UNIVERSIDADE DE SÃO PAULO

INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE FISIOLOGIA

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LOW TEMPERATURE EFFECT ON THE ENDOCRINE AND CIRCADIAN SYSTEMS OF *Danio rerio.*

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Advisor: Professor Ana Maria de Lauro Castrucci

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Advisor

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ABSTRACT

Light and temperature are abiotic factors with an important influence on the behavior of teleosts, besides metabolic and physiological processes such as the synthesis and release of hormones, the expression of gene diversity, and homeostasis at the cellular level, among others. In *Danio rerio* somatic growth is directly related to temperature; it has been demonstrated that high levels of growth hormone (GH) are induced by high temperatures, predominant in the hottest months of the year. Thermal changes also participate as stress inducers, consequently leading to an increase in metabolic levels of hormones such as cortisol, which plays a role in the organism as one of the participants in the circadian rhythm. The control of the biological rhythms begins with the activation of photosensitive and thermosensitive cells located in various organs of the fish such as brain, eye and skin. A central clock is still to be identified in teleosts, but peripheral clocks, themselves photo- and thermosensitive, have already been established. At the molecular base of these clocks lies a group of clock genes that intertwine by positive and negative feedback loops, which can be affected by light and temperature, thus adjusting the rhythm of the oscillators. In addition, temperature significantly affects the rhythm of other hormones such as melatonin, both in endo- and ectothermic organisms. Our aim was to investigate how low temperatures (23°C for 6 days) modulate the expression of melanopsins (*opn4)*, growth hormone, cortisol and melatonin pathway genes, as well as their relationship with clock genes in *D. rerio* raised and kept at 28°C. The *opn4m1* and *opn4m2* genes were more expressed in *D. rerio* eye and brain, while in peripheral tissues their expression was not homogeneous. In general, the *opn4m* genes were more expressed in *D. rerio* eye and brain, with a remarkable decrease in animals submitted to low temperature. We also observed a decrease in the expression of the clock genes *per1, per2, cry1a* and *cry1b* in animals kept at 23°C compared to animals kept at 28°C. We hypothesized that the temperature was probably perceived by melanopsin for modulation of the gene response, or indirectly by modulation of hormonal genes that participate as transcription factors regulating the molecular clock. Interestingly when we evaluated the enzymes for melatonin biosynthesis, we found higher levels of *aanat* transcripts in the brain and the eye at 23°C. The expression of *mtnr1aa/bb* in the eye showed a reduction at 23°C, eventually as a compensation for the markedly increased production of melatonin. The

growth hormonal axis was one of the most affected by the low temperature. At different levels since the production of the growth hormone and expression of the GH and IGF-1 receptors, the amount of mRNA transcripts was considerably reduced in animals submitted to 23°C. Finally, data from the cortisol axis confirmed the thermal stress (chronic stress). In these individuals, cortisol achieved higher levels (up to 1.4 µg/g weight) in relation to the control group, with a decrease in the levels of transcripts of its receptor (*gr*) in the liver, explaining a possible compensatory adjustment, thus ensuring the homeostasis of the animal. The molecular mechanism of the clock indicated a direct correlation with the endocrine systems analyzed, especially in the growth axis (*gh1, ghra, ghrb and igf1ra*), the expression of *mtnr1aa* receptor of melatonin and melanopsin in various tissues of *D. rerio*, thus establishing a direct connection between the circadian and endocrine systems in this species. With the results obtained, we conclude that changes in temperature up to ~5°C below the ideal temperature for *D. rerio* significantly affect the endocrine and circadian systems of the adults.

Keywords: Clock genes; *Danio rerio*; Hormones; Opsins; Temperature

RESUMO

Luz e temperatura são fatores abióticos com importante influência sobre o comportamento de teleósteos, além de processos metabólicos e fisiológicos como a síntese e liberação de hormônios, a expressão de diversidade de genes, a homeostase em nível celular, entre outros. Em *Danio rerio,* o crescimento somático está diretamente relacionado com a temperatura, pois tem se demonstrado que níveis altos de hormônio de crescimento (GH) são induzidos por altas temperaturas, predominantes nos meses mais quentes do ano. As mudanças térmicas participam também do rol de indutores de estresse, levando assim ao aumento nos níveis metabólicos de hormônios como o cortisol, o qual tem um papel no organismo como um dos partícipes do ritmo circadiano. O controle desses ritmos biológicos inicia-se pela ativação de células fotossensíveis e termossensíveis localizadas em diversos órgãos do peixe como cérebro, olho e pele. Um relógio central ainda está por ser identificado nos teleósteos, mas relógios periféricos, eles próprios foto- e termossensíveis já foram estabelecidos. Na base molecular destes relógios está um grupo de genes de relógio que se entrelaçam por alças de retroalimentação positiva e negativa, que podem ser afetadas por luz e temperatura, ajustando assim o ritmo dos osciladores. Além disso, a temperatura afeta significativamente o ritmo de outros hormônios como a melatonina, tanto em organismos endo- como ectotérmicos. Nosso objetivo principal foi investigar como baixas temperaturas (23°C durante 6 dias) modulam a expressão de melanopsinas (*opn4),* e genes da via do hormônio do crescimento (GH), cortisol e melatonina, assim como sua relação com genes de relógio em *Danio rerio* criados e mantidos a 28°C. De forma geral, os genes da melanopsina *opn4m* foram mais expressos no olho e cérebro de *D. rerio,* com marcada redução em baixas temperaturas. Encontramos também uma diminuição da expressão dos genes do relógio *per1, per2, cry1a* e *cry1b* em animais mantidos a 23°C em comparação aos animais mantidos a 28°C. Hipotetizamos que o anterior seja possivelmente um efeito da temperatura percebida pela melanopsina para modulação da resposta gênica, ou indiretamente pela modulação de genes hormonais que participam como fatores de transcrição regulando o relógio molecular. Interessantemente quando avaliamos as enzimas para a biossíntese de melatonina, encontramos altos níveis de transcritos de *aanat* no cérebro e olho a 23°C. A expressão de *mtnr1aa/bb* no olho demonstrou uma redução a 23°C, eventualmente como uma compensação para o marcado

incremento na produção da melatonina. O eixo do crescimento foi um dos mais afetados pela baixa temperatura. Em distintos níveis desde a produção do hormônio do crescimento, expressão dos receptores de GH e IGF-1, a quantidade de transcritos de RNAm foi notavelmente reduzida em animais submetidos a 23°C. Finalmente, os dados do eixo do cortisol confirmaram o estresse térmico (estresse crônico). Nesses indivíduos, o cortisol atingiu níveis superiores (de até 1,4 µg/g de peso) em relação aos indivíduos do grupo controle, com uma diminuição nos níveis de transcritos de seu receptor (*gr*) no fígado, explicando um possível ajuste compensatório, garantindo assim a homeostase do animal. O mecanismo molecular do relógio apresentou uma direta correlação com os sistemas endócrinos analisados, em especial o eixo de crescimento (*gh1*, *ghra*, *ghrb* and *igf1ra*), a expressão de receptor *mtnr1aa* de melatonina e de melanopsina em vários tecidos de *D. rerio*, estabelecendo assim uma conexão direta entre os sistemas circadiano e endócrino nesta espécie. Com os resultados obtidos concluímos que mudanças na temperatura de até ~5°C abaixo da temperatura ideal para *D. rerio*, afetam significativamente os sistemas endócrino e circadiano do indivíduo adulto.

Palavras-chave: *Danio rerio;* Genes do relógio; Hormônios; Opsinas; Temperatura

FIGURES

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SUMMARY

1. INTRODUCTION

1.1 Circadian rhythm and molecular clock

A variety of physiological and biochemical processes displays a daily rhythmic activity which are adjusted by environmental factors such as temperature and light (light-dark cycle), both the main temporal clues or first order *zeitgeber* (time-giver in German) (Aschoff, 1965; West & Bechtold, 2015). These rhythms are maintained, although with periods slightly different from 24 hours, by an endogenous system of temporal control, even in the absence of external clues.

The importance of internal oscillatory mechanisms of the organism, capable of regulating the cyclic temporal pattern of the physiological and behavioral processes of the animal, is the anticipatory behaviors, activation and/or inhibition of metabolic functions, all of which ensure homeostasis (Marques et al., 2003). Hastings and collaborators (2003) reviewed, several reports of cellular pacemakers, known as peripheral circadian clocks, in many tissues of all organisms.

On the base of the circadian system of vertebrates lies a set of the so-called clock genes. In mice, the heterodimer protein CLOCK: BMAL activates the expression of *Per* and *Cry* genes, resulting in the formation of PER: CRY heterodimer which inhibits the CLOCK: BMAL complex. Besides *Per* and *Cry* expression, the CLOCK: BMAL1 activates the expression of nuclear receptors (*Rev-Erb* and *Ror*) by binding to their E-box promoter elements. *Rev-Erb* and *Ror* regulate the transcription of *Bmal1* with a third feedback loop through ROR regulatory elements (Bozek et al., 2009). A similar process occurs in the molecular clock machinery of *D. rerio.*

The E-box regions, ROR response elements (RREs), cAMP response elements (CREs) and D-boxes are known sequences that centrally and peripherally regulate the expression of rhythmic genes (Bozek et al., 2009).

When light is perceived by the eye, an information transport signal is triggered. The light perception is mediated by the binding of CREB to the promoter regions of clock genes as

Per1. However, in mice it is known that clock response genes (for instance, *Dbp, Hlf, Tef, E4bp4,* and others) regulate clock-controlled genes through D-boxes (Gachon et al., 2006).

The so-called clock-controlled genes, CCGs, are responsible for integrating the clock mechanism and other tissue-specific physiological pathways, and ultimately modulate biological outputs of the circadian pathway. For example, in the mouse liver, *Rev-erbα* (transcriptional repressor) has been shown to play an important role in the recruitment of histone deacetylase 3 (HDAC3). Other CCGs for example *Atpa* and *Nrv2* encode α and β subunits of the Na^+/K^+ ATPase, a major ion pump in all mouse cells (Feng et al., 2011).

Among the most conserved properties of the mammalian central clock, the suprachiasmatic nucleus (SCN), is the ability to maintain the duration of its oscillation period, despite a wide variation of temperature, through a compensation that corrects the natural tendency of biochemical reactions to change with temperature (Sweeney & Hastings, 1960; Lahiri et al., 2005; Brown & Azzi, 2013). In addition, SCN is not affected by glucocorticoid as GR receptor is weakly expressed in this tissue. On the other hand, peripheral clocks have their rhythm modified by temperature and glucocorticoids (Balsalobre et al. 2000), as demonstrated by Moraes and colleagues (2017) in mouse melanocytes and Balsalobre and colleagues (2000) in mouse fibroblasts, respectively.

Studies in *Danio rerio* occurred in parallel to mammalian studies, and it was early shown that oscillations in the clock components were very similar to the mouse, and occurred in all tissues examined, both *in vivo* and *in vitro* (Lahiri et al., 2005). In *D. rerio*, also known as zebrafish, the protein products of *clock (clk)* and *bmal1* genes activate another group of clock genes and clock-controlled genes, through the binding of Clock: Bmal heterodimers to E-box elements in the target genes, including *per* and *cry*. The genes *period (per)* and *cryptochrome (cry)* are transcribed, translated, and upon dimerization and translocation to the nucleus, the Per: Cry heterodimer inhibits the transcription of Clock: Bmal complex which is reactivated after degradation of Per: Cry. This cycle is adjusted to a 24-hour period by the light-dark cycle (Lahiri et al., 2005; Vatine et al., 2011).

For this species, the precise nature of the signaling pathways is not yet clear. Vatine and coworkers (2011) demonstrated the presence of multiple clock genes in the teleost *Danio rerio*: six correspond to *cry* (*1a, 1b, 2a, 2b, 3* and *4),* four to *per* (*1a, 1b, 2* and *3),* and three

for *bmal (1a, 1b, 2)* and *clock (1a, 1b, 2). D. rerio* possesses light sensors and peripheral clocks in several tissues of its body, with the specific light induction of *cry1a* and *per2* genes (Hamilton et al., 2015) (Fig.1).

In turn, the Cry1a protein interacts directly with core clock components, Clock and Bmal, blocking their ability to dimerize, providing a likely mechanism for clock resetting (Tamai et al., 2007). Froland and Whitmore (2019) assigned to Cry1a protein the responsibility to prevent the oscillation of the clock molecular core. Other enhancing and controlling elements such as AP-1 have also been implicated in *cry1a* expression in *D. rerio* (Hirayama, et al., 2005).

In *D. rerio,* D-box confers light-driven expression by binding of the thyrotrophic embryonic factor (TEF). In addition, this species possesses 11 D-box-binding factors; among them nine prevail in the pineal gland, supporting the involvement of this pathway in the circadian mechanism (Martin et al., 2012).

The clock-controlled genes (CCGs) are expressed in different amounts in specific times of the day and depend on the circadian oscillator. The *clock* gene encodes bHLH (basic-helix-loop-helix) - PAS (Period-Arylhydrocarbon receptor nuclear translocator single minded) domain containing transcription factors which activate the transcription of CCGs due to their binding to E-box elements (Paibomesai et al., 2010). In mammals, *Clock* acts as a histone acetyltransferase, which promotes the modifications of chromatin in the central region of these genes (Doi et al., 2006).

It is evident that the organization of the circadian system in fish is less hierarchical than that in mammals, where the suprachiasmatic nuclei (SCNs) function as the master clock that entrains other central and peripheral oscillators (Schibler et al. 2015). Such a master clock has not yet been clearly identified in teleosts although the pineal gland plays a key role in its circadian system (Sánchez et al. 2015). The photosensitivity seems to be conferred by melanopsin that signals the intracellular phosphoinositide pathway, culminating in the production of NO and MAPK activation (Ramos et al., 2014). In *D. rerio*, five different genes encode melanopsin in the retina: *opn4x1, opn4x2, opn4m1, opn4m2, opn4m3* (Davies et al., 2011), which will be discussed in detail below.

In the case of ectothermic organisms, body temperature is strongly influenced by the environment and therefore it is important that the clock properties allow the prediction of environmental temperature. In endothermic organisms, there exist a homeostatic control of body temperature, but studies with cell culture and tissues have shown that even slight changes in temperature, such as the internal temperature circadian rhythm imposed by the central oscillator, influence the phase of peripheral clocks (Lahiri et al., 2005).

In the natural environment, temperature has a close relationship with the light-dark cycles, because the thermo-phase (high temperature) usually coincides with the photo-phase, and the cryo-phase (low temperature) coincides with the scoto-phase (Lopez & Sanchez, 2011). It is known that light or temperature exposure is required to synchronize the cellular clocks in the developing embryo of zebrafish (Dekens & Whitmore, 2008).

The zebrafish circadian clock is temperature compensated over a 10°C range however, the rhythm amplitude of *clock* gene mRNA expression (Vallone et al., 2005) and of *per3* and *per4* genes (Kaneko & Cahill 2005; López & Sanchez, 2011) is strongly influenced by the temperature. In developing larvae as well as zebrafish cell lines, temperature cycles of as little as 2°C difference are able to entrain the circadian rhythms of clock gene expression (Vallone et al., 2005). In peripheral clocks of the same species (ZEM-2S cells) the expression of *per1, per2, cry1a* and *cry1b* increased in response to a pulse of 33°C (5°C above the thermal preference) (Jerônimo et al., 2017).

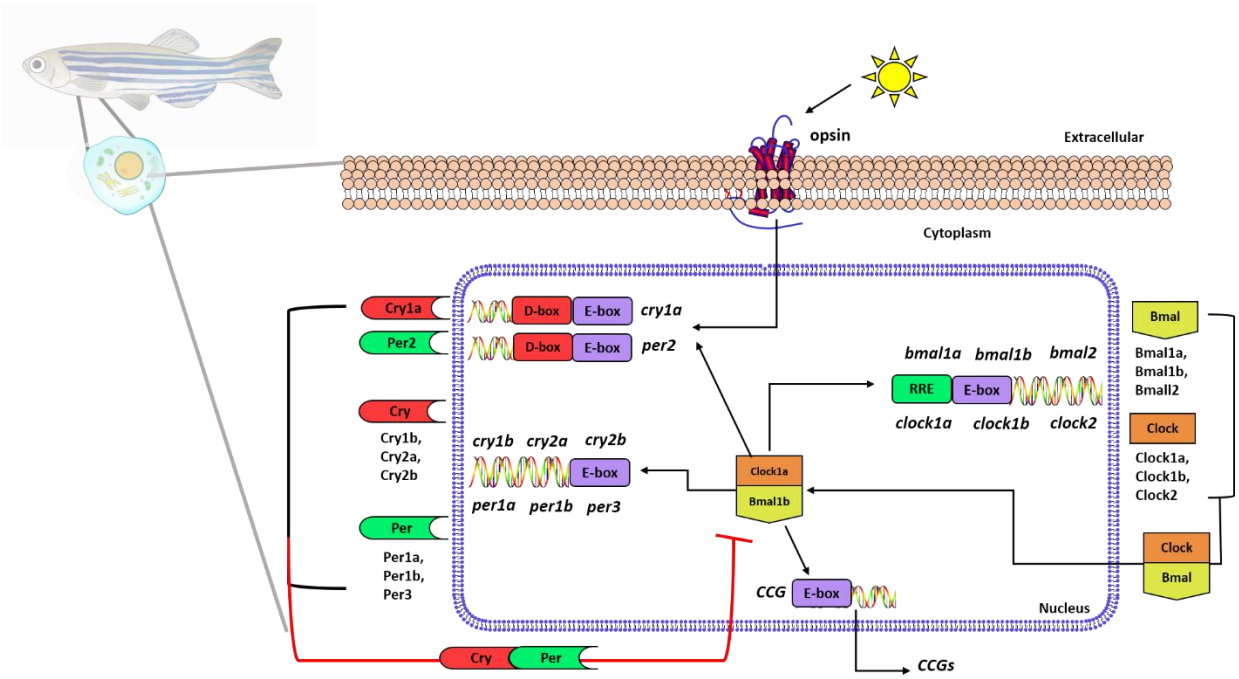

Figure 1. **Molecular clock model in *Danio rerio*.** The signaling pathway takes place upon light stimulus reception by an opsin inserted in the membrane. Per2 and Cry1a proteins start the operation of the central feedback loop, composed of multiple *clock*, *bmal*, *per* and *cry* copies. As a positive loop, *clock* and *bmal* are expressed and a heterodimer formed by their proteins. The Clock: Bmal complex binds to the E-box elements of the genes *per* and *cry*, and controls their rhythmic expression. When there are high concentrations of Per: Cry complex in the cytoplasm, the heterodimer returns to the nucleus and represses their own transcription by inhibiting Clock: Bmal. The cycle of this feedback system is completed within a period close to 24 h, thus generating a circadian pattern.

