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The melatoninergic system in the European sea bass: effects of pinealectomy

Tesi di laurea in Adattamenti degli animali all'ambiente marino

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1. INTRODUCTION

1.1. OVERVIEW

Almost all the activities that animals usually do are rhythmic. Being hungry means hunting, being sleepy means sleeping and being thirsty means drinking.

Since the Earth spins on its axis with a cycle of approximately 24 h and an orbit around the Sun almost every 365 days, it has exposed organisms to highly predictable and recurring environmental conditions. Probably as a result of millions of years of interactions between external environmental cycles and living organisms, rhythmic biological process have been widely detected, from cyanobacteria to mammals (Sharma and Chandrashekaran, 2005). In fact, many biological functions are generally synchronized with periodic annual and daily environmental variations in water salinity, rainfall or food availability, as well as with temperature, lunar and light cycles (Falcón et al., 2011; Payne et al., 2013; Simonneaux and Ribelayga 2003; Takemura et al., 2004). From an ecological point of view, the synchronization with environmental parameters (e.g. annual photoperiod) is crucial for spawning events in fish for example and for the survival of the progeny, because reproducing at the right moment means that embryonic, larval and juvenile development will occur during the best conditions for growth and survival (van Woesik et al., 2006). But, among these cues discovered as time indicators, which is considered the most informative, constant and reliable?

The alternation of light (L) and dark (D) has virtually existed since the dawn of time and represents one of the most important cues that allows animals to synchronize with (Kreitzman and Foster, 2011). The LD cycle, in temperate habitats, represents a “noise-free” signal, mainly because its duration is strictly associated with the period of the year and almost constant throughout a period of time (e.g. weeks within a season). However, in tropical areas, when the difference of photoperiods is low throughout the year, other environmental cues seem to be more important than at higher latitudes such as movements in oceanic currents, seasonal rainfall, water salinity or lunar cycles (Bradshaw and Holzapfel, 2007; Oliveira and Sánchez-Vázquez, 2010).

In some situations, the rhythms of organisms may be a switch-like (on/off) and passive response to environmental variations. In a large number of cases however, animals have developed internal time-keeping molecular clocks which drive these biological rhythms, with a period running around 24 h (circadian rhythm) or one year

(circannual rhythm) when maintained under constant conditions (Falcon, 2010). There is a wealth of information on the circadian system and comprehensive reviews on molecular clock mechanisms are available from Bell-Pedersen et al. (2005) and Harmer et al. (2001), work on the circannual system on the other hand is more scarce.

In brief, the circadian system comprises of environmental, internal or social inputs perceived by specific sensors (e.g. light sensors from the retina and pineal organ, in the case of fish) which transduce environmental information (e.g. LD cycles) to molecular clock machinery allowing the clocks to synchronise to the 24h cycle. Clocks in turn drive the output of signals in a rhythmic manner. A major and important output of the circadian system in vertebrates is melatonin, which is an indolamine hormone produced from serotonin. Rhythmic melatonin secretion is a highly conserved feature in vertebrates and is widely known as the time-keeping molecule, playing a central role in the entrainment physiological rhythms.

The aim of the next sections of introduction is to bring together current knowledge on phototransduction and the melatoninergic system in vertebrates, with emphasis on teleosts.

1.2. PHOTOTRANSDUCTION IN ANIMALS

1.2.1. Networks of phototransduction systems

In mammals, LD information is transduced by a centralized and hierarchical system in which the suprachiasmatic nucleus (SCN) of the hypothalamus has been well studied as the central circadian pacemacker (Reppert and Weaver, 2001). With about 20000 packed neurons organized in a bilateral pair of sub-nuclei on each side of the third ventricles above the optic chiasm, the SCN is a pivotal crossroads in conveying light information (Reppert and Weaver, 2001). These cells receive light signals from the retina via the Retino-Hypothalamic Tract (RHT), and, with this kind of information they are able to control circadian rhythms in the brain (pineal organ and cortex) and in other parts of the body such as the liver, kidney and hearth (Simonneaux and Ribelayga, 2003). Surprisingly the photoentrainment in the retina is not achieved by cone and rod photoreceptors but rather by a subset of ganglion cells that project to the SCN, containing the photopigment melanopsin (Hattar et al., 2002). The RHT neurotransmitters are mainly glutamate and pituitary adenylate cyclase activating peptide (PACAP) (Ding et al., 1997; Hannibal et al., 1997), while

from the SCN neurons the main neurotransmitter is GABA, which even if it is expressed by all the cells, several region-specific neurotransmitter have been identified (Hafner et al., 2012). Each SCN neuron expresses molecular clock genes and interconnected transcriptional/translational feedback loops allowing each cell in concert to generate circadian rhythms (Reppert and Weaver, 2001). From the SCN a multisynaptic way (in order, hypothalamic paraventricular nuclei [PVN], intermediolateral cells of the upper three segments of the spinal cord [IML], superior cervical ganglion [SCG]) finally connect the SCN to the pineal organ, which is the main site of melatonin production in mammals (Simonneaux and Ribelayga 2003). Once there are stimuli for synthesis, the melatonin acts on *pars tuberalis* of the pituitary and on other brain areas with a "cascade effect" and feedbacks also on the SCN (where melatonin receptors are consistently present) (Pévet et al., 2002).

In fish and in other non-mammalian organisms there is not such centralized system but rather a network-like system of inter-connecting circadian units, in which the retina and the pineal organ occupy a central role in the circadian system (Fig. 1; Falcón et al., 2007). There are several evidences that have highlighted this idea of a de-centralised system and enriched our overall understanding of differential circadian organisation among vertebrates. First, ophtalmectomized/pinealectomized fishes still responded to photoperiodic stimuli (Day and Taylor, 2005; Masuda et al.,

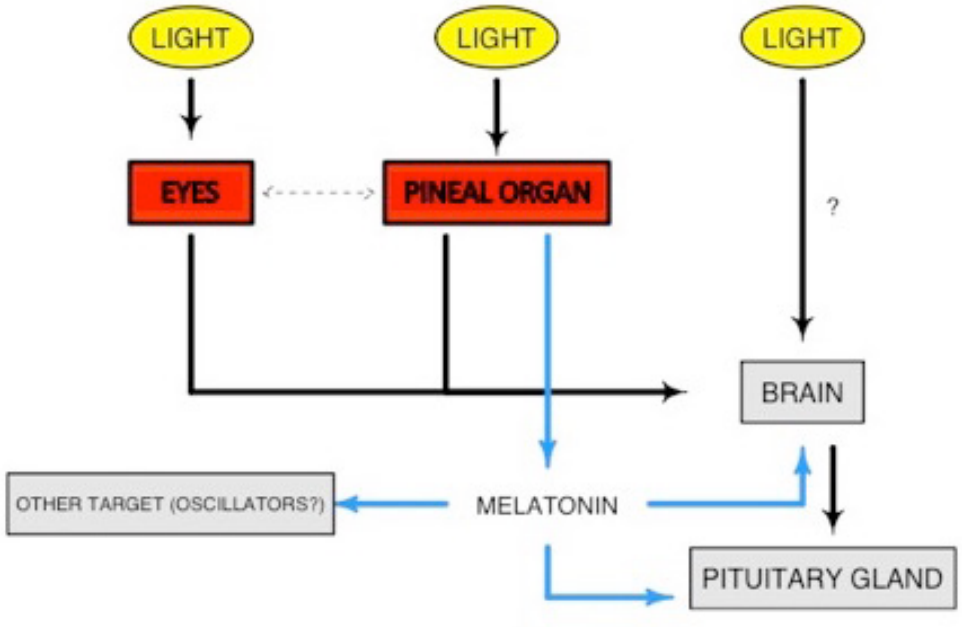

Figure 1. Photoneuroendocrine system in fish. In this case the most realistic explanation is a network of interconnected oscillators in the retina, pineal gland and maybe in some brain areas (?). Dashed line indicate hypothetical connection.

2005).

Second, light reaches deep brain areas (Foster and Hankins, 2002) and electrical activity from non-pineal and non-retinal origin have been recorded in the diencephalon and mesencephalon of the frog *Rana esculenta* (Cadusseau and Galand, 1980). Moreover, in brain areas of lampreys, fish, frogs and lizards components of the phototransduction cascade have been found such as opsins and the α subunit of the transducin (Álvarez-Viejo et al., 2004; Okano et al., 2000). With respect to the circadian basis of rhythms, the removal of eyes or pineal or both doesn't always completely abolish circadian rhythms but produces several scenarios, (i) complete loss of circadian rhythms, (ii) shift in circadian periods, (iii) splitting the circadian rhythm into different components. Thus it is probable that other circadian oscillators should exist, whose nature still remain to be elucidated.

1.2.2. The pineal organ: a brief anatomical and functional comparison

The pineal organ is basically an evagination from the roof of the diencephalon. In cold blooded animals such as fish it is located just below the skull, made by a vesicle connected with the diencephalon by a stalk and its lumen opens onto the third ventricle and is thus filled with cerebrospinal fluid (McNulty, 1984), while it becomes more glandular in mammals and folliculated in birds (Collin, 1971). In early vertebrates the pineal organ was thought to be part of a bipartite epithalamic complex, which was made by the pineal organ itself (epiphysis cerebri) and the parapineal organ; the main reason of this hypothesis is due to the extent among lizards, bony fishes (not all) and lampreys of this kind of organization (Ekström and Meissl, 2003). These changes in morphology reflect as much as deep and unique changes in cells types and connections with other organs (i.e. pituitary and hypothalamus).

In fish, but also in general in poikilotherms, the pineal organ is composed by photoreceptors cells, neurons and interstitial cells (Falcón, 1999). In the apical part, the "real" photosensitive pole is located while in the basal part one or several synaptic pedicles constitute the neurotransmitter pole in contact with dendrites of second-order neurons (Fig. 2; Collin, 1971; Ekström and Meissl, 1997; Falcón et al., 1992). The interstitial cells, or glial cells, are distributed along the whole organ since they isolate the other cell types from the blood vessels surrounding the pineal

(Falcón et al., 1992). Within this group of animals, the pineal organ can be roughly compared with the structure of cones in the retina but with much less degree of complexity. In fact, the complex network of second/third-order neurons and inter-neurons found in the retina allows the integration of visual information in the visual process, a network that is not seen in the pineal, in which the second order neurons converge on the pineal stalk to enter directly into the brain through a pinealofugal tract (Falcón, 1999). This is consistent with the idea that in cold-blooded vertebrates such as fish the pineal organ is “only” a luminance detector (Ekström and Meissl, 1997). Cone-like receptors are instead not observed in mammals, in which they have been replaced by pinealocytes *stricto sensu* with only a cell body and one or several pedicles, moreover, neurons have disappeared and glial cells represent a small amount of the total cells (Fig. 2; Collin et al., 1989). Further differences between the pineal organ of mammals and fish concern the photopigment content. In fish in fact, several photopigments have been found such as different type of opsins (also specific of the pineal organ), arrestin, recoverin, vitamin A and the α subunit of the transducin (Collin et al., 1986; Ekström et al., 1987; Pierce et al., 2008).

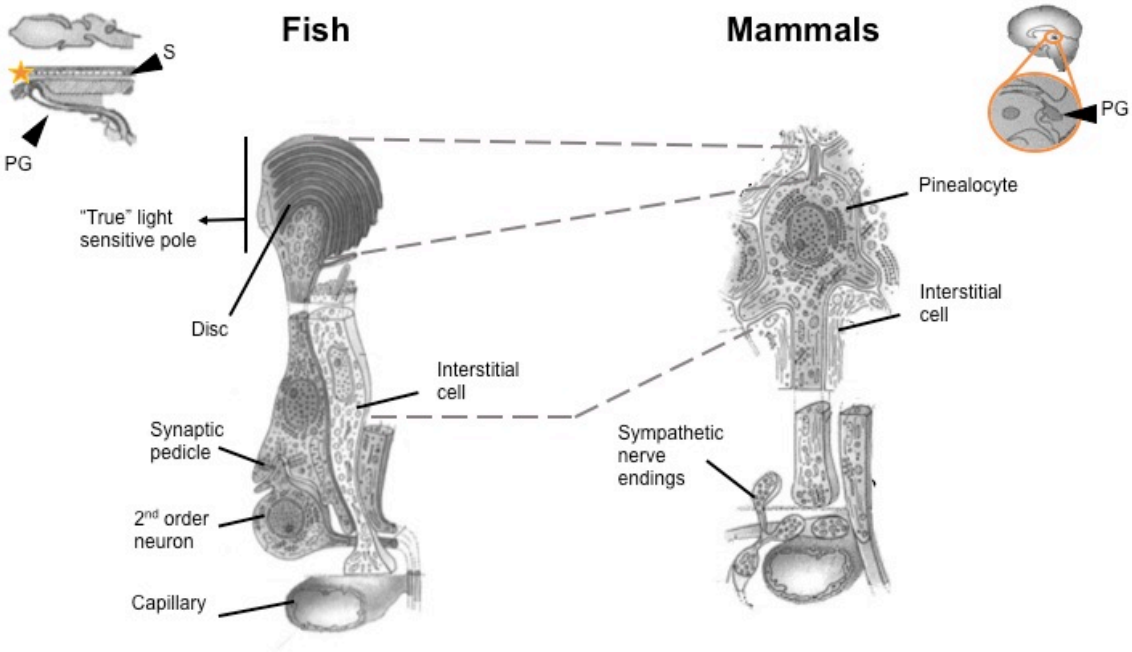

Figure 2. Anatomical comparison between the pineal of fish (central-left) and mammals (central-right) and anatomical localization in the brain (upper right and left). The pineal organ of fish is a “light” detector able to transduce the light information captured by the upper part of the organ and delivered to brain areas through second-order neurons in contact with synaptic pedicles in the basal part of the pineal. The pineal of mammals almost displays only pinealocytes. PG: Pineal Gland; S: Skull; Orange star: skull window. [from Collins et al. 1989, modified]

In trout pineal cells or dispersed trout pineal cells in culture, light reduces the cGMP levels by 30-40% with respect to the dark condition, which is know to be involved in the phototransduction cascade (Falcón et al., 1990). Further evidence comes from pharmacological studies that have tested some compounds such as pertussis toxin (that uncouples some GTP-binding proteins such as transducin) or phosphodiesterase inhibitors, showing no effect of light under these treatments and thus suggesting how light activates a phosphodiesterase through a GTP-binding protein, likely to be transducin (Falcón, 1999; Falcón et al., 1990). All in concert these results suggest how in the fish pineal organ, a phototransduction system exists, comparable with that of the retina. Although in the pineal of mammals some specific proteins related to the phototransduction cascade such as opsin, rhodopsin kinase, arrestin and recoverin have been detected (Collin et al., 1986; Korf et al., 1992; Kramm et al., 1993), it is also true that neither transducin nor cGMP gated channels have been identified (Schomerus et al., 1994). In addition, evidence indicating the absence of the chromophores, suggests that even if the photopigment would be present in the pineal of mammals the protein would be inactive (Kramm et al., 1993). All these observations support the absence of a phototransduction cascade in the pineal of mammals.

