Stress effects on brain circadian system and monoaminergic activity in rainbow trout. Mechanisms and consequences on food intake regulation

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Stress effects on brain circadian system and monoaminergic activity in rainbow trout. Mechanisms and consequences on food intake regulation

Supervised by:
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Dr. Marcos Antonio López Patiño  

DECLARE that the present work, entitled “Stress effects on brain circadian system and monoaminergic activity in rainbow trout. Mechanisms and consequences on food intake regulation”, submitted by Fatemeh Naderi to obtain the title of Doctor, was carried out under their supervision in the PhD programme “Marine sciences, Technology and management” at Universidad de Vigo.

Vigo, 17 September, 2018  

The supervisors,

Dr. Jesús Manuel Míguez Miramontes  
Dr. Marcos Antonio López Patiño
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<tr>
<td>3β-HSD</td>
<td>3β-Hydroxy steroid dehydrogenase</td>
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<tr>
<td>3-MT</td>
<td>3-Methoxy tryamine</td>
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<tr>
<td>5HIAA</td>
<td>5-Hydroxyindoleacetic acid</td>
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<tr>
<td>5HT</td>
<td>Serotonin, 5-Hydroxytryptamine</td>
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<tr>
<td>5HTOH</td>
<td>5-Hydroxytryptophanol</td>
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<td>5HTP</td>
<td>5-Hydroxytryptophan</td>
</tr>
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<td>5HTR</td>
<td>Serotonin Receptors</td>
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<tr>
<td>AAD</td>
<td>Aromatic L-amino acid decarboxylase</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<td>AgRP</td>
<td>Agouti-related peptide</td>
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<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
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<td>ALRD</td>
<td>Aldehyde reductase</td>
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<td>AVT</td>
<td>Arginine vasotocin</td>
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<td>BMAL1</td>
<td>Brain and muscle Arnt-like protein-1</td>
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<td>BSA</td>
<td>Brain-Sympathetic-Adrenal</td>
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<tr>
<td>BSC</td>
<td>Brain-Sympathetic-Chromaffin</td>
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<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
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<td>CAs</td>
<td>Catecholamines</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
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<td>CLOCK</td>
<td>Clock circadian regulator</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>COMT</td>
<td>Catechol-O-methyl transferase</td>
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<tr>
<td>CRF</td>
<td>Corticotropin-Releasing Hormone</td>
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<tr>
<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
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<td>CRY</td>
<td>Cryptochrome circadian clock</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DBH</td>
<td>Dopamine-β-hydroxylase</td>
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<tr>
<td>DHMA</td>
<td>3,4-Dihydroxymandelic acid</td>
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<tr>
<td>DOAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>FAA</td>
<td>Food anticipatory activity</td>
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<tr>
<td>FEO</td>
<td>Food entrainable oscillator</td>
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<tr>
<td>GH</td>
<td>Growth Hormone</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal Tract</td>
</tr>
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<td>GR</td>
<td>Glucocorticoid Receptors</td>
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<td>HPA</td>
<td>Hypothalamus-Pituitary-Adrenal</td>
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<td>HPI</td>
<td>Hypothalamus-Pituitary-Interrenal</td>
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<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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<td>HSC</td>
<td>Hypothalamus-Sympathetic-Chromaffin</td>
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<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
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<tr>
<td>ICV</td>
<td>Intracerebroventricular injection</td>
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<td>IGL</td>
<td>Intergenulate leaflet</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal injection</td>
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<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
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<tr>
<td>LEO</td>
<td>Light entrainable oscillator</td>
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<td>MAO</td>
<td>Monoamine oxidase</td>
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<td>MCH</td>
<td>Melanin-Concentrating Hormone</td>
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<td>MR</td>
<td>Mineralocorticoid</td>
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<tr>
<td>MSH</td>
<td>Melanocyte-Stimulating Hormone</td>
</tr>
<tr>
<td>NA</td>
<td>Norepinephrine, noradrenaline</td>
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<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAMPT</td>
<td>Nicotinamide phosphoribosyltransferase</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>ORE</td>
<td>Orphan receptor elements</td>
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<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<tr>
<td>q PCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>REV-ERB</td>
<td>Nuclear Receptor Subfamily 1 Group D, Member 1</td>
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<tr>
<td>RHT</td>
<td>Retinohypothalamic tract</td>
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<td>RORS</td>
<td>Retinoic acid receptor-related orphan receptor elements</td>
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<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
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<tr>
<td>SIRT1</td>
<td>Sirtein 1</td>
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<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
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<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<td>TPH1</td>
<td>Tryptophan hydroxylase 1</td>
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<tr>
<td>TPH2</td>
<td>Tryptophan hydroxylase 2</td>
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<td>Trp</td>
<td>Tryptophan</td>
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<tr>
<td>UCN</td>
<td>Urotensin</td>
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<tr>
<td>UI</td>
<td>Urotensin</td>
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</table>
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2. **Objectives**

3. **Experimental works**

3.1. **Experimental work 1**
Involvement of cortisol and SIRT1 in the response to stress of hypothalamic clock genes and food intake-related peptides in rainbow trout, Oncorhynchus mykiss.
3.2. **Experimental work 2**  
SIRT1 is a main mediator of the effect of stress on hypothalamic circadian oscillator and food intake-control peptides in rainbow trout, *Oncorhynchus mykiss*.

3.3. **Experimental work 3**  
Are brain monoaminergic activities and food intake control linked to a circadian oscillator in rainbow trout?

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4.3. Brain monoaminergic systems in the response to chronic stress in rainbow trout. Implication for endocrine and feeding response

5. **Conclusions**

6. **Resumen tesis / Thesis summary**

7. **References**
1. General introduction
1.1. Biological rhythms in fish

Biological rhythms are cyclic changes that perfectly fit with the temporal organization of the environment. Since most organisms perceive these environmental variations, different strategies have been developed in order to synchronize their body daily rhythms to such fluctuations (Figure 1). The response of animals to the environmental variations includes behavioral changes (sleep cycle, periods of activity-rest, locomotor activity, food activity, etc.), and internal adaptations at physiological level (body temperature, hormones secretion, cardiovascular, digestive function, reproduction, etc.) (Brock, 1991; Davidson et al., 2004). Thus, changes of the environment are an important factor for animal’s survival. Developing mechanisms that allow animals to anticipate the incidence of these variations have revealed to be an important evolutionary advantage.

Figure 1: Cyclic environmental fluctuations affect animals, which have mechanisms to adapt to changes in the environment.

Chronobiology is focused on the study of rhythmic processed developed by living organisms in response to the incidence of environmental fluctuations. A series of parameters are defined (Figure 2), in order to quantify such rhythms (Koukkari and Sothern, 2006):

- Period: the time elapsed for one complete oscillation or cycle.
- Frequency: the number of times a specified phenomenon occurs within a specified time interval.
- Amplitude: oscillation of the rhythm from the mean value or mesor (sinusoidal rhythm), or the difference between the maximum and minimum values of the rhythm (non-sinusoidal rhythm).

- Phase: temporal reference, typically the maximum value (peak), or minimum value (valley).

![Diagram showing parameters of a rhythm]

**Figure 2:** Parameters characteristic of the rhythm.

Biological rhythms are classified according to the duration of their period: Ultradian (less than 20 hours), circadian (between 20-28 hours), infradian (more than 28 hours), circamensual (1 month) and circannual (10-14 month) among others. Most of them relate to the Earth’s rotation and translation movements, which are responsible of either daily and seasonal variations of lighting conditions, temperature and other environmental factors. The physiology and behaviour of most organisms have been mostly influenced by the daily fluctuations of light intensity (light-dark, LD cycle), since they are presented with a close to 24 h periodicity. Because of that, those rhythms are known as circadian rhythms (Refinetti, 2006), and the current dissertation focuses on them.

The knowledge of the influence of the rhythmic changes of the environment is important for researchers, in order to understand the complexity of the regulatory mechanisms responsible of physiology and behaviour. In vertebrates, two patterns of response to cyclical environmental changes exist. The first one includes passive responses (on-off) that manifest as consequence of the incidence of a rhythmic fluctuation of a given environmental factor (photoperiod, temperature,...) but disappear in the absence of such fluctuation. This passive rhythmic response is known as *non-endogenous* rhythm, as it is dependent on environmental changes, thus not providing any adaptive information for individuals. The second pattern refers to a rhythmic response that persists even in the absence of cyclic external stimuli. This
rhythm is *endogenous* because internal structures with autonomous functioning generate it. These structures constitute the biological clocks (Cardinali and Golombek, 1993). Then, a biological clock can be defined as a structure that is able to integrate external temporal information, to develop a rhythmic signal, and to integrate between them both, thus transmitting the generated rhythm to a given activity/function all over the organism (Pittendrigh and Daan 1976; Halberg et al., 1977). A biological clock includes three components (*Figure 3*): 1) one or more genes that display the oscillatory pattern, and play a key role as the core clock system; 2) an “input” component, that transmits the rhythmic variation of the external cue to the clock, thus synchronizing its endogenous mechanism; and 3) an “output” signal that brings the rhythmic information all over the body (Aschoff, 1981).

![Figure 3: Schematic circadian system components (Modified from Eskin, 1979).](image)

The environmental signals that are able to synchronize the activity of the biological clocks are defined as “*zeitgebers*”. They participate in maintaining the correspondence between the environment and the endogenous rhythm, thus stabilising the interaction between biological time and geological time (Zhdanova and Reebs, 2006). The most studied zeitgeber is the LD cycle, but also other environmental cues participate as zeitbebers, such as temperature, food availability, social interaction, and pharmacological manipulations, among others. In order for the endogenous oscillator to synchronise to the environment, zeitgebers influence the level of the molecular components of the core clock system, with these variations fitting to the specific phase of the 24 h cycle. Such process is known as *entrainment*.

1.1.1. *Molecular basis of the circadian system*

In general, circadian clocks in all organisms, from cyanobacteria to vertebrates share important properties: their period is closed to 24 hours, and synchronize to
external environmental cues. The circadian CLOCK system consists of central and peripheral components. In mammals, the central one is located within the hypothalamic suprachiasmatic nucleus (SCN), typically acts as a “master” CLOCK, and is strongly influenced by the LD cycle, whereas the peripheral components behave as “slave” CLOCKs, and are distributed all over the body (Nader et al., 2010). The master clock releases hormonal/neuronal transmitters that synchronize and control peripheral clocks (Kalsbeek et al., 2006; Hastings et al., 2007), which have not been fully elucidated.

The molecular mechanism that generates the rhythmicity (Figure 4) is based on transcriptional and translational auto-regulatory feedback loops (Panda et al., 2002a,b). In the primary one, the positive elements include members of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family, clock and bmal1. Their expression leads to increased levels of the activating elements, CLOCK and BMAL1, which heterodimerize and bind to the E-box, located at the promoter region, thus enhancing the transcription of the negative elements, Period (per1, per2, and per3) and Cryptochrome (cry1 and cry2) genes (Ko et al., 2006). PER and CRY complexes inhibit their own transcription by binding to the CLOCK:BMAL1 heterodimer, thus blocking its function by making them not to be able to bind to the E-box (Kume et al., 1999; lee et al., 2001; Sato et al., 2006; Kondratov et al., 2006). This negative loop allows a daily rhythm of per and cry transcripts abundance and the respective protein products (Iuvone et al., 2005; Okamura et al., 2002), and the whole mechanism allows the rhythmic oscillation to repeat every 24 hours.

Another additional feedback loop has been described, and is dependent on nuclear receptors. It is induced by CLOCK:BMAL1 heterodimer, which activates the transcription of other clock-related genes encoding for nuclear receptors, such as rev-erb, and retinoic acid receptor-related orphan receptor α (RORα) (Akashi et al., 2005). Both, ROR and REV-ERB have been reported to display opposite transcriptional activities (Giguère, 1999), with ROR family acting as transcription activators, whereas REV-ERB family inhibits the transcription (Dumas et al., 1994; Forman et al., 1994; Retnakaran et al., 1994). Within the bmal1 promoter, REV-ERBα and RORα bind to retinoic acid-related orphan receptor response elements (ROREs) thus displaying opposite activities (Guillaumond et al., 2005; Ko et al., 2006). Once bound, RORs activates bmal1 transcription (Akashi et al., 2005, Sato et al., 2004), whereas REV-ERBs respond to this process (Preitner et al., 2002).

In poikilotherms, circadian system is not fully understood. Specifically for teleosts, some reports demonstrate the existence of several genes encoding the respective protein(s) with overlapping functions (Coon et al., 1999; Falcón et al., 2003; Forsell et al., 2002). The presence of a circadian oscillator has been reported in neural tissues of several teleost species, such as zebrafish (Cahill, 2002), goldfish (Velarde et al., 2009), sea bream (Vera et al., 2013), salmon (Betancor et al., 2014)
and rainbow trout (López-Patiño et al., 2011), and striking consistency to that reported for mammals exists. Then, positive transcriptional loop (formed by clock and bmal1) and negative transcriptional loop (formed by per and cry) have been reported. In fish, however, the existence of duplicate genes appeared as consequence of a round of whole genome duplication occurred during ray finned fish lineage, thus previous to teleosts radiation (Wang, 2008). Salmonids also suffered an additional genome duplication (Allendorf and Thorgaard, 1984). Therefore, this teleost group may have different duplicates for most genes. Regarding rhythms, salmonids display daily variations of several clock-genes mRNA abundance, such as clock, bmal1, and cry2 in the brain of Atlantic salmon (Davie et al., 2009). In rainbow trout, clock1a, bmal1, and per1 are rhythmically expressed in retina and hypothalamus (López-Patiño et al., 2011), but also in peripheral tissues such as liver (Hernández-Pérez et al., 2017).

**Figure 4**: The molecular circadian clock in mammals. CLOCK: clock circadian regulator, BMAL1: Brain and muscle Arnt-like protein-1, PERS: Periods, CRYs: Cryptochrome circadian clock, REV-ERB: Nuclear Receptor Subfamily 1, Group D, Member 1, RORS: Retinoic acid receptor-related orphan receptor, RORE: Retinoic acid-related orphan receptor response elements.
1.1.2. Circadian system organization

Mammalian circadian system is the most studied so far. Within the brain, the SCN is considered as the main pacemaker. Thus, the SCN hosts the master oscillator containing specific molecular elements (clock genes) that create/control circadian rhythms of most functions all over the organism. In support of this, lesions of SCN result in the loss of behavioral, endocrinological, and physiological circadian oscillations (Moore, 1995). SCN receives information mainly through three different pathways: The retinohypothalamic tract (RHT), the intergeniculate leaflet (IGL), and the medial nucleus of the rafe (RM). RHT pathway originates at the retina and is the main photic-related input, thus playing a critical role in generating circadian rhythms (Weaver, 1998; Gooley et al., 2001), whereas the other pathways participate as inputs of photic information to the suprachiasmatic nucleus (IGL) or non-photonic information (RM).

The existence of circadian oscillators is not limited to the SCN in mammals. Accordingly, rhythmic expression of clock genes has been found in peripheral tissues such as liver, heart, muscle, adipose tissue, pancreas, kidney, lung and ovary (Balsalobre, 2002; Mühlbauer et al., 2004; Peirson et al., 2006; Zvonic et al., 2006). These oscillators display autonomous rhythmicity but different external or internal factors (including central oscillator-driven neuroendocrine signals) are able to modulate their activity (Pando et al., 2002; Guo et al., 2006; Kalsbeek et al., 2006). Then, the presence of interactions between central and peripheral oscillators may exist in order to coordinate the circadian behavioural and physiological functions all over the body.

In non-mammalian vertebrate groups, the existence of multiple coupled central circadian oscillators is accepted. These oscillators can locate within different tissues such as retina, pineal gland and hypothalamus, but their functioning appear to remain quite similar to that of mammals (Menaker et al., 1997; Falcón et al., 2010; López-Patiño et al., 2011). In spite of hypothalamus in fish, both retina and pineal organ act as circadian oscillators, since both tissues are photosensitive, clock genes express rhythmically within them, and release an endocrine output, such as the melatonin (Falcón, 1999; Falcón et al., 2007).

Rhythmic expression of clock genes has been described in tissues other than the SCN of non-mammalian vertebrates, such as liver, heart, intestine, and muscle of birds (Chong et al., 2003), liver, heart, muscle, lung, and testis of reptiles (Della Ragione et al., 2005; Vallone et al., 2007), and liver, heart, spleen, and gall bladder of fish (Kaneko et al., 2006; Velarde et al., 2010a; Betancor et al., 2014; Hernández-Perez., et al., 2017).

The fish circadian system is typically composed by multiple oscillators located throughout the body. These oscillators are mainly entrained by external inputs such
as the LD and feeding-fasting cycles, among others. Coordination among them all leads for successfully controlling different behavioural and physiological rhythmic functions, such as locomotor activity and hormonal rhythms. The synthesis and release of specific outputs allow them to adjust all these functions.

In fish brain, the presence of a mammalian SCN-homologous neural region has not been demonstrated, but rhythms of clock genes have been described in the whole brain of Atlantic salmon (Davie et al., 2009), or within the hypothalamus including preoptic area, as reported for rainbow trout (López-Patiño et al., 2011). The photosensitive pineal organ is also playing an important role as modulator of circadian rhythms, since this organ is able to rhythmically synthesize and release one of the main endocrine output signals, melatonin (see rev. Falcón, 1999). Then, light appears to be the main environmental modulator of this process, with melatonin binding to the hypothalamic SCN-homologous region, thus transmitting the photic information to the clock. In addition, retina (where melatonin is also synthesized) as photoreceptor structure is able to also transmit photic information to SCN, as above mentioned. However, some teleost species (salmonids) have been reported not to host a functional circadian system in both tissues (Besseau et al., 2006). So, melatonin rhythm disappears in animals subjected to constant lighting condition. On the contrary, trout retina, as representative salmonid, does have such circadian system (López-Patiño et al., 2011).

The complexity of this system increases, since some studies evidence that melatonin can be synthesized in photosensitive structures other than pineal organ and neural retina of fish, such as the gastrointestinal tract (GIT), as reported for goldfish (Velarde et al., 2010b) and rainbow trout (Muñoz et al., 2016), where melatonin synthesis appears to be influenced by food as main modulator, instead of light (Muñoz et al., 2016). The same authors also indicate that the daily rhythm of melatonin synthesis persists in trout GIT even in the absence of day-night variations, which reveals the modulatory control exerted by the circadian system on this process.

Then, endogenous central clocks appear to entrain to photic information, whereas circadian oscillators located in peripheral organs (GIT, liver) can be entrained by other non-photic signals such as feeding time and metabolism-related cues, among others. Independently of the nature of the synchronizer, central and peripheral oscillators release specific outputs (melatonin, pituitary hormones, ghrelin, leptin and cortisol) in a time-dependent way (Figure 5), thus providing the temporal message to specific targets and synchronizing their daily function (Isorna et al., 2017).

Additionally, cell-autonomous and self-sustaining clock gene rhythms have been also described in several in vitro cultured cell lines of zebrafish (Tamai et al., 2005) and cavefish, Phreatichthys andruzzi (Cavallari et al., 2011).
Figure 5: The fish circadian system. This diagram only shows the most studied endocrine organs that are functionally related to the circadian system; however, other oscillators also probably exist. The simple lines indicate the connections that are currently known to exist in fishes, whereas other lines with question marks illustrate hypothetical connections that have not yet been reported. Oscillators that are targeted by: Light, are shown in yellow and blue; Feeding-fasting, are shown in green and blue; both light and feeding-fasting, are shown in purple.

The molecular functioning of peripheral oscillators is similar to that of central ones, with the existence of positive and negative feedback loops of mRNA abundance and protein levels of clock genes that oscillate on a 24 h basis (Reppert and Weaver 2002; Hastings et al., 2007; Albrecht, 2012; Schibler et al., 2015). The functioning of the teleost molecular clock has been deeply studied in zebrafish (Figure 6) (see rev. Isorna et al., 2017), but also in a variety of teleost fish (Park et al., 2007; Davie et al., 2009; Velarde et al., 2009; Huang et al., 2010; Lópe-Olmeda et al., 2010; Cavallari et al., 2011; López-Patiño et al., 2011; Azpeleta et al., 2012; Martín-Robles et al., 2012; Nisembaum et al., 2012; Vera et al., 2013; Sánchez-Bretaño et al., 2015b; Costa et al., 2016). In all of them, transcripts of the positive elements of the core clock (clockl and bmall) peak during the photophase, whereas transcripts of the negative elements (per and cry) increase at the end of the night (Cahill, 2002; Vatine et al., 2011).

Oscillators can synchronize to exogenous (light) or endogenous (hormones, nutrition...) temporal messengers through the induction or repression of specific clock genes. Accordingly, LD cycle leads to cryl and per2 induction, which involves functional E- and D-boxes together with the thyrotroph embryonic transcription factor (Tamai et al., 2007; Vatine et al., 2011). However, alternative mechanisms also participate on synchronizing the oscillators. For example, cortisol induces per1a and per1b, then repressing clock and bmall within goldfish liver, with such repression being apparently mediated by another transcription factor, Rev-erb (Sánchez-Bretaño...
et al., 2016). Also, ghrelin induces clock genes in goldfish liver (Isorna et al., 2017), with the phospholipase C-protein kinase C pathway as mediator, in addition to the adenyl cyclase-protein kinase A pathway (Figure 6) (Sánchez-Bretàno et al., 2016).

Figure 6: Hypothetical model of entrainment of circadian oscillators by light and endocrine inputs in fish: putative mechanisms for the synchronisation of endogenous clocks. Events that occur in the cytoplasm and nuclei (gene transcription) have not been separated to simplify the figure. In each box, only putative response elements that are involved in each response (light, cortisol or ghrelin) are shown. AC-PKA, adenylyl cyclase-protein kinase A; CARE, calcium response element; CCG, clock-controlled genes; CRE, CREB response element; CREB-P, CREB phosphorylated; GHR, ghrelin receptor; GR, glucocorticoid receptor; GRE, glucocorticoid response element; PLC-PKC, phospholipase C-protein kinase C; RORE, RAR-related orphan receptor response element; Tef, thyrotroph embryonic factor; TF, transcription factor; TFRE, transcription factor response element (Isorna et al., 2017).

1.1.3. Light and food as zeitgebers

A given organism needs to receive precise information in order synchronize daily behavioural and physiological functions to predictable changes of the environmental conditions. Such information has to be integrated in specialized structures, and converted in appropriate signals that spread all over the organism. A factor that displays predictable temporal changes is known as zeitgeber. The most studied zeitgeber that influences the circadian system in vertebrates is the LD cycle (Hastings et al., 2007; Albrecht 2012; Schibler et al., 2015). However, other external
cues such as food availability (Stephan, 2002; Albrecht, 2012; Patton and Mistlberger, 2013) and temperature cycles (Buhr et al., 2010; Poletini et al., 2015; Schibler et al., 2015) among others, are also important. These environmental factors are considered ‘inputs’ to the circadian system, whereas the rhythms that are generated are called ‘outputs’ or ‘overt rhythms’. Daily locomotor activity and hormonal rhythms are two examples of outputs of the vertebrate circadian system. To complete the circadian rhythm, the core clock, which synchronises to the inputs, generate and drives the output as described above (Hastings et al., 2007; Albrecht, 2012).

In mammals, two main inputs to the circadian system are the hypothalamic-pineal axis and the hypothalamic-pituitary-adrenal (HPA) axis. The first one is involved in the rhythmic regulation of melatonin secretion, which is the hormone involved in daily and seasonal adjustment of diverse cyclic processes, such as rest-activity and sleep-wake phases, body temperature or reproduction (Arendt, 1997). The second one controls the rhythmic secretion of glucocorticoids and participates in generating locomotor activity rhythms, among other functions. The role of glucocorticoids on fish circadian functions will be assessed in the following sections.

With respect to melatonin, the hormone was identified as N-acetyl-5-methoxytryptamine (Lerner et al., 1958), and was detected in vertebrates (Reiter, 1993), invertebrates, bacteria, protozoa (Hardeland and Poeggeler, 2003) and plants (Hardeland et al., 2007). It is mainly synthesized in the pineal organ, but other neural tissues, such as the retina do also synthesise it (Gern and Ralph, 1979). Synthesis of the hormone has been also reported in peripheral tissues, such as the GIT (Bubenik and Pang, 1997; Carrillo-Vico et al., 2004; Muñoz et al., 2016), thymus (Naranjo et al., 2007), cells of the immune system (Carrillo-Vico et al., 2004), the skin (Slominski et al., 2008) and gonads (Itoh et al., 1997). The daily profile displayed by melatonin, and how such profile changes among seasons (especially in poiquilotherms) directly relates the hormone with the circadian system, since the nature of such changes can be integrated there, thus releasing the appropriate output signal.

In spite of the influence exerted by melatonin and glucocorticoids on the circadian system of mammals (Pevet and Challet, 2011; Kalsbeek et al., 2012; Lin et al., 2015) and other vertebrates such as fish (Falcón et al., 2007; 2010), other mediators can also participate as inputs to the circadian system, such as those hormones and neuropeptides displaying daily oscillations (Patton and Mistlberger, 2013; Tinoco et al., 2014; Challet, 2015; Sánchez-Bretaño et al., 2015a; Schibler et al., 2015). Increasing evidence in fish suggest that the circadian system is formed by a network of central and peripheral oscillators that coordinate to each other (Albrecht, 2012; Schibler et al., 2015). In this more complex model of circadian organization, hormones may not only participate as outputs of the circadian system, but also
influence and synchronize timekeepers as inputs, as reported for mammals (Albrecht, 2012; Patton and Mistlberger, 2013; Challet, 2015; Schibler et al., 2015).

According to the model purposed fish circadian system, oscillators synchronize to different inputs (Figure 5). Among environmental zeitgebers, the LD cycle influence light entrainable oscillators (LEOs), whereas the feeding-fasting cycle is the main modulatory cue of food entrainable oscillators (FEOs). This is just a functional classification, with no specific anatomical structures being involved, since both environmental factors may influence a given oscillator, as reported for that of rainbow trout liver (Hernández-Pérez et al., 2017). The following paragraphs provide information about both zeitgebers.

**Light entrainment**

Three major properties of a light-dependent circadian system are photoreception, intrinsic oscillation, and the capacity to provide information of the circadian phase. Specialized photosensitive cells in phylogenetically advanced species show these properties. Both retinal photoreceptors and photosensitive cells of the pineal gland contain circadian clocks and secrete melatonin, the amine that can convey circadian messages either to the nearby surroundings (retinal melatonin) or to the entire organism (pineal melatonin). Furthermore, it has been shown that multiple tissues in vertebrates can directly detect light and entrain to it, and host circadian oscillators as well (Whitmore et al., 2000). Several photo-pigments have been identified in fish retina, pineal gland, brain and peripheral tissues, such as the three isoforms of vertebrate-ancient (VA) opsin (Soni and Foster, 1997; Soni et al., 1998; Kojima et al., 2000; Moutsaki et al., 2000; Minamoto and Shimizu, 2002), melanopsin (Bellingham et al., 2002; Drivenes et al., 2003), cryptochromes (Kobayashi et al., 2000; Cermakian and Sassone-Corsi, 2002), and tmt-opsin (Moutsaki et al., 2003). These molecules are candidates for a putative ‘circadian photoreceptor’. Which of them play a critical role in circadian systems still remains unclear, but it appears that several, rather than one photo-pigment, might be involved in photic entrainment. Rhythms of clock genes expression in central and peripheral tissues that depend on LD cycles were demonstrated in several teleost species, including zebrafish (López-Olmeda et al. 2010), Atlantic salmon (Huang et al., 2010), rainbow trout (López-Patiño et al., 2011), goldfish (Nisembaum et al., 2012; Sánchez-Bretaño et al., 2015a,b), sea bream Sparus aurata (Vera et al., 2013) and Nile tilapia, Oreochromis niloticus (Costa et al., 2016).

**Food entrainment**

It is not surprising that food and food-related cues can influence many biological processes, including circadian rhythms. The ability of animals to anticipate food availability is well established and persists even when food is provided at a
different time point than that expected by animals (Richter, 1922; Stephan et al., 1979). When food delivery is restricted to the same time every day, fish, like other animals, display food-anticipatory activity (FAA) under a LD cycle (Chen and Tabata, 2002; Sánchez-Vázquez et al., 2001). Even when subjected to constant lighting conditions, fish can rapidly synchronize their activity pattern to the time at which they feed (Gee et al., 1994; Naruse and Oishi, 1994). Fish may also develop such a complex separate entraining pathway, but independently of that, the existence of the modulatory action exerted by a circadian oscillator appears demonstrated. For example, trout held under constant light and food restriction display both a free-running rhythm, which might be indicative of the influence of a light-dependent oscillator, and another one corresponding to the feeding schedule (Bolliet et al., 2001).

In goldfish, results suggest that food could be as powerful synchronizer as light is (Aranda et al., 2001). Experiments with goldfish and sea bass have provided data that agree with the hypothesis of the presence of food-entrainable oscillators (Sánchez-Vázquez et al., 1995a,b; 1997).

According to that above mentioned for light and food-dependent individual oscillators, the coexistence and their interaction with each other exists in fish. In this way, FAA disappeared in golden shiners, Notemigonus crysoleucas, after LD cycle is removed and persisted for several days, when daily meals are withheld (Reebs and Laguë, 2000). Such results did not let the authors to discard the presence of linked food- and light-entrainable oscillators, and the jeopardized functioning as consequence of the LEO´s damp out after the LD cycle is removed. The mechanisms involved in FAA in fish remain unknown, as location(s) of any putative feeding-entrained oscillator does. The existence of multiple peripheral oscillators that synchronize to each other according to food intake process and/or energy expenditure may help understanding FAA. Gut distension appears to play an important role in FEOs modulation, since meal size is more effective in modulating them than the amount of dietary energy supplied (Sánchez-Vázquez et al., 2001).

In addition, a link between cell metabolism and circadian clock has been purposed (Wijnen and Young, 2006). In this way, sirtuins (SIRT) are a group of enzymes in the Sir2 family (silent information regulator 2) initially described in yeast (Saccharomyces) and other groups of animals. These enzymes are deacetylases of NAD+-dependent proteins (Doi et al., 2006) that display a unique mechanism of deacetylation. The SIRT gene sequences are highly conserved, and to date seven members (SIRT1-7) have been reported in mammals, whereas yeast only have SIRT2 (Rine et al., 1979). All the SIRTs display class III-histone deacetylase activity, which directly relate them to gene transcription (Doi et al., 2006). SIRT1 is the homolog of yeast Sir2, and a clear role of SIRT1 in cell metabolism regulation, and the response to oxidative stress has been described (Rodgers and Puigserver, 2007; Haigis et al., 2010). Among metabolic processes with SIRT1 being involved, hepatic lipid metabolism and gluconeogenesis, pancreatic insulin secretion, fat cell accumulation
and maturation, central nutrient sensing, circadian regulation of metabolism, cell survival or cell senescence have been described under stressing conditions (Longo and Kennedy, 2006).

The involvement of SIRT1 has been described for the incidence of age-related diseases, such as metabolic disorders, cancer, and neurodegeneration (Sebastian et al., 2012). Besides the role played by SIRT1 in cellular metabolism, this protein also modulates the activity of AgRP and POMC neurons (Dietrich et al., 2010) and SF-1 neurons as well (Ramadori et al., 2010; 2011), which involves SIRT1 in the modulation of energy balance within central nervous system (CNS).

SIRT1 has been also reported to participate as modulator of circadian rhythms, since SIRT1 interacts with CLOCK-BMAL1, thus directly regulating the amplitude of the clock genes expression, through deacetylation of per2 and/or bmal1 (Nakahata et al., 2009). However, whether circadian system regulates the abundance of sirt1 or its activity remains unclear. Some studies in rodent report the circadian regulation of SIRT1 activity (Ramsey et al., 2009; Nogueiras et al., 2012). The expression of NAMPT, enzyme that controls a rate-limiting step in NAD⁺ biosynthesis, is driven by CLOCK-BMAL1. This regulation leads to circadian oscillation of cellular NAD⁺ levels, resulting in a cyclical regulation of SIRT1 activity (Ramsey et al., 2009; Nogueiras et al., 2012). All these studies point to a novel feedback loop in the circadian clock that involves CLOCK-BMAL1, NAMPT, NAD⁺, and SIRT1, and provide an important link between the circadian system and cell metabolism. In mammals, this link between SIRTs and the circadian system has been described for central tissues, but also outside the CNS. Peripheral oscillators probably do not respond to light as the main synchronizer as central oscillators do. The possible interactions of SIRT1 with CLOCK could have an important role in the regulation of peripheral circadian oscillators; this effect has been analyzed in mammalian liver (Asher et al., 2008; Nakahata et al., 2008).

Little is known about the regulatory role of SIRTs in fish cell metabolism and circadian activity. Studies carried out in Gasterosteus aculeatus describe the synchrony of changes of several isoforms of sirt1 and heat shock proteins (hsp60, hsp70, hsp90) during adaptation to cold temperatures. Accordingly, a role of NAD⁺ deacetylases has been proposed in mediating the metabolism- and oxidative stress-related responses (Teigen et al., 2015). In addition, our preliminary data are indicative of sirt1 participating in the response of the circadian system to stress in liver of rainbow trout (unpublished), which makes possible the existence of interactions between SIRTs and circadian system in fish.
1.2. Stress in fish

In teleost fish, stress is a condition that threatens the dynamic equilibrium or homeostasis, as consequence of the exposure to intrinsic or extrinsic stimuli, commonly defined as stressors (Wendelaar Bonga, 1997). When stressed, most vertebrates, including fishes, display a series of marked and uniform endocrine responses, with many organs and tissues being involved. This process is in general an adaptive response that, if successful, leads to recovery and restoration of animal homeostasis, but also to increased resistance to the same stressor (Wendelaar Bonga, 2011). Different stressors (physical, chemical, biological…) can act directly and disturb the normal physiological condition. The most frequent stressors in aquatic environments that affect fish are physical and chemical changes in water (temperature, salinity, oxygen content, pH), biological interactions with other organisms (competition, predation, etc.), presence of pollutants, etc (Gesto et al., 2010).

Depending on the duration, stress can be considered as acute or chronic. Acute stressors are events that animal experiences for a short time period, for example, handling procedures. On the other hand, chronic stress refers to a given situation that persists for longer time periods, thus with the physiological response of the animal being permanently activated (Tort and Teles, 2011). Whatever the case, stress may have severe negative effects on animal’s welfare. When applied to aquaculture, some routine activities are potential stressors, such as keeping animals in high stocking density, confinement, low water renovation, transport, alteration of lighting conditions and feeding schedule, etc. Under these conditions, fish typically display decreased growth rate that leads to a significant decrease of productivity. In order to deal with such problem and solve it, more research needs to be performed, which is a priority at this moment (Barton and Iwama, 1991).

1.2.1. The physiological stress response

When subjected to stress a coordinated set of behavioral and physiological responses initiate in the animal in order to compensate and/or adapt to the new situation. Then, the animal is enabled to overcome the threat. However, if the animal is experiencing intense stress, the response may lose its adaptive value and become dysfunctional, which can result in inhibited growth, reproductive failure, and decreased resistance to pathogens, among other negative effects (Barton and Iwama, 1991). The physiological responses to a stressor are either specific for a single, or a group of related stressors (more typical), or nonspecific, commonly observed in response to many different stressors. These responses typically involve all the levels of animal organization in a process known as integrated response to stress (Wendelaar Bonga, 1997). In fish, it involves three different components: primary, secondary, and
General introduction

tertiary stress responses (Pottinger, 2008). Brief information of each is herein provided.

Specifically for teleost, the primary response has been extensively studied. This response initiates with the integration of the stress signal within specific neural centers at the hypothalamus and these neurons respond to multiple sensory signals and once activated, different neurotransmitters such as dopamine (DA) and serotonin (5HT) are released, as well as neuropeptides, such as arginine-vasotocin (AVT) (Gesto et al., 2013; 2014; 2015b). In the other hand, the activation of chromaffin cells by sympathetic nerve endings (Figure7) leads to catecholamines (CAs: adrenaline, noradrenaline) release, as consequence of the activation of the brain-sympathetic-chromaffin cells (BSC) axis (Gfell et al., 1997). Then, blood levels of CAs increase soon after animal faces the stressor. It is typically observed that acute stressors such as handling and hypoxia result in a rapid increase of plasma metabolites and CAs, which associates to the activation of the BSC axis (McDonald and Milligan 1992).

In fish, normally the CAs producing cells, (chromaffin cells), are clustered in small groups within cortisol-producing interrenal cells, surrounding the edge of the posterior cardinal veins and their branches. Together with the surrounding lymphoid tissue, the interrenal cells constitute the interrenal gland, or head kidney, which is the homologs structure of the mammalian adrenal gland. The chromaffin cells also release other substances, such as DA, enkephalin, and natriuretic peptide, which may have CAs release-related functions. Autocrine and paracrine factors as well as hormones including cortisol play a modulatory role in CA release (Wendelaar Bonga, 2011). Therefore release of CAs is an integral part of the physiological response to stress in vertebrates (Hart et al., 1989).

In parallel, a second neuroendocrine pathway is activated, the HPA axis (Wendelaar Bonga, 1997; So et al., 2011), which in fish is named the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997; Löhr and Hammerschmidt, 2011). Once activated, corticotropin-releasing factor (CRF) synthesized in the hypothalamus is released, thus activating the synthesis and release of adrenocorticotropic hormone (ACTH) from the pituitary (Mommsen et al., 1999; Son et al., 2011). ACTH derives from the proopiomelanocortin (POMC) gene whose transcript is converted in a polypeptide that, besides ACTH, generates other endocrine factors, such as melanocyte-stimulating hormone (MSHs), endorphin, and lipotropin (Gonzalez-Nunez et al., 2003). ACTH stimulates synthesis and release of glucocorticoids, such as cortisol (Figure 7), from cells located at the adrenal cortex (mammals), or the interrenal cells in fish (Mommsen et al., 1999; Son et al., 2011).
Figure 7: Diagram of the BSC and HPI axes, showing the main neurohormonal and hormonal messengers involved in the organismal stress response, and the major effects of the most important messengers of these axes, adrenaline and cortisol; arrows pointing upward (green): stimulation; arrows pointing downward (red): inhibition. (Modified from Wendelaar Bonga, 2011).

Plasma cortisol levels start rising between several minutes and one hour after stress exposure, and drop gradually to baseline levels at several hours after stress incidence (Gesto et al., 2013). However when fish are subjected to chronic stress, plasma cortisol levels can remain elevated. This profile of cortisol has been reported for many fish species and different stressing conditions, ranging from handling and
disturbance, exposure to heavy metals or organic pollutants, rapid change of water temperature or pH, and even with predator-related cues (Barton, 2002; Aluru and Vijayan, 2009; Gesto et al., 2013). Adaptation processes to chronic stress has been described, and plasma cortisol levels appear to progressively decrease up to basal levels, even when stress persists (Mommsen et al., 1999), but different profiles have been reported. However, elevated plasma cortisol levels is the most widely used indicator of stress exposure in fish (Wendelaar Bonga, 2011).

The secondary response to stress is the consequence of the physiological changes induced by the high circulating levels of CAs and cortisol. Effects of CAs occur at the cardiovascular system, such as increased heart rate and blood pressure, but increased ventilatory activity and oxygen consumption are also described (Wendelaar-Bonga, 1997). In parallel, cortisol contributes for those changes, but also plays a modulatory role in the hydro-mineral balance, modulates breathing capacity and increases blood glucose levels, mainly by increasing hepatic gluconeogenesis (Mommsen et al., 1999). Although cortisol actions on intermediary metabolism are very extensive and complex, a wide range of responses can be observed since factors such as duration of stress and the species appear to influence the nature of such response (Barton, 2002; Tort et al., 2013). The following section provides detailed information at this respect.

Finally, the tertiary response includes physiological and behavioral changes occurring at the whole animal and even the whole population. These changes can be restorative, adaptive or pathological, including abnormal behavior, such as different food intake patterns, irregular social behavior or inhibition of some processes such as growth rate, reproduction cycles and immune response, among others (Wendelaar-Bonga, 1997; Bernier and Peter, 2001; Schreck et al., 2001; Gesto et al., 2010).

1.2.2. Cortisol as main mediator of stress response

As above mentioned, cortisol is synthesized in the adrenal gland of mammals, and the interrenal cells in fish, which locate at the head kidney. These cells are close to chromaffin cells, then probably exerting a paracrine control on them and influencing their activity (Mommsen et al., 1999). Cortisol biosynthesis is a conserved process that involves the microsomal enzymatic pathways, including 21-hydroxylation (P450c21), 17-hydroxylation (P450c17), and 3-hydroxy steroid dehydrogenation (3-HSD). In addition, fish possess mitochondrial inner membrane mono-oxygenases, such as the cholesterol side-chain cleavage enzyme (cytochrome P450scc) and the 11-hydroxylase (cytochrome P450c11) that catalyzes the 11-hydroxylation of deoxycortisol/deoxycorticosterone (Lehoux et al., 1972). Cholesterol input into the internal mitochondrial membrane is the limiting step of
steroidogenic process, for which the steroidogenic acute regulatory protein (StAR) plays a key role (Stocco and Clark, 1996). After several reactions inside the mitochondria and the sarcoplasmic reticulum, 11-deoxycortisol is converted into cortisol by CYP11B1 (Alsop and Vijayan, 2009; Liu et al., 2016) (Figure 8).

**Figure 8:** Biosynthetic pathway of cortisol in teleost fish. Pink area represents inner part of the mitochondria. StAR: steroidogenic acute regulatory protein; P450scc: desmolase; CYP17A1: 17, 20 desmolase; 3β-HSD: 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145); CYP21A: 12-hydroxylase; CYP11B1: 11β-hydroxylase.

Cortisol secretion is mainly regulated by the HPI axis. Accordingly, ACTH released from the adenohypophysis binds to melanocortin type 2 receptors (MCR2) at the interrenal cells, thus stimulating cholesterol uptake. Then, cortisol synthesis and release into blood are enhanced. Due to its lipophilic nature, cortisol typically enters into the target cell through passive mechanisms, although active uptake mechanisms have been described in mammals, for example in rat liver (Allera and Wildt, 1992), and fish, such as that reported for cultured trout hepatocytes (Vijayan et al., 1997). Once in the cell, cortisol binds to specific glucocorticoid receptors and regulates a given function. If not, it is metabolized by reductases, oxidoreductases and cytochrome P-450 dependent hydroxylases. These enzymes locate at the hepatobiliary system in fish (Idler and Truscott, 1972; Redding et al., 1984; Vijayan and Leatherland, 1990; Vijayan et al., 1994 a,b; Wilson et al., 1998).
The regulatory action exerted by cortisol takes place by binding to specific receptors. Two different glucocorticoid receptors have been described in mammals, mineralcorticoid receptors (MR), as type I, and glucocorticoid receptors (GR), as type II. In most fish species, there are two GR isoforms, GR1 and GR2 (Jaillon et al., 2004). GRs are found in a large number of tissues. Specifically for rainbow trout, liver, kidney, gill, intestine, muscle and brain have been reported to express GR (Ducouret et al., 1995; Teitsma et al., 1997; 1998). Binding of cortisol to specific receptors results on its activation, migration of the ligand/receptor complex, to dimerize, and to translocate to the cell nucleus, where it binds to DNA, thus affecting the transcription of specific target genes (Jewell et al., 1995). By other hand, cortisol can modulate the activity of the HPI axis through negative feedback mechanisms in mammals (Osterlund and Spencer, 2011), although it is not fully defined in fish (Stolte et al., 2008). In addition, binding of cortisol to specific melanocortin receptors might modulate steroidogenesis at the interrenal cells (Weendelar Bonga, 1997).

Cortisol, among glucocorticoids, is essential for maintaining basal homeostasis during stress exposure. Accordingly, energy metabolism is modulated by cortisol, for example by increasing plasma glucose levels as consequence of enhanced hepatic gluconeogenesis, and/or increasing the glycolytic potential of central organs, such as the brain (Wendelaar Bonga 1997; Mommsen et al., 1999). Our previous results in rainbow trout indicate that, when exposed to acute stress, fish display increased mobilization of liver glycogen that leads to increased production of endogenous glucose, reduced use of exogenous glucose, and reduced lipogenic potential (López-Patiño et al., 2014b). Cortisol in fish has also an important role in many other processes, such as behavior, growth, reproduction, and osmoregulation (Mommsen et al., 1999; Tort et al., 2013).

Cortisol displays a daily profile that can be altered during stress exposure, but the nature and duration of the stress influence such profile even in fish. Then, under acute stress, plasma cortisol levels increase soon after stress exposure and remain elevated for up to one hour, thus gradually decreasing to those levels of non-stressed individuals. Mostly cortisol can be considered as marker of stress exposure, but it is usually measured in plasma thus requiring blood collection, then developing new non-invasive techniques may be an advantage in order to identify whether fish are subjected to stress or not (Gesto et al., 2013; 2015a,b).

On the contrary, when some species are subjected to chronic stress, the longer the duration of stress exposure, the more cortisol levels stabilize to those comparable of non-stressed individuals (Mommsen et al., 1999). As above mentioned, one of the most negative effects of chronic stress in fish is growth inhibition, for which cortisol appears to be a main responsible. In fact, cortisol administration results in inhibited growth in parallel to reduced appetite, food intake and decreased intestinal absorption of food (De Boeck et al., 2001; Pottinger, 2008; Gesto et al., 2010; Conde-Sieira et al., 2014).
1.2.3. Stress and circadian physiology

The daily profile displayed by cortisol makes the hormone a potential candidate as synchronizer of different behavioral and/or physiological functions. Accordingly, cortisol, as melatonin, is considered one of the more robust hormonal outputs of the circadian system (see rev. Isorna et al., 2017). The glucocorticoid displays daily rhythms of plasma levels in mammals, with the acrophase occurring at the onset of the activity phase, i.e., diurnal species show peaking levels of cortisol at the beginning of the light phase, whereas nocturnal species have highest levels at the day-night transition (Mohawk and Lee, 2005; Son et al., 2011; Kalsbeek et al., 2012). This pattern anticipates the active phase of the animal and relates to the role of cortisol as an arousal signal, which prepares the organism for the upcoming phase of activity (Born et al., 1999; Haus, 2007; Zisapel, 2007). The sympathetic nervous system and the SCN seem to play an important role in the control of these rhythms (Haus, 2007; Tonsfeldt and Chappell, 2012). However, increasing evidence reveal that multiple components of the circadian system (specially the adrenal clock) are involved in the regulation of glucocorticoid rhythmicity in mammals (Son et al., 2011; Spiga et al., 2014).

The conserved profile of circulating glucocorticoids is not clearly evident in fish, with a wide variety of daily patterns of plasma cortisol being described, and a dependence of factors such as species, photoperiod, season, feeding schedule, and activity rhythm being reported to influence such pattern. Regarding LD cycle, acrophase of cortisol rhythm occurs at the early photophase in gulf killifish (Fundulus grandis; García and Meier, 1973), Mozambique tilapia (Oreochomis mossambicus; Nikaido et al., 2010) and rainbow trout (Hernández-Pérez et al., 2015), at the beginning of the scotophase in sea bass (Planas et al., 1990), tench (De Pedro et al., 2005) and sole (Solea senegalensis; López-Olmeda et al., 2013) and during the scotophase in brown trout (Salmo trutta; Pickering and Pottinger, 1983) and Japanese char (Salvelinus leucomaenis; Yamada et al., 2002). By other hand, two peaks of cortisol levels were observed in sea bass (Cerdá-Reverter et al., 1998) and Atlantic salmon smolts (Ebbesson et al., 2008). Additionally, there is evidence for seasonal variations of cortisol rhythms in salmonids (Rance et al., 1982; Pickering and Pottinger, 1983; Thorpe et al., 1987), cyprinids (Kuhn et al., 1986), and perciforms (Planas et al., 1990).

Daily rhythms in the HPA axis have been reported in mammals (Kalsbeek et al., 2012, Spiga et al., 2014). However, the existence of rhythms in the HPI axis components of fish has not been deeply investigated. A daily rhythm of circulating ACTH was demonstrated in goldfish (Singley and Chavin, 1976), and rhythms of mRNA abundance of genes encoding pomc (Gilchriest et al., 1998; López-Olmeda et al., 2013), corticotropin-releasing hormone (CRH; López-Olmeda et al., 2013) and crh1 receptor (Azpeleta et al., 2012) have been described. The parallelism observed...
between crh expression and plasma cortisol in sole did lead to suggest that the daily cortisol rhythm might be a consequence of hypothalamic crh production (López-Olmeda et al., 2013).

The recent discovery of a rhythmic expression of clock genes in the adrenal tissue of mammals supports the hypothesis of the presence of a functional circadian clock in this gland (Nicolaides et al., 2014; Spiga et al., 2014). However, only one report describes in fish the rhythmic expression of clock genes in interrenal tissue, where the 24 h rhythms of per1a, per3 and cry3 in goldfish are in antiphase with that of clock1a, as expected for a functional core clock (Azpeleta et al., 2012). These data indicate that the interrenal tissue may act as a node within the circadian system network in fish.

The interaction between glucocorticoids and the circadian system has been demonstrated, as long as some experiments showed that the glucocorticoid hormone analogue, dexamethasone, is able to reset the circadian rhythm of clock genes expression in multiple peripheral cell types of rat (Balsalobre et al., 2000). Thus, a key role for the circadian output of the adrenal gland in synchronising peripheral clocks in mammals, has been proposed (Dickmeis, 2007; Kalsbeek et al., 2012; Schibler et al., 2015). There are not many data at this respect in fish, but the currently available results are indicative of the same role. In this way, bioluminescence rhythms of zebrafish and cavefish zfper1b-luc-transfected cell lines are entrained by dexamethasone (Cavallari et al., 2011). Such entrainment is apparently mediated by glucocorticoid induction of per1, as reported for goldfish liver at both in vivo and in vitro conditions (Sánchez-Bretaño et al., 2016) and for cavefish cell lines (Cavallari et al., 2011). In the same way, whereas mammalian glucocorticoid-responsive elements are found in per1 promoter (Reddy et al., 2007), dexamethasone represses the positive elements of the clock (clock1a and bmal1a) in cultured goldfish liver (Sánchez-Bretaño et al., 2016), which is in support of the resetting function exerted by glucocorticoids in fish. Finally, a widespread distribution of glucocorticoid receptors in fish is described (Prunet et al., 2006), in consistency with our preliminary data obtained in rainbow trout (unpublished), which is in support of the role of cortisol in entraining different peripheral and central oscillators.

Instead of glucocorticoids playing a role as temporal signals in that related to circadian functional organization, physiological functions that are regulated by glucocorticoid-sensitive clocks are not fully identified yet in fish. Then, cortisol is described as a component of a signalling pathway that is required for circadian cell cycle rhythmicity in zebrafish (Dickmeis et al., 2007), and does apparently participate in the regulation of the rhythmicity of melatonergic system in rainbow trout (Benyassi et al., 2001; Ceinos et al., 2008; López-Patiño et al., 2014a), catfish (Clarias gariepinus; Yanthan and Gupta, 2007) and Mozambique tilapia (Nikaido et al., 2010).
In summary, it looks like cortisol acts as an input, but may also play an important role as an output of the circadian system in fish. However, further investigation is needed in order to demonstrate such role and to explore other unsolved questions.

1.3. Food intake in fish

Animal nutrition involves different processes such as food intake, digestion, nutrients absorption and metabolism, and finally waste production. Among them, food intake regulation is a complex process that requires the interaction between different systems, such as circadian system and those involved in homeostatic control, like the CNS and GIT, among others. Then, food intake regulatory structures can locate inside or outside the CNS, where food intake-related information is integrated.

Food intake is regulated through positive and negative loops acting at different locations and time points (Langhans and Scharrer, 1992; Langhans, 1999). The positive loop (food intake stimulation) results from the relationship among prior experience with nutrient availability, status of the animal and sensory qualities of the food. The negative loop relates to metabolic and gastrointestinal inputs displaying changes prior and after nutrient absorption (Langhans, 1999). Three regulatory levels have been suggested following this model: (i) short-term regulatory factors: those influenced by the size of a single meal, (ii) mid-term regulatory factors: those operating through several days; and (iii) long-term regulatory factors: those operating through longer time periods (weeks, months, and years), thus reflecting the energy balance of the animal.

The integration of information involved in the control of food intake takes place in mammals through a circuit that mainly localizes in hypothalamic areas including arcuate, ventromedial, paraventricular, and lateral hypothalamus (Berthoud and Morrison, 2008; Zheng and Berthoud, 2008). This circuit (Figure 9) receives signals form the gut, pancreas, liver, and adipose tissue, in addition to other brain regions, and integrates these inputs (Richards and Proszkowiec-Weglarz, 2007). It is in those hypothalamic areas where the action of different food intake regulators takes place. A large number of feeding regulators that influence food intake by stimulating or inhibiting is described (De Pedro and Björnsson, 2001; Volkoff et al., 2005; 20116; Soengas et al., 2008; Delgado et al., 2017). Some of them are:

1) Appetite stimulators (orexigenic):
   - Central: Neuropeptide Y (NPY), Agouti-Related Protein (AgRP), Galanin, Orexin, Melanin-Concentrating Hormone (MCH), Apelin.
   - Peripheral: Ghrelin.
2) Appetite inhibitors (anorexigenic):

- Central: Corticotropin-Releasing Hormone (CRF), Cocaine- and Amphetamine-Regulated Transcript (CART), Melanocortins, Monoamines (CAs, 5HT).

- Peripheral: Leptin, Insulin, Cholecystokinin (CCK), Bombesin/Gastrin Releasing Peptide (BBS/GRP), Melatonin, Amylin.

**Figure 9:** A proposed model describing the long-term regulation of appetite and energy balance to achieve a stable body weight in poultry that integrates peripheral tissue and central nervous system circuits regulated by hormonal, neural, neuroendocrine, and nutrient signaling mechanisms. NPY: neuropeptide Y; AgRP: agouti-related peptide; POMC: pro-opiomelanocortin; CCK: cholecystokinin; GLP-1: glucagon-like peptide-1; PYY: peptide YY. (Denbow et al., 2015).

In fish, the mechanisms involved in food intake regulation appear to be similar to those of mammals with some differences that relate to the existence of specific processes (Kulczykowska et al., 2010; Hoskins et al., 2012; Volkoff, 2016). Early studies revealed that electrical stimulation of inferior lobes of the hypothalamus, ventral telencephalon and the optic tectum elicited a feeding response in several fish species (Demski and Knigge, 1971; Demski 1973; Roberts and Savage, 1978). It was also suggested that inputs from telencephalon and optic tectum activate hypothalamic centers and evoke feeding behavior (Kulczykowska et al., 2010). In addition, hypothalamus receives information about the nutritional, energetic and environmental status of the organism through peripheral and central inhibitory and/or stimulatory
inputs. In fish, like in mammals, the hypothalamus is the primary center of food intake regulation, thus the hypothalamus receives, integrates and transmits relevant internal and external signals (Naslund and Hellstrom, 2007), (Figure 10).

In fish, the central neural network involved in food intake regulation includes two type of neurons (Mobbs et al., 2005; Blouet and Schwartz, 2010; Waterson and Horvath, 2015). The first type synthesizes feeding stimulators (orexigenic), whereas feeding inhibitors (anorexigenic) are produced by the second one. On the following paragraphs, a brief description of the main food intake modulators is provided.

![Figure 10: Schematic representation illustrating the main signaling elements involved in the hypothalamic control of food intake in fish. CART: cocaine- and amphetamine-related transcript; CRF: corticotropin-releasing factor; POMC: pro-opiomelanocortin; NPY: neuropeptide Y.](image)

1.3.1. Food intake stimulators

**Neuropeptide Y (NPY)**

This peptide is one of the most orexigenic factors in mammals (Halford et al., 2004) and fish (López-Patiño et al., 1999). NPY is abundant in the CNS, particularly in hypothalamic nuclei such as the arcuate nucleus and paraventricular nucleus, both key areas in the regulation of feeding behavior (Halford et al., 2004). NPY belongs to a family of peptides together with peptide YY (PYY), pancreatic polypeptide (PP), and peptide Y (PY). All them peptides are reported to bind to specific G-protein-coupled receptors, the Y family, which is composed by five members, named Y1, Y2, Y4, Y5, and Y6 (Larhammar et al., 2001). It has been reported that neuropeptide Y-related peptides are potent appetite stimulators in fish and their sequence has been described as well (Liang et al., 2007; Kehoe et al., 2007).
Neuropeptide Y neurons are widely distributed in the CNS and NPY immunoreactive fibres have been identified in the pituitary and GIT of many fish species (Volkof et al. 2005; Volkof, 2006). Generally, NPY is known as a brain signal involved in feeding control in fish. In this way, npy mRNA levels in the hypothalamus of goldfish increase in food deprived animals (Narnaware and Peter, 2001a). Similar results have been reported for salmon (Oncorhynchus kisutch) and catfish (Ictalurus punctatus) (Silverstein et al., 1999). In addition, re-feeding 72 h-starved goldfish reverses the increase of npy mRNA levels observed during starvation (Narnaware et al., 2000). Intracerebroventricular injection (ICV) administration of NPY induces a dose-dependent increase of food intake in previously satiated goldfish (López-Patiño et al., 1999; De Pedro et al., 2000), salmon (Narnaware et al., 2000), and catfish (Silverstein and Plisetskaya, 2000).

The regulatory action of NPY on food intake in both mammals and fish has to be a balance between the effects of the peptide and other appetite regulators, such as CRF and cortisol (Bernier et al., 2004), CART (Volkoff and Peter, 2000), leptin (Volkoff et al., 2003), MCH (Matsuda et al., 2007), orexin and galanin (Volkoff and Peter, 2000), growth hormone (GH) (Mazumdar et al., 2007) and ghrelin (Miura et al., 2006) among others.

Other food intake stimulators that have been tested in fish are: AgRP in goldfish (Cerdá-Reverter and Peter, 2003), zebrafish (Song et al., 2003), and puffer fish Takifugu rubripes; Klovins et al., 2004); Galanin in goldfish (Volkoff and Peter, 2001) and tench (De Pedro et al., 1995); Orexiins in goldfish (Nakamachi et al., 2006) and zebrafish (Novak et al., 2005; Nakamachi et al., 2006); MCH in goldfish (Cerdá-Reverter and Peter, 2003) and Platichthys flesus (Takahashi et al., 2004); Apelin in Astyanax mexicanus (Penney and Volkoff, 2014) and goldfish (Volkoff and Wyatt, 2009). Peripheral food intake stimulators have been also reported in fish. In this way, Ghrelin stimulates food intake in goldfish, tilapia, and rainbow trout (Unniappan et al., 2002; 2004a,b; Riley et al., 2005; Matsuda et al., 2006; Miura et al., 2006; 2007).

