UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Daily rhythms of physiological processes in fish: synchronization to light and feeding cycles and effects on the molecular clock, epigenetics and welfare

Ritmos diarios en los procesos fisiológicos de peces: sincronización con los ciclos de luz y alimentación y efectos sobre el reloj molecular, la epigenética y el bienestar

D.ª Elisa Samorì

2024

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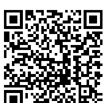

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Introduction

1 Introduction

1.1 The circadian rhythms in fish.

1.1.1 The circadian system

Fish have evolved in an environment where numerous geophysical factors follow predictable cycles due to the Earth's and Moon's movements. Geophysical events like tides, the alternation of the seasons or the variation of the day length (photoperiod) have influenced and shaped the time-keeping mechanisms that have allowed organisms to adapt to the temporal structure of the Earth (Panda et al., 2002). This synchronization provides organisms with the advantage of anticipation, contributing to their adaptive success (Krittika and Yadav, 2020). Biological rhythms were then shaped to align with Earth's complexity, characterized by their persistence even under constant environmental conditions (Cymborowski, 2010), with periods ranging from seconds to years (Goldbeter, 2008).

The most ubiquitous biological rhythm relies on a 24-hours periodicity, and it refers as circadian rhythm. The circadian system is the core of the mechanism and comprehends synchronizers (or *zeitgebers*), pacemakers (or oscillators) and output pathways (Fig. 1) (Pando and Sassone-Corsi 2002, López-Olmeda, 2017). To make this system work properly, the clock mechanism must be oscillatory and responsive to *zeitgebers* (Sharma and Chandrashekaran 2005). The mechanism starts with the reception of different stimuli by the pacemakers, which, after being entrained, can transmit the period to a specific output pathway that will display a rhythmic oscillation (Pando and Sassone-Corsi 2002). *Zeitgebers*, representing abiotic (e.g., light, temperature) or biotic (e.g., food availability) factors, play a crucial role in entraining different oscillators (Sharma and Chandrashekaran 2005, López-Olmeda, 2017). If these environmental cues are abolished, the organism can display its intrinsic free-running rhythm (*tau*), typically close to a 24-

hour period (hence circadian) and essential for the fitness of the animal (Wyse et al., 2010).

While mammals possess a master pacemaker located in the suprachiasmatic nucleus (Honma, 2018), in fish the existence of a central pacemaker is unclear, and a net of multiple oscillators without a hierarchical structure is the most representative hypothesis (Cahill, 2002, Vatine et al., 2011). Moreover, in mammal is possible to distinguish a light-entrainable oscillator (LEO) located in the SCN (Honma, 2018), and an independent food-entrainable oscillator (FEO) whose location is still debated (Schibler et al., 2003), but fish seems to lack such anatomical distinctions. Two scenarios have been proposed to explain this complexity in fish:

- The oscillators are connected but anatomically separated
- Light and food can entrain a single oscillator simultaneously, with one signal potentially stronger than the other (López-Olmeda, 2017)

The circadian system can be implicated in the regulation of different physiological pathways, like appetite regulation (Hoskins and Volkoff 2012) or stress response (Cowan et al., 2017) and recently, a connection with the epigenetic mechanism has been proposed (Stevenson 2018) which would help to explain its complexity.

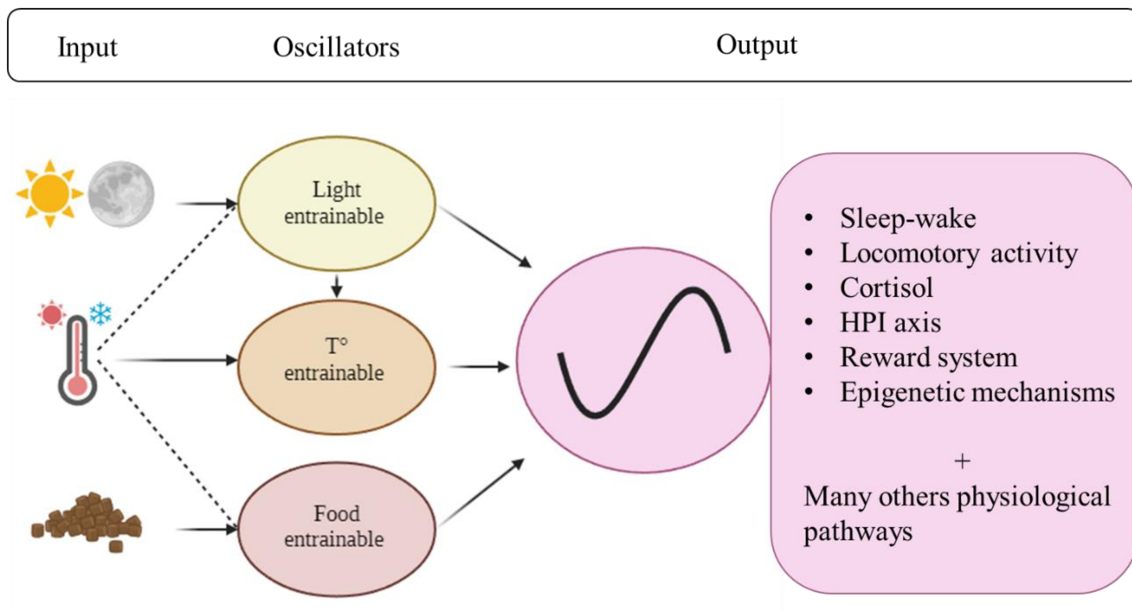


Fig.1 Schematic representation of the circadian system. Different zeitgebers can entrain the oscillators, which finally lead to the appearance of the rhythm in behavioral activity and molecular pathways.

1.1.2 The molecular organization of the circadian system

At the molecular level, the organization of the circadian system is the same in every oscillator and presents a complex organization where positive and negative feedback loops are perfectly integrated. In mammals and fish, a positive and a negative circuit can be distinguished. The first is formed by the genes *clock* (*circadian locomotor output cycles kaput*) and *bmal* (*brain and muscle ARNT like protein*), while the second is by the genes *period* (*per*) and *cryptochrome* (*cry*) (Cahill 2002, Pando and Sassone Corsi 2002, Vatine et al., 2011). The circuit starts with the transcription of *clock* and *bmal*, which form a heterodimer in the cytoplasm before returning into the nucleus and acting as a transcriptional factor for *per* and *cry* and other clock-controlled genes (CCG). Once transcribed, Per and Cry proteins become the negative regulator of the system, binding the Clock-Bmal complex and subsequently inhibiting transcription (Cahill, 2002; Pando and Sassone Corsi, 2002; Vatine et al., 2011). Simultaneously, a secondary loop takes place since the orphan nuclear receptors *rorα* and *rev-erbα* are targeted by the Clock-

Bmal complex and start their transcription. Their product can either exert a positive or negative effect since they compete for *bmal* RORE element on *bmal* promotor, thereby promoting (*rorα*) or stopping (*rev-erbα*) its transcription (Sato et al., 2004) (Fig. 2). The overall result of these mechanisms is an oscillation that approximates the 24-hours period, although the positive and negative circuits are not the only regulatory systems which ensure the oscillation this mechanism is based on a multilevel organization (Cermakian and Sassone-Corsi, 2000).

In the zebrafish multiples copies of the clock genes have been identified (Pando and Sassone Corsi 2002, Vatine et al., 2011) probably due to genome duplication events (Wang 2009):

- three *clock* genes (*clock1a, b* and *2*) and three *bmal* genes (*bmal1a, b* and *2*)
- two *per1* homologues (*per1a* and *1b*) plus other two *per* gene (*per2* and *3*) which peaks in different moment of the day and are differently regulated (i.e. *per2* is strictly dependent of light).
- six *cry* genes that can be divided in two group based on the affinity with Drosophila or mammals.

Clock gene expression can be observed in several tissues, showing different patterns of expression and acrophase. These differences can also be noticed in fish by comparing the same gene across different tissues. They can be influenced by external clues such as the light-dark cycle (Del Pozo et al., 2012), feeding time (López-Olmeda et al., 2010, Feliciano et al., 2011, Costa et al., 2016, Gómez-Boronat et al., 2018), temperature (Prokkola et al., 2018) or the season of the year (Herrero and Lepesant, 2014). In addition to these external regulators, internal factors can also entrain the clock system. One example is cortisol, which can bind the glucocorticoid responsive element (GRE) on the promoter or *per1*, modulating its transcription (Dickmeis et al., 2013).

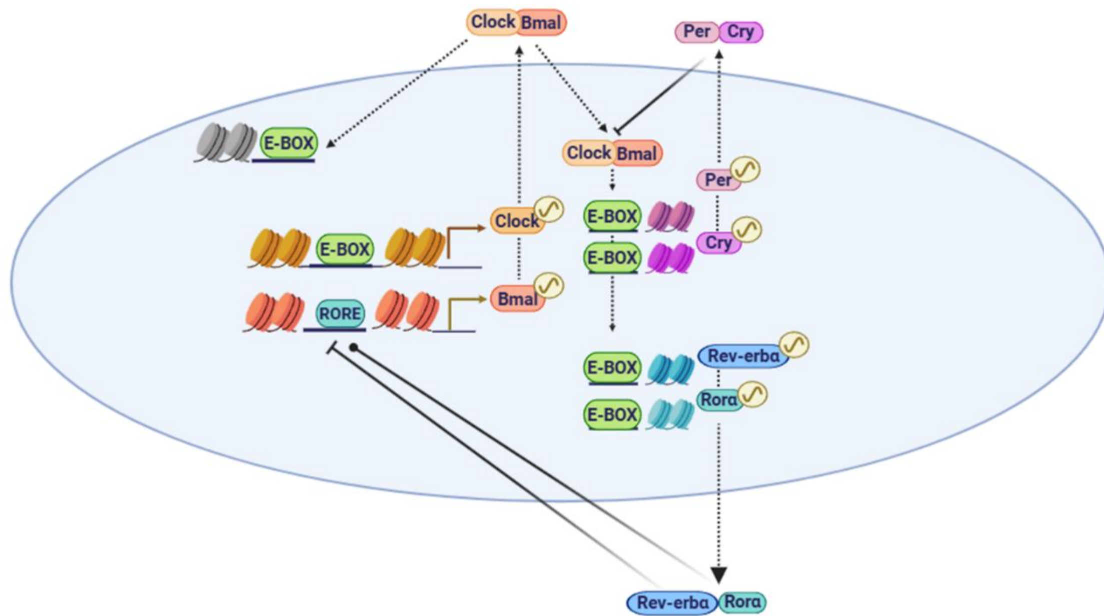


Fig. 2 The relationship between the clock genes. *Clock* and *bmal* start the circuit promoting the rhythmic transcription of *per* and *cry,* which protein will stop the complex Clock-Bmal, and *rev-erbα* and *rorα,* which can exert a either a stop or promote bmal transcription. At the same time, the complex Clock-Bmal became a transcriptional factor for several clock-controlled genes (CCG).

1.1.3 The light-dark (LD) cycle as a synchronizer

Light represents the most important *zeitgeber* for biological rhythms both in mammals (Güldür and Gül Otlu, 2017) and fish (Frøland Steindal and Whitmore, 2019). In fish, light is perceived by the eye and the pineal gland, but only the latter has a crucial function in the production and releasing of melatonin into the bloodstream, while the retina's product is metabolized *in situ* (Falcón et al., 2011). The pineal gland appears as a vesicle formed by the evagination of the roof of the diencephalon, which photoreceptive characteristic are given by the presence of *cones-like* photoreceptors, which present all the component that allow melatonin production (Falcón et al., 1992). The melatonin produced by the pineal gland represents a key hormone for biological rhythms. Its rhythmic production peaks during the night and is, at least in part, under the control of the circadian system, since *clock* and *bmal* products can bind *aanat2*'s E-box inducing its

transcription for the rate limiting enzyme in melatonin production (Appelbaum et al., 2006, Falcón et al., 2011). At the same time the hormone itself becomes an internal *zeitgeber* that can affect several physiological patterns influencing peripheral oscillators (Falcón et al., 2011). The influence of light can be registered also in terms of wavelength by the organism, potentially also influencing the clock system as proved for zebrafish (di Rosa et al., 2015), and the reason is that light spectra penetrate differently the water column, since red light (λ>600 nm) is absorbed in the first 20m of depth while blue light (λ~450 nm) can reach deeper waters. Additionally, a specific fish trait is that different tissues can be directly entrained by light as proved by experiments in vitro carried out in zebrafish, which once again demonstrate the independence of circadian pacemakers in fish (Frøland Steindal and Whitmore, 2019).

1.1.4 Photoperiod and the importance of the season

Daylights and temperatures change during the year, determining a cyclic pattern of conditions better known as seasons, which also affect the aquatic system. Seasonality refers not only to abiotic factors but also biotic ones like food availability, and for all these reasons, the ability to anticipate these annual variations was crucial during evolution (Krittika et al., 2020). The most evident external clue in seasonal changes is day length, also called photoperiod, which is registered and converted in a neuroendocrine response and is why circannual rhythms are established in *photoperiodism* (Wood and Loudon 2016). Melatonin is one of the hormones that play a fundamental role, representing a link between the change of the season and the organism's adaptation throughout the tuning of the biological rhythms, thus acting not only as a clock but also as a calendar (Sánchez-Vázquez et al., 2019). In ectothermic animals like fish, besides day length, water temperature is also considered important, and it can directly affect melatonin production

(Falcón et al., 1994). To fine-tune the biological rhythm, it is essential that light and temperature are experienced in phase, pointing out how climate change can affect fish by also targeting the circadian system (Prokkola et al., 2018). Fish exhibit various physiological and behavioral changes during the year that influence many aspects of their life, like reproduction (Migaud et al., 2010). However, for some species, the changing also affects the daily activity patterns, as observed in the European sea bass (*Dicentrarchus labrax*), which can switch from diurnal to nocturnal in winter, a behavior that is called dualism (Sánchez-Vázquez et al., 1998).

1.1.5 Food availability

In the natural environment, food alternates peaks of availability and scarceness, which can be seen on a day and year scale. To optimize food consumption, the most effective strategies imply the ability to predict feeding time using time-keeping mechanisms, which would also help to prepare the organism for digestion (Vera et al., 2007; Yúfera et al., 2012; Mata-Sotres et al., 2016). Moreover, how fish prey and actively look for food is often connected to their phase of activity since diurnal or nocturnal fish concentrate their feeding behavior mostly during the day or night, respectively (López-Olmeda et al., 2010). Some species, like the European sea bass, also have the ability to change their pattern of activity during the year, alternating months of diurnal feeding with a period of nocturnal activity, where the latter coincides with winter's months (Sánchez-Vázquez et al., 1998). Moreover, in this species, the importance of feeding time can also be seen in laboratory conditions since it helps to promote the switch from one phase of activity to another (Sánchez-Vázquez et al., 1995). Indeed, dualism has been established during the evolution as a consequence of selective forces like food availability (Daan, 1981). When food is offered at the same time of the day, a primary sign of synchronization

is visible in the behavior, trough food anticipatory activity (FAA), which consists of an increase in locomotor activity just a few hours before the meal followed by a gradual decreasing after food consumption (Vera et al., 2007). Even though food availability is considered secondary to the light and dark cycle, it can still exert an important influence, especially on the peripheral pacemakers, which seem more sensitive to food than the central oscillator. The liver is one of the organs that best respond to a change in feeding time in fish in terms of influence on the molecular circadian system (Feliciano et al., 2011; Del Pozo et al., 2012; Costa et al., 2016), becoming central in the study of the effects of different scheduled meals.

1.2 Food intake and digestion rhythms in fish.

1.2.1 The regulation of food intake

The regulation of food intake is a complex process based on the integration of different external and internal signals by the central nervous system (CNS). In this context, the hypothalamus is a key actor since it incorporates the metabolic information and neuroendocrine signals to produce hormones which can promote or diminish food intake (Volkoff et al., 2005, Delgado et al., 2017). Among the factors that modulate food intake, it is possible to distinguish between central and peripheral appetite-regulating factors, where the first refers to the hormones produced mainly by the hypothalamus and the second to the ones produced in different peripheral areas (Volkoff 2016) (Fig. 3).

Regarding the central (hypothalamic) appetite regulators, two groups of neurons have been characterized in fish. They represent the central core of the regulation: one group expresses pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which are mainly categorized as anorexigenics, while the other expresses the neuropeptide Y (NPY) and the agouti-related peptide (AgRP) which function is to promote food intake (Volkoff, 2016, Delgado et al, 2017). Although these peptides are categorized into two distinct groups in terms of roles, their function is not always fixed. Specifically, POMC can be hydrolyzed into β-endorphin or α-MSH based on the energy balance, thus promoting or inhibiting food intake (Delgado et al., 2017). CART injections can decrease food intake in goldfish, confirming its anorexigenic properties (Volkoff and Peter, 2001), but how it responds to food deprivation can vary between species (Volkoff, 2016). NPY and AgRP have been characterized in several species, including the European sea bass (Cerdà-Reverter et al., 2000; Aguillero et al., 2013). Two isoforms of AgRP (AgRP1 and 2) have been described in teleost, but only AgRP1 seems more responsive to fasting (Aguilleiro et al. 2014). Lastly, NPY is

described as one of the most essential orexigenic factors, and one study on European sea bass also suggested its involvement in the reproductive system (Cerdà-Reverter et al., 2000). Orexin is another important central appetite regulator produced mainly by the hypothalamus, and it can exert its orexigenic factors modulating the mRNA level of NPY (Yan et al., 2011).

Some of the essential peripheral factors with anorexigenic properties are cholecystokinin (CCK), which is produced in the digestive tract and increases after food ingestion (Murashita et al., 2006) and leptin, which is mainly produced in the liver and gut and is strongly associated with metabolism (Copeland et al., 2011). While among the putative orexigenic hormones, ghrelin is mainly expressed in the stomach and is one of the most controversial because its action can vary between species (Delgado et al., 2017).

1.2.1.1 Circadian regulation of feeding

Most feeding rhythms are endogenous, which means that without the external synchronizing signal of food, they are maintained with free-running periods that usually do not deviate too much from 24 hours. The hypothalamus integrates not only neuroendocrine and metabolic signals but also cyclic information from the environment, like the alternation of day and night or temperature variations, and the registration of this information drives the rhythmic production of appetite regulators (Kulczykowska and Sánchez-Vázquez 2010). Some of the appetite regulators display daily fluctuations in fish, both in the central (Vera et al., 2007; Hoskins and Volkoff, 2012) and peripheral oscillators (Tinoco et al., 2014; Sánchez-Bretaño et al., 2015) even though is difficult to discriminate which synchronizer (light or food) can affect most their regulation. Notably, the circadian system can directly affect the production of appetite regulators, but at the

same time, these factors become synchronizers for other timekeepers as well, suggesting an intricate connection of feedback (Nisembaum et al., 2014) (Fig. 3).

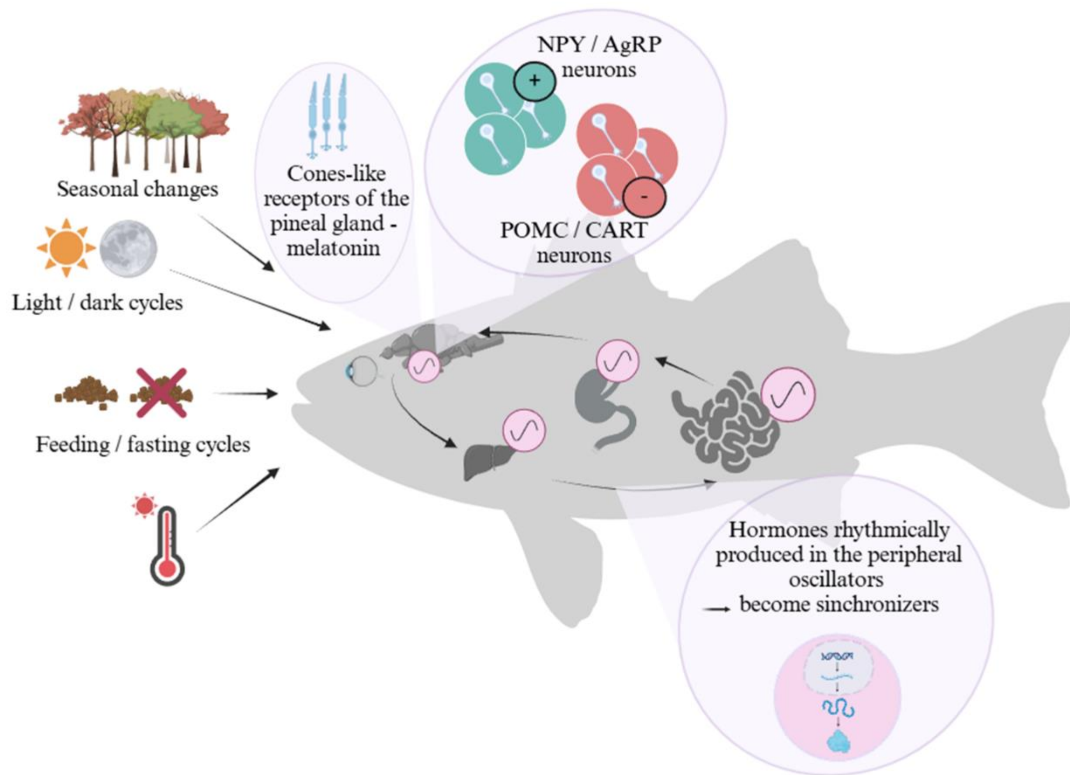


Fig. 3 The interconnection of the central and peripheral pacemaker in the regulation of the process of food intake and digestion. Central and peripheral appetite regulating factors are released rhythmically in response to different zeitgebers, which will help the fish to maximize the benefits of the food when is present, and at the same time they can become synchronizers for other time-keeping mechanisms. The hypothalamus represents the main regulators producing hormones that can stimulate or reduce appetite and food intake.

1.2.2 The reward system

The reward system plays an important role in controlling appetite, implementing the effect of appetite-regulating factors. The reward system is based on a neural network that involves different parts of the brain and can be activated by different stimuli like food (Berridge and Kringelbach, 2015). Some studies have shown that the hypothalamus could also participate in the process since anatomical projections from different hypothalamic areas have been described (Ogawa et al., 2022). As mentioned previously, the hypothalamus is crucial in food intake and appetite regulation, suggesting that the whole regulation is affected by homeostatic and hedonic mechanisms (Rossi and Stuber 2018).

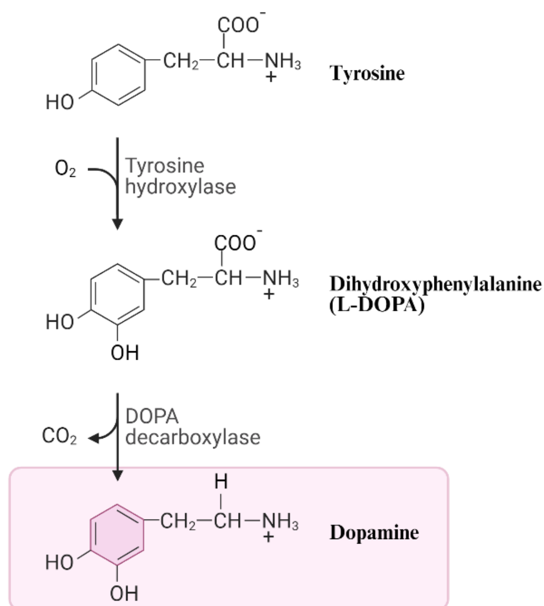


Fig. 4 Dopamine synthesis pathway.

Dopamine (3,4-dihydroxyphenethylamine) is a catecholamine that represents the principal neurotransmitter involved in the reward system. In his biosynthesis pathways, the step of the conversion of tyrosine into L-DOPA (L-Dihydroxyphenylalanina) is crucial and is catalyzed by the enzyme tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis.

After dopamine is released, the excess is taken back using a transporter (DAT) and lately broken down into DOPAC (3,4-dihydroxyphenylacetic acid) (Daubner et al. 2011) (Fig. 4). The dopaminergic pathway is involved in food intake regulation with orexigenic function in mammals. In mice, dopamine is synthesized by TH neurons in the nucleus arcuate (ARC) and can stimulate orexigenic neurons that synthesize NPY/AgRP to inhibit the neuronal population responsible for POMC synthesis (Zhang and van de Pol, 2016). Studies on fish have proven that fasting can induce the increase of TH gene expression in the brain of cavefish (*Astianax fasciatus mexicanus*) (Wall and Volkoff, 2013) and goldfish (Mandic and Volkoff, 2018). The same effect is exerted with the injections of the appetite regulators apelin and orexin which increase *TH* gene expression (Penney and Volkoff, 2014). On the contrary, the oral administration of L-DOPA in European sea bass determined a decline in the growth parameters by reducing food intake (Leal et al., 2013).

1.2.2.1 Circadian oscillation of the dopaminergic system

Concerning the circadian oscillation of DOPA or TH, the studies are more extensive in mammals. For example, the rhythm of genes involved in dopamine biosynthesis has been described in the in the midbrain of rats (Pradel et al., 2022) and the daily dopamine release has been identified as a factor involved in the rhythmic expression of *Per2* in the dorsal striatum, suggesting a multilevel connection between the clock system and dopamine (Hood et al., 2010). Conversely, results on fish are scarcer. In catfish (*Heteropneust fossilis*) brain, the enzymatic activity of TH presents oscillation during the 24 hours with acrophases mostly located at the end of the day presenting also seasonal variation (Chaube and Joy, 2003). A similar pattern has also been described in the retina of a cichlid fish, *Aequideus pulcher*, where TH and dopamine displayed higher values during the shift between the light and the dark phase (Wulle and Wagner, 1990).

1.2.2.2 The role of *bdnf*

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophins growth factor which is implicated in several neuronal roles and exerts its function by binding to his receptor tropomyosin-related kinase B (TrKB) (Lucini et al., 2018). In mammals, both BDNF and TrKB are widely distributed in the hypothalamus, suggesting that they could participate in the regulation of appetite and food intake probably with anorexigenic function (Lebrun et al., 2006). The participation of BDNF and his receptor in the regulation of food intake has been proved for zebrafish as well (Montalbano et al., 2016), but a possible orexigenic function has also been described (Blanco et al., 2020). A close relationship with the dopaminergic system is also present, since BDNF can bind TH promoter supporting its transcription (Fukuki et al., 2010). BNF is also related to circadian rhythms. In rat, *bdnf* undergo rhythmic transcription as proved in the

suprachiasmatic nucleus (Liang et al., 2000) or hippocampus (Berchtold et al., 1999, Schaaf et al., 1999) while in zebrafish larvae is essential to generate behavioral circadian rhythms (D'Agostino et al., 2020).

1.2.3 Circadian oscillation of protein digestion processes

A physiological process tightly linked and subsequent to food intake is the digestion and absorption of nutrients. The nutrients are obtained by the process of breaking down food, which occurs mechanically and enzymatically, before the can be absorbed into the bloodstream. Several studies have pointed out that enzymes involved in protein, lipid and carbohydrate metabolism display circadian rhythms in different peripheral oscillators (Paredes et al., 2014, 215; Del Pozo et al., 2012; Mata-Sotres et al., 2016). For most fish species, proteins are the primary energy source, and in aquaculture, protein levels in the food span from 30 to 45%, depending on the species or the life stage (Craig et al. 2017). The production of enzymes responds to the presence of food, but at the same time, they can exhibit circadian variations typically associated with feeding schedules. Therefore, when food is provided at a consistent daily time, there is an observable anticipation in the activation of enzymes responsible for protein digestion, as noted in various species like goldfish (Vera et al., 2007) or sea bream (*Sparus aurata*) (Yúfera et al., 2012, Mata-Sotres et al., 2016).

In terms of enzymatic activity, daily variation has been described as in Nile Tilapia (*Oreochromis niloticus*), in specific with a diurnal acrophases which followed the time of the scheduled feeding as expected (de Oliveira et al., 2022). Moreover, Nile Tilapia larvae reared under different thermal conditions (thermocycles *versus* constant temperatures), lost the rhythmic expression of proteases when constant temperature is applied, pointing out the importance of the temperature as *zeitgeber* for digestive process (Espirito Santo

et al., 2020). Some studies have also considered the rhythmicity in the process of transamination. In specific, the circulating levels of alanine aminotransferases (ALT) in the nocturnal fish *Lophiosilurus alexandri* present daily variations with two nocturnal peaks (Fortes-Silva et al., 2019). Also, aspartate aminotransferase (AST) activity presents a daily variation related to feeding in the liver of the rainbow trout (*Oncorhynchus mykiss*); a rhythm that is lost in fasted animals (Polakov et al., 2007).

1.2.4 The importance of feeding time on welfare

Most fish display a clear pattern of feeding activity that is the result of a long period of evolution in a cyclic environment (Daan, 1981). In aquaculture conditions, scheduled feeding not always matches the requirement for each species, but this practice can potentially decrease the animal welfare. Different studies have shown that providing food randomly or during the resting phase, as well as prolonged fasting periods, can lead to the disruption of the biological rhythms of digestive enzymes. For example, random feeding can disrupt the rhythm of genes involved in lipid metabolism in zebrafish (Paredes et al., 2015) and sea bream (Paredes et al., 2014)In Goldfish, random and nocturnal feeding promoted the loss of the rhythm of the leptin in liver and brain (Tinoco et al., 2014). Generally, feeding the fish in a phase that doesn't match its natural feeding activity phase can lead, in the long term, to an increase in stress and thus reduction of welfare since the fish must invest more energy to cope with this feeding schedule.

1.3 Rhythms of stress response in fish.

1.3.1 The physiological stress response in fish

Stress response is an important and well-conserved physiological mechanism that occurs when fish are subjected to an alarm situation that leads to a perturbation of the homeostasis (Ellis et al., 2012). When these conditions are maintained over a certain period, the consequences can be devastating for the organisms, affecting their growth, immune system, reproduction capacity and survival (Ellis et al. 2012). In aquaculture conditions, the sources of stress can be multiple, ranging from alteration of the physical condition of the water (temperature, salinity, or oxygenation) to the presence of chemical compounds, high level of ammonia or nitrate or inappropriate stocking density and for this reason stress is often considered as an indicator of poor welfare (Ashley et al., 2006).

Once a stress occurs, the physiological stress response takes place with the activation of the BSC (Brain-Sympathetic-Chromaffin) axis followed by the HPI (Hypothalamus-Pituitary-Interrenal) axis which leads to the release of catecholamines (mainly epinephrine) and cortisol, respectively (Mommsen et al., 1999, Faught et al., 2016). The release of epinephrine and cortisol are considered the primary response, but they are temporarily separated. Epinephrine increases rapidly in circulation while cortisol increases and, especially, decreases a bit slower (Vijayan et al., 2010), even though the process of increasing usually starts in the first five minutes after the stress (Molinero et al., 1997). Interestingly, the range of time in which cortisol reaches the peak can vary between species and life stage (Fanouraki et al., 2011, Fatira et al., 2014). Cortisol has received a lot of attention since it has been defined as one of the main biomarkers of stress in fish (Ellis et al., 2012) and even though his basal levels change between species, intraspecific differences between individual in term of responsiveness to stress have been also documented (Samaras et al., 2018). The release is the consequence of a cascade of

signals starting in the hypothalamus with the corticotropic releasing hormone (CRH), which targets the anterior pituitary and leads to the release of the adrenocorticotropic hormone (ACTH), which ultimately stimulates cortisol production after binding the melanocortin 2 receptor (MC2R) (Wendelaar Bonga, 1997, Mommsen et al., 1999, Gorissen and Flik, 2016, Faught et al., 2016). In fish, cortisol is synthesized in the interrenal cells, located in the head-kidney region close to the chromaffin, starting from cholesterol (Mommsen et al., 1999). It finally exerts its function after diffusing through the plasma membrane and binding the glucocorticoid receptor (GR), which helps the translocation into the nucleus where binds glucocorticoid responsive elements (GRE) on the promoter of specific target genes (Faught et al., 2016) (fig. 5).

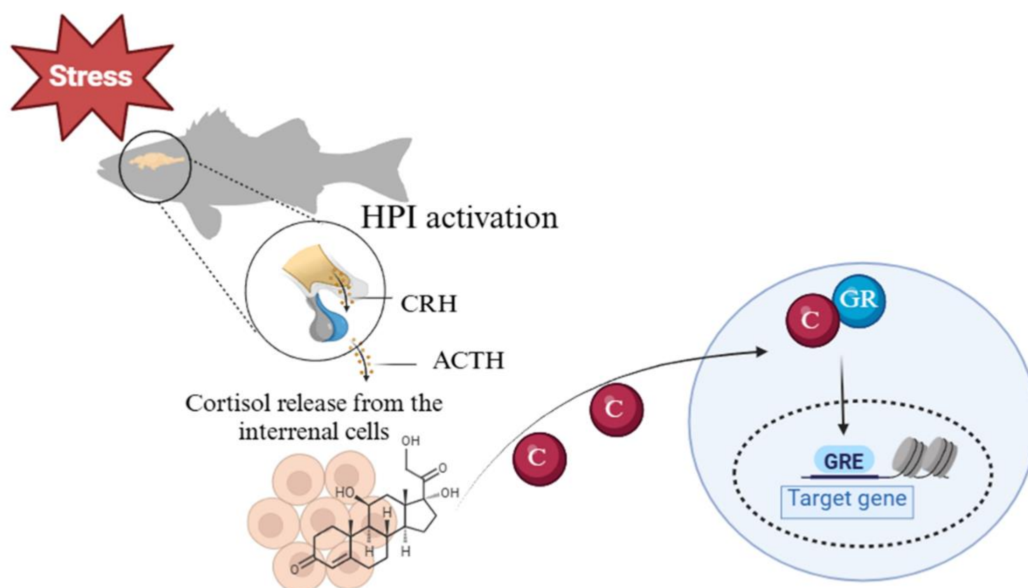


Fig. 5 Hypothalamus pituitary interrenal axis (HPI). Stress can elicit stress response through the release of CRH from the hypothalamus which stimulate the pituitary to release ACTH, which will determine an increase in cortisol production from the interrenal cells. Cortisol binds its receptor GR and target GRE regions in DNA.

The secondary response involves the increase of glucose, which is mobilized by the epinephrine, and lactate. The increase of glucose in the bloodstream is important to sustain the increase energy demand, but as for cortisol and lactate, the timing of the

release and the magnitude of the response varies between species, with European sea bass identified as one of the species with a very high response (Fanouraki et al., 2011).

1.3.1.1 The importance of the antioxidant system in the stress response

The antioxidant system produces enzymes which work in concert to eliminate ROS by a series of reaction catalyzed by specific enzymes which cooperate to transform ROS into of water and oxygens (Scandalios 2005):

- SOD (superoxide dismutase) catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide
- CAT (catalase) turns hydrogen peroxide into water and oxygen
- GSHPX and GSR cooperate to remove the hydrogen peroxide

ROS can be produced in excess for several reasons. For example, during handling for aquaculture procedures, fish are exposed to air and subsequently return to the water, a procedure that increase ROS formation due to reoxygenation (Welker et al., 2012), but also a chronic exposure to heavy metals and pollutants in water can trigger their formation (Fnghetto Fuzinatto et al., 2015), and for this reason their production must be optimized. A recent study carried out black sea bass (*Centropristis striata*) has reported the daily fluctuation on the activity of several antioxidants enzymes in plasma, and liver indicating the presence of a circadian control over the antioxidant system in fish (Ren et al., 2018)

1.3.2 Circadian regulation of the HPI axis

As many other physiological pathways of the endocrine system, the stress axis presents daily rhythmic variations in his components, showing a connection with the

circadian system, which is still difficult to elucidate completely since differences between species are often registered (Cowan et al., 2017).

Starting from the hypothalamus, the rhythm of *crh* has been documented in normal (unstressed) conditions in the Senegalese sole (*Solea senegalensis*) (López-Olmeda et al., 2013) and sea bream (Vera et al., 2014), with acrophases located at the beginning of the night and the beginning of the day respectively. Pituitary *proopiomelanocortin A* and *B*, the precursor of ACTH, peaked at the beginning of the light phase in sole (López-Olmeda et al., 2013), while in rainbow trout (*Oncorhynchus mykiss*) the two genes reported dissimilar acrophases, suggesting a different regulation (Gilchriest et al., 1998). Cortisol rhythms have been studied in many different species and different acrophases are reported, suggesting a species-specific pattern (Cowan et al., 2017). A correlation between the phase of activity and cortisol acrophases has been proposed for fish, probably as a result of an adaptative advantage (López-Olmeda et al. 2009, López-Olmeda et al., 2013). Moreover, cortisol daily variations can be affected by feeding schedule (Montoya et al., 2010), or present variations throughout the year as suggested for the European sea bass (Planas et al., 1990), probably in correlation with the reproductive phase (Cowan et al., 2017). When the stress occurs, the physiological variables of the stress axis can respond differently as well. For example, in sea bream subjected to acute stress, *crh* lost his daily rhythm (Vera et al., 2014). At the same time, cortisol response varies throughout the 24 hours, and for this reason, the time of the day in which a stressful event is experienced can differently modulate the cortisol increase (López- Olmeda et al., 2013; Vera et al., 2014).

Even though is clear that the components of the HPI axis are driven by circadian pacemakers, evidence suggest that glucocorticoids could also work as an input for the circadian system itself. In mammalian cell culture fibroblasts, dexamethasone (a

glucocorticoid analog), can induce rhythmic expression of *Per1* in just one hour after the treatment and subsequently elicit circadian oscillations in *Per2, Per3, Cry1* and *Rev-erbα* in the following hours (Balsalobre et al., 2000). In zebrafish, glucocorticoids are implicated in the generation of the daily rhythms of cell proliferation (Dickmeis et al., 2007), while in goldfish, intraperitoneal injection of dexamethasone induces the rhythmic expression of *per1*, similarly as observed in cell culture after treatment with cortisol (Sánchez-Bretaño et al., 2015). This evidence suggests the deep implications of the glucocorticoid in the regulation of the circadian system, which adds complexity to the system.

1.3.3 Acute and chronic stress: the role of epigenetics

Stressful events can be either acute or chronic. In the first case, the stress is experienced in a short period of time but with high intensity, while chronic stress represents a condition that endures in time but with a lower magnitude (Schreck and Tort, 2016).

In the natural environment, the acute stress is the most common, and when experienced in a short period of time, the stress response helps to preserve the individual and lastly confers an adaptive advantage (Gorissen and Flik, 2016). In aquaculture, some of the most frequent causes of acute stress are handling and subsequent air exposure. In the case of the air exposure, fish can experience acute hypoxia with consequent damage to gills, bradycardia and lastly asphyxia with the consequent activation of the HPI axis (Cook et al., 2015).

On the other hand, under culture conditions, fish are more likely to be exposed to chronic stress than when they are in the natural environment. Some of the most common chronic stressors are stocking density, food deprivation or inappropriate water conditions both physically and chemically (i.e. poor oxygenation, salinity, presence of nitrate or

ammonia etc.) (Ashley et al., 2006). While acute stress response can have an adaptive advantage, the chronic stress leads to a subtle but continued worsening of fish conditions from several points of view (Sadoul et al., 2021). In this context, one of the most influent parameters is the stocking density, since high stocking densities can lead to a constant stressful condition (Montero et al., 1999, Ashley et al., 2006). For example, European sea bass confined in high stocking for three months has severely modified the transcription of stress-related proteins (Gornati et al., 2004).

The new emerging epigenetic studies are showing that an event perceived as stressful during early life stages can elicit epigenetic modification, which can result beneficial in terms of increased performance later in life (Robinson et al., 2019). The following generation can also inherit this characteristic since this is a common feature of some epigenetic modifications (Granada et al., 2017). In aquaculture, although the stress response is well investigated, the effects on the epigenetic system have been started to be explored only recently (Guinand and Samaras, 2023). Recent studies have proved that one of the most common epigenetic modifications during a stressful event is a change in DNA methylation, which can be triggered by different events. For example, European sea bass exposed to a moderate temperature increase during the first stage of life present a change in the methylation patterns in genes involved in the stress response, which can persistently affect the organism during its life (Anastasiadi et al., 2017). Moreover, liver is particularly susceptible to changes in methylation. In the half-smooth tongue sole (*Cynoglossus semilaevis*), chronic low salinity exposure affects *igf1* methylation in different exons (Li et al., 2017), while 14 days of starvation modify the methylation of genes involved in metabolic pathways in liver (Lin et al. 2023). A recent study also pointed out that stress due to high density, could play a role in the process of sex

differentiation in zebrafish since it triggers masculinization probably acting on methylation of *dnmt1* (Valdiveso et al., 2023).

1.4 Rhythms of epigenetic mechanisms in fish.

1.4.1 DNA methylation and demethylation

The term *epigenetic* refers to the heritable changes in gene expression or phenotype unrelated to a change in DNA sequence. Although epigenetic modifications are quite stable and heritable, they can also be triggered by environmental factors, like diet or exposure to contaminants, creating a complex relationship with the environment (Aguillera et al., 2010).

DNA methylation represents one of the most studied epigenetic mechanisms, especially in mammals. This epigenetic modification is crucial for gene regulation (Moore et al., 2013) and the alteration of the methylation pattern has been linked to several diseases in human, indicating its importance for the organism (Jin and Liu, 2018). DNA methylation proceeds thanks to a family of enzymes called DNA methyltransferases (DNMTs), which promote the transfer of a methyl group (CH_3) to a cytosine residue to form 5-methylcytosine (5mC). Specifically, the major methylation events occur in cytosine which precede guanine in the CpG island, even though some results suggest that these are not the only site for methylation (Anderson et al., 2012, Moore et al., 2013) (Fig. 5).

The different DNA methyltransferases work with the same catalytic mechanism, but they can be divided into two categories: enzymes that maintain the methylation after the round of DNA replication or enzymes that establish a *de novo* methylation (Lyko, 2018). In mammals and fish, the gene that encodes for the first category is represented by *dnmt1*, while *de novo* methylation is mediated by the family of *dnmt3*, for which some differences were described when comparing mammals and fish. In specific, mammals present *Dnmt3a*, *Dnmt3b* and *Dnmt3L* while fish display an increased complexity due to

the whole genome duplication, as shown in zebrafish, for which three orthologs of *dnmt3b* (*dnmt3*, *dnmt4* and *dnmt7)* and two of *dnmt3a* (*dnmt6* and *dnmt8)* have been described (Kamstra et al., 2015).

Once methylation occurs, it can be retained or removed by a process of demethylation. In this sense, it is possible to distinguish between passive and active demethylation, where the first happens after DNA replication, while the second requires specific enzymes and ends with base excision repair (BER) mediated by thymine DNA glycosylase (Bochtler et al., 2017). Different mechanisms have been then described and they involve different enzymes (Fig. 6):

- Ten-eleven traslocation (Tet) enzymes convert the 5mC to 5hmC by adding a hydroxyl group. After that, Tet can continue to oxidize the 5hmC to 5fC and finally in 5caC (Ito et al., 2011).
- Alternatively, 5hmC can be deaminated by AID/APOBEC to form 5-hmU which can be excised by thymine-DNA glycosylase (TDG) and replaced by unmodified cytosine (Moore et al., 2013)
- AID/APOBEC can also convert 5mC into thymine, determining a mismatch which is then corrected by TDG and replaced by an unmodified cytosine through base excision repair (BER) (He et al., 2011; Shen et al., 2013).
- At the same time, Growth Arrest and DNA-damage-inducible Protein 45 (Gadd45a) can facilitate TDG recruitment promoting thymine excision (Niehrs & Schäfer, 2012; Li et al., 2015).
- Gadd45aa can also interact with APOBEC and MBD4 (methyl-CpG-binding domain protein 4), a BER-specific thymine glycosylase, to promote thyme excision by MBD4 itself (Rai et al., 2008).

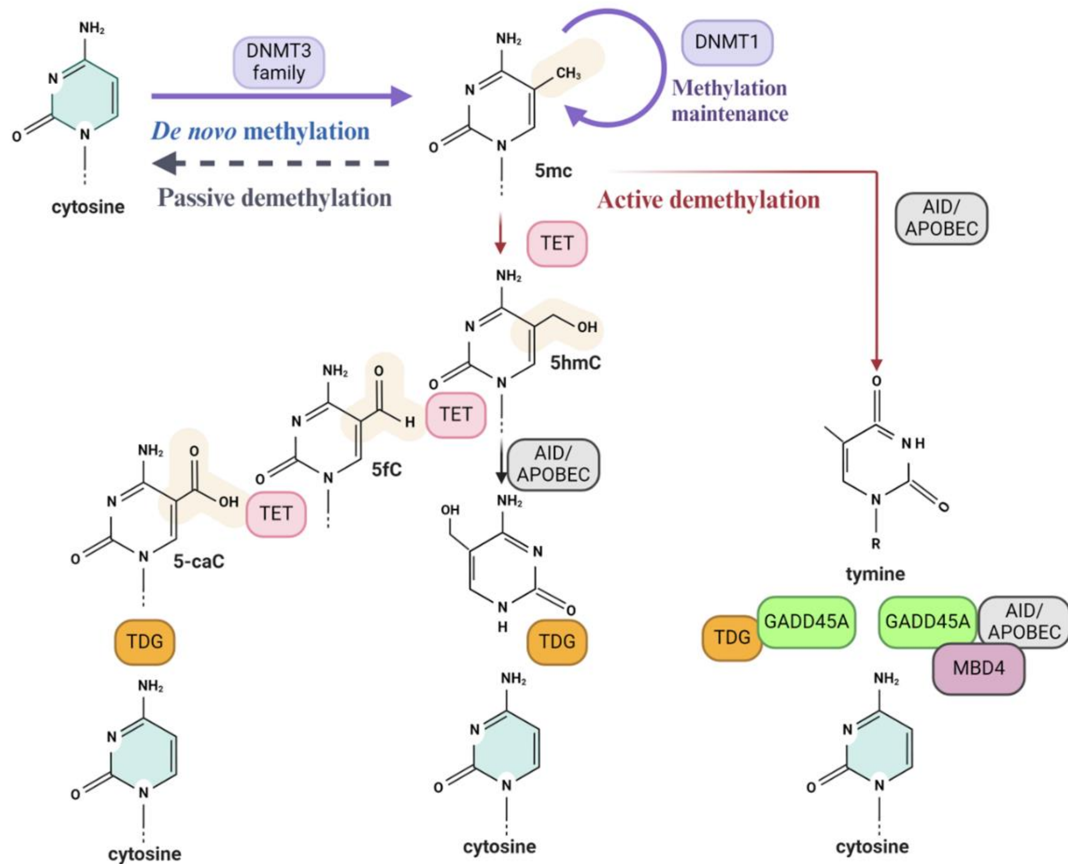


Fig. 6 Passive and active demethylation. In case of active methylation, different enzymes cooperate to oxidize and excise the methylated cytosine (5mC) or transform it into thymine before base excision repair (BER).

1.4.2 Histone acetylation and deacetylation

Histone acetylation and deacetylation represent another epigenetic modification whose output affects DNA transcription. Specifically, acetylation adds an acetyl group to the lysine residue of a histone protein within the N-terminal tail using a specific enzyme called histone acetylase (HAT). In contrast, the removal of the acetyl group is mediated by the enzyme histone deacetylase (HDAC) (Peterson and Laniel, 2004). The first modification leads to a more relaxed chromatin, which can be accessed during transcription, while the removal of the acetyl group, on the contrary, leads to a more compacted chromatin, making it inaccessible (Peterson and Laniel, 2004) (Fig. 7). In mammals, four classes of HDAC have been described based on their function and

homology (Seto and Yoshida, 2014). Among them, class III includes a group of proteins called Sirtuins (SIRT1 to 7), which can be either found in the nucleus, cytosol or mitochondria, and their function is based on the availability of NAD+ (nicotinamide adenine dinucleotide), creating an important link with cell metabolism, the metabolic state of the organism and feeding (Seto and Yoshida, 2014).

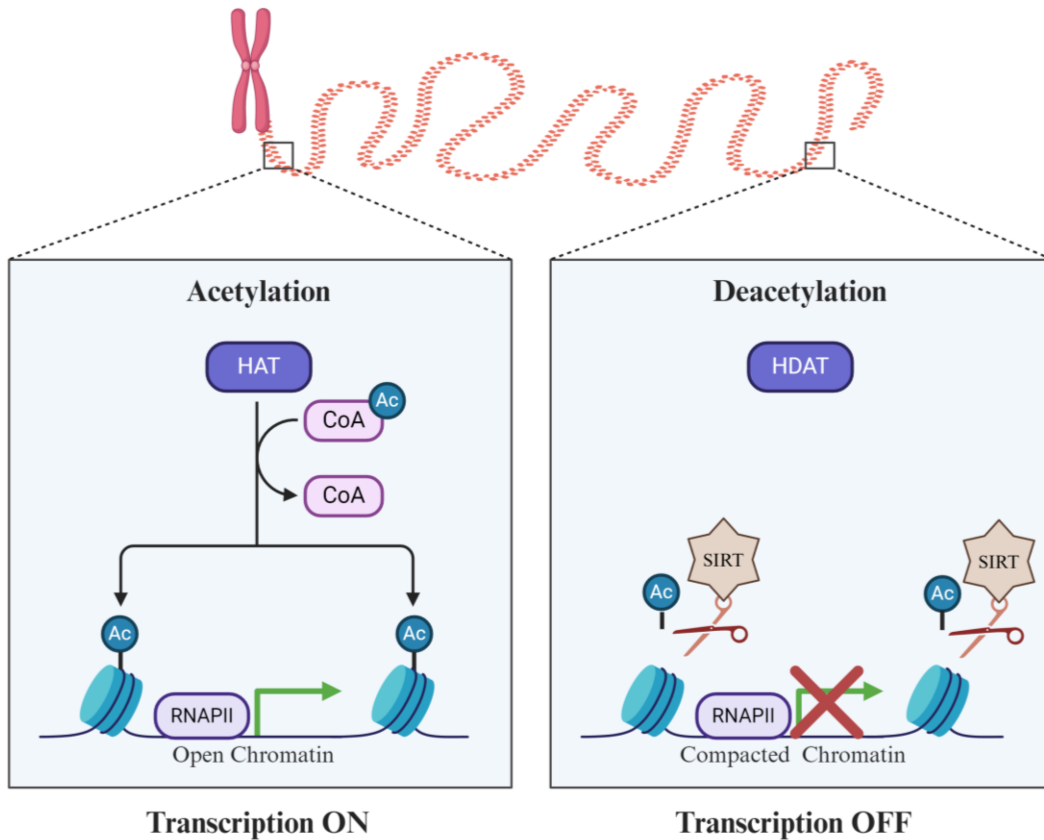


Fig. 7 Acetylation and deacetylation processes. In acetylation, the added acetyl group relax the chromatin favoring transcription, while SIRTs remove the acetyl group compacting the chromatin which become less accessible for transcription.

1.4.3 The importance of feeding for epigenetics

Methionine is an essential amino acid that participates in the one-carbon (1C) metabolism, together with vitamins B6, B9 and B12. In the epigenetic context, methionine provides the methyl group used by the DNMT to mediate DNA methylation, becoming a link between metabolism and epigenetic processes. In specific, the methyl

group is released by S-adenosylmethionine (SAM), which represents the active form of methionine and is subsequently converted into S-adenosylhomocysteine (SAH) (Anderson et al., 2012) (fig. 8). As methionine is at least in part regenerated by the folate cycle, the integration with the diet is crucial, and for this reason, it must be accurately considered when formulating a diet in aquaculture, especially the ones that are plant based which naturally lack the proper amount of this amino acid (Espe et al., 2020). Moreover, some recent studies have hypothesized that methionine assumed by the diet could influence the DNA methylation landscape (Skjaerven et al., 2018, 2020), robustly linking feeding, metabolism and the epigenetic processes.

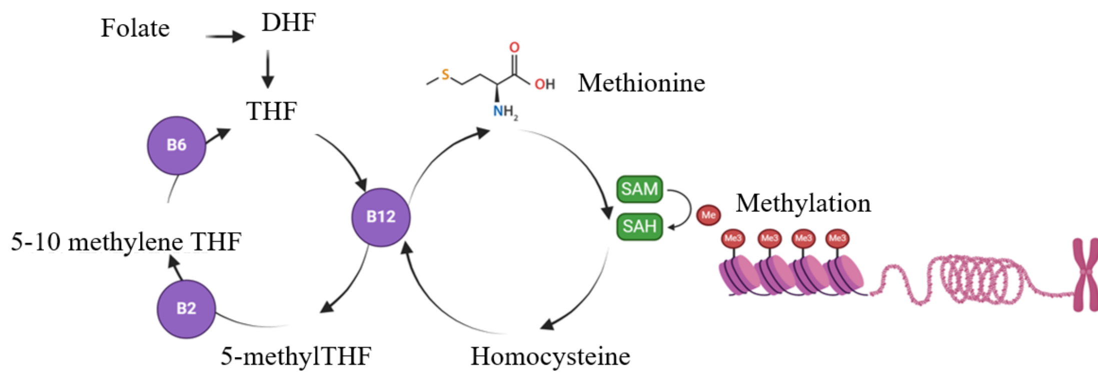


Fig. 8 1C cycle. S-adenosyl-methionine release a methyl group which is used for DNA methylation, becoming S-adenosyl-homocysteine which proceed in the 1C cycle and thanks to the folate cycle can regenerate a minimal part of methionine.

As stated before, the class III HDAC is formed by SIRTUINS (1-7), which relies on the availability of NAD+. NAD+ is central in energy metabolism and a coenzyme participating in redox reaction. Specifically, since the intracellular level of NAD+ must be maintained stable, it is synthesized from dietary resources, especially in the liver, where L-tryptophan is used for the process. Then, sirtuins use NAD+ as a cofactor in the process of removal of deacetylation, also producing nicotinamide (NAM) and acetyl-ADP-ribose (acetyl-ADRP) (Covarrubias et al., 2021). In fish, sirtuins distribution is

tissue-specific, except for *sirt1*, which is expressed in all the tissue. Sirtuins can be differently modulated by fasting, as proved for sea bream (Simò-Mirabet et al., 2017), or by food composition, as seen for *sirt1* in the liver of blunt snout bream (Yuan et al., 2019) (*Megalobrama amblycephala*) but also to specific compound which can be added in food preparation as resveratrol (Huang et al., 2021).

1.4.4 Daily rhythms in epigenetic processes

Currently, studies investigating the rhythms in epigenetic factors and mechanisms are very scarce in fish. Only one study reported the existence of daily rhythms of genes involved in epigenetic processes in zebrafish gonads (Paredes et al., 2019). Specifically, this study revealed that most of the factors involved in methylation and demethylation not only present a daily rhythm, but most of the acrophases coincide with the resting phase of the animal, which in the case of the zebrafish is the dark phase (Paredes et al., 2018). A similar pattern was also observed in mice, a nocturnal species, where the acrophases of the daily variation of *dnmt3* were located during the light phase (Xia et al., 2015) or the dawn (Maekawa et al., 2012). These articles pointed out that also global methylation fluctuates during the day, especially in correlation with *dnmt3*, which seems to be responsible for this fluctuation (Maekawa et al., 2012; Xia et al., 2015). As stated before, methionine is essential for DNA methylation since it is the principal donor of a methyl group, but the ratio between SAM and SAH gives information about the methylation potential. A study on pigs has revealed that SAM and the SAM/SAH ratio present daily variations in the liver, with different peaks during the day, which were reversely related to the daily expression of *dnmt3a* and *dnmt3b*, suggesting that these factors were affected by the SAM/SAH ratio (Zhang, 2018).

Some studies on mammals have also focused on the rhythm of sirtuins to understand if the circadian system could rhythmically drive the process of deacetylation. Sirtuins' function is strictly related to the availability of NAD+, which works as a cofactor in deacetylation (Covarrubias et al., 2021). NAD+ biosynthesis happens through the salvage pathway, which starts with the conversion of nicotinamide (NAM) to β-nicotinamide mononucleotide (NMN) operated by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), which has been shown to be under circadian control (Ramsey et al., 2009). Subsequently, NMN is converted into NAD+, which displays circadian variation due to the upstream regulation. At the same time, SIRT1 activity is regulated cyclically due to the oscillation of its coenzyme, although no differences in mRNA have been described in mammals (Bellet et al., 2013). In fish, a recent study on the liver of mackerel tuna (*Euthynnus affinis*) has described the rhythm of *sirt1* in cloudy weather condition, while no rhythm was described during sunny day, pointed out a specific metabolic feature of mackerel tuna (Wang et al., 2022)

1.4.5 The bidirectional connection between clock genes and epigenetic mechanisms

Recent observations in mammals have hypothesized the potential involvement of epigenetic mechanisms, such as DNA methylation and histone modification, in regulating the circadian system by acting at the molecular level, proposing a bidirectional connection (Stevenson et al., 2018). As previously mentioned, factors involved in DNA methylation–demethylation or histone deacetylation exhibit daily variations. *Dnmt3a* presents CLOCK:BMAL binding sites on its promoter, which allow its rhythmic transcription, but at the same time, *dnmt3a* was identified as the possible responsible for the change in the methylation pattern of *bmal1a* promoter, suggesting the existence of a complex

regulatory circuit (Satou et al., 2013, Stevenson et al., 2018). The complexity of the system is also imputable to SIRT1, which not only presents a rhythmic activity but can also associate with CLOCK to deacetylase BMAL1 (Belden and Dunlap, 2008) or direct his activity to PER2 (Asher et al., 2008). Additionally, CLOCK can exert an acetylase function which facilitates the start of the negative circuit (Doi et al., 2006), a process that can be counteracted by SIRT1 action (Nakahata et al. 2008), giving another point of regulation of the system.

1.5 Species of interest

1.5.1 European sea bass *Dicentrarchus labrax* (Linneo, 1758): biology and farming

The European sea bass (*Dicentrarchus labrax*) is a species which inhabits a wide geographic range, from the east of the Atlantic to the Mediterranean and the Black Sea, due to its high tolerance to different environmental conditions. In specific, it can be defined as eurythermal (2° – 32°C) and euryhaline (from 0‰ to 40‰). Recent studies on its genome have shown that European sea bass presents multiple copies of genes involved in the establishment of the euryhaline tract, including but not limited to genes involved in osmoregulation (Vandeputte et al., 2019). Different conditions in terms of temperature and salinity are, in fact, experienced in different stages of its life cycle. The migration pattern involves moving from costal lagoons and estuaries in the first stage of life up to one year and a half, to offshore deep water during the reproductive stage which occurs at two years of age for male and three-four for female, when they weigh at least 300-400 and 500-600 g respectively (Ortega, 2013). In specific, European sea bass can be defined as gonochoric synchronous for which the reproduction takes place from December to March in the Mediterranean, and March to June in the Atlantic, and the postlarvae will return to the coastal lagoon and estuaries during spring, where they can find good food availability (Vandeputte et al., 2019). Specifically, females arrive to produce more than 300’000 eggs/kg (1.1-1.5 mm each, approximately) with high dispersive capacity, but there is no investment in parental care (Ortega, 2013). Concerning its feeding behavior, European sea bass can be considered an opportunistic predator whose prey depends on the size of the organism; for this reason, in the first stage of like, it directs his attention to copepods or isopods to prefer crabs and small fish in adult life (Vandeputte et al., 2019).

