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INFLUENCE OF LIGHT AND FEEDING PATTERNS ON THE MOLECULAR CLOCK, DIGESTIVE PHYSIOLOGY AND BEHAVIOUR OF TWO TELEOST FISHES: SEABASS (*Dicentrarchus labrax*) AND ZEBRAFISH (*Danio rerio*).

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INFLUENCIA DE LA LUZ Y LOS PATRONES DE ALIMENTACIÓN SOBRE EL RELOJ MOLECULAR, FISIOLOGÍA DIGESTIVA Y COMPORTAMIENTO DE DOS PECES TELEÓSTEOS: LUBINA (*Dicentrarchus labrax*) Y PEZ CEBRA (*Danio rerio*).

Memoria de la Tesis Doctoral presentada por Dña. Ana del Pozo Cano para obtener el grado de Doctor en Biología por la Universidad de Murcia.

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D. **Fº Javier Sánchez Vázquez**, Catedrático de Fisiología, y Dª **Luisa M. Vera Andujar**, Investigadora Contratada Juan de la Cierva, adscritos al Departamento de Fisiología,

CERTIFICAN

Que la presente memoria de Tesis Doctoral, titulada "*INFLUENCE OF LIGHT AND FEEDING PATTERNS ON THE MOLECULAR CLOCK, DIGESTIVE PHYSIOLOGY AND BEHAVIOUR OF TWO TELEOST FISHES: SEABASS (Dicentrarchus labrax) AND ZEBRAFISH (Danio rerio)*", ha sido realizada por la doctoranda **Dª Ana del Pozo Cano** bajo nuestra dirección, y autorizamos su presentación para la obtención del Título de Doctor.

Murcia, 15 de marzo de 2013

Fdo.
F.J. Sánchez Vazquez

Fdo.
L.M. Vera Andujar

Departamento de Fisiología. Unidad Docente de Fisiología Animal
Campus de Espinardo. 30100 Murcia
Tel. 868 884 931 - Fax 868 883 963

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To my parents

A mis padres

Life oscillations are what give mean and stability to it.

Son las oscilaciones de la vida, las que le dan sentido y estabilidad a ésta.

INDEX

1. GENERAL INTRODUCTION…… 1

1.1. Justification and Ph.D. unity…….. 3

1.1.1. Molecular clock…… 5

1.1.2. Digestive physiology…….. 7

1.1.3. Behavioural patterns…… 9

1.2. Species in focus: European seabass and zebrafish. 12

1.2.1. European seabass *Dicentrarchus labrax* (Linnaeus, 1758)…….. 13

1.2.2. Zebrafish *Danio rerio* (Hamilton, 1822)…… 14

1.3. Scientific production of this doctoral Thesis…… 15

2. OBJETIVES…… 17

3. EXPERIMENTAL CHAPTERS…….. 21

3.1. **Chapter 1.** Molecular cloning, tissue distribution and daily expression of *cry1* and *cry2* clock genes in European seabass (*Dicentrarchus labrax*)…….. 23

3.2. **Chapter 2.** Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass…….. 27

3.3. **Chapter 3.** Daily rhythms of blood glucose differ in diurnal and nocturnal European sea bass (*Dicentrarchus labrax L.*) undergoing seasonal phase inversions…… 31

INDEX

3.5. **Chapter 4.** Circadian rhythms of self-feeding and locomotor activity in zebrafish (*Danio rerio*)…… 35

3.6. **Chapter 5.** Light pulses at night elicit wavelength-dependent behavioral responses in zebrafish (*Danio rerio*)…… 39

4. GENERAL DISCUSSION…….. 65

4.1. Molecular clock…… 67

4.2. Physiology…… 68

4.3. Behaviour…….. 69

4.4. Dual oscillator hypothesis of seasonal inversions…. 71

5. CONCLUSIONS…….. 73

6. GENERAL REFERENCES…….. 77

7. ANNEXS…….. 87

7.1. ANNEX I: The biological clock and dualism of European seabass…… 89

7.2. ANNEX II: *Curriculum vitae*…… 118

7.3. ANNEX III: Certificate of stay…… 131

8. RESUMEN EN CASTELLANO…… 135

GENERAL INTRODUCTION

1. General Introduction:

The present doctoral Thesis is presented as a *compendium* of scientific articles, according to the January 28th "Real Decreto 99/2011" and the February 6th R-42/2012 Ph.D. rules of Murcia University. As requested, a general introduction must be included in the doctoral thesis, "presenting and justifying the scientific unit of the Thesis".

1.1. Justification and Ph.D. unity

This doctoral Thesis investigates the effect of two synchronisers (feeding patterns and light) on the biological clock in two teleost fish (European sebass, *Dicentrarchus labrax*, and zebrafish, *Danio rerio*). To this end, overt biological rhythms have been studied across three interdisciplinary main areas of research: molecular biology (clock genes), physiology (glucose and digestive enzymes) and behaviour (dualism).

As a general introduction on biological clocks, we should start stating that Chronobiology studies the timing processes (the so-called **biological rhythms**), which have been described and recognized as a natural characteristic of living organisms at different organization levels (Cymborowski, 2001). Thus, the biological clock allows organisms to predict and adapt to the environmental changes of their habitat (Madrid *et al.*, 2001). Biological rhythms are shynchronized by biotic or abiotic cyclical environmental signals, called **synchronizers** or ***zeitgebers*** ("time givers" in german). These *zeitgebers* can show different periodicities: daily, lunar and annual. **Light** is the most important abiotic *zeitgeber* in nature, and therefore, its synchronizing ability has been investigated in the present Thesis, focusing in the effects of different photoperiods and spectral characteristics. Photoperiod (light and darkness hours of every light cycle) provides important daily and seasonal timing information, since the length of day/night changes along the year (short days in winter and long days in summer) (Lincoln, 2006). Fish often adjust their activity to the photophase or scotophase, being classified as diurnal (the greatest activity occurs during the

photophase), nocturnal (the greatest activity occurs during the darkphase) or crepuscular (activity linked to dawn and dusk) (Madrid *et al.*, 2001). However, some animals manage to shift their activity phase along their life cycle, which leads to the coexistence of diurnal and nocturnal behavioural patterns in the same species. This phenomenon is known as **dualism**, which was early detected in fish species from high latitude (Eriksson, 1978). Since then, more and more fish species have been redefined as dual species due to the high plasticity of their circadian system, including fish from temperate latitudes (Lopez-Olmeda and Sanchez-Vazquez, 2010a; Reebs, 2002). The dualism seems to be more frequent among species considered traditionally diurnal (Lopez-Olmeda and Sanchez-Vazquez, 2010a), as European seabass (*Dicentrarchus labrax*) and goldfish (*Carasius auratus*). Independent phasing appears associated with dualism, so different rhythms synchronize to different phases (light or darkness) as first described with feeding and locomotor rhythms in goldfish (Sanchez-Vazquez *et al.*, 1996).

The light **spectrum** is another property of light, which effect on zebrafish behaviour has been tested in this Thesis. In the aquatic environment, ligh spectrum is a key feature because different wavelengths are absorbed differently by the water mass. Thus, the water column acts as a powerful chromatic filter, removing rapidly wavelengths below violet and beyond infrared wavelengths, while the blue wavelengths reach deep waters (up to 150 m in the clearest ocean waters) (Jerlov, 1968; Lalli and Parsons, 1995). On the other hand, organic particles and dissolved solutes also modify the light spectrum underwater, shifting the spectrum to the green region (Blaxter, 1968; McFarland, 1986). Consequently, the aquatic organisms (fish included) have developed visual adaptations according to their spectrum niche (Patridge and Cumming, 1998; Chinen *et al.*, 2005) and melatonin (light-transucing hormone) is strongly affected by the wavelength in both seabass (Bayarri *et al.*, 2002) and zebrafish (Ziv *et al.*, 2007). Recently, light spectrum effects on genetic expression of opsins (Temple, 2011), early development (Villamizar *et al.*, 2011) and behaviour (Li *et al.*, 2012) have been reported in seabass and zebrafish.

Feeding has been considered as one of the most important biotic *zeitgebers*, and when it is perdiodically available, feeding has been reported to entrain

behavioural, physiological and clock gene expression rhythms in fish. Thus, daily feeding patterns (diurnal/nocturnal) in seabass and zebrafish have been evaluated in this Thesis. Feeding availability and the frecuency of predators are cyclic in the wild. In fish, most feeding rhythms under investigation are daily rhythms, although tidal, lunar and circannual rhthms have been also described. Moreover, daily feeding rhythms may change on a seasonal basis, as seen in seabass (Sanchez-Vazquez *et al.*, 1998). Thus, fish have developed time keeping mechanisms to predict the forthcoming meal, activating behavioural, physiological and genetic processes in advance, optimising food intake and nutrient utilization and minimizing the energy waste in comparission with fish fed at random times (Madrid *et al.*, 2001; Sanchez-Vazquez and Madrid, 2001; Vera *et al.*, 2007). Some overt rhythms (e.g. feeding rhythms) are endogenously controlled by light and/or feeding cycles through one or more oscillators. Hence, two hypotheses have been proposed: one of them suggests the existence of a couple of separate oscillators (light and food entrainable) whereas the other proposes that a single oscillator would be entrained by both light and food (Lopez-Olmeda and Sanchez-Vazquez, 2010a).

In summary, in this Thesis the influence of light and feeding on fish daily rhythms will be investigated at three levels: (1) behavioural, (2) physiological and (3) molecular clock levels, using seabass and zebrafish as models.

1.1.1. Molecular clock

In this Thesis we aimed to enlarge our knowledge on seabass clock genes by cloning and characterizing their daily rhythms of expression under different photoperiod and feeding conditions. The molecular clock is much conserved across vertebrates and is composed of clock genes organized into two feedback loops (Figure 1): a negative one, with *cryptochrome* (*Cry*) and *period* (*Per*) genes; and a positive one, with *Circadian Locomotor Output Cycles Kaput* (*Clock*) and *Brain and Muscle Aryl hydrocarbon receptor nuclear translocator (ARNT)-Like* (*Bmal*). CRY protein join PER protein to form a complex (CRY:PER), which binds to and blocks the protein complex

formed by CLOCK and BMAL (CLOCK:BMAL), thus inhibiting CRY transcription (Iuvone *et al.*, 2005; Okamura *et al.*, 2002).

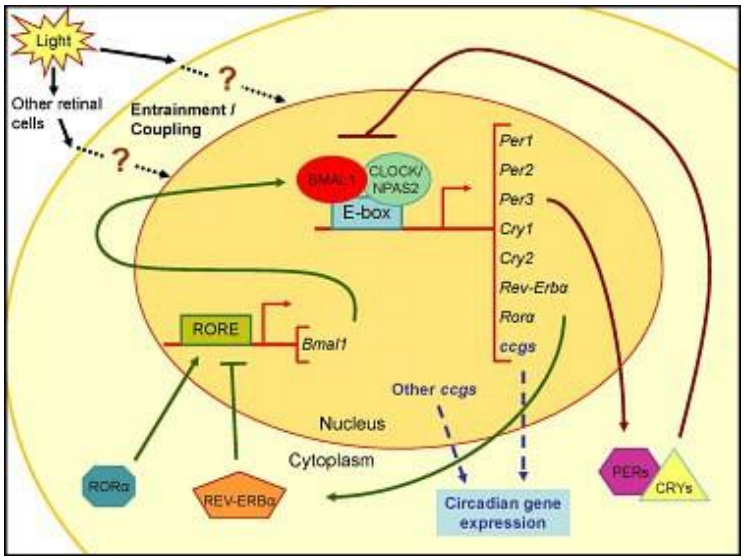


Figure 1. Simplified diagram of the molecular circadian clock. The current model of the molecular mechanism that controls circadian rhythms in eukaryotes includes at least two interconnected feedback loops of transcription and translation. Most of the genes involved ("clock genes") are transcriptional activators or repressors. *From Guido* et al*., 2010.*

The fish molecular clock differs from the mammalian one in the larger number of *cryptochrome* (*cry*) and *period* (*per*) genes, existing several subtypes, with similar, different or still unknown function in comparison to mammalian proteins (Kobayashi *et al.*, 2000; Cahill, 2002). Teleost fish is one of the most successful groups among vertebrates due to its variability of ecological time niches, providing an opportunity to investigate the flexibility of circadian clock and their adaptation to extreme environmental conditions (Iida *et al.*, 2012). In zebrafish, the components and function of the molecular clock have been well described (Kobayashi *et al.*, 2000). In contrast, in seabass, *per1* was the only clock gene already cloned, being its tissue distribution and daily expression also characterized (Sanchez *et al.*, 2010). Therefore, there is a lack of knowledge about the seabass molecular clock, in spite of being a prominent species for the Mediterranean aquaculture and a good chronobiological model due to the presence of dual behaviour. Consequently, to improve the understanding of seabass clock genes, we cloned and characterized two seabass *cry* genes in **Chapter 1**. Additionally, the tissues distribution of seabass *cry* genes was

observed, since the location of the molecular clock is uncertain. Besides the central master clock, circadian clocks in peripheral tissues have also been reported in *Drosophila*, zebrafish, mammalian cell lines and tissues, supporting the hypothesis about the existence of decentralized clocks (Tamai *et al.*, 2005). Moreover, *Drosophila* and adult zebrafish tissues, as well as zebrafish cell lines and embryos, are directly light-responsive (Whitmore *et al.*, 1998; Schibler *et al.*, 2002). In fact, seabass *per1* was also expressed in seabass liver, brain, heart, gill, muscle, digestive tract, adipose tissue, spleen and retina (Sanchez *et al.*, 2010), adjusting to circadian rhythm in brain, liver and heart with the acrophase at the end of night in all tissues.

Feeding entrainment of clock genes expression, has been recently studied in fish species such as goldfish (*Carassius auratus*) (Nisembaum *et al.*, 2012) or zebrafish (Lopez-Olmeda *et al.*, 2010). However, in seabass, this issue remained unexplored. Among zebrafish clock genes, *per2* has been described to be strictly light-entrained (Pando and Sassone-Corsi, 2002) whereas *cry1* is essensial for photic-entrainment of the molecular clock, and in brain being strongly induced and entrained by light but not by scheduled feeding (Tamai *et al.*, 2007; Sanchez and Sanchez-Vazquez, 2009). Similarly, scheluded feeding failed to entrain *per1* expression in brain under continuos light (LL) but not in liver, where restricted-time feeding also modified the *per1* expression rhythm under LD cycles (Sanchez and Sanchez-Vazquez, 2009; Lopez-Olmeda and Sanchez-Vazquez, 2010a). To conclude, different elements of the molecular clock could be entrained by different *zeitgebers* (such as light and feeding). Both photoperiod and feeding time seem to control peripheral oscillators *in vivo*, although feeding time appears to entrain more efficiently peripheral oscillators than central ones (Nisembaum *et al.*, 2012; Lopez-Olmeda et al, 2010). Therefore, in **Chapter 3** we investigate the influence of feeding patterns (diurnal/nocturnal) on the seabass dual behaviour, an issue that had not been tackled up to now.

1.1.2. Digestive Physiology

The role of light and feeding patterns on the digestive physiology of seabass has been also studied in this Thesis. The fact that seabass is a dual species

makes it an interesting model to carry out these experiments since fish physiology suffers daily variations, which in turn causes that the utilization of the nutrients is optimum during their natural feeding phase (Sanchez-Vazquez and Madrid, 2001). Accordingly, in seabass demanding food during the darkphase in winter (Sanchez-Vazquez *et al.*, 1998) the specific growth rate was higher and the ratio of feed conversion lower under nocturnal automatic- and self-feeding than when food was provided during the day (automatic-feeding) (Azzaydi *et al.*, 2000).

Daily and annual variations have been reported in several seabass digestive hormones and metabolites, such as insulin, plasma lipid levels (Fernandez *et al.*, 1989) and plasma glucose (Gutierrez *et al.*, 1984; 1987). However, up to date, the influence of feeding patterns on metabolic enzymes and hormones rhythms in seabass remained unexplored. In fasted fish, the daily rhythm of several metabolic enzymes and hormones related to feeding disappear (Lopez-Olmeda and Sanchez-Vazquez, 2010a). Thus, previous studies have showed that feeding time modified daily rhythms of several physiological parameters, such as glucose, amylase, alkaline protease and cortisol in goldfish (*Carassius auratus*) and gilthead seabream (*Sparus aurata*) (Vera *et al.*, 2007; Montoya *et al.*, 2010a) (Figure 2). Moreover, other investigations have pointed that scheduled feeding (even a single cycle) contributes to fish welfare, compared to random feeding, allowing the fish to prepare for the forthcoming feed (Sanchez *et al.*, 2009). To determine the effect of feeding time on the digestive physiology of seabass, **chapters 2** and **3** of this doctoral thesis focus on physiological rhythms of amylase and glucose in diurnal and nocturnal seabass.