1.2 Opsins

Vertebrate photoreception is often thought of as the canonical process involving the visual system and image formation, classically defined by light detection by rod and cones. However, non-visual photoreception and the use of non-visual opsins are also important in many critical biologic processes, as circadian entrainment and DNA repair (Tamai et al., 2004; Hirayama et al., 2009; Gavriouchkina et al., 2010).

Animal visual photopigments consist of membrane proteins that belong to the family of G protein coupled receptors (GPCRs), the opsins, bound to 11-cis retinal (specific form of vitamin A), which guarantee their photosensitivity (Terakita, 2005; Hankins et al., 2008). The opsins are seven-transmembrane domain proteins that use similar transduction mechanisms in both ocular and extra ocular photoreceptors (Froland & Whitmore, 2019).

Melanopsin (OPN4) is a photopigment expressed in the photosensitive ganglion cells of the mammalian retina that, together with cones and rods, send the light signal to the SCN, which transmits this information to the pineal gland (McClug, 2011). In *D. rerio,* reports have implicated the MAPK pathway with light dependent, transient induction of phosphorylated ERK and MEK (Hirayama et al., 2009). Ramos and co-workers (2014) pharmacologically demonstrated that blue light (the wavelength of melanopsin maximal absorbance) signals through the phosphoinositide pathway, which interacts with nitric oxide (NO) and the MAPK pathway.

Phylogenetic studies show that teleost genomes encode 20 different classes of opsins, reptiles 19 classes, birds 17 classes and amphibians 18 classes. In non-mammalian vertebrates there are at least two melanopsin genes, *opn4m* and *opn4x,* whose functions and inter-species diversity are largely unknown (Davies et al., 2015; Froland & Whitmore, 2019).

In *D. rerio,* Davies and collaborators (2015) identified five different melanopsin genes among 42 distinct genes coding for 10 canonic visual and 32 non-visual photopigments. According to Davies (2015), these photopigments may be grouped in visual opsins (*rh1, rh2, sws1, sws2, and lws*); non-visual opsins (*va, parapinopsin, parietopsin*); multiple tissue opsins (*tmt*), *opn3, rgr/rrh/opn5, opn6-9,* and melanopsins: *opn4m-1*, *opn4m-2, opn4m-3*, *opn4x-1* and *opn4x-2*. In particular, TMT opsin is expressed in several peripheral tissues and even in fibroblast cell lines of zebrafish (Moutsaki et al., 2003).

The zebrafish melanopsins were originally sequenced from the eye, but studies suggest that they are expressed in extra-retinal organs, such as pineal gland and brain (Bellingham et al., 2002), fin, gills, intestine, heart liver, testicles and muscles (Davies et al., 2015) and cultured embryonic cells (Ramos et al., 2014; Sousa et al., 2017).

In their study, Davies and collaborators (2011) demonstrated that *opn4m2* and *opn4x1* opsins are more similar to the classic photopigments of vertebrates, having as characteristics: mono-stability, absence of endogenous isomerase activity and the possibility to form a stable interaction with cis-like chromophores. Unlike *opn4m1* and *opn4m3* photopigments that are bi-stables and may have interactions with cis- and trans- retinal isomers, besides having higher homology with human and mouse melanopsins. In addition to their photosensitivity, opsins such as rhodopsin (OPN2) and melanopsin exhibit thermo-reception. Shen and collaborators (2011) described the thermal function of rhodopsin, which may be functionally replaced by heterologous mouse melanopsin, in *Drosophila* and Moraes and co-workers (2017) demonstrated the thermal function of native melanopsin in mouse melanocytes.

Other opsins have also gained some recent interest, Buhr and collaborators (2015) showed that neuropsin (OPN5) is critical for photo-entrainment in the mouse retina. Its expression pattern has been examined in all species, and its light sensitivity suggests that it plays an important role in cell biology (Kojima et al., 2011).

In addition to the opsins, different peripheral photoreceptors have been proposed to be involved in the light input pathway in zebrafish peripheral tissues. An example of these are the cryptochromes *cry1-4* and the flavin-containing oxidases which generate reactive oxygen species (ROS) upon exposure to light (Kobayashi, 2000; Hirayama et al., 2007).

1.3 Hormones

1.3.1 Growth hormone (GH)

In vertebrates, the growth hormone pathway begins in the hypothalamus and ends at the target level. The hypothalamus synthesizes the growth hormone releasing hormone (GHRH), which binds to its receptor on pituitary somatotropes to increase GH biosynthesis and secretion (Rosen & Yakar, 2019). The GHRH receptor is a G-protein-coupled receptor with seven

transmembrane domains and its protein has a predicted molecular weight of 47-kDa (Lin-Su & Wajnrajch, 2002).

As part of the negative feedback loop, somatostatin is a small polypeptide (14 amino acid) that inhibits synthesis and release of GH, as well as thyrotropin and pancreatic hormones (insulin and glucagon). Because the localization of its receptor, this hormone acts as an endocrine and a paracrine regulator (Rosen & Yakar, 2019).

The growth hormone, also known as somatotropin (ST), is a 22-kDa single chain polypeptide synthesized and secreted by somatotropes, located in the anterior pituitary gland of vertebrates. This hormone plays a key role in somatic growth stimulation involving regulation of protein, lipid and carbohydrates synthesis, and mineral metabolism. After its secretion, GH binds to its receptors (GHRs) located in organs such as the liver and stimulates signaling pathways that promote cell growth (Cheng et al., 1995; Choi & Geletu, 2018). These receptors are part of the superfamily of cytokine type 1 receptors (Zhu et al., 2001); they are composed of an extracellular binding domain, transmembrane region and an intracellular domain that contains a tyrosine kinase (Pierce et al., 2007). In teleosts, the presence of two types of GHR has been demonstrated, being the liver and the muscle the organs with higher expression. Both receptors differ in the number of extracellular cysteine residues and intracellular tyrosines. Although their mRNAs and proteins have been shown in various extrahepatic tissues, GHR-II is also expressed in the heart and testicles (Calduch et al., 2003; Reindl & Sheridan, 2012).

The mammalian GH signaling pathway begins when the hormone binds to its receptor, a tyrosine kinase, results in the rapid tyrosine phosphorylation of several proteins and the induction of early response genes through the activation of the STAT family of transcription factors (Herrington & Carter-Su, 2001; Di Prinzio et al., 2010). Growth-promoting effects are mostly mediated by IGFs (insulin-like growth factors) mainly IGF-1, which favor proliferation, differentiation, survival and cell migration (Butler & LeRoith, 2001; Reindl et al., 2011). IGFs are related to anabolic processes, such as the incorporation of amino acids, cell proliferation, protein synthesis, glucose transport, and muscle fiber differentiation (Mommsen, 2001). The IGF-1, a single-chain polypeptide, consists of 70 amino-acid residues, whereas IGF-2 has 67 amino acids. Both factors have three domains similar to

proinsulin (Rosen & Yakar, 2019). IGF-1 binds to IGF1R, a receptor that shares amino-acid sequence homology with the insulin receptor (Zeslawski et al., 2001).

The circulating levels of GH in humans exhibit circadian (duration proximally 24 h) and ultradian (period less than 24 h) variations, as well as sexual dimorphism (Gamble et al., 2014). In this case, woman pituitary releases GH at frequent peaks of more uniform amplitude throughout the day, whereas men show fewer pulses during the day. In both genders, GH secretion increases at night (Jaffe et al., 1998; Avram et al., 2005). A recent analysis of human GH gene (*hGH1*) locus in mice revealed that the human promoter possesses the E-box element that binds the circadian transcriptional factors *Bmal1* and *Clock.* The ability of *Bmal1* to bind the E-box was confirmed in the pituitary of these animals, providing evidence for rhythmic *GH1* levels that correlate with the rhythmic GH secretion (Vakili et al., 2016).

The increase in fish size can be induced by the action of temperature on the hormone production. Deane and Woo (2009) reported an increase in growth hormone production in teleost kept at high temperatures. For *D. rerio*, some studies relating temperature and growth were performed in larval stages (Barrionuevo & Burggren, 1999; Denvir et al., 2008), and analyzed muscle composition and development (Johnston et al., 2009), antioxidant mechanisms, immune activity and metabolic functions (Malek et al., 2004). The available information in teleost fish indicates that their growth varies according the variations in photoperiod, melatonin being a hormone that directly (on the pituitary gland) or indirectly (on the hypothalamus) affects the growth hormone release (Boeuf & Falcon, 2001).

1.3.2 Cortisol

The anterior pituitary gland is also responsible for the production of the adrenocorticotrophic hormone (ACTH), which stimulates the adrenal gland to secrete cortisol. Hormones such as glucocorticoids, vasopressin, adrenocorticotropic hormone (ACTH) and thyrotropin exhibit pronounced circadian rhythms in mammals. Moreover, several hormone receptors and the serum glucocorticoid kinase 1 (SGK1) belong to the list of clock-controlled genes (Hastings et al., 2007).

In diurnal animals, including humans, cortisol shows a circadian rhythm with maximal secretion in the morning until it reaches the minimal level during the dark phase with a subsequent increase until the next peak in the morning. In these vertebrates, cortisol is in anti-phase with melatonin release, which justifies the indoleamine inhibitory effect on the adrenal response to ACTH (Campino et al., 2011).

Nocturnal animals, in which the behavioral activity is typical during the night, peak both cortisol and melatonin during the dark phase. Despite activity profiles that are 180° out of phase, the fundamental function of the circadian system and mechanisms of the molecular clock appear conserved between nocturnal and diurnal species (Fonken & Nelson, 2014).

The expression of many hepatic genes including tissue-specific and clock related genes follows a circadian periodicity. In SCN-lesioned mice, the glucocorticoid receptor agonist, dexamethasone, rescued about 60% of the circadian gene expression in the liver (Balsalobre et al., 2000).

In teleosts, cortisol is synthesized in the interrenal cells, located in the cephalic portion of the kidney. The cortisol release is part of the endocrine response evoked by stress, together with catecholamine and pro-opiomelanocortin (POMC) secretion. Cortisol leads to decreased muscle protein and hepatic glycogen levels, hyperglycemia, increased cardiac function and blood flow, as well as ionic and osmotic disruptions in teleost tissues (Auró de Ocampo & Ocampo, 1999).

In teleosts, plasma clearance and catabolism occur in different organs such as head kidney and liver through the 11β-oxidation of cortisol, resulting in cortisone production, the biologically inactive metabolite (Sadoul & Geffroy, 2019). In the liver, cortisol is inactivated by reduction and conjugation to a glucuronide or sulphate (Sadoul & Geffroy, 2019). Cortisol uptake by cells occurs by passive diffusion, due to the lipophilic nature of the molecule. Once in the cells, cortisol binds to both glucocorticoid (GR) and mineralocorticoid (MR) receptors to modulate gene expression (Faught et al., 2016).

The NR3C1 gene that possesses a high similarity between human and *D. rerio* (Schoonheim et al., 2010; Reyer et al., 2013) encodes the glucocorticoid receptor. This receptor has two subtypes GRα and GRβ, but only GRα has transcriptional activity, and GRβ acts as a negative regulator on GRα (Faria & Longui, 2006).

Balsalobre and co-workers (2000) developed one of the first studies describing the relationship between glucocorticoids and circadian expression of the mammalian clock genes. The authors demonstrated that administration of dexamethasone induces the expression of *Per1* in mice. This information was confirmed by the identification of a glucocorticoid response element (GRE) in the promoter region of the *Per1* gene suggesting a direct genomic action of glucocorticoids on the molecular clock components (Yamamoto et al., 2005). Although the detailed molecular mechanisms are not yet recognized, Rubel e collaborators (2012) confirmed that GR is required for the regulation of circadian rhythm in rodents exposed to corticosteroids. Based on the evidence, a model of regulation of teleost molecular clock by glucocorticoids has been proposed (Dickmeis, 2009) (Fig. 2).

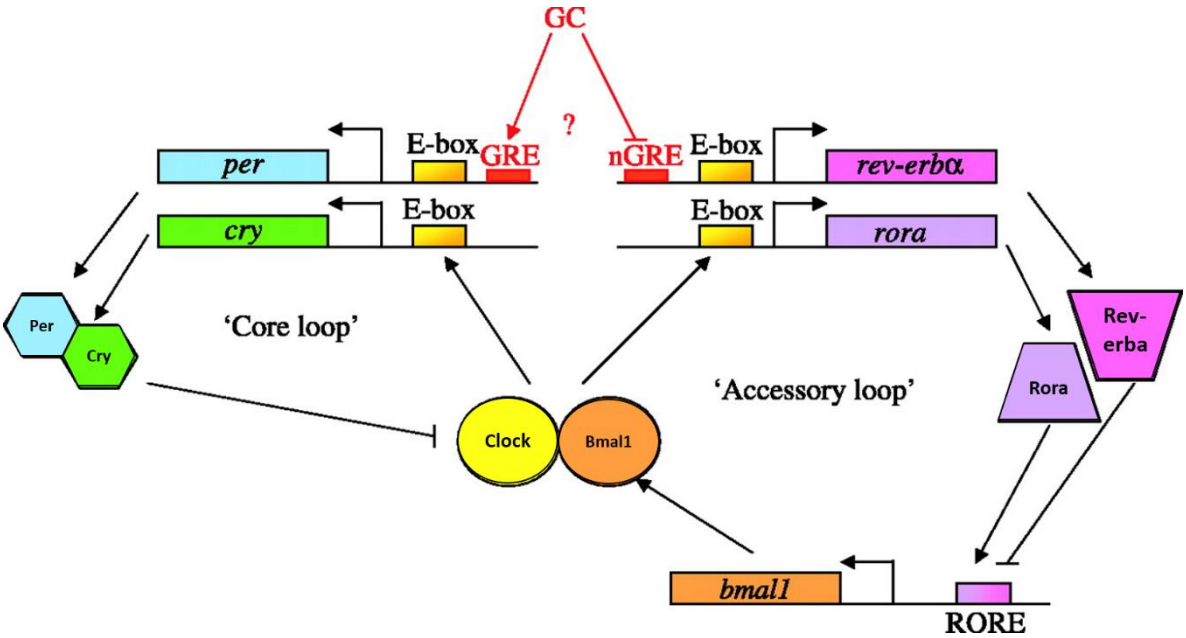

Figure 2. Glucocorticoid regulation of the molecular clock in *D. rerio*. The glucocorticoid (GC)/glucocorticoid receptor complex may modulate transcription of certain clock genes by binding to glucocorticoid response elements (GRE) in their promoters. Thus, a positive GRE element appears to mediate *per1* induction by glucocorticoids, whereas the promoter of *rev-erba* has been proposed to contain a negative GRE (nGRE) that mediates glucocorticoid-induced repression. Modified from Dickmeis, 2009.

Several studies using dexamethasone as a synthetic corticosteroid demonstrated increase in *per1, per2* and *cry1* gene expression in peripheral tissues of teleosts, and mammals as rats and mice (Mogi et al., 2017; Sousa et al., 2017; Zhao et al., 2018). In previous transcriptome studies in *D. rerio*, Zhao and co-workers observed remarkable transcriptional alterations of several clock genes such as *arntl1a (bmal), per1a* and *nr1d1 (rev-erbα)* in response to corticosteroids (Zhao et al., 2016). Dexamethasone also upregulates the transcripts of nine genes: *clock1b, arnt1a, per1a, rora, cry1a, cry2a, cry2b, nr1d1 and nr1d1a* and downregulates other four genes: *per2, rorb, cry1b* and *nr1d1* in *D. rerio* (Zhao et al., 2018). In the same study, *per1a* was significantly upregulated by all six corticosteroids evaluated.

1.3.3 Melatonin

In *D. rerio* and other teleosts, besides the retina and peripheral tissues, the pineal gland cells are also capable of sensing the light and are core components of the circadian system (Doyle & Menaker, 2007; Falcon et al., 2007). The pineal gland contains an intrinsic circadian clock that favors the rhythmic synthesis of melatonin. In teleosts, as well as other vertebrates, melatonin participates in the control of physiological process such as growth, food intake and digestion, reproduction, in addition to antioxidant activity and regulation of the immune system (Ngasainao & Lukram, 2016). Melatonin is also synthesized rhythmically in tissues as retina, gastrointestinal tract, and ovary, among others. In non-mammalian vertebrates such as avian species, ocular melatonin has a key role in circadian function (Weaver & Lockley, 2009).

Among different species of teleost fish, three types of melatonin nocturnal profiles were characterized as type A, B and C. In the type A fish, like *Gadus morhua, Melanogrammus aeglefinus,* melatonin peaks at the end of the dark phase. Type B species as *Oreochromis niloticus* exhibit a peak in the middle of the dark phase and type C such as salmonids show the melatonin peak at the beginning of dark phase (Cowan et al., 2017). Melatonin levels are higher in the dark due to light inhibition of the transcription and the stability of arylkylamine N-acetyltransferase (AANAT), an enzyme required for the hormone synthesis with activity suppressed by the light (Vatine et al., 2011). Like other teleosts, *D. rerio* has two genes,

aanat1 and *aanat2,* the first with higher expression in the retina and the second with higher expression in the pineal gland.

Melatonin is known to affect the rhythm of three clock genes *in vivo,* specifically *per1, per2* and *cry1* in the Japanese flounder, *Paralichthys olivaceus* (Mogi et al., 2017). Changes in temperature significantly affects the melatonin rhythm in ectothermic organisms (López & Sanchez, 2011). Melatonin receptors also display a circadian rhythm (Ouera & Hartlev, 2012), and were identified as MT1, MT2 and MT3 (*mtnr1aa, mtnr1bb, mtnr1ab, mtnr1al, mtnr1c*, in *D. rerio*). They can be found in the retina, adrenal gland, gastrointestinal tract, liver and kidneys, as well as in different cell types such as adipocytes, macrophages and platelets.

In both primates and humans (diurnal behavior), melatonin modulates adrenal glucocorticoid production through MT1 receptor, being able to suppress cortisol production by the reduction on cAMP production (Torres et al., 2003).

1.4. *Danio rerio* (zebrafish) as a model

The zebrafish *Danio rerio* belongs to the big freshwater Cyprinidae family, which includes carps, true minnows, barbs and related genera, it is native to northeast India, Bangladesh and Nepal. It inhabits the Ganges and Brahmaputra river basins, but is often found in shallow waters close to shore (Engeszer et al., 2007; López & Sanchez, 2011). Morphologically, the zebrafish have a laterally compressed fusiform body that reaches an average length of 25 mm; they have centrally located eyes and thin, elongated jaws with mouth upwards. *Danio rerio* has several defining characteristics, including an incomplete lateral line, two pairs of barbels and generally 5 to 7 longitudinal stripes along the body (Alberston & Kocher, 2006; Mayden et al., 2007).

