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**Effects on photoperiod and feeding regimes on Neuropeptide Y (NPY)
and Agouti-related Protein (AgRP) expression in central areas
of Senegalese sole, *Solea senegalensis***

Tesi di laurea in Adattamenti degli animali all'ambiente marino

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1 Introduction

1.1 Overview of feeding in vertebrates

Feeding is a complex phenomenon, regulated by numerous central and peripheral endocrine factors, whose actions are modulated by both external and internal variables. Environment, season, time of day, but also circulating levels of nutrients, hormones and ontogeny affect food intake which, under optimum conditions, is adequate for basal metabolic needs, growth, development, reproduction and deposition of energy store.

In all vertebrates, feeding is regulated by **orexigenic** (appetite stimulating) and **anorexigenic** (appetite inhibiting) factors, which act on feeding centers in the brain to mediate the regulation of short-term and long-term dietary intakes (Volkoff et al., 2009a). The central nervous system (CNS), particularly the hypothalamus, has a central role in the regulation of energy homeostasis, but also peripheral organs, such as gastrointestinal tract (GI), pancreas, liver and adipose tissue, are involved in the regulation of feeding (Valassi et al., 2008). Peripheral hormones, in part released in response to the presence of food in the digestive tract, carry information to central feeding centers via the vagus nerve or by crossing the blood-brain barrier acting directly through central receptors and are integrated with other neuronal signals that reflect an animal's energy status (Brightman & Broadwell, 1976; Holmgren & Olsson, 2009).

During the late 1980s and the 1990s, many brain neuropeptides related to feeding regulation were identified and characterized in mammals and then, some of these hormones or their fish homologs were examined to determine their regulatory effects on feeding in fishes (Volkoff et al., 2005; Gorissen et al., 2006). These peptides displayed similar appetite-regulating effects, showing that the regulation of food intake has been conserved along the vertebrate lineage, although differences exist: the presence of important anatomical and physiological differences between mammals (homeothermic) and fishes (ectothermic) (e.g. brain and gut morphology, caudal secretory organ in fishes) indicates that the endocrine control of feeding requires molecules and mechanism that may be specific to certain species of groups (Volkoff et al., 2009b).

Furthermore, fish represent a vast phylogenetic group, that include agnathans (jawless fishes), chondrichthyans (cartilaginous fishes), sarcopterygians (lobe-finned fishes), and actinopterygians (ray-finned fishes, which are 97% composed of teleosts). Because of this extent it shows a noteworthy level of diversity regarding morphology, ecology, behaviour and genomes (Volf, 2004; Volkoff et al., 2009a). For example it displays differences in gastrointestinal morphology and gut hormone profiles which are related to different feeding habits (Holmgren & Olsson, 2009), and furthermore variation of brain distribution of neuropeptides has been demonstrated (Cerdá-Reverter

& Canosa, 2009). Fishes also undergo several environmental challenges, that may involve long term fasting, trade-off in resource distribution between reproduction and growth (Heino & Kaitala, 1999) and this, added to indeterminate growth, has led to a number of feeding adaptations that suggests that the endocrine control of feeding in fish might also be diverse and involves species-specific molecules and mechanisms (Volkoff et al., 2009a).

Feeding might also display species-specific daily or circannual (seasonal) rhythms in fish, and 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).

1.2 Neuroendocrine regulation of energy homeostasis

In mammals, stability of body weight and body composition over long periods of time requires that energy intake matches energy expenditure, that means that dedicated mechanisms are required to avoid changes in body weight, which in mature mammals mainly concerns adipose tissue mass, since protein and carbohydrate store slightly vary (Jéquier and Tappy, 1999; Lin et al., 2000). A renowned theory, the “Lipostatic model” (Kennedy, 1953), explains the well-regulated control of body weight and food intake: hormones produced from fat cells are the key signal to the brain centers that control eating behaviour and activity to regulate feeding and body-fat deposition (Inui, 1999; Lin et al., 2000). So, when body weight exceeds a certain value, eating behaviour is inhibited and energy consumption is increased.

Leptin is thought to be the lipostatic factor that governs energy balance through a negative-feedback loop that originates in adipose tissue and acts on hypothalamic centers in the brain. (Inui, 1999). Leptin is a proteinic hormone encoded by the obese gene (*ob*) and its effects are feeding inhibition, decrease in body weight and increase in thermogenesis and locomotor activity, as showed in studies involving rodents (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Leptin and its receptor system provide an afferent negative feedback signaling system reflecting the amount of adipose energy stores to the brain hypothalamic centers (Schwartz and Seeley, 1997). A feedback regulatory loop with three distinct steps has been hypothesized, that comprise a sensor that monitors the level of energy, hypothalamic centers that receive and integrate through specific leptin receptors the intensity of the signal, and effector systems that influence energy intake and energy expenditure (Jéquier and Tappy, 1999). Leptin targets in the hypothalamus include neuropeptides, such as **neuropeptide Y** (NPY), agouti-related peptides (AGRP), **α -melanophore-stimulating hormone (α -MSH)**, **corticotropin-releasing factor (CRF)**, **galanin**, **melanocyte concentrating hormone (MCH)**, and **orexins** (Mercer et al., 1996; Cheung et al., 1997; Campfield et al., 1998; Håkansson et al., 1998, 1999), that modulate food intake and

energy expenditure. Also insulin has a similar effect on hypothalamus in the control of food intake (Inui, 1999; Schwarz et al.1992).

As mentioned before, hypothalamic sites, such as the arcuate nucleus (ARC), the lateral hypothalamus (LH), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN) and the paraventricular nucleus (PVN) have always been considered the centers involved in the control of feeding behaviour. In particular, the LH and the VMN were thought to be respectively the “feeding center” and the “satiety center”, according to the “Dual Center Model” for regulation of feeding (Anand and Brobeck, 1951; Lin et al., 2000). Over the years, new studies, which didn't employ only discrete lesions or surgical transections, have permitted to redefine the hypothalamic pathways involved in the regulation of feeding and body weight, discovering co-localization and co-production of more than one signalling molecules in neurons belonging in the same neural area and a deep interconnection between neurons belonging to different areas (Lin, 2000).

In LH, two orexigenic hormones are produced, MCH and orexins (Bittencourt et al., 1992; de Lecea et al., 1998; Sakurai et al., 1998), but also anorexigenic molecules are expressed in LH, i.e. CART (Cocaine and amphetamine regulated transcript) and CRF, which decrease food intake when is centrally administered (Koylu et al., 1997; Kristensen et al., 1998; Kelly and Watts, 1998). Also NPY terminals are abundant in the LH, in contact with orexin and MCH cells. (Williams, 2001).

ARC is situated around the base of the third ventricle and it is indicated as the site which integrates circulating metabolic signals, such as adrenal and gonadal steroids, leptin and insulin (Kalra, 1999). In ARC neurons both orexigenic (e.g. NPY, opioids, galanin, AGRP) and anorexigenic peptides (CART, α -MSH, GABA and glutamate) are produced (Kalra, 1999), which extend into various hypothalamic sites, such as VMN, DMN, PVN, preoptic area (POA) and perifornical hypothalamus. This communication between hypothalamic pathways and the caudal brainstem, responding to meal-related satiety signals, is essential for the long-term regulation of energy homeostasis (Hillebrand, 2002). When ARC is reached by adiposity signals, anorexigenic peptides are released and thus a catabolic circuit is activated. On the contrary, the activation of anabolic pathway leads to the release of orexigenic peptides and this occurs when adiposity signal concentrations in the brain are low, thus indicating the urgency to eat. There is an intense innervation of both MCH and orexin neurons in the LH by axons containing NPY, AGRP and α -MSH probably from the ARC (Broberger et al., 1998; Elias et al., 1998). The ARC also has extensively reciprocal connections with other hypothalamic regions, including the paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH).

VMN is suggested to be a receptive field that transfers information to DMN-PVN for the releasing of orexigenic signal (Kalra, 1999), rather than a productive area.

DMN is suggested as site for NPY production and for NPY and leptin interaction, it is also supposed to be involved in the attenuation or inhibition of feeding by leptin (Yokosuka et al., 1998). Along with leptin receptors (Ob-Rb), the DMN contains insulin receptors. Some ARC-NPY/AGRP neurones also terminate in the DMN.).

PVN is the area responsible for the integration and interpretation of the different peripheral and central signals that inform the hypothalamus of surrounding conditions (De Gortari, 2006). PVN receives connections from different hypothalamic areas such as the lateral, median preoptic, arcuate and dorsomedial nuclei and expresses receptors for orexigenic and anorexic peptide signals, such as neuropeptide Y (NPY), agouti related peptide (AGRP), cocaine and amphetamine regulated-transcript (CART), α -MSH, CRH, orexins (De Gortari, 2006; Kalra et al, 1991, Dube et al.)

In fish, as in mammals, several brain region are involved in the regulation of food intake. In the past, from the 1960s to the early 1980s, studies which used electrical stimulation and lesioning showed that also olfactory tracts (Peter, 1979; Demski, 1983), inferior lobes of the hypothalamus (Demski & Knigge, 1971; Demski, 1973 and 1977; Savage & Roberts, 1975; Roberts & Savage, 1978), telencephalon (Grimm, 1960, Demski & Knigge, 1971; Stacey & Kyle, 1983) and optic tectum (Peter, 1979) are all substrates involved in feeding regulation.

In fish, the main neuroendocrine regions producing appetite-regulating hormones are the telencephalon, preoptic area and hypothalamus (Cerdeira Reverte & Canosa, 2009) but the relative distribution of the peptides/mRNAs varies with the hormone considered and the species examined. For example, orexin immunoreactive (OX-ir) cells are found in the anterior hypothalamus and along the third ventricle within the preoptic area (POA) in zebrafish (Appelbaum et al., 2009) and medaka (Amiya et al., 2007), whereas in the Australian lungfish, OX-ir cells are also found in the POA, the infundibular hypothalamus and within the telencephalon (Lopez et al., 2009; Hoskins et al., 2012).

There is considerable variation in brain morphology and in the relative size of regions across species, which is often related to life history, ecology and behaviour. For example, it has been suggested that in cichlid fish, algal scrapers have small optic lobes and large telencephala whereas planktivores have enlarged optic lobes (Sylvester et al., 2010).

As mentioned before, the neuropeptides produced by hypothalamic nuclei and involved in feeding intake are divided in two main groups, regarding their action towards feeding: orexigenic, if they stimulate feeding intake, and anorexigenic, if they inhibit it. From the early 1990s, several feeding-related neuropeptides have been identified in the brain of fish, both by isolation of the

peptide or by cloning of their cDNA sequences. Table 1.1 summarizes them and their effects. So far, 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., 2009a).

1.2.1 Orexigenic hormones

1.2.1.1 Neuropeptide Y (NPY)

NPY is a 36-amino-acid peptide that belongs to the NPY-family peptides, which also includes peptide YY (PYY), pancreatic polypeptide (PP, found only in the pancreas of tetrapods), and peptide Y (Hoyle, 1999). The most important characteristic for all NPY-family peptides is that they exhibit certain amino acid residues necessary to adopt a specific three-dimensional structure, named the pancreatic polypeptide fold (PP-fold) (Blundell et al. 1981).

NPY was discovered in 1982, when Takemoto isolated it from pig brain homogenates, using a chemical assay that permitted identification of amidated peptides. The first member of this family to be discovered was the pancreatic polypeptide, but as Larhammar et al. (1993) showed, NPY had remained much more conserved throughout evolution than PP, and it seemed to be evolutionarily older than PP. Therefore, the family would be more appropriately called the NPY family than PP family, as it was at the beginning. (Cerdá reverter and Larhammar, 2000).

In mammals, NPY is considered one of the most potent orexigenic agents (Halford et al 2004; Kalra et al 1999) and this action has been demonstrated also in fish, which also produce PYY whereas only some teleosts produce PY (Cerdá-Reverter et al., 2000).

These peptides bind to a family of G-protein-coupled receptors that compose the Y family, which has seven cloned members, namely Y1, Y2, Y4, Y5, and Y6 (Larhammar et al., 2001). To date, seven NPY receptor subtypes that bind both NPY and PYY have been identified in fishes (Y1, Y2, Y4–Y8) (Salaneck et al., 2008). Y1-like receptors have been identified in several fish species (Larhammar et al., 2001), whereas Y2-like receptors have only been characterized in zebrafish *Danio rerio* and rainbow trout, *Oncorhynchus mykiss* (Fredriksson et al., 2004). Fish NPY receptors are expressed in brain but also in peripheral tissues such as eye and intestine (Fredriksson et al., 2004; Lundell et al., 1997).

In mammals, NPY is abundant in the CNS, particularly in the hypothalamic nuclei involved in the regulation of feeding, such as arcuate nucleus (ARC) and paraventricular nucleus (PVN) (Halford et al. 2004).