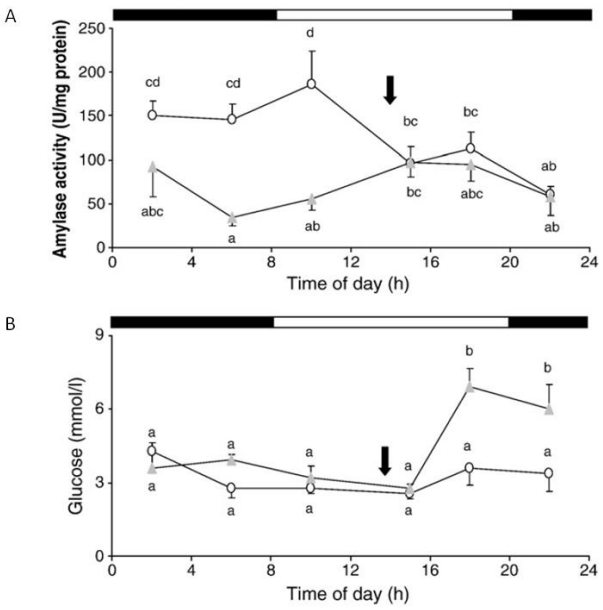


Figure 2. Amylase(A) and blood glucose (B) rhythms in seabream subjected to periodic feeding (white circles) and random feeding (grey triangles). Values represent the mean + SEM for periodic feeding and the mean - SEM for random (6 fish/time point). White and black bars above the graph show the photophase and darkphase, respectively. Black arrow indicates the time of food delivery. Letters denote statistical differences between time of day and treatment by ANOVA I ($p < 0.05$). *Modified from Montoya* et al.*, 2010a.*

1.1.3. Behaviour (feeding and locomotor)

We have investigated the daily behavioural patterns of seabass and zebrafish, undergoing photoperiod and time-restricted feeding patterns. Although European seabass had been traditionally considered as a diurnal species, several studies have described the existence of dual feeding behaviour (Sanchez-Vazquez *et al.*, 1995a, 1995b, 1998; Azzaydi *et al.*, 2007). Firstly, some authors had observed the existence of nocturnal demands (Sanchez-Vazquez *et al.*, 1994; Anthouard *et al.*, 1993; Boujard *et al.*, 1996), but this dual feeding behaviour was finally confirmed in individual and groups of seabass, irrespective of the photoperiod, intensity of light, temperature and the season in which fish are transferred to the laboratory conditions (Sanchez-Vazquez *et al.*, 1995a). Besides, the coexistence of diurnal and nocturnal patterns under the same laboratory conditions also proved the endogenous character of dualism. Subsequently, seasonal inversions in seabass feeding behaviour were described twice a year under natural conditions: diurnal fish become nocturnal in winter and *vice versa* in spring (Sanchez-Vazquez *et al.*, 1998) (Figure 3). Recently, these seasonal inversions have been also observed in locomotor behaviour of broodstock seabass, showing nocturnal locomotor activity during the spawning period (winter and early spring) and diurnal during the rest of the year (Villamizar *et al.*,

2012). Such a phase-shifting capacity of both feeding and locomotor behaviour allows the phase independence between both rhythms (Lopez-Olmeda and Sanchez-Vazquez, 2010a).

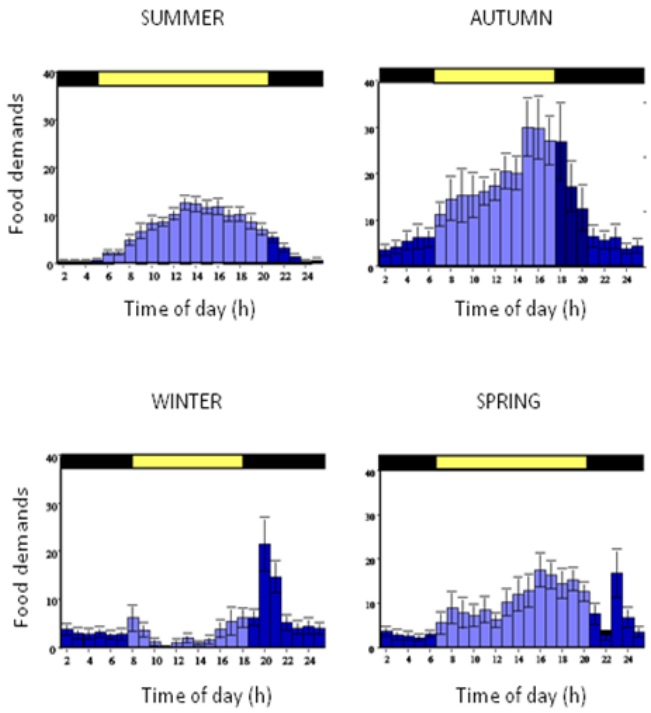


Figure 3. Food demands of seabass groups in every season, displaying seasonal phase inversions. The vertical axis shows the food demands, while the horizontal one represents the time of day (in hours). Dark and yellow bars above the graphs indicate the darkphase and photophase, respectively. *Modified from Sanchez-Vazquez* et al*., 1998.*

Regarding feeding as *zeitgeber* of behavioural rhythms, different feeding patterns altered the locomotor rhythms in a long fish list, such as seabream (Montoya *et al.*, 2010a; 2010b), Japanese sea catfish (*Plotosus japonicas*) (Kasai *et al.*, 2009), seabass (Sanchez-Vazquez *et al.*, 1995b) and zebrafish (Lopez-Olmeda and Sanchez-Vazquez, 2010b) and caused food anticipatory activity (FAA) just before the established mealtime (Lopez-Olmeda and Sanchez-Vazquez, 2010a). Futhermore, scheduled feeding synchronized locomotor activity under constant light (LL) or darkness (DD) conditions in several fish species, such as tench (*Tinca tinca*), Japanese sea catfish, goldfish, seabass, zebrafish, and even in blind fish like cavefish (Kasai et al, 2009; Lopez-Olmeda and Sanchez-Vazquez, 2010b; Vera *et al.*, 2007; Herrero *et al.*,

2005; Cavallari *et al.*, 2011). Thus, the seabass dual behaviour makes this species a very interesting chronobiological model to investigate the overt rhythms linked to dualism under natural seasonal feeding inversions (**Chapter 2**) or time-restricted self-feeding (**Chapter 3**).

Zebrafish has been reported as a strict diurnal species (Hurd *et al.*, 1998; Cahill *et al.*, 1998; 2002; Lopez-Olmeda *et al.*, 2006; Sanchez and Sanchez-Vazquez, 2009) but recently some nocturnal locomotor activity has been observed at 20°C (Lopez-Olmeda and Sanchez-Vazquez, 2009). Zebrafish is a species widely used for chronobiological studies, and the locomotor rhythm in both adults and larvae has been widely described. However, no information about self-feeding activity was available, probably due to the absence of self-feeders suitable for fish of such a size. Self-feeders are a powerful research tool to investigate feeding rhythms, since fish are free to demand food when and as much as they want, even to select the composition of their diet. Since Rozin and Mayer designed the first self-feeder in 1961, several kinds of self-feeding systems have been developed. At least, three components are required: the food container, which delivers a small amount of food when is activated; a computer system to record the demand data; and the food sensor, which is the most variable part (rigid lever or flexible string) since it needs to fit the fish characteristics and its environment, avoiding accidental activations (Adron, 1972; Boujard *et al.*, 1992; Sanchez-Vazquez *et al.*, 1994; Rubio *et al.*, 2004). All these sensors work by pulling or pushing, requiring certain strength to be activated, for which large fish in their early stages (such as seabass larvae and juvenile) and small size species (as zebrafish) are not able to trigger them (Figure 4). Therefore, in the present Thesis we aimed to design a proper self-feeder for small fish to study the zebrafish feeding rhythms (**Chapter 4**), as well as seasonal feeding inversions in early stages of seabass (**Chapter 2**).

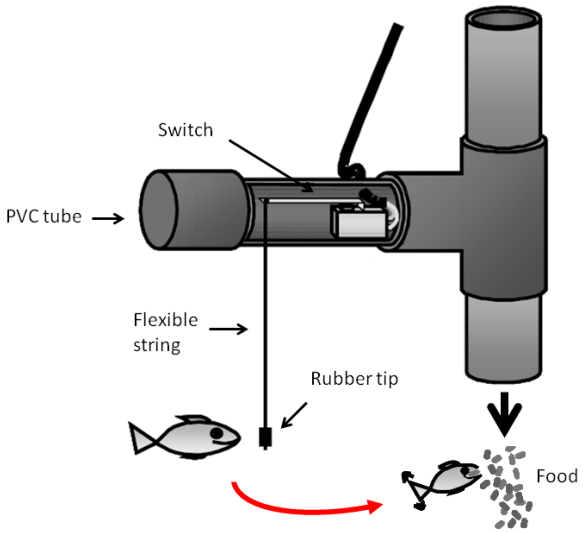


Figure 4. Squematic diagram, which shows a self-feeding system by means of a flexible string as a demand sensor. When fish pulls the flexible string, the switch is activated and then, the food is deliveried. *Modified from Rubio* et al.*, 2004.*

Finally, in this Thesis the effect of light spectrum on fish behavioural responses heve been investigated in zebrafish, which are surface swimmers in an aquatic habitat dominated by broad and shortwave spectra (Chinen *et al.*, 2005). Since light wavelengths drive locomotor behavioral responses (Muto *et al.*, 2005), in this Thesis we have studied the comparative effect of light spectrum on zebrafish behaviour. The cellular and physiological mechanisms involved on the visual sensitivity of zebrafish has already been researched (Li *et al.*, 2012; Cameron, 2002; Bilotta *et al.*, 2001), as well as the relative influence of light spectrum and feeding stimlulus (Risner *et al.*, 2006) and the optomotor response caused by spectral light (Kraus and Neumeyer, 2003). The spectral effect of the light pulse at midnight on diurnal locomotor activity and vertical position of zebrafish has been examined in **Chapter 5**.

1.2. Species in focus: European seabass (*Dicentrarchus labrax*) and zebrafish (*Danio rerio*)

The present thesis focuses on two model species with great chronobiology importance (European seabass and zebrafish). For each species we have aimed to complete the previous gaps in knowledge on dual behaviour in both species, covering different aspects (Table 1).

Table 1. *Interest and background of both species in focus on the present thesis: seabass and zebrafish. The figures shown represent the hits retrived from the "ISI web of knowledge" on 18th March 2013.*

		Seabass (marine)	Zebrafish (freshwater)
Major Interest	General	Aquaculture	Basic model / biomedicine
	Cronobiology	Dualism	Molecular clock
	ISI records	5,270	41,308
Background	Clock genes	14 (0.3%)	333 (0.8%)
	Physiological rhythms	29 (0.6%)	152 (0.4%)
	Behavioural rhythms	Locomotor: 49 (0.9%) Feeding: 113 (2.1%)	Locomotor: 61 (0.1%) Feeding: 21 (0.05%)
	Wavelenght effects	82 (0.2%)	26 (0.06%)

1.2.1. European seabass *Dicentrarchus labrax* (Linnaeus, 1758)

European seabass is one of the most important aquaculture Mediterranean species. This teleost fish was taxonomically classified by Van der Land *et al.* (2001) as follows:

Phylum: Chordata

Subphylum: Vertebrate

Superclass: Osteichthyes

Class: Actinopterygii

Orden: Perciformes

Family: Moronidae

Genus: *Dicentrarchus*

Species: *D. Labrax* (Linnaeus, 1758)

This marine species is distributed along part of the Atlantic Ocean (from Norway to Senegal), the Mediterranean Sea and the Black Sea (Wheeler, 1975), although it can be also found in coastal and brackish water and even occasionally in rivers due to its euryhaline nature (Smith, 1990). Seabass is a eurythermal species too, surviving within a wide temperature range (2—32 °C). These changes in its habitats are correlated with its different development stages, search for food and reproductive rhythms (Barnabe, 1989). Migratory movements take place in adults after sexual maturation (between 2—4 years of age for Mediterranean seabass) and until that stage they live in estuaries. From spring to summer, seabass prefers inshore waters, tidal lagoons and estuaries, where the food amount (small fish and a great variability of invertebrates according to the seabass size) is higher (Lemaire *et al.*, 2000; Varsamos *et al.*, 2001; Moretti *et al.*, 1999). In late autumn, they move to deeper (up to 70 m) waters, where the seasonal phenomenon of mating and spawning occurs in winter (December to March) in the Mediterranean population, and up to June in Atlantic populations (Moretti *et al.*, 1999).

To broaden the knowledge of the seabass biological clock and dual behaviour, see ANNEX I of this thesis.

1.2.2. Zebrafish *Danio rerio* (Hamilton, 1822)

Zebrafish is a vertebrate preferential model for chronobiology, genetics, physiological and behavioural studies (Spence *et al.*, 2008; Wang *et al.*, 2012), as well as for disease investigations and screening of therapeutic drugs (Sumanasa and Lin, 2004). This teleost fish was taxonomically classified by Fang (2003) as follows:

Phylum: Chordata
Subphylum: Vertebrate
Superclass: Osteichthyes
Class: Actinopterygii
Orden: Cypriniformes
Family: Cyprinidae
Genus: *Dario*

Species: *D.rerio* (Hamilton, 1822)

The geographical distribution of zebrafish is not clear, although the Indian subcontinent seems to be a confirmed location (Barman, 1991). It inhabits from quite to stream waters, as well as ponds in rise fields and shallow eutrophic waters (Jayaram, 1999; Daniels, 2002; Engeszer *et al.*, 2007).

Under laboratory conditions, the spawning of domesticated zebrafish strains takes place all year round, whereas in nature, this species tends to reproduce seasonally, coinciding with the onset of the monsoon season (Spence *et al.*, 2008). Temperature and food availability are monsoon cycle related, and thus could act as the external factors driving these reproductive cycles. Zebrafish is omnivorous and their diet is composed mainly by zooplankton and insects but also phytoplankton and other biological material found in their habitat.

1.3. Scientific articles that compose this Thesis.

The present doctoral thesis is a *compendium* of the following scientific papers:

1. Del Pozo A, Sanchez-Ferez JA, Sanchez-Vazquez FJ (2011). **Circadian rhythms of self-feeding and locomotor activity in zebrafish (*Danio rerio*)**. Chronobiology International, 28(1): 39–47.
2. Del Pozo A, Montoya A, Vera LM, Sanchez-Vazquez FJ (2012). **Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass.** Physiology & Behavior, 106: 446—450.
3. Del Pozo A, Vera LM, Sanchez JA, Sanchez-Vazquez (2012). **Molecular cloning, tissue distribution and daily expression of *cry1* and *cry2* clock genes in European seabass (*Dicentrarchus labrax*).** Comparative Biochemistry and Physiology, Part A, 16: 364-371.
4. Del Pozo A, Vera LM, Montoya A, Sanchez-Vazquez FJ (2012). **Daily rhythms of blood glucose differ in diurnal and nocturnal European sea bass (*Dicentrarchus***

***labrax* L.) undergoing seasonal phase inversions.** Fish Physiology and Biochemistry, DOI: 10.1007/s10695-012-9732-z.

5. Del Pozo A, Sanchez-Vazquez FJ (2013). **Light pulses at night elicit wavelength-dependent behavioral responses in zebrafish (*Danio rerio*).** In preparation.

OBJETIVES

2. Objectives:

This thesis aims at investigating the effects of light (photoperiod and spectrum) and daily feeding patterns (diurnal *vs.* nocturnal) on the biological clock of two teleost fishes: European seabass (*Dicentrarchus labrax*) and zebrafish (*Danio rerio*). Research was conducted across three organizative levels: molecular clock, physiology and behaviour.

Specific objectives:

1. Clone two *cryptochrome* genes (*cry1* and *cry2*) of European seabass and describe their tissue distribution and daily rhythm of expression in central (brain) and peripheral oscillators (heart and liver).

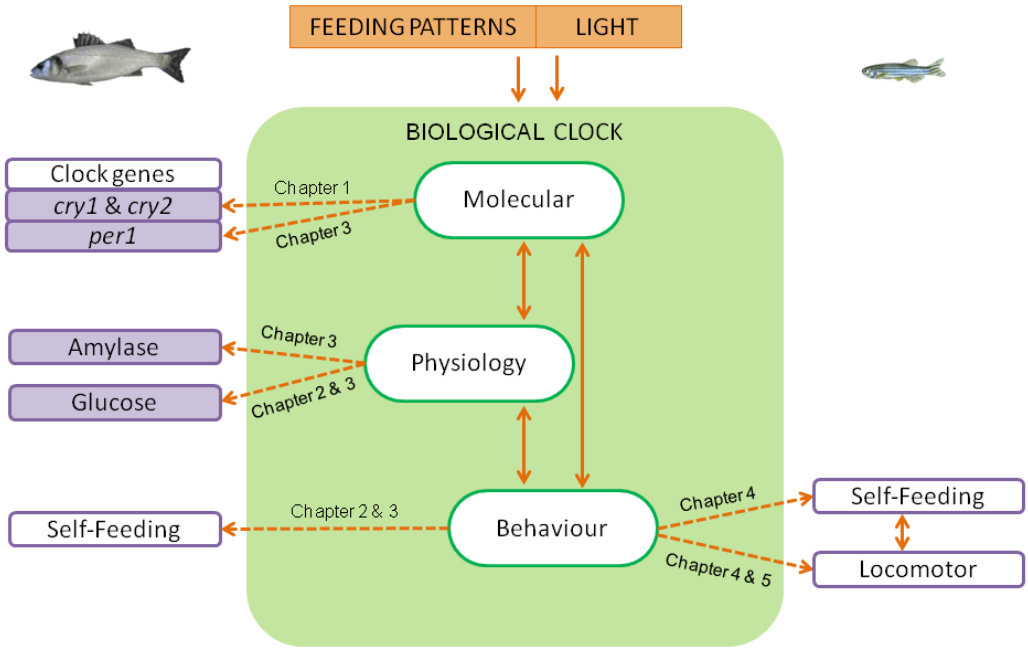
2. Evaluate the differences in the molecular clock (*period1* gene expression) and digestive physiology (blood glucose and amylase activity) in seabass with diurnal and nocturnal self-feeding behaviour.