After the period of embryonic growth, the zebrafish spend the next ~4 weeks in the larval stage. Growth during the larval stage varies widely based on temperature, density, and individual differences. Once the individuals reach a size of ~11 mm, they are classified as juveniles, and they will typically become sexually mature 10 to 12 weeks post-fertilization (Parichy et al., 2009; Meyers, 2018).

For years, researchers have been working with *Danio rerio* in biological, behavioral, physiological, pharmacological, toxicological and genetic studies, among others, basically because of its significant homology with other vertebrates, low-cost maintenance, simple reproduction in laboratory, in addition to fast embryonic development and the facility for genetic manipulation (Ingham, 2009; Collin & Martin, 2017). The zebrafish also offer the possibility to generate transgenic lines expressing fluorescent or bioluminescent reporter genes, establishing it as an excellent model for live imaging *in vivo*. Interest in *D. rerio* chronobiology dates back to the last century (Cahill, 1996), especially due to the lack of knowledge about the molecular basis of the non-mammalian circadian clock. The peripheral clocks in zebrafish are directly regulated by the light stimulus, and the species offers a diverse approach since its pineal gland may be part of the "central clock", equivalent to the SCN of mammals (Vatine et al., 2011). Zebrafish is also an attractive model to analyze the clock physiology against temperature conditions, as adults, larvae and embryos remain viable in a wide range of body temperatures that can be controlled with the water temperature. This ability to adapt is demonstrated by the wide temperature range of the water where the fish is collected, being the lowest 16.5°C and the highest 38.6°C (López & Sánchez 2011). Furthermore, this teleost offers a better combination of molecular, genetic and culture cell tools than any other ectothermic vertebrate species (Lahiri et al., 2005).

2. AIM AND HYPOTHESIS

Our study was based on the following hypothesis: Low temperature, probably sensed by melanopsins, modulates the secretion of hormones such as growth hormone, cortisol and melatonin, as well the clock system, leading to a homeostasis of the entire systems.

Our aims:

In *Danio rerio*, submitted to a temperature below (23°C) the control temperature (28°C) and at two temporal points (ZT2; ZT16), we aimed to evaluate the responses of melanopsins, and of the growth hormone, cortisol and melatonin axes.

Specifics to assess:

- the gene expression of melanopsins (*opn4m1, opn4m2*) in the brain and the eye;
- the clock gene expression (*per1, per2, cry1a, cry1b*) in the brain, eye, liver and muscle;
- the expression of the key enzyme for melatonin synthesis (*aanat2, aanat1*) and the melatonin receptors (*mtnr1aa, mtnr1bb*) in the brain and eye;
- the expression of growth hormone (*gh1*), IGF1 (*igf1a/igf1b*) and the GH (*ghra, ghrb*) and IGF1 receptors (*igf1ra, igf1rb*) in the brain, eye, liver and muscle;
- the cortisol levels and the expression of the intracellular glucocorticoid receptor (*gr*) in the liver and muscle;
- the possible interaction between low temperature and the above transcripts in the brain, eye, liver and muscle.

3. MATERIALS AND METHODS

2.1 Biological material, growing conditions and maintenance

The animals were bred and grown in the zebrafish facility of the Department of Genetics and Evolutionary Biology in the Institute of Biosciences, University of São Paulo IB-USP. *D. rerio* AB strain was donated and maintained in the Department of Physiology, IB-USP. The animals were kept in glass tanks at 28°C under photoperiod regimen of 14 hours light and 10 hours dark (14:10 LD, lights on at 8:00 AM, ~1500 lux). The water quality parameters were periodically measured (commercial test Labcon®, Camboriú, SC, Brazil) and kept as: pH 7 (using sodium bicarbonate when necessary), nitrate (<50 mg/L) and nitrite (<0.1 mg/L). Breeding was carried out in special tanks where 5 males and 3 females were placed in the afternoon. The animals were chosen according to their physical characteristics, the best females with bigger abdomen (indicating quantity of oocysts), and the largest males. Once fertilized, the oocysts were transferred to enriched medium EM3 (13.7 mM NaCl, 0.54 mM KCl, 1.0 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.025 mM Na_2HPO_4, 0.044 mM KH_2PO_4, 4.2 mM $NaHCO_3$) in Petri's dishes to be examined under the microscope (the unfertilized eggs were removed with a Pasteur pipette). The fertilized eggs were incubated at 28.5°C (incubator chamber CIT-80, MS Tecnopon Equipamentos Especiais LTDA, Piracicaba, SP, Brazil) for 72 hours and the larvae placed in glass tanks. The fish were fed with *Artemia sp.* (Artemia cyst, INVE, Salt Lake City, UT, USA, raised according to protocol established in the laboratory), during the larval phase from the 5th day post-fertilization (dpf), in addition to commercial feed of ~50 μm. The commercial adult food was from 300 to 400 μm. For larvae, juveniles and adults the food was offered three times a day. Before breeding, adults were kept fasting to avoid eggs contamination with feces. Sanitary control was carried out by the Central Vivarium of Faculty of Medicine FM-USP, ensuring the absence of pathogens according to protocol 4172.

The nomenclature used in this article follows
https://zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions

2.2 Establishment of experimental infrastructure

Two B.O.D incubators were adapted with light and temperature control. Both incubators have luminosity of ~1500 lux per tank in the light phase, and each one had different temperatures (28°C and 23°C). The water recirculating system once completed was tested to ensure its function; there were no major problems during the experiments. Each incubator has a capacity for six 7 L-tanks, and a total water flow of 60 L. Physical (grid) and biological (Bio Filter Quartz Ceramic Ring, China) filters were used. Two thermostats were placed to ensure constant temperature. Each tank had a blue background chosen because it is the species preference (Oliveira et al., 2015). Six animals were kept per tank to prevent overpopulation and to avoid stress. Only two cleanings of the system were done during the experimental period (11 days).

Figure 3. Recirculating system for *Danio rerio* during experimentation.

2.3 Experimental protocol

Adult males (~ 0.150 g and ~90 days old) were kept in 7 L-tanks under the same maintenance conditions (5 days at 28°C), with a light-dark cycle (LD 14:10), after which a group was

submitted to 23°C for 6 days (48 animals), keeping the control group at 28°C (48 animals). The light intensity was ~1500 lux (white light, 420 to 750 nm) measured with the aid of a lux meter (LX-102, Lutron Electronic Enterprise, Taipei, Taiwan). The distribution in the experimental design and the number of animals used are presented in Table 1.

Table 1. Distribution of *Danio rerio* to determine gene expression and to quantify cortisol*

Group/ replicate	Control 28°C ZT2	Control 28°C ZT16	Treated 23°C ZT2	Treated 23°C ZT16
1	8	8	8	8
2	8	8	8	8
3	8	8	8	8

* Each n experimental had a pool of 4 animals. The experimental protocol was performed twice, resulting in a final n= 3 - 6

2.4 Euthanasia

The animals were euthanized by cryo-anesthesia, 2 hours (ZT2) and 16 hours (ZT16) after the lights turned on. Fish were measured with a 6'' digital caliper (ZAAS Precision, Brazil) and weighed on a precision balance (OHAUS®, Parsippany, NJ, USA) at beginning and end of each experiment. The brains, eyes, livers and muscles were sampled and pooled from 4 animals. All procedures were performed according to the Ethics Committee on Use of Animals (CEUA) of the Institute of Biosciences, University of São Paulo, protocol N° 331/2018.

2.5 Total RNA extraction

Brain, eyes, liver and muscle were removed, stored at -80°C until processing and then homogenized with sterile pistils in 300 µL of Trizol (Ambion, Foster City, CA, USA) in 1.5

mL plastic tubes. Once homogenized 700 µL of Trizol were added for a final volume of 1 mL. After centrifugation (10 min, 1,500 x g), the supernatant was collected for further processing. RNA purification was performed by addition of 200 µL of 1-bromo-3-chloropropane-BCP (Sigma-Aldrich, St. Louis, MO, USA), followed by agitation for 15 sec and incubation for 10 min at room temperature. After incubation, samples were centrifuged at 4°C, 12,000 x g for 15 min. Subsequently, 400 µL of aqueous phase were transferred to a new 1.5 mL plastic tube, and then 600 µL of 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA) were added for RNA precipitation. After incubation for 10 min at room temperature, the samples were centrifuged at 4°C, 12,000 x g for 35 min. The supernatant was removed and the pellet washed with 1.3 mL of 75% ethanol. RNA was recovered by centrifugation at 12,000 x g, 4°C for 15 min, followed by incubation at -20°C for at least 1 h. The ethanol washing was repeated, the precipitated RNA evaporated at room temperature, and the RNA was suspended with 20 µL DEPC-water (Ambion, Foster City, CA, USA). To prevent DNA contamination, each sample was treated with DNase I according to the manufacturer's instructions (turbo DNA-free^{TM}, Ambion, Foster City, CA, USA). At the end, the samples were centrifuged at 10,000 x g, at 4°C for 2 min, and the RNA solution was transferred to a new 1.5 mL plastic tube.

2.6 Reverse transcriptase reaction (RT-PCR)

The RNA concentration and quality were determined using a spectrophotometer (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE, USA). The cDNA synthesis was performed with 1 µg of RNA, 1 µL of random hexamer oligonucleotides (100 ηg/µL), 1 µL of dNTPs (10 mM) (ThermoFisher, Waltham, CA, USA), in a reaction with final volume adjusted with DEPC water to 13 µL. The samples were heated for 5 min at 65°C, transferred to ice, and then received 4 µL of PCR buffer (5X), 1 µL of DTT (0.1 M), 1 µL of ribonuclease inhibitor (40 U/µL), 0.25 µL of Superscript III RT enzyme (200 U/µL, ThermoFisher, Waltham, CA, USA) and 0.75 µL of DEPC-water (Ambion, Foster City, CA, USA) for a final volume of 20 µL. Samples were incubated at 25°C for 5 min, 50°C for 50 min and 70°C for 15 min. The samples containing the cDNA were stored at 4°C.

2.7 Quantitative PCR (qPCR)

As a housekeeping gene for all organs 18S ribosomal RNA was chosen, tested and validated. The final concentrations of primers and probes were, respectively, 300 nM and 200 nM for the genes of interest, and 50 nM for primers and probe of 18S rRNA. Depending on the gene of interest, the samples were subject to multiplex reaction, depending on the gene of interest, the samples were subject to multiplex reaction, previously validated (Ramos et al., 2014), in two groups: *per1-cry1b-opn4m2* genes (fluorophores Cy5, HEX and FAM, respectively) and the second: *per2-cry1a-opn4m1* genes (fluorophores FAM, HEX and CY5, respectively). In these reactions, KAPA PROBE FAST 2 mix (Kapa Biosystems, Wilmington, MA, USA) and the respective primers and probes were used (Table 2). For the second group each gene was individually evaluated per well, KAPA SYBR® Fast qPCR Mix for iCycler® 2x (Kapa Biosystems, USA) and the respective pair of primers were used (Table 2). All assays were performed in a thermocycler iQ5 (BioRad Laboratories, Hercules, CA, USA) under the following conditions: SYBR® GreenER™ - 10 min at 95°C, followed by 45 cycles of 15 sec at 95°C, 1 min at 60°C and 80 cycles of 10 sec at 55°C, with gradual increase of 0.5°C; for the multiplex reaction - 3 min at 95°C followed by 45 cycles of 15 sec at 95°C and 60 sec at 60°C. The gene expressions were evaluated in the brain, eye, liver and muscle and were normalized by 18S rRNA and expressed relative to the minimal mean value of the control group (28°C). Results were statistically analyzed by two-way ANOVA, and Bonferroni's post-test.

Table 2. Distribution of plates for qPCR

Organ	Gene	Method
Brain	Clock genes + opsins	TaqMan
	mtnr1aa	SYBR
	mtrn1bb	
	aanat2	

Eye	Clock genes + opsins	TaqMan
	mtnr1aa	SYBR
	mtrn1bb	
	aanat1	
Liver	Clock genes + opsins	TaqMan
	ghra/ghrb	SYBR
	igf1ra/igf1rb	
	Gr	
Muscle	Clock genes + opsins	TaqMan
	ghra/ghrb	SYBR
	igf1ra/igf1rb	
	Gr	

The access numbers and primers' sequences are presented in Table 3. Primers and probes were designed with the PrimerBlast program or Integrated DNA Technologies (IDT, Coralville, IA, USA), according to GenBank (http://www.ncbi.nlm.nih.gov/GenBank) sequences, and synthesized by ThermoFisher (Waltham, CA, USA) (hormone receptor genes) or IDT (clock and opsins genes, and *gh1* gene).

Table 3. Primers, probes and access numbers for the qPCR assays

Template	**Sequence**

18S RNA (Several access numbers; conserved region from yeast to human)	*Forward*: 5’-CGGCTACCACATCCAAGGAA-3’ *Reverse*: 5’-GCTGGAATTACCGCGGCT-3’ Probe: 5’-/5TexRd-TGCTGGCACCAGACTTGCCCTC/BHQ_2/-3’
ghra (NM_001083578.1)	*Forward*: 5’-AGTCGTTCAGGGTTGCACTT-3’ *Reverse*: 5’-ACAGCGAACTCGCACTTCAT-3’
ghrb (NM_001111081.2)	*Forward*: 5’-CGCTTAAGTGTGCCATGCTG-3’ *Reverse*: 5-’GGGCACATTCGAAGAAAGGC-3’
igf1ra (NM_152968)	*Forward*: 5’-ACCTGAGACCAGAGTGGCTA-3’ *Reverse*: 5’-TCTTTGGATCGGAGCGAGC-3’
igf1rb (NM_152969)	*Forward*: 5’-AGGGTGGCCATTAAAACGGT-3’ *Reverse*: 5’-TTAATGGCGGCAAAGGCAAG-3’
mtnr1aa (NM_131393.1)	*Forward*: 5’-TGGGAGTTCTCTGAACAGCTC-3’ *Reverse*: 5’-TTCCAGCCCCGGTGAAATATG-3’
mtnr1bb (NM_131394.1)	*Forward*: 5’-TCCGGGATGCCAGAAAACATC-3’ *Reverse*: 5’-AGCGGGTATGGATAGAAAGCC-3’
per1 *(*NM_212439.2)	*Forward:* 5’-AGCTCAAACTCTCACAGCCCTT-3’ *Reverse:* 5’-TCAGAGCTGGCACTCAACAGA-3’ Probe: 5’-/5Cy5/TCCACCCAGCAGTTCTCTGGCATACA/BHQ_2/-3’
per2 (NM_182857.2)	*Forward:* 5'- GTGGAGAAAGCGGGCAGC-3' *Reverse:* 5'-GCTCTTGTTGCTGCTTTCAGTTCT-3'

	Probe: - 5'- /6FAM/ATGGGTTCAGGATCAAACCGCTGT/BHQ_1/-3'
cry1a (NM_001077297.2)	*Forward:* 5'-CTACAGGAAGGTCAAAAAGAACAGC-3' *Reverse:* 5'-CTCCTCGAACACCTTCATGCC-3' Probe: 5'- /5HEX/AAAGCGTGGGTTGTTTGTAGCAGC/BHQ_1/-3'
cry1b (NM_131790.4)	*Forward*: 5'-CGTCTCTGGAGGAGCTCGG-3' *Reverse*: 5'-TCTCCCCCGGGCCAC-3' Probe: 5' /5HEX/TTTGAAACAGAGGGACTGTCCACTGCTG/BHQ_1/-3'
opn4m1 (NM_001128761.1)	*Forward:* 5' GGGCAACTTCCTGGTCATCTATG 3' *Reverse:* 5'-ATGCTGGTGGTGAAGAAGATGG 3' Probe:5'-/5Cy5/AGGAGCCGGACCCTGAGGACCC/BHQ_2/-3'
opn4m2 (NM_178289.3)	*Forward:* 5'-GCGATTGTCTTCTGCCTCTGA-3' *Reverse:* 5'-AGGGAACTGACACTGGAACCA-3' *Probe*: 5'-/6- FAM/AGTGATTCTTGCTGGACTGAGAGTGAGGCT/BHQ_1/-3'
gh1 (NM_001020492)	*Forward*: 5'-ACAGCCTGACCATCGGAAAC-3' *Reverse*: 5'-AATCCTCAAAAGGCAACGGC- 3'
gr (NM_001020711.3)	*Forward*: 5'-GAGGAGAACTCCAGCCAGAAC- 3' *Reverse:* 5'-TTCACAAAGGTGTAGAAGCAGAAG-3'

	Probe: 5'- /5HEX/AGGAGTCCACCCACCAAGTCGTGC/BHQ_1/-3'
aanat1 (NM_ 200704.1)	*Forward:* 5´- GATCCATCCTCTTGTGGCGT-3' *Reverse:* 5'- CTGCCCTTTGAAGCCAGACT-3'
aanat2 (NM_131411.2)	*Forward:* 5'- TGAAGACACCCATCAGCGTT-3' *Reverse*: 5'-AGGACATTCACCAGACACCG-3'

2.8 Quantitative determination of cortisol (ELISA kit)

The cortisol extraction procedure of *D. rerio* was adapted from Alderman and Bernier (2009) and modified from Egan and coworkers (2009).

To obtain the samples, animals were euthanized, weighed and placed in 2 mL plastic tubes. The samples were homogenized in 500 uL of ice-cold 1X PBS buffer using an X1000 Homogenizer Drive (CAT Scientific, Paso Robles, CA, USA). Samples received an extra 2.5 mL of ice-cold 1X PBS buffer. During this process, all samples were kept on ice. The homogenate was transferred to 9 mL glass tubes and 5 mL of diethyl ether ($C_4H_{10}O$) (Synth, São Paulo, SP, Brazil) were added to each sample. The samples were vortexed for 1 min and centrifuged at 2,000 x g for 5 min. Following centrifugation, the organic layer containing cortisol (usually yellowish) was removed and placed in a separate glass tube; the process was repeated 2 times to ensure the maximal cortisol extraction. Once collected, samples were dried by evaporation with nitrogen and subsequently reconstituted in 1 mL of 1X PBS. The quantification was made with ELISA kit according to the manufacturer's protocol (Salivary Cortisol ELISA kit, Salimetrics, Carlsbad, CA, USA), which has a sensitivity of 0.007 µg/dL. The whole-body cortisol levels were interpolated in the standard curve, normalized with the weight of each sample (each one a pool of 4 animals) and reported as absolute cortisol concentrations (ng/g body weight).