1.3.2. Food intake inhibitors

CART

POMC neurons at the arcuate nucleus are responsible of CART synthesis. This anorexigenic peptide was isolated for the first time from ovine hypothalamus (Spiess et al., 1981). After that, CART expression has been also demonstrated in other vertebrates including fish (see rev. Subhedar et al., 2014). CART peptide was localized in catfish brain (Singru et al., 2007), and in situ hybridization studies revealed where cart expresses within zebrafish brain (Nishio et al., 2012; Akash et al., 2014). This peptide expression increased soon after cocaine and amphetamine
administration (Kuhar et al., 2002), which the anorectic properties of the peptide were proposed for mammals (Kristensen et al., 1998), and fish (Volkoff and Peter, 2000). In addition, CART mRNA abundance was reported to decrease with food deprivation in cod (Kehoe and Volkoff, 2007), goldfish (Volkoff and Peter, 2001), and Atlantic salmon (Murashita et al., 2009), and increases with re-feeding in channel catfish (Kobayashi et al., 2008). Post-prandial changes of CART have been also described in channel catfish (Peterson et al., 2012), goldfish (Volkoff and Peter, 2001), and dourado, Salminus brasiliensis (Volkoff et al., 2016).

**Melanocortins**

These compounds participate in food intake control and energy homeostasis in fish, as in mammals (Cone, 2006). Melanocortins are a group of pituitary compounds that include ACTH and α-, β-, and γ-MSH, all from the same precursor, POMC, which is subjected to cell-specific post-translational cleavage (Metz et al., 2006). Interaction of melanocortins with other food intake regulators has been reported in mammals. Accordingly, NPY inhibits POMC expression (Rahmouni et al., 2001), whereas leptin stimulates it within the arcuate nucleus (Gorissen et al., 2006). In situ hybridization analyses carried out in fish brain did lead to localize POMC-expressing neurons at the hypothalamus (Cerdá-Reverter et al., 2003). The presence of α-MSH and β-endorphin within the same POMC expressing cells have been also reported within fish hypothalamus (Forlano and Cone, 2007; Chabbi and Ganesh, 2016), which is indicative of POMC being processed into these peptides. The modulatory effect of melanocortins on food intake has been also observed in fish, since ICV administration of melanocortin 4 receptor (MC4R) agonists or antagonists to 24 h fasted fish resulted in the respective inhibition or stimulation of food intake (Cerdá-Reverter et al., 2003; Kobayashi et al., 2008).

**CRF**

CRF is, together with glucocorticoids, a component of the HPA axis. CRF stimulates the release of ACTH from the pituitary, thus enhancing cortisol secretion by the interrenal tissue (Flik et al., 2006). In addition, CRF participates in the regulation of energy balance (Richard et al., 2002; Bernier, 2006). CRF system is composed of a family of related peptides, two main receptor types, CRF-R1 and CRF-R2, and CRF-binding protein (Bale and Vale, 2004). The ligand family members include CRF, several urocortins (UCN), fish urotensin I (UI), and amphibian sauvagine (Lewis et al., 2001; Reyes et al., 2001). A role as food intake regulator was reported for CRF in mammals, as an anorexigenic peptide (see rev. Heinrichs, 1999). Similarly to that in mammals, the role CRF in food intake regulation was reported for fish, since food intake was inhibited in goldfish following ICV administration of the peptide (De Pedro et al., 1993). In support of that anorexigenic role, fasted Ya-fish,
Schizothorax prenanti, displayed decreased crf mRNA abundance (Wang et al., 2014), and exposing goldfish to fluoxetine results in inhibited food intake, together with increased crf expression (Wang et al., 2014). Specifically for rainbow trout, ICV administration of CRF or urotensin I resulted in decreased food intake (Ortega et al., 2013). Also, subjecting salmonids to hypoxia suppressed appetite and enhanced crf and urotensin 1 mRNA levels in forebrain, which is indicative of the role of CRF-related peptides on food intake regulation in this vertebrate group, by either inhibiting food intake per se, or by activating the physiological responses to stress that many result in the inhibition of food intake (Bernier and Craig, 2005).

Although some results are indicative of CRF-related peptides as mediators of the anorectic effects of other food intake regulators in fish, such as 5HT (De Pedro et al., 1998b), overall knowledge of how CRF-related peptides interact with them is still in its infancy.

Other food intake regulators

Monoamines

Hypothalamic monoamine neurotransmitters have been reported to influence food intake in fish (Kulczykowska and Sánchez-Vázquez, 2010). Both norepinephrine (NE) and DA are involved in the hypothalamic response to starvation in goldfish; the noradrenergic system is activated whereas the dopaminergic is inhibited in fasted fish (De Pedro et al., 2001). Also 5HT is involved in the control of feeding behavior in fish, acting as an anorectic neurotransmitter (Kulczykowska and Sánchez-Vázquez, 2010). Specifically for rainbow trout, administration of a 5HT-releasing agent, fenfluramine, inhibits food intake (Ruibal et al., 2002). 5HT central anorectic action may be independent or mediated, at least in part, by CRF, as reported for goldfish (Ruibal et al., 2002; De Pedro et al., 1998b). However, this effect appears to be mediated by specific 5HT receptors. Accordingly, hypothalamic ICV administration of 5HT2C receptors agonists resulted in increased expression of several anorectic peptides, including pomc, cart and crf (Pérez-Maceira et al., 2014). By other hand, the activation of 5HT1A receptors by specific agonists also inhibits food intake in trout, although this effect is apparently independent of changes in the anorectic peptides (Mancebo et al., 2013; Pérez-Maceira et al., 2016). Therefore, increased 5HT acting through different receptor subtypes and brain locations could be, at least in part, responsible of the maintenance of the endocrine response in order to modulate feeding behavior.

Peripheral food intake modulators

Other hormones participate as inputs to the central nervous system, thus bringing metabolic status-related information or that of presence/absence of food
within certain parts of the GIT. In this way, leptin and insulin influence food intake as metabolism-related signals, whereas the gastrointestinal hormones, ghrelin and CCK, supply information relative to the presence of food and its composition within the gut (Blouet and Schwartz, 2010). These hormones modulate the activity of fatty acid- and glucose-sensing systems, and mRNA abundance of food intake control-related neuropeptides within the hypothalamus (see rev. Delgado et al., 2017). Then, in fish, peripheral feedback signals, including nervous inputs, GIT peptides, leptin, cortisol, glucose and insulin are integrated by the hypothalamic food intake regulatory center (Figure 11).

1.3.3. Rhythmicity of food intake-related parameters

Energy availability is important for living organisms to cope with their daily requirements. Specialized systems, such as the digestive system, in addition to feeding-related behaviors have been developed for that purpose. Regarding feeding behaviors, most animals display food anticipatory activity (FAA), which is only possible when they are subjected to restricted temporal windows of food availability. FAA is characterized by an increase of locomotor activity and core body temperature before feeding (Davidson et al., 2003). In mammals, external cues are not needed at all for FAA to appear, and that activity is daily triggered when energy depletion reaches some threshold before feeding time.

In poikilotherms, feeding is also a rhythmic process for which the interaction of biotic (prey availability, hierarchies, social behavior), non-biotic (photoperiod, temperature) and internal (biological clocks, metabolism-related cues) factors influence (Boujard and Leatherland, 1992; Madrid et al., 2001). In fish, rhythms of food activity and metabolic requirements can strongly depend on environmental factors such as temperature and photoperiod, but also these rhythms persist even in the absence of such rhythmic environmental factors. This is indicative of a given rhythm to be generated by an internal mechanism, a biological clock. Self-sustained rhythms usually persist for many days, even while free-run, in contrast to those rhythms that disappear soon after the external cues cease (Kulczykowska et al., 2010).

Fish food intake-related behaviors (such as locomotor activity) in parallel with other rhythmic physiological processes can be affected by light (Kulczykowska et al., 2010). By other hand, some studies in fish have demonstrated that the daily rhythm of food intake is synchronized by central and peripheral clocks (Feliciano et al., 2011; Nisembaum et al., 2012; Vera et al., 2013). Central clocks are apparently responding to main circadian inputs (light, temperature), whereas feeding related cues, including feeding time, nutritional inputs, feeding/fasting cycle, and diet composition are the dominant synchronizers of peripheral clocks (Delgado et al., 2017).
Most fish species do not feed permanently during the 24 h cycle, thus displaying a specific day/night profile. According to their active phase, species can be classified as diurnal or nocturnal. However, some fish species, such as sea bass, are able to shift their activity pattern in one or another way. At this respect, it was suggested that some sort of gradual, progressive change of photoperiod or water temperature is required to trigger the phase inversions of feeding rhythms, but also supports the existence of an endogenous circa-annual clock being involved in the control of the diurnal/nocturnal feeding behavior of this fish species (Kulczykowska et al., 2010).

When food is limited to certain time of the day, feeding schedule acts as time cue for animal to temporally integrate the food intake-related rhythms. Most fish can reset the internal clock and synchronize it to feeding time. However, other species do not synchronize their rhythm of locomotor activity even when feeding time shift. For example, tench (*Tinca tinca*) feeds exclusively at night, thus displaying peaks of activity at this temporal window. When food is presented at day-time, tench continues to display a nocturnal activity pattern (Herrero et al., 2005).

Whether light or food are influencing food intake-related rhythmic patterns and the mechanisms involved is a topic that requires further investigation, but it appears that species-specific mechanisms participate in the entrainment of such rhythms. This
leads to the existence of food entrained clocks located at peripheral organs (Nisembaum et al., 2012; Sánchez-Bretaña et al., 2015a) or in central brain regions (Feliciano et al., 2011; Vera et al., 2013; Sánchez-Bretaña et al., 2015b). Research carried out in goldfish suggests that feeding time is a zeitgeber that synchronizes the central oscillator, which also drives the rhythm of food intake regulators such as NPY (Vera et al., 2007) among others. In addition, the same authors describe that NPY might participate as a signal responsible of the food anticipatory activity, since peaking levels of the neuropeptide are detected 2 hours before feeding time (for a rev., Cowan et al., 2017). Specifically for rainbow trout, it appears that a central circadian oscillator located within the photoreceptor cells of the pineal organ does not control feeding rhythm, as long as pinealectomized trout does not display jeopardized rhythms of food intake (Sánchez-Vázquez et al., 2000).

### 1.3.4. Stress and food intake

Reduced food intake is a behavioral response observed in long-fasting stressed fish (Schreck et al., 1997; Wendelaar Bonga, 1997). In addition to suppressed appetite, another feeding-related behaviors can be disrupted by stress exposure in fish, such as food searching, finding, or capturing (Beitinger, 1990). In addition, different stressors, including environmental (low pH, high ammonium concentration, low oxygen and pollutants), social (subordination and crowding), or physical challenges (handling), or stress duration (chronic or acute) have shown to inhibit food consumption in this vertebrates group (Schreck et al., 1997; Wendelaar Bonga, 1997).

Research carried out in teleosts reveals that, as in other vertebrate groups, CRF is a key modulator of the stress response (Lovejoy et al., 1999; Flik et al. 2006). CRF in vitro administration stimulates secretion of ACTH (Fryer et al., 1983), (Tran et al., 1990), and thyrotropin secretion from fish pituitary cells (Larsen et al., 1998). However, CRF-related peptides appear to also modulate locomotor activity (Lowry and Moore, 2006) and food intake (Bernier, 2006) in fish, and increasing evidence point at CRF to participate as the endogenous mediator of the stress-related inhibition of food intake (Bernier and Peter 2001a; Bernier, 2006). On the other hand, ACTH binds to specific melanocortin receptors (MC2R) at the interrenal cells of the head kidney, thus stimulating glucocorticoids release (Wenderlar Bonga, 1997; Mommsen et al., 1999), and an inhibitory effect of food intake has been reported after ACTH administration in zebrafish (Agulleiro et al., 2013). Then, stress information can be transduced by CRF and ACTH, resulting in food intake inhibition.

In fish brain, corticotropic cells are involved in both the control of food intake, and that of glucocorticoids secretion at the interrenal cells of the head kidney. At this respect, cortisol, as the main glucocorticoid in fish, is the main mediator of the stress response, thus participating in the negative effect of stress on different behavioral and
physiological functions, including food intake (Bernier et al., 2001a; Barton, 2002; Bernier, 2006; Aluru and Vijayan, 2009). Specially, chronic stress relates with food intake inhibition and somatic growth, independently of the nature of the stressor. For example, prolonged crowding (Pickering and Pottinger, 1989; Trenzado et al., 2006), repeated handling (Barton et al., 1987; Hoskonen and Pirhonen, 2006), poor water quality (Schram et al., 2009) and social subordination (DiBattista et al., 2006) are common chronic stressors that negatively affect growth or food intake. The activation of the HPI axis (Wendelaar Bonga, 1997; Bernier et al., 2009) results in the mobilization and redistribution of energy resources. Cortisol is the main responsible of such effects (Small et al., 2008), as observed following exogenous cortisol administration (see rev. Mommsen et al., 1999; Bernier, 2006).

Similarly to that observed in other vertebrate groups, monoaminergic systems also participate as mediators of the stress response in fish, and the involvement of monoamines in food intake control has been also described (Øverli et al., 2005; Winberg et al., 2016). For example, trout subjected to social stress display lower food intake than non-stressed animals, together with increased brain turnover of 5HT, DA and NE and their respective metabolites, which is indicative of increased monoaminergic activity. Preliminary results obtained in our laboratory are in the same way (unpublished). The involvement of monoaminergic systems on food intake regulation and the interaction with stress in fish was also demonstrated in goldfish subjected to ICV administration of CRF, in which reduced food intake was observed, together with decreased hypothalamic content of NE and DA, and with this effect being prevented by alpha-helical CRF<sub>(9-41)</sub>, and CRF-receptor antagonist (De-Pedro et al., 1997). Soon after, the anorectic effect of CRF-like peptides in goldfish was reported to be mediated by α<sub>1</sub>-adrenergic, and dopaminergic receptors (De-Pedro et al., 1998a). In addition, CRF release from in vitro cultured telencephalons of tilapia was enhanced by NA and 5HT addition (Pepels et al., 2004). Thus, the inhibitory effect of DA (Leal et al., 2009) and 5HT (Ortega et al., 2013; Pérez-Maceira et al., 2013) on food intake in fish and their interaction with the stress-related HPI axis (CRF) exist in this vertebrate group. Detailed information about monoaminergic systems is provided on the following section.

### 1.4. Monoaminergic systems in fish

Within the vertebrate’s brain, monoaminergic neurons (Cooper et al., 1986) participate in a wide range of functions. Monoaminergic neurotransmitters have an amino group connected to an aromatic ring by a carbon-carbon chain (Yousuf et al., 2016), and include the indoleamine 5HT, and the CAs, DA, NE and epinephrine (E).

Monoamines synthesis and metabolism are similar in all vertebrate groups. Regarding 5HT, synthesis begins when the amino acid tryptophan (Trp) is subjected
to a cascade of enzyme activities (Figure 12). In the first one, Trp is converted into 5-hydroxytryptophan (5HTP) by the enzyme tryptophan-5-hydroxylase (TPH). TPH activity is dependent of Trp availability, and its expression mainly occur in serotonergic cells. Two isoforms of TPH exist, TPH1 and TPH2, and differentially locate all over the organism in a vertebrate group-dependent way. On the following step of 5HT synthesis, 5HTP is decarboxylated into 5HT by aromatic L-amino acid decarboxylase (AAD) activity, a non-specific decarboxylase that is found all over the body. The activity rate of this enzyme determines intracellular levels of 5HTP in teleost serotonergic neurons (Winberg et al., 1993; Gesto, 2008; Gesto et al., 2008). By other hand, 5HT is metabolized in serotonergic cells following two main routes, the first one involving monoamine oxidase the consecutive activities, monoamine oxidase (MAO) and aldehyde dehydrogenase which convert the monoamine on its main metabolite, 5-hydroxyindoleacetic acid (5HIAA); the second route involves MAO and aldehyde reductase enzyme activities that lead to 5-hydroxytriptophol (5HTOH) synthesis.

On the other hand, regarding CAs, special attention is paid on DA synthesis, which iniciates with the aminoacid tyrosine begin converted in DA after consecutive enzyme activities (Figure 13), i.e. Tyrosine hydroxylase (TH), and AAD. The main route of DA metabolism includes MAO and Aldehyde dehydrogenase (ALDH) enzyme activities, with the final metabolite, 3,4-dihydroxyphenylalanine (DOPAC), being transformed into homovalinic acid (HVA) by catechol-O-methyl transferase (COMT). This enzyme can also initiate DA metabolism, thus converting the monoamine into 3-methoxytryptamine (3-MT), that metabolizes into HVA after MAO and ALDH activities (Winberg et al., 1993).

With respect to fish, previous reports do not indicate the prevalence of any main metabolite of DA (Dulka et al., 1992). Accordingly, HVA was reported as the major metabolite in crucian carp, Carassius carassius (Nilsson, 1989; 1990), whereas DOPAC seems to be the predominant one in goldfish (Sloley et al., 1992; Dulka et al., 1992). Specifically for rainbow trout, 3-MT is apparently the major DA metabolite (Saligaut et al., 1990), but our preliminary data point to DOPAC to be more relevant one. The reason for such discrepancies between studies might relate to species-specific differences in DA catabolism. For example, the rate of conjugation and/or clearance of DA metabolites from brain differs between/within species (Winberg et al., 1993).

By other hand, monoamines are stored in the cells within vesicles that release their content into the synaptic cleft by exocytosis, once the presynaptic membrane depolarizes (Figure 14). Then, monoamines bind to specific membrane receptors at the postsynaptic cell. The synaptic effects of monoamine neurotransmitters end by different mechanisms, such as the re-uptake into presynaptic terminals among others, but also with the possible involvement of glial cells (Hansson, 1983; Katz and Kimelberg, 1985).
Due to their involvement in modulating a large number of functions, monoaminergic systems have been extensively studied in mammals. Thus, it was reported for them to participate in the control of several behavioral functions, such as aggressive behavior (Mason, 1984; Miczek and Donat, 1989; Olivier et al., 1989), mating (Meyerson and Malmnb, 1978), and feeding (Leibowitz, 1992). In addition, monoaminergic systems participate in the physiological response to stress (Dunn, 1989), and the regulation of autonomic and neuroendocrine functions (Tuomisto and Mlnnisti, 1985). Compared to that in mammals, available information about brain
monoaminergic functions in non-mammalian vertebrates is scarce. Specific information is detailed on the following sections.

**Figure 13**: Pathways of dopamine metabolism. TH: tyrosine hydroxylase; AAD: aromatic L-amino acid decarboxylase; MAO: monoamine oxidase; ALDH: aldehyde dehydrogenase; ALRD: aldehyde reductase; DBH: dopamine-β-hydroxylase; PNMT: phenylethanolamine-N-methyltransferase; COMT: catechol-O-methyl transferase.
**Figure 14**: Schematic diagram of the proposed major routes of inactivation of the monoamine neurotransmitters in mammalian brain. The enzyme monoamine oxidase, located in the mitochondrial cell wall degrades 5HT, DA, and NA into the compounds 5HIAA, DOPAC, and 3, 4-dihydroxymandelic acid (DHMA), respectively. A second enzyme, catechol-O-methyltransferase (COMT) acts on DA and, to a lesser extent, NA, forming the products 3-methoxytyramine (3-MT) and normetanephrine. Transporter proteins located in the presynaptic membrane allow reuptake of monoamines into the presynaptic neuron, although DA transporters are also located extra-synaptically (Lewis et al., 2001; Modified from Barnett *et al.*, 2011).

### 1.4.1. Serotonergic system

The organization of the monoaminergic systems is apparently well conserved in vertebrates, especially that of 5HT (Lillesaar, 2011). Similarly to mammals, 5HTergic cell bodies in fish mainly localize at the raphe nucleus within the hindbrain. However, in contrast to that of mammals, teleost 5HTergic cell bodies are also found in regions others than raphe, such as pretectum, basal forebrain, ventral thalamic and hypothalamic areas (Winberg *et al.*, 1993; Lillesaar, 2011). Specifically for rainbow trout, these cell bodies also localize in diencephalon and mesencephalon (Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Lillesaar, 2011).

Serotonergic neurons in fish can be identified by assessing TPH expression, which is mostly exclusive of such neurons. However, different subtypes of TPHs exist in fish. For example, in zebrafish (*Danio rerio*) different paralogues of TPH are reported, with TPH2 expressing in the raphe, whereas TPH1a and TPH1b predominate in diencephalic cells and in the gustatory tract (Anderson and Caio, 2014). Another isoform (TPH3) expresses in several hypothalamic areas of this species (Ren *et al.*, 2013).
Independently of where 5HT synthesis occurs, the rate at which the monoamine is synthesized depends on Trp availability, which is provided with food. Then, diet composition can influence 5HT synthesis (Russo et al., 2009). Based on previous reports, the involvement of Trp (and thus 5HT system) in monitoring homeostatic challenges was reported (Russo et al., 2009), and this might be applicable in fish only in TPH2 expressing cells, within the raphe and projecting to specific hypothalamic areas (Lillesaar, 2011). Then, in fish, 5HT synthesis from raphe 5HT cells and the release within hypothalamus may also depend on Trp availability, as in mammals. However, teleost-specific hypothalamic 5HT cells, and their role in modulating the same functions than those of mammals or others need to be fully investigated.

To date, 5HT, acting as neurotransmitter, is involved in the regulation of neuroendocrine system in vertebrates (Calas, 1977; Kordon et al., 1981; Holmes et al., 1982; Jennies et al., 1982; Shannon and Moore, 1987; Cohen et al., 1990). Specifically for fish, the modulatory role of 5HT was reported for secretion of melanophore-stimulating hormone (Olivereau, 1978a), gonadotropins (Margolis-Kazan et al., 1985; Somoza et al., 1988; Somoza and Peter, 1991; Khan and Thomas, 1992; Trudeau, 1997), and GH (Somoza and Peter, 1991; Wong, 1993; Peng and Peter, 1997). In addition, it has been demonstrated the involvement of the monoamine in different behaviors, including social dominance, aggressiveness, and feeding (Winberg et al., 1997; Gesto et al., 2006; 2008; 2013; Medeiros et al., 2014), for which the ratio between its main metabolite, 5HIAA, at the monoamine has been reported as a good marker of increased 5HT neuronal activity (Winberg et al., 1997; Gesto et al., 2013).

5HT exerts the modulatory action by binding to specific receptors. Serotonergic system involves a considerable divergence, thus modulating a wide range of functions. In mammals, seven 5HT receptors (5HTRs) exist, with six of them being G-protein coupled receptors, whereas the last one is a 5HT-gated ion channel (Roth, 2006). Class 1 (5HTR1) and Class 2 (5HTR2) receptors are well characterized in mammals, and their involvement on mediating different behavioral effects of 5HT is evidenced. 5HTR1s are auto-receptors that express in 5HT neurons. Their involvement on the regulation of 5HT-mediated cell firing and 5HT release in such cells is reported, in addition to that in non-5HT neurons throughout the entire brain (Roth, 2006).

Specifically for fish, autoradiographic techniques did lead to demonstrate the presence of 5HT1A receptors within the brain (Palacios and Dietl, 1988). Soon after, the presence of 5HT1A binding sites in goldfish retina was pharmacologically demonstrated (Hansely and Cohen, 1992), and afterwards in rainbow trout (Khan et al., 1996). By other hand, three different high-affinity 5HT-binding sites were described in Arctic char (Salvelinus alpinus) brain (Winberg and Nilsson, 1996). One of them was described by the authors as 5HTR1A, since the pharmacological profile
was quite similar to that of mammals, these pointing to its presence within fish brain. In addition, 5HTRs density in fish brain is higher within the telencephalon than at the diencephalon, as reported for mammals (Khan et al., 1996).

### 1.4.2. Dopaminergic system

The brain dopaminergic system is highly complex. Cell bodies containing DA are found in large number, and localize in several nuclei. Specifically for fish, dopaminergic neurons localize at the olfactory bulb (Hornby et al., 1987; Sas et al., 1990), telencephalon (Hornby et al., 1987), preoptic area (Hornby et al., 1987), hypothalamus (Parent et al., 1984), thalamus, prefrontal area, and medulla oblongata. These cells project to a wide variety of brain regions, especially those located in telencephalon, preoptic area and hypothalamus (Øverli et al., 2005; Hornby et al., 1987; Sas et al., 1990; Parent et al., 1984).

In fish, DA participates in several functions, such as locomotor activity, cardiovascular system, sensory information, reproduction, feeding and stress response (Gesto et al., 2006; 2008; 2013; Tse et al., 2007). Since mammalian DA system, together with NE, is involved in the regulation of agonistic behavior (Mason, 1984), it was postulated that dopaminergic system may participate in intraspecific aggressive behaviour (Avis, 1974), with the same role being played in fish. Soon after, agonistic behaviour was reported to be mediated by central monoaminergic systems in fish (Munro, 1986a; Maler and Ellis, 1987; Tiersch and Griffith, 1988).

The effects of DA are mediated by different specific receptors. In mammalian brain two receptor subtypes have been described, D$_1$ and D$_2$ (Clark and White, 1987). The involvement of each receptor on specific functions in fish was also tested. For example, DA binds to specific D$_2$ receptors, thus inhibiting gonadotropin secretion in goldfish brain (Chang et al., 1984). The role played by dopaminergic system during the response to stress has been also evaluated in fish. Collected results did lead to suggest that the ratio between the main metabolite and the monoamine (DOPAC/DA) is a good indicator of increased dopaminergic neuronal activity, which typically occurs during stress exposure (Winberg et al., 1997; Gesto et al., 2008; 2013).

In addition to that of dopaminergic system, NE cells are found within the brain. These cells appear to play a role as a diffuse system that displays a wide range of projections. However, not all the noradrenergic fibers display classical synaptic connections with other target cells since, once NE is released from them, it spreads and is able to bind to receptors located at distant targets (Fillenz, 1990). Regarding NE cells located within the brain in teleosts, the presence of dopamine-$\beta$-hydroxylase (DBH) immune-reactive (DBH-IR) cell bodies has been reported in hindbrain, dorsomedial medulla, medullar tegmentum, and isthmal tegmentum, thus making such areas to be able to synthesize NE (Hornby and Peikut, 1990). Noradrenergic cell
bodies have been described within fish brain stem, where they distribute in separate groups (Fillenz, 1990), as in mammals. These noradrenergic cells mainly project to hypothalamus and telencephalon.

Different receptors have been described for NE (α1, α2, β1, and β2) in mammalian brain (Fillenz, 1990). The presence of specific receptors in fish and the role played by each of them were evaluated, and pharmacological assays evidence that NE enhances gonadotropin release from goldfish pituitary cells, and such effect is mediated by α, and β1-like adrenergic receptors (Chang et al., 1991). In addition to that reported for DA, NE is also participating in the control of agonistic behavior, but different roles are reported for mammals and fish. Whereas in mammals increased NE-ergic activity apparently relates to stress (Mason, 1984), in fish, such as the weakly electric fish, Apteronotus leptorhynchus, intracranial injection of NE significantly enhances inter-male aggressive behavior (Maler and Ellis, 1987). Social hierarchy in fish is also influenced by brain levels of NE and DA (McIntyre et al., 1979). In this way, the authors reported that dominant rainbow trout have lower NE and higher DA levels in brain than subordinate fish, and the lower ranking in the hierarchy comes with lower levels of DA content and increased frequency of attacks suffered by these animals. Then, increased levels of DA might relate with increased aggressive behavior, whereas high NE levels relate with subordinate behavior, which also comes together with stress-related noradrenergic activity, which is in agreement to that reported for mammals, with increased NE levels in brain during prolonged exposure to a stressful situation (Adell et al., 1988).

1.4.3. Monoaminergic systems and circadian oscillators

Monoaminergic and circadian systems are key regulatory networks within the central nervous system in mammals. Each of them consists on groups of cells that make a wide range of neural connections all over the brain, and a defined network of transcriptional regulators, respectively. There are connections between them both, in such a way that monoamine-related elements express in those cells hosting a circadian oscillator, and expression of clock genes exists within monoaminergic neurons (Chalet, 2007). Both systems also influence multiple neural centers involved in either affective and/or temporal-gated behaviors. Cells hosting the components of the circadian system receive monoaminergic inputs, but also generate monoamine-related outputs, thus contributing to the arrangement of internal functions to coordinate them to changes occurring in the external world. Accordingly, alteration of monoamine levels can jeopardize physiological and behavioral functions that synchronize to environmental inputs, whereas altered circadian system can lead to altered monoaminergic activity and these function modulated by this system (Chalet, 2007; Cuesta et al., 2009; Korshunov et al., 2017).
Information from both internal and environmental cues is necessary for the SCN to be able to generate and synchronize rhythmic functions. As reported earlier, rhythmic information reaches the SCN through different pathways (see rev. Delgado et al., 2017), with 5HT involved in some of them. Specially, a main role of this amine in mesencephalic raphe-SCN pathway has been clearly demonstrated (Ueda et al., 1983; Moga et al., 1997; Cavalcante et al., 2002). The SCN receives direct innervation from 5HT cells located at the raphe nucleus, but also from NPY cell at the IGL. On the way back, the SCN project to the same brain regions (Deurveilher and Semba, 2005), then making possible the existence of interaction between these regions in order to coordinate different rhythmic functions. Thus, the role played by both 5HT and NPY in modulating the activity of brain circadian oscillators is evidenced (Edgar et al., 1991, 1993; Prosser et al., 1993; Mistlberger et al., 1998; Smith et al., 2001; Glass et al., 2003; Morin and Allen, 2006). Another way for 5HT to influence the oscillator is by inhibiting retinal pathways to the SCN by acting at both presynaptic (on retinal afferents) and postsynaptic (on SCN neurons) levels (Pickard et al., 1999; Quintero and McMahon, 1999; Smith et al., 2001).

Regarding the 5HT signaling network in mammals, the presence and expression of 5HT receptors is reported in cells that host the circadian oscillator within SCN (Kiss et al., 1984; Bosler and Beaudet, 1985; Bosler, 1989; Lovenberg et al., 1993; Manrique et al., 1993; 1994; Prosser et al., 1993; Amir et al., 1998). 5HT exerts its modulatory function by binding to them. Also, clock genes are expressed in 5HT neurons within the raphe, where key enzymes of 5HT biosynthetic pathway are rhythmically expressed (Abe et al., 2002; Malek et al., 2007). Accordingly, tph mRNA abundance, and 5HT secretion display circadian rhythms within this nucleus (Cagampang et al., 1993; Malek et al., 2005, 2007), together with the secretion of 5HT from the same cells at the synaptic cleft in the SCN (Cagampang et al., 1993). Then, a link between serotonergic system and circadian system is evidenced in mammals (Kripke, 1998; Loving et al., 2002; Benedetti et al., 2003), whereas information at this respect in other vertebrate groups is scarce. Specifically for fish, information is not available.

Starting on the raphe nucleus, the nigrostriatal pathway is the main dopaminergic one all over the brain (Gerfen et al., 1992). DA cells from this pathway can influence the information carried out by another main input to the circadian oscillator, melatonin, since DA modulates melatonin synthesis. In fact, light enhances the expression of DA receptors, which participate in the inhibitory effect of light on melatonin synthesis. Thus, any interaction between DA and circadian system is expected, especially when DA has been also reported to play a role as a wake up promoting factor across vertebrate species (Ueno et al., 2012). Consequently, circadian variations of DA content, release, and turnover have been reported in rat striatum, thus resulting in changes of dopaminergic activity that lead animals to display a daily rhythm of several behavioural events (Dulzen et al., 1985; Lemmer et
Also, different aspects related to dopaminergic signalling pathway exhibit a daily rhythm (Castañeda et al., 2004; Hood et al., 2010; Imbesi et al., 2009) that is modulated by light but also is driven by the circadian system (Hampp et al., 2008; Sleipness et al., 2007). On the contrary, DA is able to modulate the expression of clock genes both in vivo and in vitro (Gravotta et al., 2011; Imbesi et al., 2009; McClung et al., 2005), whereas lesion of the nigrostriatal pathway negatively affects the daily rhythm of activity and clock genes expression (Gravotta et al., 2011). In spite of the relatively well-understood interaction between both dopaminergic and circadian systems in mammals, information at this respect in fish is still very scarce and further reference is needed in order confirm such interaction in this vertebrate group.

1.4.4. Monoaminergic systems and stress

Animals have to be prepared to cope with any potential threat, such as stress exposure. For that purpose, a complex series of behavioral and physiological mechanisms initiate (Barton, 2002). As previously reported, increased plasma CAs and corticosteroid levels occur soon after stress exposure, following the activation of the HPA-BSA (mammals) or HPI-BSC (fish) axes (Wendelaar Bonga, 1997) as primary response to stress. Several internal factors participate in this response in fish, such as CRF, ACTH, and AVT, but also the brain monoaminergic systems, which have been proposed to play a key role during, and at the very beginning of the response to stress, as reported for rainbow trout (Gesto et al., 2015b). This latest result is in concordance with that observed in mammals (Balment et al., 2006; Wendelaar Bonga, 1997; Winberg and Nilsson, 1993). Since several mediators appear to influence stress response, the interaction among them all must exist in animals subjected to any stressor. However, it is not demonstrated whether altered levels of those mediators are responsible of the following events, or are just a consequence of the activation of the stress response (Øverli et al., 2005) or both. With respect to the monoaminergic systems, a role as stress axes activators is expected even in fish, but further research is needed in order to deep into the knowledge of the underlying mechanisms. Brief information at this respect is provided on the following paragraphs.

Serotonin

Regarding serotonergic system in fish, the interaction serotonin-stress axes was described in some studies (Hoglund et al., 2000; Lepage et al., 2002), and reciprocal interactions have been reported (Chaouloff, 2000; Chaouloff et al., 1999; Heisler et al., 2007; Pottinger, 2008; Winberg et al., 1997). Accordingly, the activity monoaminergic cells (mainly serotonergic cells) increases after exposure to different stressors such as handling, isolation, predator exposure, pollutant exposure or crowding (Gesto et al., 2006; 2009; 2013; 2014; Schjolden et al., 2006; Weber et al., 2012; Winberg and Nilsson, 1993). However, mechanisms underlying the modulatory
effect of HPI axis by 5HT are not fully understood, and the specific role of brain 5HT as well. At this respect, serotonergic pathways enhance or terminate in vertebrates the adrenocortical response during stress exposure in a brain region-specific way (Markus et al., 2000). In addition, a dual response of the 5HTergic system might be suspected during stress. Thus, the amine could acts an early signal once stress response initiates, as reported for lizard (Emerson et al., 2000; Matter et al., 1998) and rainbow trout, among fish (Gesto et al., 2013) but also as a late signal that persists during chronic stress (Browne et al., 2011; Øverli et al., 2007; Summers et al., 2003). Then it is possible that brain serotonergic system is activated by exposure to stressors, but the temporal sequence of this activation is still unknown. In addition, the dynamics of serotonergic activity when animals are subjected to chronic stress and often stress causes still need to be fully evaluated in fish.

**Catecholamines**

In the same way, dopaminergic neurons are activated during stress exposure in mammals, and increased turnover of DA has been observed (Thierry et al., 1976). Results describing increased brain levels of the main metabolite (DOPAC) also support this idea (Dunn, 1988a; Roth et al., 1988). In the same way, activity of the rate-limiting enzyme of DA synthesis, tyrosine hydroxylase, is increased during exposure to stress, as in vivo (Reinhard et al., 1982) and in vitro studies (lvoone and Dunn, 1986) demonstrate. Some reports evidence the activation of the dopaminergic system during stress exposure in fish. For example, the activation of DA containing cells stimulate aggressive behavior and inhibits the physiological response of the animals to stress (Hoglund et al., 2002; Summers et al., 2006). In the same way, dopaminergic activity is increased in rainbow trout subjected to acute stress (Gesto et al., 2013; 2015b), but also in fish maintained under mild stress such as low water renovation or high stocking density, as reported for sole (López-Patiño et al., 2013). Regarding NA, although in mammals this neurotransmitter could have an important role in triggering the HPI axis response, and its levels increase in limbic areas after acute stress (Dunn et al., 2004; Morilak et al., 2005), few conclusive studies have been done on that in fish (Øverli et al., 2001; Gesto et al., 2008; 2013).

According to that above mentioned, the interaction between physiological response to stress and monoaminergic systems in fish must exist, but the underlying mechanisms for such interaction are not fully understood.

### 1.5. Rainbow trout as animal model in research

Over recent decades, the use of fish as research animals has increased significantly on a global scale and several fish species in scientific research has been
used as a model in embryology, physiology, nutrition, genetics, pharmacology, endocrinology, pathology, parasitology, reproductive behavior or toxicology studies. This increase in fish research, to a large extent, is relied to be traced back to the rapid expansion of the fish farming industry. Nowadays, aquaculture is the fastest growing food-production sector worldwide, whereas traditional fisheries are in decline. Consequently, increasing research is being conducted on the conditions that maximize the growth and quality of cultured fish, which is mainly focused on nutrition, rearing environment, management, and disease control. The great fish diversity, their ability to adapt to different environmental conditions, great reproductive captivity and low maintenance cost, as good advantages make them good animal models.

The rainbow trout (*Onchorhynchus mykiss*, Walbaum, 1792), is a euryhaline teleost species belong to the Salmonidae family whose origin is Pacific coast of North American. Its high growth rate makes this species of greatest interest in aquaculture. Therefore, since 1874, rainbow trout has been introduced into almost all continental waters for aquaculture and for fishing purposes. Adult trout usually has length range about 70 cm but could also arrive up than 1 meter, whereas averaged body weight has been reported about 4.5 kg in fresh water and 10 kg in marine water. They are generally blue-green or olive green with heavy black spotting over the length of the body (Figure 15). Rainbow trout usually inhabits and spawns in small to moderately large, well-oxygenated, shallow rivers with gravel bottoms ranging a wide range of temperatures (0-21ºC). They are predators with a varied diet and will eat nearly anything they can capture. Environmental conditions such as light has strong effect on its swimming activity and its movement during the day from surface to bottom.

**Class:** Actinopterygii  
**Order:** Salmoniformes  
**Family:** Salmonidae  
**Genus:** *Onchorhynchus*  
**Species:** *Onchorhynchus mykiss*

**Figure 15:** The rainbow trout (*Onchorhynchus mykiss*, Walbaum, 1792)

For our research we have used rainbow trout as fish model, since there is abundant information about its physiological mechanisms, in particular those related to endocrine and metabolism regulation. Studies on rainbow trout relative to environmental influence are often available, although data on circadian functioning are still scare, as for most fish species, to date the rainbow trout has been concerned in different case studies of our research group (Gesto *et al*., 2006; 2008; 2009; 2013; 2014; Hernández-Pérez *et al*., 2015; 2017; López-Patiño *et al*., 2011; 2014b;
Conde-Sieira *et al.*, 2010a,b; 2014;2017; Ceinos *et al.*, 2005; 2008; Polakof *et al.*, 2008; Naderi *et al.*, 2018). This makes more information to be available in order to undertake new studies on lesser-known aspects of the physiology of this species, in general, of teleost fish.
2. Objectives
Objectives

Much of world’s population depends on fish as a source of protein, but wild fisheries cannot meet the global demand, which means that aquaculture has to deal with this issue. In this context, animal welfare is a priority in aquaculture, since stress can compromise welfare in fish farms, thus affecting negatively food intake and other behavioural and physiological functions. Then, digging into the understanding of mechanisms that contribute to improve animal welfare is a priority. Knowing those mechanisms involved in food intake control is a key goal. Food intake regulation in fish depends on different integrative mechanisms (metabolic, circadian, nervous and endocrine) at both central and peripheral levels, which are also subjected to the influence of different aquaculture-related processes. The hypothalamus plays a key role in food intake regulation since this region integrates peripheral and central signals to modulate food intake by releasing orexigenic and/or anorexigenic factors.

Food is known to play a relevant role in synchronizing rhythmic functions in fish, by acting as a zeitgeber for the circadian system. Our previous results demonstrate in rainbow trout the presence of a circadian oscillator within the liver, and the involvement of food as synchronizer of liver circadian physiology (Hernández-Pérez et al., 2017), but also within the hypothalamus (López-Patiño et al., 2011), where food intake control takes place. The latest result leads to speculate with the existence of any interaction between the hypothalamic circadian oscillator and food intake regulators, in such a way that rhythms of food intake regulators might subordinate to the activity of the circadian oscillator. Additionally, the negative effect of stress on liver circadian physiology was also demonstrated with consequences on carbohydrates and lipids metabolism (Hernández-Pérez, 2016). This aimed to us to study the existence of similar negative effects of stress at the hypothalamus, where a circadian oscillator exists in trout and is able to control feeding rhythms (López-Patiño et al., 2011).

If stress negatively affects the circadian system, altered rhythms of behavioural or physiological functions might be expected. In fact, daily endocrine rhythms are outputs of (but also can be inputs to) the circadian system, thus influencing the synchronization between hypothalamic and peripheral clocks. Glucocorticoids, with cortisol as the main one in fish, display daily rhythms in vertebrates including fish, and is involved in synchronization of feeding behaviour (see rev. Isorna et al., 2017). Therefore, rhythmic fluctuation of cortisol is believed to play a relevant role as output of the circadian system. As consequence, altered cortisol rhythms associates to jeopardized clock genes expression, which may be indicative of the role played by cortisol as input to the circadian system. Animals exposed to any stressing condition display altered profile of plasma cortisol levels, and it may be responsible of altered rhythmic of clock genes expression.
Accordingly, our preliminary results reveal decreased amplitude of the rhythm of some clock genes mRNA abundance in liver of rainbow trout subjected to mild stress (Hernández-Pérez, 2017). Therefore, a main role of glucocorticoids in mediating the effects of stress on brain circadian oscillators in fish is plausible, in addition to that on food intake control within the hypothalamic region.

In addition to cortisol, other candidates may mediate the adverse effect of stress on food intake and the circadian system. Specifically, a role of SIRT1, a (NAD+)-dependent deacetylase, in food intake has been described in rodents in which it expresses in cells involved in food intake control (Ramadori et al., 2008; Sasaki et al., 2010). However, information at this respect in fish is very scarce. We hypothesize that SIRT1 may participate in the control of food intake in trout by itself, or by mediating the inhibitory effect of stress on food intake, or even by interacting with other stress-related mediators such as cortisol. In addition, our preliminary data reveal that SIRT1 can be a key mediator of the effect of stress on the hypothalamic circadian oscillator in fish, as it is in liver (unpublished data), suggesting that an interaction between circadian system and SIRT1 exists. Accordingly, one may expect that the alteration of sirt1 mRNA levels during stress exposure may be responsible of that of core circadian clock genes.

Finally, monoaminergic neurons participate in a wide range of functions in teleost fish, including feeding (Leal et al., 2013; Pérez-Maceira et al., 2014) and the physiological response to stress (Gesto et al., 2013). Accordingly, brain monoamines (CAs, 5HT) in rainbow trout appear to play a key role during, and at the very beginning of the response to stress (Gesto et al., 2015). In addition, these neurotransmitters appear to interact with the circadian system in mammals, either acting as inputs or modulating the input information to the circadian clock, or as output from it. At this respect, a cross-link between serotonergic and circadian systems exists in mammals (Benedetti et al., 2003), but information at this respect in fish is lacking. Similarly, DA modulates the expression of clock genes (Gravotta et al., 2010) and dopaminergic signalling pathways are rhythmically modulated in rodents (Imbesi et al., 2009), with light and the circadian system being responsible (Hampp et al., 2008). In fish, in spite of that brain monoamines to play important roles in physiology and in particular in the stress response, knowledge about the interaction among circadian system and monoaminergic neurotransmission is near null.

Taking in consideration all that above mentioned, the main aim of this study was to investigate the influence of stress on brain circadian system and monoaminergic activity, with special attention paid on its consequences on feeding.

Specific objectives were:
Objectives

- To characterize the effects of stress on daily rhythms of mRNA abundance of both clock genes and food intake regulators in rainbow trout hypothalamus, and the involvement of cortisol in mediating such effects.

- To study the role of SIRT1 as mediator of the effect of stress on the hypothalamic clock machinery and food intake control in rainbow trout, and its interaction with other mediator of stress response, cortisol.

- To evaluate the existence of daily rhythms in brain monoaminergic systems, food-intake related neuropeptides and cortisol, and to study its dependence on the circadian system.

- To dig into the knowledge of brain monoaminergic systems as a target of chronic stress, and its involvement in mediating endocrine and feeding responses associated to stress.
3. Experimental works
3.1. Experimental work 1

Involvement of cortisol and SIRT1 in the response to stress of hypothalamic clock genes and food intake-related peptides in rainbow trout, *Oncorhynchus mykiss*. 
Resumen

Papel del cortisol y SIRT1 durante la respuesta al estrés del sistema circadiano hipotalámico y péptidos relacionados con la ingesta de alimento en la trucha arco iris, *Oncorhynchus mykiss*.

El estrés condiciona el bienestar de los animales al afectar negativamente una amplia gama de funciones fisiológicas y conductuales. Esto se puede aplicar a la fisiología circadiana y a la ingesta de alimento. El cortisol, la hormona relacionada con el estrés, puede mediar el efecto del estrés, pero se pueden considerar otros mediadores indirectos, como la SIRT1. Por lo tanto, el efecto modulador independiente o la existencia de cualquier interacción entre mediadores pueden ser responsable. El sistema circadiano es el principal modulador de numerosos mecanismos integrativos rítmicos a nivel central y periférico, lo que influye en diferentes procesos como la ingesta. De esta manera, la ingesta de alimentos está controlada por el sistema circadiano, como lo demuestra la persistencia de los ritmos de ingesta de alimento en ausencia de señales externas ambientales. En este estudio evaluamos el perfil diario de la abundancia de ARN mensajero de genes reloj hipotalámicos (*clock1a, bmal1, per1* y *rev-erβ-like*) y de reguladores de la ingesta de alimento (*crf, pome-a1, cart y npy*) en la trucha arco iris (*Oncorhynchus mykiss*), el impacto del estrés en dichos ritmos y la participación de cortisol y SIRT1 como posibles mediadores. Para ello, cuatro grupos de truchas fueron sometidas a: 1) densidad normal (grupo control), 2) alta densidad, durante 72 horas (grupo de estrés), 3) densidad normal pero implantadas con mifepristona, un antagonista de los receptores de glucocorticoides, y 4) implantadas con mifepristona seguido de exposición a estrés durante 72 horas. Los animales fueron sacrificados cada 4 horas a lo largo del ciclo diario y se tomaron muestras de sangre para la evaluación de los niveles plasmáticos de cortisol, glucosa y lactato. La abundancia de ARNm hipotalámico de genes reloj, de reguladores de la ingesta de alimento, de receptores de glucocorticoides y de *sirt1* se analizaron mediante qPCR. Nuestros resultados revelan el impacto del estrés en la mayoría de los genes analizados, pero parecen estar involucrados diferentes mecanismos. El ritmo de los genes del reloj disminuyó tanto en su amplitud como en los niveles medios en las truchas estresadas, mientras que la acrofase no se vio afectada. Este efecto no fue prevenido por la mifepristona. Por el contrario, el tratamiento con mifepristona evitó el efecto del estrés sobre el perfil diario de *crf, pome-a1* y *npy*. En consecuencia, el cortisol parece mediar principalmente el efecto del estrés en los reguladores de la ingesta de alimentos a través de la unión al receptor de glucocorticoides 1 (*GR1*) dentro del hipotálamo de trucha, mientras que SIRT1 podría mediar tales efectos sobre el sistema circadiano en la misma región del cerebro. Se debe realizar más investigación para identificar los mecanismos a través de los cuales se produce el efecto del estrés sobre la ingesta de alimento y el oscilador circadiano dentro de la misma región del cerebro, el hipotálamo, en la trucha arco iris y la interacción entre todos ellos.
Abstract

Involvement of cortisol and SIRT1 in the response to stress of hypothalamic clock genes and food intake-related peptides in rainbow trout, *Oncorhynchus mykiss*.

Animal welfare can be conditioned by stress. Negative effect of stress on a wide range of physiological and behavioural functions are characterized in fish. This may be applied to circadian physiology and food intake. Cortisol, as stress-related hormone, may mediate this effect, but other indirect mediators might be considered, such as SIRT1. Therefore, either the independent modulatory effect or the existence of any interactions between mediators may be responsible. The circadian system as modulator of several integrative mechanisms is rhythmically presented at both central and peripheral levels and could influence on different processes such as food intake among others. Since such rhythms of food intake persist in the absence of environmental external cues, fish food intake is controlled by the circadian system. Our study aimed to evaluate the daily profile of hypothalamic mRNA abundance of circadian clock genes (*clock1a, bmal1, per1 and rev-erbl-like*), and food intake regulators (*crf, pomc-a1, cart, and npy*) in rainbow trout (*Oncorhynchus mykiss*), the impact of stress on such rhythms, and the involvement of cortisol and SIRT1 as mediators. Then, four groups of trout were subjected to: 1) normal stocking density (control group), 2) high stocking density, for 72 hours (stress group), 3) normal stocking density but implanted with mifepristone, a glucocorticoid receptors antagonist, and 4) stressed for 72 hours following mifepristone administration. Fish of each group were sampled every 4 h along the 24 h LD cycle, and plasma level of cortisol and metabolites were evaluated. Hypothalamic mRNA abundance of clock genes, food intake regulators, glucocorticoid receptors and *sirt1* were qPCR assayed.

Our results reveal the impact of stress on most of the genes assayed, but different mechanisms appear to be involved. In stressed trout clock genes rhythm decreased the amplitude and averaged levels, whereas the acrophase was not affected. This effect was not prevented by mifepristone. On the contrary, the effect of stress on the daily profile of *crf, pomc-a1*, and *npy* was totally prevented by mifepristone administration. Accordingly, cortisol appears to mainly mediate the effect of stress on food intake regulators through binding to glucocorticoid receptors within trout hypothalamus, whereas SIRT1 is apparently mediating this effects on the circadian system in the same brain region. Further research must be performed to clarify those mechanisms through which stress influences food intake and the circadian oscillator within the same brain region, hypothalamus, in rainbow trout, and the interaction among them.
Involvement of cortisol and SIRT1 in the response to stress of hypothalamic clock genes and food intake-related peptides in rainbow trout, *Oncorhynchus mykiss*.

**INTRODUCTION**

In living organisms circadian rhythms of behavioural and physiological processes are driven by endogenous oscillators, which ultimately entrain to environmental factors, such as the LD cycles (Pando *et al*., 2001), temperature (Rensing and Ruoff, 2002), and food availability (Mistlberger and Antle, 2011) among others. Accordingly, a wide variety of physiological functions, such as metabolism, have been demonstrated to be under circadian control in all organisms, including fish (Hernández-Pérez *et al*., 2017). Regarding the circadian signals, previous reports demonstrate the presence of circadian oscillators in different central (López-Patiño *et al*., 2011) and peripheral (Hernández-Pérez *et al*., 2017) locations. The circadian clock machinery consists on coordinated intracellular transcriptional-translational feedback loops, which involve a set of “clock genes”. The clock mechanism is also conserved throughout phylogeny, from cellular organisms and plants to insects and mammals (Panda *et al*., 2002a,b). Briefly, the expression of *clock* and *bmal1* lead to increased levels of the activating elements, CLOCK and BMAL1, which heterodimerize, thus enhancing the transcription of the negative elements, encoded by *Period* (*per1*, *per2*, and *per3*) and *Cryptochrome* (*cry1* and *cry2*) genes. PER and CRY complexes inhibit their own transcription by binding to the CLOCK: BMAL1 heterodimer, thus blocking its function. This negative loop allows a daily rhythm of *per* and *cry* mRNA abundance and the respective protein products (Iuvone *et al*., 2005; Okamura *et al*., 2002). Another additional feedback loop has been described, and depends on nuclear receptors. Specifically, ROR and REV-ERB have been reported to display opposite transcriptional activities (Giguère, 1999), with ROR family acting as transcription activators, whereas REV-ERB family inhibits the transcription (Dumas *et al*., 1994; Forman *et al*., 1994; Retnakaran *et al*., 1994).

To date, clock system in teleosts is not well understood. However, the existence of several genes encoding the respective protein(s) with overlapping functions has been demonstrated (Coon *et al*., 1999; Falcón *et al*., 2003; Forsell *et al*., 2002). In addition, the existence of duplicate genes appeared to involve a round of whole genome duplication events that occurred within the ray finned fish lineage prior to the radiation of teleost fishes (Wang, 2008). Salmonid lineage appears to have been subjected to an additional round of whole genomic duplication at approximately 25 to 100 Mya (Allendorf and Thorgaard, 1984). Therefore, salmonids may have different duplicates for most genes. From the rhythms point of view, salmonids display daily variations in the expression of several clock-genes such as
clock, cmal1, and cry2 in the brain of Atlantic salmon (Davie et al., 2009). In rainbow trout, clock1a, bmal1, and per1 are rhythmically expressed in neural tissues, retina and hypothalamus, of individuals subjected to LD or DD (López-Patiño et al., 2011), but also in peripheral tissues such as liver (Hernández-Pérez et al., 2017).

In vertebrates food intake has been also demonstrated to display a rhythmic pattern which is influenced by different biotic (prey availability, social status), abiotic (photoperiod, temperature), and internal factors (biological clocks) (Boujard and Leatherland, 1992; Madrid et al., 2001). Accordingly, food intake and other functions display rhythmic profiles that persist in the absence of environmental cues (i.e., constant lighting conditions), thus demonstrating both the endogenous nature of such rhythms, and the circadian system to modulate them. However, food intake control in fish is a complex process that involves different mechanisms within the CNS, with hypothalamus being the main regulatory center. In the same way than mammals, a complex hypothalamic circuit includes two populations of neurons: one co-expressing NPY and AgRP, and the other that co-expresses POMC and CART. In spite of the importance of this circuit in integrating information relative to the control of food intake (see rev. Delgado et al., 2017), the rhythmic profile of these food intake regulators has not been fully evaluated in fish. Such profile may ultimately control feeding behaviour and derives from integrating information of different nature, such as stress-related signals and circadian signals among others.

In fish, the endocrine stress response involves both the hypothalamic-sympathetic chromaffin cell (HSC) and the HPI axes. Their activation restores homeostasis by mobilizing fuel to make energy available to cope with increased metabolic demand (Mommsen et al., 1999; Wenderlar Bonga, 1997). Fish display jeopardized feeding behaviour in response to stress (Bernier, 2006; Bernier and Peter, 2001). Once the HPI activates, CRF synthesis is enhanced in neurones located at the preoptic area. As a consequence, the ACTH release from the corticotrophic cells in the adenohypophysis is stimulated. Binding of ACTH to specific MCR2 at the interrenal cells of the head kidney stimulates glucocorticoid synthesis and release into the blood (Mommsen et al., 1999; Wenderlar Bonga, 1997). The activation of HPI axis affects many metabolism to behavioural functions, including food intake. In this way CRF administration into fish brain reduces food intake in a dose-dependent way, as reported for goldfish (Bernier, 2006; De Pedro et al., 1993), and the administration of the receptor antagonist reverts this effect (Bernier and Peter, 2001; De Pedro et al., 1997). Cortisol, as the main glucocorticoid in fish, appears to play a key role as mediator (Aluru and Vijayan, 2009; Barton, 2002; Bernier, 2006), since treatments with either glucocorticoid antagonists or cortisol synthesis inhibitors enhance crf mRNA abundance in goldfish brain, leading to food intake inhibition. The CRF receptor antagonist also prevents this effect (Bernier and Peter, 2001). These results point to the involvement of cortisol in modulating the inhibitory effect of stress on food intake, by binding to specific
glucocorticoid receptors within the hypothalamus. In addition, the actions of cortisol in salmonids brain are mediated by glucocorticoid receptors 1 and 2 (GR1 and GR2), but also by a mineralocorticoid receptor (MR) (Bury et al., 2003; Colombe et al., 2000), which act as ligand-inducible transcription factors (Lee et al., 1992; Sturm et al., 2005). In addition, cortisol is likely the most important MR ligand in fish (see rev. Wendelaar Bonga, 1997), including rainbow trout (Columbe et al., 2000). Within trout brain, lower cortisol levels activate MR instead of GRs, which is indicative of MR to display similar role in fish brain than that of mammals (Sturm et al., 2005). Then, the activation of either MR or GR respectively results in enhanced or decreased long-term potentiation, for which the ratio GR:MR is important in controlling behavioural reactivity and memory storage in fish (Johansen et al., 2011). Specifically for GR, in spite of their important role in different behavioural and physiological functions, they play a main role in mediating the physiological response to stress exposure. However, little is known regarding the dynamics of mRNA abundance for such receptors and how such expression is altered during stress.

In case of the activation of both the HSC and the HPI axes, reduced food intake under stress appears to also relate to other parameters such as the ability of nutrient sensing systems to modulate food intake. In rainbow trout, chronic stress affects hypothalamic glucosensing mechanisms (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015), leading animals not to compensate with changes in food intake those of circulating glucose levels. Hypothalamic mRNA abundance of food intake regulators such as pomc, cart and npy is also altered (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). CRF is expected to mediate the mechanisms through which stress influences food intake control (Evans et al., 2004; McCrimmon et al., 2006), on the basis of the readjustment of glucosensing mechanisms in hypothalamus of rainbow trout induced by in vitro CRF treatment (Conde-Sieira et al., 2011) in the same way than that reported for stressed fish (Conde-Sieira et al., 2010a). Even when mRNA abundance of food intake regulators (pomc, cart, and npy) is altered during stress exposure, the nature of such variations has not been fully evaluated in fish.

Stress might negatively affect the circadian system, with the subsequent alteration of those behavioural or physiological functions that display a rhythmic daily profile. In fact, daily endocrine rhythms are outputs of the circadian system, but to date several reports also suggest that some hormones may also act as inputs in the timed signalling of hypothalamic and peripheral clocks (Challet, 2015; Coomans et al., 2015). Accordingly, plasma glucocorticoid display daily rhythms in vertebrates including fish, with that of cortisol synchronizing to both the feeding-fasting cycle and feeding time in fish (see rev. Isorna et al., 2017), leading the hormone to participate as output of the circadian system. How cortisol participates as input to the circadian system in fish is still unknown. Some studies indicate that in goldfish’s
liver cortisol stimulates the expression of per1a and per1b, and inhibits that of clock and bmall (Sánchez-Bretaño et al., 2016). Then, cortisol within the liver could link the entrainment of food-entrained clocks by both, metabolic signals and hormones. Our preliminary results in rainbow trout are in the same way, since liver of animals subjected to mild stress display decreased amplitude of the rhythm of clock1a and bmall mRNA abundance, and altered per1 rhythm (unpublished data). However, main role of glucocorticoids in mediating the effects of stress on the circadian system in fish has to be elucidated.