3. Investigate the daily rhythm of blood glucose in seabass undergoing seasonal phase inversions (from diurnal to nocturnal self-feeding behaviour and viceversa) at two different times of the year (winter and spring).

4. Design and test a self-feeder suitable for zebrafish to investigate their daily rhythm of self-feeding and locomotor activity, as well as the endogenous nature of these rhythms under constant conditions.

5. Study the effect of light spectrum on behavioural responses (swimming activity, maximum swimming speed, resting time and vertical distribution) using video tracking in zebrafish.

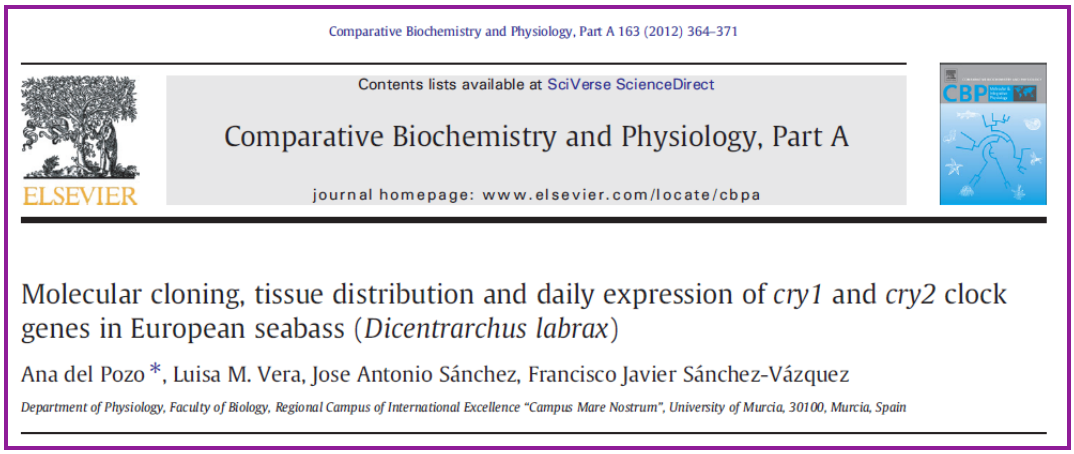
The above scientific challenges have been tackled in the following 5 chapters, which composed the doctoral thesis and are interconnected, as shows the following diagram:



At the molecular level, two seabass *cryptochromes* (*cry1* and *cry2*) genes have been cloned and their daily expression characterized (**Chapter 1**), as well as the daily expression of *period1* (*per1*) gene in diurnal and nocturnal seabass (**Chapter 3**). As regards the physiological level, the influence of feeding patterns on daily rhythms of amylase activity and blood glucose has been evaluated in seabass (**Chapter 3**); wheras the link between the glucose rhythm and diurnal/nocturnal behaviour has been studied during the natural seasonal feeding inversions occurring in seabass (**Chapter 2**). At the behavioural level, the ability of small fish to use a new self-feeder by approaching has been tested in seabass early life stages (**Chapter 2**) and adult zebrafish (**Chapter 4**), and their feeding rhythms were characterised. Moreover, the seabass capacity to shift their feeding phase when subjected to nocturnal food-reward restrictions has been investigated in **Chapter 3**. Finally, the effects of timed food-reward restriction and light wavelength on zebrafish locomotor activity were studied in **Chapter 4** and **5**, respectively.

EXPERIMENTAL CHAPTERS

3.1. Experimental Chapter 1:



Comparative Biochemistry and Physiology, Part A 163 (2012) 364–371

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Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Molecular cloning, tissue distribution and daily expression of *cry1* and *cry2* clock genes in European seabass (*Dicentrarchus labrax*)

Ana del Pozo *, Luisa M. Vera, Jose Antonio Sánchez, Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain

Published in: Comparative Biochemistry and Physiology, Part A 163, 364-371 (2012).

URL: http://ac.els-cdn.com/S109564331200414X/1-s2.0-S109564331200414X-main.pdf?_tid=0e637ed0-a614-11e2-92d9-00000aacb35f&acdnat=1366061764_cdf685c77492df67ce11e29239625a47

Impact factor: 2.235 (2011)

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
BIOCHEMISTRY & MOLECULAR BIOLOGY	290	186	Q3
PHYSIOLOGY	79	35	Q2
ZOOLOGY	146	18	Q1

Molecular cloning, tissue distribution and daily expression of *cry1* and *cry2* clock genes in European seabass (*Dicentrarchus labrax*)

Ana del Pozo, Luisa M. Vera, Jose Antonio Sánchez, Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain

ABSTRACT

Biological rhythms are driven by circadian oscillators, which are ultimately controlled by the cyclic expression of clock genes. Cryptochromes (CRY), blue light photoreceptors, belong to the negative elements of the transcriptional feedback loop into the molecular clock. This paper describes the cloning and characterization of two cryptochromes (*cry1* and *2*) in European seabass, which is considered an interesting chronobiology model due to its dual (diurnal/nocturnal) behavior. The cloned cDNA fragments encoded for two proteins of 567 and 668 amino acids, which included the FAD-binding and the DNA-photolyase domains. Moreover, both proteins had a high homology with cryptochrome proteins (Cry) of other teleost fish. These *cry1* and *2* genes were expressed in several tissues of seabass (brain, liver, heart, retina, muscle, spleen, gill and intestine). In addition, the daily expression of *cry1* was rhythmic in brain, heart and liver with the acrophase around ZT 03:15 h (after the onset of lights). Similarly, the *cry2* daily expression was rhythmic in liver, peaking at ZT 03:28 h, whereas in brain the acrophase was at ZT 11:08 h (shortly prior to the offset of lights). These findings provide new elements to help understanding the functioning of the molecular clock of seabass.

3.2. Experimental Chapter 2:

Physiology & Behavior 106 (2012) 446–450

Contents lists available at SciVerse ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass

Ana del Pozo *, Ander Montoya, Luisa María Vera, Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia Espinardo Campus, 30100 Murcia, Spain

Published in: Physiology & Behavior 106, 446-450 (2012).

URL: http://ac.els-cdn.com/S0031938412000996/1-s2.0-S0031938412000996-main.pdf?_tid=7a4f01f0-a614-11e2-8196-00000aab0f6c&acdnat=1366061945_1825a515ddf4546d381b529643e1db8d

Impact factor: 2.869 (2011).

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
BEHAVIORAL SCIENCES	48	19	Q2

Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass

Ana del Pozo, Ander Montoya, Luisa María Vera, Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia Espinardo Campus, 30100 Murcia, Spain

ABSTRACT

Seabass is a fish species with dual (diurnal/nocturnal) feeding behavior, although little is known about changes in its molecular clock, physiology and metabolism linked to this dual behavior. In the research described here possible differences in clock gene expression in central (brain) and peripheral (liver) oscillators, and in physiology (blood glucose and amylase activity in mid-intestine) were studied in seabass with diurnal or nocturnal self-feeding patterns under LD 12:12 h (light:dark) (lights on = *Zeitgeber* Time (ZT) 00:00 h). The results revealed that *per1* expression in brain shows daily rhythmicity with the acrophase (Φ) around the lights offset (ZT 12:00 h, Cosinor, $p < 0.01$) in both diurnal and nocturnal seabass. In liver, *per1* daily levels of expression were higher in diurnal fish (univariate GML, $p < 0.02$). Daily blood glucose variations were observed in both groups (ANOVA I, $p < 0.01$), with higher glucose levels occurring at night in nocturnal as well as in diurnal fish, although only diurnal seabass displayed a significant daily rhythm (Φ = ZT 16:52 h, Cosinor, $p < 0.02$). The highest values of amylase activity coincided with the feeding-phase of fish; that is, in nocturnal seabass the maximum was reached at ZT 18:00 h (ANOVA I, $p < 0.01$), whereas in diurnal seabass the Φ was ZT 03:39 h (Cosinor, $p < 0.02$). In short, our findings indicated that the feeding rhythm (diurnal *vs.* nocturnal) strongly influenced the daily patterns of digestive function and clock gene expression in the liver (feeding-entrained clock), but not in the brain (light-entrained clock).

3.3. Experimental Chapter 3:

Fish Physiol Biochem
DOI 10.1007/s10695-012-9732-z

Daily rhythms of blood glucose differ in diurnal and nocturnal European sea bass (*Dicentrarchus labrax* L.) undergoing seasonal phase inversions

A. del Pozo · L. M. Vera · A. Montoya · F. J. Sánchez-Vázquez

Published in: Fish Physiology and Biochemistry, DOI: 10.1007/s10695-012-9732-z (2013).

URL: http://link.springer.com/content/pdf/10.1007%2Fs10695-012-9732-z

Impact Factor: 1.528 (2011)

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
BIOCHEMISTRY & MOLECULAR BIOLOGY	290	231	Q4
FISHERIES	50	20	Q2
PHYSIOLOGY	79	61	Q4

Daily rhythms of blood glucose differ in diurnal and nocturnal European sea bass (*Dicentrarchus labrax* L.) undergoing seasonal phase inversions.

A. del Pozo, L. M. Vera, A. Montoya and F. J. Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia, Espinardo Campus, 30100 Murcia, Spain.

ABSTRACT

Sea bass change their feeding rhythms from diurnal to nocturnal in winter, returning to diurnal feeding in spring. Despite behavioral data, the physiological changes that take place during such changes remain unexplored. In this paper blood glucose rhythms of European sea bass with diurnal/nocturnal self-feeding rhythms were investigated during phase inversions of their feeding behavior (in winter and spring) when both diurnal and nocturnal fish coexist. Blood glucose showed daily variations in both seasons (ANOVA, $p < 0.03$), fitting a cosine function (COSINOR, $p < 0.05$) in all cases, except in diurnal fish in spring. The average blood glucose levels of nocturnal fish in winter (2.67 ± 0.09 mmol/l, mean ± SEM) were significantly (t-test, $p < 0.01$) higher than in spring (2.20 ± 0.08 mmol/l), while they were similar (~2.25 mmol/l) in diurnal fish in both seasons. These findings revealed for the first time insights into the seasonal physiological changes that accompany changes in behavioral rhythms in diurnal and nocturnal sea bass.

3.4. Experimental Chapter 4:

Chronobiology International, 28(1): 39–47, (2011)

ISSN 0742-0528 print/1525-6073 online
DOI: 10.3109/07420528.2010.530728

informa
healthcare

Circadian Rhythms of Self-feeding and Locomotor Activity in Zebrafish (*Danio Rerio*)

Ana del Pozo, Jose Antonio Sánchez-Férez, and Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia Espinardo Campus, Murcia, Spain

Published in: Chronobiology International, 28(1): 39–47, (2011)

URL: http://web.ebscohost.com/ehost/pdfviewer/pdfviewer?vid=3&sid=45c45f21-e676-4dbc-9b3a-c7684ca93e97%40sessionmgr111&hid=127

Impact Factor: 4.028 (2011)

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
BIOLOGY	85	13	Q1
PHYSIOLOGY	79	11	Q1

Circadian rhythms of self-feeding and locomotor activity in zebrafish (*Danio rerio*)

Ana del Pozo, Jose Antonio Sánchez-Férez, and Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia Espinardo Campus, Murcia, Spain

ABSTRACT

To investigate daily feeding rhythms in zebrafish, the authors have developed a new self-feeding system with an infrared photocell acting as a food-demand sensor, which lets small-size fish such as zebrafish trigger a self-feeder. In this paper, the authors used eight groups of 20 fish. Locomotor activity rhythms were also investigated by means of infrared sensors. Under a 12 h: 12 h light (L)-dark (D) cycle, zebrafish showed a clear nocturnal feeding pattern (88.0% of the total daily food-demands occurring in the dark phase), concentrated during the last 4 h of the dark phase. In contrast, locomotor activity was mostly diurnal (88.2% of total daily activity occurring in the light phase). Moreover, both feeding and locomotor rhythms were endogenously driven, as they persisted under free-running conditions. The average period length (τ) of the locomotor and feeding rhythms was shorter (τ = 22.9 h) and longer (τ = 24.6 h) than 24 h, respectively. During the time that food availability was restricted, fish could only feed during ZT0–ZT12 or ZT12–ZT16. This resulted in feeding activity being significantly modified according to feeding time, whereas the locomotor activity pattern remained synchronized to the LD cycle and did not change during this trial. These findings revealed an independent phasing between locomotor and feeding activities (which were mostly nocturnal or diurnal, respectively), thus supporting the concept of multioscillatory control of circadian rhythmicity in zebrafish.

3.1. Experimental Chapter 5:

Light pulses at night elicit wavelength-dependent behavioral responses in zebrafish (*Danio rerio*)

A. del Pozo and F. J. Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia,

Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Murcia, Spain.

In preparation

Light pulses at night elicit wavelength-dependent behavioral responses in zebrafish (*Danio rerio*)

A. del Pozo* and F. J. Sánchez-Vázquez

Department of Physiology, Faculty of Biology, University of Murcia,
Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Murcia,
Spain.

In preparation

ABSTRACT

The water column acts as a chromatic filter with different transmittance depending on wavelengths. In the present paper, zebrafish behavioral responses were investigated following the exposure at night to 1h light pulse ($1.4x10^{18}$ photons/m^2/s) of various wavelengths (violet, blue, green, orange, red and white or darkness as controls). To this end, 42 individual fish were video recorded 1 hour before, during and after the pulses. A fish tracking software analyzed the recordings, locating the fish every second. The results revealed that (i) the swimming activity raised by all wavelengths (3.3 to 6.5 fold change) with respect to their activity before the pulse, although white and short wavelengths caused the greatest increases that remaining elevated during hour after the pulse; (ii) the maximum speed and the resting time were also higher and lower at short wavelengths, respectively; and (iii) during the light pulse fish tended to swim downwards (0.7 fold change), recovering their original positions during the following hour (except for violet). No changes in the vertical position appeared under the green and red lights, just showing positive phototaxis after the red one. These profound effects on zebrafish behavior elicited by light (even after the light stimulus) suggest that dissimilar internal signals are activated by different wavelengths.

INTRODUCTION

Most living organisms have developed a circadian clock which let them keep time and predict recurrent environmental changes, being the light one of the most important synchronizers in nature (Goldman *et al.*, 2004). In the underwater environment, light spectrum changes rapidly with depth, since the water column acts as a powerful chromatic filter. Thus, extreme wavelengths (below violet, $\lambda < 390$ nm, and beyond infrared, $\lambda > 700$ nm) are quickly absorbed, while blue wavelengths ($\lambda \approx 450$ nm) reach depths of up to 150 m in clearest oceans (Lalli & Parsons, 1995). Moreover, the dissolved organic matter and suspended particles in coastal and continental waters shift the transmitted light from the blue to the greenish region of the spectra (McFarland, 1986). Fish seems to have adapted their vision and spectrum perception to their natural photo-environment (Kusmic and Gualtieri, 2000; Chinen *et al.*, 2005), containing rods and cones in accordance to the available wavelength range of their particular niche (Bowmaker, 1990; Patridge and Cumming, 1998). In addition, changes in the photic sensitivity could happen during the fish life cycle while exploring new photic environments (caused by migrations, larvae metamorphosis, etc) (Villamizar *et al.*, 2011).

Zebrafish (*Danio rerio*) has become a common model species for chronobiology to investigate the vertebrate circadian clock (Idda *et al.*, 2012). In such chronobiological studies zebrafish has traditionally been considered a diurnal species, which displays locomotor activity during daytime in both larvae and adult stages (Cahill *et al.*, 2002; 1998; Hurd *et al.*, 1998; Lopez-Olmeda *et al.*, 2006). Nevertheless, zebrafish with nocturnal activity appeared at 20 ºC (López-Olmeda and Sánchez-Vázquez, 2009) and under restricted feeding (Lopez-Olmeda *et al.*, 2010), showing independent-phasing between diurnal locomotor and noctunal self-feeding activities (del Pozo *et al.*, 2011).

Behavioral trials in fish were classically performed by direct observation (Huntingford, 2012). In spite of the existence of several commercial packages for behavioral analysis in terrestrial animals, their use in fish is very limited and not always feasible to video record in darkness underwater (Kane *et al.*, 2004; Kato *et al.*, 1996;

Miller *et al.*, 1982). Recently, novel video tracking systems have been designed and tested in our lab to evaluate the fish behavioural responses against anesthetics (Vera *et al.*, 2010, Sanchez-Vazquez *et al.*, 2011) or different lighting conditions (Blanco-Vives *et al.*, 2012).

Although many authors have evaluated the impact of several factors (i.e. shoal, age, strain, toxic exposure, water and light conditions) on zebrafish behavior (de Esch *et al.*, 2012; Paciorek and McRobert *et al.*, 2012; Padilla *et al.*, 2011; Colwill and Creton, 2011; Engeszer *et al.*, 2007), the role of light spectrum remains largely unexplored. In addition, zebrafish seems to present a double spectral sensitivity, since its visual system is especially effective at (i) detecting luminance contrast at short wavelengths (rod vision), favoring the detection into light-scanty aquatic environments (common in zebrafish habitats) against the rather bright light; and (ii) sensing chromatic contrast at middle and long wavelengths (cone vision), which could benefit the underwater vision definition in sunlight continental waters (Cameron, 2002; Losey *et al.*, 1999; Nicol, 1989; Lythgoe, 1968; Easter, 1975).