2.9 Statistical analysis

The analysis of gene expression was done by comparing the number of amplification cycles (C_Ts) between control (28ºC) and treated (23ºC) samples. The C_T value was obtained from the geometric portions of the amplification curves, by passing a threshold line that crosses these portions. Knowing the number of cycles where the threshold line (C_T) passes, the ΔC_T values were calculated (difference between the interest and housekeeping gene values). Subsequently, $\Delta\Delta C_T$ was determined subtracting the minimal mean value of the control group from each value of the control and treated groups. The $\Delta\Delta C_T$ was then used as the negative exponential of base 2 ($2^{-\Delta\Delta C_T}$). Results averaged from at least two independent experiments, each with n=3-6, were expressed as mean ± standard error of the mean (SEM). Before any statistical analysis, the normality and homogeneity of the data were evaluated using Kolmogorov-Smirnov and Bartlett tests. The log values of qPCR and hormone level values, in the control and treated groups, in both ZTs, were analyzed by two-way ANOVA, followed by Bonferroni's test, considering significant differences when $p<0.05$. To determine which of the organs had a higher expression of a given gene, we compared the mRNA levels globally (ZT2+ZT16), using the eye (clock, opsin and melatonin pathway genes) or the liver (*ghr, igfr, gr* genes) values to normalize.

For the correlation analysis, the log values of qPCR ($2^{-\Delta\Delta C_T}$). were checked for Gaussian distribution through Shapiro-Wilk test. Samples with Gaussian distribution were analyzed by Pearson correlation coefficients, and significance was set for $p < 0.05$. All analyses were carried out in GraphPad Prism Version 7.0 (La Jolla, CA, USA).

3.1 Weight and length

Total (from the tip of the snout to the tip of the longer lobe of the caudal fin) and standard length (from the tip of the mouth to the end of the last vertebrae) and weight values are presented as the initial and final values averaged of all the animals in each group. There were no statistically significant differences between the lengths and weights of the control and experimental groups (Fig. 4).

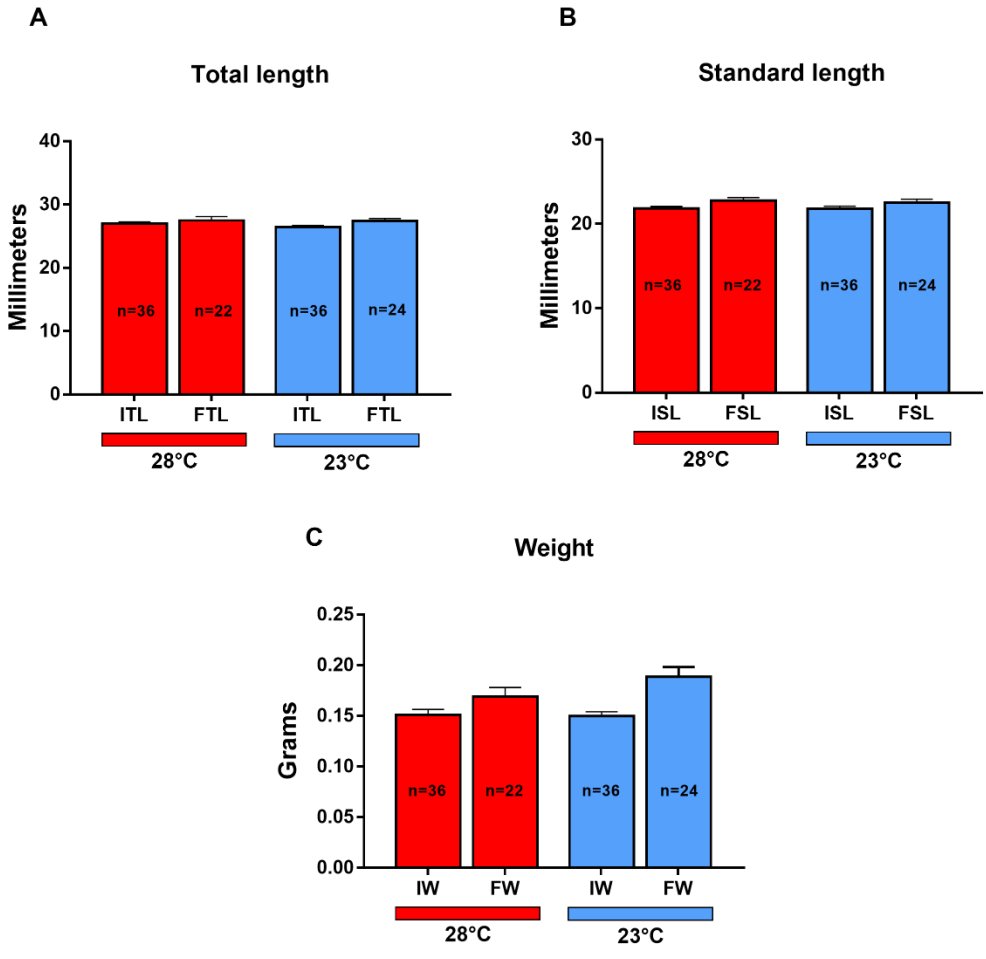

Figure 4. Length and weight of adult *Danio rerio*. (A) Total length; (B) standard length; (C) weight. ITL: Initial total length; FTL: final total length; ISL: initial standard length; FSL:

final standard length; IW: initial weight; FW: final weight. The values are expressed as mean ± SEM.

3.2 Gene expression

In Figures 5 -14, red columns correspond to control group (28°C) and blue columns correspond to experimental groups (23°C).

3.2.1 Melanopsins: *opn4m1* and *opn4m2*

Although there were no differences for *opn4m1* between temperature groups in the brain, there was a tendency to higher expression at the control temperature (28°C) at night (ZT16) with a reduction at 23°C (Fig. 5A). In the eye, no difference was found between cold-exposed and control groups (Fig. 5B), but a higher expression of *opn4m1* in the control group at ZT16 compared to ZT2 was found in both temperatures ($p < 0.05$). The *opn4m1* gene was also evaluated in liver and muscle (data not presented) with late amplifications (>34 cycles), what confirmed the literature data that this opsin has an inexpressive presence in these organs (Davies et al., 2015).

The expression of the *opn4m2* gene in the brain showed no difference between the groups and between the ZTs. (Fig. 5C). In the eye, we found a decrease in the expression at 23°C at ZT2 compared to the control group at the same time point ($p < 0.05$) (Fig. 5D). Similar to *opn4m1* expression in liver and muscle, *opn4m2* displayed late amplification cycles (data not presented), which again agrees with the literature reports of very low transcripts of the gene in these organs (Davies et al., 2015).

To determine which of the organs had a higher expression of *opn4m1* and *opn4m2*, we compared the total daily mRNA levels (ZT2+ZT16), using gene expression of the eye as a normalizer. This analysis demonstrated higher expression of both opsins in the eye, at both temperatures, when compared to the brain ($p < 0.05$) (Table 4).

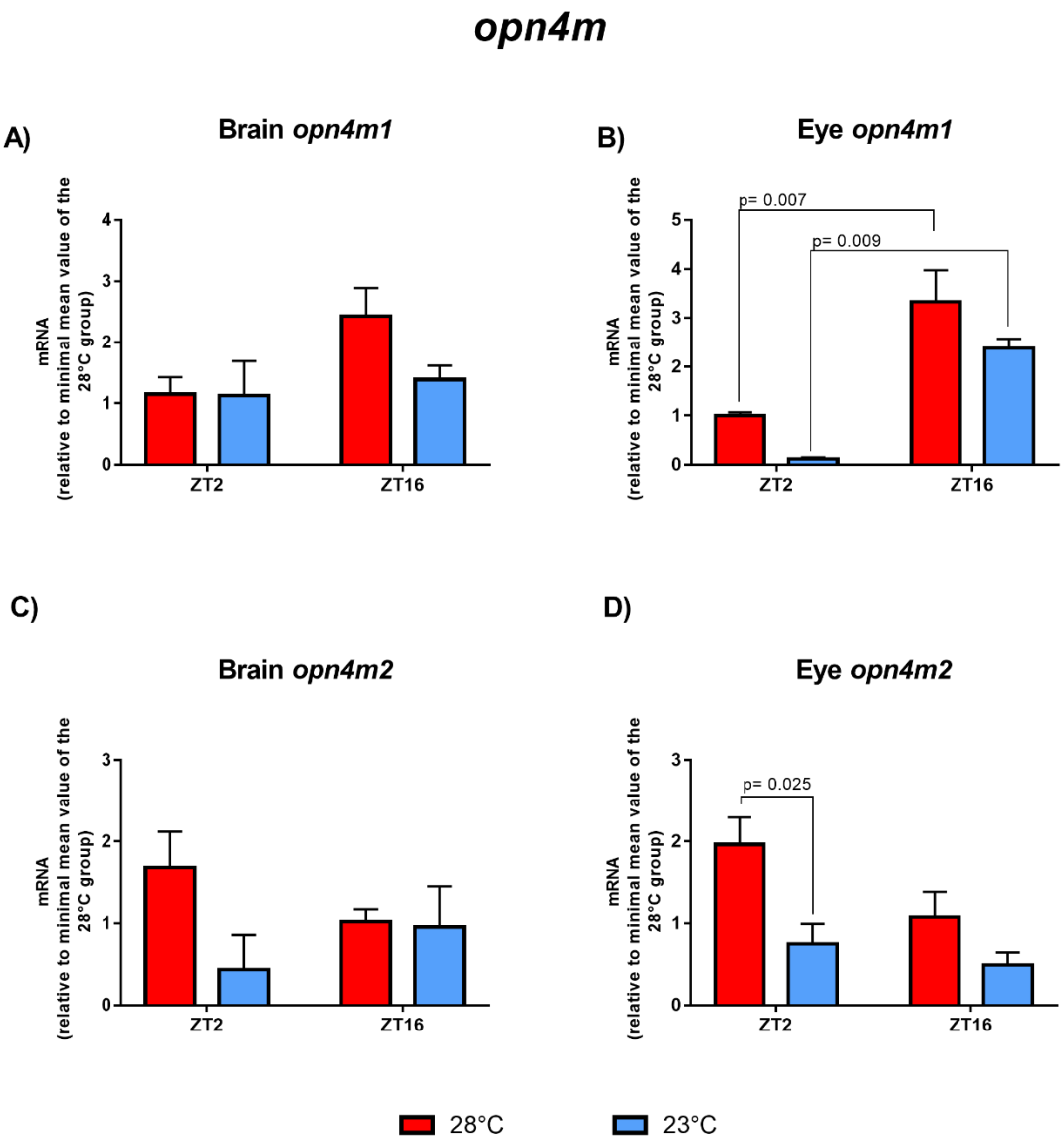

Figure 5. qPCR for the *opn4m* gene in adult *Danio rerio*. (A) Brain *opn4m1*; (B) eye *opn4m1;* (C) brain *opn4m2;* (D) eye *opn4m2*. In this and in Figures 6-12, and 14, total RNA was extracted from pools (3-4 animals), n=3-6, two hours after the lights turned on (ZT2) and two hours after the lights turned off (ZT16). The gene expression was normalized by the 18S rRNA and expressed relative to the lowest mean of the control group. The values are expressed as mean ± SEM.

Table 4. Comparison of total expression of *opn4m* transcripts (ZT2+ZT16) between the organs and the temperatures

Total *opn4m1* mRNA			
	Brain	Eye	p*
28°C	0.28 ± 0.05	2.36 ± 0.69	0.0015
23°C	0.18 ± 0.05	1.35 ± 0.47	0.015
p**	0.21	0.24	
Total *opn4m2* mRNA			
	Brain	Eye	p*
28°C	0.02 ± 0.001	1.58 ± 0.27	< 0.0001
23°C	0.002 ± 0.001	0.60 ± 0.16	< 0.0001
p**	0.28	0.005	

*p values (brain vs eye, normalized by eye value at 28ºC)

**p values (28ºC vs 23ºC in each organ, normalized by eye value at 28ºC)

3.2.2 Clock genes

3.2.2.1 *per1* and *per2*

As part of the clock system, *per* gene was evaluated. At 28°C, *per1* had the highest expression at ZT2 compared to ZT16 in the eye, brain and muscle, evidencing the diurnal rhythmic behavior of the gene peaking in the light phase (Figs. 6A, 6B, and 6D). In the brain and the eye, there was a remarkable decrease in *per1* expression at 23°C at ZT2 as compared to the controls, which demonstrate that chronic low temperatures directly affect *per1* expression (Figs. 6A and 6B). In the liver and the muscle at 23°C, we also

observed a decrease in *per1* expression at ZT16 compared to ZT2 (Figs. 6C and 6D). In summary, regardless the temperature a general pattern of expression was seen in all organs, with higher expression during the day.

Like *per1*, the *per2* gene in the brain (Fig. 6E), eye (Fig. 6F) and liver (Fig. 6G) of animals kept at 28°C exhibited a higher expression at ZT2 relative to ZT16, thus exhibiting a diurnal rhythmicity, peaking in the light phase. In all organs, except the liver, there was a significant decrease in gene expression of *per2* at ZT2 in animals kept at 23°C compared to control group (Fig. 6E, 6F and 6H).

No statistical differences were found when the total expression of *per1* transcripts (ZT2+ZT16) was compared among the organs or between the temperatures (Table 5). For *per2,* the eye was the organ with the highest expression, followed by the brain, liver, and muscle at 28ºC. Interestingly, at 23ºC, the eye presented a remarkable decrease in transcripts when compared with the control group (Table 5).

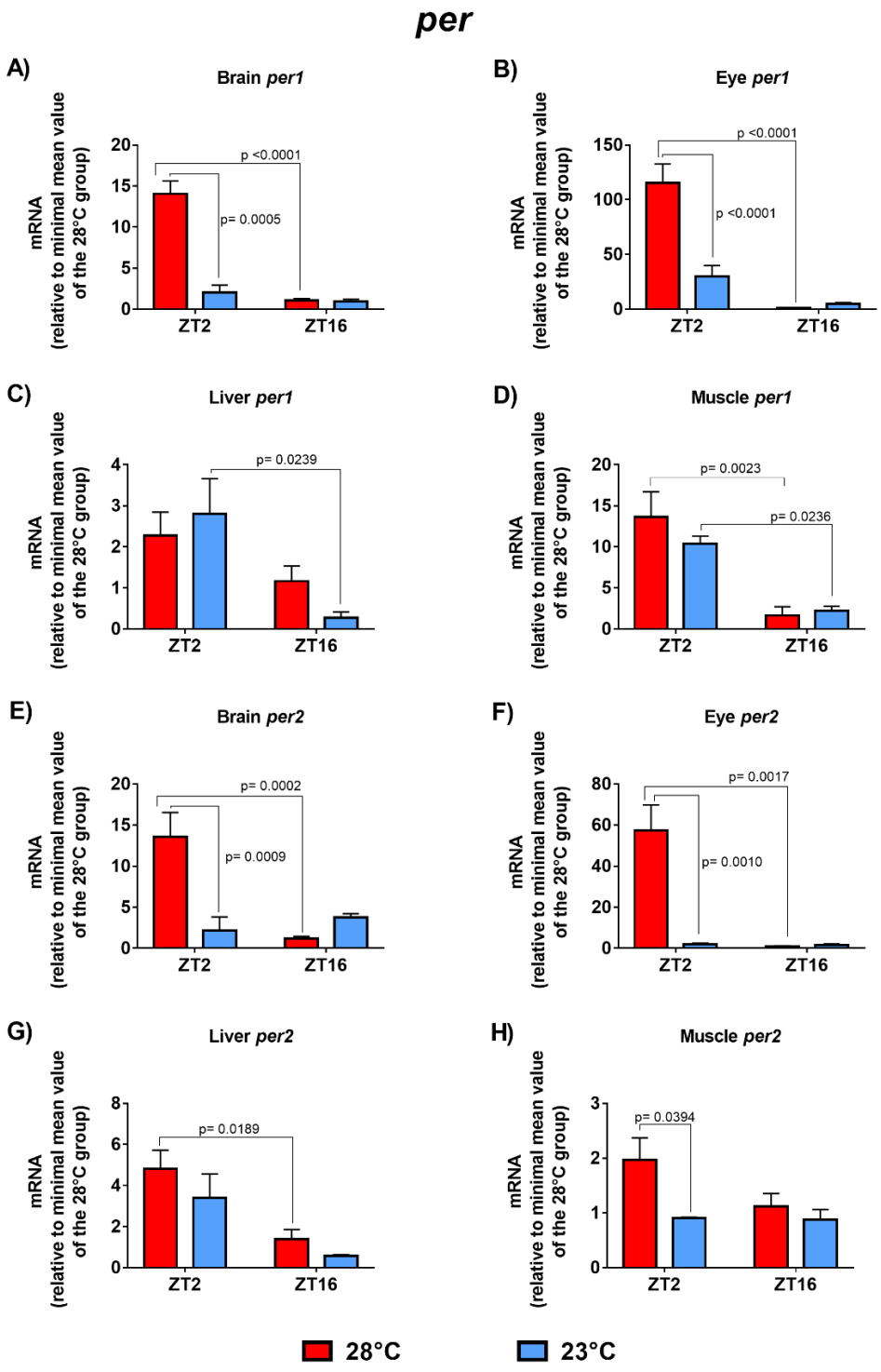

Figure 6. qPCR for the *per1* and *per2* genes in adult *Danio rerio*. (A) Brain *per1*; (B) eye *per1*; (C) liver *per1*; (D) muscle *per1*; **(**E) brain *per2*; (F) eye *per2*; (G) liver *per2*; (H) muscle *per2*.

Table 5. Comparison of total expression of *per1* transcripts (ZT2+ZT16) among the organs and between the temperatures

Total *per1* mRNA							
	Brain	p*	Liver	p*	Muscle	p*	Eye
28°C	41.39 ± 12.5	0.99	9.73 ± 2.2	0.15	19.66 ± 5.9	0.29	51.77 ± 21.3
23°C	8.20 ± 2.7	0.99	7.90 ± 3.2	0.98	13.97 ± 2.9	0.99	19.99 ± 6.97
p**	0.12		0.68		0.37		0.15
Total *per2* mRNA							
	Brain	p*	Liver	p*	Muscle	p*	Eye
28°C	6.09 ± 2.2	0.0003	2.51 ± 0.6	< 0.0001	1.32 ± 0.2	< 0.0001	33.29 ± 13.1
23°C	2.94 ± 0.8	> 0.99	1.58 ± 0.5	> 0.99	0.82 ± 0.1	> 0.99	1.77 ± 0.4
p**	0.25		0.27		0.08		0.034

*p values (normalized by the eye value at 28^{o}C)

**p values (28^{o}C vs 23^{o}C in each organ, normalized by eye value at 28^{o}C)

3.2.2.3 *cry1a* and *cry1b*

When *cry1a* was evaluated in the brain of animals kept at 28°C and 23°C, no statistical differences were evidenced between time points or between groups although at both temperatures there was an apparent oscillation with lower expression at the beginning of the night (ZT16, Fig. 7A). In the eye (Fig. 7B), liver (Fig. 7C) and muscle (Fig. 7D), the low temperature contributed to the decrease in gene expression ($p < 0.05$) at ZT2. In both liver and muscle, the expression at ZT16 at 23°C was even lower than at ZT2 at the same temperature, which suggests that despite a reduction in transcript levels, a gene oscillation still takes place in animals exposed to chronic low temperature. In all the tissues evaluated (except the brain), the expression at ZT2 was significantly higher than at ZT16 at 28°C (Fig. 7B, 7C and 7D), suggesting higher expression in the light phase.