The taxonomic hierarchy of European sea bass is the following:

Phylum: Chordata
Class: Actinopterygii
Order: Moroniformes
Family: Moronidae
Genus: Dicentrarchus
Species: *D. labrax*
(Linneaus 1758)

The European sea bass is the most farmed species in Mediterranean aquaculture, whose production was estimated at 301.420 Tn in 2022) with the expectation of an increase in the next years (APROMAR 2023). In the Mediterranean countries, it is mostly farmed in intensive and semi-intensive aquaculture systems with the aims to reach the commercial size usually higher than 250 g (APROMAR 2023). In addition to the diseases that can reduce production, one common problem in farming sea bass is the ratio male:female, favorable to males, which grow slower than females. The reason has to be researched in the high temperature applied during the larvae stage, which shifts the ratio to the male since European sea bass sex is also determined by environmental factors (Vandeputte and Piferrer, 2018). One of the main challenges in modern aquaculture is the replacement of fish source (fish meal and fish oil) with plant-based material to produce fish fed. The substitution is not always tolerated by carnivorous species like the European sea bass, but even though the domestication of this fish is recent, it has been proposed as good candidate for breeding selection to ameliorate tolerance of plant-based substitute (Montero et al., 2023).

1.5.2 Zebrafish *Danio rerio* (Hamilton, 1822)

Zebrafish (*Danio rerio*) is a freshwater shoaling species of south Asia, that can be found in stagnant water, canals, ponds, or moderate flowing water. Its distribution ranges from India, Sri Lanka, Pakistan, Bangladesh, and Myanmar, with a climate characterized by monsoons, the season of which is also preferred for reproduction (Spence et al., 2008). Zebrafish diet in the wild is based prevalently on zooplankton, insect or phytoplankton (Spence et al., 2008), and it can tolerate a wide range of temperatures which spans from 6 to 33°C in the wild (Spence et al., 2007). Even though zebrafish is described as a diurnal species, manipulation of photoperiod, temperature and feeding time in laboratory conditions can promote a change in the pattern of activity (López-Olmeda and Sánchez-Vázquez, 2009; López-Olmeda et al., 2010). The growth is usually faster during the first three months, and the maximum in size is reached around 18 months. Size is also a crucial factor in reproductive maturity. In specific, under laboratory conditions, the maturity is reached around 2/3 months with a size of approximately 2.5 cm, and the mating behavior is strongly influenced by the photoperiod, starting just after illumination and in the first hours of the afternoon (Darrow and Harris, 2004, Spence et al., 2007). Once the eggs are fertilized, the larva stage is reached in 72 hours, when the mouth is open (Kimmel et al., 1995), while the active feeding behavior starts 2-3 days after hatching (Kimmel et al., 1995). The taxonomic hierarchy of Zebrafish is the following:

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: Danio

Species: *D. rerio* (Hamilton 1882)

Regarding research purposes, zebrafish has several advantages compared to other vertebrate models, both for biomedical (Choi et al., 2021) and aquaculture research (Ribas and Piferrer, 2014). Specifically, it can be considered a good model for aquaculture research for its short life cycle, it possesses the same biological features of cultured species and many transgenic lines have been created to for many different research topics. For these reasons, it has been utilized to study reproduction, nutrition, pathology, toxicology, and stress (Ribas and Piferrer, 2014), which represent one of the main concerns in aquaculture.

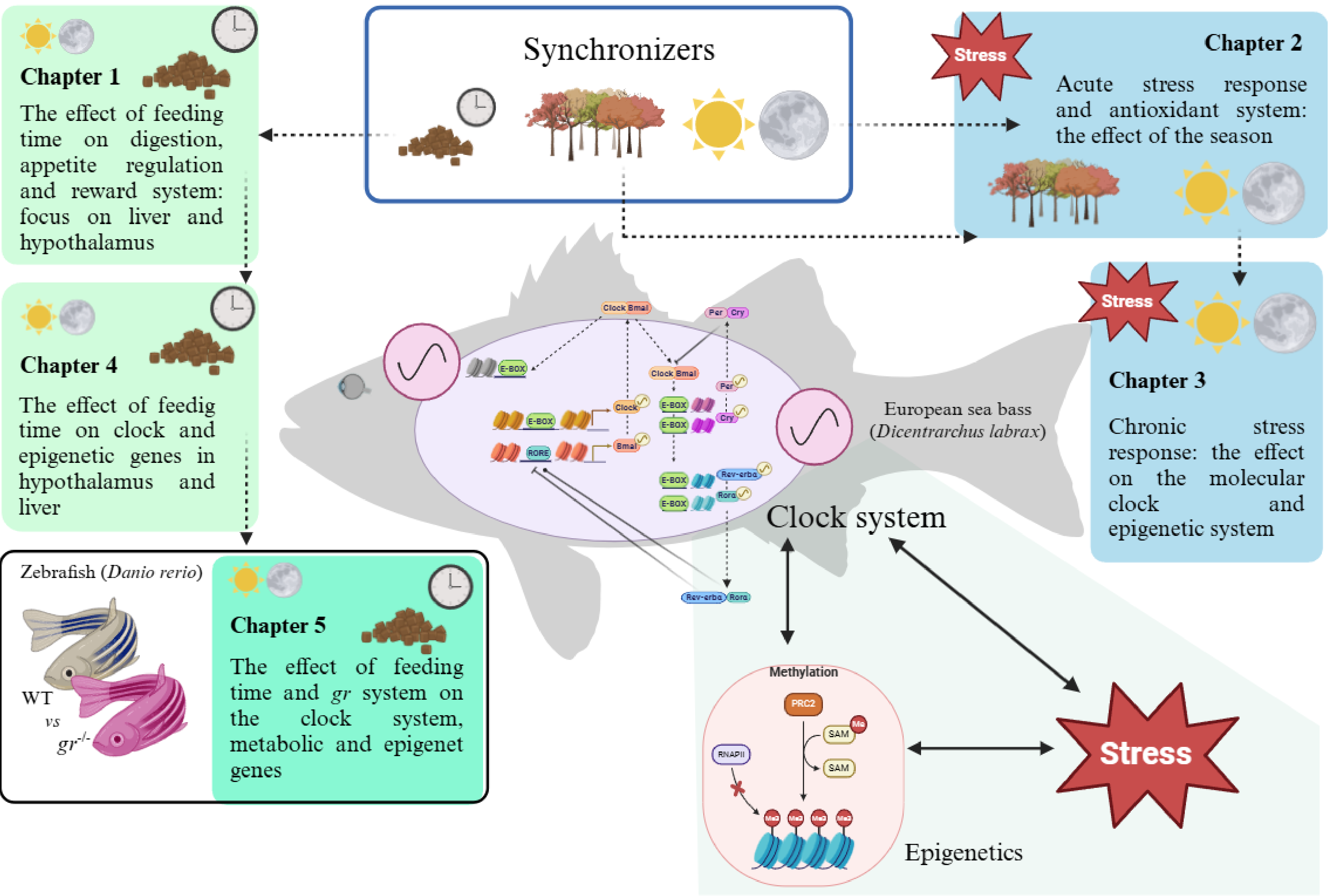
Objectives

Objectives

The main objective of this Doctoral Thesis was to evaluate the influence of feeding time and the LD cycles on the daily rhythms and the relationships between the molecular clock, the epigenetic mechanisms and physiological processes related to stress and welfare in fish, using the European sea bass as the main experimental model.

For this purpose, the following specific objectives were designed:

1. Investigate the effect of feeding time on the daily rhythms of factors involved in digestion and metabolic factors, appetite regulation and the reward system in the European sea bass.
2. Explore the effect of the time of the day and the season of the year on the physiological response to an acute stress event in the European sea bass, with a specific focus on the HPI axis, antioxidant system and mitochondrial markers.
3. Determine how the molecular clock and the epigenetic system respond to a chronic stress depending on the time of the day, and how this response changes during time in the European sea bass.
4. Elucidate how feeding time affects the rhythms of clock genes in the brain and liver and genes from the epigenetic mechanisms in the liver of the European sea bass.
5. Investigate the effect of feeding time and the glucocorticoid receptor on the daily rhythms of clock, metabolic and epigenetic genes in the brain of wildtype zebrafish and *gr* $^{-/-}$ mutants.


Synchronizers
Chapter 1
The effect of feeding time on digestion, appetite regulation and reward system: focus on liver and hypothalamus
Chapter 2
Acute stress response and antioxidant system: the effect of the season
Stress
Chapter 3
Chronic stress response: the effect on the molecular clock and epigenetic system
Chapter 4
The effect of feedig time on clock and epigenetic genes in hypothalamus and liver
Zebrafish (Danio rerio)
WT
vs
gr-/-
Chapter 5
The effect of feeding time and gr system on the clock system, metabolic and epigenet genes
European sea bass
(Dicentrarchus labrax)
Clock Bmal
Per
Cry
E-BOX
RORE
Clock
Bmal
Rev-erba
Rora
Clock system
Methylation
PRC2
SAM
Me
RNAPII
Epigenetics
Stress

Experimental chapters

Experimental chapter I

Feeding time modulates the daily rhythms of digestive enzymes in the liver and food intake regulation and reward systems in the hypothalamus of the European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

Fish exhibit daily rhythms at the molecular level across different tissues, synchronized by several *zeitgebers* such as food availability. As food is hardly constant in the environment, organisms must align their internal timekeeping systems to optimize the benefits of feeding. Previous studies on intermediary metabolism and the hypothalamic control of food intake in fish like the European sea bass (*Dicentrarchus labrax*) have underscored the significance of feeding time and daily rhythms. In the present study, we investigated the impact of two different feeding times (mid-light, *ML,* vs mid-dark, *MD*) on the rhythmic transcription of digestive enzymes in the liver and food intake regulation factors in the hypothalamus of European sea bass, as well as the connection between food intake control and the reward system. Our results revealed that, when fish were fed at ML, all genes analyzed involved in protein digestion (*tryp2, tryp3*, *ctrl* and *cpa5*) displayed daily rhythms with a peak at the beginning of the dark phase (between ZT 11.28 and 13.60), showing a delay of the acrophases when fish are fed during the night (between ZT 16.95 and 18.45). Two genes involved in transamination (*c-alt* and *m-alt*) exhibited rhythms only in ML-fed fish, with acrophases occurring during the light phase, at ZT 5.16 and 5.3, respectively. Similarly, the gene involved in lipid metabolism (*pla2*) presented a rhythm only in ML-fed fish with the acrophase located at ZT 13.96. The orexigenic genes analyzed in the brain (*npy* and *orexin*) presented daily rhythms only in the MD group, with nocturnal acrophases at ZT 13.15 and 16.1, respectively. On the contrary, the genes involved in the reward system (*th* and *bdnf*) presented daily rhythms when fish were fed during the light phase (ZT 17.93 and 11.76, respectively), with only *th* maintaining its rhythm in the MD group (ZT 15.50). These findings suggest that most of these factors that participate in digestive processes display rhythms that are significantly affected by

feeding time. Additionally, we illustrate diverse synchronization patterns to feeding time in the hypothalamus, highlighting the intricate nature of the food regulation systems.

Keywords: intermediary metabolism, digestive enzymes, hypothalamus, TH

INTRODUCTION

In the natural environment, food is not constantly available, presenting oscillations in its availability that make it a robust synchronizing factor (or *zeitgeber*) for behavior and many physiological processes (López-Olmeda 2017). The presence of a cyclic pattern in environmental cues, like light and dark cycles, temperature, and food availability itself, indeed represented a key factor for the fine-tuning of the circadian system (López-Olmeda 2017, Sánchez-Vázquez et al., 2019, Vera et al., 2023), whose ultimate benefit is the synchronization to an environment based on a 24-hour cycle. This synchronization between the organism and the environment can also be seen at the molecular level with the appearance of rhythms in mRNA expression and proteins (Pando and Sassone-Corsi 2002). Rhythms at the molecular level are mainly driven by the *clock genes,* which integrate the external stimuli in a complex system based on positive and negative feedback, regulating in a cascade a set of targets identified in several physiological pathways, among them many related to feeding and digestion (Pando and Sassone-Corsi 2002, Cowan et al., 2017). In specific, in fish, daily rhythmic variations in these processes have been reported for protein digestion (Montoya et al., 2010, López-Olmeda et al., 2012, Mata-Sotres et al., 2016, Guerra-Santos et al., 2017, Lazado et al., 2017, de Oliveira et al., 2022), carbohydrate metabolism (Montoya et al., 2010, Hernández-Pérez et a. 2015) and lipid metabolism (Gómez-Milán et al., 2007, Betancor et al., 2014, Paredes et al., 2014 and 2015, Hernández-Pérez et al., 2015, Mata-Sotres et al., 2016).

The European sea bass (*Dicentrarchus labrax*) is a carnivorous fish species in which protein metabolism and regulation are essential to obtain energy and for many physiological functions such as growth (Nejedly and Gajger 2013). In the process of protein digestion, proteases are produced in the exocrine pancreatic islets of the liver and are involved in the first steps, which allow protein breakdown by the action of

endopeptidases like trypsin and chymotrypsin, and exopeptidases like carboxypeptidase (Moraes and de Almeida, 2020). Many studies have been conducted on these proteolytic enzymes in different fish species, including the European sea bass (Kamaci et al., 2010), but only a few of them have focused on their daily rhythms (Mata-Sotres et al., 2016; Espirito Santo et al., 2020; de Oliveira et al., 2022). In addition, the liver is also the main site where transamination occurs, with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) being two of the essential enzymes for transamination reactions in fish. Indeed, these enzymes are also important in gluconeogenesis as their products may be used in this pathway (Walton and Cowey 1982). In the catfish *Lophiosilurus alexandri*, ALT displays variations throughout the day with a peak of activity during the night, although no significant daily rhythm was detected (Fortes-Silva et al., 2019). Moreover, seasonal variations have been described in the activity of ALT and in another essential enzyme, pyruvate kinase (PK), pointing to an important role of photoperiod in the modulation of the intermediary metabolism (Gómez-Milán et al., 2007bis). PK activity presents 24-hours variations depending on the feeding protocol in liver of rainbow trout (*Oncorhynchus mykiss*) (Polakof et al., 2007). Besides, in the liver is also the most important site for lipid metabolism in fish. The existence of daily rhythms in the mRNA expression of many factors involved in lipid metabolism, both lipolytic and lipogenic, has been described in the liver of several fish species (Paredes et al., 2014; Betancor et al., 2014; Paredes et al., 2015; Betancor et al., 2020; Rodriguez et al., 2021). Among the enzymes involved in lipid metabolism in the liver, phospholipase A2 (PLA2) is one of the most important as it is involved in the liberation of arachidonic acid (AA) however, no daily rhythmic changes have been reported for the European sea bass to date.

The regulation of food intake is a complex mechanism where the hypothalamus plays an essential role by combining signals from the environment and the rest of the

organism, leading to the release of orexigenic or anorexigenic factors that contribute to regulating the food intake system (Volkoff et al., 2005). Among the orexigenic factors, neuropeptide Y (*NPY*) represents one of the most important in fish (Volkoff et al., 2005; Opazo et al., 2019), including the European sea bass (Cerdá-Reverter et al., 2000). NPY function is supported by the interaction with other peptides (Volkoff and Peter, 2001) like orexin (or hypocretin), a neuropeptide identified in different brain regions, including the hypothalamus (Amiya et al., 2007). Fasting can upregulate *orexin* mRNA levels in different species (Volkoff et al., 2005; Yokobori et al., 2011; Wall and Volkoff 2013), and orexin control on food intake is also due to its endocrine action since orexin receptors are localized in the mucosa of the gastroenteric tract (D'Angelo et al., 2016). Besides the role of the hypothalamus as the main regulator of energy balance and food intake (Volkoff et al., 2005), recently a connection with the reward system has been suggested (Soengas et al., 2018). During feeding time, the homeostatic and hedonic system are simultaneously active, being even anatomically overlapped (Rossi and Stuber, 2017). One of the most important compounds of the hedonic system is dopamine (3,4-dihydroxyphenethylamine), a neurotransmitter whose biosynthesis starts with the conversion of tyrosine into L-DOPA (L-Dihydroxyphenylalanine) by the rate-limiting enzyme tyrosine hydroxylase (TH) (Daubner et al. 2011). One study on the European sea bass that involved oral administration of L-DOPA showed that this compound modifies food intake, leading to a decrease in specific growth rates and a reduction of mesenteric and hepatic fats (Leal et al., 2013). Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the catecholamine synthesis pathway and TH neurons have been described in different brain areas of teleost, including the hypothalamus (Vetillard et al., 2002). Besides the many stimuli that can modulate TH (Kumer and Vrana, 1996), brain-derived neurotrophic factor (BDNF) is involved in the modulation of *th* transcription in mammals (Fukuki et al., 2010). In mammals, BDNF is a member of the neurotrophins family of growth factors

whose main function is related to nervous system development and neuronal plasticity (Brigadski & Leßmann, 2020), but it is also involved in the control of hedonic feeding by affecting the mesolimbic dopaminergic system (Cordeira et al., 2010). Regarding the relationship between the hedonic and circadian systems in fish, only one study conducted on catfish (*Heteropneustes fossilis*) focused on the rhythm of the enzymatic activity of TH in different brain regions, revealing differences between sexes and the season (Chaube and Joy 2003). In addition, the connection between BDNF and the circadian system may be complex and affect many processes, since *bdnf* knockout zebrafish larvae present a different pattern of daily rhythms of activity and modifications in the expression of some clock genes (D'Agostino et al., 2022).

In aquaculture, feeding is one of the most important processes, and feed is the highest production cost in a fish farm. Inadequate feeding protocols and schedules may have a significant impact on fish production. Therefore, understanding feeding physiology and food utilization is crucial for optimizing fish food consumption. The European sea bass (*Dicentrarchus labrax*) is a key species in Mediterranean aquaculture. Some features of its metabolic system have been studied (Kousoulaki et al., 2015; Viegas et al., 2014) but, to date, studies focused on the relationship between the circadian system and digestion and metabolic processes of the European sea bass are still scarce. Therefore, the main objective of the present research was to study the existence of daily rhythms in factors involved in digestion, metabolism, food intake control and the hedonic system in the European sea bass and the importance of feeding time as synchronizer for these rhythms.

MATERIALS AND METHODS

The experiment was performed at the Aquaculture Lab of the University of Murcia (Naval Base of Algameca, E.N.A., Cartagena, Spain) following the European Union guidelines (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use laboratory animals. The experimental procedures were approved by the Committee of the University of Murcia on Ethics and Animal Welfare and the Government of *Región de Murcia* (license number A13191003).

Animal housing

The European sea bass specimens (N = 98, 47.3 ± 0.59 g body weight, mean ± SEM) were obtained from a local fish farm, CULMAREX (Guardamar del Segura, Alicante, Spain), and maintained in groups of 50 fish in 500-L tanks in a water flow-through system equipped with mechanical and biological filters. The water was sterilized with a UV lamp before entering the system and each tank was equipped with aeration. A timer connected to the lights (Data Micro, Orbis, Madrid, Spain) was used to set a light-dark cycle that replicated the natural photoperiod variations, with a light intensity on the water surface of 200 lx. In order to standardize the time points, avoid using of the local time of the day, in chronobiological studies the time of the light onset is set as ZT (*zeitgeber* or synchronizer time) 0h (ZT 0h) (Espirito Santo et al., 2020). Water temperature was monitored and recorded by a data logging device (HOBO PENDANT Onset Computer Corporation, Massachusetts, USA). For all the acclimation period fish were fed *ad libitum* with a commercial diet (Alterna Marine, Skretting, Burgos, Spain).

Experiment design

Fish (N=98) were divided into 14 tanks (7 fish/tank) of 150 L each. Tanks were randomly assigned to two groups (7 tanks/group) fed at different feeding times: ML (fish fed at the middle of the light phase – ZT 4.5) and MD (fed at the middle of the dark phase – ZT 16.5). The daily feed ratio provided was set at 1% of the biomass of the tank (D2 Optibream 2P, Skettring, Burgos, Spain). Feeding was administered using automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany) and the daily feed amount was divided in two rations of 0.5% each, provided 30 minutes before and after ML or MD, depending on the group. This procedure allowed to maximize food consumption and minimize waste. The photoperiod for the experiment was set at 10:14 replicating the natural environmental condition of December. After 30 days, fish were sampled during a 24 h cycle at the following sampling points: ZT 0.5, 4, 7.5, 12, 16, 20 and 24.5 h. Clove oil essence (Guinama, Valencia, Spain) previously diluted in 9 parts of ethanol was used as anesthetic at a final concentration of 50 µL/L. To minimize stress due to the repetition of the sampling, one single tank was used for each sampling point. After anesthesia, fish were sacrificed by decapitation to collect hypothalamus and liver, which were immediately frozen in dry ice and stored at -80°C until analysis. During the nocturnal sampling points (ZT 12, 16 and 20 h), a dim red light with a wavelength of 600 nm was used to avoid light contamination (de Alba et al., 2019).

Rna extraction, cDNA synthesis and Real-Time RT-PCR analysis

RNA was extracted from the collected liver and hypothalamus tissues using Trizol (Invitrogen, Thermo Fisher Scientific, Baltics UAB), following the manufacturer's instructions. RNA concentration and purity were then measured by means of spectrometry (Nanodrop® ND 1000, Thermo Fisher Scientific). Then, a DNase (Thermo Fisher) was

added to prevent genomic contamination (1U for 1 μg of RNA), and a Reverse Transcriptase commercial kit (QSCRIPT cDNA Synthesis Kit, Quantabio Beverly, USA) and a thermocycler were used to synthetize cDNA, which was diluted in a 1:10 proportion with nuclease-free water (Thermo Fisher Scientific). cDNA was then stored at -20°C until analysis. Later, gene expression levels were measured in the cDNA samples by means of quantitative PCR (qPCR) analysis, run by a thermocycler (7500 RT-PCR system, Applied Biosystem, Foster City, USA) following the next steps: 15 min at 95°C, 40 cycles of 15 s each at 95°C and 1 min at 60°C. Melting curves were run at the end of all qPCR reactions. All the samples had a final volume of 20 μl and Perfecta SYBER Green Fastmin (Quantabio) was used as master mix. The genes analyzed in the liver samples were as follows: *trypsinogen 2* and *3* (*tryp2* and *tryp3*), *chymotrypsin A-like* (*ctrl*), *carboxypeptidase A5* (*cpa5*), *cytosolic*- and *mitochrondrial-alanine aminotransferase* (*c-alt* and *m-alt*), *aspartate aminotransferase* (*ast*), *pyruvate kinase* (*pk*) and *phospholipase A2* (*pla2*). The genes analyzed in the brain samples were: *neuropeptide Y* (*npy*), *orexin*, *tyrosine hydroxylase* (*th*) and *brain-derived neurotrophic factor* (*bdnf*). The primers for liver samples had a final concentration of 400 nM, with the only exception of *ast,* which was added at a concentration of 200 nM, while primers used for hypothalamus samples had a final concentration of 200 nM. Primers were either designed using *Primer3plus* (Untergasser et al., 2012) or retrieved from literature as indicated in Table 1. Primer's efficiencies were evaluated by means of cDNA dilution curves and the 2-ΔΔCt method was used for the analysis, with the geometric mean of the reference's genes values (*ef1a* and *bactin*) for the first normalization, and the sample with the lowest expression value for the second (Livak and Schmittgen 2001).

Table 1. Genes analyzed and primers used for qPCR analyses.

Gene	FW	RV	Acc. Number / Reference
ef1a	AGTGAAGCAGCTCATCGTTG	TTGGTGATTTCCTCGAAGCG	AJ866727
bactin	TCATCACCATCGGCAATGAG	AACGTCGCACTTCATGATGC	AY148350
tryp2	TGCTGGCAAAACCCCCTTTTC	GGCATCCACTGACATAAACTGC	XM_051405811.1
tryp3	CTTGGTGAGCACAACATTGC	ATGTCATTGTCCAGGTTGCG	XM_051405637.1
ctrl	TGGCTTCACCATCAACAACG	AGACACGCATGTTCATCTGG	XM_051378204.1
cpa5	GCACCTGGTTTGCCAAAAAG	ACATACGGTTGCTGTTGTGG	XM_051382201.1
c-alt	TGAAGGAGGGGGTCAAGAAA	AGGGTAAGAACACAGAGCCA	Viegas et al., 2014
m-alt	GCAGCCAATCACTTTCTTCCG	AATGCGGCGTGCTCTACTTTT	Viegas et al., 2014
ast	GTCCAACCCACCATCTCAGG	TCTGTGCGTCTTTTTGTGCG	XM_051426694.1
pk	CAAGGTGGAAAGCCGGCAAGGC	GGTCACCCCTGGCAACCATCA	Viegas et al., 2014
pla2	ACATGTGCTGCAAGGTTCAC	ATTGTTGGAGGCTGAACAGG	XM_051423157.1
npy	TTGTGGTGATGCTGTTACCC	TCATTTGGTGTGGGAGGGTATG	XM_051391215.1
orexin	AAGTCCTGGTGCTGGTTTTG	AGCGACACAGCAACACATAG	XM_051387394.1
th	ATGTCGGAAGCTCAAGGAGA	CTTTTTGGGAACCAGTGGAA	Mazurais et al. 2020
bdnf	GCTCAGCGTGTGTGACAGTA	ACAGGGACCTTTTCCATGAC	Cerqueira et al. 2020

Data analysis

The presence of a significant daily rhythm was evaluated in all variables by means of Cosinor analysis using the chronobiology software El Temps (v. 313, Prof. Díez-Noguera, University of Barcelona, Spain). This analysis defines the statistical significance of the rhythm since an F test of the variance is described for the waveform versus a straight line of zero amplitude (null hypothesis). Cosinor is based on the least-square approximation of time-series data with a cosine function of the known period of the type (Refinetti et al., 2007, Portaluppi et al., 2008):

$$Y=M+A * (COS (\Omega t + \varphi))$$

(M = mesor, A = amplitude, Ω = angular frequency with 360°/24 h for circadian rhythm), φ = acrophases)

To check for the statistically significant differences between groups and ZT points, data were first tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test), followed by a two-way ANOVA with Tukey *post hoc* test, using the

software SPSS (v. 28.0.1.1, IBM, Armonk, USA). The significance threshold was set at $\alpha = 0.05$ and all the results are expressed as mean ± SEM. The graphical representation of the data was carried out with GraphPad Prism 8.0.1.

RESULTS

Daily rhythms on digestive and metabolic enzymes in the liver.

The mRNA expression of the factors analyzed involved in protein digestion (*tryp2*, *tryp3*, *ctrl* and *cpa5*) displayed a similar pattern in the ML group, showing significant daily rhythms with the acrophases located in the first hours of darkness (Fig. 1) at ZT 12.10 h, ZT 12.15 h, ZT 11.28 h and ZT 13.60 h (Cosinor, $p<0.05$) (Suppl. Table 1). In the MD group, significant daily rhythms were observed in *tryp2*, *ctrl* and *cpa5*, with the acrophases located shortly after mid-dark, at ZT 18.45 h, ZT 18.16 h and ZT 16.95 h (Fig. 1) (Cosinor, $p<0.05$) (Suppl. Table 1). The mRNA expression of *tryp3* and *ctrl* showed differences between time points in the ML-fed fish (Fig. 1B-C) (two-way ANOVA, $p<0.05$). In addition, comparing ML and MD feeding, all the genes presented significant differences between the two groups (two-way ANOVA, $p<0.05$), mostly located at the end of the dark phase. In specific, *tryp3*, *ctrl* and *cpa5* presented these differences at ZT 20 h and ZT 24.5 h (Fig. 1B-D), while *tryp2* only did it at ZT 20 h (Fig. 1A).

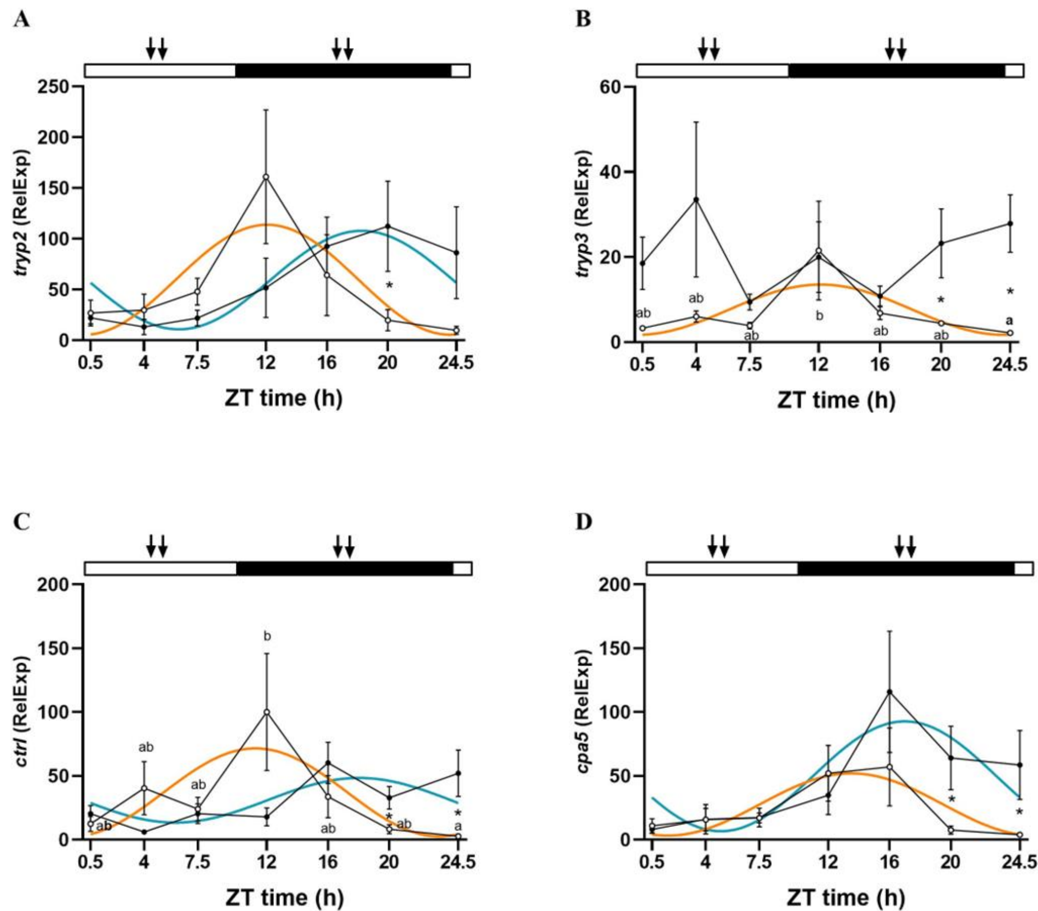

Fig. 1. Daily variations in the relative mRNA levels (fold change) of *tryp2* (A), *tryp3* (B), *ctrl* (C) and *cpa5* (D) in the liver of European sea bass. Fish were maintained under 10:14 LD lighting conditions and natural water temperature variations. Fish were divided into two groups fed at different times: in the middle of the light phase (ML, white dots ○) or in the middle of the dark phase (MD, black dots ●). Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. Orange and blue lines represent the adjustment to a sinusoidal rhythm when Cosinor analysis was significant ($p<0.05$), for ML and MD groups, respectively. White and black bars at the top of each panel represent the light and dark phases, respectively, of the LD cycle. Arrows indicate feeding times for each group.

Regarding the factors involved in metabolism in the liver, two of the genes involved in transamination, *c-alt* and *m-alt*, presented daily rhythmicity only in the ML group (Cosinor, $p<0.05$), with the acrophases occurring around the middle of the light phase, at ZT 5.15 h (Fig. 2A) and 5.30 h (Fig. 2B) (Suppl. Table 1). On the other hand, neither *ast* in both groups nor *c-alt* and *m-alt* expression in MD-fed fish displayed

significant rhythms (Cosinor, $p>0.05$) (Fig. 2A-C) (Suppl. Table 1). In addition, *c-alt* and *m-alt* expression in the ML group and *c-alt* and *ast* in MD fish showed significant differences between time points throughout the day (two-way ANOVA, $p<0.05$). In the case of both *alt* genes, the highest values were observed at ZT 4 h, whereas *ast* in the MD group showed higher values at ZT 16 h (Fig. 2A-C). Moreover, feeding time significantly affected the expression of the three transaminases analyzed as revealed by the two-way ANOVA analysis ($p<0.05$), and significant differences between ML and MD groups were described for all of them at ZT 20 h (Fig. 2A-C). Regarding the other genes involved in metabolism and analyzed in liver, *pk* exhibited a significant daily rhythm only in the ML group (Cosinor, $p<0.05$) with a diurnal acrophase located at ZT 6.26 h (Fig. 2D) (Suppl. Table 1). Similarly, the lipolytic enzyme *pla2* showed daily rhythmicity only in the ML fed fish, but in this case displaying a nocturnal acrophase located at ZT 13.96 h (Fig. 2E) (Suppl. Table 1). On the other hand, the two-way ANOVA analysis revealed a significant influence of the feeding time, sampling time, and the interaction between the two factors ($p<0.05$). Significant differences between time points were reported for *pk* in both groups, with higher values during the light phase (Fig. 2D), and for *pla2* in the ML group, with the highest values at ZT 16 h (Fig. 2E) (two-way ANOVA, $p<0.05$). Additionally, significant differences between ML and MD groups were identified at ZT 7.5 and 12 h for *pk* expression (Fig. 2D) and at ZT 12 h for *pla2* expression (Fig. 2E) (two-way ANOVA, $p<0.05$).

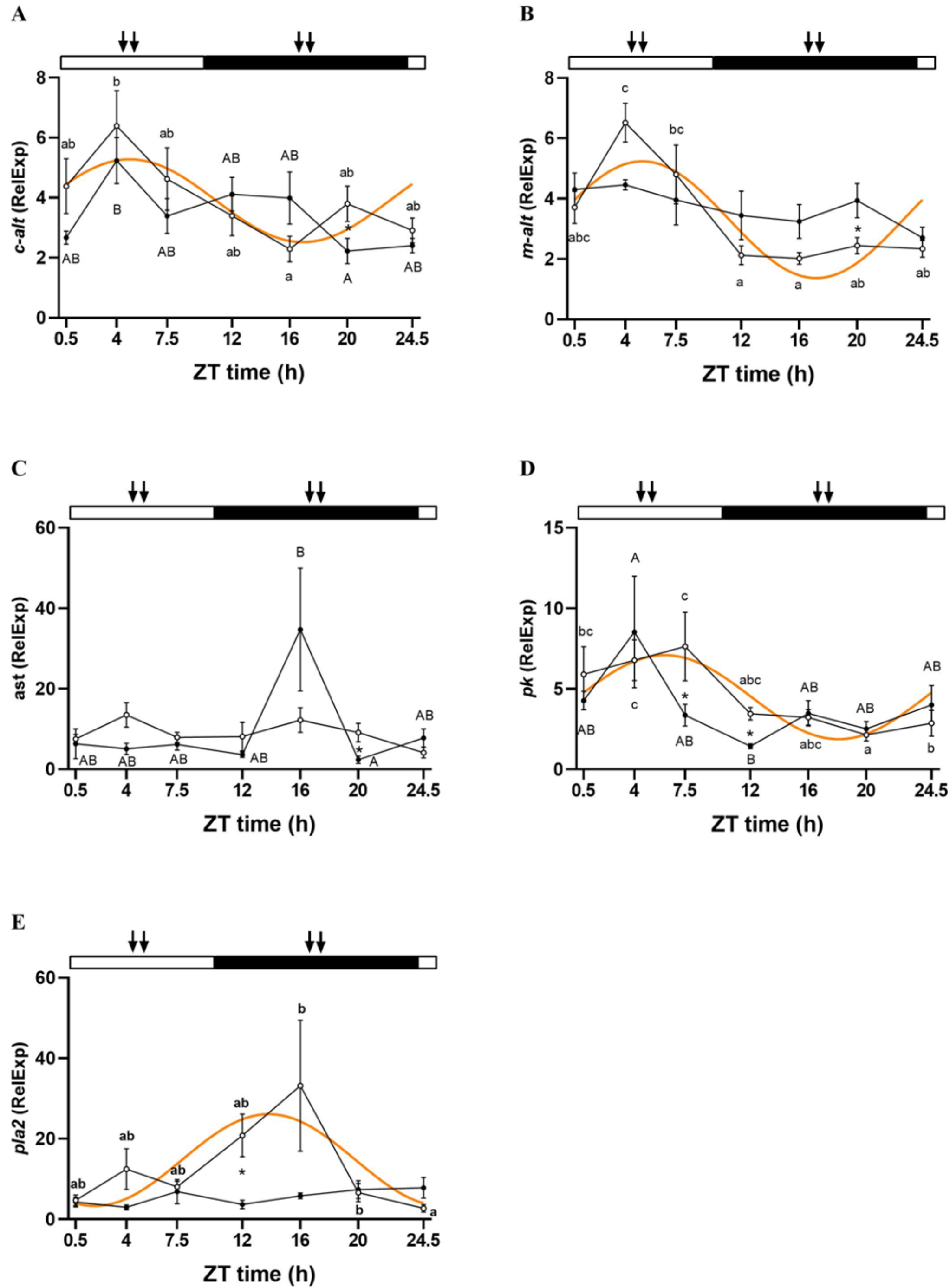

Fig. 2. Daily variations in the relative mRNA levels (fold change) of *c-alt* (A), *m-alt* (B), *ast* (C), *pk* (D) and *pla2* (E) in the liver of European sea bass. Fish were maintained under 10:14 LD lighting conditions and natural water temperature variations. Fish were divided into two groups fed at different times: in the middle of the light phase (ML, white dots ○) or in the middle of the dark phase (MD, black dots ●). Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. Orange and blue lines represent the adjustment to a sinusoidal rhythm when Cosinor analysis was significant ($p<0.05$), for ML and MD groups, respectively. White and black bars at the top of each panel represent the light and dark phases, respectively, of the LD cycle. Arrows indicate feeding times for each group.

Daily rhythms on food intake regulation factors and the reward system in the hypothalamus.

Two factors involved in food intake regulation, *npy* and *orexin*, were analyzed in the hypothalamus. These factors displayed significant daily rhythms only in MD-fed fish while no rhythm was reported for the ML group (Fig. 3A-B) (Cosinor, $p<0.05$). When the rhythm was present, a nocturnal acrophase was observed, peaking at ZT 13.15 h and ZT 16.10 h for *npy* and *orexin*, respectively (Suppl. Table 1). A significant effect of feeding time and sampling points (ZT) were described for both *npy* and *orexin* (two-way ANOVA, $p<0.05$). The mRNA expression of both genes in both groups showed differences depending on the time of the day, with the highest values being observed in all cases during the dark phase of the LD cycle (Fig. 3A-B). In addition, *npy* expression was significantly higher in the ML group than MD at all time points except for ZT 0.5 and 7.5 h (Fig. 3A), while *orexin* expression was higher in the MD group compared to the ML at ZT 12 and 20 h (Fig. 3B) (two-way ANOVA, $p<0.05$).

Finally, two factors involved in the reward system, *th* and *bdnf*, were analyzed in the hypothalamus of the European sea bass from the current experiment. Regarding *th*, the relative mRNA expression of this gene presented significant rhythms with nocturnal acrophases both in the ML (ZT 17.93 h) and MD (ZT 15.50 h) groups (Cosinor, $p<0.05$) (Fig. 3C) (Suppl. Table 1). Additionally, for *bdnf* a significant rhythm was only reported for the ML group, with the acrophase located at the beginning of the dark phase (ZT 11.76 h) (Cosinor, $p<0.05$) (Fig. 3D) (Suppl. Table 1). The expression of *th* in the MD-fed fish and *bdnf* in the ML group presented differences throughout the 24 hours (two-way ANOVA, $p<0.05$) (Fig. 3C-D). In addition, statistically significant effects were observed for feeding time (two-way ANOVA, $p<0.05$). In specific, MD fish presented significantly higher values than ML in *th* expression at ZT 16 h (Fig. 3C) and *bdnf* at ZT 4 and 20 h (Fig. 3D).

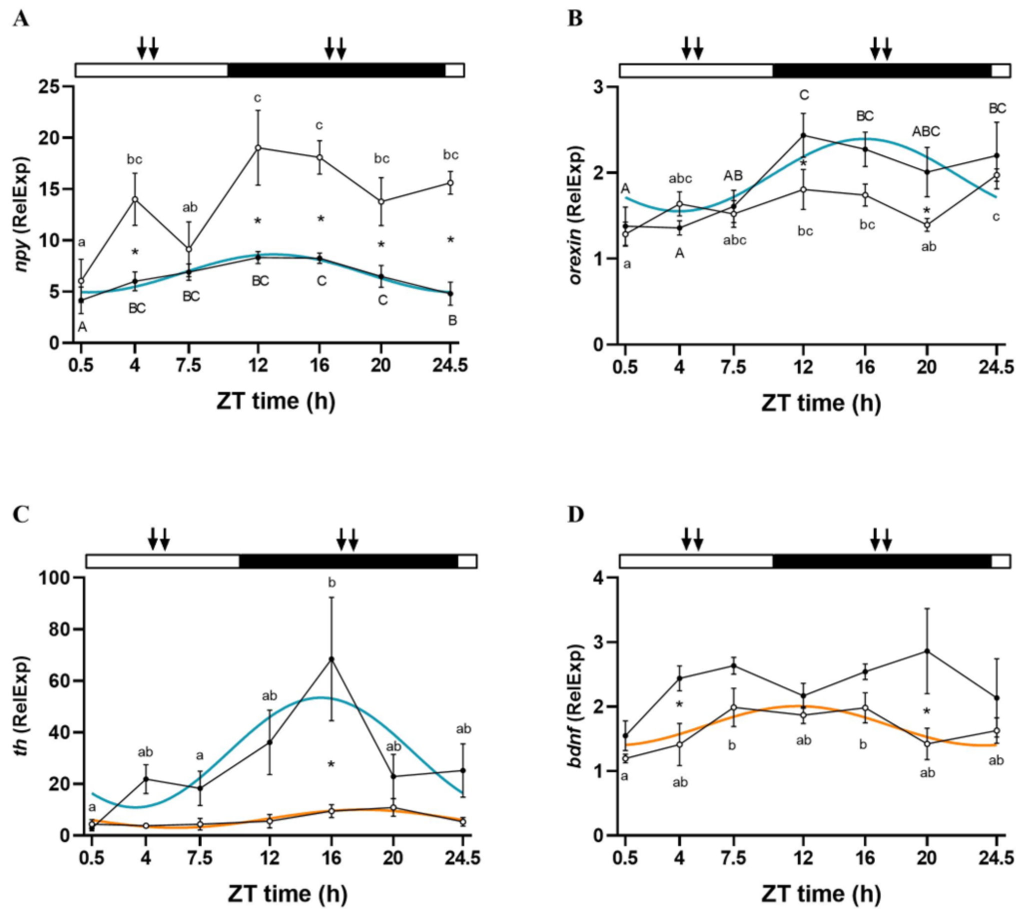

Fig. 3. Daily variations in the relative mRNA levels (fold change) of *npy* (A), *orexin* (B), *th* (C) and *bdnf* (D) in the hypothalamus of European sea bass. Fish were maintained under 10:14 LD lighting conditions and natural water temperature variations. Fish were divided into two groups fed at different times: in the middle of the light phase (ML, white dots ○) or in the middle of the dark phase (MD, black dots ●). Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. Orange and blue lines represent the adjustment to a sinusoidal rhythm when Cosinor analysis was significant ($p<0.05$), for ML and MD groups, respectively. White and black bars at the top of each panel represent the light and dark phases, respectively, of the LD cycle. Arrows indicate feeding times for each group.

DISCUSSION

In the present study, we aimed to describe the role of feeding time as a synchronizer in the peripheral (liver) and central (hypothalamus) oscillators of European sea bass, focusing on processes such digestion, regulation of food intake and the connection with the reward system. In general, feeding time played a crucial role not only in the modulation of the daily rhythms, but also influencing gene expression levels in both tissues. In the liver, most of the genes related to protein digestion presented rhythms in both ML and MD groups (*tryp3*, *ctrl* and *cpa5*). However, most genes involved in metabolism only exhibited daily rhythms when fish were fed during the light phase (*c-alt, m-alt, pk* and *pla2*) (Fig. 4), suggesting a different modulation of the two processes by the feeding time. In the hypothalamus, the genes involved in food intake control (*npy* and *orexin*) showed daily rhythms only when fish were fed during the dark phase. In contrast, regarding the reward system, *th* presented similar daily rhythms regardless feeding time whereas the rhythm for *bdnf* was observed only when fish were fed during the light phase (Fig. 4). Despite the differences, all of the genes analyzed in the hypothalamus peaked during the dark phase, suggesting a similar controlling mechanism.

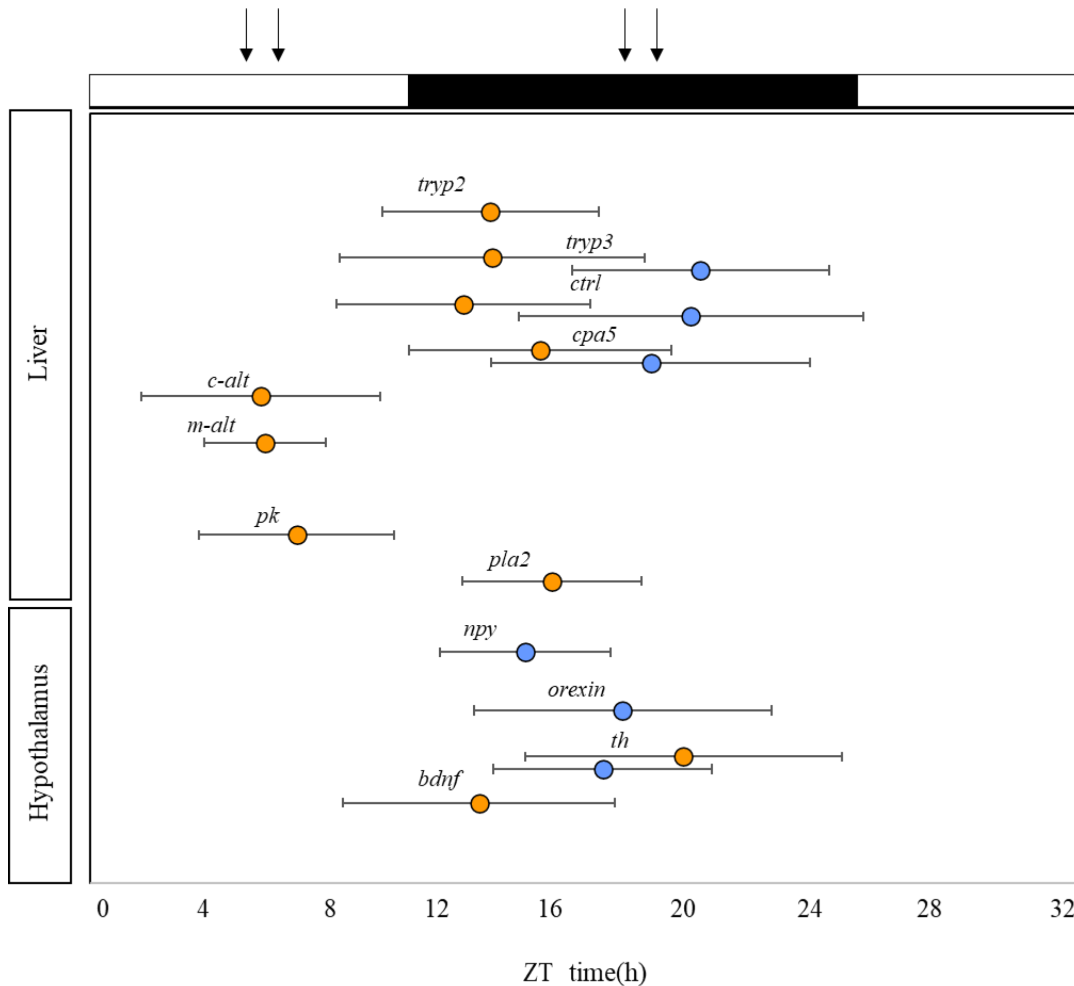

Fig. 4. Map of acrophases of genes involved in protein digestion (*tryp2*, *try3*, *ctrl*, *cpa5*), transamination (*c-alt*, *m-alt*, *ast*), glycolysis (*pk*), lipid metabolism (*pla2*), food intake regulation (*npy*, *orexin*) and reward system (*th*, *bdnf*). The achrophase is reported only when statistically significant rhythm was found by Cosinor analysis ($p<0.05$). The name of each gene is reported on the left. ML and MD groups are indicated by orange and blue circles, respectively. The x-axis represents the time scale (indicated with ZT time), where ZT 0 corresponds to light onset. White and black bars above the panel represent the light and dark phases (10:14 LD), respectively. The arrows indicate feeding times.

As a peripheral oscillator and due to its crucial role in food digestion and metabolism, liver represents one of the most susceptible organs to periodic food availability as a *zeitgeber* (López-Olmeda 2017). In the present study, all genes involved in protein digestion analyzed (*tryp2*, *tryp3*, *ctrl* and *cpa5*) presented a daily rhythm with nocturnal acrophases, occurring at the beginning of the dark phase, in sea bass fed during the light phase. In fish species, some digestive enzymes can be prepared during the night

phase, as reported for sea bream (Mata-Sotres et al., 2016) or white seabream (*Diplodus sargus*) (Yúfera et al., 2012). Consequentely, they can be used around feeding time as seen in the midgut of the European sea bass (del Pozo et al., 2012) or in the intestine of Nile tilapia (de Oliveira et al., 2022). In addition, as sea bass is a carnivorous species, a significant amount of energy is dedicated to protein digestion. Therefore, in this species, it may be beneficial to focus on enzyme preparation when the fish does not have to cope with diurnal challenges such as predation. On the other hand, fish fed during the dark phase also displayed rhythmic expression in all proteases except *tryp3*. Interestingly, the acrophases were delayed between 3-6 h compared to the fish fed at ML, but not by 12 h which was the difference between the two mealtimes. This finding is similar to the effect exerted by conflicting feeding times (ML vs. MD) in clock gene expression in the liver of other fish species (Vera et al., 2013; Costa et al., 2016) and suggests that feeding time is not the only factor that modulates this peripheral pacemaker, pointing to the LD cycle as another important *zeitgeber*.

In the fish hepatopancreas, besides the production of digestion enzymes and once amino acids from the diet are absorbed in the bloodstream, the transamination processes occur. Specifically, aspartate and alanine aminotransferases are some of the most important in fish (Cowey and Walton, 1989). In fish, the alanine aminotransferases respond to variations in dietary protein percentage (Pérez-Jiménez et al., 2007) but also to fasting and refeeding (Viegas et al., 2014), and its importance is also related to the supply of pyruvate to the TCA cycle (Cowey and Walton, 1989). In the current study, the expression of both alanine aminotransferases analyzed (*c-alt* and *m-alt*) presented a daily rhythm with diurnal acrophases (around ZT 5 h) in fish fed at ML. A daily rhythm with a similar acrophase was observed in *pk*, an essential enzyme for glycolysis (Knox et al., 1980). The fact that the expression of these metabolic enzymes peaks around around

feeding time could suggest some degree of synchronization and anticipation to feeding time, as observed in other liver enzymes and fish species like the goldfish or the gilthead seabream (Vera et al., 2007; Montoya et al., 2010). This anticipation would prepare the liver for the forthcoming metabolites, optimizing their processing. Additionally, *pla2* expression also showed a daily rhythm in fish fed at ML, but in this case the phase was located at night. *Pla2* nocturnal acrophases are similar to those described for Atlantic bluefin tuna (Betancor et al., 2020). Previous studies on the synchronization of genes involved in lipid metabolism in fish have shown that feeding time has little influence on their daily rhythms, which are mostly determined by the LD cycle (Paredes et al., 2014; Paredes et al., 2015). In contrast to ML fed fish, none of the genes involved in metabolism analyzed showed daily rhythms in sea bass fed at MD. This strong effect of feeding time could be related to the activity phase, as European sea bass used in the present study were mostly diurnal. For instance, our results revealed a diurnal peak in *alt* in sea bass fed at ML, whereas in a nocturnal catfish, *Lophiosilurus alexandri*, reported a nocturnal peak in ALT activity (Fortes-Silva et al., 2019). Therefore, feeding sea bass during the resting phase may have altered the rhythms in their metabolic enzymes to the point of suppressing them.

In fish, food intake is primarily regulated by the hypothalamus, which produces different signals given by hormones with orexigenic or anorexigenic properties. This study considered the expression of two orexigenic hormones that work together to promote feeding, *npy* and *orexin* (Volkoff et al., 2005). In our study, both *hormones* presented a daily rhythm only when fish were fed at the mid-dark phase, but no rhythm was found when fish were fed at mid-light. However, data obtained from the ML groups presented daily differences during the 24 hours with highest levels in *npy* and *orexin* expression also during the dark phase, as observed in the MD fed group.

Additionally, *npy* displayed a double peak, one just before the feeding time and another at the beginning of the dark phase, which align more closely with the acrophases of the MD group of the same gene. Specifically, the first peak corresponds with findings in other studies in goldfish, where *NPY* expression and protein levels were higher just before feeding time (Vera et al., 2007, Hoskins and Volkoff 2012). Importantly, the highest expression levels of *npy* and *orexin* observed were close to the one described for *th*. A similar coincidence of phase was observed in a previous study in the Mexican blind cavefish (*Astyanax fasciatus mexicanus*), which reported an increase in *orexin* expression 2 hours before the increase of *th* (Penney and Volkoff 2013). The connection between food intake and the reward system was previously reported in rats (Puskás et al., 2010). TH plays an crucial role in the reward system as it is crucial in the metabolic pathway of dopamine, which some studies have also highlighted as an important modulator of food intake in fish (Penney and Volkoff 2013, Wall and Volkoff 2013, Leal et al., 2013). Daily variations in TH in fish were previously reported in a study conducted in the catfish *Heteropneustes fossilis* (Chaube and Joy, 2003). In that study, the authors addressed for the first time how the activity of the enzyme pvaries in different parts of the brain, with seasonal and sex differences, describing acrophases located in the shift between light and dark period. However no other study on the daily rhythm of TH in fish brain are available. In our study, in contrast, the rhythm in *th* presented the acrophase in the middle of the dark phase, which could be species-specific. In addition, the phase was the same in both ML and MD conditions, suggesting that in the European sea bass the enzyme is prepared during the resting phase, possibly in anticipation the light period, and that its regulation is primarily governed by the LD cycle as feeding time had no effect. Besides, among other factors that can influence *th*, *BDNF* can exert its effect at the transcriptional level by inducing its transcription (Fukuki et al., 2010). Circadian variations of BDNF have been well documented in different brain regions of the rat (Bova et al., 1998, Liang et al., 1998,

Berchtold et al., 1999, Schaaf et al., 2000). In the ML-fed sea bass, *bdnf* displayed a daily rhythm with its acrophase preceding *th by around 6 hours*, suggesting a possible influence of *bdnf* on *th* transcription levels, as observed in rats (Fukuki et al., 2010).Finally, the fact that the rhythm disappears in fish fed during the MD phase implies a strong effect of feeding time on *bdnf* transcription in hypothalamus, as previously proposed for zebrafish (Blanco et al., 2020).

When comparing the rhythms and acrophases of digestive processes with those of food intake regulation and reward systems, it is evident that genes involved in different pathways in the liver do not always match the peaks observed in the hypothalamus. In fish, even before food intake itself, a series of physiological events of preparation occur (Vera et al., 2007, Montoya et al., 2010, Yúfera et al., 2012, Lazado et al., 2007). Such events are usually associated with an enhanced activity few hours before feeding, known as food anticipatory activity (Sánchez-Vázquez et al., 1995, Azzaydi et al., 2009). Additionally, the food intake network is orchestrated and directed by the hypothalamus in an organization that precedes feeding itself (Volkoff et al., 2005), followed by digestion. All these steps are synchronized to various *zeitgebers* and, although they are temporally separated and chronologically subsequent, the whole mechanism is timed to help the organism to maximize the benefit of feeding (López-Olmeda 2017). For this reason, different acrophases are expected in genes involved not only in different pathways but also located in different organs that take part on feed regulation and digestion. In conclusion, in the present study, feeding time significantly influenced the daily rhythms in all the physiological processes analyzed in the European sea bass, which also displayed different peak activity times. This study emphasizes the complex synchronization of molecular rhythms to feeding times across different tissues, highlighting the intricate nature of food regulation systems in fish. Moreover, our study underscores the importance

of considering feeding time for fish under culture conditions as one essential factor that can affects the whole physiology of the animal.

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AUTHOR CONTRIBUTIONS

Conceptualization: ES, JFLO, JAMC, JAPS; Data curation: ES, JFLO; Formal analysis: ES, JFLO; Founding acquisition: JFLO, FJSV; Investigation: ES, IR; Project administration: ES, JFLO; Supervision: JFLO, FJSV; Validation: JFLO; Visualization: ES; writing-original draft: ES; Writing-review and editing: JFLO, FJSV, JAMC, JAPS

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SUPPLEMENTARY DATA

Suppl. Table 1. P values, mesor, amplitude and acrophases of genes involved in protein digestion protein digestion (*tryp2*, *try3*, *ctrl*, *cpa5*), transamination (*c-alt*, *m-alt*, *ast*), glycolysis (*pk*), lipid metabolism (*pla2*), food intake regulation (*npy*, *orexin*) and reward system (*th*, *bdnf*) subjected to Cosinor analysis. All the acrophases are reported as ZT (*zeitgeber* time, h). Mesor and amplitudes are indicated in relative mRNA expression (fold change). Fiducial limits (set at 95%) are also indicated for mesor, amplitude and acrophase values.

