

Research Article

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

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ABSTRACT

The molecular circadian clock is synchronized by environmental stimuli, allowing fish to predict external cyclic changes. Different molecular pathways are synchronized by the clock system, which is in turn affected by their output, as proved for the glucocorticoids (GCs) activity. We used a GC receptor (*gr*) KO (null mutant) zebrafish line (*gr*^{-/-}) to investigate the importance of GCs on the molecular clock, and the involvement in metabolic and epigenetic processes. Moreover, we investigated the relevance of feeding time on these processes using different time schedules (mid-light feeding, ML; mid-dark feeding, MD). Our analysis revealed that the clock genes analyzed in zebrafish brain responded differently, since genes of the primary loop (*bmal1a*, *per1b*) conserved the rhythm in all conditions, while genes of the secondary loop (*rev-erba*, *rora*) were affected by the genotype or the feeding time. *bdnf*, a neurotrophin regulated by the circadian system and known to interact with GCs, was more affected by the feeding time, whereas *ppara*, which links the clock system to metabolism, was affected not only by the feeding time but also by the lack of Gr. Additionally, epigenetic genes were more influenced by the loss of Gr during the day when comparing the genotypes by plotting day and night data separately. Our data suggest that GCs and feeding time can interact and influence each other modulating the molecular clock system in zebrafish brain.

1. Introduction

All living organisms are subjected to environmental cyclic variables which work as synchronizing factors, promoting the establishment of biological rhythms. Specifically, the circadian clock is described as a multicomponent system where different environmental stimuli (*zeitgeber*, or time giver), entrain an oscillator, lastly determining the establishment of a 24 h-rhythm, which can be displayed from the molecular to behavioural level (Cowan et al., 2017; López-Olmeda, 2017; Sánchez-Vázquez et al., 2019; Vera et al., 2023). This mechanism has been refined during evolution and allows animals to keep track of the time or anticipating daily changes (Krittika and Yadav, 2020). In fish, a multi-oscillatory system with no hierarchical relationship between the oscillators seems to be the most representative hypothesis for the overall

organization of their circadian system (Cahill, 2002; Vatine et al., 2011; Frøland Steindal and Whitmore, 2019), with different pacemakers being sensitive to several stimuli at the same time (López-Olmeda, 2017).

To allow the perfect tuning of the circadian system, the molecular clock is governed by autoregulatory feedback loops that enclose positive and negative regulation. 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 transcriptional factors capable of inducing *per* (*period*) and *cry* (*cryptochrome*) rhythmic expressions, together with several clock-controlled genes (CCGs). The activity of *per* and *cry* is exerted by inhibiting CLOCK-BMAL complex, through a negative feedback mechanism. A secondary loop starts when the genes *rev-erba* and *rora*, transcribed by CLOCK-BMAL heterodimer, stop (*rev-erba*) or promote (*rora*) *bmal* transcription by competing for its E-box

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promoter region, conferring another level of regulation to the system (Cahill, 2002; Pando and Sassone-Corsi, 2002; Sato et al., 2004; Zhdanova and Reebs, 2005; Vatin et al., 2011; Cox and Takahashi, 2019). *Rev-erba* not only negatively regulates *bmal1* through its promoter but also exerts its function on *Nfil3* (E4BP4) (Fang et al., 2014), which is a transcriptional repressor involved in multiple pathways in mammals and fish (Keniry et al., 2014; Prokatzky et al., 2012), including the regulation of the circadian system (Mitsui et al., 2001). In fish, genes of the primary and accessory loops can be differentially sensitive to environmental synchronizers (e.g., light and dark cycle, feeding time) or internal feedback signals (e.g., glucocorticoid signalling), and distinct pacemakers may respond differently to these cues (López-Olmeda, 2017). The central pacemaker has often been reported to be less sensitive to feeding time, responding primarily to light signals (Frøland Steindal and Whitmore, 2019). However, recent findings in mice suggest that brain structures like the choroid plexus can also be sensitive to feeding time, which promoted a shift of the peak of the clock gene expression, likely mediated by changes in insulin and glucose levels (Dočkal et al., 2025). Consistently, different patterns of clock gene expression linked to feeding time have also been reported in the hypothalamus of the European sea bass, *Dicentrarchus labrax* (Samorì et al., 2025), even though not all the genes analyzed responded in the same way. These findings suggest that the interactions between circadian clock genes and metabolic pathways could be extremely complex, even within the central pacemaker. Moreover, important mutual connections are established between the clock genes and factors like *bdnf* or *ppara*, which participate in different metabolic pathways. *Bdnf* is a neurotrophin involved in multiple functions, spanning from neural plasticity and survival (Tapia-Arancibia et al., 2004; Lucon-Xiccato et al., 2022) to food intake regulation (Blanco et al., 2020). It displays circadian variation in mammals (Tapia-Arancibia et al., 2004), is involved in photic entrainment in mice (Liang et al., 2000), and in circadian rhythmicity in zebrafish larvae (D'Agostino et al., 2022). *Ppara*, a nuclear receptor expressed in different tissues and whose activation is dependent on fatty acids and eicosanoids, displays circadian rhythmicity and could represent a link between feeding, nutrient regulation and circadian control of metabolism (Duez and Staels, 2009). In specific, it exerts its function after forming a heterodimer with RXR α (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). *Ppara* 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-erba* gene expression in mammals by targeting the Rev-DRE2 sequence on its promoter, thereby inducing its transcription (Duez and Staels, 2008).

Many physiological processes present daily rhythmicity thanks to the activity of the self-sustainable clocks, and several studies have described the daily rhythm of GCs in fish (López-Olmeda et al., 2013; Vera et al., 2013; Cowan et al., 2017; Samorì et al., 2024). Moreover, the GCs could participate in the regulation of the molecular clock thus creating a bidirectional regulatory system (Reddy et al., 2007). Their function is mainly exerted by binding to the GC receptor and subsequently targeting the GC responsive element (GRE) regions located in the promoters of their target genes, thereby enhancing or repressing the transcription (Dinarello et al., 2020), although tethering mechanisms have also been observed for some genes (Lacher et al., 2025). GRE have been described in several promoter regions of clock genes like *Ror* and *Rev-erba* (Dickmeis et al., 2013) or *Nfil3* (Carey et al., 2013; So et al., 2009), while *Per1* remains one of the main targets described (Yamamoto et al., 2005). The responsiveness of clock genes to GCs has been demonstrated in both human (So et al., 2009; Balsalobre et al., 2000; Torra et al., 2000) and fish cell lines (Sánchez-Bretaña et al., 2016; Sousa et al., 2017). In fish, this regulation has also been confirmed *in vivo*, where dexamethasone, a synthetic GC analogue, was capable of stimulating *per1* expression (Sánchez-Bretaña et al., 2016; Zhao et al., 2018). In addition, other

genes functionally connected to the molecular circadian clock, such as *bdnf* has been shown to be responsive to stress (Chakravarty et al., 2013; Pavlidis et al., 2015; Tsimopolis et al., 2024), still due to the activity of GCs through the GRE (Tsimopolis et al., 2024). Differences in clock genes expression have also been found comparing zebrafish wild type and null *gr* (*gr*^{-/-}) larva and adult eye and liver (Morbiato et al., 2019), but no data are available about the expression of these targets in zebrafish brain. The same study also proved that GCs are important in the feeding entrainment of zebrafish, which was clearly dampened in *gr* mutant genotype (Morbiato et al., 2019).

Stress and feeding can potentially influence the epigenetic landscape as well, as seen in fish species like European sea bass or half-smooth tongue sole (*Cynoglossus semilaevis*) (Anastasiadi et al., 2017; Li et al., 2017). Among the epigenetic processes, DNA methylation is particularly sensitive to feeding, since this process is mediated by DNA methyltransferases (DNMTs), which promote gene silencing by adding a methyl group on CpG dinucleotides (Bird, 2002; Moore et al., 2013), and depend on methionine availability, largely assumed through the diet. Consequently, nutrient availability can significantly affect the activity of enzymes involved in epigenetic processes also in fish, with metabolic consequences that may also extend to the offspring (Skjaerven et al., 2018; Skjaerven et al., 2020).

