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Rhythms of anorexigenic neuropeptides in Senegalese sole, *Solea senegalensis*: effects of light, feeding regimes and development stage.

Tesi di laurea in Adattamenti degli animali all'ambiente marino

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1 INTRODUCTION

1.1 Biological rhythms in living organisms

1.1.1 A historical overview

The term “Chronobiology” comes from the ancient Greek χρόνος (chrónos, meaning "time"), and biology. It is a field of biology that examines the timing of rhythmic phenomena in living organisms and their synchronization to geophysical cycles.

The observation of rhythmic events in living species comes first from description on leaf movement, which has been probably known over 2300 years ago (Engelmann, 2002). Daily periodic movements of the leaves of the tamarind tree, *Tamarindus indicus,* were initially and clearly reported by Androstene of Thasos, admiral under Alexander the Great, in the island of Tylos (now Bahrain) in the Persian Gulf, 400 years before Christ (Bretzl, 1903). Much later, in 1729, the French astronomer Jean-Jacques d’Ortous de Mairan observed the daily opening and closing of the leaves of a sensitive heliotrope plant, the *Mimosa pudica*. Intrigued by this comportment, he performed an experiment to test whether this biological “behaviour” was endogenous or simply a response to the daily appearance of the sun. In order to confirm his hypothesis, he confined the plant in darkness and he observed that the daily rhythmic opening and closing of the leaves persisted even in the absence of sunlight (Gardner et al., 2006). Even if de Mairan did not concluded that *Mimosa pudica* has an internal clock, his experiment was the first scientific demonstration of the existence of circadian rhythms in plants.

In 1751, the Swedish botanist and naturalist Carolus Linnaeus (Carl von Linné) designed a *Horologium Florae* (flower clock) using certain species of flowering plants (Figure 1.1). By arranging the selected species in a circular pattern, he designed a clock that indicated the time of the day when the flowers opened (C. Linneus, 1751).

Figure 1.1. Linneus’s *Horologium florae.*

It took 30 years before further scientific investigations on this biological mechanism in plants followed de Mairan's observations. Indeed, independent research conducted by Duhamel Du Monceau and Zinn in 1759 showed that the rhythm of leaf movement in constant darkness was independent of fluctuations in environmental temperature (Robertson McClung, 2006). This means that this "clock" mechanism appeared to be endogenous.
In 1832, De Candolle gave more details on the period of this inner clock, showing that in constant light conditions, opening and closing rhythms of the leaflets of the sensitive plant *Mimosa pudica* was maintained with a period close to, but not exactly, 24 h. To make use of circadian terminology, this signifies that the endogenous period of Mimosa's clocks runs free with its own timing. De Candolle also showed that the rhythms could be inverted by manipulating the light-dark cycle. Later on, Darwin tried to understand the selective advantage of the periodic leaves movements (Bunning and Moser, 1969) and published an entire book about the Movement of Plants, arguing that the plant itself generates the daily rhythms.
In the early 1930s, Erwin Bünning, a great biologist and botanist, demonstrated the inheritance of circadian rhythms. In 1932, he started to cross bean plants with different endogenous periods, demonstrating that the next generation had periods of intermediate duration (Bünning, 1967), and thus refuting the idea that daily rhythmicity is learned, as first argued earlier Richard Semon in 1904. In 1935 he determined in plants the genetic origin of the "biological clock". Bünning also laid out the basis of photoperiodism, suggesting that plants display endogenous phases of light and dark sensitivity, important in the photoperiod response of flowering (Koukkari and Sothern, 2006).
In the 1950s, Colin Pittendrigh elaborated careful descriptions of the properties of circadian clock in *Drosophila* and other species, providing the first formal models of how circadian rhythms synchronize to local light-dark cycles (Pittendrigh, 1960). His contributions to this field were so relevant that he is considered as the founder of modern Chronobiology. In 1960, Jürgen Ashoff focused on understanding the properties of circadian rhythms and how these rhythms can change in response to stimuli specifically in diurnal and nocturnal species (Ashoff, 1960).
In recent years, considerable efforts have been done to increase knowledge in this area of science. Since the emergence of life on earth, our planet has been subjected to daily and annual rhythm of light and darkness, and also to seasonal cycles of climate changes, caused by the rotation of the Earth around its axis and around the sun. Therefore, this alternation of day and night has had deep influences in the life of the leaving organisms. Plants and animals measure time by means of oscillations in order to perform a given physiological process. The oscillations underlying life functions are now known to provide a temporal organization for physiological and behavioural activities in practically every group of organisms (Kulczykowska et al., 2010). Evolution has

favoured the development of **internal mechanisms** of measurement of time or biological clocks that may allow anticipation of these environmental cyclic changes, optimizing the biochemical and physiological processes. These internal cycles in living organisms are known as **biological rhythms**.

1.1.2 Types, properties and parameters of biological rhythms

Biochemical, physiological and behavioural parameters exhibited by organisms show periodical fluctuations and most of these rhythms persist in constant conditions, thus demonstrating that they arise within organism itself and are not imposed by the environment (Kulczykowska et al., 2010).
Biological rhythms are often classified by their period, which is the interval for completion of one cycle. Using this criterion, we can distinguish:

- **Circadian rhythms**: rhythms that persist in constant conditions with periods close to 24 h (Menaker et al., 1997). Circadian rhythms are a major characteristic of living organisms and represent one of the most evident manifestations of adaptation to the environment (Aschoff et al., 1960; Pittendrigh et al., 1960; Pittendrigh et al., 1976; Hastings et al., 1991). The term *circadian*, which was coined by Franz Halberg, comes from the Latin *circa*, meaning "around" and *dies*, "day", meaning "approximately a day". The circadian rhythm can further be broken down into routine cycles during the 24-hour day: **diurnal**, which describes organisms active during daytime; **nocturnal**, which describes organisms active in the night; **crepuscular**, which describes animals primarily active during the dawn and dusk hours.
- **Infradian rhythms**: rhythms that have periods longer than a day (>28-hour), such as the annual migration or reproductive rhythms found in certain animals or the human menstrual cycle.
- **Ultradian rhythms**: rhythms that exhibit cycles shorter than 24 hours (<20-hour), such as the 90-minute REM cycle, or the 3-hour cycle of growth hormone production (Butler et al., 2005).

Circadian rhythms have some basic features: they are widespread in nature, evolutionarily conserved and present in all eukaryotic and some prokaryotic organisms; they are generated by a self-sustained endogenous pacemaker, they are temperature-compensated and, as mentioned before, they are genetically determined. In vertebrates, circadian rhythms are generated and regulated by a circadian timing system, which consists of pacemakers and oscillators, entrainment pathways and pacemaker outputs to effector systems that express overt rhythms under circadian control (Guido et al., 2002).
By definition, in constant conditions and in the absence of environmental cues, circadian rhythms "*free-run*" presenting a period of around, but not exactly 24 hours; thus, the periods of the

endogenous rhythms actually differ from those in the nature, but exogenous synchronizing cues, commonly called ***zeitgeber*** ("timegiver" in German), adjust the biological rhythms, so that the organisms remain in phase with their surroundings (Kulczykowska et al., 2010). These external stimuli able to entrain circadian rhythms include environmental time cues such as light, temperature and other parameters such as food, noise, or social interaction, between others (Sánchez-Vázquez et al., 1995; Aranda et al., 2001; Reebs, 2002; Herrero et al., 2005). Zeitgebers help to reset the biological clock to a 24-hour day. Because its stability in period and phase, the most consistent environmental signal is the 24 hours **light-dark cycle**. As a result, almost all circadian rhythms of the species studied up to date are synchronized in a daily manner by this cue.

Circadian rhythms can be graphically represented in **cronograms,** where a sine wave can be fitted (Figure 1.2). Within each cycle, the **phase** is a particular value of the rhythm in the cycle and the time period at which the cycle peaks is called the **acrophase.** When the process is less active, the cycle is in its **bathyphase** or **trough phase**. The highest value of the rhythmic biological variable is the **peak** or **maximum** and the lowest value is the **nadir.** The difference between the peak and the mean value of a wave is measured by the **amplitude** and the **period** of the rhythm is the time between two points in the same phase (close to 24 h in the case of circadian rhythms).

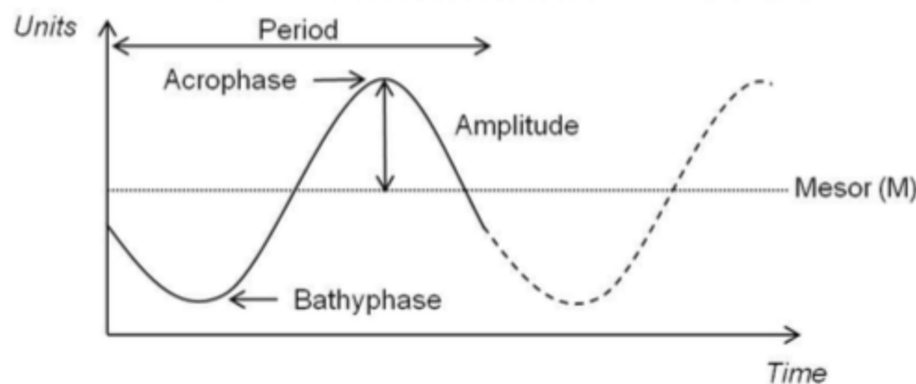

Figure 1.2. Diagram of an oscillatory process characterized by its 4 parameters.

1.1.3 Circadian organization in vertebrates

Circadian organization means the way in which the entire circadian system above the cellular level is put together physically and the principles and rules that determine the interactions among its component parts which produce overt rhythms of physiology and behaviour. All circadian systems are composed of three basic elements (Figure 1.3), an oscillator or **clock** that generates and sustains the endogenous rhythmic oscillations, an **input** that allows the synchronization of the oscillator, and an **output** by which the pacemaker regulates many physiological and behavioural processes (Guido

et al., 2002).

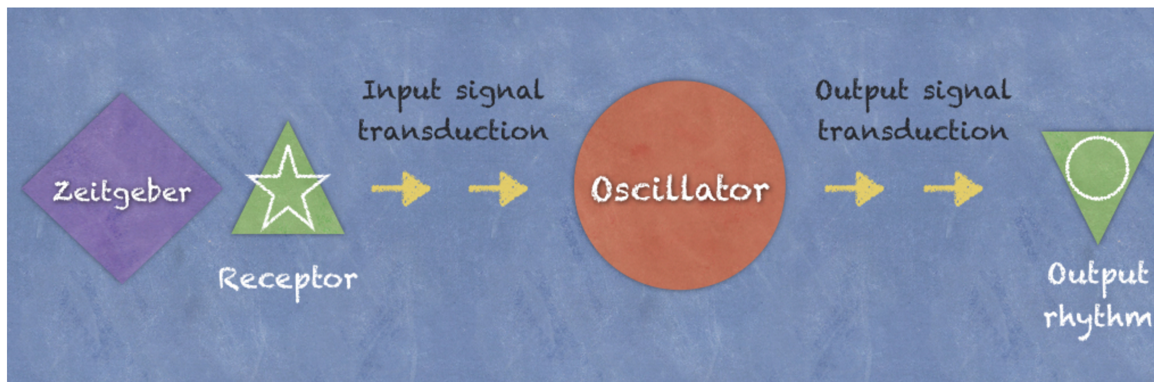

Figure 1.3. Conceptual model of the circadian system.

In vertebrates, three neural structures have been reported as oscillators or clocks.
These structures are the **retina**, the **pineal complex** and the mammalian **suprachiasmatic nucleus** (SCN) of the hypothalamus or its analogous areas in other groups (Menaker et al., 2997). Nevertheless, independent or semi-independent, light-entrainable circadian clocks appear to exist at all levels of organization from cells, through tissues to organs. Among animals, there is usually a master or **central clock**, which is located in the brain. Clocks located in other parts of the body are defined **peripheral oscillators**, in order to distinguish them from the central clock.
In mammals, entrainment of circadian rhythms depends upon photic information provided exclusively by the lateral eyes through the retina (Bertolucci et al., 2004). This photic information reaches the central oscillator localized in the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract, synchronizing SCN daily rhythmic neuronal activity (Doyle et al., 2007; Maywood et al., 2007). In turn, signals from the SCN regulate the activity of many other targets, including melatonin synthesis in the pineal gland (Korf et al., 2003), which is considered to be a peripheral oscillator (Vatine et al., 2011). In this system, oscillation signals produced by the SCN are relayed to the pineal gland via the multisynaptic efferent pathway (Falcon, 1999; Takahashi, 1994).
In non-mammalian vertebrates, the pineal complex contains all elements required for photic entrainment and circadian rhythm generation, as it is photoreceptive and contains an intrinsic circadian oscillator (Korf et al., 1998; Falcon et al., 2003). Fish pineal cells are classical photoreceptor cells with structural and functional similarities to retinal photoreceptors. Pineal and retinal photoreceptor cells share a similar set of genes, or, in certain cases, paralogues (Falcon et al., 2003). The fish pineal contains an intrinsic circadian clock that drives rhythmic synthesis of the hormone melatonin, an indoleamine with well-known effect on internal biological rhythms. Melatonin levels are high at night and low during the day as a result of regulated transcription and

stability of serotonin-N-acetyl-transferase (AANAT), the key enzyme for melatonin synthesis. Teleosts have two *aanat* genes, *aanat1* that is expressed predominantly in the retina, and *aanat2* that is expressed in the pineal gland and to a limited extent in the retina (Falcon et al., 2003; Gothilf et al., 1999). Activity of this enzyme is dictated by the circadian clock and also shows a rapid suppression in response to illumination during the night (Ziv et al., 2007; Applebaum et al., 2006). Thus, the pineal gland is considered to serve as central pacemaker, as transduce environmental light information into a neural and a neuroendocrine signal.

1.1.4 Molecular bases of circadian rhythms

The functional mechanism that all organisms on earth use for adapting their behavioural and physiological functions to daily and seasonal variation in environmental factors is built around internal biological clocks (Pevét, 2001). This time-measuring system has a number of key-molecular components necessary for normal circadian function in which are included **clock genes** and cycling proteins. The basic clock mechanism is a feedback loop in which oscillating products of specific clock genes regulate their own expression. Each complete turn of the loop takes about 24 hours to accomplish, resulting in circadian oscillations of RNA and protein levels (Nanako et al., 2012). .

There are many fundamental similarities between the well-studied *Drosophila* circadian clock and the vertebrate clock mechanism (Peschel et al., 2011; Ripperger et al., 2011). At the core of both clocks there is a transcription-translation feedback loop that cycles in a period of around 24 hours (Albrecht et al., 2004; Reppert et al., 2002). In the case of vertebrates this regulatory loop consists of positive elements (*clock* and *bmal*) that drive the expression of negative elements (*period* or *per* and *cryptochrome* or *cry*) that, in turn, feedback to down-regulate their own expression and allow the start of a new cycle of the feedback loop. The transcription activators *clock* and *bmal* bind as heterodimers to E-box elements located in the promoters of *per* and *cry* genes and thereby induce their transcription. After translation, dimerization and translocation to the nucleus, the PER and CRY proteins inhibit transcription of positive elements by physically interacting with the CLOCK:BMAL complex (Figure 1.4). Furthermore, the existence of an additional feedback loop that directs the rhythmic expression of *bmal* tends to confer robustness and stability on the core loop (Emery et al., 2004).

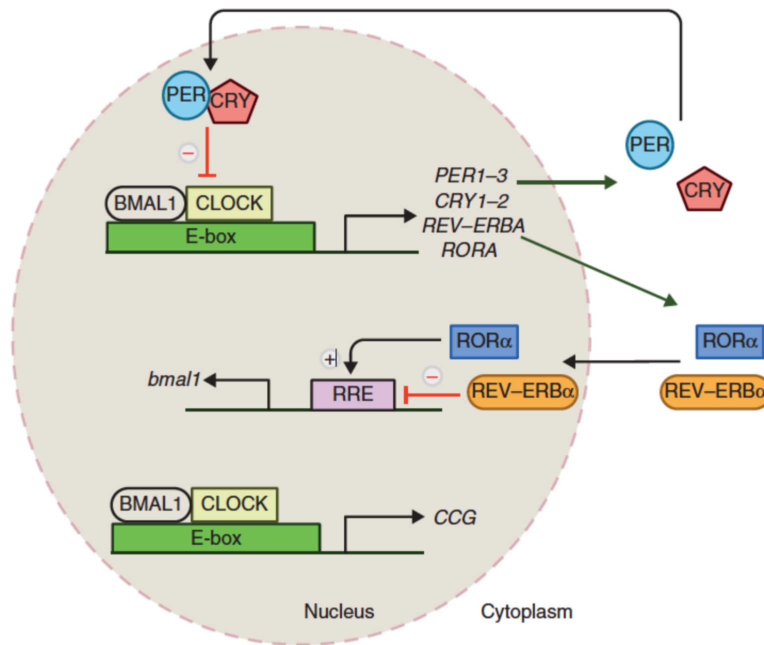

Figure 1.4. Model of the circadian molecular clock molecular mechanism in mammals (Bailey et al., 2014).

In fish, a genome duplication event that occurred during the evolution of the teleost lineage has lead to the presence of extra copies for many clock genes compared to mammals (Postlethwait et al., 1998). In some cases, duplicated gene copies have subsequently been lost during evolution but in many cases, the extra copies do persist. In zebrafish, six *cry* genes (*cry1a*, *cry1b*, *cry2a*, *cry2b*, *cry3* and *cry4*) (Kobayashi et al., 2000), three *Clock* genes (*clock 1a, clock1b* and *clock2*) and 3 *bmal* genes (*bmal1a, bmal1b* and *bmal2*) have been identified, showing complex pair-wise interactions in various heterodimeric combinations (Vatine et al., 2011). With the *per* genes, the situation is also complex, with two *per1* homologs (*per1a* and *per1b*) together with single *per2* and *per3* genes that exhibit different spatial expression patterns (Wang et al., 2009). Furthermore, while *per2* is a light-driven gene, the remaining *per* genes are predominantly clock regulated. *Per2* in particular has been shown to function in combination with *cry1a* as an element of the light input pathway as well as playing an important role in the early development of the pineal clock during embryogenesis (Tamai et al., 2007; Hirayama et al., 2007; Ziv et al., 2006; Ziv et al., 2005).

Recently, this clock machinery has been studied in other fish species such as the goldfish (*Carassius auratus*), the rainbow trout (*Oncorhynchus mykiss*), the Atlantic salmon (*Salmo salar*), the European sea bass (*Dicentrarchus labrax*), medaka (*Oryzias latipes*), the gilthead sea bream (*Sparus aurata*), the Senegalese sole (*Solea senegalensis*) and some reef fishes (*Siganus guttatus* and *Halichoeres trimaculatus*) (Davie et al., 2009, 2011; Del Pozo et al., 2012; Hur et al., 2012;

Martín-Robles et al., 2011, 2012b; Park et al., 2007; Patiño et al., 2011; Sánchez et al., 2010; Velarde et al., 2009; Vera et al., 2013). Such studies has allowed identifying genes involved in many aspects of the circadian clock system, but much more work is needed in order to understand all aspects of temporal organization within each organism.

1.2 Feeding rhythms

In the early 20th century, it was already known from von Buttel-Reepen that honeybees were able to learn the time of day when flowers secrete nectar (mentioned in Moore 2001). In 1929, lngeborg Beling, a student of the famous ethologist Karl von Frish, published the first study on the time-sense of bees. By training bees to collect a sugar solution at specific location and during specific periods of time on several consecutive days, she observed that bees continued to return to the sugar source with greatest frequency at 24 hours- intervals even if sugar was no longer present, demonstrating their ability to associate the time of the day with the presentation of food reward. She also observed that food presented at 19 or 48 hours- intervals did not train the bees. Beling and her colleague Otto Wahl demonstrated a few years later that bees shielded from external factors such as light, temperature and humidity could still be trained to collect nectar and pollen at different times of day (mentioned in Moore and Rankin 1983; Moore 2001).

Max Renner gave evidence about the presence of an endogenous biological clock entrained by feeding time a few decades later. He trained bees in his laboratory in France to collect sugar water between 8:15 p.m. and 10:15 p.m., and then he transported the bees overnight to New York, where they were placed in similar laboratory conditions. He observed that the following day of departure, bees arrived at the feeding table at the same previous French fixed time, despite having been displaced (Renner 1960). In the early 1920s, Curt Richter would have first observed a phenomenon of circadian food anticipation in rats (mentioned in Davidson 2006).

First research on daily feeding rhythms in fish was conducted by Hoar in 1942, who described that the Atlantic salmon (*Salmo salar*) and the brook trout (*Salvelinus fontinalis*) displayed preferences for feeding along the light phase of the cycle during summer. Some years later, the first self-feeder device was realized, offering an important tool for the study of feeding rhythms in fishes (Rozin and Mayer, 1961). Since these first studies, the daily patterns of feeding behaviour have been characterized in a broad variety of fish species such as the European sea bass, goldfish, rainbow trout and zebrafish (Sánchez-Vázquez et al., 1995a, 1996; Sánchez-Vázquez and Tabata, 1998).

Food is rarely constantly available in the wild and normally shows a spatial as well as a temporal distribution. Foraging behaviour is commonly restricted to a certain period in which the abundance of prey is increased and the risk of predators is reduced (Madrid et al., 2001; Reebs, 2002). Under such a cyclic environment, fish have evolved time keeping mechanisms in order to predict feeding

time and to activate in advance their physiological processes, so to avoid risk or exploit a given food source more efficiently (Madrid et al., 2001). Foraging is fundamental for an animal to survive and can be influenced by several factors such as genetics, learning and memory capacities. Another element that may help animals in their search for food resources is the circadian timing system. Although light is the most prominent zeitgeber, several non-photic stimuli, such as food, have been also shown to entrain circadian rhythms. Indeed, feeding time can act not only on the SCN and peripheral tissues, but also on an oscillator which is not yet well-defined, called **Food-Entrainable Oscillator** (**FEO**), independent from the **Light-Entrainable Oscillator (LEO)** (Meijer and Rietveld, 1989) and whose anatomical location is still unknown (Stephan et al., 1979; Stephan, 2002; Davidson, 2006). In fish, data on properties of feeding entrainment support the hypothesis of the existence of a FEO, although it is still uncertain whether fish FEO and LEO are independent (Sánchez-Vázquez et al., 1997; Aranda et al., 2001).

Most animals display an increase in locomotor activity in anticipation of the imminent meal within a few days after the feeding cycle is established; this phenomenon is known as **Food Anticipatory Activity (FAA)** (Mistlberger, 1994) and it can be defined as the increase of activity, maintained for at least 30 minutes and displayed just before feeding time (Mistlberger, 1994). Anticipation of time meals has been reported for a wide variety of animals, from bees to higher vertebrates such as monkeys (Stephan, 2002). In mammals, SCN lesions did not abolish feeding rhythms such as FAA, a fact that supports the idea of the existence of a FEO independent of the LEO (Stephan, 2002).

This synchronization to feeding time involves several advantages. For instance, when food availability is predictable, the animal can use this information to anticipate it and maximize food intake and nutrient utilization (Sánchez-Vázquez and Madrid, 2001).