Gene	Experimental group	Significance variance	Mesor	Amplitude	Acrophase (ZT hours)
tryp2	ML	0.00647	58.68 ± 23.63	53.02 ± 39.88	12.10 ± 3.6
	MD	0.01576	60.01 ± 22.91	49.11 ± 41.08	18.45 ± 3.71
tryp3	ML	0.03884	7.5 ± 3.2	5.73 ± 8.55	12.15 ± 5.31
	MD	–			
ctrl	ML	0.02069	36.36 ± 17.12	33.82 ± 29.72	11.28 ± 4.43
	MD	0.04196	31.16 ± 9.3	17.39 ± 16.9	18.16 ± 4.8
cpa5	ML	0.01553	27.33 ± 11.97	23.75 ± 19.8	13.60 ± 4.05
	MD	0.02855	47.99 ± 19.93	38.19 ± 35.27	16.95 ± 4.03
c-alt	ML	0.02499	3.89 ± 0.67	1.35 ± 1.22	5.16 ± 3.6
	MD	–			
m-alt	ML	0.00005	3.29 ± 0.52	1.90 ± 0.94	5.3 ± 1.83
	MD	–			
ast	ML	–			
	MD	–			
pk	ML	0.00385	4.46 ± 0.99	2.61 ± 1.85	6.26 ± 2.7
	MD	–			
pla2	ML	0.02279	14.57 ± 5.82	11.07 ± 9.76	13.96 ± 4.4
	MD	–			
npy	ML	–			
	MD	0.001	6.76 ± 0.65		13.15 ± 2.57
orexin	ML	–			
	MD	0.02098	1.97 ± 0.2	0.41 ± 0.36	16.1 ± 4.5
th	ML	0.02998	6.51± 1.79	3.50 ± 3.24	17.93 ± 4.05
	MD	0.02489	32.14 ± 11.14	21.10 ± 18.82	15.50 ± 4.23
bdnf	ML	0.02435	1.70 ± 0.16	0.31 ± 0.28	11.76 ± 4.84
	MD	–			

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Experimental chapter II

Daily rhythms of acute stress responses and antioxidant systems in the European sea bass (Dicentrarchus labrax): Effects of the time of the year.

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ABSTRACT

Fish reared in aquaculture face various acute stressors, including air exposure during handling. Research on the stress response in fish can provide essential insights into their physiology and help define better aquaculture practices. In this study, we investigated the daily rhythms in the stress-axis response of the European sea bass (Dicentrarchus labrax) subjected to an acute stressor consisting of air exposure (1 min), and how this response is influenced by the time of the day and the season of the year. In addition, rhythms in antioxidant systems were also assessed. The experiments were performed in late Autumn (December) and late Spring (June), with natural photoperiod (10 L:14D and 15 L:9D, respectively) and water temperature (ranging from 19.47 ± 0.17 °C in December to 22.13 ± 0.13 °C in June). Samples were collected throughout a 24-h cycle at Zeitgeber time (ZT) 0.5, 4, 7.5, 12, 16, 20, and 24.5 h at both seasons. At each sampling point, an untreated control (CTRL) group was sampled, while a STRESS group was exposed to air for one minute, returned to the tank, and sampled one hour later. Fish were sacrificed to collect plasma samples, hypothalamus and liver. Plasma samples were analyzed for cortisol, glucose, and lactate. In the hypothalamus, the mRNA expression levels of corticotropin-releasing hormone (crh) and crh-binding protein (crh-bp) were quantified using quantitative RT-PCR (qPCR). In the liver, genes related to antioxidant systems (catalase, superoxide dismutase 1, glutathione peroxidase, and glutathione reductase) and mitochondrial markers of stress (uncoupling protein 1, cytochrome c oxidase IV and peroxiredoxin3) were also analyzed by qPCR. The results revealed that most stress indicators (cortisol, *cat, sod1, gsh-px, gsr, ucp1, coxIV*) displayed daily rhythms. Furthermore, the stress response was significantly influenced by the time of day and the season in which the stressor was applied. In June, cortisol and glucose responses to stress were higher during the day than at night. The increase observed after stress in

genes related to the antioxidant system was more significant in June than in December. Conversely, the response of mitochondrial markers was greater in December. Taken together, these findings highlight that the stress response of the European sea bass is time-dependent, both on a daily and a seasonal basis. This emphasizes the importance of considering cyclic environmental factors and circadian rhythms in aquaculture procedures to enhance fish welfare.

Keywords: HPI axis; Cortisol; Glucose; Lactate; Antioxidant enzymes; Stress markers; Mitochondrial biomarkers; Acute stress.

INTRODUCTION

Fish in aquaculture face stressors such as high stock density (Montero et al., 1999), poor water quality, transport, handling or food deprivation (Ashley, 2007; Zhang et al., 2020). Among these stressors, handling represents a highly stressful operation as it often involves air exposure, triggering a significant physiological stress response (Ramsay et al., 2009; Cook et al., 2015). Furthermore, the subsequent reoxygenation, when fish return to the water after air exposure, can intensify the oxidative response and lead to liver damage (Wang et al., 2021). In teleost fish, the response to this acute stress is mediated through the activation of the Brain-Sympathetic-Chromaffin (BSC) and the Hypothalamus-Pituitary-Interrenal (HPI) axes (Wendelaar Bonga, 1997; Gorissen and Flik, 2016), culminating in cortisol secretion into the bloodstream from the interrenal tissue, specifically in cells located in the head kidney (Grassi Milano et al., 1997; Mommsen et al., 1999). These two axes collaborate to reorganize the physiological response, energy balance and, ultimately, behavior, enabling fish to cope with the stressor (Ellis et al., 2012). Similar to other acute stressful events, air exposure triggers the HPI axis, leading to cortisol release (Fanouraki et al., 2011; Fatira et al., 2014) and possibly influencing other biochemical parameters, such as the response to oxidative stress through the activation of the antioxidant system (Welker et al., 2012), which plays a crucial role in minimizing the effects of reoxygenation caused by an increase in ROS (Reactive Oxygen Species) production (Scandalios, 2005). This antioxidant system includes enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and glutathione reductase (GSR). SOD converts superoxide radicals into hydrogen peroxide, which is further broken down by CAT into water and oxygen. GSH-PX also contributes to hydrogen peroxide removal with the assistance of GSR (Scandalios, 2005). Additionally, mitochondria play a role in these processes, encoding factors like uncoupling proteins (UCP), peroxiredoxins (PRDX), and cytochrome c

oxidase 4 (COXIV), which help maintain the balance between oxidative and antioxidant mechanisms and are considered biomarkers of fish welfare (Pérez-Sánchez et al., 2011; Eissa and Wang, 2016; Vera et al., 2014).

In their natural habitat, fish experience direct influences from cyclic environmental factors, significantly impacting the adaptation process of living organisms during evolution (Krittika and Yadav, 2020). These exogenous factors act as synchronizers, or zeitgebers, for most biological rhythms within the organism. The most crucial external synchronizers include light, temperature, and food availability (Rensing and Ruoff, 2002; Lahiri et al., 2005; López-Olmeda et al., 2006). The information from these external signals is integrated by endogenous pacemakers, which coordinate all output pathways and rhythmic physiological variables (Whitmore et al., 1998; Pando and Sassone-Corsi, 2002; López-Olmeda, 2017). At the molecular level, a self-sustainable molecular clock controls all rhythmic cellular processes. This clock mechanism relies on positive and negative feedback loops: a positive loop formed by the transcriptional activators clock and bmal1, and a negative loop formed by factors per and cry (Pando and Sassone-Corsi, 2002; Cahill, 2002). Circadian oscillations in molecular clocks impact various physiological variables, controlling their rhythmic expression. Among them, most endocrine glands and hormones, including those involved in the stress response, exhibit daily rhythms (Cowan et al., 2017). Simultaneously, the relationship between the circadian system and the stress axis can be described as bidirectional since cortisol seems to play a crucial role in the negative loop of the molecular clock due to the presence of glucocorticoid responsive elements (GREs) in the regulatory regions of clock-related genes (So et al., 2009). Daily rhythms in the HPI axis, especially in plasma cortisol, have been observed in several teleost fish (Cowan et al., 2017).

Daily rhythms have also been reported in other HPI axis factors, such as transcript levels of corticotropin-releasing hormone (*crh*) and crh binding protein (*crh-bp*) in the hypothalamus, and proopiomelanocortin A and B (*pomca* and *pomcb*) in the pituitary of gilthead seabream (*Sparus aurata*) (Vera et al., 2014) and Senegalese sole (*Solea senegalensis*) (López-Olmeda et al., 2013). Alongside these daily rhythms, the stress response in these fish species appears to be time-dependent and mostly related to the activity of the species considered. Thus, the stress response seems to be greater when an acute stressor like air exposure is applied to fish at the resting phase, eliciting a greater cortisol production than when fish are stressed during the phase of activity (Vera et al., 2014; López-Olmeda et al., 2013; Sánchez-Vázquez et al., 2019). Regarding fish antioxidant defenses, they may be modulated by age, feeding behavior, or various environmental factors such as ammonia concentration or photoperiod (Martínez-Álvarez et al., 2005; Jung et al., 2016; Tian et al., 2019). However, knowledge about time-dependent differences in these factors in fish is limited (Hidalgo et al., 2017; Ren et al., 2020), although studies have reported that the circadian system regulates ROS levels by controlling the expression of certain antioxidant enzymes in mammals (Pekovic-Vaughan et al., 2014; Sato and Greco, 2021).

The European sea bass (*Dicentrarchus labrax*) is a crucial species for aquaculture, particularly in Mediterranean countries (Vandeputte et al., 2019). However, sea bass exhibits high sensitivity to acute stressors like handling and air exposure (Fanouraki et al., 2011; Fatira et al., 2014). Therefore, understanding its stress response and how the circadian system is coupled with it at various levels could aid in formulating new recommendations for handling procedures. Daily rhythms in the HPI axis of sea bass have been reported only for plasma cortisol (Fatira et al., 2014). Additionally, seasonal variations in cortisol production have been described, with oscillations coinciding with

annual variations in water temperature and photoperiod (Planas et al., 1990; Cowan et al., 2017). However, apart from cortisol, nothing is known regarding the existence of daily rhythms in other HPI axis factors or the antioxidant system in the European sea bass. Moreover, it is unknow to date whether this species presents differences in its physiological response to stress depending on the time of the day.

Therefore, the aims of this study were to evaluate: i) the existence of daily rhythms for several factors of the HPI axis, the antioxidant system and liver mitochondrial markers of the European sea bass; ii) the effect of the time of day on the response of all these factors to an acute stress challenge (air exposure for 1 min); and iii) the effect of the time of year (December and June) on these responses.

MATERIALS AND METHODS

The experiments were conducted at the Aquaculture Laboratory of the University of Murcia located at the Naval Base of Algameca (E.N.A., Cartagena, Spain). The experiments were designed according to European Union guidelines (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use of laboratory animals. They were approved by the Committee of the University of Murcia on Ethics and Animal Welfare and the Government of Región de Murcia (license number A13191003).

Animals and housing

The European sea bass specimens (N = 200; 46.2 ± 2.3 g of initial body weight, mean ± SEM) were sourced from a local fish farm (CULMAREX, Guardamar del Segura, Alicante, Spain) in July 2020. They were housed in four 500-l tanks (50 fish/tank; 18.48 kg/m3) within an open system equipped with biological and mechanical filters. UV lamps

were utilized to sterilize the water before it entered the system, and each tank was equipped with aeration. These stock fish were fed ad libitum with a commercial diet (Alterna Marine, Skretting, Burgos, Spain). The photoperiod was regulated to mimic seasonal variations through a timer (Data Micro, Orbis, Madrid, Spain) connected to the lights. For all experiments, the onset of light was designated as Zeitgeber (synchronizer) time 0 h (ZT 0). This approach is commonly employed in chronobiology studies as it allows standardizing time points without referencing specific times of the day, ensuring reproducibility (Espirito Santo et al., 2020). Fluorescent light bulbs were used to provide light and the intensity at the water surface was 200 lx. Water temperature was the same as in the natural environment, and hence paralleled the seasonal temperature variations. Water temperature was continuously monitored during the experiments (HOBO PENDANT Onset Computer Corporation, Massachusetts, USA) (Supplementary Fig. 1).

Experimental design

The experiments were designed to investigate the effects of the time of year on the daily rhythms of physiological factors related to stress and the stress response. For this purpose, two samplings were conducted in different seasons, with a 6-month interval: at the end of Autumn and at the end of Spring. One month after acclimating the stock fish to laboratory conditions, 98 animals were transferred to 150-l tanks. These fish were randomly distributed across 7 tanks (14 fish/tank) and fed using automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany). The feeders were programmed to provide a daily feed ration equivalent to 1% of the fish's body weight. To minimize feed waste and optimize consumption, food was given at the same times each day (0.5% of fish body weight at each time): one hour before and one hour after the middle of the light phase (ML). These conditions were maintained for 30 days. After this acclimation period,

fish were sampled at approximately 4-h intervals over a 24-h cycle, at the following time points: ZT 0.5, ZT 4, ZT 7.5, ZT 12, ZT 16, ZT 20, and ZT 24.5 h (Fig. 1). Different tanks were sampled at each time point to prevent stress from repeated handling. At each sampling time, fish were collected and divided into two groups: control (unstressed) and stressed. Fish from the stress group (STRESS, n = 7) were submitted to 1 min of air exposure, then returned to the tank, and sampled 1 h after the stress (Fig. 1). This stress procedure has been shown to induce a high-stress response where cortisol peaks around one hour after exposure (Fatira et al., 2014; López-Olmeda et al., 2013; Vera et al., 2014). Meanwhile, fish from the control group (CTRL, n = 7) were immediately placed in a bucket with anesthesia for sampling without the stress challenge (Fig. 1). All fish were anesthetized with clove oil essence (Guinama, Valencia, Spain) at a concentration of 50 µl/l. Clove oil was diluted first in 9 parts of ethanol and then added to water. When the fish were anesthetized, blood was collected by caudal puncture using heparinized syringes (Fragmin, Pfizer S.L., Madrid, Spain). The entire procedure, from fish capture to blood collection, took <5 min to avoid an increase in plasma cortisol due to manipulation (Molinero et al., 1997). The blood was then centrifuged at 3000 rpm for 15 min at room temperature to separate the plasma, which was collected and stored at −80 °C. Subsequently, the fish were sacrificed by decapitation to collect the hypothalamus and liver, which were snap-frozen in dry ice and stored at −80 °C until analysis. Dim red lights (λ > 600 nm) were used for sampling during the dark phase (de Alba et al., 2019).

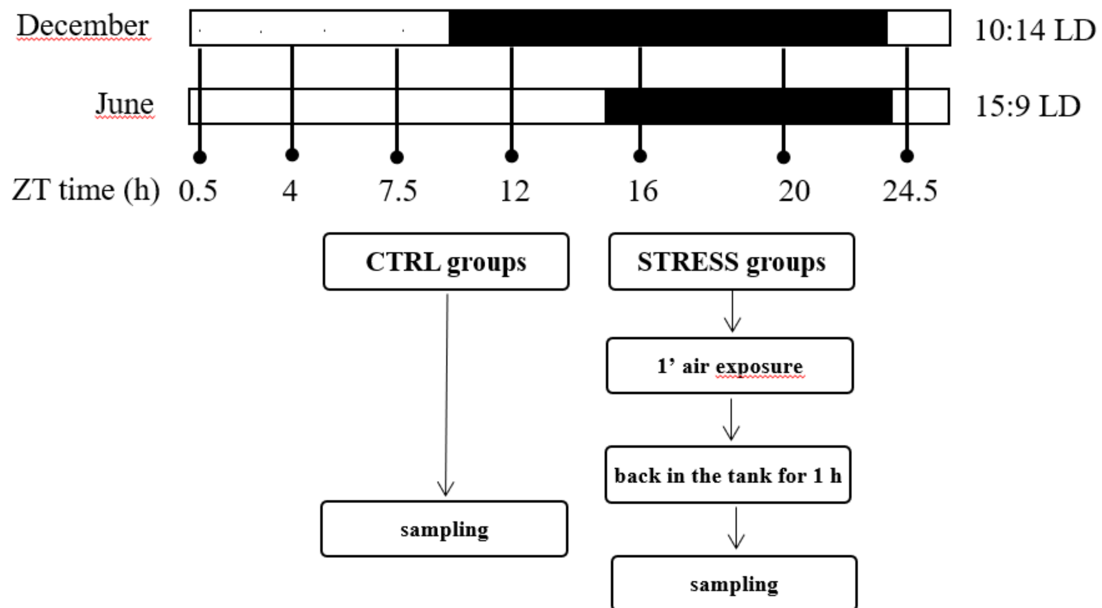


Fig. 1. Schematic representation of the experimental design. Samplings were performed at two seasons of the year: December and June. At each season, sampling points were performed around every 4 h during a 24 h cycle. CTRL groups were sampled directly at the ZT (zeitgeber time) point designed while STRESS groups were exposed for 1 minute to air, then returned to the tank and sampled 1 h later. White and black bars represent the light and dark phases, respectively (10:14 LD in December and 15:9 LD in June).

This sampling protocol was conducted in two different seasons: Late Autumn (10 December 2020) and Late Spring (19 June 2021) (Fig. 1 and Supplementary Fig. 1). The photoperiod on each sampling day was 10 L:14D and 15 L:9D for December and June, respectively. The average water temperature recorded was 19.47 ± 0.17 °C and 22.13 ± 0.13 °C for the December and June experiments, respectively. The factors analyzed were physiological stress indicators in plasma (cortisol, glucose and lactate), genes of the HPI axis (*crh, crh-bp*), genes involved in the antioxidant response (*cat, sod1, gsr* and *gsh-px*) and mitochondrial oxidative biomarkers (*prdx, ucp* and *coxIV*).

Plasma cortisol, glucose and lactic acid

Plasma cortisol was measured by means of a commercial ELISA kit (TECAN, IBL International GmbH, Hamburg, Germany, Ref. number RE52061), which was first

validated for European sea bass samples by performing parallelism and recovery tests as described elsewhere (López-Olmeda et al., 2009). The obtained recovery values accounted for 101.3%.

Plasma glucose and lactate were measured using an automated analyzer (Beckman Coulter DxC 700 AU, Beckman Coulter, Brea, USA). Glucose was measured by the hexokinase and glucose-6-phosphate assay (OSR6121, Beckman Coulter), and lactate was measured by the lactate oxidase assay (OSR6193, Beckman Coulter).

RNA extraction, cDNA synthesis and quantitative Real-Time RT-PCR analysis.

Hypothalamus and liver samples were homogenized in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, USA) using a tissue homogenizer (TissueLyser LT, Qiagen, Hilden, Germany). The homogenized samples were then mixed with 1-Bromo-3-chloropropane (BCP, 99%, Thermo Fisher Scientific) and centrifuged to obtain a supernatant containing RNA. Isopropanol (Thermo Fisher Scientific) was utilized to further separate RNA through centrifugation. The RNA was washed twice with 75% ethanol (Thermo Fisher Scientific) and dissolved in DEPC water (Invitrogen, CA, USA). RNA concentration and purity were assessed using spectrometry (Nanodrop® ND 1000, Thermo Fisher Scientific). Subsequently, 1 μg of RNA was treated with DNase I (Thermo Fisher) to eliminate potential genomic contamination (1 U of DNase I for 1 μg of RNA). Next, cDNA was synthesized using a commercial Reverse Transcriptase kit (QSCRIPT cDNA Synthesis Kit, Quantabio, Beverly, USA) and a thermocycler (MiniAmp Thermal Cycler, Thermo Fisher) and stored at −20 °C until further analysis. All samples were processed in duplicate using Perfecta SYBR Green Fastmix (Quantabio) and a real-time PCR system (7500 RT-PCR system, Applied Biosystems, Foster City, USA) following this protocol: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Each reaction had a final volume of 20 µl, and all primers were added at a final concentration of 500 nM. Melting curve analysis was performed after amplification to confirm the amplification of a single DNA species. The genes analyzed in the hypothalamus were *crh* and *crh-bp*, while in the liver, *cat, sod1, gsr, gsh-px, prdx3, ucp1*, and *coxIV* were examined. Primers (Table 1) were designed using Primer 3 Plus software (Untergasser et al., 2012), and their efficiency was evaluated using cDNA dilution curves. The 2-ΔΔCt method was employed for the analysis (Livak and Schmittgen, 2001). The geometric mean of two housekeeping gene values (*β-actin* and *ef1α*), ensured that their coefficient of variation (CV) was lower than 0.5%, was used for the first normalization, and the sample with the lowest expression value was used for the second normalization.

Table 1. Primer sequences used for the qPCR

Gene name	Fw	Rv	accession number
ef1a	AGTGAAGCAGCTCATCGTTG	TTGGTGATTTCCTCGAAGCG	AJ866727
bact	TCATCACCATCGGCAATGAG	AACGTCGCACTTCATGATGC	AY148350
crh	ACCGTGATTCTGCTAGTTGC	TGCTGGGAGTTTTGGGTTTG	JF274994.1
crh-bp	TGTGCTGCTTTCTTCATGGC	TGGGAGCTGGGAAATTTCTCTC	MG832822.1
cat	AGAGTGGTGCATGCAAAAGG	TTGCCGACATGCTCAAACA	ENSDLAT00005060237.1
sod1	TGGTCGTCCTTTGAAACTGC	TCCTGCTCAAAGTGAACGAC	ENSDLAT00005045828.1
gshpx	TGGCTGGAAACGTGAAAAGG	ACGCCACATTCTCAATGAGC	ENSDLAT00005057425.1
gsr	AAAATGGGTGCGACCAAAGC	AACGCATCGTGACAAACTCC	ENSDLAT00005017653.1
ucp1	ACTGGTGGCAAAGACAATCC	ATCTGTGGGTTGTGCAAAGG	ENSDLAT00005032761.1
prdx3	TCATTGATCCAAGCGGTGTG	AACGCCTTCACCAAACGAAG	ENSDLAT00005019248.1
coxIV	ATTGCTTTGTACCGGGTGAC	AGTTTTCCACTCTGCTGACG	DKA_LG22-25_008930

Data analysis

The SPSS software (version 24, IBM, Armonk, USA) was used to identify statistical differences, with a significance threshold set at α = 0.05 for all conducted tests. Results are presented as mean ± SEM. Data for each variable in each season were assessed for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test) before being subjected to a two-way ANOVA. Post hoc testing was conducted using the

Duncan test to identify statistically significant differences between groups and time points. Furthermore, a three-way ANOVA was employed to assess significant differences between groups (CTRL and STRESS), sampling points (ZT), and season (December and June). Data were also aggregated separately for the light and dark phases, and comparisons between these values were made using Student's t-test or Mann-Whitney analysis.

Additionally, Cosinor analysis was utilized to determine the presence of significant rhythmicity in the biological variables analyzed, using the chronobiology software CSR 3.0.2. Cosinor analysis involves the least-square approximation of time-series data with a cosine function of the known period, represented as $Y = M + A * (COS (\Omega t + \varphi))$ (M = mesor, A = amplitude, Ω = angular frequency with 360°/24 h for circadian rhythm, φ = acrophase). This analysis provides the statistical significance of the rhythm, as an F test of the variance is conducted for the waveform versus a straight line of zero amplitude (null hypothesis) (Refinetti et al., 2007; Portaluppi et al., 2008).

RESULTS

Stress markers in plasma and hypothalamus

Cortisol levels in plasma displayed daily rhythms in CTRL in December and June (Cosinor, $p < 0.005$), with nocturnal acrophases located at similar times in both seasons: ZT 19.55 and ZT 19.33, respectively. In addition, the STRESS group also displayed a rhythm in June, with the acrophase located towards the end of the night (ZT 22.83) (Cosinor, $p < 0.05$), but no rhythm was found in STRESS in December (Cosinor, $p < 0.05$) (Fig. 2A and B). At all ZT points analyzed, cortisol values varied significantly between CTRL and STRESS groups (two-way ANOVA, $p < 0.05$) (Fig. 2A and B). In general,

cortisol STRESS values were significantly higher than CTRL at every ZT point in both seasons with the exception of ZT 16 in June, and the highest cortisol levels were found in the dark phase in all groups (Fig. 2A and B). In addition, a significant effect of the season was present (three-way ANOVA, $p < 0.000$). When pooled together day or night values in each group, a significant increase in cortisol levels after exposure to stress was observed in December during both day and night (Mann-Whitney, $p < 0.05$) (Fig. 3A). However, in June, the cortisol increase was dependent on the time of day, with only a significant increase being observed only during the day (Mann-Whitney, $p = 0.4428$) (Fig. 3B).

The plasma levels of glucose showed daily rhythms in both groups in December (Cosinor, $p < 0.05$) with diurnal acrophases (ZT 8.24 and ZT 7.8 for CTRL and STRESS, respectively), but no significant rhythms were detected in June (Cosinor, $p > 0.05$) (Fig. 2C and D). In December, significant differences for sampling time, treatment and their interaction were observed between the groups (two-way ANOVA, $p < 0.05$), whereas in June significant effects were only found for the treatment (CTRL vs. STRESS). STRESS glucose levels were significantly higher than CTRL at ZT 7.5 and 20 in December and at ZT 4 and ZT 16 in June (Fig. 2C and D). The three-way ANOVA also revealed a significant effect of the season of the year, with higher glucose levels found in June (Fig. 2C and D). A significant effect of the time of day in the glucose response to stress was only found in June, where an increase was detected during the day but not at night (Mann-Whitney, $p < 0.05$) (Fig. 3D).

As for lactate levels, no significant rhythms were observed in any of the groups at any of the seasons analyzed (Cosinor, $p > 0.05$) (Fig. 2E and F). On the other hand, statistically significant effects of sampling time and treatment were observed both in December and June (two-way ANOVA, $p < 0.05$). STRESS lactate levels were

significantly higher than CTRL at every ZT point at both seasons (Fig. 2E and F). This was also observed when day or nighttime values were pooled together: STRESS groups had higher lactate values (Mann.Whitney, $p < 0.05$) regardless of the time of day in both seasons (Fig. 3E and F). Finally, a significant effect of the season was present as lactate values in June were higher than those observed in December (three-way ANOVA, $p < 0.000$).

The mRNA levels of *crh* showed significant rhythms in the STRESS group in December (Cosinor, $p < 0.01$), with a nocturnal acrophase at ZT 13.33, and in CTRL group in June ($p < 0.05$), with the acrophase in the first half of the light phase (ZT 4.40) (Fig. 2G and H). All groups, except STRESS in June, showed significant variations throughout the day and, at some time points, CTRL and STRESS were significantly different (ZT 24.5 in December and ZT 0.5, 4 and ZT 20 in June) (two-way ANOVA, $p < 0.05$) (Fig. 2G and H). In addition, higher *crh* values were observed in December compared to June (three-way ANOVA, $p < 0.05$) (Fig. 2G and H). Different effects depending on the time of day were observed when daytime and nighttime values were pooled (Fig. 3G and H). During the light phase, the stress induced a significant decrease in *crh* compared to the control group at both seasons (t-test, $p < 0.05$). In contrast, when the stress was applied during the nighttime, no effect was observed in December (t-test, $p = 0.43$) but a significant increase in *crh* expression was observed in June (t-test, $p < 0.05$). In December, the stress treatment resulted in higher crh levels at night compared to the day (t-test, $p < 0.05$) (Fig. 3G).

As for *crh-bp* expression, no rhythm was found neither in CTRL or STRESS nor any of the seasons (Fig. 2I and J). Treatment was the only variable that significantly affected *crh-bp* expression (two-way ANOVA, $p < 0.001$) (Fig. 2I and J). Values in the STRESS groups were significantly higher than CTRL at ZT 0.5, ZT 4, ZT 12, ZT 16 and

ZT 20 in December but only at ZT 4 h in June (Fig. 2I and J). In addition, gene expression of crh-bp was higher in December than in June (three-way ANOVA, $p < 0.05$) (Fig. 2I and J). In general, the stress treatment elicited an increase in *crh-bp* expression in both seasons irrespectively of the time of the day when the stress challenge was applied (t-test, $p < 0.0001$) (Fig. 3I and J). In addition, in December the stress response was different depending on the time of the day as the *crh-bp* increase after the stress was higher at night than during the day (t-test, $p < 0.05$) (Fig. 3I), as observed for *crh* in the same season (Fig. 3G).

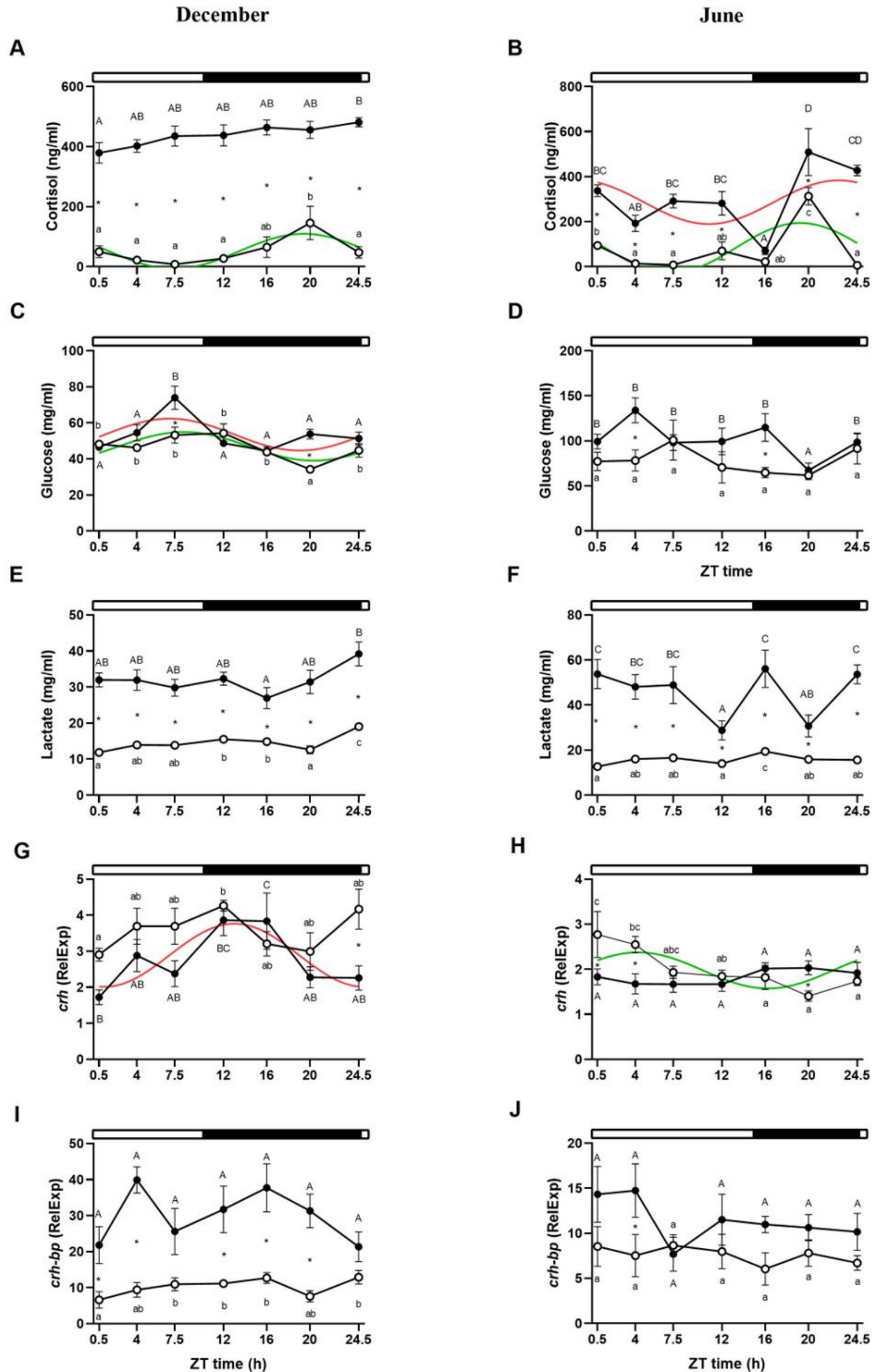
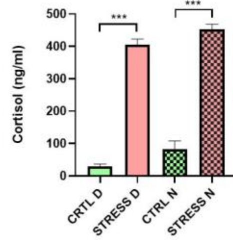


Fig. 2 Daily variations in the plasmatic levels of cortisol (A, B), glucose (C, D) and lactate (E, F), and in the hypothalamic relative mRNA levels (fold change) of *chr* (G, H) and *crh-bp* (I, J) in the European sea bass. Fish were maintained under natural annual variations of photoperiod and water temperature and samples were collected in December (left panels) and June (right panels) experiments. Photoperiod at the sampling in December was 10:14 LD while in June it was 15:9 LD.

CTRL groups were sampled directly at every Zeitgeber time (ZT) point while the STRESS groups were exposed to air for 1 min and sampled 1 h later. CTRL is represented with white dots (○) while the STRESS group is represented with black dots (●). The green and red lines represent the adjustment to a sinusoidal rhythm for CTRL and STRESS groups, respectively, whenever a Cosinor test was significant ($p < 0.05$). Different lower- and upper-case letters indicate statistically significant differences between ZT points within the CTRL and STRESS groups, respectively, and the asterisks indicate significant differences between the two groups at the same time point (two-way ANOVA, $p < 0.05$). The white and black bars above the graphs represent the light and the dark phase, respectively. The x-axis represents the time scale as ZT, in which ZT o corresponds to light onset. Data are represented as mean ± SEM ($n = 7$ per point). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

December **June**

A



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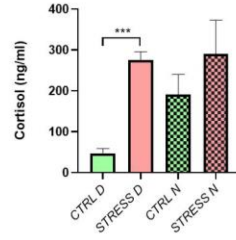

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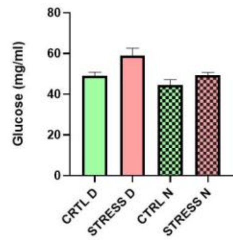

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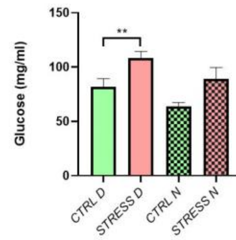

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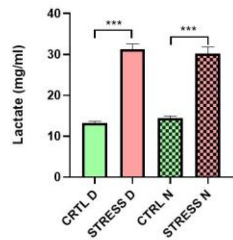

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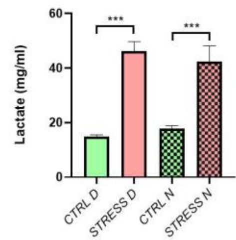

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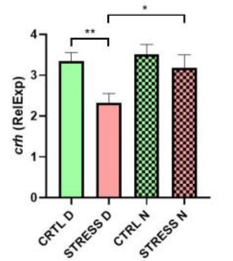

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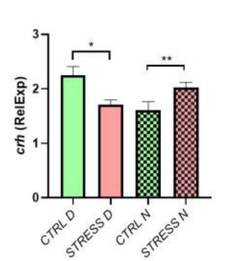

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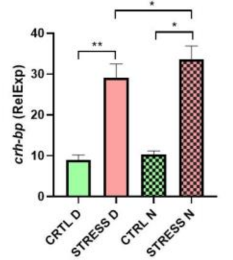

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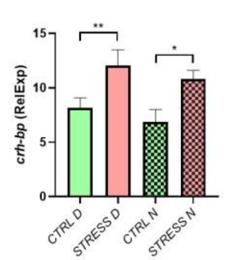


Fig. 3 Day-night differences in the plasmatic levels of cortisol (A, B), glucose (C, D) and lactate (E, F), and in the hypothalamic relative mRNA levels (fold change) of *chr* (G, H) and *crh-bp* (I, J) in the European sea bass. For each group and season, values were obtained by pooling all data from either the light phase (day, D) or the dark phase (night, N). Values from each season (December, left panels, and June, right panels) were compared by means of Student's t-test or Mann-Whitney. Asterisks indicate statistically significant differences between groups within one of the seasons.

Markers of the redox system in the liver

Cosinor analysis revealed a rhythm in *cat* expression in the CTRL groups during both seasons, with nocturnal acrophases located in the middle of the night in December (ZT 19.91) and at the beginning of the dark phase in June (ZT 15.53) (Cosinor, $p < 0.05$). This significant rhythm persisted in the STRESS group in December (ZT 17.93) but disappeared in the stressed fish in June (Fig. 4A and B). *Cat* expression showed differences due to sampling time and treatment both in December and June (two-way ANOVA, $p < 0.05$). In June, STRESS group displayed higher *cat* transcript levels than CTRL at all ZT points analyzed, whereas in December the CTRL group displayed significantly higher expression than the STRESS group only at ZT 0.5 and 20 (Fig. 4A and B). In addition, higher mRNA levels were observed in December compared with to June (three-way ANOVA, $p < 0.001$). When comparing CTRL and STRESS group after pooling daytime and nighttime *cat* values revealed that the response in the STRESS groups was higher at night than during the day in December (Mann-Whitney, $p = 0.0006$) but not in June (t-test, $p = 0.8634$) (Fig. 5A and B). In addition, in December, *cat* expression was higher in CTRL than STRESS during the day in December (t-test, $p = 0.0014$) but no significant differences were observed at night (t-test, $p = 0.1323$). In contrast, in June, *cat* expression was higher in the STRESS than CTRL both during the day and at night (Mann-Whitney, $p < 0.0001$ and t-test, $p = 0.0058$, respectively) (Fig. 5A and B).

Sod1 exhibited daily rhythms in both the CTRL and STRESS groups in December (Cosinor, $p < 0.05$), but not in June. In December, the acrophases occurred during the night in both groups, but a shift in the phase could be observed as the acrophase of CTRL was set at ZT 18.40 and for STRESS at ZT 11.78 (Fig. 4C). The expression showed differences due to sampling time (two-way ANOVA, $p < 0.05$). In December, *sod1* expression was higher in CTRL than STRESS only at ZT 20, whereas in June *sod1* transcript levels in STRESS were significantly higher than in CTRL at most ZT except at ZT 16 and ZT 20 (Fig. 4C and D). In addition, higher *sod1* values being observed in June than in December (three-way ANOVA, $p < 0.05$) (Fig. 4C and D). Comparing day and night values of CTRL and STRESS revealed significant differences only in June, where STRESS groups presented higher *sod1* mRNA levels than CTRL at both daytime and nighttime (Mann-Whitney, $p < 0.0001$ and $p = 0.043$, respectively) (Fig. 5D).

In December, *gshpx* expression showed significant daily rhythms in both the CTRL and STRESS groups (Cosinor, $p < 0.05$), with nocturnal acrophases but shifted 5 h: ZT 15.90 in CTRL and ZT 10.70 in the STRESS group (Fig. 4E and F). No significant rhythms were found in June. In general, the stress elicited an increase in *gshpx* mRNA levels, which was significant at ZT 4 in December and at all the time points analyzed in June (two-way ANOVA, $p < 0.05$) (Fig. 4E and F). A significant seasonal effect was also observed (three-way ANOVA, $p < 0.001$), with *gshpx* expression being higher in June than in December. When day and night values were pooled, significantly higher *gshpx* expression in STRESS than in CTRL was found during the day but not at night in December (Mann-Whitney, $p < 0.05$ and $p = 0.7206$, respectively), while in June STRESS transcript levels were higher both during the day and at night (Mann-Whitney, $p < 0.05$) (Fig. 5E and F).

Regarding *gsr* expression, similar to the other genes in the redox system, significant daily rhythms were detected in December for both groups (Cosinor, $p = 0.03$ and $p = 0.007$ for CTRL and STRESS, respectively). The acrophases were located at ZT 17.75 in the CTRL group (nocturnal acrophase) and at ZT 10.36 in STRESS (in the transition between light and dark phases) (Fig. 4G). No significant rhythms were found in June. Additionally, in December, *gsr* expression was higher in the STRESS group than in CTRL at ZT 12, while the opposite was observed at ZT 20 (Fig. 4G). In June, no differences due to sampling time or treatment were observed (two-way ANOVA, $p > 0.05$) (Suppl. Table 2). On the other hand, higher *gsr* expression being observed in December compared to June (three-way ANOVA, $p = 0.002$) (Fig. 4G and H). Finally, no significant differences were observed after comparing pooled data from day and night at any of the season analyzed (Mann-Whitney or t-test, $p > 0.05$) (Fig. 5G and H).

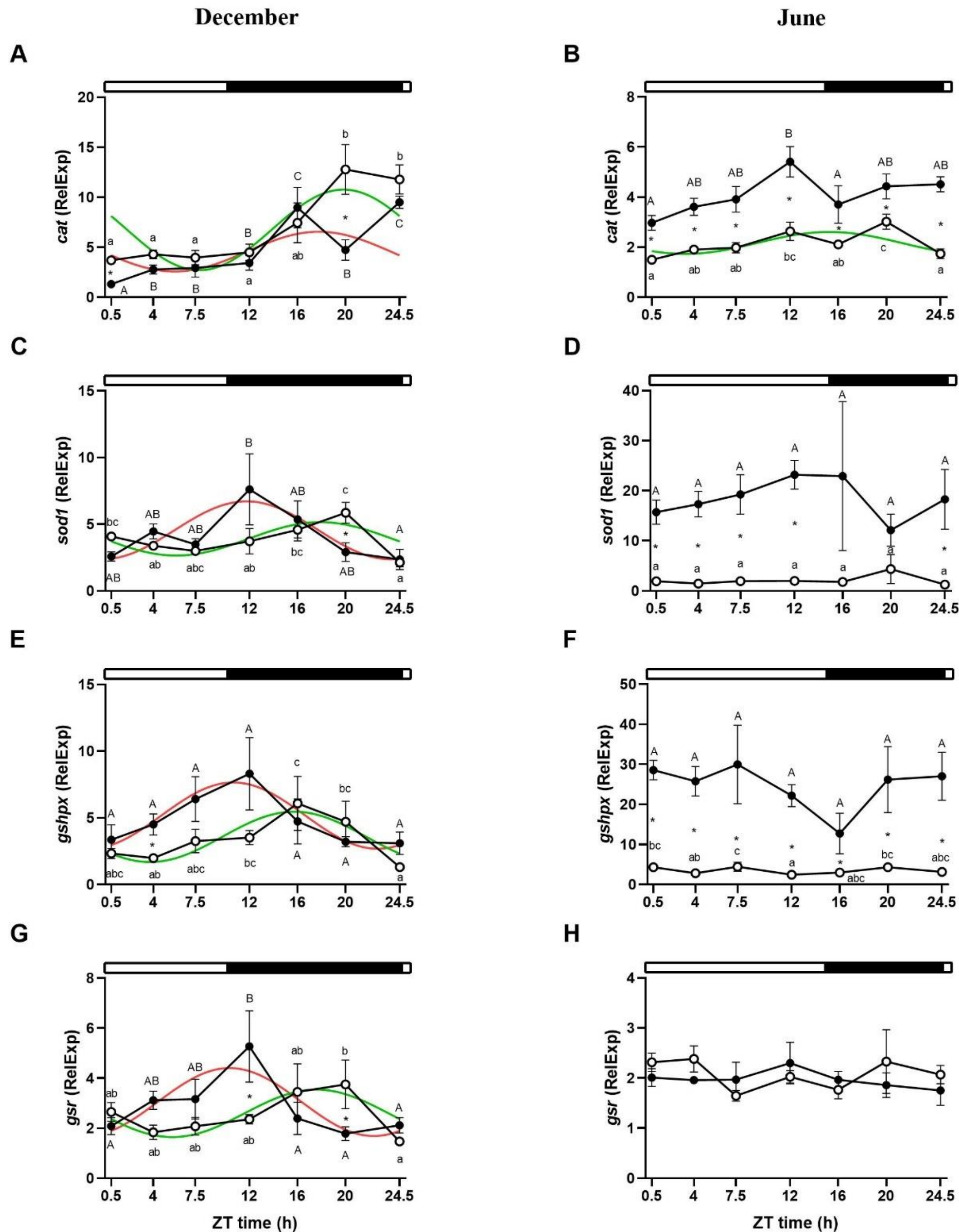


Fig. 4 Daily variations in the relative mRNA levels (fold change) of *cat* (A, B), *sod1* (C, D), *gshpx* (E, F) and *gsr* (G, H) analyzed in the liver of European sea bass in December (left panels) and June (right panels) experiments. Fish were maintained under natural variations of photoperiod and water temperature. Photoperiod at the sampling in December was 10:14 LD while in June it was 15:9 LD. CTRL groups were sampled directly at every Zeitgeber time (ZT) point while the STRESS groups were exposed to air for 1 min and sampled 1 h later. CTRL is represented with white dots (○) whereas the STRESS group is represented with black dots (●). The green and red lines represent the adjustment to a sinusoidal rhythm for CTRL and STRESS groups, respectively, whenever a Cosinor test was significant ($p < 0.05$). Different lower- and upper-case letters indicate statistically significant differences between ZT points within the CTRL and STRESS groups, respectively, and the asterisks indicate significant differences between the two groups at the same time point (one-way ANOVA, $p < 0.05$). The white and black bars above the graphs represent the light and the dark phase, respectively. The x-axis represents the time scale as ZT, in which ZT o corresponds to light

onset. Data are represented as mean ± SEM (n = 7 per point). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

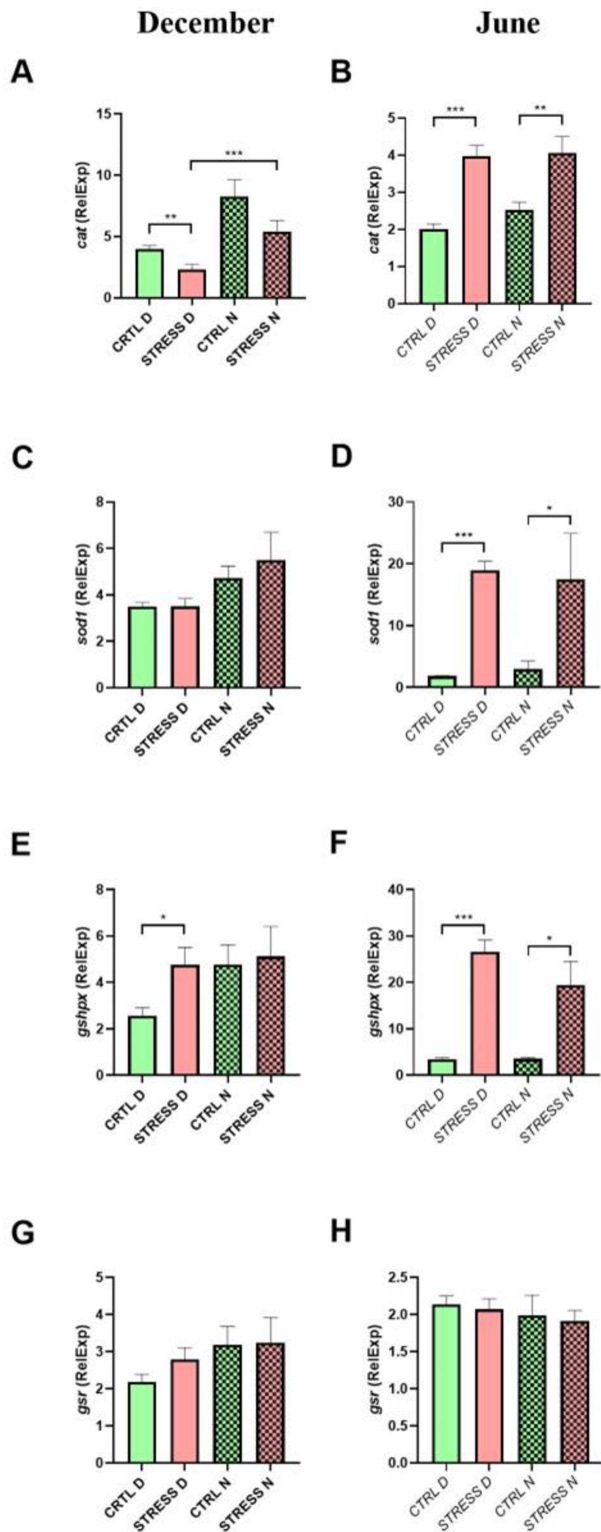


Fig. 5 Day-night differences in the relative mRNA levels (fold change) of *cat* (A, B), *sod1* (C, D), *gshpx* (E, F) and *gsr* (G, H) in the liver of European sea bass. For each group and season, values were obtained by pooling all data from either the light phase (day, D) or the dark phase (night, N).

Values from each season (December, left panels, and June, right panels) were compared by means of Student's t-test or Mann-Whitney. Asterisks indicate statistically significant differences between groups within one of the seasons.

Markers of mitochondrial stress in the liver

The Cosinor analysis revealed significant daily rhythms in *ucp1* expression in all groups and seasons (Cosinor, $p < 0.05$). All acrophases were located in the dark phase but the STRESS groups showed advanced phases compared to their respective CTRL groups: ZT 19.56 and ZT 13.40 for CTRL and STRESS in December, respectively, and ZT 9.11 and ZT 15.56 for CTRL and STRESS in June, respectively (Fig. 6A and B). The expression of *ucp1* was significantly different between CTRL and STRESS only at ZT 20 in December and at ZT 12 in June (two-way ANOVA, $p < 0.05$) (Fig. 6A and B). In addition, the season had a significant on *ucp1* transcript levels as a higher expression was observed in December than in June (three-way ANOVA, $p = 0.001$) (Suppl. Table 3). When comparing the pooled data from daytime and nighttime points, the only significant difference was a decreased *ucp1* expression at daytime in the STRESS group in December compared to the CTRL group (t-test, $p = 0.0014$) (Fig. 7A).

The relative expression levels of *prdx3* only exhibited daily rhythms in the STRESS group in December (Cosinor, $p < 0.005$), with the acrophase located in the transition from the light to the dark phase (ZT 10.38 h) (Fig. 6C). The expression of *prdx3* in the STRESS group was significantly higher than in CTRL group at ZT 0.5, ZT 4, ZT 7.5 and ZT 12 in December (two-way ANOVA, $p < 0.05$) (Fig. 6C). The season of the year had a significant effect on *prdx3* mRNA levels, and higher expression was observed in December compared to June (three-way ANOVA $p < 0.001$). When day and night values were pooled, in December the STRESS group presented higher *prdx3* expression compared to CTRL both at daytime and at nighttime (Mann-Whitney, $p = 0.0024$ and

0.013, respectively) (Fig. 7C). In June, the only significant difference was found in the STRESS group, which showed lower *prdx3* levels at night than during the day (t-test, $p = 0.0075$) (Fig. 7D).

Finally, *coxIV* expression showed significant daily rhythms in both groups in December and in the CTRL group in June (Cosinor, $p < 0.05$). The acrophases were nocturnal for both CTRL and STRESS groups in December but shifted around 7 (ZT 18.70 and 11.21, respectively) whereas in June the acrophase of the CTRL group was located at the transition between the light and dark phases (ZT 13.51) (Fig. 6E and F). Sampling time was the only variable to affect *coxIV* expression in December (two-way ANOVA, $p = 0.016$), while sampling time and treatment affected *coxIV* expression in June ($p = 0.006$ and $p < 0.001$, respectively). In December, the expression levels of *coxIV* were significantly higher in the STRESS than in CTRL group at ZT 7.5 whereas in June the CTRL group showed higher expression than the STRESS one at ZT 0.5, 7.5, 16, 20 and 24.5 (Fig. 6E and F). No significant influence of the season on *coxIV* levels was observed (three-way ANOVA, $p = 0.292$). When day and night values were pooled together, the only significant effects were observed in June, when STRESS groups showed a decrease in *coxIV* expression compared to CTRL groups (t-test, $p = 0.0006$ and Mann-Whitney, $p < 0.0001$, respectively) (Fig. 7F).

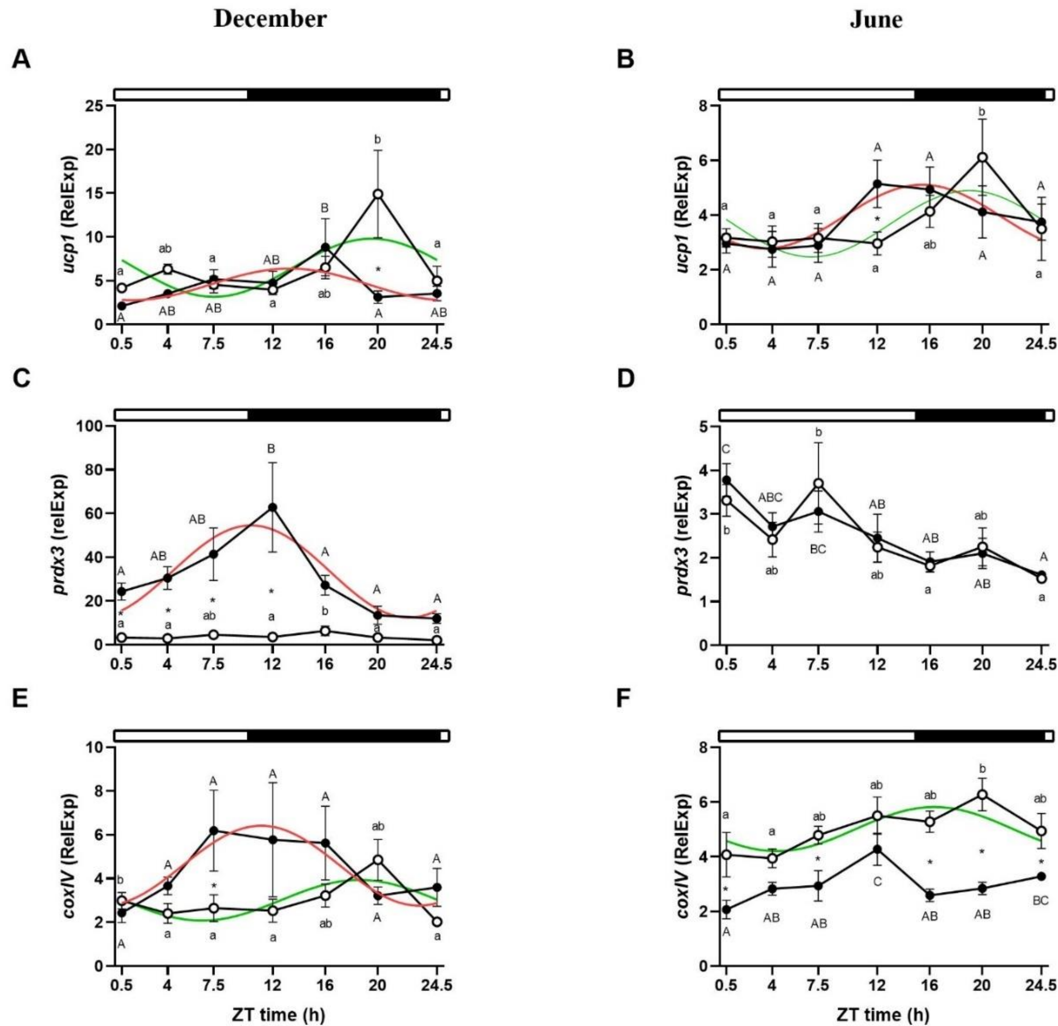


Fig. 6 Daily variations in the relative mRNA levels (fold change) of *ucp1* (A, B), *prdx3* (C, D) and coxIV (E, F) analyzed in the liver of European sea bass in December (left panels) and June (right panels) experiments. Fish were maintained under natural variations of photoperiod and water temperature. Photoperiod at the sampling in December was 10:14 LD while in June it was 15:9 LD. CTRL groups were sampled directly at every Zeitgeber time (ZT) point while the STRESS groups were exposed to air for 1 min and sampled 1 h later. CTRL is represented with white dots (○) whereas the STRESS group is represented with black dots (●). The green and red lines represent the adjustment to a sinusoidal rhythm for CTRL and STRESS groups, respectively, whenever a Cosinor test was significant ($p < 0.05$). Different lower- and upper-case letters indicate statistically significant differences between ZT points within the CTRL and STRESS groups, respectively, and the asterisks indicate significant differences between the two groups at the same time point (one-way ANOVA, $p < 0.05$). The white and black bars above the graphs represent the light and the dark phase, respectively. The x-axis represents the time scale as ZT, in which ZT o corresponds to light onset. Data are represented as mean ± SEM (n = 7 per point). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

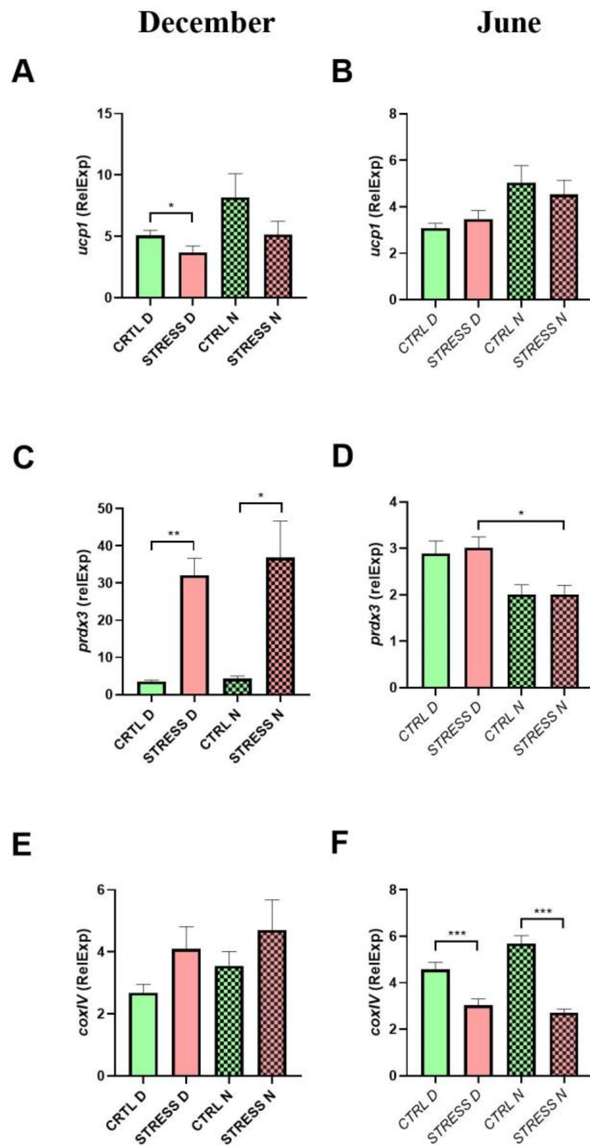
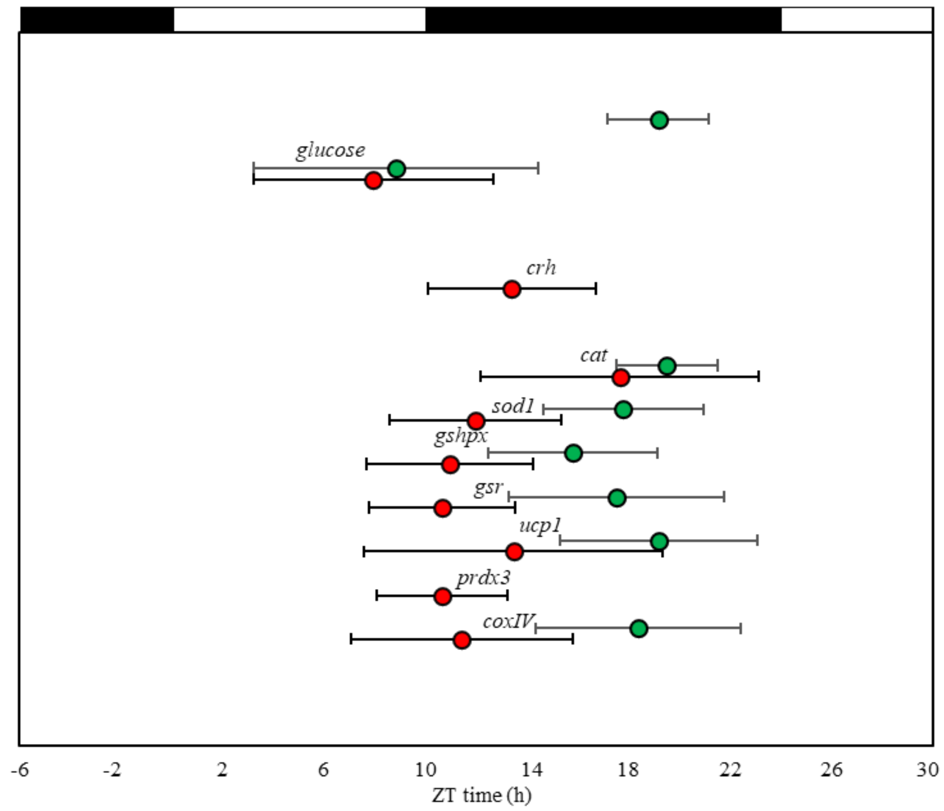


Fig. 7 Day-night differences in the relative mRNA levels (fold change) of *ucp1* (A, B), prdx3 (C, D) and coxIV (E, F) in the liver of European sea bass. For each group and season, values were obtained by pooling all data from either the light phase (day, D) or the dark phase (night, N). Values from each season (December, left panels, and June, right panels) were compared by means of Student's t-test or Mann-Whitney. Asterisks indicate statistically significant differences between groups within one of the seasons.