An additional connection between the clock system and the epigenetic mechanisms has been recently taken into consideration, also due to the possibility of a reciprocal influence (Stevenson, 2018). The effect of the circadian system on the methylation machinery is mediated by CLOCK-BMAL, which targets *Dnmt3a*'s promoter binding site initiating a rhythmic transcription (Stevenson, 2018). The existence of a daily rhythm in these processes has also been observed in zebrafish gonads (Paredes et al., 2018), and in the liver of European sea bass (Samorì et al., 2025), where different epigenetic enzymes were expressed rhythmically throughout the 24 h with acrophases mostly located during the resting phase of the animals.

Food availability in natural environments is not constantly available. Consequently, behavioural patterns and physiological processes have evolved to maximize resource utilization during periods of availability. In this study, we used the zebrafish *gr*^{ia30/ia30} mutant line (Facchinello et al., 2017). In this mutant line, the lack of a functional *Gr* results in constantly high cortisol levels (Dinarello et al., 2020) whose daily pattern diverge from that observed in wild type zebrafish, in which only *Gr* mRNA does not show a rhythmic expression (Benato et al., 2014). Using this model, we investigated the role of GC signalling pathway in regulating the expression of clock, metabolic and epigenetic genes expression in the brain of zebrafish subjected to different scheduled feeding regimes, with the purpose to better understand the relative contribution of GC signalling on the expression of genes involved in interconnected regulatory networks.

2. Materials and methods

2.1. Animal and housing

In this work we used wild-type (hereby referred as *gr*^{+/+}) and *gr* knock-out (hereby referred as *gr*^{-/-}) zebrafish of the *gr*^{ia30/ia30} mutant line described elsewhere (Facchinello et al., 2017; Morbiato et al., 2019). Fish were allocated in 200-L aquaria supplied with oxygenators, 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 times a day *ad 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.

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 Ferrara

Institutional Animal Care and Use Committee and the Italian Ministry of Health approved the experiment (Auth. num. 128/2020-PR).

2.2. Experimental design

$gr^{+/+}$ ($N = 60$; 0.30 ± 0.04 g body weight) and $gr^{-/-}$ ($N = 60$; 0.33 ± 0.02 g body weight) zebrafish with a 50–50% male-female ratio 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 of five 15-L tanks with six fish each ($N = 30$ fish each group) equipped with aerators. 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 h, and each group was sampled at ZT 3, 9, 15, 21 and ZT 3 of the second day 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 and they were processed in the following days. A red dim light was used to avoid light contamination during the dark phase sampling points ($\lambda > 600$ nm) (de Alba et al., 2019).

2.3. RNA extraction, cDNA synthesis and RT-qPCR analysis

The collected brain samples were homogenized in Trizol reagent (Invitrogen, Thermo Fisher Scientific, Baltics UAB) following manufacturer's instructions. RNA concentration and purity were evaluated with a spectrometry (Nanodrop® ND 1000, Thermo Fisher Scientific). RNA samples (1 μg) were treated with 1 U of DNase (Thermo Fisher) for elimination of possible genomic DNA contaminations and then used for cDNA synthesis with the QSCRIPT cDNA Synthesis Kit (Quantabio Beverly, USA), according to manufacturer's instruction. Every sample was then diluted in a proportion of 1:4 with nuclease-free water (Thermo Fisher Scientific) prior to the qPCR analyses in which 2 μl of 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 20 s at 60°C . After amplification, a melting curve analysis to confirm the specificity of the amplicon was performed. *Ef1a* was used as reference gene. A complete list of the primers retrieved from literature is listed in Table 1. The $2^{-\Delta\Delta\text{Ct}}$ method was used for the analysis of gene expression (Livak and Schmittgen,

2001).

2.4. 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 ($Y = \text{Mesor} + \text{Amplitude} \times \cos(2\pi(\text{t-Acrophase})/\text{Period})$) 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 significant differences between groups ($gr^{+/+}$ vs. $gr^{-/-}$ of ML and MD conditions separately) and time points (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.

3. Results

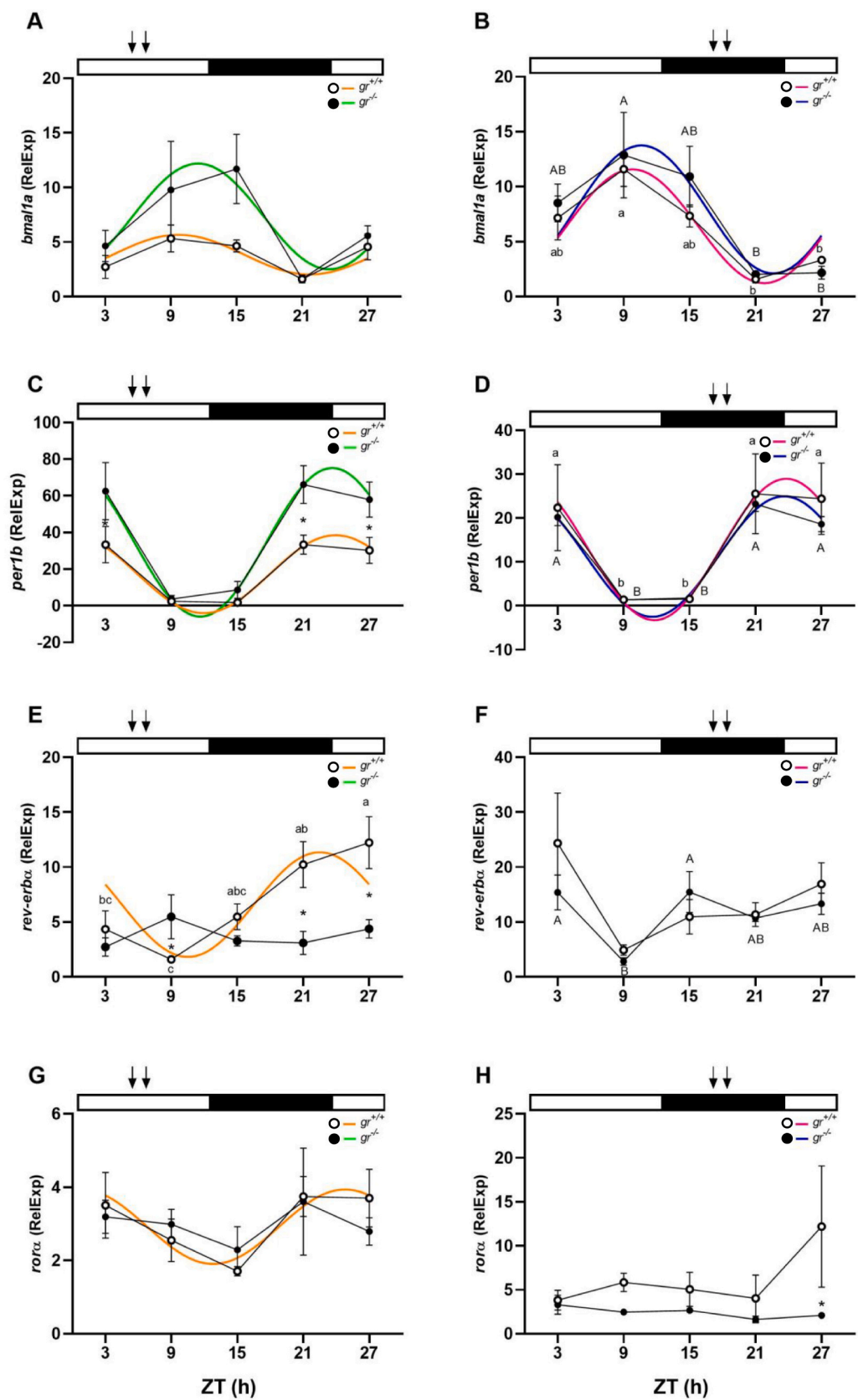
3.1. Clock primary and regulatory loops respond differently to GCs signalling and feeding time