One of the main characteristics of FAA is its gradual development, with several feeding cycles being required for its significance appearance. Moreover, after a shift of the feeding time, the time that fish require to resynchronize their FAA is directly related to the quantity of hours that the mealtime has been shifted.

Locomotor activity, that is usually seen as a complex mixture of different behavioural patterns, including feeding, reproduction and territoriality among other behavioural and physiological variables, exhibits daily rhythms that can be entrained by a feeding cycle. These behavioural patterns in many species have been fixed genetically due to the pressure generated by stable selective forces such as avoidance of predators, the availability of preys or the optimization of food (Daan, 1981) and it is often conditioned by the existence of special sensory requirements such as the dependence on the vision for the capture of preys (Madrid et al., 2001).

In addition to locomotor activities, other behavioural/physiological parameters appeared to be

controlled by the FEO in mammals. Drinking behaviour can be synchronized to the periodic food access, increasing a few hours before mealtime or being maintained during food deprivation (Boulos et al. 1980; Clarke et al. 1986). Concomitant to an increase of locomotor activity, a rise of body temperature can also be measured prior to feeding time, which can be viewed as an adaptive mechanism that prepares an organism to ingest food efficiently.

1.2.1 Dualism and endogenous origin of locomotor and feeding activities

In teleost fish, individuals from the same species can display a high flexibility in their daily feeding and locomotor activity patterns, shifting from the diurnal to the nocturnal behaviour along their life cycle. This ability is known as **dualism**, and has been reported to be a common feature among fish (Eriksson, 1978). Their locomotor and feeding behaviour can present independent phasing, as the same individual can display diurnal locomotor activity and nocturnal feeding activity or *vice versa* (Sánchez-Vázquez et al., 1995a; Vera et al., 2006). The mechanisms which drive dualism are unknown, but it has been suggested to be related to a highly flexible circadian system, which allows the animals to adapt quickly to environmental changes (Sánchez-Vázquez et al., 1996; López-Olmeda and Sánchez-Vázquez, 2009). The biological significance of dualism could be related to avoid predators, take advantages of the changes in food availability or to reproduction. The selection of one phase of the LD cycle to synchronize the feeding behaviour seems to depend essentially on an endogenous circadian pacemaker that generates oscillations in the behavioural variables and on the masking effect that exerts the light on the behaviour of fish. In diurnal species, the pacemaker is synchronized with the light phase of the light-dark cycle and they also show a positive masking by light, what means that light stimulates or allows their feeding behaviour. In nocturnal fish species, the light exerts a negative masking effect, while their circadian pacemaker is synchronized to the dark phase. In the case of dual species, their circadian pacemaker would be synchronized with one of the phases of the light-dark cycle, while the light could exert a stimulating or an inhibitory effect depending whether fish displays diurnal or nocturnal behaviour, respectively (Kasai and Kiyohara, 2010). Although the mechanisms that regulate the phase inversions of feeding patterns are still unknown, it is possible to hypothesize that, in addition to external cues, an endogenous annual clock may be involved, inducing the appearance of such behaviours in some seasons of the year (Kulczykowska et al., 2010).

As indicated before, some environmental signals such as light or temperature may influence or modulate the expression of feeding rhythms. Nevertheless, the hypothesis based on external cueing for feeding rhythms do not appear appropriate because animals maintained under a controlled environment, without variations in the environmental factors, still display feeding rhythms (Aranda

et al., 2001). Thus, a model based on an internal timing mechanism seems more plausible. Two different models have been suggested, an hourglass mechanism and a self-sustained clock or endogenous pacemaker entrainable by feeding (Mistlberger, 1994). The hourglass mechanism model, which hypothesize that the rhythms related to feeding are driven by the cycles in energy depletion and repletion, fails to explain why many rhythms such as feeding behaviour or food anticipatory activity persist in starved animals. The endogenous pacemaker mechanism model, however, is demonstrated by the persistence of a rhythm under constant environmental conditions. Under such conditions the period of the rhythm deviates slightly from the environmental cycle to which it is synchronized, displaying a free- running behaviour that matches the period of the endogenous oscillator (Aschoff, 1981).

1.3 Neuroendocrine control of feeding behaviour

Energy homeostasis is a well-regulated process with different control mechanisms that allow adjustment to maximal efficiency. Energy intake, determined by feeding behaviour, should balance energy expenditure due to basal metabolism, voluntary and involuntary exercise and thermogenesis. The organisms need to adapt their metabolic rate to cover different energy demands, and at the same time, to control their food intake in such a way that body weight is maintained within a certain range that allows quick fuel availability, so an important aim of regulation is to assure adequate energy input to the metabolic pathways (De Gortari and Joseph-Bravo, 2006).

In vertebrates, the regulation of food intake is a complex phenomenon that involves numerous central and peripheral endocrine factors. The actions of these factors are modulated by both intrinsic and extrinsic variables, including energy reserves, metabolic fuel usage, ontogeny, reproductive status and environmental conditions. In all vertebrates, energy balance is regulated through multiple pathways involving key appetite stimulating factors called **orexigenic,** or appetite inhibiting factors termed **anorexigenic**. The regulation of feeding involves not only the central nervous system (CNS), but also peripheral organs such as the gastrointestinal (GI) tract and adipose tissue (Volkoff et al., 2005). The brain, particularly the hypothalamus, plays a critical role in the regulation of energy homeostasis (Valassi et al., 2008). Originally thought to be under the influence of specific hypothalamic nuclei, feeding is now believed to be regulated by several neuronal circuits that integrate peripheral metabolic, endocrine and neuronal signals that reflect an animal energy status (Lin et al., 2000).

In mammals, stability of body weight and body composition require that energy intake matches energy expenditure (Jéquier and Tappy, 1999), and the body weight regulation mainly concerns adipose tissue mass, as protein and carbohydrate stores vary relatively little (Jéquier and Tappy, 1999). The “lipostatic model” has been proposed to explain the well-regulated control of body

weight and food intake, and predicts that secretions from fat cells are the key signal to the brain to regulate feeding and body-fat deposition (Inui, 1999). The model postulates a mechanism that inhibits eating behaviour and increases energy consumption whenever body weight exceeds a certain value (the set point); the inhibition is relieved when body weight drops below the set point. This theory predicts that a feedback signal originating in adipose tissue influences the brain centers that control eating behaviour and activity (metabolic and motor).

As indicated before, the hypothalamus has long been considered as the site of homeostatic regulation; it decodes neural influences arising from other sites of the brain as well as hormonal signals. Discrete populations of neurons in different hypothalamic nuclei express and release specific neurotransmitters and neuropeptides under different nutritional status. Among the hypothalamic nuclei involved in the control of body weight and feeding behaviour are: the **arcuate nucleus** (**ARC**), the **lateral hypothalamus** (**LH**), the **ventromedial nucleus** (**VMN**), the **dorsomedial nucleus** (**DMN**) and the **paraventricular nucleus** (**PVN**). The suprachiasmatic nucleus (SCN) senses the light period and can regulate energy storage through its connections with other nuclei as the PVN. Hunger and satiety are controlled by neuromodulators synthesized in these nuclei (Williams et al., 2007).

The ARC is located around the third ventricle at the base of the hypothalamus. Its axons are in direct contact with the bloodstream and, as the median eminence, the lack of blood brain barrier allows to sense precise hormonal concentrations (Hillebrand et al., 2002). The ARC receives information of the gastrointestinal tract and liver from vagal afferent connections through neurons of brainstem from cortical areas and from the amygdala. ARC neurons modify the endocrine responses controlled by the medial hypothalamus and the pituitary (Berthoud, 2002).

The PVN is the area responsible for the integration and interpretation of different peripheral and central signals that inform the hypothalamus of surrounding conditions. The PVN is formed by sub-nuclei interconnected and divided in the *magnocellular* and the *parvocellular regions*. The magnocellular neurons project towards the neurohypophysis from where oxcitocin and vasopressin are released. The ventral part of the parvocellular region projects to autonomic centers while the medial part includes neurons that synthesize corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). PVN receives connections from different hypothalamic areas and expresses receptors for orexigenic and anorexic peptide signals, such as NPY, AGRP, CART, α-MSH, CRH and orexins (Chalmers et al., 1995; Broberger et al. 1999; Mountjoy et al., 1994; Fekete et al., 2000a; Backberg et al., 2002).

The ARC is the chief hypothalamic area involved in the control of food intake and contains two interconnected groups of “first-order” neurons releasing NPY and AGRP, which enhance food

intake, and the anorexigenic POMC and CART, which inhibit the appetite (Figure 1.5). The axons of these neurons project to "second-order" neurons, located in part in the PVN, where the anorexigenic TRH, CRH and oxytocin are secreted, and in part in the lateral hypothalamus and perifornical area (PFA), where the orexant molecules MCH and orexins are produced. CRH is released from the median eminence to the portal blood where it reaches its receptors in the adenohypophysis inducing the synthesis and release of the adrenocorticotropic hormone (ACTH). ACTH has as target organs the adrenal cortex, where it controls the synthesis and release of corticosterone, an important hormone involved in energy homeostasis (Valessi et al., 2007).

When adiposity signals reach ARC, anorexigenic peptides are released, activating a catabolic circuit. In contrast, when adiposity signal concentrations in the brain are low, the activation of anabolic pathway leads to the release of orexigenic peptides. The adiposity signals are represented by leptin, a protein produced in adipocytes that, moving through the blood to the brain, acts on receptors in the hypothalamus to curtail appetite (Fei et al., 1997; Elmquist et al., 1998; Håkansson et al., 1998).

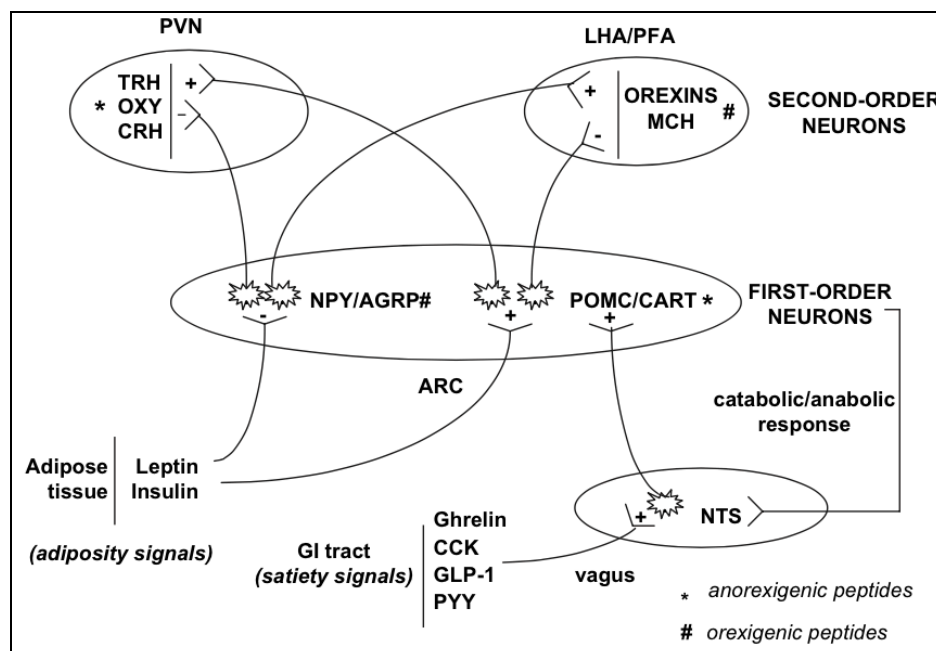

Figure 1.5. Schematic representation of the chief brain pathways involved in the regulation of feeding behaviour. ARC, arcuate nucleus; NTS, nucleus of the solitary tract; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; PYY, peptide YY. PVN, paraventricular nucleus; LHA, lateral hypothalamic area; PFA, perifornical area; NPY, neuropeptide Y; AGRP, Agouti-related peptide; POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; OX, oxytocin; MCH, melanin-concentrating hormone (Valassi et al., 2007).

It appears that the general scheme of feeding regulation in fishes is similar to that of other vertebrates in that appetite is regulated by central feeding that are themselves influenced by

hormonal factors arising from both the brain or from the periphery. As in mammals, the fish model shows redundancy, with the presence of several orexigenic or anorexigenic factors with apparently the same function, and a high degree of interaction between endocrine systems (Volkoff et al., 2009).

Evidence gathered to date seems to indicate that fish appetite-regulating neuropeptides closely interact with each other and act in parallel, rather than in a hierarchical fashion. Differences appear to exist between mammals and fishes with regards to the sites of synthesis, structure and function of some of the known endocrine regulators of appetite. Increasing evidence suggests that brain areas producing feeding-related hormones and those acting as targets for these hormones differ between fishes and mammals – and also between fishes themselves – which is not surprising, given the major differences in brain organization between groups (Cerda-Revertèr et al., 2006). The presence of specific fish tissues not present in mammals and additional gene-duplication events in ray-finned fishes (leading to more genes than in land vertebrates and thus multiple peptide forms as opposed to a single mammalian form) might hint at the presence of additional endocrine players with distinct physiological roles in fishes. To add further complexity to the piscine model, fish usually display a higher degree of heterogeneity than mammals with regards to habitats and environmental conditions, which translate into species-specific physiological adaptations, including changes in feeding behaviour. The endocrine mechanisms regulating these adaptations are to date poorly understood (Volkoff et al., 2009).

The hypothalamo-hypophysial system in fish is divided into three main areas: the *hypothalamus*, which is part of the diencephalon, the *neurohypophysis*, which derives from the ventral diencephalon and represents the neural compartment of the pituitary, and the *adenohypophysis*, which is the non-neuronal part of the gland (Cerda-Reverter and Canosa, 2009). The neurohypophysis is made up of the nerve terminals whose cell bodies are found mainly in the preoptic area and cells exhibiting glial-like properties or pituicytes, which have a supportive function. In elasmobranchs (van de Kamer and Zandbergen, 1981) and non-teleost bony ray-finned fishes (Lagios, 1968), the neurohypophysis is divided into the median eminence and the pars nervosa. The pars nervosa includes the portal system, a blood capillary network where the hypothalamic neurons release their secretory products into the vascular system for subsequent delivery to the adenohypophysis. In teleost fish there is no median eminence and no portal system, so the hypothalamic control over the adenohypophysis can be exerted through direct action upon the secretory cells. Teleost fish do not have a vascular pattern like that of tetrapod species in which a median eminence-like primary plexus leads to a portal vein which subsequently leads to a secondary capillary plexus (Gorbman et al., 1983). Adenohypophysial cells synthesize their own

hormones and remain organized in discrete domains (Pogoda and Hammerschmidt, 2007).

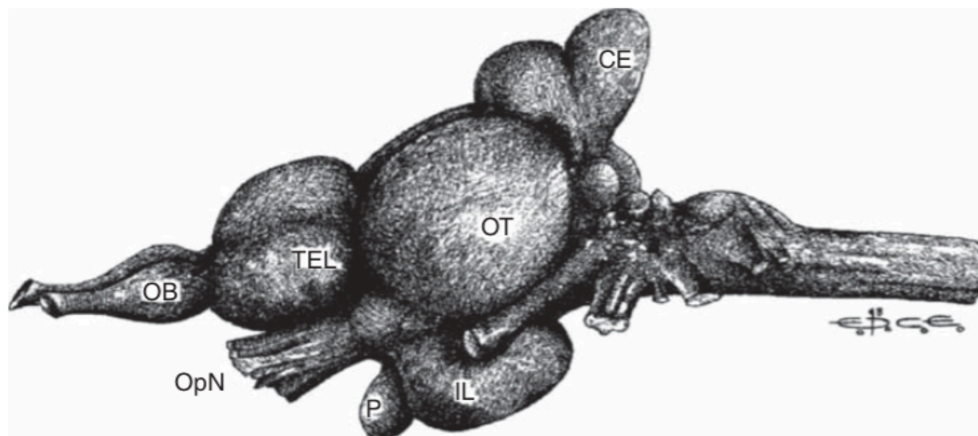

Figure 1.6. Lateral view of the brain of the sea bass (Dicentrarchus labrax). CE, cerebellum; IL, inferior lobe of the hypothalamus; OB, olfactory bulb; OpN, optic nerve; OT, optic tectum; P, pituitary; TEL, telencephalon. Scale bar 1⁄4 1 mm (Cerdà-Reverter and Canosa, 2009).

From the 1980s to the 1990s, a large number of brain neuropeptides related to feeding regulation and their receptors have been identified and characterized in mammals. Starting in the early 1990s, several feeding-related neuropeptides, such as cholecystokinin, CCK (Peyon et al., 1998), bombesin (Volkoff et al., 1999), neuropeptide Y (NPY) (Blomqvist et al., 1992; Cerdá- Reverter et al., 2000a), melanin concentrating hormone (MCH) (Baker et al., 1995), galanin (Anglade et al., 1994; Unniappan et al., 2002; Wang and Conlon, 1994), proopiomelanocorticoid (POMC)- derived peptides (Cerdá-Reverter et al., 2003a; Okuta et al., 1996), corticotropin-releasing factor (CRF) (Ando et al., 1999; Bernier et al., 1999; Okawara et al., 1988), orexins (Alvarez and Sutcliffe, 2002; Kaslin et al., 2004), cocaine- and amphetamine- regulated transcript (CART) (Volkoff and Peter, 2000), agouti-related protein (AgRP) (Cerdá-Reverter and Peter, 2003), and ghrelin (Unniappan et al., 2002) have been identified in the brain of fish by isolation of the peptide or by cloning of their cDNA sequences. However, the studies on the potential regulatory effects of these neuropeptides on food intake are very limited. Generally, these studies indicate that the brain hypothalamic area is involved in the regulation of food intake by these neuropeptides, but little work has been carried out on the precise mapping of these neuropeptides in relation to their feeding effects (Volkoff et al., 2009).

Amongst the most popular farmed fish there is a wide variation in habitats and feeding habits. Species include freshwater and marine fish as well as cold water (salmonids, cod, flounder) and warm water (sea bass, catfish, and goldfish) fish. Variations in fish feeding habits are associated

with different anatomies and physiologies. Both feeding and growth can be affected by intrinsic factors (e.g., amount of energy stores and reproductive status) and by extrinsic factors (changes in environmental cues or holding conditions). In addition, feeding might also display species-specific daily or circannual (seasonal) rhythms. The diversity in fish feeding physiology and ecology suggests that the endocrine control of feeding in fish might also be diverse and involve species-specific molecules and mechanisms (Reebs, 2002).

To date, the signaling mechanisms regulating food intake in fish are still unclear. As a consequence, optimization of food intake in farmed conditions is often carried out empirically through dietary manipulations, which might involve changes in food composition, ration size and frequency and time of feeding (Gelineau et al., 1998; Juell and Lekang, 2001; Noeske and Spieler, 1984; Petursdottir, 2002), as well as alterations of environmental factors such as day length (Biswas et al., 2006; Ergun et al., 2003). A better understanding of the endocrine mechanisms regulating feeding and growth in fish might lead to enhanced size and quality of cultured fish. Feeding is an important step for appropriate growth and reproduction. Understanding the endocrine mechanisms regulating feeding might lead not only to specific adjustments in fish holding conditions (e.g., temperature) and feeding strategies (e.g., time of feeding, frequency, rations), but also to the development of new techniques to improve feeding, food conversion efficiency, and growth in aquaculture (Volkoff et al., 2010).

1.3.1 Anorexigenic hormones: Corticotropin-releasing hormone (CRH) and Proopiomelanocortin (POMC)

Corticotropin-releasing hormone (CRH) is a 41-amino-acid neuropeptide present in the brain of vertebrates (Vale et al., 1981), and is a member of a family of related peptides that includes urotensin-I (UI), sauvagine, and urocortin/stresscopin (Lovejoy and Balment, 1999; Boorse and Denver, 2006). In mammals, CRH is known to induce the release of adenohypophyseal hormones such as adrenocorticotropic hormone (ACTH), β-endorphin, and α-melanocyte-stimulating hormone (α-MSH) from the pituitary, and there is ample evidence that CRH and its related peptides play multiple roles in animal development and also in physiological and behavioural adaptation to environmental changes and energy balance (Tonon et al., 1986; Hauger et al., 1988, 2006; Lowry and Moore, 2006; Cooper and Huhman, 2007; Denver, 2009; Papadimitriou and Priftis, 2009; Chen et al., 2012; Kubota et al., 2012).

In non-mammalian vertebrates such as amphibians and teleosts, CRH acts as a potent stimulator of corticotropin, thyrotropin, and α-MSH release (Boorse and Denver, 2004, 2006; Calle et al., 2005; Ito et al., 2006; Okada et al., 2007). CRH and its related peptides also act as regulators of feeding behaviour and stress responses (Kalra et al., 1999; Bernier and Peter, 2001; Ohgushi et al., 2001;

Hillebrand et al., 2002; Tachibana et al., 2004; Saito et al., 2005; Lowry and Moore, 2006; Carr et al., 2010; Matsuda et al., 2010; Morimoto et al., 2011; Khan et al., 2013). It has been reported that, in goldfish, intracerebroventricular (ICV) administration of CRH or UI exerts an anorexigenic action (de Pedro et al., 1997; Bernier and Peter, 2001; Volkoff et al., 2005; Matsuda, 2009). In fish, ICV administration of CRH also affects locomotor activity (Clements and Schreck, 2004; Maruyama et al., 2006; Carpenter et al., 2007; Backström et al., 2011; Ghisleni et al., 2012; Matsuda et al., 2013b), suggesting that CRH exerts psychophysiological effects in this group of vertebrates.

In fish, the hormones CRH, α-MSH, urotensin-I, pituitary adenylate cyclase-activating polypeptide (PACAP), cholecystokinin (CCK), neuromedin U (NMU), and diazepam-binding inhibitor-derived peptides such as octadecaneuropeptide (ODN) decrease food intake (Figure 1.7). These neuropeptides are not independently involved in the control of feeding behaviour, but mutually interact with each other. CRH and α-MSH mediate the actions of PACAP and NMU, and CCK and ODN, respectively. Furthermore, α-MSH-induced anorexigenic action is mediated by the CRH-signaling pathway, indicating that CRH plays a crucial role in the regulation of feeding behaviour as an integrated anorexigenic neuropeptide (Matsuda, 2013).

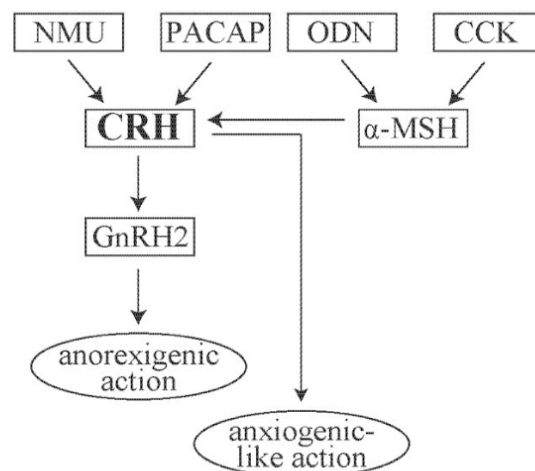

Figure 1.7. Schematic drawings of the neuronal signaling pathways of anorexigenic and anxiogenic-like action in goldfish. ODN and CCK-induced anorexigenic actions are mediated by α-MSH-signaling pathway, and the anorexigenic actions of NMU, PACAP, and α-MSH are mediated by CRH- and subsequent GnRH2-signaling pathways. CRH also evokes anxiogenic-like action. Abbreviations: NMU, neuromedin U; PACAP, pituitary adenylate cyclase-activating polypeptide; ODN, octadecaneuropeptide; CCK, cholecystokinin; CRH, corticotropin-releasing hormone; α-MSH, α-melanocyte-stimulating hormone; GnRH2, gonadotropin-releasing hormone 2 (Matsuda, 2013).