Central "Orexigenic" agents	Ingestive effects
Agouti-related protein (AgRP) (Cerdá-Reverter and Peter, 2003)	Increases food intake in fish (Schjolden et al., 2009), birds (Strader et al., 2003), and mammals (Rossi et al., 1998; Stark, 1998), and food hoarding in hamsters (Day and Bartness, 2004)
Galanin (Anglade et al., 1994; Unniappan et al., 2002; Wang and Conlon, 1994)	Increases food intake in fish (De Pedro et al., 1995a; Lin et al., 2000; Nelson and Sheridan, 2006; Volkoff et al., 2005) and rats (Kyrkouli et al., 1990)
Neuropeptide Y (NPY) (Blomqvist et al., 1992; Cerdá-Reverter et al., 2000a)	Increases food intake in fish (de Pedro et al., 2000; Lopez-Patino et al., 1999), frogs (Crespi et al., 2004), snakes (Morris and Crews, 1990), birds (Strader and Buntin, 2001), rats (Stanley and Leibowitz, 1984) and food hoarding in hamsters (Dailey and Bartness, 2009)
Orexin / Hypocretin (Alvarez and Sutcliffe, 2002; Kaslin et al., 2004)	Increases food intake in fish (Lin et al., 2000; Volkoff et al., 1999; Volkoff et al., 2005), and rats (Sakurai et al., 1998), but not in birds (da Silva et al., 2008)
Central "Orexigenic" agents	Ingestive effects
Cocaine- and Amphetamine-regulated transcript (CART) (Volkoff and Peter, 2000),	Decreases food intake in fish (Volkoff et al., 2005), birds (Tachibana et al., 2003), rats (Kristensen et al., 1998)
Cholecystokinin, CCK (Peyon et al., 1998),	Decreases food intake in fish (Himick and Peter, 1994; Volkoff et al., 2005), birds (Tachibana et al., 2012), rats (Gibbs et al., 1973) and food hoarding in Siberian hamsters (Bailey and Dela-Fera, 1995; Figlewicz et al., 1989; Teubner and Bartness, 2010)
Corticotropin-releasing factor (CRF) (Ando et al., 1999; Bernier et al., 1999; Okawara et al., 1988)	Decreases food intake in fish (De Pedro et al., 1993; Matsuda et al., 2008), amphibians (Crespi et al., 2004), birds (Denbow et al., 1999; Furuse et al., 1997), rats (Heinrichs and Richard, 1999; Levine et al., 1983; Morley and Levine, 1982; Negri et al., 1985) and food hoarding in rats (Cabanac and Richard, 1995) reviewed by (Carr, 2002)
Melanin concentrating hormone (MCH) (Baker et al., 1995)	Decreases food intake in fish (Shimakura et al., 2008), but increases food intake in rats (Presse et al., 1996)
Peripheral Orexigenic Hormones	Ingestive effects
Grelin (gut) (Unniappan et al., 2002)	Increases food intake in fish (goldfish and tilapia), but decreases food intake in rainbow trout (Jonsson, 2013; Jonsson et al., 2010), decreases food intake in birds (Kaiya et al., 2009), increases food intake in rats and mice (Tschop et al., 2000; Wren et al., 2000) and food hoarding in Siberian hamsters (Keen-Rhinehart and Bartness, 2005).
Peripheral Anorexigenic Hormones	Ingestive effects
Leptin (adipocytes, liver)	Decreases body weight, adiposity, and food intake in fish (Crespi and Denver, 2006; Murashita et al., 2008), and mice (Campfield et al., 1995; Halaas et al., 1995; Pellemounter et al., 1995) and food hoarding in Syrian hamsters (Buckley and Schneider, 2003) while increasing energy expenditure

Table 1.1: a partial list of chemical messengers with effects on ingestive behaviour in fish and other vertebrate taxa. (Adapted from Schneider et al., 2013)

NPY sequences have been determined for several fish species and show strong evolutionary conservation among vertebrate species as the goldfish NPY has only five residues different from rat NPY (Blomqvist et al., 1992). NPY neurons are widely distributed in fish CNS of dipnoans (Trabucchi et al., 2000), elasmobranchs (Chiba, 2000) and teleosts (Cerdá-Reverter et al., 2000a; Doyon et al., 2003; Leonard et al., 2001; Peng et al., 1994; Silverstein et al., 1998). The presence of NPY in fish was first demonstrated in goldfish (*Carassius auratus*) by immunological and chromatographic studies (Kah et al., 1989). NPY immunoreactive neurons are present in the ventromedial-posterior hypothalamus and hypothalamic inferior lobes (Pontet et al., 1989). Further studies have shown that NPY mRNA is expressed in ventral telencephalon, POA, olfactory bulbs and also in optic tectum, locus coeruleus and other thalamic regions (Peng et al., 1994). A similar brain distribution of NPY mRNA has been described in coho salmon (*Oncorhynchus kisutch*) (Silverstein et al., 1998). NPY-immunoreactive fibers have also been identified in fish pituitary, pancreas and gastrointestinal tract, and nerve fibers surrounding blood vessels (Cerdá-Reverter and Larhammar, 2000; Chiba et al., 1996; Danger et al., 1991)

In Senegalese sole, *Solea senegalensis*, it has been demonstrated that NPY-like matter is widely distributed in the brain, with the highest density found in the forebrain, especially in hypothalamus and the ventral telencephalon, whose NPY-containing neurons constitute the major component of the NPY- system in the Senegalese sole (Rodriguez-Gomez et al., 2001).

Several studies have demonstrated that NPY is involved in the regulation of food intake in teleosts, as central injections of mammalian or fish NPY cause a dose-dependent increase in food intake in goldfish (De Pedro et al., 2000; Lopez-Patino et al., 1999; Narnaware et al., 2000), salmon, (*Salmo salar*) and catfish, (*Ictalurus punctatus*) (Silverstein and Plisetskaya, 2000). From studies on goldfish it emerged that NPY may act centrally through Y1 and Y5 receptors, which act independently to stimulate food intake in goldfish, but not through Y2 (Narnaware and Peter, 2001b).

A further demonstration that NPY is involved in feeding is the fact that brain NPY mRNA levels increase following fasting in goldfish (Narnaware and Peter, 2001), winter skate, *Raja ocellata* (MacDonald and Volkoff, 2009), and chinook and coho salmon (Silverstein et al., 1999a) and undergo periprandial variations in goldfish. An increase in NPY mRNA levels in the telencephalon–preoptic area and hypothalamus shortly before feeding was observed, followed by a decrease in brain NPY mRNA levels after feeding (Narnaware and Peter, 2001a). Similar variations were observed also in Atlantic cod, *Gadus morhua* (Kehoe and Volkoff, 2007) and tilapia, (*Oreochromis mossambicus*) (Peddu et al., 2009). Besides in goldfish, NPY gene expression in

brain seems to be influenced by macronutrient intake (Narnaware and Peter, 2002) and by the peripheral metabolic status (Lin et al. 2000).

In fishes as in mammals, the actions of NPY on feeding occur in part by the modulation of other appetite regulators, e.g., corticotrophin-releasing factor (CRF) and cortisol (Bernier et al., 2004), cocaine- and amphetamine-regulated transcript (CART) (Volkoff and Peter, 2000), leptin (Volkoff et al., 2003), melanin-concentrating hormone (MCH) (Matsuda et al., 2008), orexins (OXs) and galanin (GAL) (Volkoff and Peter, 2001), growth hormone (GH) (Mazumdar et al., 2006) and ghrelin (Miura et al., 2006), and, in goldfish by estradiol a and testosterone (Peng et al., 1994).

1.2.1.2 Agouti-related Protein (AgRP)

Agrp1 and *Agrp2* belong to the agouti family peptides, which also include the agouti signaling proteins (ASIP) and have potent and diverse functional roles in feeding, pigmentation and background adaptation mechanisms (Västermark et al., 2012).

In mammals, AgRP is one of the most potent orexigenic peptides and is co-expressed together with NPY in the ARC. It functions by increasing appetite and decreasing metabolism and energy expenditure (Hagan et al., 2000; Sainsbury and Zhang, 2010).

AgRP has been also identified in fish (Cerdá- Reverter and Peter, 2003; Klovins and Schiöth, 2005; Kurokawa et al., 2006; Song et al., 2003b; Stütz et al., 2005). Phylogenetic analyses of the deduced proteins showed that tetrapods have two agouti family members, i.e. ASIP and AgRP, while some teleosts have four: ASIP1, ASIP2, AgRP1, and AgRP2 (Braasch and Postlethwait, 2011).

All the agouti family peptides are characterized by a C-terminal polycysteine domain (Klovins and Schiöth, 2005). Orthologs of ASIP, AgRP, melanocortin receptors MC1R and MC4R have been identified in mammalian, teleost fish and avian genomes, but not in invertebrate genomes. This may suggest that the agouti-melanocortin system evolved by gene duplication in the last 500 million years.

Studies on rats showed that stimulate hyperphagia when administered intracerebroventricularly, furthermore, it displays long-lasting effects on food intake, with animals displaying hyperphagia even 7 days following a single intracerebroventricular (i.c.v.) injection (Hagan et al., 2000a).

The orexigenic function of AgRP is primarily performed through the antagonism of central melanocortin receptors (MCRs), specifically of melanocortin receptor 3 (MC3R) and melanocortin receptor 4 (MC4R), which are directly related to metabolism and body weight control (Oyama et al., 2010; Ollmann et al., 1997; Robinson et al., 2000). Studies on the sea bass, *Dicentrarchus labrax*, revealed that AgRP antagonizes also the effect of the MC1R receptor in vitro (Sánchez et al., 2010).

However, the exact mechanism by which AgRP inhibits melanocortin-receptor signalling is not completely clear.

An mRNA encoding AgRP has been identified in goldfish (Cerdá-Reverter and Peter, 2003), zebrafish (Song et al., 2003), and pufferfish (Klovins et al., 2004; Kurokawa et al., 2006). The goldfish AgRP gene encodes a 128 amino acid precursor and is expressed in a variety of tissues including brain and peripheral tissues. Moreover, AgRP expression was identified only in hypothalamus of goldfish, while in *Takifugu rubripes* was detected throughout a wide region of brain in which suggests a species-specific expression of AgRP (Cerdá-Reverter and Peter, 2003; Ollman et al., 1997; Wei et al. 2013).

Several studies have revealed the involvement of AgRP in the control of food intake in fish: transgenic zebrafish overexpressing AgRP exhibited obesity, increased linear growth, and adipocyte hypertrophy, strongly suggesting that AgRP plays an important role in feeding control in teleost fish (Wan et al., 2012). Furthermore, AgRP levels are upregulated by fasting in different species of vertebrates, such as sheep (Adam et al., 2002), Japanese quail (Boswell et al., 2002), goldfish (Cerdá-Reverter and Peter, 2003) and zebrafish (Song et al., 2003), underlining a conserved role for AgRP in energy homeostasis. In goldfish, brain AgRP mRNA increases after 3 days of fasting, and it may exert its effect through MCRs (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003). Fasting up-regulated hypothalamus AgRP mRNA levels in *Danio rerio* (Song et al., 2003). In *Cyprinus carpio*, fasting induces an initial reduction of expression and after the initiation of re-feeding, there was a significant induction of AgRP mRNA expression (Wan et al., 2012). All together, the identification of AgRP in several teleosts and the effect of fasting on AgRP expression in goldfish, suggest that it plays a part in appetite regulation, possibly through MCRs.

1.3 Influence of intrinsic factors on feeding behaviour

1.3.1 Metabolic signals, energy reserve and feeding status

Feeding behaviour and the expression of appetite-related peptides have been shown to be influenced by circulating metabolite levels, the ingestion of food and food deprivation (Volkoff et al., 2010).

Variations in circulating metabolite levels alter food intake in fish. A decrease in food intake has been observed in trout fed with high-carbohydrate enriched diets (Banos et al., 1998) and in carp injected intraperitoneally with essential amino acids (Kuz'mina, 2005). Also, protein and lipids can modify feeding in fish. Intraperitoneal administration of amino acids decreases food intake in carp (Kuz'mina, 2005) and also a lipostatic control of food intake has been suggested after noticing that,

in chinook salmon and channel catfish, fat fish eat less than thin fish (Shearer et al., 1997; Silverstein and Plisetskaya, 2000).

Gene expression of appetite regulators can be altered by meal consumption. Usually, the expression levels of orexigenic factors (NPY and Orexin) increase before or during a meal (Narnaware and Peter, 2001; Xu and Volkoff, 2007) while the expression levels of anorexigenic factors (CCK and CART) decrease after feeding (Peyon et al., 1999; Volkoff and Peter, 2001). Fasting generally induces an up-regulation of the expression of orexigenic factors, such as brain NPY, orexins and stomach ghrelin (Narnaware and Peter, 2001; Silverstein et al., 1999; Novak et al., 2005; Xu and Volkoff, 2007; Amole and Unniappan, 2008; Terova et al., 2008) and a down-regulation of the expression of anorexigenic hormones, such as brain CART and gut CCK (Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Volkoff and Peter, 2001; MacDonald and Volkoff, 2009; Murashita et al., 2006). However, contradictory results have been reported. Fasting increases NPY mRNA expression after 3 days in goldfish hypothalamus (Narnaware et al., 2000), after 2 and 4 weeks in winter flounder hypothalamus (MacDonald and Volkoff, 2009), after 2-3 weeks in Chinook and coho salmon hypothalamus (Silverstein et al., 1999) and after 2 weeks in Brazilian flounder whole brain (Campos et al., 2010), whereas one-week fasting does not affect NPY mRNA expression in either cod forebrain (Kehoe and Volkoff, 2007) or tilapia whole brain (Riley et al., 2008). The duration of food deprivation might influence the results since, for example, in goldfish a fast of 10 days, but not of 3, decreases the hypothalamic mRNA expression of CART1 (Abbott and Volkoff, 2011). Besides, variations could be due to species-specific differences in response to fasting, as fishes might have different sets or patterns of appetite-related peptides and might respond differently to nutritional challenges or might be due to different expression analysis in different parts of the brain, as different regions of the brain might respond differently to fasting. For example, although both hypothalamus and telencephalon are brain regions that have been implicated in the regulation of feeding in fish, in skate (*Raja ocellata*), a 2-week fasting period increases NPY mRNA expression in the telencephalon but not in the hypothalamus (MacDonald and Volkoff, 2009). Moreover, different experimental conditions might influence the results (Hoskins & Volkoff, 2012).

Fish can also adapt their food intake to their energy needs and have been reported to respond with increased food intake when they are fed with low energy diets (Boujard and Medale, 1994; Geurden et al., 2006; Paspatis and Boujard, 1996; Yamamoto et al., 2000). The fact that brain NPY expression levels are influenced by macro-nutrient intake in goldfish suggests that these dietary adjustments in feeding might be mediated by variations in appetite-related hormones (Narnaware and Peter, 2002).