Clock genes from the primary (*bmal1a* and *per1b*) and secondary loops (*rev-erba* and *rora*) were analyzed in the zebrafish brain, both at ML and MD conditions, by means of RT-qPCR (Fig. 1). The mRNA abundance of *bmal1a* maintained statistically significant rhythmicity in all the analyzed conditions (Cosinor, $p < 0.05$, Table 2), with peaks at the end of the light phase (ML: $gr^{+/+}$ ZT 10.45, $gr^{-/-}$ ZT 11.60; MD: $gr^{+/+}$ ZT 9.78, $gr^{-/-}$ ZT 10.61) and an increased amplitude in the $gr^{-/-}$ ML group compared to control group (Fig. 1A and B, Table 2). *per1b* presents rhythmic expression in all the four groups (Cosinor, $p < 0.05$, Table 2), with peaks at the end of the dark phase (ML: $gr^{+/+}$ ZT 23.91, $gr^{-/-}$ ZT 23.61; MD: $gr^{+/+}$ ZT 23.80, $gr^{-/-}$ ZT 23.60), rightly in antiphase with *bmal1a* mRNA (Fig. 1C and D, Table 2). Also, *per1b* mRNA rhythmic profile in $gr^{-/-}$ compared to $gr^{+/+}$ displayed increased amplitude in ML condition (Table 2). Interestingly, analysis on *rev-erba* gene expression revealed the presence of statistically significant rhythm only in the $gr^{+/+}$ ML group (Cosinor, $p < 0.05$, Table 2) (Fig. 1E), with acrophase at the end of the dark phase. On the contrary in $gr^{-/-}$ zebrafish brain, *rev-erba* displayed an arrhythmic expression pattern both in ML and MD conditions. Moreover, food availability during the night determined the loss

Table 1
Genes analyzed and primers for the qPCR analysis.

Gene	FW	RV	Acc. Number / Reference
<i>ef1a</i>	CCTTCGTCCTCAATTTTCAGG	CCTTGAACAGCCCATG	ENSDARG00000020850
<i>bmal1a</i>	TAGAGCGCTGTTTGCTGATG	GACCCGTGGACTTCAGTGAC	NM_131578
<i>per1b</i>	CCGTGAGTTTCGCTTTTCTC	ATGTGCAAGCTGTAGATCCC	NM_001030183
<i>rev-erba</i>	GCAATTACCCCAACAATCAG	CAGGCATGGAGCCCATAGT	NM_001130592
<i>rora</i>	GCATGTACGTGACGCGGT	TGGGCCAGATGTTCCAACCTCA	ENSDARG000000031768
<i>nfil3-5</i>	CTTCCAACCCAAACAGCGG	GCAGCCTCGTTATTCTTGCGC	ENSDARG000000094965
<i>bdnf</i>	GAAGAGTGATGACCATCTCTG	ATGAACCTGCTCGAAAGTGTCGG	¹ D'Agostino et al., 2022
<i>ppara</i>	CTTCGTCATTCACGACATGG	AAGCGTACTGGCAGAAAAGG	ENSDARG000000031777
<i>dnmt4</i>	AAGATTTACCTGCAGTCCCAG	CTCGCATACTTCTGACGCAATG	NM_001025450.1
<i>dnmt8</i>	CTTTGCGCTGTAATGAAGCCCC	TGTGAAGTGTCTGTGGTTGAA	NM_001018134.1
<i>tet2</i>	TGATCGTCTCTACATGGAAGTAAAG	CCCTGACAGGCACATGTTCT	ENSDARG000000076928

The table reports the genes analyzed and the sequences of the primers with accession numbers and references. The abbreviations used are the following: *ef1a*, elongation factor; *bmal1a*, basic helix-loop-helix ARNT like 1a; *per1b*, period 1b; *rev-erba*, nuclear receptor subfamily 1 group D member 1; *rora*, RAR related orphan receptor A; *nfil3-5*, nuclear factor interleukin 3 regulated; *bdnf*, brain-derived neurotrophic factor; *ppara*, peroxisome proliferator-activated receptor alpha; *dnmt4* DNA methyltransferase 4; *dnmt8*, DNA methyltransferase 8, *tet2*, tet methylcytosine dioxygenase 2.



(caption on next page)

Fig. 1. Daily variations in the relative mRNA expression (fold change) of *bmal1a* (A, B), *per1b* (C and D), *rev-erba* (E and F) and *rora* (G and H) in the brain of $gr^{+/+}$ and $gr^{-/-}$ zebrafish genotypes 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, Suppl. Table 1). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotypes at the same time point (two-way ANOVA, Suppl. Table 1). 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of *rev-erba* oscillatory expression profile in $gr^{+/+}$ zebrafish (Cosinor, $p > 0.05$, Table 2; Fig. 1F). In the same way, our analysis revealed the presence of a daily rhythm for *rora* mRNA only for the $gr^{+/+}$ ML group (Cosinor, $p < 0.05$, Table 2) (Fig. 1G), with the acrophase at the beginning of the light phase (ZT 1.28). No statistically significant rhythm or significant variations depending on the time of the day were observed for the MD experimental groups (two-way ANOVA, Suppl. Table 1) (Fig. 1H).

Clock genes belonging to the primary circadian loop were less sensitive to GC signalling and feeding time compared than members of the secondary loop, where feeding time was strongly effective in remodeling the shape of mRNA expression.

3.2. Expression of *Nfil3-5* is modulated by GCs signalling and not by feeding time

Nfil3-5 mRNA displays rhythmicity in all the analyzed conditions (Cosinor, $p < 0.05$, Table 2), with acrophases located at ZT 9 regardless feeding time or GR expression (Fig. 2A and B, Table 2). Interestingly, we showed an increase in amplitude of *nfil 3-5* oscillations in $gr^{-/-}$ zebrafish brain (Table 2) (two-way ANOVA, $p < 0.00001$ and 0.009 respectively) (Fig. 2A and B, Suppl. Table 1).

3.3. *Bdnf* expression is sensitive to feeding time

In our study, *bdnf* mRNA abundance of both genotypes in ML condition displayed a daily rhythm, with a short delay in the acrophase in $gr^{-/-}$ brain (ZT 5.15 and 7.1 for $gr^{+/+}$ and $gr^{-/-}$, respectively; Cosinor, $p < 0.05$, Table 2) (Fig. 3A and B). Interestingly, shifting the feeding time suppressed the daily rhythm in the MD group for both genotypes (Cosinor, $p > 0.05$, Table 2). Significant differences in *bdnf* expression levels between genotypes were found under ML conditions, with $gr^{-/-}$ fish showing higher *bdnf* expression (Fig. 3A, Suppl. Table 1). As for the genes of the secondary loop, GC signalling modulated *bmal* expression levels, while feeding time mediated the loss of statistically significant circadian rhythmicity when fish were fed during the MD phase.

3.4. GCs signalling and feeding time are both regulators of *ppara*

Ppara mRNA abundance was rhythmic only in the $gr^{+/+}$ zebrafish when they were fed during the mid light phase (ML) (Cosinor, $p < 0.05$, Table 2), with peak at ZT24 (Fig. 4A and B). In the ML condition $gr^{-/-}$ fish showed upregulation of *ppara* expression levels compared to $gr^{+/+}$ group, suggesting an important effect of the GC signalling, (Fig. 4A and B, Suppl. Table 1). The analysis on *ppara* expression profiles showed both the influence of GC signal and feeding time, even though the latter appears to be a crucial factor in shaping mRNA abundance rhythm.

3.5. Methylation and demethylation mediators are not regulated by GCs signalling or feeding time in zebrafish brain

Under ML feeding conditions, the mRNA abundance of *dnmt4*, *dnmt8* and *tet2* did not display statistically significant daily rhythms (Cosinor, $p > 0.05$, Table 2) (Fig. 5A, C and E), but *dnmt4* mRNA in $gr^{+/+}$ displayed significant differences throughout the 24 h (two-way ANOVA, $p = 0.047$) (Fig. 5A, Suppl. Table 1). A difference between genotypes (Fig. 5A) was found for *dnmt4* at the end of the dark phase, at ZT 21 (two-

way ANOVA, $p = 0.001$) (Suppl. Table 1), while *dnmt8* and *tet2* showed differences due to the genotype (two-way ANOVA, $p = 0.0003$ and 0.013 respectively) at the beginning of the light phase at ZT 3 (Fig. 5C and E, Suppl. Table 1), suggesting a partial modulation mediated by the GC signalling. When comparing expression levels during the light phase ($gr^{+/+}$ vs. $gr^{-/-}$) statistically significant results were found for *dnmt4* ($p = 0.013$), *dnmt8* ($p = 0.003$) and *tet2* ($p = 0.045$) (Fig. 6A, E and I). *dnmt8* and *tet2* displayed a similar trend, with higher mRNA abundance in the $gr^{+/+}$ genotype, whereas *dnmt4* showed an opposite pattern, with higher mRNA abundance in $gr^{-/-}$. In addition, *dnmt4* presented significant differences when comparing nighttime data between the two *gr* genotypes ($p = 0.036$) (Fig. 6B, Suppl. Table 3), displaying the same pattern observed under ML conditions.

