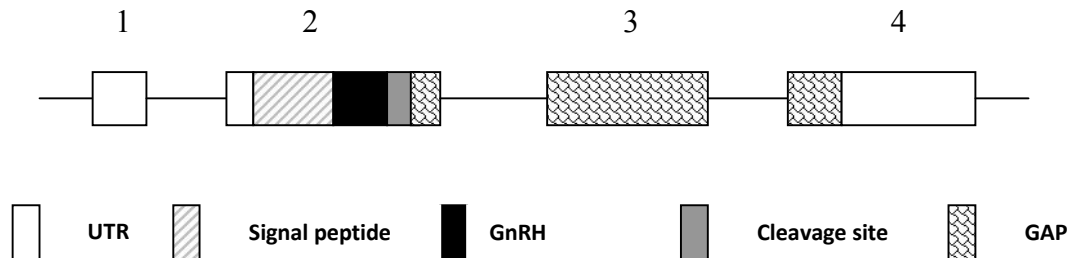


Figure 1.10. Schematic diagram illustrating the structural organization of the gonadotropin-releasing hormone (GnRH) gene in vertebrates. It is composed of four exons and three introns. Boxes represent exons and horizontal lines adjacent to exons represent introns. The exon number is shown. GnRH is encoded as part of a precursor polypeptide designated prepro-GnRH, which consists of a signal peptide, the GnRH decapeptide, a cleavage site (Gly-Lys-Arg) and the GnRH-associated peptide (GAP). UTR = untranslated region (adapted from Okubo & Nagahama, 2008).

1.4.1.2. GnRH receptors

The actions of GnRH subtypes in all vertebrates are mediated through binding to several subtypes of GnRH receptors which belong to the G protein-coupled receptor superfamily. These receptors show three main functional domains: an N-terminal extracellular domain, seven α -helical transmembrane (Tms) domains connected by hydrophilic intra- and extracellular loops and a C-terminal cytoplasmic domain (Lethimonier *et al.*, 2004; Millar, 2005). To date three classes (type 1, 2 and 3) of GnRH receptors have been identified: type 1 receptors (GnRHr1) are found in mammals and fish, type 2 receptors (GnRHr2) are found in human and amphibians and the third receptor branch type 3 (GnRHr3) in teleosts, mainly perciform species (Van

Der Kraak, 2009). More than one receptor of each GnRH receptor subtype has been identified in the studied fish species due to genome duplication (Lethimonier *et al.*, 2004; Van Der Kraak, 2009). There have been five GnRH receptors reported in pufferfish (*Fugu ruprides*) and masou salmon compared to four distinct GnRH receptors in the zebrafish (Kah *et al.*, 2007). The first cloned GnRH receptor in European sea bass has been found to be expressed in the brain and pituitary suggesting its role in mediating gonadotrophin release (Gonzalez-Martinez *et al.*, 2004b). Thereafter, four additional European sea bass cDNAs encoding GnRH receptors have been reported. They are subdivided into two GnRHR1 (A and B) and three GnRHR2 (A, B and C) subtypes (Moncaut *et al.*, 2005). The multiplicity of GnRH receptors makes it difficult to conclude as to which receptor subtypes are involved in LH and FSH release (Van Der Kraak, 2009) as for example, in Nile tilapia three forms of GnRH receptors were identified in a single gonadotrope cell type (Parhar *et al.*, 2005). In addition to the brain and pituitary, GnRH receptors are expressed in gonads, eye, olfactory epithelium, kidney and gills (Moncaut *et al.*, 2005) (Table 1.3). All the European sea bass GnRH receptors mRNAs are expressed in the anterior and mid brain, while four of them (GnRHR1A, GnRHR1B, GnRHR2A and GnRHR2C) are also expressed in the pituitary gland. These receptors showed a differential expression in the gonads, eye, olfactory epithelium, kidney and gills (Moncaut *et al.*, 2005). Clearly there is much work still to be done to characterize the functional responses mediated by different GnRH receptors in the brain and pituitary of teleosts.

Table 1.3. Pharmacological characterization, tissue distribution and classification of known GnRH receptors. Asterisk (*) indicates receptor subtype showing a higher cGnRH-II sensitivity in the species of interest (From Lethimonier *et al.*, 2004)

species	Reference	Receptor Abbreviation	GnRH-R type	Ligand selectivity	Tissue distribution
Anguilliformes Eel	Okubo <i>et al.</i> (2000 a,b)		II	ND	Pituitary>brain>testis>eye> olfactory epithelium
Cypriniformes Goldfish	Illing <i>et al.</i> (1999)	GfA GfB	II	GfA*: cGnRH-II>sGnRH>mGnRH>sbGnRH GfB: cGnRH-II>sGnRH>mGnRH>sbGnRH	Brain (but only GfA is expressed in ventral telencephalon)> pituitary (proximal pars details)> ovary (interstitial cells and theca-granulosa cell layers) = liver
Beloniformes Medaka	Okubo <i>et al.</i> (2001)	GnRH-R1	I	GnRH-R1: cGnRHII>=sGnRH=mGnRH=mdGnRH	ND
		GnRH-R2	II	GnRH-R2*: cGnRHII>sGnRH>mGnRH>mdGnRH	ND
Siluriformes Catfish	Tensen <i>et al.</i> (1997) Bogerd <i>et al.</i> (2002)	cfGnRH-R1	II	cGnRHII>cfGnRH	Pituitary>brain>cerebellum>testis
		cfGnRH-R2	II	cGnRH-II>cfGnRH=mGnRH	Brain>ovary>heart>testis>cerebellum>pituitary
Salmoniformes Rainbow trout	Madigou <i>et al.</i> (2000)	rtGnRH-R	II	ND	Brain>testis>ovary>retina>pituitary
Perciformes Sea bass	Gonzalez-Martinez <i>et al.</i> (2002c), Moncaut <i>et al.</i> (2005)	GnRHR1A	I	ND	Pituitary>brain> gonad>retina> olfactory epithelium
		GnRHR1B	I	ND	
		GnRHR2A	II	ND	
		GnRHR2B	II	ND	
		GnRHR2C	II	ND	
<i>Seriola dumerilli</i> Striped bass Astatotilapia	Alok <i>et al.</i> (2000) Robinson <i>et al.</i> (2001)	stbGnRH-R	II	cGnRH-II>mGnRH>=sGnRH>sbGnRH	Pituitary>brain>ovary
		GnRH-R1	II	cGnRH-II>sGnRH=mGnRH>sbGnRH	Brain>testis>kidney>retina>muscle> pituitary
		GnRH-R2	I	ND	ND

1.4.1.3. Seasonal fluctuation in GnRH during the reproductive cycle

Changes in the expression of GnRHs in relation to the reproductive cycle have been reported in both males and females in several fish species. For example the pituitary content of both GnRH1 and GnRH2 increased during the gonadal recrudescence and peaked around the time of spawning in maturing male and female striped bass (Holland *et al.*, 2001). Similarly, in female red seabream *Pagrus major*, the level of *GnRH1* mRNA in the brain increased in conjunction with active vitellogenesis, with peak levels obtained during spawning before levels decreased again during the ovarian regression (Okuzawa *et al.*, 2003). In female Pejerrey, the levels of *GnRH1* mRNA in brain varied with different ovarian status (Guilgur *et al.*, 2009). Equally, in male barfin flounder (*Verasper moseri*) brains, *GnRH1* mRNA expression increased in parallel with increasing GSI values, with *GnRH1* expression levels peaking at spermiation, while the two other forms (GnRH2 and GnRH3) did not show changes with spermatogenesis (Amano *et al.*, 2004). In grass puffer (*Takifugu niphobles*), *GnRH1* mRNA were substantially elevated during the spawning time in both male and females, with strong positive correlation between GnRH1 and plasma E2 and T levels (Shahjahan *et al.*, 2010a). For the European sea bass, pituitary levels of *GnRH1* and *GnRH3* mRNAs have been seen to increase along with the GnRH receptor (GnRHR2A) and follicle stimulating hormone gene expression during sexual differentiation (Moles *et al.*, 2007). Furthermore, Mateos *et al.* (2002) demonstrated that GnRH analogue injection increased mRNA levels of the common α and *lh\beta* gonadotropin subunits but not *fsh\beta* subunit.

1.4.2. Pituitary Gonadotropins

In the pituitary of fish, as in other vertebrates, gonadotroph cells (FSH/LH synthesising cells) are located in the anterior lobe (pars distalis). These cells are innervated (directly or indirectly depending on the fish species) by neurosecretory fibres (i.e. GnRH neurons & dopaminergic neurons) which release neurohormones that originate from the hypothalamus of the brain (Zohar *et al.*, 2010). It is well known that pituitary gonadotropins play a key role in the regulation of sex steroid hormones produced by steroidogenic gonadal cells and the production of gametes in both male and female teleosts. There are two main forms of gonadotropins in teleosts as in most other vertebrates (Yaron *et al.*, 2003; Weltzien *et al.*, 2004): follicle stimulating hormone (FSH, also referred to as GTH I) and luteinising hormone (LH, also referred to as GTH II). These have been shown to share a common α subunit and a hormone-specific β subunit (Yaron *et al.*, 2003). The action of these hormones is carried out through specific membrane receptors (G-protein coupled receptors) present in the cell membranes of the target tissue. Once the membrane receptors are activated, a cyclic AMP secondary messenger system will pass information to the interior of the cell (Swanson *et al.*, 2003; Yaron *et al.*, 2003). Both female and male steroidogenic cells in the gonads are the main target for LH and FSH where they stimulate steroid production. FSH is commonly found to be the main driver for vitellogenin synthesis by the liver and incorporation into the oocytes, through the process known as vitellogenesis, while LH appears to be playing a more important role in male spermiation and final oocyte maturation in female fish (Swanson *et al.*, 2003; Yaron *et al.*, 2003; Weltzien *et al.*, 2004). Expression of pituitary gonadotropin β subunits during puberty has been investigated in a number of fish species. In females which are single batch spawners such as rainbow trout, gonadotropic regulation of gametogenesis is generally

characterised by an elevation in FSH during early oocyte growth and vitellogenesis whereas LH is associated with final oocyte maturation and ovulation (Gomez *et al.*, 1999; Hassin *et al.*, 1999). Alternatively in multiple batch spawners such as Atlantic halibut (*Hippoglossus hippoglossus*), gilthead sea bream, goldfish and Japanese flounder (*Paralichthys olivaceus*), the expression of pituitary gonadotropin subunits has been seen to increase simultaneously (Sohn *et al.*, 1999; Kajimura *et al.*, 2001; Weltzien *et al.*, 2003a, b; Meiri *et al.*, 2004) as the oocytes are developing at different rates. In European sea bass males, *fsh* mRNA levels increase continuously with gonadal growth suggesting an important role in the spermatogenesis, while *lh* mRNA levels and release was maximum during late gametogenesis and spawning (Mateos *et al.*, 2003; Moles *et al.*, 2007, Carrillo *et al.*, 2010), as the LH release was more involved with later stages of gonadal development than with sex differentiation (Rodriguez *et al.*, 2000). The mRNA expression of the three gonadotropin (common α , *lh\beta* and *fsh\beta*) subunits in the pituitary of mature male sea bass increased in parallel with the GSI and declined sharply at post spermiation (Mateos *et al.*, 2003). In addition to the seasonal profile of LH levels, daily rhythms of both pituitary content and plasma LH were observed in pubertal sea bass (95 % males), these rhythms exhibit nocturnal peaks and were negatively correlated with the daily pituitary GnRH1 content (Bayarri *et al.*, 2004b).

Two gonadotropin receptors (*fshr* and *lhr*) have been described in several teleost groups (Taranger *et al.*, 2010), including the European sea bass (Rocha *et al.*, 2007, 2009). Changes in expression profiles of both receptors in gonads during the seasonal reproductive cycle and pubertal development are different according to the species (Taranger *et al.*, 2010). In the Nile tilapia, zebrafish and Atlantic cod the *fshr* and *lhr* gonadal mRNA expression is mainly associated with different phases of oocyte development with *fshr* mostly expressed during vitellogenesis and *lhr* elevating during

final oocyte maturation (Hirai *et al.*, 2002; Kwok *et al.*, 2005; Mittelholzer *et al.*, 2009). In sea bass, Rocha *et al.* (2009) analysed the expression profile of the two receptors for the reproductive cycle in both male and female gonads. The expression of *fshr* was connected with early stages of gonadal development, but also with spermiation/ovulation during spawning, while the peaks of *lhr* expression were observed only in the final stage of gamete maturation in both sexes.

1.4.3. Sex steroids and feedback mechanisms

Gonads represent the last step of the reproductive cascade and serve two main functions: the development of germ cells (oogenesis and spermatogenesis) and the production of steroids and growth factors. Steroids are involved in the stimulatory and regulatory effects of these processes, not only at the gonadal level (paracrine) but also as feedback to the brain and pituitary level (endocrine). Steroids are also important for the control of sexual behaviour (Zohar *et al.*, 2010). There are three main types of steroids: androgens, oestrogens and progestagens. Androgens including testosterone (T) and 11-Ketotestosterone (11KT), produced in the Leydig cells within the testis, are the dominant sex steroids involved in spermatogenesis in male teleosts (Kime, 1993; Borg, 1994; Weltzien *et al.*, 2004). Oestrogens are considered as female hormones but are also found in male teleosts, and it has been suggested that they have an important role in regulating gene expression in the testis (Schulz *et al.*, 2010). Progestagens have been shown to play a major role during advanced stages of gametogenesis in both male and female teleosts. In females, progestagens such as 17α , 20β -dihydroxy-4-pregnen-3-one (also called DHP) have been found to play an important role in final oocyte maturation, namely germinal vesicle breakdown (GVBD) (Nagahama *et al.*, 1987; Nagahama, 1994) which is an essential process prior to ovulation. In male rainbow trout, high

plasma levels of DHP have been found during spermiation (Ueda *et al.*, 1984) and it is thought to be involved in the acquisition of sperm motility (essential for fertilisation) by increasing the pH of the sperm duct thus increasing cAMP in sperm and facilitating motility (Miura *et al.*, 1991). Furthermore precocious spermiation can be induced by an injection of DHP (Nagahama *et al.*, 1994). As well as their role in gametogenesis and secondary sexual characteristics, sex steroids are of prime importance in the feedback control of reproductive development and provide an indicator to the brain and pituitary as to the reproductive state of the animal (Zohar *et al.*, 2010). Both the brain and pituitary have been found to contain high densities of estrogen and androgen receptors (Navas *et al.*, 1995; Blazquez & Piferrer, 2005). Whether feedback is positive or negative depends on the physiological status of the individual and the species itself (Zohar *et al.*, 2010). Seasonal profiles of gonadal steroids were studied in both adult males and female sea bass by Prat *et al.*, (1990); in females: 17α , 20β -dihydroxy-4-pregnen-3-one levels were low throughout the year, even during the spawning, while plasma testosterone and oestradiol increased significantly in advance gametogenesis period with further increase during the spawning time in parallel with the gonadal growth. In males, both plasma T and 11-KT increased with spermatogenesis and reached their peaks during the spawning. For pubertal male and female sea bass, similar profiles to the adults were observed, in which gonadogenesis was in parallel with the high increase in plasma sex steroids during spermatogenesis and vitellogenesis, remaining elevated during most of the maturation period (Rodriguez *et al.*, 2000, 2004 & 2005; Rocha *et al.*, 2009).

1.4.4. Other Brain neuropeptide and neurotransmitters regulating reproduction

Originally it was believed that GnRH was acting as the top of the BPG axis to control the endogenous reproductive cascade however, recent research in mammals has brought to light the importance of a number of upstream signal peptides that regulate GnRH expression (kisspeptin & neurokinin B) or neurotransmitters that work in association with GnRH actions to regulate gonadotropin synthesis e.g. dopamine (DA), neuropeptide Y (NPY) and gamma aminobutyric acid (GABA) (Vidal *et al.*, 2004; Van Der Kraak, 2009; Zohar *et al.*, 2010). Dopamine (DA) is known to play an inhibitory role in the neuroendocrine regulation of reproduction in a number of fish species (Van Der Kraak, 2009). Neuroanatomical investigations in the goldfish and European eel, have shown that inhibitory DA neurons which originate in the brain project directly into the pituitary where gonadotroph cells are located and exert inhibitory effects on gonadotrophin production (Chang *et al.*, 1990; Vidal *et al.*, 2004). This hypophysiotropic DA activity has been shown to vary with reproductive stage (Saligaut *et al.*, 1999) and is controlled by endogenous factors such as sex steroid feedback (Weltzien *et al.*, 2006) as well as environmental cues (Sebert *et al.*, 2008). GABA is a neurotransmitter which has been shown to have stimulatory effects on gonadotropin secretion in fish. It stimulates LH release from the pituitary (see Popesku *et al.*, 2008). *In vivo* studies showed that administration of GABA or GABA agonists increased plasma LH in goldfish (Martyniuk *et al.*, 2007). Initial research demonstrated the abundance of GABA immunoreactive fibres in the goldfish pituitary (Kah *et al.*, 1987) and this led to the discovery of its role in the stimulation of LH secretion including GnRH release and inhibition of dopamine in this species (Kah *et al.*, 1992; Sloley *et al.*, 1992). It has been hypothesised that gonadotropic stimulation by GABA can be mediated through GnRH following *in vitro* work on the goldfish pituitary where GABA

was seen to cause a dose-dependent in GnRH release (Kah *et al.*, 1992). In the rainbow trout, GABA has been seen to stimulate both basal and GnRH-induced gonadotropin secretion from pituitary cells (Mananos *et al.*, 1999). Sex, reproductive stage and ultimately sex steroids have all been shown to affect the stimulatory action of GABA on gonadotropin secretion in fish (Trudeau, 1997). NPY is a neuropeptide that stimulates LH release in some studied fish including sea bass (Cerdeira-Reveter *et al.*, 1999). NPY elevates the expression of *lhβ* but not *fshβ* in Nile tilapia (Yaron *et al.*, 2003). There is evidence that the action of NPY is associated with feeding control and reproductive status in fish with, for example, the positive energetic status in European sea bass suppressed the ability of NPY to stimulate LH secretion (Cerdeira-Reveter *et al.*, 1999). In goldfish, studies have revealed that NPY stimulates growth hormone and gonadotropin release (Kah *et al.*, 1989; Peng *et al.*, 1993) which could therefore suggest a link between feeding, growth and control of the reproductive axis (Zohar *et al.*, 2010). Kisspeptin is neuropeptide identified recently as key upstream regulators for the initiation of puberty and regulator in seasonal breeding in mammals (Smith & Clarke, 2007). Research in teleosts is somewhat behind that in mammals, however, there have been a number of advances in our understanding of the kisspeptin system in particular as a new actor in neuroendocrinology. A review focusing on the details of kisspeptin: identification, structure, and some previous research in mammals and fish will be given in the next section of this chapter.

1.5. Kisspeptin: a new signal peptide involved in the control of reproduction

Kisspeptins are a family of neuropeptides originally named metastin when they were identified in cancer studies and reported to be highly expressed in metastasis-suppressed tumour cells (Lee *et al.*, 1996). In humans the KISS1 precursor has 145 amino acids that is cleaved to yield a family of four biologically active peptides comprising 54, 14, 13 and 10 amino acids respectively, which are all able to bind and activate the KISS1R (formerly named GPR54) receptor (Ohtaki *et al.*, 2001). Figure (1.11) shows the structural features and complete amino acid sequence of human kisspeptin.

1.5.1. Mammalian Kisspeptin

In 2003, two independent laboratories discovered that mutations in *Kiss1r* in mice and men were involved with hypogonadotropic hypogonadism in which the individual fails to undergo puberty and has immature sexual characteristics (Seminara *et al.*, 2003; de Roux *et al.*, 2003). From this time KISS1/KISS1R has been suggested to be a key factor in the regulation of reproduction and initiation of puberty (Seminara, 2005; Murphy 2005; Dungang *et al.*, 2006). Studies carried out on rats indicated that metastin and kisspeptin-10 have the ability to stimulate the synthesis and release of LH and FSH hormones when administered centrally or peripherally (Gottsch *et al.*, 2004; Matsui *et al.*, 2004; Messenger *et al.*, 2005). This is true for male monkeys as well (Shahab *et al.*, 2005) (Table 1.4).

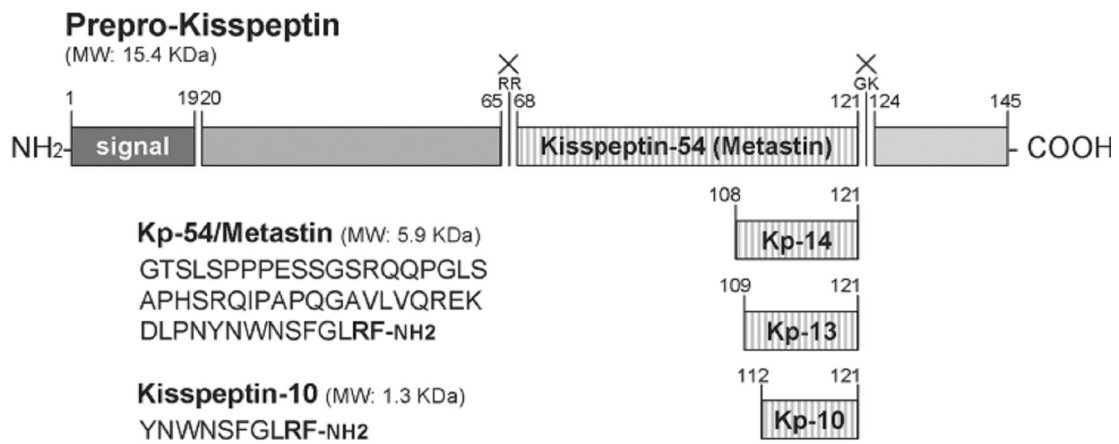


Figure 1.11: Structural features of human kisspeptin, generated by cleavage from a common precursor, the prepro-kisspeptin. Prepro-kisspeptin (KISS1 gene) is 145 amino-acid protein that contains central 54 amino acid region, flanked by two consensus cleavage sites which give rise to metastin or kisspeptin-54. Further cleavage of metastin generates low molecular weight kisspeptins: kp-14, kp-13 and kp-10 (From Tena- Sempere, 2006).

Similar effects were obtained in male humans when kisspeptin-54 was administered peripherally (Dhillon *et al.*, 2005). Kisspeptin was found to be equally effective when administered as a full length peptide or decapeptide kiss-10 (Gottsch *et al.*, 2004). KISS1/KISS1R signalling amplification takes place during pubertal maturation and could be the proximate event that stimulates GnRH neurons and activates the neuroendocrine events leading to puberty onset. Expression analysis of *Kiss1* and *Kiss1r* mRNA showed marked increase coinciding with the onset of puberty in both male and female rats (Navarro *et al.*, 2004). Likewise hypothalamic *Kiss1* mRNA levels increased in male monkeys during puberty (Shahab *et al.*, 2005).

The mechanism of how kisspeptins are able to control the gonadotrophic axis has also been a matter of intensive studies. Research has been conducted to demonstrate

whether kisspeptins are able to act on GnRH neurons and to stimulate GnRH secretion. Studies showing that the stimulatory effect of kisspeptins is blocked in the presence of GnRH antagonists proposed that kisspeptin effects on gonadotropins are GnRH dependent (Gottsch *et al.*, 2004, Irwige *et al.*, 2004, Matsui *et al.*, 2004; Shahab *et al.*, 2005). Using *in situ* hybridisation, studies have shown that mammalian GnRH neurons are potential targets for kisspeptins, as double-label *in situ* hybridisation studies in rat indicated that >75 % of GnRH neurons co-express *Kiss1r* mRNA (Irwige *et al.*, 2004). Furthermore, the brain localisation of rodent *Kiss1* expression revealed that there are two populations of *Kiss1* expressing cells both located in the hypothalamus: the two populations have different regulation potentialities by sex steroids feedback (Franceschini *et al.*, 2006; Roa *et al.*, 2008). Recently, the kisspeptin system has been proposed to have other reproductive functions such as metabolic control of fertility (Castellano *et al.*, 2009) and environmental control of reproductive ability (Roa *et al.*, 2008). Correlations between kisspeptin expression, sexual development and photoperiod have been proved in seasonal mammals through a number of studies (Revel *et al.*, 2006a; Greives *et al.*, 2007; Mason *et al.*, 2007). For example, changes in photoperiod from long to short day in the Syrian hamster, a spring breeder, inhibit sexual development and result in down regulation of *Kiss1* expression (Revel *et al.* 2006a; 2006b, Greives *et al.*, 2007). Similar results were obtained in Soay sheep, an autumn breeder, when transferred from a short to long day photoperiod (Wagner *et al.*, 2007). Kisspeptins have also been found to be involved in the cardiovascular system in human (Mead *et al.*, 2007) and were found in adipose tissue (Brown *et al.*, 2008).

Table 1.4. Studies carried on kisspeptin administration for different animals and human (From Seminara, 2005).

Animal	Sex	Age	Formulation	Dose	Route	Time point	Outcome
Mouse	M	Adult	m metastin 1–52	1 nmol	ICV	30 min	↑LH
			h metastin 45–54	1 nmol	ICV	30 min	↑LH
			h metastin 45–54	1 fmol–5 nmol	ICV	30 min	↑LH
Rat	M	Prepub (30 days)	m metastin 43–52	1 nmol	ICV	15 min 60 min	↑LH, ↓PrL ↑LH, ↓PrL
		Prepub (25 days)	m metastin 43–52	1 nmol	ICV	15 min 60 min	↑LH, ↓PrL ↑LH, ↓PrL
	F	Adult (75 days)	m metastin 43–52	1 nmol	ICV	15 min	↑LH, ↔PrL
						60 min	↑LH, ↔PrL
			m metastin 43–52	1 nmol	ICV	0–180min	↑LH
Rat	M	Adult (10 weeks)	“h metastin”	100 nmol/kg	SC	1–4 h	↑LH, ↑FSH
	F	Immature (25 days)	“h metastin”	6.7 nmol	SC	0–4 h	↑LH, ↑FSH
	F	Rx PMSG (23 days)	“h metastin”	6.7 nmol	SC	0–4 h	↑LH, ↑FSH
Rat	M		“kisspeptin 10”	0.1, 0.3, 1, or 3 nmol	ICV	60 min	↑LH (sig 1 and 3 nmol) ↑FSH (sig 1 nmol), ↑T
				3nmol	ICV	10 min 20 min 60 min	↑LH, ↔FSH, ↔T ↑LH, ↔FSH, ↑T ↑LH, ↑FSH, ↑T
				10, 30, and 100 nmol	IP	20min	↑LH (sig 100 nmol) ↔FSH, ↔T ↔LH, ↔FSH
				10, 30, and 100 nmol	IP	60 min	↑T (sig 30 and 100 nmol)
Rat	M	Pubertal (45d)	m metastin 43–52	1 nmol–10 pmol	ICV	15 min 60 min	↑LH ↑LH, ↑T (10X)(1 nmol)
				7.5 nmol	IP	15 min 30 min 60 min	↑LH ↑LH ↔LH
				7.5 nmol	IV	q 15min x 4h	↑LH max at 15-30 min
Rat	F	Immature	m metastin 43–52	1 nmol	ICV bid	Days 26–31 Day 31	Rx grp: 74% vag opening ↑uterus wt, LH, E2
Rat	M	Adult	m metastin 1–52		ICV x1	1h	↑LH, ↔FSH
Monkey	M	Juvenile 17–23mol	h metastin 45–54	30 µg	ICV	0–4 h	↑LH
				100 µg	ICV	0–4 h	↑LH
				100 µg	IV	0–4 h	↑LH
Ewes	F		h metastin 45–54	50 nmol over	ICV	4 h q 15 min x 4 h	↑LH
Humans	M	Adult	h metastin 1–54	4µmol/kg/min	IV	90 min	↑LH, ↑FSH, ↑T

1.5.2. Kisspeptin in fish

The fish kisspeptin was first identified in tilapia in 2004 by Parhar *et al.* (2004) who reported the co-localisation of the receptor *kissr4* (teleost kisspeptin receptor multiplicity is explained further below) in tilapia brain neurons and suggested an intimate physical association of *kissr4* with the GnRH system. This initial work has led to a concerted research effort in recent years to isolate and describe the kisspeptin system within a diverse range of teleost species. While only one signal peptide kisspeptin 1 (KISS1) and receptor (KISSR1, formerly referred to as G-coupled protein receptor 54 (GPR54) are present in mammals, *in silico* analysis of the sequenced teleost genomes supported by functional analysis studies have revealed two forms of both the signal peptide (Kiss1 & Kiss2) and receptor (Kissr2 & Kissr4) in fish as in non-placental mammals (Felip *et al.*, 2009; Lee *et al.*, 2009, Akazome *et al.*, 2010). Of the receptors *kissr4* has been the most common isolated isoform reported in cobia (*Rachycentron canadum*) (Mohamed *et al.*, 2007), grey mullet (*Mugil cephalus*) (Nocillado *et al.*, 2007), fathead minnow (*Pimephales promelas*) (Filby *et al.*, 2008), goldfish (Li *et al.*, 2009) and Senegalese sole (*Solea senegalensis*) (Mechaly *et al.*, 2009). The second isoform, *kissr2*, has been identified in zebrafish (Biran *et al.*, 2008), medaka (*Oryzias latipes*) (Lee *et al.*, 2009) and goldfish (Li *et al.*, 2009). *Kissr4* appeared to be fish-specific and showed homologies between fish species while *kissr2* corresponds to the mammalian sequence (Akazome *et al.*, 2010).

Isolation and identification of *kiss1* gene in fish were first done species which have full genome sequence available like zebrafish and medaka. Van Aerle *et al.* (2008) were the first to identify the *kiss1* gene in zebrafish. Recently, two forms of kisspeptins, *kiss1* and *kiss2*, have been identified in several fish species including medaka (Kanda *et al.*, 2008; Kitahashi *et al.*, 2009), zebrafish (Kitahashi *et al.*, 2009), European sea bass

(Felip *et al.*, 2009) and goldfish (Li *et al.*, 2009). Both kisspeptin signal peptides have been shown to be highly effective in activating kisspeptin receptors although showing different potencies (Lee *et al.*, 2009). The two kiss genes were suggested to be gene duplication in early vertebrate evolution, with the *kiss2* gene perhaps being lost in the mammalian lineage (Felip *et al.*, 2009). It is believed that kisspeptin performs similar roles in fish as have been reported in mammals. For example, *kiss1* has been associated with the onset of puberty in male Zebrafish (Biran *et al.*, 2008), while *kiss1* expressing neurons were significantly higher in mature than immature medaka (Kanda *et al.*, 2008). In grass puffer brain *kiss2* expression peaked during spawning in both males and females (Shahjahan *et al.*, 2010b), while in male chub mackerel (*Scomber japonicus*) *kiss1* brain expression gradually decreased during spermatogenesis and *kiss2* increased during the late spermatogenesis stages and declined during spermiation (Selvaraj *et al.*, 2010). Kisspeptin in fish has been shown to possess similar GnRH regulatory abilities as described in mammals (Elizur, 2009) as well as being susceptible to sex steroid feedback (Kanda *et al.*, 2008).

Studies on *kissr4* temporal expression in fish species such as the female grey mullet (Nocillado *et al.*, 2007) have shown high levels of *kissr4* gene expression in the brain during early puberty correlated with high expression levels of the three main GnRH types. Similar results have been found in zebrafish (van Aerle *et al.*, 2008) and Nile tilapia (Parhar *et al.*, 2004; Martinez-Chavez *et al.*, 2008), where expression levels of *kissr4* in the brain have been found to increase during the onset of puberty. These findings have led a number of authors to suggest an important role played by the kisspeptin system in the activation of the BPG axis in fish.

To confirm a potential conserved role of kisspeptin in fish as demonstrated in mammals, the biological effects of kisspeptins were studied by administration of the

core decapeptide of kiss1 and kiss2. In mature female goldfish, intraperitoneal injection of Kiss1 (10) significantly increased serum LH level within 2h post injection, while Kiss2 (10) injection showed no effects on serum LH levels (Li *et al.*, 2009). This result suggested kiss1 to be the active kisspeptin form acting on brain-pituitary axis. However, contradictory results were observed in other teleost species. Indeed, in mature zebrafish female, intraperitoneal administration of both Kiss1 and Kiss2 resulted in increases in pituitary *fsh β* and *lh β* mRNA expression 12 h post injection but did not alter *GnRH2* and *GnRH3* mRNA expression (Kitahashi *et al.*, 2009). Furthermore, the Kiss2 form was shown to be more potent than Kiss1 at stimulating *lh β* mRNA expression. In sea bass, intramuscular administration of Kiss2 to prepubertal fish elicited a four- and two-fold increase in serum LH and FSH, respectively (Felip *et al.*, 2009). However, while kiss1 injection also elicited a two-fold increase in serum LH, it had no effect on circulating FSH (Felip *et al.*, 2009). These results support previous suggestions that Kiss2 is more potent than Kiss1 for the stimulation of gonadotropin secretion. The direct action of kisspeptin- 10 on goldfish pituitary was tested *in vitro*. Results showed a significant increase above basal levels in LH, prolactin and growth hormone in pituitary cells incubated for 30-min with kisspeptin-10, suggesting that kisspeptin could act directly at the pituitary level (Yang *et al.*, 2010). Recent studies based on in situ hybridization and immunohistochemistry techniques for distribution of kisspeptin neurones have shown interesting anatomical patterns in sea bass (Escobar *et al.*, 2010) and zebrafish (Servili *et al.*, 2011). In sea bass, cells expressing *kiss1* mRNAs were in the habenular region and preoptic area while *kiss2* mRNAs were found in the dorsal, lateral and ventral hypothalamus while GNRH1 neurons were shown to express *kissr4* receptors suggesting a close association with GnRH signalling (Escobar *et al.*, 2010).

In zebra fish, localisation showed two separated neuronal systems, with *kiss2* expressed in cells mostly in the mediobasal hypothalamus and *kiss1* neurons localised in the habenular region (Servili *et al.*, 2011). They also concluded that the *kiss2* gene is likely involved in the control of reproductive function, through interaction with the GnRH, while *kiss1* is possibly involved in the perception of metabolic and environmental signals (Servili *et al.*, 2011). Taken together, the results obtained in fish suggest the significance of the kisspeptin system in the puberty and reproduction control as in mammals. This research area is attracting increasing attention due to its role as tool to understand how various regulatory signals are integrated to entrain the BPG axis. A schematic representation of the main factors controlling gonadotropin release in fish is presented in Figure 1.12.

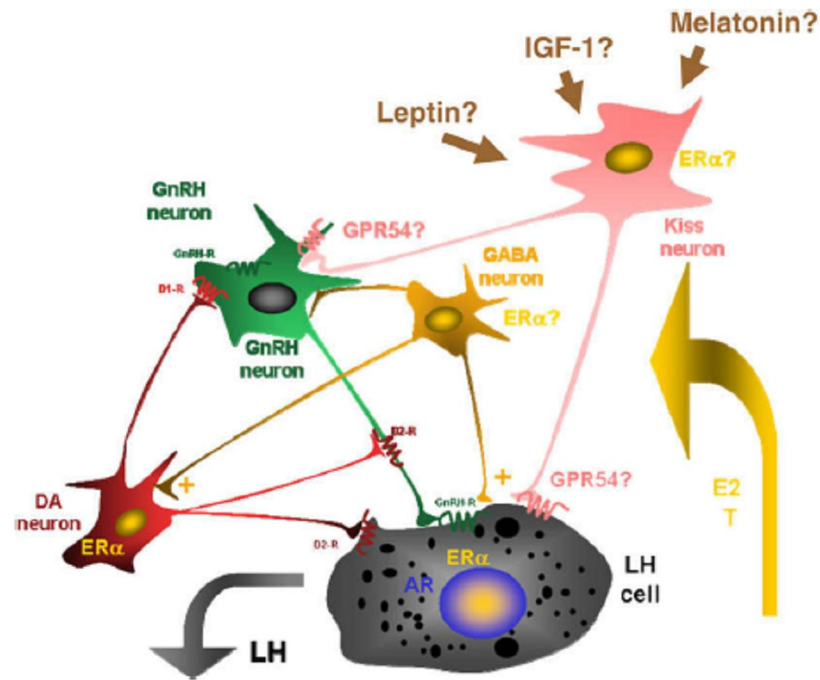


Figure 1.12. Schematic representation of the main circuits controlling gonadotropin release in fish. GnRH and dopamine (in some but not all species), respectively, stimulate and inhibit LH/FSH release directly at the gonadotrophs. These effects are modulated by GABA neurons acting to increase GnRH secretion and dopamine inhibition. Sex steroids modulate the activity of those circuits directly on dopamine and GABA neurons and indirectly, possibly through KiSS neurons, on GnRH circuits. Whether KiSS neurons also integrate photoperiodic information and nutritional status of the animals remains a hypothesis indicated by the question marks (From Zohar *et al.*, 2010).

1.6. Aims of the thesis

As is clear from the introduction, seasonality is a remarkable feature in temperate fish behaviour, particularly with regards to reproduction and spawning. There are many environmental and endocrine pathways related to biological rhythms and reproduction fairly well known in higher vertebrates. It is well understood that transduction of the photoperiodic information has a rhythmic effect on the hormonal cascade at the brain–hypothalamus–gonad axis. The acquisition of both the daily and annual rhythms of key hormones seems of critical importance to attain normal spawning at the most appropriate time of the year and maintain the reproductive function in fish. However, more work needs to be done in teleost research to improve our knowledge especially regarding the entrainment by light and its mediatory effects on reproduction. Although melatonin may not be directly initiating the reproduction, it is clear that it is playing a significant role in co-ordinating the reproductive development, as it is known as a time keeping molecule, through different suggested mechanisms with different species. However, this last aspect remains to be clarified. The rhythmic production of melatonin itself is affected by other environmental factors: in addition to light, temperature also has a significant effect, as possibly does an internal time keeping system (circadian clock). This clock rhythm was clear in some *in vitro* studies for the pineal gland with ability of self – sustaining melatonin rhythm in the absence of light; however a clear understanding of these endogenous rhythms is still lacking. The role of kisspeptin has recently come to light in fishes. It is suggested that the kisspeptin system in fish has the same functional role as in mammals in controlling reproduction and puberty. Furthermore it is thought it may act as part of the missing link between the environmental and neuroendocrine control of reproduction. Although mammalian kisspeptins primarily act at the level of hypothalamic GnRH neurons with

the possibility of kisspeptin to be a hypophysiotropic factor, it is not confirmed yet in the fish how kisspeptin interacts with the GnRH or even if it has a direct effect at the pituitary level. Clearly further research is needed in this field to discover how kisspeptin acts on the BPG axis in order to solve problems related to reproduction in farmed fish. In summary, more detailed scientific knowledge is required regarding the photoneuroendocrine control of reproduction in a temperate seasonal fish like European sea bass to improve the sustainability and profitability of the aquaculture industry and ensure a higher quality product. The overall aim of this thesis to study aspects of two interesting and highly important areas which are believed to be closely interlinked: the kisspeptin system and the endogenous melatonin production with regards to the interaction of light and temperature factors.

The specific objectives of this thesis were as follows:

1. To study the seasonal expression of kisspeptin, GnRH and gonadotropin genes in relation to the gonadal development throughout a reproductive cycle in male repeat spawning sea bass (**Chapter 3**).
2. To test the direct effect of two kisspeptin forms (kiss1 and kiss2) on the pituitary gonadotropin gene expression (**Chapter 4**).
3. To enhance the knowledge of the light perception and rhythmicity of sea bass with regards to melatonin production under the effects of both photoperiod and temperature (**Chapter 5**).

CHAPTER 2

GENERAL MATERIALS & METHODS

2. GENERAL MATERIALS AND METHODS

Below is a description of some of the general methodologies employed during the course of the PhD, accompanied by specific description of the endocrine and molecular assays employed during the course of this work.

2.1. Experimental procedure

2.1.1. Fish stock and rearing conditions

European sea bass were obtained from Machrihanish Environmental Research Laboratories of the Institute of Aquaculture (MERL, University of Stirling facilities, UK, Scotland 55° 44' N, 5° 44' W). Fish were produced in the facilities from larvae supplied by Llyn Aquaculture Ltd, UK. Fish were reared under natural photoperiod and temperature regimes and natural temperature regimes, in flow through system with stocking density of approximately 180 fish/tank (9.4m circumference, and water depth of 1.5 m). Fish were fed to satiation twice per day with commercial fish food formula (Atlantic HE 50C+ 35A/25C, Skretting, Invergordon, UK).

2.1.2. Measurement of fish growth.

To record fish size, individuals were anaesthetised using 1:10,000 2-phenoxyethanol (Sigma-Aldrich, UK), blotted with soft tissue paper and weighed individually to the nearest ± 0.1 g on a Mettler PM 6000 balance (Mettler Toledo, UK) before recording total length to the nearest mm. Fish were then transferred into a recovery tank with adequate aeration for recovery from anaesthesia. Fish were left at least two weeks prior to the next sampling.

2.1.3. Fish sacrifice

For tissue and organ sampling, fish were killed by lethal dose (1mL/L) of anaesthesia (2-phenoxyethanol, Sigma) followed by decapitation. All experimental fish were treated in accordance with the Animal Scientific Procedures (Act 1986, UK).

2.1.4. Blood sampling

Blood samples were withdrawn from the caudal dorsal aorta by using 2 ml syringes, with 21G hypodermic needles (Terumo Europe N.V., Belgium). Syringes were flushed with 560 unit/ml heparin ammonium salt (Sigma-Aldrich Co Ltd, UK). Collected blood samples were expelled into clean 1.5 ml eppendorf tubes and kept on ice until sampling was completed. Blood samples were centrifuged at 1200 g for 15 minutes at 4 °C, and then the plasma was separated into clean tubes and stored in -70 °C until time of analysis. For the blood samples taken during darkness, fish were removed from their tanks and anaesthetised in a light proof container before sampling under the illumination of a dim red light (~0.2 lux).

2.1.5. Fish identification

Passive integrated transponder tags (PIT tag, AVID, Norco, USA) were individually inserted intramuscularly in fish used for the *in vivo* melatonin trial (section 5.2.2). These tags were inserted subcutaneously through a 5 mm incision, just 2 cm below the first fin ray of dorsal fin. After the insertion of each tag, the incision was coated with a 3:1 mixture of orahesive powder (Squibb and Sons Ltd, Hounslow, UK) and cicatrin antibiotic (The Wellcome Foundation Ltd, Middlesex, UK). No mortalities were observed following the tagging procedure. When required, the tags were read by passing an extended range reader within 10 cm around the inserted tag.

2.1.6. Histology preparation

Gonads were collected from sacrificed fish and cut into 2 cm pieces. Samples were fixed in 10 % neutral buffered formalin (6.5 g disodium hydrogen phosphate (VWR International ltd, Poole, UK), 3.5 g sodium dihydrogen phosphate (VWR), 100 ml of 40 % formaldehyde (VWR) and 900 ml deionised water) and stored for later analysis.

2.1.6.1. Histological analysis

Embedding and sectioning

Fixed samples were trimmed and placed individually into cassettes that were then placed in an automated tissue processor (Shandon citadel 2000, Thermo Shandon Cheshire, UK) which then dehydrated, cleared and impregnated each sample with paraffin wax as follows:

- | | |
|----------------------------|-------------|
| 1. 50 % methylated spirit | 30 min |
| 2. 80 % methylated spirit | 90 min |
| 3. 100 % methylated spirit | 90 min(x3) |
| 4. Chloroform | 50 min(x2) |
| 5. Molten wax | 105 min |
| 6. Molten wax | 90 min (x2) |

Samples were then embedded using a histoembedder (Leica UK Ltd, Milton Keynes, and UK). After hardening the wax blocks were trimmed, before three serial sections of 5µm thickness were sliced using a rotary microtome (Leica UK Ltd, Milton Keynes, and UK) and placed on glass slides.

Staining

The sections were stained with Mayer's haematoxylin and eosin Y using modification of the procedure of Bancroft and Stevens (1991), as follows:

- | | |
|-----------------------|------------------|
| 1. Xylene | 3 min then 2 min |
| 2. Absolute ethanol | 2 min |
| 3. Methylated spirit | 1 min |
| 4. Wash in water | 30 sec |
| 5. Haematoxylin | 5 min |
| 6. Wash in water | 30 sec |
| 7. 1 % acid alcohol | 3 quick dips |
| 8. Wash in water | 30 sec |
| 9. Scott's tap water | 1 min |
| 10. Wash in water | 30 sec |
| 11. Eosin Y | 5 min |
| 12. Wash in water | 30 sec |
| 13. Methylated spirit | 1 min |
| 14. Absolute alcohol | 2 min then 1 min |
| 15. Xylene | 5 min |

Slides were kept in xylene until cover-slipping and mounted using Pertex. Identification and characterization of the different germ cell types was carried out according to Rodriguez *et al.* (2001).