In fish, CRH exerts potential effects not only in food intake, but also in locomotor and psychomotor activities, providing an example of a neuropeptide that regulates both feeding behaviour and

psychophysiological activity such as anxiogenic- or anxiolytic- like action.

The distribution of CRH in the brain of teleost fish including the goldfish, has been well-reported: CRH-containing neuronal cell bodies are localized in various hypothalamic regions, including the periventricular part of the preoptic nucleus (NPP), the lateral part of the nucleus lateralis tuberis (NLT) and the nucleus pretectalis profundus pars ventralis (NPPv), (Olivereau et al., 1984, 1988; Yulis et al., 1986; Yulis and Lederis, 1987).

In turn, proopiomelanocortin (POMC) is a pro-hormone typically composed of segments for adrenocorticotropic hormone (ACTH), β-lipotropic hormone (β-LPH) and N-terminal peptide; each segment contains one melanophore-stimulating hormone (MSH), though a single endorphin (END) also exists in the β-LPH segment (Nakanishi et al., 1979). These neuropeptides are differentially generated through successive processing steps and post-translational modifications including endo- and exo- peptidase cleavage, amidation and acetylation (for review see Slominski *et al.* 2000). Post-translational processing of POMC is cell-type-specific and depends on proteolytic cleavage by pro-hormone convertases PC1/3 and PC2.

In the pituitary gland, the *Pomc* gene is expressed in two types of cells: in the corticotrophs of the pituitary pars distalis, PC1/3 cleaves *Pomc* to yield ACTH, β-LPH and N-terminal peptide; in the melanotrophs located in the pituitary pars intermedia, PC2 processes ACTH further to α-MSH and corticotropin- like intermediate lobe peptide (CLIP), and cleaves β-LPH to β-MSH and β-endorphin (β -END) and N-terminal peptide in γ-MSH. The hypothalamus is another important site for the expression of *Pomc* where post-translational processing similar to that in the pituitary takes place: α-MSH and CLIP derive from ACTH, next to β-MSH and β-END from β-LPH (Souza et al., 2005; Pritchard et al., 2002; Tanaka et al., 2003).

Among these POMC- derived peptides, melanocortin (MC, a collective term for ACTH and MSH) have been shown to be associated with a variety of biological processes such as steroid genesis, interrenal growth, melanin synthesis, food intake, and immunomodulation in fish (Takahashi and Kawauchi, 2005).

The *Pomc* gene is well conserved among vertebrates. However, birds and mammals have only one *Pomc* gene, (Drouin *et al.* 1989) while two forms of the *Pomc* gene have been reported in fishes and amphibians (Arends et al., 1998; Gonzalez-Nunez et al., 2003; Kim et al., 2009; Takahashi et al., 2005). Duplications are not uncommon in the evolution of the *Pomc* gene family. In fact, it is thought that the plethora of biologically active *Pomc* peptides has arisen from several duplication events (Figure 1.8). The *Pomc* gene is considered to have originated from an ancestral opioid-coding gene after the first round of whole genome duplication (1R). Following the second genome duplication event (2R), the γ-MSH sequence was gained, but later, among the gnathostome, three

trends in *Pomc* evolution have been proposed of which the loss of the γ-MSH sequence for the ray-finned fish is one (Dores et al., 2011; Dores et al., 2005). A third genome duplication event (3R) occurred only in the lineage of the ray-finned fish, which most likely gave rise to duplicate copies of the *Pomc* gene (Mever et al., 2005).

Following a gene duplication, one paralogue can accumulate nonsense mutations, lose its function and become a pseudogene, or can be deleted completely from the genome (Walsh et al., 1995), although sometimes both paralogue copies are retained in the genome. The retention of the duplicated copies may be occurred when one of the paralogues acquires a new function in relation to the original gene (neofunctionalization) or, alternatively, the functions of the original gene are partitioned between the new duplicates (subfunctionalization). At least in some teleost species, POMC sequences can be subdivided in POMC*α* and POMC*β.* In general, POMC*β* is characterized by a loss of a functional β-END, which remains conserved in the POMC*α* paralogue and it has been proposed that they may have undergone subfunctionalization (Souza et al., 2005).

In fish, POMC-derived peptides exert a variety of functions. The regulation of skin pigmentation is mostly mediated by α-MSH, β-END is involved in the brain's reward system and ACTH plays an important role in the neuroendocrine stress response. In teleosts, POMC cDNA was cloned from chum salmon more than a decade ago (Kitahara et al., 1988). Since then, the POMC cDNA sequence has been determined from several fish species, including sockeye salmon, common carp, goldfish and rainbow trout (Salbert et al., 1992; Okuta et al., 1996; Arends et al., 1998; Hui et al., 1999). Similar to its mammalian counterpart, fish POMC gene encodes a precursor potentially processed into α-MSH, β-END and ACTH. However, fish POMC lacks a coding region for γ-MSH.

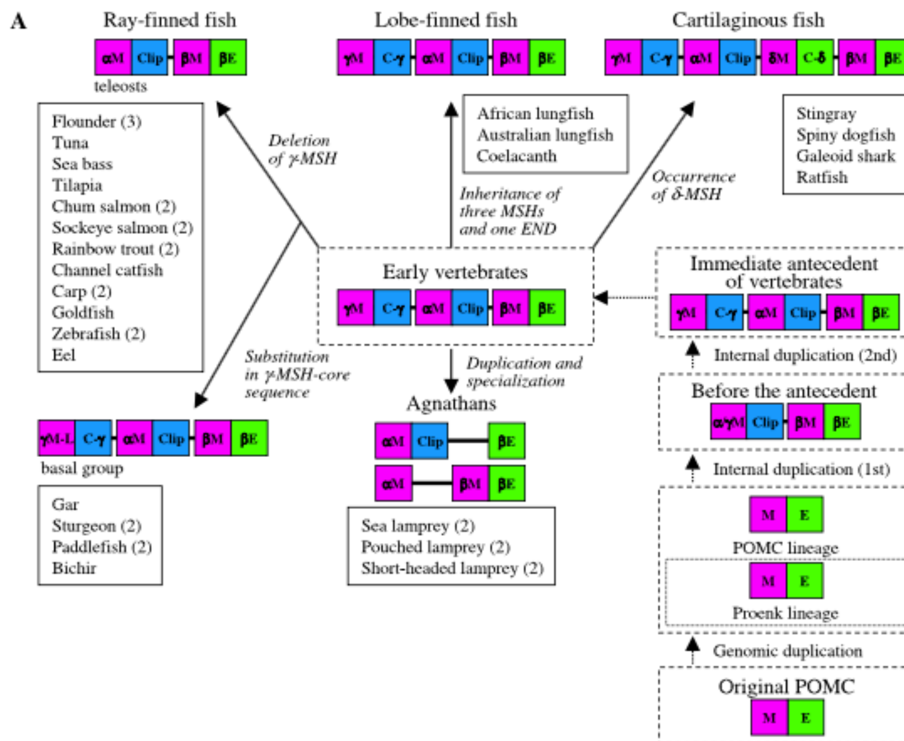

Figure 1.8. Schematic diagram of the molecular evolution of *pomc*. Diversity of *pomc* in vertebrates focuses on melanocortin and β-END. Boxes with broken line show putative *pomc* in early vertebrates and invertebrates. αM, α-MSH; βM, β-MSH; γM, γ-MSH; δM, δ-MSH, γM-L, γ-MSH-like sequence, C-γ, C-terminal segment of γ-MSH; C-δ, C-terminal extension of γ-MSH; βE, β-END. Parentheses contain the number of subtypes of *pomc* observed in each fish. Italics show epochs on the molecular architecture of *pomc* during the radiation of vertebrates (Takahashi and Kawauchi, 2005).

1.4 The Senegalese sole

The Senegalese sole (*Solea senegalensis*, Kaup, 1858) is a marine teleost fish belonging to the Class Actinopterygii, Order Pleuronectiformes, Family Soleidae (Figure 1.9).

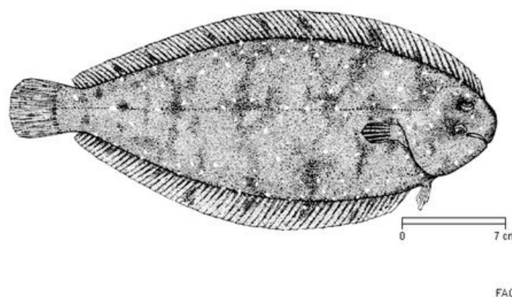

Figure 1.9. The Senegalese Sole. (Fishbase)

The Soleidae family consists of 22 genera and 89 species inhabiting brackish, marine and fresh

waters. In contrast to most vertebrates, flatfish (Pleuronectiformes) are unusual in their asymmetrical external appearance, and the left/right asymmetry of adult flatfishes is remarkable given the perfect symmetry of the larval fish (Ahlstrom et al., 1984; Youson, 1988). In adult life, they are benthic fish, characterized by an oval-shaped and flattened body, strongly compressed, with both eyes on the same right side of the head; the ocular side is slightly rounded and pigmented, while the blind side is white and totally flat. They usually mimic their background by assuming a similar coloration of the bottom, in which they normally lie down on, generally covered by sand or mud. They can protrude the small eyes above the surface of the body, in such a way that the animal can see even if buried in the substrate. This tendency to burrow into the sand, which permits them to avoid a possible aggression, represents an innate instinct of soles that does not disappear even if the individuals are kept in captivity.

The Senegalese sole inhabits mobile sand or muddy bottoms, around 100 m of depth, mainly in coastal areas, but they can also be found in salt or brackish lagoons connected to the sea, rivers and estuaries. They are located in subtropical climates, between 14°N-47°N and 1°W-19°W. The coasts of Senegal are the southern boundary in the Atlantic, the Canarian Islands represent the western limit and the shores of Brittany constitute the limit in the north. Its geographical distribution in the Mediterranean is fairly broad, covering the south and east of the Iberian Peninsula, the north of Africa and Middle East until the coast of Turkey (Figure 1.10).

Figure 1.10. Areal distribution of Senegalese sole (Fishbase).

Sole is an euryhaline and eurythermal species, adapting perfectly to changes in temperature and salinity (Rueda-Jasso et al., 2004) to 38h (Salas-Leiton et al., 2008; Ambrosio et al., 2008; Arjona et al., 2007, 2010). It is also a species that tolerates low levels of dissolved oxygen and has grown

optimally in under saturated environments (Salas-Leiton et al., 2008). Senegalese sole is a predator principally of benthic invertebrates and in its wild habitat feeds preferentially polychaetes (i. e. *Hediste diversicolor, Capitella capitata*), crustaceans (orders Tanaidacea, Amphipoda and Decapoda) and bivalve molluscs (i. e. *Scrobularia plana*).

From a reproductive point of view, the Senegalese sole is a gonochoric species with separate sex and without apparent sexual dimorphism. The first sexual maturity is reached between the second and third year of life in the case of males (first spermiation), and between the second and fourth year of life in the case of females (first oviposition), when the size reaches 30 cm (Dinis et al., 1999). They have an asynchronic ovarian development, showing oocytes in different stages of development (García-López et al., 2006, 2007). The Senegalese sole has an extended breeding season, which generally occurs in spring and autumn, with peaks in May and, to a lesser extent in October (Anguis and Cañavate., 2005; Oliveira et al., 2009). Spawning in sole has an endogenous rhythmicity that synchronizes with dusk periods. It starts after dusk and it peaks about 4 hours later. It also shows synchronization with lunar phases, peaking at the new moon (Oliveira et al., 2009b). Spawning takes place between 13°C and 23°C, with higher fecundities between 15°C and 21°C (Anguís & Cañavate, 2005).

Senegalese sole showed not only clear nocturnal habits for spawning (Oliveira et al., 2009a), but also for locomotor activity (Bayarri et al., 2004), feeding (Boluda Navarro et al., 2009) and even a higher metabolic rate during the dark phase (Castanheira et al., 2011). Senegalese sole displays clearly nocturnal self-feeding patterns under laboratory conditions, but also under farming conditions, with 77% to 85% of feed demands occurring at night. Therefore, feeding during the photophase could be incompatible with the natural feeding rhythm of sole (Boluda Navarro et al., 2009). Nutritional requirements for sole larvae are still poorly known, and big efforts are being made to maximize survival and growth. Some experiments demonstrated the ability of sole to self-feed successfully. Fish learned to operate self-feeding sensors in the laboratory, being the string sensor model the one that showed more efficiency, leading to lowest food wastes and higher demands. The same sensor proved to be operative under farming conditions, Self-feeding demand level was attained from the first day, and when reward level was modified, fish modified their feeding activity accordingly, showing an accurate compensatory feeding behaviour (Boluda Navarro et al., 2009).

1.4.1 Interest for Aquaculture and chronobiology

Senegalese sole (*Solea* senegalensis) is a flatfish of high commercial importance that is almost indistinguishable by consumers from common sole (*Solea solea*, Linnaeus, 1758). Traditionally, Southern Europe countries have been more focused in *Solea senegalensis* aquaculture due to the

lower spawning temperature requirements of *Solea solea* (Howell, 1997), and the high abundance of *Solea senegalensis* in Mediterranean and Southern Atlantic waters (Dinis et al., 1999), which makes of *Solea senegalensis* the only sole species reared in Spain or Portugal nowadays. High market demand, high market value and the adaptability of existent facilities to accommodate its rearing (Imsland et al., 2003) presents Senegalese sole as an interesting new species for diversifying Mediterranean marine aquaculture.

Ongrowing of sole is carried out under two major strategies. Traditionally, soles were reared in earthen ponds in old salt marshes in Spain and the South of Portugal, or other deltaic or estuarine environments. Fingerlings were either passively captured in these ponds. Feeding of soles could rely in the occurrence of natural prey in the bottom of the ponds, with few or nil effort from the aquaculturists, or supplementing or replacing these feed items with inert feed, taking a semi-intensive approach. On the other hand, although salt marshes are still used for semi-intensive and intensive sole aquaculture, the intensive approach is nowadays the leading trend. Sole is stocked in fiberglass or concrete tanks, often in shallow raceways (Imsland et al., 2003), for the whole production cycle, and fed inert feeds in highly controlled environments.

Although interest in farming Senegalese sole intensively in southern Europe dates back to the early 1980s, it has failed to reach successful commercial development (Flos et al., 2001; Imsland et al., 2003; FAO Fisheries and Aquaculture Information and Statistics Service, 2011). The reasons for this include lack of full control over spawning, poor fry quality and high mortality rates during the weaning stage (Cañavate & Fernández-Díaz, 1999; Anguís & Cañavate, 2005), all leading to juvenile scarcity for stocking purposes. This problem is compounded by a high incidence of skeletal malformations and pigmentation abnormalities in post-larvae and juveniles (Gavaia et al., 2002; Soares et al., 2002; Villalta et al., 2005a) and of disease outbursts affecting all ontogenetic stages caused by multiple pathogenic agents (Zorrilla et al., 2003).

Reproduction of Senegalese sole is one of the main hurdles that its domestication is still facing nowadays. There are several problems to obtain fertile spawning from second generation individuals, and this is a huge drawback, as broodstocks have to be collected from the wild as they loose reproductive performance or die (Cabrita et al., 2006; Guzmán et al., 2008, 2009a, 2011; Oliveira et al., 2011). Many approaches have been taken to investigate this problem. It has been seen that F1 females show normal vitelogenin and steroid profiles and spontaneous spawning (Guzmán et al., 2008), with egg quality parameters within normal ranges (Guzmán et al., 2009a) but typically these eggs show no fertilization in the communal reproduction tanks. To maintain genetic variation within farmed strains at levels comparable with those of wild source populations, good broodstock management practices are required, especially the use of adequate numbers of effective

parents and broodstock from different locations, or more locally if there are significant differences in the wild (Exadactylos et al., 2007).

There are only a few studies devoted to assess the optimal ongrowing photoperiod for sole, although it has been observed that sole is strongly influenced by it, as seen from a clearly nocturnal activity pattern, with locomotor activity peaking in the first part of the dark period, and progressively decreasing during the night. Recent studies show that keeping a constant photoperiod after winter solstice deters the increase in melatonin production, characteristic of shorter days and longer nights, and also increases the production of steroid hormones and vitellogenin in the pre-spawning phase, also advancing spawning (Oliveira et al., 2011). These findings point out to the possible feasibility of advancing/controlling spawning season of Senegalese sole in fish farms. A crucial step in the rearing of an aquaculture species is the ability to control maturation and, ultimately, spawning. Spawning of Senegalese sole females have been successfully induced through hormonal manipulation, although results have not been conclusive in suggesting a dosage, administration method or administration timing. The steroid of choice is an analogue of the gonadotropin-releasing hormone (GnRHa) administered either via repetitive injections or through sustained-release implants (Agulleiro et al., 2006).

The Senegalese sole is acquiring an important relevance in chronobiological studies as the number of published works focused on the sole circadian system has increased in the last few years especially focused on rhythms of locomotor activity, feeding, melatonin, sex steroids in relation to temperature and photoperiod (Anguis and Cañavate, 2005; Bayarri et al., 2004; Boluda-Navarro et al., 2009; García-López et al., 2007; Guzmán et al., 2008; Oliveira et al., 2009). The molecular mechanisms underlying sole circadian rhythms have also been explored recently, both in adults and developing sole (Martín-Robles et al., 2011, 2012a, 2012b).

1.4.2 Larval development and rearing

Larval development is fast and highly influenced by environmental cues as temperature and light (Blanco-Vives et al., 2011; Parra and Yúfera, 2001). Senegalese sole larvae suffer a dramatic metamorphosis during the first 10-30 days after hatching, involving the migration of the left eye, and the transition from a pelagic and diurnal life to the settlement in the benthos where they conduct a nocturnal life (Figure 1.11).

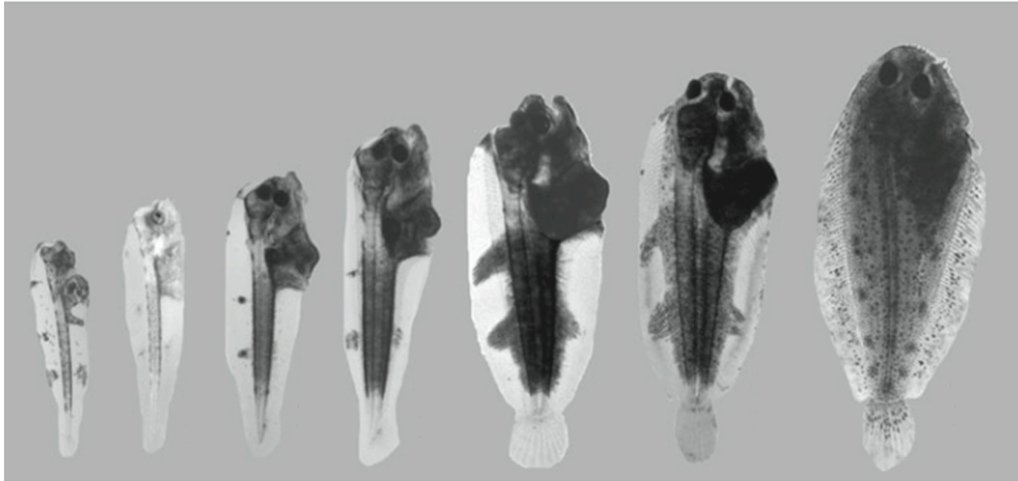

Figure 1.11. Development stages of *Solea Senegalensis* (Padròs et al., 2011).

Two days after hatching (DAH) the mouth and the anus become functional (Ribeiro et al., 1999a). On 11 DAH the metamorphosis begins and larvae start to settle. On 15 DAH larvae measure 8 mm in total length (Dinis et al., 1999). Metamorphosis is complete between 20 to 37 DAH (Padrós et al., 2011). Gastric glands appear around 27 DAH, and at 31 DAH sole larvae are capable of ingesting, digesting and absorbing nutrients, with a morphologically and enzymatically complete digestive tract (Ribeiro et al., 1999a).

An important problem that faces sole rearing during larval stages is the occurrence of malformations and pigmentation abnormalities that are observable in varying degrees in many aquaculture facilities (Flos et al., 1998). In some cases, up to 44% of larvae present at least one skeletal malformation, mainly in the caudal region and in the vertebral column. Although some authors report negligible levels of altered pigmentation under routine feeding procedures (Cañavate et al., 2006), in some cases up to 11% of juveniles show pigmentation problems in form of albinism, partial depigmentation (most of the cases) or abnormal nadiral pigmentation (Soares et al., 2002). It has been suggested that excessive eicosanoid production, or inadequate regulation of tyrosinase, could be related to deficiencies in pigmentation, as growing dosages of arachidonic acid were correlated with depigmentation (Villalta et al., 2005a).

Some of these problems during early development also seem to be related with artificial lighting conditions in aquaculture practice. In nature, the spectral characteristics of underwater light are determined by a combination of the effects of the light-scattering and absorption processes in the water column, which together define a waterbody light-attenuation capacity (Roger, 1997). The cumulative effect of the scattering and absorption processes is the attenuation of light with depth, that becomes increasingly monochromatic as it passes into the deep water (Jerlov, 1968); Thus,

higher wavelengths from visible spectrum are gradually dissipated in superficial waters, whereas blue wavelengths can reach deeper waters where sole inhabits. The effects of artificial lighting conditions on the performance, development, and welfare of some fish larvae of commercial interest have recently been reviewed by Villamizar et al, (2011), who pointed out the importance of light during early development to optimize rearing protocols at hatcheries and to sustain juvenile supply. In fish, the photosensory systems and extraretinal photoreceptors are complex and far from fully understood but it has known that they have adapted their photopigment sensitivity depending on their surrounding environment (Kusmic and Gualtieri, 2000). For the majority of teleost larvae, vision is the primary sense involved in a successful feeding response (Blaxter, 1969), therefore a lack of correlation between prey density and larval growth and survival may result from the influence of light. Recent work performed on Senegalese sole reported that light spectra, intensities, and photoperiods have a great influence in terms of larval growth, development, malformation, survival (Blanco-Vives et al., 2010), and hatching rhythms (Blanco-Vives et al., 2011). In a precedent study conducted by Blanco et al., (2012), they investigated the foraging behaviour and daily activity rhythms in sole larvae exposed to different light spectra and photoperiods, revealing for the first time the existence of daily rhythmic behavioural patterns in developing sole larvae reared under different lighting conditions and confirming that the early development of Senegal sole eggs and larvae is strongly affected by light characteristics (both photoperiod and spectrum). Light-dark cycles using blue light maximize hatching rate 94.5%±1.9, growth and development speed. On the other hand, complete darkness and light-dark cycles 12L:12D with red light result in lower hatching rate and poorer larval performance (Blanco-Vives et al., 2011a). Because "unnatural" or inappropriate environmental conditions seriously compromise the welfare of fish larvae and negatively affect their survival and performance (Villamizar et al., 2009), light/dark cycles of blue wavelengths appear to be the optimal conditions and a prerequisite for the proper development of the biological clock and behaviour of sole larvae. In most cases, aquaculture practices are conducted under established protocols that differ considerably from natural environmental conditions. Therefore, research must be directed to improve artificial breeding and to optimize rearing conditions of the cultivated species. In this sense, one of the main objectives should be to reproduce the natural conditions for each species. For this purpose, we have also focused our work on determining light spectra effects in the expression of anorexigenic genes during sole ontogeny.