This paper studies the influence of light spectrum on zebrafish behavior after exposure at midnight to one hour light pulse, which evokes hyperpolarization in dark-adapted photoreceptors (Meissl and Ekstrom, 1988; Thibault *et al.*, 1993; Meissl, 1997; Falcon and Gaildrat, 1997; Falcon *et al.*, 2001). Such a protocol (one hour light pulse at midnight) has been also used to investigate the inhibition of melatonin production in a light intensity-dependent way in tench (*Tinca tinca*) (Vera *et al.*, 2005) and wavelength-dependent way in European seabass (*Dicentrarchus labrax*) (Bayarri *et al.*, 2002) and Senegalese sole (*Solea senegalensis*) (Oliveira *et al.*, 2007). In the present paper we investigated the zebrafish behavioral responses elicited by light of different wavelengths (violet, blue, green, orange, and red) using a LED lighting technology and a novel video tracking system to assess the swimming patterns and vertical position of fish.

MATERIALS AND METHODS

Animals and housing

A total of 35 zebrafish of 0.3g of body weight and 3.3cm of total length were used in the trials. They were obtained from a local supplier (Jumipez S.A., Spain) and kept in two 60L stock aquaria (60 x 30 x 32 cm) in an isolated chamber at the chronobiology lab of Murcia University, where the experiment was conducted. These aquaria were equipped with a system to record the locomotor activity of the zebrafish groups. An infrared photocell, attached in the middle frontal side, detected the fish interruptions and sent a signal to the computer, which recorded the activity data every 10 min. A white fluorescent bulb (F15W/GRO; Sylvania GroLux, Germany) provided 400 lux at the water surface, with a 12 h:12 h light (L):dark (D) cycle, and a heater (75 W Magictherm; Prodac, China) kept the water temperature at 24 ºC. Fish were fed a commercial diet (Nutron Hi-Fi, Prodac, Italy) once a day in the middle of the photophase at approximately 2% of their body weight.

Experimental design

Zebrafish were transferred during the day from the stock aquaria to the recording tank. At night, a one hour light pulse of the five different spectra: violet (V), blue (B), green (G), orange (O) and red (R), was applied to each recording aquarium in the middle of the darkphase (MD) and fish were video recorded for 3 hours, from 1 h before the pulse (MD-1) until 1 h after the end of the pulse (MD+2), so that 3 experimental phases were defined: (i) baseline (Phase 1 = P1, from MD-1 to MD), (ii) 1 h light pulse (P2, from MD to MD+1), and (iii) after effects (P3, from MD+1 to MD+2). As a control, two fish groups were used: fish exposed to white light (W) and fish kept in darkness (D).

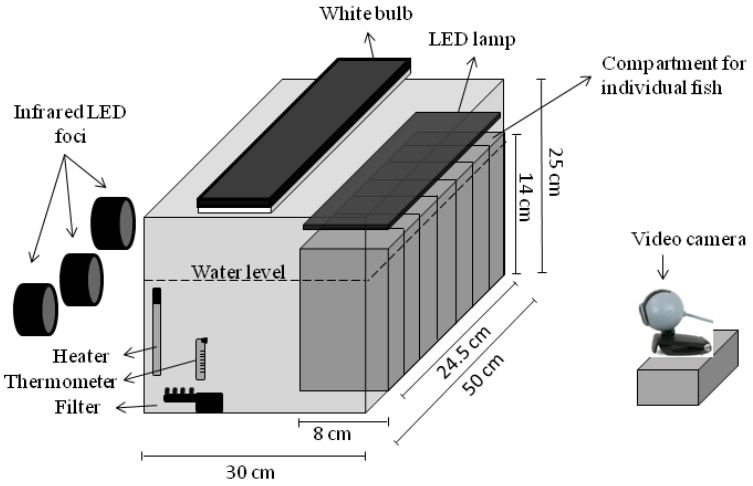

Fig. 1. Diagram of a recording aquarium. Each one was equipped with 7 compartments for individual fish; a white bulb for the normal light cycle of 12h: 12h LD; a LED lamp to apply the light pulses of every color; a video camera to record the zebrafish behavior before, during and after the light pulse; and 3 infrared LED foci, which let the recording in darkness.

Video tracking system

Hardware: swimming activity patterns were recorded in 8L aquaria divided in 7 individual compartments (3.5 x 14 x 8 cm) with white plastic separators with drilled holes which let the water flow freely (Fig. 1). These plastic aquaria were placed inside larger aquaria (50 x 30 x 25 cm) which contained a heater, filter and thermometer. Infrared (IR) LEDs (monocolor diode, model L- 53F3BT, 5 mm) covered with a blurred white panel were located behind the aquaria to record during the night in total darkness. These IR lamps emitted at λ=940 nm, which is not detected by zebrafish. Video cameras (Webcam C250, M/N: V-U0003, Logitech, Switzerland) were adapted for infrared recording by removing the UV filter located in front of the lens. Six colored LED lamps of different wavelengths (400-440 nm violet (V), 442-506 nm blue (B), 503-584 nm green (G), 569-621 nm orange (O), 620-682 nm red (R) and 400-700 nm white (W)) were built and adjusted to $1.4x10^{18}$ photons/m^2/s at the water level, using a spectroradiometer (FieldSpec®, ASD, Colorado, USA) (Fig. 2). Each lamp was placed 6 cm over the water level and turned on and off by means of a

programmable switch (Daily programmer, COATI, 13115, China). Trials were performed inside light-proof chambers and every recording aquarium was completely isolated with black plastic sheets to avoid light contamination.

Software: two specialized computer programs were used to record and later analyze the video records (MultiViewer and FishTracker, respectively, both created by G. Ros-Sánchez and G. García-Mateos, Computer Systems Department, University of Murcia). Two stations were simultaneously recorded at one frame per second by MultiViewer. The FishTracker software, which has already been validated and successfully used in sea bream (Vera *et al.*, 2010) and zebrafish (Sanchez-Vazquez *et al.*, 2011), tracked the swimming patterns of fish. The computer analysis of videos provides a text data with the coordinates (X, Y) of each fish in every second.

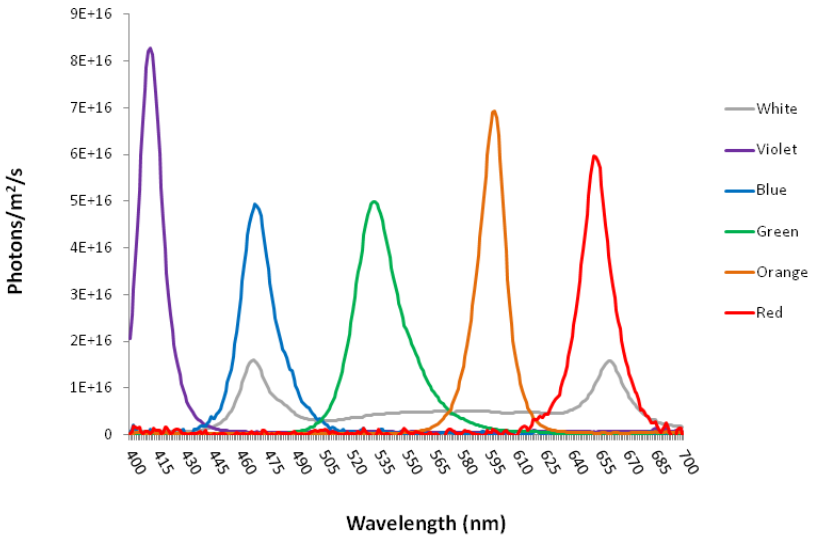

Fig. 2. Spectral composition of each experimental LED lamp (violet, blue, green, orange, red and white) expressed as photons/m2/s (Analytical Spectral Devices FieldSpec® Handheld).

Data analysis

The Chronobiology software "El Temps©" (Prof. Díez-Noguera, University of Barcelona) was used to analyze the activity data from the stock aquaria, which were recorded by the computer in 10-min bins, plotting actograms and mean waveforms.

The swimming patterns were analyzed from the video recording data, using the parameters and formulas represented in Table 1. The swimming activity of every experimental phase (P1, P2 and p3) was calculated by the summation of the swimming activity at every second, while the vertical position of every experimental phase was the mean of the vertical position at every second. To avoid possible individual differences between tests, the data were normalized according to P1. The increases of maximum swimming activity and resting time during P2 and P3 were calculated by means of their difference with P1. Moreover, the moving averages of fish activity and vertical position throughout whole recording were gathered every 5 min to smooth the fluctuations and highlight the trends of the data.

Table 1. Parameters used to define the swimming patterns of zebrafish.

Parameter	Abbreviation	Formula	Variables
Swimming activity	*SA*	$(SA_t) = \sqrt{((X_t-X_{t-1})^2 + (Y_t-Y_{t-1})^2)}$	X_t = coordinate in the horizontal axis every second (t)
Vertical position	*VP*	$VP_t = Y_t$	Y_t = coordinate in the vertical axis every second (t)
Maximum speed	*Vmax*	$Vmax = MAX(SA_t/T)$	$T = 1\ s$
Resting time	*RT*	$RT = \sum t_R / T_T$	$R = SA < 3\ mm$ $T_T = 3600\ s$
Frequency of vertical position changes	$F(C_{VP})$	$F(C_{VP})_Y = \sum T_{Y(P2)} - \sum T_{Y(P1)}$	T_Y = Time that fish spent at the vertical position *Y*.

Statistical analysis

The statistical differences of both activity and mean vertical position throughout 3 experimental phases (baseline, light pulse and 1 h after effects) for every light spectrum were checked by Generalized Linear Model (GLM) for repeated measures, in which the within-subject factors were activity or mean vertical position, with 3 levels (the 3 experimental phases), followed by a Bonferroni test. Moreover, differences in every parameter above described (activity, vertical position, increase of

maximum speed and time in repose) among different spectrum within every experimental phase were tested by one-way analysis of variance (ANOVA I) followed by a Duncan test. To assess if the swimming activity at MD+2 reached the baseline, we used Student t-test repeated measures comparing between the baseline and the last 10 min of the experiment (170-180 min). All statistical tests were carried out with the SPSS v15.0 program (SPSS Inc., USA) and the significant threshold was fixed in $p < 0.05$.

RESULTS

Daily activity rhythms

The actogram revealed a daily activity rhythms strongly synchronized to the LD cycle (Fig. 3A). Zebrafish displayed a typical diurnal activity pattern, with 94.5% ± 0.3% (mean ± SEM) of daily activity displayed during the photophase. A sudden rise of activity was detected after lights on, with a peak of activity around the middle of the day (meal time), and a drop following lights off (see daily waveform, Fig. 3B).

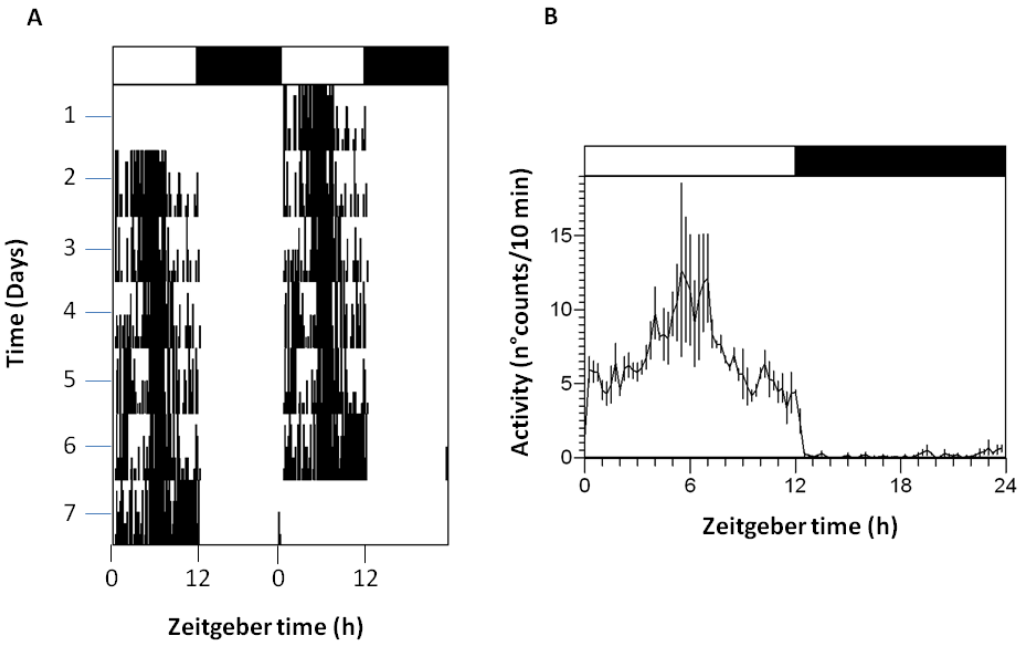

Fig. 3. A representative actogram of the diurnal locomotor activity (A) of a stock group of zebrafish. Actogram is double-plotted (time scale, 48 h) for better visualization, the height of each point representing the number of infrared light-beam (interruptions/10 min). White and black bars in the top of the graphics show the light and dark phase, respectively. Each horizontal line shows one experimental day along the vertical axis, and zeitgeber time (in hours) is represented on the horizontal axis. The mean waveform of locomotor activity from all groups (B), which displays diurnal activity. Vertical axis represents activity (interruptions/10 min) and horizontal axis zeitgeber time (h). Error bars represent the standard error about the mean (SEM).

Swimming patterns

Despite all tested spectra provoked fish relative activation, both the increase quantity and the dynamic of the swimming activity depended on the wavelengths applied (Fig. 4). When fish were exposed to 1 h light pulses at MD they generally increased their swimming activity in comparison with their previous baseline activity (in darkness). Moreover, after the white and blue light pulses such an increase in activity remained sometime after the pulse (P1 < P2=P3, GLM repeated measure, $p < 0.05$); continued increasing (P1< P2 < P3, GLM repeated measure, $p < 0.03$) after the violet and green; decreased until intermediate levels without differing statistically from neither P1 or P2 (P1 < P3 < P2, GLM repeated measure, $p < 0.05$) after the orange; and reached the baseline activity again after the red (P1=P3 < P2, GLM repeated measure, $p < 0.05$). Moreover, the activity profile along the 3 experimental phases especially differed respecting on wavelengths during the lights on and off (Fig. 4), since, the white and short wavelengths (V, B and G) activated zebrafish more sudden after the light on, and kept (or even increased after the violet light) their activities until 10-20 min after the lights off. However, fish activity gradually rose after the long wavelengths (O and R) and immediately decreased after the red light off, while the orange one kept the activity longer. At MD+2, only the fish of blue and green light did not stabilize their activity as basal levels (Student t-test repeated measures, $p < 0.05$). The fish in the D group did not change their activity throughout the trial.

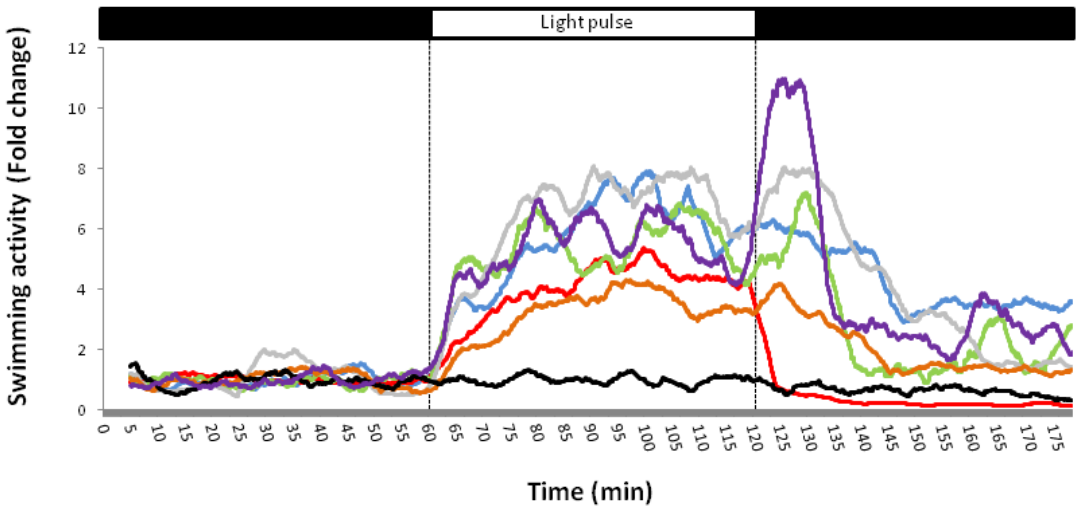

Fig. 4. Mean profile of swimming activity along the 3 experimental phases (P1, P2 and P3) of zebrafish espoused to different light wavelengths (V=violeta color, B=blue, G=green, O=orange, R=red, and W=gray and D=darkness). The vertical axis shows the activity in fold change from the baseline activity, meanwhile the horizontal axis represents the time in minutes. White and dark bars in the top of the graphics indicate the light pulse and darkness periods, respectively.

Regarding the mean activity displayed during the 1h light pulse (P2) and the following 1h in darkness (P3), differences in quantity of activity among light colors were detected, with short wavelengths showing higher effects than the long ones (ANOVA I, $p < 0.01$) (Fig. 5). During the light pulse (P2), the highest activity was observed with white (6.49 fold change from 1.00 ± 0.89, mean ± SEM), violet (5.43 ± 1.70), blue (5.71 ± 0.72) and green (6.15 ± 2.01) lights, while orange (3.25 ± 0.70) and red (4.05 ± 0.22) did not significantly differ from neither of the shorter wavelengths nor the dark control. Similarly, during P3, the highest effects were seen in the white (3.75 ± 0.71), violet (3.48 ± 0.75) and blue (4.25 ± 0.86) lights, followed by green and by orange (1.93 ± 0.74). The activity after the red pulse (1.05 ± 0.14) did not statistically differ to those under darkness (0.64 ± 0.10).