1.3.2 Ontogeny

Appetite-regulating factors might play a role in embryogenesis (pre-hatch) as well as nutrient absorption/acquisition (pre- and post-hatch) in fish larvae, as they appear early in development. A later appearance of appetite-regulating factors, especially when associated with metamorphic events or mixed feeding phases (e.g., beginning of exogenous feeding), may reflect ontogenic shifts in feeding. Gastro intestinal CCK immunoreactivity has been detected upon first hatch in herring, *Clupea harengus* (Kamisaka et al., 2005), and ghrelin has been detected 48h post fertilization in zebrafish (Pauls et al., 2007). A number of other signals involved in the regulation of feeding in fishes appear to be present prior to hatching, such as somatostatin (Xing et al., 2005; Xu and Volkoff, 2008), NPY (Volkoff et al., 2009), OX (Xu and Volkoff, 2007), α -MSH and AgRP (Forlano and Cone, 2007).

1.3.3 Gender and reproductive status

In fish, there is a close relationship between feeding, gender and reproductive parameters.

Gender-specific differences in feeding behaviour have been noticed. During the spawning season, territorial male cunners (*Tautoglabrus adspersus*) feed less often and have different diets than females (Green et al., 1984). Sexual dimorphism in growth rates and size at maturity is common in fish and could be due in part to differences in feeding activity between sexes (Davis et al., 2008; Rennie et al., 2008; Shearer et al., 2006; Toguyeni et al., 1997). Differences in levels of appetite regulating hormones have also been noted in a few species. For example, in tilapia, gastric ghrelin mRNA levels are higher in females compared to males (Parhar et al., 2003), and in salmon the number of ghrelin cells per unit area in the stomach is higher in females than in males (Sakata et al., 2004). These observations would suggest that gender, or more specifically sex steroids, might influence feeding. It seems likely that these influences would be more pronounced when fish are either breeding or preparing to breed (e.g., migrating) as a decline in feeding is often seen during spawning migrations or other reproductive behaviors (e.g. courtship, spawning, guarding, territoriality) (van Ginneken and Maes, 2005). This happens in both Atlantic cod (Fordham and Trippel, 1999) and winter flounder (MacDonald and Volkoff, 2009), as feeding is suppressed in both sexes during the spawning period and it increases after spawning. Also European eels (van Ginneken et al., 2005), Atlantic salmon (Miller et al., 2005) eat very little during spawning and spawning migration, and male domino damselfish reduce time spent feeding during courtship and nest guarding (Mann et al., 2007).

Gonadal development also influences nutrient utilization. In Atlantic cod, the investment of energy in gonad maturation results in reduced whole body dry matter and protein levels (Hemre et

al., 2002.) Seasonal changes correlated to gonadal cycles have been shown for NPY in ayu (Chiba et al., 1996) and catfish (Mazumdar et al., 2007) and CCK (MacDonald and Volkoff, 2009), suggesting that appetite-related hormones mediate in part these seasonal changes.

However, the exact roles of sex steroids on food intake are unclear as sex steroid treatment decreases food intake in some species, as seabass and eurasiatic perch (Leal et al., 2009; Mandiki et al., 2005), but increases feeding in others, as red sea bream (Woo et al., 1993). In goldfish, intracerebroventricular treatment with gonadotrophin-releasing hormone (GnRH) induces a decrease in food intake (Hoskins et al., 2008; Matsuda et al., 2008), which is in part due to down-regulation of brain OX mRNA expression (Hoskins et al., 2008). Conversely, intracerebroventricular OX-A injections induce a decrease in spawning behaviour and a decrease in GnRH mRNA expression levels in the brain (Hoskins et al., 2008). Also testosterone is thought to have an anorexigenic role, as testosterone treatment decreases food intake in male perch (Mandiki et al., 2005) and elevates MCH mRNA expression in the hypothalamus of both male and female goldfish (Cerdà-Reverter et al., 2006). Besides, castration reduces the density of NPY-immunoreactive fibers in the forebrain of tilapia (Sakharkar et al., 2005).

1.3.4 Genetic influence

In captive fish, food intake has shown genetic-based variations, such as in rainbow trout, in which different strains display variations in feeding activities and growth (Mambrini et al., 2004), feeding patterns (Boujard et al., 2007), nutrient utilization (Quillet et al., 2007) and body composition (Kause et al., 2002).

These variations have been found also in cod (Case et al., 2006) and Atlantic salmon (Glover et al., 2009) regarding variations in feeding activities and growth, while in striped bass (Wang et al., 2007) differences in body composition have been observed. However, since domestication selection has been achieved mostly empirically, very little is known about the genetic variations related to endocrine factors in these selected animals. Within the context of aquaculture, a better knowledge of these variations might lead to genetic improvement by selection for specific traits, such as better food efficiency and faster growth.

1.4 Influence of extrinsic factors on feeding behaviour

In fish, variations in several extrinsic factors have been shown to induce changes in swimming activity, feeding rhythms and growth (Boujard, 2001). Temperature and photoperiod are probably the major abiotic factors affecting feeding.

1.4.1 Temperature, salinity, hypoxia, pollutant and health status

Fish tend to increase both their food consumption and growth rates with rising temperatures within a “tolerable” range (Bendiksen et al., 2002; Kehoe and Volkoff, 2008;) and the optimal temperatures for growth usually decrease with fish size (Bjornsson et al., 2001). It has been noticed that they tend to decrease their food intake when placed in extreme temperatures (Volkoff et al. 2009). Little is known about the endocrine mechanisms regulating these temperature-induced changes in feeding. In Atlantic cod, brain NPY mRNA expression does not seem to be affected by temperature, but brain CART mRNA expression levels are higher in fish held at 2° C than in fish held at either 11° C or 15° C. This suggests that CART, but not NPY, may contribute to temperature-induced changes in appetite in this species (Kehoe and Volkoff, 2008).

Also salinity affects feeding in several fish species (De Boeck et al., 2000) but little is known about the appetite-regulating factors that may be involved. It is presumed that the somatotrophic axis might play a role in salinity-related alteration on feeding, since it is a regulator of growth and salt water tolerance in salmonids (Boeuf and Payan, 2001). In conditions of acute osmoregulatory disturbance, fishes often momentarily reduce food intake, which has been shown to be concomitant with increases in brain corticotropin-releasing factor (CRF) in trout (Craig et al., 2005).

Hypoxia has distinct appetite suppressive effects (Buentello et al., 2000; Ripley and Foran, 2007). In rainbow trout, exposure to low oxygen levels increases forebrain CRF as well as plasma cortisol, suggesting that CRF-related peptides play a physiological role in mediating at least a portion of the reduction in food intake (Bernier and Craig, 2005).

Contaminants and disease agents also have impact on feeding but the responses vary depending on the species considered as well as on the agent and the method of exposure. For example, rainbow trout exposed to elevated waterborne concentrations of metals eats less (Todd et al., 2007), on the contrary coho salmon displays increased feeding rates, if fed with a high zinc diet (Bowen et al., 2006).

1.4.2 Photoperiod, seasonal and circadian rhythms

Photoperiod can affect feeding activity (Imsland et al., 2007; Noble et al., 2005; Sunuma et al., 2007; Tucker et al., 2006) as well as the expression of appetite-regulating hormones. In fish, natural daily feeding rhythms vary among species: some species are diurnal feeders, e.g. Atlantic salmon (Paspatis and Boujard, 1996), whereas others are nocturnal feeders, e.g. catfish (Boujard, 1995). In addition, many fish species have been shown to display preferred times of feeding throughout the day or night and it's been demonstrated that alteration of normal daily feeding rhythms can induce poor performance and eventually diseases and death (Lopez-Olmeda et al., 2011)

Natural behavioural rhythms are often classified by their period, which is the interval needed to complete one cycle. Different rhythms have been studied in fish: tidal and lunar rhythms, circannual or seasonal rhythms (Bolliet et al., 2007; Boujard and Leatherland, 1992; Lall and Tibbetts, 2009).

One of the most evident expressions of adaptation to the environment, and a major characteristic of living organisms, are the **Circadian rhythms**. As the etymology of the name stresses (from the Latin *circa*, meaning "around" and *dies*, "day", meaning "approximately a day", name created by Halberg) these rhythms develop on periods close to 24 h (Menaker et al., 1997) and persist in constant conditions. The circadian rhythm can be divided into routine cycles during the 24-hour day: diurnal, which describes organisms active during daytime; nocturnal, which describes organisms active at nighttime; crepuscular, which describes animals primarily active during the dawn and dusk hours.

Besides, rhythms that last longer than a day (>28-hour) are defined **infradian rhythms** (annual migration or reproductive rhythms found in certain animals or the human menstrual cycle), while shorter ones, that last less than 20 hours, are called **ultradian rhythms** (the 90-minute REM cycle, or the 3-hour cycle of growth hormone production (Butler et al., 2005).

External stimuli are able to entrain circadian rhythms, in particular photoperiod: the 24 hours light-dark cycle is the most consistent environmental signal, because of its stability in period and phase. In fact most of the circadian rhythms of species studied until now are synchronized in a daily manner by this cue. However, periodical fluctuations exhibited by biochemical, physiological and behavioural parameters and rhythms persist in constant conditions, thus demonstrating that they arise within organism itself and are not imposed by the environment (Kulczykowska et al., 2010)

The endocrine mechanisms regulating these rhythms are poorly known. Little is known about the fluctuations in circadian-related proteins or appetite-regulating peptides in fish.

1.4.2.1 Properties and parameters of biological rhythms

Circadian rhythms are present in all eukaryotic and some prokaryotic organisms and their wide diffusion show evolutionary conservation. Circadian rhythms are temperature-compensated, genetically determined and thus generated by a self-sustained endogenous pacemaker.

In vertebrates, a circadian timing system generates and regulates circadian rhythms; it consists in pacemakers and oscillators, entrainment pathways and pacemaker outputs connected to effector systems that express evident rhythms under circadian control (Guido et al., 2002).

Circadian rhythms “free-run”, displaying a period of approximately 24 hours, when organisms are in constant conditions and without environmental cues. The periods of the endogenous rhythms actually differ from those in nature, but thanks to exogenous synchronizing cues, commonly called **zeitgeber** (“timegiver” in German), biological rhythms are adjusted in such a way that organisms remain synchronised with their environment (Kulczykowska et al., 2010), as zeitgebers help to reset the biological clock to a 24-hour day.

Circadian rhythms can be graphically represented in cronograms, where a sine wave can be fitted (Figure 1.1. Diagram of an oscillatory process characterized by its 4 parameters.). 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).

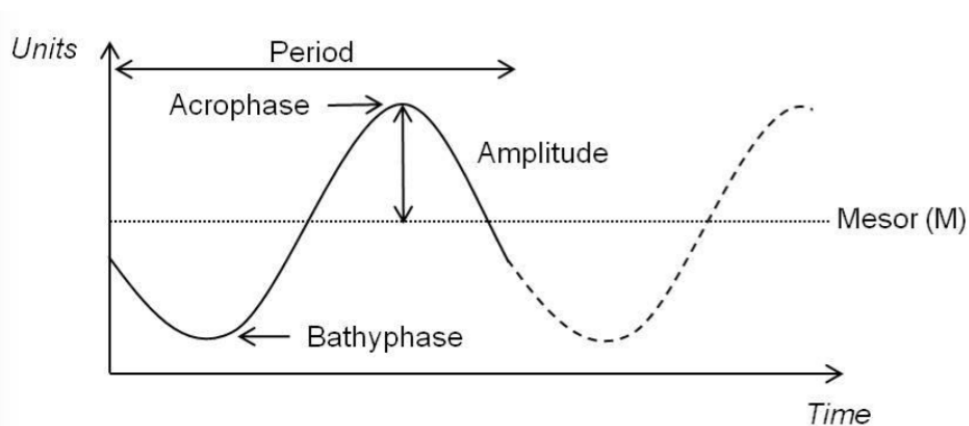


Figure 1.1. Diagram of an oscillatory process characterized by its 4 parameters.

1.4.2.2 Circadian system: structure and molecular base

All circadian systems are composed of three basic elements:

- 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).

In vertebrates, three neural structures have been reported as oscillators or clocks: the retina, the pineal complex and the mammalian suprachiasmatic nucleus (SCN) of the hypothalamus or its analogous areas in other groups (Menaker et al., 1997). Nevertheless, independent or semi-independent, light-entrainable circadian clocks appear to exist at all levels of organization from cells, through tissues to organs.

Usually in animals, a master or central clock is present and it 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). In fish the pineal gland is considered to serve as central pacemaker, as transduce environmental light information into a neural and a neuroendocrine signal. It contains an intrinsic circadian clock that drives rhythmic synthesis of the hormone Melatonin, an indoleamine with well-known effect on internal biological rhythms, whose levels are high at night and low during the day as a result of regulated transcription and stability of serotonin-N-acetyl-transferase (AANAT).

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).

Internal biological clocks allow organisms to adapt their behavioural and physiological functions to daily and seasonal variation of environmental factors (Pevét, 2001). 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).

In 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. 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).

In fish, this clock machinery has been studied in goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), 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.