2 OBJECTIVES

Increasing knowledge on the endocrine mechanisms that regulate feeding and growth in cultured fish can contribute to make improvement in fish holding conditions and feeding strategies, supporting the development of new techniques that could ameliorate feeding, food conversion efficiency and growth in aquaculture practice.

As it has been explained in the introduction section, feeding in fish is a rhythmic process that is entrained by feeding time and appears influenced by light photoperiod and spectra. Moreover, orexigenic and anorexigenic endocrine factors are regulating this rhythmic feeding behaviour. Based on these facts, the **main objective** of this study was to investigate how daily mRNA expression of three specific anorexigenic hormones, i.e. the corticotropin-releasing hormone (CRH) and the paralogues α- and β- proopiomelanocortin (POMC), is modulated by different photoperiods, light spectra and feeding regimes, in both adult and larvae of *Solea senegalensis.*

In order to reach this main objective, three main experiments were developed:

- In a first experiment, adult fish maintained under LD (12 h light/ 12 h dark) photoperiod were divided in three different tanks, each one kept at a different feeding regime (feeding during daytime, feeding during night-time and feeding at random).
- In a second experiment, adult fish were maintained under a DD (24 h dark) photoperiod; they were divided in different tanks, each one kept at a different feeding regime (feeding during daytime and feeding at random). The daily mRNA expression of selected anorexigenic hormones (CRH, POMCa, POMCb) has been studied in different central areas chosen on the basis of preliminary screening conducted on different brain areas for the three genes.
- Further experiments were carried out on larvae of Senegalese Sole, to provide a useful contribution to ameliorate artificial breeding and optimize rearing conditions of the cultivated species. Larvae were reared under a LD photoperiod and were exposed to three different light spectra, i.e. white, blue and red lights. The daily expression of the same anorexigenic genes has been studied during sole ontogeny, in order to evaluate the influence of the light features on these hormones. In addition, as Senegalese sole exhibits a shift from diurnal to nocturnal in locomotor activity and feeding habits during metamorphic process, we tried to elucidate if this shift is accompanied by relevant daily variations in the expression of these anorexigenic hormones before, during and after the completion of metamorphosis.

3 MATERIAL AND METHODS

3.1 Animal and rearing system

3.1.1 Adult fish experiment

A total of 192 adult specimens of *Solea senegalensis* from 101 to 172 g in body weight and from 20 to 25 cm in length were used in the present study. They were collected from the "Laboratorio de Cultivos Marinos" (University of Cádiz, Puerto Real, Spain) and maintained in five 250-L tanks with continuous seawater renovation and gentle aeration, at a constant temperature and salinity of 19±1°C and 39 ppt, respectively. Each tank was equipped with a lid containing two fluorescent lamps (Sylvania Gro-Lux, Germany) connected to an individual automatic photoperiod control system. The photoperiod for the "LD group" of adults was set at 12 h light:12 h dark (12L:12D) with lights on at 08:00 h local time, (Zeitgeber time 0 or ZT0), while the adults of the "DD group" were reared in constant dark condition, i.e. 0 h light: 24 h dark (0L:24D). The light intensity at the water surface was 400 lux during the illumination period. Fish were fed with commercial 3-mm dry pellets (Skretting S.A., Burgos, España) at a daily ration of 0.3% body weight. This study was approved by the Animal Experimentation and Ethics Committee of the University of Cádiz (Spain) and was conducted according to international ethical standards.

3.1.2 Larvae experiment

The experiment was carried out at the facilities of Centro IFAPA El Toruño (Puerto de Santa María, Spain). Fertilized eggs were collected at night before dawn in complete darkness from naturally spawning tanks to ensure that eggs did not receive any light before the experimental treatments. The broodstock was kept in tanks exposed to natural lighting conditions in a 12L:12D cycle. The eggs were kept at a constant temperature (20.9°C ± 1.4°C) and water was changed everyday using seawater hyperfiltered by means of a biological filter (EHEIM model 2227, Germany) and a bio-balls filter system. At 3 days post-hatching (DPH), coinciding with the onset of exogenous feeding, larvae from each experimental condition were transferred to three 1-L aquaria per treatment for four weeks. To feed the larvae, *Brachionus plicatilis* rotifers were cultured and enriched with commercially available freeze-dried green algae *Nannochloropsis* sp. (Easy Algae, Fitoplancton Marino, Cádiz, Spain) in a proportion of 300,000 cells/mL/d from 3 to 7 DPH. Enriched rotifers were added to tanks twice a day, both during the light (ZT1, with ZT0 representing the beginning of light) and dark (ZT13) phases, as an early live food at a density of 20 individuals/mL from 3 to 7 DPH. *Artemia* sp. nauplii were added to tanks twice a day both during the light (ZT1) and dark (ZT13) at a density of 2 to 3 nauplii mL^{-1}/d^{-1} from 8 to 30 DPH. Three to 5 metanauplii mL^{-1}/d^{-1} enriched for 24 hours with a mixture (ORI-GO, ORI-PRO, Skretting AS, Burgos, Spain) of

phytoproteins and highly unsaturated fatty acids (HUFAs) were provided from 27 to 40 DPH. Fish were reared and manipulated following Spanish legislation on Animal Welfare and Laboratory Practices and the study was conducted according to international ethical standards.

3.2 Experimental design and sampling

3.2.1 Adult fish experiment

This experiment was conducted to investigate the influence of restricted food access on Senegalese sole daily mRNA expression of 3 anorexigenic genes (*crh, pomc_a, pomc_b*) in 4 different neural tissues: telencephalon, diencephalon, optic tectum and pituitary gland.

3.2.1.1 Adults reared under LD conditions

The animals were kept under 12L:12D photoperiod conditions. Sole specimens were divided into three experimental groups depending on the feeding schedule: animals fed at mid-light period, i.e. middle of the light phase (ML), at mid-dark period (MD), or at random (RND), and the food was distributed by means of automatic feeders (EHEIM GmbH & co. KG, Germany) coupled to digital programmable timers (Data micro, Orbis, Spain). For ML and MD groups, the feeder was programmed to deliver food everyday at the same time 14:00 h (ZT6) and 02:00 h (ZT18), respectively. For the RND group, feeding time was chosen randomly. The feeding interval was set between 12 and 36 h, so they received the same amount of food per 24 h, as the ML and MD groups. After four weeks under these different feeding regimes, the animals were anaesthetized in MS-222 (Sigma, St Louis, MO; 100-200 mg/l of water) and sacrificed by decapitation. The adults were sampled every 4 h at six different zeitgeber time points (n=4 at each point): ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20 during a 24 h daily cycle, in which ZT 0 corresponded to the light onset and ZT12 to the light offset. The experimental design scheme is shown in figure 3.1. The selected neural tissues (telencephalon, diencephalon, optic tectum) and pituitary gland were removed, and samples were rapidly frozen in liquid nitrogen and stored at -80° C until used.

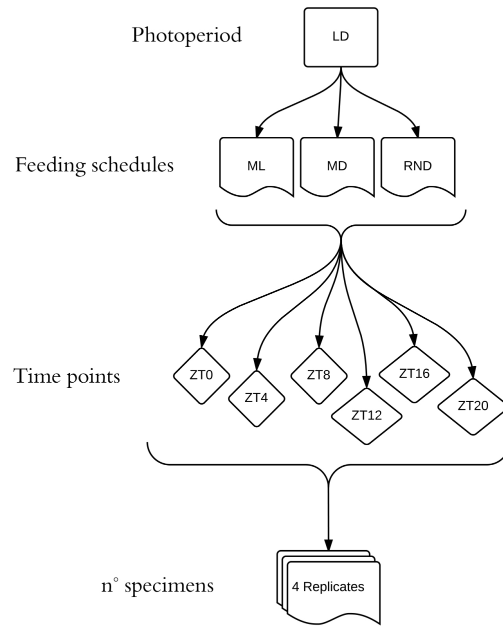

Figure 3.1. Experimental design scheme from adults reared under LD condition.

3.2.1.2 Adults reared under DD conditions

The animals were kept under constant dark (0L:24D) conditions. Sole specimens were divided into two experimental groups depending on the feeding schedule: animals fed at mid-light (ML) period and at random (RND), and the food was distributed in the same way as the previous experiment. For ML group, the feeder was programmed to deliver food everyday at the same time 14:00 h (ZT6), while for the RND group, feeding time was chosen randomly assuring that they received the same amount of food per 24 h than the ML group. After four weeks under these different feeding regimes, the animals were anaesthetized in MS-222 (Sigma, St Louis, MO; 100-200 mg/l of water) and sacrificed by decapitation. The specimens were sacrificed every 4 h at six different circadian time points (n=4 at each point): CT0, CT4, CT8, CT12, CT16 and CT20. The experimental design scheme is shown in figure 3.2. The selected neural tissues (telencephalon, diencephalon, optic tectum) and pituitary gland were removed and samples were rapidly frozen in liquid nitrogen and stored at -80° C until used.

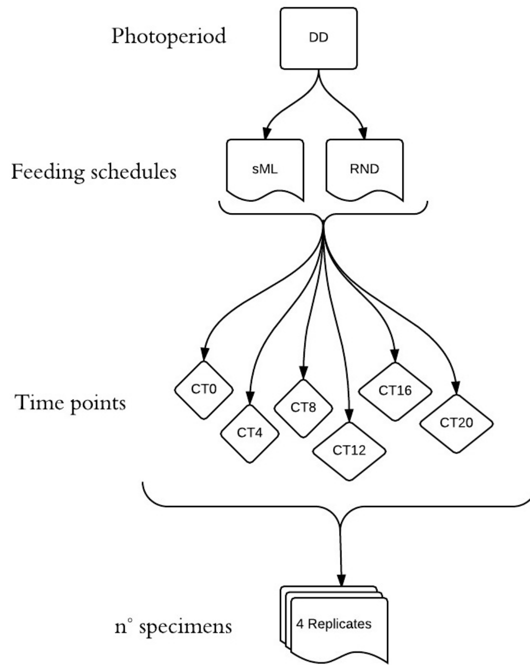

Figure 3.2. Experimental design scheme from adults reared under DD condition.

3.2.2 Larvae experiment

In order to elucidate the effects of development stage (pre-metamorphic, metamorphic, post-metamorphic) and different light spectra on the expression of anorexigenic genes (*crh*, *pomc_a* and *pomc_b*) transcript levels in Senegalese sole larvae, the eggs were distributed into 3 experimental groups: 12L:12D photoperiod exposed to red (LD_R, λ peak = 685 nm, half-peak bandwidth = 592-668 nm), blue (LD_B, λ peak = 463 nm, half-peak bandwidth = 435- 500 nm), and white (LD_W) lights. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec HH, ASD Inc., Boulder, CO). To avoid the effects of any background light on the experiments, the experimental aquaria were covered with a black light-tight screen. For the different spectral trials, lamps were constructed using light emitting diodes (LEDs) mounted on fiberglass plaques (160 × 232 mm). Both red and blue lamps had 17 homogeneously distributed LEDs. White lamps had 18 white and 4 red LEDs to produce a broader range wavelength. Each lamp was encased in a waterproof container placed approximately 50 cm above the water surface. The lamps were powered by a 3-V DC supply connected to a variable resistor (0–2 kΩ). After 9 (pre-metamorphic), 12 (metamorphic) and 25 (post-metamorphic) DPH under these light spectra conditions, the larvae of each group were sampled every 4 h at six different zeitgeber time points (n = 4 at each point): ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20 during a 24 h daily cycle, in which ZT 0 corresponded to the light onset and ZT12 to the light offset. The experimental design scheme is shown in figure 3.3.

Larvae samples were rapidly frozen in liquid nitrogen and stored at -80° C until used.

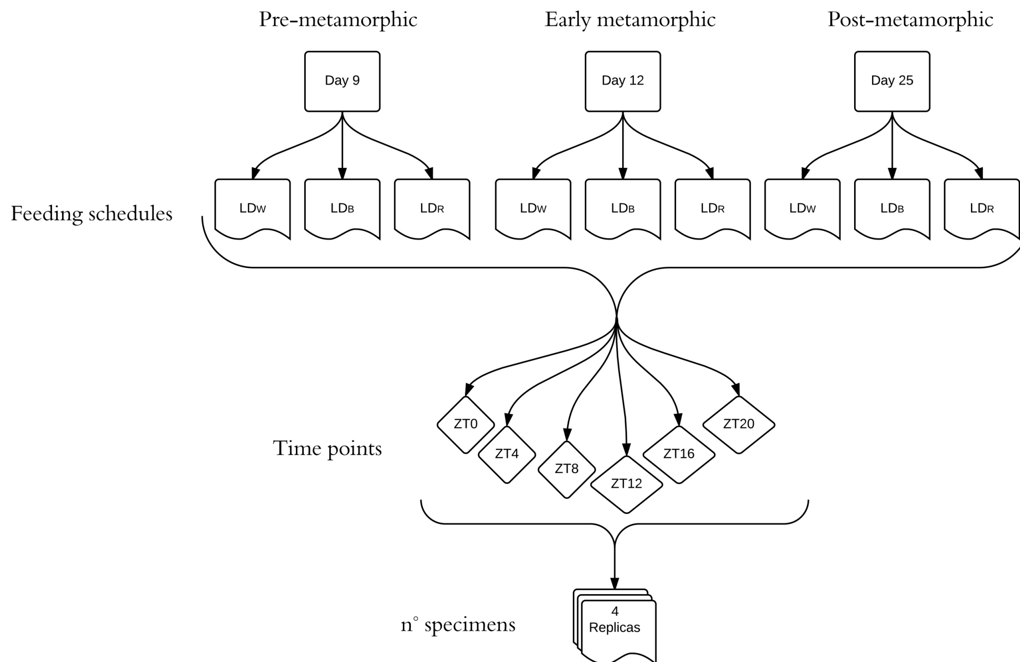

Figure 3.3. Larvae experimental design scheme.

3.3 Selection and design of specific primers

Specific primers for sole *crh* and *pomc_b* (GenBank accession numbers FR745427 and FR851916.1, respectively) used in real-time quantitative PCR (RT-qPCR) were selected according to Wunderink et al., (2012). Primers for *pomc_a* were designed from the sequence available in GenBank (accession number FR851915.1) by using Primer3 software (v. 0.4.0.) available at http://fokker.wi.mit.edu/primer3/input.htm, accessed in July 2014. Primer oligonucleotide sequences used are shown in Table 3.1.

Table 3.1. Primer oligonucleotide sequences used for RT-qPCR analysis.

Gene	Primer	Sequence (5′ →3′)
crh	qssCRH_Fw qssCRH_Rv	CCTGACCTTCCACCTGCTAC GAGATCTTTGGCGGAGTGAA
pomc_a	qssPA_Fw qssPA_Rv	TTCAGGGCGGTGTCAAAGAAGGC TTCTTGAACAGCGTGAGCAG
pomc_b	qssPB_Fw qssPB_Rv	GTCGAGCAACACAAGTTCCA GTCAGCTCGTCGTAGCGTTT
β-actin	qssBact_Fw qssBact_Rv	TCTTCCAGCCATCCTTCCTCG TGTTGGCATACAGGTCCTTACGG

In order to optimize PCR conditions, we conducted a gradient temperature PCR at 58°C, 60.8°C, 62.5°C and 64.6°C. The amplicons obtained were analysed through agarose gel electrophoresis and the DNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN Group, Inc, USA). The DNA fragment were excised from the agarose gel with a clean, sharp scalpel, put in a tube with 3 volumes buffer QG to 1 volume gel and incubated at 50°C for 10 min. Once that the gel slices had dissolved completely, the mixture was put in a collection tube and centrifuged for 1 min. The flow-through was discarded and the DNA bound at the filter of the QIAquick column was washed with 750 µL of Buffer QC. To elute the DNA, 30 µL of Buffer EB (10 mM Tris·Cl, pH 8.5) were added. Total DNA yield and quality were determined by the 260/280 nm absorbance ratio in a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, De, USA). The DNA obtained was eluted in water and sent with the primers to the SCAI – Unidad de GENÓMICA (Universidad de Córdoba, Campus de Rabanales) in order to be sequenced.

3.4 RNA extraction

Total RNA was extracted using the TRIsure Reagent® (Bioline, London, UK). Tissues were homogenized (50-100 mg) in 1 ml of reagent using 3-4 stainless steel beads (2 mm diameter) in a mixer mill MM400 (Retsch, Haan, Germany). After homogenization, 0.2 ml of chloroform D (AppliChem, Darmstadt, Germany) was added and samples were vigorously shacked by hand for 15 seconds, then incubated at room temperature for 3 minutes and centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixture was separated into lower phenol-chloroform phase containing DNA (pale green in color), an interphase containing proteins and a colorless upper aqueous phase containing RNA. The aqueous phase was transferred into a fresh tube and RNA was precipitated mixing with 0.5 ml of isopropyl alcohol (Panreac, Barcelona, Spain). Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitated formed a gel-like pellet on the bottom of the tube. The supernatant was completely removed and the RNA pellet was washed once with at least 1 ml 75% ethanol prepared with diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich, St. Louis, MO, USA). Samples were centrifuged at 7,500 x g for 5 minutes at 4°C and ethanol was removed. The RNA pellet was air-dried for 5-10 minutes and re-dissolved in DEPC-treated water (from 15 to 50 µl) and incubated for 10 minutes at 55-60°C in a water bath. Total RNA yield and quality were determined by the 260/280 nm absorbance ratio in a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, De, USA). All ratios were optimal, between 1.8 and 2.

3.5 cDNA synthesis

Aliquots of 100 ng (for the adult experiments) or 100 µg (for the larvae experiment) of total RNA were reverse transcribed into cDNA (20 µl final volume) using the QuantiTect Reverse Transcription Kit (Qiagen, Inc, USA). The procedure consisted in 2 main steps:

- *Elimination of genomic DNA*: The purified RNA samples were incubated in gDNA Wipeout Buffer containing DNAse at 42°C for 2 minutes to effectively remove contaminating genomic DNA. Then the RNA samples were directly used in the next step of reverse transcription.
- *Reverse transcription*: After genomic DNA elimination, the RNA samples were ready for reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen, Inc, USA) containing a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction took place at 42°C during 30 minutes followed by 2 min at 95°C for inactivation. Then cDNAs obtained were stored at -20°C until used.

3.6 Real Time quantitative PCR (RT-qPCR) expression analysis

Real time quantitative PCR (RT-qPCR) was performed in a Bio-Rad CFX96 Touch detection system using the SensiFAST SYBR No-ROX Kit (Bioline Ltd, UK). PCR reactions were developed in a 20µl volume containing 5ng of cDNA. Specific primers used for sole *crh, pomc_a* and *pomc_b* are indicated in Table 3.1.

The amplification protocol for the three genes was as follow (Figure 3.4.):

- Initial denaturation at 95°C for 2 min.
- 40 cycles of 95°C for 5 s and 63°C for *crh*, 64°C for *pomc_a* and 65°C for *pomc_b,* for 30 s.
- Melting curve from 70°C to 95°C, with an increment of 0.5°C.

Melting curves were generated for each sample to confirm that a single product was amplified. Non-template controls were used as negative controls. The relative expression of all genes mRNA was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) using *Solea senegalensis* β-*actin2* (Genbank accession number DQ485686) as housekeeping gene (Infante et al., 2008; Martín-Robles et al., 2012b).

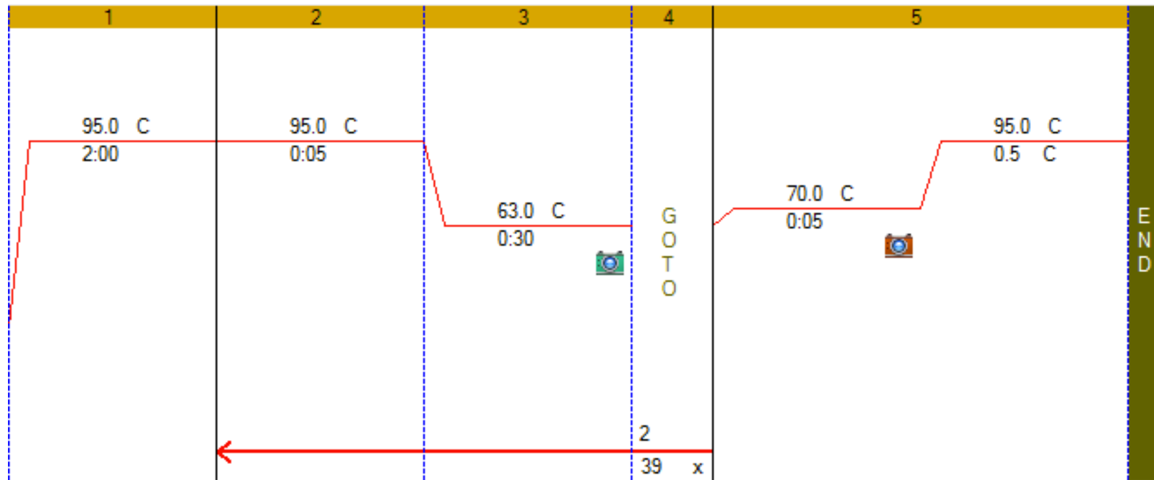

Figure 3.4. Example of the qPCR amplification protocol (Bio-Rad CFX software screen) followed for the anorexigenic gene *crh* of *Solea senegalensis* in according with Wunderink et al., (2012).

3.7 Data analysis

3.7.1 Statistical analysis:

To assess statistical differences in the expression of anorexigenic genes, mRNA levels of each gene among different daily time points were analysed by one-way ANOVA. Significant ANOVA analyses were followed by Tukey's post hoc comparison test to determine differences between means, with p <0.5 taken as the statistically significant threshold. When necessary, values were transformed to get normal distribution and homogeneity of variances. In all cases, statistical significance was accepted at p<0.05. All statistical tests were performed using the Statgraphics Plus 5.1 software (Statpoint Technologies, Warrenton, VA, USA). Data are presented as mean ± standard error of the mean (SEM). All graphics were created by means of Microsoft Office Excel 2011.

3.7.2 Cosinor analysis:

Rhythmicity in daily gene expression values was evaluated by cosinor analysis using the chronobiological software El Temps (version 1.228; www.el-temps.com) developed by Prof. A. Díez Noguera (University of Barcelona). The Cosinor method is based on the least squares approximation of time series data with a cosine function of known period and was performed to determine whether the daily expression of the studied genes showed a regular fluctuation over a defined period (24 h in the case of circadian rhythms). Cosinor analysis also provides the statistical significance of the rhythm through an F-test of the variance accounted for by the waveform versus a straight line of zero-amplitude

(null hypothesis). Therefore, if under a statistical significance of $p<0.05$ the null hypothesis was rejected, the amplitude could be considered as differing from 0, thereby constituting evidence for the existence of a statistically significant rhythm of the given period under consideration. Expression profiles were considered to display a significant daily rhythm when $p<0.05$.