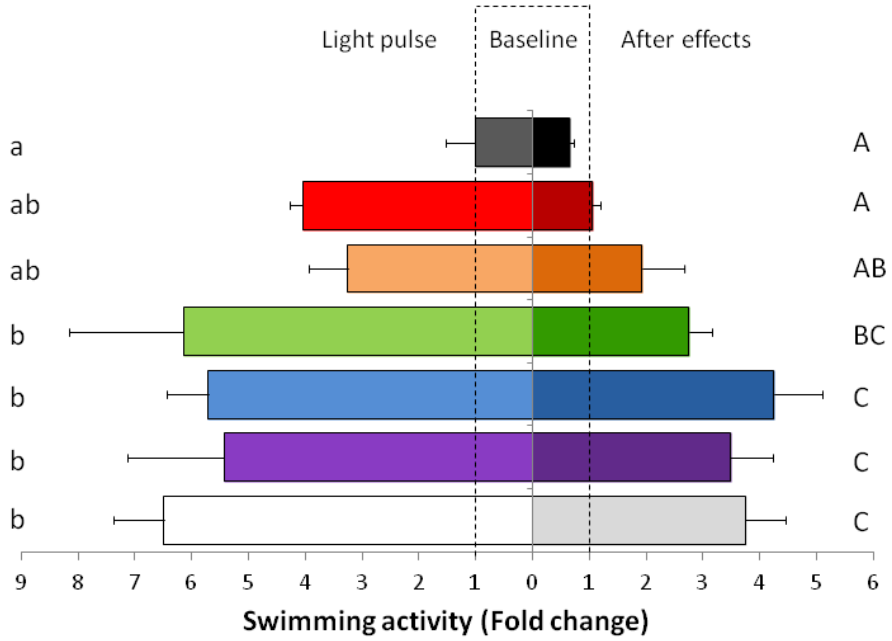

Fig. 5. Increase of swimming activity during the light pulse (left side of the graphic) and the following hour after the light pulse (right side of the graphic) for every wavelength (V=violeta color, B=blue, G=green, O=orange, R=red, and W=white/gray and D=darkness). Horizontal axis shows the increase of activity in fold change, respecting with the baseline activity (which is quantified as 1). ANOVA I followed by Duncan test were performed for each phase, so different small and capital letters denote statistical differences among wavelengths within the light pulse phase and after effects, respectively ($p < 0.01$).

Swimming speed and resting time

The maximum swimming speed during the pulse showed the highest increases under the green (5.54 cm/s ± 0.31, mean ± SEM) and violet (5.71 ± 0.90) lights (ANOVA I, $p < 0.01$), and the green light (3.50 ± 0.78) further caused the highest maximum swimming speed during P3 (G > W,D,B,V > O > R; ANOVA I, $p < 0.01$) (Fig. 6A). In contrast, the shortest resting times appeared under blue (-48.0 % ± 5.04, during the pulse, and -34.6 ± 7.8 after it) and white (-47.8 ± 5.3 during the pulse, and -25.6 ± 7.2 after it) lights during both P2 (D > V > R,O,G > B,W) and P3 (R,D > G > O,V > W > B) (Fig. 6B), and the highest one under violet light (-24.0 ± 6.8) during P2 and red (4.1 ± 14.9) during P3 (ANOVA, $p < 0.02$).

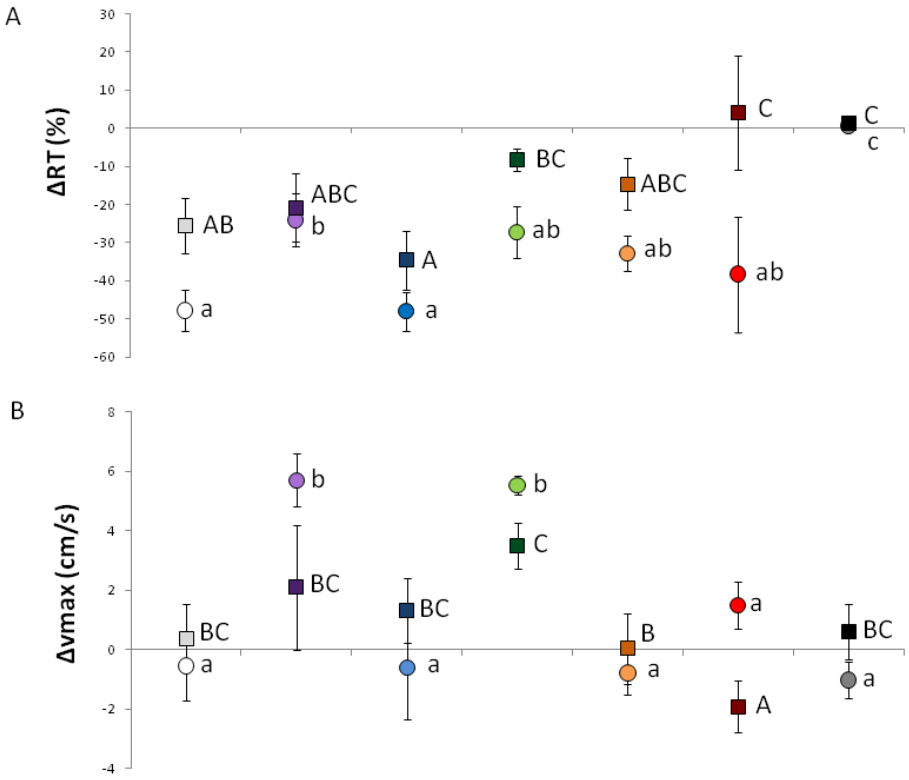

Fig. 6. Differences in the time in repose (in percentage) (A) and maximum swimming speed (in cm/s) (B) during P2 (circles) and P3 (squares), respecting with those of the P1. ANOVA I followed by Duncan test were performed for each phase, so different small and capital letters denote statistical differences among wavelengths (V=violeta color, B=blue, G=green, O=orange, R=red, and W=white/light gray and D=darkness/dark gray) within the P2 and P3, respectively ($p < 0.02$).

Vertical position

The vertical position of zebrafish also varied during and after the light pulse (P2 and P3) depending on wavelengths (GLM repeated measure, $p < 0.05$). At night zebrafish swam near the water surface, but fish showed a photophobic response during the light pulse (P2), and so they moved down towards the bottom (without reaching the lowest 3 cm of the water column though) under all spectra except the green and red lights, in which fish remained near the surface (Fig. 7). During P3, those fish which fall down during the pulse returned to their initial position, with the exception of violet light (P1 > P3 > P2), in which fish were located in a halfway between both P1 and P2 without showing significant differences (0.90 ± 0.06, mean ± SEM). Contrasting, the vertical position exceeded the initial values after the red light pulse (P1 > P2 > P3). The vertical position did not change throughout the trial in the D group.

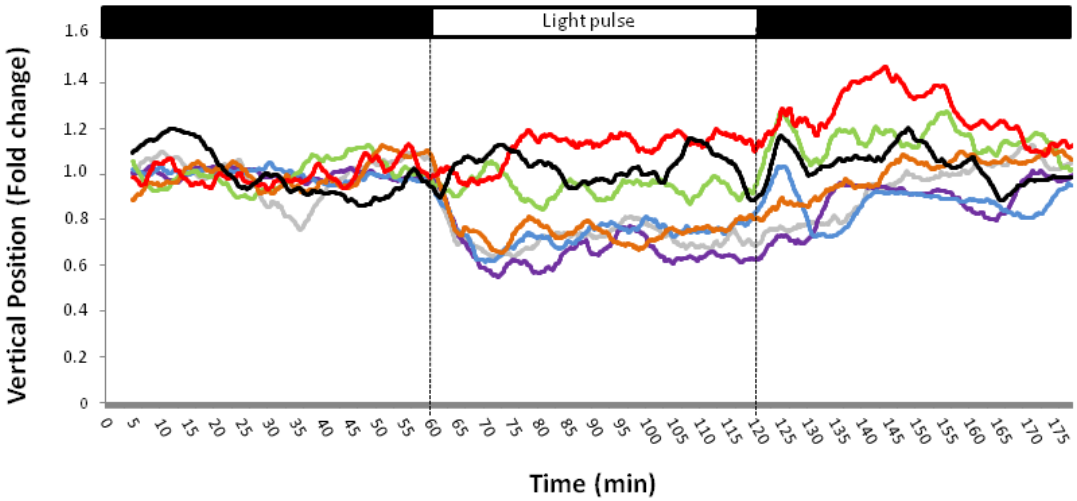

Fig. 7. Mean profile of vertical position along the 3 experimental phases (P1, P2 and P3) of zebrafish espoused to different light wavelengths (V=violeta color, B=blue, G=green, O=orange, R=red, and W=gray and D=darkness). The vertical axis shows the vertical position in fold change from the baseline lacation, meanwhile the horizontal axis represents the time in minutes. White and dark bars in the top of the graphics indicate the light pulse and darkness periods, respectively.

Regarding the preferential vertical position between P2 and P3, the white, violet, blue and orange pulses provoked fish to move from higher locations (13 and 14 cm) to lower one (4 and 5 cm), while the pattern of changes in vertical position under the red light was bimodal: fish moved from mid-positions to higher grounds (11-13 cm) and also to the lowest one (4 cm). The pattern of changes in the vertical position under green light was intermediated between the two previous ones (Fig. 8).

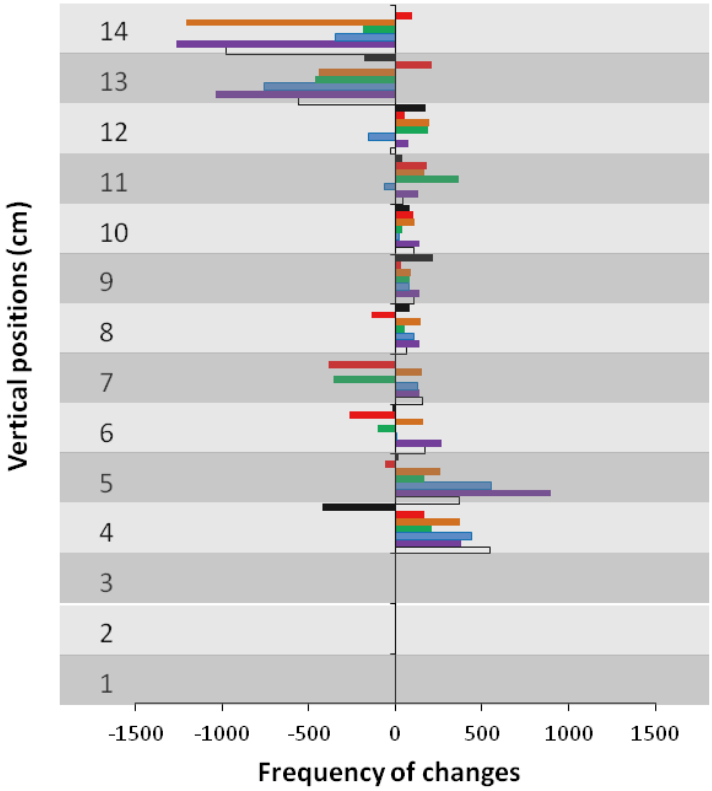

Fig. 8. Preferential vertical positions during the light pulse (P2) under every wavelength (V=violeta color, B=blue, G=green, O=orange, R=red, and W=white and D=darkness). Vertical axis shows the vertical positions (in cm), while the horizontal axis indicates the times which fish come or leave a particular position during P2 (fequency of change).

With respect to the differences in the mean vertical position among light colors during P2, short wavelengths provoked higher photonegative response than the long ones (V, W > B,O > G > D > R, ANOVA I, $p < 0.01$) (Fig. 9). Therefore, the highest descent of zebrafish into the water column appeared under the violet (-0.34 fold change from 0 ± 0.04, mean ± SEM) and white (-0.29 ± 0.04) lights, whereas the red (2.98 ± 0.22) was statistically similar to the darkness. No differences in the vertical position among wavelengths were found among wavelengths during P3 (ANOVA I, $p > 0.05$).

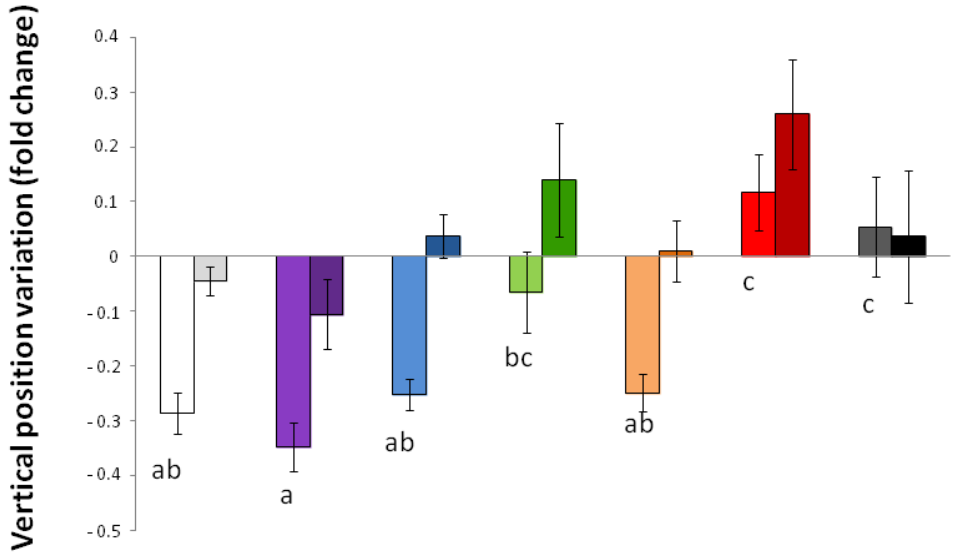

Fig. 9. Variations of vertical position during P2 (light colors) and P3 (dark colors) for every wavelength (V=violeta color, B=blue, G=green, O=orange, R=red, and W=white/light gray and D=darkness/dark gray). Vertical axis shows the variations of vertical position in centimeters, respecting with the baseline location. ANOVA I followed by Duncan test were performed for each phase, so different small letters denote statistical differences among wavelengths within the light pulse phase ($p < 0.01$), however, no differences exist during the following hour after the pulse ($p = 0.07$).

DISCUSSION

Our findings revealed zebrafish differently reacted to light exposure in a wavelength-dependent manner, which effects persisted even after lights off. In general, light exposure at night caused an increase of fish swimming activity and a photophobic response (fish swimming towards the bottom), becoming the highest variations provoked by white and shorter wavelengths (V and B). Zebrafish is considered a diurnal species, although some feeding has been detected at night (del Pozo *et al.*, 2011). In the present research, zebrafish displayed a typical diurnal rhythm of locomotor activity prior to the light pulse exposure.

There seems to be a daily rhythm in the sensitivity of the zebrafish visual system, since the behavioral visual sensitivity of zebrafish oscillates under light-darkness cycles (LD), being more sensitive to light in the afternoon than at night and early morning under white, green or red light (Li *et al.*, 2012; Li and Dowling, 1998; Ren and Li, 2004). This daily rhythm of the zebrafish visual system could be synchronized by

retinal photoreceptor cells (which contain circadian oscillators), but the pineal photoreceptor cells (as a central pacemaker) seem to be required for maintaining the overt circadian rhythms (such as physiology and behavior) (Li *et al.*, 2012). Although, it is known zebrafish possess rods and 4 types of cone photoreceptor cells with maximal sensitivity at 500 nm for rods and 360, 420, 480 and 560 nm for cones (Branchek and Bremiller, 1984; Nawrocki *et al.*, 1985; Robinson *et al.*, 1993), there is not a direct correlation between photoreceptor types and color light response influencing several factors such as photic conditions and developmental plasticity (Neumeyer, 1992; Neumeyer and Arnold, 1989).

In accordance with previous studies where a nocturnal pulse caused physiological effects on fish in a wavelength-dependent way (Bayarri *et al.*, 2002; Oliveira *et al.*, 2007), in the present paper one hour light pulses of 6 wavelengths (peaked between 410—650 nm) in MD provoked dissimilar behavioral responses in zebrafish. Zebrafish swimming activity increased during one hour light pulses of all studied spectra and it also remained elevated during the following hour after the pulse off (P3), except for the red light. However, only fish subjected to the blue or green lights did not reach their baseline activity at MD+2. This zebrafish activation followed a wavelength-dependent gradient, peaking at short wavelengths (violet, blue and green to a lesser extent) and the whole spectrum (white light) during the pulse and also during the following hour. This higher activity increase was followed by an increase of maximum speed (as happened with violet and green lights) or a decrease of resting time (white and blue lights). Similarly, Li *et al.* (2012) observed that zebrafish behavioral visual thresholds varied depending on the exposed wavelength, being higher for the 626 nm red light (that activates red cone photoreceptor cells) than 500 nm blue-green (activate both the rod and green cone photoreceptor cells) and white light (Robinson *et al.*, 1993; Cameron *et al.*, 2002; Saszik and Bilotta, 1999; Bilotta *et al.*, 2001). Contrarily, the maximal spectral sensitivity of optomotor response in adults and larvae of zebrafish appeared at long wavelengths (between green and red) (Kraus and Neumeyer, 2003; Orger and Baier, 2005), while the short wavelengths contributed to phototaxis behavior. In the current study, at midnight zebrafish swam at the water surface in darkness, but a light pulse of the short wavelengths (particularly violet) and

the whole spectrum (white) provoked the strongest negative phototaxis, fish remaining in the lowest vertical position during P3. Contrasting, mid-long wavelengths (green and red, except orange) did not descend fish in the water column, even the red light take them upper than their previous position. This negative phototaxis could be linked with the nocturnal self-feeding behavior (del Pozo *et al.*, 2011), approaching to the surface (where the plankton is more abundant) in absence of light. Our findings support the hypothesis about the particular efficiency of zebrafish visual system to detect short wavelengths, which favor the very useful vision by luminance contrast in aquatic environment, over the long wavelengths that benefic the chromatic contrast, improving the spotting of prey and predators (Cameron *et al.*, 2002; Losey *et al.*, 1999; Nicol, 1989). Differences among authors could be consequence of this double spectral sensitivity of zebrafish or variation in the type of behavioral response (of escape, optomotor, erratic…) observed and/or differences in the light stimuli, since the sort of response is decided by the stimuli properties combined with the limits of fish abilities to see, hear and move (Kim and Wardle, 2003).