1.2.2.1. The pineal organ responds slowly to light stimuli

Why is the pineal organ believed to be “only” a light detector in fish and not a third eye? Additional evidence comes from electrophysiological studies and, even if they have been performed in a very limited number of teleosts (mainly due to the small size of photoreceptor cells), they help towards the understanding of the functionality of the pineal organ. Meissl and Ekström (1988) found in the trout *S. gairdneri*, the resting (dark-adapted) membrane potential of pineal photoreceptor cells is between -20 mV and -30mV (more positive than the resting potential of the neurons), thus in dark conditions these photoreceptors are partly depolarized. Light conditions were tested through flashes of bright light, which hyperpolarized photoreceptors up to 30 mV then bringing the potential to about -60 mV (Ekström and Meissl, 1997).

Although in pineal photoreceptors of fishes the amplitude of the photoresponse increases proportionally to light intensities (thus responses between retina and pineal organ are fundamentally similar) (Meissl and Ekström, 1988), deep

differences exist in terms of the time course of the response. In the pineal organ of fish, the time to reach the potential peak is between five and six-fold longer than those measured in retinal photoreceptors in turtle (Baylor and Hodgkin, 1974) and toad (Cervetto et al., 1977) both for light intensities near the threshold and for saturating light flashes. From the same studies, results indicated that the time for membrane recovery from peak to dark resting is long, up to 60s in the rainbow trout. Thus, it is clear how the pineal photoreceptors are unable to detect rapid changes in light stimuli, but are good candidates in discriminating on/off light situations. Moreover, the amplitude of the photoresponse of pineal photoreceptors during prolonged illumination is constant irrespective of changes in light intensities (in contrast with the continuous adaptation of the retinal photoreceptors) suggesting how steady illumination decreases cell photosensitivity and indicates light adaptation (Kusmic et al., 1992); these cells are hyperpolarized just with the presence of light and their response is maintained and stable during the whole period of illumination. The maintenance of a steady membrane potential throughout the whole period of illumination could play an important role in the regulation of melatonin production (for details see chapter 1.3) and providing information of the external LD conditions since the hyperpolarization of the photoreceptors results in the inhibition of excitatory neurotransmitters as glutamate and/or aspartate, as shown in the frog (Meissl and George, 1984). The excitatory neurotransmitters reach directly the second order neurons which send their axons to the brain and as a consequence, the message delivered mainly reflect the response of the photoreceptor cells.

1.2.2.2. The double message of the pineal organ: nervous and hormonal

In teleosts the pineal organ displays bidirectional connections with brain areas (pinealofugal and pinealopetal, respectively efferent and afferent). These connections have been elucidated through retrograde tract-tracing markers such as horseradish peroxidase, lysine-cobalt and fluorescent carbocyanines such as DiI (Mandado et al., 2001; Servili et al., 2011), and the efferent projections directed to the brain can be considered the ways through which the photic message is delivered. The targets of this nervous message are several structures such as the *habenula,* ventral and dorsal thalamus, posterior commissure, periventricular *pretectum*, pretectal area, posterior *tuberculum*, paraventricular organ, posterior tuberal nucleus, dorsal synencephalon and *tegmentum* (Falcón et al., 2007) of the

brain. The preoptic area (POA), which is innervated by projections from the pineal organ and from the retina deserves particular attention. Here the message seems to be redundant since there is an overlapping of information from two different light-detector systems. But, it is not. Even if it is true that the projections of both physically overlap in the POA, the content of the message they deliver is not the same. In fact, the retina conveys nervous information in a very "real-time" system from the horizontal plan, which is the visual information, while the nervous messages from the pineal's projections concern changes in light spectrum, dusk and/or water turbidity. However, the presence of projections from the pineal organ to the POA has been found only for a limited number of teleosts and for this reason the functionality of this innervation has to be fully discovered yet (Mandado et al., 2001; Servili et al., 2005, 2011; Yáñez et al., 1993; Yáñez and Anadón, 1998). The pineal organ of the teleosts also receive axon terminals from: thalamic *eminentia*, *habenula*, dorsal thalamus, ventromedial thalamus, periventricular *pretectum*, posterior commissure, posterior *tuberculum* and dorsal synencephalon (Jiménez et al., 1995; Mandado et al., 2001; Servili et al., 2005, 2011). Some of these (ventral and dorsal thalamus, pretectal area, posterior *tuberculum*) also appear to be connected with the retina, underlying how the integration of the information between retina and pineal organ could be achieved and would mean the integration of photic information (Falcón et al., 2010), but it is also true that up to now it represents an almost unexplored field.

The other message, which is hormonal and responsible for time signalling, is **melatonin**. Importantly, melatonin is not synthesized only in the pineal, in fact another important site of production is the retina. However, more than a decade of studies has demonstrated that the melatonin produced by the pineal organ is the major source for the circulating levels in the blood stream and in the cerebrospinal fluid (Falcón et al., 2011) and, thus, is a real hormonal messenger for the rest of the body, while the melatonin produced in the inner nuclear and ganglion cell layers in the retina has rather an autocrine/paracrine function (Besseau et al., 2006).

To sum up, what looks like an apparent overlapping of information in reality is more like a very detailed and stratified message and their integration has a pivotal role in giving time information and entraining biological activities with external cues.

1.3. THE MELATONIN SIGNAL

Melatonin is known as the time-keeping molecule of vertebrates but before examining in depth the pathways of synthesis and regulation of this hormone could be worth wondering: for which reasons melatonin is defined as the time keeper in other words? The release of an hormone that represents the final output of an integrated system (in this case the circadian system) able to translate the LD and the thermal information in endogenous messages, is an enormous advantage for animals. For example, through the differential secretion of the melatonin (longer or shorter production), the internal clocks of the animals “know” indirectly in which moment of the year they are, with strong implications on behaviour, reproduction, feeding, migration and resting. Certainly there are many steps inside this system, from the perception of light by photoreceptors to the synchronization of the molecular clock machinery that finally drive the rhythmic output of messengers, but all of them operate elegantly as a system of wheels.

Thus, the melatonin is the time-keeper hormone of the biological clocks of a wide range of organisms and it is rhythmically produced in two major sites: the pineal organ and the retina (Falcón et al., 2010). The differences in melatonin synthesis between the pineal organ and the eyes are mainly two. First, the target of the melatonin produced by these organs, locally produced with autocrine/paracrine functions into eyes while released into blood stream for that pineal-produced (Falcón et al., 2010). Second, the production pattern. The production in the pineal is almost exclusively nocturnal, its duration reflects the length of the night (which also vary on annual basis depending on changes in seasons) and its amplitude is linked with the temperature in poikilotherms (i.e., higher in warmer seasons and lower in colder seasons) (Benyassi et al., 2000). Although the production pattern in the pineal is fundamentally the same among a wide range of organisms, that is nocturnal, within the group of teleosts several strategies can be identified. For example, salmonids represent an exception because no circadian clock in the pineal organ appears to be present and that reflects the production of melatonin, which simply consists in an on/off system depending on the alternation of the LD cycle (Iigo et al., 2007). The fish retina does not follow the same nocturnal pattern of the pineal because the peak of melatonin can occur at any time of the LD cycle or may not peak at all, depending on the specie or the time of the year (Besseau et al., 2006).

1.3.1. The melatonin biosynthesis pathway

The synthesis of the melatonin starts with the hydroxylation of tryptophan by the tryptophan hydroxylase (TPOH) enzyme. The product of this step, the 5-hydroxytryptophan is decarboxylated by the aromatic amino-acid decarboxylase (AAAD) to produce serotonin. The arylalkylamine N-acetyltransferase (AANAT) catalyses the conversion of serotonin to N-acetylserotonin, which is finally *O*-methylated by the action of the hydroxyindole-*O*-methyltransferase (HIOMT) to produce melatonin (Klein et al., 1997). Accordingly with the nocturnal peak of melatonin production, serotonin levels are high during the day but low during the night, when it is converted to melatonin (Bromage et al., 2001). These steps represent the general biosynthesis pathway involved in melatonin production but important differentiations exist among groups of animals.

1.3.2. Several AANAT in teleosts

Teleosts are a unique group because they possess up to four *aanat* genes (Isorna et al., 2009, 2011). The presence of the *aanat2* and *aanat1* genes, which define the two main groups of AANAT enzymes, is thought to be a result of a whole genome duplication (WGD) linked to the evolutionary history of vertebrates (Ravi and Venkatesh, 2008). Moving on, more recently in teleosts the two *aanat1* genes which have been discovered, *aanat1a* and *aanat1b*, are thought to be more likely as result of a teleost-specific WGD (Isorna et al., 2011; Cazaméa-Catalan et al., 2014). Interestingly, a tissue specificity of these enzymes also exists, AANAT1 is more expressed in the retina and in the brain, while AANAT2 is more expressed in the pineal organ (Falcón et al., 2010). Keeping in mind this picture, very recent findings are suggesting a more complex situation (see Sections 1.3.2.2 and 1.4.2). Of course, what is emerging draws a situation unique among vertebrates and probably is related with the higher fine-tuning regulation of melatonin production under photic control.

1.3.2.1. AANAT2: uncovering the basis of melatonin rhythmicity

The enzyme AANAT2 deserves special attention, because its activity, transcription, transduction and post-transduction are under circadian control with strong implications on melatonin production (Gothilf et al., 1999). This control is intrinsically rooted in a molecular circadian system, with the light at the morning that

daily resets the clock through the induction of *per2* transcription, as seen in zebrafish (Ziv et al., 2005). The *aanat2* gene is under control of the CLOCK/BMAL transcription factors, that is a complex active in absence of the repressors (PER/CRY), and thus, during the night (Zilberman-Peled et al., 2007). The synthesis of AANAT2 follows an increase in concert with the melatonin production, but, only in the absence of light. The Ca^{++} would exert a key role in regulating the AANAT2 activity. At night, the membranes of pineal photoreceptors are depolarized and the voltage-gated Ca^{++} channels allow the increase of Ca^{++} intracellular levels, followed by accumulation of cAMP (Fig. 3; Falcón, 1999). As a result, there is an increase in the activity and amount of AANAT2 through phosphorylation of the protein. The process is reversed in the presence of light, when hyperpolarization stops the income of Ca^{++}, which is subsequently depleted and the AANAT2 is degraded by proteosomal proteolysis, resulting in a fall of melatonin production (Fig. 3; Falcón et al., 2011).