4 RESULTS

4.1 Central expression of anorexigenic genes in Senegalese sole

In order to analyse the central mRNA expression of the anorexigenic genes *crh, pomc_a* and *pomc_b*, a RT-qPCR screening was conducted in Senegalese sole pituitary and brain, which was dissected in 6 central areas: olfactory bulbs, telencephalon, diencephalon, optic tectum-tegmentum, cerebellum-vestibulolateral lobe and medulla oblongata-spinal cord.

Regarding *crh* expression, the Figure 4.1 displays a column chart of *crh* mRNA relative expression examined in different central areas. *Crh* expression was detected in all neural areas analysed as well as in the pituitary (Figure 4.1), although statistical analyses did not reveal significant differences between them.

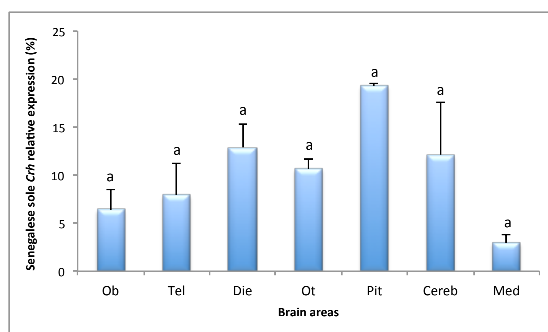

Figure 4.1. Expression of *crh* in Senegalese sole brain. Relative *crh* mRNA expression in olfactory bulbs (Ob), telencephalon (Tel), diencephalon (Die), optic tectum (Ot), pituitary (Pit), cerebellum (Cereb) and medulla oblongata (Med). RT-qPCR conditions are described in the Material and Methods section. A 135 bp specific *crh* product was detected in all areas analysed. Statistical differences between neural areas were tested by one-way ANOVA. Different letters indicate significant statistical differences.

Concerning *pomc*, central and pituitary expression of both paralogues *pomc_a* and *pomc_b* are depicted in Figure 4.2 and Figure 4.3, respectively. All central areas were found to express *pomc_a* and *pomc_b* in Senegalese sole. The analyses of mRNA expression revealed that the pituitary showed much higher transcript levels in relation in relation to the central areas analysed, being statistical differences clearly evident in this case (Figure 4.2A and Figure 4.3A). When representing the same expression data excluding the pituitary, differences in mRNA transcript levels between the central areas were also seen (Figure 4.2B and Figure 4.3B). In particular, the diencephalon showed the highest *pomc_a* mRNA levels, being this expression significantly higher than that of the olfactory bulbs (Figure 4.2B). Concerning *pomc_b*, the highest mRNA levels were

observed in the cerebellum, being significantly higher than those of the olfactory bulbs, telencephalon and diencephalon (Figure 4.3B).

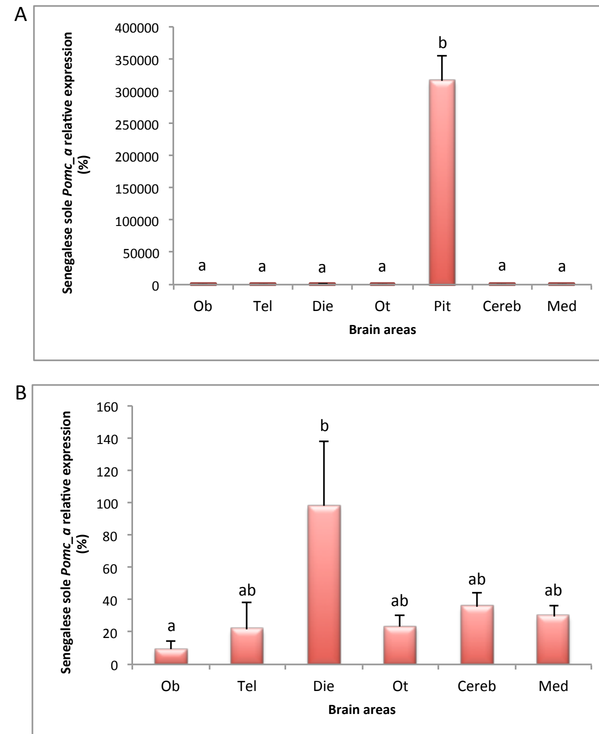

Figure 4.2. Expression of *pomc_a* in Senegalese sole brain. (A) Relative *pomc_a* expression in olfactory bulbs (Ob), telencephalon (Tel), diencephalon (Die), optic tectum (Ot), pituitary (Pit), cerebellum (Cereb) and medulla oblongata (Med). (B) Column chart of *pomc_a* mRNA transcript levels shown in A excluding pituitary. RT-qPCR conditions are described in the Material and Methods section. A 127 bp specific *pomc_a* product was detected in all areas analysed. Statistical differences between neural areas were tested by one-way ANOVA. Different letters indicate significant statistical differences.

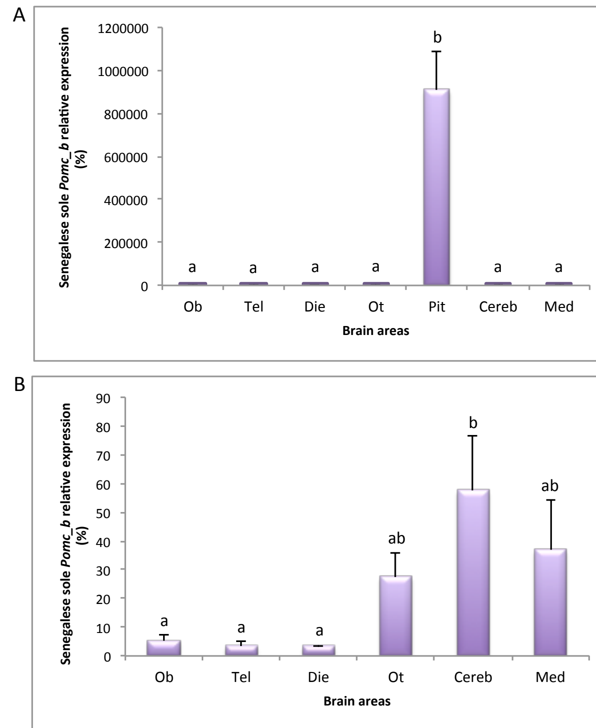

Figure 4.3. Expression of *pomc_b* in Senegalese sole brain. (A) Relative *pomc_b* expression in olfactory bulbs (Ob), telencephalon (Tel), diencephalon (Die), optic tectum (Ot), pituitary (Pit), cerebellum (Cereb) and medulla oblongata (Med). (B) Column chart of *Pomc_b* mRNA transcript levels shown in A excluding pituitary. RT-qPCR conditions are described in the Material and Methods section. A 110 bp specific *pomc_b* product was detected in all areas analysed. Statistical differences between neural areas were tested by one-way ANOVA. Different letters indicate significant statistical differences.

4.2 Influence of light and feeding time in central expression of anorexigenic genes in adult specimens of Senegalese sole

We have analysed *crh, pomc_a* and *pomc_b* daily mRNA relative expression in 3 different neural areas, i.e. telencephalon, diencephalon, optic tectum, as well as in the pituitary gland, by Real Time qPCR in adult specimens reared under LD or DD conditions and different feeding schedules: fed at ML (ZT6), MD (ZT18) and at RND times.

4.2.1 Daily expression of *crh*, *pomc_a* and *pomc_b* in Senegalese sole reared under LD conditions and different feeding schedules.

4.2.1.1 Daily rhythms of *crh* mRNA expression

According to the results obtained from the preliminary PCR screening, daily expression of *crh* mRNA was analysed in three neural areas: telencephalon, diencephalon and optic tectum (Figure 4.4, Table 4.1).

In the telencephalon, only animals fed at MD (ZT18) exhibited significant daily variations in *crh* transcript levels as evidenced by ANOVA and significant daily rhythms by Cosinor analysis (Figure 4.4, Table 4.1). *Crh* presented its acrophase at ZT13.52 (Table 4.1), coinciding with the beginning of the night. Subsequently, the expression significantly fell down until sunrise, reaching the lowest level just after feeding, at the end of the night and during the day. In contrast, daily *crh* mRNA relative expression analysed in animals fed at ML (ZT6) and RND times did not show neither statistical differences nor significant rhythms in the telencephalon (Figure 4.4, Table 4.1).

Concerning the diencephalon, *crh* expression did not show daily statistical differences and rhythms neither when the specimens were fed at ML nor when fed at MD or at RND times (Figure 4.4, Table 4.1).

Finally, in the optic tectum, *crh* mRNA expression profiles were similar in MD and RND-fed groups, with neither daily rhythms nor statistical differences between time points (Figure 4.4). However, *crh* transcript levels exhibited significant daily rhythms as evidenced by both ANOVA and Cosinor analysis in ML-fed fish (Figure 4.4, Table 4.1). In this group *crh* mRNA expression was lowest during the daytime and increased some hours after feeding, reaching the acrophase at ZT16.28 (Table 4.1), followed by a significant decrease at the end of the night until sunrise (Figure 4.4).

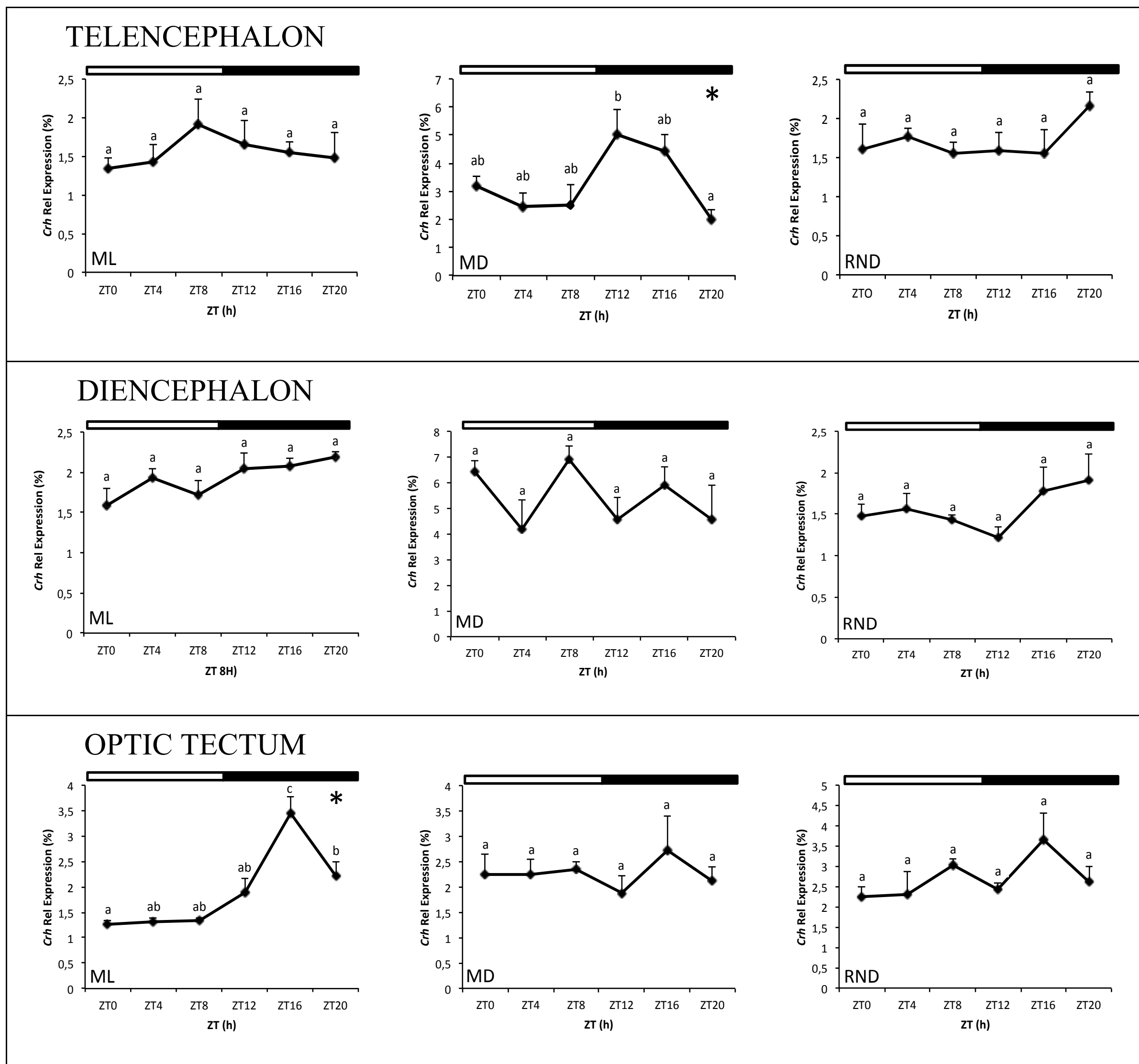

Figure 4.4. Relative expression of *crh* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under LD 12:12 cycles and fed at midligh (ML), middark (MD) or at random (RND). Different letters indicate statistically significant differences (ANOVA, p<0.05). The black and the white bars at the top of the graphics represent the light and dark phases. Asterisks represent rhythmic expression (Cosinor, p<0.05). ZT indicates zeitgeber time (hours).

Table 4.1. Parameters estimated by the Cosinor analysis for *crh* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at midlight (ML), middark (MD) or random (RND) times**.** The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant rhythm (p>0.05). Asterisks indicate significant rhythms (p<0.05).

***crh* LD CONDITIONS**					
ML GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	1.5488	0.1925	10.1781	91.9421	N.S.
Diencephalon	1.9233	0.2031	16.0218	97.4772	N.S.
Optic tectum	1.9133	0.9716	16.2830	94.0019	* 2.0960e-05
MD GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	3.2701	1.0948	13.5235	86.8365	* 0.04275
Diencephalon	5.4244	0.1809	7.2346	89.5155	N.S.
Optic tectum	2.2583	0.0712	18.4514	90.4615	N.S.
RND GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	1.7034	0.1818	21.4660	93.9559	N.S.
Diencephalon	1.5606	0.2370	20.2213	93.9267	N.S.
Optic tectum	2.7117	0.4442	14.5224	91.9725	N.S.

4.2.1.2 Daily rhythms of *pomc_a* mRNA expression

According to the results obtained from the preliminary PCR screening, the daily rhythms of *pomc_a* were analysed in telencephalon, diencephalon and pituitary gland.

In the telencephalon, daily expression of *pomc_a* did not show statistical differences by ANOVA neither when the specimens were fed at ML nor when fed at MD or at RND times. However, in the three feeding regimes *pomc_a* relative expression tends to increase at the beginning of the night followed by a decrease during the dark phase (Figure 4.5). Interestingly, the expression profile was significantly rhythmic as revealed by the Cosinor analysis in the group fed at ML, with the acrophase placed at ZT12.29 (Figure 4.5, Table 4.2).

In the diencephalon, *pomc_a* mRNA levels did not exhibited statistical differences in any of the diencephalic groups as showed by the Cosinor analysis (Table 4.2).

Regarding the pituitary gland, significant daily variations in *pomc_a* mRNA expression were revealed by ANOVA only in specimens fed at MD (Figure 4.5). The expression profile was rhythmic in the same group, as revealed by the Cosinor analysis (Table 4.2). This gene exhibited the lowest mRNA levels during the first hours of the day, then increasing its expression to reach the highest expression value during the end of light phase, at ZT9.95 (Figure 4.5). Subsequently, *pomc_a* expression significantly decreased at the beginning of the dark phase and was maintained low during the rest of the night (Figure 4.5).

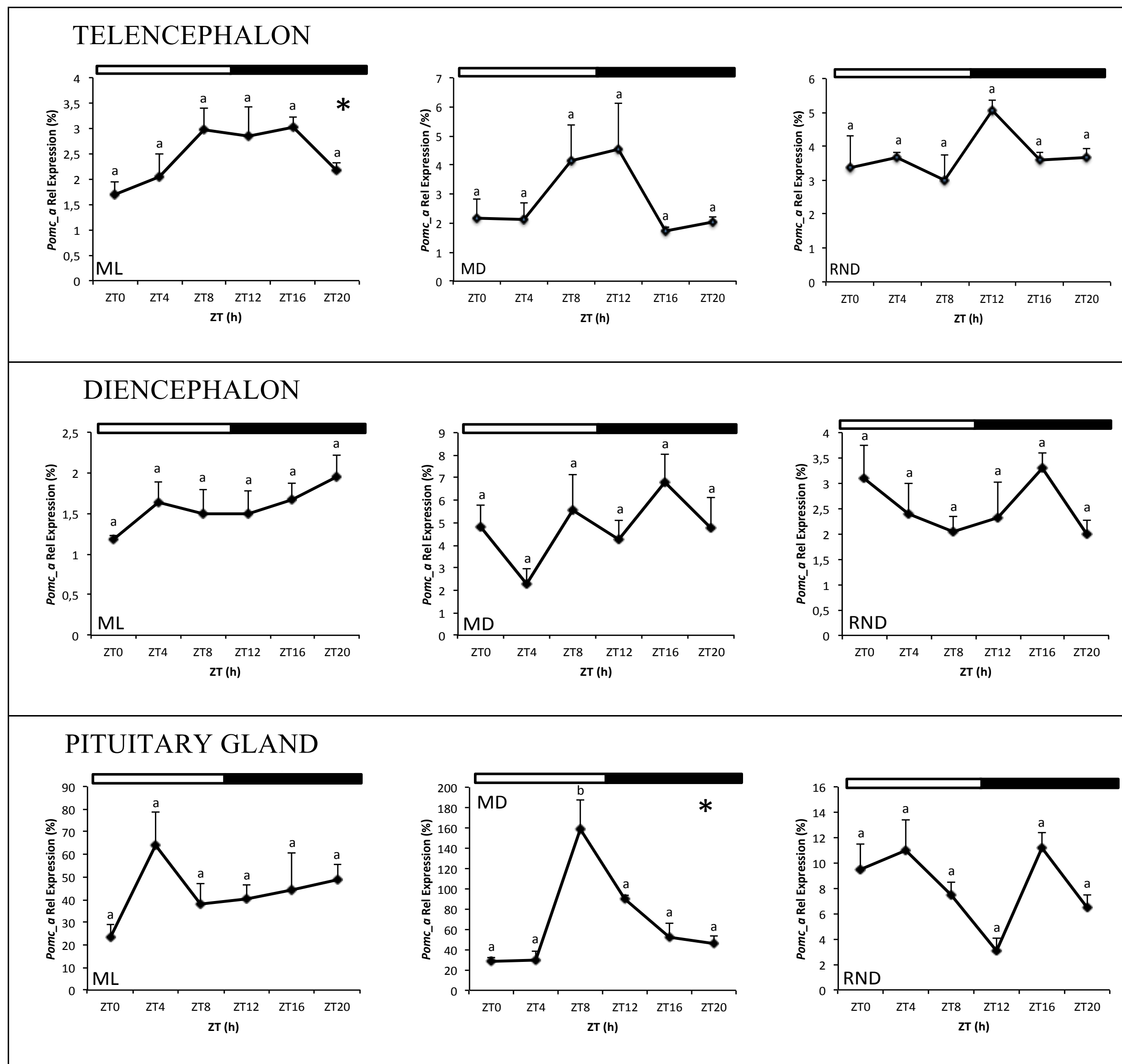

Figure 4.5. Relative expression of *pomc_a* gene in telencephalon, diencephalon and pituitary gland of Senegalese sole under LD 12:12 cycles and fed at midligh (ML), middark (MD) or at random (RND). Different letters indicate statistically significant differences (ANOVA, p<0.05). The black and the white bars at the top of the graphics represent the light and dark phases. Asterisks represent rhythmic expression (Cosinor, p<0.05). ZT indicates zeitgeber time (hours).

Table 4.2. Parameters estimated by the Cosinor analysis for *pomc_a* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at midlight (ML), middark (MD) or random (RND) times**.** The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant rhythm (p>0.05). Asterisks indicate significant rhythms (p<0.05).

***pomc_a* LD CONDITIONS**					
ML GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	2.4568	0.6772	12.2962	93.05334	* 0.0151
Diencephalon	1.5765	0.1453	16.7403	92.1599	N.S.
Pituitary	43.0250	2.6533	6.7521	79.2939	N.S.
MD GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	2.8724	1.2880	9.8146	76.0229	N.S.
Diencephalon	4.7678	1.3051	15.9505	82.6249	N.S.
Pituitary	67.3903	50.2686	9.9522	80.4059	* 0.0022
RND GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	3.7217	0.4814	13.4879	92.3608	N.S.
Diencephalon	2.5240	0.2648	19.4544	86.6890	N.S.
Pituitary	8.1096	1.9860	0.4514	77.3901	N.S.

4.2.1.3 Daily rhythms of *pomc_b* mRNA expression

The daily expression of *pomc_b* transcript was analysed in telencephalon, diencephalon and optic tectum. The results in the pituitary could not be showed because the existence of methodological problems in qPCR analysis.

In the telencephalon, daily variations in *pomc_b* mRNA expression were revealed by the statistical analysis only in specimens fed at ML (Figure 4.6). Moreover, *pomc_b* expression profile in this group displayed a significant daily rhythm, showing its highest expression value in the first half of the dark phase at ZT16.46 (Table 4.3). This peak of expression was followed by a significant decrease in transcript levels, which were maintained low during the day and the beginning of the night (Figure 4.6). No significant

daily rhythms in *pomc_b* mRNA expression were observed in fish under MD or RND feeding regimes (Table 4.3).

Concerning the diencephalon, and as reported for the other genes analysed, statistical differences in *pomc_b* mRNA levels were not observed and significant daily rhythms were neither evident in any feeding group (Figure 4.6 Table 4.3).

Finally, regarding the optic tectum, ANOVA analysis revealed significant daily variations in *pomc_b* expression in animals fed at ML and RND times (Figure 4.6). In the case of the RND group, although a significant increment in *pomc_b* transcript level was detected by the end of the light phase followed by a decrease during the night, no daily rhythm was found (Figure 4.6). In contrast, the ML group also showed a significant daily rhythm by Cosinor, reaching the acrophase at ZT14.56 (Table 4.3). After this peak, a significant reduction in transcript levels was observed at the end of the night, and low mRNA levels were maintained during the day (Figure 4.6).