In addition of cortisol effects as mediator during stress on the circadian system, other mechanisms might be also involved. SIRTs may be good candidates, based on this family to participate as a link between sensing of cellular energy status and circadian clocks. SIRTs are well characterized (NAD⁺)-dependent class III histone deacetylases involved in numerous effects on intracellular metabolism. In particular, SIRT1 and SIRT6 have been investigated as a link between cellular metabolism and circadian clocks physiology in mammals (Masri et al., 2014; Orozco-Solís et al., 2015). Specifically for SIRT1, the enzyme activity fluctuates through the feeding/fasting cycle in rodents (Cakir et al., 2009), with such rhythm being apparently modulated by the CLOCK/BMAL1 heterodimer over the enzyme nicotinamide phosphoribosyltransferase (NAMP), responsible of NAD⁺ synthesis (Ramsey et al., 2009). SIRT1 is also involved in the cyclic control of cofactors and peptides of circadian clocks by deacetylating BMAL1 and PER2 in liver (Nakahata et al., 2008) and activating the hypothalamic SCN pacemaker in mice (Chang and Guarente, 2013). In fish, the role played by molecular sensors in modulating circadian oscillators has to be elucidated, but preliminary data collected from rainbow trout demonstrate changes in mRNA abundance of sirt1 that fit with the functioning of fatty acid sensing systems (Velasco et al., 2016a,b). Since feeding-related rhythms driven by the circadian system in digestive enzymes have been reported (Vera et al., 2007; Montoya et al., 2010; Nisembaum et al., 2014), the entrainment of circadian clocks by nutrient status in fish might then be expected (see rev. Delgado et al., 2017). Considering the interaction between SIRT1 and the circadian clock machinery, it is plausible to expect that the alteration of sirt1 mRNA levels as consequence of exposure to stress may affect that of core circadian clock genes. Our preliminary data are in support of this idea, since increased sirt1 levels have been observed in liver of rainbow trout subjected to high stocking density (unpublished data).

Taking in consideration the above mentioned interactions between the circadian system and food intake regulation, and the negative influence of stress on the circadian clock machinery, the aim of the present study was to characterize in rainbow trout hypothalamus the daily rhythms of mRNA abundance of both, clock genes (clock1a, bmall, per1, and rev-erbβ-like), and food intake regulators (crf, pomc-a1, cart, and npy), and how these rhythms are affected by stress (high stocking
density). The involvement of cortisol and SIRT1 in mediating such effect was also evaluated.

MATERIAL AND METHODS

Fish

Immature rainbow trout (*Oncorhynchus mykiss*, Walbaum) weighing 93 ± 7 g were obtained from a local hatchery (A Estrada) and transferred to our facilities at the Faculty of Biology (University of Vigo). Animals were distributed in 120 L tanks with filtered and continuously renovated fresh water (13.5 ± 1°C). Feeding time was scheduled at zeitgeber time (ZT) 2 (ZT0 = light on), and consisted on a commercial (Dibaq diproteg) dry pellet diet (1% body weight). Fish were kept under 12L:12D photoperiod. Illumination was provided by means of LED light lamps (Superlight Technology Co. Ltd., China). Also, irradiance was measured with a spectro-radiometer (FieldSpec ASD, Colorado, USA) set at 1.62 E^{+18} photons m^{-2} s^{-1}. Fish were acclimated for 15 days to standard conditions before any experiment to proceed. All experiments comply with the Guidelines of the European Union Council (2010/63/EU), and of the Spanish Government (RD 53/2013) for the use of animals in research. Animal protocols were approved by the Animal Care Committee at the University of Vigo and followed international ethical standards (Portaluppi et al., 2010).

Sampling and experimental design

Fish were deeply anaesthetized by addition of 2-phenoxyethanol (0.2% v/v-Sigma Aldrich) in tank water and visual contact between fish and the manipulators was avoided. To guarantee the uniform mix of anesthetize the appropriate volume of 2-phenoxyethanol was previously diluted in 5-L of tank water and afterwards added into the fish tank. Once anesthetized, individual blood samples were collected and animals were rapidly sacrificed. From each animal 1 mL of blood was collected by caudal puncture with help of ammonium-heparinized syringes. Each individual hypothalamus, including the preoptic area was removed under sterile conditions, according to previously described (Doyon et al., 2003). Samples were placed into sterile RNase-free 1.5 ml Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80°C until qPCR assayed for mRNA abundance of clock genes (*clock1a*, *bmal1*, *per1* and *rev-erbβ-like*), food intake inhibitors (*crf*, *pomc-a1*, and *cart*) and stimulatos (*npy*) neuropeptides, SIRT1 (*sirt1*), and glucocorticoid receptors (*gr1* and *gr2*). Plasma samples were obtained after blood centrifugation and then immediately frozen on dry ice and stored at -80°C until assayed for cortisol and metabolites (glucose and lactate) levels.
To assess the daily profile of clock genes mRNA abundance and food intake-related peptides, and the effect of stress with cortisol and SIRT1 as mediators, trout were divided in four groups (N = 105 fish / group). For the study of daily variations of mRNA abundance of all the assessed genes, the first group was randomly divided in seven 120-L tanks (n = 15 trout/tank) and afterwards adapted to our laboratory conditions (see above). That number of fish/tank was selected in order to minimize the incidence of dominance and/or feeding hierarchies, even when on the day of sacrifice only 8 trout/group were sampled. Following two weeks acclimation, animals were implanted with coconut oil and immediately returned to their tanks. After 72 h, fish were sacrificed every 4 hours over the 24 h LD cycle (Control group), starting at ZT0 (lights on). Thus, scheduled sampling time points were ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT0' of the following day. At each specific time point, all fish from an assigned tank were sampled. Sacrifice and sampling procedure did never exceed 15 min / time point. Animals sampled at night were transferred to a new tank containing anesthetic solution and moved out of the housing room, which prevented any light contamination during the dark period. Then, fish were blood collected in the absence of light, and sacrificed. Dead bodies were then manipulated under dim red light.

A second group of 105 animals (n=15/group) was adapted to the same housing conditions (Stress group) and acclimation time period than control group. After acclimation, fish were implanted with coconut oil alone and immediately after that water level of each tank was adjusted to reach a high stocking density of 70 kg fish/m³, which induces stress response in trout. Reaching such water level took 60 min for each tank. In addition, water flow was readjusted in order for water quality remain indicative to that of non-stressed group. Animals remained under these conditions for the following 72 hours, and afterwards sacrificed every 4 hours (n=8/time period) over the following 24 h period, as described above. Fish were daily hand-fed as scheduled.

Following acclimation as above described for control group, the third group (RU486 group) of 105 trout (n = 15/tank) was anaesthetized in tank water and Intraperitoneal injection (IP) implanted with a volume (3 µl/g bw) of coconut oil containing 100 mg/kg bw of the general glucocorticoid receptors antagonist, mifepristone (RU486, Sigma) at ZT0, and transferred for recovery to their respective tank. That volume of implant guaranteed constant blood levels of RU486 all over the experiment. After that, fish were maintained under a regular LD cycle for 72 hours, and then sacrificed every 4 hours (n=8/time period) and sampled as described above.

To evaluate the role of cortisol in mediating the effect of stress on clock genes expression and food intake-related peptides, the fourth group of 105 fish (RU486 + Stress group, n=15 fish/tank) was acclimated to normal housing conditions as above described for control group. On the first day of the experiment, fish were anesthetized with 2-phenoxy ethanol in tank water and IP injected with
RU486 as described above. The implant guaranteed constant blood levels of RU486 up to the end of the experiment. Immediately after their recovery, water level was adjusted to reach high stocking density (70 kg fish/m³) on each tank, as described for Stress group, where fish remained for the following 72 h period. Then, trout were sacrificed every 4 hours and sampled as mentioned above (n=8/time period).

**Food intake**

To assess of food intake, every 5 min 10 g of food pellets were released in tank water until animals were satiated. After feeding, the uneaten food remaining at the bottom (conical tanks) was withdrawn, dried for 24 h at 37°C and weighed. The amount of food consumed by all fish within each tank was calculated as previously described (Polakof *et al.*, 2008a,b) as the difference from the food offered (De Pedro *et al.*, 1998b). FI values were represented as referred to baseline levels.

**Plasma assessment**

Plasma cortisol levels were measured by using a commercially available Enzyme Immunoassay kit (Cayman, Ann Arbor, MI, USA), according to manufacturer's indications. Kit was previously validated in our laboratory. Accordingly, detection limit was 6.6 pg/ml, inter-assay coefficient was 7.5%, and the intra-assay limit was 5.6%. The recovery percentage was also estimated as 92.3 ± 11.5%. Plasma glucose and lactate levels were determined enzymatically in deproteinized plasma samples by using commercial kits (Biomérieux, France and Spinreact, Girona, Spain, respectively) adapted to a microplate format.

**qPCR**

From individual hypothalamus samples total RNA was extracted by using the TRIzol® (Life Technologies, Grand Island, NY, USA) method, and treated with RQ1-DNase (Promega, Madison, WI, USA). The same RNA amount (2 µg) from individual sample was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega) and Random Primers (Promega). A negative control for each sample was assessed without reverse transcriptase in order to confirm the absence of any genomic contamination in the RNA extract.

qPCR was performed using a Maxima™ SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and a Bio-Rad MyIQ Real Time PCR system (BIO-RAD, Hercules, CA, USA). The primers were designed on the basis of previously reported sequences for rainbow trout genes, and obtained from Sigma (see Table 1), including *clock1a, bmal1, per1, rev-erbβ-like, crf, pomec-a1, cart, npy, gr1, gr2, sirt1*, and *β-actin.*
Relative quantification of the target gene transcript was assessed using $\beta$-actin gene expression as reference, since it was homogeneously expressed through the 24 h cycle with independence of the experimental condition, as previously reported (Hernández-Pérez et al., 2017). Thermal cycling was initiated with incubation at 95°C for 3 min; followed by 35 steps of PCR, each one consisting of heating at 95°C for 10 s for denaturing, and at specific annealing for 30 s and extension at 50°C for 30 s. Immediately after the last PCR cycle, melting curves were systematically monitored (50°C temperature gradient at 0.5°C/s from 50 to 95°C) to ensure that only one fragment was amplified. Relative mRNA abundance level was calculated following the Pfaffl method (Pfaffl, 2001). For each gene, samples collected at the same time point were processed in parallel and the expression was measured in triplicate within the same microplate. Efficiency values between 85% and 100% were accepted (the $R^2$ for all the genes assessed was always higher than 0.985).

### Table 1. Sequences (Forward y Reverse), annealing temperature and accession number for each gene assessed.

<table>
<thead>
<tr>
<th>Gen</th>
<th>Sequence</th>
<th>$T_A$</th>
<th>Reference</th>
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<tr>
<td>clock1a</td>
<td>F: TACACGTCTGAGATTCGTCCTC</td>
<td>57</td>
<td>GU228520.1 (GenBank)</td>
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<td></td>
<td>R: GAAACTTCAAGTCCTCCTGATT</td>
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<tr>
<td>bmal1</td>
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<td>GQ489026.1 (GenBank)</td>
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<td></td>
<td>R: TGGACATCTTCTCCACGATG</td>
<td></td>
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<tr>
<td>per1</td>
<td>F: GCTGCGAGCTCGATCTGT</td>
<td>55</td>
<td>AF228695 (GenBank)</td>
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<td></td>
<td>R: GGTCATCTCGACGGATATGG</td>
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<td></td>
</tr>
<tr>
<td>rev-erb-$\beta$-like</td>
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<td>56</td>
<td>AF342943.1 (GenBank)</td>
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<td></td>
<td>R: CGGCCAACCTAACCGAGTCTC</td>
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<tr>
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<td>AF296672 (GenBank)</td>
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<td>60</td>
<td>TC86162 (Tigr)</td>
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### Statistics

Statistical comparisons of mRNA abundance and plasma cortisol and metabolites were performed by using a two-way ANOVA test with “time” and “treatment” as main factors. When a significant effect was identified within a factor, post-hoc comparisons were carried out by using a Student-Newman-Keuls
test. In addition, the one-way ANOVA analysis was performed to determine the presence of significant differences in gene expression between time points for each experimental condition assayed. If significant differences were found, a Student-Newman-Keuls test with multiple comparisons was performed. Significance level was set at $P < 0.05$.

Daily rhythms of expression for each gene were also analysed by fitting periodic sinusoidal functions to the specific values of mRNA abundance across the seven time points by using the formula $f(t) = M + Acos(\pi t/12 - \varphi)$, where $f(t)$ was the gene expression level at a given time point, the mesor ($M$) is the mean value, $A$ is the sinusoidal amplitude of the oscillation, $t$ is time in hours, and $\varphi$ is the acrophase (time of peak). Nonlinear regression allows the estimation of $M$, $A$, and $\varphi$, and their standard error (SE) (Delgado et al., 1993). All parameters of the sinusoidal function were expressed as the average ± standard error (SE). The SE of parameters was based on the residual sum of squares in the least-squares fit. A rhythm was considered consistent if both $P < 0.05$ from the one-way-ANOVA test and $SE(A)/A < 0.3$ provided by the cosinor analysis, following the principle of a noise/signal ratio less than 0.3, the “signal” being the amplitude and the “noise” its error (Halberg and Reinberg, 1967).

RESULTS

Food intake assessment for all experimental condition is shown in Figure 1. Control group showed constant food intake all experiment, since no variation was found between that observed during the last 72 h and baseline. On the contrary, fish exposed to stress by high stocking density displayed a significant decrease of food intake up to 50% of baseline. RU486 administration prevented this inhibitory effect of stress, whereas treatment with the glucocorticoid antagonist did not affect feeding activity itself.

Plasma cortisol and metabolites levels in trout subjected to each experimental condition are shown in Figure 2. In control group, cortisol levels displayed a daily rhythm with peaking values being observed at ZT4 and basal levels at the day-night transition (ZT12). The averaged cortisol levels were 35.14 ng/mL. Stress modified the glucocorticoid rhythm in such a way that peaking values moved to the dark period (ZT16), even when a diurnal increase at ZT4 was conserved. In addition, averaged cortisol levels significantly increased, respect to control group, up to 74.08 ng/mL. IP administration of mifepristone in non-stressed trout resulted in increased cortisol levels only at the first half of the night (ZT12 and ZT16) relative to control group, but paralleled those of control during the rest of the 24 h period. As a consequence, averaged cortisol levels were enhanced (48.21 ng/mL) relative to control group. Exposing to stress those fish implanted with RU486 induced a significant increase of cortisol levels all over the 24 h period. In spite of the peaking
values for the glucocorticoid being observed within the same temporal window as the control group (ZT4-8), a huge increase of averaged cortisol levels, up to 187.79 ng/mL, was noted, relative to the other experimental groups.

A significant daily variation of plasma glucose levels was found only in control group, with maximal levels observed in samples collected at ZT4, and basal levels at the end of the night (ZT20-0’). On the contrary, averaged glucose levels were experimental condition-dependent. Hence, mean levels in control group were 6.57 mM, whereas stress increased them up to 7.01 mM, relative to control group. Averaged levels in animals receiving mifepristone decreased to 4.64 mM in RU486 group, and to 4.59 mM in RU486+stress group.

No significant daily variation for plasma lactate levels was observed among groups, similarly to that for glucose. On the other hand, a small decrease of averaged levels was found in trout subjected to stress (2.33 mM) relative to that measured in control fish (2.73 mM). Such decrease was more pronounced in those trout receiving RU486 (2.08 mM and 1.53 mM for RU486, and RU486+stress, respectively).

**Figure 1.** Averaged daily food intake of rainbow trout subjected to different experimental conditions (Control, stress, RU486, and RU486+Stress) evaluated during the last 72 h period. Data are presented as percentage of baseline levels of food intake evaluated during previous 12 days. * P < 0.05 relative to the other groups.
**Figure 2.** Daily profile of plasma cortisol, glucose and lactate levels in rainbow trout subjected to different experimental conditions (Control, Stress, RU486, and RU486+Stress). Each value is the mean ± S.E.M. of 8 fish. * P < 0.05 relative to the other groups within the same ZT time; $ P < 0.05 relative to control and RU486 groups at the same ZT time; # P < 0.05 relative to control group at the same ZT time.

Assessment of mRNA abundance in hypothalamus of rainbow trout showed distinct daily variation for the genes assessed (Figures 3-6), that were experimental condition-dependent. The parameters defining such daily rhythms are presented in Tables 2 and 3. The rhythm of mRNA abundance of clock genes (*clock1a, bmal1, per1*) and *rev-erbfβ-like* in hypothalamus is shown in Figure 3. The *clock1a* mRNA
abundance showed a consistent rhythm at any experimental condition, as revealed by the cosinor analysis. Therefore, in the control group peaking values occurred at the night onset (acrophase at ZT13.10). Amplitude and mesor values of such rhythm were estimated as 1.50 and 2.96 relative fold change units, respectively (see Table 2). Stress did not affect the acrophase, but inhibited clock1a expression in such a way that both the amplitude and the mesor values decreased, to 50% and 75% respectively, of that observed in control group. RU486 treatment was not able to counteract the effect of stress on clock1a expression. Thus, amplitude (0.85 relative fold change units) and mesor (2.12 relative fold change units) remained lower than that of control group. In addition, RU486 treatment per se did not affect the rhythm of clock1a, which was similar to that of control group.

In the same way than clock1a, the cosinor analysis revealed consistent rhythms of bmal1 mRNA abundance at all experimental condition (Figure 3). In the control group, peaking values of mRNA abundance were measured in trout sacrificed at the night onset, with the acrophase at ZT12.06. The amplitude of the rhythm was 4.15 relative fold change units, and the mesor was 5.48 units. Compared to that found in control fish, exposing fish to high stocking density resulted in a decrease of the amplitude of the rhythm (to 65%) and consequently, the averaged mRNA abundance (to 57%). No variation was found for the acrophase, which was estimated at ZT11.55. By other hand, RU486 was not effective in preventing the effect of stress on bmal1 rhythm. Accordingly, decreased amplitude (to 42%) and mesor (to 60%) were estimated in the RU486 + stress group, relative to control fish, whereas no variation in the time of the peak (acrophase at ZT11.59) was found. Mifepristone did not affect bmal1 rhythm itself.

A consistent rhythm of mRNA abundance was observed for per1 at any experimental condition (Figure 3), as revealed by the cosinor analysis (see Table 2). However, both the amplitude and averaged values were dependent of the experimental group, whereas the acrophase did not substantially change. The cosinor analysis estimated for control group peaking values during the second half of the night (acrophase at ZT19). The amplitude and averaged value were 7.44 and 8.2 relative fold change units, respectively. Stress exposure induced a decrease of amplitude and mesor of hypothalamic per1 rhythm, to 47% and 49% of that observed in control fish. In contrast, the acrophase was not affected, thus locating at ZT19. RU486 treatment did not prevent the effect of stress on per1 expression. As a result, RU486 + stress group displayed a decrease of amplitude (to 37%) and mesor (to 35%) of per1 rhythm, compared to that observed in control fish. In addition, no variation of the acrophase was observed (φ at ZT18.05). RU486 alone did not affect per1 rhythm in non-stressed trout relative to control.
Figure 3. Daily profile of circadian genes (clock1a, bmal1, per1, and rev-erbβ-like) mRNA abundance in hypothalamus of trout exposed to: a) normal conditions (Control group), b) stress by high stocking density for 72 hours (Stress group), c) mifepristone and normal conditions (RU486 group), d) RU486 and stress by high stocking density for 72 h (RU486+stress group). Each value represents the mean ± S.E.M. of 8 fish. Values are presented as the relative fold change with respect to basal levels in control group. * $P < 0.05$ relative to the other groups within the same ZT time; $* P < 0.05$ relative to control and RU486 groups at the same ZT time.
Table 2. Parameters defining the daily variations of mRNA abundance of clock genes assessed in hypothalamus of rainbow trout.

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITION</th>
<th>Control</th>
<th>Stress</th>
<th>RU486</th>
<th>RU486+Stress</th>
</tr>
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<td></td>
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</tr>
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<td>P &lt; 0.01; 0.16</td>
<td>P &lt; 0.01; 0.17</td>
<td>P &lt; 0.01; 0.12</td>
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<tr>
<td>M</td>
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<td>2.75</td>
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</tr>
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<td>A</td>
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<td>1.75</td>
<td>0.85</td>
</tr>
<tr>
<td>φ</td>
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<td>13.14</td>
<td>13.25</td>
<td>12.58</td>
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</tr>
<tr>
<td>P; SE(A)/A</td>
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<td>P &lt; 0.05; 0.18</td>
<td>P &lt; 0.01; 0.19</td>
<td>P &lt; 0.01; 0.18</td>
</tr>
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<td>5.05</td>
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</tr>
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<td>11.55</td>
<td>11.06</td>
<td>11.59</td>
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<td><strong>per1</strong></td>
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<td>P &lt; 0.01; 0.21</td>
<td>P = 0.02; 0.23</td>
<td>P &lt; 0.05; 0.22</td>
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<tr>
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<td>2.84</td>
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<td>A</td>
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<td>φ</td>
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<td>19.00</td>
<td>17.21</td>
<td>18.05</td>
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<td><strong>rev-erb-β-like</strong></td>
<td></td>
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<tr>
<td>P; SE(A)/A</td>
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<td>P &lt; 0.01; 0.19</td>
<td>P &lt; 0.01; 0.20</td>
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<td>A</td>
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</tr>
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<td>φ</td>
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<td>21.09</td>
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</tr>
</tbody>
</table>

Data represent the mesor (M) as the mean value, the sinusoidal amplitude of the oscillation (A), and the acrophase (φ; time of peak; ZT/CT) provided by the cosinor analysis. P: P value following ANOVA analysis. SE (A)/A: value of the ratio from the cosinor analysis.

The cosinor analysis revealed a consistent rhythm of *rev-erbβ-like* mRNA abundance (Figure 3) at any experimental condition (see Table 2). In fish from the control group peaking values were measured in samples from animals sacrificed during the second half of the dark phase, with the acrophase being at ZT 21.19. Amplitude and mesor values were estimated as 0.50 and 1.64 relative fold change units, respectively. Stress was not effective in changing the time of the peak (acrophase at ZT21.01), but resulted in the increase of amplitude (up to 500%) and averaged *rev-erbβ-like* mRNA abundanc (up to 800%) compared to that measured in control group. RU486 treatment partially prevented the effect of stress, but *rev-erbβ-like* mRNA levels did not fully turn back to those comparable to control group. As a result, a significant decrease of mRNA abundance was found in most of the time points relative to that measured in Stress group, but remained significantly higher than that of control group. Consequently, the acrophase was not significantly affected in RU486 + Stress group (φ at ZT18.51), but the amplitude and averaged expression remained higher than that measured in control group. RU486 treatment
resulted in no variation of each parameter from the cosinor analysis when administered to non-stressed trout.

The daily rhythms of mRNA abundance of neuropeptides related to food intake control (crf, pomc-a1, cart and npy) in hypothalamus is shown in Figure 4. In control group, crf mRNA levels displayed a consistent rhythm with peaking values during the night (acrophase at ZT16.38). Amplitude and mesor values were estimated as 1.50 and 2.57 relative fold change units, respectively. Trout subjected to stress resulted a significant increase of the mesor value (4.78 relative fold change units) compared to that of control fish. No significant variation was found for the amplitude (2.24 relative units) and the acrophase (ZT15.37) relative to Control. RU486 implant completely prevented such increase of crf mRNA abundance. Consequently, mesor, amplitude, and acrophase (ZT16.16) values were comparable to those of control group. Similar results were found in RU486 implanted but non-stressed fish (see Table 3).

As revealed by the cosinor analysis, pomc-a1 rhythm of mRNA abundance was consistent in control group, with peaking values during the night (acrophase at ZT14.26). Amplitude and mesor values were 2.32 and 3.31 relative fold change units, respectively. Trout subjecting to stress resulted in a significant decrease of pomc-a1 peaking values relative to control group, but no change was found for the acrophase, which was estimated at ZT13.32. As consequence of stress exposure both amplitude and mesor values (1.74 and 2.76 units respectively) decreased relative to that of control. RU486 administration was effective in preventing the effect of stress. Thus, amplitude, mesor, and acrophase (ZT14.58) data in RU486 + Stress group were comparable to those of control. In the same way, RU486 treatment in non-stressed group did not affect the rhythm of pomc-a1 abundance (see Table 3).

The rhythm of cart mRNA abundance was significant at any experimental condition, with peaking values at the day onset (ZT0). The cosinor analysis confirmed their significance (see Table 3). Accordingly, control group showed amplitude (0.75 relative fold change units), mesor (1.92 relative units) and acrophase (ZT23.39), with these parameters not being affected by stress (see Table 3). No significant variation of cart expression was found in those groups receiving RU486, relative to control group.

The daily rhythm of npy mRNA abundance was revealed to be consistent at any experimental condition, as indicated by the cosinor analysis. Thus, control group showed high expression during the first half of the night (ZT16), leading for the acrophase to be estimated at ZT15.56. Amplitude and mesor values were 29.16 and 30.26 relatively units, respectively. Trout subjected to stress by high stocking density resulted in the increase of the amplitude and averaged expression up to 150% of that measured in control, whereas the acrophase remained unaltered (ZT15.54). Such increase was totally prevented with RU486 treatment, with those
parameters defining the rhythm of *npy* being comparable to those of control group (see Table 3). RU486 alone did not induce any significant alteration in *npy* rhythm.

The daily profile of glucocorticoid receptors (*gr1*, and *gr2*) mRNA abundance in hypothalamus of trout at all experimental condition is shown in Figure 5. As revealed by the cosinor analysis, the rhythm of *gr1* was significant in most of the experimental conditions (Table 3), but not in Stress group. Control group displayed a significant rhythm of mRNA abundance with peaking values occurring at the dark period (acrophase at ZT18.41). The amplitude and mesor values of this rhythm were estimated as 8.00 and 9.68 relative fold change units, respectively (see Table 3). The daily profile of *gr1* was disrupted by stress at any time, with a subsequent loss of the rhythm, as revealed by the cosinor analysis. The mean values of *gr1* abundance were 225% higher than those of control group. RU486 administration completely prevented the effect of stress on *gr1* expression. Therefore, the parameters defining the rhythm remained quite similar to those of the control group, as revealed by the cosinor analysis (see Table 3). RU486 implant in non-stressed fish did not affect *gr1* rhythm. On the contrary, the rhythm of *gr2* mRNA abundance did not vary, relative to control at any experimental condition (Figure 5). Thus, peaking values were observed in samples collected at the middle of night (see Table 3). Neither the amplitude nor the averaged expression did significantly change among groups (Table 3) at any time point.

As revealed by the cosinor analysis, assessments of mRNA abundance for *sirt1* in trout hypothalamus demonstrated the existence of significant daily rhythm in all groups (Figure 6). The parameters defining such rhythms were dependent of the experimental condition (Table 3). In control fish, peaking values of *sirt1* were measured in trout sampled at ZT8. The cosinor analysis calculated amplitude and mesor values at 19.13 relative fold units, and 19.84 units respectively, and estimated the acrophase at ZT7.38. High stocking density produced a significant increase of *sirt1* expression anytime, but did not modify the phase of the rhythm. As a consequence, only amplitude (481.24 relative units) and mesor values (484.47 units) increased, compare to control, with the acrophase being estimated at ZT7.51. The administration of RU486 was able to partially prevent the effect of stress on *sirt1* expression in such a way that amplitude and mesor values (137.44 relative units, and 216.32 units, respectively) were lower than that of Stress group, but remained higher than those of control. No variation was found for the acrophase, thus being estimated at ZT7.37. RU486 alone did not affect the daily profile of *sirt1* mRNA abundance, with the parameters defining such rhythm remaining comparable to those of control group (see Table 3).
**Figure 4.** Daily rhythms of mRNA abundance of food intake-related peptides (*crf*, *pomc-a1*, *cart* and *npy*) in hypothalamus of trout subjected to different experimental conditions (Control, Stress, RU486, and RU486+Stress). Each value is the mean ± S.E.M. of 8 fish. Data are presented as the relative fold change with respect to basal levels in control group. * P < 0.05 relative to the other groups within the same ZT time.
Table 3. Parameters defining daily rhythms of mRNA abundance of food intake-related peptides, general glucocorticoid receptors and sirt1 in rainbow trout hypothalamus.

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITION</th>
<th>Control</th>
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<th>RU486</th>
<th>RU486+Stress</th>
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</tr>
<tr>
<td>P; SE(A)/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>P &lt; 0.05 ; 0.16</td>
<td>P &lt; 0.01 ; 0.16</td>
<td>P &lt; 0.01 ; 0.15</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>P &lt; 0.01 ; 0.15</td>
<td>P &lt; 0.01 ; 0.15</td>
<td>P &lt; 0.01 ; 0.13</td>
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<td>cart</td>
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<tr>
<td>P; SE(A)/A</td>
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<tr>
<td>M</td>
<td>P &lt; 0.05 ; 0.19</td>
<td>P &lt; 0.01 ; 0.16</td>
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<td>npy</td>
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<td>P; SE(A)/A</td>
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<tr>
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<tr>
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<tr>
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<td>18.43</td>
<td>18.26</td>
<td>18.31</td>
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</table>

Data represent the mesor (M) as the mean value, the sinusoidal amplitude of the oscillation (A), and the acrophase (φ; time of peak; ZT/CT). P: P value following ANOVA analysis. SE (A)/A: value of the ratio from the cosinor analysis.
Figure 5. Daily profile of general glucocorticoid receptors (gr1, and gr2) mRNA abundance in hypothalamus of trout exposed to: a) normal conditions (Control group), b) stress by high stocking density for 72 hours (Stress group), c) mifepristone and normal conditions (RU486 group), d) RU486 and stress by high stocking density for 72 h (RU486+stress group). Each value represents the mean ± S.E.M. of 8 fish. Values are presented as the relative fold change with respect to basal levels in control group. * P < 0.05 relative to the other groups within the same ZT time.

DISCUSSION

The hypothesis of this study is supported by our results, consisting on the stress effect on the daily rhythms of mRNA abundance of clock core genes and food intake regulators, and their interaction within hypothalamus of rainbow trout. Since stress affects such interaction, the role of cortisol and/or other mediators like SIRT1, has to be considered. Our results reveal that high stocking density exposure for 72 h induces a significant rise of averaged cortisol levels, but also altered daily profile of the glucocorticoid, relative to control group. Such results together with reduced food intake in stressed group validate our experimental design. However, the mean concentrations of plasma cortisol also increased with RU486 administration done, in
consistency to that previously reported for rainbow trout (McDonald and Wood, 2004), for which altered negative feedback control of cortisol release from the interrenal cells at the head kidney was proposed to be responsible (McDonald and Wood, 2004), in the same way than mammals (Bertagna et al., 1984; Gaillart et al., 1985; Healy et al., 1983), but in contrast to that described for trout receiving RU486 for 7 days (Vijayan et al., 1994b). The nature of this discrepancy might be due to the fact that fish implanted for 7 days could be missing the temporary effect of the antagonist, which persisted after 1.5 days (McDonald and Wood, 2004), but also after 72 h, as herein reported, even when cortisol levels appear to normalize at the late half of the experiment, this may be indicative of 72 h time periods or slightly longer might be needed in order to avoid such negative feedback control exerted by the glucocorticoid.

**Figure 6.** Daily rhythm of *sirt1* mRNA in hypothalamus of trout subjected to different experimental conditions (Control, Stress, RU486, and RU486+Stress). Each value is the mean ± S.E.M. of 8 fish. Data are presented as the relative fold change with respect to basal levels in control group. * P < 0.05 relative to the other groups within the same ZT time. $ P < 0.05$ relative to control and RU486 groups at the same ZT time.

Other parameters relative to secondary response to stress were observed. Accordingly increased plasma glucose averaged levels were observed, which has been previously attributed to increased production of endogenous glucose, reduced use of exogenous glucose, increased capacity of liver for releasing glucose, and enhanced gluconeogenic capacity within trout liver (López-Patiño et al., 2014b). However, different response of plasma glucose levels might be expected, since following exposure to increased cortisol levels, those of glucose can be increased,
decreased or remain unaltered (see rev., Mommsen et al., 1999). On the contrary to that found for glucose, a decrease of averaged plasma lactate levels was found in stressed trout. Such result may indicate that anaerobic metabolic response initiates shortly after stress exposure in rainbow trout (López-Patiño et al., 2014b), but rapidly dissipates in parallel to stress duration, leading to lower lactate levels than those of non-stressed fish.

Stress jeopardizes the circadian system (see rev. Koch et al., 2017). In order to evaluate how the circadian oscillator is affected by stress in trout hypothalamus, the rhythm of mRNA abundance of core circadian clock genes was evaluated in different experimental groups. According to our results, a significant rhythm of clock1a, bmal1, and per1 was found in control fish, in striking consistency with that previously reported by us (López-Patiño et al., 2011). In addition, we observed that rev-erbβ-like also displayed a significant daily rhythm with the acrophase located in antiphase with bmal1, as expected for a repressor of bmal1 expression, as demonstrated in mammals (Cho et al. 2012) and trout (Hernández-Pérez et al., 2017). No changes were found for the acrophase of herein reported circadian genes and stress exposure resulted in a significant decrease of the amplitude and averaged values for clock1a, bmal1, and per1, and enhanced expression of rev-erbβ-like. Then, the existence of any interaction between the stress response and the circadian oscillator might be responsible of such effect of stress. This interaction has been previously studied in mammals (Takahashi et al., 2013), with glucocorticoids acting as mediators of the stress response and in parallel playing an important role as inputs to the circadian oscillators, especially those located in peripheral organs such as liver, kidney, and heart (Balsalobre et al., 2000). Specific glucocorticoid receptors may have a key role in this interaction. By other hand, glucocorticoids may participate as inputs to the circadian system in fish, with cortisol acting as stimulator of per1a and per1b expression, and inhibitor of that of clock and bmal1 in liver of goldfish (Sánchez-Bretaño et al., 2016). Our preliminary results collected from rainbow trout liver are in agreement with such idea (unpublished). However, in hypothalamus of trout subjected or not to mild stress and IP administered with RU486, an inhibitory effect on clock1a, bmal1, and per1 mRNA abundance we found together with enhanced expression of rev-erbβ-like in those animals subjected to stress. This effect was not prevented by RU486 treatment, which may be indicative of the presence of mechanisms other than cortisol through binding to glucocorticoid receptors participating in the negative effect of stress on the hypothalamic circadian oscillator of rainbow trout. However, the rhythm of grl mRNA abundance was significantly affected by stress in such a way that we found increased expression and the disruption of the daily rhythm. According to our results it seems that significantly elevated cortisol levels are needed to enhance grl expression (but not gr2) and only in that situation RU486 is able to prevent such effect, with the antagonist not significantly affecting the daily profile of glucocorticoid receptors at
those lower cortisol levels typically observed in non-stressed animals. Thus, grl may mediate the stress response in rainbow trout hypothalamus, but this effect is not responsible of the altered rhythm of clock genes.

The rhythm of mRNA abundance of food intake regulators was also evaluated. Accordingly, crf, pomc-a1, and npy displayed increased expression during the early dark period and basal values at day-time, whereas cart increased during the night-day transition. Our results are mostly in agreement with that previously reported for Solea senegalensis, in which the acrophases have been reported at the same temporal window for crf and pomc-a1 (López-Olmeda et al., 2013). However, in Epinephelus coioides, peaking levels of npy mRNA abundance are reported at feeding time (Tang et al., 2013), in contrast to our results. Additionally, we demonstrate that stress has negative effect on these rhythms in such a way that crf and npy mRNA abundance enhanced in trout subjected to high stocking density for 72 h, whereas pomc-a1 decreased and cart remained unaltered. These results are consistent with that previously reported by our lab in hypothalamus of rainbow trout subjected to high stocking density (Conde-Sieira et al., 2010b), but also with that found in Literature (see rev. Delgado et al., 2017). Hence, after ICV administration of CRF food intake is reduced in goldfish (De Pedro et al., 1993) and treatment with glucocorticoid antagonists or inhibitors of cortisol synthesis increased crf mRNA brain levels but inhibits food intake, with such effect being reverted by CRF receptor antagonist (Bernier and Peter, 2001). This indicates that CRF plays a key role as physiological transductor of stress effects on food intake in fish (Bernier, 2006). Our results are consistent with this idea, and stressed trout displayed reduced food intake during the experiment, but also point to the involvement of cortisol in maintaining this response, with GR1 apparently acting as mediators, since trout administered with RU486 and subjected to stress did not show any change in hypothalamic crf mRNA content, relative to control non-stressed fish.

The negative effect of stress on food intake could also involve other mechanisms, such as the nutrient sensing systems. In support of this, rainbow trout subjected to chronic stress were reported to display a readjustment of hypothalamic glucosensing mechanisms (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015), leading the fish not be able to compensate with changes in food intake those of plasma glucose levels, which are compensated in non-stressed trout. Accordingly, the response of hypothalamic mRNA abundance of cart, pomc, and npy to glucose changes was altered in stressed trout (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). Our results are in agreement with those changes, but also describe for the first time in fish the dynamics of response to stress along the 24 h LD cycle, with the alteration of averaged levels, but the absence of changes in the phase of the herein reported rhythms of food intake-related peptides.

Subjecting trout to stress results in altered rhythms of core circadian clock genes and food intake regulators. These alterations are only prevented by the
Experimental work 1

glucocorticoid receptor antagonist, RU486, for *crf, pomc-a1* and *npy*, indicating the involvement of cortisol as mediator. The absence of variation of *gr2* among experimental groups might be indicative of cortisol, through binding to specific GR1, to be involved in such effect. Assessing variations of GR instead of gene expression may help to confirm the role of this receptor subtype. On the contrary RU486 treatment was no effective in preventing the alteration of the circadian system during stress exposure. Recent findings in mice show that stress causes altered profile of clock genes expression in peripheral organs, consisting on phase shifts, but not at central level (Bartlang *et al*., 2014; Tahara *et al*., 2015). Then it appears that the central pacemaker located within the hypothalamus is affected by stress, probably reflecting indirect mechanisms (see rev. Koch *et al*., 2017), which is in agreement with our result. Thus, other mechanisms might mediate the effect of stress on the hypothalamic circadian system in rainbow trout, such SIRT1, which play an important role as a link between sensing of cellular energy status and circadian clocks (see rev. Delgado *et al*., 2017). In spite of the rhythm of SIRT1 activity to be controlled by the CLOCK/BMAL1 heterodimer over nicotinamide phosphoribosyltransferase, responsible of NAD+ synthesis (Ramsey *et al*., 2009), SIRT1 also participates in the cyclic control of BMAL1 and PER2 in liver through their deacetylation (Nakahata *et al*., 2008), in mice also by activating the pacemaker within the hypothalamic suprachiasmatic nucleus (Chang and Guarente, 2013). According to this interaction, changes in either clock genes expression or that of SIRT1 may be responsible of the alteration of each other rhythmic profiles of mRNA abundance. Our results show a significant increase of *sirt1* expression in stressed trout with the consequent increase of amplitude and averaged levels of mRNA, but no variation was found for the phase of the rhythm. In addition, RU486 administration did partially prevent this effect, which is in support of SIRT1 mediating the effect of stress on the circadian oscillator within trout hypothalamus. Whereas, RU486 does not totally prevent such effect, the existence of any interaction between different mediators may be considered, such as SIRT1 and cortisol. By other hand, SIRT1 changes appear to reflect the alteration of nutrient status of the animal, such variation during stress exposure may modulate SIRT1 within the hypothalamus, with the subsequent effect on the rhythm of clock genes. This effect seems not to be totally mediated by cortisol, which is only participating in that related to food intake, as above discussed.

In summary, our results reveal the existence of any interaction between the daily rhythms of several clock genes and food intake-related parameters within the hypothalamus of rainbow trout. Stress by high stocking density has a negative effect in most of them. This alteration basically consists on the alteration of the averaged mRNA abundance and amplitude of each gene, with no variation of the acrophases being observed. Cortisol binding to specific GR may be responsible of such effect of stress only in those genes encoding for food intake regulators (*crf, pomc-a1*, and *npy*). In support of this hypothesis, treatment with RU486 prevents such effect of
stress. By other hand, cortisol appears not to be the only mediator of stress on hypothalamic circadian system of rainbow trout, as long as RU486 does not prevent those changes observed in the rhythm of clock genes in stressed trout. Such result is indicative of the existence of other mediators controlling the response of the circadian system to stress. SIRT1 may be one of them based on its role as a link between the cellular energy status and the circadian clock genes, and the significant increase of \textit{sirt1} mRNA abundance observed in stressed trout. This increase is partially counteracted by RU486 treatment, indicating the existence of any interaction between cortisol and SIRT1. Further experiments must be carried out in order to identify the mechanisms responsible of the differential response to stress of the circadian oscillator and food intake regulators within rainbow trout hypothalamus.
3.2. Experimental work 2

SIRT1 is a main mediator of the effect of stress on hypothalamic circadian oscillator and food intake-control peptides in rainbow trout, *Oncorhynchus mykiss*.
Resumen

Papel de la SIRT1 como mediador del efecto del estrés sobre el oscilador circadiano hipotalámico y los péptidos mediadores de la ingesta en el hipotálamo de la trucha arco iris, *Oncorhynchus mykiss*.

El estrés afecta negativamente a una amplia gama de funciones fisiológicas y conductuales (fisiología circadiana e ingesta de alimentos, entre otros), lo que compromete el bienestar de los animales. El cortisol parece mediar el efecto del estrés sobre la ingesta de alimento, bien mediante una acción directa, o bien mediante la interacción con otros posibles mediadores de dicho efecto. Un posible candidato como mediador del mismo es la SIRT1, la cual pertenece a la familia de las SIRTs. Nuestro estudio tuvo como objetivo evaluar el efecto del estrés en las variaciones diurnas y nocturnas de la abundancia de ARNm hipotalámico de los genes del reloj circadiano (*clock1a, bmal1* y *per1*) y los reguladores de la ingesta de alimento (*crf, pome-a1, cart* y *npy*), las variaciones diurnas de la abundancia de ARNm de las enzimas de la biosíntesis de cortisol (*3β-hsd, 11β-h, p450scy y star*) en el riñón anterior, y los cambios de los receptores de glucocorticoides en ambos tejidos de la trucha arco iris, junto con la participación de SIRT1 en tal efecto. Cuatro grupos de truchas fueron sometidos a: 1) densidad normal (grupo control), 2) alta densidad durante 72 horas (grupo de estrés), 3) densidad normal y tratado con el inhibidor de SIRT1 (EX527) presente en el pienso y 4) Tratado con EX527 (presente en el pienso) y estresado durante 72 horas (EX + estrés). Se tomaron muestras de los peces de cada grupo durante el día (ZT10) y la noche (ZT18) y se evaluaron los niveles plasmáticos de cortisol, glucosa y lactato. La abundancia de ARNm hipotalámico de genes reloj, reguladores de la ingesta de alimentos, receptores de glucocorticoides y *sirt1* se analizaron mediante qPCR. La ruta de síntesis del cortisol y los receptores de glucocorticoides también se evaluaron mediante qPCR en el riñón anterior. Nuestros resultados revelan un papel clave desempeñado por SIRT1 en la mediación del efecto del estrés en las variaciones día-noche de la abundancia de ARNm de los genes del reloj hipotalámico así como en los péptidos relacionados con la ingesta de alimento en el hipotálamo de la trucha. La alta densidad de stock inhibe la expresión de genes reloj, mientras que la de los péptidos relacionados con la ingesta de alimentos aumenta. El tratamiento con EX527 previene los cambios observados en los genes reloj durante la exposición al estrés, evidenciando un papel clave desempeñado por SIRT1 como mediador de dicho efecto sobre el sistema circadiano hipotalámico de la trucha. Por otro lado, los cambios de los péptidos relacionados con la ingesta de alimento se son prevenidos con la administración de EX527. Este resultado indica la existencia de interacciones entre SIRT1 y otros mediadores (cortisol) durante la respuesta al estrés. En apoyo de esta idea, nuestros resultados muestran que SIRT1 influye en la biosíntesis del cortisol durante el estrés a nivel riñón anterior. No obstante, es necesario realizar
nuevos experimentos para aclarar los mecanismos a través de los cuales SIRT1 participa en la respuesta del sistema circadiano y los péptidos relacionados con la ingesta de alimentos al estrés, así como la naturaleza de la interacción entre SIRT1 y otros mediadores.
Abstract

SIRT1 is a main mediator of the effect of stress on hypothalamic circadian oscillator and food intake-control peptides in rainbow trout, *Oncorhynchus mykiss*.

Stress negatively affects a wide range of physiological and behavioural functions (circadian physiology and food intake, among others), thus compromising animal welfare. Cortisol appears to mediate the effect of stress on food intake, but other mediators may play a key role in that related to circadian physiology. Either the independent modulatory effect or the existence of any interaction between different mediators might exist during stress exposure. One candidate to mediate such effect is the SIRT family. Then, our study aimed to evaluate the effect of stress on the day-night variations of hypothalamic mRNA abundance of circadian clock genes (*clock1a*, *bmal1*, and *per1*) and food intake regulators (*crf*, *pome-a1*, *cart*, and *npy*), the day-night variations of mRNA abundance of enzymes of cortisol biosynthesis (*3β-hsd*, *11β-h*, *p450scc*, and *star*) at the head kidney, and day-night changes of glucocorticoid receptors in both tissues of rainbow trout, together with the involvement of SIRT1 in such effect. Four groups of trout were subjected to 1) normal stocking density (control group), 2) high stocking density for 72 hours (stress group), 3) normal stocking density and fed with food pellets containing SIRT1 inhibitor (EX527), and 4) fed with pellets containing EX527 and stressed for 72 hours (EX+Stress). Fish from each group were sampled at day- (ZT10) and night-time (ZT18) and cortisol, glucose and lactate plasma levels were evaluated. Hypothalamic mRNA abundance of clock genes, food intake regulators, glucocorticoid receptors and *sirt1* were qPCR assayed. Cortisol biosynthetic pathway and glucocorticoid receptors were also qPCR assessed in head kidney. Our results agree with a key role played by SIRT1 in mediating the effect of stress on the day-night variations of mRNA abundance of core clock genes and food intake-related peptides in trout hypothalamus. High stocking density inhibits clock genes expression, whereas that of food intake-related peptides increases. EX527 treatment prevents those changes observed in clock genes during stress exposure, thus evidencing a key role played by SIRT1 as mediator of this effect on trout circadian system. On the other hand, changes of food intake-related peptides are partially prevented with EX527 treatment. This result indicates the existence of any interaction between SIRT1 and other mediators (cortisol) during response to stress. In support of that, our results show that SIRT1 influences cortisol biosynthesis during stress. Further research is needed in order to clarify the mechanisms through which SIRT1 participates in the response of the circadian system and food intake control to stress, and the nature of the interaction between SIRT1 and other mediators.
SIRT1 is a main mediator of the effect of stress on hypothalamic circadian oscillator and food intake-control peptides in rainbow trout, *Oncorhynchus mykiss*.

**INTRODUCTION**

Endogenous oscillators are responsible of circadian rhythms of a wide number of behavioural and physiological processes in living organisms. These oscillators are entrained to cyclic environmental factors, such as the LD cycles (Pando et al., 2001), temperature (Rensing and Ruoff, 2002), and food availability (Mistlberger and Antle, 2011) among others. In fish, several physiological functions have been demonstrated to be under circadian control as well (Hernández-Pérez et al., 2017). With respect to circadian signals, it has been previously reported the presence of circadian oscillators at both central and peripheral locations. For example, that previously found in our laboratory for rainbow trout (Hernández-Pérez et al., 2017; López-Patiño et al., 2011). The circadian clock machinery is well conserved throughout phylogeny (Panda et al., 2002a,b), and consists on well-coordinated intracellular transcriptional-translational feedback loops that involve a set of “clock genes”. Briefly, the expression of *clock* and *bmal1* leads the activating elements, CLOCK and BMAL1, to increase, thus heterodimerizing, and enhancing the transcription of negative elements, *Period* (*per1*, *per2*, and *per3*) and *Cryptochrome* (*cry1* and *cry2*) genes. PER and CRY complexes inhibit their own transcription by binding to the CLOCK:BMAL1 heterodimer, which blocks its function. This negative loop allows a daily rhythm of *per* and *cry* transcripts abundance and the respective protein products (Iuvone et al., 2005; Okamura et al., 2002). In addition, another feedback loop that depends on nuclear receptors has been described. Specifically, ROR and REV-ERB display opposite transcriptional activities (Giguère, 1999), with ROR family acting as transcription activators, whereas REV-ERB family inhibits the transcription (Dumas et al., 1994; Forman et al., 1994; Retnakaran et al., 1994).

Particularly in teleost lineage, this general pattern remains well conserved but remains far from being understood, since some events contributed to raise the complexity. Thus, some reports evidence the existence of several genes encoding the respective protein(s) with overlapping functions (Coon et al., 1999; Falcón et al., 2003; Forsell et al., 2002). Also, duplicate genes appeared to involve a round of whole genome duplication events occurring within ray-finned fish lineage prior to the radiation of teleosts (Wang, 2008), and finally an additional round of genomic duplication apparently occurred in salmonids at approximately 25-100 Mya (Allendorf and Thorgaard, 1984). Accordingly, salmonids may have different duplicates for most genes. Regarding rhythmicity, this teleost group shows daily variations of mRNA abundance for several clock-genes, such as *clock, bmal1*, and *cry2* in the brain of Atlantic salmon (Davie et al., 2009), and in rainbow trout.
clock1a, bmal1, and per1 are rhythmically expressed in neural tissues, retina and hypothalamus (López-Patiño et al., 2011), but also in non-neural ones, such as liver (Hernández-Pérez et al., 2017).

Food intake displays a daily rhythmic profile in vertebrates under the influence of either biotic (prey availability, social status), abiotic (photoperiod, temperature) and internal factors such as biological clocks (Boujard and Leatherland, 1992; Madrid et al., 2001). This daily profile persists even in the absence of environmental external cues (i.e., constant lighting conditions), thus evidencing both the endogenous nature of such rhythm and a key role played by the circadian system as modulator. Food intake control in fish involves different mechanisms at the CNS, with the hypothalamus as the main regulatory region where, in the same way than mammals, two neurone populations have to be highlighted: the first one, co-expresses NPY and AgRP, whereas the second one co-expresses POMC and CART. This circuit is needed for integrating information that relates to food intake control (see rev. Delgado et al., 2017). Our recent findings evidence the daily profile of mRNA abundance of most of them in rainbow trout hypothalamus (Naderi et al., 2018). Since such profile may control feeding behaviour, the integration of information of different nature might exert any influence on it. This is what is observed in trout subjected to mild stress by high stocking density, in which food intake is significantly inhibited, and the daily rhythm of mRNA abundance of food intake-related peptides (crf, pomc-a1, and npy), and clock genes (clock1a, bmal1, and per1) is jeopardized (Naderi et al., 2018). However, the mechanisms through which such influence is exerted are not fully understood.

In the same way than other vertebrates, exposure of fish to stress results in the activation of the endocrine response that involves both axes, the HSC and the HPI. Such response leads the animal to cope with the increased metabolic demand (Mommsen et al., 1999; Wenderlar Bonga, 1997), thus restoring homeostasis. When subjected to stress, altered feeding behaviour is observed in fish (Bernier, 2006; Bernier and Peter, 2001). The activation of the HPI axis results in enhanced CRF synthesis within the preoptic area. This leads to stimulated release of ACTH from the corticotrophic cells at the adenohypophysis. ACTH binds to specific MCR2 at the interrenal cells of the head kidney, leading to stimulate glucocorticoids synthesis and release into the blood (Mommsen et al., 1999; Wenderlar Bonga, 1997). Once HPI activates, food intake and other functions are affected. Accordingly, food intake is reduced by CRF in a dose-dependent way in goldfish (Bernier, 2006; De Pedro et al., 1993), with this effect being reverted by the receptor antagonist (Bernier and Peter, 2001; De Pedro et al., 1997). Cortisol is the main glucocorticoid in fish and was proposed to play a key role as mediator (Aluru and Vijayan, 2009; Barton, 2002; Bernier, 2006), since treatments with either glucocorticoid antagonists or cortisol synthesis inhibitors enhance crf mRNA abundance in goldfish brain, leading to food intake inhibition. Specifically for rainbow trout, treatments with the general
glucocorticoid receptors antagonist, RU486, prevents the inhibitory effect of stress on food intake (Naderi et al., 2018). All these findings are indicative of the main role played by cortisol in modulating the inhibitory effect of stress on food intake, through its binding to specific glucocorticoid receptors at the hypothalamus.

Recent findings point at mechanisms other than cortisol to also participate as mediators of the inhibitory effect of stress on food intake. In this way, the ability of nutrient sensing systems has been evaluated in rainbow trout, and glucosensing mechanisms have been reported to be affected by chronic stress (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). In that situation, it is noted that animals are not able to compensate with changes in food intake those of plasma glucose levels, but also hypothalamic mRNA abundance of food intake regulators (pomc, cart, and npy) is altered (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015), and their daily rhythm of expression as well (Naderi et al., 2018). CRF in vitro treatment readjusts glucosensing mechanisms in trout hypothalamus (Conde-Sieira et al., 2011), which confirms its involvement in mediating the effect of stress on food intake control (Evans et al., 2004; McCrimmon et al., 2006).

Another candidate as mediator of the effect of stress on food intake is the SIRTs family. Specifically, SIRT1 (silent mating type information regulation 2 homolog) 1 (S. cerevisiae) is an evolutionary conserved nicotinamide adenine dinucleotide (NAD+) -dependent deacetylase that has been also described as an energy sensor (Imai et al., 2000). Sirt1 expresses in a wide range of peripheral and central tissues, including the POMC neurons and AgRP neurons within the arcuate nucleus of the hypothalamus that is important for regulating food intake (Ramadori et al., 2008; Sasaki et al., 2010). Accordingly, SIRT1 has been proposed to participate as food intake regulator, with conflicting information being available at this respect. For example, ICV administration of SIRT1 inhibitor, EX527, suppresses food intake (Çakir et al., 2009; Dietrich et al., 2010), whereas the effect of hypothalamic sirt1 overexpression is not observed in normophagic mice (Sasaki et al., 2010). Information about SIRT1 and food intake in fish is very scarce. In this way, our previous study carried out in rainbow trout hypothalamus reveals that stressed trout display a significant increase of sirt1 mRNA abundance in parallel to decreased food intake (Naderi et al., 2018), which may indicate that SIRT1 is participating in food intake control in fish. However, mechanisms involved in such regulatory effect are unknown in this vertebrates group.

In addition to the impact on food intake, stress also negatively affects the circadian system in fish, as recently reported for rainbow trout (Naderi et al., 2018), which leads to altered rhythmic behavioural and physiological functions. In spite of the daily endocrine rhythms participating as outputs of the circadian system, some reports suggest that some hormones can play a role as input to the hypothalamic and peripheral clocks (Challet, 2015; Coomans et al., 2015). In this way, glucocorticoids circulating levels are rhythmic in vertebrates including fish. Among them, cortisol
appears to participate as output to the circadian system, since circulating levels synchronize to the feeding-fasting cycle and feeding time in fish (see rev. Isorna et al., 2017). Even when cortisol has been considered as an input to the circadian system in fish, such role is apparently only in that related to peripheral clocks, but not in central tissues. Accordingly, cortisol stimulates the expression of per1a and per1b, and inhibits that of clock and bmal1 within goldfish liver (Sánchez-Bretaña et al., 2016), which is in agreement with our preliminary data obtained from liver of rainbow trout subjected to mild stress (unpublished). On the contrary, our results obtained in rainbow trout reveal that cortisol is not apparently playing a key role as input to the hypothalamic circadian system since treatment with the glucocorticoid receptors antagonist, mifepristone, is not able to prevent the inhibitory effect of stress on clock1a, bmal1, and per1 daily rhythm of mRNA abundance (Naderi et al., 2018). Then, it might be interesting to identify other mediators of the effect of stress on hypothalamic circadian system in fish.

Based on our preliminary findings we speculate with SIRT1 as possible mediator of the effect of stress on the hypothalamic circadian system in fish (Naderi et al., 2018). SIRTs are well-characterized (NAD+-dependent class III histone deacetylases that are involved in a wide range of effects of intracellular metabolism. In particular SIRT1 and SIRT6 have been proposed to link cellular metabolism and circadian clock physiology in mammals (Masri et al., 2014; Orozco-Solis et al., 2015). In addition to the role proposed for SIRTs in food intake regulation, it has been described that SIRT1 enzyme activity also fluctuates through the feeding/fasting cycle in rodents (Çakir et al., 2009) with the CLOCK/BMAL1 heterodimer modulating the enzyme nicotinamide phosphoribosyltransferase (NAMP), responsible of NAD+ synthesis (Ramsey et al., 2009), thus controlling SIRT1. Our previous results are in the same way, since sirt1 displays a daily rhythm of mRNA abundance in hypothalamus of rainbow trout (Naderi et al., 2018). By other hand, SIRT1 participates in the cyclic control of cofactors and peptides of circadian clocks by deacetylating BMAL1 and PER2 in peripheral tissues such as liver (Nakahata et al., 2008), and activating the hypothalamic SCN pacemaker in mice (Chang and Guarente, 2013). The role of molecular sensors, SIRTs, in modulating circadian oscillators in fish is not fully understood, but the rhythmic profile of sirt1 mRNA abundance (Naderi et al., 2018), which perfectly fits with the functioning of fatty acid sensing systems (Velasco et al., 2016a,b) is indicative of such modulatory action to exist. In consistency with this idea, feeding-related rhythms of digestive enzymes were reported to be driven by the circadian system (Vera et al., 2007; Montoya et al., 2010; Nisembaum et al., 2014). Thus, the entrainment of circadian clocks by nutrient status in fish might be expected (see rev. Delgado et al., 2017), with SIRTs participating as mediators. Considering the interaction between SIRT1 and the circadian clock machinery, one may expect that the alteration of sirt1 mRNA levels during stress exposure (Naderi et al., 2018) may be responsible of that of core circadian clock genes in central tissues.
Taking in consideration the above mentioned interactions between the circadian system and food intake regulation, and the negative influence of stress on the hypothalamic circadian clock machinery of rainbow trout, the aim of the present study was to evaluate the role played by SIRT1 in mediating such changes. Accordingly, day-night variations of clock genes (clocka1, bmal1, and per1) and food intake-related peptides (crf, pome-a1, cart, and npy) were evaluated in hypothalamus of trout subjected or not to stress in the presence of the SIRT1 inhibitor (EX527). In addition, the cortisol biosynthetic pathway (3β-hsd, 11β-h, p450scc, and star) was also assessed in head kidney in order to evaluate the influence of SIRT1 at this level during the stress response.

MATERIAL AND METHODS

Fish

Rainbow trout (Oncorhynchus mykiss, Walbaum) weighing 80 ± 1.4 g were obtained from a local fish farm (A Estrada, A Coruña) and transferred to our facilities at the Faculty of Biology (University of Vigo). Animals were kept in 120 L tanks with filtered and continuously renovated fresh water (13.5 ± 1°C). Feeding time was scheduled at zeitgeber time (ZT) 2 (ZT0 = light on), Food consisted on a commercial (Dibaq diproteg) dry pellet diet (1% body weight). Lighting conditions were 12L:12D photoperiod. Illumination was provided by means of LED light lamps (Superlight Technology Co. Ltd., China). Also, irradiance was measured with a spectro-radiometer (FieldSpec ASD, Colorado, USA) set at 1.62 E^18 photons m^-2 s^-1. Fish were acclimated for 15 days to standard conditions before any experiment to proceed. All the experiments agreed with the Guidelines of the European Union Council (2010/63/EU), and of the Spanish Government (RD 53/2013) for the use of animals in research. Animal protocols were also approved by the Animal Care Committee at the University of Vigo, and agreed with international ethical standards (Portaluppi et al., 2010).