In accordance with the literature, blue-green wavelengths (512 nm) were the most efficient for melatonin suppressing in zebrafish pineal *in vitro*, although a lower peak of suppression appeared at middle to long wavelengths (Ziv *et al.*, 2007). As well blue-green and white wavelengths also improved zebrafish early development with respect to the longer ones (Villamizar *et al.*, unpublished data), similarly to other fish species, such as Senegalese sole (*Solea senegalensis*), European seabass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) (Villamizar *et al.*, 2011). Furthermore, blue and white (but not red) lights let the locomotor behavior change from diurnal to nocturnal during metamorphosis, when the larvae become benthonic and so change their light environment (Blanco-Vives *et al.*, 2012).

In summary, short wavelengths and white light lead to the greatest zebrafish behavioral responses: rising swimming activity and maximum swimming speed, as well as shortening resting time and decreasing the fish vertical position into the water column. Moreover, much of these effects of the shortest wavelengths persisted during the following hour after the pulse off (P3). These data indicate that

dissimilar internal signals are activated by different wavelengths of light in zebrafish, resulting in disparate behaviors.

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GENERAL DISCUSSION

4. General Discussion

4.1. Molecular clock

The results obtained in the present doctoral Thesis fill gaps in the existing knowledge of the seabass molecular clock and its components, contributing with the cloning and characterization of two *cryptochrome* genes, *cry1* and *cry2* (Figure 1). These *cry* genes together with the previously reported *period 1* (*per1*) gene (Sanchez and Sanchez-Vazquez, 2010), and recently cloned partial sequences of *period 2* (*per2*), *circadian locomotor output cycles kaput* (*clock*) and *brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-Like* (*Bmal*) (Herrero *et al.*, 2012), form the clock genes which are currently available to investigate and unravel the molecular mechanisms underlying the biological clock functions in seabass as well as its entrainment by environmental cues.

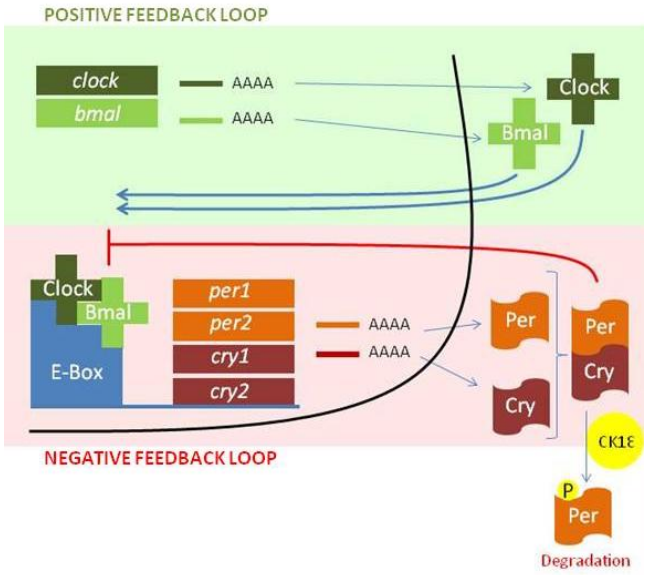

Figure 1. Proposed diagram for molecular clock in seabass, based on the molecular clock of another teleost fish (zebrafish) and the clock genes described in seabass up to now. *Modified from Del Pozo* et al.*, in press.*

of clock genes points to the existence of a decentralized clock in seabass, since *cry1* and *cry2* were expressed in a long number of tissues (brain, liver, heart, retina, muscle, spleen, gill and intestine) in this species, as reported for *per1* in brain, heart, liver, gill, muscle, digestive tract, adipose tissue, spleen and retina (Sanchez *et al.*, 2010).

Additionally, all known clock genes (*per1*, *per2*, *cry1*,*cry2*, *bmal*, *clock*) were expressed in seabass pituitary (Herrero *et al.*, 2012). Similarly, circadian clocks in peripheral tissues have been observed in zebrafish, *Drosophila* and even mammalian cell lines (Tamai *et al.*, 2005). Moreover, the daily rhythm of *cry2* expression differed between different seabass tissues, despite *per1* and *cry1* daily expression rhythms were similar in the three tissues studied (brain, liver and heart) (Sanchez *et al.*, 2010). However, under different self-feeding patterns (diurnal *vs.* nocturnal), the expression pattern of *per1* expression also differed between brain and liver tissues. Thus, the feeding pattern modified *per1* expression in liver, but not in brain where diurnal and nocturnal self-fed seabass displayed the same rhythm of *per1* expression. These results support the hypothesis of a circadian multioscillatory system in teleost fishes (Cymborowski, 2001), which may be entrained by different environmental factors. Thus, the existence of both light-entrainable oscillators (LEO) and food-entrainableoscillators (FEO) have been proposed in several fish species, such as zebrafish, in which scheduled feeding entrained *per1* expression in peripheral oscillators (liver) whereas in the brain *per1* expression was entrained by light (Lopez-Olmeda *et al.*, 2010).

4.2. Physiology

Feeding time affected daily variations of physiological parameters in seabass, such as digestive amylase and blood glucose. Phase-restricted self-feeding affected the amylase activity in mid-intestine, which peaked during the feeding phase in both diurnal and nocturnal seabass, as observed in seabream (*Sparus aurata*) and goldfish (*Carassius auratus*) subjected to feeding cycles, but not when fed at random times (Montoya *et al.*, 2010a; Vera *et al.*, 2007). Blood glucose levels also seem to be changed by feeding time, since an apparent arrhythmicity appeared when the dispersion of self-feeding time within the seabass group increased. The daily patterns of blood glucose differed between seabass subjected to phase-restricted self-feeding under stable environmental conditions (constant photoperiod and temperature), being the highest levels during the night irrespective of feeding conditions. However, seabass kept under simulated natural conditions and displaying seasonal phase inversions,

showed a peak of blood glucose during the self-feeding phase. When diurnal seabass were subjected to nocturnal feeding restriction, their activity pattern became nocturnal and presented lower blood glucose levels than those with free access to food throughout the day, which showed a diurnal activity pattern. However, both diurnal and nocturnal seabass showed the same blood glucose levels during natural feeding inversion in spring. These results suggest that seabass forced to adapt their feeding phase to the food-reward availability, displayed altered blood glucose rhythms. Furthermore, in nocturnal seabass the daily rhythm of blood glucose presented seasonal changes, showing higher amplitude, earlier acrophase and upper mean levels in winter than in spring. Therefore, blood glucose does not seem a direct reflect of feeding (although influenced by it), but a physiological parameter that may be modified by other seasonal biological processes, such as reproduction. Thus, seabass brookstock showed nocturnal activity during the reproduction season (in winter) and higher plama glucose levels during spawning (December-April) and post-spawning (May-July) than during pre-spawning (August-November), becoming diurnal out of the reproductive period (Gutierrez *et al.*, 1987; Villamizar *et al.*, 2012). Our results reinforce this hypothesis since the highest blood glucose levels were found in nocturnal seabass during the winter feeding inversion, whereas the diurnal seabass in winter and also both groups in spring (when the reproductive period should be over) presented lower and similar values.

4.3. Behaviour

In this Thesis, a new design of self-feeder suitable for small size fish was developed and tested in zebrafish and later, in seabass of early age. This self-feeder was activated by approaching, which was successfully tested in both species. Zebrafish learnt quickly how to use it, showing a clear nocturnal daily rhythm (with most of feeding events concentrated during the last 4 h of night). In contrast, the daily rhythm of locomotor activity remained diurnal, showing phase independence between both behavioural rhythms in zebrafish, which traditionally had been considered as a strict diurnal species (Cahill *et al.*, 2002; Lopez-Olmeda *et al.*, 2006; Lopez-Olmeda and

Sanchez-Vazquez, 2009; Sanchez and Sanchez-Vazquez, 2009). Interestingly, time-restriction of food availability synchronized the feeding rhythm but not the locomotor one, which agrees with the multioscillatory hypothesis in teleost fishes.

In adult seabass, the seasonal feeding inversions and their capacity to adapt the food-demands to time-restricted food reward have been widely described (Sanchez-Vazquez *et al.*, 1998; Anthouard *et al.*, 1993; Boujard *et al.*, 1996; Sanchez-Vazquez *et al.*, 1994; 1995a; Azzaydi *et al.*, 2007). However, up to now no seasonal inversions had been reported in seabass of very early stage. In the present Thesis, seabass of early age managed successfully to operate the self-feeder by approaching, exhibiting two seasonal feeding inversions per year (in winter and spring). Despite the possible link between feeding behaviour inversions and reproduction, these seasonal behaviour inversions seem to occur during the whole seabass life cycle, even when the gonad is not mature. Therefore, an endogenous biological clock must be driving and coordinating successive seasonal activities even in the absence of environmental variations, similarly to the circannual phenomenons (reproduction, moult, migrations...) in migratory birds (Gwinner, 2003).

On the other hand, a light pulse at mid-night modified the behavioural responses in zebrafish in a wavelength-dependent manner. The white light and shortest wavelengths had a stronger effect on swimming pattern, activity level and vertical position than longer wavelengths, increasing the swimming activity and causing negative phototaxis. Since these behavioural effects persisted after the lights were off, the effect of spectrum seems to be controlled by internal processes, rather than being a passive response. In agreement with these findings, other authors have reported that in zebrafish lower thresholds of behavioral visual sensitivity appeared under shorter wavelengths (Li *et al.*, 2010). Similarly, the greatest effects of light on the early development of zebrafish were observed when fish were reared under shorter wavelengths (Villamizar, 2012). However, other authors observed the maximal spectral sensitivity of zebrafish optomotor response at long wavelengths (between green and red), although short wavelengths caused phototaxis response (Kraus and Neumeyer, 2003; Orger and Baier, 2005). Our findings support previous studies indicating that zebrafish is particularly sensitive to short wavelengths, which favour the

vision by luminance contrast and therefore optimise eyesight in the aquatic environment, in contrast to long wavelengths which benefit the vision by chromatic contrast (Cameron *et al.*, 2002). This double spectral sensitivity of zebrafish could explain the differences in behavioural responses reported by different authors, depending on the spectral stimuli applied to fish.

4.4. Dual oscillator hypothesis in seasonal inversions

Seasonal processes in fish, such as migrations, feeding and locomotor phase inversions and reproductive cycles, must be associated and time-coordinated. An endogenous multioscillatory circadian system could be driving these seasonal rhythms, by means of several oscillators which interact with each other to form a diurnal or nocturnal "configuration". This hypothesis is supported by: data suggesting the existence of a decentralized clock in fish (e.g. gene and behavioural rhythms can be entrained by different *zeitgebers*), the low frequency of free-running behavioural rhythms with two free-running components under continuous darkness, the phase instability in behavioural rhythms and the fact that phase inversions do not occur at the same time in all the individuals of a fish group (Cymborowski, 2001; Aranda *et al.*, 1999; Sanchez-Vazquez *et al.*, 1998).

Therefore, a multioscillatory circadian system could lead to the appearance of seasonal dualism (Figure 2). In this way, two oscillators: "Daytime" and "Nocturnal Seasonal Reproduction (NSR)" would be coupled during almost the whole year (from spring to winter), showing a diurnal configuration held by the "Daytime" oscillator, which would suppress the "NSR" nocturnal configuration. However, during the reproductive period (from winter to spring) some external or more probably internal signals (i.e. a neuroendocrine compound from the reproductive axis) would decouple both oscillators, allowing the "NSR" expression and hereby a nocturnal configuration. A similar hypothesis has been reported to explain the zugunrhue behaviour in songbirds (Bartell and Gwinner, 2005). Zugunrhue is the nocturnal migratory restlessness, which strictly diurnal birds show during spring and autumn migratory periods. This zugunrhue is endogenously controlled and strongly coordinated with others processes such as

reproduction, moult and feeding (depending on the nutritional status) (Coppack and Bairlein, 2011; Kumar *et al.*, 2010; Gwinner, 2003). Therefore, the environmental factors would synchronise circannual rhythms that would be involved in the seasonal organization of animal behaviour.

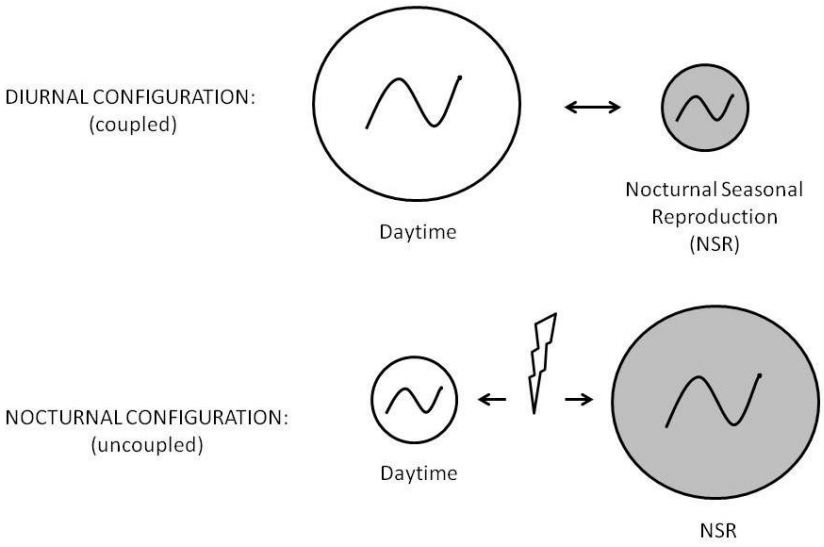

Figure 2. Theoretical diagram proposing the existence of a dual oscillator in fish species. The diurnal or nocturnal configuration depends on the coupling/uncoupling of "Daytime" and "Nocturnal Seasonal Reproduction (NSR)" oscillators. *From Del Pozo et al., in press.*

CONCLUSIONS

5. Conclusions

1. Complete sequences of two *chryptochrome* genes, *cry1* and *cry2*, have been cloned in European seabass (*Dicentrarchus labrax*), enlarging our knowledge of clock genes in this species. Both deduced proteins presented two typical domains of CRY proteins: DNA-photolyase and FAD-binding. Structural and phylogenetic analysis for these proteins have revealed high homology with Cry proteins of other fish species, clustering all Cry of teleosts together and separating Cry1 from Cry2.

2. *Cry1* and *cry2* genes are expressed in all the seabass tissues analyzed (brain, liver, heart, retina, muscle, spleen, gill and intestine), supporting the existence of multiple clocks in this species. Moreover, the daily rhythm of *cry1* expression was similar in seabass liver, heart and brain, peaking shortly after the lights were switched on. *Cry2* expression was also rhythmic in liver and brain but not in heart. In liver, the acrophase for both *cry1* and *cry2* rhythms coincided, whereas in brain the acrophase for *cry2* was at the end of the light phase. Therefore, different synchronizers could entrain the *cry* gene expression in different tissues.

3. Seabass adjusted their food-demands to the phase in which food-reward was available, although the daily rhythm of *per1* expression in brain was not affected, with maximum levels around lights onset in both diurnal and nocturnal seabass. However, in liver *per1* expression differed in diurnal and nocturnal seabass, with higher levels in diurnal fish. Therefore, feeding time seems to affect seabass *per1* expression in peripheral oscillators (liver), but not in the central one (brain).

4. The phase of self-feeding influenced differently the daily variations of digestive physiological parameters, such as blood glucose and amylase activity in mid-intestine. On one hand, diurnal and nocturnal seabass subjected to phase-restricted self-feeding showed the highest values of amylase activity during their feeding phase, although blood glucose peaked at night in both groups. On the other hand, diurnal or nocturnal seabass during the natural behavioural inversions showed the highest blood glucose levels during their self-feeding phase. Moreover, the daily rhythm of blood glucose presented seasonal variations: in winter the amplitude was

higher and the acrophase earlier and the nocturnal seabass showed the highest mean levels.

5. A new self- feeding deviced using an approach sensor was designed and developed for small fish. Both zebrafish and juvenile seabass successfully learnt to trigger this self-feeder and finely adjust food intake.

6. Although zebrafish had been considered a diurnal species, self-feeding rhythms took place at night, with most food-demands concentrated during the last 4 h of darkphase. Therefore, zebrafish showed phase independence between feeding and locomotor rhythms, which remained diurnal. Both rhythms were under circadian endogenous control and free-ran in constant conditions, with a period length shorter than 24 h for locomotor (22.9 h), and longer for feeding rhythms (24.6 h).