2.2. Hormone Analysis

2.2.1. Radioimmunoassay

Plasma levels of testosterone, 11-Ketotestosterone and melatonin as well as *in vitro* pineal culture media melatonin were measured by radioimmunoassay. The first radioimmunoassay (RIA) was developed by Yalow and Berson (1960) to measure the insulin level in humans. Later on, RIA became an important tool for the measurement of small concentrations of many important biological compounds. An RIA is based on the reaction between an antigen and a specific antibody to form the antigen-antibody complex. After a prolonged incubation time the reaction will reach equilibrium between free or unbound antigen and antibody and bound antigen/antibody complex. The technique is dependent on determining the proportion of the total amount of antigen (free and bound) that is present in bound fraction. In order to calculate the amount of antigen, known amounts of both antibody and labelled antigen are added. When equilibrium is reached the labelled and unlabelled antigen would have bound to the antibody in the same ratio as that in which they were present originally. Once the reaction has reached equilibrium, charcoal is used to separate the bound complex from the unbound material before the supernatant is taken to measure the radioactivity in the unbound material using a scintillation counter. The amount of radioactivity is inversely related to the unknown antigen amount which is calculated using a known standard curve as reference.

2.2.2. Melatonin assay

Melatonin present in both blood plasma and culture medium of pineal gland was measured using a direct radioimmunoassay adapted from Randall *et al.* (1995) as follows:

Assay buffer

Fresh buffer was made in the morning of the same day of the assay and refrigerated to 4 °C before use. The following chemicals were dissolved at 50 °C for 30 minutes in 150ml nanopure water:

2.688 g tricine [N-tris (hydroxymethyl) methylglycine] (Sigma-Aldrich, UK)

1.350 g sodium chloride (BDH, Poole, England UK)

0.150 g gelatine (BDH, Poole, England UK)

Radiolabel

A primary stock of melatonin [O-methyl-³H] (NET801250UC, Perkin Elmer, Cambridge, UK) was supplied with a specific activity of 70-85 Ci/mmol. This primary stock was used to create a stock A by diluting 20 µl in 2 ml absolute ethanol (Fisher Scientific, Leicestershire, UK). This was stored at -20°C in 20 ml high performance glass vials (Perkin Elmer Life Sciences, Cambridge, UK). A fresh working stock was then made for each assay from the stock A. Approximately 20 µl from stock A was diluted in 20 ml of buffer to give an approximate activity of 4000 disintegrations per minute (dpm)/100 µl of radiolabel.

Antibody

Frozen dried sheep anti-melatonin antiserum (Stockgrand Ltd, University of Surrey, Surrey, UK) was reconstituted in 4 ml of assay buffer. This was then aliquoted in 100 μ l and 200 μ l volumes into stoppered 3 ml polystyrene tubes and kept at -20 °C until used. The working solution was used by reconstituting one 200 μ l aliquot in 40 ml of fresh assay buffer.

Standards

A stock solution of 1 mg/ml of melatonin, N-acetyl-5-methoxytryptamine (Sigma-Aldrich, UK), was prepared by dissolving 10 mg of melatonin in 10 ml of absolute ethanol and was stored in a high performance glass vial at -20 °C until required. For each assay, two working solutions of 1 ng/ml and 2 ng/ml of standard were prepared from the primary stock by diluting aliquots in assay buffer; these were then used to create a standard curve. The standard curve was prepared to have concentrations ranging from 500 pg per tube to 0 pg melatonin per tube in duplicate tubes. In the first tube pair 250 μ l of the 2 ng/ml standard were placed (i.e. 500 pg per tube standard) followed by 250 μ l of the 1 ng/ml standard in the second tube pair (i.e. 250 pg per tube standard) and thereafter a 1:1 serial dilution was performed with fresh assay buffer to produce a range of paired standards from 500 pg per tube to 1.95 pg per tube, an additional pair of tubes was included with just assay buffer (i.e. 0 pg per tube).

Assay protocol

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

1. Prepare a series of melatonin standards in 3 ml polypropylene tubes (LP3P) (Thermo life science, UK) to give a range of dilutions from 0-500 pg / 250 μ l. Then add a further 250 μ l of assay buffer to these tubes.
2. Add 700 μ l of buffer to two tubes which are used to calculate the non-specific binding (NSB) and add 500 μ l of sample to sample tubes.
3. Add 200 μ l of antibody to all tubes except the NSB's, vortex and incubate at 20 °C for 30 minutes.
4. Add 100 μ l of labelled melatonin to all tubes, vortex and incubate at 4 °C for 18 hr.
5. On the second day after overnight incubation, dissolve 0.48 g of dextran coated charcoal (Sigma-Aldrich UK) in 50 ml of assay buffer and stir on ice for 30 minutes. Then add 500 μ l of this charcoal suspension to each tube, vortex and incubate at 4 °C for 15 minutes.
6. Centrifuge at 1730 xG for 15 minutes at 4 °C.
7. Transfer 1 ml of supernatant to 6 ml polyethylene scintillation vials (Perkin Elmer Life Sciences, Cambridge, UK) and add 4 ml of scintillation fluid (Ultima Gold, Perkin Elmer Life Sciences, Cambridge, UK).
8. Place 4 ml of scintillation fluid into 3 extra vials. Into 2 of these extras, place 100 μ l of tritiated melatonin from the previously made stock to calculate total radioactivity. Use the final vial to calculate background radioactivity (blank).
9. Vortex all vials and count the radioactivity for 10 minutes in a scintillation counter (Packard 1900 TR Liquid Scintillation Analyzer, Canberra Packard Ltd).

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

$$[(\text{pg per tube}/1000) \times 1300] \times 2 = \text{pg /ml of melatonin in plasma}$$

When measuring melatonin concentration in pineal culture media, the same protocol was followed but media samples were diluted (1:10 day sample, 1:20 night samples) in assay buffer to a final volume of 500 μl .

Quality Control & Validation

Aliquots of known melatonin concentration, (50 pg/tube of N-acetyl-5-methoxytryptamine), were stored at $-70\text{ }^{\circ}\text{C}$, and used to check the reproducibility of measurements both within and between assays. Intra- assay coefficient of variation was 8.25 % (n=5) and inter-assay coefficient variation was 8.81 % (n= 20). The assay had previously been validated for use in sea bass by Vera *et al.* (2010).

2.2.3. Testosterone Assay

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

Steroid extraction

Prior to RIA analysis, steroid extraction from plasma samples was required. Steroid was extracted using organic phase solvent (ethyl acetate) as follows:

1. Add 200 μl of plasma samples to 3 ml polypropylene tubes (LP3P)
2. Add 1 ml ethyl acetate to each tube and cap the tube
3. Spin tubes in rotary mixer for 1 hour
4. Centrifuge tubes at 770 xG, for 10 minutes at $4\text{ }^{\circ}\text{C}$

Extracted samples can be then stored at $4\text{ }^{\circ}\text{C}$ until assay.

Assay Buffer

Buffer was made fresh for every assay by dissolving the following chemicals in 250 ml of nanopure water at 50 °C for 30 minutes and was then refrigerated to 4 °C before use:

4.44 g disodium hydrogen phosphate

2.91 g sodium dihydrogen phosphate

2.25 g sodium chloride

0.25 g gelatine

Radiolabel

A primary stock of tritiated testosterone, ([1, 2, 6, 7-³H] testosterone) (Amersham, UK), was supplied in 250 µCi quantities with a specific activity of 70-105 Ci/mmol. An intermediate stock (stock A) was made by diluting 20 µl from the primary stock in 2 ml absolute ethanol and stored at -20 °C in 20 ml glass vials. A fresh working solution was then made for each assay from stock (A) by diluting 50 µl of stock (A) in 10 ml of assay buffer to give an approximate activity of 15,000 dpm/100 µl.

Antibody

Anti-testosterone antiserum (CER group, Marloie, Belgium) was reconstituted in 10 ml of nanopure water.

Standards

A stock of standard solution of 100 ng/ml of testosterone was prepared by dissolving 1 mg testosterone (Sigma-Aldrich, UK) in 10 ml of absolute ethanol and stored in glass vial at -20 °C until required. A working solution (10 ng/ml) was prepared by diluting 100 µl of stock solution in 0.9 ml of absolute ethanol and used to

prepare a standard serial dilution curve ranging from 1000 pg per tube to 1.95 pg per tube in 100 μ l absolute ethanol. The 1000 pg per tube standard was prepared from 100 μ l of standard working solution, then all subsequent standards being prepared from a series of 1:1 serial dilutions as outlined in Table 2.1.

Assay protocol

1. Add 200 μ l of sample extract into duplicate 3 ml polypropylene tube.
2. Prepare standard curve, as described in Table 2.1, in 3 ml polypropylene tubes and include 4 tubes containing only 100 μ l absolute ethanol which are used to calculate the zero testosterone level and non-specific binding (NSB's).
3. Dry down all tubes in a rotary vacuum evaporator (mivac quattro concentrator, Genovac ltd, Ipswich, UK) at less than 35 °C for approximately 45 minutes, and then cool the tubes at 4 °C.
4. Add 100 μ l of antibody solution to all tubes, except the NSB's (tubes 23&24) to which add 100 μ l of assay buffer).
5. Add 100 μ l of tritiated -Testosterone solution to all tubes, vortex and incubate at 4 °C for 18 hr.
6. Dissolve 0.48 g of dextran coated charcoal in 100 ml of assay buffer and stir on ice for 30 minutes, then add 500 μ l of the charcoal suspension to each tube, vortex and incubate at 4 °C for 15 minutes.
7. Centrifuge at 1200 xG at 4 °C for 15 minutes
8. Transfer 400 μ l of supernatant to 6 ml polyethylene scintillation vials and add 4 ml of scintillation fluid (Ultima Gold).

9. Place 4 ml of scintillation fluid into 3 extra vials. Into 2 of these extras, place 100 μ l of tritiated melatonin to calculate total radioactivity. Use the remained vial to calculate background radioactivity (blank).

10. Vortex all vials and count the radioactivity for 5 minutes in a scintillation counter.

Table 2.1. Standards of testosterone (T) and 11-ketotestosterone (11-KT), all standards were produced in duplicate of 100 μ l of working solution with subsequent being prepared from a series of 1:1 serial dilutions.

Tube No.	Standard	+ ethanol	T (ng)	11-KT (ng)
1&2	100	None	1000	3000
3&4	100	100	500	1500
5&6	100 of 3&4	100	250	750
7&8	100 of 5&6	100	125	375
9&10	100 of 7&8	100	62.5	187.5
11&12	100 of 9&10	100	31.3	93.75
13&14	100 of	100	15.6	46.9
15&16	100 of	100	7.8	23.4
17&18	100 of	100	3.9	11.7
19&20	100 of 17&18	100	1.95 (remove 100 μ l from each)	5.9 (remove 100 μ l from each)
21&22	None	100	0	0
23&24	None	100	NSB	NSB

Results calculation

The unknown samples were compared to the standard curve dpm values and converted to pg testosterone per tube using Riasmart software. Then these values were converted to pg/ml testosterone in plasma using the following formulae:

$$\left[\left(\frac{\text{pg per tube}}{400} \right) \times 700 \right] = \text{pg of testosterone per 200 } \mu\text{l of extracts} \quad (1)$$

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

Quality control and validation

Testosterone line standard, made up from known concentration (94 pg/ml), was stored in absolute ethanol at -20 °C and was used as quality control in the same assay and between assays. The intra-assay coefficient of variation was 6.22 % (n=4) and the inter-assay coefficient of variation was 9.06 % (n=8).

2.2.4. 11-KT Radioimmunoassay

The radiolabel, antibody and analysis procedure of 11- ketotestosterone radioimmunoassay were kindly provided by Dr. Alexis Fostier (Department of Animal Physiology and Livestock Systems, Rennes Research Centre, France). Plasma samples were extracted by the same procedure used for testosterone; and levels of 11- ketotestosterone were measured by RIA according to Fostier *et al.* (1982).

Assay Buffer

Buffer was made fresh for every assay by dissolving the following chemicals in 250 ml of nanopure water at 50 °C for 30 minutes before transferring to a refrigerator to cool to 4 °C prior to use:

- 250 ml nanopure water
- 3.55 g disodium hydrogen phosphate
- 3.45 g sodium dihydrogen phosphate
- 2.25 g sodium chloride
- 0.25 g gelatine

Radiolabel

A primary stock of tritiated 11-Ketotestosterone was supplied with a specific activity of 84 Ci/mmol. An intermediate stock (stock A) was made by diluting 20 μ l from the primary stock in 2 ml absolute ethanol and stored at -20 °C in 20 ml glass vials. A fresh working solution was then made for each assay from the stock (A) by diluting 200 μ l of stock (A) in to 20 ml assay buffer to give an approximate activity of 15,000 dpm/100 μ l.

Antibody

Antibody supplied in 500 μ l volumes was diluted 1:150 to make the working solution. 100 μ l of working solution was added to all tubes except the NSB as outlined below.

Standards

Reference standards were prepared from a serial dilution (1:1) of the stock standard in absolute ethanol to have standard curve ranging from 3000 to 0 ng of 11-Ketotestosterone (Table 2.1).

Assay protocol

The same protocol for testosterone RIA was used in 11- ketotestosterone determination.

Quality control and validation

The sensitivity of the assay, i.e. the minimum amount of 11-Ketotestosterone statistically distinguishable from zero, was 5.9 pg. quality control tubes with content of

1500 pg 11-KT standard approximately were used to check the reproducibility of the measurements both within and between assays. The intr-assay coefficient of variation was 8.71 % (n=4) and the inter-assay coefficient of variation was 7.87 % (n=8). Serial dilutions of pooled sea bass plasma were used to obtain an inhibition curve (Figure 2.1). When plotted against the standard curve it was observed that the curve was parallel to the standard curve and no statistical difference in the gradient was found (ANOVA), indicating that the samples were immunologically similar to the standards.

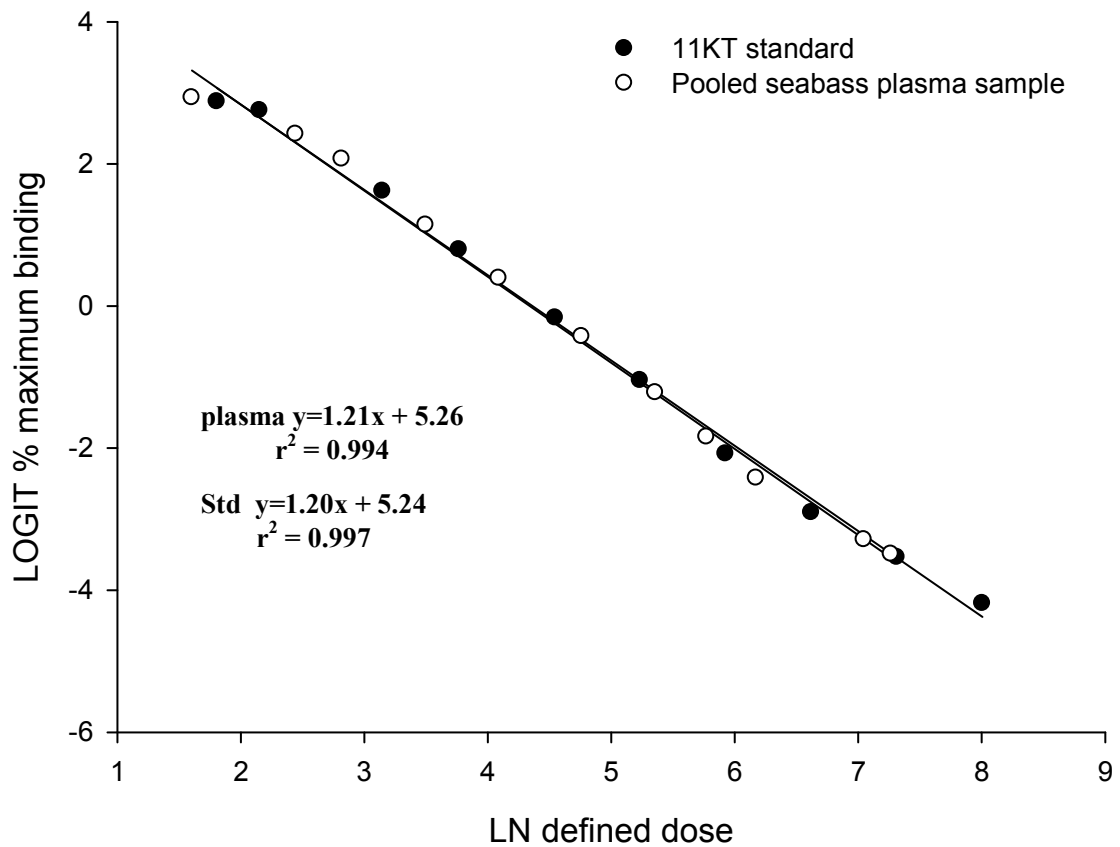


Figure 2.1 Parallelism of an inhibition curve obtained from serial dilution (1:10) of pooled plasma extract of male sea bass and standard. Each point represents the mean of duplicate measurements. The curve has been linearised using the logit transformation, with the x-axis denotes the natural log of 11- ketotestosterone content in standard.

2.3. Quantitative real time PCR

In experiments where gene expression activity was measured, quantitative real time PCR assays were established based around SYBR green chemistry using the methods outlined below.

2.3.1. Total RNA extraction

Total RNA was extracted from all tissues with the same method: fish were sacrificed by lethal dose of anaesthesia followed by decapitation, with brains and pituitaries being quickly removed and placed in DNase, RNAase free cryo vials (Alpha laboratories, UK) then frozen over liquid nitrogen vapour before being transferred to a -70 °C freezer for later total RNA extraction. For other organs, about 100- 200 mg of tissue was dissected and immediately frozen over liquid nitrogen vapour and then stored in -70 °C freezer. Thereafter the stored samples were homogenised on ice in RNA extraction buffer TRI Reagent® (Sigma-Aldrich, UK) (1 ml per 100 mg of tissue) using a rotating probe homogeniser (Ultra-Turrax®). To avoid contamination between samples, rotating probe and tweezers were washed between samples by ethanol followed by two washes of distilled water. Homogenate samples were centrifuged at 12,000 xG for 10 minutes at 4 °C in order to remove the insoluble material (extracellular membranes, polysaccharides, and high molecular weight DNA). Then, each ml of clear supernatant was transferred into a clean (DNase and RNAase free) 1.5 ml tube and samples were incubated for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. After incubation 0.2 ml of chloroform (Sigma-Aldrich, UK) was added per ml of homogenised sample, the tubes were then mixed with a vortex mixer for 10 seconds, incubated at room temperature for 10 minutes, then centrifuged at 12,000 xG for 15 minutes at 4 °C. The colourless upper

aqueous phase containing RNA was transferred to a fresh 1.5 ml tube and 0.5 ml of isopropanol (Sigma-Aldrich, UK) added to each tube and mixed well by inverting the tubes. The mixture was then incubated for 10 minutes at room temperature, then centrifuged at 12,000 xG for 10 minutes at 4 °C. Consequently, the RNA precipitated as a pellet on the side and bottom of the tube, the supernatant was discarded and the pellet was washed with 1 ml of 75 % ethanol (Sigma-Aldrich, UK) and then centrifuged at 7,500 xG for 5 minutes at 4 °C. This washing step was repeated twice. RNA pellets were air dried from ethanol for 10-15 minutes and pellets were reconstituted in 50 µl or more MiliQ water according to the pellet size. Samples were stored at -70 °C until quality check and cDNA synthesis.

2.3.2. RNA quality check

RNA quality checks were performed with a Nanodrop spectrophotometer (Labtech Int., UK) to evaluate the absorbance ratio at 260/280 and electrophoresis to check for RNA degradation: a sample with an absorbance ratio at 260/280 >1.8 indicates a high level of purity, not contaminated by protein (McKenna *et al.*, 2000). RNA samples were checked for degradation by running 2 µg of denatured total RNA by electrophoresis, on an agarose gel. Each RNA sample was mixed with 4 µl loading dye, 10 µl formamide and made up to 20 µl total volume with DNA/RNA free water, then RNA samples were incubated at 65 °C for 30 minutes after which samples were incubated on ice for 1 minute. After that samples were loaded in a 1 % agarose gel with Ethidium bromide (nucleic acid stain) (50 µg /100 ml agarose) and run for 45 minutes at 100 V and then the gel was visualized on a UV transilluminator (In Genius bio imaging, SYNGENE, UK). The total RNA quality was subjectively assessed based on

the integrity of the rRNA bands (18s and 28s) as they appeared after separation by gel electrophoresis (Figure 2.2).

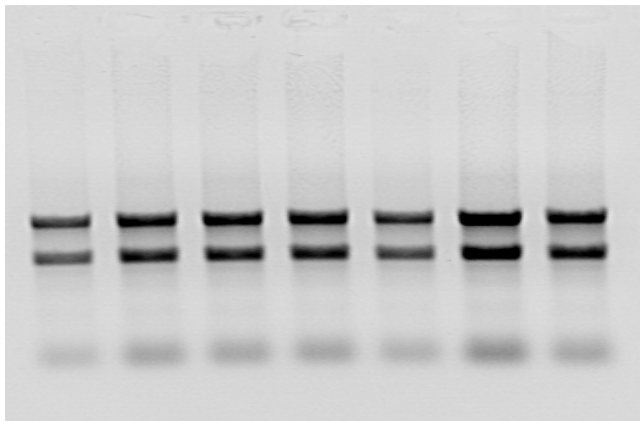


Figure 2.2. Typical total RNA quality showing rRNA 18s and 28s sharp bands after separation by gel electrophoresis.

2.3.3. DNase treatment

After the extraction of the RNA and quality checks, samples were treated to remove any contaminating DNA and the DNase and divalent cations from RNA preparations; this was done by using a commercial DNase treatment kit (*DNA-free*, Applied Biosystems, UK). The treatment was performed by adding 0.1 volume of 10x DNase1 buffer and 1 μ l of rDNase enzyme to 10 μ g of total RNA in a total reaction volume of 50 μ l. Each sample tube was mixed gently and incubated at 37 °C for 30 minutes. After incubation 5 μ l of re-suspended DNase inactivation reagent was added and mixed well, incubated 2 minutes at room temperature, centrifuged at 10,000 xG for 1.5 minute. The total RNA supernatant was then transferred to fresh tube.

2.3.4. Synthesis of sea bass cDNA

Complementary DNA (cDNA) is typically generated from RNA by the action of reverse transcriptase, which reverse transcribes a single strand of total RNA into single-stranded cDNA (Krebs *et al.*, 2011). cDNA was prepared using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) as follows: 1 µg of total RNA was mixed with master mix containing 2 µl of 10x RT buffer, 100 mM dNTPs mix, and 2 µl 10 x RT Random Primer and 4.2 µl of nuclease-free water in a final reaction volume of 20 µl. Tubes were centrifuged briefly to concentrate the contents at the base of the tube and loaded into a thermal cycler (T gradient thermocycler, Whatman Biometra, Goettingen , Germany) set on a program of 10 min at 25 °C, then 120 min at 37 °C followed by an inactivation step at 85 °C for 5 min. cDNA samples were then stored at -20 °C.

2.3.5. Cloning

To aid sequence identification and prepare stable QPCR standards PCR products (see 2.3.7) were routinely cloned into a plasmid vector (PGEM[®]-T easy vector, Promega, UK). Prior to cloning an aliquot of the target, the PCR product was checked for size and purity (i.e. single product) by gel electrophoresis and the remaining PCR product was then purified using a PCR purification kit (Illustra[®], GE Healthcare), according to the manufacturer's instructions, to remove excess primers, dNTPs and enzymes. Then the purified PCR product concentration was measured using Nanodrop spectrophotometer. The amount of PCR product to be used as insert was calculated to give a ratio of 1:3 vector to insert. The ligation of PCR product was performed according to the Promega kit manual, and then gently mixed with pipetting and incubated at room temperature for 1 hour. The plasmid containing the ligated PCR

product was then transformed into 50µl of JM109 High-Efficiency competent cells by heat shock for 45-50 seconds at 42 °C, then tubes were immediately returned to ice for 2 minutes. 950 µl of SOC medium (provided with the kit) were then added and the mixture was incubated at 37 °C for 1.5 hours on a shaker mixer (~ 150 rpm). The mixture was then spread on preheated LB agar plates and incubated overnight at 37 °C. The LB agar plates contained ampicillin (100 µg/ml), Kanamycin (50 µg/ml), X-gal (40mg/ml in dimethyleformamide) and IPTG (40µl of 100 mM for each plate) in accordance with the kit guidelines. On the following day, the white colonies only were selected as these colonies originated from cells that contained plasmids with ampicillin and kanamycin resistance genes but furthermore the white colour indicated a successful ligation of PCR product due to the disruption of the reaction of LacZα with X-gal. The white colonies were picked and grown overnight in 4 ml of LB containing ampicillin (100µg/ml). The plasmid-vector was purified using PureLink Quick plasmid Miniprep kit (Invitrogen, UK) following the kit instructions. The column was eluted with 50 µl of MilliQ water, and the plasmid was digested with EcoR1 restriction enzyme, which cut the vector before and after the cloning site. The size of the insert was checked by gel electrophoresis for the EcoR1 treated plasmid.

2.3.6. Sequence analysis

The sequence analysis was performed using a Beckman 8800 auto sequencer (Beckman Coulter, UK) following amplification of the cloned insert using a sequencing reaction kit (GenomeLab DTCS Quick Start Kit). The initial amplification reaction contained the recommended amount of the purified plasmid, 2 µl of sequencing mastermix, 1 µl of M13F or M13R (4µM) primer in a final volume of 5µl. The tubes were placed in a thermo cycler with the following cycling program: 30 cycles of 96 °C

for 20 sec, 50 °C for sec, and 60 °C for 4 min. After PCR amplification 15 µl of MilliQ water were added for each reaction tube, mixed and transferred into 1.5 ml tubes. Then reaction was stopped by adding 5 µl of stop/glycogen solution consisting of 2 µl of 100 mM EDTA (pH 8.0), 2 µl of 3M Sodium acetate (pH 5.2) and 1 µl of glycogen (supplied with the kit). DNA precipitation was performed by adding 60 µl of cold (chilled in -20°C freezer) 95 % ethanol, which was then mixed before centrifugation at 14,000 xG for 15 min. The resulting pellet was rinsed with 150 µl of cold 70 % ethanol with the last wash being removed by pipette and the pellet being left to air dry at room temperature for approximately 15 minutes. After that each sample was re-suspended in 30 µl of sample loading solution (supplied with the sequencing kit) and loaded to the sequencing plate.

Lasergene SEQman software (DNASTAR, USA) was used for editing and to assemble the DNA sequence results. The identity of the sequenced cloned PCR products were verified to be 100 % overlapping using BLAST search.

2.3.7. Quantitative PCR (QPCR)

All cDNA used in quantitative PCR (QPCR) assays was synthesised using the methods described above. Genes measured included the three different GnRH forms (*GnRH1*, *GnRH2* and *GnRH3*), two kisspeptin forms (*kiss1* and *kiss2*) and kisspeptin receptor (*Kissr4*), as well as *fshβ* and *lhβ* gene expression. The gene, elongation factor 1 alpha (*ef1α*) was used in all cases as a housekeeping reference gene, as it is stable across different stages of development (Mitter *et al.*, 2009). For all target genes, two primer pairs were designed: one was used to generate a large fragment which was cloned and acted as the assay standard and a second primer pair (smaller) was designed within this fragment and used for the QPCR assay itself. For *GnRH2* and *GnRH3*, due

to the small size of the sequence, the same primer pair was used to create the cloned standard as well as for the QPCR assay. Primers for the target genes and for the reference gene as well as accession numbers for each target are listed in Chapter 3, Table 3.1. QPCR reactions contained 1µl of each forward and reverse primers (7 pmol/µl), 5µl cDNA, 10 µl SYBR-green QPCR mix ((ABsolute™ QPCR SYBR Green Mix, ABgene, UK (consisting of Thermo-Start™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with 3 mM of MgCl₂) and 3 µl of MilliQ water in a final reaction volume of 20 µl. Reactions were run in a Quanta thermocycler (Techne, Quanta, Cambridge, UK); annealing temperature and product length for each gene are shown in Chapter 3, Table 3.1. The QPCR cycling program was the same for all reactions: first there was a “hotstart” enzyme activation for 15 min at 95 °C, followed by 45 cycles of 20 sec denaturation at 95 °C, 20 sec annealing, 30 sec extension at 72 °C, which was then followed by a melt curve temperature ramp from 70 to 90 °C with fluorescence being measured every 0.5 °C.

The copy numbers of each gene were automatically calculated using the Quanta software by a comparison to the created standard curve constructed from the results of a parallel set of reactions containing a serially diluted linearised plasmid with the insert fragment of target gene sequence. The standard for each assay was prepared by cutting the previously sequenced, cloned and purified plasmid containing the ligated target gene fragment at a single site, producing linearised plasmid. The restriction enzyme was selected following examination of the manufacturer's supplied vector map, as well as checking the inserted target gene sequence using SeqBuilder (DNASTAR, USA) to make sure that the inserted sequences lacked a restriction site for the selected enzyme. The restriction digestion was performed by adding 500 ng of plasmid to 1 µl of 10X specific enzyme (e.g. HindIII, BamH1, 0.2 µl of the specific restriction enzyme

and completed with water to a final reaction volume of 10 μ l. The reaction was then incubated at 37 °C for 2 hours followed by 10 minutes of deactivation at 70 °C. After that, 2 μ l of the reaction was checked by electrophoresis on 1 % agarose gel. After the linearization of the plasmid, the DNA concentration in the digest was measured by the Nanodrop spectrophotometer. The preparation of plasmid-based standards for each target gene was calculated using the supplier's guidance notes (Applied Biosystems, 2003). In short, the first stock standard was calculated to be 10^8 copies/ 5 μ l in a final volume of 1ml. This was made by adding the calculated amount of linear plasmid to λ -TE buffer. After this, serial dilutions were prepared from the 10^8 stock ranging from 10^7 to 10 copies per 5 μ l in 1 ml λ -TE buffer total volume. The λ -TE buffer consisted of a mixture 10 mM Tris, pH 8.0 (Sigma-Aldrich, UK), 100 mM EDTA (Sigma-Aldrich, UK) and 50 pg / μ l of λ DNA (Sigma-Aldrich, UK). The λ DNA is included within the buffer to saturate the storage tubes binding potential to free DNA and thus ensure the copy number of the target plasmid remained constant.

Following their preparation, a test assay was run including all plasmid standards, in parallel to a limited range of sample cDNA at different dilution rates. This assay helped in each case to identify the detection range and amplification efficiency of the assay. It also identified the three standards to be selected for subsequent gene expression quantification. Subsequently, each QPCR plate was prepared, including, in duplicate, three standards, non-template controls (MilliQ water) and four internal control samples (four random cDNA samples which were included in every plate to adjust for inter-assay variation) with the remainder of the plate being filled with unknown samples.

2.3.7.1 Gene copy number determination and quality assurance.

Gene copy number in each reaction was automatically calculated by the Quanta software compared to internal standards. QPCR efficiency was calculated by Quanta software for each plate run, with in all cases the efficiency being greater than 90 % (Figure 2.3 A). The Quanta software automatically determined the threshold fluorescence for crossing point determination (cp value) based on 3 times standard deviations above the mean “noise” fluorescence from the first 10 cycles (Figure 2.3 B). Following 45 amplification cycles the assay incorporated a melt curve analysis to determine the temperature at which the PCR product dissociated and thus the fluorescence dropped to background levels (Figure 2.4 A). The Quanta software then plotted this dissociation curve as a negative derivative of the fluorescence vs temperature (Figure 2.4 B). If the peaks (i.e. melting temperature) of all samples and standards were at the same temperature this indicated that the primer pairs used for the QPCR were highly specific and producing a single product. If however additional and/or shifted peaks were detected this would be an indication of primer dimers, mispriming or some form of contamination and the assay would have to be redesigned or the sample set repeated to remove this ambiguity.

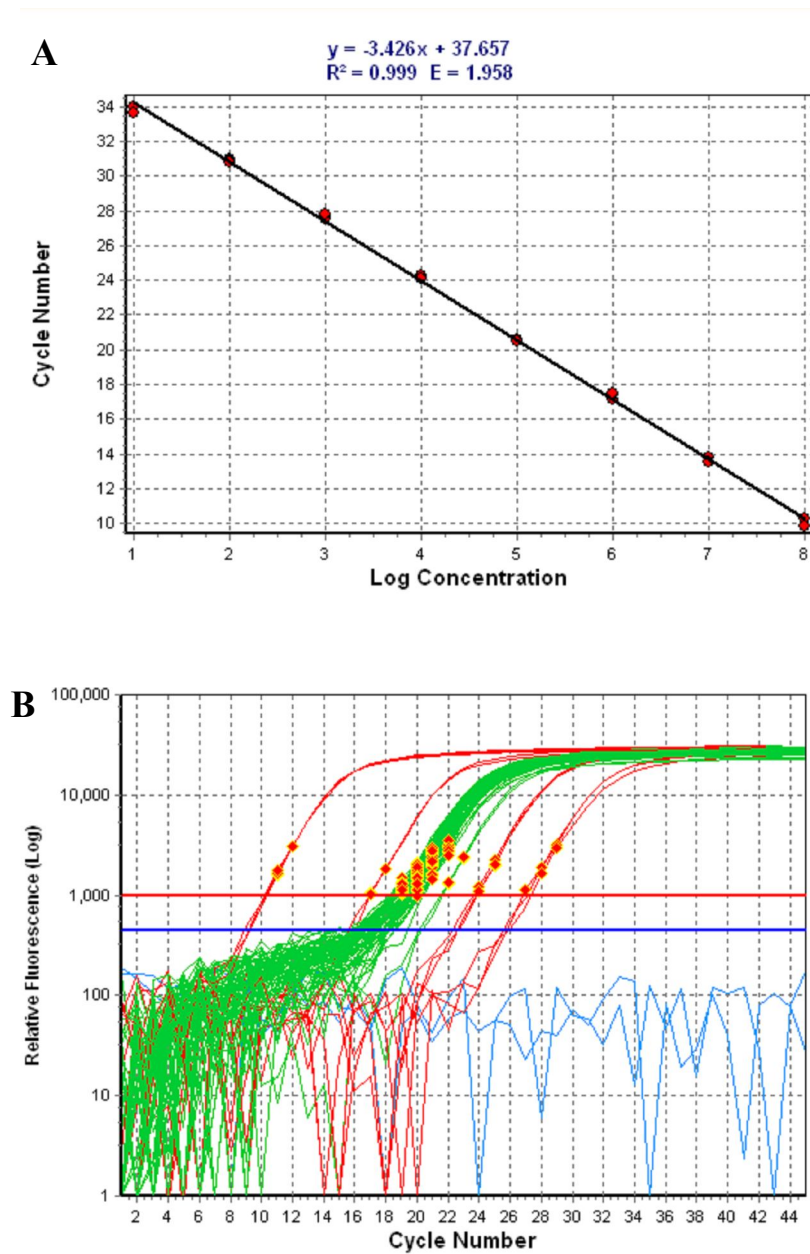


Figure 2.3. **A.** standard curve prepared from serial dilution of known amount of linearised plasmid containing the ligated target gene. **B.** log plot amplification curve: the red horizontal line is the threshold of fluorescence at which threshold cycles are crossing (red points), the blue horizontal line is the noise threshold. The samples are shown in green, standards are in red, and the non template controls are in blue.

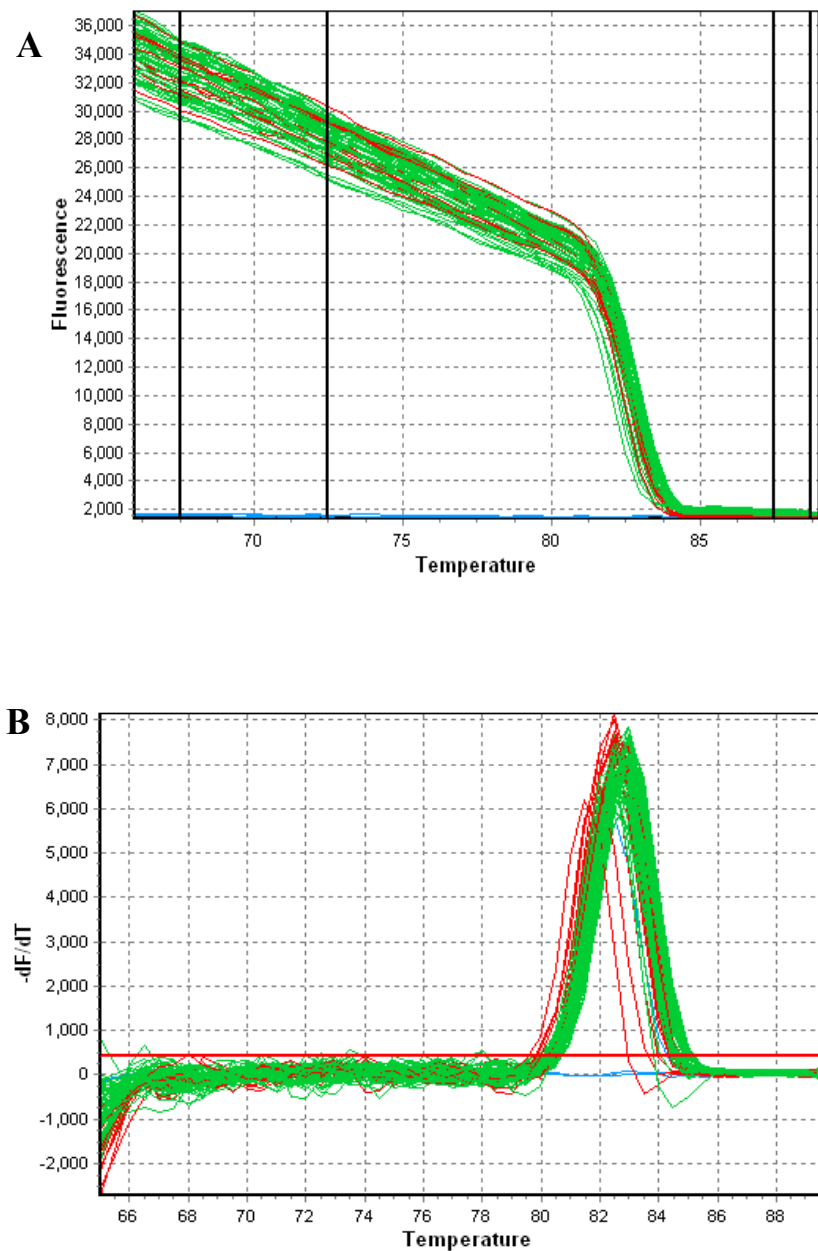


Figure 2.4. Melt curve analysis of a typical QPCR assay: **A** dissociation peak shown as fluorescence vs temperature; **B** negative derivative of fluorescence vs temperature. The red horizontal line is the automatic threshold which determines the area of the peak. Samples are in green colour, standards in red and non template controls are in blue lines.

2.4. Pineal culture protocol

In Chapter 5 the nonvisual light perception of the sea bass pineal gland was examined *ex vivo* using the following culture technique. Fish were killed between 12:00 and 16:00 and pineal glands removed after opening the skull dorsally around the pineal window and extracting the intact gland under the dissecting microscope. After removal, pineal glands were washed with culture medium before being held in sterile media prior to being installed in the culture system. The pineal culture system consisted of a continuous flow through system regulated by a peristaltic pump at a flow rate of 1.5 ml of culture medium/hour and a fraction collector automatically collecting samples every hour after passing through the culture chambers. The system was previously described by Migaud *et al.* (2006) (Figure 2.5). RPMI culture media (Sigma-Aldrich, UK) were dissolved in 1 liter of distilled water, along with 4.77 g/L HEPES sodium salt (Sigma-Aldrich, UK) with the pH adjusted to 7.4 using 2M Hydrochloric Acid. Penicillin–streptomycin (10 mg/L) (Sigma -Aldrich, UK) and Fungizone (5 mg/mL) (Sigma-Aldrich, UK) were added to the culture media to avoid bacterial and fungal development, and then culture media was filter sterilised through 0.2µm filter paper into sterile flasks and stored in a fridge. It was replaced daily. The pineal gland was placed in an individual culture chamber within the isolated culture cabinet that allowed a full control of temperature and light (intensity, spectrum and photoperiod). Media samples from each individual culture chamber were collected by an automatic fraction collector with the samples being removed daily and frozen in an ultracold freezer (-70°C) for later analysis.

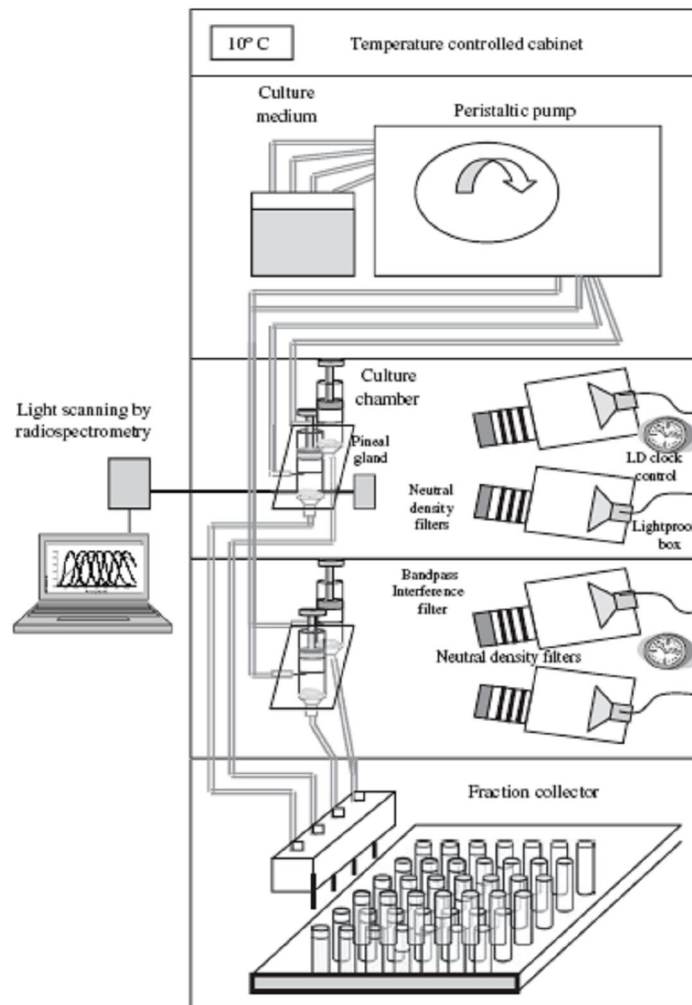


Figure 2.5. Ex vivo pineal gland culture system (Taken from Migaud *et al.*, 2006).

2.5. Pituitary cell culture

In Chapter 4 the responsiveness of isolated pituitary gonadotroph cells to synthetic stimulation was examined using the following culture methodology adapted from Chang *et al.* (1990).

Dispersion medium (DM) preparation:

Ca²⁺ and Mg²⁺ free dispersion medium was prepared from 25 % of 1x culture medium (L-15+L-glutamax, Gibco, Invitrogen), 75 % of 1x Hank's Balanced Salt (HBSS) (Gibco, Invitrogen), 25 mM HEPES (Gibco, Invitrogen), 0.5 % bovine serum albumin (BSA) (Sigma-Aldrich, UK). All the ingredients were mixed and the pH adjusted to 7.4 with 1 M NaOH (Sigma-Aldrich, UK). The medium was filtered through 0.2 µm filter, and sterility checked using tryptone soya broth (TSB) (BD biosciences, UK) for 2-3 days in 22°C and 37°C incubators including negative controls. The sterile medium was then stored in the fridge until use. On the sampling day, antibiotics (Sigma-Aldrich, UK) were added to the dispersion medium as follow: Penicillin/streptomycin (100 IU/ml of medium) (Gibco, Invitrogen), Kanamycin (100 IU/ ml of medium) (Gibco, Invitrogen) and 2.5 mg/L Amphotericin B (Sigma-, Aldrich, UK) as recommended by manufacturers.

Culture medium (CM) preparation:

The culture medium was made up from one liter of 1xL-15+L-glutamax, 25 mM HEPES and 0.1 % bovine serum albumin (BSA). All the ingredients were mixed and the pH was adjusted to 7.4 with 1 M NaOH and sterility checked as previously described. On the sampling day, 10 % of sterile FBS (Foetal bovine serum) and antibiotics

(Penicillin/streptomycin (100 IU/ml of medium), Kanamycin (100 IU/ ml of medium) and 2.5 mg/L Amphotericin B) were added as for the dispersion medium.