The expression of *cry1b* in the brain and eye of animals kept at 28°C peaked at ZT2 with a considerable reduction at ZT16 (Fig. 7E and 7F). Liver and muscle of control group showed no statistical differences between time points (Fig. 7G and 7H). In general, a rhythmic pattern during the day with a peak in the light phase was observed for this gene, as well as for *cry1a*, *per1* and *per2*, confirming the Per: Cry diurnal feedback loop.

Both the brain and the eye showed decreased *cry1b* expression at ZT2 at lower temperature (Fig. 7E and 7F). In the case of hepatic tissue, *cry1b* exhibited a decrease in animals kept at 23°C at ZT2 (Fig. 7G). In the muscle, there was no significant differences (Fig. 7H).

When compared the total daily mRNA levels (ZT2+ZT16), using gene expression of the eye as a normalizer, at 28°C, the highest expression was found in the eye followed by brain, liver, and muscle. A decrease in *cry1a* transcripts in the eye and the liver and *cry1b*, in the eye was seen at 23°C (Table 6); in the liver, *cry1b* tends to decrease at 23°C ($p=0.058$).

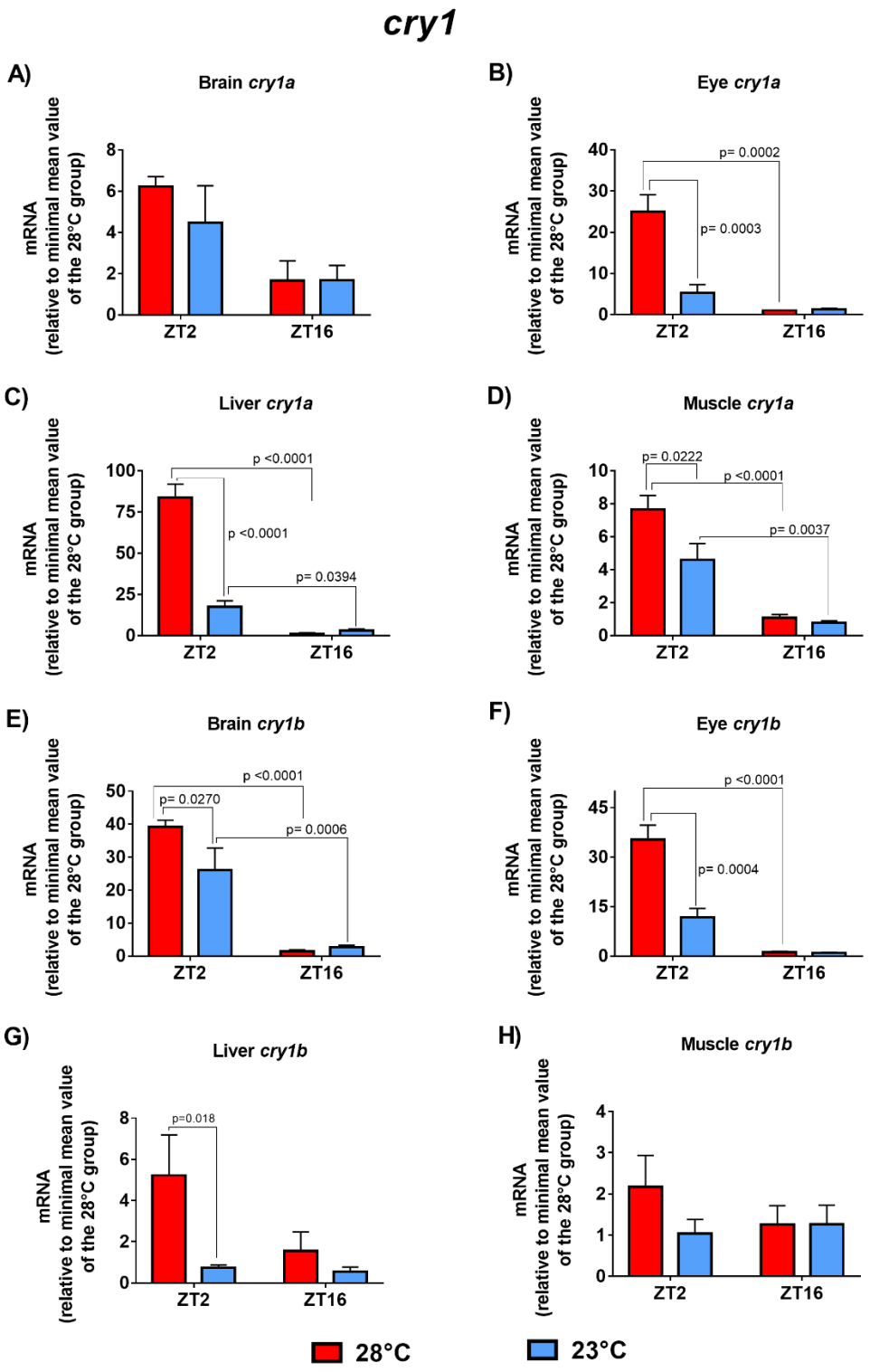

Figure 7. qPCR for the *cry1a* and *cry1b* genes in adult *Danio rerio*. (A) Brain *cry1a*; (B) eye *cry1a*; (C) liver *cry1a*; (D) muscle *cry1a*; (E) brain *cry1b*; (F) eye *cry1b*; (G) liver *cry1b*; (H) muscle *cry1b*.

Table 5. Comparison of total expression of *cry1a* and *cry1b* transcripts (ZT2+ZT16) among the organs and between the temperatures

Total cry1a mRNA							
	Brain	p*	Liver	p*	Muscle	p*	Eye
28°C	3.32 ± 0.7	0.001	1.73 ± 0.7	0.0003	0.72 ± 0.1	< 0.0001	14.72 ± 5.2
23°C	2.39 ± 0.8	0.99	0.39 ± 0.1	0.83	0.48 ± 0.1	0.86	3.52 ± 1.2
p**	0.43		0.033		0.32		0.037
Total cry1b mRNA							
	Brain	p*	Liver	p*	Muscle	p*	Eye
28°C	15.61 ± 5.6	0.99	5.69 ± 1.9	0.28	15.61 ± 3.8	0.99	20.1 ± 6.4
23°C	11.22 ± 4.1	0.99	1.21 ± 0.2	0.99	11.09 ± 2.5	0.99	6.33 ± 2.4
p**	0.54		0.058		0.36		0.07

*p values (normalized by the eye value at 28^{o}C)

**p values (28^{o}C vs 23^{o}C in each organ, normalized by eye value at 28^{o}C)

3.2.3 Pineal axis

To determine the possible effects of temperature on the pineal function, the gene expression of the enzyme arylkylamine N-acetyltransferase and melatonin receptors was evaluated.

3.2.3.1 Aanat: *aanat1* and *aanat2*

In the brain and in the eye, *aanat2* and *aanat1* expressions were evaluated, respectively, (Fig. 8A and 8B). Either gene showed no oscillation between ZTs at 28°C.There was an increase of *aanat2* mRNA in the brain and of *aanat1* in the eye of animals at 23°C at ZT16 when compared with the same ZT at 28°C (Fig. 8A). Both genes oscillated, peaking in the dark; in addition, there was an increase of the enzyme transcripts in both organs at 23ºC which may suggest an increase of melatonin levels in cold-exposed animals.

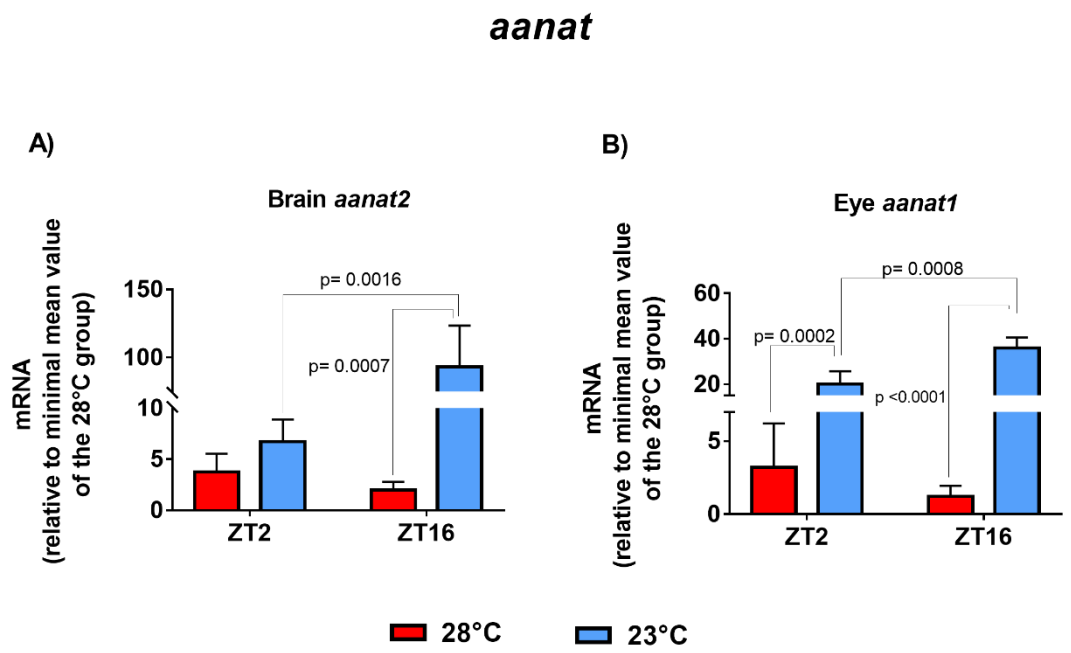

Figure 8. qPCR for the *aanat* genes in adult *Danio rerio*. A) Brain *aanat2*; (B) eye *aanat1*.

3.2.3.2 Melatonin receptors: *mtnr1aa* and *mtnr1bb*

In the brain and the eye, the melatonin receptors *mtnr1aa* and *mtnr1bb* were evaluated (Fig. 9). The only temporal variation was seen for *mtnr1aa* in the eye, with a peak in the dark phase (Fig. 9A-D). At 23ºC, this peak was abolished, and in the eye the expression of both melatonin receptors was depressed (Fig. 9A-D)

The *mtnr1aa* was more expressed in the eye than in the brain; no differences were found for *mtnr1bb* between the brain and eye. Interestingly, the eye showed a reduction of *mtnr1aa* and *mtnr1bb* total transcripts at 23°C (Table 7).

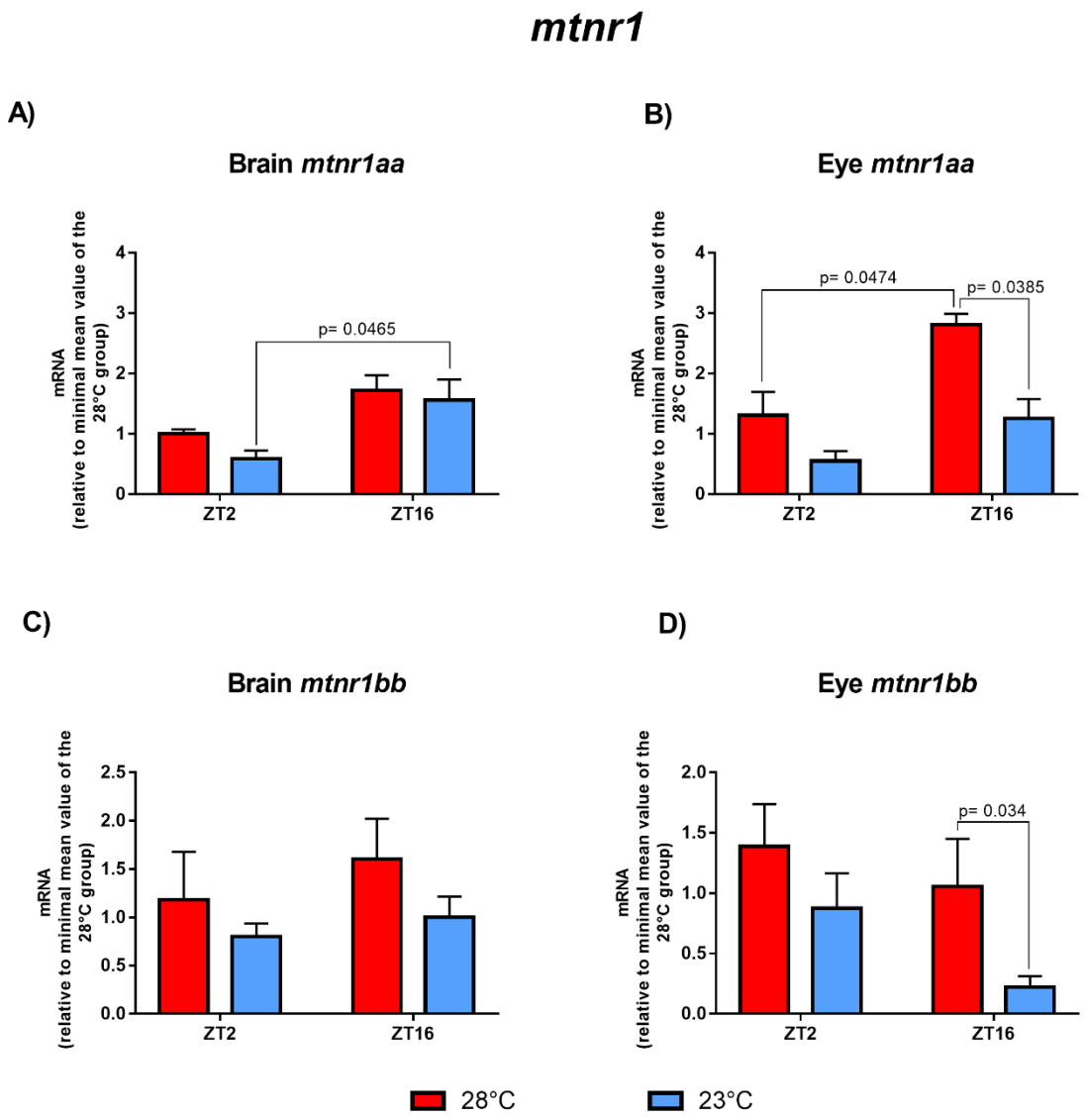

Figure 9. qPCR for the *mtnr1aa* and *mtnr1bb* gene in adult *Danio rerio*. (A) Brain *mtnr1aa*; (B) eye *mtnr1aa*; (C) brain *mtnr1bb;* (D) eye *mtnr1bb*.

Table 7. Comparison of total expression of *mtnr1aa* and *mtnr1bb* transcripts (ZT2+ZT16) between the organs and temperatures

Total *mtnr1aa* mRNA			
	Brain	**Eye**	**p***
28°C	0.62 ± 0.08	1.87 ± 0.4	0.008
23°C	0.42 ± 0.1	0.94 ± 0.2	0.06
p**	0.15	0.04	
Total *mtnr1bb* mRNA			
	Brain	**Eye**	**p***
28°C	1.13 ± 0.2	1.40 ± 0.2	0.45
23°C	0.72 ± 0.1	0.54 ± 0.1	0.34
p**	0.08	0.019	

*****p values (brain vs eye, normalized by eye value at 28ºC)

**p values (28ºC vs 23ºC in each organ, normalized by eye value at 28ºC)

3.2.4 Growth hormone axis

To determine the effects of temperature on the growth hormone axis, the expression of *gh1* gene, growth hormone receptors A and B, and the insulin-like growth factor 1 receptors was evaluated.

The levels of cerebral *gh1* are presented in Fig. 10. It was possible to note the nocturnal pattern of this hormone (ZT16 higher than ZT2), and a marked reduction in the animals of the treated group at ZT16, abolishing the oscillation at the analyzed ZTs.

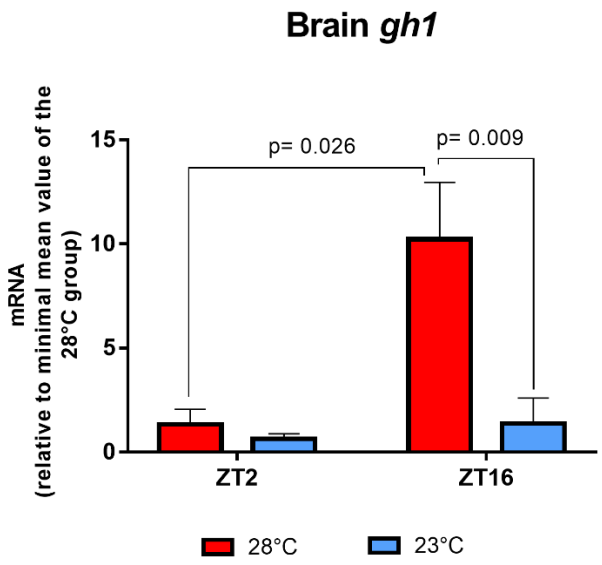

Figure 10. qPCR for the *gh1* gene in adult *Danio rerio*.

3.2.4.1 Growth hormone receptors: *ghra* and *ghrb*

In metabolic tissues (liver and muscle), genes related to growth hormone were evaluated. The growth hormone receptor *ghra* oscillated between ZTs at 28ºC only in the liver, and this temporal variation was abolished by cold temperature, mainly because the expression at ZT16 was markedly decreased compared to control group (Fig. 11A). In the muscle, *ghra* did not oscillate between ZTs at 28ºC, and exhibited a marked reduction in cold-exposed animals at ZT16 compared to the control group (Fig. 11B).

The *ghrb* in the liver and the muscle showed no temporal variation (Fig. 11C, D); its expression was not affected by low temperature in the liver, but displayed a cold temperature-induced remarkable reduction in the muscle (Fig. 11C, D).