1.4.2.3 Light entrainment and food entrainment

Although light is the most noticeable zeitgeber, several non-photoc 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, called Food-Entrainable Oscillator (FEO), which is independent from the Light-Entrainable Oscillator (LEO) (Meijer and Rietveld, 1989) but not well-defined yet, as its 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, even if it is still uncertain whether fish FEO and LEO are independent (Sánchez-Vázquez et al., 1997; Aranda et al., 2001). On the contrary in mammals studies suggested the independence of FEO from LEO (Stephan, 2002): lesions on SCN didn't abolish feeding rhythms such as FAA (Food Anticipatory Activity), which is a phenomenon that displays an increase of activity in anticipation of an imminent meal, which is maintained for at least 30 minutes

(Mistlberger, 1994) and it's clearly connected with FEO. 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 includes behavioural and physiological variables such as feeding, reproduction and territoriality, 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) 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). Anticipation of time meals has been reported for a wide variety of animals, from bees to higher vertebrates such as monkeys (Stephan, 2002) and it brings 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).

In mammals behavioural/physiological parameters appeared to be controlled by the FEO, such as rinking 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). Furthermore, along with 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

1.5 The Senegalese sole: biology and development

The Senegalese sole (*Solea senegalensis*, Kaup, 1858) is a marine teleost fish belonging to the Class Actinopterygii, Order Pleuronectiformes, Family Soleidae (**Figure 1.2**

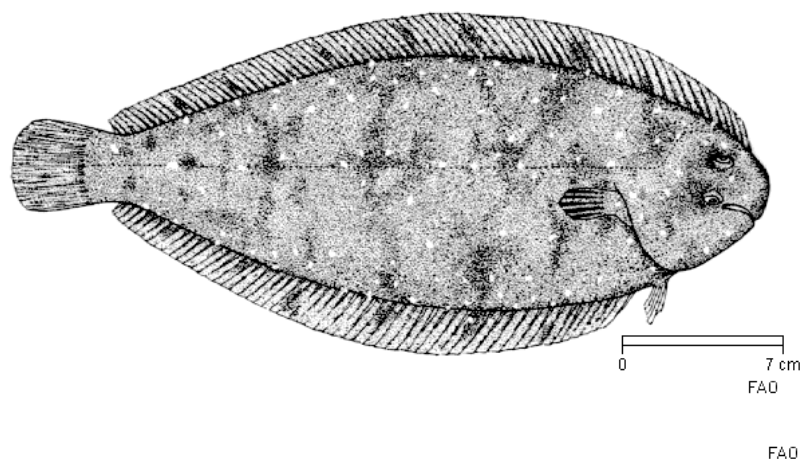


Figure 1.2: *Solea senegalensis*, Kaup 1858. (fishbase.org)

The Soleidae family, which consists of 22 genera and 89 species inhabiting brackish, marine and fresh waters, is characterized by flat bodies and unusual asymmetrical external appearance.

The larval fish display a perfect symmetry, which is lost through a metamorphosis that takes place from a few weeks/months after hatching. This determines the migration of the left eye, that will take place on the right side of the skull/head, near the the other eye.

Thus the adult fish is characterized by an oval-shaped and flattened body, strongly compressed, with an ocular side which is slightly rounded and pigmented and a blind side which is white and totally flat, facing the bottom.

As benthic fishes, they normally lie down in the bottom/seafloor, usually covered by sand or mud, and they mimic their background by assuming a similar coloration. Their habit of burrowing in the sand represents an innate instinct of soles to avoid a possible aggression and it is kept also in individuals held in captivity. Soles 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.

The Senegalese sole inhabits mobile sand or muddy bottoms, around 100 m of depth, mainly in



Figure 1.3: Computer generated distribution maps for *Solea senegalensis* (Senegalese sole), with modelled year 2100 native range map based on IPCC A2 emissions scenario. www.aquamaps.org. **Note:** Distribution range colors indicate degree of suitability of habitat which can be interpreted as probabilities of occurrence.

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 (**Errore. L'origine riferimento non è stata trovata.**).

Sole is an euryhaline and eurythermal species, adapting perfectly to changes in temperature and salinity (Rueda-Jasso et al., 2004). Sole can tolerate low levels of dissolved oxygen and grow optimally in under saturated environments (Salas-Leiton et al., 2008). Senegalese sole is a predator principally of benthic invertebrates and it 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 males (first spermiation), and between the second and fourth year of life in females (first oviposition), when the size reaches 30 cm (Dinis et al., 1999). They have an asynchronous 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 (Anguís and Cañavate., 2005; Oliveira et al., 2009). Spawning 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).

Nocturnal habits are shown not only for spawning (Oliveira et al., 2009a), but also for locomotor activity (Bayarri et al., 2004), feeding (Boluda Navarro et al., 2009) and *Solea senegalensis* displayed even a higher metabolic rate during the dark phase (Castanheira et al., 2011). Senegalese sole exhibits clearly nocturnal self-feeding patterns under laboratory and 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 and showed an accurate compensatory feeding behaviour by modifying their feeding activity accordingly to the reward level. (Boluda Navarro et al., 2009).

1.5.1 Interest in aquaculture

The Senegalese sole (*Solea senegalensis*) is a flatfish of high commercial importance that is almost indistinguishable by consumers from common sole (*Solea solea*, Linnaeus, 1758).

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

Ongrowing of sole is carried out under two major strategies: a traditional one and a more intensive one. Traditionally in Spain and in southern Portugal, soles were reared in earthen ponds in old salt marshes or other deltaic or estuarine environments. Fingerlings were either passively captured in these ponds. Feeding consisted in the occurrence of natural prey in the bottom of the ponds, with few or null effort from the aquaculturists, or supplementing or replacing these feed items with inert feed, hence taking a semi-intensive approach. On the other hand, the intensive approach is nowadays the leading trend although salt marshes are still used for semi-intensive and intensive sole aquaculture. Soles are stocked in fiberglass or concrete tanks, often in shallow raceways (Imstrand et al., 2003), for the whole production cycle, and they are 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), mainly because of juvenile scarcity for stocking purposes, caused by the lack of full control over spawning, poor fry quality and high mortality rates during the weaning stage (Cañavate & Fernández-Díaz, 1999; Anguís & Cañavate, 2005). Furthermore 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) is exhibited, along with disease outbursts affecting all ontogenetic stages caused by multiple pathogenic agents (Zorrilla et al., 2003).

Reproduction of Senegalese sole is one of the main difficulties 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 steroid profiles and spontaneous spawning (Guzmán et al., 2008), with egg quality parameters within normal ranges (Guzmán et al., 2009a) but normally these eggs show no fertilization in the communal reproduction tanks.

Good broodstock management practices are required so to maintain genetic variation within farmed strains at comparable levels to those of wild source populations, e.g. the use of adequate numbers of effective parents, broodstock from different locations, or even more local ones if significant differences are present in the wild (Exadactylos et al., 2007). There are only a few studies focusing on assessing the optimal ongrowing photoperiod for sole, although it has been observed that sole is strongly influenced by it, as it shows a evident 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 prespawning phase, also advancing spawning (Oliveira et al., 2011). These findings point out to the possible practicability 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).

2 Objectives

The general objective of this study was to investigate how different photoperiods and feeding regimes modulate daily mRNA expression of three specific orexigenic hormones, i.e. the Neuropeptide Y (NPY) and the paralogues Agouti-related Protein 1 (AgRP1) and Agouti-related Protein 2, in adult specimens of *Solea senegalensis*, a flatfish species with a growing commercial interest in Southern Europe for marine aquaculture.

It is important to increase knowledge on the endocrine mechanisms that regulate feeding and growth in cultured fish, so to improve fish holding conditions and feeding strategies in aquaculture practices. In order to achieve this and to support the development of new techniques that could enhance feeding, food conversion efficiency and growth in cultured fish, two 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 times).
- In a second experiment, adult fish were maintained under a DD (0 h light: 24 h dark) photoperiod and they were divided in two tanks, each one kept at a different feeding regime (feeding during the subjective daytime and feeding at random times).

For both experiments, daily mRNA expression of selected orexigenic hormones (NPY, AgRP1 and AgRP2) has been studied in different central areas, i.e. telencephalon, diencephalon and optic tectum, which previous studies suggested to be the main areas involved in feeding in fish and rich in neurons related to orexigenic hormones.

This main objective also allowed to attain the following specific achievements:

- To gain skills on molecular biology techniques such as RNA extraction, cDNA synthesis and real time quantitative PCR.
- To gain skills on several bioinformatic tools and specific software such as the chronobiological software El Temps and the Bio-Rad CFX Manager v 3.1.

3 Materials and Methods

3.1 Animals and rearing system

This study was carried out on a total number of 128 Senegalese sole adult specimens (*Solea senegalensis*) which were collected from the “Laboratorio de Cultivos Marinos” of the University of Cádiz (Puerto Real, Spain). The animals had length between 20 and 25 cm in length and their weight spanned from 101 to 172 g. They were kept in five 250L tanks and maintained at constant temperature ($19\pm 1^\circ\text{C}$) and salinity (39 ppt), with continuous seawater renovation and gentle aeration. Each tank was equipped with an automatic feeder (EHEIM GmbH & co. KG, Germany) connected to a digital programmable timer (Data micro, Orbis, Spain), so to provide food at scheduled times. Commercial 3 mm dry pellets (Skretting S.A., Burgos, España) were supplied at a daily ration of 0,3% body weight. The tanks were also equipped with a lid containing two fluorescent lamps (Sylvania Gro-Lux, Germany) connected to an individual automatic photoperiod control system which allowed a light intensity of 400 lux at water surface level during the illumination period.

The experimental procedures were approved by the Animal Experimentation and Ethics Committee of the University of Cádiz (Spain) and performed according to international ethical standards.

3.2 Experimental design

Two experimental groups with different photoperiod conditions were designed. The photoperiod for the “LD group” 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 animals of the “DD group” were reared in constant dark conditions, i.e. 0 h light: 24 h dark (0L:24D).

The animals reared in light-dark conditions, were subdivided in 3 groups, each one experiencing a distinct feeding regime:

- ML group: this group received food at a fixed time during the day, at 14:00h (ZT6), i.e in the middle of the light phase (ML as mid-light);
- MD group: this group comprised the animals that were fed at fixed time during the night, at 02:00h (ZT18), i.e., in the middle of the dark fase (MD as mid-dark);
- RND group, whose feeding was set at a random times, with an interval between 12 and 36 hours so they received the same amount of food per 24 h, as the ML and MD groups.

The animals kept in constant darkness, i.e., “DD group”, were divided in 2 groups with different feeding time:

- sML group: this group received food at fixed time during the mid-light point of the subjective day, at 14:00h (circadian time 6 or CT6).
- RND group: this group was fed at random times, with an interval between 12 and 36 hours, as described previously for the LD group.

3.3 Sampling

Sampling was conducted after four weeks under these different feeding regimes. during a 24 h daily cycle. The soles were anesthetized in MS-222 (Sigma, St. Louis, MO; 100-200 mg/l of water) and sacrificed by decapitation.

Animals held under LD conditions were sampled every 4 hours, starting at 8:00 h local time, which was considered ZT0 (zeitgeber time 0), at six different zeitgeber time points (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20), where ZT0 corresponded to the light onset and ZT12 to the light offset.

The soles under DD conditions were sampled every 4 hours as well, at six different circadian time points: CT0, CT4, CT8, CT12, CT16, CT20.

The selected neural tissues, i.e. telencephalon, diencephalon and optic tectum, were dissected from every specimen, rapidly frozen in liquid nitrogen and stored at -80° C until used.

3.4 Selection and design of specific primers

Several specific primer pairs for Senegalese sole *npv*, *agrp1* and *agrp2* genes analysed by real-time quantitative PCR (RT-qPCR) were designed from the partial sequences available in the SoleaDB

(http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/sessions/new?locale=es) server using the Primer3 Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).

A gradient temperature RT-qPCR was run for each primer pair, in order to optimize PCR conditions, with the following temperatures: 58°C, 60,8°C, 62,5°C and 64,6°C. The resulting amplicons were analysed through agarose gel electrophoresis and DNA was extracted by using QIAquick Gel Extraction Kit (QIAGEN Group Inc, USA) following manufacturer instructions. Briefly, DNA fragments were cut out from the agarose gel with a clean, sharp scalpel, then put in a tube with 3 volumes of buffer QG available with the kit per 1 volume gel and incubated at 50°C for 10 min. After complete dissolution of the gel slices, the mixture was put in a collection tube and centrifuged for 1 min, to let the DNA bind at the membrane of the QIAquick column. The flow-

through was discarded and the filter with DNA was washed with 750 μ L of Buffer QC. Successively, 30 μ L of Buffer EB (10 mM Tris Cl, pH 8,5) were added to elute the DNA. Total DNA yield and quality were determined by 260/280 nm absorbance ratio in a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, De, USA). The DNA obtained was sent with the primers to the SCAI (Unidad de Genómica (Universidad de Córdoba, Campus de Rabanales) in order to be sequenced and confirm the identity of amplified products. Primer oligonucleotide sequences finally used are shown in **Table 3.1**

Gene	Primer	Sequence (5'→3')
<i>NPY</i>	SsNPYqPCR-F1 SsNPYqPCR-R1	GAG GGA TAC CCG ATG AAA CC GCT GGA CCT CTT CCC ATA CC
<i>AgRP1</i>	SsAgRP1qPCR-F2n SsAgRP1qPCR-R2n	CTGGTTCATGGAAACATTCCGC TGTCCTCTTTCGATGTCAGACAGG
<i>AgRP2</i>	SsAgRP2q-F1 SsAgRP2q-R2	CAG GTC AGA CTC CGT GAG CCC GTC GAC ACC GAC AGG AGG CAC
<i>β-actin2</i>	qSsBact-Fw qSsBact-Rv	TCT TCC AGC CAT CCT TCC TCG TGT TGG CAT ACA GGT CCT TAC GG

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

3.5 RNA extraction

Total RNA was extracted by using the TRIsure Reagent® (Bioline, London, UK), following the manufacturer protocol. Briefly, 1 ml of TRIsure was added to each sample, then homogenized in a mixer mill MM400 (Retsch, Haan, Germany) with the use of 4 stainless steel beads (2 mm diameter). After homogenization, 0,2 ml of chloroform D (AppliChem, Darmstadt, Germany) was added and samples were shaken by hand for 15 s and then incubated at room temperature for 3 min. Consequently, samples were centrifuged at 12.000 x g for 15 min at 4°C, causing the mixture to separate in three phases: a pale green phenol-chloroform phase, containing DNA, at the bottom of the tube; an interphase made up of protein and, at the top of the tube, a colourless aqueous phase, containing RNA, which was transferred into a new tube. Then RNA was precipitated by adding 0,5 ml of isopropyl alcohol (Panreac, Barcelona, Spain), and letting the samples incubate at room temperature for 10 min. Samples were centrifuged at 12.000 x g for 10 min at 4°C, creating a gel-like pellet at the bottom of the tube. The supernatant was completely removed and the RNA pellet was washed with 1 ml 75 % ethanol, prepared with diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich, St. Louis, MO, USA) to avoid RNA degradation by RNAses. Samples were centrifuged at 7.500 x g for 5 min at 4°C and ethanol was removed. The RNA pellet was air-dried

for 5-10 min and re-dissolved in DEPC-treated water (from 15 to 50 μ l) and incubated for 10 min 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 between 1.8 and 2.