Primary cell culture procedure:

European sea bass pituitaries were removed using sterile dissecting instrument, kept in ice-cold dispersion medium in 50 ml Falcon tubes and washed in a Petri dish with dispersion media several times under sterile conditions. The DM was then decanted and pituitaries were cut into very small fragments using a scalpel in the laminar flow cabinet. Cells were enzymatically dispersed using trypsin/DNAse II (method modified from Chang *et al.*, 1990). Pituitary fragments were exposed to DM with 0.25 % trypsin for 40 minutes at room temperature, followed by washing with DM. Then a second treatment of trypsin was done for 40-50 minutes followed by DNAse II (0.1 mg /10ml) treatment for 10 minutes. Fragments were then mechanically dispersed by gentle suction and extrusion using a plastic transfer pipette to separate cells. Dispersed cells were harvested by centrifugation at 200 xG for 15 minutes and cells were reconstituted in 5 ml of culture medium containing 10 % foetal bovine serum. The count and viability of cells were calculated by using 100 µl of cell suspension + 100 µl of Trypan Blue dye (0.4% solution, Sigma, UK). Trypan Blue dye is an exclusion dye, viable cells remaining unstained, and enables estimation of overall culture viability. Counting was carried out using a standard Neubauer haemocytometer (0.1mm) and inverted microscope (IMT-2, Olympus).

The cell number per ml was estimated as the average count per 1 mm² square x dilution factor (dilution with trypan blue) x 10⁴. Percentage of viability was estimated as total viable cells (unstained) / total cells x100. The cell dispersion method yielded viability of 95-97 %. Dispersed cells were cultured on 12-well culture plates (Nunclon

delta, Thermo scientific) in culture medium containing 10 % foetal bovine serum. The plates were sealed with parafilm and incubated statically at 22 °C for 72 hours in an incubator for complete cell attachment; cultured cells were checked every day under an IMT-2, Olympus inverted microscope for viability and attachment percent (Fig. 2.6). After complete attachment, the culture medium was changed with fresh serum free culture medium as negative control or containing the treatment dose. All treatments and controls were performed in quadruplicate (four biological replicates). After incubation, the culture medium was removed and the cells were washed twice using 1 ml / well phosphate buffered saline 1x (PBS) (Gibco, Invitrogen), and RNA extracted from the wells using 1 ml / well of TRI Reagent® (Sigma-Aldrich, UK). Cells were scraped and moved with the TRI reagent into a clean (DNase and RNase free) 1.5 ml tube, then cells were mixed well with the TRI Reagent by pipetting up and down in the tube. Then samples were stored in -70 °C freezer for later analysis using the RNA extraction protocol described in 2.3.1.

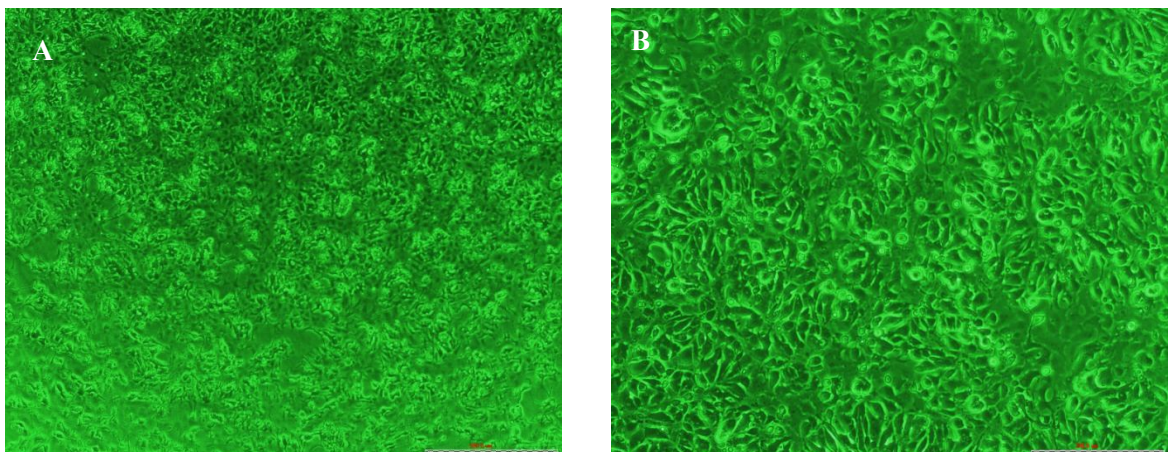


Figure 2.6. Sea bass pituitary cell after complete attachment (72 hours of incubation). (A) x 40 magnification, (B) x 100 magnification photographs were taken using IMT-2, Olympus inverted microscope with green filter.

2.6. Data and Statistical analysis

Statistical analysis was performed with MINITAB[®] version 16.0 (Minitab Ltd., Coventry, UK). Normality and homogeneity of variance were tested using Kolmogorov-Smirnov and Bartlett's tests test and data was transformed when needed. In Chapter 3, the temporal variations in gene expression, plasma steroid levels and GSI were compared using a one way ANOVA followed by Tukey's multiple comparisons post hoc test to identify significant differences. Correlations between all measured parameters were tested using Pearson's product method then the specific association with *fsh β* and *lh β* and *Kiss1/2/r4* *GnRH2*, *GnRH3* & *GnRH1* was explored using stepwise multiple regressions. In Chapter 4, for the incubation time experiment, differences between treatments and the control were analyzed using T- test for each time point, while in the second trial a one -way ANOVA was used followed by Tukey's test, while the comparisons between the two sampling times in the third experiment were analysed using two-way ANOVA. In Chapter 5, comparisons between different plasma melatonin levels at different temperatures and photoperiod (long day and short day) under in vivo and in vitro conditions were tested using two-way ANOVA followed by Tukey's test for mean comparisons. The melatonin levels under DD were tested by one- way ANOVA followed by Tukey's test for mean comparisons. In all cases data are expressed as mean \pm S.E.M values. In all cases a *p* value \leq 0.05 was considered to be statistically significant.

CHAPTER 3

KISSPEPTIN AND SEASONAL CONTROL OF REPRODUCTION IN MALE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*)

3.1. INTRODUCTION

In vertebrates, reproduction is regulated by a complex network of endocrine, paracrine and autocrine regulatory signals along the Brain-Pituitary-Gonadal (BPG) axis (Weltzien *et al.*, 2004; Zohar *et al.*, 2010). This axis is entrained by external cues, mainly photoperiod and temperature in temperate organisms (Bromage *et al.*, 2001; Pankhurst & Porter, 2003; Migaud *et al.*, 2010). The BPG axis is organised around; 1) the hypothalamus of the brain which, releases neuropeptides (gonadotropin releasing hormones, GnRHs) and neurotransmitters, which stimulate 2) the pituitary (gonadotroph cells), which synthesises and releases gonadotropins (follicle stimulating hormone, FSH and luteinising hormone, LH) which are transferred through the bloodstream and stimulate 3) the gonads to produce sex steroids (androgens, oestrogens and progestagens) that control gametogenesis and positive/negative feedback regulation of reproduction (Zohar *et al.*, 2010).

At the level of the brain (hypothalamus), the activation of the GnRH neurons and subsequent release of GnRH into the pituitary has traditionally been described as the starting point of the BPG axis controlling the onset of puberty in fish. Many studies have focused on the characterisation of this GnRH system (identification, localisation, pharmacology) in a range of teleosts and it has emerged to be more complex and diverse than first thought, with up to 24 distinct forms of GnRH identified in a variety of species ranging from invertebrates to humans, including eight variants isolated from fish brains (Kah *et al.*, 2007). Based on phylogenetic analysis of sequences and associated sites of expression, variants have been segregated into three branches named GnRH1, 2 and 3 (Kim *et al.* 2011). In the European sea bass, these three forms have previously been referred to as sea bream GnRH, sbGnRH (GnRH1), chicken GnRH-II, cGnRH-II (GnRH2) and salmon GnRH, sGnRH (GnRH3) (Zmora *et al.*, 2002;

Gonzalez-Martinez *et al.*, 2001; 2002). GnRH1 has been suggested to be the major hypophysiotropic hormone in sea bass (Gonzalez-Martinez *et al.*, 2002; 2004 a& b). whereas the significance of the GnRH2 and GnRH3 remains unclear. GnRH3 immunoreactive nerve fibres have been shown to innervate the pituitary of the sea bass although to a lesser extent than GnRH1 (Gonzalez-Martinez *et al.*, 2002; 2004a). Both *GnRH1* and *GnRH3* mRNAs have been shown in the European sea bass to increase along with the *GnRH-R* (GnRH receptor) and *fsh β* gene expression during sexual differentiation in both males and females (Moles *et al.*, 2007).

Recent research in mammals has brought to light the importance of a number of other upstream signal peptides that regulate GnRH expression (e.g. kisspeptin & neurokinin B) or neurotransmitters that work in association with GnRH's actions to regulate gonadotropin synthesis e.g. dopamine, neuropeptide Y (NPY) and gamma aminobutyric acid (GABA) (Mananos *et al.*, 1999; Vidal *et al.*, 2004; Zohar *et al.*, 2010). Kisspeptins which are peptides encoded by the *KISS1* gene, belonging to the RFamide family are ligands for the receptor Kiss1r (previously called GPR54) (Lee *et al.*, 1999; Kotani *et al.*, 2001; Muir *et al.*, 2001). The *KISS1* gene was initially identified as a metastasis suppressor in malignant melanoma cells (Lee *et al.*, 1996). *KISS1R* gene mutations were shown to impair reproductive function in human (de Roux *et al.*, 2003; Seminara *et al.*, 2003), and the targeted *Kiss1r* deletion cause serious retardation of mouse gonadal growth and fertility (Funes *et al.*, 2003), suggesting that kisspeptin system plays a crucial role in mammalian reproduction. Studies demonstrated that the Kisspeptin system responds to internal (sex steroids and metabolic factors) and external factors (environmental signals) and regulate GnRH neurons in mammals. When exogenous kisspeptin was administered to mammals, it elicited rapid increases in plasma FSH and LH levels (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Dhillon *et al.*, 2005;

Navarro *et al.*, 2005a). It is believed that kisspeptin roles have been conserved in fish and as such it has been associated with the onset of puberty (Filby *et al.*, 2008; Martinez-Chavez *et al.*, 2008). Furthermore kisspeptin was shown to have similar GnRH regulatory abilities in fish (Elizur, 2009) as well as being susceptible to sex steroid feedback (Kanda *et al.*, 2008).

While only one signal peptide kisspeptin 1 (KISS1) and receptor (KISSR1, formerly referred to as G-coupled protein receptor 54 (GPR54) are present in mammals, *in silico* analysis of the sequenced teleost genomes supported by functional analysis studies have revealed two forms of both the signal peptide (Kiss1 & Kiss2) and receptor (Kissr2 & Kissr4) in fish as in non-placental mammals (Felip *et al.*, 2009; Lee *et al.*, 2009, Akazome *et al.*, 2010). While both kisspeptin genes are expressed in zebrafish (Biran *et al.*, 2008; van Aerle *et al.*, 2008), medaka (Kanda *et al.*, 2008), goldfish (Li *et al.*, 2009) and sea bass (Felip *et al.*, 2009), only *kiss2* gene was found in the green spotted puffer fish, *Tetraodon nigroviridis*, the stickleback, *Gasterosteus aculeatus* as well as fugu, *Fugu rubripe* (Felip *et al.*, 2009; Akazome *et al.*, 2010). With regards to the kiss receptor, 4 different subtypes of *kissr* (1-4) have been described in vertebrates; only *kissr4* and *kissr2* have been reported in teleosts (Akazoma *et al.*, 2010). *Kissr4* has been reported in many teleosts and as such could be considered to be the most predominant and functionally active form (Akazome *et al.*, 2010) as the two forms (*kissr4* and *kissr2*) have been identified in only a few fish species including zebrafish (Biran *et al.*, 2008) and goldfish (Li *et al.*, 2009) and medaka (Lee *et al.*, 2009).

Mammalian Kiss1 is suggested to be key regulator for BPG axis and in teleosts its ortholog is named *kiss1*, however, its regulatory role is unclear (Kanda *et al.*, 2008; Felip *et al.*, 2009 & Li *et al.*, 2009). Kiss2 was shown to be more potent than *kiss1* to increase pituitary *lhβ* and *fshβ* mRNA expression when injected intraperitoneally to

mature female zebrafish (Kitahashi *et al.*, 2009). Similarly, intramuscularly injection of kiss2 to pre-pubertal and adult male sea bass was more potent in increasing FSH and LH over basal levels than kiss1 (Felip *et al.*, 2009). However, in the goldfish female, intraperitoneal administration of kiss1, but not kiss2, induced an increase in circulating LH (Li *et al.*, 2009). The hypophysiotropic actions of kiss1 was also confirmed *in vitro* on primary pituitary cell culture from goldfish (Yang *et al.*, 2010) and results also showed that kiss1 could stimulate prolactin and growth hormone secretion and gene expression in goldfish pituitaries (Yang *et al.*, 2010).

Studies of kisspeptin receptor temporal expression in fish species such as the female grey mullet (Nocillado *et al.*, 2007) have shown high levels of *kissr4* gene expression in the brain during the onset of puberty at the same time with high expression levels of the three *GnRH* types. Similar results have been found in zebrafish (van Aerle *et al.*, 2008), Nile tilapia (Parhar *et al.*, 2004; Martinez-Chavez *et al.*, 2008), fathead minnow (Filby *et al.*, 2008), and zebrafish (Kitahashi *et al.*, 2009). These findings have led a number of authors to suggest an important role played by the kisspeptin system in the activation of the BPG axis in fish. However, despite the recent findings obtained in fish, the role played by kisspeptin and its mode of action in the neuroendocrine regulation of reproduction is still not clearly defined especially in commercially important seasonal species like European sea bass. Furthermore, studies in fish have focused to date on the role played by kisspeptins during first reproduction (puberty) and not broodstock repeat spawning. Therefore, the aim of this study was to correlate the seasonal expression of kisspeptin genes in the brain throughout a reproductive cycle in male repeat spawning sea bass with GnRHs (*GnRH 1, 2 and 3*), gonadotropins (*fsh β* , *lh β*) gene expression and gonadal development (gonadosomatic index, sex steroid, histology).

3.2. MATERIALS AND METHODS

3.2.1. Source of the animals and sample collection

Male sea bass (total body weight: 736.78 ± 222.94 g, standard length: 37.61 ± 3.57 cm) were obtained from Machrihanish Marine Environmental Research Laboratory (Scotland, $55^{\circ} 44' N$, $5^{\circ} 44' W$). All fish were reared under simulated natural photoperiod and ambient temperature and fed to satiation twice per day with a commercial fish food formula (Atlantic HE 50C+ 35A/25C, Skretting, Invergordon, UK). Every month 10-15 fish were randomly selected, anesthetized with 1:10000 2-phenoxyethanol (Sigma-Aldrich Co. Ltd., Poole, UK), their body weight (± 0.1 g) and standard length (± 1 mm) were measured and blood was withdrawn from the caudal peduncle using heparinised syringes. Fish were dissected, pituitary and brain were snap-frozen in liquid nitrogen and stored in $-70^{\circ} C$ for later total RNA extraction and gonads were fixed in 10 % buffered formalin for histological observation. Gonadosomatic index (GSI) were calculated as $GSI = (\text{gonad weight} / \text{total body weight}) \times 100$. Blood was centrifuged at $1200 \times g$ for 15 minutes and plasma stored at $-70^{\circ} C$ for later hormonal analysis.

3.2.2. RNA extraction and cDNA synthesis

Total RNA from individual brains and pituitaries were extracted with TRIZOL reagent (Applied biosystems) following the manufacturer's protocol, RNA quality and concentration were measured with a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK) before running 1µg of total RNA in a 1 % agarose denaturing gel to check for ribosomal RNA of good quality. All RNA samples were DNase treated using Ambion DNA-free kit (Applied biosystems, Warrington, UK), cDNA was then synthesised with 1µg of total RNA and reverse transcribed in 20 µl reaction containing 2 µl of 10X RT Random primer, 50 U of MultiScribe™ Reverse Transcriptase, 2µl 10X buffer and 4mM dNTP mix (High capacity cDNA reverse transcription kit, Applied Biosystems/Ambion, Warrington, UK) and placed in a thermocycler (T gradient thermocycler, Whatman Biometra, Goettingen, Germany) using thermal cycling conditions of 25 °C for 10 mins, 37 °C for 120 mins, 85 °C for 5 mins. Samples were then diluted using MilliQ water and then stored in -20 °C until assayed by QPCR.

3.2.3. Primer design and Molecular cloning

Partial cDNA sequences for target genes were generated using primers (Eurofins MWG Operon, Edersberg, Germany) designed using PrimerSelect ver.6.1 program (DNASTAR) (Table 3.1). For sea bass *kissr4* partial sequence primers were designed based on the conserved regions of open-reading frames (ORF) of tilapia, cobia and grey mullet *Kissr4* (Accession numbers BAD34454, ABG82165 and ABG76790, respectively) sequences available from GenBank. For the other target genes, primers were designed based on previously published sequences for European sea bass. Sea bass Elongation factor -1 alpha (*ef1a*) (GenBank accession number: FJ008915) was selected as the house-keeping gene for normalization of the real time PCR quantification as recommend by Mitter *et al.* (2009). The primers for *GnRH2* and

GnRH3 were designed within the second exon, as this region contains the *GnRH* decapeptide and is thus present in all potential alternative splice variants for both genes (Zamora *et al.*, 2002). PCR reactions were performed using 2.5 µl 10x reaction buffer, 1 mM MgCl₂, 100 µM dNTPs, 0.4 µM forward and reverse primers, 1 unit of *Taq* DNA polymerase (Thermo Scientific) and 1 µl of synthesized cDNA (1:10 dilution). Thermal cycling consisted of initial denaturation at 94 °C for 3 mins followed by 35 cycles at 94 °C for 30 s, X °C for 30 s, and 72 °C for 1 minute, with final extension at 72 °C for 4 mins. The annealing temperature (X °C) for each primer pair is given in Table 3.1. Prior to cloning the PCR product was checked on 1 % agarose gel (should give a single product) and the PCR product was then purified using Wizard[®] SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA) and cloned into a pGEM-T Easy vector (Promega, Southampton, UK). The inserted fragment was then sequenced using CEQ-8800 Backman sequencer (Coulter Inc., Fullerton, USA) with the identity of the cloned PCR product verified (100% overlapping) using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

3.2.4. Sequence analysis

Sequence analysis was performed using Lasergene SEQman software (DNASTAR) to edit and assemble DNA sequences. Crustal W (Thompson *et al.*, 2000) was used to generate multiple alignments of deduced protein sequences. MEGA (ver. 4) was used (Tamura *et al.*, 2007) to deduce and bootstrap phylogenetic trees using the neighbour joining method (Saitou & Nei, 1987).

3.2.5. Quantitative RT- PCR (QPCR) assays

For all target genes quantitative PCR assays were established including a serial dilution of cloned standards which allowed absolute quantification of transcript concentration. Genes measured in the brain include the three different GnRH forms (*GnRH1*, *GnRH2* and *GnRH3*), two kisspeptin forms (*kiss1* and *kiss2*) and kisspeptin receptor (*kissr4*), while *fsh β* and *lh β* as well as *kissr4* gene expression was measured in the pituitary. Housekeeping gene Elongation factor-1 α (*ef1 α*) expression was also measured in both tissues. For all target genes apart from GnRH2 and GnRH3, two primer pairs were designed: one was used to generate a large fragment which was cloned and acted as the assay standard and a second primer pair (smaller) was designed within this fragment and used for the QPCR assay itself. For *GnRH2* and *GnRH3*, due to the small size of the sequence, the same primer pair was used to create the cloned standard as well as for the QPCR assay. Cloning and sequencing of target genes were performed as described in 2.3.5 and 2.3.6. Total RNA was extracted from sea bass male brains and pituitaries as described in 2.3.1 and cDNA generated. The qRT-PCR was performed using 1 μ l (70 nM) of each forward and reverse primers (Table 3.1), 5 μ l diluted cDNA (1:4 for brains and 1:10 for pituitaries), 10 μ l of 2X SYBR-green QPCR mix (ABSolute™ QPCR SYBR Green Mix, ABgene, UK, consisting of ThermoStart™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with 3 mM of MgCl₂) and 3 μ l of MiliQ water in a final volume of 20 μ l reaction. The reactions were run using a thermocycler (Techne, Quantica, Cambridge, UK). The thermocycler program was 95 °C for 15 mins (activation) followed by 45 cycles of 95 °C (15 s), the annealing temperature for primers (15 s) and 72 °C for extension (30 s), ending the program by a temperature ramp from 65 °C to 90 °C for melt curve analysis. Standard curves for each target gene were generated by 10- fold serial

dilutions of known concentrations of the plasmids containing the target transcripts. As part of the validation of the QPCR assays, the detection range, linearity and amplification efficiency of each primer pair were checked (Table 3.2). In each assay samples were run together with non template controls and internal controls used to correct the expression level between plates. All samples and standards were run in duplicate. Correlation coefficients (r^2) of the standard curves were ranged between 0.994 and 1.000. Gene copy number in each reaction was automatically calculated by the quantica software relative to a standard curve. Transcript levels of each target gene in brains and pituitaries were normalized against the reference gene and calculated as absolute copy numbers per μg of total RNA.

Table 3.1. List of primers used for cDNA cloning and quantitative real-time PCR standards with Gene Bank accession number, sequence, predicted amplicon size, and melting temperature (TM) of studied genes.

Primer name	Gene Bank accession No.	Nucleotide sequence	Product size	T _m (°C)
<i>kissr4F</i>		5'-TATGAGTGGAGACCGCTGTTA-3'	557	62
<i>kissr4R</i>		5'-CTATGGGGTTGACAGAGGAGTTG-3'		
<i>kissr4qPCRF</i>		5'-ATCGTTCTCCTCTTCGCTGTCTGC-3'	86	61
<i>kissr4qPCRR</i>		5'-TAGTTGGGCCGGTAGTTTGGGTAG-3'		
<i>kiss1F</i>	FJ008914	5'-ACGATGCCCCGACTCATTGT-3'	407	58
<i>kiss1R</i>		5'-TTACACTTTTCTTTTCCCCTCTGAA-3'		
<i>kiss1qpCRF</i>		5'-GCATCAATACTGGCATCAGCAAAGA-3'		
<i>kiss1qPCRR</i>		5'-TCAACCATTCTGACCTGGGAAACTT-3'		
<i>kiss2F</i>	FJ008915	5'-TCTCTGCGCTCAGGAGGAGGACTG-3'	303	60
<i>kiss2R</i>		5'-TGTTGACTTTTACCAATGGGACACA-3'		
<i>kiss2qPCRF</i>		5'-GGGAGGATTCCAGCCCGTGTCT-3'		
<i>kiss2qPCRR</i>		5'-GAGGCCGAACGGGTGAAGTTGAA-3'		
<i>GnRH1F</i>	AF224279	5'-ATGGCTGCACAAACCTTC-3'	217	59
<i>GnRH1R</i>		5'-TGATTCTCTGCACAACTAA-3'		
<i>GnRH1qPCRF</i>		5'-ACGCTGCTGGGACACTGC-3'		
<i>GnRH1qPCR</i>		5'-GGAAGCTCCCGACTATCTGATTG-3'		
<i>GnRH3 F</i>	AF224280	5'-GGTGCGGGTGTGTTGTTGGCG-3'	110	65
<i>GnRH3R</i>		5'-TGTTGCCTCTAGCTCTCCACACT-3'		
<i>GnRH2F</i>	AF2242281	5'-TGGGCTGCTTCTATGTGT-3'	86	68
<i>GnRH2R</i>		5'-CCAGCTCCCTCTTGCCTC-3'		
<i>lhβF</i>	AF543315	5'-GATGTTCCCCTTGGTGTTGAGTTT-3'	550	56
<i>lhβR</i>		5'-TGGGTATTTATTTGCACAGAGAA-3'		
<i>lhβFqPCRF</i>		5'-TTGAGCTTCCTGACTGTCCA-3'		
<i>lhβRqPCRR</i>		5'-GCAGGCTCTCGAAGGTACAG-3'		
<i>fshβF</i>	AF543314	5'-GTCATCCCACCAACATCAG-3'	275	53
<i>fshβR</i>		5'-TATGTCTCCAGGAAAGCG-3'		
<i>fshβqPCRF</i>		5'-ACCAACATCAGCATCCAAGTG-3'		
<i>fshβqPCRR</i>		5'-TTCTCTGTTCAAGCCTCTCATAGT-3'		
<i>ef1aF</i>	AJ866727	5'-AGGCTGACTGCGCTGTGCTGAT-3'	708	61
<i>ef1aR</i>		5'-ACCTGGGCGTTGAAGTTGTCTGCT-3'		
<i>ef1a qPCRF</i>		5'-CTGTGCTGATCGTTGCTGCTGGTGT-3'		
<i>ef1a qPCRR</i>		5'-CGTGCTCGCGGGTCTGTCC-3'		

Table 3.2. Details on validation assays for QPCR of *GnRH1*, *GnRH2*, *GnRH3*, *kissr4*, *kiss1*, *kiss2*, *lhβ*, *fshβ* and *ef1α*. Details include the quantification cycle (Cq) number of non-template controls (NTC), specifics of the standard curve including slope and Y intercept, QPCR efficiency calculated from the slope and r^2 value and the dynamic range including Cq variation at the lower limit.

Assay	Cq NTC (mean ± SD)	Slope	Y intercept	Efficiency (%)	r^2	Linear range	Lower limit Cq variation (coefficient of variation, %)
<i>GnRH1</i>	37.80 ± 0.96	-3.426	37.65	97.9	0.99	10-10 ⁸	0.48
<i>GnRH2</i>	30.93± 0.35	-3.166	33.51	93	99	10 ² -10 ⁸	0.44
<i>GnRH3</i>	29.34± 0.30	-3.361	35.89	98	1	10 ² -10 ⁸	1.80
<i>kissr4</i>	32.33± 0.37	-3.211	35.88	97.5	0.99	10-10 ⁸	0.43
<i>kiss1</i>	35.29± 0.70	-3.364	37.26	99	1	10-10 ⁸	0.21
<i>kiss2</i>	33.67 ± 0.20	-3.351	36.48	99.5	0.99	10-10 ⁸	1.75
<i>fshβ</i>	33.25±0.30	-3.32	36.75	99.9	0.99	10-10 ⁸	1.74
<i>lhβ</i>	30.72±0.37	-3.305	36.72	99.6	0.99	10-10 ⁸	0.83
<i>ef1α</i>	33.58±0.10	-3.162	35.89	96.4	0.99	10-10 ⁸	1.16

3.2.6. Histological preparation and analysis

Testis samples were dehydrated and embedded in wax using an automated tissue processor (Thermo Fisher, Cheshire, UK). Samples were then embedding using a histoembedder (Leica UK.Ltd, Milton Keynes, and UK). Sections were cut at 5 μ m using a rotary microtome (Leica UK.Ltd, Milton Keynes, and UK), stained using Mayers haematoxylin and eosin Y and examined under a light microscope to determine developmental stages according to Rodriguez *et al.* (2001).

3.2.7. Steroids analyses

The level of plasma Testosterone (T) and 11-ketotestosterone (11-KT) was measured by direct radioimmunoassay (RIA) according to Duston and Bromage (1987). For testosterone, tritiated T label (Amersham, UK) and anti-testosterone antisera (CER group, Marloie, Belgium) were used, the intra-assay and inter-assay coefficient of variation were 6.2 % (n=4) and the 9.1 % (n=8) respectively. The 11-Ketotestosterone RIA assay has been validated for sea bass prior to the analyses by confirming the parallelism between serial dilutions of plasma samples to the standard curve. Radiolabel and antibody were kindly provided by Dr. Alexis Fostier (Department of Animal Physiology and Livestock Systems, Rennes Research Centre, France), and levels of 11-ketotestosterone were measured by RIA according to Fostier *et al.* (1982). The intra-assay and inter-assay coefficient of variation for the 11-KT analyses were 8.7 % (n=4) and 7.9 % (n=8) respectively. All standards and samples were assayed in duplicate.

3.2.8. Statistical analyses

Statistical analysis was performed with MINITAB[®] version 16.0 (Minitab Ltd., Coventry, UK). Normality and homogeneity of variance were tested using Kolmogorov-Smirnov test and data was transformed when needed. Temporal variations within gene expression, plasma steroid levels and GSI were compared using a one way ANOVA followed by Tukey's post-hoc tests to identify significant differences. Significant differences were determined at $p \leq 0.05$. Correlations between all measured parameters were tested using Pearson's product method then the specific association with *fsh β* and *lh β* and *Kiss1/2/r4*, *GnRH2*, *GnRH3* & *GnRH1* was explored using stepwise multiple regressions. In all cases data are expressed as mean \pm S.E.M values.

3.3. RESULTS

3.3.1. Isolation of sea bass *Kissr4* partial cDNA and structural analysis

The primer pair of *Kissr4* produced a 556 bp fragment from sea bass brain cDNA samples (Fig. 3.1). This partial sequence covers almost 50 % of the target genes cds and includes sections of five of the receptor's seven trans-membrane domains when compared to other species *Kissr4* (Fig. 3.1). Phylogenetic analysis of the deduced amino acid sequence (185 aa) showed the partial fragment grouped within the *Kissr4* cluster with greatest identity (> 90 %) with orange-spotted grouper (*Epinephelus coioides*), Nile tilapia, grey mullet, cobia, bluefin tuna (*Thunnus maccoyii*) and Atlantic cod *kissr4* sequences (Fig. 3.2).

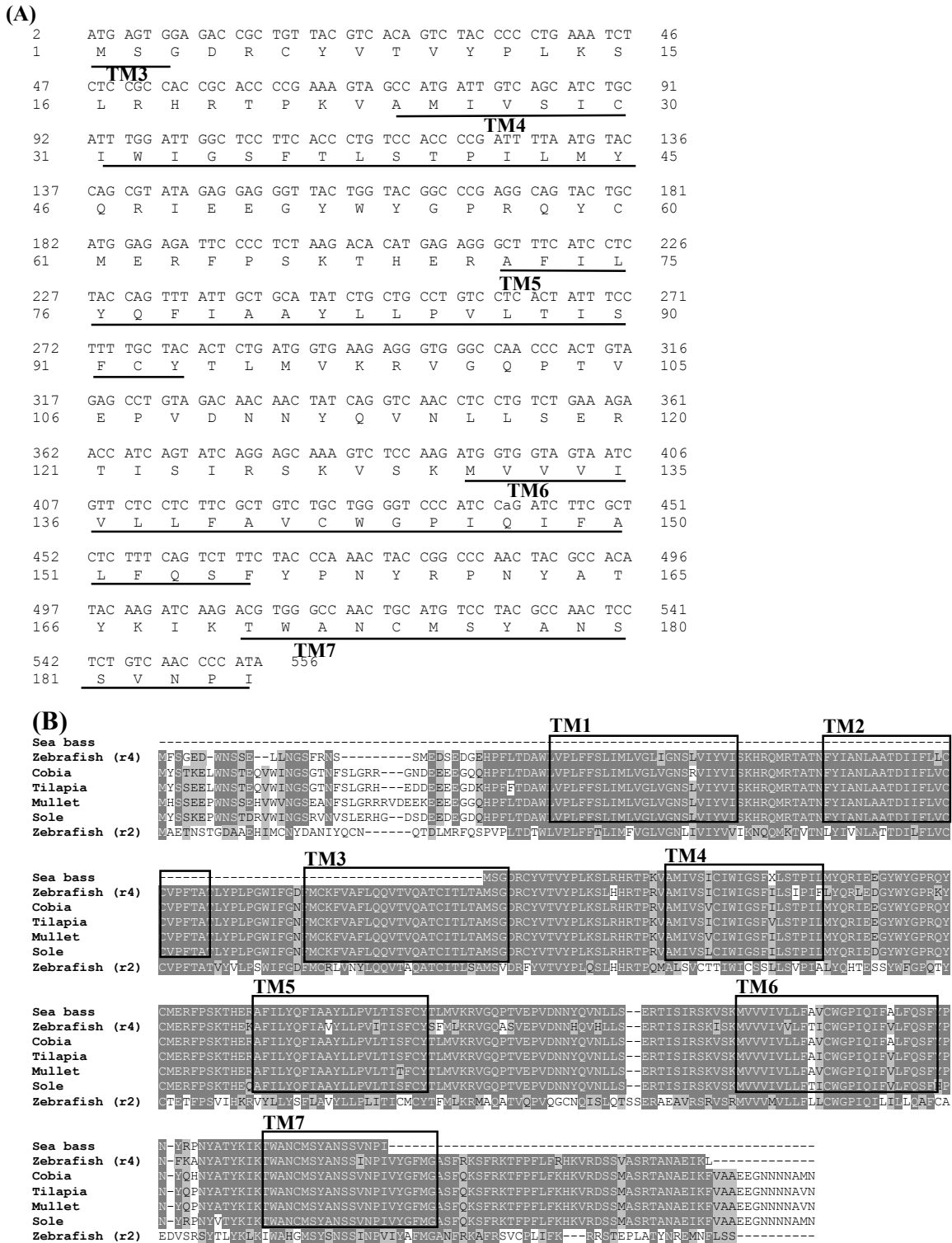


Figure 3.1. A) Nucleotide and deduced amino acid sequence of European sea bass Kissr4 partial cDNA fragment. Predicted transmembrane domains as defined in tilapia by Parhar *et al.* (2004) are underlined. B) Alignment of the deduced protein sequence for European sea bass, cobia, tilapia, grey mullet, Senegalese sole, zebrafish kissr2, zebra fish kissr4. The conserved amino acid residues are shaded. Predicted transmembrane domains as defined by Parhar *et al.* (2004) are boxed.

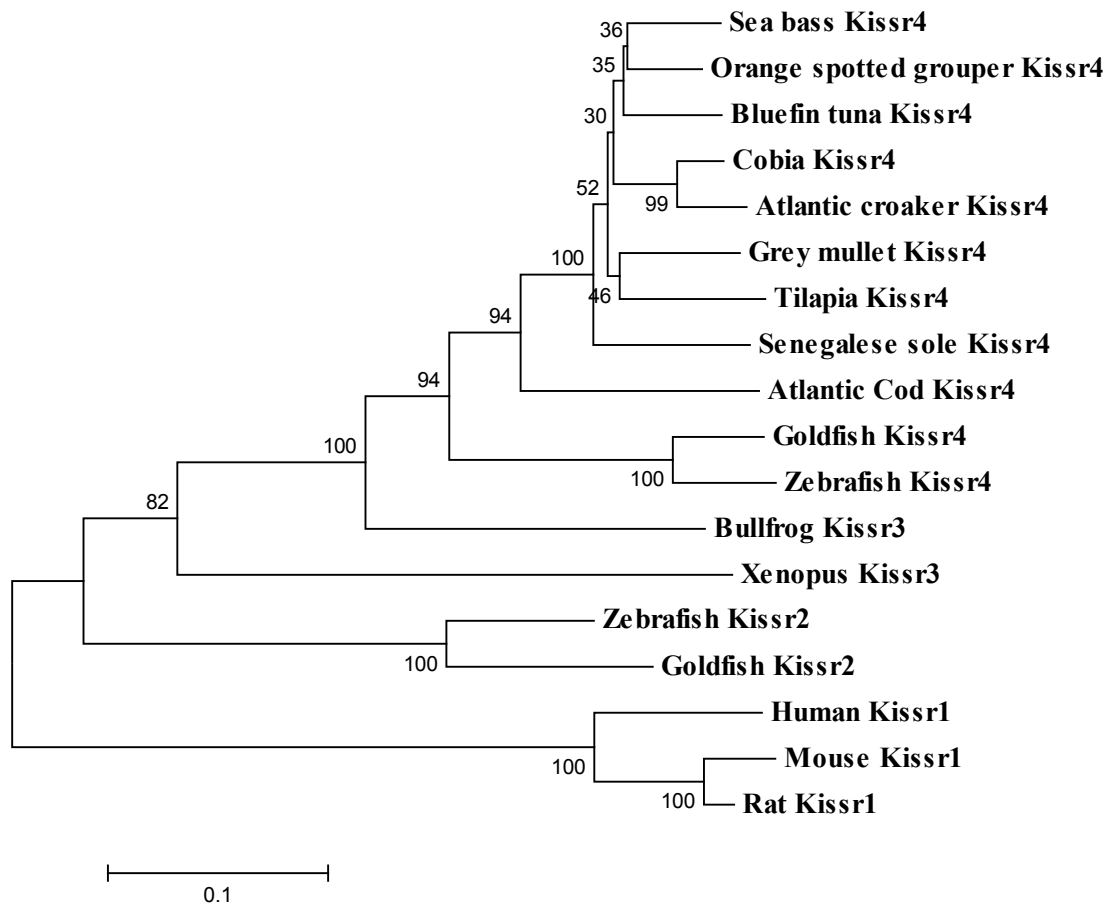


Figure 3.2. Phylogenetic tree analysis of Kissr4 sequences in vertebrates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). Accession numbers: Human KISSR1 (AAK83235), Mouse Kissr1 (AAK83236), Rat Kissr1 (AAD19664), Bullfrog kissr3 (ACD44939), Tilapia kissr4 (BAD34454), ZebraFish kissr4 (kissra) (ABV44612), Zebrafish kissr2 (kissrb) (ABV44613), Cobia kissr4 (ABG82165), Atlantic croaker kissr4 (ABC75101), Grey mullet kissr4 (ABG76790), Senegalese sole Kissr4 (ABW96362), Orange-spotted grouper kissr4 (ACT65994), gold fish kissra (ACK77792), gold fish kissr2 (kissrb) (ACK77793), Bluefin tuna kissr4 (ACT78954), Xenopus kissr1 (GPR54-1a) (EU853678) kissr3 (GPR54-2) (EU853680).

3.3.2. Gonadal development

Testicular development of adult male sea bass throughout an annual reproductive cycle was characterised histologically (Fig. 3.3). In July, August and September, the testes were in post-spermiation stage characterised by the presence of necrotic tissue and residual sperm in the lumen. In October, the presence of spermatogonia A and B as well as other cysts containing primary spermatocytes indicated the start of spermatogenesis. From November till March, the testis was in spermatogenesis with cysts containing primary and secondary spermatocytes, spermatids and spermatozoa. Full spermiation stage, characterised by cysts predominantly containing spermatozoa with some other cysts containing primary and secondary spermatocytes and spermatids, was observed from April to June (Fig. 3.4). Sea bass male gonads were smallest (*i.e.* lowest GSI) during July and August (0.5 %) then, GSI increased gradually in September and reached a peak in March (1.8 ± 0.3 %) and remained high in April and May. Thereafter, GSI dropped again to basal values in June (spent stage) (0.6 ± 0.9 %), GSI mean values for each month are represented in Figure 3.5.

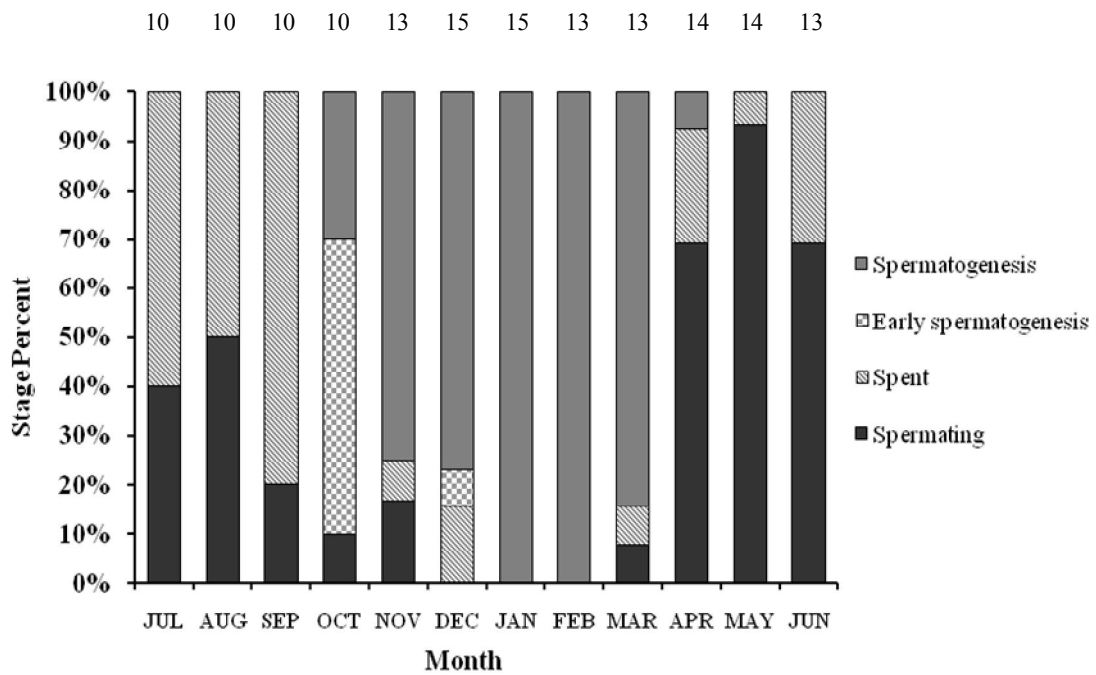


Figure 3.3. Gonadal development stage proportion of male sea bass sampled each month, during the annual reproductive cycle. Numbers above bars indicate the number of males sampled at each time point.

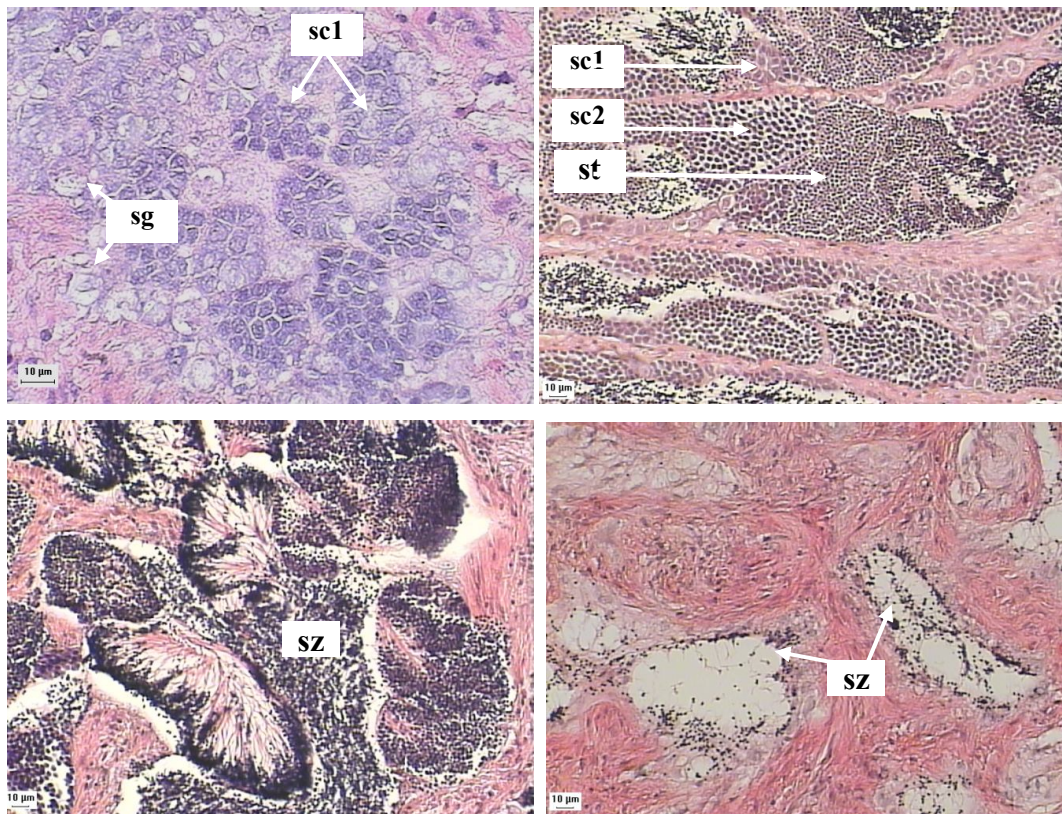


Figure 3.4. Testicular sections corresponding to the four representative stages of gonadal development during reproductive cycle of male sea bass. (A) Early spermatogenesis, lobules contain spermatogonia (sg) and primary spermatocytes (sc1) X40; (B) full-spermatogenesis, lobules contain cysts with primary spermatocytes (sc1) and secondary spermatocytes (sc2), spermatids (st) and spermatozoa (sz)X 20; (C) full-spermiation, lumen contains predominantly spermatozoa (sz) X20; (D) post-spermiation stage (spent), showing necrotic tissue with picnotic nucleus (black arrow) with residual spermatozoa (sz)X 20. Bars, 10 µm.