In MD conditions, *dnmt4* expression analysis revealed a rhythm for $gr^{+/+}$ genotype (Cosinor, $p < 0.05$, Table 2), with the acrophase located at ZT 6.23 (Fig. 5B, Table 2), but no statistically significant rhythm or variation through the day was observed for $gr^{-/-}$. *Dnmt8* expression showed no significant rhythm in both genotypes, but for $gr^{-/-}$ significant differences were detected throughout the day (two-way ANOVA) (Fig. 5D, Suppl. Table 1). A daily rhythm was described for *tet2* gene expression for $gr^{-/-}$ in MD feeding, with the acrophase located at ZT 10.13 (Cosinor, $p < 0.05$, Table 2) (Fig. 5F, Table 2). Two-way ANOVA revealed significant genotype-dependent differences for *tet2* ($p = 0.019$) at ZT 21 and at the beginning of the following day at ZT 27 (Fig. 5F), as well as time-of-day effects for *dnmt4* ($p = 0.001$), (Suppl. Table 1). When comparing day and night data separately, only *tet2* presented significant differences when plotting dark phase data together, with a higher expression in $gr^{+/+}$ fish (*t*-test, $p = 0.002$) (Fig. 6L, Suppl. Table 2). The genes involved in the epigenetic processes were differently affected by the GC signalling and feeding time, without revealing a unique trend, probably due to the high complexity of their regulation.

4. Discussion

In the present study, we described the daily rhythms in the expression of genes involved in the molecular clock (*bmal1a*, *per1b*, *rev-erba*, *rora*) together with genes involved in their regulation (*nfil3-5* and *bdnf*), and examined how these genes respond to different feeding schedules in the brains of wild type ($gr^{+/+}$) and glucocorticoid receptor null ($gr^{-/-}$) zebrafish. Additionally, we described how *ppara*, a gene involved in multiple metabolic pathways and strongly connected to the molecular clock, responds to different feeding schedules and how the GCs influence its rhythm. The genes analyzed displayed differences in daily variations, with the genes involved in the primary loop of the clock system (*bmal1a* and *per1b*) being the most conservative in terms of rhythm and acrophase, regardless of the genotype or feeding time, while the other genes responded differently (Fig. 7).

The clock genes are the core which governs the biological clocks, and they induce a cascade of rhythmic transcription of the clock-controlled genes (Pando and Sassone-Corsi, 2002; Vatine et al., 2011). Many environmental factors are responsible for clock entrainment, allowing the animal to predict cyclic environmental changes. The clock genes present a tight connection with the elements of the HPI axis, since they induce the rhythmic release of GCs into the bloodstream (Cowan et al., 2017). Cortisol, after binding its cognate receptor (Gr), interacts with the GRE present on the promoters of the clock genes themselves, thus modulating their transcription (Balsalobre et al., 2000; Sánchez-Bretaño

Table 2

Resume of the Cosinor analysis results of the genes analyzed.

Gene	Experimental group	Significance variance	Mesor	Amplitude	Acrophase (ZT hours)
<i>bmal1a</i>	ML <i>gr</i> ^{+/+}	0.0173	3.67	1.96 ±	10.45 ±
	ML <i>gr</i> ^{-/-}	0.03973	± 0.92	1.63	3.47
	MD <i>gr</i> ^{+/+}	0.00030	6.68	4.81 ± 4.6	11.60 ±
	MD <i>gr</i> ^{-/-}	0.02721	± 2.57	5.17 ± 2.9	4.1
			6.40	5.82 ±	9.78 ± 1.6
<i>per1b</i>	ML <i>gr</i> ^{+/+}	0.000	± 1.35	5.19	10.61 ±
	ML <i>gr</i> ^{-/-}	0.00005	7.93		3.83
	MD <i>gr</i> ^{+/+}	0.00138	± 2.95		
	MD <i>gr</i> ^{-/-}	0.000	17.26	21.25 ±	23.91 ±
			± 4.37	7.63	1.41
<i>rev-erba</i>	ML <i>gr</i> ^{+/+}	0.00722	34.63	40.51 ±	23.61 ±
	ML <i>gr</i> ^{-/-}	0.36373	± 9.48	17.66	1.51
	MD <i>gr</i> ^{+/+}	0.08467	12.48	16.09 ±	23.80 ±
	MD <i>gr</i> ^{-/-}	0.1486	± 5.52	9.84	2.24
			11.23	13.72 ±	23.60 ±
<i>rora</i>	ML <i>gr</i> ^{+/+}	0.03844	± 1.21	2.2	0.36
	ML <i>gr</i> ^{-/-}	0.75304	6.58	4.76 ±	22.52 ±
	MD <i>gr</i> ^{+/+}	0.65034	± 1.92	3.55	2.94
	MD <i>gr</i> ^{-/-}	0.56374	-	-	-
			-	-	-
<i>nfil3-5</i>	ML <i>gr</i> ^{+/+}	0.00199	-	-	-
	ML <i>gr</i> ^{-/-}	0.00004	2.44	1.13 ±	1.28 ±
	MD <i>gr</i> ^{+/+}	0.00002	± 0.62	1.07	5.30
	MD <i>gr</i> ^{-/-}	0.00001	-	-	-
			-	-	-
<i>bdnf</i>	ML <i>gr</i> ^{+/+}	0.04389	9.63	14.08 ±	9.46 ± 3.5
	ML <i>gr</i> ^{-/-}	0.01942	± 4.94	8.87	9.63 ±
	MD <i>gr</i> ^{+/+}	0.42028	28.69	46.21 ±	1.45
	MD <i>gr</i> ^{-/-}	0.10719	±	20.05	9.38 ±
			10.38	10.84 ±	1.32
<i>ppara</i>	ML <i>gr</i> ^{+/+}	0.0139	7.16	4.55	9.40 ±
	ML <i>gr</i> ^{-/-}	0.0620	± 2.26	22.45 ±	1.32
	MD <i>gr</i> ^{+/+}	0.0846	12.87	8.87	
	MD <i>gr</i> ^{-/-}	0.6769	± 4.61		
			-	-	-
<i>dnmt4</i>	ML <i>gr</i> ^{+/+}	0.25012	2 ±	0.68 ±	23.75 ±
	ML <i>gr</i> ^{-/-}	0.59941	0.36	0.66	5.15
	MD <i>gr</i> ^{+/+}	0.03056	0.35	1.36 ±	1.60 ± 7.1
	MD <i>gr</i> ^{-/-}	0.10926	± 0.7	1.15	-
			-	-	-
<i>dnmt8</i>	ML <i>gr</i> ^{+/+}	0.92324	-	-	-
	ML <i>gr</i> ^{-/-}	0.66765	-	-	-
	MD <i>gr</i> ^{+/+}	0.85142	-	-	-
	MD <i>gr</i> ^{-/-}	0.4777	-	-	-
			-	-	-
<i>tet2</i>	ML <i>gr</i> ^{+/+}	0.60622	-	-	-
	ML <i>gr</i> ^{-/-}	0.66765	-	-	-
	MD <i>gr</i> ^{+/+}	0.34272	-	-	-
	MD <i>gr</i> ^{-/-}	0.0462	1.72	0.35 ±	10.13 ±
			± 0.18	0.35	4.92

All the data are reported only for significant rhythm (Cosinor, $p < 0.05$): significance variance, mesor, amplitude and acrophase f (indicated in ZT time). Data are expressed as value $\pm$ fiducial limits (set at 95%).