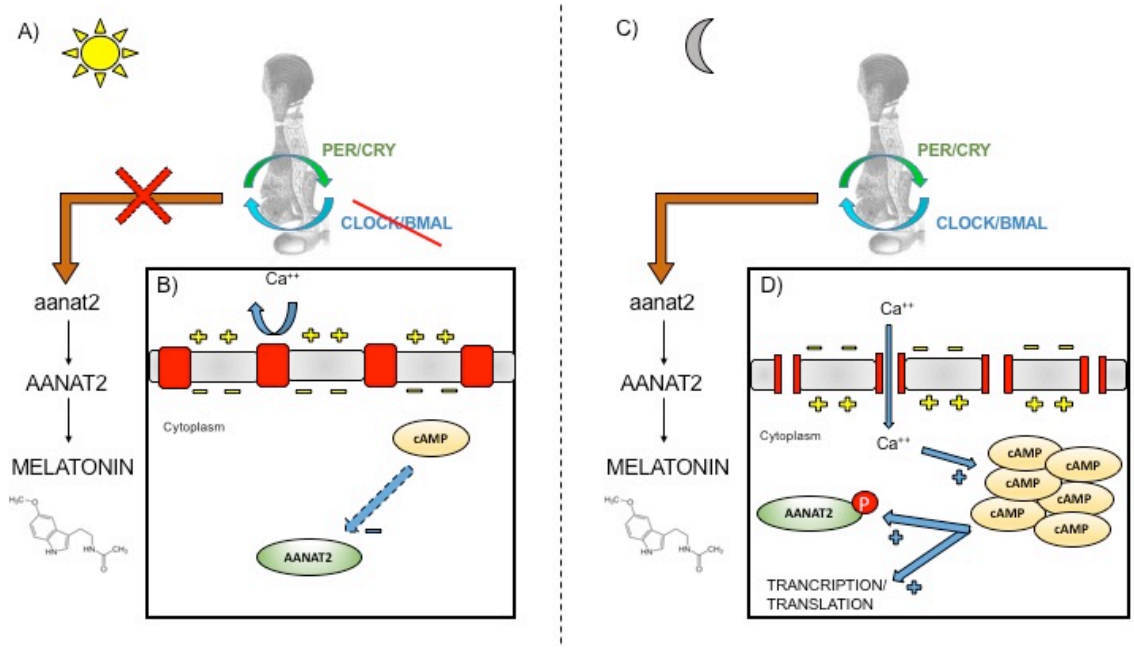

Figure 3. Schematic presentation that illustrates the main mechanisms that regulate the melatonin production in pineal photoreceptor cells in teleosts. A) During the day the light resets the clock though the induction of *per*, which is considered the first step of the molecular mechanisms that inhibits transcription of *aanat2*. B) During the day the Ca^{++} does not enter the photoreceptor cells and, as a consequence, the cAMP level does not rise, leading indirectly (no phosphorylation) to degradation of AANAT2 by proteasome. C) During the night the transcription/translation cascade of *aanat2* is fully completed and the AANAT2 can accumulate and start the production of melatonin. D) Rising intracellular Ca^{++} level during the night signals transcription/translation through cAMP dependent mechanisms and also phosphorylate AANAT2, which protect the protein from the degradation.

The photic regulation of the AANAT2 activity and transcription is central, but also other factors, both external and internal, could participate. Temperature is the

other external factor, after the light, that contributes to melatonin production. Even if a clear mechanism of action is not identified yet, evidence indicates the possible presence of temperature-sensitive membrane sensors that regulate the entry of Ca^{++} (Myers et al., 2009). It is also true that an alternative explanation could be the intrinsic response of the protein structure that affects the kinetics of the enzyme (Zilberman-Peled et al., 2004). However, internal factors such as hormones, neuromodulators and neurotransmitters could affect the AANAT2 activity and the melatonin production. In this sense, a good way to study the mechanisms of action of a substance is to locate the distribution of its receptors. Receptors for 17-β-estradiol and glucorticoids have been found in trout pineal, which are involved in inhibition of AANAT2 (cortisol) and melatonin production (cortisol and 17-β-estradiol) at night (Benyassi et al., 2000). Melatonin receptors have not been found in the fish pineal and consequently an endogenous feedback may be excluded, however melatonin receptors have been identified in many brain areas and the retina, suggesting some feedback loops in other metabolic pathways (Falcón et al., 2007).

1.3.2.2. AANAT1: distribution, catalytic activity and possible roles

To date, studies investigating *aanat1* rhythmicity have yielded a range of results, in some cases no significant rhythmicity during the LD cycle was detected, in other studies, rhythmicity depended on the species and within the same species, on the time of year (Falcón et al., 2011). At odds with *aanat2*, retinal *aanat1* presents affinity for both indoleamines and phenylethylamines and the response to temperature is similar among several species (Besseau et al., 2006). Moreover, the two isoforms (*1a* and *1b*) are expressed either both or just one among teleosts investigated so far. A pattern very different from that observed in *aanat2.*

Studies on *aanat1a* and *aanat1b* are recent and the first experimental evidences have highlighted a different expression pattern during early development and metamorphosis of *Solea senegalensis* (Isorna et al., 2011). In fact, Isorna et al. (2011) have found that *aanat1a* was more expressed before metamorphosis and did not show rhythmic pattern, while the *1b* isoform became predominant after metamorphosis and displayed a daily rhythmic pattern in the retina. Additional evidence, this time from the European sea bass, *Dicentrarchus labrax*, have enriched our understanding of how AANAT1a and 1b work (Paulin et al., 2015). In

the work performed by Paulin et al. (2015) a new and intriguing scenario has emerged: AANAT1a and 1b could be also involved in an alternative degradation pathways of monoamines (serotonin and dopamine), protecting tissues from oxidative damage. In fact, through *in situ* hybridization the two isoforms were labelled in the dorsal sac of the pineal organ (which is in contact with the cerebrospinal fluid or CSF), so not exactly in the pineal *strictu senso*. In the dorsal sac neither serotonin nor dopamine is present and *hiomt* (referred to as *asmt* in their study) transcripts were not detected, but activity of monoamine oxidase (MAO), an enzyme involved in the catabolism of serotonin and dopamine, was recorded. The hypothesis is that AANAT1a and 1b integrate the activity of MAO, eliminating serotonin and dopamine from the CSF and blood and thus reducing the formation of reactive oxygen species, which are the consequence of MAO activity. Wider functions must thus be assumed for AANAT1a and 1b and not strictly restricted to the production of melatonin, even because their tissue distribution in the sea bass is not restricted to brain areas but has been detected also in gonad, intestine, liver, muscle (AANAT1a and 1b), gills, and heart (AANAT1b).

1.3.3. Melatonin receptors

A good way to investigate the metabolic pathways involved and the mechanisms of action of the melatonin is to characterize the distribution of its receptors. Cloning and binding experiments using 2-[^{125}]iodomelatonin (^{125}Mel) have allowed the identification of three high affinity receptor subtypes belonging to the family of G-protein coupled seven transmembrane domain receptors, MT1, MT2 and Mel1c (Confente et al., 2010; Herrera-Pérez et al., 2010). The MT1 and MT2 subtypes are common among all vertebrates while the Mel1c subtype is present in nonmammalian species (Barrett et al., 2003). Combining tissue-specific expression (whose sequences has been obtained for a limited number of teleosts such as the European sea bass) and [^{125}I] iodomelatonin binding analysis, the receptors have been widely found, from nervous (retina, brain) to peripheral (hypophysis, gill, liver, small intestine, kidney, skin) tissues (Oliveira et al., 2008; Sauzet et al., 2008). Fundamentally, the central point is that in the brain, the receptors are generally associated with areas that integrate information from the sensory organs, including the olfactory bulbs, pretectal area, glomerular complex, optic tectum, torus longitudinalis, thalamus, and are involved also in neuroendocrine functions such as

the hypothalamus, telencephalon and preoptic area. The latter, which directly contribute to control the pituitary function, in particular receive innervations from both the pineal organ and from the retina (Ekström and Meissl, 1997), leading to the hypothesis that could be a centre of integration of light information.

Studies on sole and grass puffer have detected variations of mRNA levels of melatonin receptors on annual and daily basis, however the shape of the oscillations varied among brain areas (Ikegami et al., 2009; Confente et al., 2010). Upon these findings, a realistic hypothesis could be that the effects of melatonin could depend not only on the total production and release pattern, but also on the number of available receptors in a given time of the day or the year, which would play a key role in the effectiveness of melatonin action. Overall, further efforts are needed to uncover the molecular mechanisms and entrainment among oscillators behind the expression of melatonin receptors, which may involve both circadian and circannual clocks.

1.3.4. Downstream roles of melatonin

It is clearly established that melatonin is an output signal of the circadian system of vertebrates, playing an important role in transducing information from the environment. Accordingly, rhythms in melatonin production (daily and circannual) provide information about both the time of day and year. However, to date the direct downstream physiological role of melatonin remains unclear in teleosts.

1.3.4.1. Melatonin and reproduction

Despite the efforts over some decades to find correlations between melatonin and reproductive axis, a clear and general picture is still hard to draw because the results are often contradictory and unclear. Of course, one of the reason behind this could be the high number of variables not completely under control of the scientists, for example the sex, age, reproductive status, sampling time (year and day), species investigated and their reproductive strategies. Additionally, the high number of aspects to consider also likely affect the quality and reliability of the comparisons among several experiments.

Direct effects of melatonin as pro- or anti-gonadal hormone have shown very contradictory results with either strong or null effects (Mayer et al., 1997). Thus, melatonin probably exerts its effects through alternative pathways. Consistent results

have been obtained in males of masu salmon *Oncorhynchus masou* using administration of melatonin by feed. With low concentration (50 *μ*g melatonin·g^{-1} feed) and long-day photoperiod the gonadosomatic index (I_G) and the follicle-stimulating hormone (FSH) were both stimulated but no effect on luteinizing hormone (LH) was observed. These results suggest that using low melatonin administration (mimicking a short dark phase) stimulated the gonadal development but it did not activate the full brain-pituitary-gonads axis (BPG) in precocious males of masu salmon (Amano et al., 2000). However, when higher concentration of melatonin was tested (500 *μ*g melatonin·g^{-1} feed; ten fold higher) opposite effects occurred: I_G diminished, salmon gonadotropin releasing hormone (sGnRH) and LH were suppressed along with plasma testosterone (Amano et al., 2004). Thus, melatonin clearly affects the BPG at different levels (hypothalamus and pituitary) and the response are dose dependent in masu salmon. Supports to these findings come from Kahn and Thomas (1996), which have tested direct melatonin administration in the POA resulting in decrease of LH release in Atlantic croacker (*Micropogonias undulatus*) with fully developed gonads. Also in the European sea bass, melatonin appears to inhibit the reproductive axis by decreasing the expression of *gnrh-1*, *gnrh-3* and *gnrh* receptors (Servili et al., 2013).

A hypothetical mechanism of action is suggested by studies on carp, eel and goldfish, in which melatonin could affect gonadotropins (GTH) release by modulating the dopaminergic system in the basal diencephalon. Though these findings would indicate unexplored mechanisms of action of melatonin along the BPG axes, it would not be a general rule because once again results are controversial (Popek et al., 2005).

1.3.4.2. Melatonin and growth

It is widely accepted that in the wild, fish growth follows a seasonal pattern depending on food availability and favourable environmental conditions (light and temperature), as shown for example by studies on otholits, structures often used as a proxy to understand growth patterns (Morales-Nin, 2011). Body growth relies mainly on the so-called growth hormone (GH) released by the somatotrophs of the pituitary. Here, the processes could be either excitatory (dopamine, GH releasing factor, thyrotropin releasing factor) or inhibitory (norepinephrine, serotonin, somatostatin, GH itself) whereas melatonin would exert its effects on two levels:

directly on the pituitary or indirectly (Boeuf and Falcón, 2002). As in the case of the reproduction results are controversial and thus is hard to extrapolate a general pattern. As direct effects, studies investigating the localization of melatonin receptors have lead to the conclusion that they are present in the pituitary and, even if the molecular mechanisms still remain unknown, the presence of receptors in a tissue likely suggests some mechanisms directly affected by a ligand in the given tissue. Accordingly, Falcón et al., (2003) have found in cultured pituitary cells of the trout that melatonin modulated cAMP content and GH release in some period of the year but not in others. On the other hand, the indirect pathway remains to be demonstrated yet but a good candidate seems to be an upstream control through dopamine (as would be in the case of the reproduction), in agreement with the observations that melatonin administration affect the content of serotonin and dopamine in the hypothalamus (Falcón et al., 2011).

1.4. THE EUROPEAN SEA BASS *(Dicentrarchus labrax)*

1.4.1. Interest for aquaculture

The European sea bass belongs to the family Moronidae and is typically a marine species that inhabits waters ranging from hypersaline to brackish conditions. Its distribution is fundamentally temperate and, in the wild, is found off the European coast with a wide depth range (2-100 m) (Sánchez-Vázquez and Muñoz-Cueto, 2014). Over the last years, like many fish stocks, its catches have dramatically fallen, a testimony for the recent measures adopted by the European Union in restricting fishing gear methods and setting catch limits (Pawson et al., 2005). As a fish of high commercial value and interest, as well as suitable for aquaculture, it has become in few years one of the more productive species in terms of yield and profits. Accordingly, aquaculture of sea bass started in the late 1980s and early 1990s with a global yield of around 4 tonnes and almost steadily increased over years reaching 150 thousands tonnes in 2014 (FAO FishStat). Thus, a lot of money has been invested and great efforts made to enrich our knowledge about its physiology, especially for aquaculture. However, little is known about its ecology even if recent studies are unveiling its population structure and movements across European seas (Fritscha et al., 2007), projecting European fisheries toward a more sustainable activity.