Sampling

Fish were deeply anaesthetized by addition of 2-phenoxyethanol (0.2% v/v- Sigma Aldrich) to tank water. To guarantee the uniform mix of anesthetize the appropriate volume of 2-phenoxyethanol was previously diluted in 5-L of tank water and afterwards added into the fish tank. This procedure was done in the absence of visual contact between fish and the manipulators, thus minimizing the incidence of acute stress response. Once anesthetized, individual blood samples were collected and animals were rapidly sacrificed. From each animal 1 mL of blood was collected by caudal puncture with help of ammonium-heparinized syringes. After sacrifice, individual hypothalamus, including the preoptic area, was removed under sterile
conditions, according to previously described (Doyon et al., 2003), and individual head kidney as well. Samples were placed into sterile RNase-free 1.5 ml Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80°C until qPCR assayed for mRNA abundance of clock genes (clock1a, bmal1, per1), anorexigenic (crf, pomc-a1, and cart) and orexigenic (npy) neuropeptides, SIRT1 (sirt1), general glucocorticoid receptors (gr1 and gr2) and genes related to cortisol biosynthesis at the head kidney (3β-hsd, 11β-h, p450scc, and star). Plasma samples were obtained after blood centrifugation and then immediately frozen on liquid nitrogen and stored at -80°C until assayed for cortisol and metabolites (glucose and lactate) levels.

**Experimental design**

To assess the role played by SIRT1 as mediator of the physiological response to stress, trout were divided in four cohorts (N = 50 fish/cohort), and divided in 2 tanks each (n = 25 fish/tank). Following acclimation period fish from each cohort were daily fed to satiety up to food intake to stabilize among groups. Then, animals were subjected to different experimental conditions: 1) normal stocking density (control group); 2) normal stocking density receiving the SIRT1 inhibitor in food (EX527 group); 3) high stocking density (70 kg fish/m³) for 72 hours (stress group); and 4) high stocking density and fed with EX527 (EX527+Stress).

The first cohort (control group) was kept under normal stocking density all over the experimental period (15 days). Food intake was daily assessed at ZT2. On the day of sacrifice, animals from each tank were anesthetized (2-phenoxyethanol, 0.2% v/v) at two different time periods, ZT10 and ZT18 (n=15/time point). Such time points were selected according to previous results describing the daily profile of mRNA abundance for the herein assessed genes, for which high or low levels of expression was found at a given time point, but also in order to assess day/night variations for a given parameter. Sacrifice and sampling procedure did never exceed 15 min/time point. Animals sampled at night were transferred to a new tank containing anesthetic solution and moved out of the housing room, which prevented any light contamination during the dark period. Then, fish were blood collected in the absence of light, and sacrificed. Dead bodies were manipulated under dim red light for times dissection.

The second cohort (EX527 group) was kept in identical conditions than control group. However after the experiment initiated, trout were daily fed to satiety with food containing EX527 (2 µg/g of dry food). For that porpoise a 10 mM solution of EX527 was prepared, and food immediately immersed on it. Once food pellets completely absorbed this solution, food was dried out for 24 h at 37°C and then administered to the animals. Following 15 days treatment animals were sacrificed at ZT10 and ZT18, blood collected and sampled as above mentioned for control group. Food intake was daily evaluated at ZT2.
The third cohort (stress group) was maintained under identical conditions than control group and for 12 days and after that water level was readjusted in order to reach a high stocking density (70 kg fish/m³), which induces stress response in trout. Reaching such water level took 60 min for each tank. In addition, water flow was readjusted in order for water quality to be identical to that of non-stressed trout. Animals remained under these conditions for the following 72-hours, and afterwards blood collected and sacrificed at ZT10 and ZT18 (n=15/time point) as described above. Fish were daily hand-fed as scheduled, and food intake was also evaluated at ZT2.

Last group, EX527+Stress, was maintained for 12 days under identical conditions than EX527 group, i.e., fish were daily fed to satiety with food containing EX527 (2 µg/g of dry food). Then, water level was readjusted to reach high stocking density, as above described for stress group. Fish remained under such conditions for the following 72-h, but were daily fed at ZT2 with food containing EX527. Food intake was evaluated all over the experiment. On the day of sacrifice, animals anesthetised, blood collected and sacrificed at ZT10 and ZT18 as above mentioned.

Food intake Assessment

To assess food intake, food containing EX527 or not was supplied in batches of approx. 10 g every 5 min until animals were satiated. After feeding, the food uneaten remaining at the bottom (conical tanks) was withdrawn, dried for 24 h at 37ºC and weighed. The amount of food consumed by all fish within each tank was calculated as previously described (Polakof et al., 2008 a,b) as the difference from the food offered (De Pedro et al., 1998b). FI values registered after treatment are referred to those of basal values.

Plasma cortisol and metabolites

Plasma cortisol levels were measured using a commercially available Enzyme Immunoassay kit (Cayman, Ann Arbor, MI, USA), according to manufacturer's indications. Kit was previously validated in our laboratory, and detection limit was 6.6 pg/ml, inter-assay coefficient was 7.5%, and the intra-assay limit was 5.6%. The recovery percentage was also estimated as 92.3 ± 11.5%.

Plasma glucose and lactate levels were determined enzymatically in deproteinized plasma samples by using commercial kits (Biomérieux, France and Spinreact, Girona, Spain, respectively) adapted to a microplate format and following manufacturer’s indications.
Real-time quantitative RT-PCR (qPCR)

Total RNA was extracted from individual hypothalamus and head kidney samples using the TRIzol® (Life Technologies, Grand Island, NY, USA) method, and treated with RQ1-DNAse (Promega, Madison, WI, USA). The same amount of RNA (2 μg) from each sample was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega) and Random Primers (Promega). A negative control for each sample was assessed without reverse transcriptase in order for any genomic contamination to be absent in the RNA extract.

The qPCR was performed using a Maxima™ SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and a Bio-Rad MyIQ Real Time PCR system (BIO-RAD, Hercules, CA, USA). The primers were designed on the basis of previously reported sequences for rainbow trout genes, and obtained from Sigma (see Table 1), including clock1a, bmal1, per1, crf, pomc-a1, cart, npy, sirt1, gr1, gr2, 3β-hsd, 11β-h, p450scc, star, and β-actin.

Relative quantification of a target gene transcript was assessed using β-actin gene expression as reference, since no day-night variation was found among experimental groups. Thermal cycling was initiated with incubation at 95°C for 3 min; followed by 35 steps of PCR, each one consisting of heating at 95°C for 10 s for denaturing, and at specific annealing for 30 s and extension at 50°C for 30 s. Immediately after the last PCR cycle, melting curves were systematically monitored (50°C temperature gradient at 0.5°C/s from 50 to 95°C) to ensure that only one fragment was amplified. Relative mRNA abundance level was calculated following the Pfaffl method (Pfaffl, 2001). For each gene, samples collected at the same time point were processed in parallel and the expression was measured in triplicate within the same microplate. Efficiency values between 85% and 100% were accepted (the $R^2$ for all the genes assessed was always higher than 0.985).

Statistical analysis

Statistical comparisons of plasma cortisol and metabolites, as well as mRNA abundance were performed by using a two-way ANOVA test with ‘‘time’’ and ‘‘treatment’’ as main factors. When a significant effect was noted within a factor, post hoc comparisons were carried out by using a Student-Newman-Keuls test. Significance level was set at $P < 0.05$. 


Table 1: Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>clock1a (GU228520)</td>
<td>TACACGTCAGAATGTTCCC</td>
<td>GAAACTGAAGTCCTGAAT</td>
<td>57</td>
</tr>
<tr>
<td>bmal1 (GQ489026)</td>
<td>TCTTGTCCTGTCCTTTC</td>
<td>TGGACATTTCCACAGAT</td>
<td>55</td>
</tr>
<tr>
<td>per1 (AF228695)</td>
<td>GCTGCACGCTGCTGT</td>
<td>GGTGTACCGAGGAGAT</td>
<td>55</td>
</tr>
<tr>
<td>gr1 (NM001124730)</td>
<td>AGAAGCGCTGTTTGCGCCTGA</td>
<td>AGATGAGCTGACATCCTGAT</td>
<td>59</td>
</tr>
<tr>
<td>gr2 (AY495372.1)</td>
<td>CATCGCAGACGATCTGAAC</td>
<td>AGCAGCAGCAGACCAT</td>
<td>55</td>
</tr>
<tr>
<td>crf (AF296672)</td>
<td>ACAACGACTCAACTGAAGATCTCG</td>
<td>AGGAAATTGAGCTGTCAGG</td>
<td>60</td>
</tr>
<tr>
<td>cart (NM001124627)</td>
<td>ACCATGGAGAGCTCCAG</td>
<td>GGGCACTGCTCTCCA</td>
<td>60</td>
</tr>
<tr>
<td>pomc-a1 (TC86162,Tigr)</td>
<td>CTCGCTGTCAGCTCAACTCT</td>
<td>GAGTGGGTTGGAGATGGACCTC</td>
<td>60</td>
</tr>
<tr>
<td>nyp (NM001124626)</td>
<td>CTCGCTGGAGAGCTCCAG</td>
<td>GGGCACTGCTCTCCA</td>
<td>60</td>
</tr>
<tr>
<td>sirt1 (EZ774344.1)</td>
<td>GCTACTTTGGGACTGTGACG</td>
<td>CTCGAATGCAAGGCC</td>
<td>58</td>
</tr>
<tr>
<td>star (AB047032)</td>
<td>CTCCTACAGACATATGGAGAAGC</td>
<td>GGGCGGTTGTGGAGATGGACCTC</td>
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<tr>
<td>p450scc (S57305.1)</td>
<td>ATGCGTCAGGACACTAACAC</td>
<td>CAGCAGGTTGGAGGAGAAGC</td>
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<tr>
<td>3β-hsd (S72665.1)</td>
<td>TCACAGGGTGCAAGCTTACAGG</td>
<td>CTGGCAAGTTGGAGGAGAAGC</td>
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<tr>
<td>11β-h (AF179894)</td>
<td>ATTTGCCCTGTCAGGAGGTTG</td>
<td>GGATGATGGATGCTCTGACTG</td>
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<tr>
<td>β-actin (AJ438158)</td>
<td>GATGGGCCAGAAAAGACAGCA</td>
<td>TCGTCCCAGTTGGATGACGAT</td>
<td>59</td>
</tr>
</tbody>
</table>

RESULTS

Food intake of trout subjected or not to stress and fed with pellets containing or not EX527 is shown in Figure 1. Control group displayed constant food intake along the experiment, since no variation was found between that observed during the last 72 h period and baseline. Stress significantly inhibited food intake at any experimental condition. As a result, trout subjected to high stocking density displayed a decrease of food intake up to 45% of baseline levels. Oral administration of EX527 was not able to counteract such inhibitory effect of stress, with EX+Stress group displaying a 55% food intake compared to that of baseline. EX527 did not affect food intake itself.
Figure 1. Averaged daily food intake of trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress) during the last 72 h period of the experiment. Data are represented as a percentage of baseline levels of food intake consisting on averaged food intake of acclimation period and the 12-days period of the experiment. Different letters indicate significant differences among groups p<0.05.

Day-night variations of plasma cortisol and metabolites levels of fish subjected to each experimental condition are shown in Figure 2. Cortisol levels in control group did not show day-night variations. Averaged hormone levels were 13.32 ng/mL. Fish subjected to high stocking density significantly increased cortisol levels at both day and night, relative to control. Then, averaged hormone levels also increased (32.71 ng/mL) relative to that found in non-stressed trout. EX527 treatment was able to prevent the effect of stress on plasma cortisol levels, which were comparable to those of control fish. Additionally, EX527 did not influence plasma cortisol levels.

Plasma glucose levels did not display neither any significant day-night variation at any experimental condition nor significant variation among groups at a given time point. On the contrary, lactate levels in control group showed a significant day-night variation, with the highest levels being observed during the day. Averaged levels in this group were 0.99 mM. No significant day-night variation was found in the other groups, but the two way ANOVA revealed a significant variation for “treatment” as main factor (F3,65=4.452; P=0.007). Accordingly, stress enhanced lactate levels, with averaged plasma lactate being 1.28 mM. Such effect was prevented by EX527 treatment, which did not affect metabolite levels itself. Averaged lactate levels were 0.91 mM and 0.79 mM for EX+Stress and EX527 groups, respectively.
Figure 2. Plasma cortisol, glucose and lactate levels in rainbow trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress). Each value is the mean ± S.E.M. of 10 fish. Different letters indicate significant differences among groups p<0.05; * p < 0.05 between day and night.

Day-night variation of mRNA abundance of stress- and food intake-related (crf, pomc-a1, cart and npy) peptides in hypothalamus of trout subjected to each
experimental condition is represented in Figure 3. The day-night variation of \( crf \) mRNA abundance was dependent of both main factors (treatment and time) and the interaction between them did also reach significance level (two way ANOVA; \( F_{3, 24}=8.445; P<0.001 \)). Control group did not show day-night variation, and averaged levels of \( crf \) were 1.21±0.09 relative fold change units. Stress resulted in enhanced \( crf \) expression at both day and night, relative to control group, with the subsequent increase of averaged levels (3.81±0.50 relative units), compared to control. Additionally, mRNA levels were significantly higher at night in the Stress group. EX527 treatment prevented the effect of stress on hypothalamic \( crf \) expression during the day and most of it at night. Thus, averaged mRNA levels (2.03±0.13 relative units) were higher than those of control fish. EX527 administration did not affect \( crf \) expression itself. Thus, averaged mRNA levels (1.37±0.07 relative units) were comparable to those of control group.

Regarding daily \( pomc-a1 \) variation of mRNA abundance, a significant interaction between main factors was revealed by the two way ANOVA analysis (\( F_{3, 24}=9.947; P<0.001 \)), but also each main factor did individually influence such profile. Thus, control group displayed a daily variation with the highest levels being observed in those samples collected at night (ZT18). Averaged \( pomc-a1 \) levels were 1.24±0.10 relative units. Stress exposure induced a significant increase of \( pomc-a1 \) mRNA abundance either at day- and night-time, for which averaged mRNA levels (2.81±0.33 relative units) were higher than those of control. EX527 was not effective in preventing such effect of stress at day-time, but partially did at night-time. As a consequence, the day-night variation of \( pomc-a1 \) mRNA abundance was not observed in EX527+Stress group. However, a significant decrease of averaged mRNA abundance was observed (1.88±0.15 relative units) relative to Stress group (two way ANOVA; \( P<0.001 \)), but still remained higher than that of control (two way ANOVA; \( P<0.001 \)). EX527 treatment did not affect \( pomc-a1 \) day-night profile itself. Thus, averaged \( pomc-a1 \) mRNA abundance in EX527 group (1.32±0.08 relative units) remained similar to that of control group.

Assessment of hypothalamic content of \( cart \) mRNA abundance revealed that each main factor (treatment and time) did individually influence such profile, whereas no significant interaction between them both was noted. Control group showed a significant day-night variation of \( cart \) mRNA abundance, with highest levels being observed at night. Averaged levels in this group were 1.60±0.25 relative units. A significant increase of \( cart \) expression was observed in stressed trout at both time points assayed, leading to significantly higher averaged mRNA levels (2.22±0.27 relative units) than those of control. EX527 was not effective in preventing the stimulatory effect of stress on \( cart \) expression during the day, but at night-time, thus showing a significant decrease of mRNA levels (two way ANOVA; \( P=0.010 \)) relative to stressed fish. Additionally, averaged levels of \( cart \) mRNA abundance in the EX527+Stress group significantly decreased (1.95±0.22 relative units).
units) when compared to those of Stress group (Two way ANOVA, P=0.010), but remained significantly higher than those of control (Two way ANOVA, P=0.004). EX527 alone did not affect the day-night variation of *cart* mRNA abundance, thus showing similar averaged values (1.61±0.23 relative units) than control.

Hypothalamic *npy* mRNA levels in rainbow trout also displayed a day-night variation in all the experimental groups. Additionally, a significant interaction between main factors was revealed by the two way ANOVA analysis (F3, 24=8.877; P<0.001), and each main factor did individually influence such profile. A significant day-night variation of *npy* mRNA abundance was found in control group, with highest levels being observed at night. Averaged mRNA values were 3.87±1.16 relative fold change units in control. Stress exposure resulted in a significant increase of *npy* levels at both time periods, leading to a significant increase (two way ANOVA; P<0.001) of averaged *npy* levels (8.46±1.89 relative units) compared to control. However the day-night variation of mRNA abundance persisted in stressed trout. Treatment with EX527 was not effective in preventing the effect of stress on *npy* expression at day-time and partially blocked such effect at night. Thus, a significant decrease of mRNA levels was noted in EX+Stress group (two way ANOVA; P<0.001) relative to Stress group, but remained significantly higher than that of Control (two way ANOVA; P<0.001). As a consequence, averaged *npy* levels were significantly lower (6.39±1.22 relative units) than those of stressed trout (P<0.001), but higher than those in control (P<0.001). The day-night variation of hypothalamic *npy* mRNA abundance was not affected by EX527 treatment in non-stressed trout, relative to control group. Then averaged values (3.81±0.98 relative units) were similar to those of control.

Day-night variations of mRNA abundance of hypothalamic clock genes (*clock1a, bmal1*, and *per1*) are represented in Figure 4. The two-way ANOVA analysis revealed the existence of a significant interaction between main factors influencing *clock1a* expression (F3, 24=10.744; P<0.001), and each main factor did individually affect it. Control group showed significantly higher levels of mRNA during the day than at night. Averaged levels were 1.38±0.15 relative fold change units. Stress exposure resulted in the loss of such day-night variation, due to the inhibitory effect exerted on *clock1a* expression only at day-time (two way ANOVA; P<0.01 relative to control), and the subsequent decrease of averaged mRNA levels (1.21±0.05 relative units) relative to control (P=0.02). EX527 administration in food was effective in preventing the effect of stress on *clock1a* expression. Thus, a significant difference was found for mRNA levels at day-time between both groups (P<0.001). Additionally, averaged mRNA levels in EX527+Stress group (1.55±0.15 relative units) remained similar to those of control, but were significantly higher than that observed in Stress group (two way ANOVA; P<0.001). EX527 treatment alone did not influence the day-night variation of *clock1a* abundance, with animals showing similar averaged levels (1.42±0.13 relative units) than control.
Figure 3. Day-night variation of mRNA abundance of food intake related neuropeptides (crf, cart, pomc-a1 and npy) in hypothalamus of rainbow trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress). Different letters indicate significant differences among groups p<0.05; * p < 0.05 between day and night.
Similar results were observed after assessing bmal1 mRNA abundance in trout hypothalamus. A significant interaction between main factors influencing bmal1 expression was revealed by the ANOVA analysis (F_{3, 24}=8.526; P<0.001), but also each main factor did individually affect it. A day-night variation was observed for bmal1 mRNA abundance in control group, with the highest levels occurring during the day. Averaged levels were 2.20±0.48 relative fold change units. Stress exposure induced a significant decrease of bmal1 expression only at day-time relative to control group (two way ANOVA; P<0.01), thus leading for the day-night variation to disappear. As a consequence, averaged mRNA abundance did also significantly decrease (1.45±0.12 relative units) when compared to control (two way ANOVA; P=0.004). EX527 treatment totally prevented the effect of stress on bmal1 expression. Then, EX527+Stress group displayed comparable day-night variation and averaged mRNA levels (2.33±0.52 relative units) than those of control. EX527 alone had no effect on the day-night variation of hypothalamic bmal1 mRNA abundance, for which averaged levels (2.25±0.42 relative units) were comparable to those of control.

Regarding daily per1 variation of mRNA abundance, a significant interaction between main factors was revealed by the ANOVA analysis (F_{3, 24}=5.677; P=0.004), but also each main factor did individually influence such profile. Control group displayed a day-night variation of mRNA abundance, with the highest levels being observed at night-time. Averaged per1 levels were 4.68±1.45 relative fold change units. Stress exposure resulted in decreased per1 expression only at night-time (ZT18) relative to control (two way ANOVA; P<0.01), but the day-night variation of mRNA abundance still persisted (P<0.001). Such inhibitory effect of stress caused a significant decrease of averaged per1 levels (3.39±0.90 relative units) relative to those of control group (two way ANOVA; P=0.005). Administering EX527 was effective in preventing the effect of stress. This group (EX527+Stress) showed similar results than control group in both the day-night variation and averaged mRNA levels (4.89±1.43 relative units), with the latest being significantly higher than those of stressed animals (two way ANOVA; P=0.006). EX527 administration did not affect the day-night variation of hypothalamic per1 mRNA abundance itself, thus displaying averaged levels (4.75±1.38 relative units) comparable to those of control, and the day-night variation as well.

Results from the assessments of sirt1 mRNA abundance in hypothalamus of trout subjected to each experimental condition are represented in Figure 5. The two way ANOVA revealed the existence of a significant interaction between main factors (F_{3, 24}=3.426; P=0.033), and the influence of each of them on mRNA levels. Control group displayed a day-night variation with the highest levels being observed in samples collected at day-time. Averaged mRNA levels were 2.19±0.47 relative fold change units in this group. Trout exposed to stress significantly increased sirt1 expression at both time periods assayed, compared to control group (two way
ANOVA; P<0.001, each), resulting in a significant increase of averaged mRNA levels (6.38±0.61 relative units) relative to control (P<0.001). However the day-night variation of sirt1 mRNA abundance persisted in stressed fish. EX527 treatment totally prevented the effect of stress at day-time, but not at night. As a result, sirt1 abundance in the EX527+Stress group at day-time was significantly lower than that of stress group (P<0.001), and remained similar to that of control. On the contrary, a significant decrease of mRNA levels was observed at night, relative to those of stressed fish (P<0.001), but remained significantly higher than those of control (P=0.014) in the EX527+Stress group. This resulted in the day-night variation of sirt1 levels to disappear. As a consequence, averaged levels of sirt1 mRNA abundance in this group (2.76±0.16 relative units) significantly decreased respect to those of stress group (two way ANOVA; P<0.001), but remained similar to those of control. EX527 treatment alone did not affect sirt1 expression at both time points assayed relative to control group. Thus, averaged levels of sirt1 mRNA abundance (2.41±0.39 relative units) were comparable to those of control group.

Day-night changes of mRNA abundance of genes involved on cortisol biosynthesis (star, 3β-hsd, p450scc, and 11β-h) at the head kidney of rainbow trout subjected to each experimental condition are represented in Figure 6. All of them displayed similar variations that were analyzed dependent on the experimental condition. In addition, the two way ANOVA revealed the existence of a significant interaction between main factors for most of them (star; F3, 32=4.506; P=0.010; 3β-hsd; F3, 32=7.932; P<0.001; and p450scc; F3, 32=4.941; P=0.006) and the influence of each of them on mRNA levels. Thus, control group displayed significant day-night changes of mRNA abundance for each gene (star, 3β-hsd, p450scc, and 11β-h), with the highest levels being observed in samples collected at night-time. Averaged levels of mRNA were 1.66±0.27 relative fold change units (star), 2.65±0.63 relative units (3β-hsd), 2.71±0.65 relative units (p450scc), and 1.98±0.38 relative units (11β-h). When animals were subjected to stress a significant increase of each gene was observed, relative to control. In consequence, averaged mRNA levels increased as well (star: 2.97±0.19 relative units; 3β-hsd: 5.35±0.39 relative units; p450scc: 5.88±0.49 relative units; 11β-h: 3.56±0.40 relative units) relative to control (P<0.001 each). Efficiency of EX525 treatments were gene- and time point-dependent. Accordingly, those changes enhanced by stress exposure at day-time were not avoided (star) or partially prevented (3β-hsd, p450scc, and 11β-h) by EX527 administration with food. On the contrary, EX527 totally (star, and 11β-h) and partially (3β-hsd, and p450scc) prevented such effect. Averaged levels of mRNA abundance in EX527 group were 2.17±0.18 relative fold change units (star), 4.10±0.46 relative units (3β-hsd), 4.42±0.36 relative units (p450scc), and 2.72±0.35 relative units (11 β-h). EX527 alone did not affect the day-night variation of each gene relative to control. Thus, averaged expression level of each gene were comparable to those of control trout, i.e., 1.79±0.23 relative units (star), 2.65±0.59
relative units (3β-hsd), 2.74±0.59 relative units (p450scc), and 2.04±0.38 relative units (11β-h).

Figure 4. Day-night variation of mRNA abundance of core clock genes (clock1a, bmal1, and per1) in hypothalamus of rainbow trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress). Different letters indicate significant differences among groups p<0.05; * p < 0.05 between day and night.
Figure 5. Day-night variation of sirt1 mRNA abundance in hypothalamus of rainbow trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress). Different letters indicate significant differences among groups p<0.05; * p < 0.05 between day and night.

Figure 6. Day-night variation of mRNA abundance of cortisol biosynthesis pathway (star, 3β-hsd, p450scc and 11β-h) in head kidney of rainbow trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress). Different letters indicate significant differences among groups p<0.05; * p < 0.05 between day and night.
Results from the assessments of *gr1* and *gr2* mRNA abundance in hypothalamus and head kidney of trout subjected to each experimental condition are represented in Figure 7. With respect to *gr1*, the two way ANOVA analyses revealed the existence of significant interactions between main factors in both tissues (hypothalamus: $F_{3, 24}=3.025; P=0.049$; head kidney: $F_{3, 32}=8.817; P<0.001$), but also the influence of each individual factor on *gr1* mRNA levels. Control group showed a significant day-night variation of *gr1* mRNA abundance in both tissues, with the highest levels being measured in samples collected during the night. Averaged levels of mRNA were $2.06\pm0.40$ relative fold change units in hypothalamus, and $1.84\pm0.32$ relative units in head kidney. Stress exposure induced a significant increase of mRNA abundance in both tissues, relative to control group at either day- ($P<0.001$ each) and night-time ($P<0.001$ in hypothalamus and $P=0.001$ in head kidney). This effect of stress was more pronounced at day-time. As a consequence, the day-night variation of mRNA abundance disappeared in both tissues, but also averaged mRNA levels (hypothalamus: $5.89\pm0.38$ relative units; head kidney: $3.63\pm0.18$ relative units) significantly increased relative to control (two-way ANOVA; $P<0.001$ each). EX537 administration totally prevented such effect of stress in hypothalamus at both time points assessed. Then, a significant decrease of *gr1* mRNA abundance was observed in the EX527+Stress group relative to stress group at both day- and night-time ($P<0.001$ each), up to reach similar levels than control. As a consequence, averaged mRNA levels ($2.72\pm0.23$ relative units) remained similar to those of control group. At the head kidney, EX527 treatment totally prevented the effect of stress only at night, whereas a partial protective effect was observed at day-time. As a result, *gr1* mRNA abundance in head kidney of EX527+Stress trout was significantly lower than that of stressed fish at day- ($P<0.001$), and night-time ($P=0.002$), but higher than control group ($P=0.003$) only at day-time. As a consequence, averaged mRNA abundance in EX525+Stress group remained higher ($2.32\pm0.22$ relative units) than that of control (two-way ANOVA; $P=0.019$), but lower than that observed in Stress group (two-way ANOVA; $P<0.001$). EX527 alone did not affect the day-night variation of hypothalamic *gr1* mRNA abundance relative to control. Thus, averaged expression levels ($2.19\pm0.53$ relative units) were comparable to those of control. On the contrary, EX527 alone enhanced *gr1* expression in head kidney only at day-time ($P=0.024$) relative to control group. Such effect resulted in a small increase of averaged mRNA levels ($2.21\pm0.25$ relative units) relative to control (two-way ANOVA; $P=0.033$).

The two way ANOVA analyses carried out after assessing *gr2* mRNA abundance in hypothalamus and head kidney did not reveal any significant interactions between main factors in both tissues, and only *time* influenced mRNA levels (two-way ANOVA; $P<0.001$ in both tissues). Accordingly, a day-night variation of mRNA abundance was found for each experimental group, with higher mRNA levels being measured in samples from both tissues collected at night. Averaged expression levels were comparable among experimental groups in both
tissues, i.e., hypothalamus: 2.30±0.57 relative fold change units (control), 2.37±0.48 relative units (Stress), 2.21±0.44 relative units (EX527+Stress), and 2.47±0.53 relative units (EX527); head kidney: 1.66±0.28 relative fold change units (control), 1.99±0.16 relative units (Stress), 1.79±0.19 relative units (EX527+Stress), and 1.79±0.24 relative units (EX527).

**Figure 7.** Day-night variation of mRNA abundance of glucocorticoid receptors (gr1, gr2) in hypothalamus (left panels) and head kidney (right panels) of rainbow trout subjected to different experimental conditions (Control, Stress, EX527, and EX527+Stress). Different letters indicate significant differences among groups p<0.05; * p < 0.05 between day and night.

**DISCUSSION**

Our results support the hypothesis of this study, consisting on a main role of SIRT1 as key mediator of the effect of stress on the day-night changes of mRNA abundance of clock genes and food intake regulators, and their interaction within hypothalamus of rainbow trout. The protective effect of the herein reported SIRT1 inhibitor (EX527) is even reflected in cortisol biosynthetic pathway at the head kidney. We previously suggested the interaction between cortisol and SIRT1 in mediating the effect of stress in rainbow trout (Naderi et al., 2018), and our herein presented results are in support of such idea. We now point at a key role played by
SIRT1, as long as the inhibition of the enzyme in stressed animals leads for most of the parameters to remain comparable to those of non-stressed fish.

According to our results, stressing trout by high stocking density for 72 h is able to induce a significant increase of plasma cortisol levels, relative to control fish. This result, together with decreased food intake observed in stressed animals, validate our experiment. However, food intake in stressed fish receiving EX527 does not display those levels of control. This result is indicative of SIRT1 to participate as food intake regulator in fish, or the existence of other feeding behavior modulatory mechanisms. The involvement of SIRT1 as food intake regulator has been reported in mammals, since EX527 administrated rats decreased food intake (Çakir et al., 2009), but also because of SIRT1 regulates the expression of food intake related peptides, such as AgRP and POMC (Kim et al., 2000; Nillni et al., 2000). On the contrary, our results in fish point to feeding behavior is not mediated by SIRT1, since non-stressed trout receiving EX527 display comparable food intake to control animals. Then, food intake regulation and feeding behavior might be differentially influenced by specific mechanisms that need to be described.

Other parameters indicative of secondary response to stress were also assessed. For example, we have previously reported the increase of plasma glucose averaged levels in animals subjected to similar experimental conditions than those herein reported (Naderi et al., 2018). Such increase was previously attributed to enhanced production of endogenous glucose, decreased use of exogenous glucose, increased capacity of glucose release from the liver, and enhanced gluconeogenic capacity at the liver of rainbow trout (López-Patiño et al., 2014b). However, our data are not in agreement with that, since no day-night variation was found for plasma glucose levels at any experimental condition. Such discrepancy may be solved, first by the fact that we just assessed two time points in the present report, thus probably missing the daily profile of the carbohydrate levels. And second, different response of plasma glucose levels can be expected, since following exposure to increased cortisol levels, those of glucose can be increased, decreased or remain unaltered (see rev., Mommsen et al., 1999). By other hand, no significant variation was found for plasma lactate levels. A small decrease or the absence of any variation might be plausible, since anaerobic metabolic response initiates shortly after stress exposure in rainbow trout (López-Patiño et al., 2014b) but rapidly dissipates in parallel to stress duration, leading to lower or similar lactate levels than those of non-stressed fish.

According to previous results stress exposure is negatively affecting the circadian system in vertebrates from mammals (Koch et al., 2017) to fish (Naderi et al., 2018). In order to evaluate the involvement of SIRT1 in that effect, we assessed day-night variations of mRNA abundance of core circadian clock genes in hypothalamus of trout subjected to each experimental condition. Our results reveal day-night variations for clock1a, bmal1, and per1 in hypothalamus of non-stressed animals that perfectly fit with those respective rhythms reported earlier.
(López-Patiño et al., 2011; Naderi et al., 2018), thus validating our experiment. According to previously reported, stress exposure only results in decreased amplitude and averaged mRNA abundance of clock genes, clock1a, bmal1, and per1 (Naderi et al., 2018). Our herein reported results are consistent with that, since decreased mRNA abundance has been observed in hypothalamus of trout subjected to stress and afterwards sacrificed at day-time, ZT10 (clock1a, and bmal1), and night-time, ZT18 (per1). Such effect is prevented by EX527 administration, since day-night variation of hypothalamic clock genes expression observed in those fish receiving EX527 and afterwards subjected to stress was comparable to that of control fish. Based on the fact that central pacemaker at the trout hypothalamus might be affected by stress through indirect mechanisms (see rev. Koch et al., 2017) other than glucocorticoids (binding to specific receptors) as we reported (Naderi et al., 2018), our preliminary results did lead to assess SIRT1 as candidate as mediator of such effect. This family of proteins has been reported to act as a link between sensing of cellular energy status and circadian clocks (see rev. Delgado et al., 2017). In this way, SIRT1, acting as a histone deacetylase, counteracts the activity of the clock machinery (see rev. Jung-Hynes et al., 2009). In addition, SIRT1 is required for the circadian transcription of core clock genes such as bmal1, per2, and per3 (Asher et al., 2008).

The reciprocal interaction between SIRT1 and the circadian system is demonstrated, since the CLOCK/BMAL1 heterodimer controls nicotinamide phosphoribosyltransferase, responsible of NAD+ synthesis (Ramsey et al., 2009), and thus SIRT1 rhythm of activity, with SIRT1 also participating in the cyclic control of BMAL1 and PER2 through their deacetylation in peripheral tissues such as the liver (Nakahata et al., 2008), but also acting at central level through activating the pacemaker hosted at the SCN in mice (Chang and Guarente, 2013). According to this reciprocal interaction between clock genes and SIRT1, changes in mRNA abundance of each of them may result in each other to display altered expression rhythms. Our results reveal that sirt1 expression increases in trout subjected to stress, with such effect being totally (day-time) and partially (night-time) prevented by EX527 treatment. Such result is in support of SIRT1 to play a key role as mediator of the effect of stress on hypothalamic circadian system in rainbow trout.

By other hand, EX527 treatment was not totally effective at night-time, which is suggesting the existence of any interaction between SIRT1 and other mediators during response to stress in trout. At this respect, both the HSC cells and the HPI axes, activate, which leads to restoring homeostasis through mobilizing fuel to make energy available to cope with increased metabolic demand (Mommsen et al., 1999; Wenderlar Bonga, 1997). Cortisol appears to also play a key role as mediator of the effect of stress in fish (Aluru and Vijayan, 2009; Barton, 2002; Bernier, 2006) since treatments with either glucocorticoid antagonists or cortisol synthesis inhibitors enhance crf mRNA abundance in goldfish brain, which in fact leads to food intake inhibition (Bernier and Peter, 2001). Our previous report also support the role of
cortisol in rainbow trout (Naderi et al., 2018), but point to other candidates, such as SIRT1, to also mediate such effect. In order to corroborate the interaction between them both (SIRT1 and cortisol) we assessed mRNA abundance for members of cortisol biosynthetic pathway (\textit{star}, 3\(\beta\)-\textit{hsd}, p450\textit{ssc}, and 11\(\beta\)-\textit{h}) at the head kidney. To the best of our knowledge, we report for the first time in fish such day-night variations of mRNA abundance. All of the members of cortisol synthesis pathway displayed a significant increase of mRNA abundance in stressed trout, specially, at day-time. EX527 protective effect resulted more effective at night-time, thus totally preventing it (\textit{star} and 11\(\beta\)-\textit{h}), but a partial protective effect was observed for both 3\(\beta\)-\textit{hsd} and p450\textit{ssc} at both time periods. Thus, increased cell metabolic activity during exposure to stress exists within the head kidney, leading to enhanced cortisol synthesis. EX527 treatment inhibits such effect all over the body, which is in supports of the hypothesis of SIRT1 to play a key role at the very beginning of the response to stress, since it is likely that the enzyme activates, thus allowing downstream events to initiate, including the increase of mRNA observed for glucocorticoid receptors, at both hypothalamus and head kidney. Such increase is only observed for \textit{gr1}. We have previously reported the negative effect of stress on hypothalamic \textit{gr1} daily rhythm of mRNA abundance, thus displaying increased expression and the rhythm to dissapear. Such effect was not observed for \textit{gr2} (Naderi et al., 2018). Our results agree with that, since only \textit{gr1} mRNA abundance increases in both tissues in stressed trout. It is likely that elevated cortisol levels are needed to enhance \textit{gr1} expression, as found in stressed trout. However, plasma glucocorticoid levels are not enhanced in fish receiving EX527 and afterwards subjected to stress, which is in support of the key role played by SIRT1 in mediating the physiological response to stress. Accordingly, most of the herein assessed parameters do not display any variation in those animals receiving EX527 and subjected to stress, due to its inhibitory effect on \textit{sirt1} expression and activity. However we cannot underestimate that changes in mRNA levels are not necessarily accompanied by those of receptor density. Then, even when it seems that an interaction between \textit{sirt1} and \textit{gr1} may exist in mediating the physiological response to stress in rainbow trout, the role of \textit{gr2} cannot be discarded. Further research must be carried out in order to clarify the mechanisms through which SIRT1 mediate the physiological response to stress and its interaction with other mediators, such as glucocorticoids, with special attention on mRNA abundance.

The day-night variation of \textit{crf}, \textit{pomc-a1}, \textit{cart}, and \textit{npy} mRNA abundance was also assessed. Our results observed in control group are in agreement with the daily rhythm of expression previously reported for the same genes in trout hypothalamus (Naderi et al., 2018), thus validating our experimental design. In addition, our results also fit with that reported for other teleost, such as the sole (\textit{Solea senegalensis}), for which the acrophases of \textit{crf} and \textit{pomc-a1} locate within the same temporal window (López-Olmeda et al., 2013) than that reported by us. On the contrary, the day-night variation of \textit{npy} is consistent with that described earlier, but differs from that found in
Epinephelus coioides, in which peaking values occur at feeding-time (Tang et al., 2013), thus, in antiphase with that observed for rainbow trout. In addition, stressing trout by high stocking density resulted in increased expression of crf, pome-a1, cart, and npy. Such results mostly agree with that previously reported by us (Conde-Sieira et al., 2010b; Naderi et al., 2018), but also with literature (see rev. Delgado et al., 2017). These changes observed in mRNA abundance of stressed trout are also consistent with decreased food intake. In the same way, goldfish displays reduced food intake following CRF ICV administration (De Pedro et al., 1993), and treatments with either glucocorticoid antagonist or cortisol synthesis inhibitors result in both increased brain crf mRNA abundance and decreased food intake. Such effect is reverted with CRF receptor antagonist (Bernier and Peter, 2001). Our results are in striking consistency, since food intake was significantly inhibited in fish subjected to stress. On the contrary, trout receiving EX527 and exposed to high stocking density did also display decreased food intake, which lead us to discard a role of SIRT1 in mediating feeding behaviour. This result might be expected since the effect of stress on mRNA abundance of food intake regulators was not totally prevented by EX527 treatment, and thus food intake regulation remaining altered. Based on our previous findings, cortisol through binding to specific glucocorticoid receptors participates in maintaining the inhibitory effect of stress on food intake in rainbow trout, as long as the GR antagonist (mifepristone) is able to prevent such inhibitory effect (Naderi et al., 2018). Then, food intake regulation appears to be influenced by the interaction between SIRT1 and cortisol, whereas feeding behaviour is mostly mediated by cortisol in stressed trout.

By other hand, we cannot discard other mechanisms to participate in the inhibitory response of stress on food intake. In this way, nutrient sensing systems also participate, since trout subjected to chronic stress readjust hypothalamic glucosensing mechanisms (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). This leads for animals not to compensate with changes in food intake those altered plasma glucose levels whereas non-stressed fish do. In the same way, the response of hypothalamic mRNA abundance of cart, pomc, and npy to glucose changes is altered in stressed trout (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). Our results are in agreement with those changes, and also with recent findings describing the dynamic of response to stress in food intake-related peptides (Naderi et al., 2018) with altered averaged levels of mRNA abundance.

In summary, our results agree with the hypothesis of a key role played by SIRT1 as mediator of the effect of stress on the day-night variations of mRNA abundance of core circadian clock genes and food intake-related peptides within rainbow trout hypothalamus. Stress by high stocking density is able to inhibit the expression of clock genes, whereas that of food intake-related peptides is increased. EX527 treatment totally prevents those changes observed in clock genes during stress exposure. Then, SIRT1 appears to be the main mediator of such effect on the
hypothalamic circadian system. On the contrary, changes displayed by food intake-related peptides during stress are partially prevented with the SIRT1 inhibitor. Such result together with the absence of any influence of SIRT1 on trout feeding behaviour is indicative of the existence of any interaction between SIRT1 and other mediators during response to stress, such as cortisol. In support of that, our results point to SIRT1 to also influence cortisol biosynthetic pathway at the head kidney during stress, since EX527 treatment prevents most of the changes observed in this tissue of stressed trout, leading to plasma cortisol levels to be comparable to those of non-stressed fish. Further research must be carried out to clarify the mechanisms through which SIRT1 mediates the response of the circadian system to stress, and that of food intake-related peptides as well, and how the interaction between SIRT1 and other mediators is within trout hypothalamus, but also all over both the HSC and the HPI axes.
3.3. Experimental work 3

Are brain monoaminergic activities and food intake control linked to a circadian oscillator in rainbow trout?
Resumen

¿Modula el sistema circadiano las actividades monoaminérgicas cerebrales y la regulación de la ingesta de alimento en trucha arco iris?

En mamíferos, los componentes del sistema circadiano reciben información a través de diversas vías entre las que se encuentran las monoaminas (5HT y DA) y factores moduladores de la ingesta. Dichos componentes son capaces de generar respuestas que contribuyen eficazmente al ajuste temporal de las diferentes funciones comportamentales y fisiológicas del individuo. Con respecto a las monoaminas, la alteración de la neurotransmisión tanto serotoninérgica como dopaminérgica provoca cambios en la respuesta fisiológica y comportamental generada por el sistema circadiano. Se ha descrito en este grupo de vertebrados la existencia de fluctuaciones diarias en las neuronas serotoninérgicas y dopaminérgicas que están controladas por osciladores circadianos, lo que es indicativo del papel que ejercen las monoaminas como vías de salida de información procedente de dicho sistema. En cambio, en otros vertebrados, incluidos los peces, no se cuenta con información a este respecto. Por otro lado, el alimento también desempeña un papel importante en la sincronización de las diferentes funciones rítmicas en vertebrados, incluidos los peces, dado su papel como vía de entrada de información a los osciladores circadianos, independientemente de dónde estos se encuentren. Teniendo en cuenta la presencia del oscilador central en la misma región que alberga los centros moduladores de la ingesta de alimento en los peces, el hipotálamo, existe la posibilidad de que dicho oscilador pueda controlar la síntesis y liberación de los factores moduladores de la ingesta, utilizando como vías de salida de información al resto del organismo en este grupo de vertebrados. En consecuencia, el objetivo del presente trabajo fue la determinación en la trucha arco iris de la existencia de variaciones rítmicas diarias moduladoras de la ingesta (*crf, pomc-a1, cart* and *npy*) para confirmar su dependencia de la actividad de un oscilador circadiano. Para ello, ejemplares de trucha arco iris fueron adaptados a condiciones normales de alojamiento, o expuestos a 48 horas de oscuridad constante (DD), o expuestas a DD y ayunadas (DD+Fasting) y posteriormente sacrificadas a lo largo de un ciclo de 24 horas, tomándose muestras de las diferentes regiones cerebrales. Los resultados obtenidos confirmaron la existencia de ritmos diarios de abundancia de ARNm para las enzimas limitantes de la síntesis de 5HT (*tph1* y *tph2*) y DA (*th*) en todas las regiones cerebrales analizadas, si bien no se tradujeron en ritmos diarios claros en el contenido de monoaminas. No obstante, dichos ritmos de expresión se conservaron en ausencia de señales sincronizadoras externas, luz y alimento. Por otro lado, la expresión de péptidos moduladores de la ingesta también fue rítmica y se conservó en condiciones constantes. En consecuencia, en la trucha arco iris el oscilador central presente en el cerebro (hipotálamo), parece generar los ritmos de monoaminas y péptidos moduladores de la ingesta, lo que pone de manifiesto la interacción entre dichos sistemas, de modo que tanto las monoaminas como los
péptidos moduladores de la ingesta participan como salidas del oscilador hipotalámico. En cambio, tanto el papel de las monoaminas como de los moduladores de la ingesta como vías de entrada a dicho oscilador todavía debe ser evaluado en este grupo de vertebrados.
Abstract

Are brain monoaminergic activities and food intake control linked to a circadian oscillator in rainbow trout?

In mammals, components of the circadian system receive a range of inputs with monoamines, (5HT and DA) among others, such as food intake modulators. These components also generate outputs that contribute to adjust the behavioural and physiological functions. Regarding monoamines, altered serotonergic and dopaminergic neurotransmission can jeopardize physiological and behavioural responses of the circadian system. Then, mammalian dopaminergic and serotonergic neurons display daily fluctuations under the control exerted by circadian oscillators, which is indicative of the role played by monoamines as output signals from the circadian system. However, in other vertebrates, including fish, information regarding the influence of the circadian system on monoaminergic activity is still lacking. In the same way, food plays a relevant role in synchronizing rhythmic functions in fish, by acting as an input to the circadian system independently of where it is located. Since a circadian oscillator in fish locates within the hypothalamus, where food intake control takes place, a possible involvement of the circadian system in controlling food intake modulators as internal outputs is possible. Thus, the aim of the present study was to determine in rainbow trout the existence of daily variations of brain monoaminergic activities and food intake-related neuropeptides (crf, pomb-a1, cart and npy) and their dependence of a circadian oscillator. The influence of light and food as synchronizers was evaluated as well. Thus, trout adapted to normal housing conditions or subjected to 48 h constant darkness (DD) alone or together with food deprivation (DD+Fasting) respectively were sampled along the 24 h LD cycle. The results demonstrate the existence of daily rhythms of mRNA abundance of rate limiting enzymes of 5HT (tph1 and tph2) and DA (th) synthesis in all the brain regions analysed that persist in the absence of light and food as synchronizers. These rhythms are not clearly observed for monoamines content. In addition, food intake-related peptides also display daily rhythms that in general persist in those groups subjected to constant darkness. According to that herein reported, it appears that in rainbow trout, as fish model, a circadian oscillator located within the brain is able to generate the rhythms of monoamines and food intake modulators, thus evidencing the existence of a link between them all, with monoamines and food intake modulators participating as output signals of fish hypothalamic circadian oscillator. However, a role for these parameters as input to the circadian system has to be assessed in this vertebrate group.
Are brain monoaminergic activities and food intake control linked to a circadian oscillator in rainbow trout?

INTRODUCTION

A wide range of behavioural and physiological functions have been reported to be rhythmic. If so, most of them are subordinated to the activity of circadian oscillators (Aschoff, 1981; Reppert and Weaver, 2002). Accordingly, most functions in vertebrates such as locomotion, feeding, drinking thermoregulation, and hormonal secretion synchronize to environmental rhythmic events. Some internal mechanisms are involved in generating and modulating them (Dunlap, 1999). Organisms, in order to establish internal timing order, must receive environmental information, process it, and send responses to specific stimuli. Mechanisms involved in such functions constitute the circadian system (Hastings et al., 2007; Challet, 2007; 2015; Albrecht, 2012). Among others, the circadian system participate in the regulation of different functions, like food intake, but also the activity of specific neuron populations, such as the monoaminergic neurons (Cahill and Ehret, 1981; Edgar et al., 1993; Morin et al., 1999). With respect to monoamines, a network that includes both the circadian and the monoaminergic systems within the brain has special relevance. While extensively intertwined on both neuroanatomical and genetic levels, both systems influence multiple neural centres involved in either affective and/or temporal-gated behaviours (Brock, 1991; Ciarleglio et al., 2011). Specific cell within the circadian oscillator receive monoaminergic inputs, but also these cells generate monoamine-related outputs, thus proving the interaction between both systems (Morin et al., 1999; Ciarleglio et al., 2011). Then, altered monoaminergic activity can disrupt physiological and behavioral functions that synchronize to environmental cues, whereas the alteration of the circadian system can negatively affect monoaminergic activity and that modulated by this system.

The SCN is the brain region where the circadian oscillator is located. Thus, SCN receives environmental cues and transmits information in order to regulate rhythmic functions. This region receives different inputs such as direct serotonergic innervation from the raphe nucleus, but also an indirect dorsal raphe-driven NPY input from the IGL (Deurveilher and Semba, 2005). This makes possible the interaction between different functions, such as monoaminergic activity and food intake, and the involvement of the circadian system in modulating them.

Regarding monoamines, all contain one amino group connected to an aromatic ring by a carbon-carbon chain (Yousuf et al., 2016). Monoamine neurotransmitters include 5HT and the CAs, DA and NA. In most vertebrates, multiple functions involving monoamines were demonstrated, such as aggressive behavior (Mason, 1984; Miczek and Donat, 1989; Olivier et al., 1989), mating (Meyerson and Malmnb, 1978), and feeding (Leibowitz, 1992). In addition, some
studies demonstrate that monoaminergic system participates in the physiological response to stress in mammals (Dunn, 1989) and fish (Gesto et al., 2011), but also the regulation of autonomic and neuroendocrine functions (Tuomisto and Minnistii, 1985). However, functions involving monoaminergic system in non-mammalian vertebrates such as fish are not fully studied.

5HT, is a monoamine considered as a main neurotransmitter within the CNS. As in mammals and other vertebrates, serotonergic cell bodies within the teleost brain mainly localize in the hindbrain, at the raphe nucleus, but also outside the raphe area, for example in prepectum and basal forebrain (Lillesaar, 2011). Serotonergic neurons can be identified based on the expression of the rate-limiting enzyme, TPH, or levels of its acidic metabolite, 5HIAA. Also, the ratio between 5HIAA and the monoamine (5HT) has been reported as a good marker of increased serotonergic activity (Winberg et al., 1997; Gesto et al., 2013). However, the best marker of serotonergic neurons is TPH expression. In teleost species such as zebrafish, it has been reported that specific cell populations express different paralogues of TPH. For example, TPH2 expresses in the raphe, whereas TPH1a and TPH1b are expressed in diencephalon and peripheral 5HT cells, such as those within the gustatory tract (Anderson and Caio, 2014). A fourth TPH isoform, TPH3, expresses in hypothalamus of zebrafish (Ren et al., 2013). 5HT participates in the response of the circadian system to light, thus synchronizing several behaviours such as locomotor activity in mammals (Edgar et al., 1991, 1993; Prosser et al., 1993; Mistlberger et al., 1998; Smith et al., 2001; Glass et al., 2003; Morin and Allen, 2006). In the same way, 5HT receptors express in those cells hosting the circadian oscillator at the SCN (Kiss et al., 1991; Bosler and Beaudet, 1985; Bosler, 1989; Lovenberg et al., 1993; Manrique et al., 1993, 1994; Prosser et al., 1993; Amir et al., 1998), thus influencing the circadian system. On the contrary, clock genes also express in serotonergic neurons at the raphe, thus modulating the rhythms of expression of key genes in 5HT synthesis (Abe et al., 2002; Malek et al., 2007). These data evidence the existence of a link between the circadian system and serotonergic system as reported (Kripke, 1998; Loving et al., 2002; Benedetti et al., 2003). However, few data at this respect are available in other vertebrate groups, including fish, in which information is still lacking.

By other hand, DA is another well studied monoamine. In fish, the existence of dopaminergic neurons in different brain regions such as the olfactory bulb (Hornby et al., 1987; Sas et al., 1990), telencephalon (Hornby et al., 1987), preoptic area (Hornby et al., 1987), and hypothalamus (Parent et al., 1984) was demonstrated. Within the brain, the main dopaminergic pathway in the raphe nucleus (Gerfen et al., 1992). Accordingly, the influence of photic information must modulate the activity of DA cells, through the interaction with another modulator, melatonin. Accordingly, light enhances expression of DA receptors together with the inhibitory effect of melatonin synthesis and release. Because of that, a role for DA
Experimental work 3

as a wake up promotor was proposed (Ueno et al., 2012), and a link with the circadian system might be expected. In addition, a circadian profile has been proposed for DA content, release and turnover in rat striatum, which agrees with the hypothesis of the dependence of dopaminergic system on a circadian oscillator, which leads to modulate several rhythmic behavioural events (Lemmer et al., 1978). TH is accepted as a marker of dopaminergic neurons in vertebrates including fish, since it is considered the rate-limiting enzyme of DA synthesis (Linard et al., 1986; O’Connell et al., 2011). However, it was also reported for fish that the ratio between the main metabolite (DOPAC) and DA is a good indicator of altered dopaminergic activity, since this ratio changes during stress exposure (Winberg et al., 1997; Gesto et al., 2008; 2013). Similarly to that reported for serotonergic system, a link between dopaminergic and circadian systems has been proposed, since different parameters related to dopaminergic signaling pathway display daily rhythms (Castañeda et al., 2004; Hood et al., 2010; Imbesi et al., 2009), with light and subsequently the circadian system influencing such rhythms (Hampp et al., 2008; Sleipness et al., 2007). In addition, in vivo and in vitro studies carried out in mammals also demonstrate that DA administration is able to modulate the expression of clock genes (Gravotta et al., 2011; Imbesi et al., 2009; McClung et al., 2005). Even when this interaction is well-understood in mammals, information at this respect in fish is still very scarce, and further studies must be carried out in order to confirm this interaction in this vertebrate group.

By other hand, food activity is a rhythmic processes that is influenced by biotic (prey availability, hierarchies, social behavior), non-biotic (photoperiod, temperature) and internal (biological clocks, metabolism-related cues) factors (Boujard and Leatherland, 1992; Madrid et al., 2001). However, rhythms of food activity persist even in the absence of fluctuations of such environmental factors, which is indicative of the circadian system to generate them. Even when research carried out in fish demonstrates that the circadian system can generate the daily rhythm of food intake (Kulczykowska et al., 2010), the underlying mechanisms are not fully identified. In general, food intake control in fish involves different mechanisms within the central nervous system, with hypothalamus being the main regulatory brain region, where two populations of neurons exist: NPY and AgRP, and the second one POMC and CART. This brain region also hosts a circadian oscillator, as reported for rainbow trout (López-Patiño et al., 2011). Daily rhythms of mRNA abundance have been described for different food intake regulators in fish hypothalamus. Accordingly, in Experimental work 1, the rhythms of hypothalamic crf, pomc-a1, cart and npy are described for rainbow trout, with that of crf, pomc-a1, and npy displaying peaking values during the early dark period and basal levels at day-time, whereas cart levels were found to increase during the night-day transition. Those rhythms mostly agree with that reported for other teleosts, such as sole, Solea senegalensis (López-Olmeda et al., 2013). However, no information is available regarding the dependence of such rhythms on a circadian oscillator in fish, in
addition to the existence of a link between food intake modulators and the circadian system within the hypothalamus. As an example, the rhythm of a food intake stimulator such as NPY is driven by feeding time, but also appears to participate in synchronizing the central oscillator in goldfish (Vera et al., 2007).

Taking in consideration all the above mentioned, the aim of the present study was to corroborate the existence of daily rhythms monoaminergic activities and food intake regulators in rainbow trout, and their dependence on a circadian oscillator.

MATERIAL AND METHODS

Fish

Rainbow trout (Oncorhynchus mykiss Walbaum) obtained from a local fish farm (A Estrada, Spain) were acclimated to the laboratory conditions for two weeks in 120 L tanks, with continuously renovated and aerated water at 13.5±1ºC. Lighting conditions consisted on a 12L: 12D photoperiod (ZT/CT0=light/subjective light on; ZT=zeitgeber time; CT=circadian time). Fish were fed daily with commercial (Dibaq diproteg, Segovia, Spain) dry pellet diet (1% body weight) at ZT/CT=2. Illumination was provided by means of LED light lamps (Superlight Technology Co. Ltd., China). Also, irradiance was measured with a spectro-radiometer (FieldSpec ASD, Colorado, USA) set at 1.62 E+18 photons m^-2 s^-1. Trout were acclimated for 15 days to standard conditions before any experiment to proceed. All the experiments comply with the Guidelines of the European Union Council (2010/63/EU), and of the Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Animal Care Committee at the University of Vigo, and followed international ethical standards (Portaluppi et al., 2010).

Sampling

Fish were deeply anaesthetized by addition of 2-phenoxyethanol (0.2% v/v- Sigma Aldrich) to tank water. The appropriate volume of 2-phenoxyethanol was previously diluted in 5-L of tank water and afterwards added into the fish tank. This guaranteed the anesthetic to mix uniformly. Also, visual contact between fish and the manipulators was avoided, thus minimizing the incidence of any influence on the animals. Once anesthetized, animals were rapidly sacrificed, and individual hypothalamus, including the preoptic area was removed under sterile conditions, according to previously described (Doyon et al., 2003), in addition to telencephalon, optic tectum and medulla. Samples were placed into sterile RNase-free 1.5 ml Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80ºC until assayed for mRNA abundance quantification of 5HT synthesis rate limiting enzyme, tph1 and tph2, the DA synthesis enzyme, th, anorexigenic (crf, pomec-a1, and cart) and orexigenic (npy) neuropeptides. A new set of samples from each brain region
was collected and placed into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C until HPLC assayed for monoamines content.

**Experimental design**

To evaluate daily variations of mRNA abundance of monoaminergic-related enzymes, food intake-related peptides, together with the daily rhythms of monoamines, and their dependence of a circadian oscillator, a first cohort of trout (N=84) was randomly divided in 7 tanks (n=12/tank), and adapted to housing conditions (see above). On the day of sacrifice fish from each tank were sacrificed every 4 h over the 24 h light/dark cycle (LD group), starting at ZT0 (lights on). Thus, scheduled sampling time points were ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT0’ of the following day. At each specific time point, all fish from an assigned tank were netted and immediately sampled. Animals sampled at night were netted and transferred to a new tank containing the anaesthetic solution, and once anesthetized fish were sacrificed. Only during sampling at night, dead bodies were manipulated under dim red light, whereas the rest of the tanks remained light-isolated. Sample collection of different brain region was as above mentioned.

A second cohort of 84 trout (n=12/group) were maintained under the same lighting conditions and then light-isolated for 48 h (DD group) and sacrificed every 4 h over the 24 h period at the same specific time points (CT0 to CT0’; CT= circadian time). Sampling procedures were carried out under dim red light, out of the housing room, which guaranteed the absence of light contamination at the housing room during the dark period. During the exposition to constant darkness and on the day of sacrifice, fish were hand-fed as scheduled. Individual samples of each brain region were collected as mentioned above.

A third cohort of 84 trout (n=12 per group) were maintained under normal lighting conditions, and afterwards light-isolated and food deprived for 48 h (DD+F group). Following such time period, trout were sacrificed and sampled every 4 hours as above described.