7. Exposure at night to white light and short wavelengths (i.e. violet and blue) induced the strongest behavioural responses in zebrafish, increasing the swimming activity during the duration of the light pulse. Such changes persisted for one hour after lights off, except after exposure to the red light. In addition, the white, violet, blue and orange lights caused negative phototaxis during the light pulse, although fish recovered their previous vertical position during the following hour after the light off (except for violet). In contrast, green light did not cause phototaxis and red light produced positive phototaxis.

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ANNEXS

7.1. ANNEX I: The biological clock and dualism of Eurpean seabass

Chapter 1.4: The Biological Clock and Dualism

Ana del Pozo[1], Jack Falcon[2], Francisco Javier Sanchez-Vazquez[1].

[1] Department of Physiology, Faculty of Biology, University of Murcia, Campus Mare Nostrum, 30100 Murcia, Spain.

[2] Observatoire Océanologique de Banyuls sur Mer, CNRS-UPMC, UMR 7232, Avenue du Fontaule, 66650 – Banyuls sur Mer, France

CONTENT:

1.4.1. Geophysical cycles and biological rhythms

1.4.2. The molecular clock

1.4.3. Dualism (diurnal *vs.* nocturnal)

- **a) Behavioural patterns (feeding and locomotor)**
- **b) Seasonal phenomenon**
- **c) Digestive physiology**
- **d) Clock genes**

1.4.4. Mechanisms controlling dualism.

- **a) *Zeitgebers***
- **b) Reproduction rhythms and the dual oscillator hypothesis**

1.4.1. Geophysical cycles and biological rhythms

The Earth´s environment is not static, since periodic changes with different periodicities occur, driven by geophysical cycles (Figure 1). The Earth rotation around its axis imposes daily cycles of light and darkness, while the translation around the Sun provokes annual variations (i.e. seasons). Moreover, the rotation of the Moon around the Earth causes tidal as well as lunar cycles. Therefore, it should not be surprising that most living organisms have developed a biological clock to keep time and cope with these predictable changes by anticipating the forthcoming of a recurrent event in their habitat. Fish in general and seabass in particular, are no exceptions to this rule.

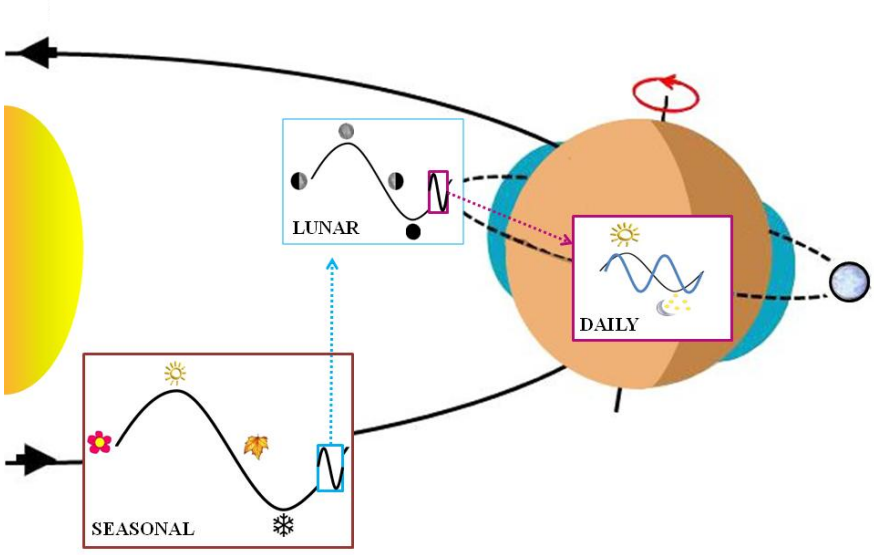

Figure 1. Seasonal, lunar and daily geophysical cycles. Geophysical phenomena, such as the Earth translation around the sun, lunar translation around the Earth and the Earth rotation, lead different periodicities cycles in the terrestrial environment: seasonal ($\tau \approx 365$ days), mensual-lunar ($\tau \approx 28$ days), daily ($\tau \approx 24$ h) and tidal ($\tau \approx 12$ h), being contained the shortest within the longest one.

One of the first evidence for an endogenous biological rhythm was observed in 1729 by De Mairan in *Mimosa* plant, which opened and closed its leaves daily (heliotropy) even indoors during constant darkness. Modern Chronobiology (from Greek: *kronos* for time; *bios* for life and *logos* for study) was established in the middle of XX century by Colin S. Pittendrigh and Jürgen Aschoff, spanning they work from fruit flies to human beings. From those days, a large number of biological rhythms has been described across the whole phylogenetic tree and

recognized as a natural characteristic of all animals including fish, providing a temporal organization for their physiological and behavioural processes (Cymborowski, 2010).

If a biological rhythm persists under constant environmental conditions, it is called an endogenous rhythm, which is generated by a self-sustainable oscillator or pacemaker. Although biological rhythms are characterized by three main parameters: period, amplitude and achrophase, they are classified in three categories depending on their periodicity (or frequency, which is the inverse relation) (Aschoff, 1981):

(i) **Circadian**: when the period (T) is around one day (20h<T>28h);

(ii) **Ultradian**: when T < 20 h, for instance, tidal (T=12,4h) rhythms;

(iii) **Infradian**: when T > 28 h. **Circalunar** (T ≈ 28 days) and **circannual** (T ≈ 365 days) are classified among infradian rhythms.

Environmental time cues synchronise endogenous oscillators, which have a similar but not identical period (tau, τ) than environmental cycles (T). These (biotic or abiotic) environmental cyclic factors are called ***zeitgebers*** ("timegivers" in German) or **synchronizers** (Aschoff, 1981). The role of a biological clock is to give an estimated internal time from the external time, with the aim of any internal process occurs at appropriated time. Therefore, biological rhythms and environmental cycles are coordinated by the following three possible ways: (i) **Synchronization**: when biological rhythms and environmental cycles display the same period and keep on a stable phase relation (phi, φ), but it does not involve necessarily the endogenous rhythmic expression of the clock. (ii) **Entrainment**: synchronization where environmental cycles lead to the biological rhythm. So when the *zeitgeber* disappears (constant conditions), the endogenous rhythm began to **free-run** from the stable phase (phi, φ) determined by the previous *zeitgeber* (Johnson *et al.* 2004). (iii) **Masking**: The environmental stimulus seems to force the rhythm expression (e.g. a photophobic response), but without entraining the endogenous clock. Therefore, the biological rhythm displays the same period and appears in phase with the environmental cycle, but in constant conditions the rhythm disappears or an endogenous rhythm began to free-run from its own phase. The anticipatory characteristic (common in entrained rhythms) is missing in masking, due to the overt rhythm responds directly to the environmental input signal without pacemaker control.

In summary, all environmental factors do not act as *zeitgebers*, even if they show a cyclic pattern. Moore-Ede *et al.* (1982) defined the characteristic to considerer an external signal as *zeitgeber*: when the animal is exposed to the synchronizer, (a) both periods from

biological rhythms and synchronizer must coincide; (b) a stable phase relation must be established between them; when the synchronizer is removed, (c) the biological rhythm must start to free-run from the phase set by the previous synchronizer.

1.4.2. The molecular clock

Over the last decade, our understanding of the basic mechanisms driving vertebrate circadian rhythms and their synchronisation to light has increased enormously. Chronobiological studies in fish have contributed most significantly to this achievement (Idda *et al.* 2012). Indeed, teleosts fish is one of the most successful groups of vertebrates, with adaptations to live in a wide range of ecological time niches. Furthermore, the particular characteristics of the fish´s molecular clock provide a unique opportunity to investigate the flexibility of circadian oscillators and their adaptation to extreme environmental conditions during evolution (Cavallari *et al.* 2011).

The vertebrate molecular clock comprises a set of clock genes classified into two feedback loops: a negative one, with *cryptochrome* (*Cry*) and *period* (*Per*) genes; and a positive one, with *Circadian Locomotor Output Cycles Kaput* (*Clock*) and *Brain and Muscle Aryl hydrocarbon receptor nuclear translocator (ARNT)-Like* (*Bmal*) (Figure 2). CRY protein join PER protein to form a heterodimer complex (CRY:PER), which binds to and blocks the protein complex formed by CLOCK and BMAL (CLOCK:BMAL), inhibiting CRY and PER transcription (Iuvone *et al.* 2005, Okamura *et al.* 2002). Zebrafish molecular clock is the best described one in fish, differing from the mammalian mechanism in the larger number of *cryptochrome* (*cry*) and *period* (*per*) genes. Several subtypes produce proteins with similar, different or still unknown function to mammalian proteins (Kobayashi *et al.* 2000).

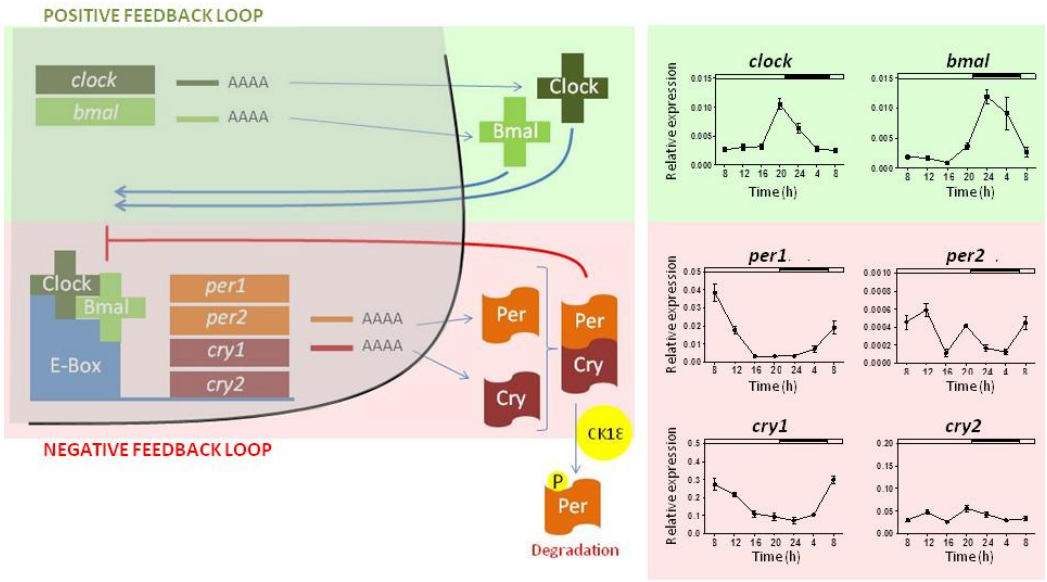

Figure 2. Seabass molecular clock diagram based on the zebrafish one. The left panel shows the diverse seabass molecular clock elements that are identified up to now are represented in according to the well-known zebrafish clock molecular interactions. The clock genes are organized in two feedback loop: (i) the positive one that is composed by clock and bmal genes, whose proteins form the Clock:Bmal complex to active (blue narrows) the negative loop clock genes; (ii) the negative loop with *per* and *cry* (*1* and *2*) genes, whose proteins inhibit their own transcription by means of blocking Clock:Bmal complex. The right panel show: the rhythmic daily expression of every clock gene (except for *Cry2*) in seabass pituitary during summer under 14L/10D (7-21h) at 20ºC (MEAN±SEM, n=5; Cosinor $p < 0.05$). The relative expression is represented in the vertical axis and the time (in hours) in horizontal one. Black and white bars in the top mark the dark and photophase, respectively.

In addition to the central master clock located in the brain, circadian clocks in peripheral tissues have been reported in *Drosophila*, zebrafish, mammalian cell lines and tissues, supporting the hypothesis on the existence of decentralized clocks (Tamai *et al.* 2005). Moreover, *Drosophila* and adult zebrafish tissues, as well as zebrafish cell lines and embryos, are directly light-responsive (Whitmore *et al.* 1998, Schibler *et al.* 2002), which makes them ideal models to investigate the ontogeny of the molecular clock and its light synchronisation pathways.

In seabass, the following clock genes have been recently characterised: *per*, *cry, clock* and *bmal*. *Period1* (*per1*) was first cloned and its expression characterized in different tissues (brain, heart, liver, gill, muscle, digestive tract, adipose tissue, spleen and retina) (Sanchez *et al.* 2010), which generates a protein of 1436 amino acids with conserved regions previously identified as a PER-ARNT-SIM (PAS) fold, a nuclear export signal (NES), carboxy terminal to PAS fold, an short-mutable domain (S/M region), and a carboxy-terminal serine/threonin-glycine (SG) repeat region. In addition, this seabass Period 1 protein (Per1) contained several

conserved Casein Kinase-I (CKI) phosphorylation regions, predictably for its degradation. The seabass Per1 was clustered together with medaka (*Orizyas latipes*) Per1 and zebrafish (*Danio rerio*) Per1a and b. Furthermore, *per1* showed a daily rhythm of expression in brain, heart and liver, with the acrophase (time of day when the rhythm values are maximum) at the end of the night in all of them.

In a recent paper, *cry1* and *cry2* were also cloned and characterized (Del Pozo *et al.* 2012a), codifying two proteins of 567 and 668 aminoacids, respectively, both with two domains: FAD-binding and DNA-photolyase (SMART, $p < 0.01$). Phylogenetic analyses grouped both seabass cryptochromes (Cry) with those of other teleost fish, separating both subtypes *Cry1* and *Cry2* in two different clusters. Moreover, *cry1* and *2* were expressed in all the analyzed seabass tissues (brain, heart, liver, gill, muscle, intestine, spleen and retina). Daily *cry1* expression oscillated rhythmically in brain, heart and liver, peaking around *Zeitgeber* time (ZT) 3:15 h, similarly to *cry2* expression in liver. However, the acrophse of *cry2* in brain was at ZT 11:08 h, and no rhythmical daily expression was observed in heart.

Most recently, partial sequences of *clock* and *bmal* and *per2* genes have been also cloned in seabass (Herrero *et al.* 2012). In this report, the daily patterns of clock gene expression of all known genes (*per1*, *per2*, *cry1*, *cry2*, *bmal* and *clock*) have been seasonally characterized in seabass pituitary along two years cycle. The results revealed that clock genes of the positive feedback loop (*clock* and *bmal*) were higher expressed around lights-off and darkphase, while those of the negative feedback loop (*per1*, *per2* and *cry1* and *cry2*) displayed elevated levels around lights-on and photophase, independently of the season (spring, summer, autumn and winter). However, the amplitude differed between seasons according to temperature, being higher under warmest temperatures (16.0—20.0 ºC) and lower under coldest temperatures (12.3—13.2 ºC). The photoperiod seems to be less important since the acrophases were raised during the warmest (16 ºC) winter. Furthermore, melatonin seems to play a role in the synchronization of gene expression (Dardente *et al.* 2003, Falcon *et al.* 2011), so in seabass pituitary melatonin modified the expression of *cry1* and *cry2* when added to the culture medium of pituitary glands kept in vitro or when added to the fish diet in vivo (Herrero *et al.* 2012).

1.4.3. Dualism (diurnal *vs.* nocturnal)

Light is the most important *zeitgeber* in nature, together with temperature (particularly among ectothermic animals, like fish).Both *zeitgebers* are often synchronised in nature, since the termophase (warmest phase) and the chryophase (coldest phase) are closely related to the photophase and the darkphase, respectively. Most fish adjust their daily activity patterns according to these daily cycles, so that they could be classified as diurnal (the greatest activity occurs during the photophase), nocturnal (the greatest activity occurs during the darkphase) or crepuscular (activity linked to dawn and dusk) (Madrid *et al.* 2001). However, the plasticity of the fish biological clock let some species change their pattern of behaviour. Thus, some individuals can be diurnal while others are nocturnal. Furthermore, the same individuals can switch from diurnal to nocturnal phase, and *vice versa*, along its life (Reebs 2002). This phenomenon is known as **dualism**, which was firstly and commonly described among fish species from high latitude by Eriksson (1978). Since then, more and more fish species have been redefined as dual species, including fish from temperate latitudes (Lopez-Olmeda and Sanchez-Vazquez 2010).

Dualism seems to be more frequent among species consider traditionally diurnal, such as Atlantic salmon (*Salmo salar*) and sharpsnout seabream (*Diplodus puntazzo*), Nile tilapia (*Oreochromis niloticus*) for locomotor activity; rainbow trout (*Oncorhynchus mykiss*) and Artic charr (*Salvelinus alpinus*) for feeding activity; goldfish (*Carassius auratus*), gilthead seabream (*Sparus aurata*), zebrafish (*Danio rerio*) and even European seabass (*Dicentrarchus labrax*), for both locomotor and feeding activities (Landless 1976, Fraser *et al.* 1995, Alanara and Brannas 1997, Sanchez-Vazquez *et al.* 1995a, 1996, Sanchez-Muros *et al.* 2003, Velazquez *et al.* 2004, Vera *et al.* 2006, 2009, Lopez-Olmeda and Sanchez-Vazquez 2009, Montoya *et al.* 2010, Villamizar *et al.* 2012). In contrast, almost no dual behaviour has been reported among traditionally considered nocturnal species, except nocturnal brown bullhead (*Ictallurus nebulosus*) which become gradually diurnal under low light intensity (Eriksson and van Veel 1980). Most nocturnal fish species never change their behaviour and remain strictly nocturnal, such as tench (*Tinca tinca*), Senegal sole (*Solea senegalensis*) or European catfish (*Silurus glanis*) (Lopez-Olmeda and Sanchez-Vazquez 2010).