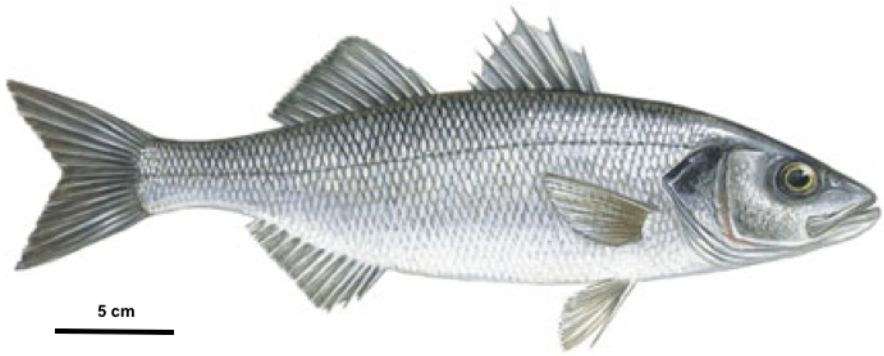

Figure 4. The European Sea Bass (source: FAO, modified)

1.4.2. Melatonin rhythms in European sea bass

Rhythmic patterns of melatonin production were first detected in the European sea bass more than twenty years ago (Sánchez-Vázquez et al., 1995). Up to now, three main conserved observations have been made: (i) the duration of the nocturnal rise in plasma melatonin content is correlated with the duration of the dark phase, (ii) there is a positive correlation between the amplitude of melatonin production at night and temperature throughout the year under natural conditions, (iii) there is an inverse daily melatonin profile between the plasma content (nocturnal acrophase) and the retina (diurnal acrophase), which reflects differences in melatonin production between the pineal organ and the retina. However, an exception does exist, where the melatonin rhythm in the retina has been seen to disappear in the summer period for not yet identified reasons (García-Allegue et al., 2001). A hypothesis could be the autocrine/paracrine actions exerted in this organ in periods of more prolonged/intense illumination. Interestingly, the inverse relationship retina/pineal is maintained also when an artificial light pulse in the middle of the night is applied, reducing melatonin plasma content but increasing its amount in the retina (Iigo et al., 1997). However, the misalignment of melatonin production in the retina and in the pineal is not a general rule among teleosts (Falcón et al., 2011).

Regarding the melatonin hormonal message, as already stated, the main enzymes that compose the machinery of the melatonin production are AANAT2, AANAT1 (1a and 1b) and HIOMT (also known as ASMT). Although they represent the fundamental steps of transformation from tryptophan to melatonin, recently have been highlighted additional roles for the new isoforms discovered (AANAT1a and 1b). Paulin el al. (2015), to better investigate their respective roles, assessed the

tissue distribution and quantitative expression of all these enzymes through RT-qPCR and *in situ* hybridization. Results demonstrated that AANAT2 was almost exclusively a pineal enzyme while ASMT was present in the pineal and in the retina. But, for the two AANAT1 isoforms the situation was more complex, in fact they were expressed in peripheral tissues such as gonad, liver, intestine, muscle (1a and 1b), gills and heart (1b). These results suggested in fact an additional role of AANAT1a and 1b in sea bass, as pointed out in Section 1.3.2. Overall the presence of all the four the enzymes in a given tissue likely could indicate that melatonin synthesis occurs, also providing that serotonin is present.

The pineal melatonin message has been widely investigated, however the nervous message is a more recent field of investigation. Bayarri et al. (2003) performed pinealectomy/ophthalmectomy (PX/OX) and eye/pineal covering, showing how in the sea bass a complete drop in melatonin plasma content occurred only when input from both the organs was disabled. At the time of the study the hypothesis was of a neuronal connection of the two organs through a yet unidentified pathway. In the last years the brain of the sea bass has been extensively investigated and such neuronal pathways characterized. In the sea bass, through carbocyanine dye tract-tracing (DiI), projections from pineal to brain areas (pinealofugal) and from brain areas to pineal (pinealopetal) have been detected. Moreover some brain areas innervated by the pineal (i.e. POA) are also reached by retinal connections in sea bass and other teleosts, suggesting that they could represent centres of integration of photic information (Servili et al., 2011; Yáñez et al., 2009; Servili and Muñoz-Cueto, unpublished).

The sea bass is an ideal model for melatonin studies and chronobiology owing to it's strong seasonality. Reproduction is timed to the winter season (Carrillo et al., 1995) in order to favour the survival of offsprings. The sea bass is able to invert its demand-feeding pattern, switching from diurnal behaviour during the summer to nocturnal feeding activity in the winter reproductive season (Sánchez-Vázquez et al., 1998). Overall, the strongest environmental information provided by the spring-summer and autumn-winter periods are the photoperiod and temperature and the interaction of the two, in which a virtually infinite set of combination of temperature and photoperiod provide information to the body through either nervous and hormonal (melatonin) messages.

2. OBJECTIVES

The overall aim of this thesis was to increase our understanding on the melatoninergic system in sea bass, with a focus on pinealectomy-ophthalmectomy effects, especially at the level of the brain. As pinealectomy alone is not able to provoke a complete depletion in melatonin plasma content in sea bass (Bayarri et al., 2010), it is possible that other extra-pineal/extra-retinal (e.g. diencephalic) sources of melatonin are contributing to maintain some local melatonin levels when the pineal melatonin secretion is lacking. To help better understand the phototransduction system and downstream role of melatonin it is important to investigate what is happening at the level of integration in the brain. This is of particular interest given the presence of the melatonin-synthesising machinery within the brain (Paulin et al., 2015). Specific objectives were defined as follows:

Objective 1: Investigate the effects of combinations of pinealectomy, opthalmectomy and melatonin implants under a day-night cycle on:

- Expression of the genes involved in melatonin production (*aanat1a*, *aanat1b*, *aanat2* and *asmt*) in the diencephalon.
- Expression of the main melatonin receptor (*mt1*) in the diencephalon.
- Plasma melatonin levels.
- Locomotor activity.

Objective 2: Optimise Digital Droplet PCR (ddPCR) technology protocols for the measurement of the genes of interest with low levels of expression. In this work, it was the first time ever in our laboratory that ddPCR had been used and because of that we had to set some specific technical sub-objectives:

- Develop a completely new operative protocol to carry out analysis with new machines and exercise problem solving/trouble shooting skills to manage initial difficulties related with this new technique.
- Design specific primer pairs for the genes of interest tailored to ddPCR technical requirements and optimise PCR reaction conditions.
- Learn how to use the specific ddPCR software (Quantasoft) to set-up plate reading instructions and perform data analyses and export.

3. MATERIALS AND METHODS

3.1. Fish stock and housing

Sea bass at the prepubescent stage (approximately 7 months prior to first maturation and average body weight of 64.66 ± 10 g) were randomly assigned to seven tanks (200-300 L volume, 16 fish per tank). Each tank of fish was designated to a different surgical treatment as listed below:

- Control (CTL).
- Sham (SHM).
- Pinealectomy (PX).
- Opthalmectomy (OX).
- Pinealectomy + melatonin implant (PXM).
- Pinealectomy + opthalmectomy (PXOX).
- Pinealectomy + opthalmectomy + melatonin implant (PXOXM).

Fish were maintained under a simulated natural photoperiod, at a water salinity of 39 ppt and a temperature of 19 ± 1°C.

3.2. Experimental procedure: surgery and sampling

The whole *in vivo* experiment ran over four months, from June to September 2014. A simplified version of the experimental working plan is shown in Fig. 5.

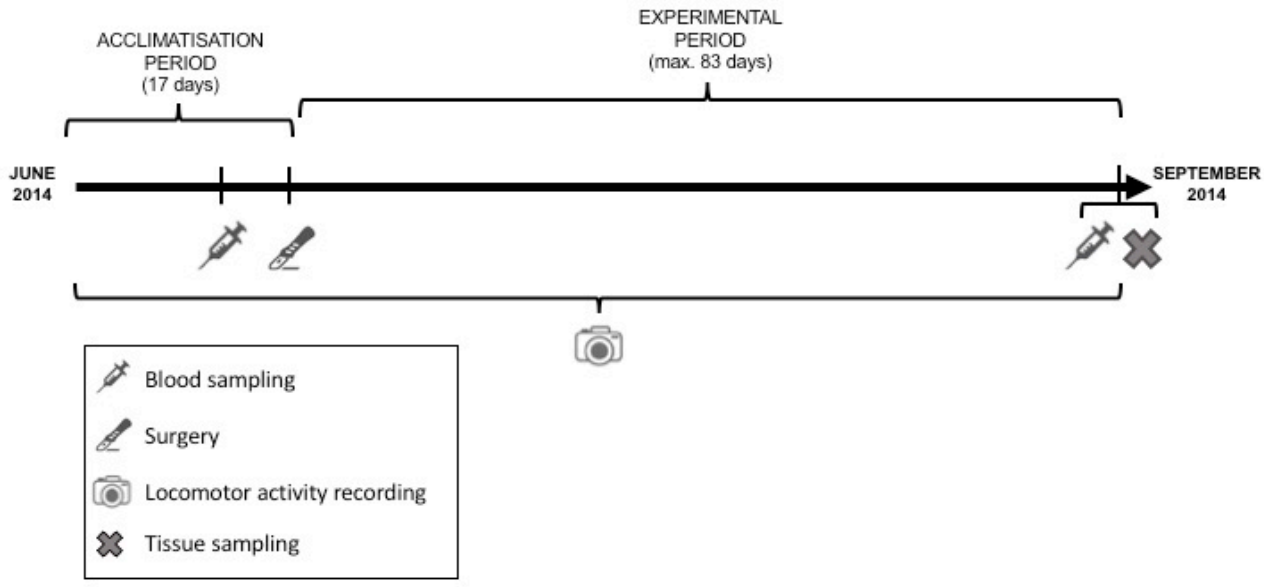

Figure 5. Schematic drawing of the experimental working plan.

Firstly, fish were acclimatised for 17 days. During acclimatisation, a blood sampling was performed at Mid-Light (ML, Day 6) and Mid-Dark (MD, Day 6/7), blood samples were then centrifuged at 1200 *g* for 15 min and the plasma aliquoted and stored at -20°C until melatonin analysis (see section 3.3). Following acclimatisation, surgery was performed. For all surgical techniques fish were kept anaesthetised through a continuous flow of anaesthetic (40 ppm) directly on the gills, supplied by a small pipe positioned towards the back of the fish mouth.

PX was performed as follows: once fish were anaesthetised with MS-222 (80 ppm, Sigma), they were wrapped in a wet cloth and placed on a malleable lead holder under a binocular loupe. Using a scalpel, a “window” of 3-4 mm wide was open on the fish cranium, exactly on the pineal window where the skin shows a lack of pigmentation. A flap of skin was lifted and a fine glass pipette attached to a siphon was used to remove the excess fat surrounding the brain thus creating a clear view of the pineal organ. Finally, the pineal organ was removed with fine forceps from the basis of the pineal stalk. Sham PX fishes were subject to the same procedure as PX fish but avoiding the step of pineal removal. The cut was finally sealed with cyanoacrylate adhesive and fish were treated with an antibiotic bath (oxytetracycline, 0.2 g/l) for 1 h prior to being returned to their experimental tanks.

OX was performed as follows: fish were anaesthetised and the membrane around the eye was cut around, the eye lifted and using surgical silk a tight knot was made around the optic nerve. The nerve was cut just above the knot, cauterized and a drop of Betadine (Laboratory Sarget, France) applied to the eye socket. Gelatine sponge (Espongostan Film, Ferrosan, Denmark) was placed into the eye socket to avoid haemorrhage and the fish were subject to an antibiotic bath (oxytetracycline, 0.2 g/l) for 1 h and then transferred to the experimental tank. Regarding the fish in which pinealectomy and ophtalmectomy had to be performed, pinealectomy was firstly performed at least seven days in advance in order to let the fish recover from the surgery and then opthalmectomy was performed.

The melatonin implants (18 mg, Melovine®/Regulin®, Ceva) were implanted intramuscularly just below the dorsal fin. An incision was made with a scalpel and an implanter was used to insert the implant. After that, two stiches were made using surgical silk (Silkam®, Braun, Spain). In those fishes subjected also to pinealectomy,

the melatonin implants were performed immediately after the pinealectomy whilst the fish were still sedated.

Following surgery, every two weeks for the first month fish were subjected to antibiotic baths. They were maintained for up to 83 days until end-point sampling. Careful daily monitoring was performed during the whole experimental period, any fish showing poor recovery or signs of distress were sacrificed. Blood and tissue sampling at the end of the experiment was performed respectively at ML and MD, within each tank half of the fish were randomly sampled at ML and the remainder at MD. For sampling, fish were sacrificed by lethal anaesthesia (MS-222, 80 ppm, Sigma), the whole body weight and total length were recorded before having their gonads dissected and weighed and a sample taken and fixed in 4% solution of paraformaldehyde. Sea bass whole brains were dissected and immediately frozen in liquid nitrogen along with pituitaries and peripheral tissues. Samples were then stored at -80ºC until molecular analysis.

3.3. Melatonin Analysis

Plasma melatonin levels were determined by a commercial radioimmunoassay kit (IBL International, Hamburg, Germany) in collaboration with Dr José Fernando López-Olmeda and Dr Javier Sánchez-Vázquez (Department of Physiology, University of Murcia, Murcia, Spain). The procedure was previously validated for sea bass samples (Bayarri et al. 2003). The assay started with a 2 h enzymatic pretreatment at 37°C and then samples were incubated for 44 h at room temperature with 50 ml of assay buffer, 50 ml of ^{125}I-tracer and 50 ml of antiserum. Subsequently, 500 ml of precipitating reagent have been added to the tubes and then centrifuged at 3000 x g. Then, after extracting the supernatant by aspiring it, tubes were placed in a gamma counter for 1 minute.

3.4. Molecular Analysis

3.4.1. Primer design, selection and validation

The highly sensitive Digital Droplet Polymerase Chain Reaction (ddPCR) technology was the technique adopted to analyse samples owing to low expression of the genes of interest. To deal with this kind of PCR new primers were designed and tested for all melatonin enzyme genes (*aanat1a, aanat1b, aanat2* and *asmt*). Primers for the melatonin receptor (*mt1*) were already available in the lab.