DISCUSSION

In this study, we investigated the stress response of the European sea bass following an acute stress challenge (1-min air exposure) and explored how it was influenced by the time of day and the season of the year. Most of the stress indicators analyzed exhibited daily rhythms in either the CTRL group, the STRESS group, or both (Fig. 8). When a rhythm was present in both groups, a shift in the acrophase between CTRL and STRESS was observed (Fig. 8). The physiological response to stress was also influenced by the time of day and the season in which the stress was applied, and these effects were different depending on the parameter evaluated (Fig. 9). For example, certain parameters of the HPI axis, such as cortisol and glucose, displayed a more significant response during the day than at night in June. In contrast, in December, the response of certain elements of the HPI axis, such as *crh* and *crh-bp* transcripts, was greater at night. The season played a crucial role as it modulated both the presence of daily rhythms, which were more prominent in December, and the amplitude of the response to stress, which was higher in June than in December (Fig. 9).


A
December
glucose
crh
cat
sod1
gshpx
gsr
ucp1
prdx3
coxIV
-6
-2
2
6
10
14
18
22
26
30
ZT time (h)

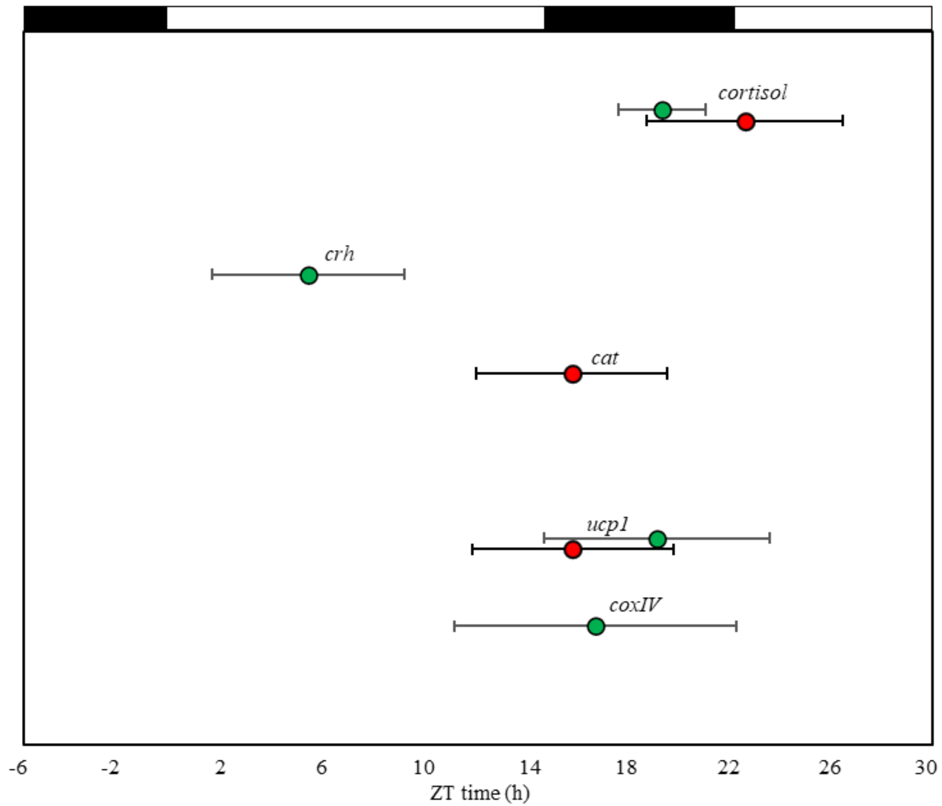

B
June
cortisol
crh
cat
ucp1
coxIV
-6
-2
2
6
10
14
18
22
26
30
ZT time (h)

Fig. 8 Map of acrophases of plasmatic levels of stress indicators and mRNA levels of genes involved in physiological stress and oxidative responses analyzed in December (A) and June (B) experiments. The acrophase is indicated only for the statistically significant rhythms (Cosinor, $p < 0.05$). The name of each represented factor is indicated to the right. CTRL and STRESS groups are represented by the green and red circles, respectively. The white and dark bars above the panels represent the light and dark phases, respectively (10:14 LD for December and 15:9 LD for June). Time scale (x-axis) is expressed as ZT, in which ZT 0 h corresponds to light onset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

	December		**June**	
	Day	Night	Day	Night
Cortisol	↑	↑	↑	—
Glucose	—	—	↑	—
Lactate	↑	↑	↑	↑
crh	↓	—	↓	↑
crh-bp	↑	↑	↑	↑
cat	↓	↓	↑	↑
sod1	—	—	↑	↑
gshpx	↑	—	↑	↑
gsr	—	—	—	—
ucp1	↓	—	—	—
prdx3	↑	↑	—	—
coxIV	—	—	↓	↓

Fig. 9 Summary of the response to an acute stress challenge of plasma stress indicators (cortisol, glucose and lactate) and relative mRNA levels of genes involved in physiological stress and oxidative responses.

Cortisol is a standard parameter for evaluating the stress response in fish (Ellis et al., 2012). European sea bass exposed to acute stress exhibits a rapid increase in cortisol levels, reaching the highest values around one hour post-stress (Fatira et al., 2014). Another stress response in fish subjected to acute stress involves increased glucose and lactate levels. Specifically, glucose levels remain elevated for up to eight hours post-stress, while high lactate levels persist for two to four hours post-stress in sea bass (Fanouraki et al., 2011). In addition, the hypothalamus represents the main area responsible for the neuroendocrine control of the stress response and is the region where crh and crh-bp genes are mainly expressed. *Crh* stimulates the production and release of adrenocorticotropic hormone (ACTH) from the pituitary and is crucial for regulating

cortisol production in fish (Wendelaar Bonga, 1997), while *crh-bp* acts as its antagonist (Huising et al., 2004). In our study, European sea bass exposed to 1 min of air exposure mainly exhibited increases in cortisol and lactate, and also glucose at different time points in both December and June. Furthermore, *crh-bp* expression indicated that a physiological response to this acute stress was triggered at various points along the sea bass stress axis. Moreover, when fish are subjected to an acute stress challenge involving air exposure followed by reoxygenation, they become particularly sensitive to oxidative damage due to increased oxygen consumption and the generation of free radicals (Welker et al., 2012; Qi et al., 2020). This effect is counteracted by an increase in enzymatic and non-enzymatic antioxidant systems, which work together to alleviate the damage caused by oxidative stress (Qi et al., 2020; Wu et al., 2023). In the present study, antioxidant enzymes (*cat, sod1*, and *gshpx*) responded similarly to stress, generally exhibiting an increase in their expression after air exposure, especially in the June sampling. In this study, we also investigated various mitochondrial factors involved in maintaining oxidative/antioxidant balance, such as *ucp1*, *prdx3*, and *coxIV* (Vera et al., 2014). However, these factors exhibited minimal effects in response to stress, with only *prdx3* showing an increase after acute air exposure and reoxygenation in December.

The presence of daily rhythms in several components of the HPI axis has been previously documented in different fish species (Cowan et al., 2017). Regarding cortisol, in the present study this hormone showed a daily rhythm in control groups at both December and June. The acrophase of cortisol is related to the behavior of the species and anticipates the onset of their active phase. For instance, nocturnal fish like Senegalese sole (*Solea senegalensis*) present the acrophase at the end of the light phase, while diurnal fish like gilthead seabream present it at the end of the dark phase (López-Olmeda et al., 2013; Vera et al., 2014). This pattern was also observed in our diurnal model, the

European sea bass, with cortisol rhythms peaking towards the end of the dark phase (ZT 20). The genes associated with the antioxidant system in the European sea bass also exhibited daily rhythms, primarily in December. In the control (non-stressed) groups, all analyzed genes displayed nocturnal acrophases. In December, the STRESS group also exhibited daily rhythms, with an acrophase occurring several hours before that in the CTRL group but still during the dark phase. This suggests that diurnal animals might prepare their antioxidant defenses during their resting period, leading to increased expression during this phase irrespective of stress conditions. Whether the expression of these enzymes in sea bass is under the control of the circadian molecular clock will require further investigation in the future. In mammals, the antioxidant response appears to be linked to the circadian system through the clock gene *bmal1*, which mediates cellular responses to oxidative stress (Khapre et al., 2011). In fish, a connection between the circadian and antioxidant systems has been identified in the gills of Atlantic salmon (*Salmo salar*) (Lazado and Voldvik, 2020), highlighting the significance of the light-dark cycle in the efficiency of antioxidant defenses. Daily rhythms were also observed in the mitochondrial markers *ucp1* and *coxIV*, with acrophases occurring during the dark phase, similar to the genes associated with antioxidant defenses. Rhythms in *coxIV* have been previously described in the gilthead seabream (Vera et al., 2014). Both CoxIV and Ucp1 play roles in oxidative phosphorylation in mitochondria, which is connected to feeding and energy availability (Krauss et al., 2005; Ramzan et al., 2021). Since the expression of *coxIV* and *ucp1* in sea bass peaked several hours after feeding, the observed rhythms in sea bass may be related not only to oxidative processes but also to feeding time, as suggested in seabream (Vera et al., 2014).

In this study, we revealed that the response of many analyzed parameters was influenced by the time of day and the season when the acute stress was applied.

Specifically, in June, the increase in cortisol and glucose was higher during the day compared to nighttime. In contrast, the increase in *crh* expression during the same season was higher at night than during the day. Similar to various physiological variables displaying circadian rhythms, their responses to external factors may exhibit variations throughout the 24-h day (Cowan et al., 2017; Sánchez-Vázquez et al., 2019). The daily variation in stress response was initially described in the cortisol production of mammalian adrenal glands and has been well characterized (Sage et al., 2001). In fish, including the European sea bass in our study, similar daily rhythms in stress response have been reported in species like the Senegalese sole, gilthead seabream, green sturgeon (*Acipenser medirostris*), and African catfish (*Clarias gariepinus*) (Lankford et al., 2003; López-Olmeda et al., 2013; Vera et al., 2014; Manuel et al., 2016). Unlike mammals, the mechanisms driving these daily variations in fish remain unexplored, although data from sole and seabream suggest that the response might be stronger when stress is applied during the resting phase (Cowan et al., 2017; Sánchez-Vázquez et al., 2019). However, in the present study, both cortisol and glucose responses to stress in the European sea bass were higher during the active phase (light phase). An alternative hypothesis would be that the daily rhythm and the phase of higher sensitivity would be species dependent and may not be related to behavior. In addition, the influence of other factors such as feeding could not be discarded. Feeding acts as a significant synchronizer, especially in peripheral tissues, when provided daily at a fixed time, as in our study (López-Olmeda, 2017; Deota et al., 2023). Further research is necessary to ascertain whether feeding time influenced the daily rhythms in stress response in sea bass.

Furthermore, differences depending on the season of the year were observed in our study. For example, in the HPI axis, the cortisol increase after an acute stress challenge was higher in December, while glucose and lactate levels were higher in June. Regarding

the antioxidant system, its response to stress was greater in June, especially for *sod1* and *gshpx*. In contrast, some mitochondrial markers like *prdx3* and ucp1 exhibited a higher response to stress in December than in June. To date, previous studies in fish have primarily focused on seasonal variations in specific parameters of the HPI axis, mainly cortisol (Cowan et al., 2017), with few studies also focusing on antioxidant defenses (Morozov et al., 2017). The idea that the season can affect the antioxidant system is also supported by another study in sea bass, which showed that photoperiod length modified antioxidant enzyme activities in different tissues (Li et al., 2021). Most of these previous studies have mainly investigated single time points of cortisol basal levels at different seasons and only a few studies have investigated how daily rhythms of cortisol vary depending on the season of the year in fish species such as the brown trout (Salmo trutta) (Pickering and Pottinger, 1983) and the Atlantic salmon (*Salmo salar*) (Thorpe et al., 1987). To our knowledge, this research is the first to investigate the influence of the season on both the stress and antioxidant responses to an acute stress challenge in a fish species. Seasonal variations in cortisol were initially hypothesized to be temperature dependent, but they appear to be more correlated with physiological processes such as reproduction and migration (Lamba et al., 1983; White and Fletcher, 1984; Thorpe et al., 1987). In our study, juvenile undifferentiated fish were used, which might explain why the rhythms in control (non-stressed) fish were similar between December and June. However, cortisol response was higher in December, which coincides with the migration and spawning season in the European sea bass (Pawson et al., 2007; Villamizar et al., 2012). Therefore, some influence from factors related to these processes or due to photoperiod length, even though the fish were not mature, may not be discarded.

In the case of genes related to the antioxidant system, temperature might have had a significant influence on the greater response observed in June in the present study.

Seasonal variations in the antioxidant system of fish have been previously observed in another species, the common bream *Abramis brama* (Morozov et al., 2017). In this species, the activity of antioxidant enzymes such as SOD, CAT, and GST was higher during the summer. Two hypotheses support a greater antioxidant activity during warmer months. Firstly, metabolic activity in fish is correlated with water temperature (Morozov et al., 2017), and free radical production from metabolism also increases at high temperatures, stimulating the antioxidant machinery. Secondly, in aquatic ecosystems, water temperature modifies dissolved oxygen levels, which, in turn, influence antioxidant defenses (van der Oost et al., 2003). In June, water is warmer compared to December, implying lower oxygen levels. When fish face low oxygen levels, they can increase antioxidant levels as a preventive measure against future reoxygenation and oxidative damage, a phenomenon known as "preparation for oxidative stress" (Hermes-Lima et al., 1998; Morozov et al., 2017). These mechanisms might explain the higher antioxidant response observed in June in the European sea bass.

Conversely, mitochondrial markers showed a higher response in December, especially *prdx3*. This protein can reduce H2O2 due to its cysteine residue at the NH2-terminal region and is considered an essential scavenger of H2O2 in cells (Rhee et al., 2005). One hypothesis for the differences observed in the response between these mitochondrial markers and antioxidant enzymes could be that both systems might coordinate their responses differently based on the season of the year (or associated factors like photoperiod or temperature). Thus, at least one system would show a greater increase in response to air exposure and subsequent reoxygenation in each season. However, further research is needed to elucidate this hypothesis.

CONCLUSIONS

Given the significance of the European sea bass in aquaculture, understanding how handling and air exposure can impact its welfare could aid in developing more effective strategies for aquaculture facilities and procedures. In this research, we have demonstrated that several stress markers in the European sea bass exhibit variations throughout the day, indicating that the stress response depends on the time of day when the stressor is applied. Moreover, the season of the year also appears to play a crucial role, as it influences the presence of daily rhythms and the magnitude of the response. Therefore, it is essential to consider not only the time of day but also the season for routine aquaculture procedures involving this species.

AUTHOR STATEMENT

ES, JFLO, JAMC, FJSV conceived and designed the experiments; ES wrote the manuscript; JFLO, JAPS, JAMC and FJSV revised the manuscript; ES, IR and LSA performed the experiment; ES and JFLO analyzed the data; JFLO and FJSV provided funding.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Elisa Samorì: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Inmaculada Rodríguez: Investigation. José Antonio Paullada-Salmerón: Conceptualization, Investigation, Writing – review & editing. Laura Sánchez-Alacid: Investigation. José Antonio Muñoz-Cueto: Conceptualization, Funding acquisition, Writing – review & editing. Francisco Javier Sánchez-Vázquez: Funding acquisition, Writing – review & editing. José Fernando López-Olmeda: Conceptualization, Formal analysis, Funding acquisition, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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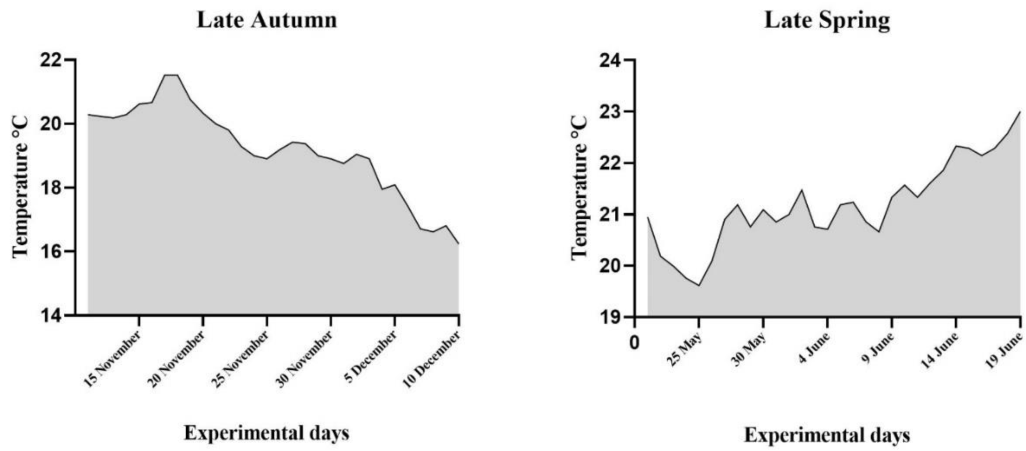
SUPPLEMENTARY DATA



Supplementary Fig. 1. Variation of the average daily water temperature in late Autumn and late Spring, during the 30 days before the samplings in December (A) and June (B), respectively.

Supplementary Table 1. Acrophases, mesor, amplitude and *p* values of plasmatic stress indicators and genes involved in physiological stress and oxidative responses subjected to Cosinor analysis. Data are expressed as value ± fiducial limits (set at 95%). The acrophases are indicated in ZT (*zeitgeber* time).

Biological parameters	Season	Experimental group	Significance variance	Mesor	Amplitude	Acrophase (ZT hours)
Cortisol	December	ctrl	0.00509	52.66 ± 22.85	59.70 ± 86.95	19.55 ± 2.92
		stress				
	June	ctrl	0.00006	85.09 ± 31.3	115.14 ± 57.9	19.33 ± 1.85
		stress	0.03143	288.84 ± 50.62	95.44 ± 88.24	22.83 ± 4.1
Glucose	December	ctrl	0.00084	46.81 ± 2.62	8.22 ± 5.03	8.40 ± 2.12
		stress	0.04361	54.71 ± 6.83	7.48 ± 7.30	7.46 ± 5.1
	June	ctrl				
		stress				
Lactate	December	ctrl				
		stress				
	June	ctrl				
		stress				
crh	December	ctrl				
		stress	0.0095	2.8 ± 0.35	0.74 ± 0.58	13.33 ± 3.57
	June	ctrl	0.02834	1.97 ± 0.20	0.41 ± 0.35	4.40 ± 4..21
		stress				
crh-bp	December	ctrl				
		stress				
	June	ctrl				
		stress				
cat	December	ctrl	0.00051	6.81 ± 1.29	4.12 ± 2.43	19.91 ± 2.15
		stress	0.049	4.58 ± 1.1	1.98 ± 1.97	17.93 ± 5.63
	June	ctrl	0.00879	2.17 ± 0.21	0.44 ± 0.33	15.53 ± 4.03
		stress				
sod1	December	ctrl	0.00901	3.92 ± 0.54	1.29 ± 1	18.4 ± 3.75
		stress	0.01299	2.13 ± 1.73	2.13 ± 1.73	11.78 ± 3.57
	June	ctrl				
		stress				
gshpx	December	ctrl	0.00594	3.58 ± 0.82	1.91 ± 1.42	15.90 ± 3.59
		stress	0.01075	5.12 ± 1.16	2.48 ± 1.97	10.70 ± 3.39
	June	ctrl				
		stress				
gsr	December	ctrl	0.02623	2.61 ± 0.48	0.97 ± 0.87	17.75 ± 4.57
		stress	0.00672	3.02 ± 0.59	1.35 ± 1.02	10.36 ± 7.26
	June	ctrl				
		stress				
ucp1	December	ctrl	0.02954	6.56 ± 1.69	3.52 ± 3.22	19.56 ± 4.18
		stress	0.04285	4.57 ± 1.04	1.80 ± 1.75	13.40 ± 5.35
	June	ctrl	0.0362	3.71 ± 0.61	1.26 ± 1.19	19.11
		stress	0.01479	3.95 ± 0.57	1.14 ± 0.96	15.56 ± 4.25
prdx3	December	ctrl				
		stress	0.00337	33.02 ± 8.48	21.10 ± 14.73	10.38 ± 2.78
	June	ctrl				
		stress				
coxIV	December	ctrl	0.02856	3.02 ± 0.47	0.97 ± 0.92	18.70 ± 4.34
		stress	0.03883	4.56 ± 1.03	1.83 ± 1.75	11.21 ± 4.7
	June	ctrl	0.04449	5.02 ± 0.45	0.82 ± 0.80	13.51 ± 3.11
		stress				

Supplementary Table 2. *P* values obtained in the two-way ANOVA analyses for the comparisons between sampling times (*zeitgeber* time, ZT), treatment (T, CTRL *vs* STRESS) and the interaction between them. Samples from December and June were analyzed separately.

	December			June		
Biological parameter	**ZT**	**T**	**ZT * T**	**ZT**	**T**	**ZT * T**
cortisol	0.025	0.000	0.240	0.000	0.000	0.002
glucose	0.000	0.000	0.001	0.101	0.000	0.300
lactate	0.000	0.000	0.153	0.000	0.007	0.023
crh	0.009	0.000	0.146	0.202	0.207	0.003
crh-bp	0.062	0.000	0.216	0.612	0.002	0.311
cat	0.000	0.000	0.031	0.000	0.000	0.456
sod1	0.035	0.717	0.086	0.031	0.842	0.279
gshpx	0.024	0.014	0.359	0.270	0.000	0.492
gsr	0.158	0.369	0.012	0.568	0.494	0.552
ucp1	0.075	0.008	0.028	0.031	0.842	0.279
prdx3	0.013	0.000	0.018	0.000	0.755	0.935
coxIV	0.294	0.016	0.090	0.006	0.000	0.223

Supplementary Table 3. P values obtained in the three-way ANOVA analyses for the comparisons between sampling times (*zeitgeber* time, ZT), treatment (T, CTRL *vs* STRESS), season (S, December *vs* June) and the interactions between them.

Biological parameter	**ZT**	**treatment (T)**	**season (S)**	**ZT * T * S**
Cortisol	0.000	0.000	0.000	0.046
Glucose	0.000	0.002	0.000	0.018
Lactate	0.000	0.002	0.000	0.026
crh	0.056	0.000	0.000	0.152
crh-bp	0.215	0.000	0.000	0.517
cat	0.000	0.806	0.000	0.018
sod1	0.582	0.000	0.000	0.996
gshpx	0.463	0.000	0.000	0.616
gsr	0.123	0.564	0.002	0.056
ucp1	0.000	0.040	0.001	0.056
cox IV	0.028	0.327	0.292	0.434
prdx3	0.008	0.000	0.000	0.015

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Influence of two different stressors (density and restricted feeding) on the circadian clock and epigenetic mechanisms in liver of European sea bass (*Dicentrarchus labrax*).

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Abstract

The physiological stress response in fish starts with the activation of the hypothalamus-pituitary-interrenal (HPI) axis, leading to a subsequent release of cortisol into the bloodstream. Cortisol exerts its function by binding the glucocorticoid responsive element (GRE) located upstream of target genes, inducing or reducing their transcription. The clock system governs different physiological pathways including the rhythmic release of cortisol into the bloodstream, but at the same time cortisol can modulate the transcription of clock genes. The connection between the stress response and clock genes can be extended to the epigenetic mechanisms since genes involved in epigenetic processes are particularly responsive to stress and they can also be rhythmically transcribed. This study was set to investigate the effect of two chronic stress, high density (HD) and low feeding (LF) on clock and epigenetic genes in liver of European sea bass at different days of treatment (5, 25 and 60) with sampling during the day and during (ML) and the night (MD). The results showed an early response of the epigenetic genes in both conditions (HD and LF) at day 5, followed by the clock genes at day 25, and concluded with the concomitant dysregulation of both epigenetic and clock genes at day 60. Even though the day- or night- response was gene specific, it was possible to identify a trend where most of the genes responded differently during the night at day 60, with density affecting the epigenetic system more and calories restriction the clock system coupled with *dnmt3a*. The Principal Component Analysis revealed that the genes that most contributed in the pattern of gene expression observed for HD trials were genes involved in DNA methylation (*dnmt1* and *dnmt3a*), deacetylation (*sirt1*) and two clock genes (*per1b* and *clock1b*) while for LF trials it was not possible to describe a significative influence. Our data suggest that genes involved in methylation and deacetylation could provide early information about the stress response of the fish.

Introduction

All the organisms are subjected to environmental variables whose cyclic occurrence entrains physiological and behavioral processes by entraining the pacemakers distributed in different animal tissues. The molecular clock represents the core of the circadian timing system. (Pando and Sassone-Corsi, 2002, Lopez-Olmeda, 2017). The genetic regulation of these mechanisms is mediated by a self-sustaining feedback loop where the main circuit starts with the transcription of *clock* (Circadian Locomotor Output Cycles Kaput) and *bmal* (Basic helix-loop-helix ARNT-like protein 1). Their products form the heterodimer CLOCK-BMAL that work as a transcriptional factor and target the E-box DNA motif of *per* (Period) and *cry* (Cryptochrome), thus mediating the start of the negative feedback loop. Finally, PER and CRY interfere with CLOK-BMAL complex after entering the nucleus stopping its activity and therefore their own transcription (Vatine et al., 2011, Zhadanova and Reebs 2005, Cox and Takahashi, 2019). The main role of this circuit is to promote the rhythmic transcription of several genes, and in mouse, more of than 40% of the genes oscillate rhythmically during the 24-hour period (Zang et al., 2014). In fish, several studies have focused on how different physiological variables vary throughout the day, suggesting that fish invest energy differently during the 24 hours, probably as a consequence of evolutionary mechanisms (Krittika and Yadav, 2019).

The molecular clock system is then linked to several physiological pathways, but also the epigenetic mechanisms. A study in the teleost model zebrafish (*Danio rerio*) has recently described the rhythm of genes involved in methylation and methylation in gonads (Paredes et al., 2018), but the link between the two systems could be more complicated because of reciprocal regulation (Stevenson et al., 2018) which, at least in mammals, could be mediated by DNMT*3a/b* activity toward *Bmal1a'*s promoter (Satou et al., 2013).

DNA methylation is one of the most studied epigenetic modifications. It is mediated by DNA methyltransferase (DNMTs), which add a methyl group received from methionine entering the 1C cycle to a cytosine, to form 5-methylcytosine and silencing transcription (Jurkowska et al., 2011, Moore et al., 2013; Friso et al., 2017). The enzymes involved in the process can be implicated in the maintaining the methylation after the round of DNA replication (DNMT1) or promote *de-novo* methylation (DNMT3s), even though they can also cooperate in the establishment of the latter modification (Fatemi et al., 2002). Methylation is directly linked to cellular metabolism, since it relies on methionine which is assumed through the diet and enters in the 1-C cycle (Friso et al., 2017), making also folate a good candidate in term of impact on the DNA methylation processes as shown in mammals (Anderson et al., 2012; Lim and Song, 2020). At the same time, in fish, different studies have shown that not only methionine and folate can modulate DNA methylation (Skjaerven et al., 2018), but also arachidonic acid (ARA) (Adam et al., 2019) or high-carbohydrate diet (Cai et al., 2020), highlighting the importance of the feeding in methylation processes. Once methylation is established, it can be retained or erased, and in the latter case, specific enzymes are involved in the active removal (Ito et al., 2010, Moore et al., 2013).

Different epigenetic factors have also been recently identified as important players in the physiological stress response, like the deacetylase SIRT1 which has been found to physically interact with the glucocorticoid receptor (GR) by being recruited to the glucocorticoid responsive element (GRE), enhancing the GR induced transcription (Suzuki et al., 2018). SIRT1 is a NAD+-dependent class III HDAC primarily located in the nucleus with diverse roles. In specific, SIRT1 is involved in the epigenetic mechanisms, possessing deacetylase activity towards lysine residues (Seto and Yoshida, 2014), whose variations throughout the 24 hours have been described in fish liver (Wang

et al., 2022), suggesting a regulation mediated by the circadian system. Moreover, SIRT1 can counterbalance the acetylation activity of CLOCK (Doi et al., 2006, Nakahata et al., 2008), or deacetylate PER2 (Asher et al., 2008) or BMAL1 (Masry et al., 2014) thus creating a bidirectional connection with the circadian clock. Additionally, sirtuins are also involved in the regulation of glucose and lipid metabolism, as seen in fish (Mirabet et al., 2018, Mu et al., 2024), and its dependency on the NAD+ coupled with enhanced GR-activity, creates a link between cellular metabolism, epigenetic, and stress response.

In aquaculture conditions, fish can be exposed to different stressors, with stocking density and inadequate feeding conditions being the most common (Montero et al., 1999, Ashley et al, 2006, Gornati et al., 2004). Even though short-term and prolonged stress response rely on the same physiological mechanism, their consequences can be extremely different. In specific, chronic stress is the one that can lead to a wide range of maladaptive responses which can include growth impairment, reproductive issues, compromised immune functions, diseases or even death (Ashely et al., 2006, Dai et al., 2022, Murugananthkumar and Sudhakumari 2022) and represents one of the main issues in aquaculture. The stress response represents one of the most important adaptive mechanisms allowing the organism to cope with an external cue that disturbs the homeostasis (Ellis et al., 2012). The stress response is characterized by three response levels, where the primary refers to the activation of the brain-sympathetic-chromaffin (BSC) and hypothalamus-pituitary-interrenal axis (HPI), the secondary is characterized by compensatory physiological processes which are the consequences of the released hormones, while the tertiary response represents the behavioral changes and the whole animal performance (Wenderlaar Bonga, 1997, Ellis, 2012, Gorissen and Flik, 2016). One of the main stress indicators used to assess fish welfare is cortisol (Ellis, 2012), which in teleost fish corresponds to the last step of the neuroendocrine stress response. Cortisol

exerts its functions after binding to its receptor (GR), which dissociates from heat proteins and translocate into the nucleus to target glucocorticoid responsive element (GRE) located upstream of specific target genes, eventually modulating their transcription (Faught, 2016). These well-conserved sequences have been identified upstream several genes, including genes that are part of the molecular clock (Balsalobre et al., 2000, Yamamoto et al., 2000, Dickmeis et al., 2013) and genes involved in methylation in mammals (Urb et al., 2019).

European sea bass (*Dicentrarchus labrax*) represents one of the most farmed species in the Mediterranean sea (Vandeputte et al., 2019), which exhibits a high physiological stress response (Fanouraki et al., 2011, Fatira et al., 2013, Samorì et al., 2024), but how its circadian system and epigenetic processes respond to chronic stress is still unclear. Due to the importance of these molecular pathways and their involving in multiple processes, this study aimed to investigate the chronic stress response of European sea bass by exploring a panel of genes potentially affected by stress, which share multiple connections. Specifically, gene of the circadian system and epigenetic mechanisms were considered after submitting European sea bass to two different types of chronic stress: high density and low feeding. The additional purpose was to understand how these genes respond both during the day and night, since a previous study has shown that stress response changes during the day in European sea bass (Samorì et al., 2024) For this purpose, we exposed fish to different density and feeding conditions for 60 days collecting samples at different time points during the day and during the night.

Materials and methods

Ethical statement

The experiments were designed according to European Union guidelines (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use of laboratory animals. They were approved by the Committee of the University of Murcia on Ethics and Animal Welfare and the Government of *Región de Murcia* (license number A13191003).

Animal housing

The European sea bass specimens (N = 900; 49.0 ± 1.94 g, mean ± SEM) were retrieved from the local fish farm CUPIBAR S.L. (Cádiz, Spain) in April 2022 and housed in 450 l tanks (8 kg/m^3) equipped with biological and mechanical filters subjected to a daily change of part of the water. A commercial diet (Alterna Marine, Skettring, Burgos, Spain) was used to feed fish during the entire housing period considering 1.5% of their body weight. The light was provided by fluorescent bulbs (intensity at the surface: 200 lx) and the photoperiod was set according to the seasonal variation throughout April and May by means of a timer (Data Micro, Orbis, Madrid, Spain). The temperature (19°C) and dissolved oxygen (6 mg/l) were controlled via The Pacific Oxyguard system (Pentair, Apopka, USA). Fish were maintained in these conditions for two months before the start of the treatment.

Experimental design

The experiment was designed to test the effect of two different chronic stressors (high density and low feeding) applied separately for a total of 60 days, monitoring the

response at day 5, 25 and 60 of the treatment. For this purpose, at the starting day of the trial, fish were randomly assigned to three different experimental groups considering density (D) and feeding ratio (F) as variables: high feeding-low density (HF – LD or CTRL group), high feeding-high density (HF – HD, density trial), low feeding-low density (LF – LD, feeding trial). The fish were then divided into different tanks which differed in volume and helped to create different densities. For this purpose, were used six tanks of 300 l containing 30 fish (three tanks for the control and three for LF-LD), and three tanks of 120 l containing 120 fish (for HF-HD condition), to create the condition of 5 kg/m^3 or 50 kg/m^3 respectively which represent low (LD) or high density (HD). Low feeding (LF) was set at 0.5% while high feeding (HF) was set at the 1.5% of the initial body weight (49.0 ± 1.94 g, mean ± SEM). The ratio given during the experiment was provided randomly during the day by the operator and changed accordingly to increasing fish weight across the experiment, which was monitored at the sampling days. For the experiment, the light onset was set at *Zeitgeber* (synchronizer) time 0 h (ZT 0, corresponding in our experiment at 8 a.m.) while light offset at ZT 13 (9 p.m.), creating 13:11 Light/Dark conditions. To evaluate the effects of high density and low feeding over a chronic period, and to determine if the time of day affects the response to these treatments, we established different sampling points: days 5, 25, and 60. On each of these days, we collected samples at two times: in the middle of the light phase (ML, ZT 6.5) and in the middle of the dark phase (MD, ZT 18.5). For each sampling point, we collected samples from 3 randomly selected fish from each tank (total n = 9 per experimental group). Fish were anesthetized with clove oil essence (Guinama, Valencia, Spain) which was diluted in 9 parts of ethanol and then added at a concentration of 50 µL/L to the water. Fish were then sacrificed by decapitation to collect liver, which was snap-frozen in dry ice and subsequently stored at -80°C until analysis. During the nocturnal sampling, a red dime light (λ > 600 nm) was used to avoid light contamination as in others

chronobiological studies (de Alba et al., 2019). After each sampling, density conditions were adjusted modifying water volume to ensure the same conditions during the whole experiment.

Rna extraction, cDNA synthesis and Reat-Time RT-PCR analysis

Liver samples from the different time points (day 5, 25 and 60) were homogenized in Trizol reagent (Invitrogen, Thermo Fisher Scientific, Baltics UAB) prior RNA extraction which were performed following manufacturer's instructions. A spectrometer was (Nanodrop® ND 1000, Thermo Fisher Scientific) used to check for concentration and purity of the RNA and 1U of DNase I (Thermo fisher) each 1 µg of RNA was then added to each sample to avoid genomic contamination. The commercial kit Reverse Transcriptase (QSCRIPT cDNA Synthesis Kit, Quantabio Beverly, USA) and a thermocycler (MiniAmp Thermal Cycler, Thermo Fisher) were used to produce the cDNA. To perform the q-PCR analysis, the obtained cDNA was diluted in the proportion 1:10, and 2.5 µl were add to every reaction with the master mix Perfecta SYBER Green Fastmin (Quantabio) and selected primers in a final volume of 10 µl. The following steps were then run by a thermocycler: 10 min at 95 ◦C, followed by 40 cycles of 15 s at 95 ◦C and 1 min at 60°C. Primer used for the selected genes are listed in table 1. The primers sequences were retrieved from literature or designed with *Primer3plus* (Untergasser et al., 2012) and their efficiency was evaluate using dilutions curves of cDNA. The geometric means of the reference genes *ef1a* and *bact* was used in the first normalization of the 2-ΔΔCt method followed by the sample with the higher value for the second normalization (Livak and Schmittgen 2001).

Table 1 Primers used for the qPCR analysis

Gene name	Fw	Rv	Acc. Numbers
ef1a	AGTGAAGCAGCTCATCGTTG	TTGGTGATTTCCTCGAAGCG	AJ866727
bact	TCATCACCATCGGCAATGAG	AACGTCGCACTTCATGATGC	AY148350
clock1b	CCACAGAGCTCCACCCATTA	AAATCCACTGCTGCCCTTTG	ENSDLAG00005012393
bmal1a	TGACGCTAAAACTGGCCTTC	TGCAGAAAAACGACCGTCTG	ENSDLAG00005026433
per1b	CATGGTGAAGACGAACGGAC	CTTTGGGTGGTTTCGTCAGG	ENSDLAG00005015065
cry1a	AGACCACGACGACAAGTTTG	AAGCCTTCCTCTCCAAATGC	ENSDLAG00005023868
dnmt1	ATCAAGCTTGCAGGTGTCAC	TTTGTTGGGTGACGAATGGC	ENSDLAG00005018520
dnmt3	TCATGTGCGGAAACCACAAC	TCTTTGATGGCTGCATGTGC	ENSDLAG00005018806
tet2	TGCCAACAAGAATGCCATGC	AGTGCCAGCTTTTGACTTGG	ENSDLAG00005008342
sirt1	ACGCAAAGTCCCAATGTCAC	ACACTGGGCATTTGGACAAG	ENSDLAG00005013704

Data analysis

Statistical analyses were performed with RStudio, (R version 4.3.1 (2023-06-16) -- "Beagle Scouts" and Rstudio version 2023.06.2+561 RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, URL http://www.rstudio.com). Descriptive statistics are reported as mean ± standard deviation, and significant threshold of statistical test was set at $p = 0.05$. Data were tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test) of the variance before computing the parametric analysis.

We evaluated the effect of density and sampling time on chronic stress response in two steps.

First, we assessed differences on single gene expression levels (number of individual gene = 8) among groups by performing the parametric test 1-way ANOVA followed by T-test or the non-parametric Wilcoxon test to assess the influence of density and low feeding (planned contrast: HF – LD *vs* HF-HD / LF-LD) separated for each sampling time (ML and MD).

In the second step, we compared the effect of treatment on a panel of multi-gene expression levels. Since most of these genes that we analyzed are involved in the same

physiological pathways their response can be due to the co-expression of these genes. To investigate this, we hypothesized that the treatments (HF – HD / LF – LD), could affect not only the single gene but also on the co-expression of multiple genes in a related way. For this reason, we performed a Principal Component Analysis (PCA) to study the effect of the stressors on the panel of multi-genes involved in the stress response. This approach allowed us to summarize the eight correlated genes into a smaller set of new variables, known as principal components, which capture most of the variation on the original data. We performed a nonparametric PCA based on the Spearman rank correlations among pairs of gene expression, using the "PCA" function from the "EFA.dimensions". PCA was computed on the scaled and centered dataset for each sampling point (Day 5, Day 25 and Day 60) and time of the day (Day and Night). We performed 6 separate PCA analyses (2 sampling time point × 3 sampling days) and found that the first two Components explained 62.33 ± 1.85 % of the total variance, with eigenvalues greater than 1.60. Differences among groups, were assessed based on the score extracted from the two Component ("EFA_SCORES" function from "EFATOOLS" R package) followed by Tukey *post hoc* test as previously described (Suppl. Table 1).

Results

Gene expression analysis

At day 5, 1-way ANOVA revealed significant differences for *per1b* (p = 0.023), *dnmt3a* (p = 0.002) and *sirt1* (p = 0.008) during the day (ML), and *dnmt1* (p = 0.017) during the night (MD) (Suppl. Table 1). Post hoc analysis on evaluating the effect of the density as chronic stressor (HF – LD *vs* HF-HD) revealed significant differences during the day in *dnmt3a* (p = 0.014) and *sirt1* (p = 0.021) expression, while the other genes didn't change

significantly respect to the control condition ($p > 0.05$) (Fig. 1, Suppl. Table 2). When considering the effect of feeding condition (HF – LD *vs* LF – LD), post hoc analysis revealed that the feeding affected the expression of *Dnmt3a* mRNA ($p = 0.0004$) and *clock1b* ($p = 0.02$) (Fig. 1, Suppl. Table 2). Data collected during the night displayed only one significative difference, in specific for *dnmt1* ($p = 0.016$) due to the feeding conditions (T-test, $p > 0.05$) (Fig. 1, Suppl. Table 2)

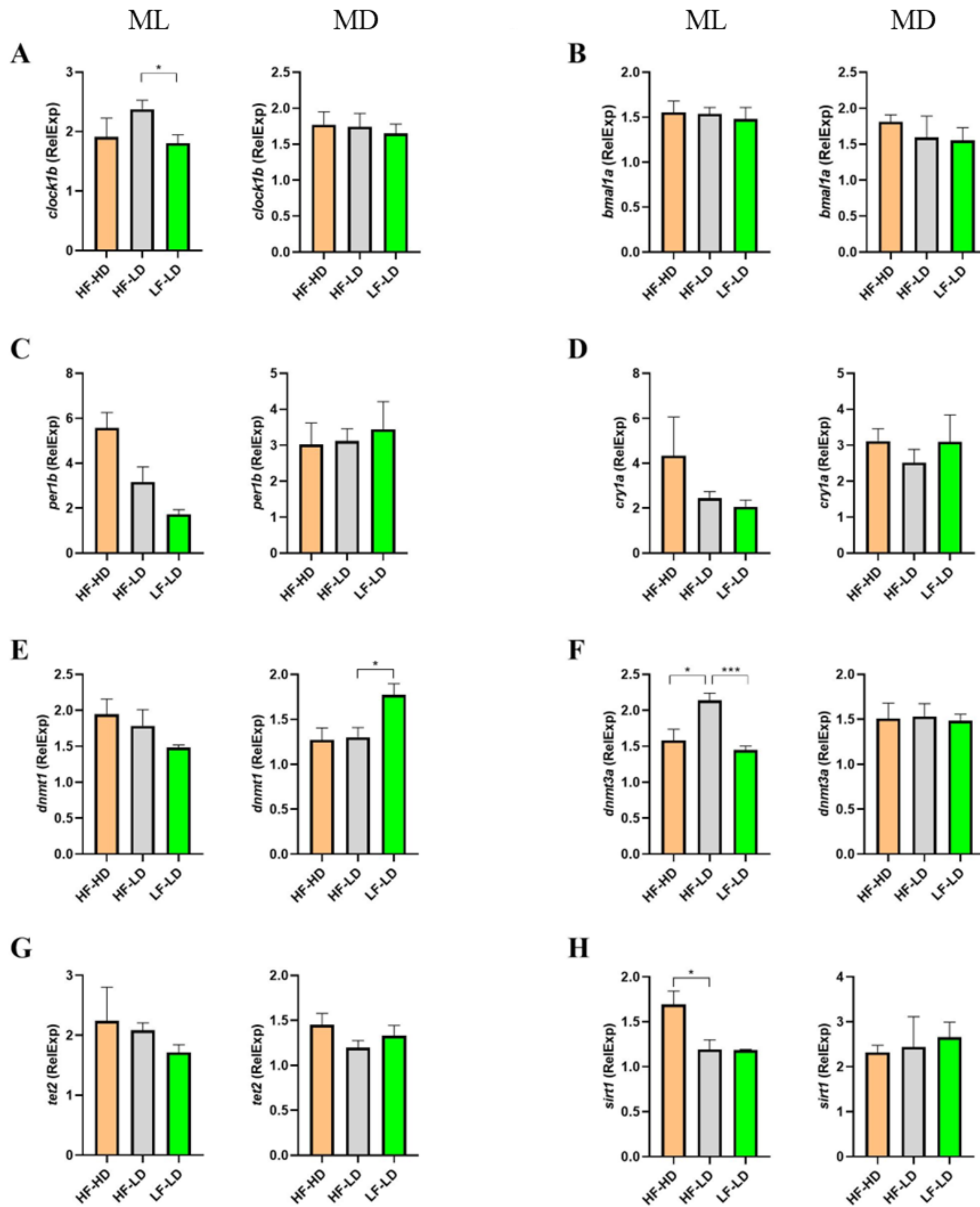


Fig. 1 Relative mRNA expression of *clock1b* (A), *bmal1a* (B), *per1b* (C), *cry1a* (D), *dnmt1* (E), *dnmt3a* (F), *tet2* (G) and *sirt1* (H) in European sea bass liver collected at day 5 in ML (mid-light, ZT 6.5) and MD (mid-dark, ZT 18), left and right panel of each letter respectively. Fish were submitted to two different stressors: HF-HD indicated with orange bars and LF-LD indicated with green bars and compared with control conditions where no stressor were applied for all the experiment (HF-LD, indicated with gray bars). Asterisk indicated statistically significant differences between control conditions and treatments (T-test, $p < 0.05$). Data ($n = 9$) are represented as mean ± SEM

At day 25, significant differences were found for *clock1b* (p = 0.044), *bmal1a* (p = 0.0008), *per1b* (p = 0.008), *cry1a* (0.023) and *tet2* (0.016) during the day (ML) (1-way ANOVA), and *bmal1a* (p = 0.031) during the night (MD) (Suppl. Table 1). The effect of the density (T-test, HF – LD *vs* HF – HD) determined significant differences in the expression between the groups in the genes *clock1b* (p = 0.0005), *per1b* (p = 0.004) and *cry1a* (p = 0.02), while when submitted to low feeding condition (T-test, HF – LD *vs* LF – LD) the genes for which it was possible to describe a significant variation were *bmal1a* (p = 0.007) and *tet2* (p = 0.01) (Fig. 2, Suppl. Table 2). During night, the comparison between control and high-density group (T-test, HF – LD *vs* HF – HD) revealed a significant variation only for *per1b* (p = 0.004). Moreover, submitting the fish to a low-feeding treatment didn't affect the gene analyzed (T-test, HF – LD *vs* LF – LD, p > 0.05) (Fig. 2, Suppl. Table 2).

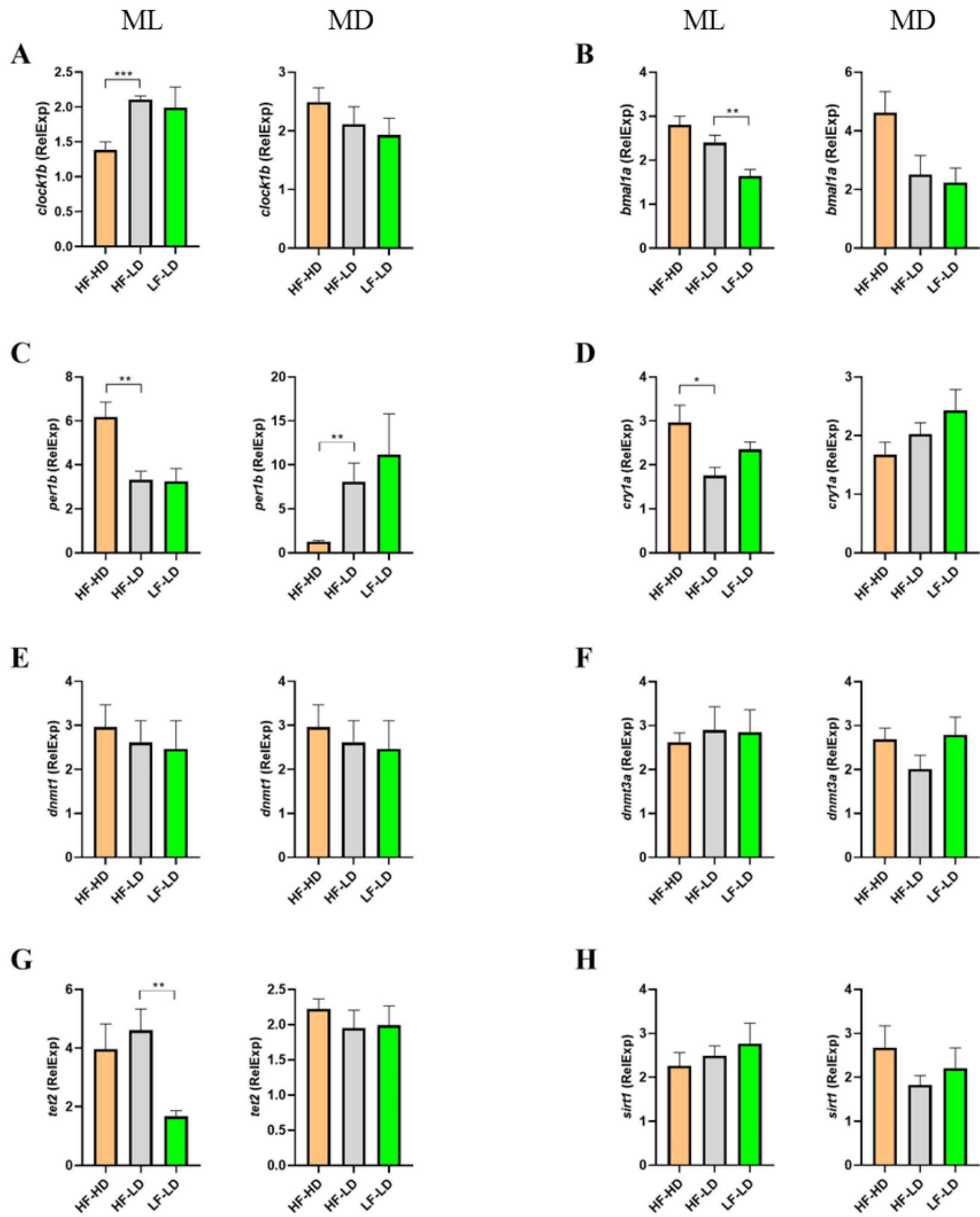


Fig. 2 Relative mRNA expression of *clock1b* (A), *bmal1a* (B), *per1b* (C), *cry1a* (D), *dnmt1* (E), *dnmt3a* (F), *tet2* (G) and *sirt1* (H) in European sea bass liver collected at day 25 in ML (mid-light, ZT 6.5) and MD (mid-dark, ZT 18), left and right panel of each letter respectively. Fish were submitted to two different stressors: HF-HD indicated with orange bars and LF-LD indicated with green bars and compared with control conditions where no stressor were applied for all the experiment (HF-LD, indicated with gray bars). Asterisk indicated statistically significant differences between control conditions and treatments (T-test, $p < 0.05$). Data ($n = 9$) are represented as mean ± SEM.

At day 60, 1-way ANOVA revealed significant differences for *bmal1a* (p = 0.007), *per1b* (p = 0.047), *cry1a* (p = 0.016), *dnmt1* (p = 0.0004) during the day (ML), and *block1b* (p = 0.017) during the day (ML), while during the night significative differences were found for *clock1b* (p = 0.017), *per1b* (0.0004) and *dnmt3a* (p = 0.003) (Suppl. Table 1). The effect of the density (T-test, HF – LD *vs* HF – HD) significantly altered gene expression of *bmal1a* (p = 0.04), *cry1a* (p = 0.008) and *dnmt1* (p = 0.0009) during the day, while low feeding treatment (T-test, HF – LD *vs* LF – LD) significantly affected only *cry1a* (p = 0.045) but no other were significantly affected in terms of expression (Fig. 3, Suppl. Table 2). During the night, *per1b* and *sirt1* mRNA levels were significantly affected by the high-density treatment (p = 1.6E-05 and 0.01 respectively) (T-test, HF – LD *vs* HF – HD), while low feeding treatment (T-test, HF – LD *vs* LF – LD), significantly impact *clock1b* (p = 0.025), *per1b* (p = 0.038) and *dnmt3a* (p = 0.005) gene expression (Fig. 3, Suppl. Table 2).

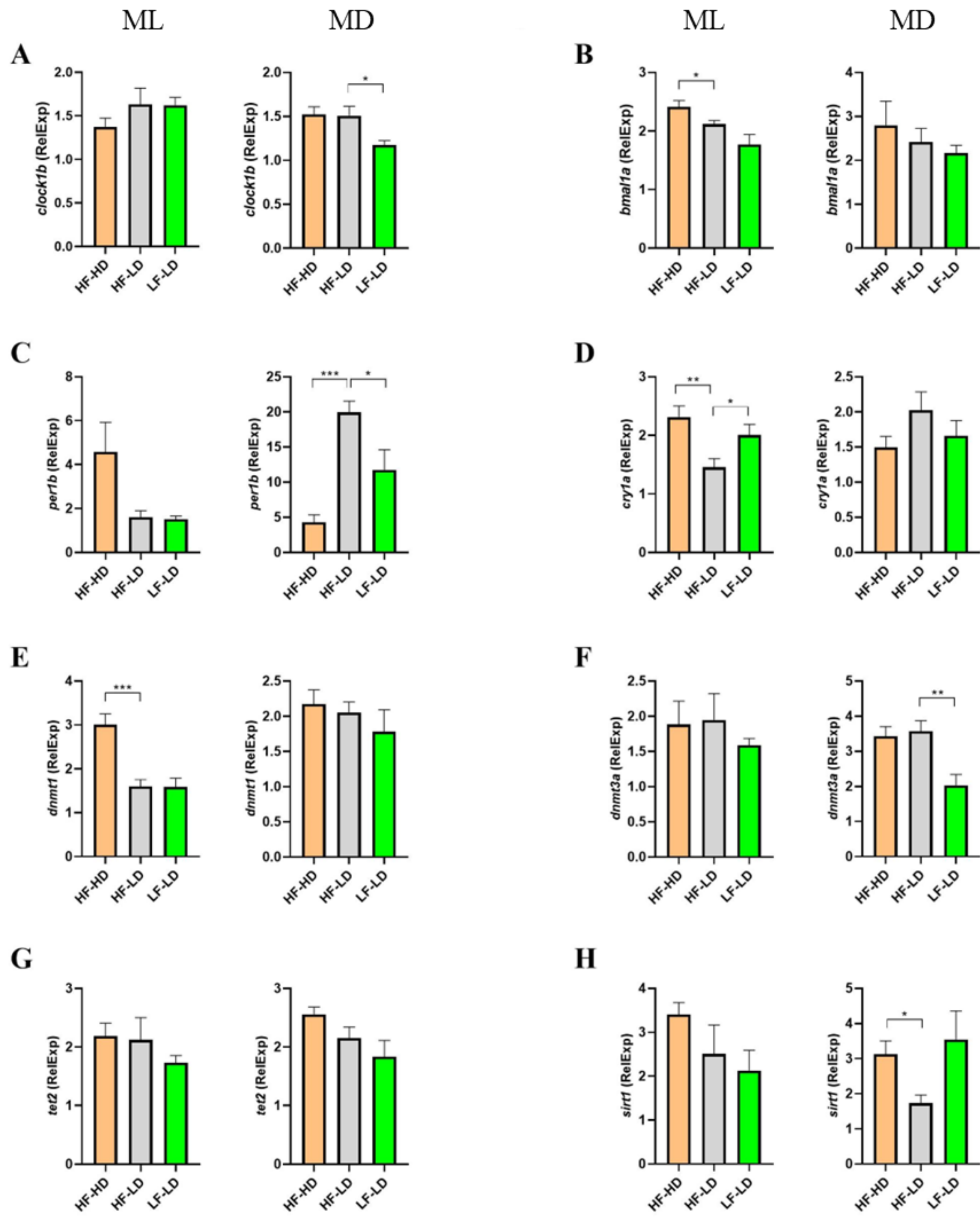
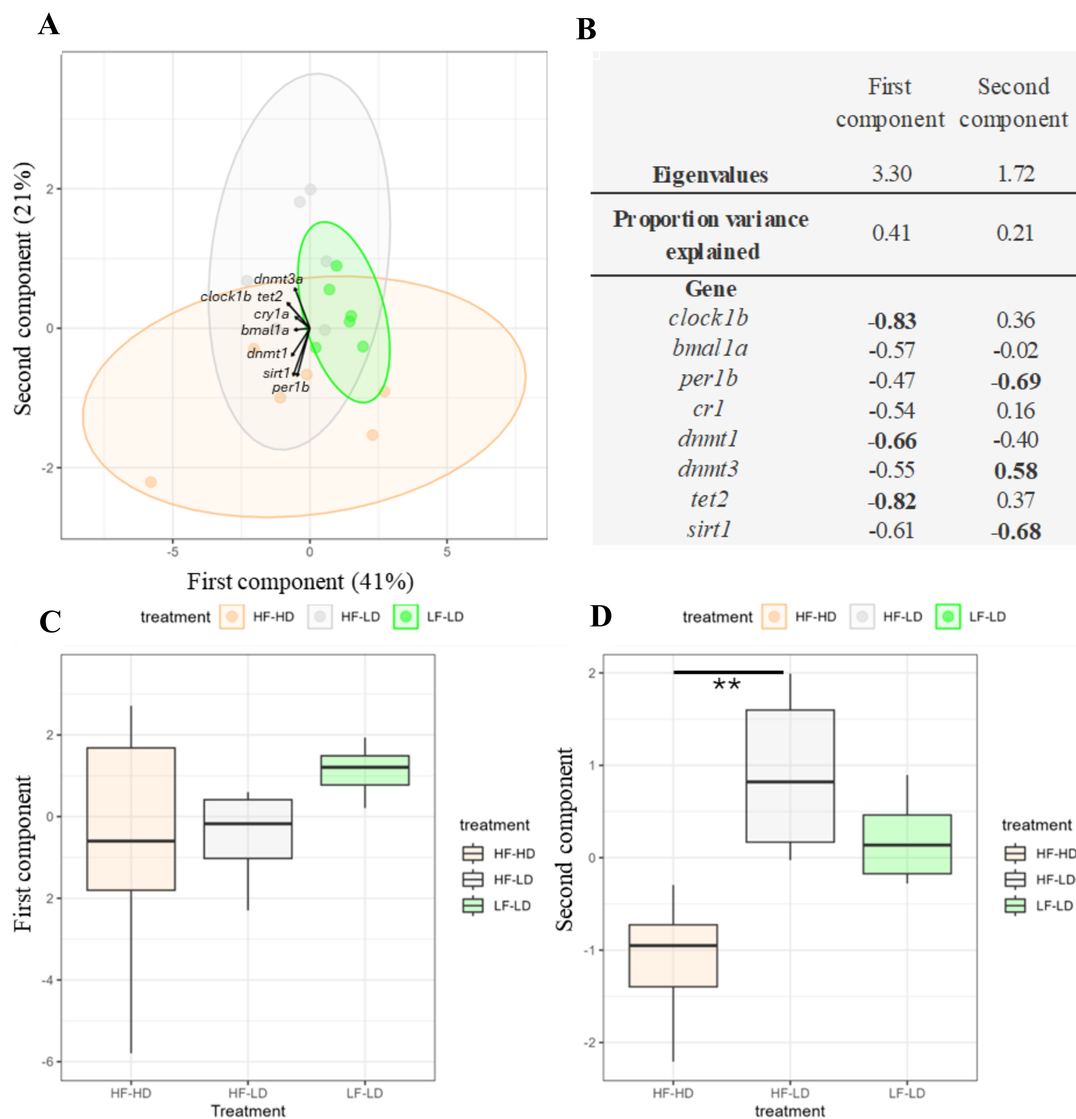


Fig. 3 Relative mRNA expression of *clock1b* (A), *bmal1a* (B), *per1b* (C), *cry1a* (D), *dnmt1* (E), *dnmt3a* (F), *tet2* (G) and *sirt1* (H) in European sea bass liver collected at day 60 in ML (mid-light, ZT 6.5) and MD (mid-dark, ZT 18), left and right panel of each letter respectively. Fish were submitted to two different stressors: HF-HD indicated with orange bars and LF-LD indicated with green bars and compared with control conditions where no stressor were applied for all the experiment (HF-LD, indicated with gray bars). Asterisk indicated statistically significant differences between control conditions and treatments (T-test, $p < 0.05$). Data ($n = 9$) are represented as mean ± SEM.