Abbreviations: *ef1a*, elongation factor; *bmal1a*, brain and muscle arnt-like 1a; *per1b*, period 1b; *rev-erba*, nuclear receptor subfamily 1 group D member 1; *rora*, RAR related orphan receptor A; *nfil3-5*, nuclear factor, interleukin 3 regulated; *bdnf*, brain-derived neurotrophic factor; *ppara*, peroxisome proliferator-activated receptor alpha; *dnmt4* DNA methyltransferase 4; *dnmt8*, DNA methyltransferase 8, *tet2*, tet methylcytosine dioxygenase 2.

et al., 2016). Cortisol rhythm can be also modulated by feeding time (Vera et al., 2007; Montoya et al., 2010; López-Olmeda, 2017) indicating that feeding can act as a synchronizer. In our study, two of the genes involved in the main loop, *bmal1a* and *per1b*, were rhythmic both in ML and MD conditions with similar acrophases when comparing the same genes in different conditions. This pattern suggests that an altered GC signalling, consequent to the loss of Gr, and shifted feeding time, are not enough to interfere with the rhythm of the principal players of the molecular clock in the central pacemaker. In goldfish, the feeding schedule was essential for synchronizing the central pacemaker only in the absence of an LD cycle (Feliciano et al., 2011) and the maintaining of a rhythm regardless of feeding schedule has already been described in fish (López-Olmeda et al., 2013; Vera et al., 2013). Even though the rhythm is maintained, some differences between the two genotypes are present for *per1b* under ML conditions. Among the clock genes, *per1* represents the main target for GCs 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 even in goldfish liver (Sánchez-Bretaña et al., 2016). We found that the brain of *gr*^{-/-} zebrafish exhibits increased expression of *per1b*, consistent with the results obtained in the whole larvae of the same line (Morbiato et al., 2019), but contrasting with data from the eye and liver of the same line (Morbiato et al., 2019) and from 5 dpf larvae of the *gr*^{s37} line (Jaikumar et al., 2020). In the *gr*^{s37} line, the disruption of GRE-dependent activity does not prevent the signalling by protein-protein interaction (Facchinello et al., 2017), which could in part explain the difference observed in larvae. Moreover, we cannot exclude a different regulation between the genes *per1* analyzed in zebrafish larvae by Jaikumar and colleagues (Jaikumar et al., 2020), even though GCs seem to be involved in both their regulation in fish (Sánchez-Bretaña et al., 2016).

Rev-erba and *rora* 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 GCs is exerted through the GRE region which is present on the *Rev-erba* regulatory region (Dickmeis et al., 2013). However, although dexamethasone modulates the phase of *Rev-erba* in the liver of rats, GCs could not be the only signal to entrain this peripheral clock (Balsalobre et al., 2000). In our study, the rhythm observed under ML feeding for *gr*^{+/+} genotype, was subsequently lost in *gr*^{-/-} in both analyzed genes from the secondary loop, *rev-erba* and *rora*. Moreover, the expression pattern of *rev-erba* over the 24 h in wild type zebrafish, appears to be inverted in the *gr*^{-/-} genotype. The absence of a rhythmic expression of *rev-erba* and *rora* in *gr*^{-/-} zebrafish suggests that GCs could be key components in regulating the secondary loop of the circadian clock in zebrafish brain, also involving different mechanisms. In specific, the GRE region present in the *Rev-erba* regulatory region (Dickmeis et al., 2013) could explain the pattern observed. However, we cannot exclude possible regulation throughout tethering mechanisms, which may be impaired in the *gr*^{-/-} genotype. In addition, an effect of the feeding was present as shown by the results from the MD group, where neither genotype exhibited rhythmic transcription of *rev-erba* and *rora*. Specifically, *Rev-erba* is considered one of the main regulators of the crosstalk between the circadian system and metabolism in mammals, due to its involvement in several metabolic processes (Duez and Staels, 2009). Moreover, its deletion impairs food entrainment in mice (Delezie et al., 2016). In our case, shifting the feeding schedule was sufficient to induce an arrhythmic profile based on Cosinor analysis, with almost no differences between genotypes. This suggest that feeding time is the primary factor shaping the rhythmic mRNA expression under MD conditions and indicates a degree of sensitivity to feeding also in the central pacemaker, at least in the secondary loop. Considering the multiple connections between the clock genes and different molecular pathways, the connection between *Rev-erba* and *Nfil3* represents an important link, due to the involvement of the latter in the regulation of the molecular clock, whose expression is in turn regulated by *Rev-erba* (Fang et al., 2014). In our study, *nfil3-5* maintained a rhythmic expression in both feeding

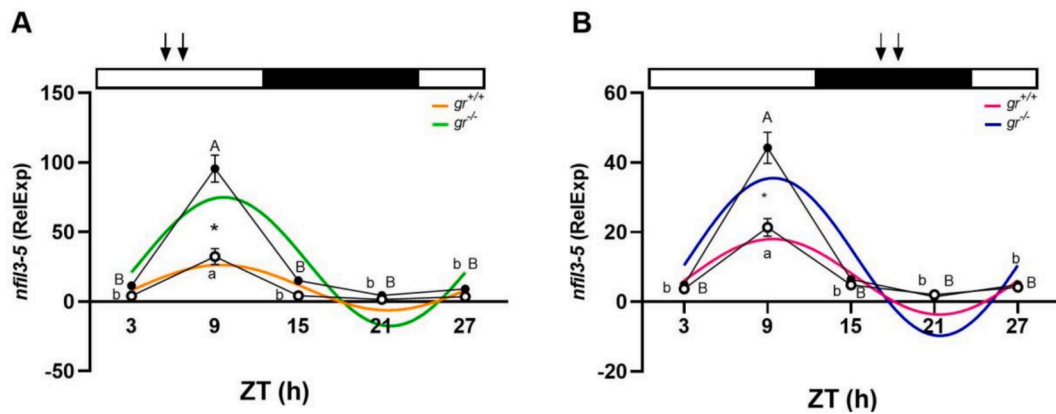


Fig. 2. Daily variations in the relative mRNA expression (fold change) of *nfil3-5* (A, B) in the brain of $gr^{+/+}$ and $gr^{-/-}$ zebrafish genotypes maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles ($\circ$) represent the $gr^{+/+}$ genotype, while the $gr^{-/-}$ is represented with black dots ($\bullet$). 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, Suppl. Table 1). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotypes at the same time point (two-way ANOVA, Suppl. Table 1). 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