Steps to design primers involved in order: research and acquisition of the available sequences of the target genes on the National Centre of Biotechnology for Information (NCBI) website, the open reading frame (ORF) of each target sequence was then deduced through the bioinformatics resource portal ExPASy (Expert Protein Analysis System, available at: http://www.expasy.org). Primers were designed on the ORF of sequences using Primer3Plus version 2.4.0 (Untergasser et al., 2007) where the required technical parameters of the primers could be input and possible forward and reverse primers were then designed and presented. Two sets of primers (forward and reverse) were ordered for each of the melatonin enzyme genes of interest.

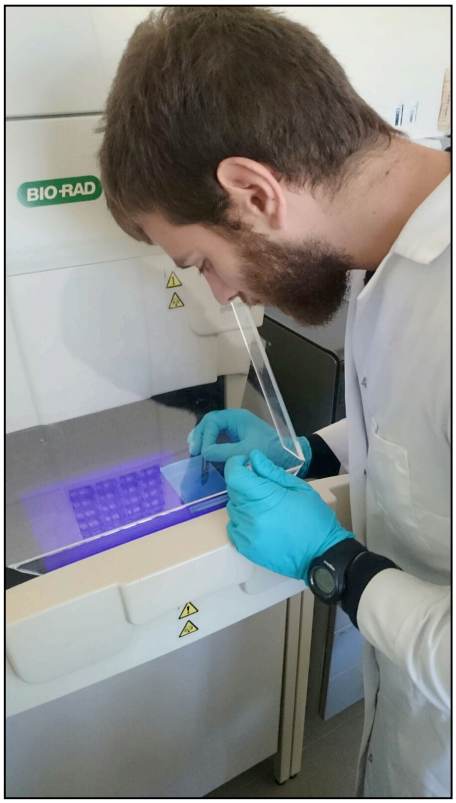

Figure 6. One of the step before DNA extraction: after amplification samples were analysed through agarose gel electrophoresis, the bands checked under UV light and then excised using a scalpel for the next DNA extraction

All primer sets were tested for each gene of interest through Real Time quantitative PCR (RT-qPCR). For the RT-qPCR reaction mix, each 19 μl well included: Takara SYBR Green mix (Takara Bio Inc., USA), cDNA template, primers and sterile distilled water. Amplification and acquisition of results was carried out with the CFX96 System and the coupled CFX Manager Software (Bio-Rad Laboratories Inc., USA). Primer tests involved a temperature gradient which was performed to obtain optimal amplification of targets and to avoid undesirable secondary structure of the primer reaction (i.e. primer dimers).

Melting curves were checked every time to confirm that a single product was amplified and non-template negative controls were included in all assays. Final selected primer oligonucleotide sequences used are shown in Table 1.

As an additional validation, the products of the RT-qPCR were run through an agarose gel by electrophoresis to check that amplicons were the correct size (number of base pairs). The DNA band was then extracted using the QIAquick Gel Extraction Kit (QIAGEN Group Inc., USA). Briefly, DNA bands were excised from the

agarose gel with a clean, sharp scalpel (Fig. 6), put in a tube with 3 volumes of provided buffer QG to 1 volume gel and incubated at 50°C for 10 min, shaking on a vortex each 2-3 min. Once the slices had dissolved, 1 gel volume isopropanol was added left for 2 min to facilitate precipitation of the nucleic acid, the column placed into a collection tube, centrifuged for 1 minute and the flow-through discarded. At this point we slightly modified the manufacturer's instructions as we discovered that we needed a more purified product for sequencing. In fact, the washing step which involved addition of 600 µl of buffer PE, the wait for 5 min and the centrifugation for 1 minute were repeated not once as the provided protocol said, but twice. To finally elute DNA, 27 µl of water was added exactly to the centre of the membrane and the column centrifuged for 1 minute. DNA yield and quality were checked by 260/280 nm absorbance ratio on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The DNA obtained was further eluted in water to obtain the correct dilution and sent with the primers to the
SCAI-Unidad de GENÓMICA (Universidad de Córdoba, Campus de Rabanales) for being sequenced.

Table 1. Primer oligonucleotide sequences tested through RT-qPCR

Gene	GeneBank Accession No.	Primer sequences (5'-->3')	Optimal annealing/ extension T°	Amplicon Size (bp)
aanat1a	EU378922	FW: GCCATCAGTGTGTTCGAGATCG	62 °C	125
		RV: TCAAACCAGCCAAGAGACAGC		
aanat1b	EU378923	FW: TGTTTCGCCTGGCATACAAAGG	62 °C	82
		RV: GACGCTTATGGCATCTTGCG		
aanat2	KP954654	FW: ACGCCGCAGGATGCCATCAGTGTA	57 °C	185
		RV: TCCTTGTCCCAGCCAGAGCCAATG		
asmt	KP986446	FW: ACTGGACTGACGAACGCAGCATAGA	59.5 °C	188
		RV: GTGCACTGCTTGGCTAATGC		
mt1	EU378918	FW: GGTGGCCATCTATCCGTACCC	59.5 °C	110
		RV: TGACGCTGACACCCATGAGG		
l13a	DT044539	FW: TCTGGAGGACTGTCAGGGGCATGC	62 °C	148
		RV: AGACGCACAATCTTGAGAGCAG		
elfα	AJ866727	FW: CTGTGCTGATCGTTGCTGCTGGTGTT	59 °C	75
		RV: CGTGCTCGCGGGTCTGTCC		

The reason why primers were primarily tested by RT-qPCR is because in the process of ddPCR, droplets are directly discarded after fluorescence detection thus it is not possible to further analyse the amplified product by a final melt-curve (to check for the amplification of a single product) or by running through an agarose gel and sequencing. It was thus essential to validate the specificity of primers before using them for ddPCR.

3.4.2. RNA extraction and cDNA synthesis: from RNA to cDNA

Total RNA was extracted from brains, which were divided into three main regions (telencephalon, diencephalon, mid-hindbrain), using TRIsure reagent® (Bioline, London, UK) according to the manufacturers guidelines. Tissues were homogenized (50-100 ng) in 1 ml of reagent using 4 stainless steel beads in a mixer mill MM400 (Retsch, Haan, Germany). After homogenization, for each ml of reagent used, 0.2 ml of chloroform was added, samples manually shacked for 15 s, then incubated at room temperature for 3 min and centrifuged at 12.000 x g for 15 min at 4°C. During this step the sample was separated into a pale green organic phase containing DNA, an interphase containing protein and a colourless upper aqueous phase containing RNA. The latter was carefully removed and RNA precipitated by mixing it with cold isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml of reagent used) then incubated at room temperature for 10 min and centrifuged at 12.000 x g for 10 min at 4°C. At this point the RNA precipitated formed a gel-like pellet on the bottom of the tube. The supernatant was completely removed, the RNA pellet washed once with 1 ml 75% ethanol prepared with diethylpyrocarbonate (DEPC)-treated water per ml of reagent used. Samples were shaked on a vortex and centrifuged at 7500 x g for 5 min at 4°C and ethanol was removed. To re-dissolve the RNA, samples were air-dried and dissolved in DEPC-treated water and incubated for 10 min at 60°C in a water bath. Total RNA yield was quantified and RNA quality checks were performed by the 260/280 nm absorbance ratio in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Samples were then further diluted with DEPC water in order to obtain a concentration 250 ng RNA/ µl water.

In order to get cDNA from the previous extracted RNA, a PrimeScript™ kit (Takara Bio Inc., USA) was used following the manufacturer's instructions. The genomic DNA elimination reaction through a specific genomic DNA eraser was the

first necessary step. To effectively remove contaminating genomic DNA, 4 µl per sample (corresponding to 1000 ng of RNA) was incubated with 6 µl of master mix (including a gDNA eraser, a buffer and sterile distilled water) at 42°C for 2 min. Then the gDNA free samples were directly used in the next step. The reverse transcription reaction was performed by adding 10 µl of a second master mix (including dNTPs, the reverse-transcription enzyme, oligo dT primers, an RNase inhibitor and sterile distilled water) and incubated at 37°C for 15 min then 85°C for 5 s.

3.4.3. Digital Droplet PCR (ddPCR): gene expression analysis

Four main treatments from the experiment were selected for melatonin enzyme and melatonin receptor gene expression analysis: SHM, PX, PXOX, PXOXM. Expression analysis was performed specifically on the diencephalic region of brain samples.

Within our laboratory ddPCR technology was officially used for the first time in the present work and consequently a PCR optimisation and a complete new protocol had to be developed to understand how to manage machines, improve techniques (i.e. pipetting, software settings), maximize the reaction efficiency and also interpret the results.

3.4.3.1. Experimental workflow

Instead of one machine like the conventional RT-qPCR, three machines are necessary for ddPCR, in order: the QX200™ Droplet Generator, the C1000™ Touch Thermal Cycler and as last the QX200™ Droplet Reader coupled with QuantaSoft™ software (Bio-Rad Laboratories Inc., USA).

Even if the principles are the same as the standard PCR, the functioning of the ddPCR is more complicated, delicate and slower. In summary, the first machine split the reaction volume in thousands of 1 nL volume droplets (up to 20.000 droplets), the thermal cycler amplified the target inside each droplet (simplifying, it performs thousands of PCR reactions simultaneously) while the last machine read and computed the results. The latter, that is the detection of the fluorescence, is virtually the same as the qRT-PCR as the labelled nucleotides used during the reaction are detected to quantify the amplification of targets, of course the resolution of the two PCRs is different.

3.4.3.2. PCR optimisation

Regarding the ddPCR reaction solution for PCR, guidelines were followed according to the QX™ 200 ddPCR™ EvaGreen Supermix (Bio-Rad Laboratories Inc., USA) kit used. Primer concentrations were fixed at 100 nM for both forward and reverse primers. In order to deal with the particular requests of the technique it was necessary to find out the best temperatures of annealing/extension per primer. The ddPCR works better at slightly lower temperature than the RT-qPCR owing to the properties of the oil thus further temperature gradient tests were performed for each primer using extra cDNA samples dedicated only to this purpose. The best annealing/extension temperatures for each couple of primers are shown in Table 1.

The amount of DNA template for each reaction varied according to the genes being measured. For all the genes of interest, owing to low expression, 50 ng of template was used whereas for the housekeeping genes *elfα* and *l13a* which had high expression, it was necessary to perform dilution series to determine the best concentration of cDNA into the samples that allowed good outputs on the ddPCR (see Appendix 1). During the first test the starting concentrations ranged from 50 ng of cDNA to 0.75 ng of cDNA, while in the second test dilutions were more severe, and ranged from 1 ng of cDNA to about 0.015 ng of cDNA. Among the several dilutions performed a 1:200 dilution (2 µl of template diluted into 398 µl of sterile distilled water) was selected for both of the housekeeping genes.

3.4.3.3. ddPCR working protocol

The developed working protocol started with the preparation of a 22 µl solution for each sample, using a QX™ 200 ddPCR™ EvaGreen Supermix (Bio-Rad Laboratories Inc., USA) kit. The final solution included EvaGreen supermix, primers, sterile distilled water and cDNA template. 20 µl of each sample solution was then transferred to a well within a DG8™droplet generator cartridge with 70 µl of Droplet Generator Oil (Bio-Rad Laboratories Inc., USA) aliquoted into a corresponding well. Exactly 20 µl of sample were required by the Droplet Generator to effectively generate sufficient droplets. The loaded cartridge was covered with a gasket and placed into the QX200 Droplet Generator, where in about 2 min samples and oil are combined within the microchannels of the cartridge to create an emulsion of up to 20,000 monodispersed, nanoliter-sized droplets for each sample. Theoretically each

droplet thus contains all the basic components to perform a standard PCR within the droplet. Following droplet generation, the droplets were very carefully pipetted into a standard 96-well PCR plate, which was immediately sealed when fully loaded and then placed into the C1000™ Touch Thermal Cycler, basically a standard thermal cycler. The protocol adopted for the thermal cycler is presented in Table 2. It is worth pointing out that pipetting from the loaded cartridge to the standard 96-well PCR plate was a key step of the protocol since it was necessary to do it very slowly one well at a time to keep droplets intact and to pipette them with a certain angle respect to the bottom of the wells to maximize the yield. After PCR amplification the plate was loaded into the QX200™ Droplet Reader using Absolute Quantification Experiment (ABS) settings and the run started. The droplet reader is able to analyse independently each well by aspiring it, streaming the droplets in a single file under a two-fluorescence detector working with the fluorescent dye FAM and VIC/HEX.

Table 2. Example of ddPCR amplification protocol used for the thermal cycler

Cycling step	Temperature,°C	Time	Ramp rate	Number of cycles
Enzyme activation	95	5 min	2°C/sec	1
Denaturation	95	30 sec		40
Annealing/Extension	See Table 1	1 min		40
Signal stabilization	4	5 min		1
	90	5 min		1

The detector reads the droplets to define which contain the amplified target (+) and which do not (-). Only the presence or the absence of the target matters. A non-template control and a quality control check were included in all assays.

3.5. Locomotor activity monitoring

Tanks were each fitted with two photoswitches (see Appendix 1) (model E3S-AD62, Omron, Japan), one near the water surface (11 cm below the surface, 28 cm from the bottom of the tank) and another at the bottom of the tank (9 cm from the bottom of the tank). Photoswitches in turn were connected to a computer and worked by emitting a continuous infrared light beam. Interruptions in the beam caused by fish movements within 20 cm were recorded on the computer and organized into 10

minute bins using specialized software (DIO98USB, University of Murcia, Spain). Locomotor activity rhythms were captured for the whole duration of experiments.