3.6 cDNA synthesis

Aliquots of 100 ng of total RNA were reverse transcribed into cDNA (20 μ l final volume) using the QuantiTect Reverse Transcription Kit (Qiagen, Inc, USA). The procedure consisted of 2 main steps:

1. *Elimination of genomic DNA*

The purified RNA samples were incubated in gDNA Wipeout Buffer available with the kit, containing DNase, at 42°C for 2 min. This step is required to effectively remove contaminating genomic DNA.

2. *Reverse transcription*

After genomic DNA elimination, the RNA samples were prepared for reverse transcription, adding 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 min followed by 2 min at 95°C for enzyme inactivation. Then cDNAs were stored at -20°C until used.

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

Real time quantitative PCR (RT-qPCR) was performed in a Bio-Rad CFX96 Touch detection system (Bio-Rad, Alcobendas, Spain), using the SensiFAST SYBR No-ROX Kit (Bioline). PCR reactions were developed in a 20 μ l volume containing 5 ng of cDNA. Duplicates of each sample were analysed in the same assay. Specific primers for sole *npv*, *agrp1*, *agrp2* and *β -actin2* are shown in **Errore. L'origine riferimento non è stata trovata.**

The amplification protocol for the three genes was as follow (**Errore. L'origine riferimento non è stata trovata.**)

1. Initial denaturation at 95°C for 2 min;
2. 40 cycles of 95°C for 5 s and 60°C for 30 s for *npv*;
40 cycles of 95°C for 5 s and 62,2°C for 25 s for *agrp1*;
40 cycles of 95°C for 5 s and 64°C for 20 s for *agrp2*;
3. 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 was calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) with *Solea senegalensis* β -actin2 (Genbank accession number DQ485686) as housekeeping gene (Infante et al., 2004; Martín-Robles et al., 2012b).

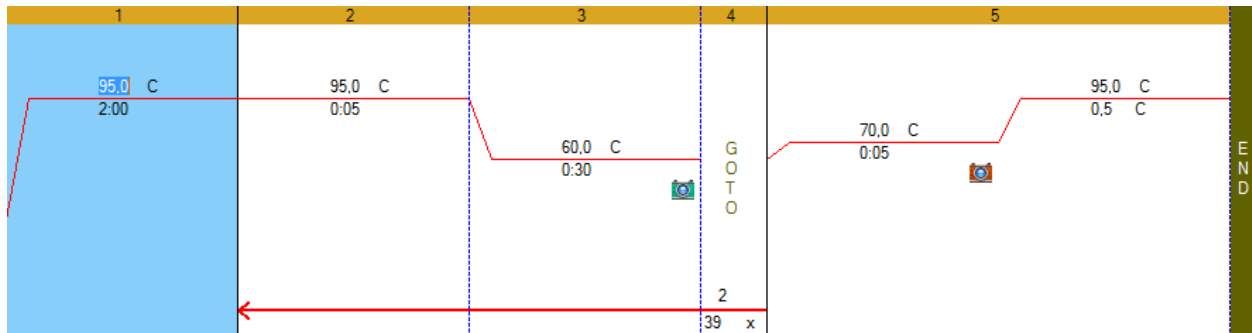


Figure 3.1: Example of the RT-qPCR amplification protocol (Bio-Rad CFX software screen) followed for the orexigenic gene *NPY* of *Solea senegalensis*.

3.8 Data analysis

3.8.1 Statistical analysis

To evaluate statistical differences in the expression of orexigenic genes, mRNA levels of each gene were analysed, among different daily time points, by one-way ANOVA. Significant ANOVA analyses were followed by Tukey's post hoc comparison test, to determine differences between means, with $p < 0.05$ taken as the statistically significant threshold. When necessary, values were transformed to achieve normal distribution and homogeneity of variances. In all cases, statistical significance was accepted at $p < 0.05$.

All statistical tests were performed using Statgraphics Plus 5.1 software (Statpoint Technologies, Warrenton, VA, USA). All graphics were created by means of Microsoft Office Excel 2011 and 2016.

3.8.2 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 Daily expression of NPY, AgRP1 and AgRP2 in Senegalese sole reared under light-dark (LD) conditions and different feeding schedules.

In order to assess the influence of light and feeding time in central expression of orexigenic genes, daily mRNA expression of *npy*, *agrp1* and *agrp2* was investigated by real time qPCR in three different neural areas, i.e., telencephalon, optic tectum and diencephalon of Senegalese sole adult specimens maintained under LD conditions and different feeding schedules: fed at ML (ZT6, 14h local time), MD (ZT18, 02h local time) and at RND (random) times. These areas were selected because they have been implicated in the neuroendocrine control of feeding behaviour in fish and previous studies have shown that these brain regions are rich in neurons producing these hormones (Rodríguez-Gómez et al. 2001). Moreover, these neural areas exhibited a high expression of anorexigenic genes *crh*, *pomc_a* and *pomc_b* in sole (Muñoz-Cueto et al. unpublished).

4.1.1 Daily expression of NPY mRNA under LD conditions

As stated before, daily expression of *npy* mRNA was analysed in three neural areas: telencephalon, diencephalon and optic tectum (**Figure 4.1**).

In telencephalon and diencephalon, statistical analysis revealed that mRNA expression of *npy* did not show significant differences in any of the different feeding regimes used in this study (**Figure 4.1**). Cosinor analysis neither evidences rhythmic expression of *npy* in these central areas.

However, in the optic tectum *npy* daily expression did not show statistically significant differences in groups fed at mid light (ML group) and mid dark (MD group) times, while it significantly differed in the group fed at random times (RND group). In this group, a significant increase in *npy* transcript levels was detected at the beginning of the dark phase (**Figure 4.1**). Moreover, *npy* expression was revealed as rhythmic by Cosinor analysis (**Table 1.1**), reporting a peak of expression (acrophase) near the day-night transition, at ZT11.18 (**Table 4.1**). The peak was followed by a significant decrease toward the end of the night. The lowest expression values were displayed around ZT4, in the first half of light period, and at ZT20, in the second half of the dark phase (**Figure 4.1**).

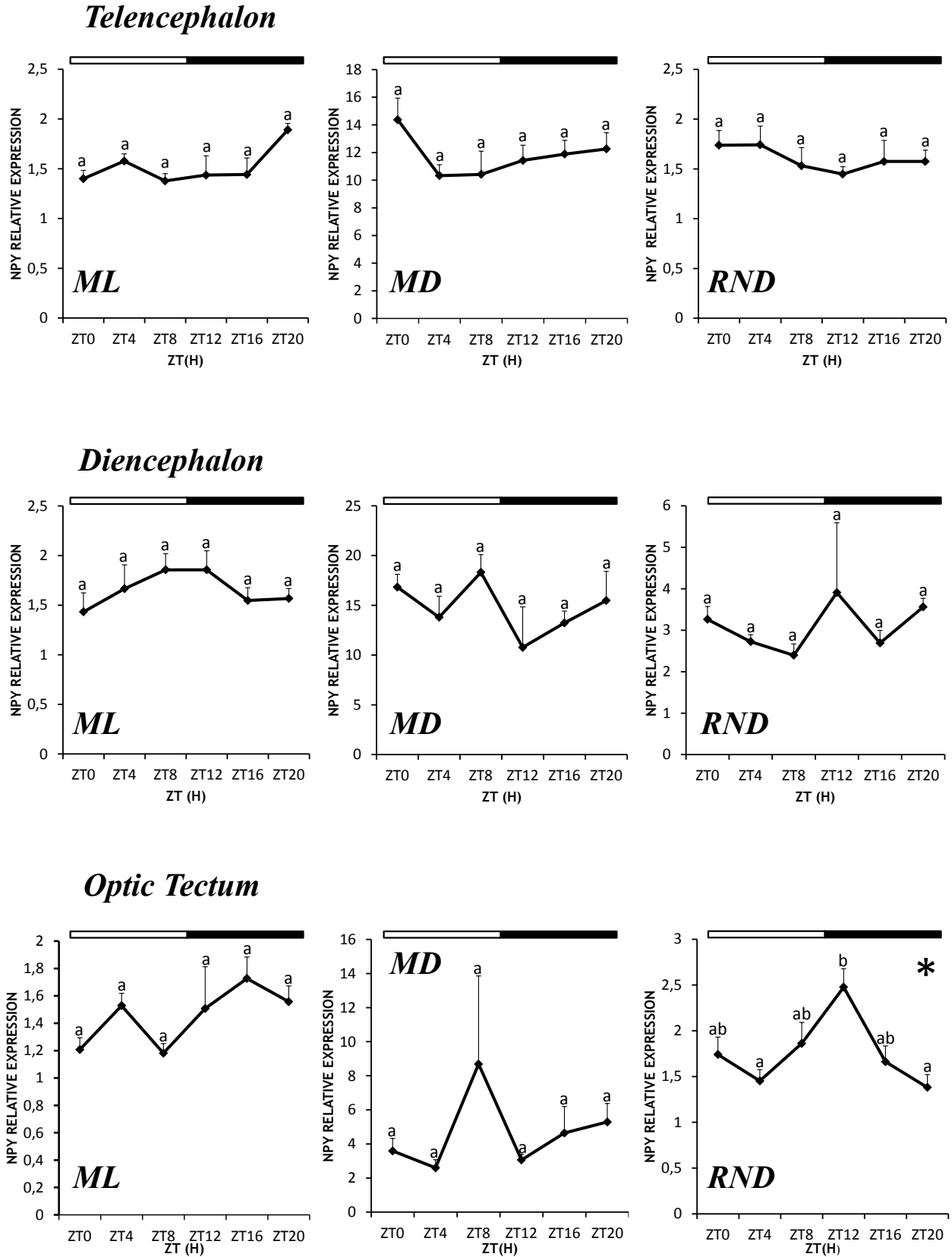


Figure 4.1 Relative expression of *npy* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under LD 12:12 cycles and fed at mid light (ML), mid dark (MD) or at random times (RND). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$). The black and 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).

NPY LD Conditions

ML GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	1.5206	0.1453	20.7533	97.3207	N.S.
Diencephalon	1.6537	0.2065	9.6794	96.6170	N.S.
Optic tectum	1.4514	0.1802	16.4665	95.5640	N.S.
MD GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	11.8502	1.1691	21.3975	96.0389	N.S.
Diencephalon	14.9723	1.3930	0.0897	91.4069	N.S.
Optic tectum	4.6437	0.8297	10.1213	53.0952	N.S.
RND GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	1.6017	0.1369	1.0081	96.9177	N.S.
Diencephalon	2.0316	0.4115	13.1619	70.7865	N.S.
Optic tectum	1.7623	0.3687	11.1833	95.2597	0.0233 *

Table 4.1: Parameters estimated by the Cosinor analysis for *npv* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at ML, MD and RND times. The variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., non significant rhythm ($p > 0.05$). Asterisks indicate significant rhythms ($p < 0.05$).

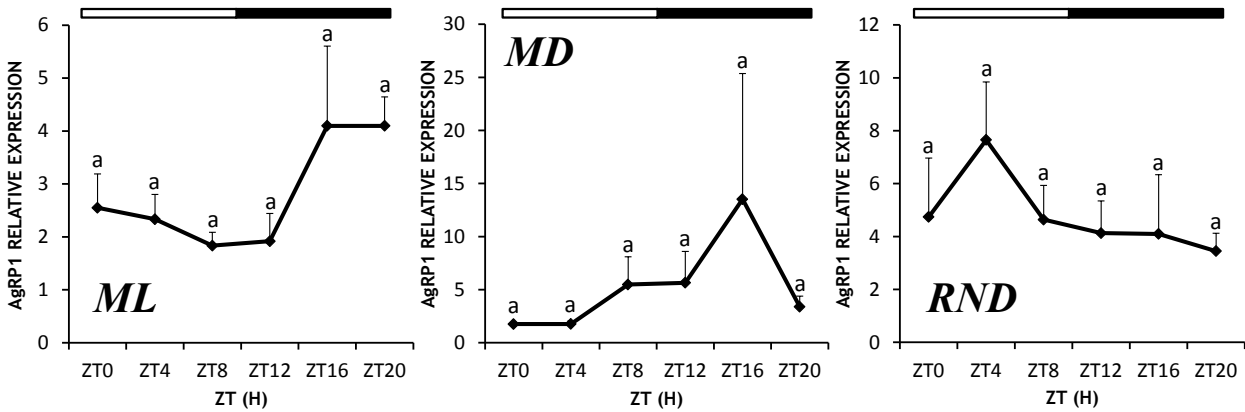
4.1.2 Daily rhythms of *AgRP1* mRNA expression

As for *npv*, daily rhythms of *agrp1* were analysed in telencephalon, diencephalon and optic tectum of Senegalese sole. (**Figure 4.2**).