**Real-time quantitative RT-PCR (qPCR)**

Total RNA was extracted from individual samples of hypothalamus, telencephalon, optic tectum and medulla by using the TRIzol® (Life Technologies, Grand Island, NY, USA) method. RQ1-DNAse (Promega, Madison, WI, USA) was added to each sample. The same amount of RNA (2 µg) from each individual sample was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega) and Random Primers (Promega). A negative control for each sample was assessed without reverse transcriptase in order to discard any genomic contamination in the RNA extract.
The qPCR assays were performed by using a Maxima™ SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and a Bio-Rad MyIQ Real Time PCR system (BIO-RAD, Hercules, CA, USA). The primers were designed on the basis of previously reported sequences for rainbow trout genes, and obtained from Sigma (see Table 1), including tph1, tph2, th, crf, pomc-a1, cart, npy, and β-actin. Specifically for tph1 and tph2, partial sequencing and primers design were carried out in our laboratory. Specific information at this respect is provided in Experimental work 4.

Relative quantification of the target gene transcript was assessed by using β-actin gene expression as reference. Thermal cycling was initiated with incubation at 95°C for 3 min; followed by 35 steps of PCR, each one consisting of heating at 95°C for 10 s for denaturing, and at specific annealing for 30 s and extension at 50°C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (50°C temperature gradient at 0.5°C/s from 50 to 95°C) to ensure that only one fragment was amplified. Relative mRNA expression level was calculated by using the standard comparative delta Ct method, according to the Pfaffl method (Pfaffl, 2001). At each microplate, for each gene, samples from each time point were assessed in triplicate and in parallel. Only efficiency values between 85% and 100% were accepted (the R2 for all the genes assessed was always higher than 0.985).

Analysis of monoamines and metabolites

To assess NA, DA, DOPAC, 5HT, 5HIAA contents in trout brain (telencephalon, hypothalamus, optic tectum and medulla), high performance liquid chromatography with electrochemical detection (HPLC-EC) was used, following a previously described method (Gesto et al., 2006). Individual samples from each brain region were homogenized by ultrasonic disruption in 0.4 ml of mobile phase (63.9 mmol l⁻¹ NaH₂PO₄, 0.1 mmol l⁻¹ Na₂EDTA, 0.80 mmol l⁻¹ sodium 1-octanesulfonate, and 15.3% (v/v) methanol). Mobile phase was pH-adjusted to 2.95 with ortho-phosphoric acid and afterwards filtered (0.20 μm) and degassed. Each homogenate was centrifuged (16,000 × g, 10 min), the supernatant diluted with mobile phase, and afterwards injected into the HPLC system for the analysis of monoamines content. The HPLC system consisted on a Jasco PU-2080 Plus pump, a 5 μm analytical column (Nucleosil C18, 150 mm length×4.6 mm diameter; Phenomenex), a Jasco AS-2057 auto-sampler and an ESA Coulochem II detector. The detection system included a double analytical cell (M5011) with oxidation potentials set at +40 mV and +340 mV. For each sample, running time was 15 min at a 1.0 ml min⁻¹ flow rate at 24°C. Detection limit for each compound was between 0.5 and 1.5 pg/injected sample, with a signal-to-noise ratio of 3. Acquisition and integration of chromatograms were performed by using ChromNAV version 1.12 software (Jasco Corp). The peak area of each tissue sample was compared with that
of the appropriate standard in order to estimate the quantity of each compound. Data obtained from each sample were normalized by protein content.

Table 1: Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Ta</th>
</tr>
</thead>
<tbody>
<tr>
<td>tph1 (MG015697)</td>
<td>AGGGAAAGATGAGAGGCTACG</td>
<td>CCAGAGTGCATGCTTCAG</td>
<td>57</td>
</tr>
<tr>
<td>tph2 (MG015698)</td>
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<td>ATCTTCTGGGGGAAAACGGGA</td>
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</tr>
<tr>
<td>th (XM_021564247.1)</td>
<td>GTCTGAGACGATGCTACCCCTT</td>
<td>GGGATCTGTTGAATCTCCTGGTG</td>
<td>61</td>
</tr>
<tr>
<td>cfr (AF296672)</td>
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<td>AGGAAATAAGCACTAGTGCAGGG</td>
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</tr>
<tr>
<td>cart (NM001124627)</td>
<td>ACCATGGAGAGCTCCAG</td>
<td>GGCGCTGCTCTCAA</td>
<td>60</td>
</tr>
<tr>
<td>pome-a1 (TC86162.Tigr)</td>
<td>CTACGTCAGCAAGCTCAACTCT</td>
<td>GAGTGGGTTGGAGAGTGACCTC</td>
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</tr>
<tr>
<td>npy (NM001124266)</td>
<td>CTGCTCTGGACCTTTATATGC</td>
<td>GTCATCATATCTGGACTGTTG</td>
<td>58</td>
</tr>
<tr>
<td>β-actin (AJ438158)</td>
<td>GATGGGCCGAGAAAAGACAGTA</td>
<td>TCGTCCCAGTGTTGGAGCATG</td>
<td>59</td>
</tr>
</tbody>
</table>

Statistical analysis

To determine the presence of significant differences among groups (LD, DD and DD+F), two-way ANOVA tests were performed with “experimental condition” and “time” as main factors. If significant differences were found, a Student-Newman-Keuls post-hoc test for multiple comparisons was performed. Significance level was set at P<0.05.

Daily rhythms of mRNA abundance for each gene were also analyzed by fitting the periodic sinusoidal function to the specific values of gene expression across the seven sampling time points, by using the formula f(t)=M+Acos(πt/12− φ), where f(t) was the mRNA abundance at a given time point; the mesor (M) was the mean value; A was the sinusoidal amplitude of the oscillation.; t was the time in hours; and φ was the acrophase (estimated time of the peak). Estimation of M, A, and φ, and their standard error (SE) was obtained with help of a non-linear regression (Delgado et al., 1993). All the parameters obtained from the sinusoidal function were expressed as average±standard error (SE). The SE of parameters was based on the residual sum of squares in the least-squares fit. A rhythm of expression was considered consistent if both P<0.05 from the ANOVA test and SE(A)/A < 0.3 provided by the cosinor analysis, following the principle of a noise/signal ratio less than 0.3, the “signal” being the amplitude and the “noise” its error (Halberg and Reinberg, 1967).

RESULTS

Assessment of daily rhythms of mRNA abundance of serotonergic- (tph1 and tph2) and dopaminergic-related (th) enzymes in hypothalamus, telencephalon, optic tectum and medulla of rainbow trout subjected to normal housing conditions or in the absence of light or/and food are shown in Figure1. The parameters defining such
rhythms as estimated by the cosinor analysis are presented in Table 2. Daily rhythms of expression persisted in all the brain regions, but a dependence of the experimental condition and the region was observed for most of the genes. In control group, a consistent rhythm of tph1 mRNA abundance was observed in all the brain regions assayed, but some differences were found. Accordingly, both hypothalamus and telencephalon displayed similar averaged mRNA levels (1.54 relative fold change units and 1.74 relative units respectively), and acrophase (ZT12.22 and ZT13.15), but the amplitude of the rhythm was 50% higher in telencephalon (see Table 2). Averaged levels and amplitude in optic tectum (1.46 relative units, and 0.46 relative units respectively) were comparable to that of hypothalamus, but a 2 h phase advance (ZT10.35) was found in this region, compared to that of hypothalamus and telencephalon. The highest averaged levels of tph1 mRNA abundance (1.99 relative units) and amplitude of the rhythm (0.88 relative units) were observed in medulla, together with a 12 h phase shift of the acrophase (ZT0.52). Subjecting trout to DD resulted in a significant increase of averaged tph1 levels together with a 3- to 5 h phase delay of the acrophase in hypothalamus and telencephalon, compared to that found in control (see Table 2). By other hand, a 3 h phase delay and a 4 h phase advance were found for the acrophase in optic tectum and medulla relative to that of control. Both constant darkness and fasting only resulted in enhanced expression of tph1 in hypothalamus and telencephalon, whereas the acrophase of the rhythm did not substantially change, relative to control group. In optic tectum, only a 3 h phase delay was found in trout subjected to DD and food deprived, relative to control (see Table 2). Similar results were observed in medulla, where subjecting fish to DD and fasting resulted in a phase advance of tph1 mRNA rhythm, up to 5 hours relative to control group.

In the same way than that found for tph1, in control group, analysis of tph2 mRNA abundance in all the brain regions revealed the existence of consistent rhythms of expression, even when some differences were observed. The highest levels of tph2 expression within control group were found in hypothalamus, thus with the highest mesor (3.53 relative fold change units) and amplitude of the rhythm (3.04 relative units). The cosinor analysis also estimated the acrophase at ZT9.16, i.e., during the second half of the day. By other hand, both telencephalon and optic tectum displayed significant rhythms of tph2 mRNA abundance, but either the averaged levels of mRNA (telencephalon: 1.55 relative units; optic tectum: 1.42 relative units) and the amplitude of the rhythm (telencephalon: 0.92 relative units; optic tectum: 0.46 relative units) were lower than that found in hypothalamus. In addition, cosinor analyses revealed a 2 h and 7 h phase delay in the rhythm of tph2 expression in telencephalon (acrophase at ZT11.02) and optic tectum (acrophase at ZT16.04) respectively, compared to that found in hypothalamus. A significant rhythm of tph2 mRNA levels was also observed in medulla, with averaged levels (1.88 relative units) being lower, up to a 50%, than those of hypothalamus, in the same way than that for the amplitude (0.88 relative units), thus a 75% lower than
that in hypothalamus, but being comparable to that of telencephalon. The acrophase of the rhythm of tph2 mRNA abundance in medulla occurred at ZT7.41, i.e., 2 h advanced to that of hypothalamus.

Subjecting trout to constant darkness resulted in no changes in averaged tph2 mRNA levels in hypothalamus (4.35 relative units), optic tectum (1.62 relative units) and medulla (2.03 relative units), and a small decrease in telencephalon (0.99 relative units), relative to that observed in LD (see Table 2). In the same way, amplitude of the tph2 rhythms remained comparable to that observed in LD at the hypothalamus (3.52 relative units), optic tectum (0.68 relative units) and medulla (0.97 relative units), whereas a decrease was observed in telencephalon, up to 50% (0.48 relative units). The acrophase of thp2 rhythm was 3 hours delayed in hypothalamus (CT12.03) and telencephalon (CT14.59), and 2 hours advanced in optic tectum (CT14.58), relative to that of control, whereas no variation was revealed in medulla (CT8.59). Constant darkness and food restriction resulted in increased expression of tph2 in hypothalamus and telencephalon, relative to that found in control group. Then, averaged levels were higher (hypothalamus: 4.72 relative units; telencephalon: 2.33 relative units), whereas the amplitude of the rhythms did not substantially change (hypothalamus: 2.95 relative units; telencephalon: 1.60 relative units), relative to that of control group. In addition, the acrophase of the rhythms remained comparable to that of control (see Table 2). In optic tectum, neither averaged tph2 levels (1.60 relative units) nor the amplitude of the rhythm (0.66 relative units) changed relative to that of control, whereas the acrophase advanced 5 hours (CT11.06). In medulla, a significant decrease of averaged tph2 levels (0.95 relative units), up to 50%, and amplitude (0.25 relative units), up to 75%, relative to LD was found. The cosinor analysis estimated the acrophase 4 hours earlier (CT3.33) than that of control.

The cosinor analysis revealed the existence of consistent rhythms of th mRNA abundance in all the brain regions, but some differences were found between brain regions and experimental conditions. In control group, no significant variations of averaged levels were observed between brain regions (hypothalamus: 1.49 relative fold change units; telencephalon: 1.95 relative units; optic tectum: 1.11 relative units; medulla: 1.75 relative units). On the contrary, the amplitude of the rhythm did depend on the brain region in such a way that highest amplitude was found in telencephalon (1.24 relative units), followed by medulla (0.75 relative units), and hypothalamus (0.48 relative units), whereas the lowest amplitude was observed in optic tectum (0.26 relative units). The daily profile was similar among brain regions, with the acrophase occurring during the early day in them all (hypothalamus: ZT1.13; telencephalon: ZT3.02; optic tectum: ZT2.56; medulla: ZT3.29). Light isolation altered in a brain-region dependent way the daily rhythm of th. Accordingly, the expression increased in hypothalamus, telencephalon and optic tectum, which resulted in the increase of averaged levels (hypothalamus: 3.46
relative units; telencephalon: 3.00 relative units; optic tectum: 1.33 relative units), relative to control (see Table 2). On the contrary, expression decreased in medulla of trout exposed to DD (1.48 relative units) relative to control group. Amplitude of the rhythms paralleled those changes described for averaged mRNA levels. Thus, an increase was found in hypothalamus (1.74 relative units), telencephalon (2.11 relative units), and optic tectum (0.34 relative units), and a decrease was observed in medulla (0.20 relative units), relative to control. The main change was observed in the acrophase of the rhythms, for which with light isolation resulted in a phase delay in hypothalamus (7 hours; acrophase at CT8.51), telencephalon (2 hours; acrophase at CT5.24) and optic tectum (4 hours; acrophase at CT6.47), and a 5 h phase advance in medulla (acrophase at CT22.53).

Light isolation together with food restriction enhanced th expression in hypothalamus and telencephalon, where averaged mRNA levels significantly increased (hypothalamus: 4.89 relative units; telencephalon: 4.68 relative units) together with the amplitude (hypothalamus: 3.25 relative units; telencephalon: 3.77 relative units), compared to those of control. Such effect was also observed in optic tectum, but was not that pronounced (mesor: 1.41; amplitude: 0.53).

On the contrary, the inhibitory effect of light isolation and food privation on th expression was also observed in medulla, resulting in the decrease of averaged expression (1.36 relative units) and amplitude of the rhythm (0.30 relative units), compared to that observed in control group (see Table 2). The profile of the rhythm changed in a brain region-dependent way, but peaking mRNA levels were estimated at day-time in all brain regions. Accordingly, the cosinor analysis revealed a phase delay that was more pronounced in optic tectum (6 hours; acrophase at CT8.08) and medulla (7 hours; acrophase at CT10.33) relative to control, whereas a 3 h and a 2 h delay was found in hypothalamus (acrophase at CT4.16) and telencephalon (acrophase at CT5.20) respectively.

Assessment of daily rhythms of mRNA abundance of hypothalamic food intake regulators (crf, pome-al, cart and npy) in of rainbow trout subjected to normal housing conditions or in the absence of light or/and food are shown in Figure 2. The parameters defining such rhythms following the cosinor analysis appear in Table 3. Analysis of hypothalamic content of crf in control group revealed the existence of a daily rhythm with peaking values occurring during the first half of the night (acrophase at ZT14.30). The cosinor analysis estimated the mesor value as 2.28 relative fold change units and the amplitude as 1.37 relative units. Trout subjected to DD revealed an overall increase of crf levels. In consequence, mesor value (2.99 relative units) and amplitude (1.89 relative units) increased up to 20% of that found in control group, whereas the acrophase (CT15.25) was not affected by light isolation. When trout were light isolated and food restricted crf expression increased (mesor: 5.34 relative units), but the rhythmic profile completely disappeared (see Table 3).
Figure 1: Daily rhythms of mRNA abundance of \( th \), \( tph1 \) and \( tph2 \) in trout brain (hypothalamus, telencephalon, optic tectum and medulla) subjected to different experimental conditions (LD, DD and DD+ F). Data are presented as the relative fold change with respect to basal levels in control group. \( n=4 \); *, significant difference respect to LD group; #, significant difference respect to DD group; \( P<0.05 \).
Table 2: Data represent the mesor as the mean value (M), the sinusoidal amplitude of the oscillation (A), the acrophase (φ, time of peak; ZT/CT) and SE (A)/A (value of the ratio from the cosinor analysis) and P value of tph1, tph2 and th in trout brain (hypothalamus, telencephalon, optic tectum and medulla) in all experimental groups (LD, DD and DD+F).

<table>
<thead>
<tr>
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<th>LD</th>
<th>DD</th>
<th>DD+F</th>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>A</td>
<td>φ</td>
</tr>
<tr>
<td><strong>Hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tph1</td>
<td>1.54</td>
<td>0.42</td>
<td>12.22</td>
</tr>
<tr>
<td>tph2</td>
<td>3.53</td>
<td>3.04</td>
<td>9.16</td>
</tr>
<tr>
<td>th</td>
<td>1.49</td>
<td>0.48</td>
<td>1.13</td>
</tr>
<tr>
<td><strong>Telencephalon</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tph1</td>
<td>1.74</td>
<td>0.83</td>
<td>13.15</td>
</tr>
<tr>
<td>tph2</td>
<td>1.55</td>
<td>0.92</td>
<td>11.02</td>
</tr>
<tr>
<td>th</td>
<td>1.95</td>
<td>1.24</td>
<td>3.02</td>
</tr>
<tr>
<td><strong>Optic tectum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tph1</td>
<td>1.46</td>
<td>0.46</td>
<td>10.35</td>
</tr>
<tr>
<td>tph2</td>
<td>1.42</td>
<td>0.46</td>
<td>16.04</td>
</tr>
<tr>
<td>th</td>
<td>1.11</td>
<td>0.26</td>
<td>2.56</td>
</tr>
<tr>
<td><strong>Medulla</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tph1</td>
<td>1.99</td>
<td>0.88</td>
<td>0.52</td>
</tr>
<tr>
<td>tph2</td>
<td>1.88</td>
<td>0.88</td>
<td>7.41</td>
</tr>
<tr>
<td>th</td>
<td>1.75</td>
<td>0.75</td>
<td>3.29</td>
</tr>
</tbody>
</table>
As revealed by the cosinor analysis, *pomc-a1* rhythm of mRNA abundance was consistent in control group, with mesor and amplitude being 3.13 and 2.20 relative units, respectively. Peaking values were observed during the night (acrophase at ZT14.59). Constant darkness exposure resulted in the enhancement of *pomc-a1* expression relative to that observed in LD. Thus, mesor (6.16 relative units) and amplitude (5.19 relative units) increased up to 100% of that in control. However, no variation was found for the acrophase (CT13.35). Trout subjected to constant darkness and food restriction displayed a pronounced alteration of *pomc-a1* rhythm in such a way that mRNA abundance significantly increased, thus with mesor values (12.61 relative units) being 400% higher than those of control group. This also resulted in the increase of the amplitude of the rhythm (4.48 relative units) up to 200% of that in control. The profile of the rhythm changed as well, since the acrophase (CT7.56) occurred with a 7 h advance compared to that of control (see Table 3).

![Figure 2: Daily rhythms of mRNA abundance of food intake-related peptides (cart, pomc-a1, cart, and npy) in hypothalamus of trout subjected to different experimental conditions (LD, DD and DD+ F). Data are presented as the relative fold change with respect to basal levels in control group. n=4; *, significant difference respect to LD group; #, significant difference respect to DD group; P<0.05.](image)

The rhythm of *cart* mRNA abundance was significant in control group. Mesor value was 1.69 relative units, amplitude was 0.61 relative units, and the acrophase was estimated at ZT23.40, i.e., during the night-day transition. Trout subjected to DD displayed a significant rhythm of *cart* mRNA abundance, but the profile changed relative to that of control fish. Accordingly, neither the averaged
expression (1.62 relative units) nor the amplitude (0.75 relative units) changed relative to control, but the acrophase phase shifted to the day-night transition (CT14.51), thus being in antiphase with control group. Similarly to that found for crf, the rhythm of cart in hypothalamus of animals exposed to DD and food restriction disappeared (see Table 3).

Table 3: Data represent the mesor as the mean value (M), the sinusoidal amplitude of the oscillation (A), the acrophase (φ, time of peak; ZT/CT) and SE(A)/ A (value of the ratio from the cosinor analysis) and P value of food intake related neuropeptide (crf, pome-α1, cart and npy) in trout hypothalamus, in all experimental groups (LD, DD and DD+F).

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>DD</th>
<th>DD+F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>A</td>
<td>φ</td>
</tr>
<tr>
<td>crf</td>
<td>2.28</td>
<td>1.37</td>
<td>14.30</td>
</tr>
<tr>
<td>pome-α1</td>
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<td>2.20</td>
<td>14.59</td>
</tr>
<tr>
<td>cart</td>
<td>1.69</td>
<td>0.61</td>
<td>23.40</td>
</tr>
<tr>
<td>npy</td>
<td>19.02</td>
<td>18.61</td>
<td>14.43</td>
</tr>
</tbody>
</table>

Analysis of hypothalamic content of npy in control group revealed the existence of a daily rhythm in all the experimental groups. In control fish, the cosinor analysis estimated peaking levels at the first half of the night (acrophase at ZT14.43). Mesor and amplitude values were 19.02 relative units, and 18.61 units, respectively. The expression of npy significantly decreased in trout subjected to constant darkness alone of together with food restriction. Thus, mesor values were 4.30 relative units (DD group) and 5.34 relative units (DD+F group). Amplitude of the rhythms also decreased in both experimental (DD group: 3.68 relative units; DD+F group: 3.49 relative units) groups compared to control. However, the profile of npy rhythm was not affected in both DD and DD+F groups, thus with the acrophase occurring at CT14.47 (DD group) and CT15.33 (CT+F group), being in phase with that of control group.

Content of monoamines and their main metabolites in different brain regions of trout subjected to normal conditions or subjected to light isolation together or not with food restriction appears in Figures 3-7. NA content displays daily variations and, in general, the two-way ANOVA (see Table 4) analyses reveal their dependence on “time” and “experimental condition” as main factors, and an interaction among them exists (Figure 3) in each brain region analyzed. However, cosinor analysis discarded the significance of such variations. Then, in hypothalamus of control group highest levels of NA were found in samples collected at the night onset (ZT12), and lower levels all over the night. Such profile was apparently conserved in trout subjected to DD, with peaking values being delayed
4h, and lower averaged levels relative to control. In trout subjected to DD and food deprivation this profile was blunted, thus with basal levels of NA being observed all over the 24 h cycle. In telencephalon, only “experimental condition” significantly affected the daily profile of NA content (ANOVA, F\(\text{2, } 130 \) = 6.732; P=0.002). Control group displayed higher NE levels at day-time and basal levels at night. This tendency disappeared in DD group, but also in DD+F group, in which lowest averaged NA (relative to LD and DD groups) were observed. No day-night variation was found for NA content in optic tectum at any experimental condition, but “experimental condition” influenced this profile (ANOVA, F\(\text{2,136} \) = 3.959; P=0.021) in such a way that trout subjected to DD and food restriction displayed an increase of mean NA levels (see Table 4). In contrast to that found in hypothalamus and telencephalon, a day-night variation of NA content was observed in medulla of control fish, with higher levels occurring at night, and basal levels during the day. Thus, a dependence on “time” and “experimental condition” as main factors exists, whereas the interaction between both factors was not significant. DD exposure resulted in the increase of mean NA levels, relative to LD group. Also, DD and food restriction enhanced NA content (relative to both LD and DD groups), and modified the daily profile in such a way that peaking levels were found in samples collected at night onset (CT12), and basal levels occurring at the end of the night (CT0’).

Content of DA, DOPAC, and the ratio DOPAC/DA in each brain region of rainbow trout is presented in Figures 4 and 5. DA content in hypothalamus of control fish did not show any significant daily variation. Exposure to constant darkness resulted in no significant change in DA content. However, a significant variation was observed with “experimental condition” as main factor (see Table 4), due to the decrease of DA content observed in animals from DD+F group (ANOVA, F\(\text{2,140} \) = 47.899; P<0.001). No significant change was found for DOPAC content in hypothalamus of control fish. However, “experimental condition” as main factor significantly affected this profile (ANOVA, F\(\text{2,140} \) = 9.552; P<0.001) in such a way that a day-night variation was observed in DD group, with peaking values occurring at the subjective night onset (CT12), whereas in DD+F group only decreased DOPAC levels were found, relative to LD and DD groups. No significant differences were found for the DOPAC/DA ratio among experimental groups in trout hypothalamus.

In telencephalon, DA content in control group revealed the existence of a day-night variation, with high levels being observed at day time, whereas basal levels occurred at the end of the night (ZT0’). Constant darkness resulted in a decrease of averaged DA levels (relative to control) together with a shift of peaking levels, thus being observed during the subjective night (CT16). This effect was even more pronounced in trout exposed to DD and fasted, thus showing the lowest averaged DA levels (relative to control and DD groups), and peaking levels occurring with a 12 h (relative to control) or 4 h delay (relative to DD). These results were supported by the existence of a significant interaction between both main factors, “time” and “experimental condition” (ANOVA, F\(\text{2,127} \) = 2.107; P=0.021). DOPAC content did not reveal the existence of a day-night variation, but was dependent on the experimental
condition (ANOVA, $F_{2, 126} = 3.963; P=0.021$). Accordingly, mean DOPAC levels were lower in DD+F group, compared to both DD and LD groups. No significant changes were observed for DOPAC/DA ratio among experimental groups in trout telencephalon.

![Graph showing daily variation of NA in trout brain (hypothalamus, telencephalon, optic tectum and medulla) in different experimental conditions (LD, DD and DD+F). $n=8$; *, significant difference respect to LD group; #, significant difference respect to DD group; $P<0.05$.]

**Figure 3**: Daily variation of NA in trout brain (hypothalamus, telencephalon, optic tectum and medulla) in different experimental conditions (LD, DD and DD+F). $n=8$; *, significant difference respect to LD group; #, significant difference respect to DD group; $P<0.05$. 

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Table 4: Table of two-way ANOVA results for CAs, Time (ZT) and Condition (LD, DD, DD+F) as main factor in trout brain (Hypothalamus, telencephalon, optic tectum and medulla). *, Indicate the existence of significant daily variation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Parameter</th>
<th>Time</th>
<th>Condition</th>
<th>Time x Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td>NA</td>
<td>F(6,138):2.074 P:0.060</td>
<td>F(2,138):25.708 P&lt;0.001*</td>
<td>F(12,138):3.286 P&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>F(6,140):0.504 P:0.804</td>
<td>F(2,140):47.899 P&lt;0.001*</td>
<td>F(12,140):1.573 P:0.106</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>F(6,140):0.348 P:0.910</td>
<td>F(2,140):9.552 P&lt;0.001*</td>
<td>F(12,140):1.805 P:0.053</td>
</tr>
<tr>
<td></td>
<td>DOPAC/DA</td>
<td>F(6,140):0.229 P:0.967</td>
<td>F(2,140):0.938 P:0.394</td>
<td>F(12,140):2.123 P:0.019*</td>
</tr>
<tr>
<td>Telencephalon</td>
<td>NA</td>
<td>F(6,130):0.552 P:0.767</td>
<td>F(2,130):6.732 P&lt;0.001*</td>
<td>F(12,130):0.973 P:0.478</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>F(6,127):1.192 P:0.315</td>
<td>F(2,127):10.444 P&lt;0.001*</td>
<td>F(12,127):2.107 P:0.021*</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>F(6,126):3.141 P&lt;0.001*</td>
<td>F(2,126):3.963 P:0.021*</td>
<td>F(12,126):0.835 P:0.615</td>
</tr>
<tr>
<td></td>
<td>DOPAC/DA</td>
<td>F(6,119):0.254 P:0.957</td>
<td>F(2,119):2.040 P:0.135</td>
<td>F(12,119):1.299 P:0.228</td>
</tr>
<tr>
<td>Optic tectum</td>
<td>NA</td>
<td>F(6,136):2.146 P:0.052</td>
<td>F(2,136):3.959 P&lt;0.021*</td>
<td>F(12,136):1.244 P:0.259</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>F(6,129):2.342 P&lt;0.001*</td>
<td>F(2,129):3.997 P:0.021*</td>
<td>F(12,129):1.409 P:0.169</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>F(6,128):2.645 P:0.019*</td>
<td>F(2,128):21.569 P&lt;0.001*</td>
<td>F(12,128):2.557 P:0.005*</td>
</tr>
<tr>
<td></td>
<td>DOPAC/DA</td>
<td>F(6,120):3.366 P:0.004*</td>
<td>F(2,120):5.587 P:0.005*</td>
<td>F(12,120):1.854 P:0.047*</td>
</tr>
<tr>
<td>Medulla</td>
<td>NA</td>
<td>F(6,133):3.749 P&lt;0.002*</td>
<td>F(2,133):37.817 P:0.001*</td>
<td>F(12,133):1.328 P:0.210</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>F(6,138):1.744 P:0.115</td>
<td>F(2,138):4.939 P:0.008*</td>
<td>F(12,138):1.003 P:0.450</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>F(6,130):1.569 P:0.161</td>
<td>F(2,130):1.222 P:0.298</td>
<td>F(12,130):1.249 P:0.257</td>
</tr>
<tr>
<td></td>
<td>DOPAC/DA</td>
<td>F(6,128):0.935 P:0.473</td>
<td>F(2,128):5.082 P:0.008*</td>
<td>F(12,128):1.250 P:0.257</td>
</tr>
</tbody>
</table>
DA content in optic tectum of control group did not display any significant variation. However, light isolation resulted in a decrease of DA levels during the early subjective day and late subjective night. This effect was more robust in trout exposed to constant darkness and fasting. As consequence, “time” and “experimental condition” independently influenced the DA daily profile, whereas no significant interaction between both main factors was observed (see Table 4). DOPAC content in control group displayed a daily variation, with peaking levels being observed at the second half of the day. The absence of light resulted in the loss of such fluctuation and a significant decrease of mean DOPAC levels, independently of feeding condition, relative to that of control. In the same way, the ratio DOPAC/DA paralleled the daily variation observed for DOPAC content in control group. Light isolation resulted in a 4-8 hours advance of peaking values, and a decrease of averaged values, relative to control. Such effect was not apparently dependent on food.

![Figure 4: Daily variation of DA, DOPAC and DOPAC/DA ratio in hypothalamus and telencephalon in different experimental conditions (LD, DD and DD+F). n=8; *, significant difference to respect to LD group; #, significant difference respect to DD group; P<0.05.](image-url)
Figure 5: Daily variation of DA, DOPAC and DOPAC/DA ratio in optic tectum and medulla in different experimental conditions (LD, DD and DD+F). n=8; *, significant difference respect to LD group; #, significant difference respect to DD group; P<0.05.

In medulla, DA content in control group was higher during day-time than at night. Such fluctuation dissipated in trout exposed to DD. However, exposure to both DD and fasting resulted in a significant increase of DA averaged levels and a 12 h shift of peaking values relative to control group, thus occurring at the subjective dark phase (CT16). By other hand, DOPAC content did not reveal the existence of any day-night variation among groups. Even when no significant daily changes were observed for DOPAC/DA ratio among experimental groups in trout medulla, a significant difference was found when “experimental condition” was analyzed (ANOVA, $F_{2,128} = 5.082; P=0.008$), with the lowest mean values being observed in DD+F group (see Table 4).
Hypothalamic and telencephalic content of 5HTP, 5HT, 5HIAA, and the ratio 5HIAA/5HT in all the experimental groups is represented in Figure 6. Regarding 5HTP content in hypothalamus of control group, no significant daily variation was found, but higher levels were observed during the day, including a slight increase at ZT8. Such oscillation disappeared in trout exposed to DD, together with an increase of averaged HTP levels, relative to control. When analyzing “experimental condition” as main factor, a significant difference was observed, consisting on animals being subjected to DD and fasted to display decreased levels of 5HTP (ANOVA, $F_{2,138} = 21.620; P<0.001$), relative to control and DD groups (see Table 5). In addition, the one way ANOVA analysis revealed the existence of a significant daily variation of 5HTP content in this group (ANOVA, $F_{6,47} = 4.991; P<0.001$), with the highest levels occurring at ZT8, and basal levels all over the day.

The two way ANOVA analysis revealed the existence of a significant difference for “experimental condition” as main factor (ANOVA, $F_{2,140} = 13.157; P<0.001$) for 5HT content. Accordingly, control group displayed a daily variation (ANOVA, $F_{6,46} = 4.804; P<0.001$) with peaking values during the day-night transition (ZT12) and basal levels all at night. Exposing trout to DD resulted in the increase of averaged 5HT levels, a 4 h delay of peaking values (at CT16), relative to control, and the loss of the significance level of the daily fluctuation. Averaged 5HT levels in hypothalamus of fish subjected to DD and fasted were lower than those of control and DD groups. No significant differences were found in 5HIAA content for both main factors, and no differences were found among experimental groups at any time point. However, the one way ANOVA analysis revealed the existence of a daily variation only in control group (ANOVA, $F_{6,46} = 4.710; P<0.001$), with peaking values at ZT12. With respect to the 5HIAA/5HT ratio, a significant difference was observed for “experimental condition” as main factor (ANOVA, $F_{2,137} = 3.985; P=0.021$). Accordingly, the control group showed a significant daily variation with peaking levels at ZT16 (ANOVA, $F_{6,46} = 2.456; P=0.038$). Such profile was not observed when trout were subjected to DD alone, in contrast to that observed in DD+F group, in which high values for the ratio were observed at the subjective day-time, and lower levels at subjective night, thus with a 8 h shift in peaking values, relative to control. In addition, averaged ratio was significantly higher in DD+F group than DD group.
Figure 6: Daily variation of 5HTP, 5HT, 5HTP, 5HIAA and 5HIAA/5HT ratio in hypothalamus and telencephalon in different experimental conditions (LD, DD and DD+F). n=8; *, significant difference respect to LD group; #, significant difference respect to DD group; P<0.05.
Table 5: Table of two-way ANOVA results for indole amines, Time (ZT) and Condition (LD, DD, DD+F) as main factor in trout brain (Hypothalamus, telencephalon, optic tectum and medulla). *, Indicate the existence of significant daily variation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Parameter</th>
<th>Time</th>
<th>Condition</th>
<th>Time x Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothalamus</td>
<td>5HTP</td>
<td>F(6,138):2.145, P:0.052</td>
<td>F(2,138):21.620, P&lt;0.001*</td>
<td>F(12,138):1.162, P:0.316</td>
</tr>
<tr>
<td></td>
<td>5HT</td>
<td>F(6,140):0.630, P:0.706</td>
<td>F(2,140):13.157, P&lt;0.001*</td>
<td>F(12,140):0.980, P:0.467</td>
</tr>
<tr>
<td></td>
<td>5HIAA</td>
<td>F(6,140):1.897, P:0.085</td>
<td>F(2,140):0.205, P:0.980</td>
<td>F(12,140):0.411, P:0.957</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
<td>F(6,137):0.517, P:0.795</td>
<td>F(2,137):3.985, P:0.021*</td>
<td>F(12,137):1.803, P:0.053</td>
</tr>
<tr>
<td>Telencephalon</td>
<td>5HTP</td>
<td>F(6,136):2.463, P:0.027*</td>
<td>F(2,136):9.776, P&lt;0.001*</td>
<td>F(12,136):2.593, P:0.004*</td>
</tr>
<tr>
<td></td>
<td>5HT</td>
<td>F(6,132):0.249, P:0.959</td>
<td>F(2,132):6.803, P:0.002*</td>
<td>F(12,132):0.924, P:0.525</td>
</tr>
<tr>
<td></td>
<td>5HIAA</td>
<td>F(6,131):0.798, P:0.573</td>
<td>F(2,131):4.461, P:0.005*</td>
<td>F(12,131):1.052, P:0.406</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
<td>F(6,125):0.694, P:0.655</td>
<td>F(2,125):4.120, P:0.019*</td>
<td>F(12,125):1.776, P:0.059</td>
</tr>
<tr>
<td>Optic tectum</td>
<td>5HTP</td>
<td>F(6,136):2.463, P:0.027*</td>
<td>F(2,136):9.776, P&lt;0.001*</td>
<td>F(12,136):2.593, P:0.004*</td>
</tr>
<tr>
<td></td>
<td>5HT</td>
<td>F(6,130):1.057, P:0.392</td>
<td>F(2,130):2.106, P&lt;0.001*</td>
<td>F(12,130):1.783, P:0.057*</td>
</tr>
<tr>
<td></td>
<td>5HIAA</td>
<td>F(6,135):0.430, P:0.858</td>
<td>F(2,135):48.492, P&lt;0.001*</td>
<td>F(12,135):1.455, P:0.149</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
<td>F(6,130):1.664, P:0.135</td>
<td>F(2,130):11.928, P&lt;0.001*</td>
<td>F(12,130):1.414, P:0.017</td>
</tr>
<tr>
<td>Medulla</td>
<td>5HTP</td>
<td>F(6,138):1.174, P:0.324</td>
<td>F(2,138):7.520, P&lt;0.001*</td>
<td>F(12,138):1.818, P:0.051*</td>
</tr>
<tr>
<td></td>
<td>5HT</td>
<td>F(6,134):2.302, P:0.038*</td>
<td>F(2,134):48.140, P&lt;0.001*</td>
<td>F(12,134):0.723, P:0.727</td>
</tr>
<tr>
<td></td>
<td>5HIAA</td>
<td>F(6,130):1.049, P:0.397</td>
<td>F(2,130):13.504, P&lt;0.001*</td>
<td>F(12,130):1.592, P:0.010</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
<td>F(6,138):2.145, P:0.052</td>
<td>F(2,138):21.620, P&lt;0.001*</td>
<td>F(12,138):1.162, P:0.316</td>
</tr>
</tbody>
</table>
Telencephalic content of 5HTP did not show daily variation among experimental groups. In addition, experimental condition did not affect this profile. On the contrary, 5HT content was significantly affected by the experimental condition (ANOVA, F$_{2,132} = 6.803$; P=0.002). Accordingly, control group showed high levels of 5HT during the day and basal levels at night onset. Trout exposed to DD increased averaged 5HT content compared to that of control, but also the highest levels were found at the early subjective night, i.e., with a 12 h delay, relative to that of control group. Constant darkness and food restriction resulted in an overall decrease of averaged 5HT content, in addition to a 12 h shift in the profile, relative to control, thus with high levels occurring at subjective night. No daily variations were observed for 5HIAA content among groups. However, when analyzing “experimental condition” as main factor (see Table 5), a significant difference was observed, consisting on increased levels of the metabolite in trout subjected to DD (ANOVA, F$_{2,131} = 4.461$; P=0.005), relative to that observed in control group. With respect to the 5HIAA/5HT ratio, a significant difference was observed for “experimental condition” as main factor (ANOVA, F$_{2,125} = 4.120$; P=0.019). Accordingly, averaged values were higher in trout subjected to constant darkness and food restriction, relative to control. No daily variations were observed for the 5HIAA/5HT ratio among groups.

Content of 5HTP, 5HT, 5HIAA, and the ratio 5HIAA/5HT in optic tectum and medulla in all the experimental groups is shown in Figure 7. In optic tectum, no daily variations were observed for 5HTP content at any experimental condition, but “time”, “experimental condition”, and the interaction between both factors resulted significant following the ANOVA analysis (see Table 5). According to that, averaged 5HTP levels decreased in trout subjected to DD alone, relative to control fish, with this effect being even more robust in DD+F group (ANOVA, F$_{2,136} = 9.776$; P<0.001). The one-way ANOVA analysis revealed a significant daily variation of 5HT content in control group (F$_{6, 47} = 2.435$; P = 0.039) with peaking levels at ZT0. Similar results were observed in trout subjected to DD, but peaking levels shifted to CT4. On the other side, light isolation and food restriction resulted in the alteration of the 5HT daily profile in such a way that averaged 5HT levels significantly increased (ANOVA, F$_{2,130} = 23.106$; P=0.019) relative to control group, and a 12 h shift occurred for peaking levels, thus being observed during the subjective night onset (CT12). Similar results were observed for the metabolite 5HIAA, with control group showing a daily variation with peaking levels during the night (ZT20). A significant variation among groups was observed after assessing “experimental condition” as main factor (ANOVA, F$_{2,135} = 48.492$; P<0.001), in such a way that DD group showed a significant decrease of 5HIAA content, relative to control group, leading the daily variation to disappear. In addition, subjecting trout to DD and fasting resulted in the daily variation to persist, but with an advance of peaking levels relative to control, up to the subjective night onset (CT12). This result came together with the increase of averaged 5HIAA levels, relative to control.
group. With respect to the 5HIAA/5HT ratio, a significant difference was observed for “experimental condition” as main factor (ANOVA, $F_{2,130} = 11.928; P<0.001$). Accordingly, control group showed increased values at night and basal values during the day. Trout exposed to DD displayed a significant decrease of averaged values, relative to control group, but the daily variation persisted, even when peaking values advanced 4 hours (CT0) relative to control. Constant darkness and fasting resulted in no apparent variation of averaged values of the 5HIAA/5-HT ratio relative to control, but altered profile of the daily variation in such a way that peaking values were found during the subjective night-day transition, whereas basal values were observed during the subjective night onset (CT12).

Regarding 5HTP content in medulla, no significant daily variation was found at any experimental condition. However, ANOVA analysis revealed the existence of significant differences (see Table 5) for “experimental condition” as main factor (ANOVA, $F_{2, 138} = 7.520; P<0.001$). Accordingly, averaged content of 5HTP was lower in DD+F group, relative to control and DD groups. 5HT content in medulla displayed a daily variation in control group consisting on high levels of the monoamine during the night (ZT16) and basal levels during the day (ZT8). Significant differences were observed for “time” (ANOVA, $F_{2, 140} = 13.157; P = 0.038$) and “experimental condition” (ANOVA, $F_{2, 134} = 48.140; P<0.001$) as main factors. Thus, DD group displayed a significant increase of averaged 5HT levels relative to both, control and DD+F groups. In addition, the profile changed relative to that of control, then with high monoamine levels occurring during the subjective day-night transition, i.e., from CT8 to CT16. A similar change of the daily profile was observed in trout subjected to constant darkness and fasting, in addition to a significant increase of averaged 5HT levels relative to control, but such increase was not as pronounced as that of DD group. The daily profile of 5HIAA content in medulla of control group remained similar to that of the monoamine, with peaking values being observed at night (ZT16) and basal levels during the day (ZT8). Exposure to DD resulted in a significant increase of averaged metabolite levels in medulla relative to control (ANOVA, $F_{2, 130} = 13.504; P<0.001$) and the daily variation to disappear. On the contrary, the daily variation persisted in DD+F group, but peaking levels shifted to subjective day-time (CT0), whereas such variation did not reach significance level. In addition, increased mean content was observed in this group, relative to control. Finally, the analysis of the 5HIAA/5HT ratio revealed the existence of significant differences for “experimental condition” as main factor (ANOVA, $F_{2, 138} = 21.620; P<0.001$). In spite of control group not displaying any daily variation, both groups of trout subjected to DD did. Thus, in DD group peaking values were measured in samples collected during the subjective night-day transition, and basal values during the subjective night onset (CT12). Additionally, averaged values were also significantly lower than those of control. Similar results were also observed in medulla of DD+F group, with the only difference consisting on the time for basal values, which was CT16 in this group.
**DISCUSSION**

The hypothesis of this study is mainly supported by our results, consisting on brain monoaminergic activities and food intake regulators to display daily rhythms that persist even in the absence of light and food as synchronizers in rainbow trout. These results are indicative of the control exerted by a circadian oscillator that
generates these rhythms. Then, the existence of a link between brain monoaminergic systems, food intake control and the circadian system exists in this teleost species.

With respect to monoaminergic activity the enzyme TPH is the rate-limiting enzyme of 5HT biosynthesis within the serotonergic neurons (Grahame-Smith, 1964; Lovenberg et al., 1967; Jequier et al., 1969). This enzyme displays rhythmic variations at both cell soma (within the raphe) and cell terminals, with the latest projecting to those cells hosting the circadian system, such as the SCN and the IGL, as reported in rat (Barassin et al., 2002; Malek et al., 2004). This leads, the rhythm of 5HT synthesis to correlate with that of the monoamine release within these brain regions (Dudley et al., 1998; Barassin et al., 2002; Grossman et al., 2004). Even when in early studies carried out in mammals, 5HTergic neurons were reported to display daily rhythms of activity, no information was available regarding the elements participating in these rhythms. Soon after, a second isoform of TPH was isolated and described as TPH2 (Walther et al., 2003). This isoform was finally found to be predominant in the mammalian central nervous system, thus with the tph2 mRNA expressing at the raphe nuclei (Patel et al., 2004; Clark et al., 2006), but both isoforms also co-express in other brain regions such as the pineal gland (Patel et al., 2004; Sugden et al., 2004). Studies in fish, regarding tph expression and its regulation are still lacking. Therefore, we have cloned and sequenced in our laboratory a fragment of rainbow trout tph1 and tph2 isoforms, in such a way, the specific primers for each gene are available in order to evaluate tph expression in studies on serotonergic activity. Detailed description of the technical approaches is provided in experimental work 4. Nowadays, we prove that both tph genes express in trout brain, since quantification of mRNA abundance of tph1 and tph2 was consistent in different brain regions such as hypothalamus, telencephalon, optic tectum and medulla.

In addition, we report the existence of significant daily rhythms of both tph genes in brain of rainbow trout. Accordingly, peaking levels of mRNA abundance are mainly occurring at the same temporal window in all brain locations, from late day to first half of the night. These results are in line with previously reported in other neural regions of mammals, such as rat retina (Liang et al., 2004), but also in the raphe nucleus in the same species (Malek et al., 2007). As far as we are aware, this is the first time in fish, the daily rhythms of mRNA abundance of tph are described, which limits the comparison among fish. Moreover, in each brain region of trout subjected to light isolation together or not with food restriction these rhythms persisted with characteristics (phase, amplitude) that in general varied little in relation with fish kept under normal lighting and feeding conditions. Similarly to our study, tph activity showed a circadian variation in the IGL pathway in rat (Malek et al., 2004), which corroborates the existence of daily variations of 5HT inputs to the IGL (Pasquier and Villar, 1982; Villar et al., 1988). Accordingly, we also speculate with the hypothesis of 5HT synthesis to be under circadian control in
trout 5HTergic neurons. Therefore, we highlight two important results of this study. First, the existence of a daily profile of mRNA abundance for each \textit{tph} gene, suggesting that 5HT synthesis is regulated through the control exerted over \textit{tph1} and \textit{tph2} expression; Second, at least in the brain regions analysed, it also evidenced that a circadian oscillator might control and modulate mRNA abundance of both genes.

In consistency with this, the control exerted by the SCN over the raphe neurons has been reported in mammals (Malek \textit{et al.}, 2005). However, the circadian control exerted by the central clock in mammals is not limited to the one hosted in the SCN, since circadian rhythms were also described in isolated brain regions (Abe \textit{et al.}, 2002), suggesting the existence of multiple oscillators. It is likely that these oscillators subordinate to the main SCN clock and probably regulate over locally generated functions (Akhtar \textit{et al.}, 2002; Yoo \textit{et al.}, 2004). Therefore, given that in fish brain the location of a possible chief oscillator is unknown, we speculate with that existence of TPH rhythms in the different brain regions, as we herein demonstrate, could be due to regulatory activity exerted by multiple oscillators distributed in the brain of this species at transcriptional level.

As for the most remarkable data about rhythms of \textit{tph} expression, both \textit{tph1} and \textit{tph2} displayed peaking values at the day-night transition in hypothalamus, telencephalon, and optic tectum (ZT 16.04), with the latter showing a small delay in \textit{tph2} towards midnight (ZT 16.04). Interestingly, in medulla \textit{tph1} peak appeared at the daytime onset (ZT 0.52), whereas \textit{tph2} continued to peak at midday (ZT 7.41). This suggests that \textit{tph2} rhythmic expression at the regions level, predominantly receivs serotonergic terminals such as hypothalamus, telencephalon and optic tectum, might be relatively similar to that accounted in medulla, where most serotonergic cell bodies locate. Similar conclusion cannot be expected for \textit{tph1} since rhythm in both cell bodies- and neuronal terminals-containing regions are near to 180° out of phase. Further studies will be needed to understand whether 5HT synthesis is differentially regulated in the different brain regions, and how projections from mesencephalic nuclei are related to monoamine synthesis and release in forebrain regions.

When comparing the expression rhythms of each enzyme exhibited under different lighting or feeding conditions, some differences could be outstanding. Thus, light isolation for fish during 48 h had little effects on the phase of the rhythms, since generally peak levels remain in a similar temporal window than that of controls. Specific increases or decreases took place on each enzyme expression and brain region assessed. For instance, a remarkable phase delay in \textit{tph1} expression was noted in hypothalamus, telencephalon and optic tectum under DD, in such a way that peak shifted for near to 3 h (hypothalamus, optic tectum) and 5 h (telencephalon) towards the night period, whereas in medulla it was 3 h phase advanced. With respect to \textit{tph2}, a phase delay of 3 h was noted in hypothalamus and telencephalon of trout kept under DD, whereas no shifts occurred in optic tectum.
addition to changes in rhythmic phase, amplitude was also affected by DD condition and \textit{tph1} values were near to three fold higher in hypothalamus and telencephalon of trout kept under DD than in those of the LD group. As for \textit{tph2}, the most remarkable effect on rhythm amplitude was a decrease observed in the telencephalon of the DD group, which did not appear in the other brain regions. When DD and fasting were applied together a higher increase in amplitude was noted for \textit{tph1} in hypothalamus, and \textit{tph2} in telencephalon, as compared to DD-exposed group. Decreases in relation to the DD group were noted for amplitude of \textit{tph1} in telencephalon and \textit{tph2} in medulla in fish kept under DD+fasting condition, with the last region showing a very remarkable attenuation in the rhythm of the enzyme.

The TH is the rate-limiting enzyme of NE and DA biosynthesis (Levitt \textit{et al.}, 1965). Similarly to TPH, TH can display daily rhythms in different brain regions of mammals, as demonstrated in very early studies in rodents that described for the first time circadian rhythms of TH activity in the pineal gland (McGeer and McGeer, 1966), years later extended to brain stem (Otten \textit{et al.}, 1975; Cahill \textit{et al.}, 1981). In fish, significant rhythmic profiles of TH activity were reported in Midas cichlid (\textit{Cichlasoma citrinellum}) retina with peaks located at night, which are under light and circadian modulation (McCormack \textit{et al.}, 1993). In contrast, in medulla of rat peaking levels of TH activity mainly were observed during daytime (Otten \textit{et al.}, 1975). TH enzyme activity was not assessed in our study, but significant daily rhythms of mRNA abundance of this enzyme were found in all the brain regions (telencephalon, hypothalamus, optic tectum and medulla). Clear rhythmic profiles were found in all these regions of trout in LD group with peaks generally located at the first part of the light phase (ZT between 2 and 3), with the exception of the hypothalamus, where it was even earlier (ZT 1.13). In absence of light as external synchronizer of clock system, these rhythms in \textit{th} expression persisted in all brain region assessed. In addition, they remained in light-isolated fasted fish, which is suggesting the daily rhythms of \textit{th} expression are endogenous and are subordinated to a circadian oscillator, as above described for \textit{tphs}. However, clear changes were noted in rhythm parameters when light or food were not present. Thus, phase delays of rhythmic enzyme expression were observed in fish kept under DD, at least in hypothalamus (CT 8.51), telencephalon (CT 5.24) and optic tectum (CT 6.47), but not in medulla, where a phase advance seems to occur (peak at CT22.53). When light isolation and fasted were applied, significant increases occur in the amplitude of the rhythm of \textit{th} expression in hypothalamus and telencephalon, with minor changes in optic tectum and medulla. Also, this combined treatment induced a phase delay of \textit{th} expression rhythm in hypothalamus, compared to DD group, thus returning to values near to control LD group. In medulla this effect was even higher, since rhythm acrophase shifted from the end of the night (DD group, CT22.53) toward the end of the day (DD+fasting group, CT 10.33).
Under the best of our knowledge, we describe for the first time in fish, that a circadian oscillator drives the daily rhythms of mRNA abundance of enzymes strictly related monoamine synthesis, and probably to activity of monoaminergic neurons. In spite of the existence of such rhythmic fluctuations in both tph isoforms and th all over the brain of rainbow trout, unclear daily variations were noted for the monoamines content, their main metabolites, and the ratio metabolite/monoamine at any experimental condition. In mammalian brain, circadian rhythms of NE, DA, and 5HT were reported to exist (Dixit and Buckley, 1967; Manshardt and Wurtman, 1968; Friedman and Walker, 1968; Scheving et al., 1968; Reis and Wurtman, 1968; Collu et al., 1973; Simon and George, 1975; Friedman and Piepho, 1979; Owasoyo et al., 1979). However, whether these fluctuations are due to changing rates of monoamine synthesis or/and changes in the rates of their release is still not clarified.

The rhythmic secretion of NA in neurons of the mammalian brain has been described very early (Akersted and Levi, 1978). Peaking levels occur at noon and basal levels appear during sleep-time. This rhythm disappears in animals subjected to sleep deprivation or constant lighting conditions, and when environmental synchronizers are experimentally phase-shifted NA secretion rapidly re-adjusts (Akersted and Levi, 1978). This suggests that environmental factors play a key role in generating of NA rhythm, this effect being independent on the modulatory action of a circadian oscillator. Our results in rainbow trout are in the same way, at least in hypothalamus, since even daily variation in content is not significant, high NA averaged levels are observed at day-time, with low averaged levels at night, but with such variation disappearing in groups of trout subjected to constant darkness (DD/DD+fasting). Daily variation in other brain regions were modest, except for medulla, where a significant change exist, with higher values at night. This fluctuation disappeared in the DD group but remained in the LD+fasting group, suggesting that both factors could interact to modulate daily changes in neurotransmitter content. Then, light and food play a key role in modulating daily variations of NA content in some brain regions of trout, even when the main enzyme of the biosynthetic pathway, TH, seems to be subordinated to a circadian oscillator.

In brain of mammals, DA and its main metabolite, DOPAC clearly display daily rhythms (Smith et al., 1992; Paulson et al., 1996; O'Neill et al., 1985). The profile of such fluctuations in DA content was disrupted by constant darkness in rat brain (Khalidy et al., 2002). In the same way, DA content in rainbow trout displays a day-night fluctuation in telencephalon and medulla, but not in the other regions, with high levels during the day and basal levels at night. This agrees with the described rhythmic fluctuation of th expression, suggesting that daily changes in amine content are specifically modulated by activation/deactivation of its synthesis. However, a similar clear variation did not exist for DOPAC content and the ratio DOPAC/DA, suggesting that changes in DA synthesis are not immediately reflected in neurotransmitter turnover or, alternatively, that catabolism to DOPAC does not
follow a circadian profile. In trout subjected to DD, DA rhythmic profile disappears, which indicates that light cycle is important to modulate metabolism of DA in rainbow trout, thus this factor masking the influence that circadian synthesis might have on amine content.

Daily rhythms in mammalian brain were more deeply studied for 5HT than for CAs, due to the leading role that 5HT has as neurotransmitter that modulates circadian rhythms (Prosser et al., 1993; Morin and Allen, 2006). At extracellular level within the striatum of rat, 5HT concentrations have been reported to display day-night variations as also occurs in its main oxidative metabolite, 5HIAA (Rueter et al., 1997; Paulson et al., 1996; Rueter and Jacobs, 1996). In general, when such rhythm exist, peaking levels of 5HT are detected during the light to dark transition, at least in rat SCN (Barassin et al., 2002), but also in melatonin synthesising tissues, such as the pineal gland of rat (Dudley et al., 1998) and several species of hamster (Sun et al., 2002; Míguez et al., 1995; 1996). To our knowledge, the rhythmic variation of 5HT content in fish were only described in the pineal organ of rainbow trout (Ceinos et al., 2005). In this tissue, 5HT rhythm is in antiphase with that of melatonin, which is explained by 5HT being actively metabolized at night to form melatonin, thus reducing pineal 5HT content during the night.

The particular relationship between 5HT and melatonin in the pineal organ made that changes in the amine content in this tissue are not necessarily similar to that of other brain tissues. Accordingly, our results show discrete daily changes in 5HT content in hypothalamus, optic tectum and medulla. In these regions, significant time-dependent changes occurred in 5HT content, which were accompanied by daily variations of 5HIAA levels. Moreover, the 5HIAA/5HT ratio displayed a significant fluctuation in hypothalamus of trout kept under LD conditions, with peak levels at the end of the light phase. Interestingly a similar rhythmic profile was found for the levels of its precursor (5HTP), which was clearly related to expression of tph1 and particularly to tph2, which peaked at the end of the day. This data are consistent with brain serotonergic function being activated rhythmically during the last phase of the light period, whith is consistent with that reported for rat striatum (Paulson et al., 1994; 1996; Rueter and Jacobs, 1996) and SCN (Barassin et al., 2002). This clock tissue-linked rhythm remains in constant darkness, although it is influenced by lighting cues (Sun et al., 2002; Huether et al. 1993; Snyder et al., 1965). Our data show that, in the absence of light (together or not with food) a tendency to higher levels of 5HT at daytime and lower levels at night was found in optic tectum and medulla, with even lower fluctuations in the other regions. Since the robust rhythms found for tph expression in trout brain and its persistence when animals remained under DD, is presumable that a specific circadian control is exerted on neurotransmitter synthesis. This process could be modulated by light and food, although this is not clearly reflected in rhythms of the neurotransmitter content. This discrepancy might be related to the low number of
serotonergic neurons in the fish brain, as compared to mammals (Lillesaar, 2011), and the diffusing effect that occurs when assessing their concentrations in a large mass of tissue, as we did. The use of more specific techniques, such as brain microdialysis, could aid to bring light to the variations that occur in the amine at synaptic level as consequence of rhythms in the neuronal activity.

This study also demonstrates the existence of rhythms of brain tph1, tph2 and th mRNA abundance. Most of them persist in the absence of light and food as entraining cues, which is suggesting that they subordinate to the control of a circadian oscillator. Additionally we show that rhythms of monoamine synthesis are influenced by both light and food, in such a way that rhythmic profile is altered in trout subjected constant darkness, combined or not with fasting. On the contrary, other parameters, such as brain monoamine levels, their metabolites and the ratio metabolite/monoamine, show weak daily changes that relate in some cases to those rhythms in monoamines synthesis or/and release. However, whether changes in amines and metabolites are related or not to the activity of the circadian system should be assessed in studies by using more refined neurochemical techniques.