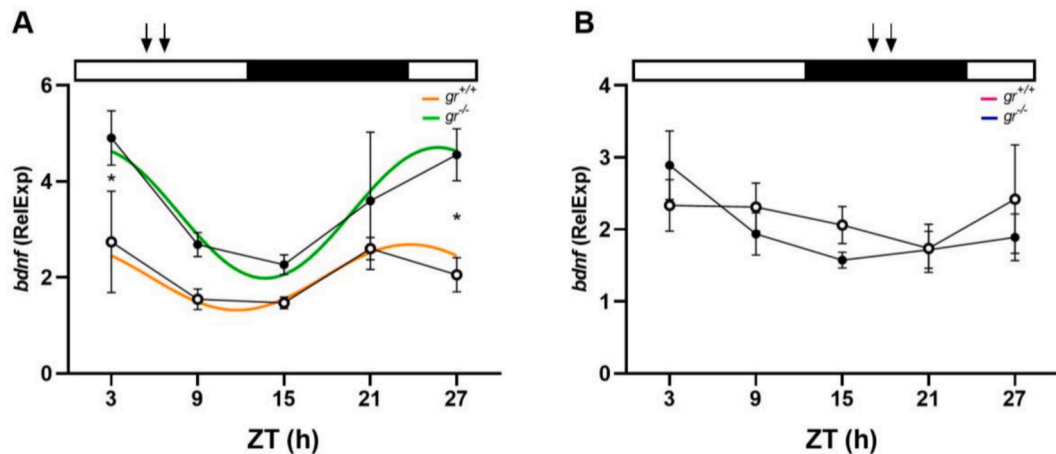


Fig. 3. Daily variations in the relative mRNA expression (fold change) *bdnf* (A and B) in the brain of $gr^{+/+}$ and $gr^{-/-}$ zebrafish genotypes maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles ($\circ$) represent the $gr^{+/+}$ genotype, while the $gr^{-/-}$ is represented with black dots ($\bullet$). The adjustment to a sinusoidal rhythm (Cosinor, $p < 0.05$), when significant, is represented by green and orange lines for $gr^{+/+}$ and $gr^{-/-}$ genotypes 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, Suppl. Table 1). The asterisks indicate significant differences between $gr^{+/+}$ and $gr^{-/-}$ genotype at the same time point (two-way ANOVA, Suppl. Table 1). 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions and genotypes, presenting a different pattern compared to *rev-erba*, suggesting that additional regulatory factors influence its rhythmic gene expression in fish. Specifically, when comparing $gr^{+/+}$ and $gr^{-/-}$ under both feeding conditions, the acrophase appears to increase in $gr^{-/-}$ zebrafish during the light phase. In mammals, *Nfil3* presents GRE regions on its regulatory sites which are activated by dexamethasone (Carey et al., 2013; So et al., 2009). In our case, the pattern observed in correspondence of the acrophases shown that $gr^{-/-}$ presents higher mRNA expression compared to the wild type at least during the light phase, suggesting a potential additional level of gene regulation. *Nfil3-5* is involved in multiple processes, ranging from light entrainment to metabolism (Keniry et al., 2014) and it could be hypothesized that its regulation could be more complex to be only attributed to GCs. Interestingly, it does not lose the rhythm in the $gr^{-/-}$ or when fish are fed during the dark phase. This specific trend could be

attributed to a direct effect of the LD cycle in which fish were kept during the trials. Moreover, light has also been shown to be crucial in remodelling the curve described into the 24 h also in a different mutant line (D'Agostino et al., 2022). Analysis in mice have reported different effects of GCs on the clock genes, whose up- or down-regulation was tissue specific (Soták et al., 2016). In addition, in our study we did not differentiate between various zebrafish brain regions where Grs are expressed (Natsaridis et al., 2023). Therefore, we cannot rule out the possibility of different trends in different brain regions.

Bdnf is a neurotrophin with multiple functions (Tapia-Arancibia et al., 2004; Blanco et al., 2020) for which a connection with the zebrafish circadian system has been recently discovered (D'Agostino et al., 2022). *bdnf* is also particularly responsive to acute and prolonged stress, usually displaying an upregulation in zebrafish (Chakravarty et al., 2013; Pavlidis et al., 2015; Lucon-Xiccato et al., 2023).

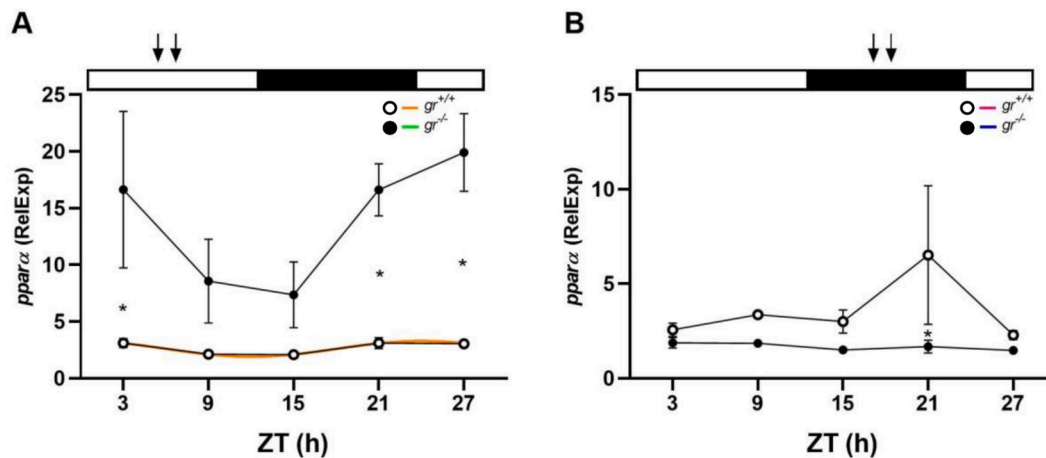


Fig. 4. Daily variations in the relative mRNA expression (fold change) of *ppara* (A, B) in the brain of *gr*^{+/+} and *gr*^{-/-} zebrafish genotypes maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) phase. White circles (○) represent the *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, Suppl. Table 1). The asterisks indicate significant differences between *gr*^{+/+} and *gr*^{-/-} genotypes at the same time point (two-way ANOVA, Suppl. Table 1). 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Additionally, using a different zebrafish *gr* mutant line, *bdnf* has been found to be directly regulated by the GCs, with possible implication for behaviour (Eachus et al., 2023). In our study, *bdnf* exhibited rhythmic expression when fish were fed during the light phase in both genotypes, and an upregulation in the *gr*^{-/-} was observed at the beginning of the day. This indicates at least partial involvement of the GCs in its regulation, as previously described (Eachus et al., 2023). In contrast, 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, as its expression increases during starvation, and peptide administration leads to food intake and upregulation of genes involved in orexigenic processes in the brain (Blanco et al., 2020). In line with these recent findings, our data suggest an involvement of food intake in its regulation. The two different feeding conditions highlight that GC signalling can fine-tune the expression but is not capable of establish a statistically significant circadian rhythm when feeding occurs during the dark phase, a trend described also for *rev-erba* and *rora*, which are also involved in metabolic pathways.

To better understand the crosstalk between Gr signalling and metabolism in the zebrafish brain, we examined the daily gene expression of *ppara*. In our study, *gr*^{-/-} genotype presented a significant increase in the expression of this gene under ML feeding. This increase was also associated to a loss of a statistically significant rhythm, in contrast to the daily rhythm described in *gr*^{+/+} phenotype. An involvement of GCs in the regulation of *ppara* expression was previously described in zebrafish larvae liver, where dexamethasone increased *ppara* expression acting through Gr (Chang et al., 2024). Our results may suggest a different regulation in the brain, as *ppara* expression was lower in *gr*^{+/+} fish. Moreover, feeding the fish during the dark phase led to the loss of the rhythmic expression regardless 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 *ppara* revealed that feeding time alone was less important than the LD cycle in the establishment of the rhythm (Paredes et al., 2014, 2015). In our case, although we focused the central pacemaker, which is generally less sensitive to feeding time, we found that this gene clearly responds to different feeding schedules. A similar pattern was observed for *rev-erba*, whose expression is also regulated by *ppara* (Duez and Staels, 2008), as well as for *rora* and *bdnf*. These results suggest that, for genes connected to the metabolic pathways, the individual contribution of feeding may not be negligible, even within the

central pacemaker.

The results of the analysis of epigenetic genes involved in the methylation and demethylation processes (*dnmt4*, *dnmt8* and *tet2*) exhibited a different pattern compared to the other genes. Specifically, none of the analyzed genes presented rhythmicity in the ML feeding group. In contrast, in the MD feeding group, rhythmic expression was observed for *dnmt4* in *gr*^{+/+} and for *tet2* in *gr*^{-/-} fish. Statistically significant differences, without evidence of circadian rhythmicity, were instead observed in the transcript of *dnmt8* knockout line. The inconsistent rhythmic pattern observed could be attributed to different factors and their complex connection, spanning from the transcriptional control mediated by the clock genes, the effect of GC signalling, feeding, and consequently nutrient availability.