Principal component analysis

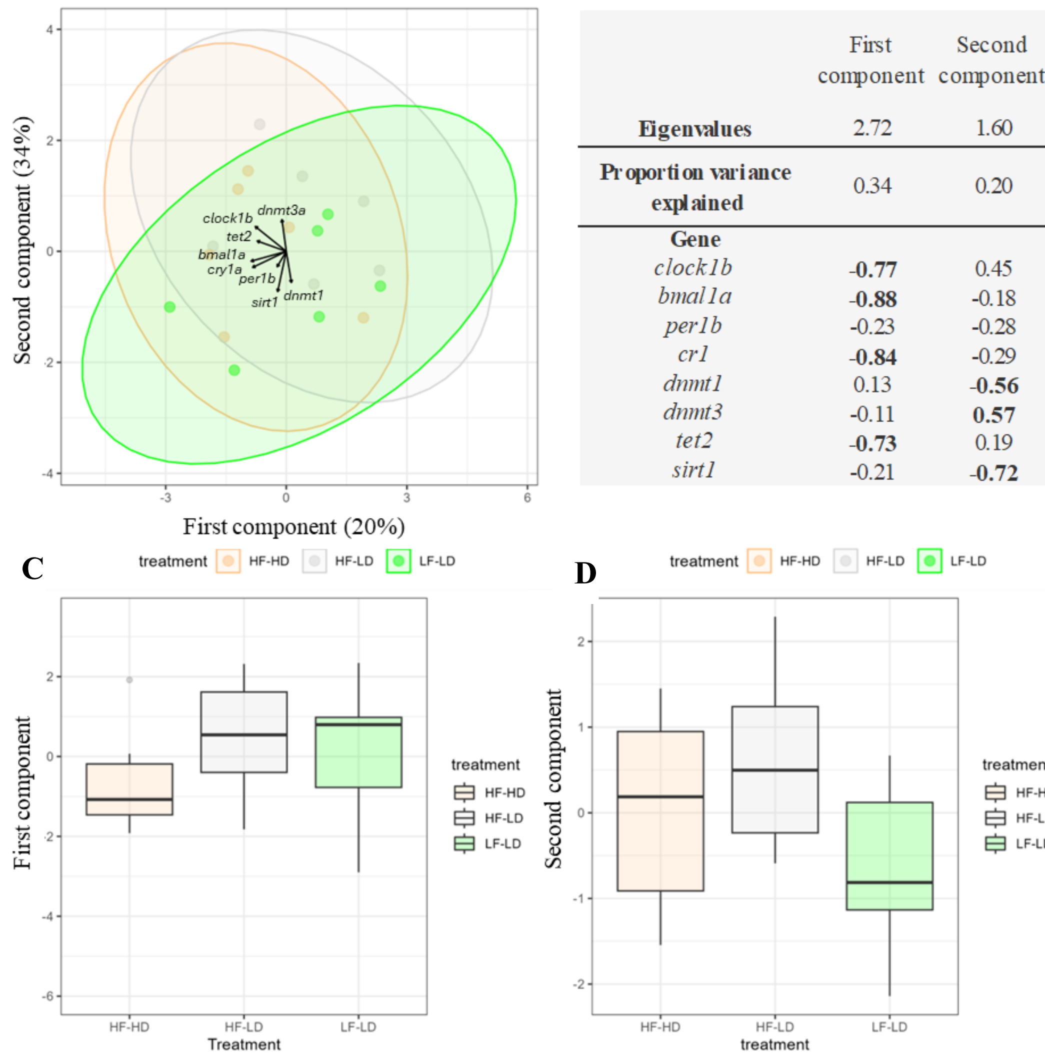
At day 5, during the day (ML), the PCA found that the First and Second Components explained 41% and 21% of the total variance, respectively (Fig. 4B). The First Component was mainly loaded by *clock1b*, *dnmt1*, and *tet2*; while *per1b*, *dnmt3a* and *sirt1* with an opposite pattern were the major contributor to the Second Component (Fig. 4A, B). The ANOVA did not find a significant difference on the First Component among groups (1-way ANOVA; $p = 0.2491$). However, a significant effect of treatment on the Second Component was found (1-way ANOVA; $p < 0.001$). The post hoc test revealed a significant difference between HF-LD and HF-HD (Tukey *post hoc*; $p = 0.004$), indicating an early effect of the density. On the contrary, the treatment condition "feeding" did not differed among control and treated group (HF-LD vs LF-LD, Tukey *post hoc*; $p = 0.2114$) (Fig. 4C, D; Suppl. Table 3).



	First component	Second component
Eigenvalues	3.30	1.72
Proportion variance explained	0.41	0.21
Gene		
clock1b	**-0.83**	0.36
bmal1a	-0.57	-0.02
per1b	-0.47	**-0.69**
cr1	-0.54	0.16
dnmt1	**-0.66**	-0.40
dnmt3	-0.55	**0.58**
tet2	**-0.82**	0.37
sirt1	-0.61	**-0.68**

Fig. 4 Principal component analysis for day 5, ML sampling, which comprehend the relative mRNA expression of the genes *clock1b, bmal1a, per1b, cry1a, dnm1, dnmt3a, tet2* and *sirt1*. (A) Biplot of the two components obtained from the PCA. (B) Eigenvalues, proportion of the variance and values for the first and second component for each gene. (C) (D) Boxplot representing the 1-way ANOVA and planned contrast between group ($p < 0.05$). Gray represents the control condition (HF-LD), orange represents the high-density trial group (HF-HD) and green the low-feeding trial group (LF-LD). Data are represented as mean ± SEM.

During the night (MD), the PCA found that the First and Second Components explained 34% and 20% of the total variance, respectively (Fig. 5B). *Clock1b, bmal1a, cry1* and *tet2* were the major contributor to the First Component, while the Second Component was mainly loaded by *sirt1* and secondarily by *dnmt1* and *dnmt3a* which had a different pattern (Fig. 5A, B). No differences emerged among groups on the First (1-way ANOVA; p = 0.5185) and on the Second Component (1-way ANOVA; p = 0.1801) (Fig. 5C, D; Suppl. Table 3).

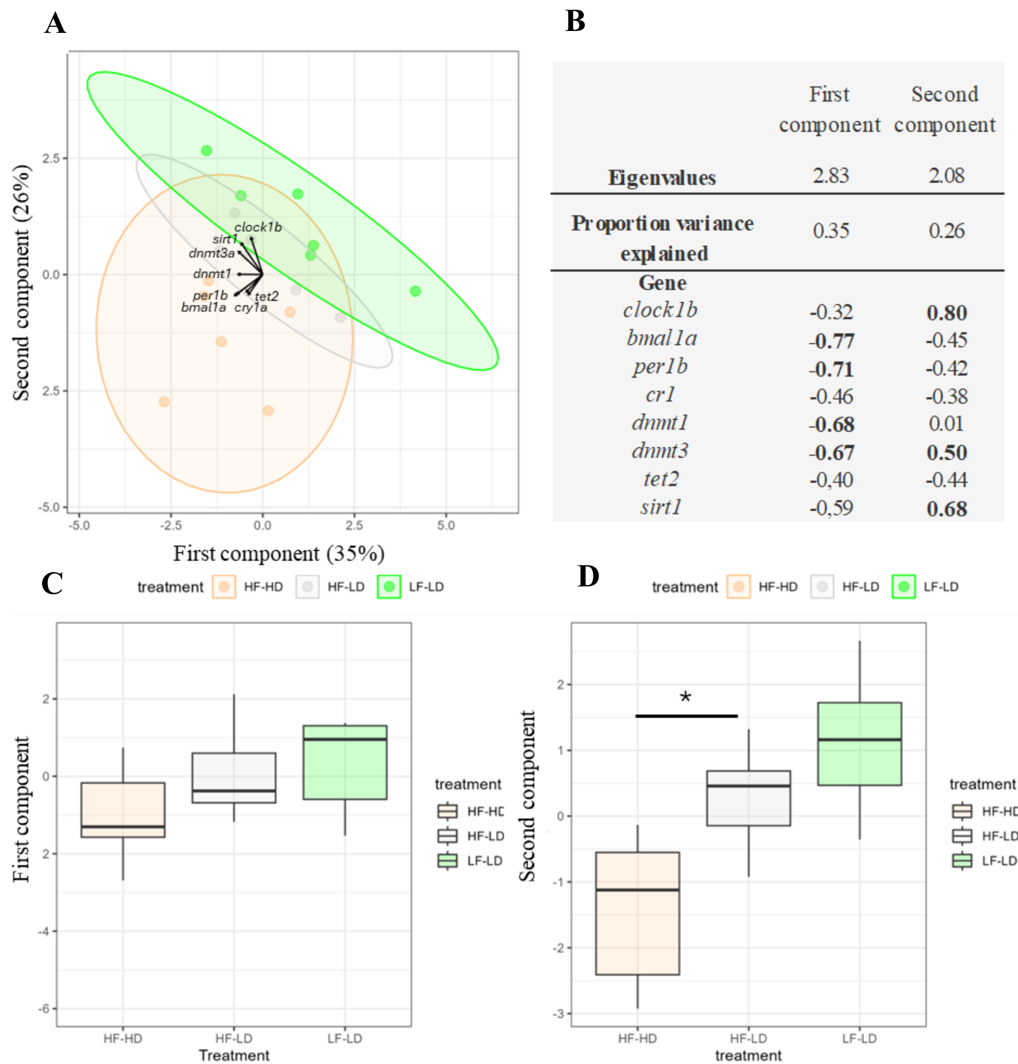


	First component	Second component
Eigenvalues	2.72	1.60
Proportion variance explained	0.34	0.20
Gene		
clock1b	**-0.77**	0.45
bmal1a	**-0.88**	-0.18
per1b	-0.23	-0.28
cr1	**-0.84**	-0.29
dnmt1	0.13	**-0.56**
dnmt3	-0.11	**0.57**
tet2	**-0.73**	0.19
sirt1	-0.21	**-0.72**

Fig. 5 Principal component analysis for day 5, MD sampling, which comprehend the relative mRNA expression of the genes *clock1b, bmal1a, per1b, cry1a, dnm1, dnmt3a, tet2* and *sirt1*. (A) Biplot of the two components obtained from the PCA. (B) Eigenvalues, proportion of the variance and values for the first and second component for each gene. (C) (D) Boxplot representing the 1-way ANOVA and planned contrast between group ($p < 0.05$). Gray represents the control condition (HF-LD), orange represents the high-density trial group (HF-HD) and green the low-feeding trial group (LF-LD). Data are represented as mean ± SEM.

At day 25, during the day (ML), the PCA found that the First and Second Components explained 35% and 25% of the total variance, respectively (Fig. 6B). The First Component was mainly loaded by *bmal1a, per1b, dnmt1* and *dnmt3*, which highly correlated with the same pattern. *Clock1b* was the major contributor to the Second Component followed by *sirt1* and *dnmt3* (Fig. 6A, B). Although no differences on the First Components emerged among groups (1-way ANOVA; $p = 0.1178$), the ANOVA

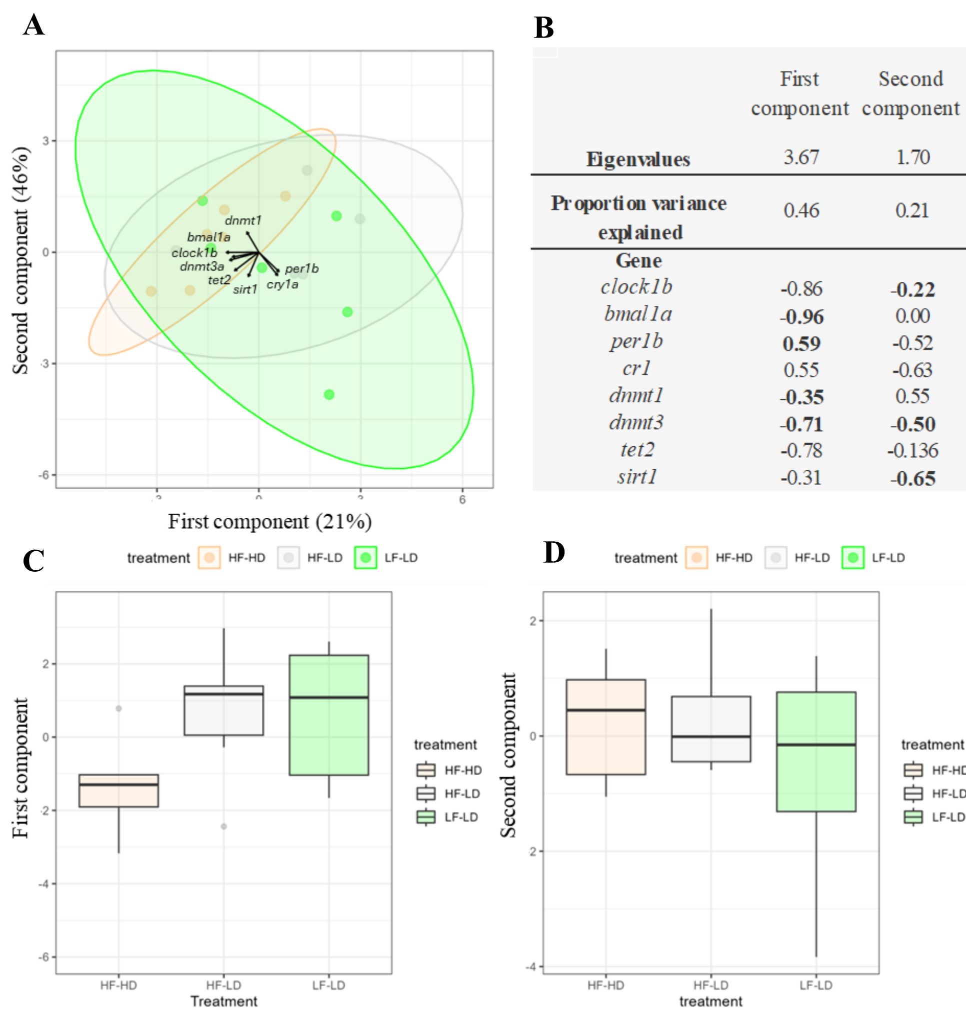
found a significant effect of treatment on the Second Component (p = 0.002). Similarly to the first sampling point (Day 5) during the day, the *post hoc* test revealed a significant difference between HF-LD and HF-HD (p < 0.0309). No effect of “feeding” was found for the Second Component (HF-LD vs LF-LD, Tukey *post hoc*; p = 0.3627) (Fig 6C, D; Suppl. Table 3).



	First component	Second component
Eigenvalues	2.83	2.08
Proportion variance explained	0.35	0.26
Gene		
clock1b	-0.32	**0.80**
bmal1a	**-0.77**	-0.45
per1b	**-0.71**	-0.42
cr1	-0.46	-0.38
dnmt1	**-0.68**	0.01
dnmt3	**-0.67**	**0.50**
tet2	-0,40	-0.44
sirt1	-0,59	**0.68**

Fig. 6 Principal component analysis for day 25, ML sampling, which comprehend the relative mRNA expression of the genes *clock1b, bmal1a, per1b, cry1a, dnm1, dnmt3a, tet2* and *sirt1*. (A) Biplot of the two components obtained from the PCA. (B) Eigenvalues, proportion of the variance and values for the first and second component for each gene. (C) (D) Boxplot representing the 1-way ANOVA and planned contrast between group (p < 0.05). Gray represents the control condition (HF-LD), orange represents the high-density trial group (HF-HD) and green the low-feeding trial group (LF-LD). Data are represented as mean ± SEM.

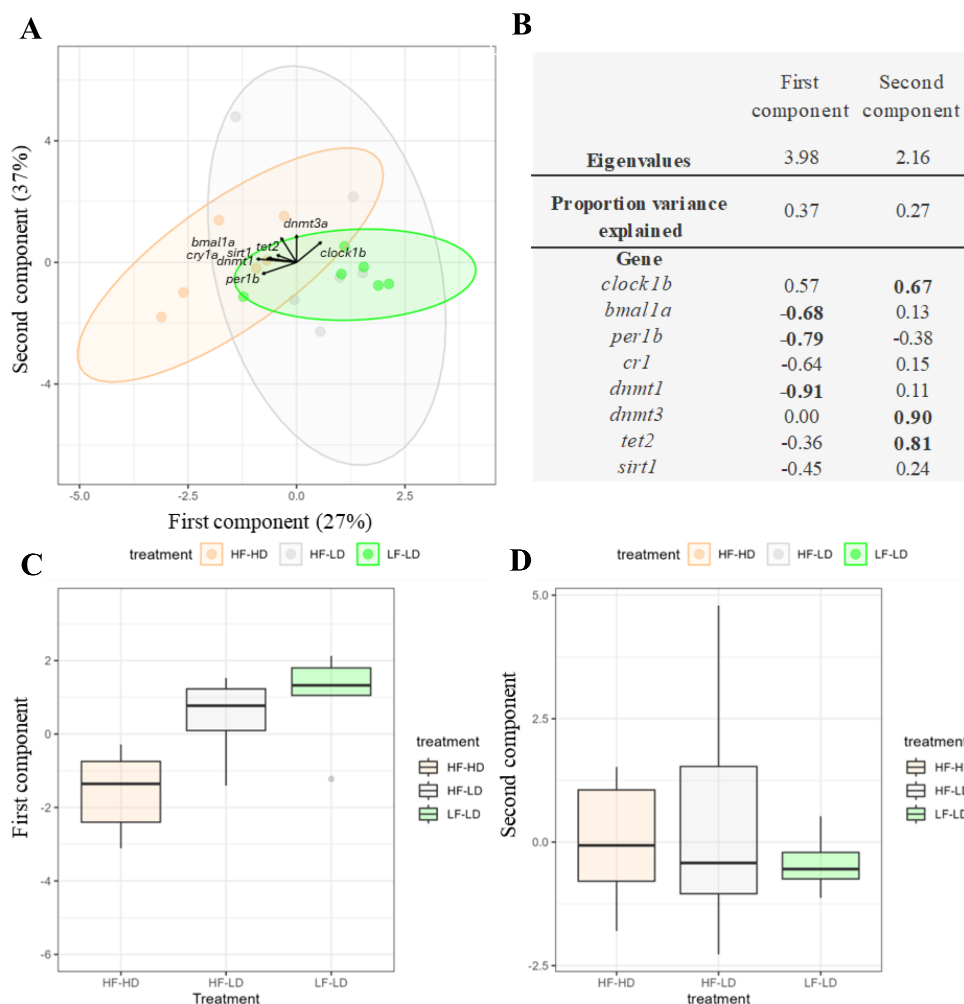
During the night (MD), the PCA found that the First Components explained 46% of the total variance, while the Second Component 21% (Fig. 7B). *Clock1b* and *bmal1a* were the major contributor to the First Component followed by *tet2* and *dnmt3a* with the same pattern. The Second Component was mainly loaded by *sirt1* and *cry* (Fig. 7A, B). No differences emerged among groups on the First (1-way ANOVA; p = 0.0964) and on the Second Component (1-way ANOVA; p = 0.503) (Fig 7 C, D; Suppl. Tab. 3).



	First component	Second component
Eigenvalues	3.67	1.70
Proportion variance explained	0.46	0.21
Gene		
clock1b	-0.86	**-0.22**
bmal1a	**-0.96**	0.00
per1b	**0.59**	-0.52
cr1	0.55	-0.63
dnmt1	**-0.35**	0.55
dnmt3	**-0.71**	**-0.50**
tet2	-0.78	-0.136
sirt1	-0.31	**-0.65**

Fig. 7 Principal component analysis for day 25, MD sampling, which comprehend the relative mRNA expression of the genes *clock1b, bmal1a, per1b, cry1a, dnm1, dnmt3a, tet2* and *sirt1*. (A) Biplot of the two components obtained from the PCA. (B) Eigenvalues, proportion of the variance and values for the first and second component for each gene. (C) (D) Boxplot representing the 1-way ANOVA and planned contrast between group ($p < 0.05$). Gray represents the control condition (HF-LD), orange represents the high-density trial group (HF-HD) and green the low-feeding trial group (LF-LD). Data are represented as mean ± SEM.

At day 60, during the day (ML), the PCA found that the First and Second Components explained 37% and 27% of the total variance, respectively (Fig. 8B). The First Component was mainly loaded *dnmt1* and secondary by *per1b* and *bmal1a* showing the same pattern, while the second component was mainly loaded by *dnmt3a* and *tet2* followed by *clock1b*. *Clock1b* was the major contributor to the Second Component followed by *sirt1* and *dnmt3* (Fig. 8A, B). Similarly to the first two daily time point (Day 5 and Day 25), the ANOVA found a significant effect of treatment on the First Component (1-way ANOVA; p = 0.003), which was mainly attributed to density (Tukey *post hoc*; p < 0.0182). No differences on the Second Component were found among groups (1-way ANOVA; p = 0.685) (Fig. 8C, D; Suppl. Table 3).



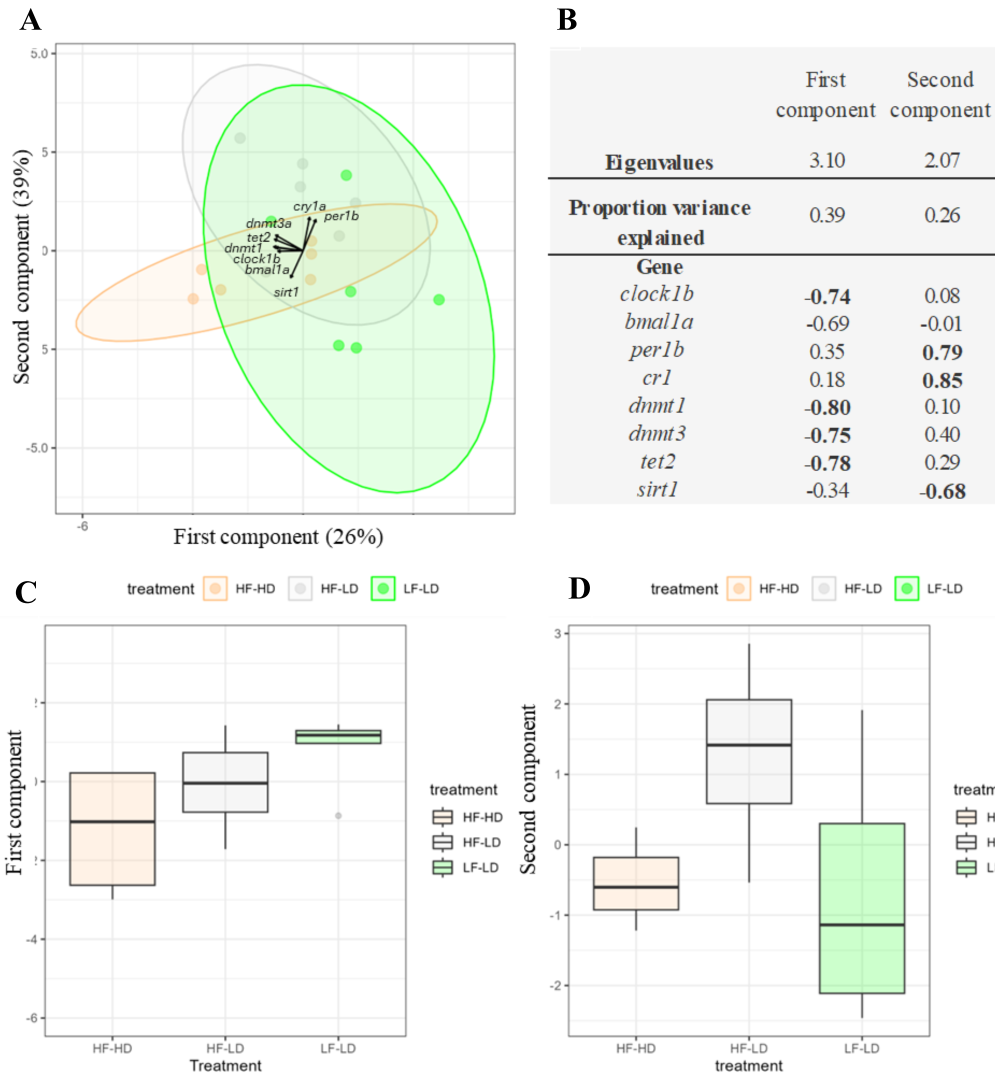
B

	First component	Second component
Eigenvalues	3.98	2.16
Proportion variance explained	0.37	0.27
Gene		
clock1b	0.57	**0.67**
bmal1a	**-0.68**	0.13
per1b	**-0.79**	-0.38
cr1	-0.64	0.15
dnmt1	**-0.91**	0.11
dnmt3	0.00	**0.90**
tet2	-0.36	**0.81**
sirt1	-0.45	0.24

Fig. 8 Principal component analysis for day 60, ML sampling, which comprehend the relative mRNA expression of the genes *clock1b, bmal1a, per1b, cry1a, dnm1, dnmt3a, tet2* and *sirt1*. (A)

Biplot of the two components obtained from the PCA. (B) Eigenvalues, proportion of the variance and values for the first and second component for each gene. (C) (D) Boxplot representing the 1-way ANOVA and planned contrast between group ($p < 0.05$). Gray represents the control condition (HF-LD), orange represents the high-density trial group (HF-HD) and green the low-feeding trial group (LF-LD). Data are represented as mean ± SEM.

During the night (MD), the PCA found that the First Components explained 39% of the total variance, while the Second Component 26% (Fig. 9B). The first component was mainly explained by *dnmt1, tet2, dnmt3a* and *clock1b* which all loaded in the same direction. On the contrary, the second component was explained by *cry* and *per1b*, and by *sirt1* in the opposite direction (Fig. 9A, B). Although we found significant differences on the First Component among groups (1-way ANOVA; $p = 0.026$), *post hoc* tests did not reveal a significant effect of the density (Tukey *post hoc*; HF-LD vs HF-HD $p = 0.3617$) and feeding (Tukey *post hoc*; HF-LD vs LF-LD $p = 0.2536$) on the first component. Contrary to the previous data collected during the night, a significant difference on the Second Component emerged (1-way ANOVA; $p = 0.027$). The effect of feeding was significant (Tukey *post hoc*; $p =0.0365$) but not the density (Tukey *post hoc*; $p = 0.0618$) (Fig 9C, D; Suppl. Table 3).



	First component	Second component
Eigenvalues	3.10	2.07
Proportion variance explained	0.39	0.26
Gene		
clock1b	**-0.74**	0.08
bmal1a	-0.69	-0.01
per1b	0.35	**0.79**
cr1	0.18	**0.85**
dnmt1	**-0.80**	0.10
dnmt3	**-0.75**	0.40
tet2	**-0.78**	0.29
sirt1	-0.34	**-0.68**

Fig. 9 Principal component analysis for day 60, MD sampling, which comprehend the relative mRNA expression of the genes *clock1b, bmal1a, per1b, cry1a, dnm1, dnmt3a, tet2* and *sirt1*. (A) Biplot of the two components obtained from the PCA. (B) Eigenvalues, proportion of the variance and values for the first and second component for each gene. (C) (D) Boxplot representing the 1-way ANOVA and planned contrast between group ($p < 0.05$). Gray represents the control condition (HF-LD), orange represents the high-density trial group (HF-HD) and green the low-feeding trial group (LF-LD). Data are represented as mean ± SEM

Discussion

In this study we separately examined the effect of two different chronic stressors, high density and low feeding, on the clock and epigenetic mechanisms in the liver of European sea bass also considering the time of the day. We found that the expression of individual genes involved in the clock system and epigenetic pathways varies based on the duration of the administered chronic stressor, with an early activation of the epigenetic system (Suppl. Figure 4). Additionally, the factorial analysis on the multi-genes expression pattern revealed differences in the relationships of genes among groups, specifically, genes involved in the methylation and deacetylation processes were particularly important to explain the overall patter of gene expression observed under high-density stress condition.

The effect of the density.

At day 5, *dnmt3a* and *sirt1* showed significant differences during the day in terms of mRNA abundance compared to control group. The early response of the genes involved in epigenetic pathway was subsequently lost at day 25, while the clock genes began to respond differently. Lastly, at day 60, we observe a response from both clock and epigenetic systems, revealing the concomitant effect of the chronic treatment on both.

DNA methyltransferase activity and the overall DNA methylation can be modulated by different stress such as temperature (Anastasiadi et al., 2017; Valdivieso et al., 2022) or rearing density (Valdivierso et al., 2023) sometimes determining long lasting modifications (Robinson et al., 2019). In our study, we observed an immediate response at day 5 of *dnmt3a*, that reduced its mRNA abundance, and *dnmt1* at day 60 that on the contrary increase its mRNA abundance. Although *dnmt3a* and *dntm1* mediate the same

process, the first is involved in de-novo methylation, while the second maintain the established pattern of methylation after the DNA replication (Anderson et al., 2012; Moore et al., 2013). They both rely on the active form of methionine as methyl donor, which is part of the 1C cycle and it requires ATP for its completion (Anderson et al., 2012; Duker and Rabinowitz, 2017). Under stressful conditions, fish must reallocate energy quickly to cope with the new challenge (Schreck and Tort, 2016), and the depletion of ATP could have led to a reduction of the *dnmt3a* abundance at the very beginning of the trial, especially during the day when fish are more active. Alternatively, the different pattern that we observe for *dnmt3a* and *dnmt1* throughout the trials could be due to the activity of glucocorticoids toward GRE regions of DNA methyltransferases, since their presence has been previously describe in mammals' brain (Urb et al., 2018), even though no records are available in fish that could confirm which effect cortisol promote by binding the *dnmt*'s promoters (Faught et al., 2016). Epigenetic processes are flexible and reversible and the up- or down-regulation of the genes involved in similar mechanism can depend on the tissue (Giannetto et al., 2013), life stage (Anastasiadi et al., 2017) or also the stressor applied (Hu et al., 2021), making difficult to generalize, but their altered expression along time could lead to different patterns of methylation in the genome which is commonly a marker of several pathologies in fish (Mirbahai et al., 2011). In addition, these effects were observed in the samples at ML, but not at MD, pointing also to differences in the response depending on the time of the day, as observed in the sea bass for the acute stress response (Samorì et al., 2024).

As seen for methylation enzymes, *sirt1* also presents significant differences in mRNA relative values, with an increase at days 5 and 60, during the day and night respectively. *Sirt1* is an NAD+ dependent enzyme involved in epigenetic processes, generally reducing gene expression (Jing and Lin, 2015). *Sirt1* is also linked to *dnmt1*

since their proteins can physically interact to maintain the nuclear structure of the cell and they are usually found in the same hypermethylated areas in mammals (Jing and Lin, 2015), which may explain the simultaneous increase of abundance of mRNA at day 60. An additional link between *sirt1* and one of the DNA methyltransferases, the *dnmt3a,* is also reported by PCA analysis, since they are the main contributors to the pattern that we observed in gene expression at day 5 and 25 during the day, partially matching the differences observed in gene expression. The regulatory role that *sirt1* and *dnmt*s impose to chromatin (Moore et al., 2013; Jing and Lin, 2015) suggests that stress could have led to chromatin modifications which have affected other genes on a cascade. *Sirt1* also interacts with the elements of the clock system in several ways, including deacetylating PER2 (Asher et al., 2008) and BMAL1 (Masry et al., 2014) and physically interacting with CLOCK-BMAL complex on circadian genes promoters (Nakahata et al., 2008). In rainbow trout submitted to high stoking density for 72 hours, an increase on *sirt1* mRNA in the brain was observed, similarly to the result obtained in our study at day 5 (Naderi et al., 2018). The same study reported decreases in the mRNA of *clock1a* and *bmal1* during the day and *per1* during the night, which seemed to be mediated by *sirt1*. In our study, we also observed significant changes in *clock1b* and *per1b* during the day and night, respectively, but these were noted at day 25. These differences between the trout and sea bass might be determined by a species-specific response, but also due to the tissue considered, since central and peripheral pacemakers can respond differently to stress (Sanchez-Vazquez et al., 2019). Additionally, clock genes are particularly sensitive to cortisol, due to the presence of the GRE elements on their promoter (Balsalobre et al., 2000, Yamamoto et al., 2000, Dickmeis et al., 2013), so variations in the cortisol response might also explain these differences, but this hypothesis should be tested in further analyses on cortisol production. In addition, the PCA suggested the involvement of *per1b* at day 5 and 60. Since *per1b* is part of the feedback circuit (Pando and Sassone-Corsi,

2002), its alteration can potentially lead to modify the expression of other clock genes later in time. At day 25 *per1b* is replaced by *clock1b.* Hence the initial involvement of *per1b* could have contributed to affect *clock1b*. Lastly, the PCA shown that at day 60, *per1b* cooperate also with *bmal1a* and *dnmt1*, confirming that, after all the chronic stress period, both positive and negative circuits of the molecular clock and methylation processes have been reorganized.

The effect of the feeding.

The pattern observed due to the effect of the feeding was different from that observed for density. At day 5, differences were detected on *clock1b* and several epigenetic genes, were the reduction of mRNA abundance of *dnmt3a* was coupled with the increase of *dnmt1* during the night. At day 25, only *bmal1a* and *tet2* responded differently, both showing decreased mRNA abundance. At day 60, *clock1b*, *per1b* and *cry1a* presented differences in the expression, along with *dnmt3a*, but only during the night, with the exception of *cry1a*. Interestingly, *dnmt1* and *dnmt3a* responded immediately to restricted feeding. The DNA methyltransferases rely on the availability of methionine, which is provided by the diet and enter in the 1C cycle (Duker and Rabinowitz, 2017). Different studies have shown that micronutrient deficiencies can alter locus-specific DNA methylation (Skjaerven et al., 2018), likely mediated by DNA methyltransferases activity. In our case, we observed an increase of mRNA abundance of *dnmt1,* especially during the night, and a decrease in *dnmt3a* mRNA during the day. Due to the low food ratio that was provided, it is possible that most of the energy was allocated to maintaining methylation processes to ensure cell stability, as methionine activation requires ATP (Duker and Rabinowitz, 2017). The fact that this differences in *dnmt1* were evident during the night align with the recent chronobiological studies, which locate the

acrophase of DNA methyltransferases during the night (Paredes et al. 2018). A different trend is observable at day 25, where only *tet2* presents a reduction in mRNA transcript, and *dnmt3a* returns to lower mRNA levels only at day 60. Methylation and demethylation process are very flexible and inducible by different stressors (Mirbahai et al., 2011; Anastasiadi et al., 2017; Valdivieso et al., 2022), but in case of calories deficiencies due to low food ratio, it is also possible that methionine assumed by diet was progressively insufficient to maintain proper induction of methylation enzymes.

Regarding clock genes, the early response of *clock1b* at day 5, is followed by *bmal1a* at day 25, and *clock1b*, *per1b* and *cry1a* at day 60. From a chronobiological perspective, the liver is considered one of the most important peripheral oscillators, where the clock genes are particularly sensitive to feeding time (Lopez-Olmeda et al., 2010; Feliciano et al., 2011;Vera et al., 2013). At the same time, recent studies on mice have pointed out that not only feeding time but also feed composition (Honma et al., 2016) or calories restriction (Patel et al., 2016) could participate in their regulation. Specifically, 30% of calories restriction affected the rhythms of clock genes in mice liver, whose effect was mediated by *bmal1* as proved using knockout mice (Patel et al., 2016). Therefore, the effects observed in the clock genes of sea bass subjected to low feeding stress may be probably generated by a direct effect due to caloric restriction, as observed in mammals.

The time of the day

All genes analyzed in the present study have been reported to display daily rhythms, either in the European sea bass or in other fish species (Del Pozo et al., 2012; Morbiato et al., 2019; Paredes et al., 2018, Wang et al., 2022). Consequently, the regulation and response of these genes throughout the day may vary in response to physiological stress. These variations have been previously observed in the response to

acute stress in the HPI axis and the antioxidant system of the European sea bass (Samorì et al., 2024). In our study, only few genes responded with different mRNA abundance during the night at day 5 (*dnmt1*) and 25 (*per1b*), while at day 60 most of the genes analyzed displayed differences (*clock1b, per1b, dnmt1, dnmt3a* and *sirt1*). Hence the response to chronic stress of the physiological processes analyzed showed a greater response during the day than at night. The study on European sea bass mentioned above, revealed that the amplitude of the stress response can be either higher during the day or during the night when an acute stress occurs, depending on the factors analyzed (Samorì et al., 2024). These differences could be related to the different pathways analyzed which are targeted by stress, but further research should be performed to elucidate the reasons of the differences among different pathways.

Conclusions

Clock and epigenetic genes are linked to each other in a double way, and they can be both modulated by stress, even though different stress could elicit different response. In our study, the epigenetic genes analyzed displayed an early responsiveness to both stressors, while the majority of the clock genes presented different mRNA level only after 25 days of treatment. The perfect coordination of both system is essential for the well-being of the fish, since their alteration is the gateway to different disease. Due to the early activation of the epigenetic genes, they could also represent early biomarker to assess stress even though more studies are necessary to understand their dynamics in fish. Moreover, the clock genes were particularly responsive to feeding treatment making them

good indicators to understand the potential effect of calories restriction in aquaculture but information on how the diet modulate their daily rhythm is still missing.

Supplementary materials

Suppl. Table 1 Resume of the *p* values obtained from the 1-way ANOVA for ML and MD sampling at day 5, 25 and 60.

	Day 5		Day 25		Day 60	
	ML	MD	ML	MD	ML	MD
clock1b	0.179	0.864	**0.044**	0.370	0.326	**0.017**
bmal1a	0.895	0.650	**0.0008**	**0.031**	**0.007**	0.507
per1b	**0.023**	0.872	**0.003**	0.058	**0.047**	**0.0004**
cry1a	0.273	0.660	**0.023**	0.161	**0.016**	0.238
dnmt1	0.269	**0.017**	0.102	0.884	**0.0004**	0.485
dnmt3a	**0.002**	0.970	0.892	0.226	0.624	**0.003**
tet2	0.546	0.263	**0.016**	0.674	0.435	0.073
sirt1	**0.008**	0.088	0.589	0.371	0.434	0.072

Suppl. Table 2 Resume of the *p* values of the T-test analysis for ML and MD sampling at day 5, 25, and 60. HF-HD columns represent the results for the contrast HD-LF while LF-LD columns represent the results for the contrast HD-LF vs LF-LD.

	Day 5				Day 25				Day60			
	ML		MD		ML		MD		ML		MD	
	HF-HD	*LF-LD*	*HF-HD*	*LF-LD*	*HF-HD*	*LF-LD*	*HF-HD*	*LF-LD*	*HF-HD*	*LF-LD*	*HF-HD*	*LF-LD*
clock1b	0.214	**0.02**	0.913	0.688	**0.0005**	0.713	0.355	0.658	0.255	0.97	0.906	**0.025**
bmal1a	0.913	0.715	0.505	0.906	0.146	**0.007**	0.052	0.747	**0.04**	0.309	0.558	0.502
per1b	0.089	0.084	0.891	0.709	**0.004**	0.913	**0.004**	0.572	0.082	0.786	**1.6E-05**	**0.038**
cry1a	0.303	0.383	0.263	0.509	**0.02**	0.405	0.247	0.346	**0.008**	**0.045**	0.112	0.306
dnmt1	0.614	0.236	0.818	**0.016**	0.433	0.154	0.623	0.866	**0.0009**	0.961	0.644	0.454
dnmt3a	**0.014**	**0.0004**	0.918	0.774	0.633	0.947	0.124	0.161	0.91	0.937	0.733	**0.005**
tet2	0.791	0.065	0.118	0.367	0.58	**0.008**	0.37	0.913	0.891	0.36	0.104	0.359
sirt1	**0.021**	0.943	0.874	0.786	0.545	0.609	0.151	0.475	0.212	0.652	**0.01**	0.179

Suppl. Table 3 Resume table of the 1-way ANOVA and planned contrast for PCA analysis. The two components are reported for ML and MD conditions at day 5, 25 and 60. Planned contrast are reported only when result is significant confronting control condition HF-LD vs HF-HD / LF-LD.

		ML		MD	
		First component	Second component	First component	Second component
Day 5	Principal effect	F(2,15)= 1.5269, P=0.2491	**F(2,15)=13.073, P<0.001**	F(2,15)=0.6865, P=0.5185	F(2,15)=1.926, P=0.1801
	Planned contrast		HF-LD vs HF-HD P<0.0004		
Day 25	Principal effect	F(2,15)=2.4752, P=0.1178	**F(2,15)=9.4017, P=0.002**	F(2,15)=2.7451, P=0.0964	F(2,15)=0.7196, P=0.503
	Planned contrast		HF-LD vs HF-HD p<0.0309		
Day 60	Principal effect	**F(2,15)=8.8931, P=0.003**	F(2,15)=0.388, P=0.685	**F(2,15)=4.685, P=0.026**	**F(2,15)=4.627, P=0.027**
	Planned contrast	HF-LD vs HF-HD P<0.0182			

Suppl. Table 4 Increase or decrease of the mRNA abundance of clock (*clock1b, bmal1a, per1b, cry1a*) and epigenetic genes (*dnmt1, dnmt3a, tet2, sirt1*) considered in the analysis at day 5, 25 and 60 for M and MD sampling.

Gene		mRNA aboundance HF-HD		mRNA aboundance LF-LD	
	Day	ML	MD	ML	MD
clock1b	5			↓	
	25	↓			
	60				↓
bmal1a	5				
	25			↓	
	60	↑			
per1b	5				
	25	↑	↓		
	60		↓		↓
cry1a	5				
	25	↑			
	60	↑		↑	

Gene		mRNA aboundance HF-HD		mRNA aboundance LF-LD	
	Day	ML	MD	ML	MD
dnmt1	5				↑
	25				
	60	↑			
dnmt3a	5	↓		↓	
	25				
	60				↓
tet2	5				
	25			↓	
	60				
sirt1	5	↑			
	25				
	60		↑		

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Experimental chapter IV

Influence of feeding time on daily rhythms of locomotor activity, clock genes and epigenetic mechanisms in the liver and hypothalamus of the European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

The circadian system plays a crucial role in most physiological processes. The molecular clock is linked to epigenetic mechanisms, which are responsive to nutrient status. This research investigated how feeding times (mid-light, ML, *vs*. mid-dark, MD) synchronize daily rhythms of behavior, clock genes and epigenetic mechanisms in the European sea bass (*Dicentrarchus labrax*), focusing on hypothalamus and liver to assess the impact on central and peripheral pacemakers. Feeding at MD influenced the molecular clock of the hypothalamus, causing shifts in acrophases (peaks) for genes of the negative loop (*per1b*, *per2*, *cry1a*). In the liver, the ML fed group showed rhythmic expression for all clock genes, whereas only *per2* maintained the rhythms in the MD group. Epigenetic genes related to methylation (*dnmt1*, *dnmt3a*) and demethylation (*tet2*, *gadd45aa*, *mbd4*) in the liver displayed rhythmic expression in the ML group, but only *dnmt3a* maintained the rhythm in the MD group. Nutrient-related factors (SAM and SAH) showed differences between day and night, suggesting a different utilization based on feeding times. Finally, *sirt1*, a gene involved in deacetylation, displayed a clear circadian rhythm in the ML group. All epigenetic genes peaked during the night (resting phase). Overall, these findings indicated feeding time serves as a potent *zeitgeber*, synchronizing circadian clock and epigenetic rhythms in the liver, with peaks during the resting phase, pointing out this phase as the right time for epigenetic modifications.

Keywords: central and peripheral pacemakers, clock genes, methylation, acetylation, methylation potential

INTRODUCTION

Living organisms present biological rhythms in their functions or processes at various levels, ranging from behavioral to molecular. These rhythms are synchronized to different external time cues, known as *zeitgebers*, such us the light-dark (LD) cycle (Vatine et al. 2011, Paredes et al. 2015, Steindal and Whitmore 2020), feeding (Lewis et al. 2020, López-Olmeda 2017) and temperature (Rensing and Ruoff 2002, Lahiri et al. 2005 and 2014; López-Olmeda and Sánchez-Vázquez 2011). In this context, the pacemakers represent functional anatomic regions capable of integrating these external stimuli, generating oscillations and transmitting them to output pathways, thereby generating overt rhythms (Pando and Sassone-Corsi 2002). In fish, the circadian system appears to be composed of a network of pacemakers present in most tissues and cells (Whitmore et al. 2000), lacking the hierarchical organization that is a distinctive feature of mammals, where the master pacemaker is located in the suprachiasmatic nucleus (SCN) (Mohawk et al. 2012).

The generation of all these rhythms relies on the molecular machinery within cells. The core molecular system of the pacemakers is referred to as the molecular clock and exhibits a fundamentally similar organization in both central and peripheral tissues of mammals and fish (Kumar and Sharma 2018). The molecular clock operates through positive and negative feedback loops that confer pacemakers their self-sustaining activity and drive the development of rhythmicity. The positive loop initiates with the transcription of *clock* and *bmal* genes, whose proteins form a heterodimer functioning as a transcription factor binding to E-box domains in the promoter regions of many genes. Among these genes, Clock and Bmal promote the transcription of *per* and *cry*, which constitute the negative loop responsible for the downregulation of *clock* and *bmal* expression (Pando and Sassone-Corsi 2002; Mohawk et al. 2012; Kumar and Sharma

2018). The circadian molecular clock is regulated at various levels, relying on E-box binding domains (Hardin et al. 2004), post-transcriptional modifications (Mehra et al. 2009), and epigenetic regulation (Eckel-Mahan and Sassone-Corsi 2013; Satou et al. 2013). The circadian system and epigenetic mechanisms are closely related. In recent years, the existence of a bidirectional pathway between these two systems has been proposed, although it remains controversial which one controls the other (Stevenson 2018). Among the epigenetic systems, DNA methylation and histone acetylation/deacetylation have been reported to be associated with the circadian system (Doi et al. 2006; Nakahata et al. 2008; Satou et al. 2013; Stevenson 2018). DNA methylation involves the addition of a methyl group to a cytosine followed by a guanine (CpG dinucleotide), particularly in CpG islands. This process facilitates DNA silencing by preventing transcription factor binding and recruiting methyl-binding proteins that act as inhibitory elements (Bird et al. 2002; Moore et al. 2013). The components involved in silencing belong to the DNA methyltransferase (Dnmts) family enzyme, which includes enzymes responsible for maintaining methylation after DNA duplication (Dnmt1) and those that promote de novo methylation (Dnmt3s) (Jurkowska et al. 2011). All genes involved in DNA methylation are S-adenosyl methionine-dependent, utilizing the methyl group produced in the conversion of S-adenosyl methionine (SAM) into S-adenosyl homocysteine (SAH) and adenine. SAH itself serves as an important inhibitor of most methyltransferases, and the SAM/SAH ratio is used to describe methylation potential (Mirbahai et al. 2013; Xia et al. 2015).

The establishment of a methylation pattern represents a crucial aspect for the cell. The methylation process is dynamic and reversible, as it involves multiple mechanisms for active removal. Demethylation can occur through oxidative and repair-based mechanisms. In the oxidative pathway, the ten-eleven-translocation enzyme (Tet) successively hydroxylates 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC),

followed by 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani et al. 2009; Ito et al. 2010). Subsequently, 5caC can be excised by thymine-DNA glycosylase (Tdg) and replaced with an unmodified cytosine through base excision repair (BER) (He et al. 2011; Shen et al. 2013). Simultaneously, Growth Arrest and DNA-damage-inducible Protein 45 (Gadd45a) can interact with Tdg to facilitate its recruitment to target loci for subsequent excision (Niehrs & Schäfer 2012; Li et al. 2015). Gadd45aa can also interact with the deaminase Apobec (Apolipoprotein B RNA-editing catalytic component) and Mbd4 (methyl-CpG-binding domain protein 4), a BER-specific thymine glycosylase, in a coupled mechanism where the deaminase converts 5-mC to thymine, followed by final base excision of the T:G mismatch by Mbd4 (Rai et al. 2008).

Recently, the interplay between DNA methylation and the circadian system has garnered attention to elucidate the complexity and regulation of biological rhythms. This interest starts from the observation that *Dnmt3a* harbors a high-density Bmal1-binding site on its promoter, potentially rendering it a target for Bmal1 (Stevenson 2018). Moreover, *Dnmt3a/3b* may be implicated in the methylation of *Bmal1*'s promoter in various diseases, leading to its silencing (Satou et al. 2013). Furthermore, DNA methylation is not the sole epigenetic process associated with the clock machinery; histone deacetylation also plays a role. *Clock* possesses histone acetyltransferase (HAT) activity, which facilitates histone acetylation, thereby initiating the negative circuit (Doi et al. 2006). To counterbalance this activity, the histone deacetylase SIRT1 is crucial, as it targets *Clock*'s HAT action preferentially (Nakahata et al. 2008, 2009). Moreover, SIRT1 relies on NAD+ as a cofactor (Suave et al. 2006), making histone deacetylation depending upon the cell's energy status. Additionally, the function of Dnmts enzymes is dependent on the availability of the amino acid methionine, which serves as a methyl donor following its conversion to SAM (Niculescu and Zeisel 2002). Consequently,

feeding patterns and nutritional status exert significant influence on epigenetic regulation, a phenomenon observed in fish as well (Skjaerven et al. 2018).

Regarding feeding, food provided in a periodic manner can also serve as a potent synchronizer for fish, influencing food entrainable oscillators (FEOs), clock gene expression, particularly in peripheral pacemakers, and overt rhythms such as the daily patterns of locomotor activity (López-Olmeda et al. 2010; Feliciano et al. 2011; Vera et al. 2013; Costa et al. 2016; Gómez-Boronat et al. 2018). The daily rhythm of clock genes and their regulation by LD cycles and feeding time has been described in various fish species, both in central and peripheral tissues (López-Olmeda et al. 2010; Feliciano et al. 2011; Nisembaum et al. 2012; Vera et al. 2013), including the European sea bass (*Dicentrarchus labrax*) (Sánchez et al. 2010; Del Pozo et al. 2012; Herrero and Lepesant 2014). However, research on the presence of rhythms in epigenetic processes in fish is limited, with only a single study on zebrafish (*Danio rerio*) demonstrating that genes involved in methylation exhibit a rhythm with peak values mainly occurring during the night phase (Paredes et al. 2018). Furthermore, although the circadian clock and epigenetic mechanisms appear to be interconnected at various levels in mammals, the situation in fish models, as well as the influence of feeding time, remains largely unknown.

The aim of this research was to investigate the interplay between circadian rhythms of genes involved in the clock and epigenetic mechanisms in the European sea bass, and the synchronizing effect of two different feeding times. In order to deepen into the role of nutrient utilization, we also tested SAM, SAH and the methylation potential. Finally, locomotor activity was recorded to elucidate if the appearance of the rhythm at the molecular level is linked to the phase of activity.

MATERIALS AND METHODS

The experiments were conducted at the Aquaculture Laboratory of the University of Murcia, located within the Naval Base of Algameca (E.N.A., Cartagena, Spain). The experimental design followed the European Union guidelines (2010/63/UE) and the Spanish legislation (RD 53/2013 and Law 32/2007) regarding the use of laboratory animals. Approval for the study was obtained from the Committee on Ethics and Animal Welfare of the University of Murcia and the Government of the *Región de Murcia* (license number A13191003).

Animals and housing

Juvenile European sea bass specimens (N = 100) were obtained from CULMAREX (Guardamar del Segura, Alicante, Spain) and divided into two 500-L tanks (50 fish/tank) within an open system equipped with biological and mechanical filters, and UV lamp that sterilized the water before entering the system. A commercial diet (Alterna Marine, Skettring, Burgos, Spain) was used for this stock fish, which were fed *ad libitum.* The photoperiod was controlled to simulate the seasonal variation by means of a timer connected to the lights (Data Micro, Orbis, Madrid, Spain) and light onset set at *Zeitgeber* time 0 (ZT 0 h) (Espirito Santo et al. 2020). Fluorescent light bulbs were employed to grant illumination, with an intensity of 200 lx at the water surface. Water temperature was monitored for all the experiment (HOBO PENDANT Onset Computer Corporation, Massachusetts) and mirrored that of the natural environment.

Experimental design

After one month of acclimation, 98 animals (47.3 ± 0.59 g body weight, mean ± SEM) were randomly divided into 14 tanks (7 fish/tank) and two groups of 7 tanks each were set considering the feeding time: one group was fed around the middle of the light phase (ML, ZT 5 h) and the second group was fed around the middle of the dark phase (MD, ZT 17 h). All tanks were fed daily with the 1% of the biomass of the fish (D2 Optibream 2P, Skettring, Burgos, Spain) and the feed ration was provided by automatic feeders (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany) set to provide half of the ration (0.5% of the body weight) 30 minutes before ML or MD, depending on the group, and the other half 30 minutes after ML or MD, in order to optimize feed consumption and reduce waste. Fish were kept under these conditions for 30 days. During this period, locomotor activity was continuously recorded by means of infrared photocells (Omron, mod E3S-AD62, Kyoto, Japan) placed 10 cm under the water surface and connected to a computer that registered the numbers of light interruptions occurred every 10 minutes, as described elsewhere (Vera et al. 2014). After this period, fish were sampled at ZT 0.5, 4, 7.5, 12, 16, 20 and 24.5 h (Suppl. Fig. 1). At each sampling point, one tank (n=7 fish) from both groups (ML feeding/MD feeding) was sampled. Fish were anaesthetized with clove oil essence (Guinama, Valencia, Spain) at a concentration of 50 μL/L, which was previously diluted in 9 parts of ethanol to improve the dissolution in water. After anesthesia, fish were sacrificed by decapitation to collect samples of hypothalamus and liver, which were frozen in dry ice and subsequently stored at -80°C until analyzed. To avoid light contamination during the dark phase, all the sampling procedures at these time points (ZT 12, 16 and 20 h) were performed under a red dim light ($\lambda > 600$nm) (de Alba et al. 2019). The sampling was performed in December 2020

and the photoperiod inside the Aquaculture Lab at the sampling time was 10:14 LD (light:dark) and the average water temperature was 19.47 ± 0.17 °C.

RNA extraction, cDNA synthesis and Real-Time RT-PCR analysis

The hypothalamus and liver were homogenized in Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, USA) before being mixed with BCP (1-Bromo-3-chloropropane, 99%, Acros Organics, Thermo Fisher Scientific) and centrifuged to obtain a supernatant containing RNA. RNA was then extracted by centrifugation with the addition of isopropanol (Fisher BioReagents, Thermo Fisher Scientific). The RNA was washed twice with 75% ethanol and diluted with DEPC water (Invitrogen, CA, USA). RNA concentration and purity were assessed using spectrometry (Nanodrop® ND 1000, Thermo Fisher Scientific). 1U of DNase I (Thermo Fisher) per 1 µg of RNA was added to each sample and incubated at 65ºC for 10 minutes. Subsequently, cDNA was synthesized using a commercial Reverse Transcriptase kit (QSCRIPT cDNA Synthesis Kit, Quantabio, Beverly, USA) and a thermocycler (MiniAmp Thermal Cycler, Thermo Fisher). The cDNA was utilized for quantitative PCR analysis using Perfecta SYBR Green Fastmin (Quantabio) for the master mix and a real-time thermocycler (7500 RT-PCR system, Applied Biosystem, Foster City, USA). All samples were run in duplicate, with each reaction having a final volume of 20 µl. *Primer 3 Plus software* was used to design *forward* and *reverse* primers (Untergasser et al. 2012) (Table 1). In this study, we analyzed clock genes from both the positive and negative loops (*clock1b*, *bmal1a*, *per1b*, *per2*, *cry1* and *cry2*) in both the hypothalamus and liver, and genes involved in epigenetic mechanisms like DNA methylation (*dnmt1* and *dnmt3a*), demethylation (*tet2*, *gadd45aa* and *mbd4*) and deacetylation (*sirt1*) in the liver samples. All primers were used at a final concentration of 500 nM except *sirt1,* which was added at a final concentration of 200

nM. *β-actin* and *ef1a* were selected as housekeeping genes. The $2^{-\Delta\Delta Ct}$ method was employed for the analysis of the mRNA expression using the geometric means of the reference genes for the first normalization, while for the second normalization we used the sample with the lowest expression value (Livak and Schmittgen 2001).