Together with the existence of rhythms in monoaminergic systems of trout brain we evaluated rhythms in hypothalamic food intake-related peptides, and their dependence on an oscillator. This is important since food intake is a rhythmically driven process linked to the circadian system (Kulczykowska and Sánchez-Vazquez, 2010; Delgado et al., 2017; Isorna et al., 2017). Accordingly one can expect factors regulating feeding activity to display circadian rhythms, including those of regulatory hypothalamic neuropeptides and also 5HT, as already shown, which has been associated to feeding regulation (De Pedro et al., 1998b). Our results on food intake-related neuropeptides revealed the existence of significant daily rhythms in crf, pomec-a1, cart and npy in trout hypothalamus, in agreement with our previous studies in this species (Naderi et al., 2010). These rhythms persisted in the absence of light, although specific changes were observed in acrophase (phase-advance in npy) and amplitude (increase of crf and pomec-a1, decrease of npy) of the rhythms. In addition, trout that were kept under DD+fasting showed an increase of averaged mRNA levels for pomec-a1, decreased amplitude of npy, and the loss of rhythmicity for crf and cart. These results suggest that a circadian control is exerted over some peptides such as pomec-a1 and npy, but it is unclear over other peptides (crf and cart). Specifically for NPY, it has been reported a role as input to the circadian oscillator within the hypothalamus of goldfish (Vera et al., 2007), which complement our data, suggesting a role of this peptide in circadian physiology. Nonetheless, this control seems not to be so strict for crf and cart, which leads us to suggest that food-related cues could influence the rhythms of this neuropeptides in trout hypothalamus, thus driving them or masking the influence exerted by the circadian clock on them.
In summary, we demonstrate in rainbow trout the existence of rhythms of brain monoaminergic function that are particularly evident in *tph1*, *tph2* and *th* transcripts, but also in critical food intake modulators such as *crf*, *pomc-a1*, *cart* and *npy*. The persistence of most of these rhythms (including those of *pomc-a1* and *npy*) in constant conditions has been demonstrated by submitting fish to light isolation and fasting, which also corroborates their dependence on the circadian system. The rhythmic fluctuations of other neuropeptides (*crf* or *cart*) seems to be more influenced in origin by factors other than circadian oscillator, then being hidden under constant environment conditions. Since monoamines have been reported to modulate food intake in teleost through acting on hypothalamic neuropeptides (De Pedro *et al*., 1998b; Leal *et al*., 2011; Pérez-Maceira *et al*., 2014), it would be interesting to know if monoamines are also involved in modulating the rhythms of these neuropeptides. In this context, the role of 5HT is particularly important since 5HT actions on food intake are mediated by specific 5HT receptors, in particular 5HT$_{2c}$-like that seems to locate in POMC neurons (Pérez-Maceira *et al*., 2014). Moreover, 5HT has been suggested to regulate HPI axis by activating 5HT$_{1a}$ receptors, mainly located on CRF neurons (Medeiros *et al*., 2010; 2014). Due to all these interactions, it is likely that circadian changes in 5HT synthesis and those in the amine released from the neuronal terminals to the synaptic clef can influence the rhythmic fluctuations of food intake-related neuropeptides. Finally, an effect of the daily monoaminergic rhythms on HPI axis rhythmicity is also possible since 5HT, acting through hypothalamic CRF neurons (De Pedro *et al*., 1998b), modulate the rhythms of this peptide. Accordingly, parallelism for rhythms of mRNA abundance of 5HT synthesis enzymes (*tph1* and *tph2*), and *crf* in trout hypothalamus is noted, which deserves further research.
3.4. Experimental work 4

Changes in brain catecholaminergic and serotonergic systems in rainbow trout (*Oncorhynchus mykiss*) during chronic stress and its recovery. Relation with changes in cortisol axis and feeding behaviour.
Resumen

Cambios en los sistemas catecolaminérgicos y serotoninérgicos del cerebrales en la trucha arco iris (Oncorhynchus mykiss) durante el estrés crónico y el retorno a las condiciones normales. Relación con el cortisol y la ingesta.

En los peces, los sistemas serotoninérgicos y dopaminérgicos cerebrales parecen desempeñar un papel importante en el inicio y el mantenimiento de la respuesta al estrés. Esta respuesta involucra la activación de los ejes cerebro-sistema simpático-tejido cromafín e hipotálamo-hipófisis-tejido interrenal y lleva a un aumento de las concentraciones plasmáticas de catecolaminas y cortisol, pero también de la actividad monoaminérgica cerebral. Mientras que el aumento de las catecolaminas es de corta duración, los niveles de cortisol en sangre continúan elevados cierto tiempo, aunque tienden a disiparse progresivamente hasta retornar a la normalidad. Además, en situaciones de estrés crónico la respuesta del cortisol puede desvanecerse, lo que añade complejidad a los mecanismos implicados en su regulación. En este sentido se sabe poco sobre el papel de los neurotransmisores monoaminérgicos cerebrales durante el estrés crónico e incluso no se han desarrollado estudios centrados en la fase de post-stress, cuando cesan los estímulos estresantes y se recuperan los valores normales de cortisol circulantes. Para abordar estas situaciones, un grupo de truchas arco iris (Oncorhynchus mykiss) fue sometido durante 10 días a estrés por alta densidad, mientras que otro grupo se sometió a la misma situación de estrés durante 7 días y a continuación se expuso a la densidad normal. Estos peces fueron sacrificados a las 2 h, 6 h, 24 h y 72 h tras el cese del estrés. Se tomaron muestras de sangre (para evaluar niveles de cortisol y metabolitos), de regiones cerebrales (para evaluar mediante HPLC los niveles de monoaminas y mediante q-RT PCR la abundancia de ARNm de tph1, tph2 y th, así como de diversos neuropéptidos hipotalámicos implicados en el control de la ingesta) y del riñón anterior (parámetros relacionados con la síntesis de cortisol: expresión de star, 3β-hsd, p450ssc y 11β-h). Los resultados mostraron un aumento en los niveles de cortisol en plasma y en las actividades dopaminérgica y serotoninérgica en telencéfalo, hipotálamo, techo óptico y róbemenciéfalo de los peces estresados. También se observó una mayor abundancia de ARNm de star, 3β-hsd, p450ssc y 11β-h en riñón anterior, y de th, tph1 y tph2 en cerebro. Las actividades monoaminérgicas disminuyeron a valores basales a las 2-6 horas de cesar la situación de estrés, algo similar a lo que ocurrió con el cortisol plasmático. El estrés también causó un aumento de la expresión de cart, crf y pomeca1 en el hipotálamo, el cual desapareció de forma muy rápida tras el retorno a la situación normal (no estrés). Por tanto, la respuesta fisiológica que se pone en marcha por el estrés crónico en la trucha arco iris parece disiparse en un periodo de tiempo relativamente corto (2-6 h) cuando cesa el estrés. La dinámica temporal de los cambios que ocurren en las monoaminas cerebrales, especialmente en las regiones diencefálicas y telencefálicas, tiene un gran paralelismo como los que ocurren en el eje de estrés y
en los neuropéptidos hipotalámicos que actúan de forma anorexigénica. Por otro lado, el estrés crónico aumentó la expresión de los receptores de glucocorticoides \textit{gr1}, sin producir cambios en los \textit{gr2}. Tras el cese del estrés se produjo una recuperación de los niveles basales de expresión que, en el caso del hipotálamo y telencéfalo, mostró un gran paralelismo temporal con los cambios encontrados en los sistemas monoaminérgicos. Estos datos sirven de apoyo al papel que ejercen las monoaminas cerebrales en el inicio de la respuesta fisiológica al estrés, el cual parece ser operativo en el reconocimiento de la situación estresante y en mantener la activación neuroendocrina a lo largo del tiempo, pero también en la modulación de su desactivación cuando el pez regresa a una situación de ausencia de estrés.
Abstract

Changes in brain catecholaminergic and serotonergic systems in rainbow trout (Onchorhynchus mykiss) during chronic stress and its recovery. Relation with changes in cortisol axis and feeding behaviour.

In fish, brain serotonergic and dopaminergic systems appear to play a main role in initiating and maintaining the response to stress. Such response involves the activation of two neuroendocrine axes: the HSC and the HPI tissues, leading to increased plasma CAs and cortisol levels, but also in brain monoaminergic activity. While increased CAs is short, blood cortisol levels remain elevated for longer period. In addition, under chronic stress the response of cortisol may fade even if the stress remains, thus increasing the complexity of its regulation. After stress exposure, the physiological response tends to dissipate, with cortisol levels, among other parameters, turning back progressively to normal. However, little is known regarding the role of brain monoamine neurotransmitters during chronic stress and indeed no studies focus on what happens after stress, when cortisol decays to non-stress levels. To address these situations, a cohort of rainbow trout (Onchorhynchus mykiss) was subjected to stress by high stocking density for up to 10 days and then sacrificed, whereas another cohort was stressed for 7 days and afterwards unstressed and sacrificed following 2 h, 6 h, 24 h and 72 h after stress exposure. Food intake was estimated throughout the experiment. Individual samples of blood (for cortisol and metabolites assessments), several brain regions (for HPLC assessment of monoamines content and qPCR assessment of th, tph 1, tph 2 mRNA abundance, as well as hypothalamic neuropeptides involved in food intake control), and head kidney (for the assessment of parameters related to cortisol synthesis: star, 3β-hsd, p450ssc and 11β-h) were collected. Our results show enhanced plasma cortisol levels, and dopaminergic and serotonergic activities in telencephalon, hypothalamus, optic tectum and hindbrain of stressed fish. Increased mRNA abundance of star, 3β-hsd, p450ssc and 11β-h (head kidney) and th, tph 1 and tph 2 (brain) were also observed. After stress, monoaminergic activities decreased to basal values from 2 to 6 h post-stress in parallel to plasma cortisol. Stress also increased the expression of cart, crf and pomc-a1 in hypothalamus, which down-regulate very soon after returning fish to unstressed condition. Thus, the above described physiological response to chronic stress in rainbow trout dissipates in a relatively short time period (6 h) after stress is over. A strong parallelism was observed between the temporal dynamic of the changes that occur in brain monoamines, especially in the diencephalic and telencephalic regions, and those that occur in the stress axis and in the hypothalamic anorexigenic neuropeptides. Furthermore, chronic stress increased brain expression of glucocorticoid gr1 receptors (but not gr2), which recovery to basal levels in hypothalamus and telencephalon followed a similar trend to that of monoamines systems. These data support a role of brain monoamines in mediating the upstream physiological response to stress, which
seems to be operative to recognize a stressful situation and to maintain the neuroendocrine activation signal over time, but also to modulate its deactivation after returning to a non-stress situation.
Changes in brain catecholaminergic and serotonergic systems in rainbow trout (*Oncorhynchus mykiss*) during chronic stress and its recovery. Relation with changes in cortisol axis and feeding behaviour.

**INTRODUCTION**

Stress response in an animal is a complex series of behavioural and physiological (cellular, endocrine, biochemical) components that operates at different body levels in order to cope with a potentially harmful or dangerous external stimulus (Barton, 2002). Two endocrine axes, the HPI and the BSC axes, command the actions against the stressor. Their activation trigger a homeostatic response that leads to mobilize fuel and make energy available to cope with increased metabolic demand (Wendelaar-Bonga, 1997; Mommsen et al., 1999). Although the physiological stress coping is considered a homeostatic process that tries to re-establish the functional balance altered by the stressor, when this is repeated, or chronic, may become maladaptative, i.e., malfunction, with brain and neuroendocrine centers responding to stress. This usually entails risk of physiological deterioration and the incidence of diseases. Then, understanding the underlying mechanisms of the stress response is important in order to establish strategies that mitigate the impact of stress during breeding and handling in farms.

Similar to mammals, CAs and cortisol are the representative main end-products of the two major pathways coordinating the stress response in fish (Mommsen et al., 1999). Several biochemical mechanisms participate in the normal function and regulation of the hormonal axis, mainly including the CRF released from neurons located in the preoptic area, and the ACTH, which is released from the pituitary in response to CRF and acts as the main corticosteroids secretagogue (Wendelaar Bonga, 1995; Barton, 2002). It has also been proposed that CRF stimulates the melanotropic cells to release α-MSH and β-endorphin. Similarly other brain peptides such as AVT and thyrotropin releasing hormone may activate the hypothalamic-pituitary cascade in response to stressors (Tort, 2013; Balment et al., 2006). The monoamine neurotransmitters, including the CAs, DA and NA, and the indoleamine 5HT could also have a role in the regulation of the neuroendocrine axis (Heisler et al., 2007; Gesto et al., 2009; 2013).

Stressors are firstly recognized, and then processed in the CNS before the neuroendocrine response initiates. According to time as criteria, two main types of stressors affecting fish can be defined, i.e., the acute and the chronic stress, whereas stress intensity resides in more qualitative parameters like mild, moderate or high stress. Whereas acute stress can be of high intensity and short duration, chronic stress is normally of lower intensity but prolonged in time. Accordingly, the hormonal changes during stress are graduated in time, so that the secretion of CAs occurs very quickly in the presence of the stressor, and is of short duration,
facilitating the fish to escape, and the immediate supply of nutrients (glucose, lactate) for vital organs such as heart, gills or kidney. Meanwhile, the cortisol response is slower but more continuous over the time, sustaining the high energy flow in blood. Then the fish can cope with the high metabolic and functional demand during days or even weeks (Barton, 2002), while the stressor persists. However, changes in circulating CAs and cortisol at long-term are largely dependent of fish species, age, social status, type and duration of the stressor, among others (Barton, 2002) in such a way that a more complex scenario may be considered.

Studies on the involvement of brain monoamines in stress response point to the activity of these neurotransmitters to increase in response to different type of stressors, including handling, chasing, crowding, hypoxia, salinity exposure and pollutant exposure (see Table 1, for a brief references list). Behavior-related stressor such as isolation, predator exposure and social hierarchies have also been shown to perturb monoaminergic activity in several teleost species, including rainbow trout, Arctic charr (Salvelinus alpinus) and sea bass (Dicentrarchus labrax) (Winberg et al., 1997; Höglund et al., 2001; Schjolden et al., 2006). However, the causes of these changes within the stress response along with the dynamics of the monoaminergic response to stress are not well understood. For instance, the serotonergic system, which has received more attention than others (dopaminergic and noradrenergic), could have a role in the initial steps of the stress response. In fact, serotonergic neurons of the forebrain (hypothalamus, telencephalon) rapidly increases their activity following a brief (15 s) chasing stimulus (Gesto et al., 2013), and recover basal levels several hours later. Studies also pointed to that enhances in the activity of the serotonergic system shortly after handling disturbance occur independently of the duration, and possibly the intensity of the stressor (Gesto et al., 2015b). Dopaminergic activity also increases after stress but this response seems to be more limited within the brain, and the time they occur after exposure to stressor. Indeed, increased dopaminergic levels are usually associated with social stress responses in fish (Summers and Winberg, 2006; Höglund et al., 2001).

Additionally, studies in mammals report that increased and sustained serotonergic activity are considered as an output signal in chronic stress (Browne et al., 2011). In fish, recent contributions in rainbow trout kept under low- and high-stocking densities for 28 days suggest that increases in serotonergic turnover occur independently of fish storing condition, and are important to maintain high cortisol response (Moltesen et al., 2016). It has been suggested that these long-term changes in 5HT are related to chronic stress and could indicate compromised fish welfare.
Table 1: Brief summary of studies on the involvement brain monoamines in stress response.

<table>
<thead>
<tr>
<th>Type of stressor</th>
<th>Duration</th>
<th>Species</th>
<th>Dopaminergic</th>
<th>Serotonergic</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>High or low post-stress cortisol values (high stocking density)</td>
<td>8 Day</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>Øverli et al., 2001</td>
</tr>
<tr>
<td>Net handling</td>
<td>1 min chase, 2 min rest and 2 min chase</td>
<td><em>Solea senegalensis</em></td>
<td>x</td>
<td>x</td>
<td>Weber et al., 2015</td>
</tr>
<tr>
<td></td>
<td>15 s, 2 min, 5 min and 15 min. Recovery period of 15 min, 45 min, 2 h, 4 h and 8 h.</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>Øverli et al., 2013; 2015b</td>
</tr>
<tr>
<td>Air exposure</td>
<td>3 min</td>
<td><em>Solea senegalensis</em></td>
<td>x</td>
<td>x</td>
<td>Weber et al., 2015</td>
</tr>
<tr>
<td></td>
<td>3 min for 14 and 28 days</td>
<td><em>Solea senegalensis</em></td>
<td>x</td>
<td>x</td>
<td>Costas et al., 2012</td>
</tr>
<tr>
<td>Social stress</td>
<td>1–21 days</td>
<td><em>Salvelinus alpinus</em></td>
<td></td>
<td>x</td>
<td>Winberg &amp; Nilsson, 1993</td>
</tr>
<tr>
<td></td>
<td>1 or 7 days</td>
<td><em>Oncorhynchus mykiss</em></td>
<td></td>
<td>x</td>
<td>Winberg &amp; Lepage, 1998</td>
</tr>
<tr>
<td></td>
<td>3 and 7 days</td>
<td><em>Oncorhynchus mykiss</em></td>
<td></td>
<td>x</td>
<td>Øverli et al., 2001</td>
</tr>
<tr>
<td></td>
<td>5 min, 3 h, and 24 h</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>Øverli et al., 1999</td>
</tr>
<tr>
<td></td>
<td>13 and 29 weeks</td>
<td><em>Salmo salar</em></td>
<td></td>
<td></td>
<td>Cubitt et al., 2008</td>
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<td></td>
<td>5 days</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>DiBattista et al., 2005</td>
</tr>
<tr>
<td>Exposure to chemicals compound</td>
<td>3, 24 and 72 h</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>Øverli et al., 2001</td>
</tr>
<tr>
<td></td>
<td>12 and 24 h</td>
<td><em>Solea senegalensis</em></td>
<td>x</td>
<td>x</td>
<td>Weber et al., 2012</td>
</tr>
<tr>
<td></td>
<td>28 days</td>
<td><em>Betta splendens</em></td>
<td>x</td>
<td>x</td>
<td>Ethan et al., 2010</td>
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<tr>
<td></td>
<td>1 to 6 h and 1 to 5 days</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>Øverli et al., 2001</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td><em>Oncorhynchus mykiss</em></td>
<td>x</td>
<td>x</td>
<td>Melnyk-Lamont et al., 2014</td>
</tr>
<tr>
<td></td>
<td>9 weeks</td>
<td><em>Ictalurus punctatus</em></td>
<td></td>
<td>x</td>
<td>Heidi et al., 2011</td>
</tr>
<tr>
<td>Temperature</td>
<td>26°C, 29°C and 32°C, for 3 weeks.</td>
<td><em>Oreochromis mossambicus</em></td>
<td></td>
<td>x</td>
<td>Tsai and Wang, 1997</td>
</tr>
<tr>
<td>Salinity</td>
<td>Increased levels of salinity (1 % NaCl) at 25 °C and 30 °C for 2 week</td>
<td><em>Cyprinus carpio</em></td>
<td>x</td>
<td>x</td>
<td>De Boeck et al., 1996</td>
</tr>
</tbody>
</table>
The consequences of changes occurring in brain serotonergic and dopaminergic systems after stress are also matter of interest. The neurotransmitter 5HT plays an important role in the regulation of the HPI axis, either by interacting with CRF release in the hypothalamus or by acting directly on the pituitary to regulate ACTH secretion (Winberg et al., 1997). As consequence, increases in serotonergic activity in forebrain and blood cortisol levels usually correlate in teleost (Winberg et al. 1997; Gesto et al., 2013; Winberg and Thörnqvist, 2016). Moreover, 5HT and CRF transmission are under feedback control of glucocorticoids and interact with brain and neuroendocrine systems during chronic and repeated stress, which is likely to modulate HPI axis response (Carpenter et al., 2007; Medeiros et al., 2014). In addition, studies in fish report that brain monoamines (5HT or DA) are involved in food intake regulation (Bernier and Peter, 2001). Experiments following ICV administration of selective 5HT and DA receptor agonists and antagonists, and with oral administration of monoamine precursors (L-DOPA) evidence an inhibition of food intake in teleost species, trout and goldfish (De Pedro et al., 1998a; Leal et al., 2013; Pérez-Maceira et al., 2014; 2016), by acting through specific postsynaptic receptors. According to pharmacological evidence, it has been proposed that 5HT reduction of food intake is mediated by the activation of hypothalamic 5HT<sub>2C</sub> receptors, resulting in increased expression of anorexigenic neuropeptides such as pomc, cart and crf (Pérez-Maceira et al., 2016). Meanwhile, effects of increased DA release on food intake have been associated to D<sub>1</sub> receptors and a mediating role of CRF is reported for sea bass (Leal et al., 2013). Since the direct involvement of CRF in the stress response in fish, it is plausible that related changes in food intake, i.e., inhibition of food intake after long-term stress, are due to monoaminergic pathways acting downstream on CRF neurons of the hypothalamus.

Understanding the dynamics of brain monoaminergic systems during chronic stress exposure might allow to better know how external information that initiates the neuroendocrine signals may also prime it at long-term in order to allow the fish to cope with the continuously increased metabolic demands. Additionally, if stress ceases, it is important to know how these systems can re-establish their function, and whether it can be an element to consider in order to achieve neuroendocrine and metabolic homeostasis. This also would help to establish critical elements for further studies trying to propose procedures that mitigate the negative effect of stress in fish.

The present study aimed to investigate in rainbow trout changes in brain monoaminergic (CAs, 5HT) neurochemistry during typical chronic stress, such as high stocking density, for several days. In addition, we wanted to know if these changes reverted when fish subjected to stress return to normal conditions, establishing a relationship with changes in cortisol levels as an expression of the activity of the neuroendocrine axis of stress.
MATERIAL AND METHODS

 Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (A Estrada, Spain) and transferred to our facilities at the Faculty of Biology, University of Vigo (Spain). Fish were kept in 120 L water tanks at a density of 10 kg body mass/m³, with continuously fresh and aerated water, and controlled temperature (13.5 ± 1°C) for at least twenty days before any experiment initiated. Fish were maintained under artificial photoperiod (12L: 12D) and were fed handily once per day (1% body mass; 10 a.m.) with commercial dry pellets (Dibaq-Dipotg SA, Segovia, Spain; proximate food analysis: 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). During acclimation, all fish tanks were inspected several times every day in order to detect dominant individuals, which were removed from the experiment. Additionally, tanks contained more fish than required in the experimental groups in order to reduce dominance hierarchies, which occur in most species when subjected to low stocking density. The experiments comply with the Guidelines of the European Union Council (2010/63/EU), and Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Animal Welfare Committee of the University of Vigo.

 Food intake Assessment

Food intake was assessed in a preliminary experiment with the same protocols as described before. Food was supplied in batches of 10 g every 5 min until animals were satiated. After feeding, the food uneaten remaining at the bottom (conical tanks) was withdrawn, dried for 24 h at 37°C and weighed. The amount of food consumed by all fish within each tank was calculated as previously described (Polakof *et al*., 2008 a,b) as the difference from the food offered (De Pedro *et al*., 1998b). FI values registered after treatment are referred to those of basal values.

 Experimental groups and sampling

Fish were randomly distributed in 11 experimental tanks (120 L) with 20 fish per each one. After acclimation (at least 10 days) fish were initially assigned to one of these two groups: i) control unstressed group (NSD, 4 tanks), ii) stressed group by exposure to high stocking density (HSD, 7 tanks). Stress was induced by lowering water levels in the tanks to reach a fish density of 70 Kg body mass/m³, which was previously demonstrated as a stressful situation (Conde-Sieira *et al*., 2013). Fish from NSD and HSD tanks were sampled on days 3, 7 and 10, respectively (one tank of each group at each time), after starting the experiment.

The remaining four tanks of stressed fish were used to evaluate the recovery after stress exposure into the same experimental protocol. For this purpose, and in
parallel to the control and stressed fish sampled at day 7, four tanks of stressed trout that remained from day 0 under HSD were returned to a NSD situation by increasing progressively the water level to the tank, which did not take longer than 10 min. Subsequently fish density in these tanks returned to values similar to those of control fish tanks (10 Kg body mass/m³). According to scheduled sampling times fish from recovery tanks were anesthetized and sampled at +2 h, +6 h, +24 h, and +72 h, after removing the stress condition (post-stress groups). Following this protocol, fish of the +72 h post-stress group were sampled at the same time as the NSD (control unstressed) and HSD groups of fish killed at day 10, and then these fish served as controls (unstressed and stressed fish) to evaluate the recovery response.

During the experiment fish from all the groups were daily fed in a normal condition but were fasted on sampling day. Sampling process did never exceed 2min/fish. Individuals were directly anesthetized in the tanks by adding 2-phenoxyethanol (0.2% v/v - Sigma Aldrich), in such a way that the calculated volume of the drug was diluted in 5 litres of tank water and added into the tank, rapidly and avoiding visual contact of the fish with the manipulator. Once anesthetized fish were collected with a net and transferred to an annexed sampling room to avoid any disturb of the remaining fish during sampling procedure. During sampling 1 mL of blood from each fish was obtained by puncturing caudal peduncle with a 1 ml disposable heparinized syringe and afterwards animals were killed by spinal transection. Immediately, brains were removed and the hypothalamus, telencephalon, optic tectum and medulla were separated under sterile conditions, placed into Eppendorf tubes, frozen in liquid nitrogen and stored at −80°C until assayed. Plasma samples were obtained after blood centrifugation (6000g for 10 min at 4°C) and then 50 µl separated and immediately stored at -80°C to be used for cortisol assessment. An aliquot of 300 µl plasma was deproteinised with 75 µl of 0.6 M perchloric acid. After centrifugation (14,000 g for 4 min at 4°C) the supernatant was neutralized with 75 µl of 1M potassium bicarbonate and stored at -80°C until analysis of metabolites levels.

**Plasma cortisol and metabolites assays**

The levels of cortisol in plasma were quantified by mean of an enzyme-linked immunosorbent assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) previously validated for trout samples in our lab. Commercially available kits (Spinreact, Girone, Spain) were used to assess the plasma glucose and lactate levels.

**Brain monoamines**

Brain tissues were thawed and homogenized by ultrasonic disruption in 0.4 ml of HPLC mobile phase. The homogenates were centrifuged (16,000 g, 10 min) and supernatants were further diluted with mobile phase to HPLC analysis. Protein
content of the tissues was measured with the bicinchoninic acid method and was used for data normalization. The content of NA, DA, DOPAC, 5HT and 5HIAA in hypothalamus, telencephalon, optic tectum and medulla was quantified by HPLC with electrochemical detection (HPLC-EC), as previously described (Gesto et al., 2006; 2013). The HPLC system consisted on a Jasco PU-2080 Plus pump, a 5 μm analytical column (Nucleosil C18, 150 mm length×4.6 mm diameter; Phenomenex), a Jasco AS-2057 autosampler and an ESA Coulchem II detector. The detection system included a double analytical cell (M5011) with oxidation potentials at first electrode set at +40 mV and +340 mV for the second one. Mobile phase was a mixture of 63.9 mmol l⁻¹ NaH₂PO₄, 0.1 mmol l⁻¹ Na₂EDTA, 0.80 mmol l⁻¹ sodium 1-octanesulfonate, and 15.3% (v/v) methanol; pH was adjusted to 2.95 with ortho-phosphoric acid. The mobile phase was filtered (0.20 μm) and degassed under vacuum for 5 minutes before used for chromatographic assays at a flow rate of 1.0 ml min⁻¹ and 24°C. The analytical run time for each sample was 15 min. Detection limit for the monoamines and metabolites was 0.5-1.5 pg per injection, with a signal-to-noise ratio of 3. Each day of assay, monoamine standard samples of known concentration were run in parallel to tissue samples, and peak area values were used to estimate the monoamine content in the tissue samples. Acquisition and integration of chromatograms were performed using ChromNAV version 1.12 software (Jasco Corp).

**Cloning tryptophan hydroxylase 1 and 2**

Partial cloning of tryptophan hydroxylases 1 and 2 from *O. mykiss* was performed by RT-PCR using primers designed on fish conserved nucleotide regions of the homologue transcripts from *Poecilia mexicana*, *Danio rerio*, *Esox lucius*, *Oryzias latipes*, *Oreochromis niloticus* and *Takifugu rubripes*, using the programme Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). Accordingly, designed primers were:

- **tph1**: Fw: 5’-GAGGACAACATCCCCCAGCTGGA-3’  
  Rev: 5’-GTGTACGCGTGTACCTCAGTTC-3’

- **tph2**: Fw: 5’-AGATCTACGCCAGCTGCACTGC-3’  
  Rev: 5’-CCTCCTCTGCGGTGATTACATTAT-3’

Total RNA was isolated from hypothalamus using Trizol (Ambion) method. The quality and concentration of total RNA was assessed by using Ge NanoVue plus-2264. Contamination of genomic DNA was removed from 900 ng of total RNA using RQ1 Dnase (Promega). cDNA was synthesized with M-MLV RT using random primers following manufacture’s recommendations (Promega) and PCR products with the predicted amplicon size were gel purified using a commercial kit (Quiagen). For sequencing analyses, the considered PCR products were ligated using pGEM T easy vector (Promega) and inserted in JM109 Competent cells (Promega). To ensure
the bacteria incorporated the inserts a screening on the LBA/X-Gal/IPTG/ampicillin was carried out. Then white clones were isolated in LB/ampicillin and those clones containing plasmid with insert were selected using the Eco RI restriction enzyme (Promega). Finally, plasmids were sequenced in both directions with SP6 and T7 by the University’s services (CACTI, Univ. Vigo). The identity of the sequenced fragments was confirmed by submitting against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the program BLASTX 2.8.0. Both sequences were send to the Bankit where identification numbers were procured for tph1 (BankIt2051269_Seq1_MG015697) and tph2 (BankIt2051269_Seq2_MG015698).

**Real-time quantitative RT-PCR (qPCR) assays**

Total RNA from the brain tissues (hypothalamus, telencephalon, optic tectum, and medulla) was extracted following the TRIzol® (Invitrogen, USA) method following manufacturer indications, and treated them with RQ1-DNase (Promega). 2 µg of mRNA from each sample was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) and Random Primers (Promega). To confirm the absence of any genomic contamination of the RNA extract, negative controls without reverse transcriptase were included in the assays of cDNA synthesis. The cDNA samples were amplified in triplicate by real-time reverse transcription-PCR using a Bio-Rad MyIQ real-time PCR system (Bio-Rad, USA).

PCR reactions were performed in a total volume of 15 µl containing 7.5 µl of MyMaximaTM SYBR Green qPCR Master Mix (K0253, Thermo Scientific, USA), 1 µl of first strand DNA or no RT controls, and the corresponding concentrations of forward and reverse primers. The primers were designed on the bases of previously reported sequences for rainbow trout genes. These included th, tph1, tph2, food intake regulatory neuropeptides (crf, pomc-a1, cart, npy), glucocorticoid receptors 1 and 2 (gr1 and gr2), steroidogenic acute regulatory protein (star), cytochrome p450 cholesterol side chain cleavage (p450scc), 3-β-hydroxysteroid dehydrogenase (3β-hsd), cytochrome P45011β (11β-h). Relative quantification of the target gene transcripts was carried out using β-actin gene expression as reference, which was stably expressed in the tissues assayed in the study. Table 1 includes the sequences of the sequences for forward and reverse primers assayed.

Thermal cycling initiated with incubation at 95°C for 3 min; followed by 35 steps of PCR, each one consisting of heating at 95°C for 10 s for denaturing, and at specific annealing temperature (see Table 2) for 30 s and extension at 50°C for 30 s. To ensure that only one fragment was amplified, following the final PCR cycle, melting curves were systematically monitored (50°C temperature gradient at 0.5°C/s from 50 to 95°C). Relative mRNA expression was evaluated according to the standard comparative delta-Ct method, based on the Pfaffl method (Pfaffl, 2001). For each gene, individual samples at the same time point were processed in parallel
and the expression was measured in triplicate within the same microplate. Only efficiency values between 85% and 100% were accepted (the $R^2$ for all the genes assessed was always higher than 0.985).

**Statistical analysis**

Data are presented as mean ± S.E.M. of each experimental group. Based on the levels of amines and the respective oxidative metabolites for each sample according to the treatment, the ratios DOPAC/DA and 5HIAA/5HT were calculated. Before any comparison was done, the variance homogeneity into each group was assessed following Levene test. Comparisons were made between HSD and NSD groups at each time assayed (3, 7, 10 days), for which the one-way ANOVA test was used. Subsequently, comparisons were made for each measured parameter between all the control groups included in the experiment (days 3, 7 and 10). Since no differences were found, data of the four control groups were pooled to make a single control value ("average control"), which was used for comparisons with the post-stress groups (+2 h, +6 h, +24 h, +72 h after 7-days exposure to HSD). This improved the global vision of the results and avoided the duplication of the control groups for each stress recovery time, also facilitating the results plotting. One-way ANOVA was applied to determine the existence of significant differences among the average control, stress and post-stress groups. When significant differences were found, the post hoc Student-Newman-Keuls test was used. Differences between means were considered significant when $P < 0.05$. SPSS-SigmaPlot V11.0 (Systat Software Inc., USA) was used for all statistical analysis.

**RESULTS**

As above mentioned, values of the assessed parameters in control groups sampled at days 3, 7 and 10 remained stable. In order to facilitate data comparison these data were pooled, and the mean values of each assessed parameter were represented in the graphs as the mean control values line for the whole experimental period.

**Food intake**

Trout kept under HSD exhibited a decrease of food consumption as monitored at 3, 7 and 10 days (Figure 1). This effect was maximum at day 7 reaching a 53% inhibition with respect to the basal intake level estimated in the days prior to the experiment, and in comparison with the respective control fish at the same time. After 10 days of exposure to HSD, a small recovery was observed in food intake (40% inhibition with respect to baseline) although it remained significantly lower than control group at that time. Fish returned to normal density.
situation after 7 days of exposure to HSD showed a rapid recovery so that at +24 h post-stress their intake was comparable to that observed before exposure to HSD. A small increase of food intake was noticed at +72 h post-stress, although it was not significant from control group at this time (10 days).

Table 1: Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tₐ</th>
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</thead>
<tbody>
<tr>
<td>tph1(MG015697)</td>
<td>AGGGAAAGATGAGAGCCTACG</td>
<td>CCAGAGTGCAATGCTTCAG</td>
<td>57</td>
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<tr>
<td>tph2 (MG0156998)</td>
<td>CCTCAACACGCCTCAAAAACC</td>
<td>ATCTCTGGGGGAACCAAGA</td>
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<tr>
<td>th (NM02154247.1)</td>
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<tr>
<td>crf (AF296672)</td>
<td>ACAACGACTCAACTGAGATATCG</td>
<td>AGGAAATTGAGCTTATGTCAGG</td>
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<tr>
<td>cart (NM001124627)</td>
<td>ACCATGGAGAGCTCCAG</td>
<td>GGGACACTGTCTCACC</td>
<td>60</td>
</tr>
<tr>
<td>pomc-al1(TCS86162,Tigr)</td>
<td>CTCGCTGTCAAGACACTCAACTCTC</td>
<td>GAGTTGGGAGAGATGGACACCTC</td>
<td>60</td>
</tr>
<tr>
<td>npy (NM001124266)</td>
<td>CTCGCTTGCACCTTTATATGC</td>
<td>GTTCATCATCTCTCGAGTTCG</td>
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<tr>
<td>gr1 (NM001124730.1)</td>
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<td>AGATGAGCTGCAATCCATGTCCTGAT</td>
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<tr>
<td>gr2 (AJ495372.1)</td>
<td>CATCGGACAGACTGCTGAAAC</td>
<td>AGCAGACGAGACACTTCATC</td>
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<tr>
<td>star (AB047032)</td>
<td>CTCCTACAGACATAGGGGAGAAC</td>
<td>GCCCTTCTTCTTCCCTCCTCAC</td>
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<tr>
<td>p450scc (S57305.1)</td>
<td>ATGCGTCAGGACACTAAAC</td>
<td>CAGGGTATCATCTCTCCAGCA</td>
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<tr>
<td>3β-hsd (S72665.1)</td>
<td>TCACAGGGCTCAACGTCAAGAIGG</td>
<td>CCTCCTTCTTGGTCTGCTG</td>
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<tr>
<td>11β-act (AF179894)</td>
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<tr>
<td>β-actin (AJ438158)</td>
<td>GATGGGCCAGAAAGACACTA</td>
<td>TCGTCCCCAGTGGGTAGGCGG</td>
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</table>

Plasma levels of cortisol, glucose and lactate

Plasma cortisol, glucose and lactate levels are shown in Figure 2. Cortisol concentration in stressed fish significantly increased all over the experiment, compared to control unstressed groups at each time, although this increase gradually decayed from day 3 (2.5 times higher than control) until day 10 (two times higher vs control).

After seven days of HSD stress, water volume was returned to normal level in several tanks, thus allowing trout to have a similar environmental condition than control groups. In this condition, cortisol levels remained elevated for the first 2 hours, and decreased progressively, so that at +4 and +6 h post-stress they exhibited similar values to those of unstressed groups. Specifically, comparison of cortisol levels between the HSD group at 10 days and the +72 h post-stress group clearly reveal that normalization of cortisol levels was fully dependent on the presence/absence of stress.

Plasma glucose content in fish exposed to HSD for 3, 7 and 10 days were similar to those of unstressed groups at these times. In the post-stress assay (from day 7 to 10 after removing from HSD to NSD) the only change observed in glucose levels was a decrease at +6 h, which disappears at +24 h in that condition.
Plasma lactate levels increased at days 3 and 10 in trout exposed to HSD but not at day 7, in which lactate levels were even lower than those of the respective control group. Regarding the dynamic after stress removal at day 7, low levels of lactate were found in all times assayed (from +2 h to +72 h of post-stress time). A clear trend to increase was noticed at +72 h post-stress since lactate concentration was very close to the control (unstressed) group at the same time (10 days).

Figure 1: Averaged daily food intake of rainbow trout subjected to different experimental conditions (NSD, HSD, and Post-stress). Different letters: P<0.05 significant difference between all experimental groups; * p < 0.05 significant difference between each NSD and HSD. Data are presented as percentage of baseline levels of food intake evaluated during previous 12 days.

Expression of genes involved in cortisol synthesis in head kidney

Figure 3 shows the changes induced by exposure to HSD for 7 days in mRNA abundance of *star*, *p450sc*, *3β-hsd* and *11β-h* in head kidney. All of them exhibited a significant increase in trout kept for 7 days to HSD, as compared to the corresponding control trout group that remained in NSD, which in some cases doubled the expression values. When trout held for 7 days in HSD were exposed to NSD, there was a rapid decrease in *star* expression and, similarly in the expression of the enzymes involved in steroidogenesis. All these parameters returned in 6 h post-stress to values similar to those of fish that did not kept under HSD.

Brain neuropeptides in trout held under HSD and after changing it to NSD

The expression of several neuropeptides involved in the hypothalamic regulation of food intake (*crf, pome-a1, cart* and *npy*) was assessed, and results are shown in Figure 4. A significant increase in the expression of *cart* and *pome-a1* was observed at 3, 7 and 10 days in trout exposed to HSD, while *crf* were significantly
increased on the 7th and 10th days of HSD exposure. Fish that remained for 7 days under HSD and afterwards exposed to NSD showed a gradual fall in the expression of these three peptides, being faster in *cart* and *crf* (values at +6 h post-stress similar to averaged controls) than in *pomc-a1* (values at +24 h post-stress similar to averaged controls). As for the expression of *npy*, neither it did respond to HSD stress nor undergo immediate alterations when the situation changed from HSD to NSD. However, a sharp increase was noticed in *npy* expression in fish changed to NSD after 24 h, which persisted at least until 72 h, being in this time clearly higher to that of respective group of HSD exposed fish (10 days).

**Figure 2:** Plasma cortisol, glucose and lactate levels in rainbow trout subjected to different experimental conditions (HSD, NSD, and Post-stress). Each value is the mean ± S.E.M. of 10 fish. Different letters: P<0.05 significant difference between HSD and post-stress groups; * p < 0.05 significant difference between each NSD and HSD; #: P<0.05 significant difference between average NSD and post-stress groups.
Figure 3: mRNA abundance of cortisol biosynthesis pathway (*star*, 3β-hsd, p450scc and 11β-h) in head kidney of rainbow trout subjected to different experimental conditions (HSD, NSD, and Post-stress). Different letters: P<0.05 significant difference between HSD and post-stress groups; * p < 0.05 significant difference between each NSD and HSD; #: P<0.05 significant difference between average NSD and post-stress groups.
Figure 4: mRNA abundance of food intake related- neuropeptides (crf, cart, pomc-a1 and npy) in hypothalamus of rainbow trout subjected to different experimental conditions (HSD, NSD, and Post-stress). Different letters: P<0.05 significant difference between HSD and post-stress groups; * p < 0.05 significant difference between each NSD and HSD; #: P<0.05 significant difference between average NSD and post-stress groups.
Stress and post-stress related changes in brain monoaminergic activity

Analysis of the partial tph1 and tph2 genes

Tph1 and 2 were partially sequenced from the hypothalamus of rainbow trout. The partial tph1 cDNA has a 593bp open reading frame (ORF) and encodes a partial protein of 190 amino acids. The partial tph2 has a 254bp ORF encoding a partial protein of 69 amino acids. Using smart blast, a Biopterin-dependent aromatic amino acid hydroxylase conserved domain was detected in both tph1 and tph2. Nonetheless, these partial proteins only share 17% identity (See Figure 5). The alignment (data not shown) of O. mykiss tph1 Biopterin-dependent aromatic amino acid hydroxylase domain with homologous sequences reveals a 99% identity with the homologous tph1 from Danio rerio (NP_840091.2), a 95% with Homo sapiens (NP_004170.1), and 86% with Drosophila melanogaster (NP_612080.1). Regarding to tph2 the partial predicted amino acid sequence showed 69% of identity with Danio rerio (NM_214795.2), 67% with Homo sapiens (NM_173353.3) and 50% with Drosophila melanogaster (NM_138236.2). This data suggest that tryptophan hydroxylases are much conserved along groups.

Figure 5: Alignment of the partial proteins Tph1 and Tph2 from O. mykiss cloned in this study

Note: Recent progress in the methods of genome sequencing has made it possible that currently a full-sequence of the predicted tryptophan 5-hydroxylase 1-like (XM_021586410) and tryptophan 5-hydroxylase 2-like (CDQ65319.1) from O. mykiss are available in Genbank.

Tph1, tph2 and th expression

The results of tph1, tph2 and th mRNA abundance in hypothalamus, telencephalon, optic tectum and medulla are shown in Figure 6. Tph1 expression was significantly enhanced after 3-days exposure to HSD stress in hypothalamus,
telencephalon, optic tectum and medulla, with a 2-3 fold higher values relative to control fish at the same time. This effect was also evident at 7 and 10 days under HSD in hypothalamus, telencephalon and optic tectum, and at 10 days in medulla. Additionally, increased tph1 expression appeared after 10 days of stress in all brain regions studied, relative to control fish at this time. Removing the stress condition at 7 days induced a clear restoration in tph1 expression, as compared with mean values of the unstressed control fish, which was clearly noticed at +6 h post-stress in all brain regions, except to the medulla, in which it occurred at +2 h post-stress.

Stress by exposure to HSD induced a significant increase of tph2 mRNA abundance in all regions studied, although some differences were noticed. Thus, tph2 mRNA levels were not affected at 3 days in medulla and were slightly increased in hypothalamus and telencephalon, whereas a significant increase was found in optic tectum of the HSD group, as compared to respective unstressed fish at this time. In contrast, tph2 expression increased after 7 days of stress, relative to the respective unstressed fish, in hypothalamus, telencephalon and medulla, but not in optic tectum. Regarding stress effect at 10 days, tph2 expression was significantly higher in hypothalamus and telencephalon, but this effect was attenuated in the other regions. Fish exposure to normal density after 7 days HSD resulted in decreased tph2 expression in all brain regions, allowing the restoration of values similar to unstressed fish at +2 hours post-stress in medulla and optic tectum, with the latest decreasing even more than averaged control values. A little slower recovery was noticed in telencephalon (+6 h post-stress) and hypothalamus (+24 h post-stress).

With respect to th mRNA abundance, it was clearly affected by the HSD and showed a sharp increase in hypothalamus at 3 and 7 days, relative to values of the respective NSD groups. Results for this parameter were less consistent in the other brain areas since th expression increased after 7 days of stress in telencephalon and medulla, but it was not significantly altered at 3 days in these two regions and in optic tectum at any time. Moreover, stress-induced increase of th mRNA abundance was evident after 10 days in hypothalamus of HSD group but not in the other brain regions, in which values were not significantly different to those of unstressed fish.

In relation to the evolution of th expression when the stressful condition disappears it was found to remain increased at +2 h post-stress in several brain regions (hypothalamus, telencephalon, medulla), then immediately decreasing to normal values. Data obtained at +6 h after removing HSD were not significantly different than mean values of unstressed fish in any region. No significant changes with stress removal were noticed for th expression in optic tectum.

**Monoamines content and turnover**

Table 3 shows the content of monoamines (NA, DA, 5HT) and their metabolites (DOPAC and 5HIAA) in fish exposed for 10 days to HSD stress, as well
as during the recovery phase after 7 days of stress. Additionally Figure 7 shows the changes observed in the ratios of DOPAC/DA and 5HIAA/5HT, which were used as functional indexes of dopaminergic and serotonergic neuronal activities, respectively.

HSD induced a clear and sustained increase in dopaminergic and serotonergic activities in hypothalamus and telencephalon, as evidenced by significantly higher values of DOPAC/DA and 5HIAA/5HT ratios all over the exposure to HSD, as compared to respective unstressed groups (Figure 7). A two fold increase in these parameters was noticed at 3 days of stress, with an apparent reduction along the experimental period but still being significantly increased after 10 days of stress (about 50% increase for both DOPAC/DA and 5HIAA/5HT ratios, relative to control group at this time). As for the amines and metabolites levels, HSD exposure enhanced the hypothalamic DOPAC (3 and 10 days) and 5HIAA (3 days) levels, whereas opposite changes were found for DA (3 and 7 days) and 5HT (3 days), relative to the respective control groups (Table 3). In telencephalon, increases were also notorious in DOPAC and 5HIAA contents after 3 days of stress, as compared to the respective control group, together with decreases in the contents of DA (7 days) and 5HT (3, 7 and 10 days). Similarly, hypothalamic NA content decreased significantly after 10 days of stress in HSD group, whereas a similar effect occurred in the telencephalon after 3 days.

In both hypothalamus and telencephalon, returning fish kept for 7 days under HSD to NSD caused a fall in the DOPAC/DA and the 5HIAA/5HT ratios, which was evidenced in some cases at +2 hours and, in general, at +6 hours of post-stress. Regarding DOPAC/DA ratio, the decrease was immediate (+2 h post-stress) in both hypothalamus and telencephalon and, indeed, reached at +6 hours post-stress values lower than those observed in fish never stressed. For the 5HIAA/5HT ratio, recovery was slower, especially in telencephalon, thus reaching values similar to the unstressed fish at +6 hours post-stress, thus stabilizing.

With respect to amines and metabolites content in these two regions (hypothalamus and telencephalon), a reduction was generally observed for 5HIAA and DOPAC, which were elevated in the previous situation of HSD. After removal the fish from the HSD to NSD condition a drop in the level of the metabolites was observed, which was almost immediate (+2 h post-stress) and persistent (lower values from +24 to +72 hours than in previous times). As exception, levels of 5HIAA in the telencephalon remained elevated at +2 h after removal the HSD condition, although a significant drop was found at +6 h post-stress. NA content increase by HSD in hypothalamus (only at 7 days) and then decreased during post-stress time in both hypothalamus and telencephalon, relative to mean control values.
Figure 6: mRNA abundance of Tryptophan hydroxylase 1 and 2, Tyrosine hydroxylase (tph1, tph2 and th) in hypothalamus, telencephalon, optic tectum, medulla of rainbow trout subjected to different experimental conditions (HSD, NSD, and Post-stress). Different letters: P<0.05 significant difference between HSD and post-stress groups; * p < 0.05 significant difference between each NSD and HSD; #: P<0.05 significant difference between average NSD and post-stress groups.
Table 3: Brain monoamines levels: NA (ng/gP), DA (ng/gP), DOPAC (ng/gP), 5HTP (ng/gP), 5HIAA (ng/gP), 5HT (ng/gP), DOPAC/DA and 5HIAA/5HT ratios (%) in different experimental conditions ((HSD, NSD, and Post-stress). Different letters: P<0.05 significant difference between HSD and post-stress groups; * p < 0.05 significant difference between each NSD and HSD; #: P<0.05 significant difference between average NSD and post-stress groups.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Condition</th>
<th>NA (ng/gP)</th>
<th>DA (ng/gP)</th>
<th>DOPAC (ng/gP)</th>
<th>5HT (ng/gP)</th>
<th>5HIAA (ng/gP)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypothalamus</strong></td>
<td>Control</td>
<td>28.37±3.25</td>
<td>23.85±0.61</td>
<td>0.80±0.04</td>
<td>41.64±1.44</td>
<td>0.35±0.04</td>
</tr>
<tr>
<td></td>
<td>3 day stress</td>
<td>35.85±5.85*</td>
<td>18.26±1.10*</td>
<td>1.25±0.11*</td>
<td>39.67±1.08*</td>
<td>0.82±0.11*</td>
</tr>
<tr>
<td></td>
<td>7 day stress</td>
<td>29.60±2.82*</td>
<td>21.07±0.50*</td>
<td>0.94±0.05*</td>
<td>55.25±1.50*</td>
<td>0.60±0.02*</td>
</tr>
<tr>
<td></td>
<td>+ No stress</td>
<td>23.44±1.88*</td>
<td>24.00±1.00*</td>
<td>0.79±0.04*</td>
<td>65.22±2.91*</td>
<td>0.61±0.03*</td>
</tr>
<tr>
<td></td>
<td>+6 h</td>
<td>19.76±2.92*</td>
<td>25.06±0.47*</td>
<td>0.52±0.05*</td>
<td>60.39±1.81*</td>
<td>0.39±0.03*</td>
</tr>
<tr>
<td></td>
<td>+24 h</td>
<td>22.63±2.29*</td>
<td>25.17±0.39*</td>
<td>0.64±0.04*</td>
<td>60.86±1.75*</td>
<td>0.35±0.01*</td>
</tr>
<tr>
<td></td>
<td>+72 h</td>
<td>25.91±1.41*</td>
<td>19.84±0.93*</td>
<td>1.13±0.05*</td>
<td>47.2±1.48*</td>
<td>0.69±0.06*</td>
</tr>
<tr>
<td><strong>Telencephalon</strong></td>
<td>Control</td>
<td>52.18±7.49</td>
<td>13.14±1.08</td>
<td>1.12±0.09</td>
<td>13.66±0.84</td>
<td>1.83±0.09</td>
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<tr>
<td></td>
<td>3 day stress</td>
<td>50.34±6.04*</td>
<td>10.62±0.59*</td>
<td>1.91±0.17*</td>
<td>9.90±0.75*</td>
<td>2.49±0.13*</td>
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<tr>
<td></td>
<td>7 day stress</td>
<td>26.9±2.82*</td>
<td>8.49±0.50*</td>
<td>0.97±0.02*</td>
<td>11.11±0.86</td>
<td>1.90±0.07*</td>
</tr>
<tr>
<td></td>
<td>+ No stress</td>
<td>33.85±5.26*</td>
<td>9.78±0.43*</td>
<td>0.77±0.04*</td>
<td>10.97±0.94</td>
<td>2.10±0.09*</td>
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<tr>
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<td>+6 h</td>
<td>39.51±3.23*</td>
<td>12.32±0.74*</td>
<td>0.70±0.05*</td>
<td>13.34±0.58</td>
<td>1.57±0.10*</td>
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<tr>
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<td>+24 h</td>
<td>32.77±5.89*</td>
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<td>13.52±0.62</td>
<td>1.63±0.05*</td>
</tr>
<tr>
<td></td>
<td>+72 h</td>
<td>31.92±1.98*</td>
<td>12.20±0.67*</td>
<td>0.70±0.06*</td>
<td>13.35±0.81</td>
<td>1.54±0.05*</td>
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<tr>
<td><strong>Optic tectum</strong></td>
<td>Control</td>
<td>42.52±3.98*</td>
<td>10.98±0.64*</td>
<td>1.38±0.06*</td>
<td>10.25±0.64*</td>
<td>2.15±0.10*</td>
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<tr>
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<td>3 day stress</td>
<td>18.39±3.50</td>
<td>2.50±0.20</td>
<td>4.19±0.33</td>
<td>6.69±0.34</td>
<td>2.36±0.18</td>
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<tr>
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<td>7 day stress</td>
<td>30.66±3.39</td>
<td>2.85±0.29</td>
<td>7.65±0.36</td>
<td>9.9±0.88</td>
<td>4.53±0.29</td>
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<tr>
<td></td>
<td>+ No stress</td>
<td>21.51±5.29</td>
<td>2.67±0.27</td>
<td>6.17±0.49</td>
<td>7.68±0.60</td>
<td>3.02±0.23</td>
</tr>
<tr>
<td></td>
<td>+6 h</td>
<td>20.22±1.57</td>
<td>2.28±0.27</td>
<td>5.81±0.66</td>
<td>7.5±0.70</td>
<td>3.28±0.14</td>
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<tr>
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<td>+24 h</td>
<td>29.84±2.77</td>
<td>2.68±0.14</td>
<td>5.73±0.56</td>
<td>8.44±0.65*</td>
<td>3.09±0.19</td>
</tr>
<tr>
<td></td>
<td>+72 h</td>
<td>21.62±2.87</td>
<td>2.30±0.19</td>
<td>6.04±0.98</td>
<td>8.75±2.66</td>
<td>3.05±0.31</td>
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<tr>
<td><strong>Medulla</strong></td>
<td>Control</td>
<td>14.86±1.61</td>
<td>2.17±0.13</td>
<td>6.14±0.78</td>
<td>7.61±0.75</td>
<td>3.09±0.23</td>
</tr>
<tr>
<td></td>
<td>3 day stress</td>
<td>12.57±0.98</td>
<td>5.10±0.29</td>
<td>4.92±0.32</td>
<td>5.01±0.33</td>
<td>0.71±0.07</td>
</tr>
<tr>
<td></td>
<td>7 day stress</td>
<td>17.23±0.99*</td>
<td>6.18±0.42*</td>
<td>4.61±0.45*</td>
<td>6.37±0.31*</td>
<td>1.00±0.09*</td>
</tr>
<tr>
<td></td>
<td>+ No stress</td>
<td>10.66±0.89</td>
<td>3.74±0.23*</td>
<td>9.99±1.01*</td>
<td>4.28±0.35*</td>
<td>0.47±0.07*</td>
</tr>
<tr>
<td></td>
<td>+6 h</td>
<td>8.01±1.31*</td>
<td>3.15±0.24*</td>
<td>7.33±0.72*</td>
<td>3.69±0.23*</td>
<td>0.40±0.04*</td>
</tr>
<tr>
<td></td>
<td>+24 h</td>
<td>9.83±1.49*</td>
<td>3.29±0.26*</td>
<td>8.14±0.62*</td>
<td>3.79±0.25*</td>
<td>0.41±0.05*</td>
</tr>
<tr>
<td></td>
<td>+72 h</td>
<td>11.22±0.90*</td>
<td>3.42±0.22*</td>
<td>8.56±0.42*</td>
<td>3.86±0.18*</td>
<td>0.50±0.03*</td>
</tr>
<tr>
<td></td>
<td>10 day stress</td>
<td>11.51±2.32*</td>
<td>2.99±0.24*</td>
<td>7.67±0.71*</td>
<td>3.58±0.34*</td>
<td>0.44±0.05*</td>
</tr>
</tbody>
</table>
Figure 7: DOPAC/DA ratio and 5HIAA/5HT ratio levels in percent in hypothalamus, telencephalon, optic tectum, and medulla of rainbow trout subjected to different experimental conditions (HSD, NSD, and Post-stress). Different letters: $P<0.05$ significant difference between HSD and post-stress groups; * $p < 0.05$ significant difference between each NSD and HSD; #: $P<0.05$ significant difference between average NSD and post-stress groups.
In the other two brain regions, optic tectum and medulla, changes observed under the stress condition were less consistent. In particular, increased NA and DOPAC levels were observed in this region after 7 days of HSD stress, whereas no changes occurred in DOPAC/DA and 5HIAA/5HT ratios (Figure 7). In medulla, however, the levels of DOPAC were higher in the HSD group at 7 and 10 days, simultaneously with a decrease in DA content. Regarding the serotonergic system, the only change in this region was a decrease in the levels of 5HIAA in the HSD group at 7 days (Table 3). These alterations in the content of amines and metabolites in medulla caused an increase in the ratio DOPAC/DA of stressed trout after 7 and 10 days, in relation to the respective controls, while the 5HIAA/5HT ratio increased at 3 and 10 days of exposure to HSD.

Regarding the dynamics of the monoaminergic systems when fish were removed from HSD to NSD, no remarkable effects were found in DOPAC/DA and 5HIAA/5HT ratios within optic tectum, in accordance with the lack of effect that the previous exposure to HSD had in this region. At the level of metabolite concentrations, there were also no significant differences between the different post-stress times and the mean values of the control groups, with the exception of the concentration of NA, in which a slight increase was noticed at +24 hours after returning to NDS. On the other hand, values of DOPAC/DA ratio in medulla increased at 7 days of HSD exposure and remained high during all the post-stress period. Finally, changes of 5HIAA/5HT ratio in medulla were similar to those found in hypothalamus and telencephalon, with increasing effects in fish held in HSD. A significant decrease in this parameter took place after removing the stress condition, then showing values similar to control unstressed fish.

**Glucocorticoid receptors in brain regions of trout during exposure to HSD and after switching to NSD**

Figure 8 shows the evolution over the time of the expression of the glucocorticoid receptor subtypes gr1 and gr2 in different brain regions of trout exposed for 10 days to HSD, and after returning from HSD to NSD (days 7 to 10). These conditions induced a significant alteration of mRNA content in gr1 subtype receptors, while no effect was observed for gr2 receptors, whose expression was constant all over the experiment at any brain region.

Increased expression of gr1 receptors was found in hypothalamus, telencephalon and optic tectum of fish kept during 3, 7 and 10 days under HSD, whereas not significant changes were found in medulla. The effect of stress at 7 days on gr1 receptors disappeared when fish were exposed to NSD conditions, so that in hypothalamus, a complete restoration was found at +6 h post-stress, while in telencephalon a partial recovery occurred at +2 and+ 6 h, and it was full at +24 h
post-stress. A faster response occurred in optic tectum since gr1 expression at +2 h post-stress was already similar to that of control unstressed fish.

![Graph](image)

**Figure 8:** mRNA abundance of glucocorticoid receptors (gr1, gr2) in hypothalamus, telencephalon, optic tectum, medulla of rainbow trout subjected to different experimental conditions (HSD, NSD, and Post/stress). Different letters: P<0.05 significant difference between HSD and post-stress groups; * p < 0.05 significant difference between each NSD and HSD; #: P<0.05 significant difference between average NSD and post-stress groups.
DISCUSSION

Evidence exists in vertebrates regarding the involvement of brain monoaminergic neurotransmitters in the response to stress (Chaouloff et al., 1999; Flügge et al., 2004; Belujon and Grace, 2015; Puglisi-Allegra and Andolina, 2015). In fish, both CAs and 5HT display significant changes in the presence of a stressor. Then, these neurotransmitters were postulated as the origin and/or target of endocrine changes that characterize the stress response (Winberg et al., 1997; Winberg and Thörnqvist, 2016). Previous studies in rainbow trout subjected to acute stress highlight their role as initiators of the stress response, with a possible involvement in decoding those environmental stimuli that could be harmful, thus acting as stress inductors, compared to other stimuli to which fish are often exposed (Gesto et al., 20013; 2015b). Although some studies focused on the neuroendocrine effects of chronic stress in this species, evidence of the involvement of aminergic neurotransmitters in the long-term stress response do not exist. Our study contributes to the knowledge of the role of these neurotransmitters in chronic stress, demonstrating that trout kept under HSD display a sustained increase in brain monoaminergic activity that correlates with enhanced cortisol synthesis (interrenal tissue) and plasma levels. Likewise, this strong correlation is also maintained once the stressor disappears with a rapid recovery (2 and 6 hours) in the monitored neural and hormonal parameters, thus disclosing the role that monoaminergic systems can have in the activation-maintenance-deactivation of the long-term stress response.