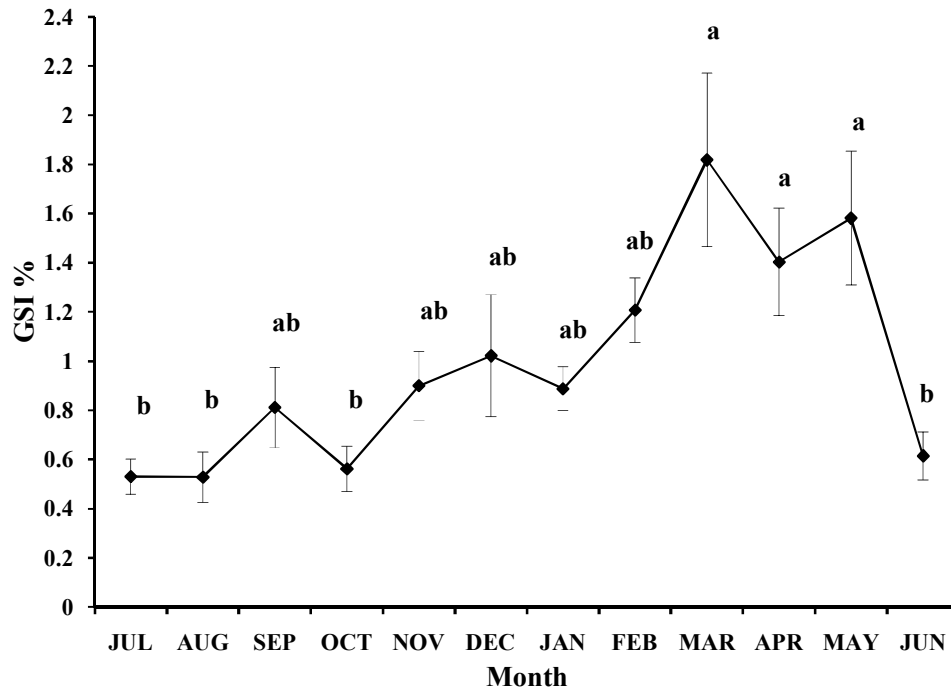


Figure 3.5. Changes in the gonadosomatic index (GSI, %) of male sea bass during the annual reproductive cycle (Mean \pm SEM, n= 10-15/month). Superscripts denote significant differences between months.

3.3.3. Plasma steroids

The plasma testosterone (T) levels showed very low levels in July (<0.1 ng/ml, spent stage) and increased significantly to reach a peak in October (spermatogenesis) (0.97 ± 0.08 pg/ml). Thereafter, T levels showed a second peak in February-March (1.56 ± 0.22 pg/ml) and then decreased again sharply to basal levels in April (spawning) (Fig. 3.6 A). The plasma levels of 11- ketotestosterone (11-KT) were basal in July (<1 ng/ml). 11-KT levels then gradually increased to reach a peak in October (7.83 ± 0.77 ng/ml) before slightly decreasing in the following months and then increased again to reach the maximum in March (15.55 ± 1.98 ng/ml). Levels then dropped to basal levels in June (Fig. 3.6 B).

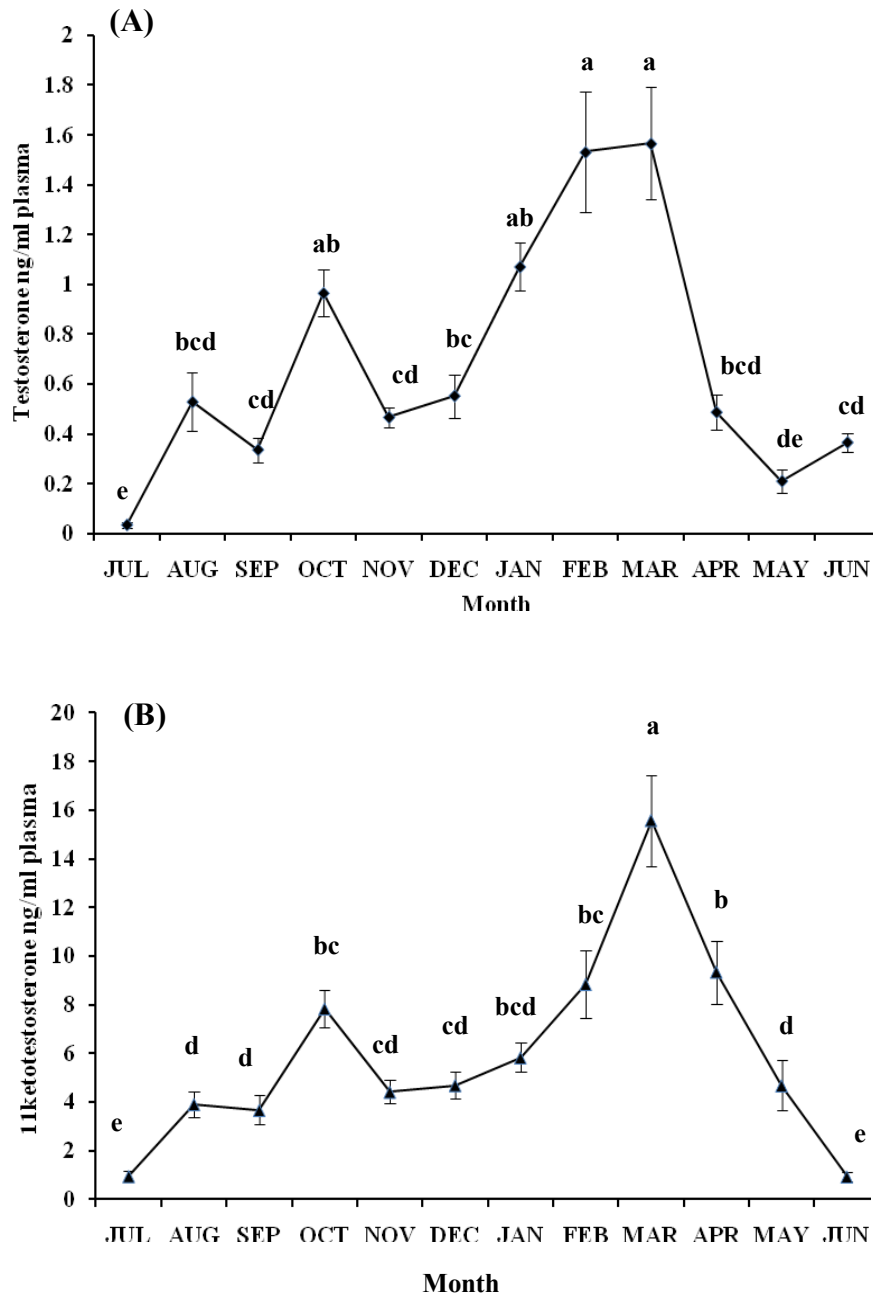


Figure 3.6. Monthly changes (Mean ± SEM, n= 10-15/month) in plasma levels of Testosterone (A) and 11-ketotestosterone (B) during the annual reproductive cycle. Superscripts denote significant differences between months.

3.3.4. Temporal expression of *GnRH* genes in the brain

Overall the variation of expression of *GnRH1* (Sea bream GnRH) was low (<4 fold difference between highest and lowest levels) and a clear seasonal pattern in expression was not apparent. However expression in September (spermatogenesis onset), January (spermatogenesis) and May (full spermiation) (>10,000 copies/ μ g total RNA) was significantly higher than in June (3,000 copies/ μ g total RNA) (Fig. 3.7 A). For *GnRH2* (Chicken *GnRHII*) expression profile showed a significant peak in March (spermatogenesis) (~2.5 fold) from basal expression levels in September and October (Fig. 3.7 B). However, for *GnRH3* (salmon GnRH) gene expression remained steady throughout the annual cycle (Fig. 3.7 C). Mean expression levels of *GnRH1* were up to 5 times lower than for *GnRH2* and *GnRH3*. By comparing the expression levels of *GnRHs* for the four developmental stages, *GnRH1* expression levels during spermatogenesis and spawning stages are significantly higher than expression level during spent stage (Fig. 3.10 A), while *GnRH2* and *GnRH3* expression levels did not vary significantly when compared between developmental stages (Fig. 3.10 B&C).

3.3.5. Temporal expression of kisspeptin genes in the brain

Expression of *kissr4* in the brain showed the first peak in September (spent/beginning of spermatogenesis) with significant differences from the lowest levels in whole reproductive cycle (August), then expression levels increased gradually and reached a peak in January (full spermatogenesis) (140,000 copies/ μ g of total RNA, a ~3.5 fold increase from basal levels observed in June and August) (Fig. 3.8 A). When the expression was considered in relation to developmental stage, *kissr4* expression peaked in the spermatogenesis stage with the expression being lowest in the spent stage (Fig. 3.10 D). Expression of *kiss1* was the lowest in October-November and then

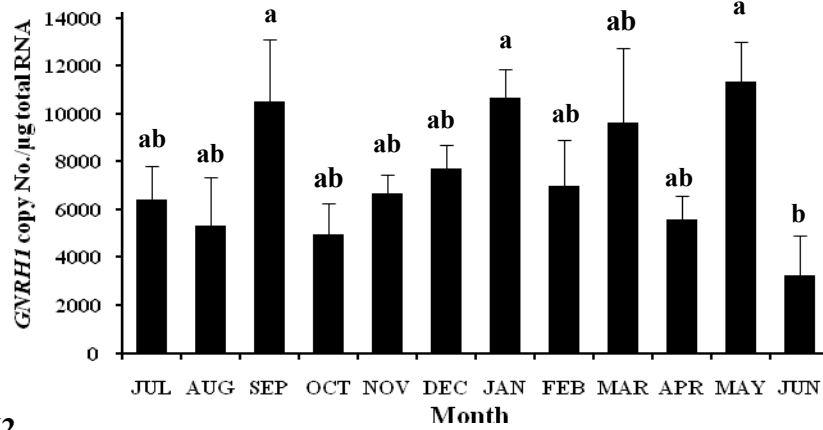
increased to have the first significant rise in January (spermatogenesis), then the expression increased gradually to reach peak expression levels in March-May (full spermiation) (~3 fold increase when compared to the minimum expression). *kiss1* expression then decreased sharply in June to similar levels as in July-December (start of the study) (Fig. 3.8 B). Comparing the expression levels of *kiss1* in the four developmental stages, spermiating males have significantly higher expression levels than early spermatogenesis stage (Fig. 3.10 E). *kiss2* expression showed a similar profile to that seen for *kiss1*, the first significant rise in January (spermatogenesis), then a significant peak appeared in March (~2 fold increase) and then a gradual decrease to basal levels in June (Fig. 3.8 C). No significant differences were found for *kiss2* expression levels between the four identified developmental stages (Fig. 3.10 F).

3.3.6. Temporal expression of *fsh β* , *lh β* and *kissr4* in the pituitary

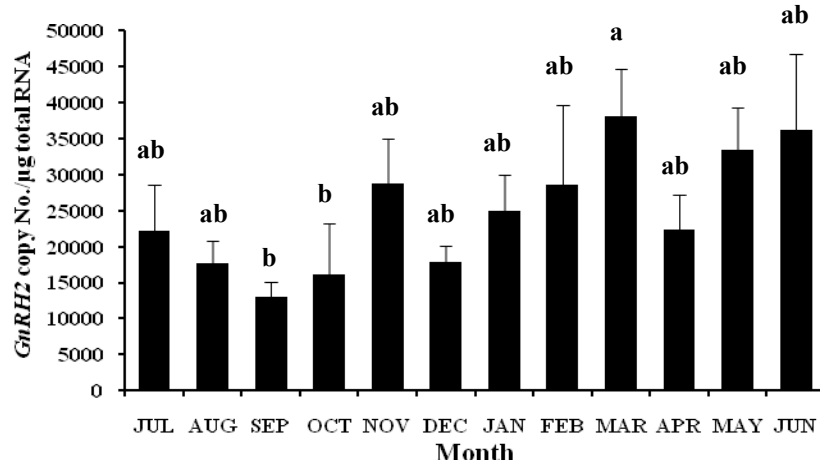
Both *fsh β* and *lh β* gene expression had the lowest expression levels in June and July (spent stage), then a transient but significant elevation of *fsh β* expression was observed by August and September, then both *fsh β* and *lh β* mRNA expression increased sharply between November and January (~3 fold increase in both cases when compared to basal levels seen at the start of the study) (Fig. 3.9 A& B). Thereafter *fsh β* remained elevated before returning to basal levels in June (Fig. 3.9 A), while *lh β* mRNA expression declined more gradually to reach its lowest expression levels in June (Fig. 3.9 B). The absolute *fsh β* mRNA levels were found to be consistently lower than those of *lh β* during the whole annual cycle. The *fsh β* mRNA expression level for spermatogenesis stage was significantly higher than those for early spermatogenesis and spent stages, with no significant differences to the spermiating stage expression levels (Fig. 3.10 G). When developmental stages were compared for *lh β* mRNA expression, no significant differences were observed between early spermatogenesis,

spermatogenesis and spermiating stages, while the spent stage was significantly lower than the expression levels for spermatogenesis and spermiating stages (Fig. 3.10 H). *kissr4* mRNA expression was detected in the pituitary during the whole reproductive cycle with very low levels of expression, except for the October samples, in which expression level was significantly higher (32003.32 ± 26430 copies/ μg total RNA) compared to the rest of annual cycle (mean = 338.55 ± 36.8 copies/ μg total RNA) ($P < 0.05$) (Fig. 3.9 C). The *kissr4* expression was significantly higher in the early spermatogenesis stage than the other developmental stages (Fig. 3.10 I).

(A) *GnRH1*



(B) *GnRH2*



(C) *GnRH3*

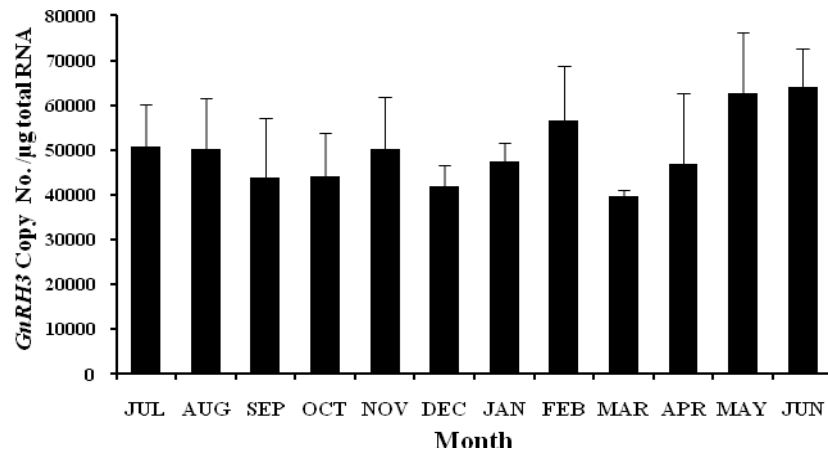
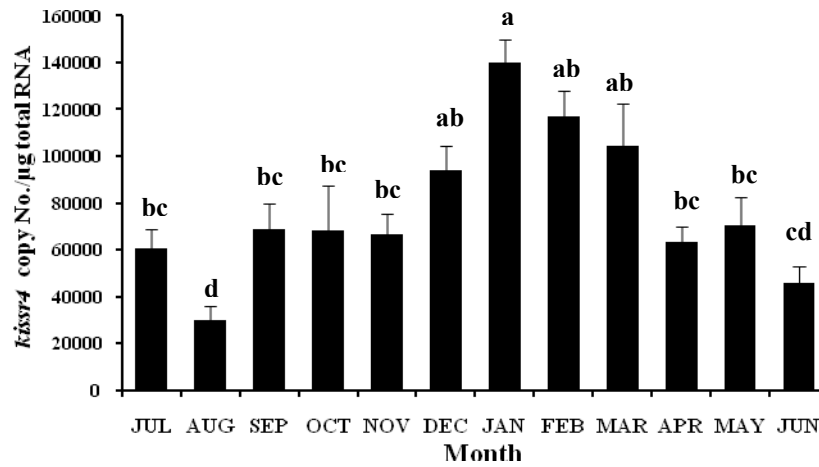
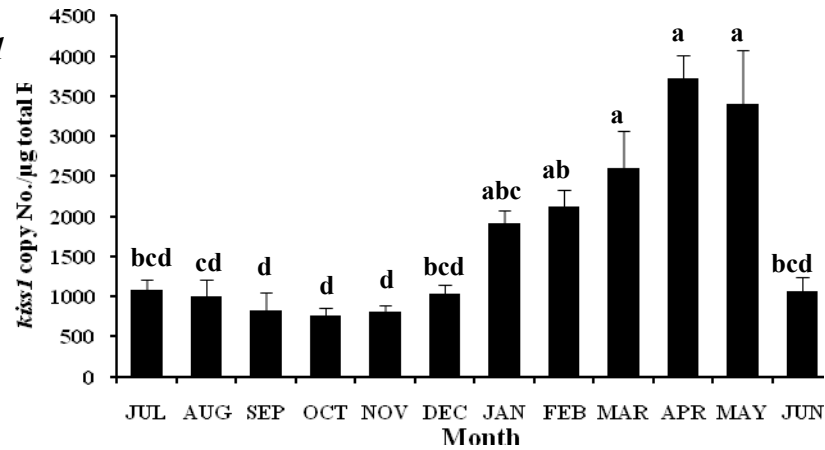


Figure 3.7. Absolute copy numbers of *GnRH1* (A), *GnRH2* (B) and *GnRH3* (C) genes in the brain of male sea bass during the annual reproductive cycle (mean ± SEM, n= 6/month). Superscripts denote significant differences between months.

(A) *kissr4*



(B) *kiss1*



(C) *kiss2*

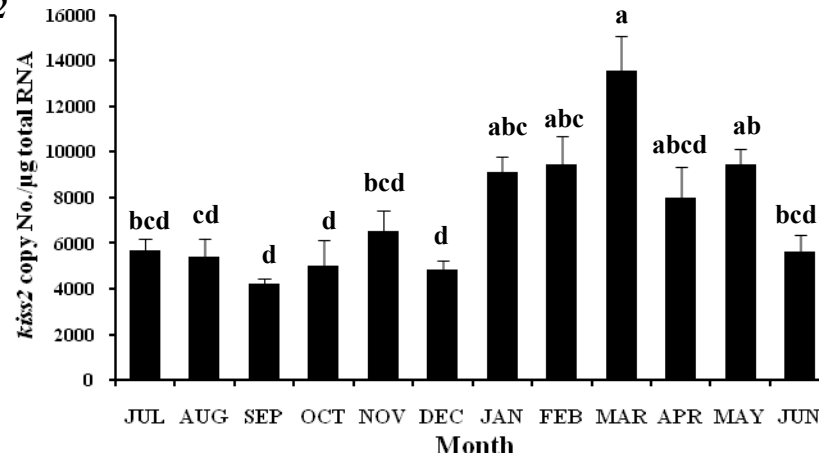


Figure 3.8. Absolute copy numbers of *kissr4* (A), *kiss1* (B) and *kiss2* (C) gene in the brain of male sea bass during the annual reproductive cycle (mean ± SEM, n= 6/month). Superscripts denote significant differences between months.

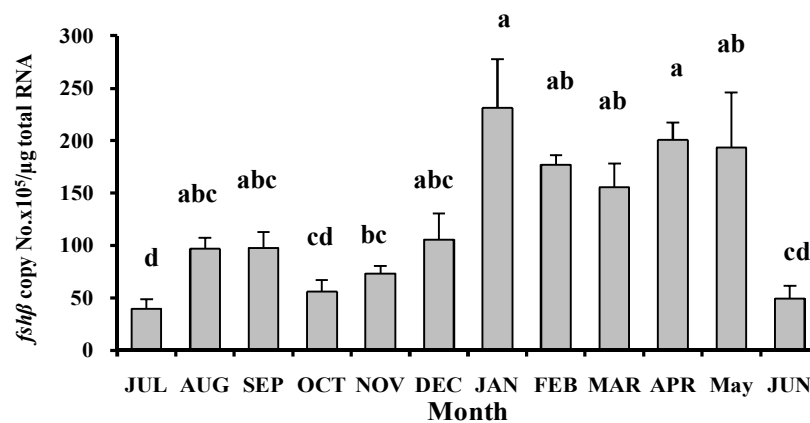
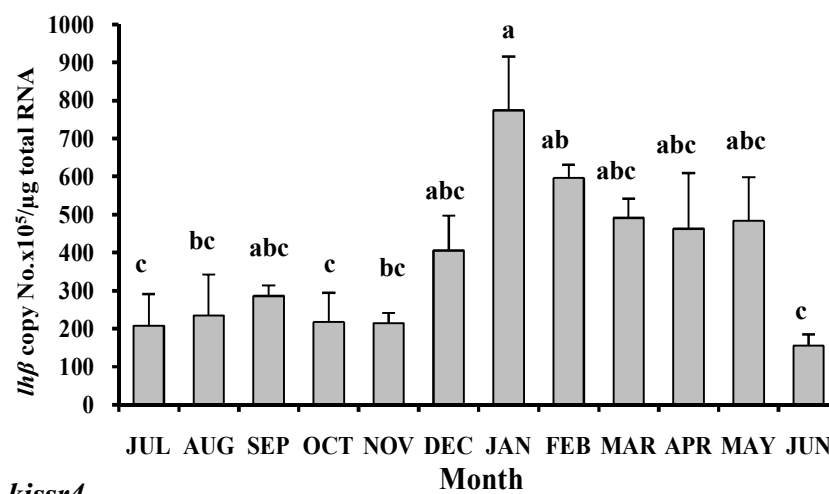
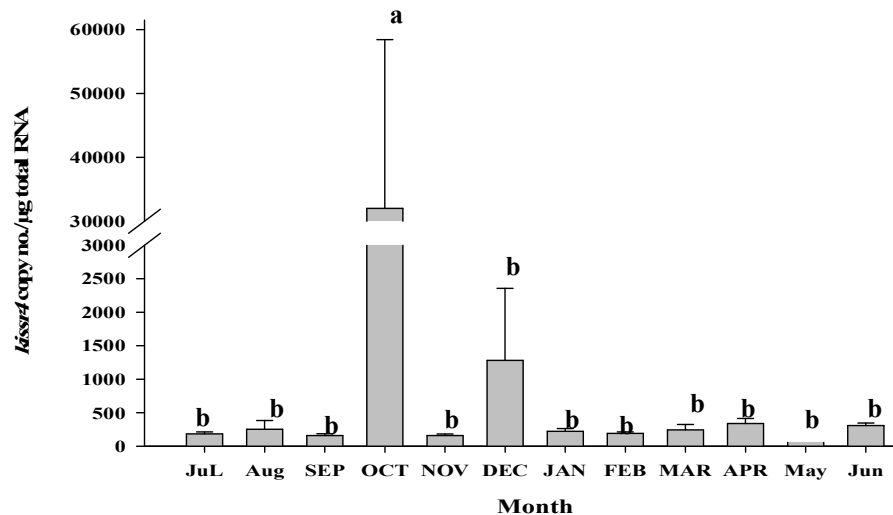
(A) *fsh β* (B) *lhβ*(C) *kissr4*

Figure 3.9. Absolute copy numbers of *fsh β* (A), *lh β* (B) and *kissr4* (C) genes in the pituitary of male sea bass during the annual reproductive cycle (mean \pm SEM, n= 6/month). Superscripts denote significant differences between months.

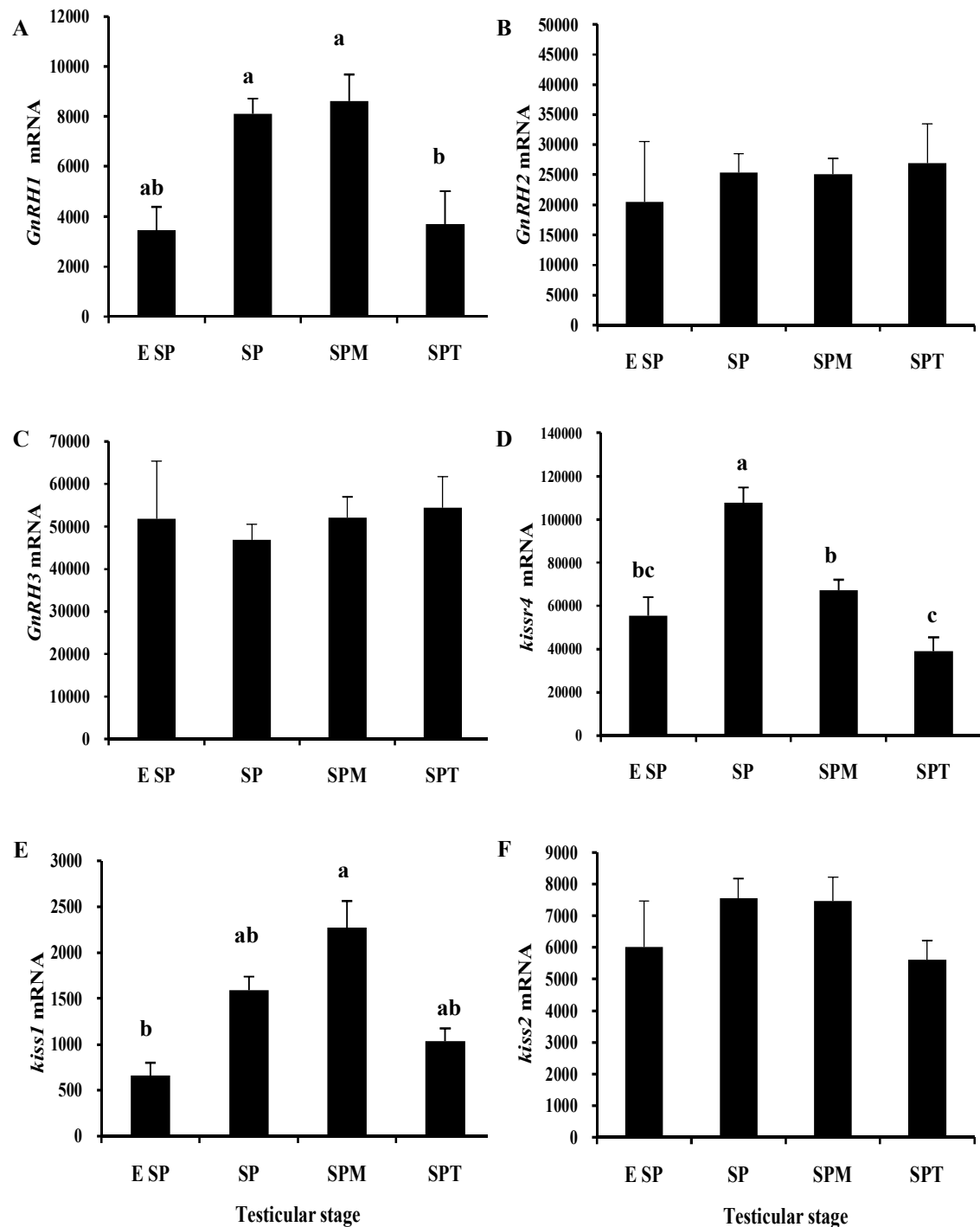


Figure 3.10. Mean absolute copy numbers of *GnRH1* (A), *GnRH2* (B), *GnRH3* (C), *kissr4* (D), *kiss1* (E), *kiss2* (F) genes in the brain and *fsh β* (G), *lh β* (H) and *kissr4* (I) in the pituitary of European sea bass based on gonadal staging. Values are mean \pm SEM. Superscripts denote significant differences between gonadal stages, ESP, early spermatogenesis; SP, spermatogenesis; SPM, spermiating; SPT, spent.

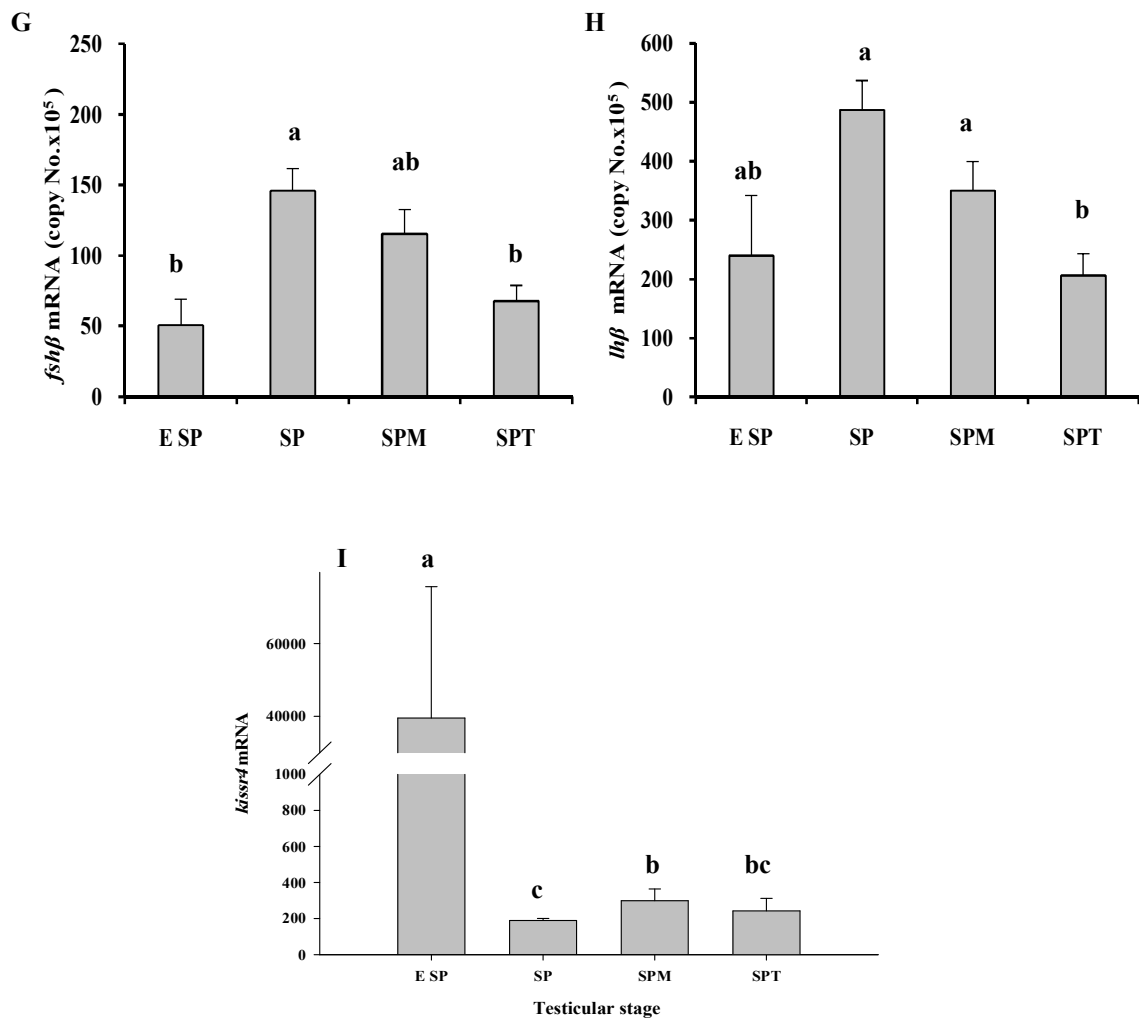


Figure 3.10 Contd. Mean absolute copy numbers of *GnRH1* (A), *GnRH2* (B), *GnRH3* (C), *kissr4* (D), *kiss1* (E), *kiss2* (F) genes in the brain and *fshβ* (G), *lhβ* (H) and *kissr4* (I) in the pituitary of European sea bass based on gonadal staging. Values are mean \pm SEM. Superscripts denote significant differences between gonadal stages, ESP, early spermatogenesis; SP, spermatogenesis; SPM, spermiating; SPT, spent.

3.3.7 Correlation analysis of gene expression levels.

To explore the potential relationship between gene expression and the measured endocrine and physiological changes a full factorial correlation matrix was created (Table 3.3). In total of 27 significant correlations were identified, however it must be acknowledge that the data set was not subjected to a multiple comparison correction, e.g. Bonferroni which may remove some of these correlations as false positive result, notwithstanding this, *Kiss1* & *Kiss2* show a strong positive correlation (Fig. 3.11) neither showed a significant association with their receptor (*Kissr4*). With regards to the different GnRH isoforms, *GnRH1* correlated with *kissr4* while *GnRH2* correlated with *Kiss2* (Fig. 3.11). However, *GnRH3* showed no significant correlation with any measured parameter except GSI. Both *fshβ* and *lhβ* correlated with most parameters thus a stepwise (backwards) multiple regression was tested which identified the main contributing parameters. In both cases *Kissr4* and *Kiss1* were the only parameters that contributed significantly to the model which accounted for >85 % of the variation in *fshβ/lhβ* expression:

$$fsh\beta = 3635971 + 117.28(kissr4) + 3622.9(kiss1)$$

$$p = 0.0002, r^2 = 85.29$$

$$lh\beta = 1.157e^7 + 461.99(kissr4) + 7765.6(kiss1)$$

$$p = <0.0001, r^2 = 87.69$$

When the data are plotted in three dimensions they display three distinct groups (April/May, Jan/Feb/Mar and all other months) of expression (Fig. 3.12).

Table 3.3. Correlation matrix of all possible linear regressions for all measured parameters over the course of the experiment.

	Kiss 1	Kiss 2	Kiss r4	sbGnRH	cGnRHII	sGnRH	FSH	LH	T	11KT	GSI
Kiss 1											
Kiss 2	$r^2 = 0.52$ $p = 0.0081$										
Kiss r4	n.s.	n.s.									
sbGnRH	n.s.	n.s.	$r^2 = 0.398$ $p = 0.0279$								
cGnRHII	n.s.	$r^2 = 0.52$ $p = 0.0072$	n.s.	n.s.							
sGnRH	n.s.	n.s.	n.s.	n.s.	n.s.						
FSH	$r^2 = 0.54$ $p = 0.06$	$r^2 = 0.39$ $p = 0.0297$	$r^2 = 0.51$ $p = 0.0093$	$r^2 = 0.38$ $p = 0.03$	n.s.	n.s.					
LH	$r^2 = 0.36$ $p = 0.045$	$r^2 = 0.34$ $p = 0.056$	$r^2 = 0.72$ $p = 0.0017$	$r^2 = 0.39$ $p = 0.047$	n.s.	n.s.	$r^2 = 0.9215$ $p = <0.0001$				
T	n.s.	$r^2 = 0.39$ $p = 0.03$	$r^2 = 0.45$ $p = 0.0169$	n.s.	n.s.	n.s.	n.s.	$r^2 = 0.26$ $p = 0.032$			
11KT	$r^2 = 0.30$ $p = 0.0631$	$r^2 = 0.65$ $p = 0.0015$	n.s.	n.s.	n.s.	n.s.	$r^2 = 0.27$ $p = 0.084$	$r^2 = 0.335$ $p = 0.0624$	$r^2 = 0.65$ $p = 0.022$		
GSI	$r^2 = 0.69$ $p = 0.0009$	$r^2 = 0.72$ $p = 0.0005$	n.s.	$r^2 = 0.3$ $p = 0.065$	$r^2 = 0.3$ $p = 0.065$	n.s.	$r^2 = 0.46$ $p = 0.015$	$r^2 = 0.37$ $p = 0.0348$	n.s.	$r^2 = 0.54$ $p = 0.0068$	

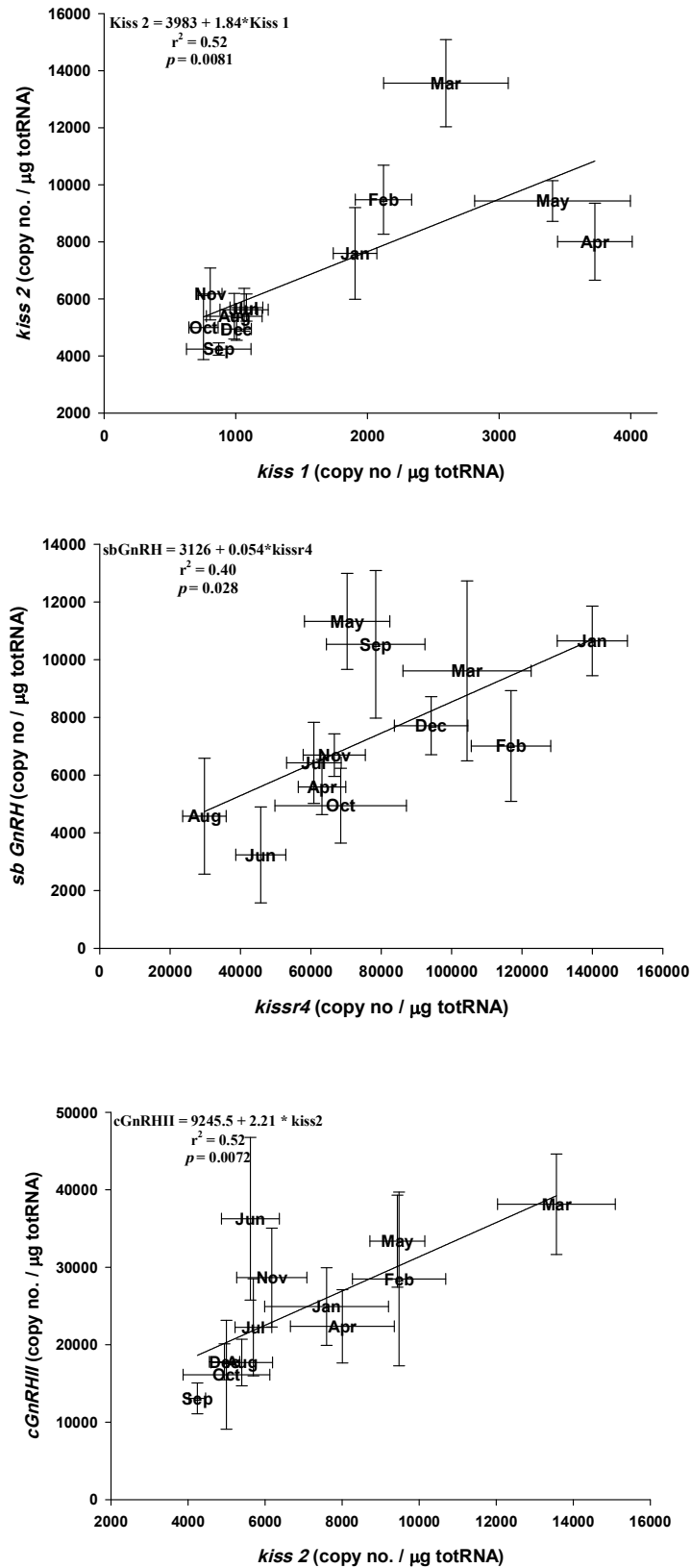


Figure 3.11. Linear regression of monthly mean *kiss1* v.s *kiss2*, *kissr4* vs. *sbGnRH* and *kiss2* vs. *cGnRHIII* expression.

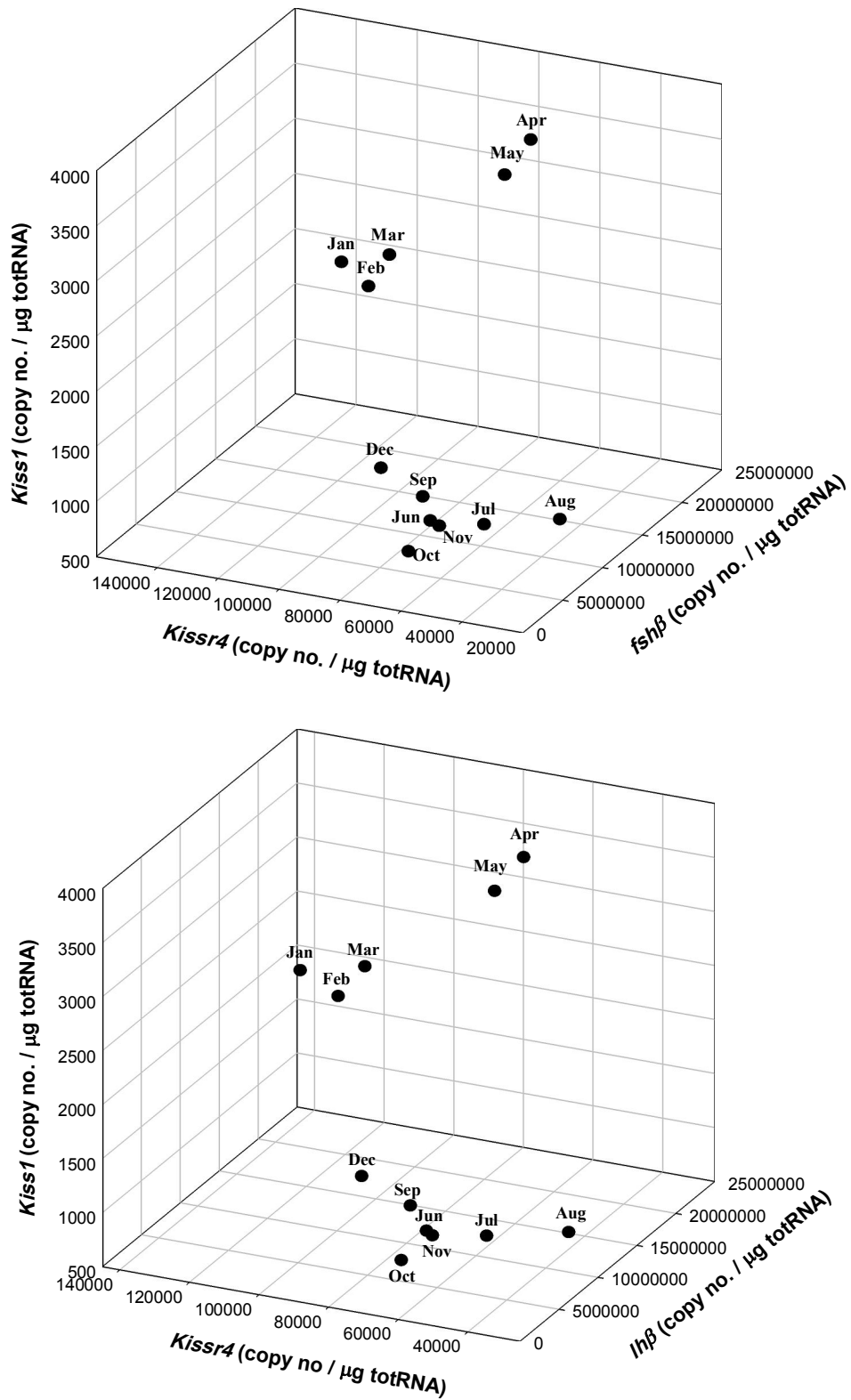


Figure 3.12. Three dimensional plot of monthly mean *fsh β* (top) and *lh β* (bottom) expression with respect to *kiss1* and *kissr4* expression.

3.4. DISCUSSION

In the present study we profiled mRNA expression of *kiss1*, *kiss2* and *kissr4* in the brain of repeat spawning male sea bass through a complete annual reproductive cycle along with *GnRH* (1,2,3) expression in the brain, *fsh β* , *lh β* and *kissr4* expression in the pituitary, plasma sex steroids and testes development. Kisspeptin mRNA expression levels (*kiss1*, 2 and *kissr4*) in the brain significantly increased towards the end of the annual reproductive cycle (late spermatogenesis to spermiation) and in some cases correlated with other BPG markers (*GnRHs*, *fsh β* and *lh β*). These results suggest a possible involvement of kisspeptin genes in the seasonal control of reproduction in repeat spawning sea bass.

European sea bass gonadal development and associated sex steroid profiles have been well characterised and show clearly that male spermiation usually takes place from December to March with a peak in GSI in February (Prat *et al.*, 1990; Navas *et al.*, 1998; Mananos *et al.*, 1997) in the Mediterranean area. In the present study, spermiation was observed in March-June and GSI peaked from March to May. Accordingly, plasma testosterone (T) and 11 ketotestosterone (11-KT) levels peaked in February-March and then returned to basal levels. The shifted reproductive cycles observed in this study compared to Mediterranean stocks are in line with previous reports from British waters (Pawson & Pickett, 1996) and are most likely due to subtle shifts in the ambient temperature/photoperiod profiles in higher geographic latitudes (Bruslé & Roblin, 1984).

Gene expression analyses performed in the present study confirmed that all three forms of GnRH (*GnRH1*, *GnRH2* and *GnRH3*) are expressed in the brain of male sea bass. However, GnRH3 showed no significant variation while both *GnRH1* and *GnRH2* showed significant changes in expression during the annual cycle with peak

levels in September, January and May (*GnRH1*) and March (*GnRH2*) when GSI and sex steroid plasma levels peaked and most individuals examined were spermiating. The subsequent correlation analysis helped to clarify the potential roles of the different isoforms, however it must be acknowledged that correlation does not, in itself, imply causation and the identified associations must be specifically tested. *GnRH1* correlated with *kissr4*, *fsh β* , *lh β* as well as GSI while *GnRH2* correlated with *kiss2* and GSI while *GnRH3* showed no significant correlation with any other parameter. The weak correlation with GSI for both *GnRH1* and *GnRH2* implies changes in GnRH signalling with developmental state, as has been shown in pituitary GnRHs levels of first time spawning male sea bass (Rodriguez *et al.*, 2000) as well as in brain and pituitaries of male and female viviparous grass rockfish *Sebastes rastrelliger* (Collins *et al.*, 2001). Otherwise the association of *GnRH1* with *kissr4* and the GTH's suggest that of the three, *GnRH1* (*sbGnRH*) plays a more active role in the reproductive cycle. This compliments previous reports that proposed it to be the main hypophysiotropic form in European sea bass (Gonzalez-Martinez *et al.*, 2002; 2004a,b; Fornies *et al.*, 2003) and other species including barfin flounder (Amano *et al.*, 2004), grass puffer (Shajahan *et al.*, 2010a), and red seabream (Okuzawa *et al.*, 2003). The *GnRH2* correlation with *Kiss2* is unexpected as while GnRH2 has been associated with sexual behaviour (Kauffmann *et al.*, 2005; Millar, 2005) it has more recently been specifically linked to pineal-specific melatonin signalling in sea bass (Servili *et al.*, 2010). It is proposed that future work should at least consider both GnRH forms to further decipher their precise roles.