No differences were found in the total transcripts of *ghra* and *ghrb* between the muscle and the liver at 28°C. In the cold exposed animals, the *ghrb* mRNA was higher in the liver than in the muscle (Table 8). Both *ghra* and *ghrb* transcripts in the muscle exhibited a significant decrease at 23ºC when compared with control group (Table 8).

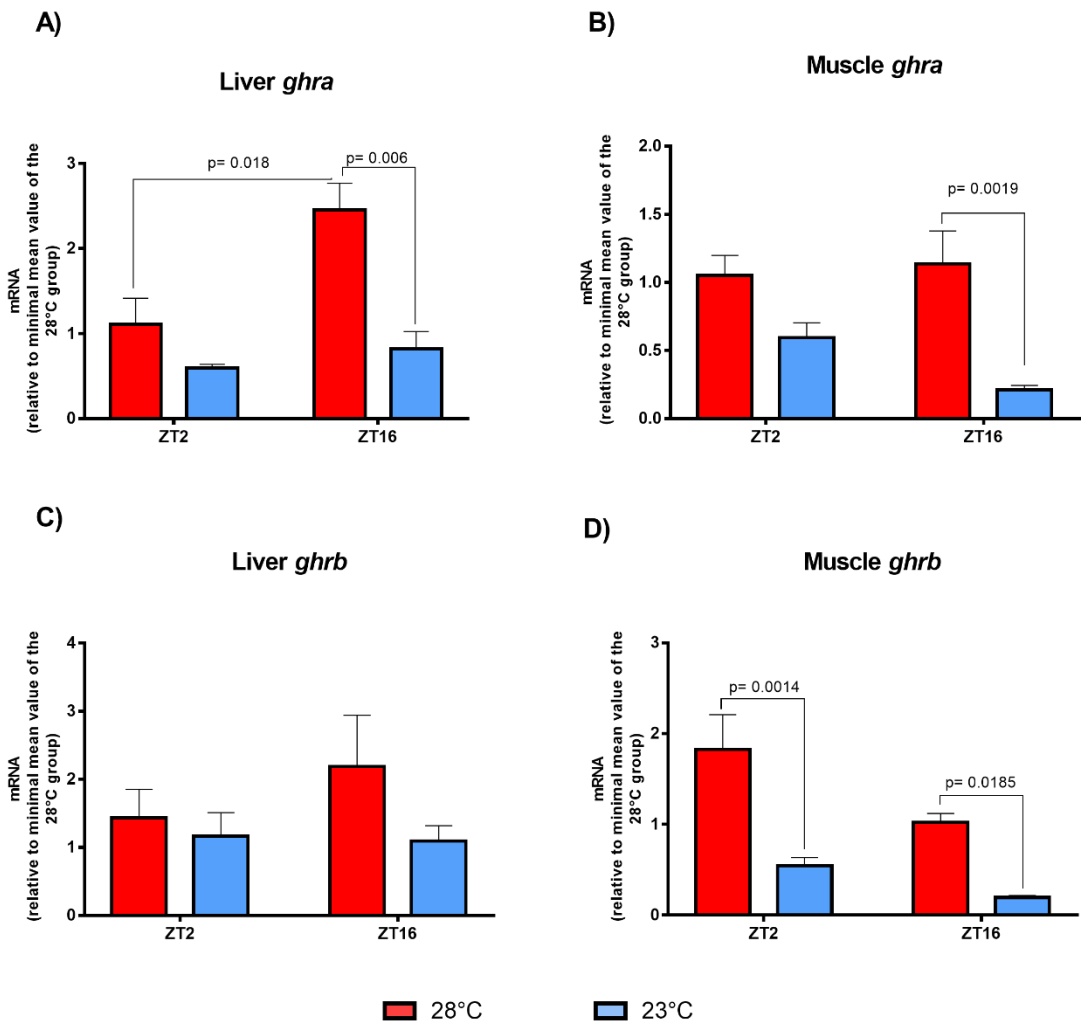

Figure 11. qPCR for the *ghra* and *ghrb* genes in adult *Danio rerio*. (A) Liver *ghra*; (B) muscle *ghra*; (C) liver *ghrb*; (D) muscle *ghrb*.

Table 8. Comparison of total expression of *ghra* and *ghrb* transcripts (ZT2+ZT16) between the organs and temperatures

Total *ghra* mRNA			
	Liver	**Muscle**	**p***
28°C	1.99 ± 0.9	2.13 ± 0.3	0.849
23°C	1.26 ± 0.4	0.78 ± 0.1	0.194
p**	0.42	0.0002	
Total *ghrb* mRNA			
28°C	1.78 ± 0.4	1.15 ± 0.2	0.159
23°C	1.12 ± 0.2	0.29 ± 0.05	0.0005
p**	0.15	0.0002	

*****p values (liver vs muscle, normalized by the liver value at 28^{o}C)

**p values (28^{o}C vs 23^{o}C in each organ, normalized by the liver value at 28^{o}C)

3.2.4.2 IGF1a – IGF1b

We evaluated other components of the growth hormone pathway such as the insulin-like growth factors (IGFs) and its receptors, specifically *igf1ra* and *igf1rb*. The expression of

igf1a in the liver had no statistical differences in both groups. In the muscle was observed a decrease in the mRNA for this gene associated to low temperature at ZT16 (Fig. 12B).

In the liver, the *igf1b* had the highest expression at ZT16 compared to ZT2 at 28°C evidenced a possible nocturnal peak subsequent at GH secretion (Fig. 12C). No statistical differences were found in the muscle

No statistical differences were found when the total expression of *igf1a/b* transcripts (ZT2+ZT16) was compared among the organs or between the temperatures (Table 9).

igf1

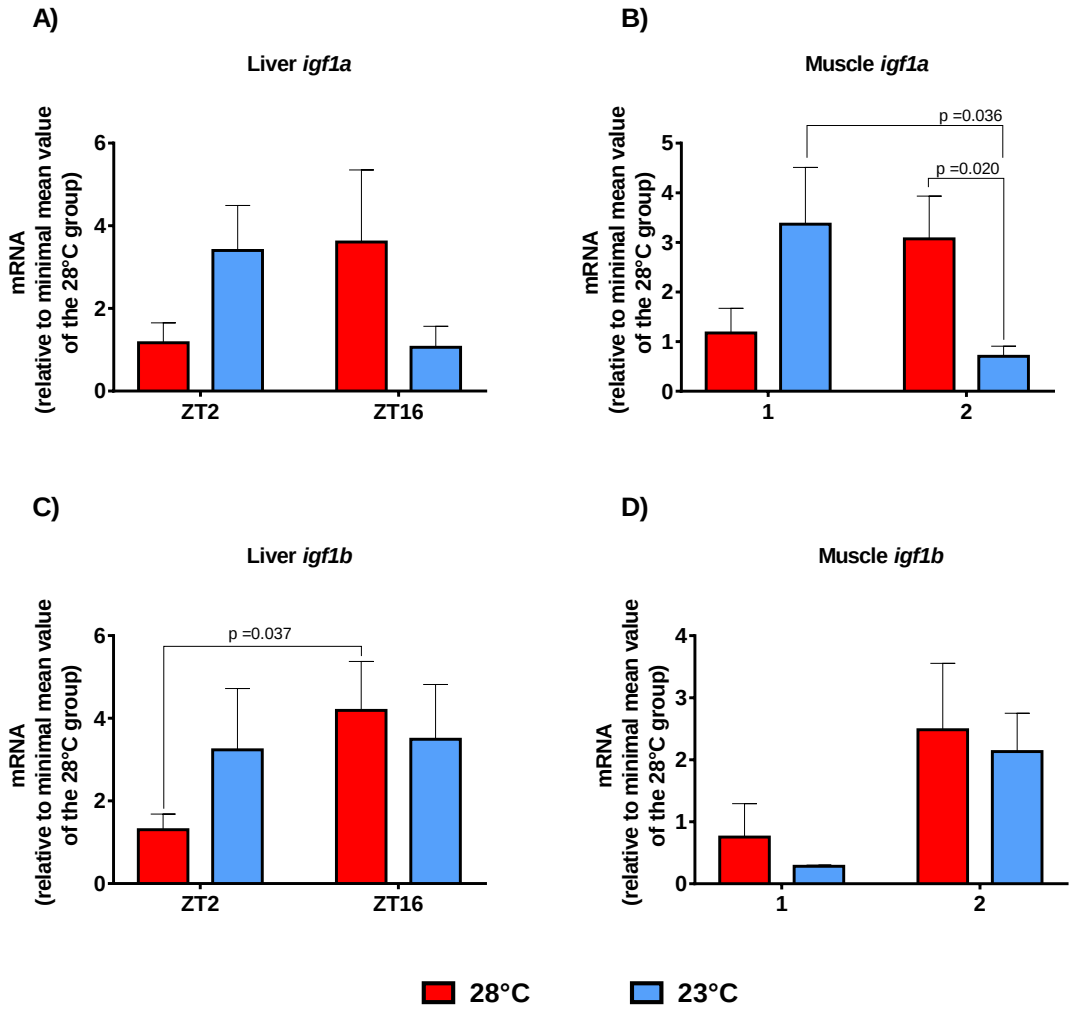

Figure 12. qPCR for the *igf1a* and *igf1b* genes in adult *Danio rerio*. (A) Liver *igf1a*; (B) muscle *igf1a*; (C) liver *igf1b*; (D) muscle *igf1b*.

Table 9. Comparison of total expression of *igf1ra* and *igf1rb* transcripts (ZT2+ZT16) between the organs and temperatures

	Total *igf1a* mRNA		
	Liver	**Muscle**	**p***
28°C	2.69 ± 1.1	2.26 ± 0.6	0.999
23°C	2.06 ± 0.6	1.88 ± 0.7	0.999
p**	0.999	0.999	
	Total *igf1b* mRNA		
28°C	2.58 ± 0.72	1.83 ± 0.7	0.999
23°C	3.35 ± 0.95	1.60 ± 0.5	0.87
p**	0.999	0.999	

*****p values (liver vs muscle, normalized by the liver value at 28^{o}C)

**p values (28^{o}C vs 23^{o}C in each organ, normalized by the liver value at 28^{o}C)

3.2.4.3 IGF1 receptors: *igf1ra* and *ifg1rb*

The *igf1ra* and *igf1rb* presented the highest hepatic expression at ZT16 at 28°C compared to ZT2, with a remarkable reduction in the cold-exposed group at ZT16, what abolished the variation between ZTs (Fig. 13A and 13C). In the muscle, there was no temporal or temperature differences of *igf1ra* and *igf1rb* expression in both groups (Fig. 13B and 13D).

There were no differences of global mRNA levels for *igf1ra* between liver and muscle or between temperatures in each organ; but *igf1rb* mRNA levels were higher in the muscle than in the liver at 23°C ($p < 0.05$). In addition, the liver presented a significant reduction of total *igf1rb* transcripts at 23°C as compared to the control group (Table 10).

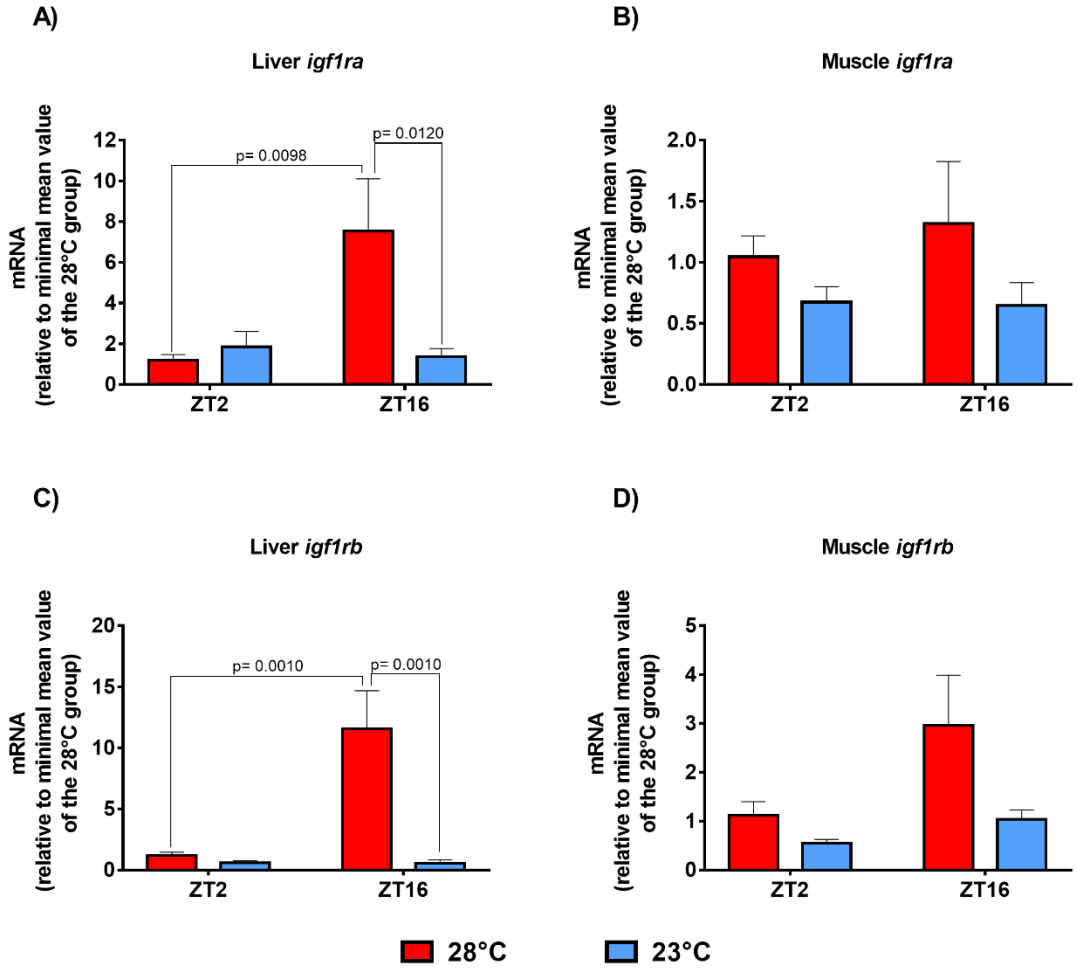

Figure 13. qPCR for the *igf1ra* and *igf1rb* genes in adult *Danio rerio*. (A) Liver *igf1ra*; (B) muscle *igf1ra*; (C) liver *igf1rb*; (D) muscle *igf1rb*.

Table 10. Comparison of total expression of *igf1ra* and *igf1rb* transcripts (ZT2+ZT16) between the organs and temperatures

	Total *igf1ra* mRNA		
	Liver	**Muscle**	**p***
28°C	4.01 ± 1.5	3.60 ± 0.7	0.813
23°C	1.60 ± 0.4	2.04 ± 0.3	0.436
p**	0.13	0.07	
	Total *igf1rb* mRNA		
28°C	5.8 ± 2.2	5.74 ± 1.6	0.984
23°C	0.57 ± 0.1	2.23 ± 0.4	0.001
p**	0.04	0.08	

*****p values (liver vs muscle, normalized by the liver value at 28^{o}C)

**p values (28^{o}C vs 23^{o}C in each organ, normalized by the liver value at 28^{o}C)

3.2.5 Cortisol axis

3.2.5.1 Cortisol determination

Since temperature changes are stressful factors, we analyzed cortisol concentration in the whole-body extract and the glucocorticoid receptor (*gr*) expression in liver and muscle. There were no statistical differences between ZT2 and ZT16 of the control (Fig. 14). Unexpectedly, the control group did not show higher levels at ZT2 possibly because its peak preceded the time of sample collection. However, we observed an increase in cortisol (µg/g) in animals at 23°C at ZT2 as compared to the control group at the same ZT, and a reduction at night (ZT16) compared to ZT 2 of cold-exposed animals, exhibiting in this condition an oscillatory profile (Fig. 14).

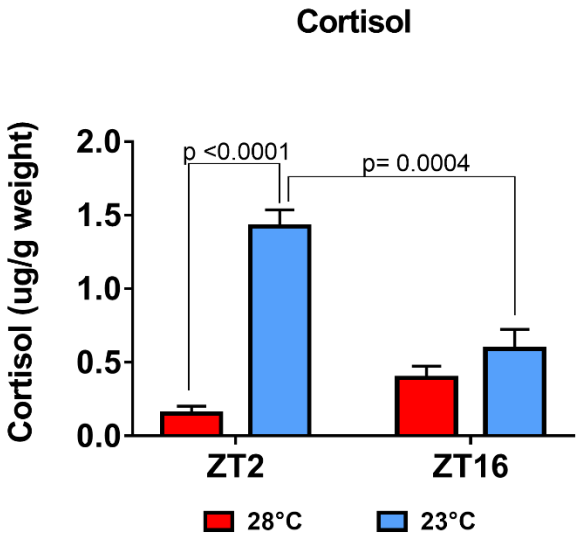

Figure 14. Whole body cortisol in adult *Danio rerio*. The animals were kept for 5 days at 28°C, followed by 6 days 23°C (treated). Cortisol extraction was performed two hours after lights were turned on (ZT2) and two hours after the lights were turned off (ZT16). The hormone concentration (µg) was normalized by sample weight (g). The values are expressed as mean ± SEM. Pool (3-4 animals); n=6.

3.2.5.2 Glucocorticoid receptor: *gr*

We analyzed the glucocorticoid receptor (*gr*) expression in liver and muscle. In the liver. we found a significant peak in *gr* expression at night (ZT16) in animals at 28ºC (Fig. 15A), whereas on the muscle there was no difference between ZTs (Fig. 15B). At 23ºC, there was a decrease of liver *gr* at ZT16 when compared with the control group, what abolished the oscillatory pattern at low temperature (Fig. 15A). There were no significant differences between time points in either temperature or between temperatures at the same time point in the muscle *gr* expression (Fig. 15B). There was no statistical difference of the global mRNA levels of *gr* between the liver and the muscle or in each organ between temperatures (Table 11).