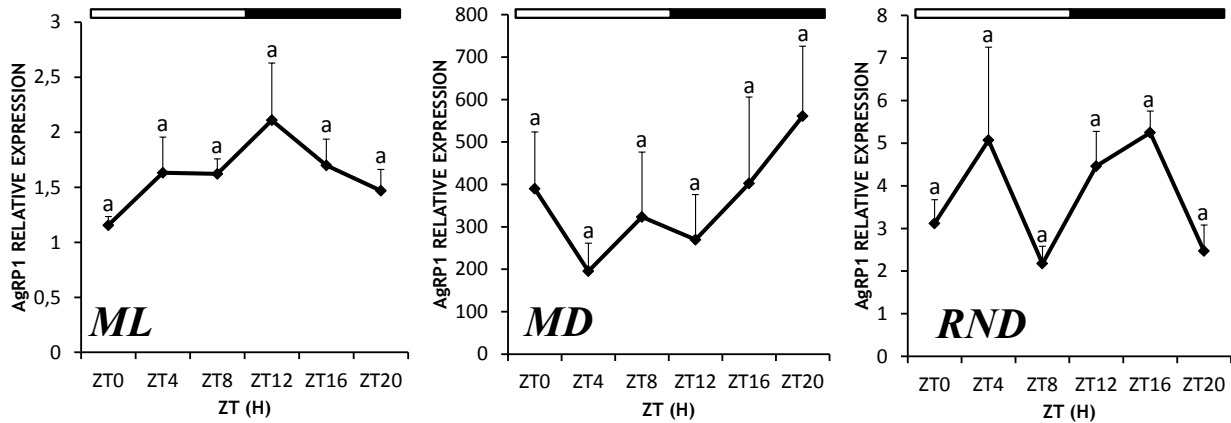
Statistically significant variations in *agrp1* daily mRNA levels were not evident in the neural areas analysed.

In contrast, Cosinor analysis revealed a significant daily rhythm in *agrp1* levels in optic tectum for the ML group, i.e., animals fed at mid light (ZT6), showing its acrophase during the early night at ZT14.49 (**Table 4.2**).

Telencephalon



Diencephalon



Optic Tectum

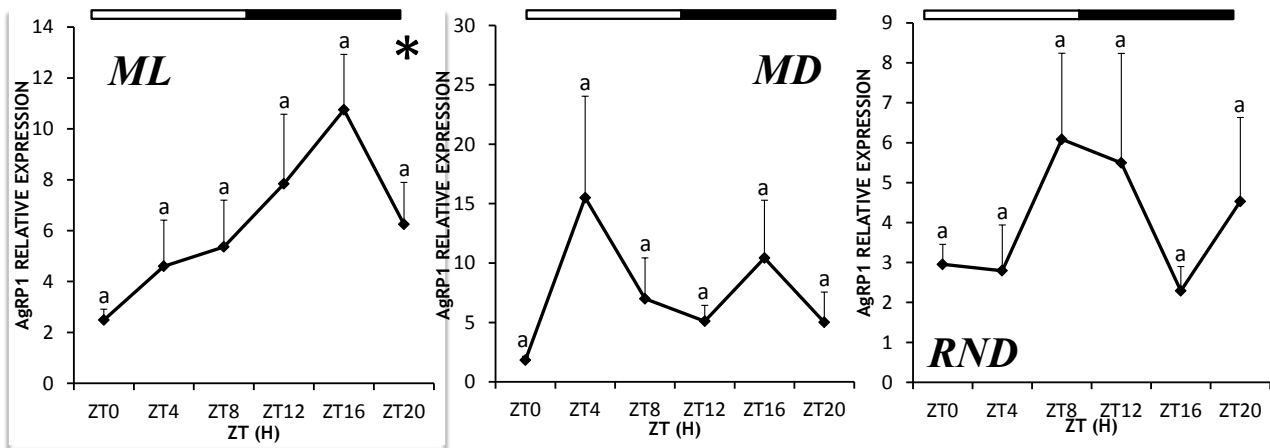


Figure 4.2: Relative expression of *agrpl* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under LD 12:12 cycles and fed at mid light (ML), mid dark (MD) or at random times (RND). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$). The black and 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).

AgRP1 LD Conditions

ML GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	2.6207	0.8503	18.4937	78.2809	N.S.
Diencephalon	1.6339	0.3349	12.1067	91.1133	N.S.
Optic tectum	6.2146	3.3481	14.4906	78.6266	0.0180 *
MD GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	5.2610	4.5574	14.5170	30.9600	N.S.
Diencephalon	356.9160	136.0000	19.2928	67.1501	N.S.
Optic tectum	7.4784	2.1157	7.0512	43.4575	N.S.
RND GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	4.7606	1.4770	4.5432	68.3113	N.S.
Diencephalon	3.7575	0.4496	13.1782	75.3085	N.S.
Optic tectum	4.0920	1.1765	9.5759	61.5924	N.S.

Table 4.2: Parameters estimated by the Cosinor analysis for *agrp1* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at ML, MD or RND times. The variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., non significant rhythm ($p > 0.05$). Asterisks indicate significant rhythms ($p < 0.05$).

4.1.3 Daily expression of AgRP2 mRNA under LD conditions

The daily expression of *agrp2* transcript was analysed in telencephalon, optic tectum and diencephalon. (**Figure 4.3**)

In both telencephalon and diencephalon ANOVA and Cosinor analysis did not reveal significant daily variations of *agrp2* expression.

In optic tectum, *agrp2* daily profile displayed significant differences only in Senegalese sole specimens fed at mid light (ZT6). Also, Cosinor analysis showed a significant daily rhythm in *agrp2* relative expression (**Table 4.3**). Transcript levels remained low during the day, until ZT12, when they rapidly increased, reaching a peak of expression at ZT16 (acrophase at ZT15.35), and then they significantly decreased quickly during the night. In groups fed at MD and RND times, no significant differences were observed neither by ANOVA or Cosinor (**Figure 4.3**).

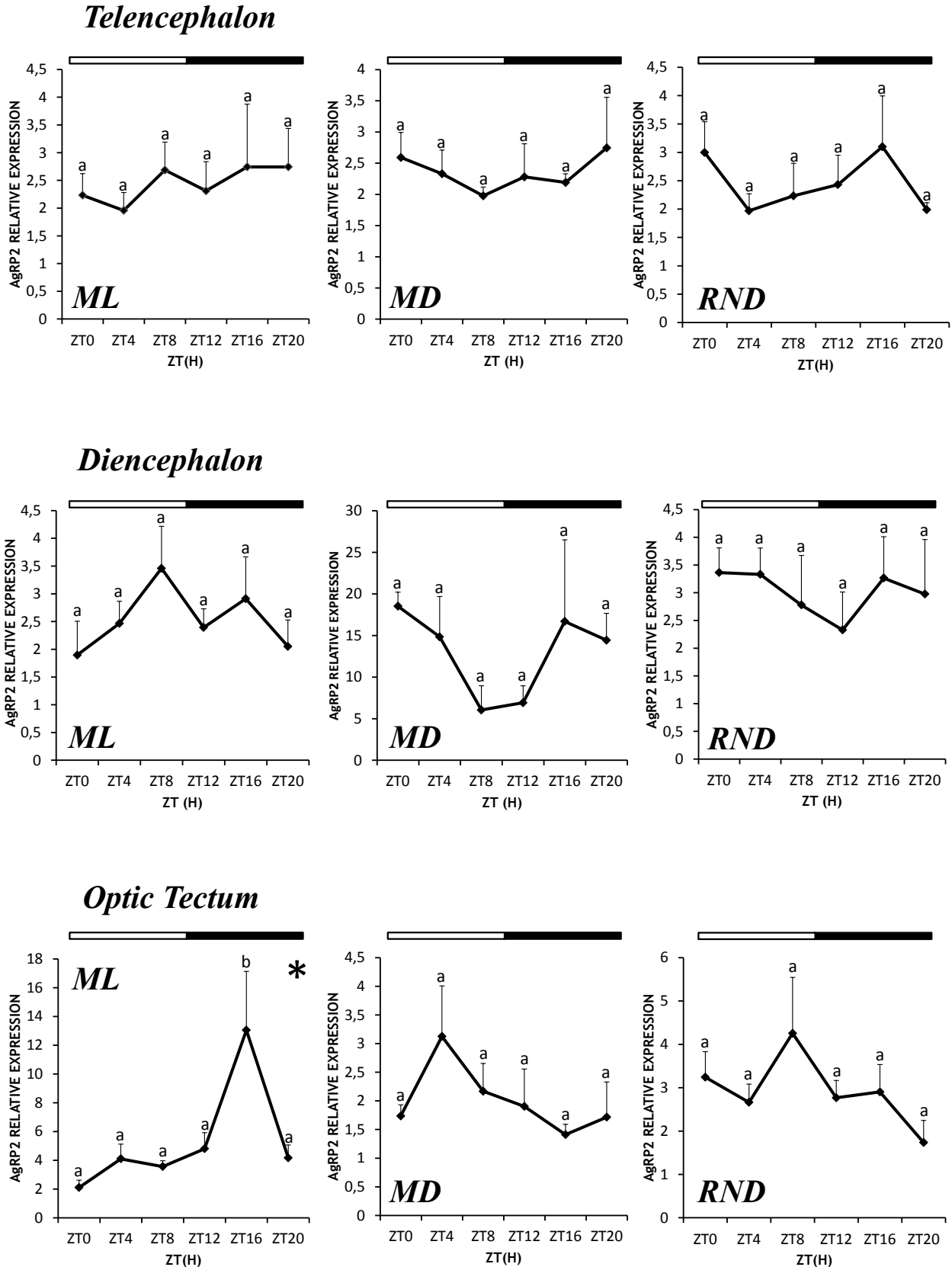


Figure 4.3 Relative expression of *agrp2* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under LD 12:12 cycles and fed at mid light (ML), mid dark (MD) or at random times (RND). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$). The black and 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).

AgRP2 LD Conditions

ML GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	2.6207	0.8503	18.4937	78.2808	N.S.
Diencephalon	2.5298	0.5499	9.9755	85.2463	N.S.
Optic tectum	5.2924	3.5824	15.3533	67.6872	0.0314 *
MD GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	2.3524	0.3136	21.6353	89.4243	N.S.
Diencephalon	12.9068	5.7720	21.9450	69.9576	N.S.
Optic tectum	2.0106	0.6443	5.0749	80.4041	N.S.
RND GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	2.4748	0.2552	18.0434	84.4484	N.S.
Diencephalon	3.0065	0.3907	23.6290	84.5050	N.S.
Optic tectum	2.9281	0.7242	7.6406	83.0351	N.S.

Table 4.3: Parameters estimated by the Cosinor analysis for *agrp2* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under LD conditions and fed at ML, MD or RND times. The variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., non significant rhythm ($p > 0.05$). Asterisks indicate significant rhythms ($p < 0.05$).

4.2 Daily expression of NPY, AgRP1 and AgRP2 in Senegalese sole reared under constant dark (DD) conditions and different feeding schedules.

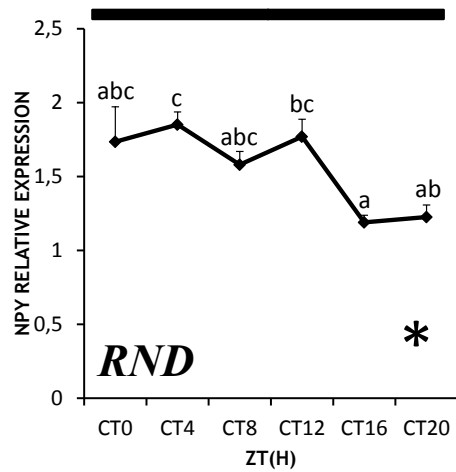
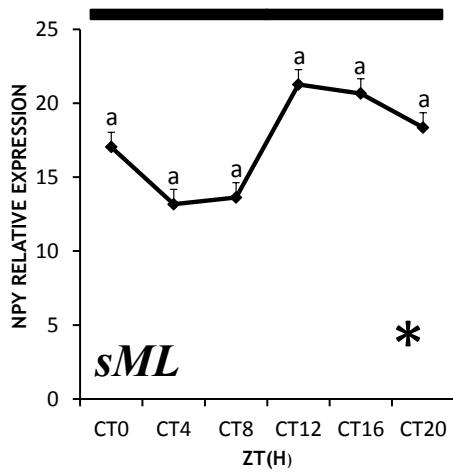
As for LD conditions, daily expression of *npv*, *agrp1* and *agrp2* was analysed in telencephalon, diencephalon and optic tectum of Senegalese sole kept under DD conditions and different feeding schedules: animals fed during the subjective mid light period (CT6, sML group) and animals fed at random times (RND group).