Endocrine and metabolic effects of chronic stress and evolution of the post-stress recovery

In teleost, as in other vertebrates, the endocrine response to stress is structured as an immediate adrenergic response, the mobilization of CAs, and a somewhat slower activation of the HPI hormonal axis that synthesizes and releases glucocorticoids. At the central level, the response of the HPI axis begins with the synthesis and secretion of CRF from the preoptic area and some other hypothalamic areas (Flik et al., 2006; Bernier et al., 2009), which binds to corticotropic cells of the pituitary gland thus activating ACTH synthesis. Increased blood levels of ACTH and its binding to MCR2 at the interrenal cells initiates cortisol production. This is mediated by a cascade of intracellular events involving cholesterol mobilization from the internal mitochondrial membrane, through the StAR (Mommsen et al., 1999; Aluru and Vijayan, 2008). Cholesterol is transformed in pregnenolone by the rate limiting enzyme of cortisol synthesis (Mommsen et al., 1999), the cytochrome p450 that cleavages the side chain (P450scc).

The response to stress in different fish species implies imminent changes in the activity of the HPI axis, whose magnitude correlate with the severity and duration of stress (Barton and Iwama, 1991). In our study, trout exposed to a high density
Fatemeh Naderi  

(HSD, 70 Kg.m3) showed significant increased cortisol levels anytime (3, 7 and 10 days), compared to those of non-stressed animals (10 Kg / m3). Interestingly, a significant reduction (-30%) was observed in averaged plasma cortisol levels of trout subjected to HSD between days 3 and 10, which suggests the existence of an attenuation in the cortisol response to the long-lasting stress action. These results agree with several studies in teleost, and suggests that fish exposed to physical, chemical or social stressors for a long time display altered behavioral and physiological response (Barton, 1997; Colombo et al., 1990). This usually leads to a gradual decrease in circulating cortisol levels (Rotllant et al., 2000a; b; 2001) or a lower response to a subsequent acute stress stimulus (Øverli et al., 1999; Jeffrey et al., 2014; Wunderink et al., 2014). Then, a downregulation of the HPI axis associated with high levels of cortisol for a long time must exist. However, few studies have focused on the underlying mechanisms. On the other hand, it is known that repeated exposure to a stressor, discontinuous and repeated over time, or continuously maintained, originates habituation, which may lead to the attenuation of the endocrine stress response (Fernandes -de-Castilho et al., 2008; Koakoski et al., 2013; Conde-Sieira et al., 2018). Finally, it has been pointed out the existence of divergent responses to stress among individuals of the same species (Ruiz Gómez et al., 2011), which could affect the whole response observed in trout exposed to chronic stress.

Our results in rainbow trout agree with those reported by other authors in this species and other teleost subjected to similar or longer-lasting experimental conditions. Thus, high cortisol levels were found in trout kept in HSD for 3 days (Gesto et al., 2008) and 10 days (Sieira et al., 2012). Balm and Pottinger (1995) show that after 96 days of stress cortisol levels are still elevated, although with a drop in comparison with previous time points, in which a significant decrease in circulating ACTH levels were found as well. Moreover, reduced cortisol synthesis has been reported in brown trout (Salmo trutta L.) submitted to confinement for 35 days, which could be considered as an adaptation to long stress situation (Pickering and Stewart, 1984). With this in mind, some authors suggested the existence of decreased sensitivity of interrenal cells to ACTH (Vijayan and Leatherland, 1990; Rotllant et al., 2000b; Sloman et al., 2002; Medeiros and McDonald, 2012), although it was not corroborated by other studies in which cortisol was administered as for a situation of chronic stress (Rotllant et al., 2000a).

The results obtained after quantifying mRNA abundance of various enzymes of cortisol biosynthesis at the head kidney do not support a low sensitivity of interrenal cells during chronic stress. Thus, at both 7 and 10 days after HSD exposure, high star, p450scc expression persists, which indicates accelerated cortisol synthesis. Similar increases in mRNA and protein content corresponding to both enzymes were observed in rainbow trout exposed to acute stress induced by handling and subsequent anesthesia, which caused a marked increase of cortisol levels (Gerlin and Auperin, 2004). Moreover, the same authors show that situations of moderate
Experimental work 4

stress, such as netting, have little effect on *star* and *p450* mRNA abundance, suggesting that both enzymes have similar regulatory mechanisms likely associated with transcriptional and post-transcriptional processes that could depend on the intensity of cortisol production. On the other hand, data obtained from *in vitro* cultured head kidney showed that ACTH treatment induces the activation of enzymes downstream the steriodogenic pathway, such as P450-11β hydroxylase that catalyzes the passage of cholesterol to pregnenolone and the 3β-HSD that mediates its dehydrogenation to form 17-OH progesterone (Hagen *et al*., 2006; Conde-Sieira *et al*., 2013). Our data in rainbow trout chronically exposed to HSD show increased mRNA content of these two enzymes, which supports a role in the maintenance of the biosynthetic steroid pathway. It has reported that these enzymes have a greater abundance and half-life than those that initiate cortisol biosynthesis (*star* and *p450scc*), so they are good candidates to keep the increase of the biosynthetic capacity of cortisol during long-term stimulation (chronic stress) (Hagen *et al*., 2006), which agrees with our results.

Elevated cortisol levels during stress might benefit the animal, since mobilization of energy resources is facilitated thus coping with such adverse situation (Barton and Iwama, 1991; Pankhurst, 2011). However, if the stress situation persists for long time periods it turns very negative for the animal, due to metabolic reserves being depleted, the inhibitory effect on food intake, and the harmful effects of cortisol on the immune system, all together leading to a weakened organism (Barton and Iwama, 1991). In our study, metabolites (glucose, lactate) plasma levels showed little noticeable changes in HSD-exposed trout, compared to those of fish kept in NSD. In fact, no significant changes in circulating glucose levels were found at 3, 7 and 10 days, whereas lactate levels increased at 3 and 10 days but not at 7 days in HSD. Usually, metabolites increased in response to CAs (few minutes) and cortisol (hours and days after stress) actions on carbohydrate metabolism, as reported for different teleost species subjected to acute stress (Barton and Iwama, 1991; Pottinger, 2008; López-Patiño *et al*., 2014b). These metabolites are ready to use during stress, to cover the incipient energy demand of metabolically active tissues. Then, one might expect that in the long term, the greater use of the catabolic processes that provide glucose and lactate to blood would be counteracted by the permanent use of these metabolites in other tissues. The necessity of a balance between glucose formation and expenditure can explain the absence of variations in glucose levels in stressed fish. Then, fish are still able to maintain the metabolic homeostasis after 10 days of HSD. Regarding lactate, its levels usually increase in stress situations due to the greater use of oxidative glycolysis pathways (Van Ham *et al*., 2003), which explains the higher levels of this metabolite at 3 and 10 days. The fact that there were no changes in lactate between fish kept for 7 days in HSD and NSD could be indicative of a temporary reorganization in the metabolic substrates as consequence of the elevated cortisol levels, but further specific studies are required to confirm this.
When cultured, fish suffer a wide range of stress situations, thus being the subject of numerous experimental designs. However, little attention is paid on how does animal recover after a stressful situations takes place. This may be important to predict the rate of response level of the fish, and to manage its subsequent phase of culture. For instance, stress reduces food intake, which has an impact on the rate of fish growth (Bernier, 2006). Therefore, if the stress situation ceases it is important to know if those mechanisms that inhibit food intake in response to stress turn back to normal values and when, in order to restore a certain food ration. Our study shows a rapid recovery in most of the endocrine and metabolic parameters examined after stress is over, with 6 h post-stress being needed to turn back to those values of non-stressed fish. This is partially noticeable in plasma cortisol, whose levels at 6 h are intermediate between stressed and non-stressed fish, as well as those of the post-stress group sampled at 24 h, which display similar values than control non-stressed animals. Therefore, we conclude that cortisol levels in crowding stressed trout return to normal values between 6 and 24 h post-stress.

There are few studies in rainbow trout for our results to compare with. Naderi et al. (2018) have reported that enhanced cortisol levels in rainbow trout subjected to high stocking density takes up to 24 h to return to pre-stress level. On the other hand, Gesto et al (2015b) observed in trout stressed by chasing (5 sec, 15 sec, 3 min) a graduated increase of cortisol levels that relates to the duration of the stimulus, with recovery occurring at 4 h after stress exposure. These authors also show that 15-min chasing causes a sharp increase in cortisol levels that returns to baseline after 4 to 8 h (Gesto et al., 2013). With respect to social stress induced by forced contact between individuals of different hierarchies, Culbert and Gilmour (2016) showed that high values of cortisol in stressed trout (subordinate) return to normal within 48 hours after moving them away. Jeffrey et al (2014) also reported that cortisol recovery in socially subordinated trout subjected to netting stress for 1 h occurs between 1 and 4 h after stress. In other fish species, cortisol recovery period is highly variable. Lim and Hur (2018) reported that olive flounder (Paralichthys olivaceus) subjected to stress by 5-min air exposure recovers normal cortisol levels after 12 h post-stress, whereas when repeated during 70 days cortisol needs at least 20 days to turn back to normal levels after stress is over. Finally, sea bream subjected to handling and confinement needs several days to recover normal cortisol levels after stress is eliminated (Rotllant et al., 2001).

Therefore, when compared to acute stress-related studies, it is possible to define the response of cortisol associated with a continuous stress, as we do in our study, as a quick recovery once the stress ends. The aforementioned habituation of the endocrine stress axis during chronic stress might contribute to accelerate the recovery after chronic stress. In fact, during the post-stress period the expression of the enzymes of the steroidogenic pathway in the head kidney return to values of non-stressed fish after 6 h, thus showing an even faster recovery than that of
circulating cortisol levels. The discrepancy between the dynamic of the hormone levels and the mRNA content of the enzymes involved in the steroidogenic pathway could be related to the half-life of the circulating hormone, which allows to keep high plasma cortisol levels during a given time period, whereas its secretion from the interrenal tissue is reduced. On the other hand, metabolism-related data were unclear, due to the lack of reliable effects of stress on blood glucose levels, and that of lactate levels at 7 days, just prior to post-stress period initiates. It is notorious the low lactate levels during most of the post-stress period, which appears to recover towards the end (72 h post-stress), even when remains lower than that observed in trout subjected to HSD for 10 days. These data suggest that the metabolic effect of HSD on anaerobic glycolytic pathways rapidly restores in NSD retorned fish, in which the aerobic pathways of glucose metabolism would prevail. However, it looks like some changes affecting these metabolites levels are a consequence of reduced food intake during the stress period, which may also affect carbohydrate metabolism during recovery.

It is generally assumed that the central and peripheral actions of cortisol in fish are mediated by glucocorticoid receptors 1 and 2 (GR1 and GR2), which have an important role in different behavioral and physiological functions (Mommsen et al., 1999). Cortisol is also the main ligand of mineralcorticoid receptors in some teleost species, including the rainbow trout (Columbe et al., 2000). All these receptors are ligand-inducible transcription factors, and appear to play a key role in mediating the physiological response to stress (Mommsen et al., 1999), but also in the regulation of cortisol signaling in brain or pituitary (Stolte et al., 2008; Alderman et al., 2012). Our study demonstrates that gr1 mRNA abundance increases during exposure to HSD, especially in hypothalamus, telencephalon and optic tectum. In contrast, gr2 expression does not change, suggesting that the effect of stress-induced cortisol levels in forebrain could take place (at least in part) through the modulatory effect exerted over the transcription of gr1 receptors.

As far as we know the dynamics of gr mRNA abundance during stress exposure and its recovery in fish are not known. Therefore, we show that increased gr1 expression persists in trout exposed to HSD, whereas it falls to basal levels shortly after stress ceases. Similarly to that found for cortisol, restoration of gr1 mRNA content after stress was very fast, i.e., 2-6 hours in optic tectum and hypothalamus, and 24 h in telencephalon. Overall the data suggest that increased cortisol levels up-regulates gr1 expression in forebrain regions when stress is present, which would otherwise have lower expression. Also, regarding the lack of changes during stress and the post-stress period in the gr2 receptors, their involvement in the response to stress remains unknown. However, we cannot assume that changes in mRNA levels are necessarily accompanied by those of receptor density, so the role of gr2 cannot be fully discarded.
Chronic stress effects on food intake and feeding-related hypothalamic neuropeptides

In the present study, trout kept in HSD for 10 days showed a significant reduction of food intake. This agrees with the fact that inhibited food intake is one of the generic consequences of chronic stress in teleost (Leal et al., 2011; Conde-Sieira et al., 2010; Madison et al., 2015). Since the HPI axis activates under stress, it was postulated that several of its components might play a key role in feeding regulation. Hypothalamic CRF, and cortisol released from the interrenal tissue are apparently the main candidates (Bernier et al., 2001; Madison et al., 2015).

The profiles of several of the anorexigenic and orexigenic hypothalamic neuropeptides were examined in trout exposed to HSD, and then after return to NSD. As expected, increased hypothalamic expression of crf occurs in trout subjected to HSD. This peptide plays a critical role in the control of the HPI axis and the coordination of the autonomic and behavioral responses to stress (Cheng et al., 2007; Fick et al., 2006). Different environmental and social stresses result in crf mRNA increase in POA-anterior hypothalamus of teleost (Bernier et al., 2008). The abundance of crf was also reported to change after exposure to a stressor in a brain region-, stressor-, and time-dependent way (Doyon et al., 2005). CRF is a potent anorexigenic factor in goldfish (De Pedro et al., 1993; 1997). Studies carried out in trout highlight the key role of CRF in mediating the inhibitory effect of stress on food intake (Conde-Sieira et al., 2010; 2011). In addition, research assessing the interactions between CRF and other anorexigenic/orexigenic systems suggests that CRF-related peptides may modulate a variety of appetite-regulating pathways. For instance, in mammals CRF inhibits NPY-induced food intake, and partially mediates the anorexigenic effect of other feeding modulators (Volkoff et al., 2005). Studies in goldfish (De Pedro et al., 1998a,b) and trout (Pérez-Maceira et al., 2016) also demonstrate that increased CRF could mediate the inhibitory effects of 5HT on food intake. Regarding changes in CRF during chronic stress, studies are more limited. However, chronic cortisol treatments associate with a reduction of crf mRNA content in telencephalon-POA (Bernier et al., 2004; Doyon et al., 2006), which suggests the existence of an autoregulatory feedback loop of cortisol on hypothalamic centers that might participate in the regulation of appetite/satiety processes. Our data showing that crf expression remained elevated for 10 days in the hypothalamus of trout kept under HSD suggest that the inhibitory effect of stress-induced cortisol increase could not be high enough to reduce crf expression, then remaining elevated all the time the stress persists.

Chronic HSD stress also increased gene expression of pome-al and cart all over the experiment. These are anorexigenic neuropeptides, as reported for teleost (Volkoff et al., 2005). The action of cart seems to be largely dependent of its interaction with other neuropeptides such as npy and orexin (Volkoff et al., 2005),
whereas *pomc-a1* could induce a tonic inhibitory effect of food intake via its cleavage to form $\alpha$-MSH (Cerdá-Reverter *et al*., 2003). In contrast to the effects of chronic stress on these anorexigenic peptides, *npy* expression was not responsive to HSD. NPY is a powerful orexigenic peptide in fish (López-Patiño *et al*., 1999), and plays a key role in the appetite sensation (Volkoff *et al*., 2005). However, the effect of stress on *npy* expression in fish is somewhat controversial. In rainbow trout, increased hypothalamic *npy* abundance is observed in 6 h HSD-exposed fish (Conde-Sieira *et al*., 2012), similarly to that found in 72 h isolated trout (Doyon *et al*., 2006). Moreover, chronic cortisol treatment in rainbow trout increased *npy* expression in the hypothalamus at 14 and 28 days but not at 42 days (Madison *et al*., 2015). These data, together with our results, suggest that the effect of stress on *npy* expression can depend on the duration of stress, the stressor, and the magnitude of the increase of cortisol levels. Overall, our data do not support a relevant role of *npy* in the reduction of food intake displayed by rainbow trout during chronic stress.

During recovery, once the HSD-induced stress is over, a fast restoration of all the neuuropeptides expression occurs, reaching those of control fish after 6 h (*cart, crf*) and 24 h (*pomc-a1*). Interestingly, *npy* increased at 24 h post-stress, with values high enough to guarantee a significant stimulation of food intake by the time the stressor disappears. Therefore, the restoration of food intake in fish returned to NSD after 7 days in HSD might not only involve a reduction of anorexigenic peptides, but also an increase of *npy* expression, leading to compensate the reduced feeding during stress, and to reach as soon as possible the nutritional balance.

**Chronic stress effects on brain monoaminergic systems and evolution of the post-stress recovery**

When fish are exposed to stress the sensory information (visual, olfactory, physical contact, etc) accessing the brain is integrated into the CNS in order to initiate the endocrine response that leads to CAs and cortisol release to blood (Wendelaar Bonga, 1997). In a large extent, the brain must discriminate the received stimuli and decide if they are of a degree enough (greater or lesser intensity and duration) to cause a harmful effect on the organism. If so, the mechanisms that enable the physiological stress response activate. In acute stress, the response is limited in time (Gesto *et al*., 2015b) and fish must be able to verify the absence of the stressor and calm/stop the activation of the brain. Under long-lasting stress (several days or weeks) information can be integrated in several ways: the animal is still aware of the continuous influence of the stressor and the neuroendocrine mechanisms that rise cortisol are still active; On the other hand, the response must be proportional to the intensity of the stimulus in order to reduce the high metabolic and behavioral costs of the stress response. Finally, after stress is over, the central integratory mechanisms must be able to perceive this situation and deactivate the stress response, restoring the function in order to initiate a new event if needed. Previous studies reveal that
monoaminergic systems have an important role in the upstream regulation of the stress response. In fact, 5HT and DA are important regulators of the endocrine and behavioral response to stress in vertebrates, although with differential roles (Winberg and Nilsson, 1993; Gesto et al., 2008). Previous studies by Gesto et al. (2008; 2013; 2015b) showed that in acute stress serotonergic activity increases and, in lower degree, dopaminergic activity as well, from the very beginning of exposure to stress, which is indicative of a key role of these systems as primary mechanisms during response to stress. However, little information is available regarding the changes of these monoamines during prolonged stress and the relationship with the stress endocrine response. Less information is even available with respect to the recovery ability of these neurotransmitters following long-lasting stress.

In order to evaluate the monoaminergic response during HSD stress in rainbow trout and the subsequent return to NSD, our study analyzed different parameters, including i) expression of genes related to 5HT (tph1 and tph2) and CAs (th) synthesis; ii) content of amines (NA, DA, 5HT) and some of their oxidative metabolites (DOPAC, 5HIAA); iii) metabolite/amine ratios that were used as indexes of dopaminergic (DOPAC/DA) and serotonergic (5HIAA/5HT) turnover.

The TPHs and THs are the rate-limiting enzymes of 5HT and CAs synthesis. In mammals, there are two tph paralog genes; thp1, which expresses in the pineal gland and in peripheral tissues (thymus, spleen and intestine) (McKinney et al., 2005; Zhang et al., 2004) and tph2, that expresses mainly in telencephalon and mesencephalon, and is responsible of 5HT synthesis in the brain (Zhang et al., 2004). In teleost, the existence of two paralogical forms of tph is evidenced (Bellipanni et al., 2002), although their expression seem to differ from that of mammals, since both express at the same level in fish brain. However, there are still few fish species in which the sequences and characteristics of these genes is investigated. In our study, we have cloned and sequenced a fragment of both genes, tph1 and tph2, since at the time of the experiment there were no references in the databases on this species (now, more information is available). Using specific primers, we determined changes in their expression (tph1 and tph2) during stress. We found a strong increase all over the HSD period. This effect was more prominent in hypothalamus and telencephalon, where it doubled that expression of fish kept under NSD. In optic tectum, tph1 abundance in trout exposed to HSD increased continuously, but it was only evident after 3 days for tph2. In medulla, tph1 abundance increased at 3 and 10 days, whereas tph2 increased at 3 days in HSD fish.

Research on the expression of tph under stress is limited to hypoxia, and decreased expression, protein content and activity of both enzymes (TPH1 and TPH2) is reportedin hypothalamus of Micropogonias undulatus. Since oxygen is a cofactor for the TPH activity, it is likely that the decrease observed under hypoxia is very specific to the experimental condition, and not generalizable to other types of
stress. In fact, studies on stress effect in some rodent models report increased expression and enzymatic activity of TPH in dorsal raphe nuclei, where most of the neuronal soma that project to the telencephalon and diencephalon locate (Yamaguchi et al., 2016; Donner et al., 2018). In contrast to that reported in mammals, where diencephalic 5HT comes largely from extraneuronal reuptake, studies in zebrafish indicate a greater tph expression in diencephalic areas than that of the raphe (Bellipanni et al., 2002), suggesting the existence of important synthesis in these brain regions. Our results in trout reveal increased 5HT synthesis in telencephalon and hypothalamus during chronic stress, which may be indicative enhanced usage of the neurotransmitter at the synaptic level.

The expression of th in brain also increased in chronic stress, especially in hypothalamus and, in a less consistent way, telencephalon and medulla (in both cases with significant increases only at 7 days under HSD). These results agree with previous reports obtained from zebrafish subjected to social stress, in which the caudal hypothalamus (containing a high abundance of TH-positive cells) presents increased th2 expression (Pavlidis et al., 2011; Semenova et al., 2014), together with higher contents of DA and its metabolites (Teles et al., 2013). Also, in rodents subjected to different types of stress, increased th mRNA content and TH enzyme activity are found in several areas containing dopaminergic endings, particularly the hypothalamus (Kiss et al., 2008; Wong and Tank, 2007).

Changes in mRNA abundance of the enzymes of synthesis of monoamines during chronic stress were consistent with those of the amines and metabolites content. Using metabolite/amine ratios allows us to summarize changes and to apply them as indexes of neurotransmitter turnover in relation to their use in neuronal activity. Significant increases were found in HSD situation, for both 5HIAA/5HT and DOPAC/DA ratios, particularly within hypothalamus and telencephalon. This is in agreement with previous studies in trout subjected to acute (Gesto et al., 2008; 2013; 2015b; Moltensen et al., 2016) and chronic stress (Gesto et al., 2008; Conde Sieira et al., 2014), as well as that for the 5HIAA/5HT ratio, which increased in trout exposed to several social stress situations (Winberg et al., 1991; 1997). Increased monoaminergic activity was also reported in other teleost species, Arctic charr (Salvelinus alpinus) (Höglund et al., 2001), gilthead seabream (Skrzynska et al., 2018) and sole (Conde-Sieira et al., 2018), among others. Our study shows that the increase of the serotonergic ratio was more robust after 3 days of keeping trout in HSD, with a slight decrease at 7 days, although being significantly higher than that of controls. No significant variations were found for monoaminergic activity in the optic tectum, which emphasizes the low sensitivity of this region to stress. The metabolite/amine ratios also increased in medulla, although changes were less consistent, with increases at 7 days, but not at 3 days (DOPAC/DA), and at 3 days, but not at 7 days (5HIAA/5HT). Interestingly, in the three regions affected by stress (hypothalamus, telencephalon, medulla), relevant increases were noticed at 10 days,
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thus highlighting the persistence of enhanced monoaminergic activity when stress persists.

After 7 days in HSD some trout returned to NSD. This resulted in a rapid recovery of the monoaminergic activity to values of non-stressed fish. For 5HT, the expression tph1 and tph2 in hypothalamus and telencephalon (increased during stress exposure) returned to normal values at 6 hours post-stress, except that of tph2 in hypothalamus that showed slower recovery (normal values at 24 hours post-stress). Also, in optic tectum, tph1 expression fell to values of control group at 6 hours post-stress, and even to lower values in the case of tph2. In medulla, expression of tph1 and tph2 at 2 hours post-stress was comparable to that of the control group, thus showing a fast recovery. Furthermore, restoration of the enzyme gene expression after chronic HSD exposure is over coincided with that noticed for amines and metabolites levels. Thus, the analysis of the 5HIAA/5HT ratio in hypothalamus and telencephalon allowed to establish a similar temporal range (between 2 and 6 hours) for post-stress recovery, being the monoamine turnover in these regions the most affected by stress. In optic tectum and medulla, regions in which no significant changes in serotonergic activity were observed after 7 days of stress, there were no noticeable effects after exposing fish to NSD.

Regarding the dopaminergic system, post-stress changes were quite similar to those of the serotonergic one. Then, increased th expression during stress was still apparent at 2 h post-stress in hypothalamus and telencephalon, but dissipated at 6 h, thus with values in NSD-returned fish being comparable to those of control unstressed group. Medulla did need more time to restore normal values, and it was noticed at 24 h post-stress. In relation to the DOPAC/DA ratio, unlike the serotonergic system, values returned rapidly to those of non-stressed trout in hypothalamus and telencephalon, for which only 2 h were necessary. However, in medulla the increase of DOPAC/DA ratio by exposure to HSD during 7 days persisted after returning fish to NSD even after 72 hours. Finally, regarding NA levels, inconsistent changes were noticed all over the experiment, so exposure to HSD produced specific increases or decreases that did not reflect a clear evolution of the applied paradigm. However, during stress recovery, a tendency to reduce NA levels was noticed in different brain regions, compared to that observed in animals that remained under HSD. In literature, only few references describe changes in brain noradrenergic activity in fish subjected to stress. Most of them refer to the effect of aquatic contaminants and offer inconclusive results (Gesto et al., 2006; 2008; Weber et al., 2015).

In summary, our data reveal the activation of dopaminergic and serotonergic systems in trout kept in chronic (HSD) condition. Such effect persist throughout the exposure to stress. The more noticeable changes relate to the expression of the regulatory enzymes of the monoamines synthesis, thus indicating that mechanisms
that replenish the levels of the neurotransmitters in the neuronal endings during chronic stress remain activated. In addition, these changes were coincident with a higher use of the neurotransmitters, as evidenced by the increase of the metabolite/amine ratios. Since stress-induced increase of extracellular monoamines precedes those of synthesis (Chauloff et al. 1999), it is likely that the latter increases only serve to counterbalance neuronal depletion of the monoamine due to the release. Taking that in mind, our results support an overall increase of monoaminergic neuronal activity during chronic stress, with telencephalon and hypothalamus being the more affected regions. Our results also allow us to conclude that the deactivation of this neurochemical response to stress occurs quickly, thus supporting a role for brain monoamines as primary response elements when the stressor is present, but also when it is no longer present.

**Brain monoamines as a link for chronic stress effects on endocrine response and feeding behavior. Focusing on serotonin.**

Our study reveals a strong correlation among dynamic of changes that occur in trout exposed to HSD, affecting brain monoamines, HPI axis, and feeding regulation. Enhanced serotonergic and dopaminergic activities paralleled the increase of cortisol synthesis (interrenal tissue), and altogether occurs simultaneously with reduced food intake. Normal feeding restored shortly after returning fish to NSD conditions, in parallel with the deactivation of monoaminergic function and HPI axis. Particular attention requires 5HT since the neurotransmitter could play a pivotal role in a complex neuroendocrine loop in order to maintain homeostasis and promote acclimation during physiological or environmental challenges (Chaouloff, 1993; Winberg et al., 1997). 5HT plays a key role in regulating stress response in fish, and its effects on the HPI axis in teleost are usually inhibitory (Winberg et al., 1997; Medeiros, 2010), as the influence on food intake is (De Pedro et al., 1998a,b; Winberg and Thörnqvist, 2016; Pérez-Maceira et al., 2016). However, little information is available regarding the level at which 5HT is acting and the signaling mechanisms involved. Specific class 1 and 2 receptors seem to be important for the behavioral effects of 5HT, and are likely involved in changes occurring during stress. In mammals 5HT<sub>1A</sub> receptors are emphasized since stressors specifically affect them. Thus, treatment with 5HT<sub>1A</sub> receptor agonists induced the activation of the adrenal stress-response in mammals (Chaouloff et al., 1999), as proposed for some fish species, such as rainbow trout (Winberg et al., 1997) and Gulf toadfish (*Opsanus beta*) (Medeiros et al., 2014). These auto-receptors express in 5HT neurons, where they regulate 5HT-mediated cell firing and 5HT release, and in non-5HT neurons all over the entire brain (Roth, 2006). Activation of 5HT<sub>1A</sub> receptors by the specific agonist 8-OH-DPAT in teleost resulted in a significant increase of CRF precursor mRNA abundance and ACTH release in the pituitary (Medeiros et al., 2014). With that in mind, our results suggest that stress-induced increase in synaptic 5HT might
result in the stimulation of cortisol secretion as consequence of the enhanced activity of the HPI axis, then stimulating 5HT and CRF expression and release. In hypothalamic-POA, also growing evidence point to 5HT to act directly on pituitary cells to induce ACTH release. In mammals this effect was assigned to the activation of 5HT$_{1A}$ and 5HT$_2$ receptors (Dinan et al., 1996; Winberg et al., 1997). Additionally, 5HT$_{1A}$ receptors are reported as target for cortisol during stress in fish, in such a way that chronic elevation of plasma cortisol mediates the decrease of brain 5HT$_{1A}$ receptor mRNA and protein levels, via the glucocorticoid receptor (Medeiros et al., 2012; 2014). We observe elevated mRNA content of grl receptor in trout brain after chronic stress, thus suggesting that sustained increase of serotonergic activity also involves central effects of cortisol. A population of GR-immunoreactive cells was reported in the anterior hypothalamic region of trout, supporting that the autororregulatory cortisol feed-back loop might operate at this location, then down-regulating the long-term CRF and ACTH activation (Rotllant et al., 2000). We postulate that 5HT$_{1A}$ receptors could be part of this action, since the inhibitory effect of sustained elevated cortisol would result in increased 5HT neuronal activity, with the subsequent negative influence on the HPI axis. In addition, our data reveal the absence of habituation of stress-induced 5HT, probably due to cortisol promoting serotonergic function all over the time the stressor is acting.

The increase of crf levels in trout exposed to long-term HSD could be responsible of the inhibitory effect observed on food intake. 5HT can participate on this effect since the amine inhibits food intake in teleost (De Pedro et al., 1998a,b; Ruibal et al., 2002), with this effect being blocked by CRF antagonists (De Pedro et al., 1998a,b). In addition, hypothalamic administration of 5HT$_{2C}$ receptor agonists results in increased expression of several anorectic peptides, including pomc, cart and crf (Pérez-Maceira et al., 2014), in striking consistency with that herein reported for trout subjected to HSD-induced stress. The activation of 5HT$_{1A}$ receptors by agonists also inhibit food intake in trout, although this effect seems to be independent on those changes of anorectic peptides (Mancebo et al., 2013; Pérez-Maceira et al., 2016). Accordingly, we observed that chronic stress (HSD) did not affect hypothalamic npy mRNA content, which appears not to participate in the inhibitory effect of stress on food intake. Interestingly, npy abundance increases in fish returned to NSD (after stress), suggesting that the peptide might play a role in restoring the feeding behavior. Whether changes in 5HT during recovery of stress mediate the increase of npy when stressor disappears or not has to be evaluated.

In summary, our data on serotonergic function following exposure of trout to chronic stress evidence a critical role of the neurotransmitter in integrating stress-related information. Increased 5HT could be, at least in part, responsible of the persistence of the endocrine response to long-term stress, by acting through different receptor subtypes and brain regions, but also of the effect on feeding behavior. CAs (DA, NA) are also interesting, since the increased content and neuronal activities
seem to be part of the stress response in fish (Gesto et al., 2013), as we report for trout. In particular, evidence in teleost points to DA to play an inhibitory role on food intake, probably mediated by D₁ and D₂ receptors (De Pedro et al., 1998a). However, the interaction DA-brain neuropeptides seems to be fish species-dependent. In Chinese perch DA decreased cart expression (He et al., 2018), but did not affect that of pome and npy, whereas in sea bass the inhibitory effect of DA on food intake is not apparently mediated by changes in anorectic neuropeptides (Leal et al., 2013). However, the presence of DA in fish hypothalamus up-regulates crf expression, suggesting that the activation of the dopaminergic system during stress occurs by stimulation of CRF neurons (Leal et al., 2013). Whether this effect of DA could have a modulatory role on the HPI axis in response to stress in fish is unknown. By other hand, increased forebrain CAs under stress was associated to the modulation of other functions, such as aggression, social status and learning (Summers and Winberg, 2006), but also to inhibition of reproduction (Chabbi and Ganesh, 2016), which are all critical for fish adaptation and survival in a stressful environment.
4. General discussion
4.1. Stress effects on the hypothalamic clock system. The role of cortisol and SIRT1

A number of behavioral and physiological functions have been reported to be controlled by clock systems in animals. The functioning of the oscillatory systems extends from individual cells to the whole body, and is affected by cyclic synchronizers that mediate external or internal inputs to adjust the oscillators. The light-dark and feeding-fasting cycles are recognized as major entraining cues for vertebrate circadian clocks (Sánchez-Vázquez et al., 2001; Hastings et al., 2007), with relative importance among them depending on species and tissues in which clocks are located. The molecular core mechanism of the circadian oscillators works in a circadian cyclic (24 h basis) phase that is driving by positive or negative feedback loops among clock genes and their post-transcriptional products (Reppert and Weaver, 2002; Hastings et al., 2007; Albrecht, 2012; Schibler et al., 2015). In fish, daily profiles of clock genes have been investigated in a number of species and in different tissues, such as retina, pineal gland, brain, pituitary gland, liver, gut, gonads and head kidney (see Isorna et al., 2017, for a review). In most of these tissues, the transcripts of the positive elements of the core clock (bmal1 and clock1) peak during the photophase, whereas the transcripts of the negative elements (per and cry) increase at the end of the scotophase (Cahill, 2002, Vatine et al., 2011). According to our results in rainbow trout, a significant rhythm of clock1a, bmal1, and per1 exists at the level of the hypothalamus, in consistency with previously reported (López-Patiño et al., 2011). In addition, we show that rev-erβ-like displays a significant daily rhythm with the acrophase occurring in antiphase with bmal1, which agrees with the postulated role of rev-erβ-like as repressor of bmal1 expression in mammals (Cho et al., 2012) and trout liver (Hernández-Pérez et al., 2017).

In addition, to synchronize their activities to day/night and feeding/fasting cycles, fish are continuously facing short- and long-term changes in the environment, some of which could act as stressors to jeopardize welfare or integrity of the animal (Chrousos, 2009). To cope with stress and enhance the surviving chance, teleost activate two main neuroendocrine axis arising from the hypothalamic neurons: the HSC and the HPI, whose ending-products are the hormones CAs and cortisol, respectively (Wendelaar Bonga, 1997; Chrousos, 2009). The stress response tends to restore the internal homeostasis by regulating many biological activities, including those of the CNS, cardiovascular system, intermediary metabolism, immunity or reproduction (Barton and Iwama, 1991; Barton, 2002; Chrousos, 2007; 2009). In this context the clock and stress systems are both fundamental for survival, and thus communicate with each other at multiple levels to adjust numerous physiological activities. Interestingly, dysregulation one of these systems can lead to similar pathologic conditions. Therefore, the circadian system has been proposed to be jeopardized by stress (see rev. Koch et al., 2017).
In fish, cortisol is the main glucocorticoid and displays a major role in mediating the effects of stress response at metabolic and behavioural levels (Barton, 2002; Bernier, 2006; Aluru and Vijayan, 2009). It appears that cortisol participate as output of the circadian system, since circulating levels synchronize to light-cycle in some teleost species (Pickering and Pottinger, 1983; López-Olmeda et al., 2013; Hernández-Perez et al., 2015), whereas in others the feeding-fasting cycle and feeding time also seem to have a role in adjusting circulating cortisol rhythms (see rev. Isorna et al., 2017). In our study, a significant cortisol rhythm was noticed in control group of trout with peaking values being found at the early morning (ZT4), and basal levels at the day-night transition (ZT12). The exposure to HSD stress for 72 h significantly enhanced the averaged cortisol levels, but also altered the daily profile of the glucocorticoid, relative to control group, with peak values of stress group in the dark period (ZT16) even when a diurnal increase at ZT4 was conserved. Moreover, the IP administration of RU486 to the control unstressed group increased mean plasma cortisol concentrations, in consistency to that reported for rainbow trout (McDonald and Wood, 2004), which was even more evident in stressed group. This effect has been associated to negative feedback control of cortisol released from the interrenal cells acting at the head kidney (McDonald and Wood, 2004), as also shown in mammals (Bertagna et al., 1984; Gaillard et al., 1985; Healy et al., 1983), but in contrast to that described for trout receiving RU486 for 7 days (Vijayan et al., 1994a,b). The nature of this discrepancy might be due to the fact that fish implanted for 7 days could be missing the temporary effect of the antagonist, which persisted after 72 h (our present study). Therefore, it might indicate that 72 h time periods or slightly longer might be needed in order to avoid such negative feedback effects exerted by the glucocorticoid on its synthesis at the interrenal cells. In addition to changes in cortisol, stress also altered rhythmic profiles of metabolism-related parameters, like those of plasma glucose and lactate by increasing the daily averaged values of the first and decreasing those of the second one. Taking together, the data suggest that a mild stress condition like HSD is able to modulate the rhythms of hormonal and metabolic parameters related to the stress response in rainbow trout, thus affecting animal welfare and adaptability to the environment.

In order to evaluate how stress affects the circadian oscillator in rainbow trout hypothalamus, the rhythms of mRNA abundance of core circadian clock genes in different experimental groups were assessed. Based on our result no changes occurs in the acrophase of circadian genes assayed, but stress exposure induced a significant decrease of the amplitude and averaged values of clock1a, bmal1, and per1, and enhanced expression of rev-erβ-like. It is likely that the inhibitory effect of stress on core-clock genes involves an interaction of stress-related hormones and the hypothalamic circadian oscillator. In fact, inhibitory effects of stress on circadian system has been proposed in mammals (Takahashi et al., 2013) with glucocorticoids pointed out as inputs to the circadian oscillators, especially those located in peripheral
organs such as liver, kidney, and heart (Balsalobre et al., 2000), but also at hypothalamic level (Challet, 2015; Coomans et al., 2015).

The herein reported data suggest that cortisol acts as input to the trout circadian system. This is in agreement with the reported stimulation induced by cortisol on the expression of per1a and per1b in liver of goldfish, whereas it inhibits that of clock and bmal1 (Sánchez-Bretaño et al., 2016), and also with preliminary data of our laboratory obtained from liver of rainbow trout subjected to mild stress (unpublished). Our results following IP administration of the glucocorticoid receptor antagonist RU486 also agree with this hormone acting to modulate hypothalamic circadian system. Thus, trout subjected or not to mild stress and treated with RU486, showed an inhibitory effect on clock1a, bmal1, and per1 mRNA abundance in hypothalamus as compared to stress groups. In contrast, stress-induced enhanced expression of rev-erbβ-like was not prevented by treatment with RU486, which may indicate that beside cortisol, there are other mechanisms involved in the stress effects on the hypothalamic circadian oscillator in rainbow trout.

Generally, glucocorticoids act via MR and GR with broad expression patterns throughout the body. Therefore, GR signalling can mediate phase resetting of central and peripheral clocks, pointing at a special role of glucocorticoids rhythms in the coordination of the organism's circadian network (Balsalobre et al., 2000; Hernández-Pérez, 2016). Stress effects on daily rhythm of gr1 mRNA abundance were rather pronounced with increased expression and the disruption of the daily rhythm. In contrast, no changes were observed in gr2 mRNA abundance of trout exposed to stress neither in mean expression values nor in the rhythmic profile. According to our result significantly elevated cortisol levels might be needed to enhance gr1 expression. Only in that situation RU486 seems to be able to prevent such effect, with the antagonist not significantly affecting the daily profile of glucocorticoid receptors at those lower cortisol levels typically observed in non-stressed animals. However, we cannot underestimate that changes in mRNA levels are not necessarily accompanied by those of receptor density. Thus, even when it seems that gr1 may mediate the response to stress in rainbow trout hypothalamus, the role of gr2 cannot be discarded. Further research will be performed to evaluate the dynamics of both receptors (GR1 and GR2) during exposure to stress.

The inhibition of food intake is considered as a canonical effect of stress in fish. Specifically, increased CRF levels are a major player in such effect (De Pedro et al., 1993; Bernier, 2006), although changes in other brain neuropeptides are also involved in the negative effects of stress on feeding (Conde-Siera et al., 2010a,b; Kulczykowska and Sánchez-Vázquez, 2010; Delgado et al., 2017; Ronnestad et al., 2017). In our study, clear rhythms of mRNA abundance of food intake regulators were noticed, with those of crf, pomc-a1 and npy displaying increased expression during the early night period, whereas cart showed a peak during the night to day transition. We also showed that stress has a severe impact on the amplitude of these rhythms, so
it enhanced daily averaged mRNA abundance of crf and npy, reduced pomc-a1, and did not affect cart, all that without changes in the phase of the rhythms. The results agree with that previously reported by our lab in hypothalamus of rainbow trout subjected to HSD (Conde-Sierra et al., 2010a,b) and with others (see Delgado et al., 2017, for a review) pointing to a critical role of CRF in stress-induced food intake inhibition. In addition, cortisol has been pointed as a relevant suppressor of food intake in fish when they are submitted to chronic stress (Madison et al., 2015). Taking that in mind, our results did not discard an involvement of cortisol itself in the inhibition of food intake induced by HSD in rainbow trout exposed to HSD. Apparently, GR1 might act as mediator since trout administrated with RU486 and exposed to HSD did not show any change in hypothalamic crf mRNA content, relative to control non-stressed fish. In addition, the effects of stress on several food intake regulators (crf, pomc-a1, npy) did not occur in fish treated with the cortisol antagonist RU486, supporting a role of cortisol on central modulatory effects of stress on these peptides, and also that GR1 has a keyrole in mediating the effects of cortisol.

Another candidate to mediate of the effect of stress on the hypothalamic circadian system in fish is the SIRT family. These are a group of well-characterized (NAD\(^+\))-dependent class III histone deacetylases that are involved in a wide range of regulatory effects in the intracellular metabolism. Specially, SIRT1 and SIRT6 have been postulated to link cellular metabolism and circadian clock physiology in mammals (Masri et al., 2014; Orozco-Solis et al., 2015). In addition, the rhythm of SIRT1 activity is to be controlled by the CLOCK/BMAL1 heterodimer acting over nicotinamide phosphoribosyl transferase (NAMPT), responsible of NAD\(^+\) biosynthesis (Ramsey et al., 2009). SIRT1 also participates in the cyclic control of BMAL1 and PER2 in liver through their deacetylation (Nakahata et al., 2008), but also by activating the pacemaker within the hypothalamic SCN in mice (Chang and Guarente, 2013). According to that interaction, changes in either clock genes expression or in that of SIRT1 may be responsible for the alteration of the respective rhythmic profiles of gene transcripts abundance. Our results demonstrated a significant enhancement of sirt1 expression in stressed trout with the consequent increase of amplitude and averaged mRNA levels. On the contrary, no variation in the phase of the sirt1 rhythm was noted. The treatment with RU486 did partially prevent this effect, which is in support of the role played by SIRT1 in mediating the effect of stress on the circadian oscillator within trout hypothalamus. The fact that RU486 does not totally prevent the effect of stress on sirt1 mRNA abundance may be also indicative of the existence of multiple interactions between mediators, such as SIRT1, cortisol, and possibly several others (Koch et al., 2017, for a review). For instance, alterations in the nutrient sensing system were reported in rainbow trout kept under chronic stress, displaying a readjustment of the hypothalamic glucosensing mechanisms This leads the fish to be unable to compensate with changes in food intake those of glucose levels in plasma, which in control trout are compensated (Conde-Siera et al., 2010a,b; Otero-Rodiño et al., 2015; Velasco et al., 2016a,b).
Accordingly, the response of hypothalamic neuropeptides controlling food intake (crf, pomc-a1, npy) was altered in stressed trout. Our present data agree with such a possibility showing that stress did not affect the daily phase of brain neuropeptides, but modified the averaged levels in a way that agrees with a reduction of food intake. Therefore, the possibility exists that stress-induced changes in daily rhythm of sirt1 also reflects, in some way, an altered cellular metabolic environment as consequence of changes in the hypothalamic nutrient systems. In such a situation, the entrainment of circadian clocks by nutrient status in trout hypothalamus would be expected (see rev. Delgado et al., 2017), and SIRT1 could participate as a relevant mediator in the effects of stress, on the circadian system.

Based on this hypothesis, we assessed in a subsequent experiment the day-night variations of mRNA abundance of core circadian clock genes in hypothalamus of control and stressed trout fed with pellets containing SIRT1 inhibitor EX527. Our results in trout sacrificed at two time points (day, ZT10; night, ZT18), which were chosen according to specific times in which clock genes peaked in the previous study, revealed significant day-night variations for clock1a, bmal1, and per1 in hypothalamus of non-stressed animals, thus supporting the respective rhythms reported earlier (López-Patiño et al., 2011). On the other hand, trout that were exposed to HSD exhibited decreased amplitude and averaged mRNA abundance of clock genes clock1a and bmal1 (ZT10) and per1 (ZT18), also in agreement with previous results. Additionally, the effect of stress was totally (daytime) or partially (nighttime) prevented by EX527 administration, since day-night variation of hypothalamic clock genes expression observed in those fish receiving EX527 and afterwards subjected to stress was generally comparable to that of control fish.

In our experimental design, EX527 treatment was not completely effective at night-time, which is suggesting that interaction between SIRT1 and other mediators related to stress exposure could be present. Changes in cortisol levels that account during stress are a candidate to modulate EX527 response at the level of circadian clock gene expression, as it influences endocrine and metabolic status, likely acting on the regulation of clock gene and sirt1 expressions. In order to corroborate the possible interaction between SIRT1 and cortisol we assessed levels of this hormone in plasma whose increases in stressed fish were fully reversed when this condition was combined with EX527. In addition, we assessed mRNA abundance for main enzymes of cortisol biosynthetic pathway (star, 3β-hsd, p450scc, and 11β-h) at the head kidney and we report, for the first time, an interaction of SIRT1 with day-night variations of these enzymes in fish. In stressed trout, all of the genes related to cortisol biosynthesis pathway displayed a significant increase of mRNA abundance, specially during day-time. EX527 protective effect resulted more obvious at night, thus totally preventing the effects of stress on star and 11β-h, although a partial protective effect for both 3β-hsd and p450scc was noticed during both day- and night-time points assessed. Then, as expected the metabolic activity in steroidogenic cells of the head
kidney is increased after stress exposure with the associated increase of expression in enzymes involved in cortisol synthesis, leading to enhanced cortisol synthesis and release to blood. The administration of EX527 inhibits the stress effect also at this tissue, which might be indicative that drug effects extended all over the body tissues. Taking together, the data suggest that SIRT1 might play a key role at the very beginning of the response to stress culminating with the increase in cortisol synthesis at the head kidney. It is likely that, when the enzyme activates, a battery of genes associated to stress response is expressed, thus allowing downstream events that participate in such response to initiate. These could include the increased expression of GR at both the hypothalamus and kidney, although only evident for gr1, with also a presumptive role of cortisol in such a receptor regulation.

Interestingly, we observed that trout treated with EX527 and subsequently subjected to stress display decreased food intake as untreated stressed trout did, which lead us to discard a role of SIRT1 in mediating feeding behaviour. However, EX527 was effective to reverse the increased effects of stress on mRNA levels of hypothalamic peptides involved in food intake control, including those of crf. Taking together the data suggest that cortisol, through binding to specific GR participates in maintaining the inhibitory effect of stress on food intake in rainbow trout, as long as its influence is blocked by treatment with the GR antagonist (Naderi et al., 2018). Then we speculate with a main role of cortisol in the effects that SIRT1 produce on food intake, whereas both SIRT1 and cortisol collaborate to influence food intake regulation at central neuropeptides level.

In summary, most of the assessed parameters did not display any variation in animals receiving EX527 and subjected to stress, supporting a role of SIRT1 in stress-activated processes. After HSD the expression of clock genes in inhibited in the hypothalamus, whereas those of food-related peptides is increased. SIRT1 seems to be a modulator of such an effect on hypothalamic circadian system since treatment with EX527 is effective to prevent those changes. On the contrary, the SIRT1 inhibitor partially prevent changes in food intake-related peptides during stress but fails to reverse the reduction of food intake. It seems, therefore, that SIRT1 interacts with other mediators to promote inhibitory effects of stress on feeding. Cortisol is a serious candidate for such an interaction since decreased cortisol synthesis at the head kidney occurs in stressed fish receiving EX527, leading to plasma cortisol levels to restore at those levels of the non-stressed fish. This study also points to gr1 receptors to be involved in stress effects on each the expression of circadian clock genes, the HPI axis regulation and the food intake control. Whether SIRT1 can interact with GR to mediate the physiological response to stress should be taken into account for future in-depth research.
4.2. Brain monoaminergic systems as rhythmic outputs of the circadian system in rainbow trout

In mammals, circadian system components receive a variety of inputs through monoamines, such as CAs and 5HT, some of them participating in the response of the circadian system to entrainment by external or internal cues (Morin and Allen, 2006; Glass et al., 2003; Gravotta et al., 2011). Therefore, alterations of dopaminergic and serotonergic neurotransmission can jeopardize physiological and behavioural responses modulated by the circadian system (Ueno et al., 2012; Gravotta et al., 2011). Additionally, the catecholaminergic and serotonergic neuron activity display daily fluctuations under the endogenous control of circadian oscillators (Benedetti et al., 2003; Hamp et al., 2008), which is indicative of the important role that monoamines can play as output signals from the circadian system in mammals.

Regarding serotonergic neurons, TPH is considered as the rate-limiting enzyme of 5HT biosynthesis (Grahame-Smith, 1964, Lovenberg et al., 1967; Jequier et al., 1969). In rodents, this enzyme was reported to display rhythmic variations at both cell soma (within the raphe nuclei) and cell terminals, with the latest projecting to cells that host the circadian system or connect with it, such as the SCN and the IGL (Barassin et al., 2002; Malek et al., 2004). This leads to a rhythm of 5HT synthesis, which correlates with that of the monoamine release in brain regions containing serotonergic nerve terminals (Dudley et al., 1998; Barassin et al., 2002; Grossman et al., 2004). Even when in early studies carried out in mammals 5HTergic neurons were reported to display daily rhythms of activity, little information is available regarding the elements participating in those rhythms. Soon after, a second isoform of TPH was isolated and described as TPH2 (Walther et al., 2003), with the first described isoform named as TPH1. Tph2 expression was found to be predominant in the mammalian CNS with mRNA abundance being higher at the raphe nuclei (Patel et al., 2004; Clark et al., 2006). However, both tph isoforms co-express in other brain regions such us the pineal gland (Patel et al., 2004; Sugden et al., 2003). Since studies on tph expression and its regulation are lacking in fish, we cloned and sequenced a fragment of both rainbow trout tph1 and tph2 genes. After that, we can report that both isoforms display consistent expression in different brain regions of rainbow trout (hypothalamus, telencephalon, optic tectum and medulla).

In addition, significant daily rhythms of both tph genes exist in all the brain regions assessed, with peaking levels occurring in the same temporal window, from late in the day to first half of the night. These results agree with that reported for mammals (Liang et al., 2004; Malek et al., 2007). However, we report for the first time in fish the existence of daily rhythms of mRNA abundance of both tph isoforms that persist even in the absence of environmental cues such as light and food, with some small variation. The existence of circadian variations of TPH activity in the rat IGL pathway (Malek et al., 2004), corroborates the existence of daily variations of
5HT inputs to the SCN circadian clock (Pasquier and Villar, 1982; Villar et al., 1988). This leads us to speculate with 5HT synthesis to be under circadian control in trout 5HTergic neurons, thus subordinating to a circadian oscillator, at least in the brain regions assessed, as reported for mammals (Malek et al., 2005) and mammalian isolated brain regions (Abe et al., 2002).

The rhythm of both tph isoforms displays peaking values at the day-night transition in hypothalamus, telencephalon, and optic tectum, with some delay in the latest. By other hand, medulla displayed different peaking values for both isoforms, with that of tph2 occurring at midday. This is indicative of tph2 expressing rhythmically in those regions that receive serotonergic terminals (hypothalamus, telencephalon and optic tectum) in a similar way than that of medulla, where most of serotonergic cell bodies locate (Lillesaar, 2011). This is not applicable to tph1, since a 180º phase shift occur between cell bodies- and neuronal terminals. Further research is needed in order to understand whether 5HT synthesis is regulated in a brain-region dependent way, and how projections from mesencephalic nuclei are related to monoamine synthesis and release in forebrain regions.

After comparing the rhythms of both enzymes following constant conditions light isolation and/or food restriction we observe that light isolation has little effect on such profiles, with peaking values remaining comparable to those of controls, even when specific changes on averaged mRNA levels occurred on each brain region (see Experimental work 3). For example, a phase delay is observed for tph1 in hypothalamus, telencephalon and optic tectum of trout kept under DD, and a phase advance was noted in medulla. Increase of averaged expression in hypothalamus and telencephalon was also observed. With respect to tph2, such phase delay occurs only in hypothalamus and optic tectum. Also, decreased averaged amplitude of tph2 rhythm is found only in telencephalon of light-isolated trout. When DD and fasting were applied together, an increase in amplitude was noted for hypothalamic tph1 and telencephalic tph2, relative to DD group. Decreased amplitude relative to that of DD group was observed for tph1 in telencephalon and tph2 in medulla of DD+Fasted fish.

Regarding TH, the rate-limiting enzyme of NE and DA biosynthesis (Levitt et al., 1965), daily rhythms have been reported in different brain regions of mammals, such as the pineal gland (McGeer and McGeer, 1966), and the brain stem (Otten et al., 1975; Cahill et al., 1981). In fish, rhythms of TH activity were reported in retina of a Midas cichlid (Cichlasoma citrinellum) with the peaks locating at night, which are under light- and circadian modulation (McCormack et al., 1993). This the contrary of that found in mammals, with TH activity being observed at daytime in medulla of rat (Otten et al., 1975). We have not assessed TH enzyme activity in trout, but mRNA abundance, which displays significant rhythms in all the brain regions, shows peaking values mainly occurring during daytime. Such rhythms persist in animals subjected to DD, which is indicative of th expression being endogenous and to be subordinated to the control of a circadian oscillator, as above described for tphs. However, light and
food strongly influence such rhythms, since the parameters defining each them changed in a tissue- and brain region-specific way, when animals were subjected to DD and/or fasting, as reported in Experimental work 3.

To the best of our knowledge we report for the first time in fish that a circadian oscillator drives the rhythms of mRNA abundance of enzymes strictly related monoamine synthesis, and probably the activity of monoaminergic neurons all over trout brain. In spite of the rhythms of mRNA abundance reported for *tphs* and *th*, monoamines contend display unclear daily variations, in contrast to that reported for 5HT, NE and DA in mammalian brain (Dixit and Buckley, 1967; Manshardt and Wurtman, 1968; Friedman and Walker, 1968; Scheving *et al.*, 1968; Reis and Wurtman, 1968; Collu *et al.*, 1973; Simon and George, 1975; Friedman and Piepho, 1979; Owasoyo *et al.*, 1979). Whether these fluctuations are due to changing rates of monoamine synthesis or/and to changes in the rates of their release is still not clarified.

Rhythms of NA secretion in mammalian neurons was described early (Akersted and Levi, 1978), with peaking levels occurring at noon and basal levels during sleep-time. These rhythms disappear in animals subjected to sleep deprivation or constant lighting conditions, and when environmental synchronizers are experimentally phase-shifted NA secretion rapidly re-adjusts (Akersted and Levi, 1978). This is indicative of environmental cues to play a key role in generating NA rhythm, independently on the modulatory action exerted by a circadian oscillator. Our results support this hypothesis (at least in hypothalamus) since even when no significant rhythms are observed, NA content displays daily variations that disappear in trout subjected to DD (DD and DD+fasting groups). In medulla the fluctuation disappeared in DD trout, but persisted in animals subjected to DD+Fasting. Then, both light and food can interact in order to modulate daily variations of NA content in some brain regions of trout, independently of the modulatory action exerted by the circadian system over *th* expression, as the main enzyme of NA biosynthesis.

DA and its main metabolite, DOPAC clearly display daily rhythms in brain of mammals (Smith *et al.*, 1992; Paulson *et al.*, 1996; O'Neill *et al.*, 1985), and DD disrupts such rhythms in rat brain (Khaldy *et al.*, 2002). In trout, DA content displays day-night fluctuations only in telencephalon and medulla. Such variation fits with the rhythm of *th* mRNA abundance, indicating the modulatory action exerted by activation/deactivation mechanisms over its synthesis. Such day-night variation does not exist in DOPAC content and the ratio DOPAC/DA. Thus, changes in DA synthesis are not immediately reflected in neurotransmitter turnover or, alternatively, catabolism to DOPAC does not follow a circadian profile, then adjusting changes in DOPAC/DA ratio. Also, since DA daily profile disappears in trout subjected to DD, suggesting that the light-dark cycle is a key factor that modulates DA metabolism in rainbow trout, thus covering the influence that circadian synthesis might have on the amine content.
Daily rhythms in mammalian brain 5HT content were more deeply studied than those of CA*s, in part due to the leading role that 5HT has as a neurotransmitter that modulates circadian rhythms (Prosser et al., 1993; Morin and Allen, 2006). Extracellular 5HT levels in rat striatum display day-night variations, in addition to those of its main oxidative metabolite, the 5HIAA (Rueter et al., 1997; Paulson et al., 1996; Rueter and Jacobs, 1996). Peaking 5HT levels are detected during the light to dark transition, at least in rat SCN (Barassin et al., 2002), and in melatonin synthesising tissues, such as the pineal gland of rat (Dudley et al., 1998) and several hamster species (Sun et al., 2002; Míguez et al., 1995; 1996). In fish, 5HT rhythms are only reported for rainbow trout pineal organ (Ceinos et al., 2005), which are in antiphase with those of melatonin. This result is expected as long as 5HT is actively metabolized at night to synthetize melatonin, thus reducing 5HT content of this tissue at night.