3.6. Data analysis

For gene expression data, raw absolute quantification results from QuantaSoft™ were initially computed using the automatic calculation of thresholds provided by the software. However, in some cases this tool didn't work well and thresholds were set up manually. Next, using housekeeping genes as references, gene expression data were normalized through an Excel sheet provided by the company BioRad.

One-way and two-way ANOVAs were the main statistical analyses performed for plasma melatonin and gene expression analyses. Significant interaction between the factors of treatment and time (day or night sampling), detected by the two-way ANOVA, was followed by the Student Newman-Keuls (SNK) test, comparing all groups independently of factor. If interaction was not detected by two-way ANOVA, one-way ANOVAs were performed, testing the factor treatment, at night and at day separately. Within each treatment t-test was performed as additional analysis. In all cases, $p<0.05$ was taken as significant statistical threshold. Before performing statistical analysis, homogeneity of variance was checked by Levene's test and outliers that were identified after the Bonferroni p-value correction of the Studentized residuals were removed.

All statistical analyses were performed using the software RStudio (R Core Team, 2016) and the specific packages car (Fox and Weisberg, 2011) and Agricolae (De Mendiburu, 2016). All the graphics were created with the R packages ggplot2 (Wickham, 2009).

4. RESULTS

The ANOVA tables can be found in Appendix 2.

4.1. Plasma melatonin

Figure 7 shows the levels of plasma melatonin for each treatment analysed at the experimental end-point.

One-way ANOVAs detected significant differences between treatments at day

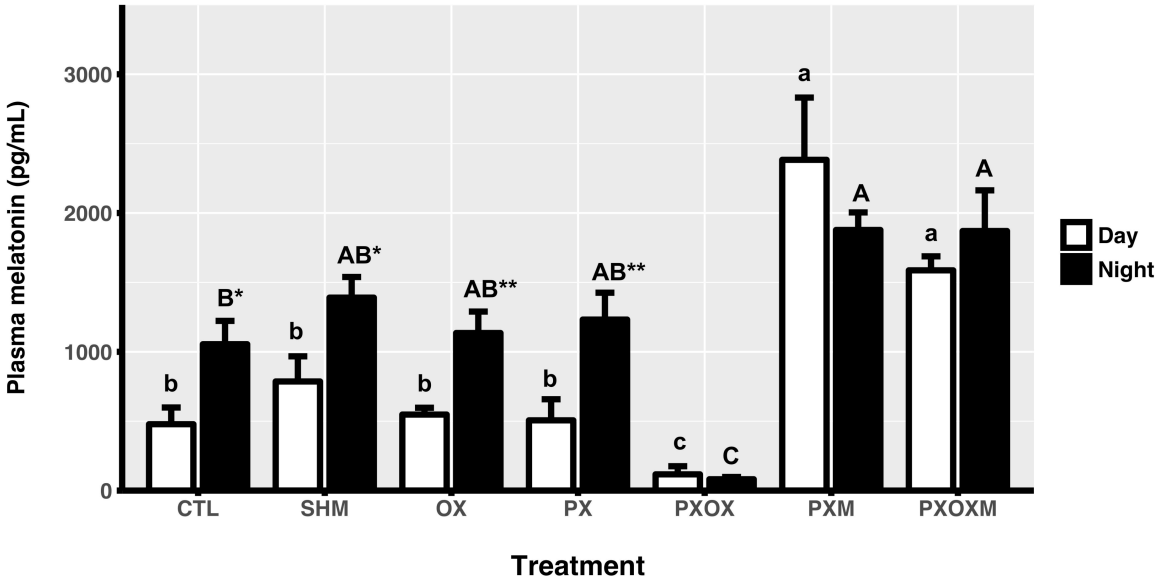

Figure 7. Plasma melatonin levels for each treatment. White and black bars represent day and night levels, respectively. Each bar represents the mean + S.E. Lowercase lettering indicates significant differences between treatments at day, uppercase lettering indicates significant differences between treatments at night (one-way ANOVAs followed SNK tests). Significant day *versus* night differences for each treatment are indicated by asterisks (T test, *=p<0.05, **=p<0.01, ***=p<0.001). CTL: control, SHM: sham, OX: opthalmectomy, PX: pinealectomy, PXOX: pinealectomy+opthalmectomy, PXM:pinealectomy+melatonin implant, PXOXM: pinealectomy+opthalmectomy+melatonin implant.

($F(6,33)$=14.16, p=6.53*10^{-8}) and at night ($F(6,43)$=14.51 p=5.72*10^{-9}). Student-Newman-Keuls (SNK) tests run separately at day and at night allowed three main assemblages of results to be identified on the graph. The first one, included the CTL, SHM, OX and PX groups which all showed similar levels of melatonin during the day-time and similar elevated levels during the night. Individual t-tests indicated that the increase in night-time melatonin levels was significant for each of these groups. The second assemblage, contained groups in which melatonin implants had been

inserted (PXM and PXOXM) and showed the highest mean concentrations of plasma melatonin. Levels of melatonin between these groups and between day and night did not differ. The remaining PXOX group exhibited significantly lower levels of plasma melatonin with respect to others, there was no significant variation between day and night levels.

4.2. Expression of melatonin enzyme genes and the melatonin receptor in the diencephalon

As indicated in Material and Methods, the expression analysis was performed in SHM, PX, PXOX and PXOXM groups (Figure 8).

4.2.1. Expression of *aanat1a*

A two-way ANOVA was performed and revealed a strong interaction between factors (treatment by time) in influencing *aanat1a* expression ($F(3,36)$=7.04, p=7.56*10^{-4}). A one-way ANOVA and post-hoc SNK test was then performed, comparing all groups independent of treatment or time, and results are shown in Figure 8A.

At day, *aanat1a* expression in all surgical treatments was comparable to that of the SHM control. However, between experimental treatments alone, PX fish displayed higher *aanat1a* expression *versus* PXOX and PXOXM individuals. This expression was significantly greater than the nocturnal expression in the same group. The standard error for the PX group mean at day was larger than other treatments indicating greater variation between individuals.

At night, *aanat1a* expression was higher in the PXOXM group *versus* all other groups. This expression was also significantly greater than day-time expression within the same group.

4.2.2. Expression of *aanat1b*

A two-way ANOVA indicated no significant interaction between factors (treatment by time) in *aanat1b* expression (Figure 8B).

Moreover, one-way ANOVA comparing treatments during the day-time also indicated no significant differences in *aanat1b* expression. Significant differences were found between treatments at night (one-way ANOVA: $F(3,19)$=5.04, p=0.009) where expression in the PXOX group was lower than that of PX and PXOXM

individuals. No significant differences between day and night within each treatment were found by t-tests.

4.2.3. Expression of *aanat2*

A two-way ANOVA indicated no significant interaction between factors (treatment and LD) in *aanat2* expression (Figure 8C). One-way ANOVAs indicated no significant differences between treatments at day or at night. There were also no significant day-night differences within treatments, as indicated by t-tests.

Although no significant differences were found, mean expression at night-time was higher than at day-time, particularly for PX, PXOX and PXOXM treatments, showing a trend towards a night-time elevation. These night-time groups showed wide dispersion of the data around as shown by high standard errors, a point in contrast with the compact dispersion of the rest of the data. There appeared to be higher nocturnal variation between individuals that had been pinealectomised (PX, PXOX, PXOXM) versus the SHM group.

4.2.4. Expression of *asmt*

A two-way ANOVA indicated no significant interaction between factors (treatment by time) in influencing *asmt* expression (Figure 8D). One-way ANOVAs indicated no significant differences between treatments at day or at night. There were also no significant day-night differences within treatments, as indicated by t-tests.

4.2.5. Expression of *mt1*

A two-way ANOVA was performed and revealed a significant interaction between factors (treatment by time) in influencing *mt1* expression. A one-way ANOVA and post-hoc SNK test was then performed, comparing all groups independent of treatment or time, and results are shown in Figure 8E.

There were no significant differences between the SHM control and treatments during day-time. At night-time, expression was significantly higher in the PX and PXOXM treatments *versus* the SHM and PXOX groups. Day-night differences within PX and PXOXM were also significant.

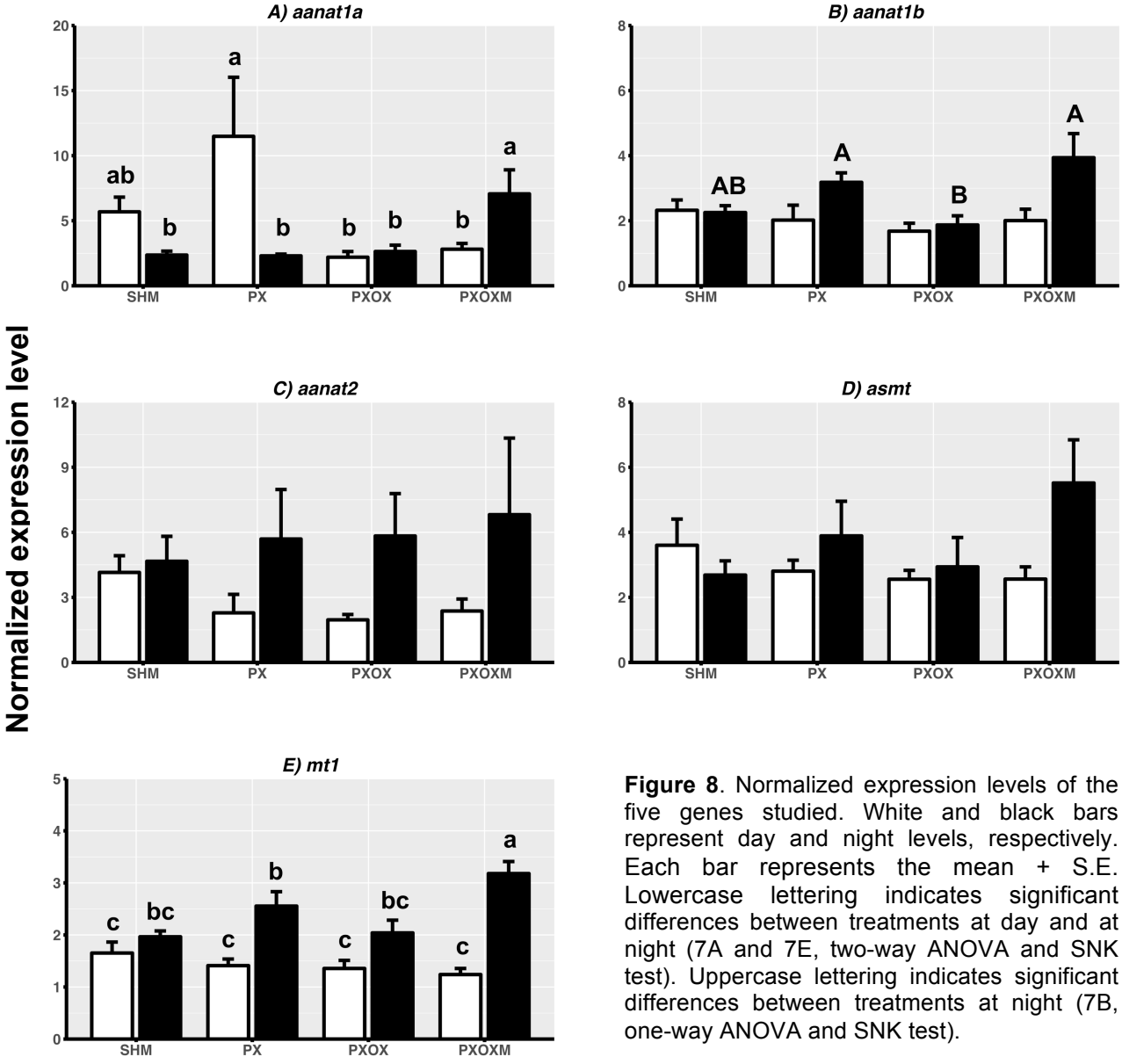

Figure 8. Normalized expression levels of the five genes studied. White and black bars represent day and night levels, respectively. Each bar represents the mean + S.E. Lowercase lettering indicates significant differences between treatments at day and at night (7A and 7E, two-way ANOVA and SNK test). Uppercase lettering indicates significant differences between treatments at night (7B, one-way ANOVA and SNK test).

4.3. Locomotor activity

Locomotor activity recordings are displayed in Figure 9. The most clearly defined patterns were displayed in both the CTL and SHM groups (Figure 9A, B) where fish showed diurnal activity in both the upper and lower parts of the water column throughout the duration of the trial.