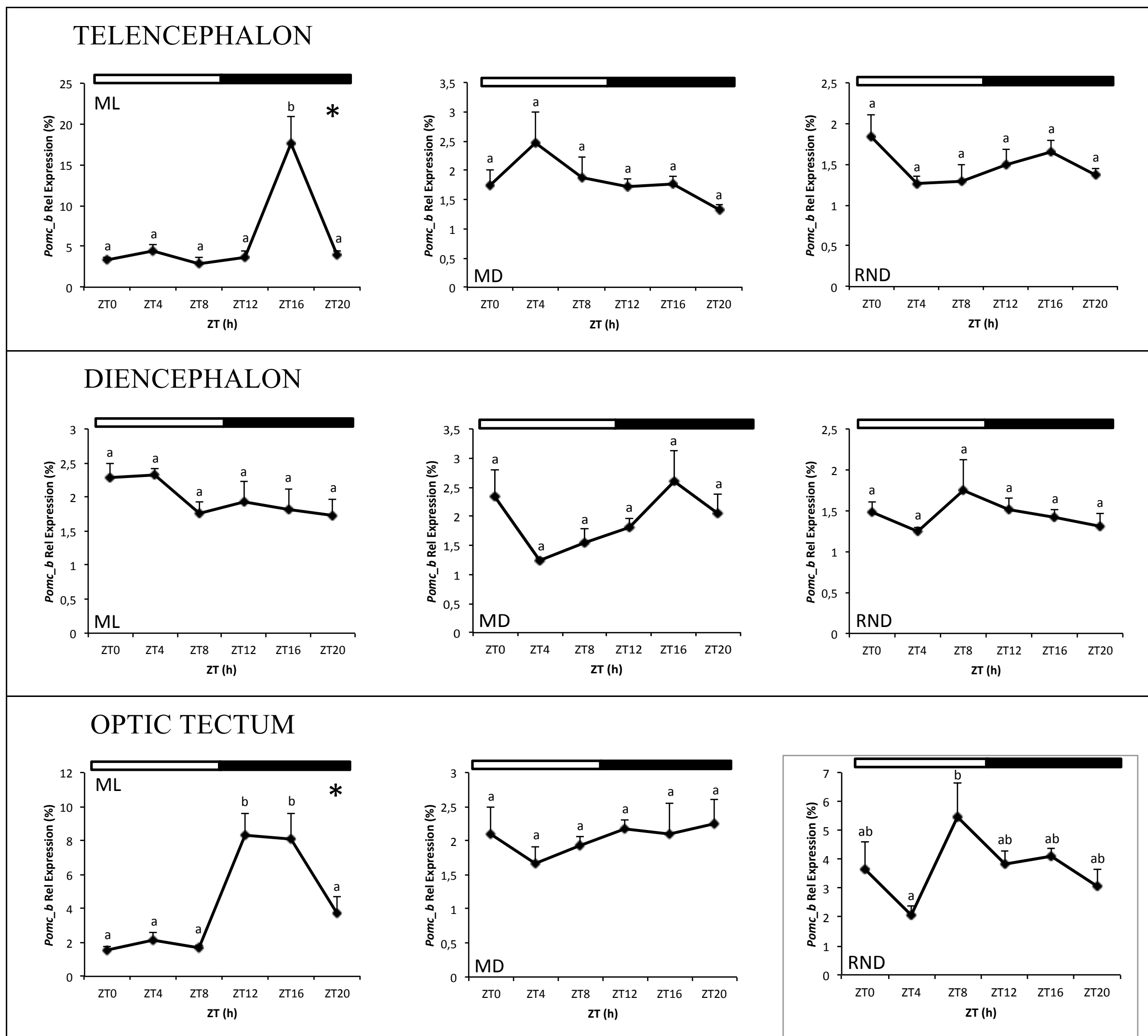

Figure 4.6. Relative expression of *pomc_b* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under LD 12:12 cycles and fed at midligh (ML), middark (MD) or at random (RND). Different letters indicate statistically significant differences (ANOVA, $p<0.05$). The black and the white bars at the top of the graphics represent the light and dark phases. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). ZT indicates zeitgeber time (hours).

Table 4.3. Parameters estimated by the Cosinor analysis for *pomc_b* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at midlight (ML), middark (MD) or random (RND) times**.** The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant rhythm (p>0.05). Asterisks indicate significant rhythms (p<0.05).

***pomc_b* LD CONDITIONS**					
ML GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	6.1974	4.9164	16.4672	68.1288	* 0.0140
Diencephalon	1.9675	0.2641	2.3933	95.7421	N.S.
Optic tectum	4.2358	3.7034	14.5695	86.3146	* 2.4943e-05
MD GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	1.8004	0.3816	5.5481	91.2331	N.S.
Diencephalon	1.9319	0.5405	18.2784	89.0192	N.S.
Optic tectum	2.0339	0.2227	17.2564	93.1225	N.S.
RND GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	1.4855	0.1451	19.5830	94.7057	N.S.
Diencephalon	1.4528	0.1341	9.6755	94.3584	N.S.
Optic tectum	3.6850	0.8029	11.4910	85.6888	N.S.

4.2.2 Daily expression *of crh, pomc_a* and *pomc_b* in Senegalese sole reared under DD conditions and different feeding schedules.

4.2.2.1 Daily rhythms of *crh* mRNA expression

As for the LD experiment, the daily rhythms of *crh* were analysed in telencephalon, diencephalon and optic tectum, according to the results obtained from the preliminary PCR screening (Figure 4.7).

In the telencephalon, only animals fed at RND exhibited significant daily variations in *crh* transcript levels as evidenced by ANOVA and Cosinor analyses (Figure 4.7, Table 4.4). *Crh* mRNA expression was low during the subjective daytime and increased

progressively at the end of the day and at the beginning of the subjective night, reaching a peak at the end of the subjective night (CT19.16).

Concerning the diencephalon, *crh* mRNA levels did not show statistical differences (ANOVA $p>0.05$) neither when the specimens were fed at sML (subjective mid light) nor when fed at RND (Figure 4.7). Cosinor analysis also revealed the absence of rhythmic expression under both experimental conditions in this tissue (Table 4.4).

In the optic tectum, statistical analyses (both ANOVA and Cosinor) revealed significant daily and rhythmic variations in *crh* expression in animals fed at RND, but not in those fed at sML (Figure 4.7, Table 4.4). The gene expression was maintained low during the subjective daytime and increased during the first half of the subjective night (acrophase at CT12.62), decreasing significantly thereafter (Figure 4.7, Table 4.4).

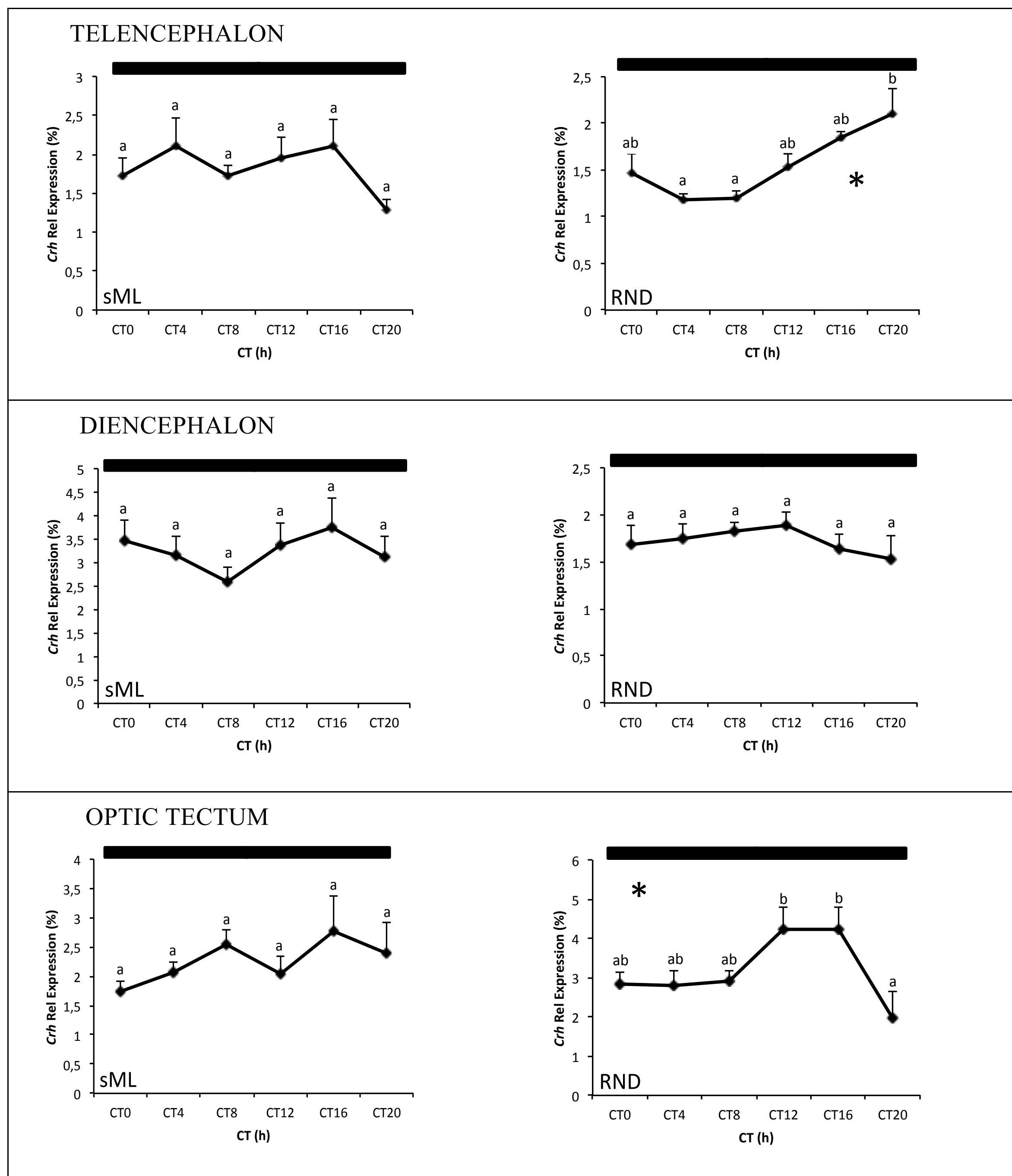

Figure 4.7. Relative expression of *crh* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under constant darkness condition DD and fed at subjectieve (sML) or at random (RND). Different letters indicate statistically significant differences (ANOVA, $p<0.05$). The black bars at the top of the graphics represent the constant dark phase. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). CT indicates circadian time (hours).

Table 4.4. Parameters estimated by the Cosinor analysis for *crh* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under DD conditions and fed at the subjective midlight period (sML group) or at RND times (RND group)**.** The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant rhythm (p>0.05). Asterisks indicate significant rhythms (p<0.05).

***crh* DD CONDITIONS**					
sML GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	1.8160	0.1956	9.2376	92.4606	N.S.
Diencephalon	3.2367	0.3207	18.1864	93.5546	N.S.
Optic tectum	2.2542	0.2938	14.2697	91.1357	N.S.
RND GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	1.4821	0.3709	19.1639	95.7664	* 0.0047
Diencephalon	1.7210	0.1544	8.5817	97.1078	N.S.
Optic tectum	3.1655	0.8825	12.6238	91.2536	* 0.0314

4.2.2.2 Daily rhythms of *pomc_a* mRNA expression

As for the LD experiment, the daily rhythms of *pomc_a* under DD were analysed in telencephalon, diencephalon and pituitary gland (Figure 4.8).

In the telencephalon, only animals fed at sML exhibited significant daily variations in *pomc_a* transcript levels as evidenced by ANOVA analysis (Figure 4.8) but rhythmic *pomc_a* expression was demonstrated in both sML and RND groups by Cosinor (Table 4.5). In sML group, *pomc_a* reached its highest expression value in the subjective early morning (CT0.87), immediately followed by a significant reduction in transcript levels at CT8 and CT12, and then remaining at intermediate levels along the subjective night (Figure 4.8). In contrast, rhythmic telencephalic expression of *pomc_a* in RND group reached a peak at the beginning of the subjective night (CT15.25, Table 4.5).

Concerning the diencephalon, statistical analyses revealed daily variations in *pomc_a* expression in animals fed at both sML and RND times (Figure 4.8). Cosinor analysis also evidenced significant rhythms in both feeding regimes at DD condition (Table 4.5). In the

sML group, *pomc_a* reached its highest expression value at the subjective night, peaking at CT16.88 (Table 4.5). Under RND feeding regime the highest expression value was slightly advanced in relation to the sML group (acrophase at CT13.59). Subsequently, *pomc_a* expression significantly decreased in both groups (Figure 4.8).

Finally, in the pituitary gland, significant statistical differences in *pomc_a* daily mRNA expression were observed in animals fed both at sML and at RND, as evidenced by ANOVA (Figure 4.8). However, daily profiles in both conditions were markedly different. In animals fed at sML, a significant increase in *pomc_a* relative expression was observed from CT0 (lowest value) to CT20 (peak) whereas in animals fed at RND transcript levels peaked at CT0, decreasing thereafter (Figure 4.8). No apparent rhythmic profile was evident by Cosinor neither in sML nor RND group (Table 4.5).

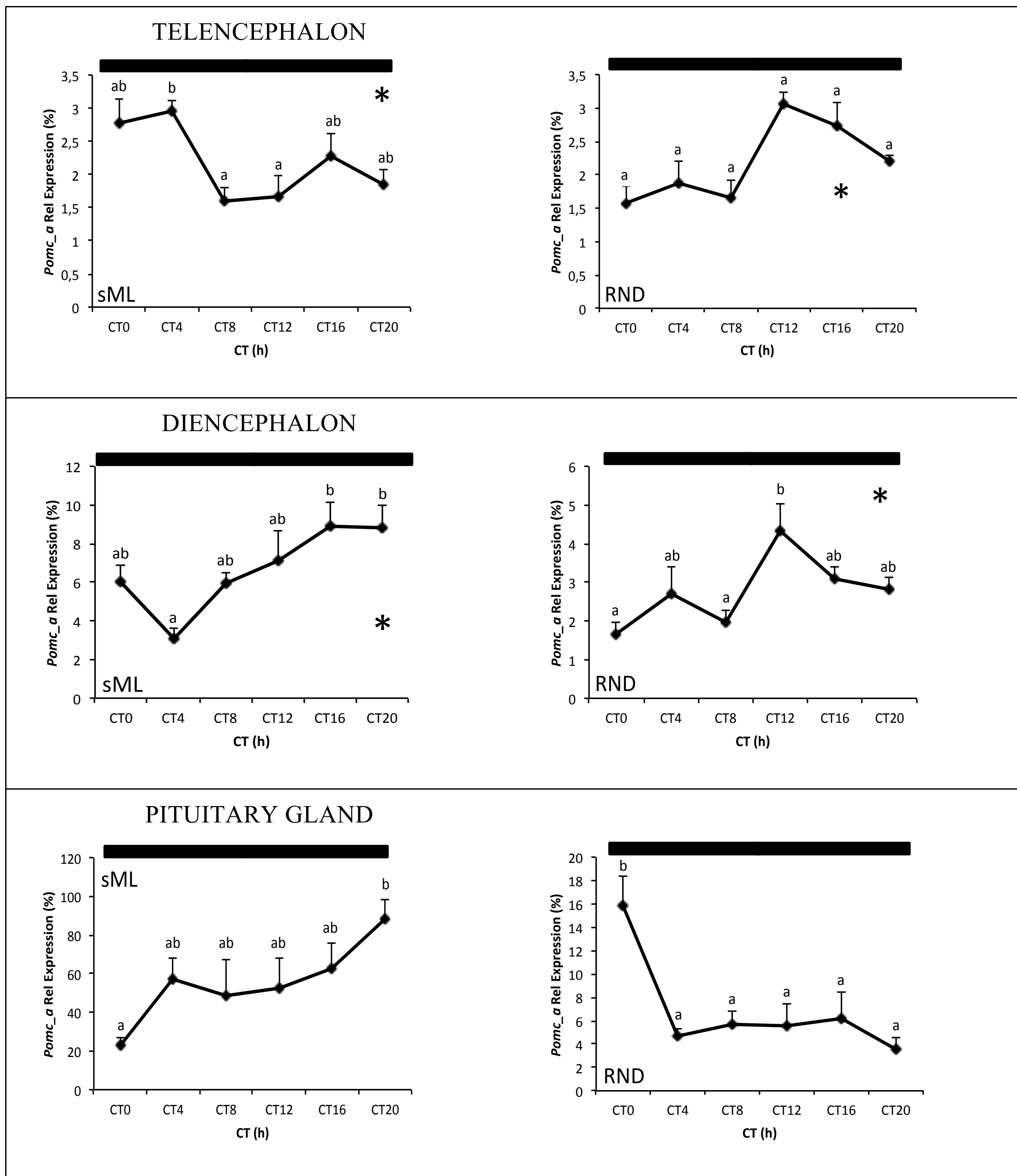

Figure 4.8. Relative expression of *pomc_a* gene in telencephalon, diencephalon and pituitary gland of Senegalese sole under constant darkness condition DD and fed subjective ML (sML) or at random (RND). Different letters indicate statistically significant differences (ANOVA, $p<0.05$). The black bars at the top of the graphics represent the constant dark phase. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). CT indicates circadian time (hours).

Table 4.5. Parameters estimated by the Cosinor analysis for *pomc_a* mRNA relative expression in telencephalon, diencephalon and pituitary of *Solea Senegalensis* under DD conditions and fed at the subjective midlight period (sML group) or at RND times (RND group)**.** The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant rhythm (p>0.05). Asterisks indicate significant rhythms (p<0.05).

***pomc_a* LD CONDITIONS**					
sML GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	2.1842	0.5311	0.8738	93.0083	*0.0334
Diencephalon	6.5984	2.7043	16.8765	92.5852	*0.0012
Pituitary	54.6305	14.3413	16.4252	78.7987	N.S.
RND GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	2.2600	0.7105	15.2472	90.9568	*0.0170
Diencephalon	2.7558	0.8899	13.5902	88.5342	*0.0333
Pituitary	6.8243	2.5228	0.7007	71.8086	N.S.

4.2.2.3 Daily rhythms of *pomc_b* mRNA expression

As for the LD experiment, the daily rhythms of *pomc_b* were analysed in telencephalon, diencephalon and optic tectum (Figure 4.9).

In the telencephalon, daily expression of *pomc_b* transcript levels did not show statistical differences (ANOVA, p>0.05) neither when the specimens were fed at sML nor when fed at RND (Figure 4.9). As for the telencephalon, statistical differences in *pomc_b* expression were neither evident in the diencephalon or the optic tectum, in fish fed under both (sML and RND) regimes (Figure 4.9). Although no significant differences were detected in the three central areas analysed, very similar daily *pomc_b* expression profiles were observed in the sML group in these tissues, with a peak in transcript levels at CT 16 (subjective night, Figure 4.9).

Cosinor analysis did not reveal rhythmic *pomc_b* expression in none of the neural tissues analysed irrespective of the feeding regime (Table 4.6).

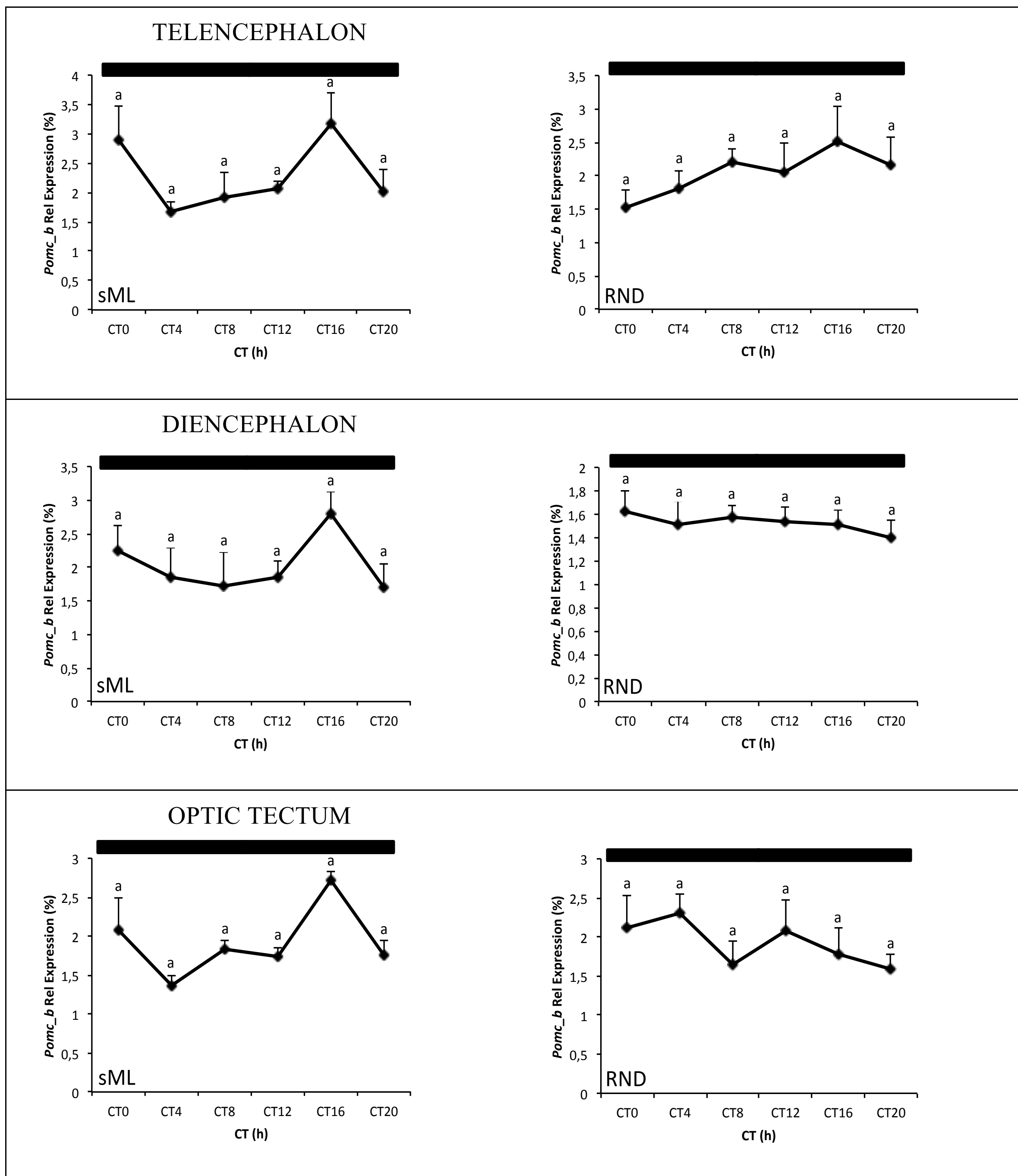

Figure 4.9. Relative expression of *pomc_b* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under constant darkness condition DD and fed at subjective ML (sML) or at random (RND). Different letters indicate statistically significant differences (ANOVA, $p<0.05$). The black bars at the top of the graphics represent the constant dark phase. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). CT indicates circadian time (hours).

Table 4.6. Parameters estimated by the Cosinor analysis for *pomc_b* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at the subjective midlight period (sML group) or at RND times (RND group). The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant rhythm (p>0.05). Asterisks indicate significant rhythms (p<0.05).

***pomc_b* LD CONDITIONS**					
ML GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	2.2896	0.4566	18.3814	88.4706	N.S.
Diencephalon	2.0259	0.2676	17.6590	88.1352	N.S.
Optic tectum	1.9114	0.3871	16.7574	88.2233	N.S.
RND GROUP	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
Telencephalon	2.0443	0.3582	14.0978	90.3413	N.S.
Diencephalon	1.5226	0.0510	6.0219	96.9131	N.S.
Optic tectum	1.9156	0.1915	4.1725	89.8554	N.S.