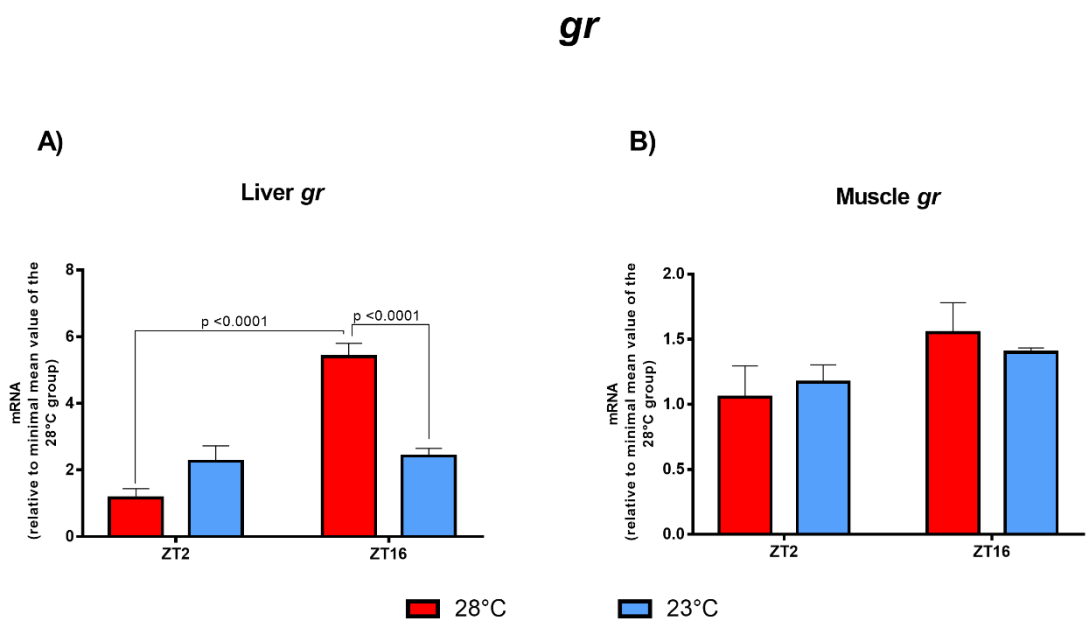

Figure 15. qPCR of the *gr* gene in *Danio rerio* adults. (A) Liver *gr*; (B) muscle *gr*.

Table 11. Comparison of total expression of *gr* transcripts (ZT2+ZT16) between the organs and temperatures

	Total *gr* mRNA		
	Liver	**Muscle**	**p***
28°C	3.28 ± 0.7	1.54 ± 0.3	0.065
23°C	2.34 ± 0.2	1.53 ± 0.3	0.058
p**	0.21	0.99	

*p values (liver vs muscle, normalized by the liver value at 28^{o}C)

**p values (28^{o}C vs 23^{o}C in each organ, normalized by the liver value at 28^{o}C)

3.3 Correlation analysis

Once the gene expression values were obtained, we performed a Pearson correlation analysis to determine the level of association between the expression of clock genes and all others in the different tissues. A heat map was performed to present the results, samples correspond to the same time points (ZT2 or ZT16) and its conditions (28°C or 23°C) ($p < 0.05$) (Figs. 16, 17, 18 and 19).

In the brain, at 28ºC, a positive correlation was observed between *cry1b:gh1* (at ZT2, 96%) ($p < 0.05$). At 23ºC positive correlations were found between *per2: opn4m1* (at ZT2, 95.5%); between *cry1b: mtnr1aa* (at ZT2, 95%) ($p < 0.05$) (Fig. 16). A negative correlation was observed between *per1: mtnr1aa* (at ZT16, -96.6%) at 28° group ($p < 0.05$); at 23°C a negative correlation was observed between *cry1a:gh1* (at ZT16, -100%) ($p < 0.05$) (Fig.16).

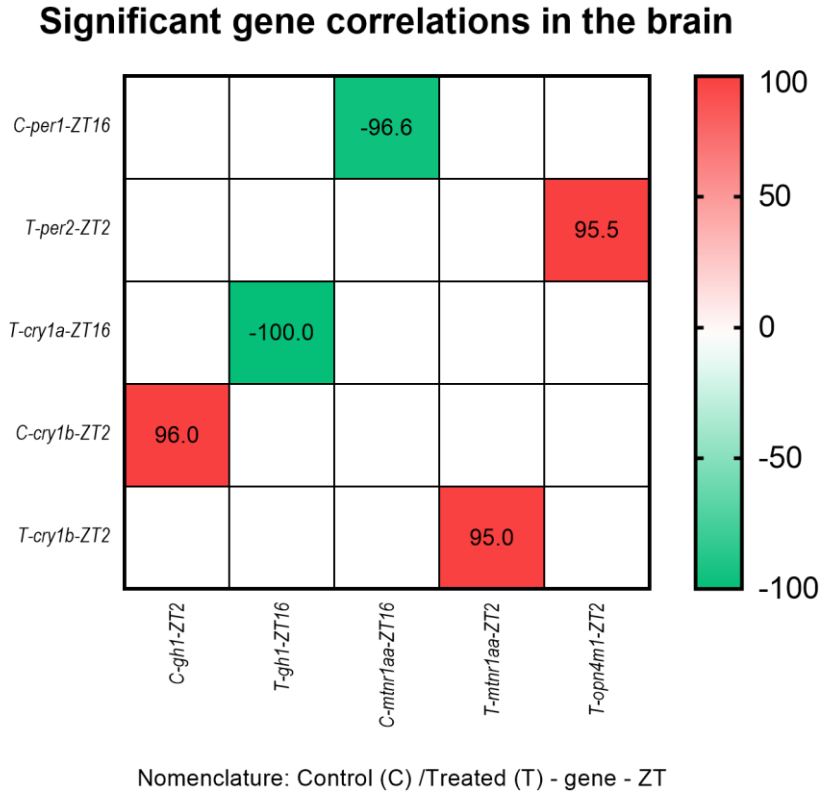

Figure 16. Gene correlation in the brain of adult *Danio rerio*. Using the data from the same sample and *zeitgeber* time, this analysis shows 3 positive and 2 negative correlations. In this and in the following figures, samples with Gaussian distribution were analyzed by Pearson correlation coefficients. The values are expressed as percentage. Significance was set for $p < 0.05$.

In the eye, only positive correlations were observed: between *per2: opn4m1* (at ZT2, 98.9%) and *cry1a: opn4m1* (at ZT2, 99.8%) at 28°C group ($p < 0.05$). Another positive correlation was observed at 23°C between *per1: opn4m2* (at ZT2, 81.7%); *per2: opn4m1* (at ZT2, 97.8%) and *cry1a: mtnr1aa* (at ZT2, 69.9%) ($p < 0.05$) (Fig.17).

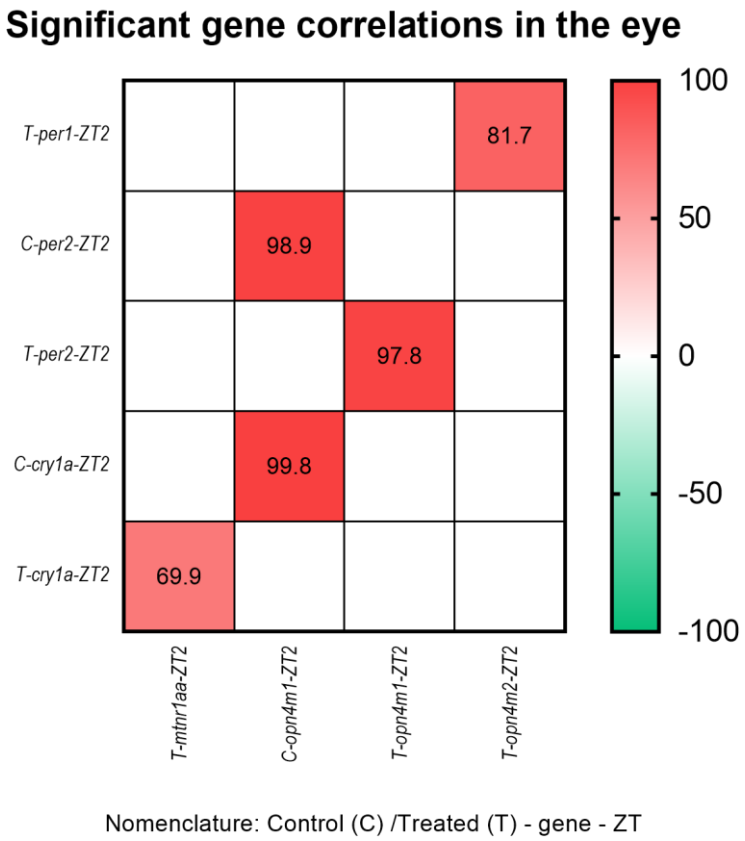

Figure 17. Gene correlation in the eye of adult *Danio rerio*. Using the data from the same sample and *zeitgeber* time, this analysis shows 5 positive correlations.

In the liver, positive correlations were observed at 28°C between *per1: igf1a* (at ZT16, 98.8%), *cry1a: ghra* (at ZT2, 99.8%) and *cry1a: ghra* (at ZT16, 100%) and *per1: igf1b* (at ZT16, 96%) in the treated group ($p < 0.05$) (Fig. 18). No negative correlation was found between the clock genes and the other genes evaluated in the liver.

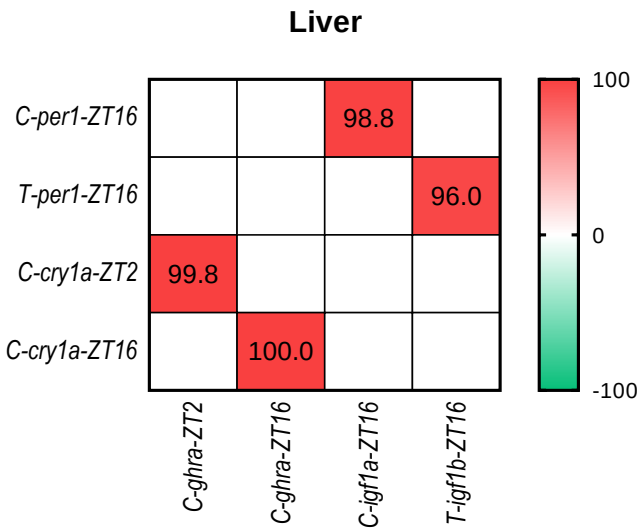

Figure 18. Gene correlation in the liver of adult *Danio rerio*. Using the data from the same sample and *zeitgeber* time, this analysis shows 2 positive correlations.

In the muscle, positive correlations were observed at 28°C between *per1: igf1ra* (at ZT16, 95.1%); *cry1a: igf1ra* (at ZT2, 88.4%); *cry1a: ghra* (at ZT16, 87.4%); cry*1b: ghrb* (at ZT2, 99.8%); and *cry1b: ghra* (at ZT16, 95.7%) ($p < 0.05$) (Fig. 19). At 23°C was observed a correlation between *per2: igf1a* (at ZT2) (Fig. 19)

At 23°C, a negative correlation was observed between *cry1a: ghrb* (at ZT16, -96.6%) ($p < 0.05$) (Fig. 19).

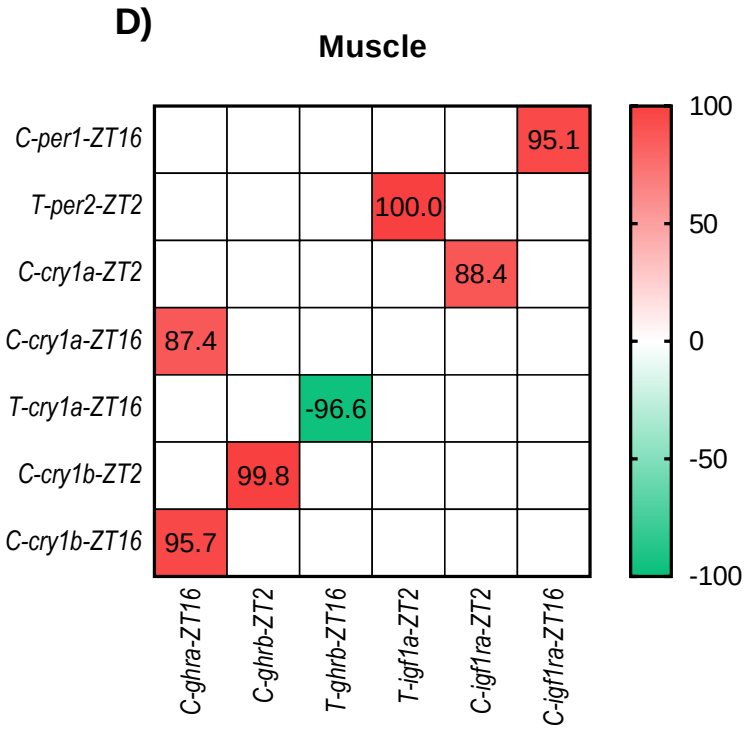

Figure 19. Gene correlation in the muscle of adult *Danio rerio*. Using the data from the same sample and *zeitgeber* time, this analysis shows 5 positive correlations and 1 negative correlation.

5. DISCUSSION

The organisms are able to anticipate the challenges imposed by the environment by adjusting innate temporal programs to these variable environmental clues, known as *zeitgebers*, among them light and temperature (Pattering, 1993; Buhr et al., 2010). In the case of temperature, the ability of cyclic changes (known as thermo-cycles) to entrain biological rhythms has been demonstrated for a wide variety of organisms from bacteria, algae, fungi, plants and ectothermic animals (Rensing & Ruoff, 2002). Similarly, transitions from low to high temperatures are associated with dawn and transitions from heat to cold with dusk (Johnson et al., 2004). Rensing and Ruoff (2002) demonstrated that even slight temperature changes (between 1 and 2°C) have the capacity to entrain biological rhythms in ectotherms, and to affect a number of molecular reactions such as protein phosphorylation (Lahiri et al., 2005).

The knowledge of the basic mechanisms and functions of the circadian clock of vertebrates greatly progressed during the last decades. It is important to highlight the success of the starting-point study that made possible to determine the clock mechanism in *Drosophila spp* (Peschel & Helfrich-Förster, 2011). The Nobel Prize in Physiology or Medicine 2017 was awarded jointly to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for their discoveries of molecular mechanisms controlling the circadian rhythm.

Rupp and co-workers (2019) demonstrated that melanopsin is critical for the acute effects of light on thermoregulation in mice. A pioneering study, almost a decade ago, showed for the first time that rhodopsin acts as a thermosensor in *Drosophila*, a groundbreaking concept at the time (Shen et al., 2011). In addition, our group showed that melanopsin is required for thermo-reception, acting as thermo-opsin that ultimately feeds the local circadian clock in mouse melanocytes and melanoma cells (Moraes et al., 2017).

Knowing that melanopsin acts as a receptor of temperature, we decided to evaluate the possible changes in its expression *in vivo*, regarding its functionality. For that, we analyzed the expression of *opn4m1* and *opn4m2* in the brain, eye, liver, and muscle. Although *Danio rerio* has 5 different melanopsin genes (*opn4m1*, *opn4m2*, *opn4m3*, *opn4x1*, and *opn4x2*),

previous results from our group identified *opn4m1* and *opn4m2* as the most widely expressed in a cell line of *D. rerio* (ZEM-2S) (Sousa et al., 2017).

In most vertebrates, melanopsin is not only expressed in the retina but also in many other organs. A study on *D. rerio* embryos showed the expression of *opn4m1* and *opn4m2* in the brain prior to retinogenesis (which starts between 28-32 hours post-fertilization). It was reported that *opn4m1* is expressed continuously from 1 to 3 days post-fertilization (dpf) in the presumptive preoptic area. *opn4m2* was not expressed in the brain until 3 dpf, when transcripts were located at the juncture between the caudal hindbrain and anterior spinal cord (Matos et al., 2011). Davies and collaborators (2015) studied the distribution of *Danio rerio* opsins in different tissues; similarly, *opn4m2* was weakly expressed in the brain (not detected by instruments), and *opn4m1* exhibited variable levels of expression in diverse tissues. In Davies group study, transcriptome data did not show expression of *opn4m2*, but revealed exorh (exo-rhodopsin, non-visual opsin similar to cones) and rgr1 (rgr/rrh/opn5 type opsins) in the brain. However, we were able to demonstrate *opn4m1*-2 by qPCR in the brain and the eye.

Our data coincide with those obtained by Davies and collaborators in 2015, regarding the expression of *opn4m1* and *opn4m2* in the eye. We found that in animals kept at 28ºC *opn4m1* and *opn4m2* display higher expression at night (ZT16) and at morning (ZT2), respectively. In both cases, there was a decrease, not always demonstrated by statistics, in the expression in animals kept at 23°C. These opsins have specific retinal cellular localizations and discrete functional properties in the zebrafish: o*pn4m1* is more expressed in the retinal bipolar cells and *opn4m2* in the horizontal cells. With the exception of multiple tissue opsins (tmt/opn3) and non-visual cone-type opsins, the eye presents a significant expression of all opsins (Davies et al., 2011).

For metabolic tissues like liver and muscle, we obtained interesting data that corroborate those obtained by Davies and collaborators (2015). For the muscle, Davies group pointed a small expression of the *opn4m* opsins while *parapinopsin* and *rgr2* displayed high level of transcripts. When we evaluated the opsins in the muscle, there was no homogeneous expression and, in many cases, the C_T of amplification was between 35 and 40. Likewise,

when we evaluated *opn4m1* and *opn4m2* in the liver we obtained variable expressions with amplification cycles > 35.

It should be noted that *D. rerio* possesses a sophisticated system of opsins with different expression patterns according to the organ, in addition to the ability of retinal cells to regulate the expression of *opn4* based on the duration of the photoperiod. Our data are similar to those obtained by Matos and collaborators (2011) in the retina of *D. rerio*. In this study, the *opn4* genes presented different rhythmic expressions. The peak expression of *opn4m2* was at ZT13 (light cycle dark 14:10), while the expression of *opn4m1* was reduced. In the same way when the level of *opn4m1* was high (ZT21), no transcripts of *opn4m2* were detected. Therefore, they inferred that melanopsin presents a rhythmicity proper to each subtype and dependent on the cell type where it is expressed.

Interestingly we obtained positive correlations between the *opn4m1* and *per2* and *cry1a* genes at ZT2. Several studies showed the modulation of *cry1a* and *per2* genes by the melanopsin signaling cascade after light stimulation (Ziv et al., 2005; Tamai et al., 2007; Froland & Whitmore, 2019). Here we evidenced a decrease in both genes at 23°C suggesting that low temperatures downregulate the mRNA expression of *opn4m* genes and subsequently may lead to a reduction in the expression of clock genes as *per2* in adult *D. rerio*.

Although most studies on the rhythmicity in *D. rerio* are focused on the effect of light, the influence of temperature has also been evaluated. Lahiri and collaborators (2005) reported that changes of 2°C modified the expression of clock genes in *D. rerio* cells and larvae. Temperature cycles of 27.5°C:23.5°C decreased *per1* and *cry1-3* expression at high temperature and an increase in mRNA of these genes at low temperature in cultured cells. Curiously, previous studies in our laboratory have shown that the expression of *per1, per2, cry1a* and *cry1b* clock genes increased after heat shock in cells of *D. rerio* maintained in dark-light cycle (Jerônimo et al., 2017).