4.2.1 Daily expression of NPY mRNA under DD conditions

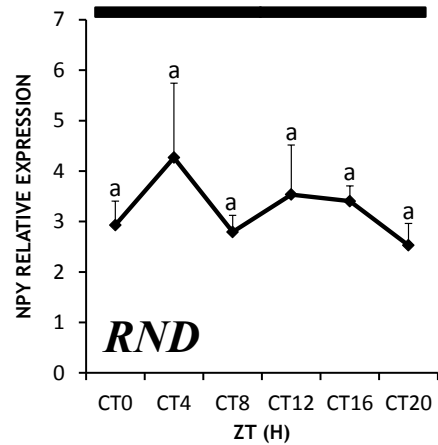
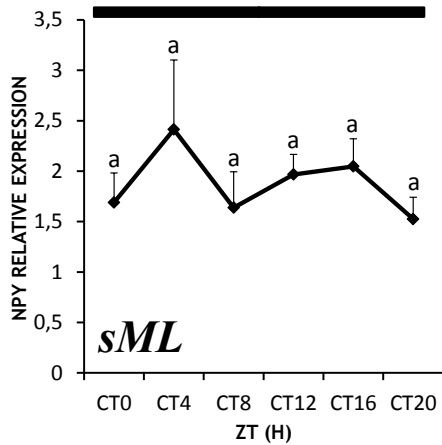
In telencephalon, only animals fed at random times showed significant daily variations in *npv* mRNA expression, as revealed by ANOVA. It displayed the highest value at CT4, during the subjective day, and the lowest transcript levels at CT16, during the subjective night (**Figure 4.4**). On the other hand, Cosinor analysis showed a significant rhythm for both sML and RND groups, displaying the acrophases at CT 16.13 (subjective night) and CT5.48 (subjective day), respectively (**Table 4.4**).

In diencephalon and in optic tectum of soles held in DD conditions, *npv* relative expression did not show any significant differences neither in animals fed at sML (subjective mid light) nor in those fed at random times (RND group). Significant daily rhythms were neither observed in these neural areas (**Figure 4.4; Table 4.4**).

Telencephalon



Diencephalon



Optic Tectum

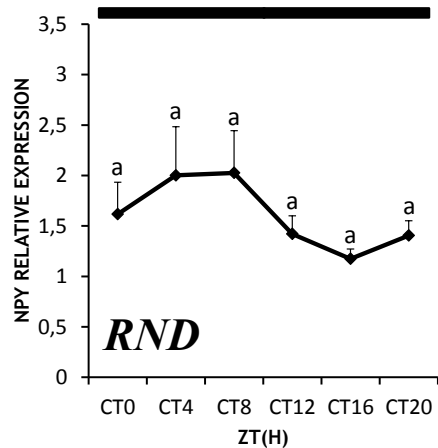
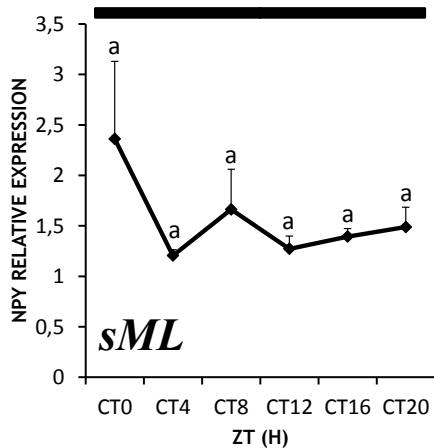


Figure 4.4 Relative expression of npy gene in telencephalon, diencephalon and optic tectum of Senegalese sole under DD constant darkness cycle and fed at subjective mid light (sML) or at random (RND). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$). The black bars at the top of the graphics represent the constant dark phases. Asterisks represent rhythmic expression (Cosinor, $p < 0.05$). CT indicates circadian time (hours).

NPY DD Conditions

sML GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	17,3523	3.9938	16,1384	93.8581	0.0305 *
Diencephalon	1.9020	0.2082	5.7924	85.1544	N.S.
Optic tectum	1.5639	0.3027	23.9540	83.0394	N.S.
RND GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	1.5585	0.2959	5.4854	97.1229	0.0066
Diencephalon	3.2428	0.3416	7.1579	81.3781	N.S.
Optic tectum	1.6075	0.4300	5.1058	90.1625	N.S.

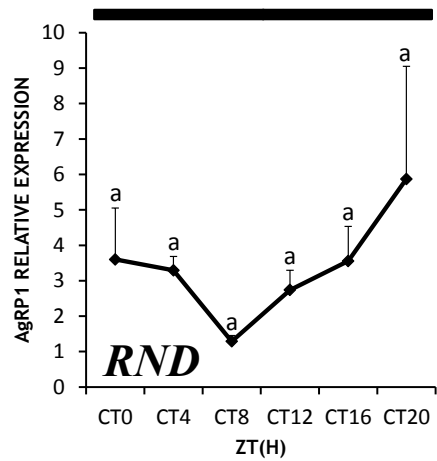
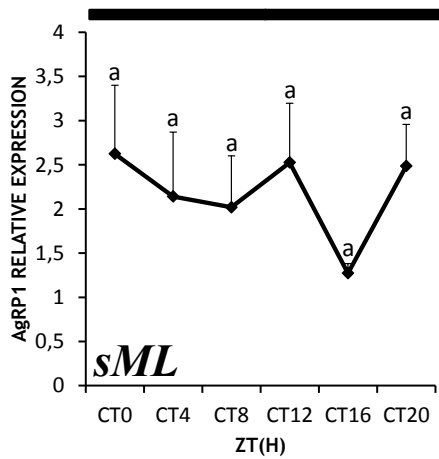
Table 4.4: Parameters estimated by the Cosinor analysis for *npy* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under DD conditions and fed at the subjective midlight period (sML group) or at RND times (RND group). The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., non significant rhythm ($p>0.05$). Asterisks indicate significant rhythms ($p<0.05$).

4.2.2 Daily expression of AgRP1 mRNA under DD conditions

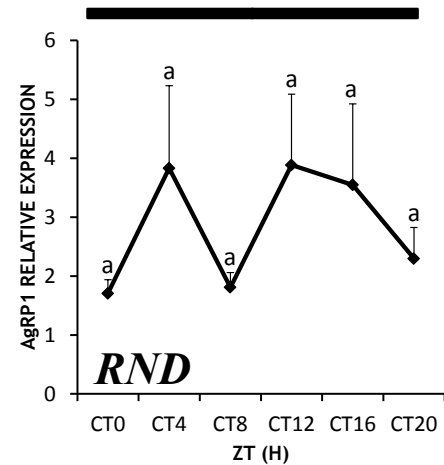
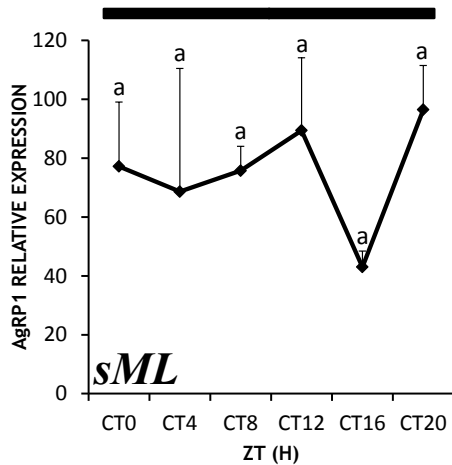
In telencephalon, diencephalon and optic tectum, no significant variations of *agrp1* mRNA relative expression were observed neither by ANOVA nor by Cosinor analysis under DD conditions (**Figure 4.5; Table 4.5**).

However, in sML groups in telencephalon and diencephalon, transcripts showed a similar trend with a rapid decrease after CT12 and a quick increase starting at CT16, which displayed the lowest expression (**Figure 4.5**).

Telencephalon



Diencephalon



Optic Tectum

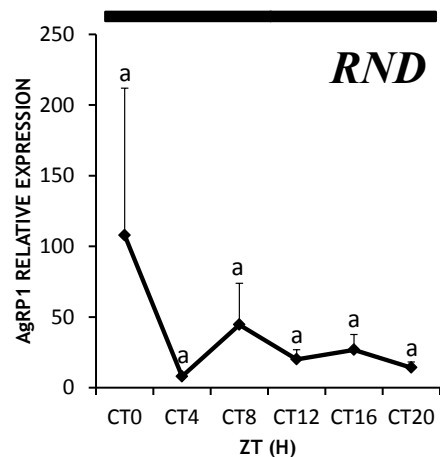
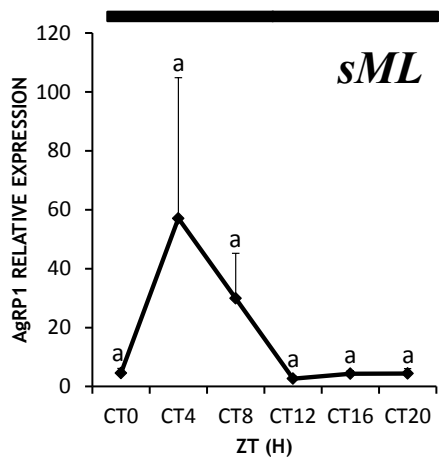


Figure 4.5 Relative expression of *agr1* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under DD constant darkness condition and fed at subjective mid light (sML) or at random times (RND). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$). The 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).

AgRP1 DD Conditions

sML GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	2.1776	0.2802	1.6293	79.2689	N.S.
Diencephalon	75.0459	6.5330	1.4828	70.6290	N.S.
Optic tectum	17.1670	23.1766	5.1403	28.7001	N.S.
RND GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	3.3912	1.7228	20.3842	64.3412	N.S.
Diencephalon	2.8453	0.6001	12.3792	68.6344	N.S.
Optic tectum	36.9143	21.3405	0.5968	18.8287	N.S.

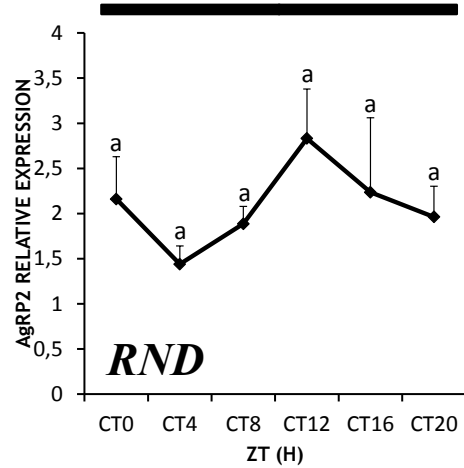
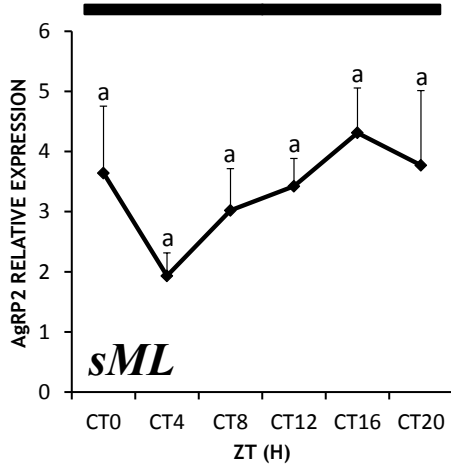
Table 4.5: Parameters estimated by the Cosinor analysis for *agrp1* mRNA relative expression in telencephalon, diencephalon and optic tectum of *Solea Senegalensis* under DD conditions and fed at the subjective midlight period (sML group) or at RND times (RND group). The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. Significance, p-value. N.S., non significant rhythm ($p>0.05$). Asterisks indicate significant rhythms ($p<0.05$).

4.2.3 Daily expression of AgRP2 mRNA under DD conditions

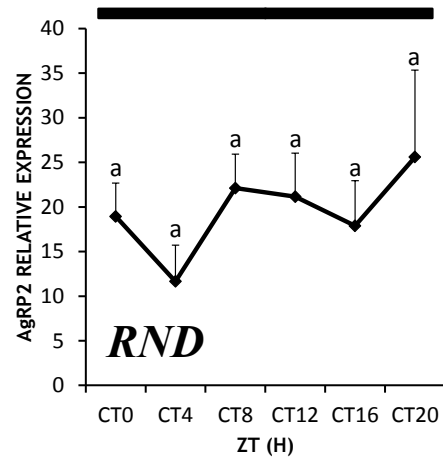
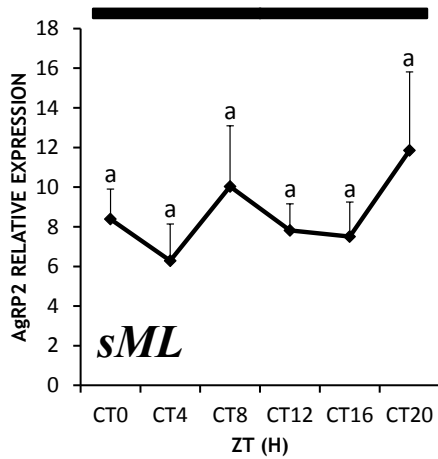
agrp2 mRNA relative expression did not exhibit statistical differences in any neural area and in any of the examined groups, as revealed by ANOVA (**Figure 4.6**). Furthermore, Cosinor analysis did not reveal any significant rhythms in *agrp2* transcript levels (**Table 4.6**).

However, both in telencephalon and diencephalon (sML and RND), as well as on RND group from the optic tectum, *agrp2* transcripts showed similar trends, with the lowest value of relative expression at CT4 (subjective daytime), and the highest mRNA levels in the subjective night time, at different time points (**Figure 4.6**).

Telencephalon



Diencephalon



Optic Tectum

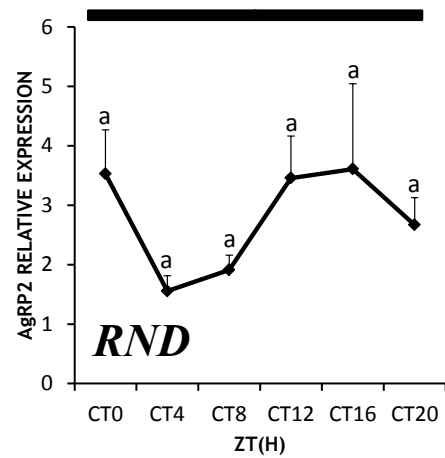
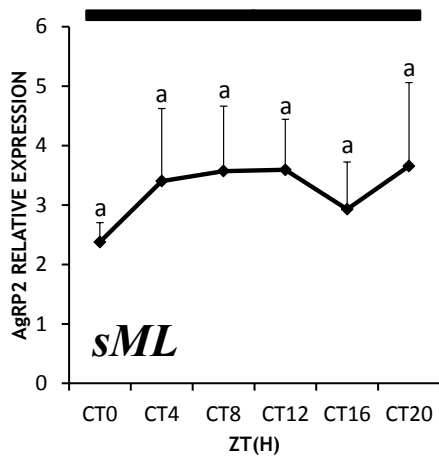


Figure 4.6 Relative expression of *agr2* gene in telencephalon, diencephalon and optic tectum of Senegalese sole under DD constant darkness condition and fed at subjective mid light (sML) or at random (RND). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$). The black 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).