4.3 Influence of light spectra and developmental stage in daily expression of anorexigenic genes in Senegalese sole larvae

We have investigated the impact of light spectra on *crh, pomc_a* and *pomc_b* mRNA daily expression in *Solea senegalensis* during early development. To this purpose, Senegalese sole larvae were reared under LD (12L:12D) cycles of white, blue and red lights, and they were collected every 4 hours at 9, 12 and 25 days post hatching (DPH).

4.3.1 Influence of light spectra on *crh* mRNA daily expression

Larvae sampled at 9 DPH displayed statistical differences in *crh* mRNA expression when reared under LD cycles of white light (LD_W), blue (LD_B) and red (LD_R) wavelengths (Figure 4.10). The highest expression value was observed during the light phase in larvae under LD cycles of white light and LD cycles of blue wavelengths. *Crh* expression then significantly decreased until reaching the lowest levels during the night (Figure 4.10). However, the highest expression value in larvae maintained under LD cycles of red wavelength was detected during the night phase, reaching the lowest expression values at the end of the night and during the day (Figure 4.10). Moreover,

daily expression profiles were found to be significantly rhythmic for both larvae maintained under LD cycles of blue and red lights, as revealed by Cosinor analysis, being the acrophases placed at ZT3.92 and ZT12.86 respectively (Table 4.7).

Larvae sampled at 12 DPH exhibited significant daily variations in *crh* mRNA expression when kept in LD_W and LD_B but not in LD_R conditions, where statistical differences were not observed (Figure 4.10). Under LD_W, a significant increase in *crh* expression level was found at the end of the light phase (ZT8-ZT12), reaching its lowest value near midnight, which was maintained until sunrise (Figure 4.10). In the case of LD_B conditions, high *crh* expression levels were maintained during the light phase and a significant decrease was also found until reach the nadir near midnight (Figure 4.10). Cosinor analysis revealed a rhythmic *crh* expression in all three light spectra conditions, with the acrophases found during the light phase at ZT8.17, ZT6.47, ZT7.33 for LD_W, LD_B and LD_R respectively (Table 4.7).

At 25 DPH, only larvae under LD_W conditions displayed statistical differences between time points, showing a significant rise at the beginning of the night, as revealed by ANOVA (Figure 4.10). Low expression values were maintained during the rest of the night and during the light phase. This profile was also the only one found to be significantly rhythmic, peaking at ZT10.09 (Table 4.7).

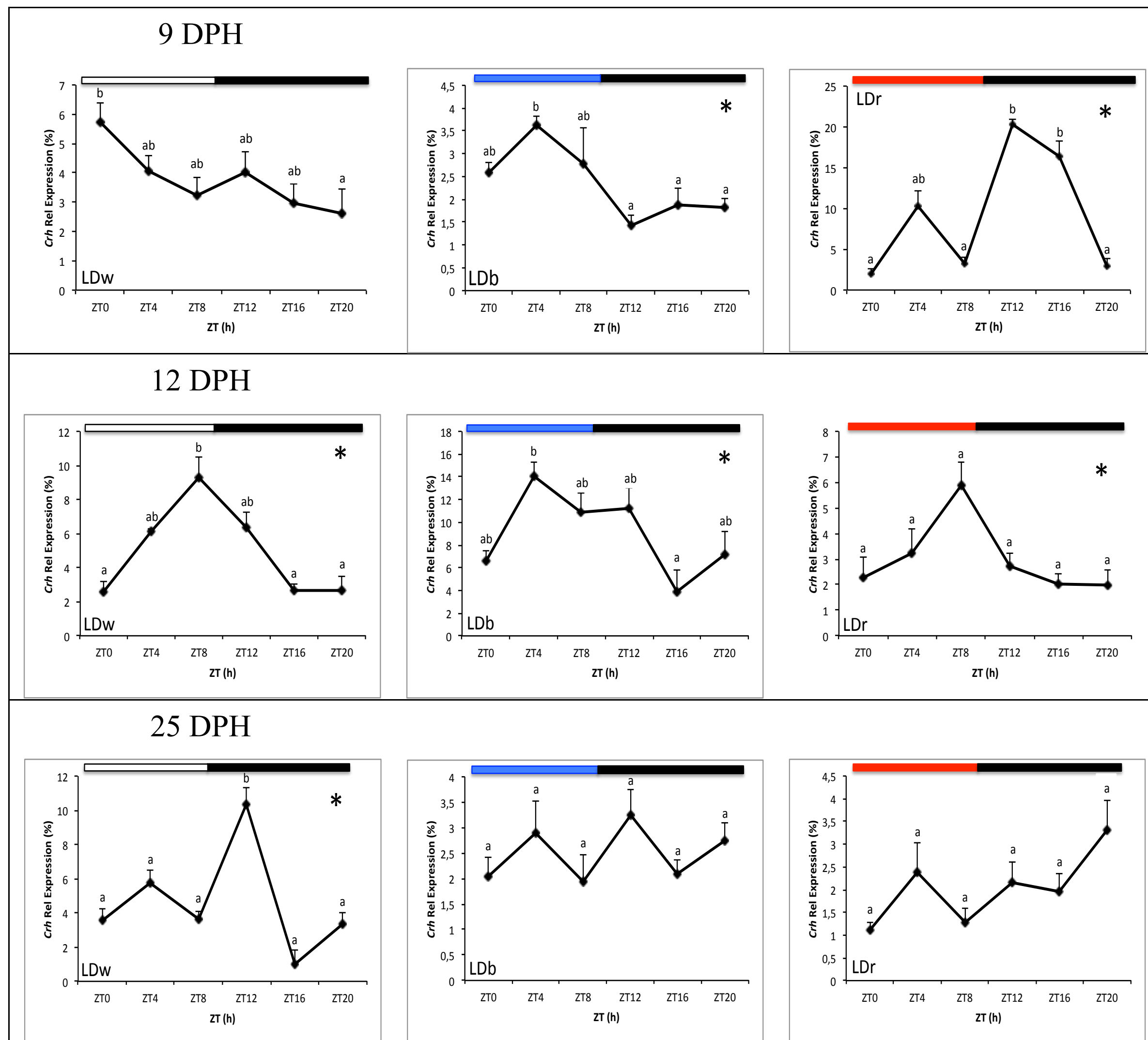

Figure 4.10. Relative expression of *crh* gene in Senegalese sole larvae reared under LD 12:12 cycles with white (LD_W), blue (LD_B) or red light (LD_R) spectra. The letters indicate statistically significant differences (ANOVA, $p<0.05$). The black bars at the top of the graphics represent the dark phases, while the white, blue and red bars indicate the different light spectra of the light phases. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). ZT indicates zeitgeber time (hours).

Table 4.7. Parameters estimated by the Cosinor analysis for *crh* mRNA relative expression in *Solea Senegalensis* larvae kept under 12L:12D cycles of white (LD_W), blue (LD_B, λ peak=463nm) and red (LD_R, λ peak=685nm) lights. Samples during 9, 12 and 25 days post hatching (DPH) were obtained every 4 hours. The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant daily rhythm (Cosinor p-value>0.05). Asterisks indicate significant daily rhythms (Cosinor p-value<0.05).

crh					
LD_W	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	3.7675	0.8217	2.5373	87.5201	N.S.
12 DPH	4.9767	3.4024	8.1768	92.5929	* 5.4181e-06
25 DPH	4.6225	1.9168	10.0937	83.942	* 0.0263
LD_B	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	2.3727	0.9202	3.9266	92.6363	*0.0020
12 DPH	8.9660	4.0613	6.4756	86.4265	*0.0075
25 DPH	2.4918	0.1306	11.9657	87.7638	N.S.
LD_R	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	9.2057	7.3473	12.8621	76.9929	* 0.0019
12 DPH	3.0039	1.5214	7.3394	84.405	* 0.0127
25 DPH	2.0579	0.5096	18.2605	78.0064	N.S.

4.3.2 Influence of light spectra on *pomc_a* mRNA daily expression

Senegalese sole larvae sampled at 9 DPH, displayed significant daily variations in *pomc_a* mRNA expression under LD_W, LD_B and LD_R conditions (Figure 4.11). In LD_W and LD_B groups, *pomc_a* reached its highest value during the light phase, whereas the lowest transcript levels were observed during the night (Figure 4.11). However, in the LD_R group a significant increase in expression was observed during the night, being mRNA levels lower during daytime. Furthermore, Cosinor analysis revealed

that *pomc_a* profiles were rhythmic only in larvae reared under LD_B and LD_R, showing their acrophases at the beginning of the day (ZT2.36) and the night (ZT15.03), respectively (Table 4.8).

At 12 DPH, statistical analysis revealed significant differences in all three light conditions analysed (Figure 4.11). Concerning LD_W and LD_B regimes, *pomc_a* expression displayed a significant increase throughout the day and peaks at the beginning of the night, decreasing thereafter (Figure 4.11). However, under LD_R conditions highest mRNA levels were achieved in the first half of the light phase (ZT4), being *pomc_a* expression values lower in the remaining time points (Figure 4.11). *Pomc_a* profiles were significantly rhythmic in LD_W and LD_B groups, with similar acrophases observed in the second half of light phase, at ZT8.96 and ZT9.53, respectively (Table 4.8). No significant daily rhythm in *pomc_a* expression was observed under LD_R conditions (Table 4.8).

Larvae sampled at 25 DPH displayed, as in metamorphic animals, statistical differences in *pomc_a* daily expression under LD_W, LD_B and LD_R conditions (Figure 4.11). A progressive increment of *pomc_a* mRNA levels was observed from the onset of the day to the beginning of the night, peaking at the day-night transition, i.e. at the beginning of the dark period in larvae kept under LD_W and LD_B cycles and at the end of the light period in the LD_R group (Figure 4.11). In contrast to that observed in LD_W and LD_B conditions, nocturnal transcript levels were maintained high in LD_R regime (Figure 4.11). Cosinor analysis revealed significant daily rhythms in *pomc_a* expression only in larvae kept under LD cycles of white and blue lights, with acrophases observed at ZT10.83 and ZT14.78, respectively (Table 4.8).

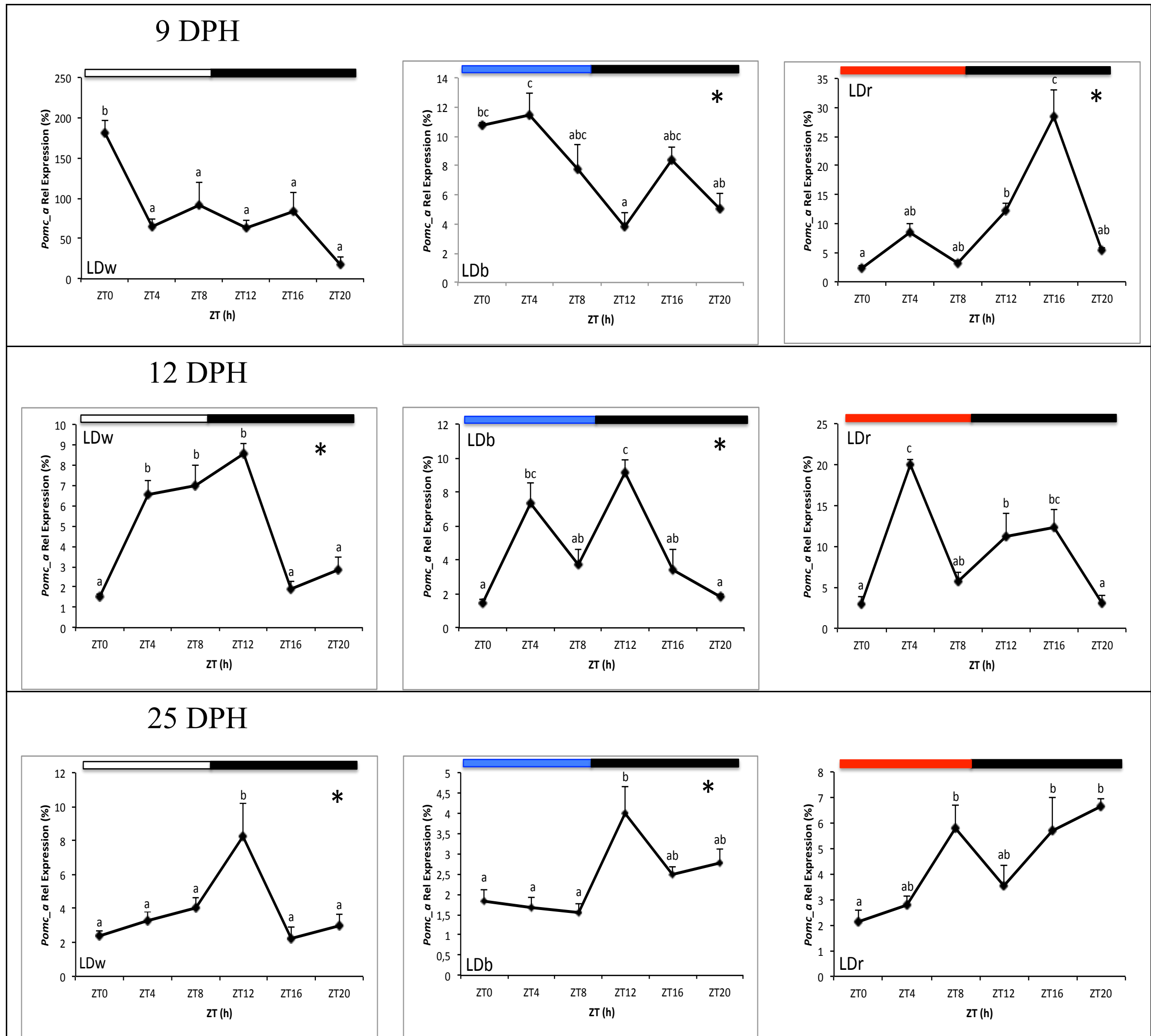

Figure 4.11. Relative expression of *pomc_a* gene in Senegalese sole larvae reared under LD 12:12 cycles with white, (LD_W), blue (LD_B) or red light (LD_R) spectra. The letters indicate statistically significant differences (ANOVA, $p<0.05$). The black bars at the top of the graphics represent the dark phases, while the white, blue and red bars indicate the different light spectra of the light phases. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). ZT indicates zeitgeber time (hours).

Table 4.8. Parameters estimated by the Cosinor analysis for *pomc_a* mRNA relative expression in *Solea Senegalensis* larvae kept under 12L:12D cycles of white (LD_W), blue (LD_B, λ peak=463nm) and red (LD_R, λ peak=685nm) lights. Larval pools during 9, 12 and 25 days post hatching (DPH) were obtained every 4 hours. The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant daily rhythm (Cosinor p-value>0.05). Asterisks indicate significant daily rhythms (Cosinor p-value<0.05).

pomc_a					
LD_W	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	83.8840	28.7777	2.2350	71.5378	N.S.
12 DPH	4.8045	3.3437	8.9690	89.3868	*5.4518e-05
25 DPH	3.8746	2.0569	10.8350	78.5726	*0.0197
LD_B	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	7.8609	2.8929	2.3661	87.6931	*0.0199
12 DPH	4.4774	2.7578	9.5390	79.0116	*0.0117
25 DPH	2.3784	0.8726	14.7896	89.1778	*0.0123
LD_R	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	9.9781	9.0094	15.0367	72.9139	*0.0023
12 DPH	8.7493	3.2228	9.1207	69.3485	N.S.
25 DPH	4.28538	1.2824	14.7060	85.5004	N.S.

4.3.3 Influence of light spectra on *pomc_b* mRNA daily expression

At 9 DPH, statistical differences were revealed by ANOVA in *pomc_b* mRNA daily expression in larvae reared under LD_W, LD_B and LD_R conditions (Figure 4.12). In the first two light regimes, higher expression values were detected during the light phase, being the lowest levels found during the night. In contrast, *pomc_b* expression profile was inverted in larvae kept under LD_R cycles, with highest values detected during the night and lowest transcript levels during the light phase (Figure 4.12). *Pomc_b* expression profile exhibited a significant daily rhythm only in LD_R group, as evidenced by Cosinor analysis, with the acrophase placed during the night at ZT13.60 (Table 4.9).

Regarding 12 DPH, *pomc_b* mRNA daily expression also displayed statistical differences in larvae reared under the three light regimes (Figure 4.12). This gene reached its highest expression level during the day-night transition (LD_W and LD_B groups) or in the first half of the dark phase (LD_R group) (Figure 4.12). Cosinor analysis revealed the existence of a significant daily rhythm in *pomc_b* expression only in larvae maintained under LD_W, peaking during the end of the light phase at ZT10.17 (Table 4.9).

Finally, Senegalese sole larvae sampled at 25 DPH showed statistical differences when reared under LD_W and LD_B conditions (Figure 4.12). In both cases, *pomc_b* expression reached its maximum level during the dark phase, although in LD_W condition it occurred at the end of the night whereas in LD_B the expression peaked in the day-night transition (Figure 4.12). Cosinor analysis revealed a significant rhythmic profile of this gene in the LD_B group, with the acrophase at ZT13.91, whereas LD_W and LD_B groups did not exhibit significant daily rhythms in *pomc_b* expression at this post-metamorphic stage (Table 4.9).

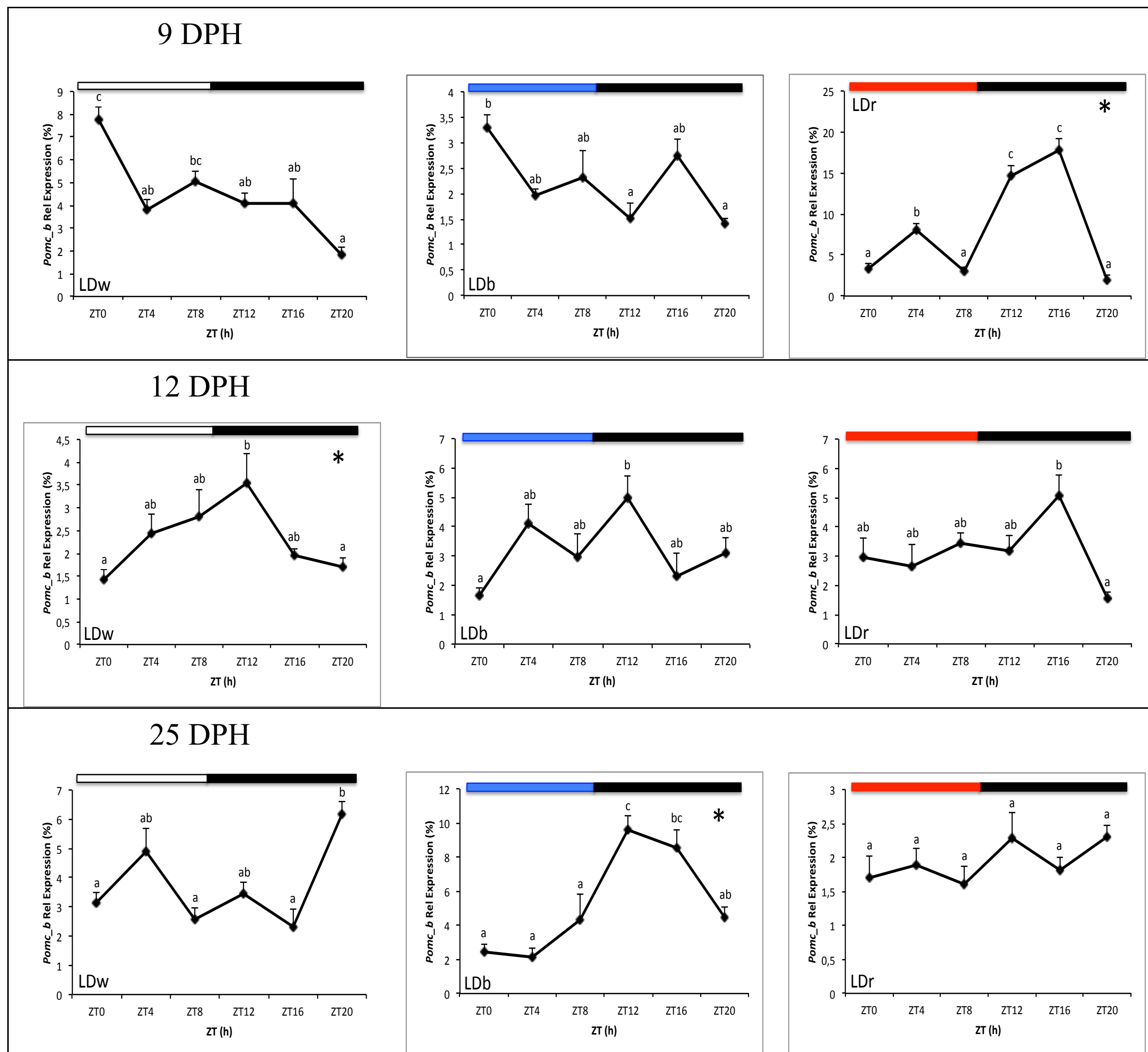

Figure 4.12. Relative expression of *pomc_b* gene in Senegalese sole larvae reared under LD 12:12 cycle with (LD_W), blue (LD_B) or red light (LD_R) spectra. The letters indicate statistically significant differences (ANOVA, $p<0.05$). The black bars at the top of the graphics represent the dark phases, while the white, blue and red bars indicate the different light spectra of the light phases. Asterisks represent rhythmic expression (Cosinor, $p<0.05$). ZT indicates zeitgeber time (hours).

Table 4.9. Parameters estimated by the Cosinor analysis for *pomc_b* mRNA relative expression in *Solea Senegalensis* larvae kept under 12L:12D cycles of white (LDw), blue (LDb, λ peak=463nm) and red (LDr, λ peak=685nm) lights. Larval pools during 9, 12 and 25 days post hatching (DPH) were obtained every 4 hours. The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., no significant daily rhythm (Cosinor p-value>0.05). Asterisks indicate significant daily rhythms (Cosinor p-value<0.05).

pomc_b					
LD_W	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	4.4273	1.0533	3.5098	84.4802	N.S.
12 DPH	2.3273	0.9395	10.1724	90.6433	* 0.0035
25 DPH	3.7547	0.9775	22.8362	84.5607	N.S.
LD_B	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	2.2082	0.3109	0.4215	87.9143	N.S.
12 DPH	3.1879	0.9396	9.9569	83.7599	N.S.
25 DPH	5.2251	3.9278	13.9162	92.0899	* 1.5985e-06
LD_R	**Mesor**	**Amplitude**	**Acrophase**	**Variance %**	**Significance**
9 DPH	8.1301	6.1142	13.6025	80.1718	* 0.0012
12 DPH	3.1055	0.8821	12.6469	85.3162	N.S.
25 DPH	1.9333	0.1889	16.7821	90.8885	N.S.

5 DISCUSSION

In this study, we have tried to enlarge our knowledge about the endocrine mechanisms that regulate feeding and growth in cultured fish, which could be relevant for the improvement of fish farming conditions and feeding strategies. In order to reach this goal, we have investigated some anorexigenic hormones (CRH, POMCa, POMCb) in *Solea senegalensis*, an important species in the Mediterranean aquaculture, by focusing on their synchronization to different feeding regimes (diurnal *vs* nocturnal and random feeding) and photoperiod (light-dark cycle *vs* constant darkness). Therefore, results obtained could also be relevant from a chronobiological perspective.