In mammals a direct connection between the circadian system and epigenetic mechanisms has been hypothesized, potentially mediated by BMAL1 binding to E-box regions in genes involved in methylation (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). In other fish like European sea bass, a daily rhythm of epigenetic genes has been described in liver (Samorì et al., 2025), while in mammals, the expression profiles of these enzymes depend on the tissue (Stevenson, 2018). Different studies have thus suggested a clear connection between the two systems, which could be also bidirectional (Stevenson, 2018). However, since methylation and demethylation are flexible processes (Bochtler et al., 2017), the clock system may not be the only regulatory factor. This is also suggested by the data from our study, where no similarity was found between the trend described in the clock and epigenetic genes. Another study focused on the analysis of the transcriptomic of genes involved in methylation in a different mutant line, the *gr*³⁵⁷/*gr*³⁵⁷ (Eachus et al., 2023), to test the importance of GC signalling. This line, maintaining the protein-protein interactions (tethering activity), determining a lower overall impact on GC-regulated genes compared to the mutant lines used in our study (Facchinello et al., 2017). In that case, an upregulation of the genes encoding *dnmt3aa* (or *dnmt8*) and *dnmt3bb.3* (or *dnmt5*) was observed in brain samples collected at a single time point at the beginning of the day (Eachus et al., 2023), suggesting an important regulatory mechanism mediated by GC signalling. This is consistent with the alteration of the methylation processes observed in fish under stress (Anastasiadi et al., 2017; Li et al., 2017). However, our 24-h analysis revealed an opposite

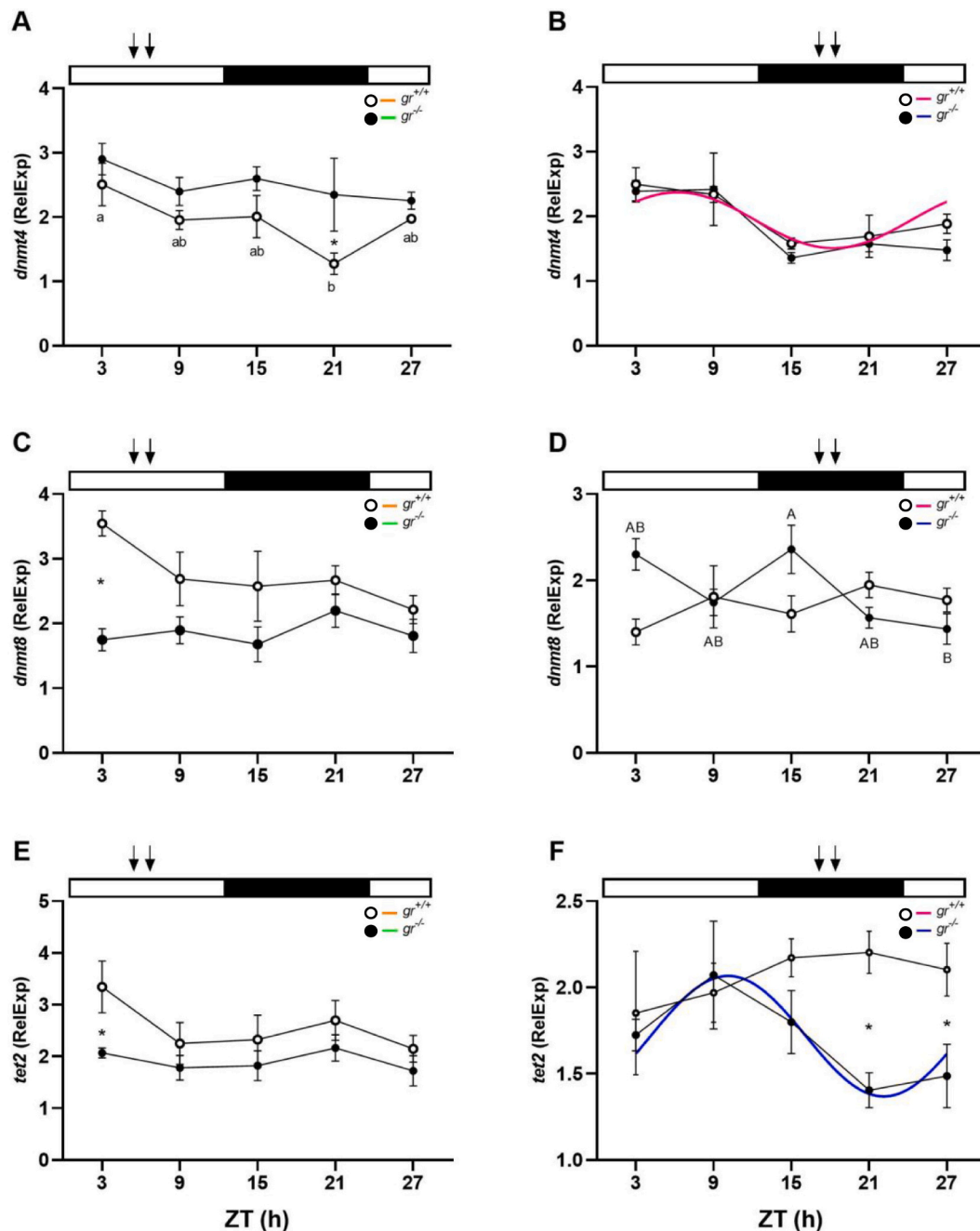


Fig. 5. Daily variations in the relative mRNA expression (fold change) of *dnmt4* (A, B), *dnmt8* (C and D), and *tet2* (E and F) in the brain of *gr*^{+/+} and *gr*^{-/-} zebrafish genotypes maintained in a 12:12 LD cycle and fed during the middle of the light (ML, left panel) or dark (MD, right panel) 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*^{-/-} genotypes 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, Suppl. Table 1). The asterisks indicate significant differences between *gr*^{+/+} and *gr*^{-/-} genotype at the same time point (two-way ANOVA, Suppl. Table 1). 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

trend for *dnmt8*, showing higher transcript levels in WT compared to *gr*^{-/-} at least at the beginning of the light phase. The same result is obtained by plotting day and night data separately for that gene, while *dnmt4* presents a similar pattern described by the previously mentioned study. It is important to note that the study by Eachus et al. (2023) did not consider the 24-h variation of the expression of the epigenetic genes examined, as samples were collected at only one time point, at the beginning of the day. Although this time point is comparable to our first sampling point, in our study it corresponds only to the first hour of the

light phase. Additionally, the differences between the two mutant lines could explain the diverse regulatory patterns that we observed. Notably, the day and night data analysis revealed significant differences in the analyzed epigenetic genes during the light phase, confirming an influence of GC on the methylation and demethylation enzymes at the transcriptional level. This result may suggest the presence of GRE elements on the *dnmts* genes, as previously described in Wistar rat brain (Urb et al., 2019). However, these differences mostly disappeared when fish were fed during the MD phase. Since *dnmt* genes are strongly