Table 1. Genes analyzed and primers sequences used for the quantitative PCR analyses

Gene name	Fw	RV	Acc number
ef1a	AGTGAAGCAGCTCATCGTTG	TTGGTGATTTCCTCGAAGCG	AJ866727
bact	TCATCACCATCGGCAATGAG	AACGTCGCACTTCATGATGC	AY148350
clock1b	CCACAGAGCTCCACCCATTA	AAATCCACTGCTGCCCTTTG	ENSDLAG00005012393
bmal1a	TGACGCTAAAACTGGCCTTC	TGCAGAAAAACGACCGTCTG	ENSDLAG00005026433
per1b	CATGGTGAAGACGAACGGAC	CTTTGGGTGGTTTCGTCAGG	ENSDLAG00005015065
per2	AGCTCCAATGCCTTCAGTCT	ACACATCGCAGCGCATATTT	ENSDLAG00005020824
cry1a	AGACCACGACGACAAGTTTG	AAGCCTTCCTCTCCAAATGC	ENSDLAG00005023868
cry2	AGCGCTTTCAGACCATTGTG	TGCGACATTTGTCCATCTGC	ENSDLAG00005019570
dnmt1	ATCAAGCTTGCAGGTGTCAC	TTTGTTGGGTGACGAATGGC	ENSDLAG00005018520
dnmt3a	TCATGTGCGGAAACCACAAC	TCTTTGATGGCTGCATGTGC	ENSDLAG00005018806
tet2	TGCCAACAAGAATGCCATGC	AGTGCCAGCTTTTGACTTGG	ENSDLAG00005008342
gadd45aa	AATTCCAAAGCGCTGCTGAC	TGACAGACGCAACTCCAAAC	ENSDLAG00005028099
mbd4	AGGGCCAAAACACTTGTTCG	TGCAACTCAATGGGGTAACG	ENSDLAG00005035111
sirt1	ACGCAAAGTCCCAATGTCAC	ACACTGGGCATTTGGACAAG	ENSDLAG00005013704

S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH)

Tissue samples (0.088 ± 0.002 g) were first homogenized in cold PBS and centrifuged at 10,000 x g for 15 minutes at 4°C, and the resulting supernatants were stored at -80 °C until analyzed. Levels of S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH) in the liver samples were measured using a commercial ELISA kit (Cell Biolabs Inc, San Diego CA USA, ref number MET-5151-C), following the manufacturer's instructions.

Data analysis

To assess statistically significant differences between time points (ZT) and groups (ML and MD), all the biological variables studied underwent two-way ANOVA analysis followed by a Tukey *post hoc* test using SPSS software (v. 28.0.1.1, IBM, Armonk, USA). Additionally, data obtained from SAM and SAH analysis were pooled separately for light and dark phases to compare ML and MD groups during the same phase using a Student's t-test. Daily patterns of locomotor activity (percentage of diurnalism) data were subjected to a Student's t-test to evaluate statistically significant differences between ML and MD tanks. The significance threshold was set at $\alpha = 0.05$ for all tests, and results are expressed as mean ± SEM. The presence of a daily rhythm was assessed using Cosinor analysis with El Temps software (v. 313, Prof. Díez-Noguera, University of Barcelona, Spain). The Cosinor analysis defines the statistical significance of the rhythm since an F test of the variance is described for the waveform versus a straight line of zero amplitude (null hypothesis) (Refinetti et al. 2007, Portaluppi et al. 2008). El Temps software was also employed to analyze locomotor activity recorded during the experiment and to plot actograms and waveforms. Figures were generated using GraphPad.

RESULTS

Daily patterns of locomotor activity

All European sea bass groups exhibited a predominantly diurnal activity pattern regardless of feeding time. However, fish fed at ML showed 72.59 ± 1.57% of their daily activity during the light phase, whereas fish from the MD group displayed a lower percentage of total activity during the day, 55.33 ± 6.75% (Fig. 1). This difference was statistically significant when comparing the activity of ML and MD groups during the

light phase (% of diurnalism) (p < 0.0001) (t-test p < 0.05). Additionally, the shape of the daily average of activity differed, as it was centered around feeding time in the ML group, while the MD group exhibited two peaks during the light phase, both close to lighting transitions (Fig. 1).

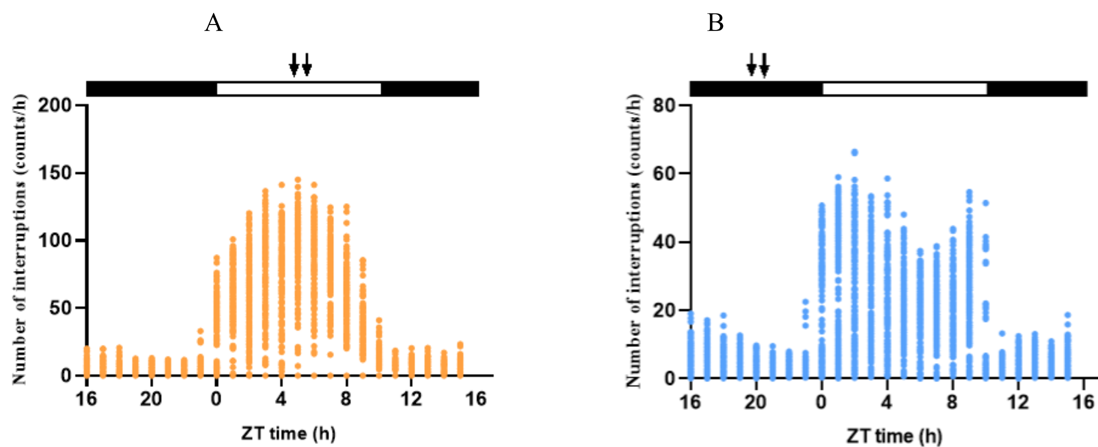

Fig 1 Average diel profile of the locomotor activity of two groups of European sea bass maintained in a 10:14 LD cycle and fed during the middle of the light (ML) (A) or dark (MD) phase (B). Each point in the mean waveform has been calculated as the mean ± S.D. from 10-min binned data across all the experimental days (n=30) and tanks (n=7). The white and black bars above represent the light and dark phases, respectively, while the arrows represent the feeding times (ML and MD)

Hypothalamic molecular clock

Clock genes from the positive (*clock1b, bmal1a*) and negative loops (*per1b, per2, cry1a* and *cry2*) of the molecular clock were analyzed in the hypothalamus of sea bass from the two groups. Regarding the genes from the positive loop, *clock1b* and *bmal1a* exhibited a similar pattern. Both genes displayed daily rhythms in both ML and MD groups (Cosinor, p< 0.05) with similar nocturnal acrophases, located around 3 h after light offset (ML: *clock1b* ZT 13:21 h, *bmal1a* ZT 13:46 h; MD: *clock1b* ZT 13:18 h, *bmal1a* ZT 13:06 h) (Fig. 2a and b; Suppl. Table 1). Significant differences throughout the 24-hour cycle between time points were observed for both groups except for *clock1b* in MD feeding (two-way ANOVA, p<0.05) (Fig. 2a and b). Moreover, feeding influenced

clock1b and *bmal1a* similarly, with higher expression levels in the ML feeding group compared to MD (two-way ANOVA p<0.05) (Fig. 2a and b) (Suppl. Table 2).

As for the negative loop*, per1b* and *per2* exhibited daily rhythms in both ML and MD feeding groups (Cosinor, p <0.05) but with different acrophases. In both groups, *per1b* peaked during the dark phase (ZT 23:34 h and 15:51 h for ML and MD, respectively) while *per2* peaked during the light phase (ZT 7:08 h and 4:46 h for ML and MD, respectively). In both cases, an advance of the acrophases in the MD group compared to the ML group was evident (Fig. 2c and d, Suppl. Table 1). This was mainly evident for *per1b*, which displayed a shift of around 7 h between ML and MD feeding groups (Fig. 2c). Like the positive loop, both genes showed significant differences between time points throughout the 24-hours cycle (two-way ANOVA, p<0.05) (Fig. 2c and d). Additionally, a statistically significant interaction between feeding time and sampling points was observed for both genes (two-way ANOVA p<0.05) (Suppl. Table 2).

The other two components of the negative loop of the hypothalamic circadian clock exhibited a different pattern. *Cry1a* showed rhythms both in ML and MD groups (Cosinor, p<0.05), with partially shifted acrophases since in the ML group it peaked during the light phase, at ZT 5:05 h, while in the MD group *cry1a* expression peaked at the beginning of the dark phase (ZT 10:23 h) (Fig. 2e and f; Suppl. Table 1). Conversely, *cry2* did not exhibit any significant rhythm (Cosinor, p>0.05). Moreover, significant differences between sampling points were observed only for *cry1a* in the ML group (two-way ANOVA, p<0.05). Significant differences between ML and MD groups were also found at ZT 12 h for *cry1a* and ZT 20 h for *cry2* (Fig. 2e and f). Finally, a significant interaction between feeding and sampling times was observed for *cry1a* mRNA expression (two-way ANOVA, p<0.05) (Suppl. Table 2).

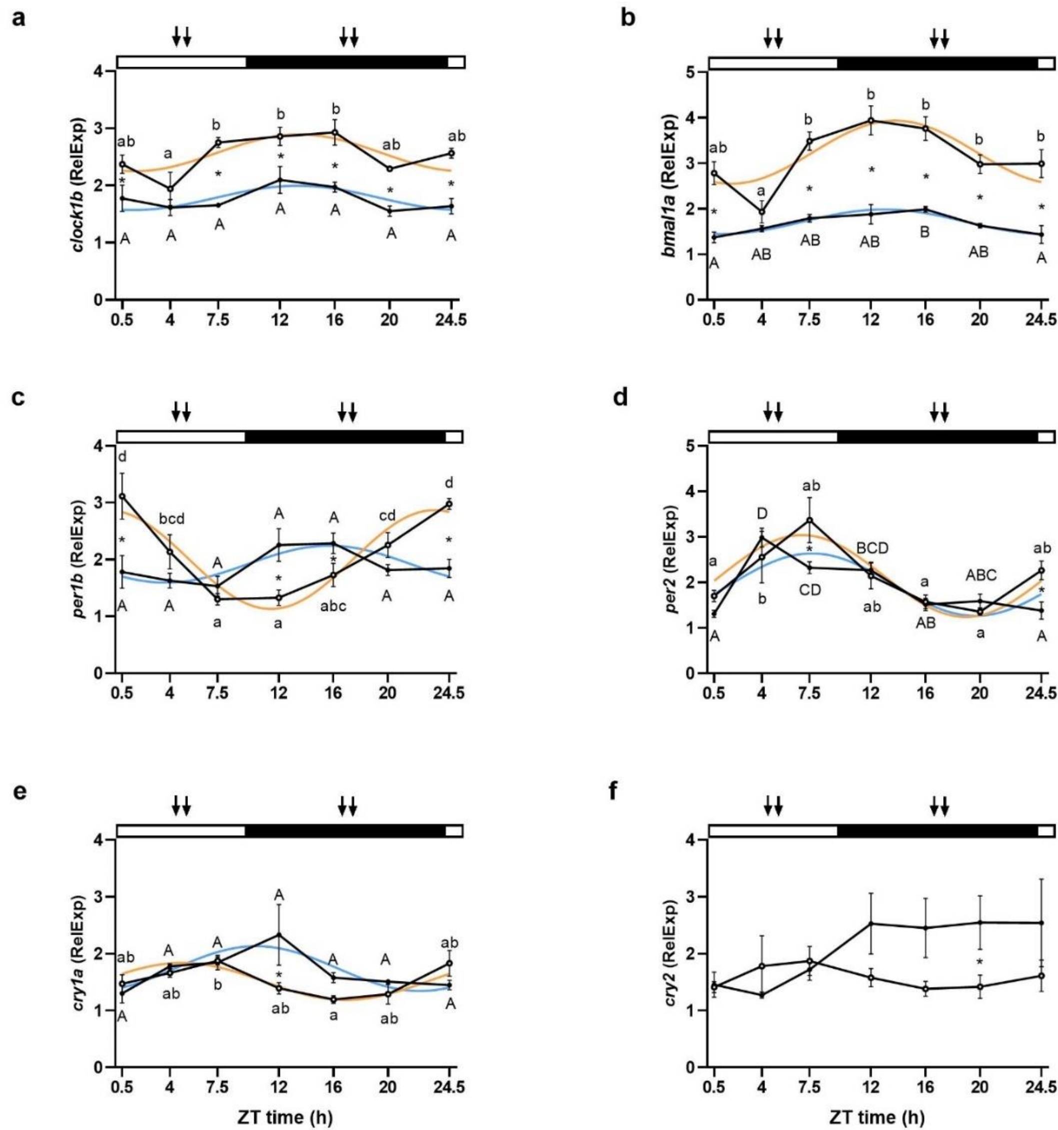

Fig. 2 Daily variations in the relative mRNA expression (fold change) of *clock1b* (A)*, bmal1a* (B), *per1b* (C), *per2* (D), *cry1a* (E) and *cry2* (F) in the hypothalamus of two groups of European sea bass maintained in a 10:14 LD cycle and fed during the middle of the light (ML) or dark (MD) phase. White circles (○) represent the ML group, while the MD group is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, $p<0.05$), when significant, is represented by orange and light blue lines for ML and MD groups, respectively. Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. The asterisks indicate significant differences between ML and MD groups at the same time point (two-way ANOVA). The white and black bars above the graphs represent the light and dark phases, respectively, while the arrows represent the feeding time for each group. The x-axis represents the time scale as ZT (*zeitgeber* time, h). All data are represented as mean ± SEM (n=7 fish per point)

Liver molecular clock

Regarding the molecular clock in the liver, the genes from the positive loop, *clock1b* and *bmal1a*, presented different patterns depending on the feeding regime used.

Both genes presented a significant rhythm in the ML group (Cosinor, $p<0.05$) with nocturnal acrophases located at ZT 12:49 h for *clock1b* and ZT 14:58 h for *bmal1a* (Fig. 3a and b, Suppl. Table 1). In contrast, no significant rhythms were observed in the MD group (Cosinor, $p>0.05$). However, significant differences between time points were found for *clock1b* and *bmal1a* in the ML group and *bmal1a* in the MD group (two-way ANOVA, $p<0.05$). Concerning the differences between ML and MD, statistically significant differences were found for *clock1b* at ZT 0.5, 7.5 and 20 h, while differences in *bmal1a* expression were found only at the beginning of the day (ZT 0.5 and 4 h) (two-way ANOVA, $p<0.05$) (Fig. 3a and b). Feeding time and sampling points significantly affected both genes, but not the interaction between these factors (two-way ANOVA $p<0.05$) (Suppl. Table 2).

Regarding the negative loop of the liver molecular clock, *per1b* and *per2* mRNA expression presented rhythms in the ML group (Cosinor, $p<0.05$), with nocturnal acrophases located at ZT 18:43 h and 23:17 h, respectively. However, only *per2* displayed rhythmicity in the MD group, showing a diurnal acrophase located at ZT 5:09 h (Fig. 3c and d, Suppl. Table 1). The two-way ANOVA analysis revealed significant differences between sampling times for *per1b* (ML and MD groups) and *per2* (only MD). Furthermore, significant differences between ML and MD groups on the mRNA expression of these genes were found at some ZT points: 0.5, 7.5 and 20 h for *per1b*, and 4, 7.5 and 24.5 h for *per2* (Fig. 3c and d) (two-way ANOVA $p<0.05$) (Suppl. Table 2).

Finally, *cry1a* and *cry2* presented a similar trend as observed for the other clock genes in the liver since a significant rhythmicity was only found in the ML group (Cosinor, $p<0.05$). In this group, the acrophases were located at the transition between light and dark phases for *cry1a* (ZT 9:50 h) and at the beginning of the dark phase for *cry2* (ZT 11:55 h) (Fig. 3e and f, Suppl. Table 1). Additionally, significant differences

between sampling points were found only for *cry1a* at the MD group, but the differences between the two feeding conditions (ML vs. MD) were observed at several sampling points for both genes: ZT 4, 12, 16, 20 and 24.5 h for *cry1a*, and ZT 4, 16 and 24.5 h for *cry2* (two-way ANOVA, p<0.05) (Fig. 3e and f).

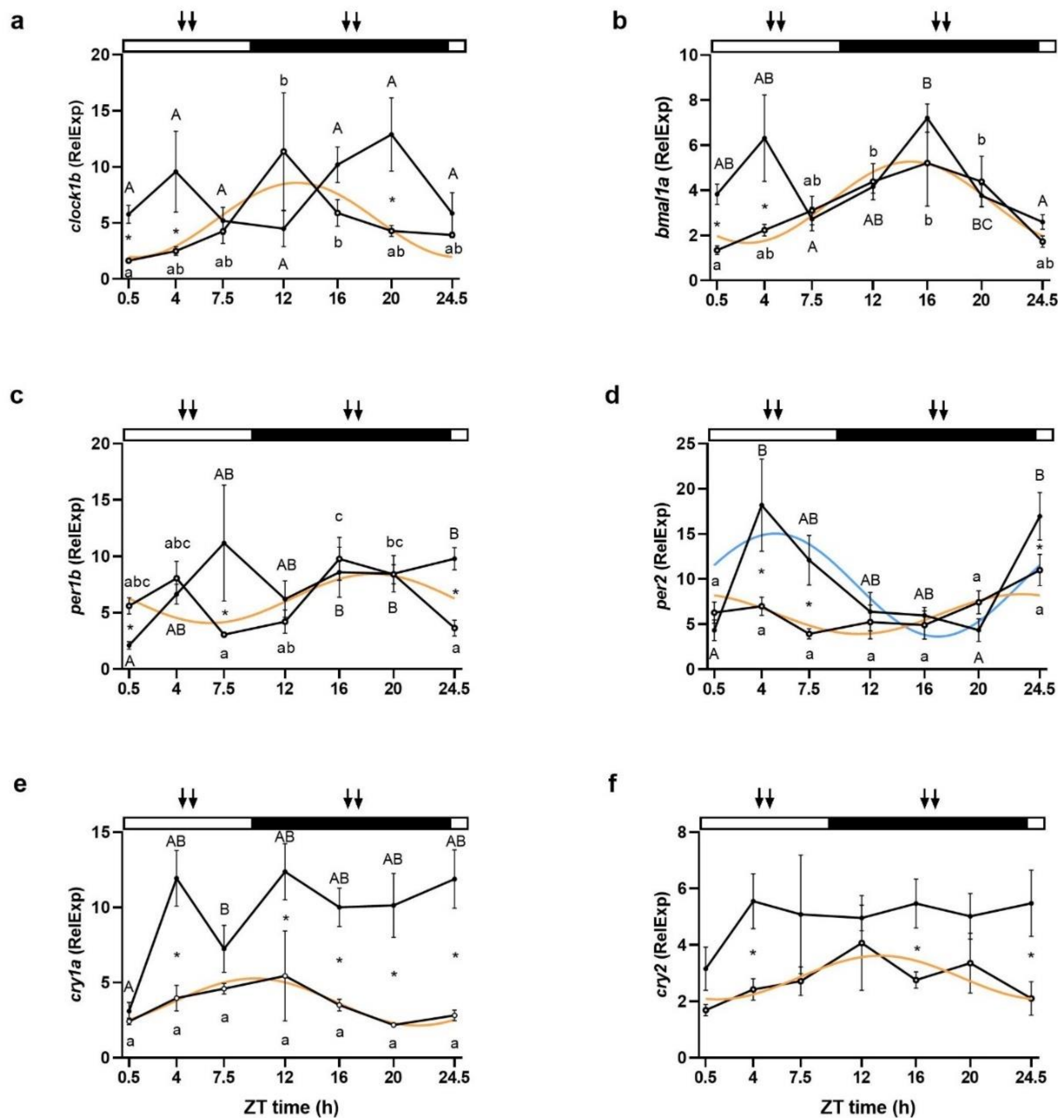

Fig. 3 Daily variations in the relative mRNA expression (fold change) of *clock1b* (A), *bmal1a* (B), *per1b* (C), *per2* (D), *cry1a* (E) and *cry2* (F) in the liver of two groups of European sea bass maintained in a 10:14 LD cycle and fed during the middle of the light (ML) or dark (MD) phase. White circles (○) represent the ML group, while the MD group is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, p<0.05), when significant, is represented by orange and light blue lines for ML and MD groups, respectively. Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. The asterisks indicate significant differences between

ML and MD groups at the same time point (two-way ANOVA). The white and black bars above the graphs represent the light and dark phases, respectively, while the arrows represent the feeding time for each group. The x-axis represents the time scale as ZT (*zeitgeber* time, h). All data are represented as mean ± SEM (n=7 fish per point)

Genes involved in methylation (*dnmt1, dnmt3a*), demethylation (*tet2, gadd45aa, mbd4*) and deacetylation processes (*sirt1*) in the liver

Genes involved in methylation and demethylation processes were analyzed in the liver. Concerning methylation, *dnmt1* and *dnmt3a* displayed daily rhythms with some distinction. *Dnmt1* displayed a significant rhythm solely in the ML group (Cosinor, $p<0.05$) whereas *dnmt3a* exhibited a rhythm in both ML and MD groups (Cosinor, $p<0.05$). The acrophases for both genes were located during the first half of the dark phase. In the ML group, *dnmt1* and *dnmt3a* presented very close acrophases, which were located at ZT 15:24 h and ZT 15:10 h, respectively. The acrophase of *dnmt3a* in the MD group was located at ZT 17:29 h, slightly delayed when compared to ML group (Fig. 4a and b, Suppl. Table 1). Both genes displayed significant differences between sampling points (two-way ANOVA, $p<0.05$) and the differences between ML and MD group at the same time point were significant at ZT 4 and 16 h for *dnmt1* and ZT 24.5 h for *dnmt3a* (Fig. 4a and b). Feeding and sampling time had a significant effect on methylation genes, but not the interaction between these two factors (two-way ANOVA, $p<0.05$) (Suppl. Table 2).

Regarding the daily rhythms in demethylation processes, all genes analyzed (*tet2, gadd45aa* and *mbd4*) displayed a similar pattern. The rhythm was only present in the ML group for all three genes, with nocturnal acrophases occurring closely together and situated towards the middle of the dark phase. Specifically, *tet2* peaked at ZT 16:37 h, *gadd45aa* at ZT 16:10 h and *mbd4* at ZT 16:25 h (Cosinor $p<0.05$) (Fig. 4c–e, Suppl. Table 1). In addition, all genes presented significant differences between time points

throughout the 24 hours in the ML group, but only *gadd45aa* conserved this pattern also in the MD group (two-way ANOVA, $p<0.05$) (Fig. 4c–e). All genes presented significant differences between ML and MD groups at ZT 0.5, 4, 7.5, 20 and 24.5 h, with the exception of *gadd45aa* at ZT 20 h (two-way ANOVA, $p<0.05$) (Fig. 4c-e). Feeding time significantly influenced the three genes, while only *gadd45aa* was also affected by the interaction between feeding and sampling times (two-way ANOVA, $p<0.05$) (Suppl. Table 2).

In addition to methylation and demethylation, the mRNA expression of *sirt1*, a gene involved in acetylation processes, was examined in the liver. This gene presented a significant daily rhythm solely in the ML group (Cosinor, $p<0.05$), with its acrophase occurring at the beginning of the dark phase (ZT 12:05 h) (Fig. 4F, Suppl. Table 1). Significant differences between time points throughout the 24 hours were observed in both feeding regimes. Furthermore, significant differences in *sirt1* expression between the ML and MD groups were found at ZT 0.5, 4, 16, 20, and 24.5 h (Fig. 4f) (two-way ANOVA, $p<0.05$). The mRNA expression of *sirt1* was significantly influenced by feeding and sampling times, but not by their interaction (two-way ANOVA, $p<0.05$) (Suppl. Table 2).

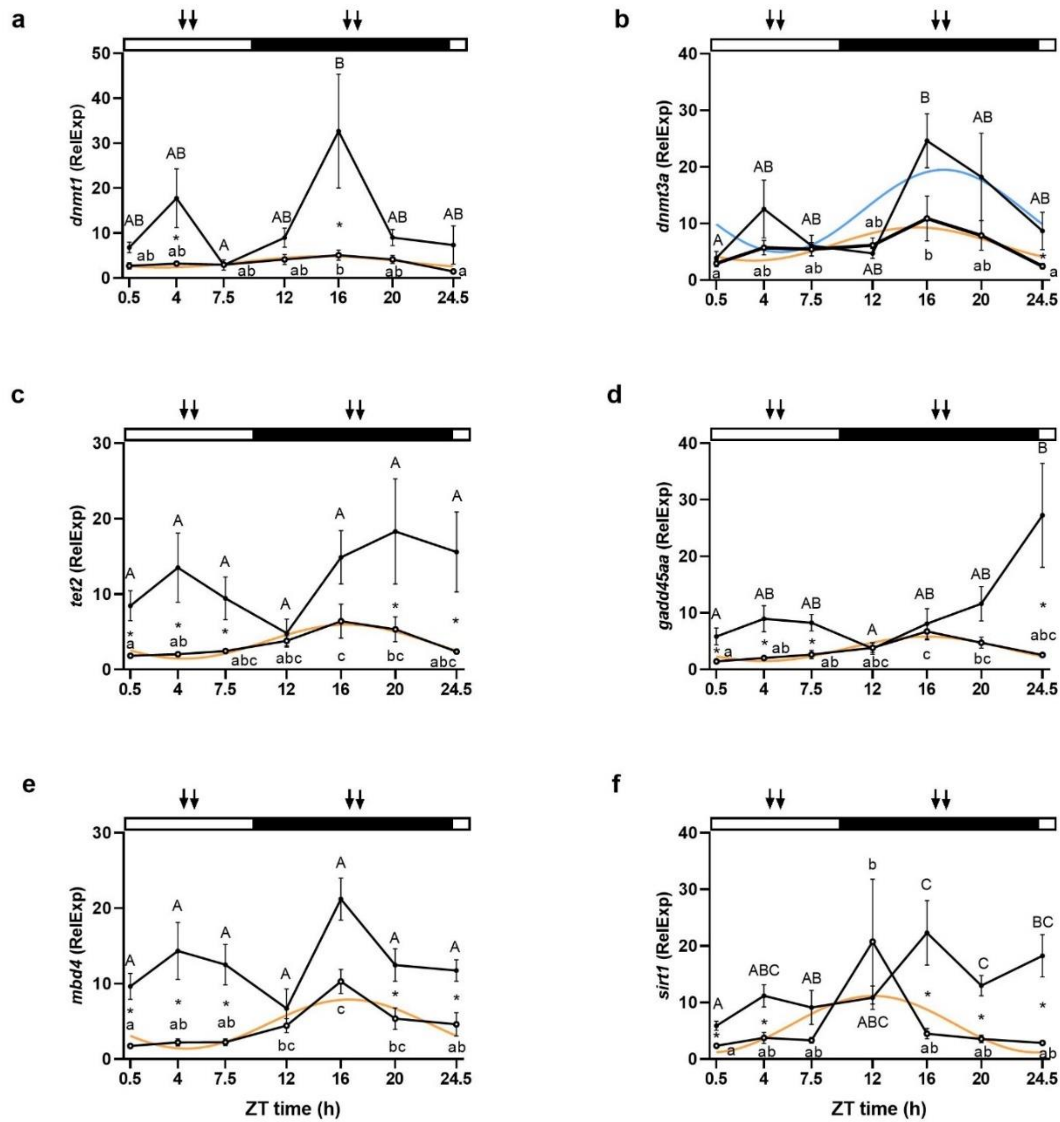

Fig. 4 Daily variations in the relative mRNA expression (fold change) of *dnmt1* (A)*, dnmt3a* (B), *tet2* (C), *gadd45aa* (D), *mbd4* (E) and *sirt1* (F) in the liver of two groups of European sea bass maintained in a 10:14 LD cycle and fed during the middle of the light (ML) or dark (MD) phase. White circles (○) represent the ML group, while the MD group is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, $p<0.05$), when significant, is represented by orange and light blue lines for ML and MD groups, respectively. Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. The asterisks indicate significant differences between ML and MD groups at the same time point (two-way ANOVA). The white and black bars above the graphs represent the light and dark phases, respectively, while the arrows represent the feeding time for each group. The x-axis represents the time scale as ZT (*zeitgeber* time, h). All data are represented as mean ± SEM (n=7 fish per point)

S-Adenosyl methionine (SAM), S-Adenosyl homocysteine (SAH) and methylation potential (SAM/SAH) in the liver

Besides gene expression, SAM and SAH abundance, as well as their ratio (SAM/SAH), were analyzed in the liver. None of these factors presented any significant rhythmicity (Cosinor $p>0.05$) (Fig. 5, Suppl. Table 1). SAH (ML and MD groups) and the SAM/SAH ratio (in the ML group) presented significant differences between time points (two-way ANOVA, $p<0.05$) (Fig. 5, Suppl. Table 1). In addition, significant differences between ML and MD groups were observed for SAM at ZT 16 and 24.5 h (Fig. 5). which was also affected by the feeding regime, with fish from the ML group showing higher SAM levels than MD (two-way ANOVA $p<0.05$) (Suppl. Table 2). This difference could also be observed by pooling the data from the day (d) or night (n) phases (Fig. 5, right). SAM levels were higher in the ML group than MD at both day and night (t-test, $p<0.05$) (Fig. 5A), while SAH levels were higher in the ML group than MD only during the light phase (t-test, $p<0.05$) (Fig. 5B). On the contrary, the SAM/SAH ratio presented no differences neither during the day or at night (t-test, $p>0.05$) (Fig. 5C).

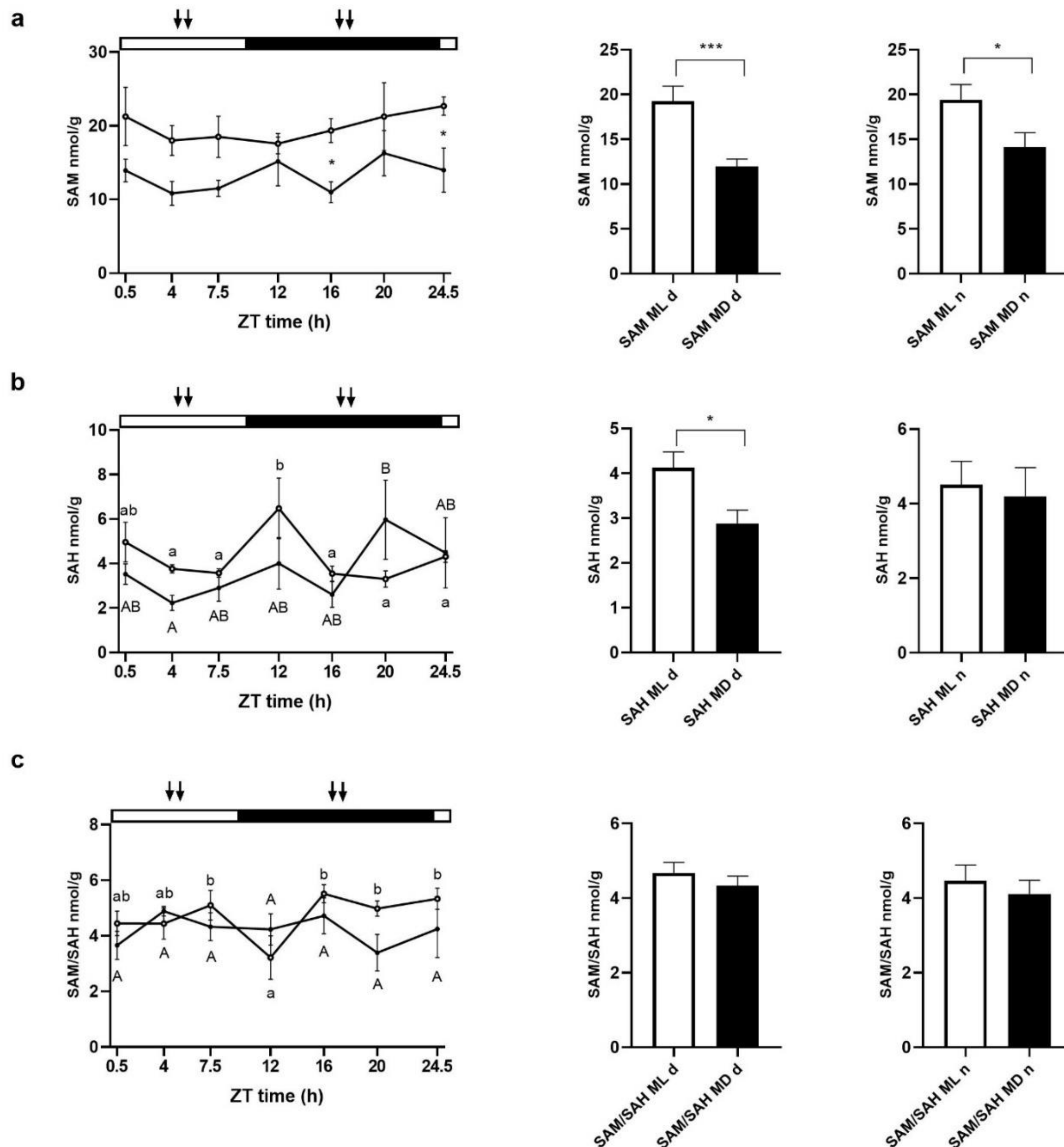

Fig. 5 Daily variations of S-Adenosyl methionine (SAM) (A), S-Adenosyl homocysteine (SAH) (B) and the methylation potential represented by the SAM/SAH ratio (C) in the liver of two groups of European sea bass maintained in a 10:14 LD cycle and fed during the middle of the light (ML) or dark (MD) phase. White circles (○) represent the ML group, while the MD group is represented with black dots (●). Statistically significant differences between ZT points within the ML and MD groups are represented by different lower- and upper-case letters (two-way ANOVA), respectively. The asterisks indicate significant differences between ML and MD groups at the same time point (two-way ANOVA). The white and black bars above the graphs represent the light and dark phases, respectively, while the arrows represent the feeding time for each group. The x-axis represents the time scale as ZT (*zeitgeber* time, h). In addition, to the right, day-night differences in SAM (A), SAH (B) and the SAM/SAH ratio (C) have been represented. Data from ML and MD groups are represented by white and dark bars, respectively. Values were obtained by pooling all data from the light (d) or the dark phase (n) and were compared by means of Student's t-test. Statistically significant differences between groups ($p<0.05$) are indicated with an asterisk. All data are represented as mean ± SEM (n=7 fish per point)

DISCUSSION

In the present research we examined the positive (*clock1b, bmal1a*) and negative (*per1b, per2, cry1, cry2*) loops of the molecular clock in both central (hypothalamus) and peripheral (liver) tissues of the European sea bass, along with the effects of feeding time on these factors and on behavior. Our analyses revealed that feeding significantly influences the clock in peripheral tissues but has a lesser impact on the brain of the sea bass (Fig. 6). Additionally, we demonstrated the existence of daily rhythms in the expression of various factors involved in epigenetic processes, such as methylation (*dnmt1, dnmt3a*), demethylation (*tet2, gadd45aa, mbd4*) and deacetylation (*sirt1*) in the liver of the sea bass (Fig. 6). Feeding time had also a strong influence on the rhythms of these factors, revealing a possible interplay between the circadian system and the epigenetic processes.

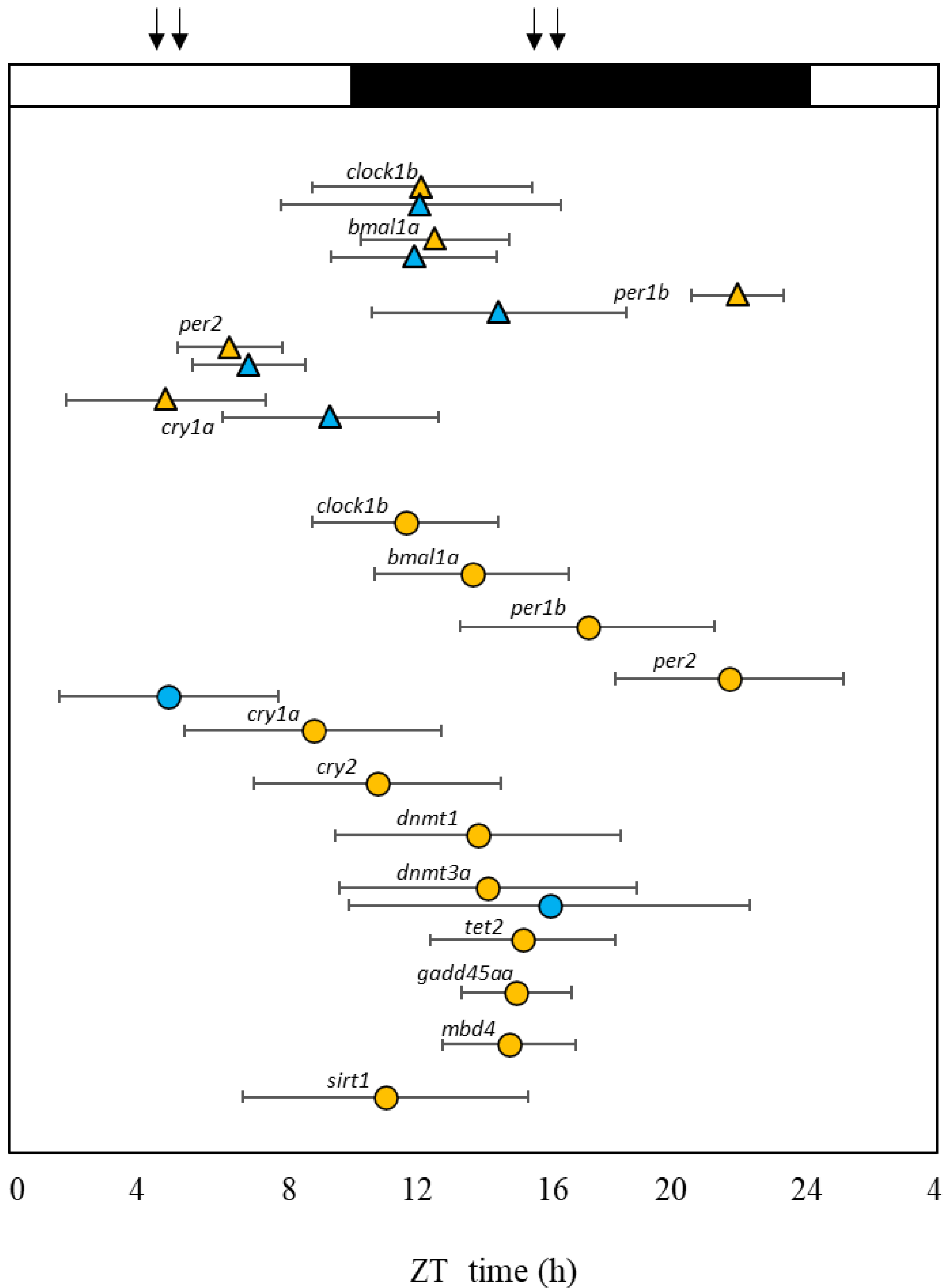

Fig. 6 Map of acrophases of the genes and proteins analyzed in the present study and involved in the molecular clock, methylation, demethylation, acetylation and methylation potential. The acrophase is indicated only for the statistically significant rhythms (Cosinor $p < 0.05$) and the name of each factor is indicated near the correspondent marker. ML group parameters are represented with orange triangles (hypothalamus) or circles (liver), while MD group parameters are represented with blue triangles (hypothalamus) or circles (liver). The x-axis represents the time scale, which is expressed as ZT (zeitgeber) time and where ZT 0 corresponds to the light onset. The white and dark bars above the figure represent the light and dark phases of the LD cycle (10:14 LD), respectively, and the black arrows represent the feeding times

The clock genes work at the molecular level creating a self-sustainable molecular clock based on positive and negative feedback loops (Vatine et al. 2011). Food can be a powerful synchronizer for the molecular clock of peripheral oscillators both in mammals and fish, but central oscillators in the brain do not seem to respond in the same way to feeding time (López-Olmeda 2017). In our study, in the hypothalamus the rhythm is present in most of the clock genes analyzed (*clock1b, bmal1a*, *per1b, per2* and *cry1*) in both ML and MD groups. In sea bass fed at ML, genes from the positive loop (*clock1b* and *bmal1a*) present an acrophase around the beginning of the dark phase, while the acrophases of genes from the negative loop are shifted and peak around the end of the night (*per1b*) or during the light phase (*per2, cry1a*). These rhythms and their phases are conserved among teleost fish and have been reported for several species such as the zebrafish (Vatine et al. 2011), Nile tilapia (Costa et al. 2016), gilthead seabream (Vera et al. 2013), goldfish (Gómez-Boronat et al. 2018), and also previously for the European sea bass (Sanchez et al. 2010, Del Pozo et al. 2012, Herrero and Lepesant 2014). Regarding fish fed at MD, *clock1b* and *bmal1a* presented similar acrophases to the ML group, pointing that feeding time alone probably is not enough to affect the positive loop. In the genes of the negative loop (*per1b, per2* and *cry1*), in contrast, we observed a shift in the acrophases of *cry1a* and *per1b* when comparing ML and MD, with *per1b* presenting the most significant difference with an eight-hour phase shift between the two groups. Although the circadian system of fish is more likely to be a multi-oscillatory system, the fish hypothalamic clock usually behaves in a similar way of the central clock in mammals, located in the SCN of the hypothalamus (López-Olmeda 2017). Thus, the clock in this tissue entrains mainly to light, with food having little influence on it (Vera et al. 2013; Costa et al. 2016). However, the responsiveness of the molecular clock in this tissue to feeding may be different depending on the fish species. For instance, a similar phase advance of the clock genes in the hypothalamus, as we have observed for *per1b,* was

previously reported in goldfish fed at MD compared to ML feeding (Gómez-Boronat et al. 2018). Hypothalamic *per1* seems to be more affected by feeding time than other clock genes also in Wistar rats, where a specific food-restricted access determined a phase advance of its acrophase in the hypothalamus (Miñana-Solis et al. 2009) or a change in the patterns of expression (De Araujo et al. 2016), suggesting that this gene may serve as the gateway through which feeding exerts its effect on the circadian clock.

In the liver, feeding time significantly influenced the molecular clock, as all the analyzed genes from both the positive and negative loops exhibited rhythms in the ML group, while only *per2* maintained the rhythm in the MD group. Notably, *per2* has been previously characterized as light-dependent in zebrafish (Pando et al. 2001, Vatine et al. 2009), suggesting a similar regulation in the European sea bass. Interestingly, nocturnal feeding in diurnal sea bass suppressed the rhythmicity of certain clock genes in the liver, analogous to observations in some clock genes in other species such as Nile tilapia (*bmal1a* and *per1b*) and gilthead seabream (*per2*) (Vera et al. 2013, Costa et al. 2016). The complexity of the circadian system suggests that additional factors such as seasonality might be involved, as evidenced in previous studies on the European sea bass pituitary (Herrero and Lepesant 2014). Furthermore, we examined the daily rhythms of locomotor activity as an indicator of the output signal from the circadian system in sea bass. Previous studies have demonstrated that periodic feeding alone serves as an important *zeitgeber* for the locomotor activity rhythms of fish (López-Olmeda 2017). When conflicting with the LD cycle, nocturnal feeding may lead to a shift from diurnal to nocturnal activity, as observed in certain fish species, such as the gilthead seabream (Montoya et al. 2010). However, in some species, the alteration in behavioral patterns is not consistently complete. For example, goldfish predominantly exhibited diurnal locomotor activity regardless of feeding time, although a significant reduction in diurnalism was noted in fish fed during the middle of the night compared to those fed

during the light phase (Gómez-Boronat et al. 2018). This aligns with the findings of the current research on European sea bass behavior, where both groups remained primarily diurnal, but nighttime feeding diminished the percentage of diurnalism and altered the shape of the daily rhythm of activity. Collectively, these results underscore the influence of feeding time on clock genes and its impact on overt rhythms such as the daily patterns of locomotor activity.

Recently, there has been growing interest in the relationship between the circadian system and the epigenetic mechanisms of DNA methylation. These mechanisms are implicated in the transcriptional regulation of clock genes at various levels. Previous studies have suggested that Dnmt3a may play a role in methylating the promoter of *Bmal1* in certain diseases, leading to its silencing (Satou et al. 2013). Additionally, DNA methyltransferases are intertwined with cellular metabolism, as they depend on the availability of methyl groups, thus linking this epigenetic mechanism to feeding behavior. In our investigation, we aimed to determine whether genes involved in the epigenetic mechanisms of the sea bass exhibit daily rhythms and how feeding time may influence this epigenetic system. To the best of our knowledge, rhythms in the expression of factors involved in DNA methylation and demethylation in fish have only been previously demonstrated in zebrafish gonads (Paredes et al. 2018). In our study, European sea bass fed during the light phase displayed rhythmic expression of genes involved in methylation (*dnmt1, dnmt3a*) and demethylation processes (*tet2, gadd45aa,* and *mbd4*). In contrast, only *dnmt3a* exhibited rhythms in the liver of fish fed during the dark phase, similar to what was observed for the clock genes, where most of their rhythms were suppressed in the liver of these fish. In all cases, the acrophases were situated during the dark phase, which corresponds to the resting phase of the sea bass used in the experiment, as evidenced by the locomotor activity records. This finding aligns with the results of a previous study in zebrafish ovaries, where genes involved in methylation and

demethylation also exhibited daily rhythms with peak values during the dark/resting phase (Paredes et al. 2018). In mouse liver, the peak expression of *dnmt3a* occurs during the light phase, correlating with the variation in DNA methylation levels (Xia et al. 2015). Mice, unlike zebrafish or sea bass, are nocturnal animals and are more active during the night, resting during the light phase (Robinson-Junker et al. 2018). Thus, in both mammals and fish, the acrophase of genes involved in methylation processes appears to be inversely related to their activity phase, suggesting that the epigenetic landscape may undergo more significant remodeling during the animal's resting phase.

The acrophases of genes involved in methylation and demethylation closely resemble those displayed by *clock1b* and *bmal1a*, supporting the idea of a connection between the two systems in the liver of sea bass, as previously found in mice (Maekawa et al. 2012). Among all genes analyzed in the epigenetic mechanisms, *dnmt3a* was the only gene exhibiting a rhythm in fish fed during the dark phase, thereby escaping the effects elicited by MD feeding that seemed to suppress the rhythms in the other genes analyzed. This suggests that *dnmt3a* rhythms may not respond to food signals and could be regulated by other systemic mechanisms. For instance, in mammals, *Dnmt3a* rhythms seem to be regulated by the daily variations in SAM levels, as well as the SAM/SAH ratio (Zhang et al., 2018). The fact that *dnmt3a* expression was not altered by feeding time and maintained its rhythmicity also suggests the high importance of this gene in circadian mechanisms. In mammals, *dnmt3a* is identified as a potential transcriptional regulator for *bmal1a* (Satou et al. 2013). Moreover, knocking out DNMT3a in a cell line induced differences in the circadian periods of this cell line (Li et al. 2020), supporting the idea of the importance of this factor in the maintenance or stability of the molecular clock. On the other hand, the rhythm of *dnmt1* was not maintained in the MD group, suggesting that, at least in the sea bass liver, the maintenance of methylation driven by *dnmt1* could follow a different regulation than de novo methylation driven by *dnmt3a*.

Acetylation plays an important role in regulating the circadian system through *sirt1*, whose histone deacetylase activity compensates for CLOCK's acetylase function (Nakahata et al. 2006). In mammals, SIRT1 is described as a NAD+-dependent cytoplasmic enzyme that relies on cellular energy (Suave et al. 2006), suggesting an essential role of feeding in its regulation. Moreover, *Sirt1* gene expression and its protein levels do not display rhythmicity in mice (Nakahata et al. 2008), but NAD+ presents a clear oscillation in this species, ultimately regulating the daily activity of SIRT1 (Ramsey et al. 2009; Bellet et al. 2013). In our study, contrary to observations in mammals, *sirt1* mRNA expression exhibited a daily rhythm in the European sea bass. Additionally, daily rhythms in *sirt1* expression were suppressed in fish fed during the MD period, indicating a significant effect of feeding and possibly of the metabolic state through NAD+ levels, as observed in mammals. However, further research would be required in the future to test this hypothesis regarding the role of NAD+ in fish.

Finally, SAM, SAH, and the methylation potential (SAM/SAH ratio) did not display a circadian rhythm, but there were some differences observed when comparing the phase of the LD cycle and feeding time. SAM showed differences between ML and MD fed sea bass, suggesting a distinct utilization of energy provided by the diet, as ATP is required for the process of methionine activation to synthesize SAM (Froese et al. 2019). Additionally, SAH exhibited nocturnal peaks, with higher values at night than during the day, at ZT 12 and 20 h for ML and MD groups, respectively. Similar day-night differences have been previously reported in mammals (Xia et al. 2015). These peaks were inversely related to the lowest values in the methylation potential indicated by SAM/SAH in the sea bass liver. The low values in the methylation potential during the dark phase would also suggest a higher methyltransferase activity at this time of day, correlating with the higher *dnmt3*a expression observed during this phase.

CONCLUSIONS

This paper provides insights into the intricate interplay between the external synchronizers (light and feeding times), the circadian system and epigenetic mechanisms in the European sea bass (*Dicentrarchus labrax*). Our findings revealed that feeding time exerts differential effects on the molecular clock of the hypothalamus, causing notable shifts in the acrophases of key genes of the negative feedback loop. Interestingly, the positive loop genes exhibited no significant differences depending on feeding time. The liver, a crucial peripheral pacemaker, displayed rhythmic expression of clock genes influenced by feeding times, emphasizing the role of feeding as a potent *zeitgeber* for this organ. Moreover, this research uncovered the rhythmicity of epigenetic genes associated with methylation and demethylation processes, describing also a tight connection between feeding time and epigenetic regulation. Notably, the synchronization of circadian and epigenetic processes in the liver, with peak activity during the resting phase, highlights the potential significance of this phase for epigenetic modifications. Overall, this research contributes to our understanding of the intricate temporal dynamics governing the circadian and epigenetic landscape in the European sea bass, offering a foundation for future studies exploring the broader implications of these findings in the context of fish physiology and chronobiology.

SUPPLEMENTARY MATERIALS LEGENDS

Suppl. Fig 1 Schematic representation of the experimental design and sample collection. Fish were maintained under a 10:14 LD cycle and divided into two groups (7 tanks/group) fed a two different time points: around the middle of the light phase (ML group) or around the middle of the dark phase (MD group). After 30 days of acclimation, fish were sampled in a 24-hours cycle (approximately every 4 hours). White and black bars represent the light and dark phases, respectively. Feeding times are indicated by the arrows

Suppl Table 1 Resume of the Cosinor analysis results of the biological variables analyzed in the present study. Data are only indicated for significant rhythms (Cosinor, $p<0.05$): p value, mesor, amplitude and acrophase (indicated in ZT time). Data are expressed as value ± fiducial limits (set at 95%)

Tissue and biological factor	Experimental group	*p* values	Mesor	Amplitude	Acrophase (ZT hours)
Hypothalamus					
clock1b	ML	0.0072	2.57 ± 0.15	0.32 ± 0.24	13:21 ± 3:33
	MD	0.03208	1.78 ± 0.11	0.21 ± 0.19	13:18 ± 4:31
bmal1a	ML	0.00024	3.24 ± 0.23	0.69 ± 0.37	13:46 ± 2:33
	MD	0.00109	1.71 ± 0.10	0.27 ± 0.17	13:06 ± 2:40
per1b	ML	0,000	2.01 ± 0.18	0.87 ± 0.32	23:34 ± 1:24
	MD	0.01396	1.92 ± 0.15	0.32 ± 0.26	15:51 ± 2:07
per2	ML	0.00005	2.13 ± 0.23	0.90 ± 0.44	7:08 ± 1:42
	MD	0.00007	1.94 ± 0.18	0.68 ± 0.35	4:46 ± 1:50
cry1a	ML	0.00445	1.51 ± 0.12	0.32 ± 0.23	5:05 ± 2:23
	MD	0.01844	1.73 ± 0.18	0.39 ± 0.23	10:23 ± 3:30
cry2	ML	0.19821			
	MD	0.11578			
Liver					
clock1b	ML	0.00219	4.95 ± 1.45	3.49 ± 2.49	12:49 ± 3:01
	MD	0.12196			
bmal1a	ML	0.00412	3.47 ± 0.72	1.8 ± 1.21	14:58 ± 3:00
	MD	0.15100			
per1b	ML	0.02025	6.29 ± 1.07	2.27 ± 1.96	18:43 ± 4:07
	MD	0.65381			
per2	ML	0.02067	6.16 ± 1.09	2.21 ± 1.92	23:17 ± 3:22
	MD	0.00628	9.29 ± 2.45	5.68 ± 4.25	5:09 ± 3:32
cry1a	ML	0.03223	6.68 ± 0.8	1.57 ± 1.46	9:50 ± 3:09
	MD	0.34997			
cry2	ML	0.02714	2.61 ± 0.48	0.93 ± 0.84	11:55 ± 3:01
	MD	0.83407			
dnmt1	ML	0.01397	3.61 ± 0.61	1.25 ± 1.03	15:10 ± 4:06
	MD	0.08701			
dnmt3a	ML	0.02922	6.33 ± 1.62	3.02 ± 2.76	15:29 ± 4:50
	MD	0.04385	11.28 ± 3.18	5.57 ± 5.44	17:29 ± 4
tet2	ML	0.00178	3.72 ± 0.85	2.27 ± 1.5	16:37 ± 3.01
	MD	0.34229			
gadd45aa	ML	0.00005	3.65 ± 0.63	2.18 ± 1.1	16:10 ± 2:10
	MD	0.11877			
mbd4	ML	0.00001	4.49 ± 0.84	3.40 ± 1.47	16:25 ± 1:48
	MD	0.33270			
sirt1	ML	0.03910	6.06 ± 2.69	4.84 ± 4.62	12:05 ± 4:32
	MD	0.07929			
SAM	ML	0.31358			
	MD	0.55693			
SAH	ML	0.51030			
	MD	0.23000			
SAM / SAH	ML	0.36302			
	MD	0.54500			

Suppl Table 2 P values obtained in the two-way ANOVAs analysis for the comparisons between feeding time (FT) (ML *vs* MD), sampling time (ZT) and the interaction between the two factors (FT * ZT)

Gene	FT	ZT	ZT * FT
Hypothalamus			
clock1b	<0.001	0.014	0.036
bmal1a	<0.001	<0.001	0.043
per1b	0.335	<0.001	0.001
per2	0.118	<0.001	0.006
cry1a	<0.001	<0.001	0.008
cry2	<0.001	0.088	0.588
Liver			
clock1b	<0.001	<0.001	0.403
bmal1a	<0.001	<0.001	0.14
per1b	0.157	<0.001	<0.001
per2	0.118	<0.001	0.006
cry1a	0.08	0.243	0.05
cry2	0.025	0.573	0.143
dnmt1	<0.001	<0.001	0.156
dnmt3a	0.008	<0.001	0.522
tet2	<0.001	0.118	0.187
gadd45aa	<0.001	<0.001	0.002
mbd4	<0.001	<0.001	0.088
sirt1	<0.001	0.002	0.113
SAM - SAH			
SAM	<0.001	0.515	0.907
SAH	0.116	0.223	0.145
SAM/SAH	0.111	0.239	0.316

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Experimental chapter V

Feeding entrainment of the primary and accessory loops of the molecular circadian clock and epigenetic processes in Zebrafish brain: the importance of the glucocorticoid system

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ABSTRACT

The molecular clock is synchronized by environmental stimuli, allow the fish to predict external changes and is organized as a loop system which integrate positive and negative feedback. Different molecular pathways in the cells are then synchronized by the clock system, but at the same time, they can be involved in the modulation of the molecular clock as well, creating a reciprocal interaction. The molecular clock is not only sensitive to external factors, but also regulated by internal signaling. Between them, the glucocorticoids system is one of the main candidates in their regulation. In this study, we used glucocorticoid receptor null Zebrafish to investigate the importance of glucocorticoids on the molecular clock, as they are considered key regulators, involved in the metabolic pathways or in epigenetic processes. Moreover, we investigate the importance of feeding time in the synchronizations of these genes submitting fish to different time schedules (mid-light feeding, ML and mid-dark feeding, MD). Regarding the molecular clock system, our results reveled that *bmal1a* and *per1b* expression presented rhythm in both genotypes ($gr^{+/+}$ and $gr^{-/-}$) and feeding conditions analyzed, with similar acrophases between the groups (*bma1a* between ZT 9.78 and 11.60, and *per1b* between 23.60 and 23.91). On the contrary, *rev-erbα* and *rorα* which are part of the secondary regulatory loop, presented rhythm in gene expression only in $gr^{+/+}$ genotype in ML condition, respectively at ZT 22.52 and 1.28 h. *Nfil3-5*, involved in the negative loop of the molecular clock, presented rhythm in all genotype and conditions with acrophases located between ZT 9.38 and 9.63 h. *Bdnf,* a neurotrophin was analyzed for its involvement in the regulation of the circadian system, and presented rhythm only when fish were fed during the light phase, at ZT 23.75 and 1.60 h in $gr^{+/+}$ and $gr^{-/-}$ genotypes, respectively. A similar acrophase was described for *pparα,* that peaked at ZT 24 $gr^{+/+}$ in ML conditions, but on the contrary, it didn't conserve the rhythm in any other conditions.

Lastly, among the genes involved in the epigenetic mechanisms, two of them presented rhythm when fish were fed during the night, respectively d*nmt4* $gr^{+/+}$ genotype (ZT 6.23 h), and *tet2* $gr^{-/-}$ genotypes (ZT 10.13 h). Moreover, plotting day and night data, revealed differences between the genotypes especially during the day when fish are fed during the light phase. Our data suggest that glucocorticoids and feeding time can modulate the molecular clock system. Additionally, epigenetic processes seem to be more affected during the day when comparing the two genotypes.