AgRP2 DD Conditions

ML GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	3.3492	0.9250	17.1737	84.0141	N.S.
Diencephalon	8.5507	1.0233	17.9040	77.7249	N.S.
Optic tectum	3.2545	0.3314	10.6824	76.7933	N.S.
RND GROUP	Mesor	Amplitude	Acrophase	Variance %	Significance
Telencephalon	2.0858	0.4262	14.4163	85.0193	N.S.
Diencephalon	19.5552	3.0451	16.4631	77.7618	N.S.
Optic tectum	2.7870	0.8339	17.1097	79.9316	N.S.

Table 4.6: Parameters estimated by the Cosinor analysis for *agrp2* 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., non significant rhythm ($p>0.05$). Asterisks indicate significant rhythms ($p<0.05$).

5 Discussion

In the present study, we have tried to expand our knowledge about the neuroendocrine 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 orexigenic hormones (NPY, AgRP1, AgRP2) in *Solea senegalensis*, an important species for Mediterranean aquaculture. We focused on hormone synchronization to different feeding regimes (diurnal vs nocturnal and random feeding) and photoperiod conditions (light-dark cycle vs constant darkness). Therefore, the achieved results could also be relevant from a chronobiological perspective.

There is a growing number of studies on neural control of feeding in fish (Peter, 1979; Demski, 1983; Volkoff et al. 2005). Nonetheless, fish are a diverse phylogenetic group and relatively few species have been studied to date, with regards to feeding regulation. Information concerning the possible influence that external cues can have on the daily expression of food intake related hormones is rather scarce...In particular, studies regarding the effects of light and feeding time on the daily expression of orexigenic/anorexigenic hormones are really limited, although light is considered the most prominent zeitgeber, and together with other non-photoc stimuli such as feeding time, have been proved to be responsible for entraining circadian rhythms. Moreover, the hypothesis of the existence of a FEO, along with a LEO, has been supported in fish by data on properties of feeding entrainment, but its anatomical location and its independence from LEO are still unknown (Sánchez-Vázquez et al., 1997; Aranda et al., 2001).

In fish, daily rhythms regarding orexigenic hormones have been described along with phenomena of Feeding Anticipatory Activity (FAA), (Vera et al., 2007; Yan et al., 2011; Hoskins and Volkoff, 2012; Nisembaum et al., 2014), but only a few studies were about their modulation by restricted food access in different feeding regimes and different photoperiods, as most of them regarded their modulation by fasting. Moreover, the interpretation of experiments of circadian endocrine rhythms is limited by the collection of samples only from animals held in a LD cycle. This approach does not allow to determine whether daily rhythms are triggered by circadian endogenous clocks or driven by environmental cues, being necessary to hold animals in constant darkness as well, so to assess if hormone rhythms are entrained by feeding time and /or photoperiod as external cues. Therefore, in this experiment *Solea senegalensis* specimens were reared in two different photoperiods, i.e. LD or light-dark conditions and DD or constant dark conditions along with different feeding regimes (fed at ML, MD and RND times), so to determine if mRNA expression of orexigenic hormones (NPY, AgRP1 and AgRP2) is controlled by the endogenous clock and is entrained by feeding time and/or photoperiod.

Under LD conditions, NPY mRNA expression was rhythmic and showed statistical differences in optic tectum of animals fed at random times (RND group). The acrophase occurred at ZT 11.18, at the beginning of the dark phase, which is coincident with the active phase of this species, as the Senegalese sole shows a nocturnal behaviour (Bayarri et al. 2004). In telencephalon and diencephalon, no significant variations of NPY mRNA relative expression were observed neither by ANOVA nor by Cosinor analysis. Our results are not coincident with previous studies concerning NPY daily variations performed on animals held in natural or LD conditions, that suggest that NPY relative expression displays prandial variations in fish brain. In goldfish and in Atlantic cod, NPY expression increased a few hours before mealtime (Narnaware et al. 2000; Kehoe & Volkoff, 2007). Besides, studies on Brazilian flounder (*Paralichthys orbignyanus*) demonstrated that hypothalamic NPY augmented at mealtime and decreased significantly after two hours (Campos et al, 2012). This does not coincide with NPY variations in sole, which do not exhibit statistical differences in diencephalon under LD conditions. According to our results, no daily variations in NPY expression after a meal was observed in the telencephalon of snakesin gourami, *Trichogaster pectoralis* (Boonanuntanasarn et al., 2012). However, in this latter species a reduction in NPY expression was found after a meal in the diencephalon and mesencephalon while no daily differences were observed in our study in sole in these brain areas. After better exploring the expression in all feeding conditions, it is possible to notice that in MD groups of each neural tissue the relative expression was slightly increased at mealtime (ZT 18). This profile was not evident in ML groups and it might suggest that light is interfering with feeding cues; as sole is a strictly nocturnal fish, receiving food during the light phase might create a masking effect that does not let NPY to express properly. This could be stressed also by the differences exhibited in the optic tectum of RND group, in which NPY showed clear daily rhythms and variations that reflect the nocturnal behaviour of sole, this is, it peaked in the dark phase, independently from the feeding time. The fact that in the optic tectum under LD conditions NPY mRNA expression peaked during the dark phase in the RND group suggests that this rhythm is entrained by the light-dark cycle and exhibit some independence from the feeding time. However, under DD conditions, the rhythms in optic tectum were not evident in both sML and RND groups, suggesting that these rhythms are not circadian. The crosstalk between two external cues light and feeding cycles could lead to the disappearance of the NPY rhythms in the optic tectum of ML and MD groups. Further experiments are needed to firmly conclude the circadian nature of NPY rhythms in the optic tectum in sole.

In animals kept under DD conditions, NPY expression displayed statistical differences in telencephalon in the RND group and daily rhythms were observed in both sML and RND groups, with the acrophases placed at CT16.13 and CT5.48 respectively. This result suggest a circadian

nature of NPY rhythms in the telencephalon of sole, i.e, rhythms in NPY expression are driven by the endogenous clock in this cerebral area, as no synchronizing external cues are present in the RND group. However, the different acrophases observed in both groups suggest a role of feeding time in the expression of NPY in sole telencephalon. This is consistent with previous studies that affirmed that telencephalon is the main brain area involved in NPY expression in fish (Narnaware et al., 2000; Cerda-Reverter et al. 2000). Cosinor analysis revealed an acrophase of NPY expression 10 hours after feeding in the sML group, which is not consistent with its orexigenic role. In general, orexigenic factors (NPY and Orexin) increase before or during a meal (Narnaware and Peter, 2001; Xu and Volkoff, 2007) while the expression levels of anorexigenic factors (CCK and CART) increase after feeding (Peyon et al., 1999; Volkoff and Peter, 2001). An increase in NPY mRNA levels in the telencephalon–preoptic area and hypothalamus shortly before feeding has been observed in goldfish, followed by a decrease in brain NPY mRNA levels after feeding (Narnaware and Peter, 2001a). Similar variations were also observed in Atlantic cod (Kehoe and Volkoff, 2007) and tilapia (Peddu et al., 2009). In Senegalese sole, the acrophase of NPY in the telencephalon of the sML group is coincident with the active phase of this species, even when animals were fed during the subjective day (Bayarri et al., 2004). A combination of light and feeding cues seem to mask expression and rhythms in NPY under some particular photoperiod conditions and feeding schedules. Moreover, the duration of food deprivation might influence the expression of orexigenic/anorexigenic factors in fish. It has been reported that for example in goldfish, a fast of 10 days, but not of 3, decreases the hypothalamic mRNA expression of CART1 (Abbott and Volkoff, 2011). Besides, variations could be due to species-specific differences in response to fasting, as fishes might have different patterns of appetite-related peptides and might respond differently to nutritional challenges or might be due to different expression in different parts of the brain, as different regions of the brain might respond differently to fasting. Further studies on food deprivation will be carried out and in other brain areas related to feeding behaviour in sole.

Interestingly, in this study the diencephalon, which contains the light-entrainable oscillator (LEO) in mammals, did not exhibit significant daily NPY rhythms in sole at any of the feeding regimes analysed under LD and DD conditions, suggesting that this area is not responsible for NPY rhythmicity in this species. It has been demonstrated that fish can exhibit several paralogues of encoding genes, as a result of duplication events during fish evolution. It is known that Senegalese sole displays 7 paralogues for the CART genes (Bonacic et al., 2015) and in *Takifugu rubripes* 2 paralogues of NPY has been confirmed (NPYa and NPYb), but only one was related to food intake, showing variations associated to mealtime (Kamijo et al.; 2011). According with this fact, and also considering that in this study NPY showed neither variation in expression, nor rhythms in

diencephalon, which is usually strictly connected with feeding behaviour, further studies concerning the presence of NPY paralogues could be suggested in Senegalese sole.

AgRP is present in *Solea senegalensis* in two different forms: AgRP1 and AgRP2. In this study, both genes showed similar profiles. Regarding AgRP1, its expression did not show differences in any of the groups or brain area examined, but it exhibited a significant daily rhythm in optic tectum in ML group under LD conditions, showing the acrophase during the night (ZT14.49). ML group was fed at ZT6 and the acrophase at ZT14.49 might suggest that AgRP1 expression is more related to photoperiod than feeding time as the active phase of sole coincide with the dark phase (Bayarri et al., 2004) This could also be stressed by the common tendency in showing a slight increase in the AgRP1 transcripts in ML group at the beginning of the dark phase. AgRP1 expression of animals held under DD conditions showed neither statistical differences nor rhythms in any of the examined brain areas and groups. The absence of daily variations in AgRP1 indicates that its expression is not related with feeding time in sole brain, at least in this experimental conditions. However, the involvement of AgRP in the control of food intake should not be discarded in sole. AgRP levels are upregulated by fasting in different fish species such as goldfish *Carassius auratus* (Cerdá-Reverter and Peter, 2003), zebrafish *Danio rerio* (Song et al., 2003) and *Cyprinus carpio* (Wan et al., 2012) and different approaches taking into account several fasting periods should be carried out in Senegalese sole.

AgRP2 exhibited statistical differences and significant rhythms in ML group in optic tectum under LD conditions. The peak of expression was displayed at ZT15.35, during the dark phase. As for AgRP1, this could suggest that AgRP2 expression is linked with the nocturnal nature of sole and is related with photoperiod. AgRP2 expression of animal under DD conditions did not exhibit statistical differences and rhythms in any of the neural tissues. This result indicates that also AgRP2 expression is not entrained by feeding time and that there are no endogenous rhythms of this gene. Our results on Senegalese sole AgRP1 and AgRP2 showed the same pattern of expression, exhibiting statistical differences and significant rhythms only in the optic tectum of the ML group. As mentioned before, this fact could indicate that expression of AgRPs is related to photoperiod in this visually related area, instead to feeding time. Previous studies on goldfish demonstrated that AgRP increased in animals fasted from 3 to 7 days (Cerdá-Reverter and Peter, 2003); other studies on *Schizothorax prenanti* suggested that AgRP expression is not affected by less than 14 days of studying, suggesting as well a species specific regulation (Wei et al., 2013). This reinforce the need to perform different approaches taking into account several fasting periods in Senegalese sole.

Surprisingly, no significant variations or daily rhythms were observed in diencephalon, as it contains hypothalamus that is usually considered the homeostatic and feeding center (Forlano and

Cone, 2007). In this study the diencephalon, which also contains the light-entrainable oscillator (LEO) in mammals, did not exhibit significant daily NPY, AgRP1 and AgRP2 rhythms in sole at any of the feeding regimes analysed under LD and DD conditions, suggesting that this brain area could be less involved in the neuroendocrine control of food intake in fish than previously thought.

In summary, our findings have shown that daily expression of orexigenic factors NPY, AgRP1 and AgRP2 is related to photoperiod in visually related structures such as the optic tectum, exhibiting peaks of expression during the active phase of the animal (nocturnal). Moreover, our results reinforce the role of the telencephalon as the main neural area involved in the neuroendocrine control of food intake in fish, where endogenous NPY rhythms have been found. The involvement of AgRP1 and AgRP2 in feeding behaviour should not be discarded in sole, as further research will be carried out with specimens maintained under different fasting conditions.

6 Conclusions

1. Under LD conditions, NPY mRNA expression was rhythmic and showed statistical differences in optic tectum of animals fed at random times, peaking during the dark phase. This suggests that this rhythm is entrained by the light-dark cycle and exhibit some independence from the feeding time. In animals kept under DD conditions, NPY expression displayed statistical differences in telencephalon in the RND group and daily rhythms were observed in both sML and RND groups, suggesting a circadian nature of NPY rhythms in the telencephalon of sole.
2. Our results on Senegalese sole AgRP1 and AgRP2 showed the same pattern of expression, exhibiting statistical differences and significant rhythms only in the optic tectum of the ML group. This fact could indicate that expression of AgRPs is related to photoperiod in this visually related area, instead to feeding time.
3. Surprisingly, no significant variations or daily rhythms were observed in diencephalon, which is usually strictly connected to feeding behaviour . In this study the diencephalon did not exhibit significant daily NPY, AgRP1 and AgRP2 rhythms in sole at any of the feeding regimes analysed, suggesting that this brain area could be less involved in the neuroendocrine control of food intake in fish than previously thought

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