The lack of significant variation in expression of the GnRHs as measured could be due to alternative splicing of the GnRHs. The assays for both *GnRH2* and *GnRH3* used primers which were designed on the second exon of these genes, as this region

contains the GnRH decapeptide and thus should be present in all splice variants. Pilot work on both genes using primers outwith this region identified at least two forms in both genes which agrees with the work of Zamora *et al.*, (2002) who reported two transcripts for *GnRH2* and *GnRH3* in sea bass brain. Alternative splicing can lead to the production of different proteins from a single pre-RNA (nuclear messenger RNA precursors) or can function as an on/off switch during development (Maniatis, 1991). In rainbow trout brains, two transcripts were found for *GnRH2* but only the mRNA expression of the long variant was related to a specific stage of sexual maturation (Gray *et al.*, 2002). It has also been shown that GnRH expression in gonads can be alternatively spliced in relation to the sex, and stage of development in both rainbow trout and sea bream (Nabissi *et al.*, 2000; Uzbekova *et al.*, 2001&2002).

In the present study the genes encoding for pituitary *lhβ* and *fshβ* were expressed throughout the year, with lower expression of *fshβ* relative to *lhβ*. A significant correlation between both GTHs and GSI was observed which was explained by a clear rise in the mRNA expression levels of the two genes during spermatogenesis with peak levels in January and high levels maintained through to May corresponding to the spermiation period when the testis were at their largest. Such a correlation to gonadal development has previously been reported in European sea bass (Mateos *et al.*, 2003), perjerrey (Miranda *et al.*, 2007) and male Senegalese sole (Cerdeira *et al.*, 2008). *fshβ* expression showed a transient but significant elevation by the end of the spent stage and just prior to the spermatogenesis onset; this elevation was found in FSH levels in sea bass plasma as well and can be linked to spermatogonial proliferation (Moles *et al.*, 2011) as in other teleosts (Schulz *et al.*, 2010). This increase of *fshβ* expression coincided with the first significant peaks of *GnRH1*, *kissr4* mRNA and was just prior to the first peaks of plasma 11KT, T and the significant peak of pituitary *kissr4*,

suggesting a likely regulation of *GnRH1*, *kissr4* and *fsh β* during the onset of the gametogenesis period (September-October) of sea bass. In an attempt to explore the drivers of both *fsh β* and *lh β* expression a stepwise multiple regression was performed which considered the potential association with *kiss1/2/r4* and *GnRH1*, *GnRH2* as well as *GnRH3*. In both cases the variation in *fsh β* and *lh β* expression was explained by *kiss1* and *kissr4* expression alone. As discussed further below it has been commonly suggested that *kiss2* was the most functionally important in fish due to its greater potency in eliciting a LH response (Felip *et al.*, 2009), however the present results suggest that their roles are potentially not as clear cut. For some studied mammals, the administration of the core sequence of kisspeptin decapeptide (Kp-10) *in vivo* stimulates both LH and FSH release, when given centrally or peripherally as in mouse (Gottsch *et al.*, 2004; Messenger *et al.*; 2005), and stimulates both LH and FSH release in male and female rat (Matsui *et al.*, 2004). The LH and testosterone releasing effect of kisspeptin has also been proven in human males when kisspeptin-54 was peripherally administered (Dhillon *et al.*, 2005). Furthermore, kisspeptin has been shown to be ineffective in directly stimulating LH release in ovariectomized hypothalamo-pituitary-disconnected sheep (Smith *et al.*, 2008b), and for the GnRH antagonist pre-treated male rats (Matsui *et al.*, 2004), supporting the hypothesis that kisspeptin induces the secretion of hypothalamic GnRH, which in turn elicits gonadotropin release. In teleosts, the decapeptide of *kiss2* has shown effect on the regulation of both FSH and LH synthesis and release, while *kiss1* was less effective on the pituitary gonadotropins in European sea bass (Felip *et al.*, 2009) and zebrafish (Kitahashi *et al.*, 2009). Under *in vitro* conditions the core decapeptide of *kiss1* (kp-10) induced gene expression and the subsequent secretion of luteinizing hormone (LH), prolactin (PRL) and growth hormone (GH) in goldfish pituitary (Yang *et al.*, 2010). Clearly further work is required

to define the specific roles of both kisspeptin peptides in fish. For pituitary *kissr4*, mRNA was very low during all the cycle but it showed significantly high levels in October and for early spermatogenesis stage in the with *kissr4* in the brain, As far the authors know it is the first time that temporal expression of *kissr4* to be done using QPCR.

The main objective of the current study was to profile kisspeptin gene expression in repeat spawner male sea bass. While full sequences for both kisspeptin signal peptides were available at the start of the study (Felip *et al.*, 2009), no kisspeptin receptor sequence had been reported in sea bass to date. In the present study, a partial *kissr4* cDNA sequence was isolated in sea bass. It contained an open reading frame of 556 bp resulting in a predicted protein of 185 amino acid peptide. Phylogenetic analyses confirmed the identity of the genes as being *kissr4* with high similarity (>90 %) with other teleost *kissr4* sequences such as Nile tilapia (Parhar *et al.*, 2004), grey mullet (Nocillado *et al.*, 2007), cobia (Mohammed *et al.*, 2007) and Atlantic halibut (Mechaly *et al.*, 2009). Most of the fish *Kissr4* sequences cluster in a tight group while zebrafish *kissrb* and goldfish *kissrb* are located on a separate branch (*Kissr2*) as for mammalian *kiss1r* (*kissr1*) (Akazome *et al.*, 2010). In some fish species two distinct subtypes of kisspeptin receptor have been reported (*Kissr2* and *Kissr4* paralogs) as in zebrafish (Biran *et al.*, 2008), medaka (Lee *et al.*, 2009) and goldfish (Li *et al.*, 2009), while only one form has been found in mammals (Akazome *et al.*, 2010). In fish species that belong to Pleuronectiformes, Tetraodontiformes and Acanthopterygian, only one kisspeptin receptor was found so far suggesting the loss of one *kissr* form (Mechaly *et al.*, 2010). Studies failed to detect a *kissr2* in some fish species as Senegalese sole (Mechaly *et al.*, 2009) and Atlantic halibut (Mechaly *et al.*, 2010).

In the present study, the expression of kisspeptin receptor (*kissr4*) and the two known ligands (*kiss1* and *kiss2*) have been profiled during an annual reproductive cycle. *Kissr4* expression level increased just prior the spermatogenesis (September) and have its peak during spermatogenesis (January) and declined towards the end of the spermiation window (April). However, *kiss1* and *kiss2* expression in the brain started to increase in January to reach peak expression levels during spermiation in March (*kiss2*) and April (*kiss1*). These results came as a surprise given the suggested stimulating role of kisspeptin on GnRH neurons (Irwig *et al.*, 2004, Parhar *et al.*, 2004, Servili *et al.*, 2011). In the present study, *kissr4* expression level increased during spermatogenesis stage similar to the peak of *kissr4* in the brain of male fathead minnow (Filby *et al.*, 2008) in male fathead minnow shown during early spermatogenesis, which then declined during the advanced stage of spermatogenesis (Filby *et al.*, 2008). Similarly, in male and female grass puffer brain *kissr4* expression peaked during spawning (Shahjahan *et al.*, 2010b). The present positive correlation between *GnRH1* and *kissr4* expression and the peaking of both in the same month is in good agreement with the findings of Zebrafish (Kitashi *et al.*, 2009), cobia (Mohammed *et al.*, 2007) and grey mullet (Nocillado *et al.*, 2007). Localisation work helps clarify this relationship as it has been shown that the great majority of GnRH1 neurons have been found to express *kissr1* in adult rat (Irwig *et al.*, 2004) or mouse (Han *et al.*, 2005) while the same neurons in tilapia express *kissr4* (Parhar *et al.*, 2004) and most recently this has also been reported in European sea bass (Escobar *et al.*, 2010).

To date there have been contrasting results on the expression of kisspeptins during reproduction. Similar results to the present findings were found in grass puffer in which brain *kiss2* expression peaked during spawning in both males and females (Shahjahan *et al.*, 2010b). Furthermore, the number of brain nucleus ventralis tuberculi

(NVT) *kiss1* expressing neurons was significantly higher in mature than immature medaka (Kanda *et al.*, 2008). However in male chub mackerel, *kiss1* brain expression gradually decreased during spermatogenesis, while *kiss2* increased during the late spermatogenesis stages and declined during spermiation (Selvaraj *et al.*, 2010). The contrasting results obtained between studies might be related to as yet undefined species differences and divergent reproductive strategies e.g. timing of sexual differentiation (late in sea bass compared to most other fish species), age at puberty (early in male sea bass), spawning pattern ovarian organisation (group-synchronous in sea bass [Prat *et al.*, 1990] vs. asynchronous in chub mackerel [Shiraishi *et al.*, 2008]) among others. Other factors could also explain differences seen such as fish reproductive state when samples were performed (first reproductive cycle in most previous studies vs. repeat spawning in the present study) and evolutionary differences in functional kisspeptin forms which are, in most species, not known yet. For all these reasons, direct comparisons between species to unravel the role played by the kisspeptin system in fish reproduction are not straightforward.

In the present study *kiss2* expression in the brain was correlated with high steroid levels, suggestive of an involvement of *kiss2* genes in gonadal steroid positive feed back control of reproduction. The relation between the kisspeptin system and sex steroids has been studied in some mammals as Kiss1 neurons are suggested to be sex-steroid sensitive neurons that can receive and then transmit steroid feedback to the GnRH neurons (Kauffmann, 2010). In rodents, Kiss1 neurons have been found to express both estrogen and androgen receptors, which explains how gonadal steroids can have both stimulatory and inhibitory effects on Kiss-1 expression (Smith *et al.*, 2005; Kauffmann *et al.*, 2007). Furthermore in sheep, both positive and negative feedback of sex steroids are mediated by kisspeptin neurons (Estrada *et al.*, 2006). Clarkson *et al.*

(2010) proposed that *Kiss1* neural sensitivity to estrogen not only provides a critical estradiol-dependent amplification mechanism to activate GnRH neurons and complete the puberty onset, but also then subsequently facilitates the regular preovulatory GnRH /LH surge in adult females. Similarly for teleosts, in the female medaka, *kiss1* neurones of the nucleus ventral tuberries (NVT) were found to be involved in the positive feedback effect of ovarian estrogens, as the NVT neurons were found to express estrogen receptor- α in medaka (Kanda *et al.*, 2008; Mitani *et al.*, 2010). Recent work in female zebrafish has similarly shown that *kiss2* neurons of the ventral hypothalamus are estrogen sensitive (Servili *et al.*, 2011), with an estradiol treatment causing a significant increase in both *kiss1* and *kiss2* mRNA expression in the brain of juvenile zebrafish (Servili *et al.*, 2011). It is clear from the above results in mammals and teleosts that at least in females, steroids regulate the kisspeptin signalling, however the role of androgens in male puberty and reproductive cycling is far less clear (Clarkson *et al.*, 2010). The current results suggest that such a relationship may well exist, thus the potential for androgen regulation in males reproductive physiology should perhaps be explored in greater detail.

In conclusion, the present study revealed that mRNA expression of the two kiss genes (*kiss1*, *kiss2*) and their receptor *kissr4* showed clear seasonal profiles and correlated well to other BPG markers (*GnRHs*, *fsh β* and *lh β*), suggesting their possible involvement of kisspeptin genes in the seasonal control of reproduction in repeat spawning male sea bass.

CHAPTER 4

KISSPEPTIN EFFECTS ON *LHB* AND *FSHB* mRNA EXPRESSION

LEVELS BY SEA BASS, *DICENTRARCHUS LABRAX*,

GONADOTROPH CELLS

4.1. INTRODUCTION

Reproduction in fish, as in all vertebrates, is controlled by a suite of neuropeptides and hormonal signals at the brain-pituitary-gonadal axis (BPG) (Zohar *et al.*, 2010) that respond to environmental changes and initiate/regulate the gametogenesis process. Among these, gonadotropin releasing hormones (GnRHs) produced by discrete neurons in the brain, gonadotropins synthesised by gonadotroph cells in the pars distalis of the pituitary and sex steroids synthesised by gonads play pivotal roles. Unlike mammals, fish GnRH neurons innervate the anterior pituitary and create a physical and functional connection between the brain and pituitary (Yamamoto *et al.*, 1998; Gonzalez-Martinez *et al.*, 2002a). The functional role of GnRH on pituitary gonadotropin release has been demonstrated in many teleost species including the striped sea bass, *Morone saxatilis* (Hassin *et al.*, 1998), the European sea bass (Mateos *et al.*, 2002; Fornies *et al.*, 2003) and the common carp (Kandel-kfir *et al.*, 2002). Recent research has brought to light the importance of a number of upstream neuropeptides that regulate GnRH expression and neurotransmitters that work in association with GnRH's actions to regulate gonadotropin synthesis (Vidal *et al.*, 2004; Zohar *et al.*, 2010).

The neuropeptide Kisspeptin as a regulator of GnRH function controlling puberty and sexual maturation at the hypothalamus level has been the object of many studies over recent years particularly in mammalian model species. Studies suggested that kisspeptin, namely the signal peptide Kiss1 and its receptor Kissr (formerly referred to as G-coupled protein receptor 54. GPR54), are key factors in the regulation of reproduction and initiation of puberty (Seminara, 2005; Murphy 2005; Dungang *et al.*, 2006). Studies carried out on laboratory animals indicated that kisspeptin-10 (derivative of Kiss1) has the ability to stimulate the synthesis and release of LH and

FSH hormones from the pituitary when administered centrally or peripherally (Gottsch *et al.*, 2004; Matsui *et al.*, 2004; Navarro *et al.*, 2005a and Shahab *et al.*, 2005). Importantly, kisspeptin was found to be equally effective when administered as a full length peptide or decapeptide Kiss-10 (Gottsch *et al.*, 2004). In mammals, kisspeptin clearly stimulate the synthesis of gonadotropins from the pituitary although this stimulation appears to be mediated through GnRH neurons in the preoptic area of the hypothalamus (Roa *et al.*, 2009). This indirect role is supported by many authors. A direct innervation of Kiss1 nerve fibers and *Kissr1* mRNA was demonstrated within GnRH neurons (Roseweir & Millar, 2009). Kisspeptin was shown to trigger GnRH secretion in rat hypothalamus when applied *in vivo* (Messenger *et al.*, 2005) and *in vitro* as well (Nazian, 2006; Quaynor *et al.*, 2007). Both FSH and LH were secreted in response to *in vivo* kisspeptin treatments while this secretion was inhibited when GnRH antagonists were used (Matsui *et al.*, 2004; Irwig *et al.*, 2004). Although mammals express only one signal peptide kisspeptin (KISS1) and receptor (KISSR1) as for some teleost species, in other fish two forms of both the signal peptide (*kiss1* & *kiss2*) and receptor (*kissr2* & *kissr4*) have been found (Felip *et al.*, 2009; Lee *et al.*, 2009; Akazome *et al.*, 2010). Most of the work done in teleosts focused mainly on the kisspeptin receptor (*Kissr*) as it is well conserved across fish species as compared to the signal peptides. Results suggested a conserved role played by kisspeptin in fish as reported in mammals with an association to the onset of puberty (Filby *et al.*, 2008; Martinez-Chavez *et al.*, 2008), similar GnRH regulatory abilities (Elizur, 2009) as well as being responsive to sex steroid feedbacks (Kanda *et al.*, 2008). However, as it was found with the GnRH system, the kisspeptin system has emerged to be more complex and diverse than first thought with potentially multiple roles played (by several gene

variants) at different levels of the BPG axis for the control of reproduction as well as other functions.

Although the hypothalamus appears to be the primary site of kisspeptin action for the regulation of reproduction, kisspeptin could also act directly at the pituitary or gonadal levels as suggested by previous published work. Indeed, *Kissr* mRNA expression in the pituitary gland of rat (Richard *et al.*, 2008; Gutiérrez-Pascual *et al.*, 2007), in addition to the release of kisspeptin in ovine hypophyseal portal blood, are rising the possibility that role of kisspeptin as a hypophysiotropic neuromodulator in the pituitary (Smith *et al.*, 2008b). However, contrasting results have been obtained in mammals when kisspeptin was applied *in vitro* to pituitary cells. In rat and bovine for example, kisspeptin has direct stimulatory actions on LH when applied to pituitary cells (Gutiérrez-Pascual *et al.*, 2007; Suzuki *et al.*, 2008). However, in other studies, kisspeptin was ineffective as it failed to elicit basal FSH release directly at the pituitary level in adult rat pituitary cells (Navarro *et al.*, 2005b), or pituitary fragment (Thompson *et al.*, 2004). Kisspeptin treatment on pituitary cells was also shown to stimulate growth hormone (GH) and prolactin (PRL) in bovine anterior pituitary (Kadokawa *et al.*, 2008), and growth hormone (GH) in cultured rat pituitary cells (Gutiérrez-Pascual *et al.*, 2007). The direct action of kisspeptin on the pituitary in fish is scarce and gave contradictory results. In goldfish pituitary cell culture, no effects of kisspeptin on LH contents were found by Li *et al.* (2009) whereas Yang *et al.* (2010) showed a stimulation of LH, PRL and GH mRNA following incubation with decapeptide kiss10. In European sea bass, an *in vivo* study has shown that kiss10 peptide injection for both kiss1 and kiss2 increased the secretion of plasma LH and FSH with more potency for kiss2 (Felip *et al.*, 2009).

The aim of this study was to examine the potential direct action of the kisspeptin forms (Kiss1 and Kiss2) on gonadotropin (*lhβ* and *fshβ*) gene expression in male European sea bass pituitary cells in culture. This was done by testing the kisspeptin decapeptide derivatives on the *lhβ* and *fshβ* transcript expression in primary culture of sea bass pituitary cells using quantitative RT-PCR technique. Three experiments were performed to test incubation doses, time course and fish stage of development.

4.2. MATERIALS AND METHODS

4.2.1. Animals and sampling

In experiment 1, one year old mixed sex sea bass originating from stock available at Machrihanish Environmental Research Laboratories (MERL, University of Stirling facilities, 55° 44' N, 5° 44' W) were used. Fish were produced in the facilities from larvae supplied by Llyn Aquaculture Ltd, Ireland, and reared under a constant regime of 12L: 12D photoperiod and temperature of 14 °C and fed to satiation twice per day. For experiments 2 and 3, adult sea bass males were also obtained from MERL, but fish were reared under a natural photoperiod and temperature regime and fed to satiation twice per day with a commercial fish food formula (Atlantic HE 50E+ 35A/25C, Skeriting, Invergordon, UK). When fish reached the targeted gonadal developmental stage, fish were transferred to recirculating marine facilities on the University of Stirling campus at least 1 week prior to sampling. Sampling consisted of sacrificing fish by a lethal dose of anaesthesia (2-phenoxyethanol, Sigma-Aldrich Co. Ltd., Poole, UK) and dissecting the pituitary using a sterile dissecting set. Pituitaries were immediately placed in dispersion medium kept on ice and brought back to the laboratory. Body weight and standard length were recorded. All trials were carried out in accordance with the UK Home Office Animal Scientific Procedures, Act 1986.

4.2.2. Hormones and chemicals

To test for the pituitary actions of two kisspeptin encoded genes in sea bass, the decapeptides (10 amino acid core sequence) were produced by Peptide Synthetics (Fareham, United Kingdom) using the standard procedures for solid phase peptide synthesis with purity of >95 % determined by HPLC analysis. The amino acid sequences were sbKiss1 NH₂-YNLNSFGLRY-CONH₂ and sbKiss2 NH₂-FNFNPFGLRF- CONH₂ according to the published sequence in Felip *et al.* (2009). Newly synthesised peptides were named sbkiss1 and sbkiss2. Both peptides were dissolved in 100 % sterile DMSO (Dimethyl Sulfoxide) (final DMSO concentration < 0.1%) to make stock concentration of 1μM. For testing different doses of both kisspeptin forms, the prepared stock diluted with culture media to have final concentrations of 10 nM, 100 nM and 1000 nM. Forskolin (Planta natural products, Austria) was prepared at 10 μm by dissolving in sterile DMSO and diluting with culture media. Forskolin was selected as a positive control because it is commonly used to raise levels cyclic AMP (cAMP), which is an important signal carrier for biological response of cells to hormones and other extracellular signals as previously described (Evans *et al.*, 1985; Ding *et al.*, 2001).

4.2.3. Experiment 1: Incubation time trial

To test the effect of the duration of incubation, 28 sea bass (weight of 333.5 ± 18.1 g, length of 30.2 ± 0.5 cm and GSI of 0.4 ± 0.1 %) pituitaries were collected from the immature mixed sex population in June 2010, Cells were dispersed and cultured as described in Chapter 2 and incubated at a density of 4 x 10⁵ cells/well (2 ml), and then left for 72 hours to attach to the plates in the dark in an incubator at 22 °C. After attachment, doses of 100 nM of decapeptide 10 for sbkiss1 or sbkiss2 were tested in

quadruplicate wells (4 wells/incubation times) with untreated wells kept as controls for each tested time. Five incubation times were tested: 1, 3, 6, 12 and 24 hours.

4.2.4. Experiment 2: Dose-response trial

To test the dose response effects, pituitaries were collected from mature male sea bass (weight of 580.3 ± 25.6 g, length of 34.6 ± 0.4 cm and GSI of 1.8 ± 1.2 %) in March 2010. Fish were at the spermiation stage with high plasma steroid levels (see Chapter 3). Cells were dispersed and incubated at a density of 7.5×10^5 cells/well (2 ml) and then left for 72 hours to attach to the plates. After attachment, 3 doses of decapeptide 10 for *sbkiss1* or *sbkiss2* were tested (10, 100, 1000 nM) together with 10 μ M forskolin as positive controls with untreated wells kept as negative controls. Samples (medium and cells) were collected after 24 and 48 hours of incubation. The incubation times for this trial were chosen independently from (prior to) the results of the first experiment.

4.2.5. Experiment 3: Gonadal development stage trial

To test the effect of stage of development, pituitaries were collected from mature male sea bass in March (full spermiation with high plasma steroid levels) (weight of 580.3 ± 25.6 g, length of 34.6 ± 0.4 cm and GSI of 1.8 ± 1.2 %) and in August (spent stage with low plasma steroid levels) (weight of 772.8 ± 60.0 g, length of 38.9 ± 0.6 cm and GSI of 0.8 ± 0.1 %). Cells were dispersed and incubated at a density of 7.5×10^5 cells/well (2 ml) and then left for 72 hours to attach to the plates. After attachment, 2 doses of decapeptide 10 for both *sbkiss1* or *sbkiss2* were tested (10 and 1000 nM), together with 10 μ M forskolin as positive controls. Samples were collected after 24 hours incubation. Due to limited number of wells and the replicates needed for

each dose or time and based on results obtained in experiments 1 and 2, only two doses of each kisspeptin for only one incubation time were used.

4.2.6. RNA extraction and quantitative RT-PCR

After incubation, the culture medium was removed and the cells were washed twice using 1 ml/well phosphate buffered saline 1x (PBS) (Gibco, Invitrogen). RNAs from individual wells were collected by adding 1 ml of TRI reagent/ well and scraping out the cells which was transferred into a clean Eppendorf tube (DNase and RNase free) (Axygen, Fisher Scientific,UK). Samples were frozen at -70 °C for later extraction. Total RNA extraction was performed as described in Chapter 2 (section 2.3.1), RNA quality was checked and cDNA was synthesised as described in Chapter 2 (sections 2.3.2 and 2.3.4) using 1 µg of total RNA, cDNA samples were diluted 1:10 with MiliQ water RT-QPCR validation and assay of *lh* and *fsh* genes were performed as described in Chapter 2. Absolute copy numbers were normalized against sea bass *eflα* as a reference gene using the same specific primers (Table 3.1).

4.2.7. Data analysis and statistics

Pituitary hormone transcripts were expressed in absolute copy number of target genes. Data were routinely normalized with *eflα* mRNA in the same sample to control the potential variation in cell number between wells, as no significant differences were noted for *eflα* mRNA expression in the experiments. Normalized data were transformed as a percentage of the control group within the same trial for the graph presentation. In all experiments, data were presented as mean ± SEM of four wells for individual treatments. Normality of variances and homogeneity were tested using the Kolmogorov-Smirnov test and when appropriate, data were transformed using the

natural logarithm. For the incubation time experiment, differences between treatments (including control) were analyzed using a T- test each time, followed by Tukey's multiple comparisons post-hoc test to compare between the different sampling times for both control and treated samples. In experiment 2 (the dose-response trials) data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test. The comparisons between the two sampling times in experiment 3 were done using two-way ANOVA. When significant interaction was found, inspection of mean values was done using one-way ANOVA for each sampling time. For all tests, significance was set at $P < 0.05$. All analyses were performed with MINITAB[®] version 16.0 (Minitab Ltd., Coventry, UK).

4.3. Results

4.3.1 Experiment 1: Effects of incubation time on *sbkiss1* and *sbkiss2* stimulation of *fshβ* and *lhβ* mRNA levels in pituitary cells.

In this trial, the effect of different incubation durations (1, 3, 6, 12 and 24 hours) with a single dose of 100 nM of either *sbkiss1* or *sbkiss2* was tested. The results showed no significant effect of *sbkiss1* on *fshβ* and *lhβ* expression levels when incubated with the pituitary cells for one or three hours (Fig. 4.1 A, B, C and D). After 6 hours, *fshβ* but not *lhβ* mRNA expression appeared to increase in pituitary cells incubated with *sbkiss1* but with no significant difference when compared to the control. There was a significant effect after 12 hours of incubation for both *fshβ* and *lhβ* (60 and 40 % increase, respectively). After 24 hours of incubation, while *lhβ* mRNA was still significantly higher (10 %) in the exposed cells than in the control, no significant difference was seen in *fshβ*. While *fshβ* mRNA remained relatively steady throughout the incubation period (80-120 x 10⁴ copy number/μg total RNA) in both treated and non-treated cells, *lhβ* mRNA decreased significantly over time from 490 x 10⁴ copies /μg total RNA at the first hour sampling to 172 x 10⁴ copies /μg total RNA after 24 hours of incubation (Fig. 4.1 A&C).

When pituitary cells were exposed to *sbkiss2*, a significant increase was observed in *fshβ* mRNA after only 1 hour of incubation (30 %) although the effect was not sustained thereafter, as no significant differences were seen after 3 and 6 hrs of incubation (Fig. 4.2 A, B, C and D). Another significant surge in *fshβ* was then measured at 12 hours (100 % increase, Fig 4.2B). No difference could then be found at 24 hrs. Similarly, a significant increase at 12 hrs was measured for *lhβ* mRNA (70 % increase, Fig. 4.2D). However, no other differences were observed throughout the incubation period. During the whole experimental period *fshβ* mRNA remained

relatively steady throughout the incubation except for the sampling points of 6 and 12 hours comparing to the sampling point for first and third hours (Fig. 4.2 A). While, as seen for the *sbkiss1* results, *lhβ* decreased significantly over time in both treated (*sbkiss2*) and non-treated (control) cells (Fig. 4.2 C).

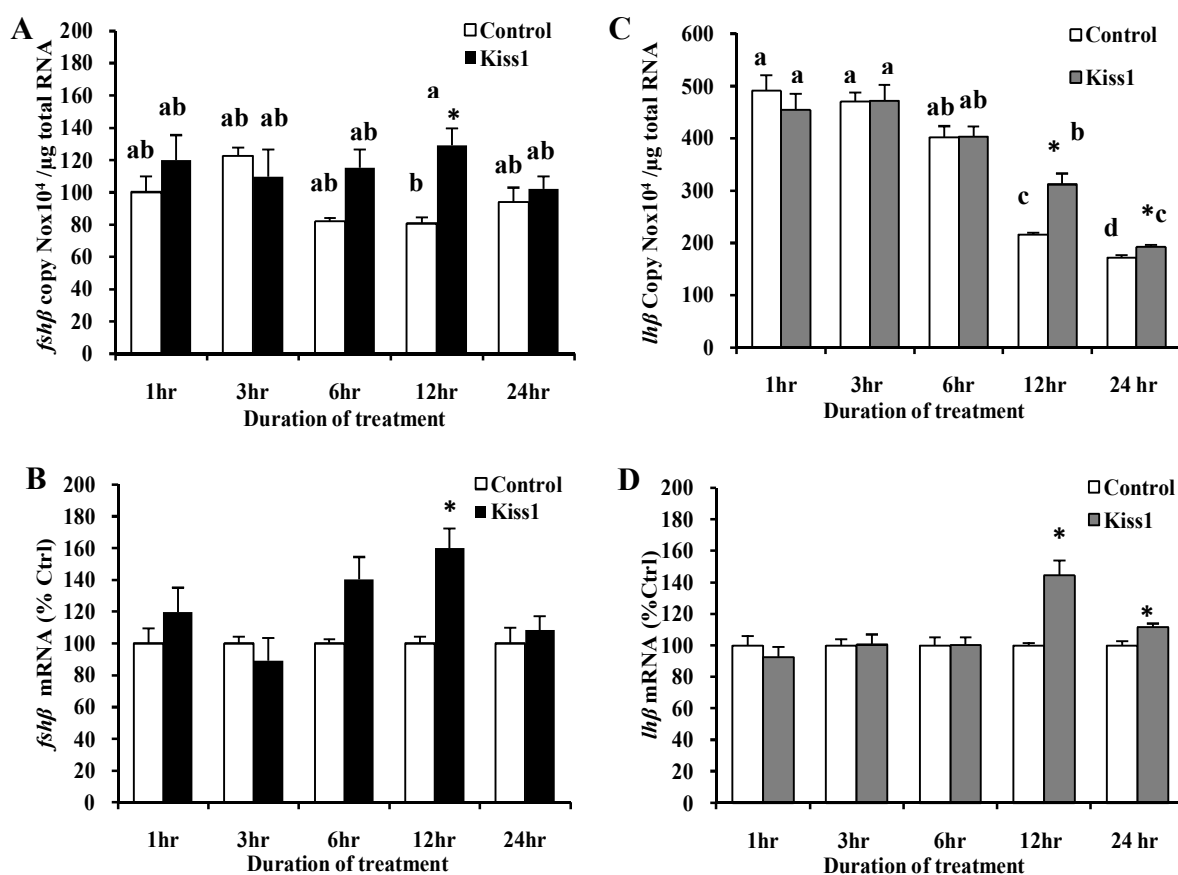


Figure 4.1. Time course effect of sbkiss1 (100 nM) on *fshβ* (A&B) and *lhβ* (C&D) mRNA in dispersed pituitary cell cultures taken from mixed sex immature sea bass. Cells were treated with sbkiss1 and harvested after 1, 3, 6, 12 or 24 hrs of incubation. Data are presented as absolute copy numbers (A&C) or relative values to control (B&D). Data are expressed as the mean \pm SEM (n = 4). Asterisks denote significant differences between the treatment and its control for the same incubation time, while superscripts denote significant differences between the expression levels at different time points for both control and treated wells.

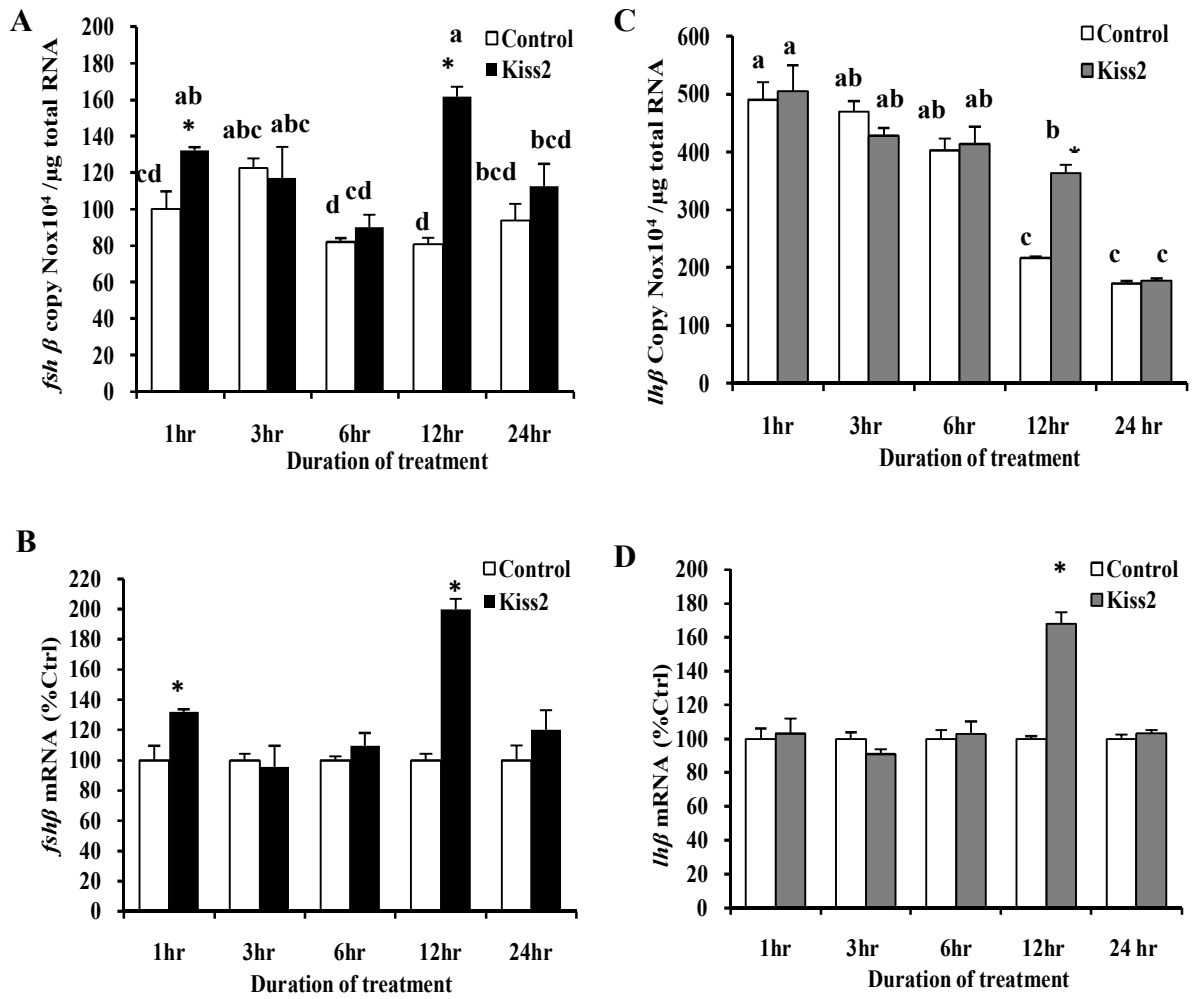


Figure 4.2. Time course effect of sbkiss2 (100 nM) on *fshβ* (A&B) and *lhβ* (C&D) mRNA in dispersed pituitary cell cultures taken from mixed sex immature sea bass. Cells were treated with sbkiss2 and harvested after 1, 3, 6, 12 or 24 hrs of incubation. Data are presented as absolute copy numbers (A&C) or relative values to control (B&D). Data are expressed as the mean ± SEM (n = 4). Asterisks denote the significant differences between the treatment and its control for the same incubation time while superscripts denote significant differences between the expression levels at different time points for both control and treated wells.

4.3.2. Experiment 2: Dose-response effects of sbkiss1 and sbkiss2 on *fshβ* and *lhβ* mRNA levels.

Dose- response effects of sbkiss1 and sbkiss2 core sequences on the levels of *fshβ* and *lhβ* mRNA were examined in March, when both steroids levels and pituitary *fshβ* and *lhβ* mRNA were the highest (Chapter3) to check the responsiveness to different doses of both sbkiss1 and sbkiss2. Male pituitary cells were exposed to increasing concentrations (10, 100 and 1000 nM) of sea bass kisspeptin -10. After 24 hours of incubation, no significant effects of sbkiss1 or sbkiss2 on *fshβ* mRNA expression levels were observed when compared to controls (Fig. 4.3.A and C). However, relative expression to controls showed a significant decrease in *lhβ* when kisspeptide concentrations (both kiss 1 and 2) were increased from 10 to 100 nM for sbkiss1 and 10 to 1000 for sbkiss2 (Fig. 4.3 B and D). *lhβ* mRNA levels appeared to increase in cells treated with both peptides although it was significant only for sbkiss1 (60 % increase, Fig. 4.3B). Forskolin appeared to have no effects on both *fshβ* and *lhβ* mRNA expression relative to controls (except a slight decrease in *lhβ* in cells treated with sbkiss1, Fig. 4.3 B). After 48 hours, significant differences in *fshβ* mRNA levels were measured in cells treated with 10 and 100 nM of sbkiss1, while no significant differences were found for 1000 nM of dose (Fig. 4.4A). No significant effects of sbkiss2 on *fshβ* mRNA was observed (Fig. 4.4C). However, when cells were exposed to sbkiss1, significant differences in *lhβ* expression were measured between cells treated with either 10 or 100 nM of sbkiss2 and 1000 nM (Fig. 4.4B) although no significant differences to controls were seen. For the three doses of sbKiss2, no significant differences with the controls could be seen in *lhβ* mRNA levels (Fig. 4.4D). Forskolin did not have any effect on either gonadotropin gene expression after 48 hrs of incubation.

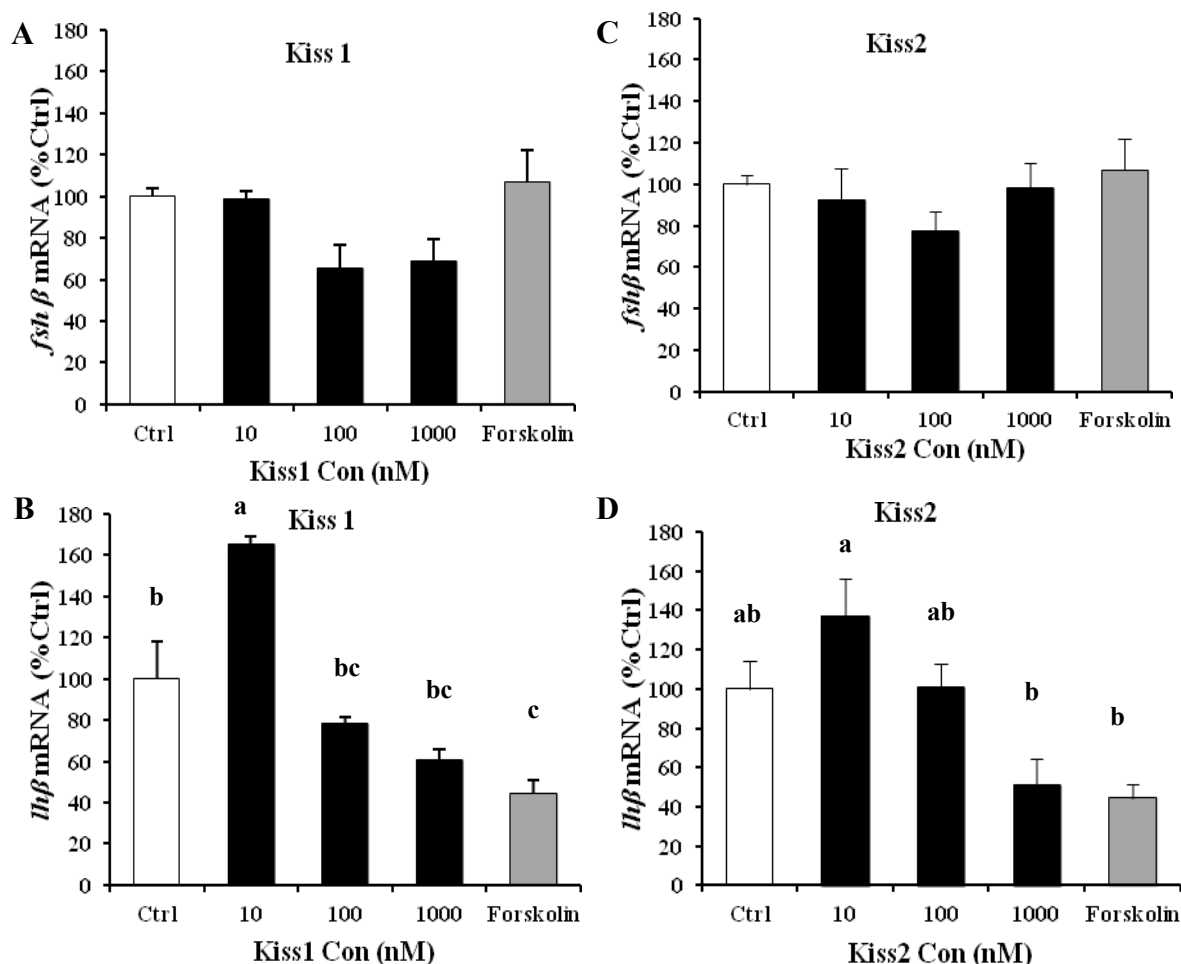


Figure 4.3. Dose-response effects of sbkiss1 (A&B) and sbkiss2 (C&D) on *fshβ* (A&C) and *lhβ* (B&D) mRNA in cells harvested after 24 hours of incubation. Male sea bass pituitary cells were collected in March and plated at density of 7.5×10^5 cells /well/2ml. Cells were treated with sbkiss1 or sbkiss2 (10, 100 and 1000 nM) and 10 μ m forskolin for 24 hrs of incubation. Data are presented as relative values to control. Data are expressed as the mean \pm SEM (n = 4). Superscripts denote significant differences between treatments (doses).

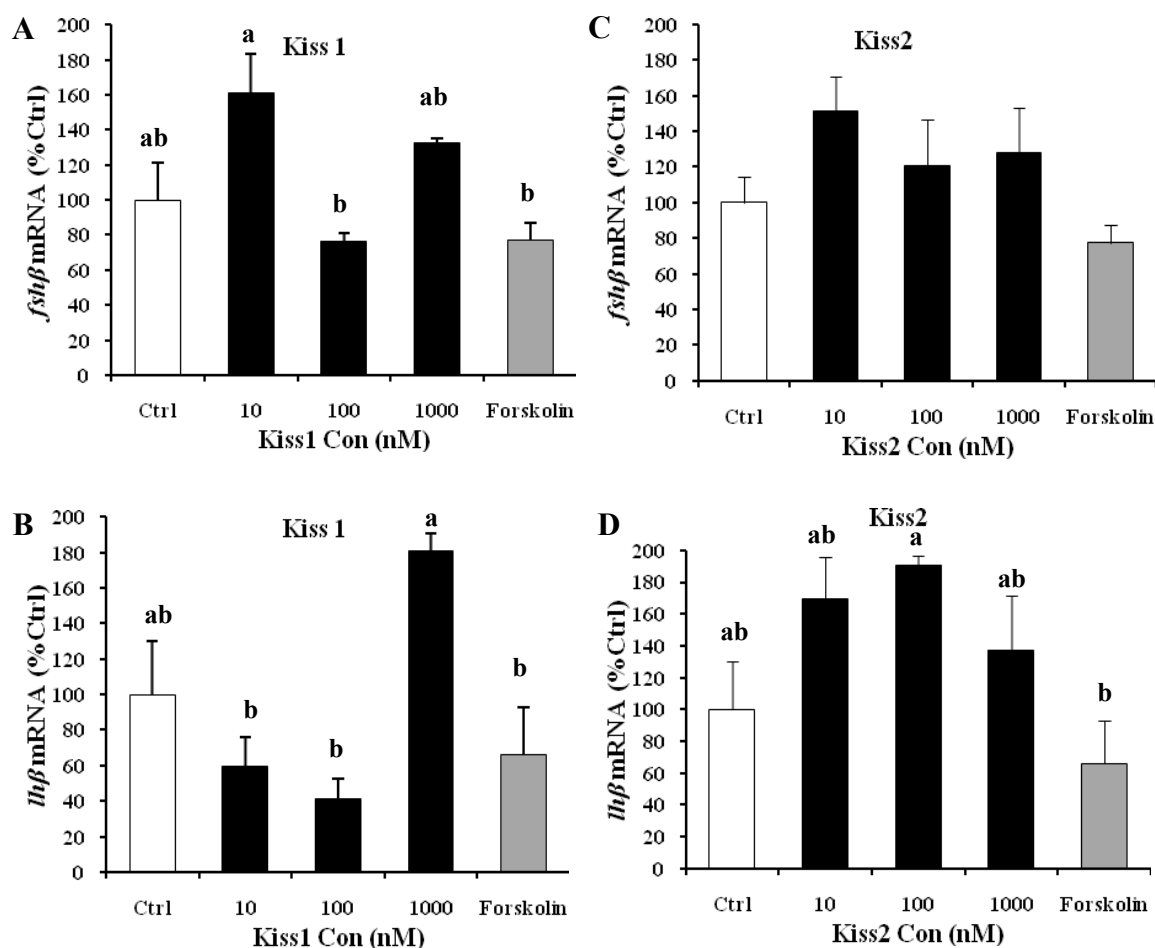


Figure 4.4. Dose-response effects of sbkiss1 (A&B) and sbkiss2 (C&D) on *fshβ* (A&C) and *lhβ* (B&D) mRNA in cells harvested after 48 hours of incubation. Male sea bass pituitary cells were collected in March and plated at density of 7.5×10^5 cells /well/2ml. Cells were treated with sbkiss1 or sbkiss2 (10, 100 and 1000 nM) and 10 μ m forskolin for 48 hr of incubation. Data are presented as relative values to control. Data are expressed as the mean \pm SEM (n = 4). Superscripts denote significant differences between treatments (doses).