INTRODUCTION

All living organisms are subjected to environmental and cyclic variables which work as synchronizing factors, promoting the establishment of the biological rhythms. In specific, is possible to describe a multicomponent system where different environmental stimuli (*zeitgebers*, or time giver), entrain an oscillator, lastly determining the establishment of a rhythm, which can be displayed at several level from behavioral to molecular (López-Olmeda, 2017). This mechanism has been refined during the evolution and allow the fish to keep track of the time or anticipating the daily changes (Krittika and Yadav, 2020). In fish, even though there are different hypotheses on the overall organization of the circadian system, a multi-oscillatory system with no hierarchical relationship between the oscillators seems to be the most representative (Cahill, 2002, Vatine et al., 2011), with different pacemakers being sensitive to several stimuli at the same time (López-Olmeda, 2017).

To allow the perfect tuning of the circadian system, a molecular control is the key, and is represented by an autoregulatory feedback loop that comprehend positive and negative regulation. This positive regulation is based on the activity of the genes *clock* (Circadian Locomotor Output Cycles Kaput) and *bmal* (Brain and muscle arnt-like), which encode for transcriptional factors capable of inducing a second circuit formed by *per* (Period) and *cry* (Cryptochrome). The activity of *per* and *cry* is exerted by interfering with CLOCK-BMAL complex, negatively regulating the system. Another secondary loop starts when the genes *rev-erbα* and *rorα* are transcribed by CLOCK-BMAL heterodimer. They can either stop (*rev-erbα)* or promote (*rorα) bmal* transcription by competing for its E-box region, conferring another level of regulation to the system. Additionally, CLOCK-BMAL dimer binds different clock-controlled genes (CCG) activating they rhythmic expression by imposing a circadian regulation over them (Cahill, 2002; Pando and

Sassone Corsi, 2002; Sato et al., 2004; Zhadanova and Reebs 2005; Vatine et al., 2011; Cox and Takahashi, 2019). In fish, multiple copies of the clock genes have been described, probably due to the whole genome duplication event (WGD) (Idda et al., 2012). This organization represents the main core of the molecular clock, but the system is also subjected to a multilevel organization process where different checkpoints ensure its function, as if multiple loops would be integrated in a more complex network (Cermakian and Sassone-Corsi, 2000). For examples in mammals, *Rev-erbα* not only negatively regulates *Bmal1* through its promoter but exerts its function also on *Nfil3* (E4BP4) (Fang et al., 2014). *Nfil3* is a transcriptional repressor involved in multiple pathways related to metabolisms and immune cell differentiation which aberrant expression is linked to many types of cancer (Zeng et al., 2023), and in the clock system it targets and inhibits *Per2* gene expression, thus adding another level of regulation of the circadian system (Doi et al., 2001). Additionally, others important mutual connections are established between the clock genes and other factors, like *bdnf* or *pparα*. *Bdnf* is a neurotrophin involved in multiple function, spanning from neural plasticity and survival (Tapia-Arancibia et al., 2004) to food intake regulation (Blanco et al., 2020), which display circadian variation in mammals (Tapia-Arancibia et al., 2004). At the same time, *Bdnf* involvement in photic entrainment was shown in mice (Liang et al., 2000) and in circadian rhythmicity in zebrafish larvae (D'Agostino et al., 2022). On the other hand, *pparα* represents the link with metabolism (Duez and Staels, 2009). *Pparα* is nuclear receptor expressed in different tissues and its activation is dependent on fatty acids and eicosanoid. It exerts its function after forming a heterodimer with RXRα (retinoid X receptor) (Gearing et al., 1993) by binding the PPRE (peroxisome proliferator response element) on the regulatory regions of genes involved in different biological processes (Louet et al., 2001, Bougarne et al., 2018). *Pparα* has been proved to be under circadian control in the liver of different fish species (Paredes et al., 2014, and 2015), but at the same time it can modulate *Rev-erbα*

gene expression in mammals by targeting the Rev-DR2 sequence on its promoter, inducing its transcription (Duez and Staels, 2007).

Many physiological processes present daily rhythm thanks to the activity of the self-sustainable clock and several studies have described the daily rhythm of glucocorticoids in fish (López-Olmeda et a., 2013, Vera et al., 2013, Cowan et al., 2017, Samorì et al., 2024). Their function is exerted by binding the glucocorticoid receptor (GR), and subsequently targeting the glucocorticoid responsive element (GRE) located on the promoter of his target genes, promoting, or repressing the transcription (Faught et al., 2016). Several evidence suggest that glucocorticoids themselves could participating in the regulation of the molecular clock thus creating a bidirectional regulatory system (Reddy et al., 2007). In zebrafish larvae, glucocorticoids are involved in cell proliferation rhythm (Dickmeis et al., 2007) and dexamethasone (a glucocorticoid analog) induce *per1* rhythmic expression in mammalian cell culture fibroblast just one hour after the treatment (Balsalobre et al., 2000). Specifically, *per1* gene is one of the main targets of glucocorticoids in mammal (Yamamoto et al., 2005) and fish, (Sánchez-Bretaño et al. 2016), but is not the solely, since GRE region have been found in *ror* and *rev-erbα* (Dickmeis et al., 2013) or *nfil3* (Carey et al., 2013; So et al., 2009). Difference in clock gene expression have also been found confronting Zebrafish wild type and null corticoid receptor (*gr*$^{-/-}$) larva and adult eye but no data are available on the brain (Morbiato et al., 2019). The same study has also proved that GC (glucocorticoid) are important in the feeding entrainment of Zebrafish, which was clearly dampened in mutant genotype (Morbiato et al. 2019).

The stress response can potentially affect different physiological pathway, but also epigenetic landscape, as seen for the change in the methylation pattern of European sea bass (*Dicentrarchus labrax*) or half-smooth tongue sole (*Cynoglossus semilaevis*)

exposed to different stress (Anastasiadi et al., 2017; Li et al., 2017). Epigenetic processes are central in the organisms and especially the methylation processes are linked to several physiological pathways. The connection between the clock system and the epigenetic mechanisms has been recently taken into consideration, also due to the possibility of a reciprocal influence, with particular attention to methylation processes (Stevenson, 2018). The process of DNA methylation is mediated by a group of enzymes called DNA methyltransferases (DNMTs), which add a methyl group on the CpG dinucleotide, promoting the silencing by preventing the transcription (Bird, 2002; Moore et al. 2013). Nevertheless, these enzymes promote the same effect, but is possible to distinguish between *dnmt1*, which maintain the methylation after the round of DNA replication, and the *dnmt3*s, a group of genes which encode for enzymes involved in de-novo methylation, even though some studies have pointed out that this classification could be outdated and the overall effect could be due to an interaction between the enzymes (Hsieh, 2000; Fatemi et al., 2002). The overall process displays a high grade of flexibility, and the added methyl group can be erased in the process of de-methylation that can proceed by passive erasing (during DNA replication) or due to the action of specific enzymes (Bochtler et al., 2017). The latter possibility is initiated by the Ten-eleven translocation (Tet) enzymes, which convert the 5mC to 5hmC by adding a hydroxyl group. This first oxidation can be followed by other rounds of oxidation mediated by TETs to finally obtain a 5caC which is excised by the thymine-DNA glycosylase (TDG) and replaced by unmodified cytosine in a bas excision repair process (BER) (Ito et al., 2010, Moore et al., 2013). As other many physiological processes, DNA methylation is influenced by the circadian machinery. Specifically, the heterodimer CLOCK-BMAL target *dnmt3a*'s promoter binding site initiating a rhythmic transcription (Stevenson, 2018). The existence of a daily rhythm has been observed in Zebrafish gonads, where different *dnmt*s were expressed rhythmically throughout the 24 hours with acrophases mostly located during the resting phase of the

animals (Paredes et al., 2018). Additionally, in mammals, *Dnmt3a* could be the responsible of a change in the methylation status of *Bmal1*'s promoter (Satou et al., 2013), determining a bidirectional link. Several studies have already proved how methylation can be susceptible to external clues, and beside them, feeding play a central role (Skjaerven et al., 2018). Despite the slight difference in terms of function, all the DNMTs rely on the presence of a methyl group (Hermann et al., 2004), which is released from S-adenosyl-methionine (SAM) before reentering the methionine cycle as S-adenosyl-homocysteine (SAH), providing a strong connection between methylation processes and feeding.

Among the external synchronizer capable of entraining the different pacemaker, food availability or feeding time can be considered among the most important (Roenneberg et al., 2003). Food is not always constants and available in the wild, and for this reason, behavioral patterns and physiological processes have been optimized to take advantage of it. The aim of this study was to investigate the importance of glucocorticoid signaling pathway on clock, metabolic and epigenetic genes in the brain of Zebrafish subjected to different scheduled feeding time. For this purpose, we focused on the zebrafish glucocorticoid receptor $gr^{ia30/ia30}$ mutant line (referred as $gr^{-/-}$) which is characterized by high levels of body cortisol and a dysregulated expression of *crh* and *pomca*, two important players of the HPI axis (Facchinello et al., 2017).

MATERIALS AND METHODS

The animal housing and experimental procedure was performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and the Italian animal protection standards (D.lgs. 26/2014). The

University of Padova and University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health (auth. num. 112/2015 and 801/2017-PR) approved the experiment (auth. num. 112/2015 and 801/2017-PR).

Animal and housing

Wild-type (hereby referred as *gr*$^{+/+}$) and GR knock-out (hereby referred as *gr* $^{-/-}$) Zebrafish from the (9×GCRE-HSV.Ul23:EGFP)ia20 transgenic line were obtained from Dalla Valle Lab and housed at the Fish Chronobiology Laboratory of the University of Ferrara from one month of age. Fish were allocated in 200-L aquaria supplied with oxygenator, biological and mechanical filters. The photoperiod was set to provide 12:12 h of light and dark, with light onset at Zeitgeber time 0, ZT0 at 8:00 a.m. The water temperature was monitored and kept at 27°C. Fish were fed three time as day at libitum using *Artemia salina* nauplii and commercial dry food (Vipan Nature, Sera GmbH, Heinsberg, Germany) and maintained in this condition until they reached nine months of age.

Experimental design

Gr$^{+/+}$ (N = 60; 0.30 ± 0.04 g body weight) and *gr*$^{-/-}$ (N = 60; 0.33 ± 0.02 g body weight) Zebrafish were randomly divided into four groups based on the feeding time. Two groups were fed during the mid-light phase (ML groups, *gr*$^{+/+}$ and *gr*$^{-/-}$) and the others during the mid-dark phase (MD groups, *gr*$^{+/+}$ and *gr*$^{-/-}$). Each experimental group was composed by five 15L tanks with six fish each (N=30 fish each group) equipped with aerator. Fish were fed at 2% of body weight using a commercial dry food (Vipan Nature, Sera GmbH, Heinsberg, Germany) and an automatic feeder (Eheim GmbH & Co. KG,

model 3581, Deizisau, Germany) set to provide food at ZT6 (ML groups) or ZT18 (MD groups). The photoperiod was kept at 12:12 LD, with light onset at ZT0 (8 a.m.) and light offset at ZT12 (8 p.m.). The experimental conditions were maintained for four weeks. At the end of the experimental trial, fish were fasted for 24 hours, and each group was sampled at ZT 3, 9, 15, 21 and 27, considering one tank each sampling point. Before collecting brain samples, fish were anesthetized with an overdose of tricaine methane sulfonate (Sigma-Aldrich, Milan, Italy) and killed by decapitation. The brain samples were conserved in RNA later at -20°C until analysis. A red dim light was used to avoid light contamination during the dark phase (λ>600nm) (de Alba et al., 2019).

Rna extraction, cDNA synthesis and Real-Time RT-PCR analysis

The collected brain samples were homogenized in Trizol reagent (Invitrogen, Thermo Fisher Scientific, Baltics UAB) prior RNA extraction which proceeded following manufacturer's instructions. RNA concentration and purity were evaluated with a spectrometry (Nanodrop® ND 1000, Thermo Fisher Scientific). 1U of DNase (Thermo Fisher) each 1 µg of RNA was used to prevent DNA contamination in the samples prior the use of the Reverse Trascriptase commercial kit (QSCRIPT cDNA Synthesis Kit, Quantabio Beverly, USA) and a thermocycler to obtain the cDNA. Every sample was then diluted in a proportion of 1:4 with nuclease-free water (Thermo Fisher Scientific) prior the qPCR analysis where 2 µl of every sample were used in each reaction, with a final volume of 10 µl. The following program was used for the qPCR analysis using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Italia): 3 min at 95 ◦C, followed by 40 cycles of 15 s at 95 ◦C and finally 20 s at 60°C. *Ef1a* was used as reference gene. A complete list of the primers retrieved from literature is listed in Table 1. The 2-ΔΔCt method was used for the analysis of gene expression (Livak and Schittgen 2001).

Table 1 Primers used for the qPCR analysis

Gene	Forward	Revers	Acc. Number
ef1a	CCTTCGTCCCAATTTCAGG	CCTTGAACCAGCCCATG	ENSDARG00000020850
bmal1a	TAGAGCGCTGTTTGCTGATG	GACCCGTGGACTTCAGTGAC	NM_131578
per1b	CCGTCAGTTTCGCTTTTCTC	ATGTGCAGGCTGTAGATCCC	NM_001030183
rev-erbα	GCAATTCACCCAACAAATCAG	CAGGCATGGACGCCATAGT	NM_001130592
rorα	GCATGTCACGTGACGCGGT	TGGGCCAGATGTTCCAACTCA	ENSDARG00000031768
nfil3-5	CTTCCAACCCAAAACAGCGG	GCAGCCTCGTTATTCTTGCGC	ENSDARG00000094965
bdnf	GAAGAGTGATGACCATCCTG	ATGAACCTGCTCGAAAGTGTCCG	[1]
pparα	CTTCGTCATTCACGACATGG	AAGCGTACTGGCAGAAAAGG	ENSDARG00000031777
dnmt4	AAGATTTACCCTGCAGTCCCAG	CTCGCATACTTCTGACGCAAT G	NM_001025450.1
dnmt8	CTTTGCCTGTTAATGAAGCCCC	TGTGAAGTGTCCTGTGGTTGAA	NM_001018134.1
tet2	TGATCGTCTCTACATGGAACTAAGTGA	CCCTGACACGCACATGTTCT	ENSDARG00000076928

[1] D'Agostino et al, 2022

Data analysis

To evaluate the significance of a daily rhythm in the relative gene expression we performed the Cosinor analysis using the software El Temps (v. 313, Prof. Díez-Noguera, University of Barcelona, Spain). This analysis uses the last square methods to fit a time series to a cosine function of a known period (Y = Mesor + Amplitude * cos (2π(t-Acrophase)/Period) and provide the statistical significance of the rhythm comparing the F-test of the variance of the described waveform to a straight line which represent the null hypothesis and has zero-amplitude (Refinetti et al. 2007, Portaluppi et al. 2008). To analyze the presence of a statistically significative differences between groups (*gr*$^{+/+}$ *vs* *gr*$^{-/-}$ of ML and MD conditions separately) and time point (ZT), the data obtained from the relative gene expression were tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test) of the variance before performing a two-way ANOVA followed by Tukey *post hoc* test. Additionally, data obtained from *dnmt4*, *dnmt8* and *tet2* gene analysis were pooled separately dividing light and dark phase to compare the genotype respectively in ML and MD group and analyzed with Student's t-test. All the analyses have been performed with R studio version 4.3.3.

RESULTS

Clock genes and regulatory loops

Clock genes from the positive (*bmal1a*) and negative loops (*per1b* and *rev-erbα*) have been analyzed in the Zebrafish brain together with the circadian rhythm inhibitor *nfil3-5* (Fig. 1).

Looking at the positive loop, *bmal1a* presents and conserves rhythm in all the analyzed conditions (Cosinor, $p < 0.05$), with acrophases located in a range of two hours distance between each other's, near the end of the light phase (ML: $gr^{+/+}$ ZT 10.45 h, $gr^{-/-}$ ZT 11.60 h – MD: $gr^{+/+}$ ZT 9.78 h, $gr^{-/-}$ ZT 10.61 h) (Fig. 1A and B, Suppl. Table 1). Comparing $gr^{+/+}$ and $gr^{-/-}$ in both treatments, no differences were found (two-way ANOVA $p > 0.05$), while statistical significative differences were detected due to the sampling time for the MD group (two-way ANOVA, $p = 0.0001$) but no effect of the interaction of these two parameters where registered in nether in ML or MD (two-way ANOVA $p > 0.05$) (Suppl. Table 2).

The genes analyzed for the primary (*per1b*) and secondary (*rev-erbα*) negative loops displayed different patterns. *Per1b* present rhythm in all the four groups (Cosinor, $p < 0.05$), with close acrophases located at the end of the dark phase (ML: $gr^{+/+}$ ZT 23.91 h, $gr^{-/-}$ ZT 23.61 h – MD: $gr^{+/+}$ ZT 23.80 h, $gr^{-/-}$ ZT 23.60 h), in antiphase of the ones described for *bmal1a* as expected as being part of two opposite loops (Fig. 1C and D, Suppl. Table 1). Two-way ANOVA analysis revealed significative differences between $gr^{+/+}$ and $gr^{-/-}$ ($p = 3.21E-05$) only in the ML group (specifically at ZT 3, 21 and 27 h) (Fig. 1C), where also the sampling time exerted a significative effect ($p = 1.25E-07$) as observed also for MD group ($p = 9.15E-07$), while the interaction between the factors wasn't significant for both treatment ($p > 0.05$) (Suppl. Table 2). Analysis on *rev-erbα*

gene expression revealed the presence of a circadian rhythm only in *gr*$^{+/+}$ of the ML group (Cosinor, $p < 0.05$) (Fig. 1E, Suppl. Table 1), but significative differences between time point were detected in the gr$^{-/-}$ of MD group, revealing a certain variation throughout the day (two-way ANOVA, $p = 0.0099$) (Fig. 1F, Suppl. Table 2). Analysis on the ML groups revealed significative differences between *gr*$^{+/+}$ and *gr*$^{-/-}$ ($p = 0.003$) specifically located at ZT 21 and 27 h (Fig.1E, Suppl. Table 2), due to the sampling time ($p = 0.0036$) and the interaction between the two factors ($p = 0.001$). On the contrary, MD condition results were affected only by the sampling time (Suppl. Table 2).

Rorα is part of the accessory loops and exerts its effect promoting *bmal1* transcription. Our analysis revealed a similar trend descripted for *rev-erbα,* with the presence of a daily rhythm only for *gr*$^{+/+}$ of the ML group (Cosinor, $p < 0.05$) (Fig. 1G, Suppl. Table 1), with acrophase located at the beginning of the light phase at ZT 1.28 h, while no other rhythm or significant variations were observed for the others experimental groups (two-way ANOVA) (Fig. 1H, Suppl. Table 2). Analysis on the MD groups revealed significative differences between *gr*$^{+/+}$ and *gr*$^{-/-}$ ($p = 0.0087$) specifically located at ZT 27 (Fig.1H, Suppl. Table 2), but no differences were found for ML group. Additionally, two-way ANOVA didn't report any difference due to the sampling time or the interaction between genotype and sampling time in both feeding conditions (Suppl. Table 2).

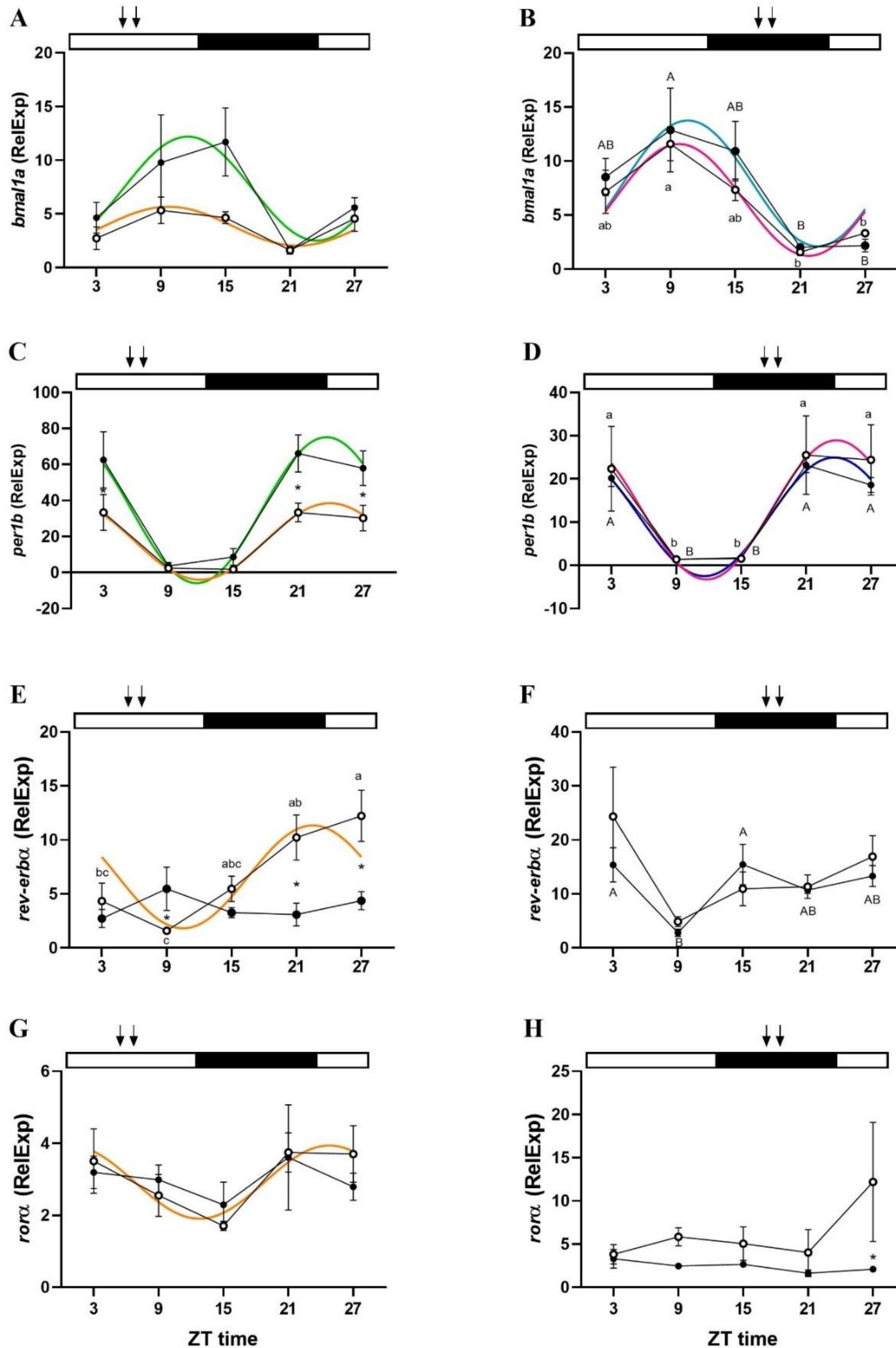

Fig. 1 Daily variations in the relative mRNA expression (fold change) of *bmal1a* (A, B), *per1b* (C and D), *rev-erbα* (E and F) and *rorα* (G and H) in the brain of $gr^{+/+}$ and $gr^{-/-}$ Zebrafish genotype maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles (○) represent the $gr^{+/+}$ genotype, while the $gr^{-/-}$ is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, $p<0.05$), when significant, is represented by green and orange lines for $gr^{+/+}$ and $gr^{-/-}$ genotype respectively in ML condition, or pink and blue lines for $gr^{+/+}$ and $gr^{-/-}$ in MD conditions. Lower and uppercase letters are used to mark statistically significant differences between ZT points within the $gr^{+/+}$ and

$gr^{-/-}$ in each feeding conditions (two-way ANOVA). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotype at the same time point (two-way ANOVA). Time as ZT (zeitgeber time, h) is represented in the x-axis and white and black bars above the graph represent light and dark phase. Arrows represent feeding time.

Additional regulatory system of the clock machinery

Nfil3-5 is part of the additional regulatory system which affect clock genes expression. In our experiment, the gene display rhythm in all the analyzed condition (Cosinor, $p < 0.05$), with similar acrophases locate at ZT 9.43 h (ML, $gr^{+/+}$), 9.63 h (ML, $gr^{-/-}$), ZT 9.38 h (MD, $gr^{+/+}$) and ZT 9.40 h (MD, $gr^{-/-}$) (Fig.2A and B, Suppl. Table 1). Differences between the two genotypes were found for ML and MD groups (two-way ANOVA, p = 4.74E07 and 0.0001 respectively) and due to sampling time specifically at ZT 9 h in both ML and MD conditions (two-way ANOVA, p = 1.67E07 and 0.0099 respectively) (Fig. 2A and B, Suppl. Table 2). Only in ML group the interaction between the genotypes and sampling time resulted significant (two-way ANOVA p = 2.06E-08) (Suppl. Table 2).

Bdnf is a neurotrophin which presents circadian regulation but like *nfil3-5* is involved in the regulation of the molecular clock system. In our study, both genotypes in ML condition displayed a daily rhythm, with a short delay in the acrophase of $gr^{-/-}$. In both cases the acrophase were located around the middle of the light phase, at ZT 5.15 and 7.1 h for $gr^{+/+}$ and $gr^{-/-}$ respectively (Cosinor, $p < 0.05$) (Fig. 1C and D, Suppl. Table 1). On the contrary, no daily rhythm nor daily variation were assessed in the MD group for both genotypes (Cosinor, $p > 0.05$). Significative differences between the genotypes were found in ML conditions at ZT 3 and 27 h (two-way ANOVA, p = 0.0002) but not for MD condition (two-way ANOVA, p = 0.4161) (Fig. 2C and D, Suppl. Table 2). The two-way ANOVA failed to reveal differences due to the interaction between the genotype and the sampling time in both conditions (Suppl. Table 2).

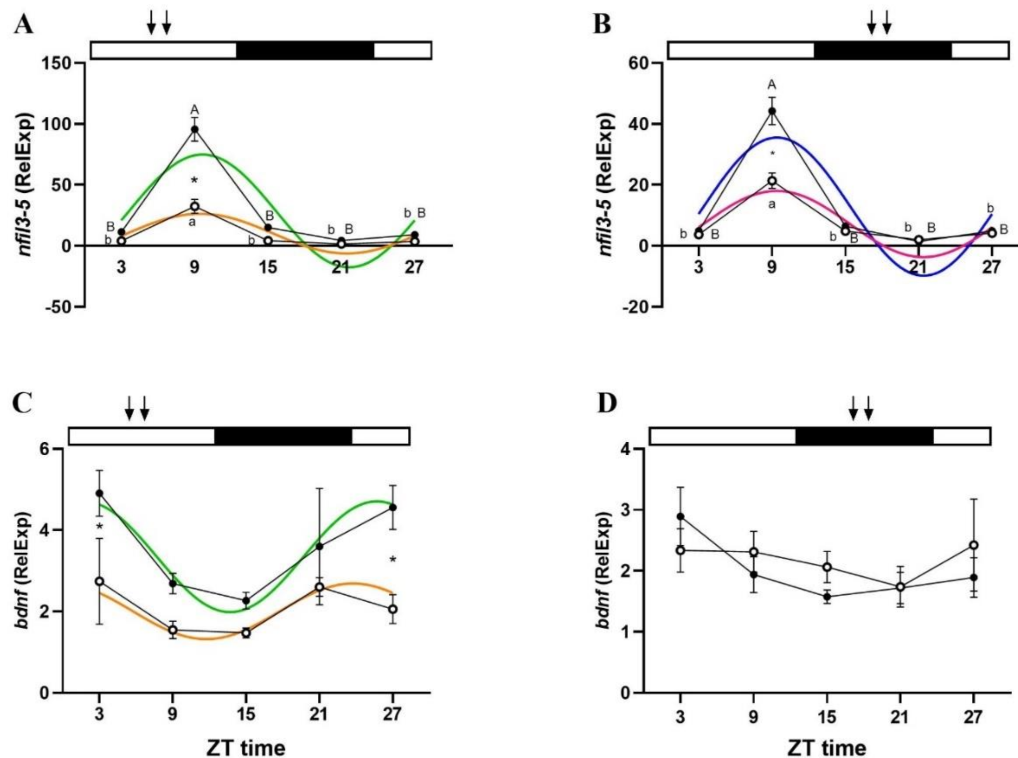

Fig. 2 Daily variations in the relative mRNA expression (fold change) of *nfil3-5* (A, B) and *bdnf* (C and D) in the brain of $gr^{+/+}$ and $gr^{-/-}$ Zebrafish genotype maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles (○) represent the $gr^{+/+}$ genotype, while the $gr^{-/-}$ is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, $p<0.05$), when significant, is represented by green and orange lines for $gr^{+/+}$ and $gr^{-/-}$ genotype respectively in ML condition, or pink and blue lines for $gr^{+/+}$ and $gr^{-/-}$ in MD conditions. Lower and uppercase letters are used to mark statistically significant differences between ZT points within the $gr^{+/+}$ and $gr^{-/-}$ in each feeding conditions (two-way ANOVA). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotype at the same time point (two-way ANOVA). Time as ZT (zeitgeber time, h) is represented in the x-axis and white and black bars above the graph represent light and dark phase. Arrows represent feeding time.

Metabolic link of the clock system

The analysis of *ppara* gene expression revealed rhythm only in the genotype $gr^{+/+}$ when fish were fed during the mid light phase (ML) (Cosinor, $p < 0.05$) with acrophase at ZT24 (Fig. 3A and B, Suppl. Table 1), moreover no variation throughout the day have been detected in the other group (two-way ANOVA, $p > 0.05$). Significative differences due to the treatment between genotype have been found for both ML and MD conditions (two-way ANOVA, p = 8.11E-07 and 0.0046 respectively), at ZT 3, 21 and 27 in the first case, and ZT 3 in the second (Fig. 3A and B, Suppl. Table 2).

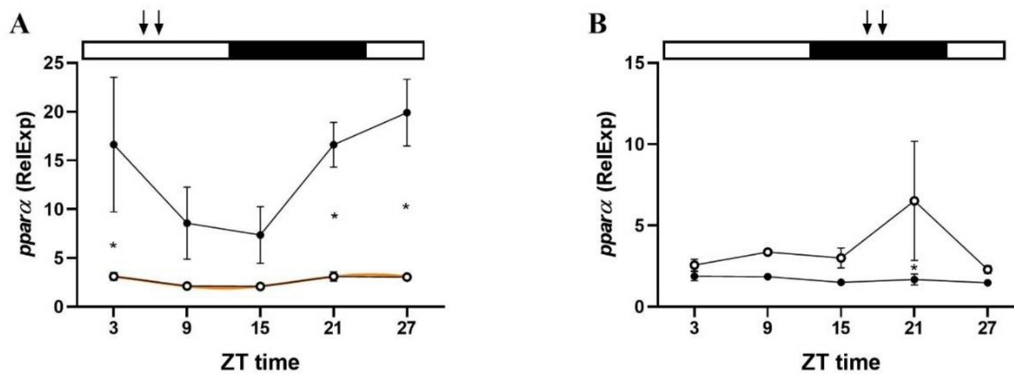

Fig. 3 Daily variations in the relative mRNA expression (fold change) of *pparα* (A, B) in the brain of $gr^{+/+}$ and $gr^{-/-}$ Zebrafish genotype maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles (○) represent the $gr^{+/+}$ genotype, while the $gr^{-/-}$ is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, $p<0.05$), when significant, is represented by green and orange lines for $gr^{+/+}$ and $gr^{-/-}$ genotype respectively in ML condition, or pink and blue lines for $gr^{+/+}$ and $gr^{-/-}$ in MD conditions. Lower and uppercase letters are used to mark statistically significant differences between ZT points within the $gr^{+/+}$ and $gr^{-/-}$ in each feeding conditions (two-way ANOVA). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotype at the same time point (two-way ANOVA). Time as ZT (zeitgeber time, h) is represented in the x-axis and white and black bars above the graph represent light and dark phase. Arrows represent feeding time.

Methylation and demethylation processes

To test how genes involved in process of methylation and demethylation respond to different feeding time in the different genotype we analyzed *dnmt4*, *dnm8* and *tet2* (Fig. 2).

In ML conditions, none of the genes analyzed presented rhythm when submitted to Cosinor analysis (Cosinor, $p > 0.05$) (Fig. 4A, C and E, Suppl. Table 1), but *dnmt4* revealed significative differences throughout the 24 hours (two-way ANOVA) (Fig. 4A, Suppl. Table 2). Differences between genotypes (at ZT 21) (Fig. 4A) and due to the sampling time were found for *dnm4* (two-way ANOVA, $p = 0.0013$ and 0.0475 respectively) (Suppl. Table 2), while *dnmt8* and *tet2* presented differences due to the genotype (two-way ANOVA, $p = 0.0003$ and 0.0134 respectively) at ZT 3 (Fig. 4C and E, Suppl. Table 2). Comparing day data ($gr^{+/+}$ vs $gr^{-/-}$) with T-test analysis, significative

results were found for *dnmt4* ($p = 0.0126$), *dnmt8* ($p = 0.0029$) and *tet2* ($p = 0.0451$) Fig. 5A, E and I), but only *dnmt4* presented a significative difference when comparing night data (*gr*$^{+/+}$ vs *gr*$^{-/-}$) ($p = 0.0355$) (Fig. 5B, Suppl. Table 3).

In MD conditions, genes displayed different patterns. *Dnmt4* expression analysis revealed rhythm for *gr*$^{+/+}$ genotype (Cosinor, $p < 0.05$) , with acrophase locate at ZT 6.23 h (Fig. 4B, Suppl. Table 1), but no rhythm or variation through the day were described for *gr*$^{+/+}$. *Dnmt8* analysis didn't reveled any rhythm in the expression in both genotypes, but for *gr*$^{-/-}$ were detected significative differences during the day (two-way ANOVA) (Fig. 4D, Suppl. Table 2). A daily rhythm was finally described for *tet2* gene expression for *gr*$^{-/-}$ in MD condition, with acrophase located at ZT 10.13 h (Cosinor, $p < 0.05$) (Fig. 4F, Suppl. Table 1). Two-way ANOVA revealed significative differences in function of the genotype for *tet2* ($p = 0.0195$) located at ZT 21 h (Fig. 4F), or the sampling time for *dnmt4* ($p = 0.0005$), but only for *dnmt8* the interaction between the factors was significative ($p = 0.0377$)(Suppl. Table 2). Comparing day and night data separately, only *tet2* presented significative differences when plotting dark phase data together (T-test, $p = 0.0017$) (Fig. 5L, Suppl. Table 3).

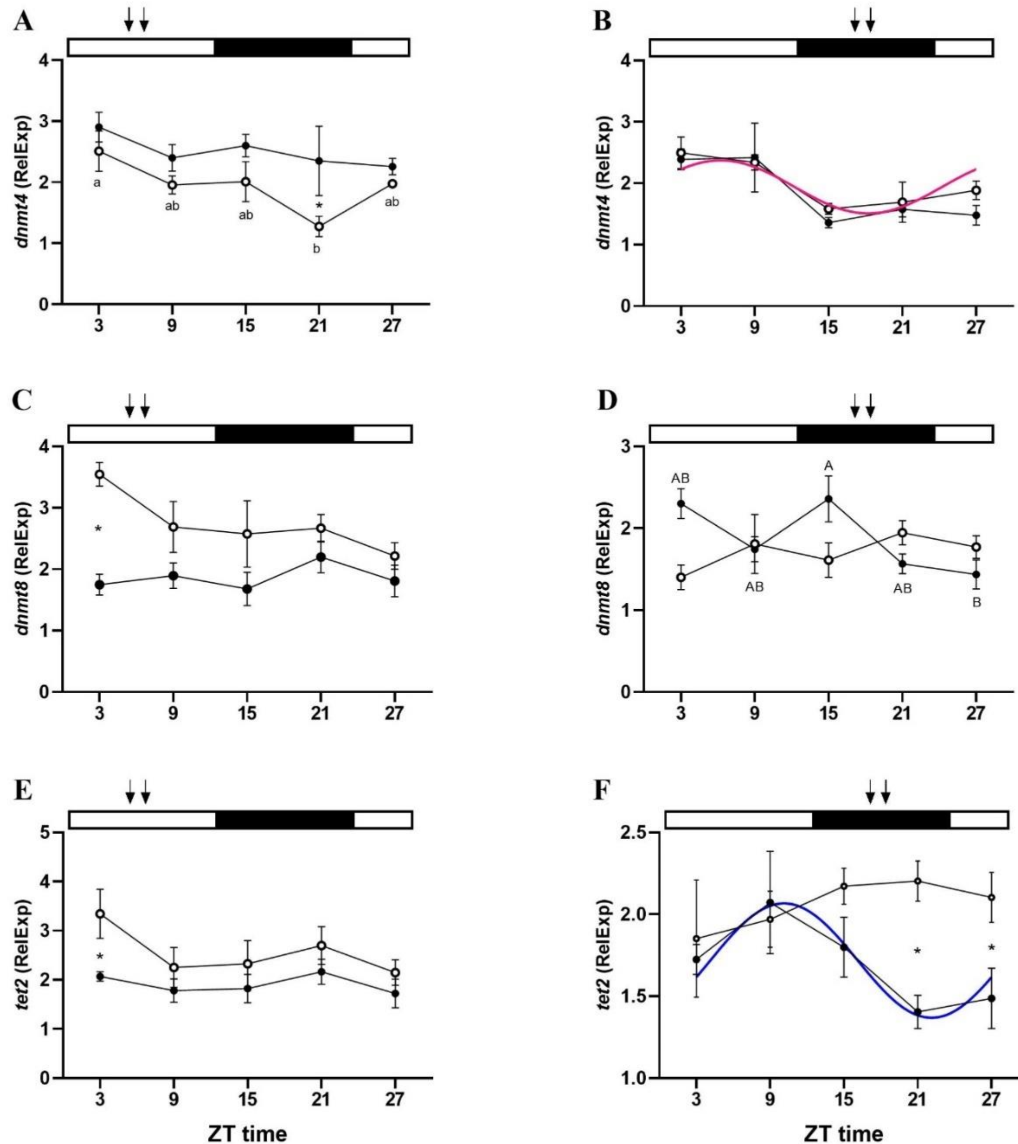

Fig. 4 Daily variations in the relative mRNA expression (fold change) of *dnmt4* (A, B), *dnmt8* (C and D), and *tet2* (E and F) in the brain of $gr^{+/+}$ and $gr^{-/-}$ Zebrafish genotype maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles (○) represent the $gr^{+/+}$ genotype, while the $gr^{-/-}$ is represented with black dots (●). The adjustment to a sinusoidal rhythm (Cosinor, $p<0.05$), when significant, is represented by green and orange lines for $gr^{+/+}$ and $gr^{-/-}$ genotype respectively in ML condition, or pink and blue lines for $gr^{+/+}$ and $gr^{-/-}$ in MD conditions. Lower and uppercase letters are used to mark statistically significant differences between ZT points within the $gr^{+/+}$ and $gr^{-/-}$ in each feeding conditions (two-way ANOVA). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotype at the same time point (two-way ANOVA). Time as ZT (zeitgeber time, h) is represented in the x-axis and white and black bars above the graph represent light and dark phase. Arrows represent feeding time.

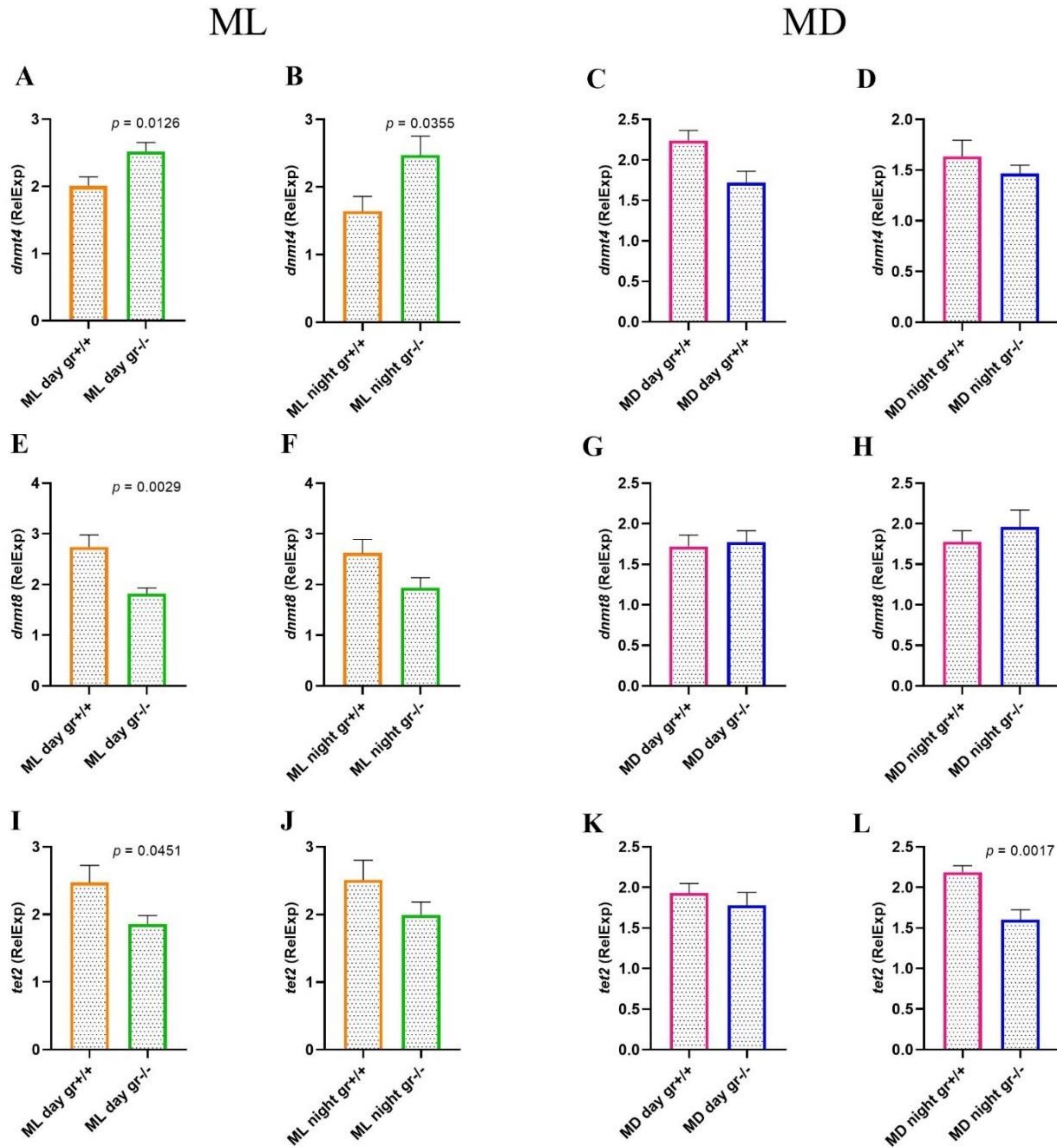

Fig. 5 Day-night differences in *dnmt4* (A, B, C and D), *dnmt8* (E, F, G and H) and *tet2* (I, J, K and L) analyzed in ML (left panel) of MD (right panel) conditions. Data from $gr^{+/+}$ and $gr^{-/-}$ genotypes are represented by orange / green and pink and blue panel respectively for ML and MD conditions. Values were obtained by pooling all data from the light or the dark phase ($gr^{+/+}$ *vs* $gr^{-/-}$) in both ML and MD conditions and analyzed by means of Student's t-test ($p<0.05$). All data are represented as mean ± SEM (n=6 fish per point).

DISCUSSION

In the present study, we described the daily expression rhythm of genes involved in the molecular clock (*bmal1a*, *per1b*, *rev-erbα, rorα)* coupled with genes which affect their expression (*nfil3-5* and *bdnf*) and how they respond to different feeding schedule in the brain of Zebrafish wild type ($gr^{+/+}$) and null corticoid receptor ($gr^{-/-}$). Additionally, we described how *pparα,* a gene involved in multiple metabolic pathways and strongly connected to the molecular clock, responds to different feeding schedule to understand how glucocorticoid system interact with the establishment of its rhythm. Lastly, we investigated the daily variations of genes involved in the epigenetic processes of methylation (*dnmt4* and *dnmt8*) and demethylation (*tet2*) due to their double connection with the molecular clock and sensitiveness to feeding. (Fig. 6).

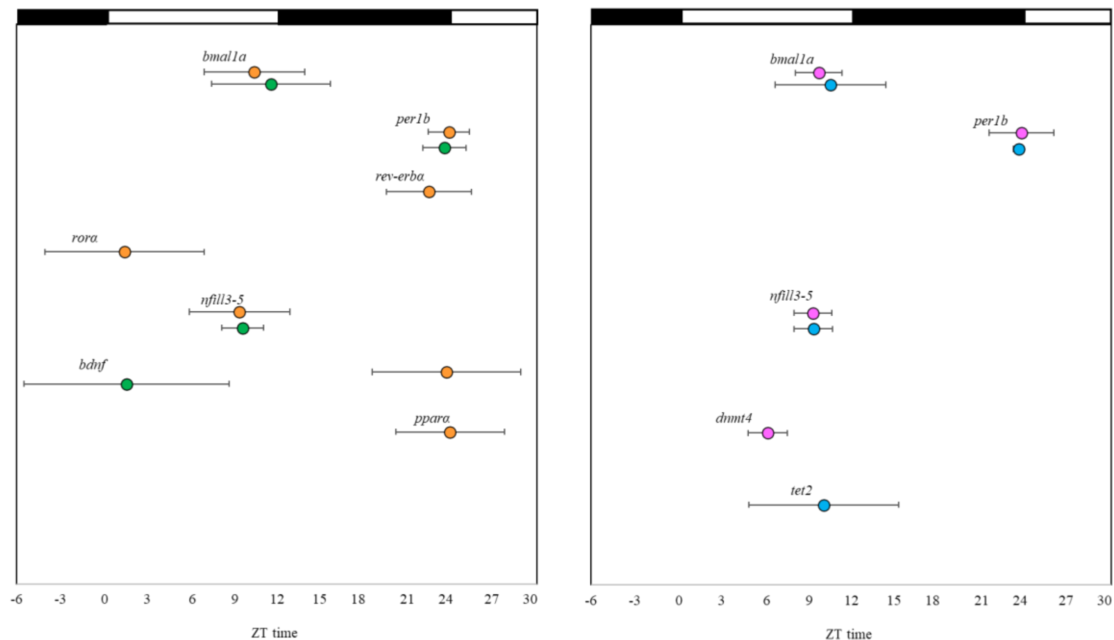

Fig. 6 Maps of acrophases of the genes analyzed and involved in the molecular clock, methylation and demethylation processes and metabolism. The acrophase is only indicated when Cosinor analysis revealed a statistical significative rhythm (Cosinor, $p<0.05$). ML group is reported in the left panel while MD in the right, with arrows indicating feeding time. For ML group, the acrophases of $gr^{+/+}$ and $gr^{-/-}$ genotypes are indicated with orange and green dots respectively, while for MD group, they are indicated with pink and blue dots. The name of each gene is reported near the correspondent marker. All data are plotted against the time which is reported as ZT (Zeitgeber) time in the x-axis with ZT 0 representing the light onset. Light and dark phases are represented with white and black bars above the graph (12:12 LD).

The clock genes are the core which govern the biological clocks and they induce cascade of rhythmic transcription of the clock-controlled genes (Pando and Sassone-Corsi, 2002, Vatine et al., 2011). Many environmental factors are the responsible of clock entrainment, allowing the animal to predict cyclic environmental changes. The clock genes present a tight connection with the element of the HPI axis, where they induce the rhythmic release of glucocorticoids into the bloodstream (Cowan et al., 2017), which after binding the specific receptor (Gr) interact with the glucocorticoid responsive element (GRE) on clock genes modulating their transcription (Balsalobre et al., 2000, Sánchez-Bretaño et al. 2016). In our study, two of the genes involved in the main loop, *bmal1a* and *per1b*, presented rhythm both in ML and MD conditions with similar acrophases when comparing the same genes in the different conditions. This pattern suggests that, in the central pacemaker, an altered glucocorticoid system and feeding time, are not strong enough to interfere with the rhythm of the principal players of the molecular clock. In Goldfish, the feeding schedule resulted essential to synchronize the central pacemaker only in absence of a LD cycle (Feliciano et al., 2011) and the maintaining of a rhythm regardless of feeding schedule has been already described in fish (López-Olmeda et al., 2013, Vera et al., 2013). Even though the rhythm is maintained, some differences between the two genotypes is present for *per1b* in ML conditions, as observed in the Zebrafish eye and liver of the same mutant line (Morbiato et al., 2019), but in contrast with the found in the *gr^{s537}* line larvae (Jaikumar et al., 2020). Among the clock genes, *per1* represents the main target for glucocorticoids, due to the presence of GRE on its regulatory region (Dickmeis et al. 2007), as previously observed in cultured rat fibroblast (Balsalobre et al., 2000) or Goldfish liver (Sánchez-Bretaño et al. 2016), but their influence is stronger in the peripheral clock than in the central pacemaker, as seen in the suprachiasmatic nucleus (SCN) of the rat hypothalamus (Sánchez-Bretaño et al. 2016). Moreover, GRs are

essential to mediate the effect on the GRE of *per1* gene (Balsalobre et al., 2000). In our study, we didn't discriminate between different brain region where GRs are expressed (Natsaridis et al., 2023), and we found that $gr^{-/-}$ zebrafish display an increase expression of *per1b*, which is in contrast with the data in eye and liver of the same mutant Zebrafish (Morbiato et al., 2019).

Rev-erbα and *rorα* are part of the secondary loop of the molecular clock system whose activation depends on the CLOCK-BMAL dimers (Sato et al., 2004). As for *per1*, in mammals the influence of glucocorticoids is exerted through the GRE region which is present on the *rev-erbα* regulatory region, mediating in this case an inhibitory regulation (Dickmeis et al., 2013). In a previous studies conducted in rat, dexamethasone has induced a phase shift of *Rev-erbα* liver, but the study concluded that glucocorticoids couldn't be the only signal to entrain peripheral clock (Balsalobre et al., 2000). In our study, the rhythm found in ML condition for $gr^{+/+}$ genotype, is subsequently lost in $gr^{-/-}$ in both the genes analyzed from the secondary loop, *rev-erbα and rorα*. Moreover, the shapes described by *rev-erbα* data collected during the 24 hours, appears to be inverted. The absence of a rhythm in the $gr^{-/-}$ in both *rev-erbα* and *rorα* suggest that glucocorticoids are key components in the regulation of the secondary loop of the clock mechanism of Zebrafish brain, and feeding time coupled with a LD cycle weren't enough to maintain the rhythmic expression of the gene. An effect of the feeding was in any case present as shown by the results from the MD group, where none of the genotype reported rhythm in *rev-erbα* and *rorα* expression. In specific, *Rev-erbα* can be considered one of the main responsible of the cross talk between circadian system and metabolisms in mammals, due to its implications to several metabolic processes (Duez and Staels, 2009); moreover, its deletion damages food entrainment in mice (Delezie et al., 2016). In our case, the shift of the feeding schedule was a sufficient stimulus for the loss of the rhythm. *Rev-erbα* is also

directly related to *nfil3* modulating its expression in mice (Fang et al., 2014). In our study, *nfil3-5* display rhythm in both feeding condition and genotypes, presenting a different pattern compared to *rev-erbα,* suggesting that other regulatory factors influence the rhythmic gene expression in the brain. In specific, when comparing $gr^{+/+}$ and $gr^{-/-}$ in both feeding condition, the acrophase is visible dampened in wildtype Zebrafish. In mammals, *nfil3* present GRE regions on its regulatory sites which are activated by dexamethasone (Carey et al., 2003, So et al., 2009) but in our case the pattern observed in correspondence of the acrophases suggest an inhibitory regulation by glucocorticoids, as observed for *per1b*. Analysis in mice have reported different effect of glucocorticoid on the clock genes, which up- or down-regulation was tissue specific, and for this reason we can exclude a different regulation in other tissue (Soták et al., 2016).

Bdnf is a neurotrophins with multiple function (Tapia-Arancibia et al., 2004, Blanco et al., 2020) for which a connection with the circadian system has been recently discovered (D'Agostino et al., 2022). Bdnf is also particularly responsive to acute and prolonged stress, usually display an upregulation in Zebrafish (Chakravarty et al., 2013, Pavlidis et al., 2015) and has been also identified as GR regulated with implication on behavior using another GR mutant line (Eachus et al., 2023). In our study, bdnf presented rhythmic expression when fish were fed during the light phase in both genotypes, but an upregulation in the $gr^{-/-}$ was described at the beginning of the day, suggesting at least in part an implication of the glucocorticoids system on its regulation as previously described (Eachus et al., 2023). On the contrary, no rhythm was described when fish were fed during the mid-dark phase. An emerging metabolic role of bdnf has been recently described in Zebrafish, since its expression increase during starvation and the peptide administration leads to food intake and upregulation of genes involved in orexigenic processes in the

brain (Blanco et al., 2020). Our data suggest an involvement of food intake on its regulation, which appear as a loss of the circadian rhythm.

To better understand the crosstalk between Gr signaling and metabolism, we examined the daily gene expression of *pparα*. In our study, *gr*$^{-/-}$ genotype presents a significant increase in gene expression in ML condition, associated with the loss of the rhythm that was described in *gr*$^{+/+}$ phenotype. A similar result was observed in rat liver and primary hepatocyte culture treated with dexamethasone, suggesting a similar regulation between mammals and fish (Pineda Torra et al., 2000). Moreover, feeding the fish during the dark phase, promoted the loss of the rhythm irrespective of the genotype. In two different studies conducted in the liver of Zebrafish and Gilthead Sea bream (*Sparus aurata*), the analysis of the rhythm of *pparα* revealed that feeding time alone was less important than LD cycle in the establishment of the rhythm (Paredes et al., 2014, Paredes et al., 2015). In our case, even though we considered the central pacemaker which would be less sensitive to feeding time, we found that this gene clearly responds to different feeding schedule as observed also for *rev-ervα*, which expression is also regulated by *pparα* (Duez and Steals, 2007). Looking at the results, these two genes present the same pattern in terms of response of feeding, suggesting that the central pacemaker is also sensitive to the change of feeding schedule.

The results of the analysis of epigenetic genes involved in the processes of methylation and demethylation (*dnmt4*, *dnmt8* and *tet2*) present a different pattern if compared to the other genes. In specific, none of the genes analyzed presented rhythm in the ML group, and only *dnmt4* revealed diurnal variation throughout the 24 hours in *gr*$^{+/+}$ genotype, while in MD group the rhythm was displayed by *dnmt4 gr*$^{+/+}$ and *tet2* for *gr*$^{-/-}$ genotype. A direct connection between the circadian system and the genes involved in methylation has been hypothesized in mammals by being mediated by the binding of the

E-box region of the genes involved in methylation by *bmal1* (Stevenson, 2018). In Zebrafish, the existence of a daily rhythm of these genes has been proved in gonads, with mostly nocturnal acrophases (Paredes et al., 2018), while in mammals, different profiles can be displayed by the enzymes based on the tissue (Stevenson, 2018). A study focused on the transcriptomic of different Zebrafish GR-mutant line, finding an upregulation of the genes encoding *dnmt3aa* (or *dnmt8*) and *dnmt3bb.3* (or *dnmt5*) in brain samples collected in a single time point at the beginning of the day using a different Zebrafish mutant line (gr^{357}/gr^{357}) (Eachus et al., 2023). Looking at the 24-hours result of our experiment, regarding *dnmt8* we described an opposite trend, with $gr^{+/+}$ transcript being upregulated compared to $gr^{-/-}$. The same result is obtained by plotting separately day and night data, where only *dnmt4* present a similar pattern described by the study mentioned earlier. The line used int the previous study presents an impair GRE-dependent activity but conserves the protein-protein interactions, and present lowest range of influence on GC controlled genes if compared to the lines that we used (Facchinello et al., 2017), differences that could be the reason of a different regulation that we observed. Notably, day and night data plot, reveled significative differences in the epigenetic genes analyzed, where the differences between the genotypes is more significant during the day, confirming an influence of GC on the epigenetic of methylation and demethylation. These differences, tend to disappear when fish are fed during the MD phase, where only *tet2* responded with significative differences between night data. The *dnmt*s genes are strongly dependent on the methionine availability that is assumed through the diet (Hermann et al., 2004) and feeding the fish during the night could have cause a reallocation of the energy in different processes, making disappear the differences appreciated in ML feeding.

CONCLUSIONS

In this study we described how clock, metabolic and epigenetic genes respond in zebrafish wild type and null glucocorticoids receptor, and how feeding time influence their expression. Our study suggests an implication of the glucocorticoid especially in the regulation of genes of the secondary loop and related to metabolism while less effect was observed on the epigenetic genes. Moreover, even though the central pacemaker is less sensitive to feeding time, this zeitgeber was still capable of inducing change in the rhythm and gene expression as part of the complexity of the regulation of the central pacemaker.