Further experiments were carried out on larvae of Senegalese Sole, in order to evaluate for the first time the influence of the light features (spectra) on the anorexigenic hormone modulation during early development and metamorphosis.

5.1 Central expression of anorexigenic genes in Senegalese sole

In a first approach, we have conducted a preliminary brain screening to identify the expression of each gene in 6 different cerebral areas, i.e., olfactory bulbs, telencephalon, diencephalon, optic tectum, cerebellum and medulla, as well as in the pituitary gland.

Concerning the *crh* gene, although the diencephalon exhibited one of the highest transcript levels, *crh* expression was evident in most central areas analysed and the statistical analysis did not reveal any significant difference between them. Our results agree with those obtained in zebrafish (*Danio rerio*) by whole-mount *in situ* hybridization histochemistry, revealing the presence of CRH-synthesizing cells in the telencephalon, preoptic region, hypothalamus, posterior tuberculum, thalamus, epiphysis, midbrain tegmentum, and rostral hindbrain (Chandrasekar et al., 2007). The localization of *crh* mRNA within the diencephalic preoptic area and hypothalamus is consistent with the central role of CRH in the teleost stress response and feeding control. The widespread distribution of CRH-synthesizing cells outside these diencephalic areas suggests additional functions of CRH in the fish brain. Relying on these results and on those obtained from experiments conducted in goldfish (*Carassius auratus*) by Matsuda (2013) and Wong et al., (2013), in zebrafish *(Danio rerio*) by Chandrasekar et al., (2007), and in *Oreochromis mossambicus* by Pepels et al., (2002), we have decided to analyse the effects of photoperiod and feeding regimes on *crh* mRNA expression in the telencephalon, diencephalon and optic tectum of Senegalese sole.

POMC is the precursor of several hormones involved in physiological functions including food intake. The presence of two *pomc* paralogues has been reported in Senegalese sole (Wunderink et al., 2012) as well as in other fish species such as carp (*Cyprinus carpio*), zebrafish and the pleuronectiforms japanese and barfin flounder (*Paralichthys olivaceus* and *Verasper moseri*) (Arends et al., 1998; González-Nuñez et al., 2003; Kim et al., 2009; Takahashi et al., 2006). In this study, the *pomc* expression resulted much more evident in the pituitary gland, in accordance with results reported by other authors (Castro and Morrison, 1997; Smith and Funder, 1988; Arends et al. 1998; Hui et al. 1999). Regarding the *pomc* genes, few studies have focused on *pomc* expression on fish brain. Our Real Time quantitative PCR analysis showed an important expression of both *pomc_a* and *pomc_b* genes in all central areas analysed including olfactory bulbs, telencephalon, diencephalon, optic tectum, cerebellum and medulla. These results are consistent with previous studies performed in fish, where *pomc* is highly expressed in the pituitary gland but also in the central nervous system, although at lower levels (Arends et al., 1998; Cardoso et al., 2011; González-Nuñez et al., 2003; Karsi et al., 2006; Kim et al., 2009). More detailed studies have shown that a diencephalic structure as the hypothalamus represents one of the main extra-pituitary *pomc*-expressing areas in carp, snakeskin gourami (*Trichopodus pectoralis*), goldfish and trout (Arends et al., 1998; Hui et al. 1999; Boonanuntanasarn et al., 2015; Leder and Silverstein, 2006). Accordingly, this area is also the neural region showing the highest *pomc_a* expression in Senegalese sole. The pituitary gland is closely attached to the hypothalamus via the neurohypophysary stalk, and both structures have a close relationship in endocrine control of almost all the physiological processes (Treier et al., 1996). Moreover, *pomc* expression has been detected in other discrete brain regions belonging to the telencephalon, mesencephalon and metencephalon of snakeskin (Boonanuntanasarn et al., 2015) and in the telencephalon, cerebellum, hypothalamus and optic tectum of rainbow trout (*Oncorhynchus mykiss*) (Leder and Silverstein, 2006). Interestingly, these brain areas also exhibited a conspicuous *pomc* mRNA expression in sole. Some of these areas could be linked to control feeding behaviour as it has been suggested in previous studies (Peter, 1979). Based on these results, and those obtained from the species mentioned before, we decided to analyse the effects of photoperiod and feeding regimes in *pomc a* and *pomc_b* mRNA expression in the telencephalon, diencephalon and pituitary of sole. Unfortunately, we had a methodological problem that prevented us from being able to address the analysis of *pomc_b* expression in the pituitary. Therefore, we decided to

enlarge *pomc b* study to the optic tectum of Senegalese sole, permitting us to compare *crh* and *pomc_b* expression in the same neural tissues.

5.2 Influence of photoperiod conditions and feeding time on daily expression of anorexigenic genes in Senegalese sole adult specimens

In vertebrates, the control of food intake is a complex phenomenon that involves numerous central and peripheral endocrine factors that regulate energy balance through multiple pathways involving orexigenic and anorexigenic hormones. In fish, there is a growing number of studies dealing with the neural control of feeding (see Peter, 1979; Demski, 1983 and Volkoff et al., 2005 for review). However, information about the possible influence of external cues on the daily expression of these hormones is rather scarce although considerable progress has been made in the analysis of the orexigenic and anorexigenic genes from a board taxonomic group of vertebrates and invertebrates (Takahashi and Kawauchi, 2006). Although light is the most prominent zeitgeber, several non-photic stimuli, such as food, have been also shown to entrain circadian rhythms. Indeed, feeding time can act not only on the SCN (the mammalian light-entrainable oscillator or LEO) and peripheral tissues, but also on the food-entrainable oscillator (FEO), which is not yet well-defined (Meijer and Rietveld, 1989), and whose anatomical location is still unknown (Stephan et al., 1979; Stephan, 2002; Davidson, 2006). In fish, data on properties of feeding entrainment support the hypothesis of the existence of a FEO, although it is still uncertain whether fish FEO and LEO are independent (Sánchez-Vázquez et al., 1997; Aranda et al., 2001).

In mammals, daily rhythms in the hypothalamus-pituitary-adrenal (HPA) axis have been previously described (Haus, 2007). The suprachiasmatic nucleus (SCN) plays an essential role in the control of HPA-axis rhythms, as ablation of this nucleus generates a loss of HPA-axis factors rhythms (Dickmeis, 2009). For instance, the rhythm in CRH secretion is thought to be controlled by the projections of the suprachiasmatic nucleus (SCN) to the CRH-producing neurons (Girotti et al., 2007). In fish, the knowledge of HPA-axis and its modulation by photoperiod and restricted food access is very limited. Most of the available works take into account the effects of food deprivation on the stress and the role of CRH as stress modulator (Wunderink et al., 2012; Bernier, 2006; Cachat et al., 2010) and as anorexigenic factor (de Pedro et al., 1997; Bernier and Peter, 2001; Volkoff et al., 2005; Matsuda, 2009). The role of POMC in the darkening camouflage, blind-side hypermelanosis, and appetite or the function of its POMC-derived peptides have also

been approached in several studies (Takahashi and Kawauchi, 2006; Kang and Kim, 2015). However, studies on their possible modulation by restricted food access are rather scarce and none of them has been conducted using different feeding regimes. To fill these gaps, in this study we have investigated how the mRNA expression of *crh* and *pomc* is influenced by photoperiod (LD vs DD) and different feeding regimes (fed at ML, MD or at RND times) in different brain areas of adult Senegalese sole. These anorexigenic genes have been recently cloned and characterized in this species in studies conducted by Wunderink et al., (2011, 2012).

Regarding *crh,* its expression was only rhythmic in schedule-fed fish in both telencephalon and optic tectum in animals reared under LD conditions, with the acrophases occurring during the dark phase irrespective of the feeding time. The acrophases, placed at ZT13.52 and ZT16.28 in telencephalon and optic tectum respectively, were coincident with the active phase of this species (Bayarri et al., 2004). These results are consistent with previous reports obtained in sole, where *crh* expression showed a rhythmic profile peaking at the beginning of the dark phase (López-Olmeda et al., 2013). However, sole *crh* expression profiles differed from diurnal species such as sea bream (*Sparus aurata*), where a daily rhythm of *crh* was observed, with the acrophase at the beginning of the light phase (Vera et al., 2014). Interestingly, in this study the diencephalon, which contains the light-entrainable oscillator (LEO) in mammals, did not exhibit daily *crh* rhythms in sole at any of the feeding regimes analysed under LD conditions suggesting that this area is not responsible of *crh* rhythmicity. The fact that in all examined tissues under LD conditions *crh* mRNA expression peaked almost always during the dark phase in both schedule-fed and random-fed fish suggests, at a first glance, that these rhythms are entrained by the light-dark cycle and exhibit some independence from the feeding time. After better exploring the expression in all feeding conditions, it is possible to observe differences between feeding groups. In animals fed at ML the gene expression was low during the day when they were fed, and afterwards it increased during the night. In specimens fed at MD, the expression was strongly decreased immediately before the feeding time (ZT18), thus anticipating and advising an influence of the feeding cue on *crh* daily expression, in addition to that determined by light-dark cycle. The crosstalk of light and feeding cycles, and the importance of feeding cues in the establishment of *crh* daily rhythms is reinforced by the fact that animals maintained under RND feeding regime lack *crh* rhythmic expression. However, CRH is not only related to

feeding, being also involved in the stress response, which increases *crh* expression in sole (Wunderink et al., 2011, 2012). Therefore, the absence of *crh* rhythms under RND feeding in sole could be related with the stress provoked by the randomly food supply.

The interpretation of studies of circadian endocrine rhythms is limited by the fact that samples are often collected from animals held in a LD cycle. This method does not allow for the determination of circadian endogenous control *vs* environmental driven daily rhythms. In this work, we have reared *Solea senegalensis* specimens in constant dark conditions, thus allowing us to evaluate if the anorexigenic hormones are entrained by the feeding time and/or photoperiod as external cues. The existence of daily rhythms in telencephalon and optic tectum in specimens reared under DD conditions and fed at random times suggest an independence of gene expression from the feeding time. Furthermore, this fact could be indicating that rhythms in *crh* expression are driven by the endogenous clock in this cerebral area, as no synchronizing external cues are present in this group. The differences in the acrophases detected between both neural areas in DD as well as the absence of *crh* significant rhythms in the diencephalon require further research.

In sole, the *pomc_a* gene expression has shown significant daily variations only in the pituitary gland in fish fed at MD. However, Cosinor analysis demonstrated the existence of *pomc_a* daily rhythms also in the telencephalon of ML-fed fish. The oscillatory profile of *pomc_a* resulted more evident in the pituitary gland than in the other brain areas examined in this study. This finding is in accordance with several experiments performed in order to localize the areas in which this gene is produced, such as those conducted by Castro and Morrison (1997) and Smith and Funder (1988). Even if the pituitary gland has been indicated as the mayor organ expressing the *Pomc* genes, Arends et al., (1998), Hui et al., (1999), Leder and Silverstein (2006) and Boonanuntanasarn et al., (2015) between others, have found out that *pomc* mRNAs are expressed in hypothalamus and in other regions of the brain in several teleost species such as common carp, goldfish, snakeskin or rainbow trout. In sole pituitary, the peak occurred at the end of the light phase, eight hours before feeding whereas in the telencephalon *pomc_a* peaked at the beginning of night, 4 h after feeding. In the case of *pomc b*, daily rhythms were only observed in the optic tectum and telencephalon of fish fed at ML, peaking in the first half of the night between 6-8 h after feeding time. In addition, daily variations but not daily rhythms in *pomc b* transcript levels were observed in the optic tectum of randomly-fed fish, with a

peak at the end of the day. In contrast to our results, peaks of pituitary *pomc_a* and *pomc_b* expression at the beginning of the light phase (ZT1.2 and ZT2.4, respectively) were found in a previous study performed in this species (López-Olmeda et al., 2013). Nevertheless, the feeding protocol from this study (food provided at random restricted to night-time) was different to that used in our study, which could explain this different daily expression profiles.

Our results on Senegalese sole *pomc_a* and *pomc_b* expression showed some similarities but also marked differences. On one hand, as mentioned before, all brain areas analysed together with the pituitary seem to express both *pomc* paralogues. However, excluding the pituitary, the diencephalon showed the highest mRNA expression in the case of *pomc_a* whereas the cerebellum was the central area exhibiting the most important expression values for *pomc_b*. On the other hand, significant rhythms were maintained or were even more robust under DD conditions for *pomc_a*, but were completely abolished for *pomc_b*. The differential transcript expression in the experimental groups reinforces different physiological roles for both POMC paralogues and the existence of different regulatory mechanism in different brain areas. This fact has been already suggested in Senegalese sole, where *pomc_a* expression is down-regulated in specimens undergoing chronic stress, whereas *pomc_b* expression levels remain unaffected, suggesting different roles for both paralogues (Wunderink et al., 2012). Moreover, studies conducted in trout have shown that the two genes can be differently regulated in hypothalamus and pituitary (Salbert et al., 1992; Gilchriest et al., 1998). Both genes showed a diurnal pattern but differed in the expression levels, expression sites within the pituitary gland and diurnal changes (Gilchriest et al., 1998). Differential expression of *pomc* mRNA subtypes has also been observed in other fish that contain two or more paralogues, further suggesting distinct functions of each subtype (Varsamos et al., 2003; Cardoso et al., 2011). *Pomc* paralogues have been also found in many peripheral tissues related with immunity, osmoregulation and reproductive system in fish, suggesting its contribution to multiple physiological functions (Boonanuntanasarn et al., 2015).

POMC-derived peptides bind to melanocortin receptor resulting in an anorexigenic effect (Bertile et al., 2006). Moreover, circadian fluctuations and/or expression levels of *pomc* expression differed between rats with free access to food and those with food restrictions (Xu et al., 1999; Girotti et al., 2009), although no significant changes in *pomc* expression in relation to food intake were demonstrated in mice (Stutz et al., 2007). The relationship

between *pomc* expression and food intake thus appears to differ among organisms and feeding habits. There is some controversy concerning the role of different POMC subtype genes with respect to food regulation also in fish (Takahashi et al., 2005; Leden and Silverstein, 2006; Valen et al., 2011; Kang and Kim, 2015). In another flatfish, *Paralichthys olivaceus*, the expression of pituitary of *pomc*2 significantly increased upon fasting on a dark-green background, but its expression was not influenced by food deprivation on a white background (Kang and Kim, 2015). Therefore, these authors suggested that *pomc* expression is influenced to a greater extent by background colour than food intake. In addition, hypothalamic POMC neurons do not appear to respond to progressive fasting in goldfish (Cerdá-Reverter et al., 2003), zebrafish (Song et al., 2003) and sea bass (Sánchez et al., 2009). In contrast, the *pomc-a1* expression increased 3 h post-feeding, while *pomc-b* increased 0.5 and 6 h post-feeding, indicating that these isoforms might play a role in the short-term control of food intake in *Salmo salar* (Valen et al., 2011). The present study demonstrated different patterns of *pomc* expression in discrete brain regions of Senegalese sole. It would be expected that low levels of *pomc* expression were detected before mealtime, being higher after it. However, a clear relationship between feeding time and *pomc* expression was not evident in this species. In trout, significant decreasing levels of *pomc* were observed 6-12 h after mealtime (Boonanuntanasarn et al., 2015). This fact might be explained, at least in part, by interactions between physiological responses, given that *pomc* can generate multiple POMC-derived hormones. Other factors such as stress, nutritional status, age of the specimens or photoperiod could be also affecting expression profiles in brain areas. Further studies appear necessary to elucidate the involvement of different *pomc* genes in food intake regulation in fish and to corroborate their anorexigenic actions.

Regarding DD conditions, *pomc_a* was rhythmic in telencephalon and diencephalon both in animals fed during the subjective light phase (sML) or at random times. The different acrophases observed in random groups compared with sML groups in both brain areas could suggest a role of feeding time as synchronizer when light-dark cycles are not present. The rhythmic expression of *pomc_a* in specimens kept under DD conditions and fed at random times could be indicating an endogenous control of *pomc_a* expression by the molecular clock in these brain areas. In contrast, *pomc_a* rhythms are lost under DD conditions in the pituitary, suggesting that *pomc_a* expression is not clock-controlled in this gland, as occurred for *pomc_b* in all neural areas analysed. Interestingly, rhythmic

clock gene expression has been described in some brain areas of Senegalese sole including the diencephalon, reinforcing the possibility that circadian function of the HPA elements could depend on intrinsic clock gene expression in these areas (Martín-Robles et al., 2011; 2012). However, ongoing research is being carried out at analysing clock gene expression in DD conditions in sole brain areas that could be an important clue to understand DD results in this study. The existence of daily rhythms in *pomc_a* expression in diencephalon when animals were kept under DD conditions and the lost of the rhythms in specimens under LD conditions were surprising. In trout, daily variations were found in *pomc* expression in diencephalon/pituitary, with decreasing levels after mealtime (Boonanuntanasarn et al., 2015). This could be related with the fact that both external cues photoperiod and feeding time are interacting under LD conditions and restricted food access to mask the endogenous rhythms of the sole diencephalon. However, further studies with different restricted feeding schedules are needed to firmly conclude it.

5.3 Influence of light spectra on anorexigenic hormone expression in Senegalese sole larvae

Light plays a key role in the development and the onset of biological rhythms in fish. The effect of light on fish biology appears to be species specific. For example, European sea bass larvae exposed to LD cycles of blue light (463 nm) show increased feeding activity, earlier weaning, and better spatial distribution, which coincides with the distribution of their live prey (*Artemia* sp.) (Villamizar et al., 2011b). Recent research in Senegalese sole has revealed that spawning and hatching rhythms, larval development, and growth performance are strongly influenced by lighting conditions (Blanco-Vives et al., 2011; Blanco-Vives et al., 2012). A key aspect of the effect of light exposure during early development is the potential for stress. The elements of the hypothalamus–pituitary–interrenal axis play a key role during ontogeny in the response to environmental stressors. The production and release of corticotropin-releasing hormone (CRH) from the hypothalamus is induced in response to exposure to stress and, in turn, CRH stimulates the secretion of the adrenocorticotropic hormone (ACTH) from the pituitary (Alsop et al., 2009). ACTH is generated by the proteolytic cleavage of pro-opiomelanocortin (POMC) and activates the signalling pathway leading to corticosteroid biosynthesis (Aluru et al., 2008).

Although the actions of light photoperiod and spectra on different morphological, physiological and molecular parameters have been largely studied in sole at early

developmental and metamorphic stages (Blanco-Vives et al., 2011, 2012; Martín-Robles et al., 2012, 2013), the effect of exposure to different light wavelengths on *crh* and *pomc* function has not been approached yet. Therefore, one of the aims of this thesis was to investigate the impact of white, blue, and red lights on *crh*, *pomc_a* and *pomc_b* daily expression before, during and after the metamorphosis of *Solea senegalensis*. Interestingly, the peaks of *crh, pomc_a* and *pomc_b* expression shift during development from diurnal (9 DPH, pre-metamorphosis) to nocturnal (25 DPH, post-metamorphosis) under LDw and LDb conditions, whereas these peaks were maintained at night under LDr in both pre-metamorphic and post-metamorphic (25 DPH) animals. In a previous study, it was demonstrated the existence of a light-dependent switch in Senegalese sole locomotor activity and feeding behaviour from diurnal to nocturnal (Blanco-Vives et al., 2012). Animals reared under LDw and LDb wavelengths exhibit this shift while animals maintained under LDr regime remain nocturnal along all development. Therefore, different locomotor and feeding behaviour patterns could be on the basis of these light-dependent changes in *crh, pomc_a* and *pomc_b* expression during sole ontogeny. In addition, daily expression patterns of *crh, pomc_a* and *pomc_b* observed in post-metamorphic animals (25 DPH) under LDw condition agree with those reported in adults (maintained under the same light wavelengths), which also exhibited higher nocturnal expression levels. Taken together, our results suggest that the adult daily pattern of expression of these genes is already attained soon after the metamorphosis.
We also found higher expression of these genes under LDr, suggesting a greater stress response in this condition. This higher stress response could explain previous data obtained by Blanco-Vives et al. (2012) in Senegalese sole, showing that larvae reared under LDr cycles exhibited the poorest development (reduced weight and length, delayed opening of mouth and development of fins, higher malformation rates) and feeding rates. These results also agree with those obtained by Villamizar et al., (2013), who observed that zebrafish larvae reared under red lights also showed higher expression of *crh* and *pomc_a* genes.

Our findings confirm the significant influence that ambient lighting has on larval growth and development in Senegalese sole, revealing the strong effect of light spectra upon functional elements of this species. Our results also emphasize the importance of maintaining cycling light-dark conditions of the adequate wavelengths in aquaculture practices during early development of sole. Since “unnatural” or inappropriate

environmental conditions seriously compromise the welfare of sole larvae and negatively affect their stress, survival and performance (Blanco-Vives et al., 2010), adequate ambient lighting appear critical for the culture of this species and a prerequisite for the proper maturation and development of larvae.

6 CONCLUSIONS

1. Senegalese sole anorexigenic genes *crh*, *pomc_a* and *pomc_b* are expressed in all central areas analysed (olfactory bulbs, telencephalon, diencephalon, optic tectum, cerebellum and medulla) and in pituitary gland of adult specimens.

2. Daily rhythms have been revealed in some cerebral areas including the telencephalon, optic tectum and pituitary gland when fish were maintained under light-dark cycles or constant dark conditions and different feeding regimes. *Crh* daily expression is rhythmic in schedule-fed fish in both telencephalon and optic tectum in animals reared under LD conditions, with the acrophases occurring during the dark phase irrespective of the feeding time, suggesting an independence of gene expression from the feeding time. Under DD conditions, daily rhythms are found in fish fed at random times, suggesting an endogenous control of *crh* expression in sole.

3. Both *pomc* paralogues showed significant daily rhythms under LD conditions. The rhythms were maintained or were even more robust under DD conditions for *pomc_a*, but were completely abolished for *pomc_b.* The rhythmic expression of *pomc_a* in specimens kept under DD conditions and fed at random times could be indicating an endogenous control of *pomc_a* expression by the molecular clock in these brain areas. In contrast, *pomc_a* rhythms are lost under DD conditions in the pituitary, suggesting that *pomc_a* expression is not clock-controlled in this gland, as occurred for *pomc_b* in all neural areas analysed. The existence of daily rhythms in *pomc_a* expression in diencephalon when animals were kept under DD conditions and the lost of the rhythms in specimens under LD conditions could be related with the fact that both external cues photoperiod and feeding time are interacting under LD conditions and restricted food access to mask the endogenous rhythms of the sole diencephalon.

4. The results obtained from the experiment conducted on Senegalese sole larvae suggest for the first time that different locomotor and feeding behaviour patterns could be related with the light-dependent changes in *crh, pomc_a* and *pomc_b* expression during sole ontogeny. Our results suggest that the adult daily pattern of expression of these genes is already attained soon after the metamorphosis. Our findings confirm for the first time, the significant influence that ambient lighting

has on larval growth and development in Senegalese sole, revealing an important effect of light spectra upon functional elements of this species. Our results also emphasize the importance of maintaining cycling light-dark conditions of the adequate wavelengths in aquaculture practices during early development of sole.

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