4.3.3. Experiment 3: Changes in the *fshβ* and *lhβ* mRNAs in cells treated with *sbkiss1* and *sbkiss2* in relation gonadal developmental stage

Direct effects of *sbkiss1* and *sbkiss2* on *fshβ* and *lhβ* mRNAs were examined using primary pituitary cell cultures from sea bass sampled at two different periods of their reproductive cycle (March and August 2010). The cells were treated with either *sbkiss1* or *sbkiss2* at two different concentrations (10 or 1000 nM) or forskolin (10 μM) as a positive control. After 24 hrs of incubation, no significant effects of *sbkiss1* at 10 or 1000 nM were found on the *fshβ* mRNA expression levels from the March or August sampling. The effects of forskolin appeared to differ between sampling times with no significant effect in March and a significant decrease relative to untreated cells in August (Fig. 4.5). When comparing sampling times using two-way ANOVA, a significant difference was observed between March and August ($p < 0.05$, $F = 166.16$), with no significant effect of the *sbkiss1* dose, while there was a significant interaction of time and dose ($p < 0.05$, $F = 4.93$). In *sbkiss2* treated cells, no significant differences between treatments were measured in the cells sampled in March (Fig. 4.6 A, B). In cells sampled in August, a significant decrease in *fshβ* mRNA was measured when cells were exposed to a dose of 10 nM of *sbkiss2* or forskolin (approx. 30 % decrease); however, no difference relative to control was observed when the dose was increased to 1000 nM (Fig. 4.6 A, B). A significant difference was observed between March and August ($p < 0.05$, $F = 195.6$) with no significant effect of the *sbkiss2* dose ($p = 0.10$, $F = 2.27$) and no significant dose/time of sampling interaction ($p = 0.26$, $F = 1.4$) was found in *kiss2* treated cells when two-way ANOVA was applied. The *fshβ* mRNA levels in both treated and control cells measured in August were significantly lower (ranging from 100 to 200 × 10⁴ copy numbers/μg total RNA) than in March (ranging from 300 to

500 x10⁴ copy numbers/μg total RNA) when both negative controls were compared (Figs. 4.5 A and 4.6 A).

lhβ mRNA expression results in treated cells differed from *fshβ* (Figs. 4.7 and 4.8). No significant changes in *lhβ* mRNA levels were observed between treated (sbkiss1 or sbkiss2) and control cells harvested in August. However, a significant increase in *lhβ* mRNA was seen in cells harvested in March and treated with 10 nM of sbkiss1 (60 % increase) (Fig. 4.7 A, B). When the dose was increased to 1000 nM, levels significantly decreased relative to controls and 10 nM (40 and 100 %, respectively) as shown in Figure 4.7 B. Similar effects were observed when cells were treated with sbkiss2, although the increase in *lhβ* mRNA in the 10 nM treated cells and decrease in the 1000 nM treated cells were not significant compared to the untreated control wells (Fig. 4.8 A, B). For the sbkiss1 treated wells, no significant differences were observed for *lhβ* mRNA between March and August levels ($p = 0.25$, $F = 1.37$), while the doses were significant ($p < 0.05$, $F = 12.25$), with significant interaction for both time and dose ($p < 0.05$, $F = 14.28$) when two-way ANOVA was applied. When *lhβ* mRNA expression levels for the sbkiss2 treated cells were analysed by two-way ANOVA, no significant differences were observed for *lhβ* mRNA between March and August levels ($p = 0.057$, $F = 4.00$), while the doses were significant ($p < 0.05$, $F = 7.40$) and significant dose by time of sampling interaction was found ($p < 0.05$, $F = 5.76$). Finally, overall, irrespective of treatments, higher absolute expression levels were observed in *lhβ* (200-1100 x 10⁴ and 500-600 x 10⁴ copy numbers/μg total RNA, respectively for March and August) (Figs. 4.7 and 4.8) compared to *fshβ* (300-500 and 100-200 x 10⁴ copy numbers/μg total RNA, respectively for March and August) (Figs. 4.5 and 4.6).

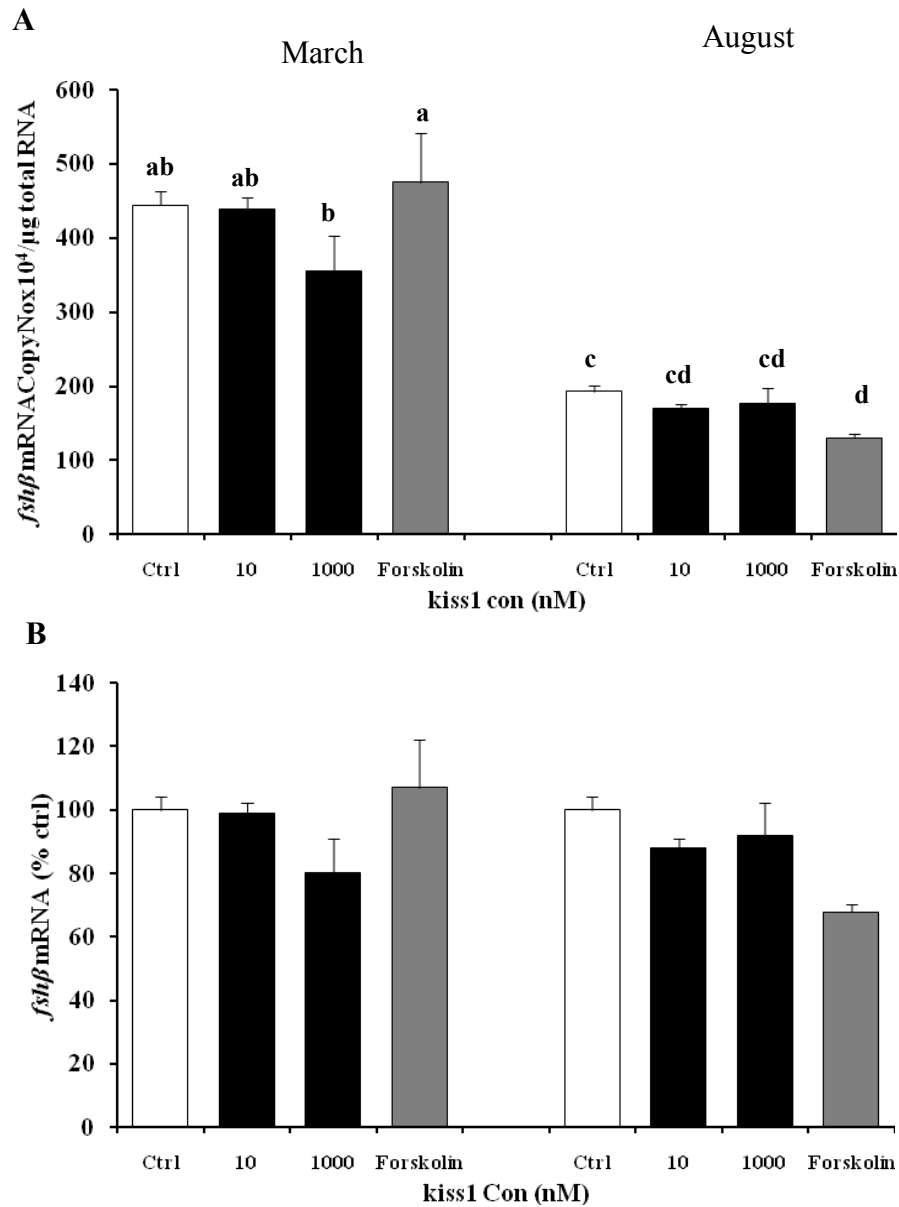


Figure 4.5. Effect of sbkiss1 on *fshβ* mRNA in cells harvested at two sampling points (March & August). Cells were treated with sbkiss1 (10 and 1000 nM) and 10 μm forskolin for 24 hr of incubation. Data are presented as absolute copy numbers (A) or relative values to control (B). Data are expressed as the mean \pm SEM ($n = 4$). Superscripts denote significant differences between treatments (doses).

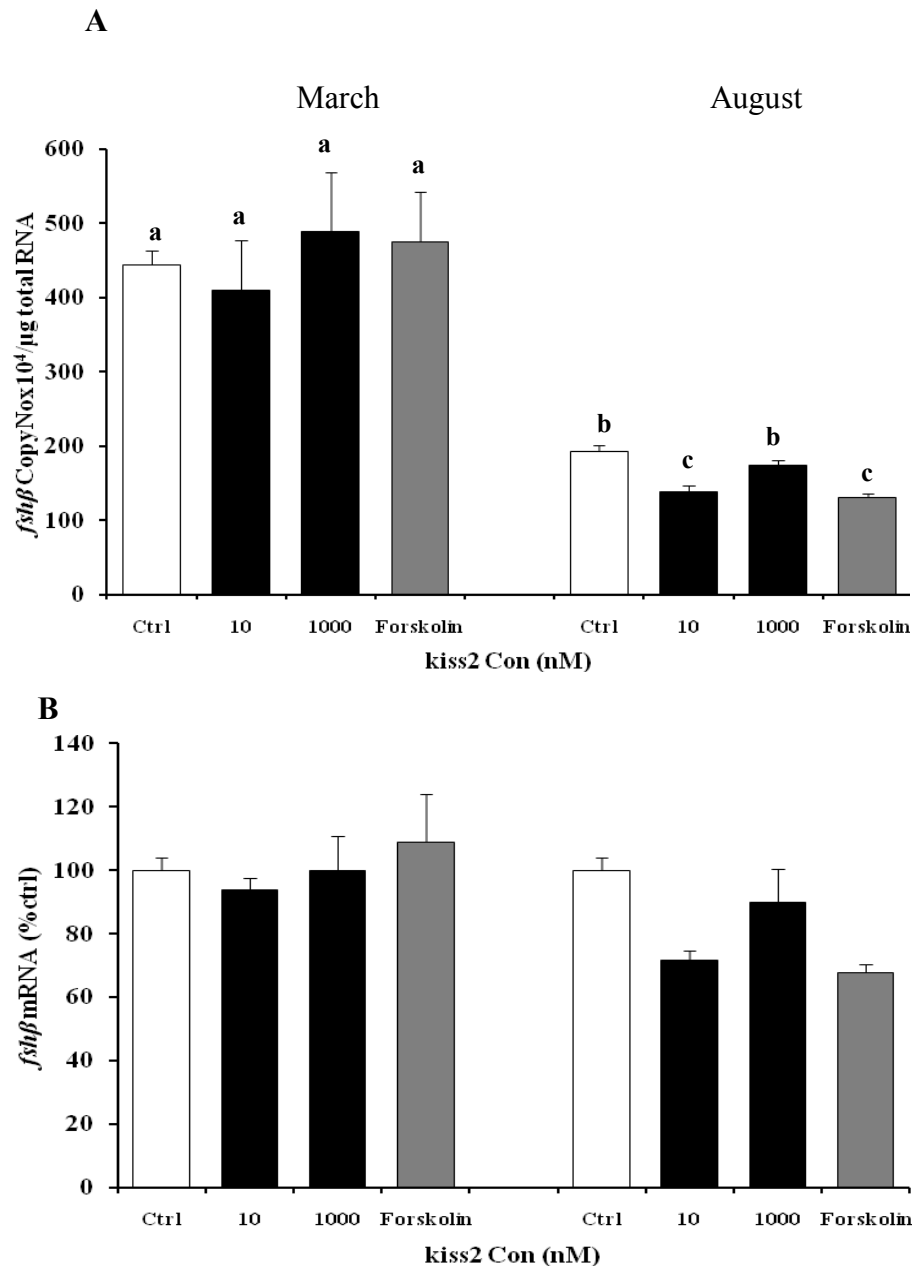


Figure 4.6. Effect of sbkiss2 on *fshβ* mRNA in cells harvested at two sampling points (March & August). Cells were treated with sbkiss2 (10 and 1000 nM) and 10μm forskolin for 24 hr of incubation. Data are presented as absolute copy numbers (A) or relative values to control (B). Data are expressed as the mean ± SEM (n = 4). Superscripts denote significant differences between treatments (doses).

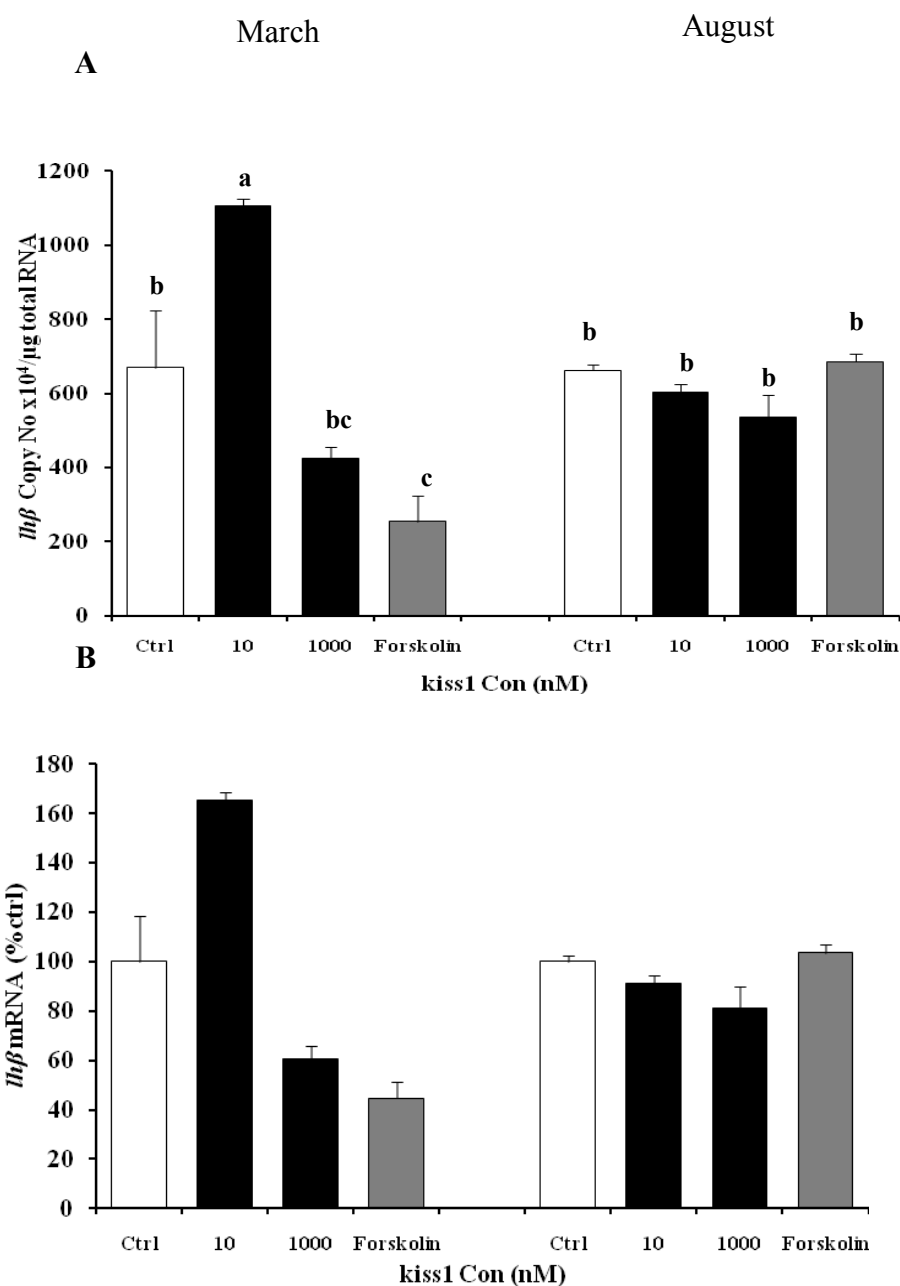


Figure 4.7. Effect of sbkiss1 on *lhβ* mRNA in cells harvested at two sampling points (March & August). Cells were treated with sbkiss1 (10 and 1000 nM) and 10 μ m forskolin for 24 hr of incubation. Data are presented as absolute copy numbers (A) or relative values to control (B). Data are expressed as the mean \pm SEM (n = 4). Superscripts denote significant differences between treatments (doses).

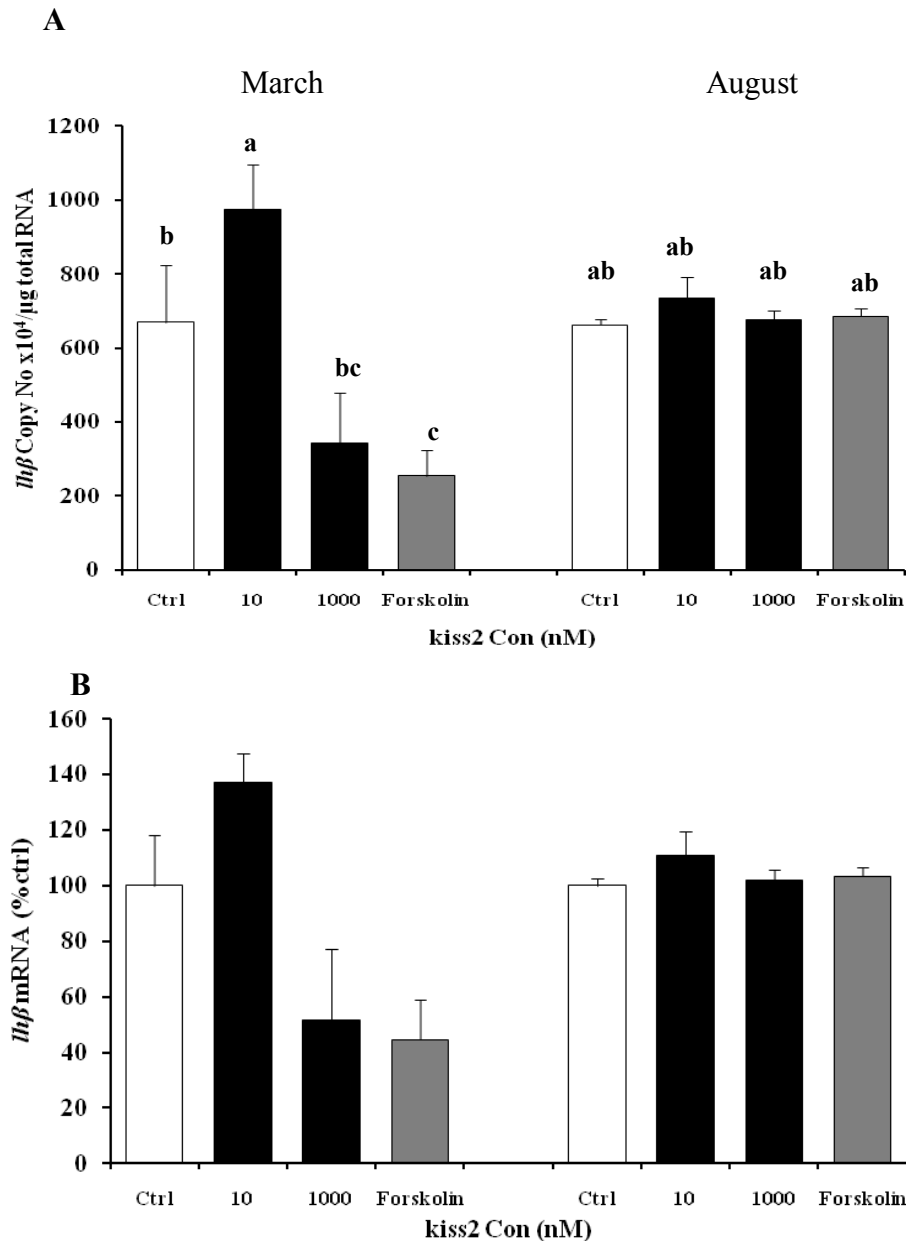


Figure 4.8. Effect of sbkiss2 on *lhβ* mRNA in cells harvested at two sampling points (March & August). Cells were treated with sbkiss2 (10 and 1000 nM) and 10μm forskolin for 24 hr of incubation. Data are presented as absolute copy numbers (A) or relative values to control (B). Data are expressed as the mean ± SEM (n = 4). Superscripts denote significant differences between treatments (doses)

4.4. DISCUSSION

The role of kisspeptin and its receptor has recently started to be investigated in fish with the aim to confirm its action on the reproductive function in fish, as shown in mammals. However, the long evolution time in fish and adaptation to a vast range of environments has resulted in a large diversity of reproductive strategies and several gene variants, due to genome duplication events, involved in the control of reproduction through the BPG axis. Studies have focused on several fish species so far including data rich model species (e.g. zebrafish), but sea bass and tilapia have also been at the forefront of the kisspeptin research due to the commercial importance of controlling their reproductive cycles. However, although progress has been made, notably with the cloning of two kisspeptin signal peptides in sea bass (Felip *et al.*, 2009), the co-localisation of *kissr4* (cited as *kiss1r*) in tilapia (Parhar *et al.*, 2004) and both the receptors and kisspeptin in sea bass (Escobar *et al.*, 2010) and zebrafish (Servili *et al.*, 2011) with the GnRH neurons, and the profiling of kisspeptin receptor expression during ontogeny (Mohamed *et al.*, 2007) and puberty (Martinez-Chavez *et al.*, 2008; Filpy *et al.*, 2008), the mode of action of kisspeptins at the BPG level remains unknown. Most studies have focused on the kisspeptin-GnRH interaction at the brain level based on mammalian discoveries. If a direct connection appears to be conserved in fish as seen in mammals, this is based on indirect evidence showing either a correlation between kisspeptin and gonadotropin expression (both circulating protein levels and gene expression) or sex steroids (see Chapter 3). Studies carried out on mammalian species, including humans, indicated that kisspeptin or its derivative decapeptide sequence (kisspeptin-10) have the ability to stimulate release of LH and FSH hormones when administered centrally or peripherally (Gottsch *et al.*, 2004; Messenger *et al.*, 2005; Navarro *et al.*, 2005a; Shahab *et al.*, 2005; Dhillon *et al.*, 2005) in a dose-

dependent manner. Only a few studies looked at the direct effects of kisspeptin on GnRH expression due to the lack of specific assays in most species and the multitude of forms (3 types and 8 GnRH gene variants) found so far in fish. It has therefore been difficult until now to ascertain if the gonadotropin surge in response to kisspeptin stimulation was operating through GnRH neurons that are well known for their hypophysiotropic action, or directly at the pituitary cell level. Infusion of kisspeptin into the third ventricle of sheep was shown to induce the release of GnRH into the cerebrospinal fluid (Messenger *et al.*, 2005). Thus, it is strongly suggested that kisspeptin acts directly on GnRH neurons in the hypothalamus, where kisspeptin elicits GnRH secretion, which in turn activate LH and FSH secretion from the pituitary. However, as suggested in mammals (Richard *et al.*, 2009), kisspeptin could also have a direct hypophysiotropic effect on gonadotropin producing cells in the pituitary. The direct effect of kisspeptin on pituitary gonadotropin cells has revealed contrasting results so far in mammals, with in some cases stimulatory effects (Gutiérrez-Pascual *et al.*, 2007; Suzuki *et al.*, 2008) or no effects (Matsui *et al.*, 2004; Navarro *et al.*, 2005a). Irrespective of these findings, high kisspeptin signal peptide and receptor expression has been shown in human and rat pituitaries (Ohtaki *et al.*, 2001; Gutiérrez-Pascual *et al.*, 2007; Richard *et al.*, 2008) as well as fish like tilapia (Martinez-Chavez *et al.*, 2008), fathead minnow (Filby *et al.*, 2008); grey mullet (Nocillado *et al.*, 2007); grass puffer (Shahjahan *et al.*, 2010) and goldfish (Yang *et al.*, 2010). This could suggest a direct involvement of kisspeptin in the pituitary. The present study therefore aimed to test the effects of the two kisspeptin signal peptides identified in sea bass on gonadotropin mRNA expression in pituitary gonadotroph cells. The work first studied the duration of incubation, the second the dose response and finally the time of harvesting the pituitary cells corresponding to different reproductive status.

The results showed that sbkiss1 and sbkiss2 have a stimulatory effect on *lhβ* and *fshβ* subunits mRNA expression after 12 hours of incubation. Similar results were previously reported from a study on goldfish pituitary cells where short term incubation with 100 nM kiss10 failed to show an effect on *lhβ* gene expression while an elevation in mRNA level was observed after 24 hours of incubation, compared to a short-term (30 minutes) rise in LH in the culture medium (Yang *et al.*, 2010). When the same decapeptides (sbkiss1 and sbkiss2) were injected into prepubertal sea bass *in vivo*, it resulted in increased plasma LH levels only 2 hours post-injection, with great potency of sbkiss2 (Felip *et al.*, 2009), while FSH plasma levels increased 1 and 2 hour post-injection respectively in response to sbkiss-2 and sbkiss1 (Felip *et al.*, 2009).

The study then looked at the dose response of sbkiss1 and sbkiss2 on *fshβ* and *lhβ* mRNA expression. After 24 hours of incubation, no effects of either peptide were seen for any dose on the *fshβ* mRNA levels, however a dose response of both peptides was observed for *lhβ*, with a significant increase in cells treated with 10 nM relative to untreated cells and decreases in cells treated with increased doses (100 and 1000 nM). These results clearly showed the importance of the peptide concentration when performing such *in vitro* experiments. However, results obtained after 48 hours of incubation differed as a reverse dose response was observed for *lhβ* with increased mRNA expression in cells treated with the highest dose (1000 nM) compared to 10 and 100 nM doses. Similar results were obtained in goldfish where *lhβ* mRNA levels increased only in response to the highest dose of kiss10 (1000 nM) after 24 hours of incubation (Yang *et al.*, 2010). Furthermore, LH secretion levels from bovine anterior pituitary cells in culture was shown to increase in response to increasing kiss1 doses (Suzuki *et al.*, 2008) as in rat pituitary cells (Gutiérrez-Pascual *et al.*, 2007). The contrasting results observed between 24 and 48 hours of incubation suggest that the

prolonged treatment duration can either reduce the magnitude of stimulation in a time-dependent manner or completely abolish the stimulatory effect found in the low doses. These results raise the possibility that the *kissr4* expressed in sea bass pituitary may be able to receptor desensitization (decline in receptor response) by time. Furthermore, differences seen between the incubation duration and dose-response trials could come from the differences in reproductive status of the fish sampled as shown by GSI values (immature with GSI < 0.5 % and mature with GSI > 1.7 % in trials 1 and 2, respectively). Due to these differences in the reproductive stages, the incubation times used in second trial were tested independently from the results obtained in the first trial. Indeed, pituitary cells sensitivity to kisspeptin could vary with the reproductive status of the fish depending on the availability of kisspeptin receptors in pituitary cells. In bovine cultured pituitary cells, kisspeptin -10 showed different stimulatory effects for growth hormone production depending on age and pubertal stage (Kadokawa *et al.*, 2008), and for LH release as well (Suzuki *et al.*, 2008). Unfortunately, kisspeptin receptor expression was not assessed in this trial. However, irrespective of these, these results suggest that kisspeptin could act directly on pituitary cells and modulate gonadotropin expression.

In the view of the results obtained, a final trial tested the effect of reproductive status on pituitary cell responsiveness to kisspeptin. Two batches of fish were compared, sampled in March and August, corresponding to spermatogenic and spent stages respectively. Interestingly, *fshβ* mRNA levels differed between sampling times with higher expression in fish at the spermatogenesis stage (sampled in March) than spent stage (sampled in August) as previously described in Chapter 3. These results agree with the well known role of *fshβ* in the spermatogenesis process (Schulz *et al.*, 2010). However, no such effect was seen for *lhβ* expression between stages of development despite the

role played by LH on spermiogenesis. It is clear that gonadotropins release and gene expression are related to the gonadal stage, and the response of the pituitary cells as well. In masu salmon, the effect of sGnRH treatment on *lhβ* and *fshβ* genes expression was different between male and female and gonadal stage (Ando *et al.*, 2004). The present results suggest that the response to kisspeptin in the pituitary level is affected by the reproductive state of the fish. However, more comparisons to other times of the year are needed to assess more accurately the response of the gonadotropins for different developmental stages. More work is needed to test the effect of both kisspeptin forms on the mRNA expression and LH and FSH release in sea bass pituitary cell culture, as that the effect of kisspeptins on the gonadotropins subunits mRNA may be dependent on the sex, gonadal state, incubation times and different doses. The present study is a preliminary approach; different developmental stages, doses, males in two trials and mixed sex in the third one have been used; however, more justification for the experimental protocols is needed to discriminate the real action of kisspeptins.

Throughout the present studies, the use of forskolin as a positive control was unsuccessful as it did not promote *fshβ* and *lhβ* mRNA expression. This came as a surprise as forskolin is a well known agent that increases intracellular levels of cAMP and stimulates pituitary cells to express gonadotropin genes and release hormones such as LH and also GH as shown in rat pituitary cells (Evans *et al.*, 1985; Voss *et al.*, 2001). The use of 10 μ M forskolin activated directly the adenylate cyclase and the growth hormone mRNA to 163 % in rat pituitary cell line after 2 days (Voss *et al.*, 2001). In goldfish, using 5 μ M forskolin as positive control in primary pituitary cells was sufficient to increase the expression levels of *lhβ*, *gh* and *prl* mRNA after 24 hours (Yang *et al.*, 2010). In the present study, the forskolin dose used was 10 μ M which was

higher than that used in the primary cell culture in goldfish and could be a superphysiological level.

In conclusion, the results obtained in the present study clearly showed that kisspeptin can act directly at the pituitary level and modulate *fshβ* and *lhβ* mRNA expression in sea bass although the effects were limited (two-fold change in mRNA at most). The present findings support suggestions made in mammals according to which kisspeptin could act directly on the pituitary to regulate gonadotropin synthesis. However, many factors such as exposure time (duration of incubation), peptide dose and reproductive status of the fish sampled can impact on the responsiveness of the pituitary cells to kisspeptin. Further studies are needed to confirm these results in sea bass and other species and better understand the variability seen especially in relation to kisspeptin receptor expression.

CHAPTER 5

**SEA BASS MELATONIN RHYTHMS: EFFECTS OF LIGHT AND
TEMPERATURE**

5.1. INTRODUCTION

Melatonin, produced by the pineal gland, is known as the biological time keeping hormone in vertebrates and it displays circadian and circannual rhythms (Falcon *et al.*, 2010). Melatonin has been suggested to play an important role in synchronising most physiological processes in vertebrates. However, if direct evidences are available in mammals (Arendt, 1998), the mechanisms by which melatonin control reproduction, growth and behaviour (locomotor, feeding, shoaling, and migration) in fish and birds remains unclear (Gwinner *et al.*, 1997; Mayer *et al.*, 1997; Falcon *et al.*, 2007). Melatonin rhythms are clearly under the control of external (environmental) and internal (clocks) signals. A better understanding of these key conserved mechanisms in fish would help unravel the environmental control of seasonal physiology in fish and develop protocols for aquaculture.

The production of melatonin by the pineal gland exhibits a distinct diel rhythm with elevated levels during the hours of darkness and low basal levels during the day (Falcon *et al.*, 2010). In all vertebrates, elevated melatonin production accurately reflects the length of the light: dark cycle (Ekstrom and Meissl, 2003), however, unlike mammals, the pineal gland in fish appears to be directly photosensitive (Max and Menaker, 1992; Falcon *et al.*, 2010). Arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase (HIOMT) are known as the rate limiting enzymes of melatonin production as they catalyse the formation of *N*-acetylserotonin, and then the conversion of *N*-acetylserotonin into melatonin (Falcón *et al.*, 2007). Light inhibits AANAT activity and melatonin release (Falcon *et al.*, 2010). At night, photoreceptor depolarization allows calcium (Ca^{2+}) entry (through voltage-gated Ca^{2+} channels) and cyclic AMP (cAMP) accumulation (Falcón, 1999). Both contribute to increase AANAT

amount and activity through phosphorylation of the AANAT protein. This process is reversed by illumination, which induces sequentially photoreceptor hyperpolarization, dephosphorylation and degradation of AANAT through proteasomal proteolysis, resulting in a drop of melatonin production (Falcón *et al.*, 2010). Interestingly, the organization of the circadian system that controls melatonin production has changed dramatically in the course of vertebrate evolution (Migaud *et al.*, 2010). In mammals, photoentrainment is exclusively mediated by retinal photoreceptors and as such pineal photoreceptors have lost their direct light sensory abilities (Ekstrom and Meissl, 2003). In fish, differential photic regulation of pineal melatonin synthesis have been suggested with at least three different organisations: 1) salmonids with a “decentralized” system in which the pineal gland responds directly to light independently of the eyes; 2) seabass and cod in which both the eyes and the pineal gland are required to sustain full night-time melatonin production and 3) tilapia and catfish in which the pineal gland is not light sensitive (or only slightly) and the eyes required to perceive light and inhibit melatonin synthesis during the day (Migaud *et al.*, 2007). These have been shaped through the 500 million years of evolution to diverse environments occupied by fish.

Other external signals than light have been shown to regulate melatonin production, mainly temperature and salinity but also feeding (Ceinos *et al.*, 2008). In many of investigated fish species, photoperiod clearly controls the duration of melatonin production while ambient temperatures can dictate the amplitude (Falcon, 1999; Garcia-Allegue *et al.*, 2001). In the pike, temperature had no effect on the phase and period of the circadian melatonin rhythm but it had an impact on the amount of melatonin synthesized (Falcón *et al.*, 1994). As fish are ectotherms, they are directly influenced by the external temperature, which fluctuates on a daily and seasonal basis.

However, temperature effects depend on the fish species. In the white sucker, *Catostomus commersoni*, low temperature stimulates the nocturnal melatonin surge while high temperature inhibits it (Zachman *et al.*, 1992). In contrast, in pike, high summer water temperatures stimulate melatonin produced at night while low winter temperatures inhibit the nocturnal surge (Falcon *et al.*, 1994). Furthermore, there is an optimum temperature for melatonin biosynthesis above or under which melatonin production is suppressed as found in rainbow trout (12 °C, Iigo *et al.*, 2007). The mechanisms by which temperature acts on melatonin secretion are not fully understood.

It has been suggested that such effects would be mediated through direct temperature regulation of AANAT2 enzymatic activity although this might be species-specific (Coon *et al.*, 1999; Falcon, 1999; Benyassi *et al.*, 2000). Interestingly, a good correlation between the peak of AANAT response and preferred physiological temperature was found in rainbow trout (12 °C), pike (20 °C), seabream (27 °C) and zebrafish (30 °C) (Falcon *et al.*, 1994). Furthermore, the same response curves were obtained when AANAT2 activities were measured from cultured pineal gland or recombinant AANAT2 enzymes which indicate that the response to temperature is an intrinsic property of the enzyme itself. Thus, the concurrent action of photoperiod, that determines the duration of the melatonin signal, and of temperature, that determines its amplitude, provide accurate definitions of both the daily and annual cycles. In addition, salinity was recently shown to act on pineal melatonin production (Lopez-Olmeda *et al.*, 2009). In this study, plasma melatonin was reported to be higher in sea bass reared in full strength seawater as compared to freshwater while the density of melatonin binding sites (receptors) in the brain was higher in fish reared in freshwater. These results suggest that salinity could also play a role in the entrainment of seasonal events

such as migration in fish.

In most cases, daily and annual rhythms are also driven by internal clock mechanisms that free-run with a period close to 24h (circadian) or a year (circannual) when exposed to constant conditions. Circadian rhythmicity is a very conserved feature observed from photosynthetic prokaryotes to mammals (Menaker *et al.*, 1997; Ekstrom and Meissl, 2003). Clocks are key components of the circadian system together with photoreceptors involved in light perception. In teleosts, the circadian organization and clock controlled rhythms are poorly understood with studies mainly performed on model species such as zebrafish (Lopez-Olmeda *et al.*, 2006; Dekens and Whitmore, 2008). However, intrapineal oscillators, capable of self-sustaining melatonin rhythms *in vitro* in the absence of light stimuli, have been found in numerous species including pike (Falcon *et al.*, 1989), goldfish (Iigo *et al.*, 1991; Kezuka *et al.*, 1989), white sucker (Zachmann *et al.*, 1992), zebrafish (Cahill, 1996; Cahill, 2002; Vallone *et al.*, 2005), golden rabbitfish (Takemura *et al.*, 2006), ayu (Iigo *et al.*, 2004), Nile tilapia (Martinez-Chavez *et al.*, 2008) and sea bass (Bayarri *et al.*, 2004; Ron, 2004). The role played by these oscillators in the timing of seasonal events remains to be found.

European sea bass is a temperate species that displays strong seasonality in its physiology and behaviour (feeding habits, reproduction, and migration). Interestingly, sea bass has a dual feeding behaviour being diurnal during the summer and nocturnal during the winter (Sanchez-Vasquez *et al.*, 1998). In addition, sea bass can undergo seasonal migrations in both the Mediterranean and Atlantic seas from the open sea during Autumn/winter where fish mature and spawn to estuaries and lagoons in spring and summer where food is more abundant (Lopez Olmeda *et al.*, 2009). Over the last decade, sea bass, as for other warm-water fish species (red mullet and tuna), is

becoming more commonplace in Northern parts of Europe (Scotland and also Norway) with established breeding populations (Henderson, 2007). This is thought to be associated with the climate change which resulted in a 0.7 °C rise in the North Sea over the last decade (Hiddink & Hofstede, 2008). However, the mechanisms controlling these changes in behaviour in relation to the external environment are not understood yet and it is very likely that these changes are mediated through melatonin. So far, studies carried out on sea bass melatonin system have shown: 1) a classical daily melatonin profile *in vivo* with low levels during day time and higher levels at night (Sanchez-Vazquez *et al.*, 1997; Gracia-Allegue *et al.*, 2001); 2) that both pineal and eyes are needed to sustain daily circulating melatonin rhythms (Bayarri *et al.*, 2003, Migaud *et al.*, 2007); 3) inverse plasma and ocular melatonin daily rhythm profiles with high melatonin levels in the retina during the day (Sanchez-Vazquez *et al.*, 1997; Iigo *et al.*, 1997); and 4) seasonal melatonin profiles with large differences in nocturnal melatonin levels (plasma levels from 144 pg/ml in summer to 23 pg/ml in autumn) (Garcia-Allegue *et al.*, 2001). In addition, *in vitro* studies have suggested that the sea bass pineal gland is directly photosensitive and has intrapineal oscillators regulating melatonin production (Bayarri *et al.*, 2004; Ron, 2004), although only four out of seven pineal glands showed significant circadian rhythm under continuous darkness in the study of Bayarri *et al.* (2004).

All the above make sea bass an interesting species for the study of external and internal regulatory mechanisms of melatonin production. The aims of this study were: 1) to investigate the effects of both photoperiod and temperature on melatonin daily rhythms by combining both *in vivo* and *in vitro* conditions for the first time to enhance the knowledge of the light perception and rhythmicity in sea bass; and 2) study/confirm

the presence or absence of intrapineal oscillators in sea bass in comparison to the previous work. To do so, a series of trials under *in vivo* and *in vitro* conditions were carried out, firstly by applying different temperatures and two photoperiod regimes, secondly by examining the endogenous melatonin rhythms under constant darkness (DD), and two applied temperatures.

5.2. MATERIALS AND METHODS

5.2.1. Animals

European sea bass males (total weight of 580.2 ± 137.4 g and standard length of 36.0 ± 2.1 cm) were obtained from Machrihanish Environmental Research Laboratories of the Institute of Aquaculture (MERL, Scotland, $55^{\circ}44'N$, $5^{\circ}44'W$). Fish were acclimated to constant 12L: 12D artificial photoperiod (light on at 7:00, lights off at 19:00) and a temperature of 14 ± 1 °C for a period of at least 1 month prior to the start of the experiments. Fish were fed twice per day to satiation with commercial fish food (Atlantic HE 50C+ 35A/25C, Skretting, Invergordon, UK). In all experiments, fish were either anesthetized with 2-phenoxyethanol (Sigma, 1:10000) or killed using a lethal dose (1ml/L). All experiments were carried in accordance with the Animals (Scientific Procedures) Act 1986, UK.

5.2.2. Experiment 1. Effects of varying photoperiod and temperature on daily *in vivo* plasma melatonin levels

The aim of the experiment was to determine the diel plasma melatonin profiles under different photoperiod and temperatures. Sea bass males were PIT-tagged and transferred into 500L light-proof circular tanks placed in light-proof and temperature-controlled rooms. Water was filtered by external bio-filter canisters (Fluval FX5) matured using a nitrifying pack (ABIL) with a flow through rate of 2000L/h. Water quality was monitored twice weekly using commercial kits (C-Test kits, New Aquarium systems, Mentor, Ohio, USA) and ammonia, nitrite, nitrate and pH levels remained within safe limits throughout the trial. Lighting was provided by one light unit/tank (100 watt bulbs inside bulkhead) fixed on each tank cover giving a light intensity of 0.6 watts /m² at the water surface (measured by single channel light sensor, Skye Instruments, Powys, UK). Two light regimes were tested under different temperatures: long day (18L: 6D, light on at 07:00 and off at 01:00); or short day (6L: 18D, light on at 09:00 and off at 15:00). For each light regime two replicate tanks each containing 14 fish were used. Tanks were kept in controlled temperature rooms and subjected to gradual temperature changes for acclimation over 2-3 weeks interval, starting from 10 °C. Once the tanks water temperature reached the required experimental temperature (10 °C, 14 °C, 18 °C and 24 °C), fish were kept under this temperature for at least two weeks and then blood sampled. At each sampling time, 4 fish were anaesthetised and blood withdrawn by superficial venepuncture using heparinized syringes, every 4 hours for a 24 hour period for both long and short day treatments and each temperature (10 °C then 14 °C, 18 °C and 24 °C). Sampling times were 10:30, 14:30, 18:30, 22:30 on day 1 and 02:30, 06:30 and 10:30 on day 2 for both light regimes. Extreme care was made to the

experimental lighting regimes and night sampling to avoid potential light pollution. During night time sampling, when lights were switched off sampling was performed using a red dim light.

5.2.3. Experiment 2. Effect of varying photoperiod and temperature on daily *in vitro* melatonin rhythms

The aim of this trial was to determine the effect of photoperiod and temperature on daily melatonin profiles from pineal glands in culture. The fish used for this trial were from the same stock as for experiment 1. Fish were killed by lethal dose of 2-phenoxyethanol (Sigma, 1 ml /L) between 12:00 and 16:00 and pineal glands removed under a dissecting microscope after opening the skull dorsally around the pineal window and extracting the pineal according to Migaud *et al.* (2006). After removal, pineal glands were washed with sterile culture medium, then placed in the pineal culture chambers in the culture system; The pineal culture system consisted of a continuous flow through system regulated by a peristaltic pump at a flow rate of 1.5 ml of culture medium/hour and a fraction collector automatically collecting samples (1.5 ml/ tube) every hour after passing through the culture chambers, as previously described by Migaud *et al.* (2006) (see Chapter 2 for further details). HEPES sodium salt (Sigma, ref: H3784, 4.77 g/L) was added the culture media (RPMI 1640, Sigma) to adjust the pH (7.4). Penicillin–streptomycin (10 mg/L) and Fungizone (5 mg/mL) were also added to the culture media to avoid bacterial and fungal development, and then culture media filtered for sterility through 0.2 µm filter paper and stored in the fridge and replaced every day for the culture system. Each trial consisted of exposing the pineal glands to a light/dark (LD) cycle at the temperature at which the fish were reared, followed by 2

LD cycles per temperature tested (total of 7 LD cycles, 168 hours). After the first complete LD cycle, the cabinet temperature was decreased gradually (for 3 hours) to 10 °C and pineals were left for two complete LD cycles under this temperature, then the temperature was increased to 14 °C. Once the temperature was stable pineals were left for two complete LD cycles under this temperature. The same was done for the last tested temperature, 18 °C, and pineals were kept under this temperature for the last two complete LD cycles. Two photoperiods were tested: long day (light switched on from 5:00 to 23:00) and short day (light switched on from 9:00 to 15:00). A total of 8 pineal glands per photoperiod treatment were tested in two replicats trials per photoperiod (4 pineal glands in each). Selected culture medium samples were first selected from the first and the last LD cycle (3 from day and 3 from night periods for both LD cycle) to confirm the viability of each pineal gland and the samples produced from the dead pineals were eliminated from the analysis. Because of the large number of samples generated (8 pineal glands x 2 photoperiodic treatments x 7 LD cycles x 24 hours, total of 2688 samples) and the high costs and time required for the analyses, only selected samples were analysed from 6 pineal glands every 2 to 4 hours corresponding to three and four time points assayed during the dark phase for the LD (00:00, 02:00, 04:00) and SD (18:00, 22:00, 02:00, 06:00) treatment, respectively and vice versa during the day (3 and 4 sampling points for SD and LD respectively). Temperature changes were monitored throughout the trials (every 5 minutes) using a temperature logger (DigiTag, Farnell, UK) placed inside the pineal culture cabinet (Fig. 5.1 A and B).