SUPPLEMENTARY MATERIALS

Suppl Table 1 Resume of the Cosinor analysis results of the genes analyzed. All the data are reported only for significant rhythm (Cosinor, $p<0.05$): p value, mesor, amplitude and acrophase (indicated in ZT time). Data are expressed as value ± fiducial limits (set at 95%)

Gene	Experimental group	*p* values	Mesor	Amplitude	Acrophase (ZT hours)
bmal1a	ML $gr^{+/+}$	0.0173	3.67 ± 0.92	1.96 ± 1.63	10.45 ± 3.47
	ML *gr* $^{-/-}$	0.03973	6.86 ± 2.57	4.81 ± 4.6	11.60 ± 4.1
	MD $gr^{+/+}$	0.00030	6.40 ± 1.35	5.17 ± 2.9	9.78 ± 1.6
	MD $gr^{-/-}$	0.02721	7.93 ± 2.95	5.82 ± 5.19	10.61 ± 3.83
per1b	ML $gr^{+/+}$	0.000	17.26 ± 4.37	21.25 ± 7.63	23.91 ± 1.41
	ML *gr* $^{-/-}$	0.00005	34.63 ± 9.48	40.51 ± 17.66	23.61 ± 1.51
	MD $gr^{+/+}$	0.00138	12.84 ± 5.52	16.09 ± 9.84	23.80 ± 2.24
	MD $gr^{-/-}$	0.000	11.23 ± 1.21	13.72 ± 2.2	23.60 ± 0.36
rev-erbα	ML $gr^{+/+}$	0.00722	6.58 ± 1.92	4.76 ± 3.55	22.52 ± 2.94
	ML *gr* $^{-/-}$	0.36373			
	MD $gr^{+/+}$	0.08467			
	MD $gr^{-/-}$	0.1486			
rorα	ML $gr^{+/+}$	0.03844	2.44 ± 0.62	1.13 ± 1.07	1.28 ± 5.30
	ML *gr* $^{-/-}$	0.75304			
	MD $gr^{+/+}$	0.65034			
	MD $gr^{-/-}$	0.56374			
nfil3-5	ML $gr^{+/+}$	0.00199	9.63 ± 4.94	14.08 ± 8.87	9.46 ± 3.5
	ML *gr* $^{-/-}$	0.00004	28.69 ± 10.38	46.21 ± 20.05	9.63 ± 1.45
	MD $gr^{+/+}$	0.00002	7.16 ± 2.26	10.84 ± 4.55	9.38 ± 1.32
	MD $gr^{-/-}$	0.00001	12.87 ± 4.61	22.45 ± 8.78	9.40 ± 1.32
bdnf	ML gr+/+	0.04389	2 ± 0.36	0.68 ± 0.66	23.75 ± 5.15
	ML *gr* $^{-/-}$	0.01942	3.35 ± 0.7	1.36 ± 1.15	1.60 ± 7.1
	MD $gr^{+/+}$	0.42028			
	MD $gr^{-/-}$	0.10719			
pparα	ML $gr^{+/+}$	0.0139	2.61 ± 2.29	0.69 ± 0.13	24 ± 3.75
	ML *gr* $^{-/-}$	0.0620			
	MD $gr^{+/+}$	0.0846			
	MD $gr^{-/-}$	0.6769			
dnmt4	ML $gr^{+/+}$	0.25012			
	ML *gr* $^{-/-}$	0.59941			
	MD $gr^{+/+}$	0.03056	1.94 ± 1.22	0.43 ± 0.39	6.23 ± 4.95
	MD $gr^{-/-}$	0.10926			
dnmt8	ML $gr^{+/+}$	0.92324			
	ML *gr* $^{-/-}$	0.66765			
	MD $gr^{+/+}$	0.85142			
	MD $gr^{-/-}$	0.4777			
tet2	ML $gr^{+/+}$	0.60622			
	ML *gr* $^{-/-}$	0.66765			
	MD $gr^{+/+}$	0.34272			
	MD $gr^{-/-}$	0.0462	1.72 ± 0.18	0.35 ± 0.35	10.13 ± 4.92

Suppl Table 2 P values obtained in the two-way ANOVAs analysis for the comparisons between genotypes (gr+/+ vs gr-/-), sampling time (ZT time) and the interaction between the two factors (G * ZT).

Gene	Group	$gr^{+/+}$ *vs* $gr^{-/-}$	ZT time (ZT)	G * ZT
bmal1a	ML	0.0519	0.0759	0.4424
	MD	0.3546	0.0001	0.8227
per1b	ML	3.21E-05	1.25E-07	0.169
	MD	0.520	9.15E-07	0.957
rev-erbα	ML	0.0030	0.0036	0.0010
	MD	0.3955	0.0099	0.4747
rorα	ML	0.889	0.243	0.0845
	MD	0.0087	0.43317	0.1960
nfil3-5	ML	4.7E-07	1.67E-15	2.06E-08
	MD	0.0001	0.0099	0.4747
bdnf	ML	0.0002	0.0119	0.5708
	MD	0.4161	0.1082	0.4752
pparα	ML	8.11E-07	0.130	0.218
	MD	0.0046	0.2222	0.2117
dnmt4	ML	0.0013	0.0475	0.6079
	MD	0.3312	0.0005	0.9005
dnmt8	ML	0.0003	0.4586	0.2929
	MD	0.4451	0.5313	0.0377
tet2	ML	0.0134	0,2246	0,7646
	MD	0.0195	0.4871	0.1692

Suppl Table 3 P values obtained in the Student's t-test analysis for the comparisons between day and night data (gr+/+ vs gr-/-) in both ML and MD conditions.

Gene	Group	DAY data	NIGHT data
dnmt4	ML	0.0126	0.03552
	MD	0.5828	0.3633
dnmt8	ML	0.0029	0.0638
	MD	0.802	0.417
tet2	ML	0.0451	0.1615
	MD	0.4478	0.0017

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General discussion

4 General discussion

4.1 General discussion

The present PhD thesis explored the effect of different synchronizers (light/dark cycle and feeding time) on physiological processes in European sea bass. The main focus was on pathways that share links and influence each other, such as the clock gene system (chapters 3 and 4), epigenetic mechanisms (chapters 3 and 4), the physiological stress response (chapters 2 and 3), and the modulation of feeding time and digestion (chapter 1). Additionally, to better understand the effect of feeding time and elucidate the link between stress, the clock gene system and the epigenetic system, zebrafish mutant for the glucocorticoid receptors (gr) were subjected to different scheduled feedings (chapter 5).

The clock genes represent the core of the molecular clock that governs biological rhythms. They are rhythmically expressed in every pacemaker, being part of a self-sustaining circuit (Pando and Sassone-Corsi, 2002; Vatine et al., 2011), and they promote the rhythmic expression of CCGs involved in several physiological pathways (Cahill, 2002; Pando and Sassone-Corsi, 2002; Sato et al., 2004; Zhdanova and Reebs, 2005; Vatine et al., 2011; Cox and Takahashi, 2019). Even though the clock genes are expressed in every pacemaker, they can be affected differently by the synchronizers. Specifically, the clock genes of the central pacemaker are more sensitive to the light/dark cycle, while in the peripheral pacemakers, feeding time exerts the major effect (López-Olmeda, 2017). In Chapter 4, we described the same differences between central and peripheral tissues, since most of the clock genes analyzed in the brain of European sea bass subjected to different feeding schedules presented a rhythm when feeding was scheduled during the ML and MD phases. On the contrary, most of the clock genes analyzed in the liver lost their rhythm in the MD group, with the only exception of *per2*, which is usually light-dependent (Pando et al., 2002; Vatine et al., 2009). A similar result was then confirmed in

Chapter 5, where the genes of the primary clock loops (*bmal1a* and *per1b*) presented and conserved a rhythm in the zebrafish brain, both wildtype and mutant, when fish were subjected to different feeding schedules. In the same study, a different response from the genes of the secondary loop (*rev-erbα* and *rorα*) was observed in wildtype fish, as they displayed a rhythm only when fish were fed during the ML phase, suggesting a different regulation probably due to their metabolic role, which is considered a link between the circadian system and metabolism in mammals (Duez and Staels, 2008; Delezie et al., 2016).

The clock genes exert their function by inducing the rhythm of several physiological factors (Cowan et al., 2017). Among them, cortisol is known to promote a bidirectional circuit, as it can directly affect the clock machinery (Reddy et al., 2007). In Chapter 5, we analyzed the importance of GR in the zebrafish brain as part of the signaling system of cortisol to the clock genes. This signaling is exerted through GRE regions, which have been already described for *per1b* (Balsalobre et al., 2000; Yamamoto et al., 2005; Sánchez Bretaño et al., 2016), *rev-erbα* and *rorα* (Dickmeis et al., 2013), and also *nfil3*, that is part of the accessory loop of the clock system (Doi et al., 2001; Carey et al., 2013; So et al., 2009). In this study, using a zebrafish null glucocorticoid receptor ($gr^{-/-}$), we reported that when fish are fed during their active phase, *bmal1a, per1b*, and *nfil3-5* are not affected in terms of rhythm, which is conserved in both wild type and mutant genotypes. On the contrary, *rev-erbα* and *rorα* did not reveal any rhythm in the $gr^{-/-}$ fish, suggesting that the glucocorticoid system in the brain mostly interferes with the secondary loop of the clock genes. An interference with the primary loop was found in the liver of goldfish, suggesting a different regulation between the tissues (Sánchez Bretaño et al., 2016).

To deepen the effect of stress mechanisms on the clock genes, in Chapter 5 we described how they respond when a chronic stressor is applied, focusing on the effects of high density and calorie restriction in the European sea bass liver, since they represent two of the most common stressors in aquaculture (Montero et al., 1999; Ashley et al., 2007; Zhang et al., 2020). In this study, high-density conditions elicited a response from the clock genes after 25 days, while calorie restriction affected fewer genes during the treatment, but *clock1b* responded immediately on day 5. These differences suggest that even though different chronic stressors can elicit the same physiological activation of the stress axis, the type of stress can differently modulate the clock genes. A study conducted on the effect of high density on rainbow trout described significant changes in mRNA levels of different clock genes in the brain after 72 hours (Naderi et al., 2008), which is not completely consistent with our results, due to the early response. However, the differences could be attributed to the different tissues analyzed, since central and peripheral pacemakers respond differently to stress (Sánchez-Vázquez et al., 2019). Additionally, the rapid response of *clock1b* observed on day 5 due to calorie restriction highlights that clock genes in fish could be influenced not only by the feeding time, and that calories and food composition could play a role as reported in mice liver (Patel et al., 2016).

Besides high density and calorie restriction, another important source of stress in aquaculture is air exposure due to handling during routine procedures. This can lead to considerable physiological stress or intensify the oxidative response once the fish are returned to the tank (Ramsay et al., 2009; Cook et al., 2015). In Chapter 2, we studied how European sea bass responds to acute stress caused by air exposure at different times of the day and year. Our study revealed that the elements of the HPI axis analyzed (*crh* and *chr-bp*), cortisol, glucose, and lactate responded differently to stress. Specifically,

while cortisol showed the highest levels at the end of the dark phase, as expected for diurnal species (Vera et al., 2014; Sánchez-Vázquez et al., 2019), glucose and lactate displayed different diurnal peaks. This suggests that sensitivity to stress could be species-dependent and not solely linked to the phase of activity, contrary to other studies that attribute the highest physiological stress responses to the resting phase (Cowan et al., 2017; Sánchez-Vázquez et al., 2019). Generally, the resting phase represents a crucial period when energy is invested to prepare the organism for the active phase. This idea is confirmed by the mRNA levels of the antioxidant genes (*cat, sod1, gshpx*, and *gsr*) and mitochondrial markers (*ucp1, prdx3*, and *coxIV*) analyzed in December. The acrophases of these genes, when present, were always located during the dark phase. Interestingly, most of the rhythms observed in December were subsequently lost in June, likely due to changes in temperature or photoperiod length, as previously reported for antioxidant enzyme activity in European sea bass (Li et al., 2021). The effect of the season was also evident in the amplitude of the antioxidant response, which was higher in June than in December. This increase aligns with the greater need to prepare enzymes to cope with a possible increase of oxygen level that can determine oxidative stress (Hermes-Lima et al., 1998), as also previously observed in the common bream (*Abramis brama*) (Morozov et al., 2017). Conversely, mitochondrial markers showed a higher response in December, especially *prdx3*, ensuring that at least one system is always highly active against oxidative damage (Rhee et al., 2005).

Recent studies on stress have clarified the potential epigenetic modifications that can trigger. Depending on the stage of life when stress is experienced, it may differently influence fish physiology (Guinand and Samaras, 2023), and in some cases, even enhance the fish performance (Robinson et al., 2019). Epigenetic modifications can be triggered by various events, and several studies have suggested that methylation and demethylation

processes are among the most common (Anastasiadi et al., 2017; Li et al., 2017; Lin et al., 2023; Valdiveso et al., 2023). Additionally, *sirt1*, which mediates deacetylation, is directly involved in the stress response due to its physical interaction with GR (Suzuki et al., 2018). In Chapter 3, we examined the impact of chronic stressors on epigenetic genes, finding differences related to high density and calorie restriction. During the day, high density affected the mRNA levels of *dnmt3a* and *sirt1* after 5 days of treatment, while at day 60, differences were observed for *dnmt1* and *sirt1*, with the latter showing effects only during the night. No significant differences in mRNA levels were noted at the midpoint of the experiment on day 25. The immediate response of *dnmt3a* may be attributed to reduced ATP availability due to stressful conditions, causing the fish to quickly reallocate energy (Schreck and Tort, 2016), which is also needed for DNA methylation processes (Anderson et al., 2012; Duker and Rabinowitz, 2017). The presence of GRE regions in the promoter of *dnmt1* in mammals (Urb et al., 2019) might explain the differences in mRNA abundance observed for this gene on day 60. Interestingly, *sirt1* responded on both days 5 and 60, and PCA analysis indicated that *sirt1*, along with *dnmt3a*, significantly contributed to the gene expression patterns observed. Both genes help regulate chromatin stability (Moore et al., 2013; Jing and Lin, 2015), suggesting that long-term stress could lead to chromatin remodeling. When the stressor applied was a low feeding ratio, we observed both an early (day 5) and late (day 60) response of the *dnmt* genes. The alterations in mRNA abundance are consistent with studies demonstrating the impact of feeding on DNA methylation (Skjaerven et al., 2018), likely through the modification of methionine availability provided by the diet, which is involved in the 1C cycle (Moore et al., 2013; Duker and Rabinowitz, 2017).

To further explore the connection between the physiological stress response and epigenetic pathways, in Chapter 5, we analyzed genes involved in methylation (*dnmt4*

and *dnmt8*) and demethylation (*tet2*) in the brains of wildtype Zebrafish and null glucocorticoid receptor mutants (*gr*$^{-/-}$). Additionally, we examined the effect of feeding on the presence of rhythmic gene expression. When fish were fed during the light phase (ML), no rhythm was observed in either *gr+/+* or *gr-/-,* but some differences emerged between the two genotypes when day and night data were plotted separately for all genes, suggesting an influence of glucocorticoids on epigenetic mechanisms. The influence of glucocorticoids has been previously reported in the brains of another Zebrafish mutant (gr357/gr357) (Eachus et al., 2023). In that case, the authors described an increase of mRNA in all the epigenetic genes investigated, which is only partially consistent with our results, as only *dnmt4* exhibited the same trend. This discrepancy could be due to the different mutant lines used (Facchinello et al., 2017). Notably, when fish were fed during the night phase, the analyzed *dnmt*s genes did not show any changes when day and night data were plotted separately. This suggests that feeding the fish during their resting phase could have eliminated the differences between genotypes, likely due to the reallocation of energy to different processes.

The activity of *dnmt*s genes depends on the availability of ATP and methionine from the diet (Hermann et al., 2004; Froese et al., 2019). In chapter 3 and 5 we demonstrated that genes involved in epigenetic mechanisms are particularly sensitive to feeding. In chapter 4, we used different scheduled feeding time (ML and MD) to test the effect on the epigenetic genes in the liver of European sea bass, finding most of the time a recurrent pattern. All the epigenetic genes analyzed in the liver (*dnmt1, dnmt3a, tet2, mbd4* and *gadd45aa*) displayed rhythm when fish were fed during the light phase (ML) but lost the rhythm in MD conditions with the only exception of *dnmt3a*. Moreover, all of them reached the peak during the resting phase of the animal, as confirmed by the analysis of the locomotory activity. For genes involved in methylation and demethylation,

a similar pattern was described in Zebrafish ovaries (Paredes et al., 2018) but also mouse liver (Xia et al. 2018). The fact that *dnmt3a* was the only gene to maintain the rhythm when fish were fed during the dark phase, suggests a different regulation, maybe related to the availability of SAM (S-adenosyl-methionine) during the day as proposed for mammals (Zhang et al., 2018). In our study, even though no rhythm was registered for SAM, SAH (S-adenosyl-homocysteine) or the ratio SAM/SAH, a difference was described for SAM when comparing day and night data separately for ML and MD fish, suggesting a different energy investment between the group. Moreover, SAH presented a nocturnal peak inversely related to the methylation potential's lowest values. Our data are consistent with the founding in mammals (Xia et al., 2015) and suggest that the enzymes prepared during the night could be used during the day when the methylation potential is higher. Among the epigenetic genes analyzed, we also considered *sirt1* due to its link with metabolism and the clock genes (Suave et al., 2006; Nakahata et al. 2006). In mammals, the rhythm was described only for its cofactor, (Ramsey et al. 2009; Bellet et al. 2013) while in our study we could describe a daily rhythm when the fish were fed during the light phase, with a nocturnal achrophase and the subsequent loss of the rhythm for the MD group. These results suggest a different regulation between mammals and fish and reveals the same pattern of the genes related to methylation and also the clock genes. The latter connection is also seen in the chapter 3, where *sirt1* was indicated by the PCA analysis as one of the main contributors in the establishment of the pattern of gene expression observed.

Feeding time can be also an important synchronizer for all the processes related to feeding and digestion. In chapter 1, we analyzed genes involved in digestion in liver and genes involved in food intake and reward system in brain of European sea bass. In liver, almost all genes involved in protein digestion present rhythm both ML and MD

condition with nocturnal acrophases. For all of them, the differences in terms of acrophase never cover the 12 hours of distance between ML and MD feeding, suggesting that probably feeding time is not the only synchronizer in liver. Additionally, the fact that the acrophase were always nocturnal, suggests that enzymes produced for protein digestion are prepared during the night as seen for other digestive enzymes in sea bream (Mata-Sotres et al., 2016) or white seabream (*Diplodus sargus*) (Yúfera et al., 2012), and consistent with the idea that they could be used during the day (Del Pozo et al., 2012; de Oliveira et al., 2022). On the contrary a different regulation is shown for gene encoding transamination enzymes (*c-alt* and *m-alt*) and glycolysis (*pk*), which present rhythm only when fish are fed during the light phase, with similar acrophase. These enzymes participate also in the TCA cycle (Walton and Coway, 1982) that can probably explain the same achrophase displayed. Lastly, *pla2* which is involved in the lipid metabolism presented rhythm only in ML group, with a similar acrophase reported for Atlantic blue tuna (Betancor et al., 2020), but in contrast with the founding in Sea bream and Zebrafish where it was suggested that light/dark cycle could be a stronger synchronizer for the genes of the lipid metabolism (Paredes et al., 2014; Paredes et al. 2015). The genes analyzed in the brain of European sea bass related to feeding time and reward system display dissimilar pattern revealing different effect of the feeding. In specific, *bdnf* presented rhythm only when fish were fed during the light phase, a result also confirmed in the brain of Zebrafish in chapter 5, which confirm the strong effect of feeding time on its transcription as previously reported for Zebrafish (Blanco et al., 2020). *Bdnf* is a neurotrophins involved in different function in fish included food intake (Blanco et al., 2020) for which a connection with TH has been established since it exerts an influence on its transcriptional level (Fukuki et al., 2010). In our study, the early acrophase of *bdnf* compared to *th* reinforce the idea of this connection, even though the maintenance of the rhythm of *th* in MD group suggest an additional regulation in terms of rhythmic

transcription, which is in part affected by feeding time as suggested by the anticipation of the acrophase of MD group.

Conclusions

5 Conclusions

5.1. Conclusions

1. In the European sea bass, feeding time is a potent synchronizer for a peripheral oscillator like liver. The genes involved in digestion and metabolism were always rhythmic when the feeding time matched the active phase of the animal. On the contrary, feeding the fish during their resting phase led in most cases to a loss of the rhythm or, alternatively, a shift in the acrophase. In the brain, genes involved in the food intake and reward systems were affected by feeding time in a gene-specific way, suggesting an intricate connection between the synchronizers and the physiological pathways which regulate appetite.

2. The stress response of the European sea bass was significantly affected by the time of the day. The response of genes related to the antioxidant system and mitochondrial markers in this species was also time-dependent. Moreover, for all the parameters analyzed, not only the time of the day but also the season of the year plays a crucial role, suggesting that both factors should be taken into consideration to maximize welfare in aquaculture, especially during routine procedures involving acute air exposure.

3. The chronic stress affects the molecular clock and the epigenetic system in the European sea bass. In general, the epigenetic genes display an early response compared to the clock genes, but both the systems are affected by stress of it persists over time. The type of stressor can affect differently the two systems as their response was different to high density and caloric restriction. Due to the early

activation of the epigenetic genes, they could also represent an early biomarker to assess stress. On the other hand, the clock genes were particularly responsive to feeding treatment making them good indicators to understand the potential effect of calories restriction in aquaculture.

4. As observed for digestion and metabolism, feeding time affected the rhythms of the clock and epigenetic genes in the liver of the European sea bass, especially when fish were fed during the resting phase. The rhythm observed on the clock genes from the positive loop (*clock* and *bmal*) and epigenetic genes presented similar acrophases, located during the resting phase of the animal, suggesting a connection between them and a potential role of this phase for the epigenetic stability of the genome. In the central pacemaker, feeding time has less effect and the main synchronizer seemed to be the light, since all the clock genes presented similar rhythms mostly synchronized to the LD cycle, although a slight shift was described for some of them.

5. The glucocorticoid system plays an important role on the rhythmicity of the clock genes of the secondary loop in the brain of zebrafish. Moreover, even though the central pacemaker should be less sensitive to feeding time, this synchronizer was capable of modifying the rhythmic expression of genes involved in the control of the clock system, pointing to a link between the molecular clock and the metabolic system. The epigenetic genes show differences between day and night but did not seem to be influenced by both the glucocorticoid system and feeding time.

General bibliography

6 General bibliography

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Annexes

7. Annexes

7.1. Scientific publications and publications in progress

Samorì, E., Rodríguez, I., Paullada-Salmerón, J. A., Sánchez-Alacid, L., Muñoz-Cueto, J. A., Sánchez-Vázquez, F. J., & López-Olmeda, J. F. (2024). Daily rhythms of acute stress responses and antioxidant systems in the European sea bass (Dicentrarchus labrax): Effects of the time of the year. *Aquaculture*, *584*, 740616.

Samorì, E., Rodríguez, I., Oliver J.A., Sánchez-Vázquez, F. J., & López-Olmeda, J. F.. Influence of feeding time on daily rhythms of locomotor activity, clock genes and epigenetic mechanisms in the liver and hypothalamus of the European sea bass (*Dicentrarchus labrax*). Sent to Fish Physiology and Biochemistry.

Samorì, E., Rodríguez, I., Paullada-Salmerón, J. A, Muñoz-Cueto, J. A., Sánchez-Vázquez, F. J., & López-Olmeda, J. F. Feeding time affect daily rhythms of digestive enzymes liver, food intake regulation and reward system in hypothalamus of European sea bass (*Dicentrarchus labrax*). In preparation.

Samorì, E., Gatto E., Paullada-Salmerón, J. A., Muñoz-Cueto, J. A., Sánchez-Vázquez, F. J., & López-Olmeda, J. F. Influence of two different stressors (density and restricted feeding) on the circadian clock and epigenetic mechanisms in liver of European sea bass (*Dicentrarchus labrax*). In preparation.

Samorì E., Frigato E., dalla Valle L., Sánchez-Vázquez F.J., López-Olmeda J.F., Bertolucci C. Feeding entrainment of the primary and accessory loops of the molecular circadian clock and epigenetic processes in Zebrafish brain. In preparation.

Gatto E., **Samorì E**., Frigato E., Bertolucci C., Lucon-Xiccato T. Analysis of neural plasticity genes' expression in fish brain reveals the basis of individual differences in learning. In preparation

7.2 Congress contributions

7.2.1 Oral communication

European Aquaculture 2023, Vienna 18-21/09/2023. **Elisa Samorì**, Inmaculada Rodríguez Carretero, Francisco Javier Sánchez-Vázquez, José Fernando López-Olmeda. Feeding time effects on epigenetic mechanisms and the circadian clock in liver and brain of the European sea bass (*Dicentrarchus labrax).*

European Aquaculture 2022, Rimini 27-30/9/2022 – **Elisa Samorì**, Inmaculada Rodríguez Carretero, Jose Antonio Paullada-Salmerón, Laura Sánchez-Alacid, José Antonio Muñoz-Cueto, Francisco Javier Sánchez-Vázquez, Jose Fernando López-Olmeda. Daily rhythms in the stress response of the European sea bass (*Dicentrarchus labrax*) and the effects of seasonality.

AIEC on line 16-17/19/2021 - **Elisa Samorì**, J.A. Paullada-Salmerón, I. Rodríguez, L. Sánchez-Alacid, J.A. Muñoz-Cueto, F.J. Sánchez-Vázquez, J.F. López-Olmeda. Daily rhythms in the stress response of the European sea bass (*Dicentrarchus labrax*): effects of seasonality and feeding time.

7.2.2 Poster

10th international Symposium on Fish Endocrinology, Baltimore, MD USA 15-19/09/2024. Paullada-Salmerón, José A., Vergès-Castillo, Alba, **Samorì, Elisa**, López-Olmeda, José F. , Muñoz-Cueto, José A. Does acute stress affect the reproductive neuroendocrine systems of the European sea bass? a regional and daily study. Poster accepted

European Aquaculture 2024, Copenhagen 26-30/8/2024 – **Elisa Samorì**, Elena Frigato, Tryrone Lucon-Xiccato, Cristiano Bertolucci, Jose Fernando López-Olmeda, Elia Gatto. Understanding the contribution of individual variation in stress-response for improving fish welfare in aquaculture: a case of study in a diurnal and nocturnal species. Poster accepted

European Aquaculture 2023, Vienna 18-21/09/2023. **Elisa Samorì,** José Antonio Paullada-Salmerón, José Antonio Muñoz-Cueto, José Ángel López-Jiménez, Francisco Javier Sánchez-Vázquez, José Fernando López-Olmeda. The influence of two different stressors (density and restricted feeding) on the epigenetic system, the circadian clock and lipid metabolism in the european sea bass (*Dicentrarchus labrax*): the importance of the time of the time of the day.

European Aquaculture 2022, Rimini 27-30/9/2022 – **Elisa Samorì**, Inmaculada Rodríguez Carretero, José Antonio Paullada-Salmerón, José Antonio Muñoz-Cueto, Francisco Javier Sánchez-Vázquez, José Fernando López-Olmeda. How feeding time affects the daily rhythms of food intake control and digestive factors in the European sea bass *Dicentrarchus labrax*.

European Aquaculture 2022, Rimini 27-30/9/2022 – **Elisa Samorì**, Inmaculada Rodríguez Carretero, Francisco Javier Sánchez-Vázquez, José Fernando López-Olmeda. How feeding time affects clock and epigenetic mechanisms in liver and brain of European sea bass (*Dicentrarchus labrax).*

European Aquaculture 2022, Rimini 27-30/9/2022 – Inmaculada Rodríguez, **E. Samori**, J.A. López-Jiménez, Marta Conde-Sieira, Sara Comesaña, José L Soengas, F.J. Sánchez-Vázquez, J.F. López-Olmeda. Daily rhythms of lipid metabolism and fatty acids profile in the liver of the European seabass (*Dicentrarchus labrax*): influence of feeding time and the season of the year.

European Aquaculture 2022, Rimini 27-30/9/2022 - Inmaculada Rodríguez, **E. Samori**, J.A. López-Jiménez, González-Silvera, F.J. Sánchez-Vázquez, J.F. López-Olmeda. Fatty acids profile of liver, plasma, muscle and perivisceral fat in the European sea bass (Dicentrarchus labrax): influence of feeding time and season of the year.

AIEC 16-17/19/2021 - **Elisa Samorì**, I. Rodríguez, J.A. Paullada-Salmerón, L. Moreno Zapata, J.A Muñoz-Cueto, F.J. Sánchez-Vázquez, J.F. López-Olmeda. How seasonality and feeding time affect daily rhythms of food intake and digestive factors in the European sea bass.

Summary in Italian

8. Summary in Italian

Lo scopo di questa tesi è stato studiare l'effetto di due agenti sincronizzatori (luce e momento della alimentazione) su diversi processi fisiologici che riguardano il branzino, in quanto specie di particolare interesse per l'acquacultura. Inoltre, l'effetto dei sincronizzatori e dello stress sui geni orologio è stato preso in considerazione a causa del loro ruolo centrale nella regolazione del sistema circadiano. L'obiettivo comune di ogni capitolo è stato quello di studiare la presenza di ritmi circadiani nelle variabili analizzate, e di variazioni durante l'alternanza giorno / notte. La specie modello Zebrafish è stata inoltre utilizzata per chiarire quale sia l'effettivo collegamento tra i geni clock, i meccanismi epigenetici ed i meccanismi dello stress.

Per investigare questi aspetti sono stati stabiliti i seguenti obiettivi:

1. Determinare l'effetto del momento dell'alimentazione in fattori coinvolti nei meccanismi digestivi, regolazione dell'appetito e sistema ricompensa nel branzino.
2. Studiare la risposta allo stress acuto da parte del branzino e quale sia il ruolo della stagione. In specifico sono stati analizzati componenti dell'asse ipotalamo-ipofisi-ghiandola surrenale, e del sistema antiossidante e marker mitocondriali nel fegato.
3. Determinare come i geni orologio assieme a geni coinvolti in processi epigenetici rispondono in seguito ad uno stress cronico (alta densità o bassa alimentazione) nel fegato di branzino.
4. Descrivere come il momento dell'alimentazione condiziona i geni orologio nell'ipotalamo e fegato del branzino, e quale sia l'effetto sui geni coinvolti in processi epigenetici nel fegato.

5. Determinare l’effetto del momento della alimentazione sui geni orologio, geni coinvolti in processi epigenetici e metabolici nel cervello di Zebrafish mutanti per il recettore dei glucocorticoidi ($gr^{-/-}$).

Capitolo 1. Il momento della alimentazione influisce sul ritmo di geni coinvolti nei processi digestivi del fegato, regolazione della alimentazione e sistema ricompensa nell’ipotalamo del branzino.

I pesci presentano ritmi circadiani a livello molecolare di diversi tessuti grazie alla sincronizzazione dovuta all’effetto di diversi *zeitgeber*, uno dei quali è rappresentato dalla disponibilità di cibo in natura. Essendo quest’ultimo raramente costantemente disponibile, l’evoluzione ha indotto gli organismi a sincronizzare i propri processi metabolici e digestivi con l’ambiente esterno affinché si traesse il maggior beneficio dall’alimentazione nella ristretta finestra di disponibilità dell’alimento stesso. Nei pesci, la regolazione dei processi di alimentazione è molto complessa e l’ipotalamo riveste un ruolo cruciale, ma poco si conosce di come il sistema ricompensa intervenga nella regolazione del suddetto sistema. In questo studio, abbiamo investigato come il momento dell’alimentazione possa effettivamente influire sulla trascrizione di alcuni enzimi digestivi nel fegato del branzino, e come la stessa possa influenzare l’ipotalamo nella trascrizione di ormoni coinvolti nella regolazione dell’appetito o di fattori coinvolti nel sistema ricompensa. Per questo scopo i branzini sono stati suddivisi in un gruppo alimentato a metà della fase luce (ML) ed un gruppo alimentano a metà della fase buio (MD) e successivamente i campioni sono stati raccolti in diversi momenti delle 24 ore per poter descrivere l’eventuale espressione ritmica dei fattori analizzati. I risultati hanno dimostrato che i geni analizzati coinvolti nella digestione delle proteine (*try2, tryp3, ctrl*

e *cpa*) presentano ritmo in condizioni ML con acrofase notturna (tra le ZT 11.28 e 13.60) e che tutti ad eccezione di *try2*, conservavano la ritmicità anche quando i pesci venivano alimentati in MD, anche se un certo ritardo nel raggiungere l'acrofase era presente (da ZT 16.95 a 18.45). Al contrario, i geni coinvolti nel processo di transaminazione (*c-alt* e *m-alt*) hanno presentato ritmo nel il gruppo ML con acrofasi diurne (ZT 5.16 e 5.3), similmente a quanto accaduto per *pk*, anch'esso ritmico solo nel gruppo ML con una simile acrofase (ZT 6.26). L'enzima lipolitico *pla2*, per il quale il ritmo è stato descritto solo nel gruppo ML, ha invece fatto registrare la sua acrofase a ZT 13.96. Nell'ipotalamo, i geni coinvolti nella regolazione della alimentazione (*npy* e *orexin*) hanno mostrato ritmo solo nel gruppo MD con le acrofasi rispettivamente a ZT 13.15 e 16.1. I geni coinvolti nel sistema ricompensa hanno invece mostrato pattern diversi in quanto *th* ha mostrato ritmicità in entrambe lo condizioni di alimentazione con acrofasi notturne a (ZT 17.93 e 15.50 rispettivamente per ML e MD), mentre *bdnf* ha riportato ritmo solo nel gruppo ML con acrofase a ZT 11.76. Nel complesso questo studio ha descritto come il momento della alimentazione possa condizionare la trascrizione ritmica di geni coinvolti nei processi di alimentazione e digestione e ne sottolinea l'importanza per i sistemi di acquacultura per migliorare le condizioni di welfare dell'organismo.

Capitolo 2. Ritmi giornalieri della risposta allo stress acuto e del sistema antiossidante nel branzino: l'effetto della stagione.

Negli allevamenti di acquacultura i pesci sono spesso sottoposti a diverse fonti di stress, tra cui l'esposizione all'aria, la quale si può verificare durante le normali procedure di routine. Questo evento viene percepito come uno stress acuto, il quale determina sia l'attivazione dell'asse dello stress (ipotalamo-ipofisi-ghiandola surrenale) sia una serie di risposte in termini di difese antiossidanti nel fegato, le quali sono volte a contenere il danno dovuto al successivo evento di re-ossigenazione. In questo studio abbiamo valutato

la risposta allo stress dovuto alla esposizione all'aria da parte del branzino, ripetendo l'esperimento in due momenti diversi dell'anno, ovvero a dicembre (9L:15D) e giugno (15L:9D). La ragione per cui si sono analizzati due momenti differenti dell'anno, è da riferire al fatto che, nel branzino, il cortisolo presenta valori diversi durante l'anno, suggerendo quindi che anche la risposta allo stress possa cambiare. Per mimare la condizione di stress i pesci sono stati esposti all'aria per un minuto e successivamente reintrodotti in acqua prima di effettuare il campionamento, il quale è avvenuto un'ora dopo per permettere al cortisolo di raggiungere il picco nel sangue. Al contrario, il gruppo di controllo è stato campionato immediatamente al punto di campionamento stabilito. Per evidenziare non solo l'ampiezza della risposta ma anche la presenza di un ritmo circadiano, i campioni sono stati raccolti in 7 punti della giornata. Per valutare la risposta dell'asse dello stress, campioni di plasma sono stati utilizzati per analizzare i valori di cortisolo, glucosio e lattato, mentre nell'ipotalamo sono stati analizzati i geni *crh* e *crh-bp*. Per valutare la risposta del sistema antiossidante, i geni *cat, sod1, gshpx* e *gsr* sono stati analizzati nel fegato, assieme ad alcuni marker mitocondriali quali *ucp1, prdx3* e *coxIV*. I risultati hanno rivelato che molti indicatori dell'asse dello stress e della risposta anti-ossidativa presentano ritmo, e la risposta allo stress è risultata essere significativamente influenzata dal momento del giorno in cui sono stati raccolti i campioni. Anche la stagione ha giocato un ruolo fondamentale in quanto molti degli elementi presentanti ritmo in dicembre, lo hanno successivamente perso in giugno. Inoltre, la risposta di cortisolo e glucosio in dicembre è risultata essere significativamente superiore se comparata alla risposta registrata in giugno. Al contrario, la risposta dei geni appartenenti al sistema antiossidante è risultata maggiore in giugno, un risultato probabilmente riconducibile ad un meccanismo di preparazione allo stress ossidativo. Nel complesso questi dati suggeriscono che la risposta allo stress da parte del branzino è fortemente condizionata dal momento del giorno in cui lo stress viene applicato, e

suggerisce l'importanza di prendere in considerazione i ritmi circadiani della specie allevata per ridurre al minimo l'effetto negativo di un evento stressante.

Capitolo 3. Influenza di due tipi di stress cronici (alta densità e bassa alimentazione) sull'orologio circadiano e meccanismi epigenetici nel fegato del branzino.

La risposta fisiologica allo stress viene mediata dalla attivazione dell'asse dello stress che porta al rilascio di cortisolo. Quest'ultimo riesce ad esercitare la sua influenza a livello molecolare solo dopo aver legato con il suo recettore (GR), attraverso il quale promuove o reprime la trascrizione genica legando con specifiche aree del promotore genico chiamate GRE (glucocorticoid responsive element). Il rilascio del cortisolo nel flusso sanguigno è regolato ritmicamente dai geni orologio, ma allo stesso tempo il cortisolo stesso diventa un fattore di trascrizione in grado di influire sui geni orologio creando quindi un doppio circuito. Lo stress è in grado di influenzare anche i processi epigenetici, in specifico, diversi studi riportano il suo effetto sul processo di metilazione, il quale a sua volta si trova sotto controllo circadiano. Questo studio aveva come obiettivo quello di studiato l'effetto di due diversi stress applicati in modo cronico per una durata totale di 60 giorni, concentrandosi in specifico sull'alta densità e bassa alimentazione, in quanto rappresentano due degli aventi stressanti più comuni in acquacoltura. Sono stati creati tre gruppi sperimentali: alta densità e alta alimentazione (HF-HD), bassa alimentazione e bassa densità (LH-LD) e gruppo di controllo con bassa densità e alta alimentazione (HF-LD). I campioni di fegato sono stati successivamente raccolti al giorno 5, 25 e 60 a metà della fase luminosa (ML) e a metà della fase oscura (MD). I geni analizzati sono stati i geni orologio (*clock1b, bmal1a, per1b* and *cry1a*), geni coinvolti nei processi di metilazione e de-metilazione (*dnmt1, dnmt3a* e *tet2*) e de-acetilazione (*sirt1*). I risultati hanno dimostrato una rapida risposta dei geni epigenetici in entrambe le condizioni sperimentali al giorno 5, seguiti dai geni clock al giorno 25 mentre al giorno

60 entrambi i sistemi dimostravano alterazioni in termini di mRNA. Inoltre, nonostante la risposta giorno-notte sia stata gene-specifica, al giorno 60 molte delle variazioni in termini di mRNA sono state registrate durante la notte. Comparando l'alta densità e la bassa alimentazione, la prima condizione è stata quella che ha dimostrato il maggior impatto nel sistema clock e nei geni coinvolti nei meccanismi epigenetici. La Principal Component Analysis (PCA) ha inoltre evidenziato che i geni che più hanno concorso nel determinare il pattern di espressione genica osservata per l'alta densità sono stati *dnmt1* e *dnmt3a* per i processi di metilazione, *sirt1* per la de-acetilazione e *per1b* and *clock1b* per i geni orologio. I dati raccolti nel complesso sottolineano come lo stress cronico possa influenzare anche processi molecolari importanti in grado di condizionare a cascata tanti alti pathway fisiologici.

Capitolo 4. Influenza del momento dell'alimentazione sui ritmi giornalieri di attività locomotoria, geni orologio e meccanismi epigenetici nel fegato ed ipotalamo del branzino.

L'orologio circadiano regola molteplici funzioni nell'organismo. In specifico, studi recenti suggeriscono che l'orologio circadiano ed i meccanismi epigenetici possano condividere un collegamento bidirezionale, in quando da un lato geni appartenenti ai meccanismi epigenetici sono espressi ritmicamente, e dall'altro gli stessi possono regolare i geni orologio. Questo studio ha avuto due obiettivi che hanno preso in considerazione due pacemakers differenti considerando due distinti momenti di alimentazione, ovvero ML (mid-light; metà della fase luminosa) e MD (mid-dark; metà della fase oscura). Il primo obiettivo è stato valutare l'impatto del momento della alimentazione su fegato per capire come i geni orologi (*clock1b, bmal1a, per1b, per2, cry1a, cry2*), geni coinvolti in meccanismi epigenetici (*dnmt1, dnmt3a, tet2, mbd4,*

gadd45aa) e la disponibilità di SAM (S-adenosil-metionina) e SAH (S-adenosil-omocisteina) rispondano in termini di ritmo. Il secondo obiettivo è stato verificare la risposta dei geni orologio nel cervello, in modo da descriverne il ritmo e l'eventuale influenza della alimentazione nel branzino. Inoltre, l'attività locomotoria è stata monitorata per verificare il se momento della alimentazione avesse una influenza sul pattern di attività. Nel fegato, i geni orologio si sono mostrati molto sensibili alla alimentazione, dove tutti i geni analizzati (con l'eccezione di *per2*) hanno mostrato ritmo solo nel gruppo ML ma non ne gruppo MD, con acrofasi collocate durante la fase notturna. Similmente, i geni connessi ai processi di metilazione (*dnmt1*, *dnmt3a*) de-metilazione (*tet2, mbd4, gadd45aa*) e de-acetilazione (*sirt1*) hanno mostrato ritmo nel gruppo ML con acrofase notturna, il quale è stato successivamente perso nel gruppo MD, con la solo eccezione di *dnmt3a*. SAM e SAH non hanno mostrato alcun ritmo in entrambi i gruppi, ma differenze tra giorno e notte sono state evidenziate suggerendo che l'alimentazione possa influenzare il potenziale di metilazione. Nell'ipotalamo, i geni orologio hanno invece mostrato ritmo in entrambi i gruppi, ma caratterizzato da un leggero shift nella acrofase tra i due gruppi. Il nostro studio ha rivelato che il momento della alimentazione agisce in modo diverso sul pacemaker centrale ed il fegato, ed in particolare esercita una certa influenza sui geni coinvolti nei meccanismi epigenetici.

Capitolo 5. Sincronizzazione del circuito principale ed accessorio dell'orologio circadiano e dei meccanismi epigenetici nel cervello di Zebrafish: l'importanza del sistema dei glucocorticoidi.

L'orologio molecolare non è solo sensibile a sincronizzatori esterni come il momento della alimentazione, ma anche a sincronizzatori interni, tra i quali spiccano i glucocorticoidi. I glucocorticoidi sono inoltre strettamente collegati con i meccanismi

epigenetici, in quando lo stress può determinare cambiamenti a livello epigenetici quali a loro volta possono influenzare i geni clock. Si crea così un circuito formato da molteplici interazioni. Per investigare l'importanza del sistema dei glucocorticoidi nella regolazione dei geni clock ed epigenetici, in questo studio è stato utilizzato Zebrafish mutante per il recettore dei glucocorticoidi ($gr^{-/-}$), il quale rappresenta il mediatore nella segnalazione del cortisolo nei pesci. In aggiunta, è stata verificata l'influenza del momento della alimentazione alimentando i pesci durante la metà della fase luminosa (ML) o metà della fase oscura (MD). Riguardo il circuito principale dei geni orologio, *baml1a* e *per1b* hanno presentato ritmo in entrambi i genotipi ($gr^{+/+}$ *e* $gr^{+/+}$*)* con acrofasi speculari e non dissimili tra loro. Al contrario i geni appartenenti al circuito accessorio *rev-erbα* and *rorα*, hanno presentato ritmo solo nel gruppo ML del genotipo $gr^{+/+}$. *Nfil3-5,* il quale è coinvolto nella regolazione dei geni clock, ha presentato ritmo in entrambi i genotipi e condizioni testate con acrofasi tra le ZT 9.38 e 9.63, mentre *bdnf* si è dimostrato più sensibile al momento della alimentazione in quanto il ritmo è stato descritto solo per le condizioni di ML di entrambi genotipi, alle ZT 23.75 ($gr^{+/+}$) e 1.60 ($gr^{+/+}$*)*. *Pparα* invece ha presentato ritmo solo nel genotipo $gr^{+/+}$ alimentato il ML. Infine, per quanto riguarda i geni coinvolti nei meccanismi epigenetici, solo *dnmt4* (genotipo $gr^{+/+}$) e *tet2* (genotipo $gr^{-/-}$) hanno riportato ritmo, rispettivamente alle ZT 6.23 e 10.13, ma nella condizione MD. Confrontando però i dati raccolti di giorno e quelli raccolti di notte, sono emerse differenze tra i due genotipi per quanto riguarda i geni coinvolti nei processi epigenetici in condizione ML. I nostri dati suggeriscono quindi che il sistema dei glucocorticoidi ed il momento della alimentazione hanno una discreta influenza sui geni orologio, e che i geni coinvolti nei meccanismi epigenetici sono più soggetti ad alterazione durante il giorno.

Summary in Spanish

9. Summary in Spanish

El propósito de esta tesis doctoral fue estudiar el efecto de diferentes sincronizadores (luz y hora de la alimentación) sobre diversos procesos fisiológicos de la lubina, una especie de particular interés para la acuicultura. El efecto de los sincronizadores y el estrés sobre los genes reloj se consideró debido a su papel central en la regulación del sistema circadiano. El objetivo general fue estudiar la presencia de ritmos circadianos en las variables analizadas y sus variaciones durante la alternancia día/noche. Además de la lubina, se utilizó el pez cebra para investigar cuál es la conexión molecular entre los genes reloj, los mecanismos epigenéticos y los mecanismos de estrés.

Para investigar estos aspectos, se establecieron los siguientes objetivos:

1. Determinar el efecto de la hora de la alimentación en los mecanismos digestivos, la regulación del apetito y el sistema de recompensa en la lubina.
2. Estudiar la respuesta al estrés agudo por parte de la lubina y verificar si hay diferencia entre dos momentos distintos del año. Específicamente, se analizaron componentes del eje HPI, del sistema antioxidante y de marcadores mitocondriales en el hígado.
3. Determinar cómo los genes reloj, junto con los genes de procesos epigenéticos, responden a un estrés crónico (alta densidad o baja alimentación) en el hígado de la lubina.
4. Describir cómo la hora de la alimentación condiciona los genes reloj en el hipotálamo y el hígado de la lubina, y cuál es el efecto sobre los genes involucrados en procesos epigenéticos en el hígado.

5. Determinar el efecto de la hora de la alimentación sobre los genes reloj, los genes de procesos epigenéticos y metabólicos en el cerebro de pez cebra mutantes para el receptor de glucocorticoides (gr-/-).

Capítulo 1. La hora de la alimentación influye en el ritmo de genes involucrados en los procesos digestivos del hígado, la regulación de la alimentación y el sistema de recompensa en el hipotálamo de la lubina.

Los peces presentan ritmos circadianos a nivel molecular en diversos tejidos gracias a la sincronización debida al efecto de diferentes *zeitgebers*, uno de los cuales es la disponibilidad de alimento. Este alimento no está disponible de manera constante, de modo que la evolución ha llevado a los organismos a sincronizar sus procesos metabólicos y digestivos con los ciclos de disponibilidad de comida para maximizar la alimentación. En los peces, la regulación de los procesos de alimentación es muy compleja, y el hipotálamo es crucial, pero se sabe menos sobre cómo el sistema de recompensa interviene en la regulación. En este estudio, investigamos cómo la hora de la alimentación puede influir en la transcripción de algunas enzimas digestivas en el hígado de la lubina, así como su influencia en la transcripción de hormonas involucradas en la regulación del apetito o de factores involucrados en el sistema de recompensa en el hipotálamo. En este experimento, las lubinas se dividieron en un grupo alimentado a la mitad de la fase de luz (ML) y un grupo alimentado a la mitad de la fase de oscuridad (MD) y posteriormente se recogieron muestras de hígado e hipotálamo a diferentes momentos del día. Los resultados mostraron que los genes involucrados en la digestión de las proteínas (*try2*, *tryp3*, *chymotrypsin A-like* y *cpa*) presentan ritmo en el grupo ML con acrofases nocturnas (entre ZT 11.28 y 13.60) y que todos, excepto *try2*, conservaban la acrofase incluso cuando los peces se alimentaban en MD, aunque mostraban un cierto retraso en la fase

(entre ZT 16.95 a 18.45). A diferencia de los genes que participan en el metabolismo inicial de las proteínas, aquellos involucrados en el proceso de transaminación (*cALT* y *mALT*) mostraron ritmo solo para el grupo ML, con acrofases diurnas (ZT 5.16 y 5.3). También *PK* presentó ritmo diario solo en el grupo ML, con una acrofase similar (ZT 6.26). La enzima lipolítica analizada, *PLA2*, solo mostró ritmo en el grupo ML, presentando su acrofase a ZT 13.96. En el hipotálamo, los genes de la regulación de la alimentación (*NPY* y *orexin*) presentaron ritmo diario solo en el grupo MD, con sus acrofases a ZT 13.15 y 16.1, respectivamente. Los genes involucrados en el sistema de recompensa mostraron patrones diferentes, ya que *TH* mostró ritmo en ambas condiciones de alimentación con acrofases nocturnas a ZT 17.93 y 15.50, respectivamente, para los grupos ML y MD, mientras *bdnf* mostró ritmo solo en el grupo ML, con su acrofase a ZT 11.76. En conjunto, este estudio ha descrito cómo el momento de la alimentación puede condicionar la transcripción rítmica de genes involucrados en los procesos de alimentación y digestión, y subraya su importancia para los sistemas de acuicultura, con el objetivo de mejorar las condiciones de bienestar de los organismos en cultivo.

Capítulo 2. Ritmos diarios de respuesta al estrés agudo y del sistema antioxidante en la lubina: el efecto de la época del año.

En acuicultura, los peces están expuestos a diversas fuentes de estrés, incluida la exposición al aire, siendo común durante los procedimientos rutinarios como la clasificación o el transporte. Este evento se percibe como un estrés agudo, que provoca tanto la activación del eje del estrés como una serie de respuestas de defensas antioxidantes en el hígado, que están destinadas a contener el daño debido al evento posterior de reoxigenación. En este estudio, evaluamos la respuesta al estrés agudo debido a la exposición al aire en la lubina, repitiendo el experimento en dos momentos diferentes

del año, en diciembre (10L:14D) y junio (15L:9D), para evaluar el efecto de la época del año. Las muestras se recogieron en 7 puntos distintos a lo largo del día, para evaluar también la existencia de ritmos diarios. Para generar el estrés, los peces se expusieron al aire durante un minuto y luego se reintrodujeron en el agua. La toma de muestras se realizó una hora después para permitir que el cortisol alcanzara su pico en la sangre. Un grupo de control (no estresado) se muestreó también en cada punto de muestreo. Para evaluar la respuesta del eje del estrés, se utilizaron muestras de plasma para analizar los niveles de cortisol, glucosa y lactato, mientras que en el hipotálamo se analizaron los genes *crh* y *crh-bp*. Para evaluar la respuesta del sistema antioxidante, se analizaron en el hígado los genes *cat, sod1, gshpx* y *gsr*, junto con algunos marcadores mitocondriales como *ucp1, prdx3* y *coxIV*. Los resultados revelaron que muchos indicadores del eje del estrés y de la respuesta antioxidante presentan ritmo diario y que la respuesta al estrés se vio significativamente influenciada por el momento del día, mostrando valores diferentes en los distintos puntos de muestreo. La estación también jugó un papel fundamental, ya que muchos de los elementos que presentaban ritmo en diciembre lo perdieron en junio. Además, en junio, la respuesta de cortisol y glucosa fue significativamente más alta durante el día. También en junio, la respuesta de los genes pertenecientes al sistema antioxidante fue mayor en comparación con diciembre. En conjunto, estos datos sugieren que la respuesta al estrés de la lubina está fuertemente condicionada por el momento del día en que se aplica el estrés, y sugieren que es importante tener en cuenta los ritmos circadianos de la especie cultivada para minimizar el efecto negativo de un evento estresante.

Capítulo 3. Influencia de dos tipos de estrés crónico (alta densidad y baja alimentación) en el reloj circadiano y los mecanismos epigenéticos en el hígado de la lubina.

La respuesta fisiológica al estrés activa el eje del estrés, que conduce a la liberación de cortisol. Este último puede ejercer su efecto a nivel molecular solo después de haberse unido a su receptor (GR), a través del cual promueve o reprime la transcripción génica al unirse a áreas específicas de los promotores, llamadas GRE (*glucocorticoid responsive element*). La liberación de cortisol al flujo sanguíneo está regulada rítmicamente por los genes reloj pero, a su vez, el propio cortisol se convierte en un factor de transcripción capaz de influir en estos genes reloj. Por otro lado, el estrés también puede influir en los procesos epigenéticos. Varios estudios han descrito su efecto sobre el proceso de metilación, el cual a su vez está bajo control circadiano. Este estudio tuvo como objetivo investigar el efecto de dos tipos diferentes de estrés, aplicados de manera crónica durante un total de 60 días: la alta densidad y la baja alimentación. Se crearon tres grupos experimentales: alta densidad y alta alimentación (HF-HD), baja alimentación y baja densidad (LH-LD) y un grupo de control con baja densidad y alta alimentación (HF-LD). Las muestras de hígado se recogieron en los días 5, 25 y 60, en mitad de la fase de luz (ML) y en mitad de la fase de oscuridad (MD). En todas las muestras se analizó la expresión de genes reloj (*clock1b, bmal1a, per1b* y *cry1a*), genes de procesos de metilación y desmetilación (*dnmt1, dnmt3a* y *tet2*) y desacetilación (*sirt1*). Los resultados mostraron una rápida respuesta de los genes epigenéticos en ambas condiciones experimentales a día 5, seguidos de los genes reloj en el día 25, mientras que en el día 60 ambos sistemas mostraban alteraciones en la expresión de mRNA. Además, aunque las diferencias día-noche fueron específicas para cada gen, en el día 60 muchas de las variaciones en el mRNA se observaron principalmente durante la noche. Comparando la alta densidad y la baja alimentación, la primera condición fue la que demostró el mayor

impacto en el reloj molecular y en los genes involucrados en los mecanismos epigenéticos. El Análisis de Componentes Principales (PCA) también destacó que los genes que más contribuyeron a determinar el patrón de expresión génica observado para la alta densidad fueron *dnmt1* y *dnmt3a* para los procesos de metilación, *sirt1* para la desacetilación y *per1b* y *clock1b* para los genes reloj. En conjunto, los datos recopilados subrayan cómo el estrés crónico puede influir en importantes procesos moleculares, capaces de afectar en cascada muchos otros procesos fisiológicos.

Capítulo 4. Influencia de la hora de la alimentación en los ritmos diarios de actividad locomotora, genes reloj y mecanismos epigenéticos en el hígado y el hipotálamo de la lubina.

El reloj circadiano regula múltiples funciones en el organismo. En particular, estudios recientes sugieren que el reloj circadiano y los mecanismos epigenéticos pueden compartir una conexión bidireccional, ya que por un lado los genes pertenecientes a los mecanismos epigenéticos se expresan rítmicamente y, por otro lado, estos pueden regular los genes reloj. En este estudio, se evaluó la expresión de distintos genes en lubinas alimentadas a dos horas distintas: ML (mid-light; mitad de la fase de luz) y MD (mid-dark; mitad de la fase de oscuridad). El primer objetivo de este trabajo fue evaluar la existencia de ritmos diarios y del impacto de la hora de la alimentación en los genes reloj (*clock1b, bmal1a, per1b, per2, cry1a, cry2*), los genes involucrados en los mecanismos epigenéticos (*dnmt1, dnmt3a, tet2, mbd4, gadd45aa*) y la disponibilidad de SAM (S-adenosil-metionina) y SAH (S-adenosil-homocisteína) en el hígado de estos animales. El segundo objetivo fue verificar la respuesta de los genes reloj en el cerebro, describiendo sus ritmos diarios y las diferencias debidas a la alimentación. Además, se registró la actividad locomotora para verificar si el momento de la alimentación tenía una influencia en el patrón de actividad. En el hígado, los genes reloj se mostraron muy sensibles a la

alimentación, de modo que todos los genes analizados (con la excepción de *per2*) mostraron ritmo solo en el grupo ML, pero no en el grupo MD. De manera similar, los genes relacionados con los procesos de metilación (*dnmt1, dnmt3a*), desmetilación (*tet2, mbd4, gadd45aa*) y desacetilación (*sirt1*) mostraron ritmos diarios en el grupo ML, los cuales se perdieron en el grupo MD, con la única excepción de *dnmt3a*. La disponibilidad de SAM y SAH no mostró ningún ritmo en ambos grupos, pero se evidenciaron diferencias entre el día y la noche, sugiriendo un uso diferente basado en la hora de la alimentación. En el hipotálamo, los genes reloj mostraron ritmos en ambos grupos, caracterizados por un ligero desplazamiento en la acrofase entre los dos grupos. Nuestro estudio reveló que el momento de la alimentación actúa de manera diferente sobre el marcapasos central del cerebro y el hígado y, en particular, que ejerce influencia sobre los genes involucrados en los mecanismos epigenéticos.

Capítulo 5. Sincronización de los bucles principal y accesorio del reloj circadiano y de los mecanismos epigenéticos en el cerebro del pez cebra: la importancia del sistema de glucocorticoides.

El reloj molecular no solo es sensible a sincronizadores externos como la hora de la alimentación, sino también a sincronizadores internos, entre los cuales destacan los glucocorticoides. Los glucocorticoides están estrechamente relacionados con los mecanismos epigenéticos, ya que el estrés puede inducir cambios a nivel epigenético que, a su vez, pueden influir en los genes reloj. Esto crea un circuito formado por múltiples interacciones. Para investigar la importancia del sistema de los glucocorticoides en la regulación de los genes del reloj y epigenéticos, este estudio utilizó el pez cebra mutante para el receptor de glucocorticoides (gr-/-), que es el mediador en la señalización del cortisol en los peces. Además, se evaluó la influencia de la hora de la alimentación, proporcionando de forma periódica el alimento a los peces durante la mitad de la fase de

luz (ML) o la mitad de la fase de oscuridad (MD). En cuanto al bucle principal de los genes reloj, *bmal1a* y *per1b* mostraron ritmo diario en ambos genotipos ($gr^{+/+}$ y $gr^{-/-}$), con acrofases similares. En cambio, los genes pertenecientes al bucle accesorio, como *rev-erbα* y *rorα*, mostraron ritmo solo en el grupo ML del genotipo $gr^{+/+}$. *Nfil3-5*, involucrado en la regulación de los genes reloj, exhibió ritmo en ambos genotipos y condiciones estudiadas, con acrofases entre ZT 9.38 y 9.63, mientras que *bdnf* mostró ser más sensible a la hora de la alimentación, mostrando un ritmo diario solo en las condiciones de ML en ambos genotipos, a ZT 23.75 ($gr^{+/+}$) y 1.60 ($gr^{-/-}$). Por otro lado, *pparα* mostró ritmo solo en el genotipo $gr^{+/+}$ alimentado en ML. En cuanto a los genes involucrados en los mecanismos epigenéticos, solo *dnmt4* (genotipo $gr^{+/+}$) y *tet2* (genotipo $gr^{-/-}$) mostraron ritmo, respectivamente a ZT 6.23 y 10.13. Al comparar los datos recogidos durante el día y la noche, surgieron diferencias entre los dos genotipos en relación con los genes implicados en los procesos epigenéticos. Nuestros datos sugieren que el sistema de glucocorticoides y la hora de la alimentación tienen un impacto significativo en el reloj molecular, y que los genes involucrados en los mecanismos epigenéticos son más susceptibles a las alteraciones durante el día.