Such relationship between 5HT and melatonin in the pineal organ is not necessary to occur in other tissues. Accordingly, our results show discrete daily changes in 5HT content in hypothalamus, optic tectum and medulla. Thus, significant time-dependent changes in 5HT content but also in 5HIAA levels were observed within these regions. Moreover, the 5HIAA/5HT ratio displayed a significant fluctuation in the hypothalamus, with peaking values during the end of the day. Interestingly, 5HTP (the 5HT precursor) displays a similar profile, which clearly relates to the expression of tph1 and particularly tph2. Such results agree with brain serotonergic function being activated rhythmically during the end of the light period, as reported for rat striatum (Paulson et al., 1994; 1996; Rueter and Jacobs, 1996) and SCN (Barassin et al., 2002). Since these rhythms persist in constant darkness a circadian oscillator must control them, even when lighting cues strongly influence them as well (Sun et al., 2002; Huether et al 1993; Snyder et al., 1965). Our data show a tendency for the daily profile to persist in DD-subjected trout (independently of the feeding condition) in optic tectum and medulla, with high 5HT levels during the subjective day. Since robust rhythms of tphs occur in trout brain, even in DD, specific circadian control exists over the neurotransmitter biosynthesis, but food and light might modulate this process, even when the neurotransmitter content does not display significant rhythms. This discrepancy may relate to the low density of serotonergic neurons in the fish brain, be related to that of mammals (Lillesaar, 2011), and the diffusive effect that occurs while assessing monoamines concentration in large mass of tissue, as in our study. More accurate techniques (such as brain microdialysis) could help to corroborate the existence of amine daily variations at synaptic level as consequence of the daily rhythm of neuronal activity.

Our study reveals the existence of rhythms of brain tph1, tph2 and th mRNA abundance. Most of them persists in the absence of environmental synchronizers, light and food, thus being controlled by a circadian oscillator. We also show a strong influence of light and food on the rhythms of monoamines biosynthesis. On the
contrary, other parameters, such as brain monoamines levels, their metabolites, and the ratios metabolite/monoamine display weak daily variations that might relate to those rhythms of monoamines synthesis or/and release. Whether changes in amines and metabolites relate or not to the activity of the circadian system deserves further approaches by using more refined neurochemical techniques.

By other hand, we assessed rhythms of hypothalamic food intake-related peptides, and their dependence on an oscillator. This is important since food intake is a circadian system-linked rhythmic process (Kulczykowska and Sánchez-Vazquez, 2010; Delgado et al., 2017; Isorna et al., 2017). Then, food intake regulatory hypothalamic neuropeptides, and 5HT as well (as herein reported) should display circadian rhythms, thus associating to feeding regulation (De Pedro et al., 1998b). Our results confirm the existence of circadian rhythms of hypothalamic crf, pomc-a1, cart and npy in trout, in agreement with our previous studies in this species (Naderi et al., 20108) but also with that reported for other teleost (López-Olmeda et al., 2013). These rhythms persist (although altered) in the absence of light (see Experimental work 3). Interestingly, trout subjected to DD and fasting displayed increased averaged mRNA levels (pomc-a1), decreased amplitude of npy rhythm, and the loss of rhythm (crf and cart). These results suggest that a circadian control exists over some peptides (pomc-a1 and npy), but unclear not over others (crf and cart). Regarding NPY, this neuropeptide participates as input to the hypothalamic circadian oscillator in goldfish (Vera et al., 2007), which agrees with our data suggesting a role of the peptide in trout circadian physiology. However, such control is not that strict for crf and cart, which leads us to suggest that food-related cues might influence the rhythms of these neuropeptides in trout hypothalamus, then driving directly them, or masking the influence that circadian clock exerts on them.

In summary, we demonstrate in rainbow trout the existence of rhythms in brain monoaminergic function that are particularly evident in tph1, tph2 and th transcripts, but also in critical food intake modulators such as crf, pomc-a1, cart and npy. The persistence of most of these rhythms (including pomc-a1 and npy expression) in constant conditions is demonstrated by submitting fish to light isolation and fasting, that corroborates their dependence on the circadian system. Rhythmic fluctuations of other neuropeptides (crf or cart) seem to be influenced by factors other than the circadian oscillator, then disappearing under constant environment conditions. Since monoamines modulate food intake in teleost by acting on hypothalamic neuropeptides (De Pedro et al., 1998b; Leal et al., 2011; Pérez-Maceira et al., 2014), it would be interesting to know whether monoamines are also involved in modulating the rhythms of these neuropeptides or not. In this context, the role of 5HT is particularly important since actions of the amine on food intake are mediated by specific 5HT receptors, in particular 5HT2c-like, which seems to locate in POMC neurons (Pérez-Maceira et al., 2014). Moreover, 5HT has been suggested to regulate HPI axis by activating 5HT1a receptors within CRF neurons (Medeiros et al., 2010; 2014). Due to all these
interactions, it is likely that circadian changes in 5HT synthesis and release from the neuronal ending to the synaptic cleft can influence the rhythmic fluctuations of food intake-related neuropeptides. Finally, an effect of the daily monoaminergic rhythms on HPI axis rhythmicity is also possible in order to modulate CRF, by acting through hypothalamic CRF neurons (De Pedro et al., 1998b). Accordingly, a great parallelism is noted for the rhythms of mRNA abundance of 5HT synthesis enzymes (tph1 and tph2) and crf in trout hypothalamus, which deserve further research in future studies.

4.3. Brain monoaminergic systems in the response to chronic stress in rainbow trout. Implication for endocrine and feeding response

In teleost, monoaminergic systems, 5HT and CAs, are important brain neurotransmitters working to modulate a variety of functions such as locomotor activity, endocrine status and reproductive activity, stress response, immunity, and different behaviors including social behavior, aggressiveness, and feeding among others. Different roles have been proposed for the different monoamines in fish (Winberg and Nilsson, 1993; Gesto et al., 2008).

The response to different kind of stressors is the objective of numerous studies in fish because of the negative impact that could produce in animal health and growth. Fish behavior is also strongly affected by stressors, in such a way that stress alters routine activity and individual behavioral patterns, and collectively the social interaction with conspecifics. All these disadvantages impact on fish welfare and finally determine farm productivity. Most of the physiological studies concerning the stress response in fish were made on the acute stress model since the facility to handle the stress situation, to control the intensity of the stressor, and to compare different types of stress or the response among different species. It has also advantage in order to know the neuroendocrine mechanisms that activate when stress affects individuals. A number of such studies clearly support the involvement of brain monoaminergic neurotransmitters in the response to stressors in fish (Winberg and Nilsson, 1993; Backström and Winberg, 2017). Previous research of our laboratory suggest that specifically responses of 5HT and DA are important to recognize stress stimuli (Gesto et al., 2013), and perhaps to integrate and/or coordinate a graduated response to stress (Gesto et al., 2015b). It seems in stress conditions that the sensorial information transmits to the fish brain and integrates into the monoamine neuronal network. These conjointly with other neurotransmitters, initiate the neuroendocrine response that finally provides CAs and cortisol to the blood (Wendelaar Bonga, 1997). Therefore, brain must discriminate the received stimuli, which might be done on the basis of their intensity and duration. Accordingly, increase in brain CAs and 5HT activity occurs after acute stressors is present, thus suggesting a role as primary mechanisms of
response to stress (Gesto et al., 2008; 2013; 2015b). Furthermore, the stress response is limited in time (Gesto et al., 2015b), which implies that the fish must be able to verify the absence of the stressor and calm or stop the activation of the brain mechanistic. Even, long-time stress requires a more complex response, since the animal should be aware of the continuous effect of the stressor and the neuroendocrine mechanisms should remain active leading rise the plasma cortisol levels. Moreover, fish adapt or reduce the metabolic and behavioral cost of the stress response at long-term in order to make it proportional to the duration of the stimulus. Finally, when stressor disappears, stress response should be deactivated to restore normal functioning within a reasonable time, allowing it to respond to possible future events (Barton, 1991; Herman, 2013; Tort, 2013). As for acute stress, a role of brain monoamines is also presumed for long-term response in chronic stress; however, it has been less studied in fish.

With the aim of better understand the role of brain monoaminergic systems in chronic stress experiments were performed by submitting fish for 10 consecutive days to HSD or alternatively to normal conditions. In addition, recovery from stress was investigated by using fish that remained for 7 days under HSD and were exposed to NSD for 3 consecutive days. In all cases samples were obtained at different times to draw the temporal dynamic of changes occurring at several physiological levels of fish.

The stress induced by exposure to HSD is often used in chronic fish studies (Barton, 1991), and it can be considered as a mild stress model since its effects do not threaten the life of the fish (Gesto et al., 2010). Moreover, the responses obtained at the neuroendocrine level are far from dramatic, although they are sufficient to allow its study during the time the stressor is present. All these circumstances facilitate that response to HSD are generally repetitive among studies, and even among different teleost species (Barton and Iwama, 1991; Mommsen et al., 1999; Conde-Sieira et al., 2010a,b). In our study, the time course of plasma cortisol levels in rainbow trout kept under HSD condition displayed a significant increase in all time points assessed (3, 7 and 10 days of HSD exposure) in relation to non-stressed animals. These results are in agreement with previous data obtained in our laboratory for this species (Gesto et al., 2008; Conde-Sieira et al., 2012a,b), thus validating the proposed design. Interestingly, fish subjected to HSD showed a significant reduction (~30%) in the mean plasma cortisol values, between day 3 and day 10, which suggests that an attenuation in the cortisol response is possible and could be associated to long-lasting stress conditions. This could be associated with a gradual decrease in circulating cortisol levels due to a limited production in the interrenal tissue (Rotllant et al., 2000a; b; 2001). Several hypothesis were handle in relation to the attenuated cortisol response, including the deactivation by cortisol of upstream mechanisms in the HPI axis (Rotllant et al., 200b), and the habituation to stressors that maintain continuously or repeat over time (Fernandes-de-Castilho et al., 2008; Koakoski et al., 2013;
Additionally, the existence of divergent response to stress among individuals might be also relevant since it could affect the whole response observed when fish are stressed for a long time (Ruiz Gómez et al., 2011).

The response to stress includes changes in the activity of the HPI axis, which correlate with the intensity and duration of stress (Barton and Iwama, 1991). As in mammals, the biosynthesis of cortisol in fish involves the stimulation of the interrenal cells by ACTH released from the pituitary to blood. The activation of the steroidogenic pathway involves the participation of several microsomal enzymes, including StAR (Mommsen et al., 1999; Geslin and Auperin, 2004). It is believed that increases in the activity of both enzymes are indicative of an accelerated synthesis of cortisol, as demonstrated previously in rainbow trout submitted to different kinds of acute stress (Geslin and Auperin, 2004), although the authors also indicate that a moderate stress such as that induced by netting, was without effect on the expression of star and p450scc. It seems, that both enzymes have similar regulatory mechanisms likely associated with transcriptional and post-transcriptional processes that could depend on the intensity of cortisol production. Our results obtained in rainbow trout exposed to a mild stress, such as HSD, for 10 days do not fully agree with such a possibility since high star and p450scc expression were found during all the time of exposure, suggesting that processes that initiate the cortisol synthesis are plenty activated even at long-term. By other hand, in vitro studies with tissues of anterior kidney showed an activation by ACTH treatments of enzymes downstream in the steroidogenic pathway, such as P450 11β hydroxylase that catalyzes the passage of cholesterol to pregnenolone and the 3β-HSD that mediates its dehydrogenation to form 17-OH progesterone (Hagen et al., 2006; Conde-Sieira et al., 2013). In agreement, rainbow trout chronically exposed to HSD displayed increases in the mRNA abundance of these two enzymes, which support a role in the maintenance of the biosynthetic steroid pathway. Although, these enzymes have a greater abundance and half-life than those that initiate the biosynthetic pathway (star and p450scc), so they are good candidates to keep the increase of the biosynthetic capacity of cortisol during long-term stimulation (chronic stress) (Hagen et al., 2006), which agrees with our results.

Whereas most studies were focus on the effect of a variety of stressors present in aquaculture facilities, how the recovery of animals after stressful situations takes place, has paid little investigation. Our experimental design allowed us to study the dynamic of endocrine changes during stress recovery since trout initially kept under HSD for 7 days returned then to a NSD condition remaining for the next 3 days (post-stress period). Plasma cortisol levels at 6 hours of the post-stress period were intermediate between stressed fish and non-stressed fish, whereas those of the post-stress group sampled at 24 hours already had values similar to the control non-stressed group. Therefore, it seems that cortisol levels restored to normal values between 6 and 24 hours post-stress. To compare this result with literature is difficult
since the diversity of stress situations and species. Recently, Naderi et al. (2018) reported enhanced cortisol levels in rainbow trout subjected to high density in the tanks take up 24 hours to return to pre-stress level. Whereas, Gesto et al. (2013) observed that trout stressed by chasing for brief times (5 sec to 3 min) displayed a graduated increase in plasma cortisol levels according to the duration of the stimulus, which recover in all cases at 4 h after ending the stressor action. Similarly, cortisol increase induced by 15-min chasing in rainbow trout takes between 4 and 8 hours to return to baseline (Gesto et al., 2013). In relation with social stress, high values of cortisol reported in subordinate trout returned to normal within 48 hours after removing fish of different hierarchical ranks (Culbert and Gilmour, 2016). A more dramatic response was obtained in Olive flounder (Paralichthys olivaceus) in which air exposure for 5 min allowed cortisol values to remain elevated for up to 12 h in the post-stress period, whereas stress applied chronically for 70 days elevated cortisol levels for at least 20 days after the stress finished (Lim and Hur, 2018). Similarly, in the sea bream subjected to handling and confinement, increased cortisol levels remain elevated for several days once the stressor has been eliminated (Rotllant et al., 2001).

Therefore, according to our results in rainbow trout it seems that cortisol response associated with a long-term stress, such as that induced by HSD, show a fast recovery. In agreement to that, the gene expression of all enzymes of the steroidogenic pathway that were assessed in this study also returned to values of non-stressed fish in the first 6 h, showing an even faster recovery at enzymes transcriptional level than for circulating cortisol content. The half-life of the circulating hormone could explain of the discrepancy between the dynamic of the hormone levels and the mRNA content of the enzymes involved in the steroidogenic pathway. Thus, despite the fact that secretion of cortisol from the interrenal tissue is reduced rapidly after stress removed, the hormone maintains its high levels in plasma for a longer time.

The metabolic effects of cortisol form part of the secondary response to stress in fish (Barton and Iwama, 1991), and it is characterized by mobilization of energy resources to cope with the adverse effects of stress, thus facilitating functional adaptive homeostasis (Gesto et al., 2010; Tort, 2013). When stress persists for a long time, the metabolic reserves can be empty, thus endangering the needed physiological responsiveness. Moreover, stressed fish reduce food intake, which together with the adverse effects of cortisol on immune system increases the weakness of the animal and the risk of pathologies (Tort, 2013). Unlike expected, we found that chronic HSD produced no changes in plasma glucose levels as compared to fish exposed to NSD, whereas those of lactate increased at 3 and 7 days of stress. The lack of changes in glucose contrasts with other reported data in the same and other teleost species kept under acute stress, in which the carbohydrate responds to CAs and afterwards to cortisol actions increasing its levels in plasma (Mommsen et al., 1999; López-Patiño et al., 2014b). However, in chronic stress the metabolic effects are often more complex and changes in metabolites levels are more variable (Barton and Iwama,
This is due to that carbohydrates are of immediate use in the different tissues, such as brain, heart, muscle, kidney, etc., whose activity increase during stress whereas liver glycogen stores in liver and muscle are practically empty. Therefore, it is supposed that lack of changes in blood glucose levels observed in our study derive from the balance established between glucose formation and expenditure. With respect to lactate, its levels usually increase during stress due to the activation of oxidative glycolysis pathways (Van Ham et al., 2003; López-Patiño et al., 2014b; Skrzynska et al., 2018), which can explain the increased levels of this metabolite at 3 and 7 days in our study. Interestingly, after returning stressed fish to normal density, plasma lactate levels were immediately reduced and remain low until the end of the post-stress period (72 hours), then restoring to normality. These could suggest that the metabolic effects of stress on anaerobic glycolytic pathways restored rapidly when fish return to a non-stress condition, in which the aerobic pathways of glucose metabolism would prevail. Nonetheless, it also needed to consider that changes affecting the levels of these metabolites during HSD are likely influenced by the reduction of food intake, which could also affect the carbohydrate metabolism when fish return to a normal situation.

Central and peripheral actions of cortisol are mediated by GR1 and GR2, which have a key role in different behavioral and physiological functions in fish, including the response to stress (Mommsen et al., 1999; Alderman et al., 2012). It seems that they also participates in brain and the pituitary as elements of regulation of cortisol signaling (Stolte et al., 2008; Alderman et al., 2012). However, the dynamic of mRNA abundance for gr receptors during stress exposure and stress-recovery in fish is little known. Our data demonstrated that brain gr1 expression is increased by exposing to HSD stress with major changes found in hypothalamus, telencephalon and optic tectum. Such as plasma cortisol, restoration of gr1 mRNA content in brain regions after stress was very fast, since it occurred at 2-6 hours in optic tectum and hypothalamus, and something later in telencephalon (24 hours post-stress). These data suggest the expression of gr1 in forebrain regions of trout up-regulates by enhanced cortisol levels, when stressful is present, which would otherwise have a lower expression. By contrast, the expression of gr2 did not show change during the stress and post-stress periods. Therefore, central effects of increased cortisol levels could be, at least partially, mediated by acting the steroid to modulate at transcriptional level gr1 receptor expression, whereas the involvement of gr2 in such effects seems to be unlikely. However, changes in mRNA receptor levels are not necessarily accompanied by those in receptor density and therefore a role of gr2 cannot be ruled out.

One of most remarkable consequences of chronic stress in fish is the inhibition of food intake (Conde-Sieira et al., 2010a,b; Madison et al., 2015). Accordingly, trout that were kept in HSD condition for 10 days showed a strong reduction in food intake. Given the activation of the HPI axis under stress, it has been postulated that several of
its components can play a decisive role in the regulation of feeding, with hypothalamic CRF and cortisol released from the interrenal tissue as main candidates (Bernier et al., 2001; Madison et al., 2015). We examined the expression of several anorexigenic (crf, pomc-a1 and cart) and orexigenic (npy) hypothalamic neuropeptides in trout exposed to HSD, and then after return to NSD. As expected, increased in expression of crf in the hypothalamus. This peptide is considered as a potent anorexigenic factor in goldfish (De Pedro et al., 1993; 1997) and studies on trout has also highlighted the key role of CRF in mediating the inhibitory effect of stress on food intake (Conde-Sieira et al., 2010; 2011). In addition, it has been suggest that crf can interact with other anorexigenic and orexigenic systems to modulate a variety of appetite-regulating pathways (Volkoff et al., 2005). Studies in goldfish (De Pedro et al., 1998b) and trout (Pérez-Maceira et al., 2016) have also demonstrated that increased CRF could be involved in mediating the inhibitory effects of 5HT on food intake, but also central effects of cortisol (Bernier et al., 2004; Doyon et al., 2006). Both stimulatory and inhibitory effects of cortisol treatments were described in fish (Bernier et al., 2004), with the hormone modulating NPY/CRF systems to increase food intake; However, the anorexigenic effects of cortisol in fish has not yet been elucidated (Bernier et al., 2004; Madison et al., 2015). Therefore, in our study cortisol could also be responsible for reduction of food intake in trout submitted to HSD. Since crf expression remains high all along the duration of the exposure to stress (10 days), it seems unlikely that cortisol action, if so, was not mediated by alteration of hypothalamic CRF-releasing system.

An increased gene expression of cart and pomc-a1 was also noted in HSD-stressed trout, which remained throughout the time of fish exposure to stress. These are anorexigenic neuropeptides as has been demonstrated in several studies in teleost (Volkoff et al., 2005). By contrast, npy expression was not responsive to HSD. This neuropeptide exerts powerful orexigenic effects in fish, playing a key role in appetite (Volkoff et al., 2005). The effect of stress on the expression of npy in fish is somewhat controversial. Thus, in rainbow trout stress by HSD exposure during 6 h increased the hypothalamic expression of npy (Conde-Sieira et al., 2012), which was similar to that found in this species after 72 h isolation (Doyon et al. 2006). Longer chronic cortisol increased npy expression in the hypothalamus of rainbow trout at 14 and 28 days but not at 42 days (Madison et al., 2015). These data, together with those obtained in our study suggest that effects of stress on npy expression depend in a great extent on variables affecting stress such as the type of stressor, its duration, and the increase that occurs in cortisol levels. Overall, our data do not support a relevant role of npy in the reduction of food intake that operates under a situation of chronic stress in the rainbow trout.

As for the recovery phase once the HSD-induced stress ended, we noticed a fast restoration of these neuropeptides with values of control fish after 6 h (cart, crf) and 24 h (pomc-a1). Interestingly, npy expression was increased 24 h post-stress, reaching
values high enough to guarantee a significant stimulation of food intake by the time at which stressor disappeared. Therefore, it is plausible that restoration of food intake in fish returned to NSD after 7 days in HSD does not only involve a reduction of anorexigenic peptides but also an increased npy expression that could operate to compensate the reduced feeding during the stress period, and reach as soon as possible the nutritional balance.

In this study, the monoaminergic response to chronic stress was evaluated in several brain regions of rainbow trout exposed to HSD for 10 and then after returning the fish to NSD. Different parameters related to neurotransmitter activity were measured, including the gene expression of limiting enzymes involved in CA (th) and 5HT (tph) synthesis. As for the latest, the existence of two paralog genes of tph has been reported on both mammals and fish. The sequences and characteristics of these genes being investigated in few teleost species. It has been proposed that both are expressed in all fish brain (Bellipanni et al., 2002), in contrast to mammals in which tph1 express in pineal gland and peripheral tissues, and tph2 express preferably in telencephalon and the mesencephalic raphe nuclei (McKinney et al., 2005; Zhang et al., 2004). Therefore, tph2 activity is believed to be responsible for most 5HT synthesis in the mammalian brain (Alenina et al., 2009; Savelieva et al., 2008). In our study, we cloned and sequenced a fragment of both genes (tph1 and tph2) in rainbow trout and, by using specific primers, we were able to measure gene expression in all brain regions assessed.

As for the effects of stress, we demonstrate that chronic stress by exposure to HSD results in an increased expression of tph1 and tph2. This effects was very consistent in hypothalamus and telencephalon, with expression of tph1 and tph2 significantly increased at 3, 7 and 10 days in HSD-exposed trout in comparison with control non-stressed fish. In addition, enhanced expression of tph1 was found to be persistent in optic tectum (3, 7, 10 days) and medulla (3 and 10 days) of stressed trout, whereas tph2 increased less consistently (at 3 days in optic tectum; at 7 days in medulla). As far as we know, existing data in fish regarding changes in serotonergic enzymes during stress are limited to hypoxia where decreases in expression, protein content and activity in hypothalamus of Atlantic croacker (Micropogonias undulatus) have been reported (Rahman and Thomas, 2009). In contrast, studies in rodents showed increased expression and enzymatic activity of TPH dorsal raphe nuclei, which contain most of the neuronal soma that project to the forebrain regions (Yamaguchi et al., 2016; Donner et al., 2018). In fish, at difference to mammals, there is evidence of a greater expression of tph in diencephalic areas than in the raphe (Bellipanni et al, 2002), suggesting the existence of an important synthesis in neuronal ending-containing regions. Therefore, from our results it is likely that TPH activation in the brain could potentially be used as an additional approach for the assessment of neurochemical effects of stress in fish.
The existence of an increased TPH activity in brain could be interpreted as that activity of the serotonergic neurons is being activated during stress to produce 5HT levels enough to fulfill the neurotransmitter synaptic function. Moreover, we demonstrate that the higher neuronal activity in several brain regions is followed by increased 5HIAA/5HT ratio, which also corroborates that stress induces a higher mobilization of the neurotransmitter, increasing its turnover likely as consequence of a higher utilization in the synapse (Browne et al., 2001). Changes in the 5HIAA/5HT ratio were especially evident in hypothalamus and telencephalon, whereas it appeared only at specific times in medulla and neither occurred in optic tectum. This is in agreement with previous studies in trout exposed to several types of acute (Gesto et al., 2008; 2013; 2015b) and chronic stress (Gesto et al., 2008; Conde Sieira et al., 2014), as well as those exposed to social stress (Winberg et al., 1991; 1997). In other teleost, such as the Arctic char (Salvelinus alpinus) (Hoglund et al., 2001), the gilthead seabream (Skrzynska et al., 2018) and the sole (Conde-Sieira et al., 2018), increases of brain monoaminergic activity after stress has been also reported. Interestingly, the three regions most affected by stress (hypothalamus, telencephalon, medulla) in our study showed relevant increases at 10 days, thus highlighting the persistence of an enhanced monoaminergic activity at every time when stress was affecting the fish.

We also reported an enhanced expression of th in brain of rainbow trout exposed to chronic stress, thus suggesting that synthesis of CAs is also been activated. This effect was especially consistent in the hypothalamus since the enzyme expression remained elevated all along the experiment, as compared to non-stressed fish. It was also evident at 7-days of stress in telencephalon and medulla, but not in the other time points evaluated. Again, as for tph expression, no effects were noted for this enzyme in optic tectum, highlighting the low sensitivity of this region to stress. Interestingly, the measured concentration of the monoamines (DA, 5HT) in this region is much lower than in regions in which the effect of stress was more evident, like hypothalamus and telencephalon. This suggests that monoaminergic innervation in the optic tectum is likely less dense than in the others and, as consequence, effects of stress on processes relative to monoaminergic neurons are much more difficult to discriminate and quantify. Nonetheless, our results are in accordance with studies in mammals subjected to different types of stress, with elevated th mRNA content and TH enzyme activity observed in several areas rich in dopaminergic endings, particularly at hypothalamus (Kiss et al., 2008; Wong and Tank, 2007). Similarly, studies in zebrafish subjected to a social stress reported increased th2 expression in ventral hypothalamus, a region that contains a high abundance of TH-positive cells (Pavlidis et al., 2011; Semenova et al., 2014), together with high contents of DA and its metabolites (Teles et al., 2013). In agreement with that and with the present data, and according to changes in DA and its main oxidative metabolite, clear increases of the DOPAC/DA ratio appeared in the hypothalamus, telencephalon and medulla of stressed rainbow trout that remains all along the exposure period, whereas the ratio
was not modified in optic tectum. In comparison with the serotonergic one, there is not much information on changes in dopaminergic system in trout, although several studies have reported a stimulatory action of different kinds of stressors (Backström et al., 2011; Gesto et al., 2008; Weber et al., 2012). Changes in this neurotransmitter seem to be operative in the regulation of activities like aggression, social status, motor activity, motivation and reward (Summers and Winberg, 2006), and even it has been suggested to interact antagonistically with serotonergic system in the neural circuitry controlling aggression, which could overlap with that regulating stress responses (Summer and Winberg, 2006). However, in the present study we denote that changes induced by chronic stress in the dopaminergic system were generally concordant with those affecting serotonergic one. Also in a similar way to 5HT, the increased th expression observed in stressed trout clearly points to that dopaminergic neurons are being activated, this process being specially striking in the hypothalamus. It would be interesting to know if activation of hypothalamic dopaminergic system observed herein could have role in processes that, such as reproduction, are critically affected by stress and in which changes in DA have been involved (Chabbi and Ganesh, 2015).

The brain noradrenergic system also system seems to be involved in the stress response in mammals, where NA has a role in triggering the activation of the HPA axis (Dunn et al., 2004). Meanwhile, a limited number of data also support a role for NA in fish exposed to stressors (Overli et al., 2001; Gesto et al., 2006; 2008; Weber et al., 2015), most of them offering inconclusive results. In our experiment NA levels showed inconsistent changes, so that exposure to HSD produced specific increases or decreases were observed in stressed fish. However, a general trend was evident in the different brain regions to reduce the levels of NA in trout exposed to HSD, reaching in very specific cases the significance as compared to the levels of the non-stressed group. Although TH activity is also relevant for NA formation, in this work we were not able to notice the existence of a correlation between stress-induced changes in th expression and NA content in the studied brain regions. This suggests that changes affecting NA content are not directly related to those affecting th expression, or alternatively that levels of this neurotransmitter are modulated multifactorially during the chronic stress situation.

This study also pursued the objective of analyzing the dynamics of recovery of stress-induced changes in monoamine systems once the presence of the stressor ceased. Following our experimental design, after 7 days of stay under HSD the trout returned to NSD, which resulted in a rapid recovery in monoaminergic activity to non-stressed values. In hypothalamus and telencephalon, 5HT and the expression of synthesis enzymes, tph1 and tph2, which were increased by the stress situation, returned to normal values at 6 h post-stress, with the exception of tph2 in hypothalamus that showed a slower recovery (normal values at 24 h post-stress). In optic tectum it appears that tph1 drops down to control values at 6 h post-stress, and even to lower values in the case of tph2. Regarding the medulla, a very fast recovery
was noted, with decreased expression of \textit{tph1} and \textit{tph2} at 2 h post-stress when values similar to the control group were found. Furthermore, the restoration of the enzyme gene expression after chronic exposure to HSD coincided generally with those noticed for the levels of amines and metabolites. Thus, in hypothalamus and telencephalon, similar temporal range (between 2 and 6 h) as for \textit{th} expression occurred for 5HIAA/5HT during the post-stress recovery time, thus supporting that monoamine turnover in these regions was highly affected by stressor presence and normalize immediately in its absence. No significant changes during the post-stress period were found in the serotonergic activity of optic tectum and medulla, which also points to these regions being less responsive to the presence of the stressor.

In relation with the dopaminergic system, the restoration of normal values after stress followed a similar pattern to the serotonergic one, with slight differences. Hence, in the hypothalamus and telencephalon stress-induced increase in \textit{th} expression was still apparent at 2 h post-stress, but at 6 h disappeared, with values at this time similar to controls. In medulla, however, this restoration to normal values was slower (noticed at 24 h post-stress). Unlike the serotonergic system, DOPAC/DA ratio shows a very rapid decrease during the post-stress phase. Thus, in hypothalamus and telencephalon the values of the DOPAC/DA ratio were at 2 h post-stress similar to non-stressed group. By contrast, in medulla, the increased DOPAC/DA ratios that was present in the trout exposed to stress during 7 days remained after returned the fish to NSD, in such a way that values at 72h post-stress values were similar to those of the fish continuously held in HSD. These data, together with those described for the serotonergic system, suggest that monoaminergic activities, which are highly increased by exposure to HSD, are critically dependent on the presence of the stressor.

Therefore, we show that following exposure of trout to chronic HSD condition the dopaminergic and serotonergic systems are activate, this effect being persistent along the time in which stressor was present. Interestingly, increased expression of the regulatory enzymes monoamine synthesis was remarkable all along the stress exposure, noting the relevance of this parameter as stress marker. In particular, the hypothalamus and the telencephalon are the regions more responsiveness to stress, in agreement with previous studies in this and other teleost species submitted to stress (Winberg \textit{et al.}, 1992; Gesto \textit{et al.}, 2013; 2015; Conde-Sieira \textit{et al.}, 2018), and also supporting that mechanisms promoting monoaminergic neuronal activity at short-term remain activated for a long time. In addition, changes in amine synthesis occurred simultaneously with a higher use of the neurotransmitter, evidenced by an increase in the values of the metabolite/amine ratios. This suggests that under chronic stress monoaminergic neuronal activity increases, with the telencephalon and hypothalamus as the most affected areas. The obtained results also allows to conclude that deactivation of neurochemical response to stress occurs quickly once stressor is removed, thus emerging the role of brain monoamines as primary response elements,
but also addressing the end of its effects when the stressor is no longer present in the environment.

**Linking chronic stress effects on HPI axis response and feeding behavior through modulation of the monoaminergic systems**

We clearly demonstrate that stress enhances serotonergic and dopaminergic activity, which correlates in time with increased cortisol synthesis and the reduction in food intake. After changing fish from stress to unstress condition feeding rate rapidly restored, which also correlates with deactivation of the monoaminergic function and the HPI axis. Currently, 5HT receives an especial attention since this neurotransmitter could play a pivotal role in a complex neuroendocrine loop that maintains homeostasis and promote acclimation during physiological or environmental challenges (Chaouloff, 1993; Winberg *et al*., 1997). 5HT also is important factor in regulating stress response in fish, with reported inhibitory effects (Winberg *et al*., 1997; Medeiros, 2010). It also influences food intake in such a way that 5HT treatments result in a reduction of feeding rate (De Pedro *et al*., 1998b; Winberg and Thörnqvist, 2016; Pérez-Maceira *et al*., 2016). However, information regarding mechanisms mediating 5HT signaling and its location is lacking. It has been suggested that 5HT effect in stress are mediated by receptors belonging to the 5HT1 and 2 types (Hoglund *et al*., 2002). Thus, in mammals a particular emphasis in the role of 5HT1A receptors was made, since treatment with specific 5HT1A receptor agonists induced a potent activation of the adrenal stress-response in mammals (Chaouloff *et al*., 1999). A similar role has been described for 5HT1A receptors in some teleost species, like rainbow trout (Winberg *et al*., 1997) and the Gulf toadfish (*Opsanus beta*) (Medeiros *et al*., 2014). These are autoreceptors that down-regulate serotonergic neuronal firing and 5HT release, but also it might be located in non-5HT neurons through the whole brain (Roth, 2006). Thereby, activation of 5HT1A receptors by the specific agonist 8-OH-DPAT in teleost resulted in significant increases in CRF precursor mRNA levels and release of ACTH in the pituitary (Medeiros *et al*., 2014). With all the above in mind, our results suggest that stress-induced increase in synaptic 5HT would result in stimulation of cortisol secretion due to enhanced activity of the HPI axis. It is also likely that 5HT exerts this effect by increasing crf expression and release in hypothalamus-POA, which has been demonstrated in fish (Pérez-Maceira *et al*., 2016). Still, there is growing evidence that 5HT acts directly on pituitary cells to induce ACTH release, being this effect attributed to activation of 5HT1A and 5HT2 receptors (Dinan *et al*., 1996; Winberg *et al*., 1997). These receptors could also be a target for central cortisol effects during stress in fish, in such a way that chronic elevations of plasma cortisol mediate decreased brain 5HT1A receptor mRNA and protein levels via GR (Medeiros *et al*., 2013; 2014). A population of GR-immunoreactive cells has been reported in the anterior hypothalamic region of trout, supporting that the autorregulatory loop of cortisol could operate at this location.
to down-regulate at long-term CRF and ACTH activation (Rotllant et al., 2000b). We postulate that serotonin 5HT1A receptors could be part of this action since its inhibition by sustained elevated cortisol would result in increased 5HT neuronal activity, with the subsequent negative influence on the HPI axis. In addition, our data suggest that stress-induced 5HT does not display habituation, which could be justified by cortisol promoting serotonergic function during all the time in which the stressor is acting at CNS level.

The increased levels of CRF in hypothalamus of stressed trout could also explain the inhibitory effect that chronic stress exerts on food intake. 5HT can be part of the stress-induced effects on feeding since this amine exerts an inhibitory effect on food intake in teleost (De Pedro et al., 1998b; Ruibal et al., 2002), and CRF antagonists counteracted this effect (De Pedro et al., 1998b). In addition, treatment with agonists of 5HT2C receptors increased expression of several anorectic peptides, including pomc-a1, cart and crf (Pérez-Maceira et al., 2014), which were also increased by HSD-induced stress in our study. Additionally, the agonistic activation of 5HT1A receptors has been shown to inhibit food intake in trout, this effect likely being independent on changes in the anorectic peptides (Mancebo et al., 2013; Pérez-Maceira et al., 2016). Accordingly, we denoted that chronic HSD-induced stress did not affect hypothalamic npy mRNA content, which apparently are not involved in inhibitory stress effect on food intake. Interestingly, npy expression increases in fish after stress cessation, thus suggesting this peptide could have a role in restoration of feeding rate.

Finally, according to our results, we postulate a critical role of serotonergic function in the integration of the stress information. Increased 5HT acting through different brain receptor subtypes and in different locations could be, at least partially, responsible for maintaining the endocrine response of stress at long-term and, simultaneously, to modulate feeding behavior. A role of CAs is also possible since increased contents of DA and NA seem to be part of the stress response in fish (Gesto et al., 2013), as also revealed in our study in trout. It is also possible the interaction of DA with food-related neuropeptides such as cart, pomc-a1 and npy, although it seems to depend on fish species (Leal et al., 2013; He et al., 2018). Interestingly, the presence of DA in fish hypothalamus induced an up regulation of crf expression, suggesting that the activation of the dopaminergic system that takes place under stress occurs by stimulation of CRF neurons (Leal et al., 2013). Whether this effect of DA could have a role in modulating the HPI axis in response to stress in fish is actually unknown. Additionally, CAs are involved in the modulation of much other important functions, such as aggression, social status, learning and reproduction (Summers and Winberg, 2006; Chabbi and Ganesh, 2016), which are important in the environmental adaptation and the social interaction of fish. We hope that further studies will be focused toward better understanding the neurochemical mechanisms that mediate brain monoaminergic function in response to stress response, but also on its physiological consequences at the fish organism.
5. Conclusions
Conclusions

Based on the results obtained from the different experiments, we elaborate the following conclusions:

1. Stress by high stocking density has a negative impact on the daily rhythms of clock genes expression in hypothalamus of rainbow trout, all of them displaying a decrease in averaged mRNA abundance and rhythm amplitude, whereas the acrophase was not affected. Cortisol is a serious candidate for such a regulation, although the glucocorticoid receptor antagonist, mifepristone, fails to reverse the effects of stress on circadian system. This suggests that cortisol is not the only mediator of stress effects on clock oscillatory system in trout brain.

2. Stress inhibits food intake and modifies the daily rhythmic expression profile of hypothalamic food intake-related neuropeptides, which can be summarized as increased averaged mRNA abundance (crf), or increased (npy) or decreased (pomc- a1) amplitude of the rhythm. Stress also increased daily expression of glucocorticoid receptors type 1 (gr1) but not that of type 2 (gr2). Most of these changes are prevented by mifepristone, which indicates a key role of increased cortisol levels in mediating effects of stress on food intake regulatory peptides, probably through binding to the GR1 in trout hypothalamus.

3. Associated to its role in cellular energy homeostasis, Sirtuin 1 appears to act as a mediator of the effects of stress on hypothalamic circadian clock genes network, and partially on food intake-related peptides. Experiments with a Sirtuin1 inhibitor demonstrate that reduces most of stress effects on circadian system and food intake-related peptides. Increased cortisol biosynthesis at head kidney is also shortened, suggesting that both Sirtuin and cortisol may interact at different levels to mediate effects of stress on both circadian and food intake regulatory systems, in particular in the hypothalamus. It remains our interest to know the underlying molecular mechanisms through which the Sirtuin and cortisol act on those systems.

4. We demonstrate the existence of daily rhythms of tryptophan hydroxylase (tphl and tph2) and tyrosine hydroxylase (th) mRNA abundance in several brain regions (hypothalamus, telencephalon, optic tectum and medulla) of rainbow trout, suggesting that serotonergic and catecholaminergic activities could be organized on the basis of daily rhythms in fish. Changes in the contents of amines and their metabolites are evident, likely, which is likely due to the dynamics of amine turnover or, alternatively, to limited neurochemical technics used in this study.
The rhythms of expression of monoamines synthesizing enzymes seem to be endogenous since they persisted in the absence of rhythmic changes in light and/or food as main environmental synchronizers. Our results support that rhythms of serotonergic and dopaminergic activities are complex, and participate as strong outputs of the circadian system in rainbow trout, like in mammals, as it has been shown in mammals, thus affecting a wide range of physiological processes and behaviors, including feeding regulation.

Together with the daily rhythms of monoamines, we describe for the first time in rainbow trout hypothalamus the existence of rhythmic fluctuations in food intake regulatory peptides, with a clear evidence of some of them (npy and pomc-a1) being subordinated to the control of a circadian oscillator. However, rhythms of crf and cart mRNA abundance disappear in constant conditions, with food-related cues appearing to influence drastically their rhythms influence their daily variations. Altogether, our data are indicative of both, monoamines and food intake regulators, to participate as output signals of the hypothalamic circadian oscillator in rainbow trout.

Brain monoaminergic (serotonergic, catecholaminergic) systems respond to chronic stress by increasing the expression of the enzymes of the respective amine synthesis and monoamine turnover, thus indicating the existence of increased neurotransmitter synthesis and utilization that is particularly notorious in hypothalamus and telencephalon. These changes remain all over the time the stressor is present, and disappear shortly after stress ceases, thus supporting a role of monoamines at the upper-level regulation of the stress response in fish.

Our results, together with previous evidence, indicate that brain monoamines are operative to recognize a stressful situation and to maintain the neuroendocrine activation signal over the time, but also to modulate its deactivation after returning to a non-stress situation. Interestingly, changes in expression of monoamine synthesis enzymes were consistent along the stress/de-stress periods, and therefore it should be considered when define reliable central neurochemical markers that link to the stress response in fish.

A strong parallelism among changes induced by chronic stress and its recovery in parameters related to cortisol biosynthesis, food intake neuropeptides, and brain monoaminergic systems leads us to suspect the later to play a key role in mediating stressor information that is integrated in neuronal circuitry, which ultimately regulates HPI axis and feeding behavior in fish.
6. Resumen tesis / Thesis summary
Resumen tesis / Thesis summary

Una gran parte de la población mundial depende del pescado como principal fuente de proteína animal tomada en la dieta. Sin embargo, la industria pesquera es incapaz de proveer dicha creciente demanda, al encontrarse los caladeros al límite de su explotación. Por este motivo se han tenido que desarrollar nuevas estrategias que puedan garantizar el suministro de pescado, fundamentalmente ligadas al desarrollo de la acuicultura (FAO, 2014). No obstante, la acuicultura es un proceso costoso, sobre todo debido a las exigencias de la alimentación, por lo que es prioritario desarrollar estrategias que la hagan más eficiente con objeto de minimizar el coste económico y medioambiental (asociado al alimento no ingerido). Por ello es necesario conocer los mecanismos que modulan la ingesta, sabiendo que ésta es un proceso complejo que resulta de la integración a nivel central de diferentes factores tales como el propio comportamiento alimentario de la especie (Guillot et al., 2016; Stengel et al., 2011), la capacidad de los peces para detectar los niveles circulantes de nutrientes y su estatus energético (Conde-Sieira y Soengas, 2017), así como la capacidad de generar respuestas predictivas de un modo rítmico (ver Hardin y Panda, 2013). Distintos factores externos e internos, tales como el estrés, pueden afectarla. En este último caso, resulta prioritario garantizar el bienestar de los animales, dado que si esto no ocurre, la eficacia del proceso de alimentación se ve negativamente afectada, tal y como ocurre en una situación de estrés. Por tanto, conocer perfectamente los mecanismos implicados en el control de la ingesta de alimento en los peces es importante, dado que puede contribuir a la mejora en su bienestar y en su tasa de crecimiento.

A nivel fisiológico, la regulación del comportamiento alimentario en los peces depende de diversos mecanismos integradores (metabólicos, circadianos, nerviosos y endocrinos) que actúan tanto a nivel central como periférico, los cuales a su vez están sujetos a la influencia de los procedimientos que a diario acontecen en una explotación acuícola. El hipotálamo es la región cerebral que desempeña el papel clave en la regulación de la ingesta de alimento, dado que en él se lleva a cabo la integración de numerosas señales internas y externas, elaborándose una respuesta generalmente mediada por la liberación de factores orexigénicos y/o anorexigénicos que determinan, respectivamente, el apetito y la saciedad.

Por otro lado, el alimento (disponibilidad, hora a la que se lleva a cabo,...) desempeña un importante papel como señal sincronizadora del sistema circadiano, responsable de la mayoría de las funciones rítmicas del organismo. En este sentido, nuestros estudios previos llevados a cabo con la trucha arco iris pusieron de manifiesto la existencia de un oscilador circadiano en tejidos periféricos tales como el hígado, así como el papel del alimento como señal sincronizadora de la fisiología rítmica hepática (Hernández-Pérez et al., 2017), pero también en tejidos centrales como el hipotálamo (López-Patiño et al., 2011), región en la que se regula la ingesta de alimento. En este
último caso, la existencia de un oscilador circadiano hipotalámico ha llevado a postular la existencia de relaciones recíprocas entre el sistema circadiano y sistema de control de la ingesta, de modo que los ritmos diarios de los factores moduladores de la ingesta de alimento estarian subordinados a la actividad del oscilador circadiano.

Por otro lado, el estrés puede afectar negativamente al organismo, de modo que numerosas funciones fisiológicas y comportamentales que presentan un perfil rítmico diario pueden verse seriamente comprometidas. Dicho efecto negativo ha sido corroborado en tejidos periféricos de la trucha arco iris, como es el caso del hígado, en donde se apreció un aumento de la movilización de glucógeno que conduce a una mayor disponibilidad endógena de glucosa, un descenso de su uso en este órgano y en general en el potencial lipogénico hepático (Hernández-Pérez, 2016), así como alteraciones generales en la ritmicidad metabólica en este órgano. Estos resultados llevaron a especular con la existencia de efectos adversos del estrés en áreas cerebrales clave en el control del comportamiento alimentario y la fisiología rítmica diaria, como es el caso del hipotálamo.

Teniendo en consideración que el estrés condiciona el bienestar animal y que bajo esta circunstancia se alteran numerosas funciones conductuales y fisiológicas del individuo, nuestro trabajo se centró, en primer lugar, en identificar dichas alteraciones a nivel del sistema circadiano hipotalámico de la trucha arco iris (Oncorhynchus mykiss), así como en el control de la ingesta y los posibles mediadores de dichos efectos. Para ello, se evaluó el perfil diario de abundancia de mRNA hipotalámico de genes del reloj circadiano (clock1a, bmal1, per1 y rev-erbβ-like) y reguladores de la ingesta de alimento (crf, pomc-a1, cart y npy) en dicho teleósteo, el impacto del estrés en dichos ritmos y la participación de cortisol y sirtuina1(SIRT1) como posibles mediadores. Nuestros resultados revelaron el efecto del estrés en la mayoría de los genes analizados, si bien diferentes mecanismos parecen estar implicados. Los ritmos diarios de expresión de los genes reloj disminuyeron su amplitud y sus valores medios en los peces estresados, si bien no se observaron cambios significativos en las acrofasies. Este efecto no fue prevenido por la mifepristona, un antagonista de los receptores de glucocorticoides (GR). Por el contrario, el efecto del estrés sobre el perfil diario de crf, pomc-a1 y npy fue totalmente contrarrestado por la administración del antagonista. En consecuencia, el cortisol parece mediar principalmente el efecto del estrés sobre los reguladores de la ingesta de alimentos en el hipotálamo través de la unión al receptor GRI. El estudio también aportó datos que hacían sospechar que SIRT1, un mediador ligado al estado redox y la respuesta de estrés a nivel celular, podría mediar los efectos sobre el sistema circadiano en esta misma región cerebral, hipótesis que debía ser evaluada.

Para corroborar el papel mediador de SIRT1 en el efecto del estrés sobre el sistema circadiano hipotalámico de la trucha, se llevó a cabo un nuevo experimento con cuatro grupos de truchas controles y estresadas (72 h en elevada densidad) y tratadas o no con un inhibidor de la SIRT1 (EX527). Junto con la abundancia de
mRNA de los genes mencionados anteriormente relativos al sistema circadiano y el control de la ingesta, también se evaluaron las variaciones día-noche en la abundancia de las enzimas de la biosíntesis de cortisol (3β-hsd, 11β-h, p450scc y star) en el riñón anterior, así como en la de los GR en ambos tejidos (hipotálamo y riñón anterior), con objeto de evaluar la participación de la SIRT1 en la respuesta endocrina de estrés y la interacción entre SIRT1 y cortisol como potenciales señales mediadoras. Nuestros resultados concuerdan con la hipótesis de un papel clave desempeñado por SIRT1 como mediador del efecto del estrés sobre las variaciones diarias en la abundancia de ARNm de los genes del reloj hipotálámico en la trucha arco iris, así como en los péptidos relacionados con la ingesta de alimento en el mismo tejido. El estrés por alta densidad de stock inhibe la expresión de genes de reloj, mientras que la de los péptidos relacionados con la ingesta de alimento aumenta. El tratamiento con EX527 previene los cambios observados en los genes reloj durante la exposición al estrés, lo que evidencia el papel clave desempeñado por SIRT1 como mediador del efecto negativo del estrés sobre el sistema circadiano hipotálámico de esta especie.

Por otro lado, los cambios de los péptidos relacionados con la ingesta de alimento durante el estrés se contrarrestaron parcialmente con el tratamiento con EX527. Este resultado es indicativo de la existencia de interacciones entre SIRT1 y otros mediadores (cortisol) durante la respuesta al estrés. En apoyo de esta idea, nuestros resultados muestran que SIRT1 influye en la biosíntesis del cortisol a nivel del riñón anterior durante la exposición a una situación de estrés. No obstante, es necesario realizar nuevos experimentos con objeto de identificar los mecanismos a través de los cuales SIRT1 participa en la respuesta del sistema circadiano y los péptidos reguladores de la ingesta de alimento al estrés, aun como la naturaleza de la interacción entre SIRT1 y otros mediadores.

Algunos estudios llevados a cabo en diferentes especies de vertebrados sugieren que el sistema circadiano interactúa con otros sistemas moduladores, tales como los sistemas monoaminérgicos cerebrales y ciertos factores moduladores de la ingesta, entre otros. Respecto a los sistemas monoaminérgicos cerebrales, estudios en mamíferos señalan que éstos parecen participar en dicha interacción, en base a que poblaciones de células hipotálámicas que albergan un oscilador circadiano reciben señales procedentes de células monoaminérgicas, a la vez que generan señales efectoras dirigidas a estas últimas. El resultado es una acción compleja que permite modular la coordinación de las numerosas funciones internas con los cambios que se producen en el ambiente externo del animal. En este sentido, se ha descrito la existencia de un nexo de unión entre los sistemas serotonínérgico y el sistema circadiano en mamíferos (Kripke, 1998; Loving et al., 2002; Benedetti et al., 2003). Además, también han sido descrita la existencia de ritmos diarios en las rutas de señalización dopaminérgica (Castañeda et al., 2004; Hood et al., 2010; Imbesi et al., 2009), los cuales son dirigidos tanto por la luz como el propio sistema circadiano (Hampp et al., 2008; Sleipness et al., 2007). También se ha visto la acción moduladora
ejercida por la DA sobre la expresión de genes reloj in vivo e in vitro (Gravotta et al., 2011; Imbesi et al., 2009; McClung et al., 2005), lo que corrobora el papel de los sistemas monoaminérgicos como vía de entrada al sistema circadiano. No obstante, la información disponible a este respecto en otros grupos de vertebrados como es el caso de los peces, o bien no existe o es muy escasa.

Con respecto a los factores moduladores de la ingesta, se sabe que el alimento desempeña un papel importante en la sincronización de las diferentes funciones rítmicas en vertebrados, incluidos los peces, dado su papel como vía de entrada de información a los osciladores circadianos, independientemente de dónde se encuentren estos (Delgado et al., 2017). Teniendo en cuenta la presencia del oscilador central en la misma región que alberga los centros moduladores de la ingesta de alimento en los peces, el hipotálamo, existe la posibilidad de que dicho oscilador pueda controlar la síntesis y liberación de los factores moduladores de la ingesta, utilizándolos como vías de salida de información al resto del organismo en este grupo de vertebrados. Conocida la naturaleza rítmica diaria encontrada para ciertos péptidos moduladores de la ingesta en la trucha arco iris, tal y como se ha mencionado anteriormente, la subordinación de dichos ritmos a la actividad del oscilador circadiano hipotalámico de la trucha debe ser confirmada.

Con objeto de corroborar en los peces el papel desempeñado por los sistemas monoaminérgicos cerebrales y los factores moduladores de la ingesta, tanto actuando como vías de entrada de información al sistema circadiano, como vías señalizadoras de salida del mismo, al cual se subordinan, se diseñó un experimento con truchas arco iris, las cuales fueron adaptadas a condiciones normales de alojamiento, o expuestas a 48 horas de oscuridad constante (DD), o bien expuestas a DD y ayunadas (DD+Fasting). Posteriormente fueron sacrificadas a lo largo de un ciclo de 24 horas, tomando muestras de diferentes tejidos cerebrales (telencéfalo, hipotálamo, techo óptico y médula). Los resultados obtenidos confirman la existencia de ritmos diarios de abundancia del ARNm de las enzimas limitantes de la síntesis de 5HT (tph1 and tph2) y DA (th) en todas las regiones cerebrales analizadas, si bien dichos ritmos no se vieron reflejados claramente en el contenido de monoaminas en ninguna de las regiones analizadas. Los ritmos de expresión observados se conservaron en ausencia de señales encarriladoras externas, tales como la luz y el alimento. Por otro lado, la expresión de péptidos moduladores de la ingesta (crf, pomic-a1, cart y npy) también fue rítmica y se mantuvo en condiciones de ambiente constante. En consecuencia, en la trucha arco iris como modelo de peces, el oscilador central presente en el cerebro (concretamente el hipotálamo), parece generar los ritmos de monoaminas y péptidos moduladores de la ingesta, lo que sugiere una interacción entre dichos sistemas, de modo que tanto las monoaminas como los péptidos moduladores de la ingesta podrían participar como salidas del oscilador central hipotalámico. En cambio, el papel de las monoaminas y de los moduladores de la ingesta como vías de entrada a dicho oscilador todavía debe ser evaluado en este grupo de vertebrados.
Finalmente, se sabe que los sistemas monoaminérgicos cerebrales ejercen su acción moduladora sobre un amplio rango de funciones entre las que se incluye el comportamiento alimentario (Leibowitz, 1992) así como durante la respuesta fisiológica al estrés en vertebrados (Dunn, 1988), incluidos los peces (Gesto et al., 2013). En este último caso, concretamente en la trucha arco iris, se ha especulado con la posibilidad de que los sistemas monoaminérgicos desempeñen un papel crítico durante el inicio de la respuesta al estrés, así como durante el tiempo en el que el animal permanece en dicha situación (Gesto et al., 2015). Se sabe que la respuesta fisiológica al estrés conlleva la activación de los ejes cerebro-sistema simpático-tejido cromafín e hipotálamo-hipófisis-tejido interrenal que conduce al aumento de las concentraciones plasmáticas de catecolaminas y cortisol, así como de la actividad monoaminérgica cerebral. Mientras que el aumento de las catecolaminas es de corta duración, los niveles de cortisol en sangre continúan elevados cierto tiempo (Barton, 2002). Además, cuando la situación de estrés se mantiene en el tiempo, es decir, se convierte en crónico, la respuesta del cortisol puede desvanecerse, lo que añade aún mayor complejidad a los mecanismos implicados en su regulación. En este sentido, los conocimientos relativos al papel de los neurotransmisores monoaminérgicos cerebrales durante el estrés crónico son bastante escasos, e incluso no se han desarrollado estudios centrados en la fase de post-stress, cuando cesan los estímulos estresantes y se recupera los valores circulantes normales de cortisol.

Con objeto de abordar la dinámica de los cambios ocasionados por la exposición a estrés, y una vez que desaparece el agente estresante, un grupo de truchas arco iris (Oncorhynchus mykiss) fue sometido durante 10 días a estrés por alta densidad, mientras que otros grupos se sometieron a la misma situación de estrés durante 7 días y posteriormente se devolvieron a densidad normal. Los animales de estos últimos grupos fueron sacrificados a las 2 h, 6 h, 24 h y 72 h tras el cese del estrés. Se tomaron muestras de sangre (para evaluar niveles de cortisol y metabolitos), de regiones cerebrales (para evaluar los niveles de monoaminas y la abundancia de tph1, tph2 y th, así como de neuropéptidos moduladores de la ingesta: crf, pomc-a1, cart y npy) y del riñón anterior (expresión de star, 3β-hsd, p450ssc y 11β-h). Los resultados mostraron que los peces estresados sufrieron un aumento en los niveles de cortisol en plasma, así como en las actividades dopaminérgica y serotonínérgica, especialmente en telencéfalo e hipotálamo y en menor medida en techo óptico y cerebro posterior. También se observó una mayor abundancia de ARNm de star, 3β-hsd, p450ssc y 11β-h en el riñón anterior, y de tph1, tph2 y th en el cerebro en general. Las actividades monoaminérgicas disminuyeron a valores basales a las 2-6 horas de cesar la situación de estrés, algo similar a lo que ocurrió con el cortisol plasmático. El estrés también causó un aumento de la expresión de crf, pomc-a1 y cart en el hipotálamo, el cual desapareció rápidamente tras el cese del estrés. En cambio, no se apreciaron cambios significativos en los niveles de npy en los peces estresados, si bien dicho péptido aumentó significativamente tras el retorno de los animales a una situación normal (no estrés). Estos resultados revelan que, a diferencia de los péptidos
anorexigénicos (*crf, pomc-a1, cart*) el *npy* no parece tener un papel clave en la reducción de la ingesta de la trucha arco iris durante el estrés crónico, pero en cambio su aumento durante la recuperación del estrés ayuda al animal a compensar más rápidamente el estatus nutricional previo a la exposición de estrés.

Por tanto, la respuesta fisiológica que se pone en marcha por el estrés crónico en la trucha arco iris parece disiparse en un periodo de tiempo relativamente corto (2-6 h) cuando cesa el estrés. La dinámica temporal de los cambios que ocurren en las monoaminas cerebrales, especialmente en las regiones diencefálicas y telencefálicas, tiene un gran paralelismo como los que ocurren en el eje de estrés y en los neuropéptidos hipotalámicos que actúan de forma anorexigénica. Por otro lado, el estrés crónico aumentó la expresión de receptores *gr1*, sin producir cambios en los *gr2*. Tras el cese del estrés se produjo una recuperación de los niveles basales de expresión de *gr1* que, en el caso del hipotálamo y telencéfalo, mostró un gran paralelismo temporal con los cambios encontrados en los sistemas monoaminérgicos. Estos datos sirven de apoyo al papel que ejercen las monoaminas cerebrales en el inicio de la respuesta fisiológica al estrés, la cual parece ser operativa en el reconocimiento de la situación estresante y en mantener la activación neuroendocrina a lo largo del tiempo, pero también en la modulación de su desactivación cuando el pez regresa a una situación de ausencia de estrés.

En resumen, los resultados obtenidos durante la realización de la presente Tesis Doctoral han permitido caracterizar en el hipotálamo de la trucha arco iris los ritmos diarios de abundancia de mRNA de genes del oscilador circadiano (*clock1a, bmal1, per1 y rev-erbβ-like*), así como de péptidos reguladores de la ingesta de alimento (*crf, pomc-a1, cart y npy*), la interacción entre ambos sistemas, así como el efecto negativo que el estrés ocasiona en su funcionamiento, bien a través de la acción mediadora del cortisol (fundamentalmente sobre el control de la ingesta), o de la SIRT1 (mayoritariamente sobre el oscilador circadiano), si bien no se descarta la existencia de interacciones entre ambos mediadores. Además, se han caracterizado los ritmos diarios de parámetros relacionados con las actividades monoaminérgicas cerebrales, básicamente las enzimas limitantes de la síntesis de 5HT (*tph1 y tph2*) y DA (*th*) y su dependencia del oscilador circadiano, el cual los genera. Finalmente, se ha podido demostrar en la trucha arco iris el papel clave desempeñado por dichos sistemas monoaminérgicos durante la respuesta fisiológica al estrés, así como durante la recuperación del animal una vez el estrés cesa.
7. References
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