5.2.4. Experiment 3. Effect of constant darkness on daily *in vivo* plasma melatonin rhythms

The aim of this trial was to test if daily plasma melatonin rhythms seen in fish exposed to a 12L:12D photoperiod could be sustained endogenously under constant darkness at two different water temperatures. A total of 120 fish were placed in 500L light proof circular tanks (20/tank), three replicat tanks were used with total 60 fish for each temperature and kept in light proof and temperature controlled rooms with the same water filtration system as described in exp. 1. Fish of the same origin as exp. 1 and 2 were used and first acclimatised for 2 weeks to a 12L: 12D photoperiod (light switched on at 8:00 and off at 20:00) at two different temperatures (10 or 18 °C ± 1). Sampling consisted of killing 5 fish at random by lethal anaesthesia (2-phenoxyethanol, 1 ml/L) and collecting blood by venepuncture. The experiment ran for 5 days (day 1 under LD, day 2 to 4 under DD and day 5 under LD) with one sample during the day (2pm) and one sample during the night (2 am) every day (subjective day and night). Lighting was provided by one light unit/tank (100 W bulbs inside bulkhead) fixed on each tank cover giving a light intensity of 0.6 W/m² at the water surface (measured by single channel light sensor, Skye instruments, Powys, UK). During DD sampling was performed using a red dim light.

5.2.5. Experiment 4. Effect of constant darkness on daily *in vitro* melatonin rhythms

The aim of this experiment was to confirm *in vivo* results obtained in exp. 3 in cultured pineal glands exposed to constant darkness (DD) and two different temperatures (10 °C and 18 °C) to determine the presence or absence of functional

intrapineal oscillators controlling melatonin production in the absence of a day/night cycle. Fish used in the experiment were from the same stock used in previous experiments. A total of 6 pineal glands were analysed for each temperature regime. Pineal glands were removed as previously described and exposed *in vitro* to the same photoperiod and temperature regime to avoid any endogenous rhythm or thermal shock to the pineal glands that could compromise the results. Pineal glands were exposed to first a light/dark (LD) cycle followed by four subjective cycles under DD and another LD cycle at the end to confirm that the pineal glands were still alive with a normal melatonin secretion pattern. Pineal glands were in culture for a total of 6 LD cycles (144 hours) and culture medium samples were collected every hour (total of 864 samples). However, due to the cost and time constraints, only samples collected every four 4 hours for each pineal gland over the 6 cycles were analysed (total of 36 samples per pineal). Temperature changes were monitored and recorded daily (Figs. 5.1C & D).

5.2.6 Melatonin assay

Blood samples were centrifuged at 1200 g for 15 min at 4 °C and plasma stored at -70 °C until analysed for melatonin. Culture medium samples were immediately stored in -70 °C. Melatonin level was determined by radioimmunoassay (RIA) according to Vera *et al.* (2010), as detailed in Chapter 2 (2.2.2). Samples from pineal culture were diluted 1:10 and 1:20 for light and dark samples respectively with assay buffer prior to assay while dark plasma samples were diluted 1:2 and no dilution was done for the light plasma samples. All standards and samples were assayed in duplicate. Intra- assay coefficient of variation was 8.25 % (n=5) and inter-assay coefficient variation was 8.81% (n= 20). A known concentration of melatonin (50 pg/ml) was used to check

reproducibility of measurements between assays (quality control).

5.2.7. Statistical analysis

All data was analyzed using MINITAB® version 16.0 (Minitab, Ltd, UK). When necessary, data were transformed to improve normality and homogeneity of variance (Kolmogorov-Smirnov and Bartlett's tests). Melatonin levels were analysed using General Linear Model (GLM) followed by Tukey's post-hoc tests to identify significant differences. Data is expressed as mean \pm S.E.M values. Significant differences were determined at $p \leq 0.05$.

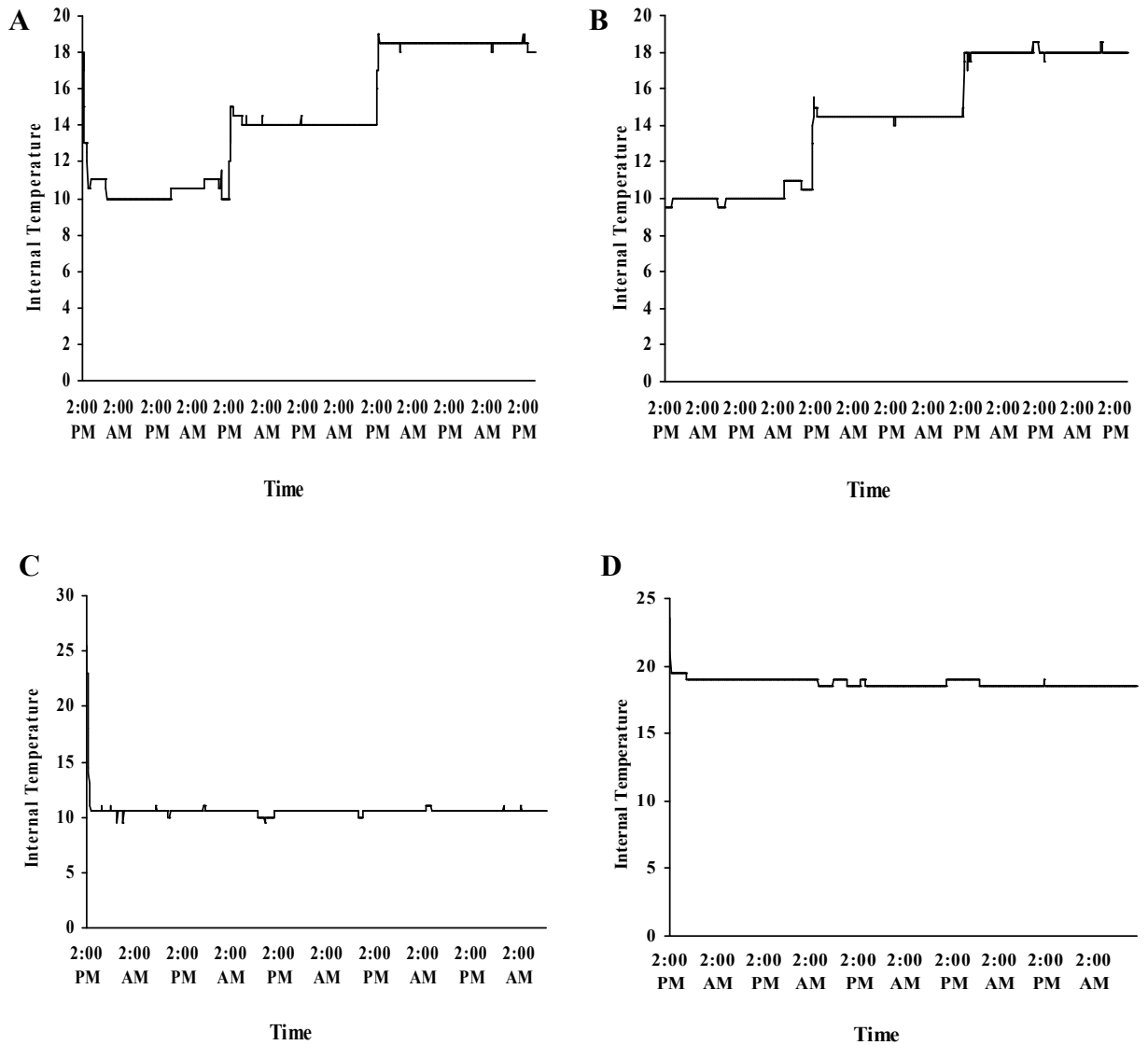


Figure 5.1. Temperature changes during the experimental period of *in vitro* trials. A and B related to experiment 2 for long day and short day, respectively. C and D relate to experiment 4 for 10 °C and 18 °C respectively. Temperature was recorded every 5 minutes using a data logger.

5.3. RESULTS

5.3.1. Experiment 1. Effects of varying photoperiod and temperature on daily *in vivo* plasma melatonin levels

Plasma melatonin of sea bass displayed a clear diurnal rhythm for both long and short day in all four studied temperatures. Dark phase melatonin levels increased significantly comparing to light phase (Fig. 2A&B). Under a LD regime (18L: 6D), day-time plasma melatonin levels were significantly higher in fish exposed to 24 °C (70.7 ± 7.9 pg/ml) than all other temperatures tested (32.6 ± 11.6 , 29.3 ± 7.8 and 27.1 ± 12.9 for 10, 14 and 18 °C, respectively) with no significant differences between 10, 14 and 18 °C (Figure 5.2A). Night plasma melatonin levels showed a significant increase from basal day levels (on day 1 and 2) for all temperature treatments under LD, except for fish exposed to 24 °C sampled at 22.30. Significant differences were measured at night for both sampling points (2.30 and 6.30) with fish exposed to 24 °C displaying higher plasma melatonin levels (150-170 pg/ml) than fish exposed to the other temperatures (mean melatonin ranging from 88 to 110 pg/ml). Levels in each temperature treatment remained high at both night sampling points with no significant differences and returned to basal levels in fish sampled on day 2 following the light being switched on at 7.00.

Similarly, under a SD regime (6L: 18D), day-time plasma melatonin levels were significantly higher in fish exposed to 24 °C (76.0 ± 7.9 pg/ml) than all other temperatures tested (31.8 ± 7.6 , 36.2 ± 9.5 , and 35.0 ± 10.8 pg/ml for 10, 14 and 18 °C, respectively) with no significant differences between 10, 14 and 18 °C (Figure 2B). Night plasma melatonin levels showed a significant increase from basal day levels (on day 1 and 2) for all temperature treatments under LD. Significant differences were

measured at night for all night sampling points (18.30, 22.30, 2.30 and 6.30) with fish exposed to 24 °C displaying higher plasma melatonin levels (212.0 ± 19.2 pg/ml) than fish exposed to all the other temperatures (mean melatonin ranging from 88 to 144 pg/ml). In addition, significant differences were observed between the 10, 14 and 18 °C treatments. Plasma melatonin levels in fish exposed to 10 °C were significantly lower than all other treatments throughout the night period except for 18 °C at both the 18.30 and 02.30 sampling points and 14 °C at the 22.30 sampling point. A clear gradual increase in melatonin levels was observed when temperature increased (10 °C < 14-18 °C < 24 °C). Levels in each temperature treatment remain high throughout the night period with no significant differences and returned to basal levels in fish sampled on day 2 following the light being switched on at 7.00.

Relative night-time melatonin levels are presented in Figure 5.3. In fish exposed to long days, relative night melatonin levels represent 350, 300, 250 and 224 % (for 10, 14, 18 and 24 °C respectively) of the day-time levels at the same temperature, while for short days these percentages were 290, 308, 318 and 285 % (for 10, 14, 18 and 24 °C respectively) of the day-time levels at the same temperature (Fig. 5.3). The melatonin levels at 10, 14 and 18 °C relative to the levels produced at 24 °C for light and dark samples under long and short days are presented in Table 5.1.

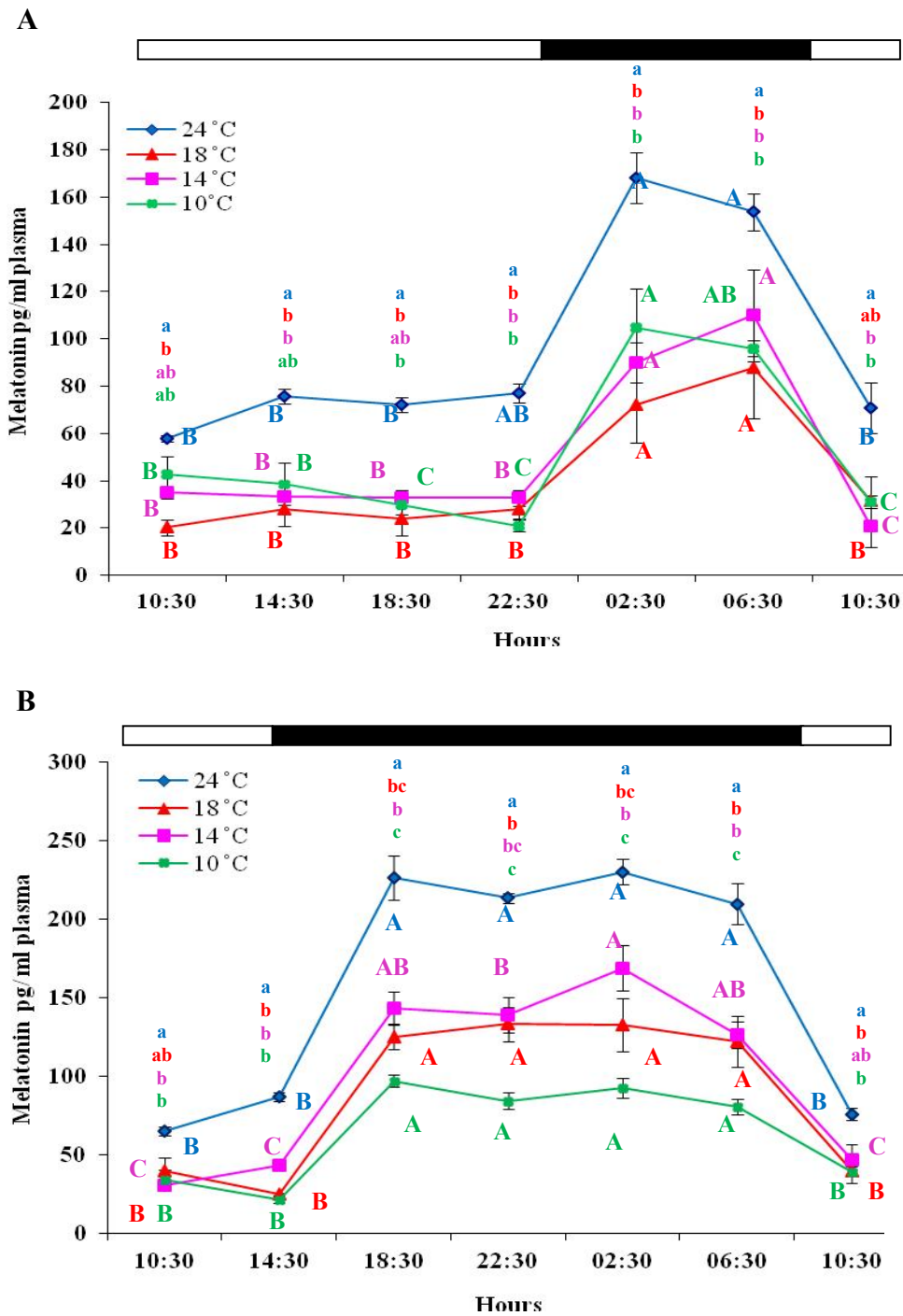


Figure 5.2. Effect of temperature (10, 14, 18 or 24 °C) on plasma melatonin profiles measured in sea bass subjected to 18L: 6D (A) and 6L: 18 D (B) regimes. Values are expressed as mean \pm SEM (n=4 fish/sampling point). Lower-case letters indicate significant differences between temperature treatments at a given sampling point while upper-case letters indicate significant differences between sampling points for a given temperature treatment. Open and filled boxes indicate day and night periods respectively.

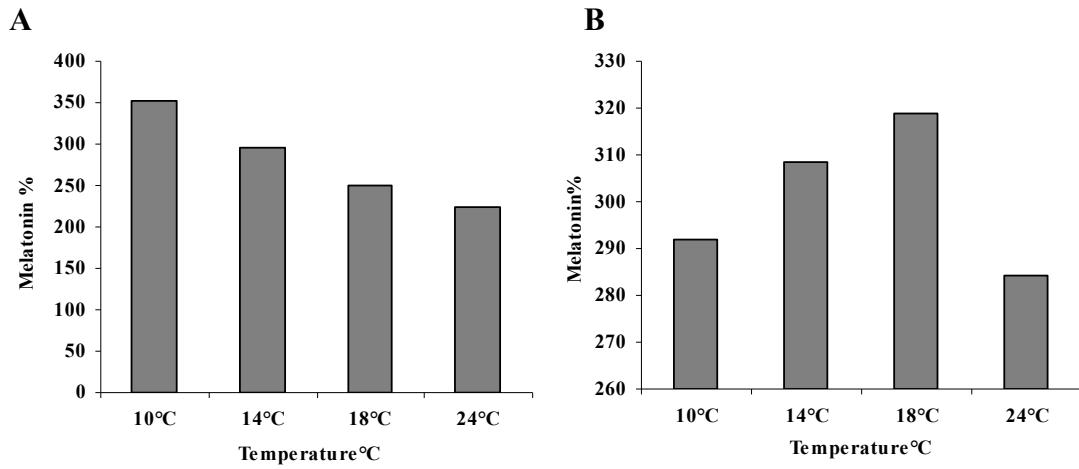


Figure 5.3. Effect of different temperatures on *in vivo* relative night-time melatonin levels in fish exposed to long day (A) and short day (B) for the four different temperatures. The melatonin mean levels are presented as a relative percentage of the day-time mean levels.

Table 5.1. Relative differences between temperature treatments at both day and night for long day (LD) and short day (SD). The melatonin mean levels are presented as a relative percentage of the day or night mean levels at 24 °C.

Treatments	Day		Night	
	LD	SD	LD	SD
10 °C	47.10	40.75	74.23	41.87
14 °C	42.65	48.63	56.27	52.80
18 °C	39.35	44.25	43.89	49.61
24 °C	100	100	100	100

5.3.2. Experiment 2: Effect of varying photoperiod and temperature on daily *in vitro* melatonin rhythms

Melatonin production by pineal glands in culture at 10, 14 and 18 °C showed marked diurnal rhythm for both LD and SD. Dark phase melatonin levels increased significantly relative to basal day-time levels for pineal glands exposed to either LD or SD photoperiods or any of the three temperatures tested (Figs. 5.4- 5.6). Under both LD and SD regimes, melatonin levels remained below 2000 pg/ml during the light phase for all pineal glands tested whereas they significantly increase during the dark phase to reach peak levels of >12,000 pg/ml in some of the pineal glands. Individual profiles for 6 complete cycles (2 cycles/temperature) for each of the 5 (LD) or 6 (SD) pineal glands are shown in Figs. 5.4 and 5.5. Inter-pineal variability was apparent in both the day and night time melatonin levels reached for both LD and SD (from 1300 pg/ml to 2900 pg/ml at day and 5000 pg/ml to 9316 pg/ml at night). However, most pineals showed a gradual increase in night time melatonin peak levels with increasing temperatures (Figs. 5.6 A, B) as seen when means of the 5 (LD) or 6 (SD) pineal glands are presented (values for 10, 14 and 18°C for both photoperiod, Fig. 5.7).

Day melatonin values for short days were higher than day melatonin values under long days for the three tested temperatures (Fig. 5.7A), while night values were not significantly different between long day and short day for the same tested temperature (Fig. 5.7B). Relative night-time melatonin levels from pineal glands exposed to LD showed an increase when temperature increased (354.26 ± 33.6 , 448.5 ± 31.0 and 500.2 ± 28.5 % of day-time levels for 10, 14 and 18 °C, respectively) (Fig. 5.8). However, under SD, no significant difference was seen between 10 and 14 °C with relative values of 270.0 ± 9.3 and 258.9 ± 11.8 % respectively, but a significant increase

(347.7 ± 9.9 %) was observed when pineal glands exposed to 18 °C (Fig. 5.8).

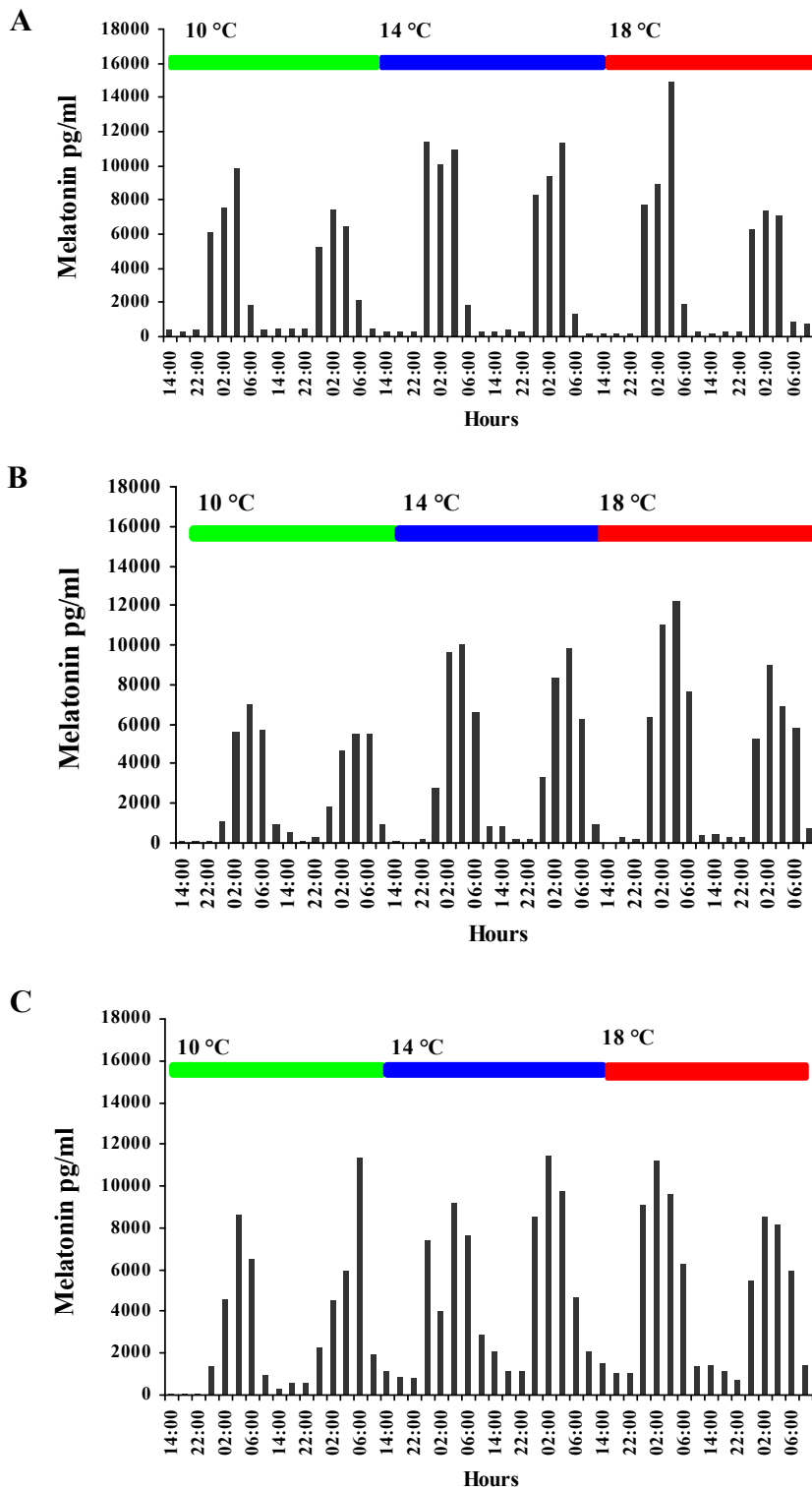


Figure 5.4. *In vitro* melatonin production (pg/ ml) by individual sea bass pineal gland exposed to LD (18L: 6D) (n=5) under 10 °C (green), 14 °C (blue) and 18 °C (red). Graph A to E show individual pineal profiles. Data expressed as mean of the duplicates.

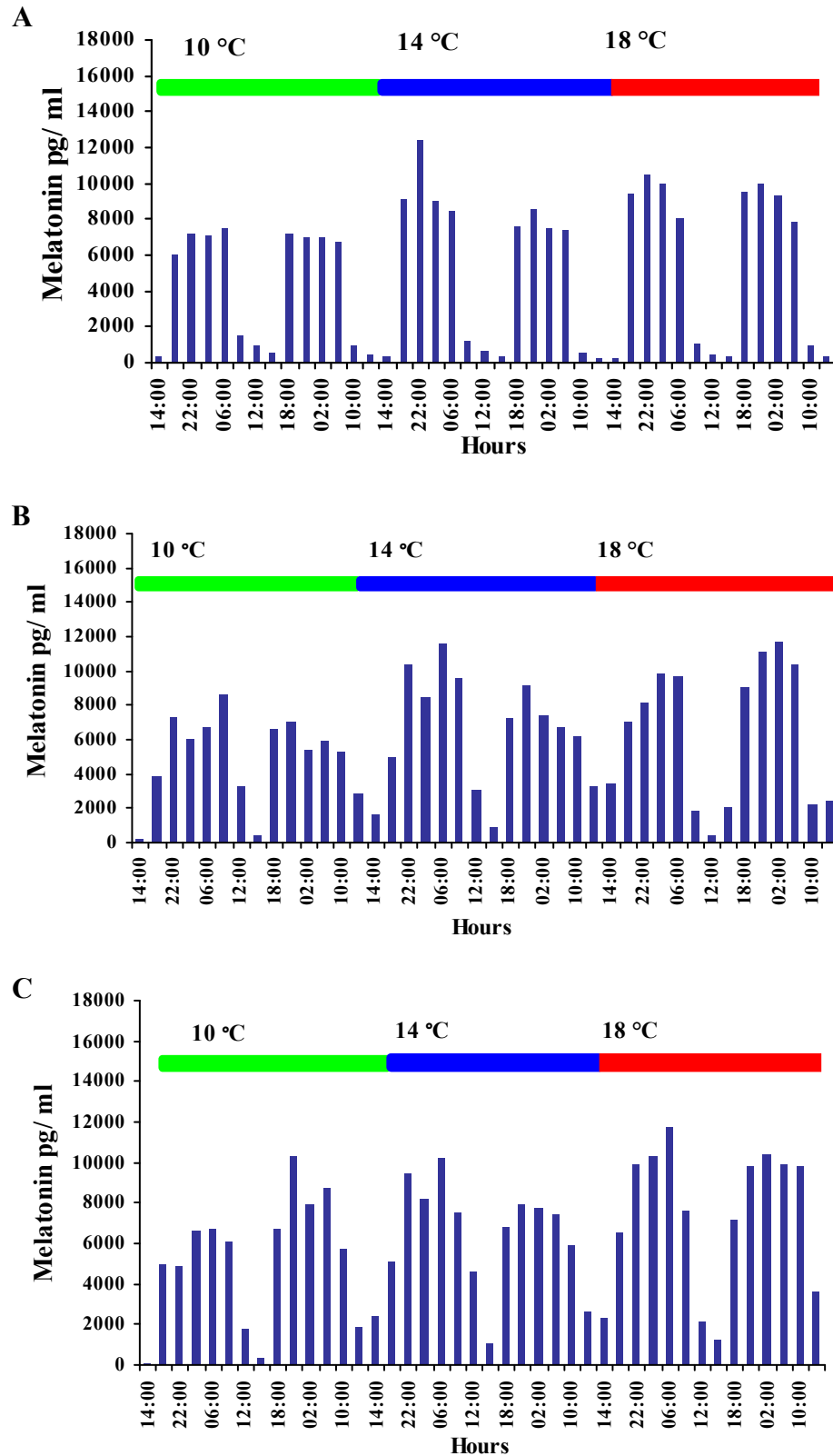


Figure 5.5. *In vitro* melatonin production (pg/ ml) by individual sea bass pineal gland exposed to SD (6L: 18 D) under 10 °C (green), 14 °C (blue) and 18 °C (red). Graph A to F show individual pineal profiles. Data expressed as mean of the duplicates.

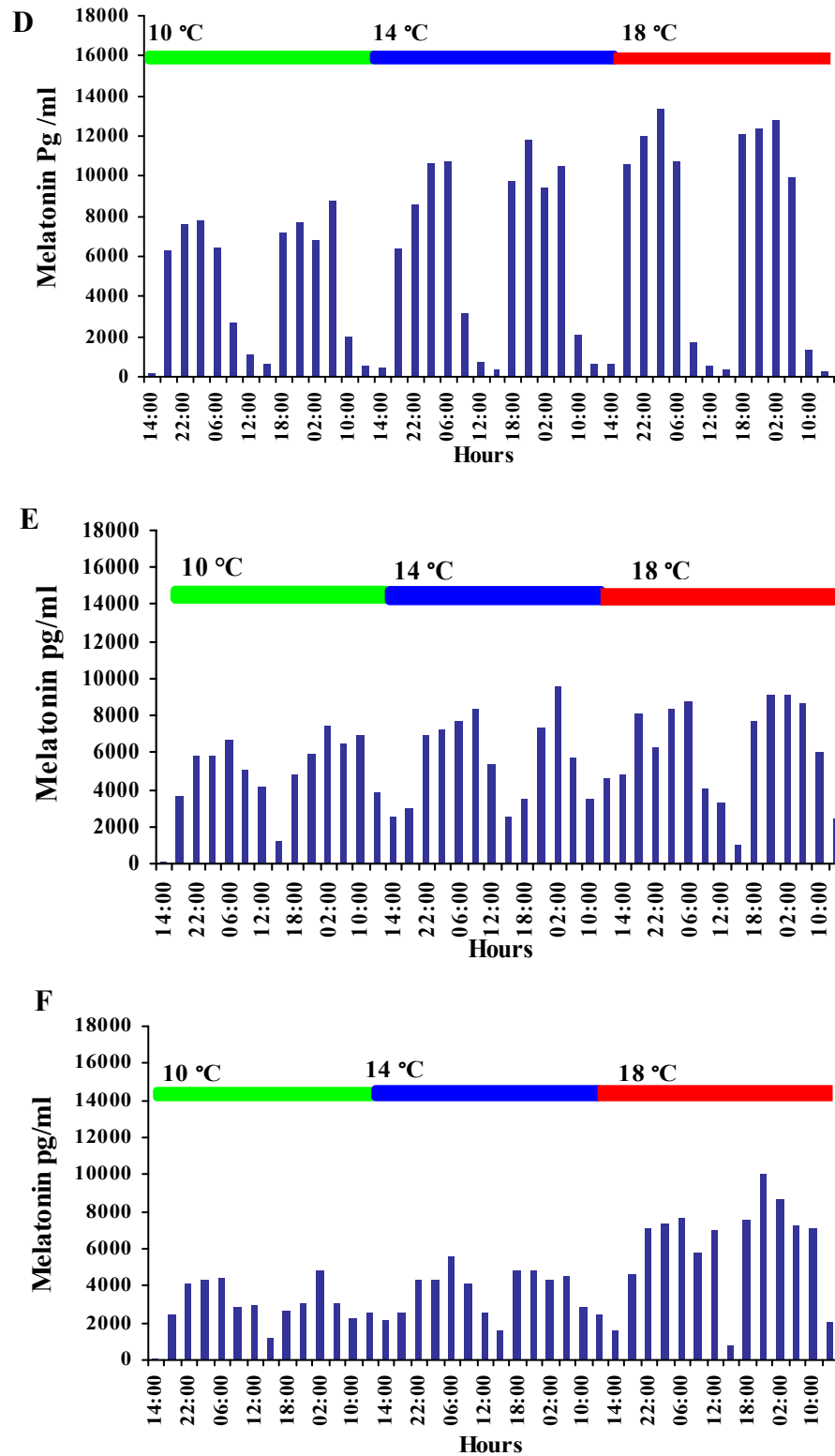


Figure 5.5 Cont'd. *In vitro* melatonin production (pg/ml) by individual sea bass pineal gland exposed to SD (6L: 18 D) under 10 °C (green), 14 °C (blue) and 18 °C (red). Graph A to F show individual pineal profiles. Data expressed as mean of the duplicates.

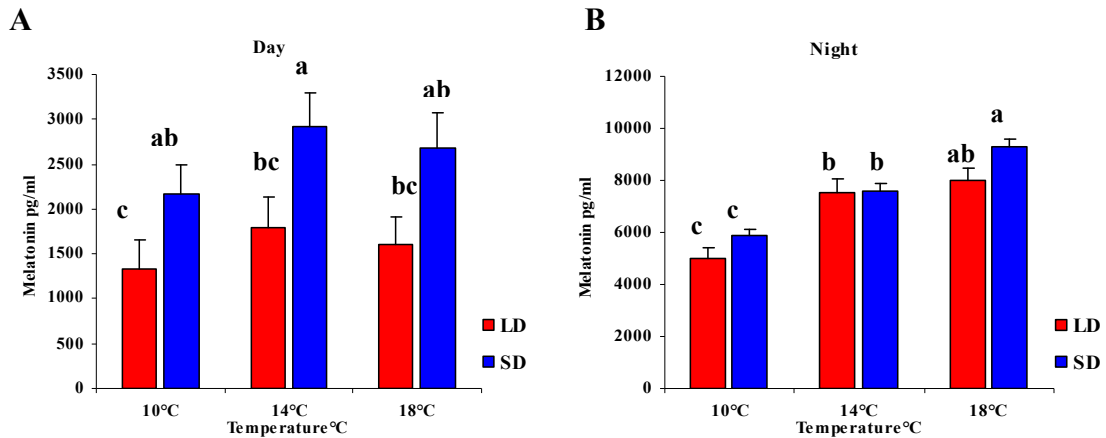


Figure 5.7. Mean melatonin levels measured in culture medium when sea bass pineal glands were exposed to either LD or SD and three different temperatures (10, 14 and 18°C). Values are expressed as means \pm SEM (n=5 or 6 pineal glands for LD and SD, respectively). Values for each pineal represent the mean of day-time (4 and 2 sampling points for LD and SD, respectively) (A) or night-time (2 and 4 sampling points for LD and SD, respectively) (B) for two cycles under a given temperature. Superscripts denote significant differences if letters are different.

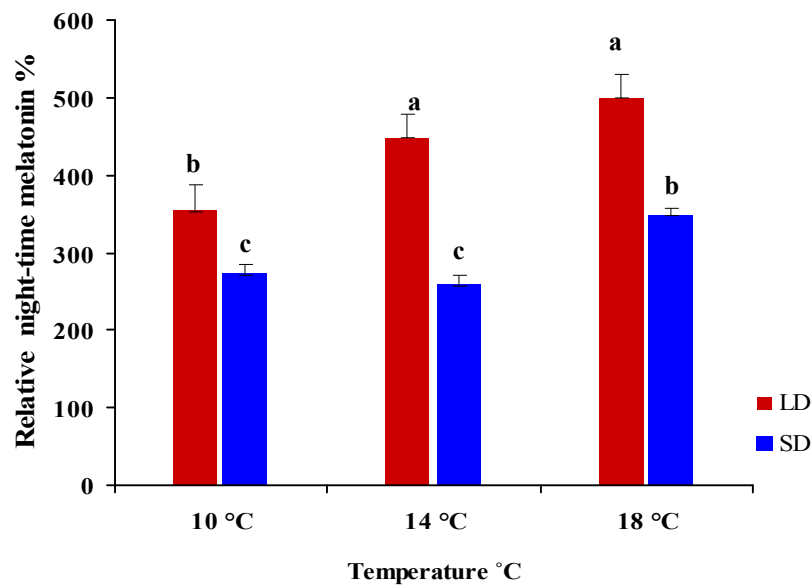


Figure 5.8. Effect of different temperatures on *in vitro* relative night-time melatonin levels. The melatonin levels are presented as a relative percentage of the day-time levels. Data are expressed as mean \pm SEM (n=5 or 6 pineal glands for LD and SD, respectively). Superscripts denote significant differences if letters are different.

5.3.3. Experiment 3. Effect of constant darkness on daily *in vivo* plasma melatonin rhythms

When sea bass were exposed to complete darkness (DD), plasma melatonin levels remained high throughout the subjective LD cycles with no significant differences, indicating the lack of any circadian rhythm (Figs. 5.9 A and B). The plasma melatonin levels produced under DD at 18°C were higher than the levels produced at 10 °C with mean values 54.0 ± 3.9 pg/ml and 35.5 ± 2.0 pg/ml respectively, while levels were similar during the light period at both temperatures.

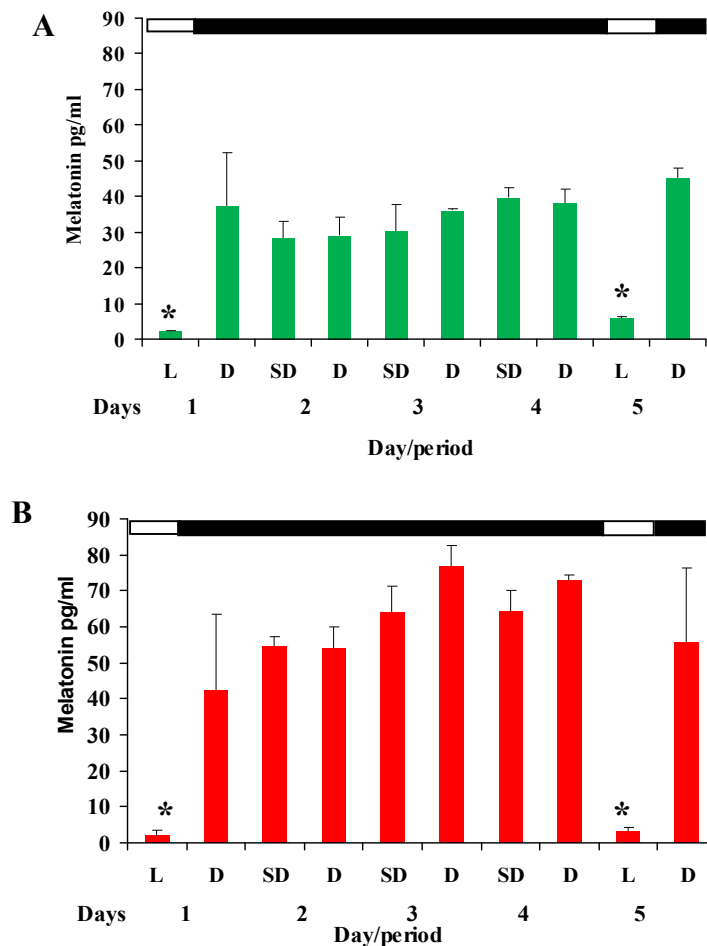


Figure 5.9. *In vivo* plasma melatonin production (pg/ml) of sea bass exposed to DD at either 10 °C (A) or 18 °C (B). Values are expressed as mean \pm SEM (n=5). Samples were taken for light time (L), dark (D) and subjective day (SD). No significant differences were observed except for day levels (L) as indicated by asterisks.

5.3.4. Experiment 4. Effect of constant darkness on daily *in vitro* melatonin rhythms

When sea bass pineal glands were exposed to complete darkness (DD), melatonin levels in the culture media remained high throughout the subjective LD cycles, indicating the lack of any circadian rhythm (Figs. 5.10 A and B). Similar results were obtained at 10 °C or 18 °C although, surprisingly, no differences were seen in melatonin levels when pineal glands were exposed to a higher temperature (8528.7 ± 1950.9 and 8232.4 ± 2919.6 pg/ml for 10 and 18 °C, respectively). When light was returned, melatonin levels significantly decreased under both temperatures (3249.4 ± 3270.6 and 2656.7 ± 3237.6 pg/ml for 10 and 18 °C, respectively). When the night-time melatonin levels under DD was calculated as relative percentage of the day-time melatonin (at last LD cycle) under the same temperature, a significant difference was found between the two temperatures, with higher relative night-time melatonin levels in pineal glands incubated at 18 °C than at 10 °C with 550 % and 450 % of day-time levels, respectively (Fig. 5.11).

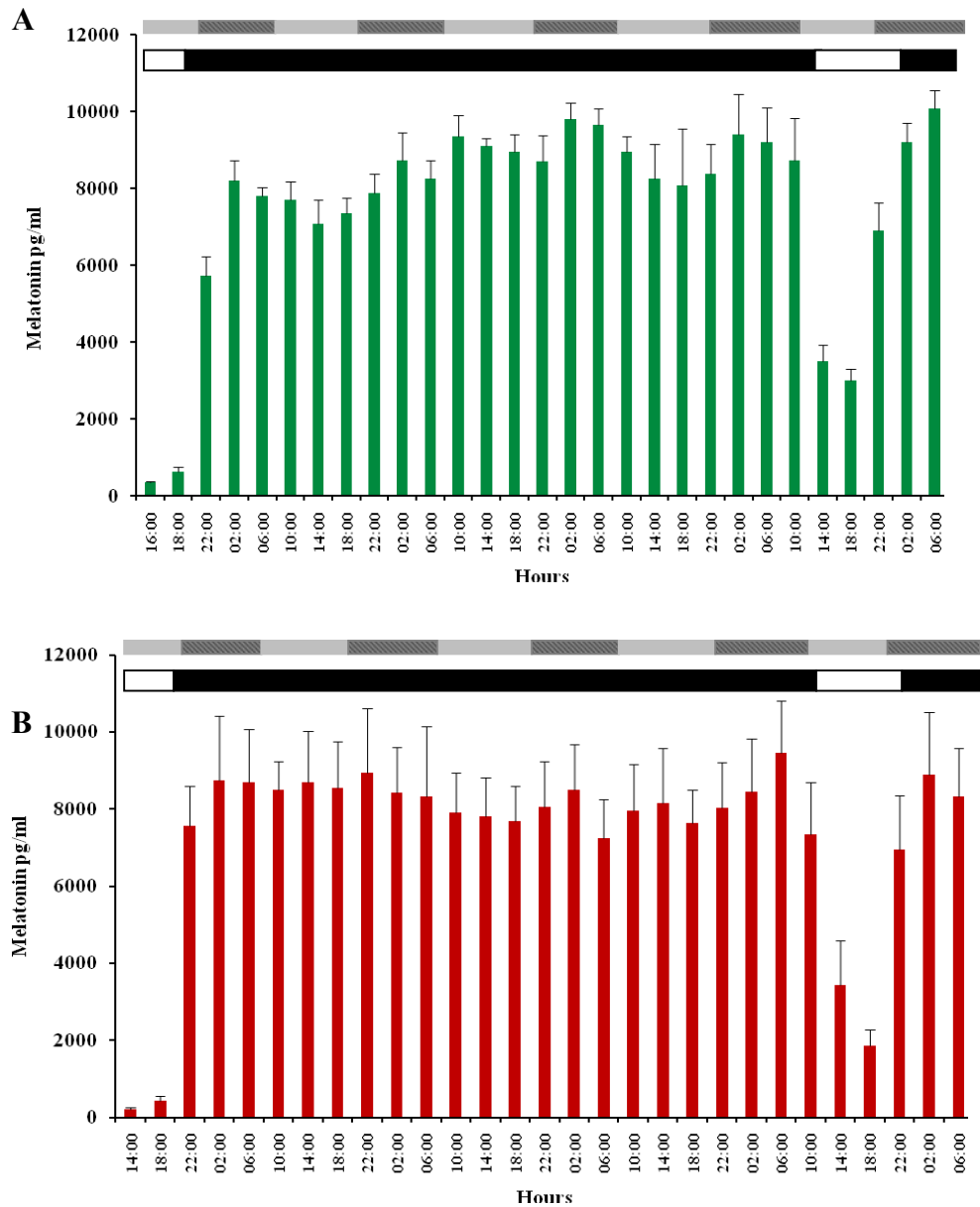


Figure 5.10. *In vitro* mean melatonin production (pg/ml) by sea bass pineals exposed to DD under either 10 °C (A) or 18 °C (B). Values are expressed as mean \pm SEM (n= 6). Open (light grey) and filled (dark grey) boxes indicate day (subjective day) and night (subjective night) periods respectively.