Based on the evidence, we evaluated the effects of low temperatures on the expression of clock genes (*per1, per2, cry1a* and *cry1b*) in several organs. In summary, there was a reduction in the total mRNA levels (ZT2 +ZT16) of the clock genes in the treated group (23°C) in all organs analyzed. Jerônimo and collaborators (2017) demonstrated a relationship between heat shock proteins (HSP) and rhythm generation. The heat stimulation causes

dissociation of the HSP90/HSF1 complex allowing free HSF1 to migrate to the nucleus and activate the transcription of genes such as *per2* which have the HSE (Heat Shock Elements) sequence in their promoters.

Several authors described a higher expression of the clock genes *per1*, *per2*, *cry1a* and *cry1b* of *D. rerio* and other teleosts during the light phase or in the dark-light transition. In the specific case of *per2* and *cry1a*, the light stimulus is sufficient to trigger their expression, being generally at ZT0 (Delaunay et al., 2003; Wang, 2008). The previous results corroborate our data, as independently of the organ, the peak expression was at ZT2, assuming that the *per* and *cry* transcription as well as the protein heterodimer formation (Per: Cry) were achieved; during ZT16, smaller expressions were obtained, corresponding to the decrease of the transcripts in the dark phase. These findings demonstrate that the molecular clock is functioning in control animals while in cold-exposed ones it may be suggested that the phase was altered or a reduction of transcripts levels took, in fact, place. To confirm this, a 24 h long gene expression analysis is needed. Nevertheless, we can suggest that cold exposure significantly affect the molecular clock.

Likewise, our *in vivo* data are similar to those previously obtained by our laboratory in *in vitro* assays. In embryonic cells of *Danio rerio* (ZEM-2S) challenged by a cold pulse (25.5°C) in DD, there was a decrease in the mRNA of *per1* and *cry1b* genes six hours after the end of the pulse (unpublished data). This result is similar to our findings in animals kept at 23°C. We can relate the above data with the presence/absence of light, since in the natural environment; the temperature sustains a close relationship with the light-dark cycles: the thermophase (high temperature) usually coincides with the photophase, and the cryophase (low temperatures) coincides with the scotophase (López & Sánchez, 2011).

Once the clock machinery and opsins were evaluated, we focused our question on physiological responses that the animal could present when kept at chronic low temperatures. We focused on the endocrine system: melatonin, cortisol and growth hormone. Melatonin, mainly produced in the pineal gland, is one of the molecules that acts as an indicator of environmental changes (Falcon et al., 2007). Its level fluctuates showing a peak during the dark and change seasonally under temperature conditions, acting as a clue for physiological and behavioral processes (Chemineau et al., 2007). In addition to diurnal and seasonal

photoperiod cues, ambient temperature affects melatonin levels. A study in rainbow trout (*Oncorhynchus mykiss*) showed maximal melatonin peak during summer when water temperature recorded 16.5°C (Masuda et al., 2003). In tropical teleosts such as *Solea senegalensis*, the highest values of melatonin also were observed with the highest temperatures (Vera et al., 2007). One has to bear in mind, however, that different seasons not only show different temperatures, but also the length of photoperiod change accordingly. In our protocol, photoperiod length and light intensity were kept similarly constant at both temperatures.

There are two types of highly conserved melatonin receptors among vertebrates: MT1 and MT2; a third form called Mel1c has been found, exclusively in non-mammalian species (Falcon et al., 2010). In mammals, MT1 is the subtype expressed in the suprachiasmatic nucleus and is involved in the modulation of circadian rhythms; it has been observed in other regions of the encephalon and in some peripheral organs (Kadekaro et al., 2004). Several studies discuss the correlation between melatonin concentrations and the expression levels of its receptors (Guerrero et al., 2000). In our study, the expression of eye *mtnr1aa* was higher at ZT16 at the control temperature, and remarkably depressed at low temperature. Loganathan and colleagues (2018) found that teleosts challenged by high temperatures had an increase in *mtnr1aa* and *mtnr1bb* mRNA levels in the brain. As for *mtnr1bb*, we did not find any difference between time points at both temperatures or between temperatures, either in the brain or the eye, what may indicate that this receptor type is constitutive and is not affected by temperature changes. These data, therefore, show tissue-specific effects of low temperature in *D. rerio*, demonstrating that the melatonin receptor *mtnr1aa* in the eyes is more sensitive to the ones in the brain. Likewise, our data suggest expression peaks of brain and retina *mtnr1aa* in the dark, as the results of Yumnamcha and collaborators (2017) in *Danio rerio* females.

The activation of the melatonin receptor 1 inhibits cAMP formation, protein kinase A (PKA) activity, and phosphorylation of the cAMP-responsive element binding protein (CREB) (required for the transcription of clock genes such as *per1*) (Masana & Dubocovich, 2001). When we evaluated both genes, we found a negative correlation between *mtnr1aa* and *per1* levels in the brain at ZT16, suggesting that other genes and their respective proteins would

be inhibiting *per1* in this organ during the dark phase; different of the *per2* that is directly responsive to light stimulus in teleost fish.

Studies have reported circadian fluctuations in the expression of *mtnr* genes, with higher expression during the light or dark phase depending on the tissue or species and gene. In teleosts such as salmon (*Oncorhynchus keta*), *mtnr1a* mRNA levels increased during the day (Ciani et al., 2019). These diurnal variations in mRNA levels of melatonin receptors were also described in the brain, retina, and pineal gland of the gold rabbitfish (*Siganus punctatus*) (Park et al., 2006; Shang & Zhdanova, 2007). Our data agree with the report by Sharma (2011) who showed that *mtnr1aa* of the eye presents fluctuations throughout the day with its peak expression during the dark phase.

It is known that the mammalian melatonin can be synthesized locally in various peripheral organs such as bone marrow, gastrointestinal tract, lymphocytes, and various regions of the eye including the retina, ciliary body, and lacrimal gland (Lundmark et al., 2007). In the eye, melatonin participates in the regulation of retinal cell photoactivity, dopamine synthesis and release, among others, besides being an effective antioxidant against free radicals (Wiechmann & Rada, 2003; Lundmark et al., 2007).

To relate the expression of receptors to the levels of melatonin, we decided to evaluate the expression of AANAT gene; an enzyme required for melatonin production. We found that cold-exposed animals had increased *aanat1* and *aanat2* levels compared to control. These data show that cold exposure leads to a considerable increase on *annat* levels and possibly melatonin levels in both the eye and the brain. Studies demonstrated the rhythmic secretion of melatonin in *D. rerio* and its maximal release at the second third of the dark phase (Appelbaum et al., 2009; Ben-Moshe et al., 2016). Considering that the lowest expression during the dark in animals at 28°C occurs at a ZT different from ZT16, we suggest that the *aanat* and consequently of melatonin peak occurs at a ZT subsequent to the one evaluated. If this hypothesis is true, there was a phase advance in the expression of both *aanat1* and *aanat2* in the brain and eye at 23°C.

Similar to melatonin other hormones are regulated by light and temperature. We investigated two targets of the hypothalamic-hypophysis-growth axis: the growth hormone and its receptors as well as the receptors of insulin-like growth factors (IGFRs). Growth hormone

(GH) can promote hepatic production of IGF-1 by binding to its *ghra* and *ghrb* receptors in the teleost liver. In addition, GH distributed throughout the body can elicit growth by regulating IGF-1 production, IGFR receptor functionality, and systemic and local production of IGF-binding proteins (IGFPs) (Dang et al., 2018).

The increase in fish growth induced by high temperatures may be mediated by the action of temperature on hormonal production. Water temperature does not seem to modulate the cell differentiation or GH expression in the early stages of life, but rather in post-harvest development (Gabillard et al., 2005). Several studies in *D. rerio* focused on the effect of temperature on muscle composition (as an output of growth), analyzing the number of muscle fibers: higher in animals kept at 31°C and 26°C than in animals kept at 22°C (Johnston et al., 2009). In several teleost species, GH secretion is higher at higher temperatures (Deane & Woo, 2009), suggesting higher growth during spring/summer and reduced GH levels in winter. When *gh1* in the brain was evaluated, we confirmed the above: the expression peaked in the dark in control animals kept with a notable reduction in animals kept at low temperature.

We found a positive correlation between *gh1* and *cry1b* transcript levels at ZT2. In fish, the cAMP signaling pathway leads to increased *gh1* mRNA transcription and release through the activation of transcription factors such as *c-fos* (Klein & Sheridan, 2008). In addition, the cryptochrome (*Cry*) genes of mammals are known to inhibit the accumulation of cAMP (Zhang et al., 2010). Interestingly we found low levels of *gh1* during the day suggesting a direct relationship between *gh1* and the clock genes (specifically *cry1b*) by inhibiting CREB. This inhibition seems to happen in GH receptors as well. We found a positive correlation between *cry1a* and *cry1b* levels (high in ZT2) and *ghra* (low in ZT2). In the muscle, we observed a positive correlation between several genes. The correlation between: *cry1b* and *ghrb* (99.8%) as well as *cry1b* and *ghra* (95.7%), at 28°C, is remarkable. In both cases there seems that the *cry* gene also modulates the expression of GH receptors.

In our study, we found that the muscle *ghra* and *ghrb* receptors exhibited a marked decrease in animals kept at the low temperature. Due to their ectothermic physiology, tropical fish in cold waters decrease their metabolism, resulting in a reduction of growth, probably due to a decrease in both GH production and the sensitivity and/or number of their receptors. No

difference in size and weight was found in animals from the two temperatures, probably due to the short (6 days) stay at 23ºC. Molecular alterations occurred before the reflex of those changes on the effective growth.

For many species, Deane and Woo (2009) indicate that the increase in GH levels may be associated with thermal stress and not directly with the growth of the animal. In the muscle, we found the highest expression of *ghra* at 28ºC, which agrees with what was reported by Gabillard and collaborators (2006) who kept rainbow trout at two temperatures (12°C and 4°C) and obtained an increase in the amount of *ghra* transcripts when the water temperature was raised. Our data also showed a remarkable decrease of the muscle *ghra* at 23°C.

IGF signaling induces proliferation and regulates cell cycle duration (Yuan et al., 2015). In some situations, they act in association with other growth factor signaling such as epidermal growth factor receptor (EGFR) (Alagappan et al., 2014). The levels of IGF receptors presents a variation in organ distribution (Obermann et al., 2019). To evaluate the functionality of an IGF cascade component in the target tissue we chose to assess the expressions of *igf1ra* and *igf1rb* in liver and muscle.

A recent work by Breit and co-workers (2018) demonstrated the relationship between the circadian clock and the growth hormone pathway in mice. The authors suggested that IGF-1 is able to entrain hypothalamic clock gene expression to a 24 h rhythm, and that glycogen synthase kinase-3β (GSK-3β) is required for the IGF-1 mediated regulation of the *Bmal1* promoter in hypothalamic cells.

Unlike our results (higher *igf1ra* and *igf1rb* expression in the liver than in the muscle), Mohammed and collaborators (2015) described the *Sparus aurata* muscle as one of the organs with higher *igf1r* followed by the pituitary gland. This because fish, unlike mammals, possess a more active IGF-1 muscle binding, replacing the active insulin binding in mammalian muscles (Dai et al., 2015).

Nornberg and co-workers (2016) demonstrated relative gene expression between 1.0 and 1.5 for the *igf1ra* and *igf1rb* in the muscle of *Danio rerio*. For all cases, the highest levels of expression were obtained at ZT16, indicating a greater need for functional receptors because of nocturnal growth hormone peak and IGF-1 synthesis.

A third hormone that can be regulated by temperature and photoperiod is cortisol. In general, changes in water temperature cause stress in fish, which include from physiological and behavioral adaptive changes to the death of the individual (Gordon, 2005). The metabolic effects of glucocorticoids are primarily mediated by the activation of the glucocorticoid receptors (*grs*), transcription factors associated with the binding agent, highly conserved in vertebrates (Stolte et al., 2006). Unlike other teleosts with two subtypes of *gr*, called *gr1* and *gr2*, *Danio rerio* contains only one encoding gene for Gr (Vazzana et al., 2010) corresponding to the *gr2* receptor of other teleosts (Schaaf et al., 2008).

The data for whole body cortisol showed remarkably higher amounts (µg/g weight) in animals kept at 23°C, with a noticeable reduction at ZT16. The study conducted by Ramsay and collaborators (2009) in adult *D. rerio* demonstrated high levels of cortisol in animals stressed by handling, with a recovery of baseline levels 24 h post-stimulation. Unexpectedly, animals kept at 28°C had low hormonal levels at both ZTs, not showing the well-known anticipatory peak in the early morning. Because of the pattern of our data, we suggest that the peak release of cortisol in animals kept at 28°C may have occurred at a ZT prior to ZT2 and that the high levels in animals at low temperatures correspond to a maintained stress response, with a phase advance of the rhythm.

We decided to determine the expression of *gr* in metabolic tissues such as liver and muscle of animals kept at different temperatures. In the liver but not in the muscle, there was a reduction in the mRNA of these receptors in animals kept in lower temperature at ZT16 when compared to the control. Reports from the 90's showed that the activation of the hypothalamic-hypophysis-adrenal axis in heat stress-induced responses was accompanied by a peripheral adaptation in the amount and affinity of glucocorticoid receptors (Molijn et al., 1995).

In a study conducted by Pavlidis and collaborators (2015), it was demonstrated a rapid adaptation of *gr* transcripts in animals challenged to acute stress, presenting up-regulation in the first 15 minutes and a return to baseline levels after 30 minutes. In our protocol, in which the stress was chronic (6 days at 23°C), it would be harmful to the organism to generate and keep high levels of *gr* mRNA, as it would trigger an exacerbated response to cortisol and consequently homeostasis disruption.

6. GRAPHICAL ABSTRACT

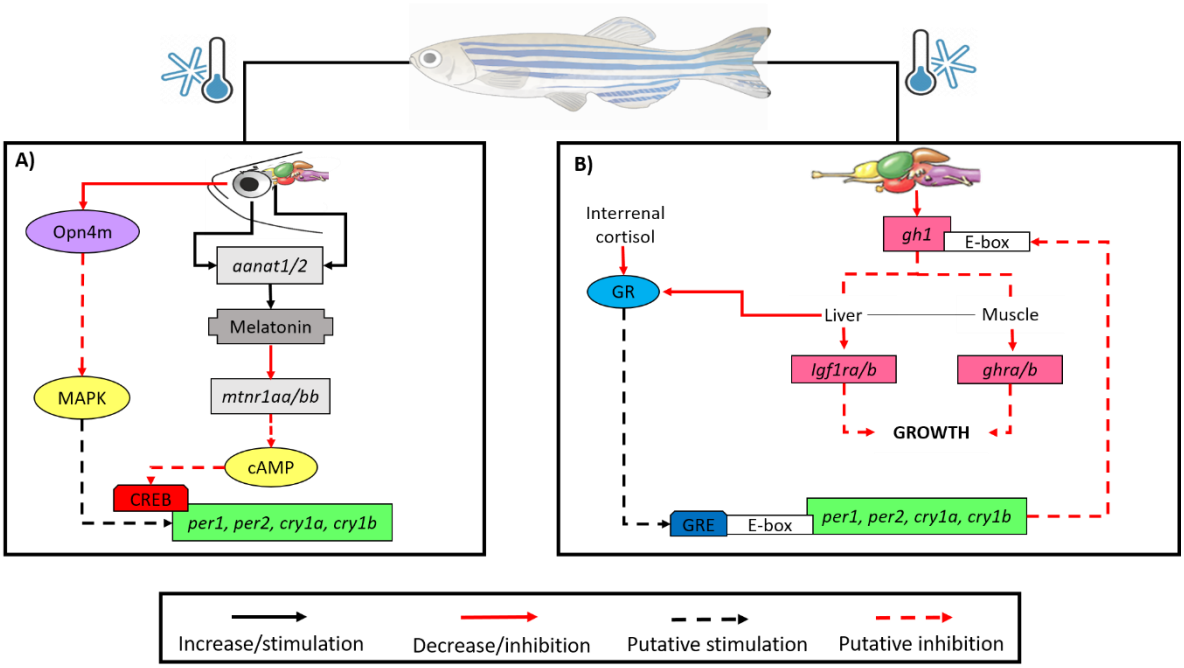

Figure 20. A). Brain and eye of *D. rerio* submitted to low temperatures show reduced levels of *opn4* mRNA leading to a possible inhibition of the MAPK pathway (blue light-activated pathway) that participates in the modulation of the clock genes. In both organs, there has been an increase in the *aanat* transcripts causing an increase in melatonin levels. To ensure homeostasis, the *mtnr1aa/bb* expression decreased. In turn, it is known that Mtnr1aa/bb activation ultimately leads to the inhibition of CREB phosphorylation, a transcription factor required for clock gene expression, resulting in a reduction of their transcripts. B) A reduction in the *gh1*, *ghra/b*, *igf1a* and *igf1ra/b* transcripts suggest a later delay in the animal growth. On the other hand, there was an increase in the cortisol concentration and a compensatory reduction of its receptor transcripts. The glucocorticoid (GC)/glucocorticoid receptor complex modulates the expression of clock genes by binding to glucocorticoid response elements (GRE) in their promoters. Consequently, with a decrease of *gr* transcripts, we evidenced a decrease in the expression of clock genes. In turn, clock genes are capable to bind to the promoter E-box region of genes as *gh1* thus modulating its expression.

7. CONCLUSIONS

- The melanopsin *opn4m* presented a detectable expression in brain and eye, but *opn4m1* in the eye oscillates exhibiting higher expression in the dark phase, and *opn4m2* in the eye was reduced in low temperature.

- The clock genes in *D. rerio* were downregulated by low temperatures, being a modulating clue of the mRNA transcripts; confirming that tissue clocks do not show temperature compensation.

- The low temperature induces a marked increase of both *aanat2* and *aanat1* in the brain and eye respectively, involved in the production and secretion of melatonin, but depressed melatonin receptor *mtnr1aa* in the eye, eventually as a way to compensate the exacerbated melatonin production.

- Like other tropical teleost species, low temperatures negatively affect the transcripts of GH, GHRa and b in the muscle, and IGF1Ra and b in the liver, what might result in diminished growth and metabolism.

- Cortisol secretion peak in *D. rerio* probably occurs in a ZT prior to ZT2. There was a phase delay in cold temperatures since the individuals in the cold exhibited their peak at ZT2. Although *D. rerio* supports a wide temperature range, 5°C below its maintenance temperature represented a thermal stress, as seen by the remarkable increase of whole-body cortisol, thus modulating its physiological responses.

- Temperature changes of as low as ~5°C below the ideal temperature were able to modulate the endocrine and circadian systems in adult *Danio rerio*.

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