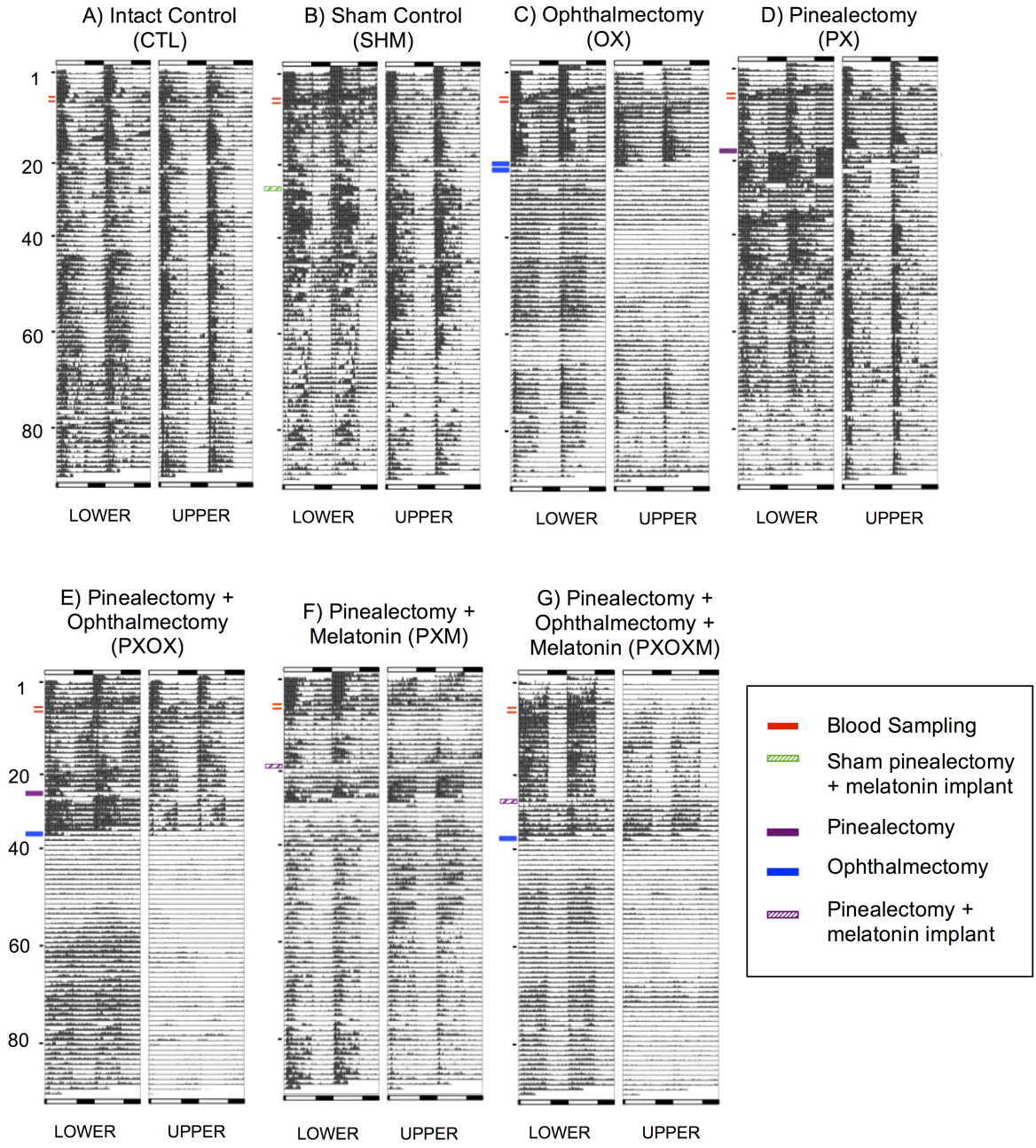

Figure 9. Actograms based on raw data of sea bass activity. Each pair of actograms represents the recording of group activity within a tank/treatment, the left actogram of each pair represents recording from the lower photocell, the right actogram represents recording from an upper photocell. Data have been double plotted for convenient visualization. The number of days from the start of the experiment is plotted on the left of the actogram. Horizontal bars above/below each actogram indicate day-time (open bar) and night-time (solid bar). Sampling and surgery procedures are shown in the left part of each actogram.

Regarding experimental treatments, prior to surgery, diurnal behaviour similar to SHM and CTL treatments was seen. Immediately following surgery, all experimental groups appeared to be disturbed and lost their diurnal rhythmicity. Rhythmicity was then recovered in all treatments within the last month of the experiment, however the patterns were less clear than for control groups. In the OX group (Figure 9C) there was a strong decrease in activity in the upper part of the tank after the surgery. Only a peak in activity in the upper water column could be seen at the time of light onset and feeding. A similar loss in activity in the upper water column was seen in the other ophthalmectomised groups (PXOX and PXOXM, Figure 9E, G respectively). Conversely, a different behaviour was reported for fish belonging to PX and PXM groups in which recordings after the surgery were reported also in the upper part of the tanks which indicated higher activity than for ophthalmectomised fish, and more similarity to control fish both in the upper and bottom recordings. The loss in rhythmicity immediately following surgery, was most pronounced in the PXOX group (without the main natural sources of melatonin or an implant). Interestingly, the group implanted with melatonin (PXOXM) recovered the rhythm in locomotor activity almost 10 days before the PXOX group, at least in the bottom part of the tanks.

5. DISCUSSION

In the present study, specific primers were successfully designed for use with ddPCR in order to measure the expression of the four main melatonin enzyme genes (*aanat1a, aanat1b, aanat2, asmt*) and the melatonin receptor (*mt1*) in the diencephalon of European sea bass, *Dicentrarchus labrax.* Detection of these genes was accomplished because of the use of ddPCR, which exhibits higher sensitivity, accuracy and resolution than conventional RT-qPCR. Our efforts uncovered the expression of these genes following different treatments: sham control (SHM), pinealectomy (PX), pinealectomy+opthalmectomy (PXOX) and pinealectomy+opthalmectomy+melatonin implant (PXOXM) under a light-dark cycle. Moreover, under the same experimental conditions, in order to further increase our knowledge on the output of the melatoninergic system in sea bass we investigated plasma melatonin levels and locomotor activity, with analyses of additional conditions included: intact control (CTL), ophthalmectomy (OX) and pinealectomy + melatonin (PXM).

5.1. Plasma melatonin levels

Our findings on plasma melatonin for control groups (CTL, SHM) confirmed a nocturnal elevation as reported by previous authors (Bayarri et al., 2003). Groups with melatonin implants (PXM, PXOXM) showed the highest elevations of both nocturnal and diurnal levels, in accordance with continuous melatonin release from such implants. Importantly, removal of both the pineal and eyes (PXOX) completely abolished the night-time melatonin elevation. In spite of the existence of some extra-pineal and extra-retinal sources of melatonin production such as the intestine and hypothesised hypothalamic production, the idea of these as significant contributors to circulating levels of melatonin is unlikely and evidence that in teleosts the major source of circulating melatonin is the pineal has been extensively reported (Falcón et al., 2011). This consideration is supported by earlier studies which have shown that pinealectomized fish lost plasma melatonin rhythms, although some discrepancies among species still remain suggesting that pinealectomy would act in a species-dependent manner (Ekström and Meissl, 1997).

We found no consistent differences in circulating melatonin levels between controls (CTL and SHM) and the OX group, thus removing light information provided

by the eyes did not affect the nocturnal melatonin elevation when pineal is intact (Fig. 10 C and D). In the past it has been suggested that the active machinery of the pineal organ of sea bass was made of two types of pinealocytes: photoreceptors able to transduce the light information upon the light-dark (LD) cycle and pinealocytes "*sensu stricto"* that without light information provided by the eyes would have lowered melatonin production. At that time authors formulated this conclusion because eye removal and optic nerve sectioning caused plasma melatonin levels to fall to approximately a half of control levels (Bayarri et al., 2003; Migaud et al., 2007), which suggest that only a part of the whole pineal organ would have been working without light information from the eyes (Fig. 10 A and B). It is important to that non-homogenous experimental procedures such as the weight of fish, acclimatisation/experimental period, or sex may have accounted for the differences in results between these experiments and ours. The season in which the experiments were performed. i.e., winter in the case of Bayarri et al. (2003), summer in our experiment, could also be on the basis of these differences. In fact, it has been shown that plasma melatonin rhythms in sea bass are basically driven by the photoperiod length (duration of the nocturnal rise) and water temperatura (amplitude of the rhythm), generating for each season a specific melatonin profile (García-Allegue et al., 2001). It is our opinion however the circadian system organisation (where rhythmic pineal melatonin production requires input from the eyes), described in the experiment by Bayarri et al. (2003) and Migaud et al. (2007), is not always the case in sea bass, as indicated by our study. Considering that in some brain areas an overlapping of information occurs between projections of eyes and those of the pineal and that the ventral thalamus (also a retinorecipient and relay center to the optic tectum) appears bidirectionally connected with the pineal organ (Servili et al., 2011), it seems that light information provided by the eyes would reach the pineal organ through the brain but wouldn't trigger a significant drop of melatonin production because of direct light sensitivity of the whole pineal. However, it is possible the eyes could have a role at a smaller scale, by fine tuning the melatonin production and also in programming the regeneration of the pineal system.

Pinealectomized fish (PX) exhibited an unexpected melatonin elevation at night, comparable with that of control groups. This was an entirely contrasting outcome with respect to previous pinealectomy studies on sea bass and other teleosts (Falcón et al., 2010) and a short-term pinealectomy study performed in the

host lab. There were two possible mechanisms which may have been responsible for this unexpected production: 1) a compensatory mechanism of production, however this seemed unlikely especially given the lack of melatonin in the PXOX group and absence of rhythmic expression in diencephalic melatonin enzymes (section 5.2), or 2) pineal regeneration. The more likely explanation would be the regrowth of pineal tissue during the experimental period, a feature previously observed in some teleosts (Garg, 1988; Kavaliers, 1979), but yet to be demonstrated in the European sea bass. During the tissue collection at the experimental end-point, partial re-growth of the pineal stalk was observed in some individuals, a totally unexpected finding.

The question remains as to why if there were normal levels of melatonin production in the PX group (potentially due to regeneration), this wasn't the case for

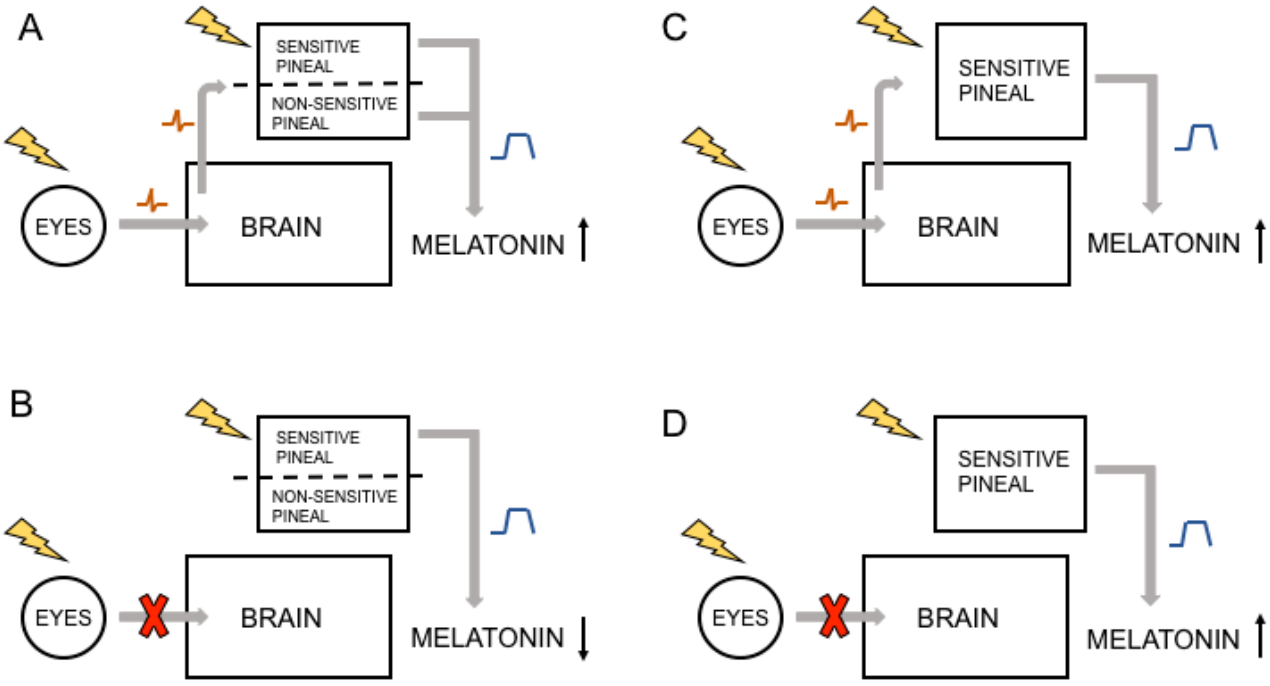

Figure 10. Schematic comparison between our hypothesis and that one suggested by Bayarri et al. (2003) about the effects of opthalmectomy on melatonin production in the pineal organ.
On the left side (A,B) the pineal organ is supposed to be divided into a sensitive and a non-sensitive portion. A full production of melatonin is assured by the nervous signal provided by the eyes and its lack would result in diminishing levels of melatonin production (Bayarri et al. 2003).
On the right side (C,D) our hypothesis in which the pineal organ is considered full sensitive. The removal of the nervous message from the eyes would not affect melatonin production in an intact pineal because of its intrinsic sensitivity.

the PXOX group? It is possible that the input from the eyes may have influenced the programming of pineal regeneration. It is also possible that the eyes may have affected the melatonin production from already exisiting/regrown pineal stalk in the

PX group for example. Results from Herrera-Pérez et al. (2011) showed that melatonin-synthesising cells and cone opsin-like and rod opsin-like photoreceptor cells were present in the pineal stalk and vesicle, indicating that both the pineal regions are photosensitive structures. However, the comparison between labelling obtained by anti-cone/anti-rod opsin sera and by antiserum against serotonin was not uniform and further studies are needed to elucidate if there is a matter of anti-opsine antibodies or if do exist non-sensory pinealocytes together with sensory pinealocytes.