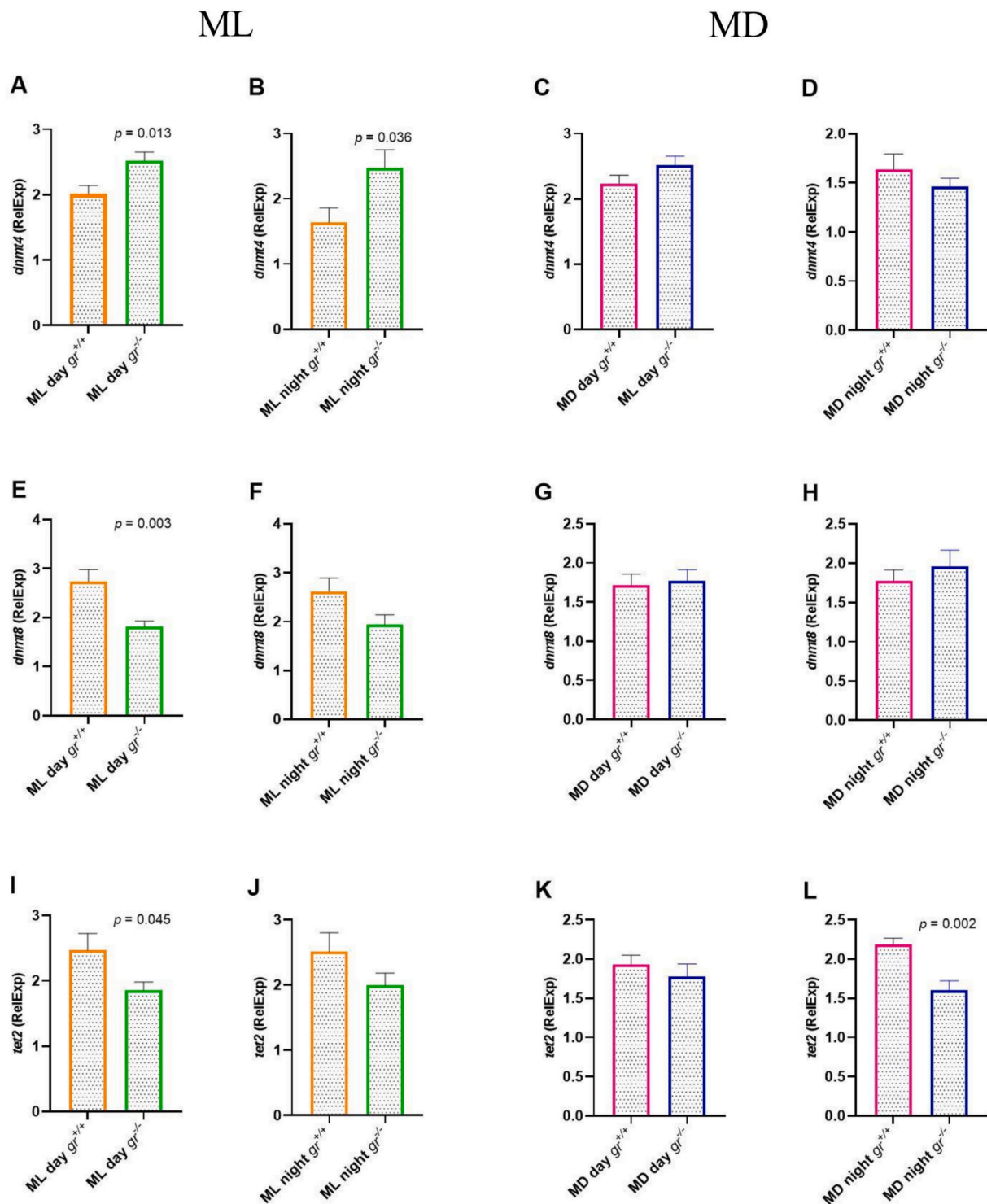


Fig. 6. Day-night differences in *dnmt4* (A, B, C and D), *dnmt8* (E, F, G and H) and *tet2* (I, J, K and L) analyzed in ML (left panel) of MD (right panel) conditions. Data from *gr*^{+/+} and *gr*^{-/-} genotypes are represented by orange/green and pink/blue panel respectively for ML and MD conditions. 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 ($p < 0.05$, Suppl. Table 2). All data are represented as mean $\pm$ SEM ($n = 6$ fish per point). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

influenced by methionine availability, which is obtained through the diet (Hermann et al., 2004), feeding fish during the night may have led to a reallocation of energy toward different biological processes, resulting in the loss of the differences between genotypes. A similar trend was described in the liver of European sea bass in the availability of SAH (s-adenosyl-homocysteine) (Samorì et al., 2025), which is

generated after releasing the methyl group used by the methylation enzymes. This suggests that the methylation process in fish could be extremely sensitive to feeding time. Additionally, although the transcriptional level of epigenetic genes may be influenced by multiple factors, we cannot exclude that the methylation landscape is affected by the integration of different signal, including feeding. Functional

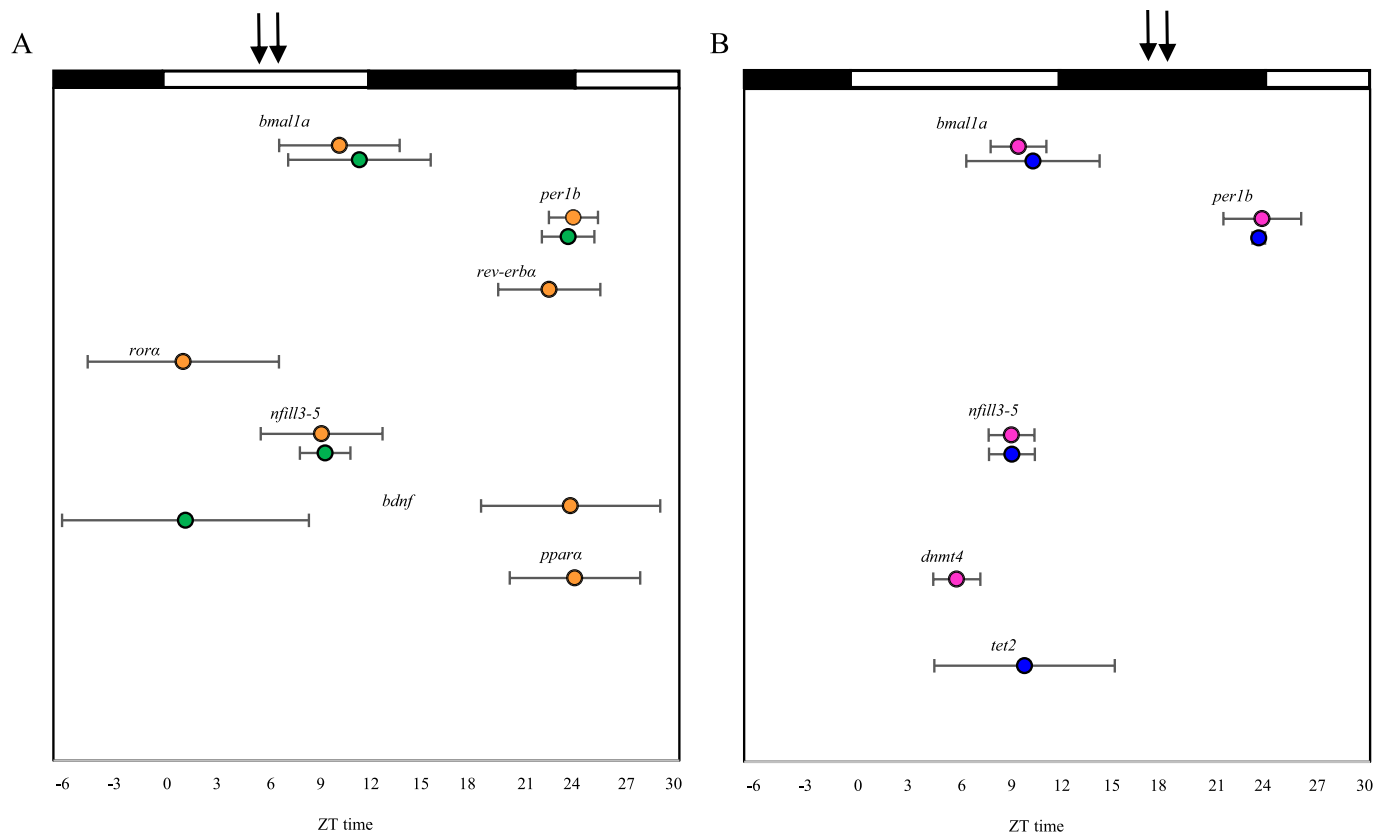


Fig. 7. Maps of acrophases of the genes analyzed of the molecular clock (primary and secondary loop), additional regulatory system of the molecular clock, metabolism and methylation/demethylation processes. The acrophase is only indicated when Cosinor analysis revealed a statistical significant rhythm (Cosinor, $p < 0.05$, Table 2). ML group is reported in the left panel (A) while MD group in the right (B), 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epigenetic changes should be further investigated to clarify whether one of these signals can be considered the main effector.

5. Conclusions

In this study we described how clock, metabolic and epigenetic genes respond in zebrafish brain when GC signalling is altered, and how feeding time influences their expression. Our study suggests that the core of the molecular clock is relatively stable when GC signalling is modified or feeding time is moved to the dark phase. On the contrary, GC signalling seems to be implicated in the regulation of the genes of the secondary loop and the ones with metabolic implications but only when fish are fed during the light phase. Instead, feeding time becomes the primary factor shaping gene expression when scheduled during the dark phase. A more complex trend was described for the epigenetic genes, where the influence of the circadian system, GC signalling, feeding and nutrient availability could have concurred. The results obtained suggest a complex regulation of the central pacemaker, but we cannot rule out the presence of specific differences related to different areas of the brain, especially the ones which are strictly related to feeding regulation.

CRediT authorship contribution statement

Elisa Samorì: Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Frigato:** Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Luisa Dalla Valle:** Writing – review & editing.

Annachiara Tesoriere: Writing – review & editing. **Francisco Javier Sánchez-Vázquez:** Writing – review & editing. **José Fernando López-Olmeda:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Cristiano Bertolucci:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpa.2026.112018>.

Data availability

Data will be made available on request.

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