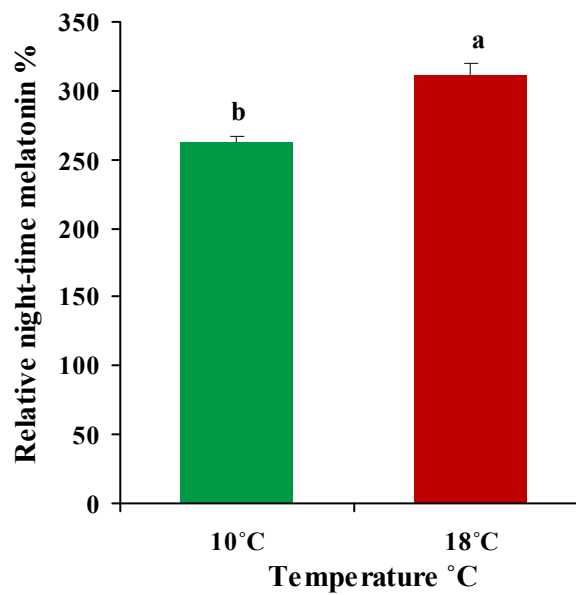


Figure 5.11. Relative night-time melatonin levels of *in vitro* pineal glands in culture exposed to constant darkness (DD) and two different temperatures (10 or 18°C). The night-time melatonin levels are presented as a relative percentage of the day-time values at the same temperature. Data are expressed as mean \pm SEM (n=6). Superscripts denote significant differences.

5.4. DISCUSSION

In temperate teleosts, the integration of the daily and seasonal variations in both photoperiod and temperature play a crucial role to synchronize physiological and behavioural activities (Migaud *et al.*, 2010). Melatonin is the main endocrine output in photoperiod signalling and it has been shown to be directly involved in the transduction of daily and seasonal environmental changes in vertebrates. However, in fish, despite a likely conserved role as seen in mammals, there is a lack of direct evidence for such an important role (Migaud *et al.*, 2010). This study aimed to better understand the combined roles of photoperiod and temperature in regulating the daily melatonin production in sea bass, known for its strong seasonality in reproduction, migration and feeding behaviour.

The present results first confirmed the daily variations in plasma melatonin with high levels secreted throughout the night period and low levels during the day according to the prevailing photoperiod (LD or SD). Such a strong seasonal melatonin rhythm has been reported in most teleost fish studied to date including sea bass (Falcon, 1999; Falcon *et al.*, 2007; Garcia-Allegue *et al.*, 2001; Bayarri *et al.*, 2010). In addition, as suggested in previous studies (Garcia-Allegue *et al.*, 2001), if photoperiod appears to drive the phase of the daily melatonin profile, temperature appears to control the amplitude (Falcon *et al.*, 1996). Accordingly, in the present study, temperature showed a significant effect on the absolute melatonin levels secreted and released in the blood stream *in vivo* or culture medium when pineal glands were incubated *in vitro*. Higher temperatures (24 °C) stimulate the pineal gland to produce increased amount of melatonin. *In vivo*, a 135 % (LD group) and 240 % (SD group) increase in night-time melatonin levels, on average, were observed when fish were reared under 24 °C as

compared to 10 °C. The difference between both photoperiod regimes could be explained by the time required for the pineal gland to reach full stimulation and produce peak physiological levels. This cannot be easily seen on the graphs as the first night sample (at 2:30) in LD was performed 1.5 H after the light was switched off (at 1:00) while it was 3.5 H in the SD regime (sampling at 18:30, light switched off at 15:00). Furthermore, the temperature effect was direct as the pineal gland in fish is unable to store melatonin which is thus immediately released, when synthesised, in the blood and cerebrospinal fluid (as shown in mammals by Tricoire *et al.*, 2002). Interestingly, temperature also had an effect on day-time melatonin release (with an average of 250 % and 230 % increase for LD and SD under 24 °C, respectively) when compared to the day-time melatonin under other lower temperatures. The temperature selected for this experiment aimed to recreate seasonal ambient changes. The results are in agreement with the study of Garcia-Allegue *et al.* (2001) who showed that the amplitude of the daily plasma melatonin profile of sea bass exposed to ambient conditions was the highest in summer conditions, with peak melatonin levels reaching 200 pg/ml (photoperiod of 15L: 9D and temperature of 28.2 °C) and the lowest in winter conditions with peak melatonin reaching 50 pg/ml (photoperiod of 9L:15D and temperature of 14.2 °C). The same patterns have been observed for a number of fish species *in vivo* including rainbow trout, Atlantic salmon, goldfish and Senegalese sole (Iigo & Aida, 1995; Falcon, 1999; Bromage *et al.*, 2001). In Senegalese sole, water temperature was shown to affect the nocturnal melatonin levels, with the highest levels reported at 25 °C, but temperature did not affect diurnal levels (Vera *et al.*, 2007). In Atlantic salmon, plasma melatonin levels were significantly higher in fish maintained at 12 °C than 4 °C (Porter *et al.*, 2001). As most fish are poikilothermic, their body

temperature will impact on many metabolic and enzymatic pathways including the melatonin biosynthesis pathway. Studies have shown that temperature acts directly on the pineal organ to modulate melatonin secretion, through the regulation of AANAT2 activity which is the main melatonin rate-limiting enzyme (Falcon *et al.*, 1994, 1996; Zachmann *et al.*, 1992; Coon *et al.*, 1999; Falcón, 1999; Benyassi *et al.*, 2000). Teleosts, unlike all other vertebrates, possess two AANAT genes, probably as a result of genome duplications (Falcon *et al.*, 2010). While AANAT-1, more specifically expressed in the retina and brain in fish, seems to display classical kinetics for vertebrates with an increased activity at temperatures of 35 to 37 °C, the pineal-specific AANAT-2 appears to have species-specific properties (Falcon, 1999; Falcon *et al.*, 2010). However, contrasting results were reported in sea bass under a natural photoperiod regime with an increased daily melatonin amplitude in November (14.5 °C) and February (12.6 °C) and reduced in September (24.2 °C) and May (19.0 °C) (Bayarri *et al.*, 2010). Differences between the two studies are probably related to the stage of development of the fish at the time of the sampling. Indeed, in the present study, fish were in the spermatogenesis stage while in the study by Bayarri *et al.* (2010), development stages differed between sampling dates as fish reached puberty. Therefore, it appears very clearly that internal factors such as reproductive status, age and size of the fish can modulate melatonin amplitude, although the mechanisms at work remain unknown.

The results obtained *in vitro* also confirmed the effect of temperature on the melatonin production as melatonin production at night was significantly higher when pineal glands were incubated at 18 °C than 10 and 14 °C under both LD and SD. Interestingly, melatonin production from isolated sea bass pineal glands in culture

medium was in average 50 times higher than plasmatic levels. This has been widely observed in other *in vitro* studies and may be explained by the large proportion of melatonin produced *in vivo* directly released into the cerebrospinal fluid (CSF) although the relative amount released into the CSF vs. blood is not known in fish (Migaud *et al.*, 2007; Falcon *et al.*, 2010). *In vitro*, all the melatonin produced by the pineal gland is released in the culture medium. Similar results on temperature-dependent melatonin production were reported *in vitro* in other teleost species including rainbow trout, pike and white sucker pineal glands (Max & Menaker, 1992; Zachmann *et al.*, 1992; Bolliet *et al.*, 1994; Falcon *et al.*, 1994). For example, in white sucker, *in vitro* melatonin production at 20 °C was higher than that at 10 °C (Zachmann *et al.*, 1992). Furthermore, temperature is not only affecting the amplitude of melatonin production but can also act on the circadian rhythm of melatonin production. In pike, endogenous rhythms in melatonin production were shown in pineal glands exposed to DD when the temperature was higher than 15 °C but the rhythm was lost if the temperature was maintained below 15 °C (Bolliet *et al.*, 1994). Similarly in white sucker, a circadian-like pattern was detected under DD in pineals kept at 20 °C; while at 10 °C no circadian melatonin rhythm was observed (Zachmann *et al.*, 1992). Melatonin rhythm can also be lost under LD cycles when temperatures were kept low as shown in Arctic lamprey, *Lampetra japonica* (Samejima *et al.*, 2000).

The mode of action of temperature changes on melatonin production is not understood yet in fish. If temperature can directly modulate the activity of melatonin rate-limiting enzymes as previously discussed, it can also act on the second nervous message produced and released by fish photoreceptors as shown in rainbow trout, in which the ganglion cell's activity is temperature-dependent, affecting the melatonin

discharge rates (Tabata & Meissl, 1993). The nervous message is an excitatory neurotransmitter in the retina as well as in the pineal gland (Ekstrom & Meissl, 1997; Falcon *et al.*, 2007) that is released in the dark at the synaptic junction between photoreceptor end terminals and second order neurons. Temperature can also influence molecular and cellular processes such as membrane properties, ion homeostasis, calcium influx and signal cascade (cAMP, cGMP and protein kinases), which may affect protein phosphorylation processes of the circadian clock mechanism (Rensing & Ruoff, 2002) while *Aanat2* gene expression is controlled by the clock machinery (Appelbaum *et al.*, 2006).

While melatonin circadian rhythms are clearly regulated by environmental conditions, they can also be self-sustained under the control of circadian clocks (Migaud *et al.*, 2010). In order to further characterise the circadian control of melatonin production in sea bass, melatonin rhythms under constant darkness were investigated *in vitro*. Constant photoperiods (LL/DD) have been commonly used to study endogenous rhythmic melatonin production in many vertebrates including teleosts. By doing so, the existence of oscillators intrapineal capable of self-sustaining melatonin production *in vitro* in the absence of light stimuli have been demonstrated in many fish species except in salmonids (Gern & Greenhouse 1988; Migaud *et al.*, 2006; Iigo *et al.*, 2007). In sea bass, previously published findings suggested the existence of such intrapineal oscillators as circadian melatonin rhythms were sustained in pineal glands exposed to DD (Bayarri *et al.*, 2004a; Ron, 2004). However, only four out of seven pineal glands showed significant circadian rhythm under continuous darkness in the study of Bayarri *et al.* (2004a). Therefore, the present study aimed to confirm these results obtained in sea bass through both *in vivo* and *in vitro* studies. When fish were exposed to DD

following an acclimation to 12L:12D at two different temperatures (10 and 18 °C), no endogenous daily plasma melatonin rhythms were apparent. Similarly, when sea bass pineal glands were exposed to DD in culture at either a temperature of 10 or 18 °C, no circadian melatonin rhythmicity could be observed. It is difficult at this stage to explain these contrasting results, which may be due to differences in experimental protocols, fish strain used, fish background history (photoperiod, temperature) and different environmental conditions. In a recent study, when male European sea bass were kept under continuous light from early stages, plasma melatonin rhythm was lost, while hypothalamus melatonin binding sites (K_d and B_{max}) exhibited seasonal variations, indicating that these variations are controlled by internal circadian and circannual clocks (Bayarri *et al.*, 2010). Limitations with the present *in vivo* results should be acknowledged as one single night-time melatonin assessment is not enough to confirm the presence or absence of an endogenous rhythm. However, if a melatonin rhythm was sustained under DD, significant decrease in melatonin levels should have been seen during the subjective day which was not the case with levels maintained above 30-40 and 50-80 pg/ml, respectively for 10 and 18 °C treatments. While a more acute melatonin assessment was done *in vitro* (every 4 hours during 3 subjective LD cycles), still no endogenous rhythm was apparent. Importantly, sea bass circadian organisation has been shown to differ significantly to other teleost species as, *in vivo*, both the pineal and eyes are needed to sustain melatonin production (Bayarri *et al.*, 2003; Migaud *et al.*, 2007). Furthermore, if an oscillator capable of driving melatonin production in DD conditions exists, which still requires confirmation, its localisation remains unknown (pineal, eye, brain) and should be further studied. This therefore highlights the importance of studying the circadian system as a whole (*in vivo* and *in vitro*).

In conclusion, these results further enhance our knowledge of light perception and circadian rhythmicity in fish, as sea bass exhibited daily variations in plasma melatonin with nocturnal elevations. Temperature affected these nocturnal levels both *in vivo* and *in vitro*, confirming previous work. The combination of both *in vivo* and *in vitro* studies in sea bass for the first time has enhanced our knowledge of the circadian rhythmicity of melatonin production in sea bass. No circadian endogenous melatonin production was observed under DD conditions; however, further work is needed especially for *in vivo* to confirm the presence or absence of an endogenous rhythm. Mechanisms by which environmental signals regulate/entrain seasonal sea bass physiology are relevant to both commercial farming, where various regimes (continuous light, altered temperature regimes...) are used throughout the production cycle to manipulate reproduction, enhance/increase growth, and fisheries as well, especially given the climate change and its potential effects on wild populations (migration, natural range, breeding).

CHAPTER 6
GENERAL DISCUSSION

6. GENERAL DISCUSSION.

6.1. Summary of findings

In this section, the main findings of the thesis are summarised:

- GnRHs mRNA expression throughout an annual cycle in male sea bass did not show clear seasonal profiles with the exception of some peaks for *GnRH1* and *GnRH2* coinciding with the high levels of steroids and GSI, however *GnRH3* showed no significant variations.
- Clear seasonal patterns were observed for the genes encoding pituitary mRNA expression of *lhβ* and *fshβ*, with a significant correlation between both subunits expression and GSI and steroids levels.
- Brain Kisspeptin mRNA expression levels (*kiss1*, *2* and *kissr4*) showed clear seasonal profiles and correlated well to other BPG markers (*GnRHs*, *fshβ* and *lhβ*), supporting a possible involvement of kisspeptin genes in the seasonal control of reproduction in repeat spawning sea bass.
- Kisspeptin can act directly at the pituitary level and modulate *fshβ* and *lhβ* mRNA expression in sea bass although effects were limited and not uniform.
- No effects of the reproductive stage on pituitary response to kisspeptin treatments were observed.
- Diel melatonin rhythm in sea bass was similar to that seen in previous studies in the same species and other teleosts.
- Temperature showed clear effect on the amplitude of the night-time melatonin production under both *in vivo* and *ex vivo* conditions for both long day and short day photoperiods.

- No circadian endogenous melatonin rhythms were found under darkness in sea bass, contrasting with previous reports.

6.2. A note on the fish used in the thesis

The European sea bass is one of the most commercially important species in Europe, with approx. 11933 tonnes from the wild stocks (FAO, 2011) and 113,653 tonnes from aquaculture, mainly from cage farming, harvested in 2009 (FAO, 2011). Sea bass farming is a well advanced industry with knowledge gained and technologies developed on larviculture used as a reference for most other commercial marine species in the world. Sea bass broodstock management under captivity is well established since the 80s and photoperiod regimes have been developed to manipulate spawning windows (Zanuy *et al.*, 1985; Carrillo *et al.*, 1989). Due to its strong seasonality and commercial importance, sea bass has been the object of many studies focusing on the photo-neuroendocrine control of reproduction and growth (Zanuy *et al.*, 2001; Carrillo *et al.*, 2009). However, problems remain in hatcheries, where survival can be low and deformity prevalence high, and during ongrowing stage, where fish reach puberty early especially as sex ratios are usually skewed towards males (>80%) (That mature early) due to sex differentiation being driven by temperature (Carrillo *et al.*, 1995; Piferrer *et al.*, 2005). Interestingly, sea bass natural distribution is spread from the Mediterranean Sea, Black sea and along the Eastern Atlantic coast from Great Britain to Senegal, and it is also becoming more commonplace in Northern parts of Europe (Scotland and also Norway) with established breeding populations (Henderson, 2007). This is thought to be associated to the climate change which resulted in a 0.7 °C rise in the North Sea over the last decade (Hiddink & Hofstede, 2008). However, environmental conditions in

these Northern habitats are different with cooler water temperatures over the year (ranging from 6 to 18 °C) and higher amplitudes in photoperiod (from 21L: 3D in summer to 7L: 18D in winter). The fish used in this thesis were all from the same domestic broodstock produced by Llyn Aquaculture Ltd (Ireland), brought as juveniles and reared in Machrihanish Environmental Research Laboratories (MERL, 55°44'N, 5°44'W, Scotland). Due to the low temperature experienced in the facilities (flow through system with water directly pumped from the sea), sea bass growth performances differed from the classical growth curve seen in the Mediterranean. To illustrate this, stock fish used in this study were aged 6+ for a weight ranging from 500 to >1Kg. It is therefore important to acknowledge that stock used might not be considered as a reference population for sea bass as a species.

6.3. Some comments on the methods used and additional experiments

The main objectives of this PhD thesis were to better understand the molecular and endocrine drivers that control the Brain-Pituitary-Gonadal axis in repeat seasonal spawner sea bass, and expand our knowledge of sea bass light and temperature regulation of melatonin production. For this purpose, a range of laboratory tools were used: radioimmunoassay for hormonal assessment, histology for gonadal staging, cell culture and several molecular techniques including reverse transcriptase polymerase chain reaction (RT-PCR), cloning, quantitative RT-PCR (QPCR). Several bioinformatic tools were used including PrimerSelect, BLAST, ClustalW, genetic and genomic data bases (GenBank in NCBI, ENSEMBL). In addition to sequence alignments, phylogenetic relationships were reconstructed using ClustalW, Bioedit and MEGA. Pituitary cell culture protocol including cell dispersion, cell culture and harvesting were

used to assess the direct effect of kiss1 and kiss2 core peptide on the gonadotropin gene expression. Pineal gland culture protocols have also been used to investigate the circadian rhythm of melatonin production. Besides these techniques and protocols, during the course of experiments, general fish experimental techniques have been used (handling, anaesthesia, blood sampling, PIT-tagging, dissection of targeted organs and general husbandry).

Gene expression analyses have been used extensively in this doctoral project with a total of 9 genes studied throughout an annual cycle in sea bass broodstock as well as in pituitary cell culture. Real-time QPCR was used to analyze mRNA expression as this method is truly quantitative and preferred to the semi-quantitative RT-PCR which relies on the assessment of band intensity in an agarose gel stained with ethidium bromide. While it took more resources it was decided to use internal standards of a cloned sequence to allow absolute quantification of product abundance in preference to relative quantification (i.e. target expression expressed as a ratio to a reference gene) as it was felt the output was more comparable to other work when it is published. Both real-time PCR methods can be performed using different chemicals, the most common being SYBR Green or probe based assays e.g. TaqMan. SYBR green is an easy method that requires a double-stranded DNA dye in the PCR reaction, which binds to the newly synthesized DNA, and detection of fluorescent signal occurs during the PCR cycle. Since the aim was to study the variation in mRNA expression levels of candidate genes for different developmental stages, absolute QPCR was performed using SYBR Green, which is easy to use with low reaction cost compared to other fluorescent probes. One of the key points in the QPCR quantification is the choice of the reference gene (house keeping gene); usually the reference genes are selected due to their uniform expression

across the sample set. In the present studies we designed primers for three housekeeping genes, β actin, 18S and elongation factor 1 alpha (*ef1a*) and tested them by RT-PCR, to confirm that they were suitable to use. However, *ef1a* was selected for normalization as Mitter *et al.* (2009) demonstrated that it is more stable across various stages of development. Had time and resources allowed it would have been preferable to confirm this selection of the housekeeper in the present sample sets by comparing the stability of a suite of potential genes using the geNorm methodology (Vandesompele *et al.*, 2002). Where possible I have tried to comply with the MIQE guidelines for QPCR data (Bustin *et al.*, 2009), with my greatest challenge being the confirmation of RNA quality. As I did not have access to an Agilent Bioanalyzer, RNA quality had to be subjectively assessed by gel electrophoresis however in all cases this indicated ribosomal RNA of good quality thus it is fair to assume my sample integrity was good.

One main objective of this thesis is to investigate the role of kisspeptin in the reproduction of adult seasonal repeated spawner sea bass; *kissr4* partial sequence was first cloned in our laboratory because when the study was initiated there was no sea bass specific sequence information available. However, during the course of the work, sequences of both sea bass *kiss1* and *kiss2* were published (Felip *et al.*, 2009) which helped to give the complete image for the temporal changes in kisspeptin as a whole system. There was however unconfirmed reports of a second receptor form, but as discussed below, this did not become available until after my work was completed.

6.4. Seasonal expression of GnRHs genes during an annual reproductive cycle

Until recently, GnRH was regarded as the uppermost level of the BPG control of reproduction in vertebrates including fish, and it is described as the starting point

corresponding to the onset of puberty and reproduction. However, this changed since the discovery of kisspeptin peptides which have been recently identified as key catalysts for the initiation of puberty and regulator in seasonal breeding in mammals (Revel *et al.*, 2007; Smith & Clarke 2007). Kisspeptin is now considered as the main driver of reproductive events sitting at the top of the BPG axis with a stimulatory role on GnRH signalling, sexual development and successive reproductive events in mammals. This was first demonstrated by the fact that the loss of *Kiss1r* through gene mutation resulted in impaired reproductive function e.g. delay in gonadal growth and decrease in fertility (Funes *et al.*, 2003; de Roux *et al.*, 2003; Seminara *et al.*, 2003). In fish, kisspeptin has received much attention from scientists all over the world in an attempt to unravel the underlying mechanisms controlling the seasonal entrainment of reproduction in commercially important species. However, most of the published literature has so far focused on the characterisation of the kisspeptin system itself and its relationship to GnRH neurons without clear links to other well-known paracrine and autocrine regulatory signals along the BPG axis. One of the most likely reasons is the lack of available molecular and endocrine assays (e.g. GnRH, FSH, LH...) in most fish species. Sea bass, in this regards, is a very good “model” among temperate commercial species as a range of tools has already been developed or sequences are available, thanks to Spanish colleagues (Zamora *et al.*, 2002; Mateos *et al.*, 2003; Felip *et al.*, 2009). One of the main objectives of this PhD was to analyse a suite of known endocrine and molecular messengers at the brain, pituitary and gonadal levels that play key roles in the initiation and completion of gametogenesis throughout a reproductive cycle in male repeat spawning sea bass including GnRHs (*GnRH 1, 2 and 3*), gonadotropins (*fsh β* , *lh β*) gene expression and gonadal development (gonadosomatic index, sex steroid,

histology). It must be acknowledged that while these data are mostly correlative, they bring further support to the potential roles played by kisspeptin in the reproductive axis as part of a complex network of regulatory signals. Importantly, if most studies to date have focused their effort on understanding how puberty is regulated, the present work took a different route by looking at repeat spawners (“broodstock”).

In the present study, gene expression analyses performed on the brain GnRHs confirmed that all three forms of GnRH (*GnRH1*, *GnRH2* and *GnRH3* corresponding to the sea bream, chicken and salmon forms in sea bass) are expressed in the brain of male sea bass. The variation of GnRHs expression between months did not show clear seasonal profiles lacking significant differences between months with the exception of few significant peaks for *GnRH1* and *GnRH2*. Both *GnRH1* and *GnRH2* mRNA expression have peaks coinciding with the high levels of steroids and GSI and most individuals examined were spermiating, however, *GnRH3* showed no significant variations. A correlation with GSI in both *GnRH1* and *GnRH2* implies changes in GnRH signalling with developmental state although such correlation was weak and not significant (borderline, $P=0.065$). Furthermore, *GnRH1* appeared to be more potent as it was correlated to *kissr4* and FSH/LH when multiple regression was done. This is in agreement with the well known hypophysiotropic role of GnRH1 (Gonzalez-Martinez *et al.*, 2002a; 2004a, b; Fornies *et al.*, 2003; Amano *et al.*, 2004; Shajahan *et al.*, 2010a). The *GnRH2* expression variation shed the light on its possible involvement in the reproductive cycle; whereas it has been associated with sexual behaviour (Kauffmann *et al.*, 2005; Millar, 2005), other possible roles for sea bass GnRH2 have been suggested such as modulation of sea bass pineal function (Servili *et al.*, 2010).

Comparing our results of GnRHs expression to previously published data in sea

bass was not straightforward as most previous studies were conducted on pubertal fish or larval stages. Of note, in the present work, QPCR assays to measure the expression levels of the three GnRHs mRNA forms found in sea bass was done for the first time. Other techniques and methods have been used to study the GnRH system in sea bass such as *in situ* hybridization using primers designed on sea bass GnRH associated protein coding sequence (GAP) (Gonzalez-Martinez *et al.*, 2002b), immunohistochemistry using specific antibodies raised against the GAPs, specific competitive enzyme linked immunosorbent assay (EIA) (Moles *et al.*, 2007), and semi quantitative PCR technique to measure *GnRHs* mRNA expression levels (Moles *et al.*, 2007). Crucially, sea bass *GnRH2* and *GnRH3* showed at least two isoforms for each genes resulting from alternative splicing. This is a common feature in many gene families in fish. It is a mechanism by which the coding diversity of the genome can be greatly increased according to the evolutionary strategies of a particular group of teleosts (Lu *et al.*, 2010). It is also one of the most important mechanisms that regulates gene expression in fish (Izquierdo & Vacarcel, 2006) and can translate into the production of truncated and non-functional proteins from a single pre-RNA (nuclear messenger RNA precursors). The lack of clear seasonal profiles in GnRH expression in this study could be due to alternative splicing of the GnRHs as QPCR assays were developed from primers designed on conserved coding regions from available sequences and were not specific to any variants. Thus, future studies should analyze the GnRHs gene structure to identify isoforms and using specifically designed primers, identify the different transcripts of GnRHs in sea bass and their differential expression during the reproductive cycle.

Regarding genes encoding pituitary *lh β* and *fsh β* , a clear rise in the mRNA

expression levels was observed during spermatogenesis and spermiation with a significant correlation between both subunit expression and GSI, as previously reported (Mateos *et al.*, 2003; Miranda *et al.*, 2007; Cerda *et al.*, 2008). It has been suggested that FSH could have a key role at early stage of male gonadal development (Moles *et al.*, 2008) by promoting the synthesis of sex steroids involved in spermatogonial proliferation in male fish (Schulz & Miura, 2002). The change of gonadotrophs expression during the annual reproductive cycle of male sea bass clearly demonstrates their involvement in the BPG axis in repeat spawners.

6.5. Sea bass *kissr4* phylogenetic relationships: presence of two kisspeptin receptors in sea bass

In Chapter 3 we reported on the identification of sea bass specific transcripts for the kisspeptin receptor *kissr4*, while the peptides *kiss1* and *kiss2* genes were available at the time of the study (Felip *et al.*, 2009), this led to the development of quantitative assays to measure their expression for the first time in sea bass. Phylogenetic analyses confirmed the identity of the genes as being *kissr4* with high similarity to sequences found in other teleost species, such as Nile tilapia, grey mullet, cobia, and Atlantic halibut. While only one form has been found in mammals, in some fish species, two distinct subtypes of kisspeptin receptor have been reported (*Kissr2* and *Kissr4* paralogs), although *kissr4* appeared to be the most predominant and functionally active form (Akazome *et al.*, 2010). A recent *in situ* hybridization study using specific probes revealed that two kisspeptin receptors are expressed in sea bass brain (Escobar *et al.*, 2010). By the end of the present study, the whole genome of sea bass became available showing 2 paralogs for sea bass kisspeptin receptors (*Dicentrarchus labrax* Whole

Genome Shotgun sequencing project, 2010). The sequence isolated in the present study is 100 % identical to one of them.

6.6. Expression of kisspeptin genes during the annual reproductive cycle: implications for the BPG axis and control of reproduction in fish

While most studies have focused to date on the role played by kisspeptins during first reproduction (puberty), few others were done at different developmental stages including mature fish. Gene expression studies of the kisspeptin system in fish supported its involvement in the regulation of puberty. For example *kissr4* levels showed significant differences in the brain between different pubertal stages as in grey mullet (Nocillado *et al.*, 2007), cobia (Mohamed *et al.*, 2007). In Chapter 3, results revealed a clear seasonality in *kissr4* expression levels. These findings are supported by the differences in expression levels of brain *Kissr4* between the gonadal developmental stages as shown in male fathead minnow (Filby *et al.*, 2008) and in male and female grass puffer (Shahjahan *et al.*, 2010b). For the kisspeptin ligand, both *kiss1* and *kiss2* also displayed seasonal expression patterns with significant increases towards the end of the annual reproductive cycle (late spermatogenesis to spermiation). To date, contrasting results in *kiss1* and *kiss2* expression patterns during the gonadal development have been reported according to the studied species. Similar results to the present findings were found in grass puffer in which brain *kiss2* expression peaked during spawning in both males and females (Shahjahan *et al.*, 2010b), and *kiss1* and *kiss2* mRNA levels in zebrafish brain (Kitahashi *et al.*, 2009). In contrast, in chub mackerel, the highest expression levels were observed in pre-pubertal fish (Selvaraj *et al.*, 2010). The discrepancies found between studies for different species are probably

due to the different kisspeptin paralagous genes detected in the last few years (Akazome *et al.*, 2010), which were unknown at the time when other studies were carried out. Furthermore, it could be due to the presence of a second isoform still undetected in some species. A third reason may relate to the precise definition of the developmental stage in each sex and species. For example, some studies referred to immature fish without precisely explaining whether the stage corresponds to juvenile immature or adult regressed fish. In order to avoid confusion in future researches, the following recommendations are suggested: 1) sexual maturation stage for each individual to be precisely determined using histological classification, 2) gene structure studies to be conducted and paralog genes identified using phylogenetic and synteny analyses, 3) alternative splice variants to be identified and analysed, 4) localisation studies to be performed for genes and proteins in central (different brain areas or nuclei if possible) and peripheral tissues including different stages of sexual maturation throughout the seasonal reproductive cycle.

Recent studies using *in situ* hybridization helped to localise the expression of kisspeptins in fish and speculate on their modes of action (Kanda *et al.*, 2008; Lee *et al.*, 2009; Servili *et al.*, 2011). Adding to the clear seasonal profile of kisspeptin system in sea bass, the positive correlation between kisspeptin genes expression and *GnRH1*, *GnRH2*, GSI and the expression levels of pituitary gonadotropins is in agreement with the suggestion that kisspeptin system is involved in the regulation of sea bass reproductive axis. The *GnRH1* correlation with *kissr4* is strongly supported in the recent *in situ* hybridization studies of sea bass brain in which GnRH1 expressing neurons are co-expressed with *kiss2r* (*kissr4* in the present study) messengers (Escobar *et al.*, 2010).

Kisspeptin research in teleost fish has mainly focused on reproduction and in particular the control of puberty, it is possible that kisspeptin can regulate other physiological functions as suggested in mammals (Oakley *et al.*, 2009). A recent study in zebrafish showed that *kiss2* is involved in the control of reproduction, while *kiss1* is probably implicated in the perception of environmental and metabolic signals (Servili *et al.*, 2011). Future studies are needed to unravel the other possible functions of the kisspeptin system in teleosts, kisspeptin could be involved in sex determination or differentiation. Molecular assays developed for kisspeptin can be used as tools to measure maturity status and assess population reproduction status and dynamics of population growth in wild stocks. The field of reproductive endocrinology is moving at a fast pace and many new exciting findings will soon become available involving other upstream signal peptides still poorly described in vertebrates.

6.7. Direct stimulatory actions of kisspeptin peptides on pituitary FSH and LH gene expression

In order to test the direct effect of kisspeptin on the reproductive axis, the specific core sequences of both sea bass *kiss1* and *kiss2* were applied directly on cultured pituitary cells. For this purpose, cell culture techniques were done in the virology laboratory (IoA, Stirling University), with the technical support from Mrs Fiona Muir (Chief technician of virology lab). Results showed that kisspeptin can act directly at the pituitary level and modulate *fsh β* and *lh β* mRNA expression in sea bass (chapter 4), although effects were limited and not uniform. The present findings support suggestions made in mammals according to which kisspeptin could act directly on the pituitary to regulate gonadotropin synthesis. Kisspeptin administration *in vivo* was shown to be

potent in other studied fish, including sea bass (Felip *et al.*, 2009), medaka and zebrafish (Kithashi *et al.*, 2009), as well as goldfish (Yang *et al.*, 2010). The present study relied on the analyses of mRNA expression levels by QPCR. One limitation to this study is the fact that released LH and FSH in the culture media could not be assessed due to the lack of available commercial antibodies for ELISA analyses. Such antibodies have been developed by the past and published (Moles *et al.*, 2007; Moles *et al.*, 2008). Absence of tools to measure key endocrine factors represents a limiting factor in this type of study in fish. In pituitary cells of goldfish, the response to kisspeptin administration was greater for the LH release than for *lh β* mRNA expression, whereas expression levels increased only in response to the highest dose of kisspeptin after 24 hours of incubation (Yang *et al.*, 2010). Kisspeptin treatment on pituitary cells was also shown to stimulate growth hormone (GH) and prolactin (PRL) release and mRNA expression as well (Yang *et al.*, 2010). Further studies are needed to confirm our findings in sea bass and other species and better understand the variability seen. Kisspeptin receptor expression analyses together with LH/FSH medium analyses, not studied in this experiment, could prove to be very informative. In addition, studies should look at kisspeptin effects on other hormonal output of the pituitary such as growth hormone, somatolactin or prolactin.

6.8. Additional work done on the use of kisspeptin as a hormonal therapy for fish spawning

One of the important applications for aquaculture is to synchronize natural spawning and induce spawning out of season. Reproduction in fish kept in captivity can be controlled by environmental manipulations, however it is not always effective and,

in these instances, the use of hormonal therapies has been effective as a means to induce final oocyte maturation, ovulation and spawning. A lack of pituitary LH secretion and stimulation is the main reason for reproductive failure under culture conditions (Mylonas *et al.*, 2010). Therefore, hormonal therapies have been developed using exogenous LH that can act directly at the gonadal level or more recently using Gonadotropin Releasing Hormone analogues (GnRHa) that stimulate the endogenous production of pituitary LH (Mylonas *et al.*, 2010).

Another experimental approach, although data are not presented in this thesis, was to test the direct effect of kisspeptin on the induction of ovulation/spawning in fish. The concept of this study was that if kisspeptin operates as shown in mammals (master regulator of puberty directly stimulating the GnRH system involved in the onset of maturation) then it could be used as a more potent hormonal therapeutant to improve broodstock spawning and gamete quality in fish. To do so, African catfish, available at the IoA, was selected instead of sea bass for scientific reasons. Female catfish, once puberty has been reached, remain all year long at a postvitellogenic gonadal stage under constant environmental culture conditions and do not spawn spontaneously without being induced hormonally (Richter *et al.*, 1987; Bromage & Roberts, 1995). These fish can be hormonally induced and spawn at any time of the year which makes African catfish an ideal candidate to test the potency of alternative hormonal preparations such as kisspeptin. The standard method to induce African catfish ovulation is to use GnRHs together with a dopamine antagonist administered by injection (Baidya & Senoo, 2002). A commercial preparation, Ovaprim (Syndel, Vancouver, Canada, containing 20 mg salmon GnRHa and 10 mg domperidon per ml), has been shown to be very efficacious at a concentration of 0.5 ml/kg body weight. Importantly, catfish responds to the

hormonal induction within 24 hours with eggs being stripped and hatching occurs 24 hrs later. Catfish males and females were obtained from the Tropical Aquarium facilities, Institute of Aquaculture, University of Stirling. Two trials were carried out using the 10 amino acids core sequence of kisspeptin. The first trial consisted in testing a single dose of a mix of 4 kisspeptin forms (mammalian Kiss1, zebrafish kiss1, sea bass kiss1 and sea bass kiss2). Eight fish (250-300g, 4 males and 4 females) were used for each treatment, and fish were injected with either: a) saline + dopamine antagonist (domperidone) as control, b) Ovaprim (0.5 ml/kg), c) Kisspeptin mix (containing 250ng/100g fish body weight of each kisspeptin forms) + domperidone. Before injection blood samples were taken from all fish. In the second day, 18 hours following the injection, gonadal samples as well as blood from both males and females were collected. The second trial was similar but in this case two successive injections (24 hours apart) were tested. Gonadal samples as well as blood from both males and females were collected. Plasma samples were analysed for sex steroids (testosterone and estradiol). Histological sections were done on both male and female gonads, and female oocyte diameters were determined. In both experiments kisspeptin injection failed to induce spawning of catfish, no significant differences were observed in the testosterone, estradiol and oocyte diameter between injected and control fish, while estradiol and oocyte diameter were significantly increased in fish treated with ovaprim (positive control). In this trial, the administration of kisspeptin did not show effect on spawning induction, however, lacking of the methods to analyse the plasma LH after injection is a limitation in order to investigate the effects of kisspeptin injection on the pituitary level with the measured gonadal steroids, also kisspeptin core sequences are different between species and might be species-specific in their stimulatory effects.

6.9. Environmental signalling through circadian melatonin production in sea bass: temperature and photoperiodic effects

Sea bass, as with many other temperate fish species, display strong seasonality in its physiology and behaviour and is therefore an excellent candidate for the study of calendar mechanisms. As such, the circadian melatonin system is thought to play a key role in the entrainment and synchronisation of most physiological functions including reproduction, migration and feeding behaviour. Both photoperiod and temperature seasonal signals are known to regulate melatonin production by the pineal gland in sea bass. The present work aimed to study the synergistic effects of both key environmental factors on the daily phase and amplitudinal changes in melatonin production through both *in vivo* and *in vitro* studies. The results first confirmed the low at day and high at night melatonin profile, as seen in most teleost species studied to date. Then, a clear effect of temperature on daily melatonin amplitude between day and night was reported with enhanced melatonin production at higher temperatures. In many of the investigated fish species, photoperiod clearly controls the duration of melatonin production while temperatures can dictate the amplitude (Falcon, 1999). Furthermore, there is an optimum temperature for melatonin biosynthesis and it is species-dependent. The mechanisms by which temperature acts on melatonin secretion are not fully understood. However, it has been suggested that temperature effect would be mediated through the regulation of AANAT2 enzymatic activity in a species-dependent manner (Falcon, 1999).

As fish are ectotherms, they are directly influenced by the external temperature, by acting directly on their metabolism, physiology and behavior. Temperature varies on daily and seasonal bases as in the case of photoperiod. Integration of these seasonal and

daily variations is an essential factor in the adaptation of organisms to the natural fluctuations of their environment. In the last few decades global temperature has increased by 0.5 to 3.5 °C, depending on the geographic area (Hiddink & Hofstede, 2008), and therefore fish have to adapt to this change. The impact of recent increases in temperature includes shifting of natural distribution areas and migration patterns. Sea bass, as for other warm-water fish species (e.g. red mullet and tuna), is now found in Northern parts of Europe (Scotland and also Norway) with established breeding populations (Henderson, 2007). This is may be due to climate change, which resulted in a 0.7 °C rise in the North Sea over the last decade (Hiddink & Hofstede, 2008). The initiation of distribution area displacement towards colder regions is an alternative way to compensate for temperature changes; however this will induce other changes like photoperiod or ecosystems, thus adaptation to the diel and annual changes for the new environment will be required. Furthermore, in sea bass sex ratio is temperature-dependent. When larval and juvenile sea bass are reared at 19-22 °C instead of the more typical spawning temperature (~ 14 °C), it can result in a high male sex ratio (as high as 90 %) due to the temperature-sensitive sex determination mechanism present in the species (Piferrer *et al.*, 2005), which affects the sea bass aquaculture industry. It is clear that temperature changes are involved in the mechanisms controlling physiology and behaviour of fish, however, more studies are needed to understand the effects of environmental factors on these mechanisms.

In the present study, we confirmed the low at day, high at night melatonin levels in sea bass which is also found in all teleost and vertebrates studied to date. This is in accordance with the role of melatonin as a “Zeitgeber” in entraining the physiology of animal to seasonal day length changes. However, unlike mammals, the mechanisms by

which melatonin controls reproduction, growth and behaviour (locomotor, feeding, shoaling, and migration) in fish and birds remain unclear (Falcon *et al.*, 2007). This mechanism is thought to be controlled in mammals directly by light at the transcriptional level through specialized light regulated promoter regions in the AANAT gene. The daily and annual rhythms are also driven by internal clock mechanisms that free-run with a period close to 24h (circadian) or a year (circannual) when exposed to constant conditions, while melatonin circadian rhythms are clearly entrained by environmental conditions, they can also be self-sustained under the control of circadian clocks, through the existence of intrapineal oscillators (Migaud *et al.*, 2010). Contrasting to the previous findings, sea bass did not show any endogenous melatonin production under DD in both *in vivo* and *in vitro* conditions. Further confirmation for the presence of an oscillator capable of driving melatonin production in DD conditions is needed. Importantly, sea bass circadian organisation belongs to the fish group in which both the pineal and eyes are needed to sustain melatonin production (Bayarri *et al.*, 2003; Migaud *et al.*, 2007). More studies are required to define the circadian system in sea bass and its localization (in the brain, eye or pineal gland?). Due to the importance of AANAT activity, future studies are needed to quantify sea bass AANAT protein and *aanat* gene expression as well as its localisation. The understanding of mechanisms by which environmental signals regulate/entrain seasonal sea bass physiology will help the commercial farming where photoperiodic regimes are used throughout the production cycle to manipulate reproduction, enhance/increase growth and enable all year long supplies of seed (eggs/fry). It can also help to better understand/predict the effects of climate change on wild populations. It was also our intention in this PhD project to investigate the direct effect of different photoperiodic

regimes on the spawning time of sea bass broodstock. To do so, three different photoperiodic regimes were applied to adult males and females. However, due to a technical failure in the rearing system leading to fish mortalities, the experiment could not be completed.

6.10. Importance of the environmental/neuroendocrine control of reproduction for fish aquaculture

Research in reproductive physiology/endocrinology has direct implications for aquaculture where farmers continuously search for new means of improving fish growth, producing all year long product of constant high quality and ensuring fish welfare. Applied research has mainly focused on synchronizing the natural spawning and inducing spawning out-of-season through the use of hormonal manipulations (Mylonas *et al.*, 2010) and photoperiodic regimes (Carrillo *et al.*, 1993; Zanuy *et al.*, 1995), controlling the timing of puberty (Carrillo *et al.*, 2009; Taranger *et al.*, 2010) and sex ratio (Piferrer *et al.*, 2005) of populations to promote growth and reduce production time using a range of environmental and genetic techniques and optimising the hatchery phases. To date, a significant part of the scientific knowledge gained over the last decade or so remains to be implemented in farming conditions and problems remain to be addressed in commercial settings related to reproduction, especially. Photoperiod is one of the most common tools used to control reproduction and puberty. Photoperiod manipulations have been used to control sea bass spawning time (Carrillo *et al.*, 1989; Carrillo *et al.*, 1993; Zanuy *et al.*, 1995) or control puberty and growth (Begtashi *et al.*, 2004, Felip *et al.*, 2008). The characterisation of kisspeptin as an assay for puberty could prove to be essential in defining windows of decision (onset of puberty) and

optimising the timing of application which will significantly improve profitability of the industry. In some cases the use of exogenous hormone is an effective way to induce reproductive maturation and produce fertilized eggs (Mylonas *et al.*, 2010). Kisspeptin administration can be an efficient tool in inducing spawning, as it can elicit the plasma LH as observed in previous studies on species including sea bass (Felip *et al.*, 2009), medaka and zebrafish (Kitahashi *et al.*, 2009). However, hormone administration in fish aquaculture is labour intensive, time consuming and therefore costly, and the control of spawning under culture conditions is not standardized. Therefore, further work is important to study environmental regulations of the neuroendocrine axis to develop protocols for the aquaculture industry.

6.11. Conclusions and future work

Although the present findings clearly support previous suggestions according to which kisspeptin would play a key role in the neuroendocrine regulation of reproduction by the BPG axis in fish, the exact mode of action at the brain and pituitary levels remains unclear. The work carried out in this thesis provided correlative evidence supporting such a role. Further studies should now focus on dissecting the pathway by which kisspeptin acts on GnRH neurons and pituitary cells. Importantly, the mechanisms by which environmental signals regulate the BPG axis remain unknown and if melatonin is clearly involved in transducing environmental changes to the brain and tissues, direct evidence and mode of action of such an important endocrine signal still have to be ascertained. It has been hypothesised that kisspeptin could act as the missing link between environmental stimuli and GnRH stimulation in mammals and also in fish and this warrants further studies on this interesting research avenue. Further experiments on

the interplay between melatonin and kisspeptin would be of great interest. Circulating melatonin (and kisspeptin) levels could be manipulated *in vivo* through injection, the use of melatonin antagonists or surgery (e.g. pinealectomy), photoperiodic regimes and *in vitro* assays, and their effects on kisspeptin, GnRH and reproduction analysed. Also, localisation of kisspeptin and melatonin expressing cells in the brain as well as neural projections would help to better define mechanisms at work. Importantly, both melatonin and kisspeptin might have other important functions than regulating seasonal reproduction and these should be investigated. Furthermore, given the large diversity in physiology (growth, reproduction), organisation (circadian systems, light perception), life strategies (puberty, reproduction feeding, migration) and natural distribution seen in fish, comparative physiology, endocrinology and molecular phylogenetic studies would be of great interest to unravel the roots of seasonal mechanisms shaped through millions of year of evolution. A better understanding of the control of reproduction has also direct implications in commercially important fish species for aquaculture as well as wild fish stocks that are under increased pressure from fishery activities and a changing environment (climate change). New knowledge would allow designing regimes and protocols that can be used to manipulate sea bass physiology (and other aquaculture species) in a farming context (optimised photoperiod/temperature regimes to enhance growth, suppress puberty or produce eggs out of season; new hormonal preparations to stimulate fish spawning). In addition, new tools developed along the BPG axis could be used to improve wild stock modelling based until now on gross physiological observations and better understand/predict how fisheries and climate change impact on fish recruitment, migration and overall population dynamics.

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