The regeneration of the pineal organ represents a fascinating topic. Deeper insight on this topic would elucidate which mechanisms turn stem cells into photoreceptors in fish with potential applications to human diseases such as vision loss. In spite of this interest however, regeneration may also represent a drawback for pinealectomy trials, and we would point out that it is important to adopt procedural controls. First, a control at the end of the period over the regeneration of the pineal is necessary in order to discard those samples exhibithing tissue regrowth. Second, in case of long period of testing, capturing the broader amount of variability by repeating blood sampling may improve understanding of results and also follow various parameters over time. One one hand, in our study some blood samplings along the experimental period would have allowed a stricter control over the experiment. On the other hand, repeated blood sampling, handling and anaesthetics would have been signified sources of stress for fish by affecting physiological parameters during recovery (Zahl et al., 2012) or even compromise surgical stitches and cicatrizing processes. As a result, we would say that there were the need to find a compromise between experimental procedures and sources of stress in order to avoid some possibile confounding effects, factors that we have taken into account while planning and that have lead us to the sampling strategy adopted in this work.

5.2. Influence of pinealectomy/opthalmectomy and day-night cycle on the expression of genes involved in melatonin biosynthesis in the diencephalon of the European sea bass and on its melatonin receptor MT1

A recent study hypothesized a possible production of melatonin in the diencephalon of sea bass suggesting that the presence of *aanat1 or annat2* genes in combination with *asmt* could implicate its production, providing serotonin is present (Paulin et al. 2015). In the current study, since the expression of these genes has

been confirmed, in particular the presence of *asmt* encoding the final enzyme in the biosynthesis pathway, we tentatively support the hypothesis that melatonin synthesis could occur locally in the diencephalon of sea bass. That being said, it must be considered that expression was low and we would point out that a significant contribution to circulating levels of melatonin is unlikely. Furthermore, caution must be taken regarding the expression of *aanat2* which may have been an indicator of pineal residue, rather than local diencephalic expression.

We came to the conclusion that none of the melatonin enzyme genes analysed in the diencephalon were significantly affected by removing the pineal organ or eyes (PX and PXOX groups), as the expression levels of all genes were comparable to that of the SHM control at day and at night. There was also a lack of rhythmicity in gene expression in the SHM, PX and PXOX groups, with no clear pattern of day-night variation for *aanat1b, aanat2* and *asmt.* Regarding *aanat2* expression, although the average expression for surgical treatments at night was higher than day-time, in agreement with it's known rhythm in the pineal (Falcón et al., 2011), this was not significant owing to high individual variability. Regarding *aanat1a* expression, it was noted that the PX group had significantly higher expression during the day. This is the inverse pattern to pineal melatonin production. In contrast, significantly higher nocturnal transcript levels of *aanat1a* were observed in the PXOXM group, suggesting a differential day-night regulation of *aanat1a* expression when the eye is also lacking and under sustained high levels of melatonin.

The roles of AANAT1a and AANAT1b are unknown, it is possible that their presence in the diencephalon eludes to alternative roles in the catabolism of serotonin and/or dopamine, features that would be further confirmed by their wider distribution in other tissues compared to ASMT and AANAT2 (Paulin et al. 2015). Alternatively, if they are responsible for melatonin production in the diencephalon, then the melatonin may perform a more autocrine/paracrine role, similar to the scenario in the retina, rather than a time-keeping role. It would be of interest to further investigate the biological role of these enzymes.

Interestingly, in the PXOXM group where natural sources of melatonin were replaced by a constant external source, *aanat1a* and *aanat1b* expression were higher compared to the PXOX treatment at night, with a significant day/night difference for *aanat1a*. This may indicate a time-dependent response of *aanat1a* and *aanat1b* to melatonin treatment. Also of note, is that melatonin receptor (*mt1*)

expression for this group was highly elevated at night and may have been indirectly responsible for the effect on *aanat1a* and *aanat1b.* Again, it would be interesting to further elucidate the role of these two enzymes considering this night-time elevation in response to melatonin implant.

The downstream effects of melatonin depend not only on its rhythmic release but on the availability of receptors. MT1 is a high affinity melatonin receptor, and widely expressed in the diencephalon of sea bass (Herrera-Pérez et al., 2010). As discussed, our results revealed a night-time elevation in *mt1* expression in the PXOXM group, a significant increase in night-time expression was also present in the PX group. Indeed, the night-time average was also higher in SHM and PXOX groups, although not significant. In many fish species, studies of ^{125}I-melatonin (^{125}I-Mel) binding in the brain have revealed significant day-night variations (Falcón et al., 1996; Oliveira et al., 2008). Studies on receptor expression have also revealed significant day-night differences and circadian rhythms in the brain (Gaildrat et al. 1998; Park et al. 2007). Specifically, in sea bass, under constant light, melatonin receptors Bmax (the maximum number of receptors bound by a ligand) showed variations, an outcome that would suggest the existence of a circadian rhythm (Bayarri et al. 2010). The results of the PXOXM group were of particular interest as even though melatonin levels were constant between day and night (as seen in the plasma melatonin analysis) the day-night variation in *mt1* expression was highly significant. This suggests that melatonin up-regulated the expression of its own receptor in a time-dependent manner. Therefore, the existence of a circadian rhythm in receptor expression cannot be ruled out, though, post-transcriptional processes involved in shaping this pattern must also be taken into account. It is also true that a possible role of photoreception in deep brain areas should not be totally excluded because opsin-immunoreactive cells were detected in the diencephalon of sea bass (Herrera-Pérez and Muñoz-Cueto, unpublished) and other teleost (Alvarez-Viejo et al., 2004). In addition, the night-time expression of *mt1* in the melatonin implant group (PXOXM) was significantly higher than other groups. Past studies on melatonin administration have shown results for up-regulation or down-regulation of the melatonin receptor. Bayarri et al. (2010) have found in the hypothalamus and the optic tectum of sea bass that under natural photoperiod melatonin receptors Bmax reached its minimum level when melatonin was high, result that agrees with the idea that melatonin could contribute to down-regulate its own receptors. However, this is

not a general rule because Kd (the dissociation constant at the equilibrium), Bmax and melatonin levels have been found in phase in masu salmon under natural photoperiod (Amano et al., 2003). How *mt1* expression can be entrained in a daily basis in the absence the photoreceptor structures and under sustained constant melatonin levels will require further investigation in the near future.

5.3. Effects of pinealectomy/opthalmectomy on locomotor activity

Locomotor activity recordings indicated that control groups (CTL, SHM) were rhythmic and displayed diurnal activity throughout the duration of the experiment. This diurnal activity was in agreement with that of previous reports of sea bass behaviour, where they exhibited diurnal behaviour from spring to autumn (Sánchez-Vázquez et al., 1998). Pinealectomised groups (PX, PXM) recovered rhythmicity soon after surgery. Early studies on the circadian system of fish suggested that the pineal organ could act as the central pacemaker, however, further research has shown that many rhythms are maintained in PX fish (Sánchez-Vázquez et al., 2000; Shedpure and Yadu, 2002) as observed in the sea bass in this study. This eludes to the role of the pineal more in the entrainment of an oscillator involved in controlling the behavioural rhythms. Altogether, these evidences reinforce the idea that fish circadian system could be composed of an extended net of oscillators in central and peripheral locations, being less hierarchical than the mammalian one (Moore and Whitmore, 2014). It must also be noted that the rhythmicity may have been the result of a masking effect owing to positive phototaxis mediated by the eyes. Alternatively, pineal regeneration would have exerted a role in restoring rhythmicity but it is unlikely that tissue regrowth would have occurred in the early stages after surgery.

The PXOX group which had both the pineal and the eyes removed, thus eliminating the masking effect of eyes, showed a longer loss in rhythmic activity (up to 20 days). Interestingly, rhythmic behaviour did return in this group suggesting that it was not dependent on the input of the eyes or pineal. Nevertheless, melatonin appeared to aid in restoring rhythms sooner. A similar group, but implanted with melatonin (PXOXM), recovered rhythmic activity 10 days earlier than the PXOX group. It must be considered that feeding time could have been act as a zeitgeber to entrain activity in these groups. There is however increasing evidence that light can penetrate deeply into the brain (Vígh et al., 2002), and fish may have been receiving a “direct” message at the level of the brain, encoding day and night. In fact, this

group also exhibited day-night differences in the expression of *aanat1a* and *mt1*, which evidence that the melatoninergic system is also entrained with the daily cycle.

In the ophthalmectomy only group (OX), fish still had the pineal organ intact and recovered rhythmicity soon after surgery, implying the important role of the pineal in entraining locomotor activity. There was a reduction in activity in the upper part of the water column, which was thought to be due to reduced feeding activity owing to loss of vision.

Little is known about the direct effects of melatonin on locomotor activity in teleosts. In general in vertebrates it is considered a hormone that promotes a sleep-like behaviour in diurnal species (Zhdanova, 2005) whereas these effects on nocturnal species appear to be different. Accordingly, a lack of such effect has been seen in nocturnal species such as rat (Tobler et al., 1994) and owl (Murakami et al., 2001) while more robust results have been found in goldfish (a diurnal teleost) in which intraperitoneal melatonin injections diminished locomotor activity (Azpeleta et al., 2010). Moreover, López-Olmeda et al. (2006 a,b) have observed decreases in locomotor activity in goldfish but not in tench (a nocturnal teleost) after intraperitoneal injections of melatonin. Furthermore, Zhdanova et al. (2001) have investigated how the locomotor activity and arousal threshold were affected in larvae of zebrafish by testing several compounds including melatonin. Basically, their findings were in line with other studies indeed the exposure to a wide range of melatonin concentrations reduced proportionally both the locomotor activity and the arousal threshold. It is particularly complex to unravel the effect of melatonin on behaviour in sea bass as this species shows dual behaviour with a seasonal phase-shift from diurnal (spring to autumn) to nocturnal behaviour (winter), coinciding with the onset of the reproductive period (Sánchez-Vázquez et al., 1998). In our study, performed in summer, we showed that animals implanted with melatonin (PXM and PXOXM) showed more robust daily locomotor activity rhythms that their counterparts non implanted (PX and PXOX), which suggest a different behavioural sensitivity to melatonin in particular moments of the daily cycle. If there is also a differential sensitivity to melatonin in terms of behaviour, in other moments of the annual cycle (e.g. in winter) remains to be elucidated.

Considering the evidence that behavioural rhythms were present in all treatments including the PXOX group, it appears that rather than the pineal acting as a central pacemaker, it may play more of a role in the input pathway used by light to

entrain the oscillator (or oscillators) involved in behavioural rhythms of sea bass. Furthermore, although the exact role of melatonin is unknown, it appears to be important in this pathway, given the effect of the melatonin implant in restoring diurnal activity and the strong day-night variation of the melatonin-synthesising enzyme *aanat1a* and melatonin receptor (*mt1*) in response to melatonin. Nevertheless, further research is required to elucidate the role of the pineal organ (nervous and endocrine effects) and eyes in the circadian system of sea bass. Improved knowledge on such a system and the environmental control of biological rhythmicity is important in basic and applied research as responses to external factors may vary depending on the time of day. For example, stressors (i.e. netting, air exposure) in aquaculture may have greater negative impact at certain times of day (Vera et al., 2014).

6. CONCLUSIONS

1. Opthalmectomy performed in summer did not affect plasma melatonin levels and day-night variation. We hypothesise that even if connections between lateral eyes and the pineal through the brain are confirmed, the light information provided by the eyes is not a key step in regulating melatonin production in the pineal because of the direct light sensitivity of the whole pineal organ. Input from the eyes may however act at the level of fine-tuning of melatonin production or influence the program of pineal regeneration. Studies aimed at elucidating the role of melatonin by PX should also consider removing the eyes.
2. Unexpected normal plasma melatonin levels at night in PX fish were observed, this result indirectly suggests that pineal regeneration may occur in the European sea bass after long term pinealectomy. This regeneration seems to be dependent on the presence of functional eyes because depleted melatonin levels were found in PXOX animals. In PX experiments this could impair results and a control over this potential drawback should be performed in future studies in order to collect reliable results. On the other hand, pineal regeneration represents a fascinating field in itself, by elucidating mechanisms of tissue re-growth their application to biomedical research could be achieved in the future.
3. Expression of the four genes involved in melatonin production was observed in the diencephalon of sea bass. However caution must be applied when interpreting their significance as expression levels were low:
    - There was no clear effect of the removal of light sensitive organs on any of the melatonin enzymes in the diencephalon, there didn't appear to be a compensatory mechanism of melatonin production.
    - There was no clear pattern in rhythmicity of melatonin enzyme expression under the light-dark cycle, except for high day-time expression of *aanat1a* in PX individuals and higher nocturnal transcript levels in PXOX animals. This leads us to support the hypothesis that alternative roles, other than time-keeping, may be considered for melatonin enzymes in the diencephalon. If melatonin is produced in the

diencephalon, it may play an autocrine/paracrine role, as in the case of the retina.

- Melatonin appeared to act at night as a signal to enhance expression of the genes involved in its production within PXOXM group. It is likely to accomplish that by interacting with other circadian rhythms or pathways not studied here.

4. Day-night variation in the melatonin receptor (*mt1*) under constant conditions of melatonin input, may provide circadian information to downstream systems, even in the absence of pineal organ.

5. Locomotor behaviour appeared to be rhythmic even in the absence of input from the main photosensitive organs (pineal and eyes). The pineal organ and possibly the eyes are more likely to play a role in the input pathway used by light to entrain an oscillator (or oscillators) involved in behavioural rhythms of sea bass. Furthermore, administration of melatonin helped to restore behavioural rhythms.

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