Another interesting feature of the dualism is the independent phasing displayed by feeding and locomotor rhythms, which could happen during different phases (light or darkness) at the same time in the same fish (Sanchez-Vazquez *et al.* 1996, Del Pozo *et al.* 2011, Fortes-Silva *et al.* 2010). For instance, some goldfish and zebrafish may display diurnal locomotor activity while nocturnal feeding activity. This phenomenon appears associated to dualism as a consequence of the high flexibility of the circadian system of fish (Reebs 2002).

(a) Behavioural patterns (feeding and locomotor)

Feeding behaviour has been more widely studied than their locomotor behaviour in seabass. The development of self-feeders for fish has supported this research, as they are a powerful tool to study feeding rhythms, meal size as well as diet selection when given the choice to choose among different feeds. The reason is these self-feeders let fish feed freely, demanding food from a feeder whenever and as much they want. Since the pioneering self-feeder designed by Rozin and Mayer (1961), different kinds of self-feeders have been created along the last three decades. The self-feeding system is made of three main parts: (i) a feeding sensor, which should be adjust to the fish particular characteristic to active it and avoid the accidental activations, being so, the most variable part (solenoid, rigid lever, flexible string, infrared sensor) (Adron, 1972, Boujard *et al.* 1992, Sanchez-Vazquez *et al.* 1994, Rubio *et al.* 2004, Del Pozo *et al.* 2011); (ii) a food container, which delivery small amount of food after its activation; (iii) a recording system, which monitors the feeding activity. European seabass have used very skillfully both rigid/flexible rods and infrared sensors (Rubio *et al.* 2004, Del Pozo *et al.* 2012b), showing high learning potential and associative abilities (Rubio *et al.* 2003). Moreover, the individual feeding activity within a fish group could be monitored, coupling a PIT tag monitoring device with a self-feeding system (Di-Poi *et al.* 2008, Coves *et al.* 2006). Such a device proved very useful to investigate social interactions and different diurnal/nocturnal feeding patterns in seabass within a group (Millot and Begout 2009).

European seabass was traditionally considered a diurnal fish species till some food demands were recorded during the darkphase (Sanchez-Vazquez *et al.* 1994). Later, the endogenous character of the feeding circadian rhythm was revealed in groups and in single seabass (Figure 3), as well as confirming the dualism under controlled and identical experimental conditions (regarding temperature, light intensity, photoperiod and salinity) (Sanchez-Vazquez *et al.* 1995a). That was the first time where a fish species displayed a dual behaviour indoors independently of seasonal variation in the photoperiod, changes associated with the intensity of light or temperature, or related with the season during which fish are transferred to the laboratory conditions.

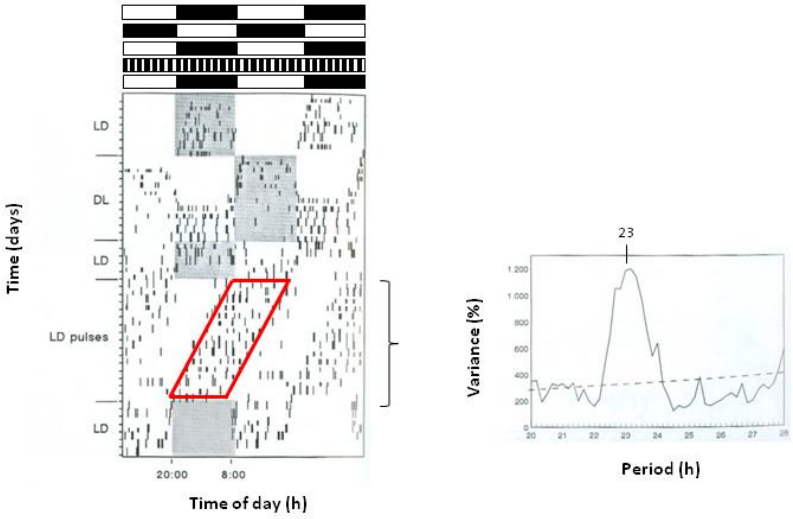

Figure 3. Self-feeding activity of seabass groups (n = 4 fish). In the left panel: the actogram represents the 5 experimental phases along the time (vertical axis): (i) 12 light:12 dark (LD 12:12 h) cycles, (ii) a first photoperiod revision (doubling the light phase), (iii) a second photoperiod reversion (doubling the dark phase), (iv) LD 40:40 min pulses, evidence the endogenous character of seabass feeding rhythm, and (v) the restoration of LD 12:12 h where phase inversion phenomenon (from nocturnal to diurnal) occurred. The horizontal axis signifies the time of day (in hours). The upper black and white bars indicate the dark and light phase for every experimental phase. In the right panel: the free-running periodigram under LD 40:40 min pulses that show a circadian rhythm with τ = 23 h. Vertical axis shows the percentage of the variety of the data that can be explained by fitting the Sokolove-Bushell periodogram method and horizontal axis time (in hours). Modified from Sanchez-Vazquez et al. (1995a).

The seabass group feeding behaviour is not just the sum of individual fish behaviour, but the output of complex social interactions. For instance, in seabass groups provided with a self-feeder, food demands are led by one or a few high-triggering individuals with a well defined circadian rhythm, which feed the rest of the seabass group (Coves *et al.* 2006, Millot *et al.* 2008, Millot and Begout 2009). The feeding activity of the leader synchronizes the food demands of the low-triggering fish, which could copy the feeding behaviour of the high-triggering fish or being stimulated by the food delivered (Millot and Begout 2009). Moreover, when more than one high-triggering fish cohabit in the same tank, they activated the self-feeder following the same hourly rhythm (Coves *et al.* 2006). However, the feeding behaviour of a seabass group is not a single fish reflect, as the leadership may change. Transition to another leader lasted around 4 days (Millot *et al.* 2008). The reasons by each fish acquires a social status within the group and it changes by the time remain unknown. No differences of length, mass or physiological status (muscle composition, plasma and tissues biochemistry)

between high and low triggering fish, either the sex or an initial low specific growth rate (SGR) of the leader seem determine its status (Millot *et al.* 2008, Benhaim *et al.* 2012).

Regarding fish locomotor activity, it can be quantified (i) by an infrared sensor placed in a specific location in a tank, which detects the fish interruptions and sends a signal to a recording computer, or (ii) by video recording the total activity of fish in a tank, and analysing the fish movements with specialised tracking software. Phase inversions in locomotor activity have been observed recently in seabass. Adult seabass showed clearly nocturnal locomotor activity (68% of daily activity occurred at night) during the spawning season (in winter and early spring), but mostly diurnal (65.5% of the daily activity occurred during the daytime) out of the studied reproductive period (April to June) (Villamizar *et al.* 2012). Seasonal phase inversions of both locomotor and feeding behaviour in adult seabass have been also monitored under natural conditions, finding independent phasing between feeding and locomotor rhythms (unpublished data). In that trial, 3 out of 8 groups shifted from nocturnal to diurnal locomotor activity diurnal, while one group remained nocturnal and the other half were arrhythmic. Contrasting, all seabass groups displayed their food demands during the daytime.

b) Seasonal phenomenon

When first reported in fish species from high latitude (Eriksson, 1978) the dualism was considered a phenomenon typical of fish inhabiting extreme photoperiodic conditions (constant light/dark during summer/winter, respectively), so that fish were forced to become diurnal in summer. Jorgensen and Jobling (1989, 1990) also reported seasonal changes in the daily feeding patterns of Arctic charr. The dualism was however reported in fish from temperate regions, such as European seabass (Sanchez-Vazquez *et al.* 1995a), therefore ruling out this “high latitude” hypothesis.

In seabass, changes in their daily feeding pattern were reported. Several authors observed different feeding patterns in seabass depending on the season when the experiments were performed (Anthouard *et al.* 1993, Boujard *et al.* 1996). Anthouard *et al.* (1993) found nocturnal seabass, which became diurnal during the course of the experiment from January to May. Discrepancies in the feeding phase also occurred when comparing April, May and June (Boujard *et al.* 1996). Finally, the seabass seasonal inversions were found by Sanchez-Vazquez *et al.* (1998), who monitored groups of adult seabass along a whole year

under natural environmental conditions (water temperature and photoperiod). In these conditions, seabass shifted from diurnal to nocturnal in winter and *vice versa* in spring, remaining mostly diurnal during the rest of the year. Maximum diurnal feeding activity occurred coinciding with the longest photoperiod (June), while maximum nocturnalism occurred in February (annual lowest temperature). Such changes in feeding behaviour had also practical consequences for fish performance, as observed in a follow up experiment where seabass fed at night in winter had better growth and feed conversion rate (Azzaydi *et al.* 2000).

Although seasonal feeding phase inversions were reported in groups of juvenile seabass, recent research also found such phase inversion in adult seabass (Villamizar *et al.* 2012, unpublished data). It should be noted that phase inversion do not occur simultaneously in all groups, so that some fish advance/delay their switch from one type of phasing to another (Figure 4). This fact is important because it indicates that the switch is not triggered by sudden changes in the environment (critical photoperiod or abrupt temperature changes), but points to an endogenous control.

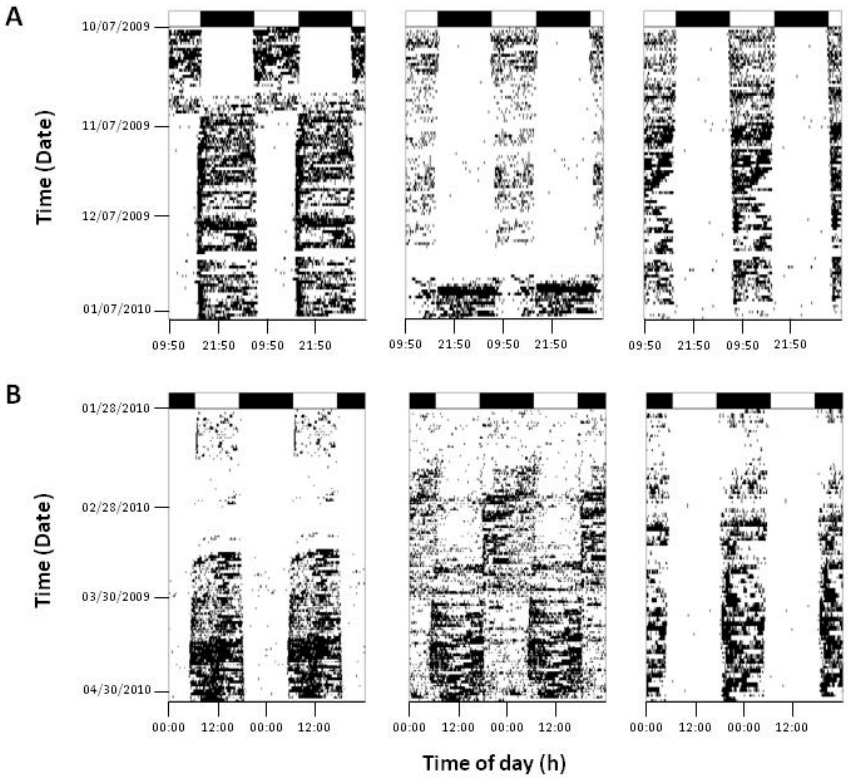

Figure 4. Both seasonal inversions of seabass feeding behavior (A) from diurnal to nocturnal in winter and (B) vice versa in spring. Actograms represent the feeding activity of fish groups, which shift their feeding phase gradually (the earliest to latest inversions are painted from left to right side). Vertical axis shows the time (by date) and the horizontal one the time of day (in hours). Dark and white bars in the top indicate the dark and light phase at the trial beginning. Extracted from Del Pozo et al. 2012b.

Curiously, the pattern of macronutrient self-selection (from three diets with pair of macronutrients) also seems to have a seasonal effect (Vivas, 2007). On one hand, seabass defended the same diet composition along the whole year (64.0% protein, 26.8% lipids and 9.3% carbohydrates). On the other hand, seasonal variability was found in daily profile of macronutrient self-selection: seabass demanded all macronutrients during daytime in summer, while they did at night in winter. In autumn and spring, lipids were demanded during daytime in both seasons, while carbohydrates and proteins were demanded at the beginning of the night.

(c) Digestive physiology

Food availability and prey/predator activity are hardly constant in the wild. Therefore, fish display feeding rhythms synchronised to a specific period of the day or night that best suit their daily way of life. As stated before, the existence of clock-controlled feeding rhythms represents an evolutionary advantage because fish can thus anticipate a meal and prepare their digestive physiologically and metabolism, that entail a better nutrition utilization (Sanchez-Vazquez and Madrid 2001). Consistently, both automatic nocturnal feeding (2 meals: pre-dawn and post-dusk) and (nocturnal) self-feeding in seabass from January to April increased the specific growth rate and reduced feed conversion ratio with respect to automatic diurnal feeding (3 meals: morning, noon and afternoon), the common practice in aquaculture farms (Azzaydi *et al.* 1998). Feeding protocols in fish farms should take in account the seabass dualism, and the seasonal changes in feeding patterns, to improve their production rates.

Annual variations have been reported in several seabass hormones and metabolites, such as insulin, plasma glucose (Gutierrez *et al.* 1987), melatonin (Garcia-Allegue *et al.* 2001), testosterone, estradiol (Prat *et al.* 1990) and plasma lipid levels (Fernandez *et al.* 1989). However, daily physiological differences in seabass with diurnal and nocturnal behavioural patterns have been little explored. Blood glucose has been recently observed in diurnal and nocturnal seabass during both seasonal inversions, when both feeding patterns coexist spontaneously (Del Pozo *et al.* 2012b). Seasonal differences in the daily blood glucose rhythms were found in nocturnal seabass, presenting higher amplitude, earlier acrophase and upper mean levels in winter than in spring. Daily differences were also detected between both self-feeding patterns, starting to increase the glucose levels of both at night but peaking during the respective self-feeding phase (Figure 5A). In another recent trial with diurnal/nocturnal

restricted self-fed seabass, blood glucose rose during the dark phase in both diurnal and nocturnal feeding groups, but they reached higher mean levels in diurnal ones which fitted a circadian rhythm (Del Pozo *et al.* 2012c). Moreover, significant differences were found in mid-intestine amylase activity of both diurnal/nocturnal fish, which increased during the feeding phase (Figure 5B). In short, these results revealed that self-feeding time affects physiological rhythms. Further research is necessary to understand the cause/effect relationship between behaviour and physiology during the dualism.

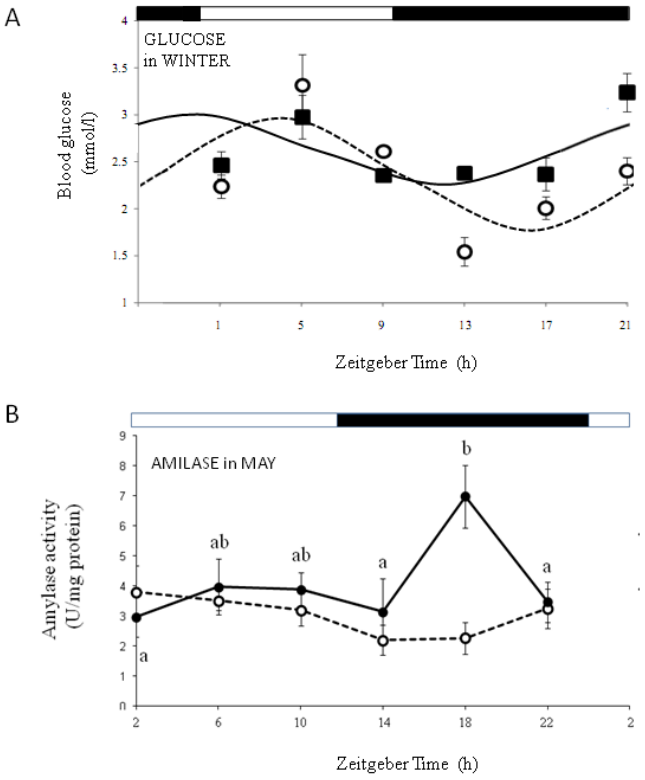

Figure 5. Daily variations in physiological parameter levels of diurnal and nocturnal seabass groups. (A) Mean blood glucose levels (mmol/l) of five fish in winter are presented by dark squares for nocturnal fish and white circles for diurnal. The cosinor adjustment ($p < 0.03$) for diurnal fish is represented by dotted sinusoidal line and continuous sinusoidal line for nocturnal. (B) The mean amylase activities (U/mg protein) of seven fish in May are represented by a continuous line for the nocturnal fish and dotted line for diurnal. Different letters denote significant differences between time points in the nocturnal fish (ANOVA I, $p < 0.01$, followed by Tukey test). Modified from Del Pozo et al. (2012a, b).

(d) Clock genes

As described previously, the key molecular elements involved in the seabass biological clock have been recently reported. Most interestingly, subtle changes in the expression pattern of these rhythms have been found in diurnal/nocturnal seabass. The impact

of diurnal or nocturnal self-feedings on the daily pattern of expression of *per1* has been characterized in brain and liver (Figure 6). Self-feeding time influences *per1* expression in the peripheral oscillator involved in digestive processes (liver), but not in the central oscillator (brain) (Del Pozo *et al.* 2012c). In liver, *per1* expression levels were higher in diurnal than nocturnal seabass, without fitting a cosinor curve under any feeding condition. Contrasting, rhythmic expression in brain was identical in both diurnal and nocturnal fish, with the acrophase around the lights onset as had been previously reported in diurnally fed seabass (Sanchez *et al.* 2010) and other teleost fish (Vallone *et al.* 2004, Lahiri *et al.* 2005, Velarde *et al.* 2009, Sanchez and Sanchez-Vazquez 2009).

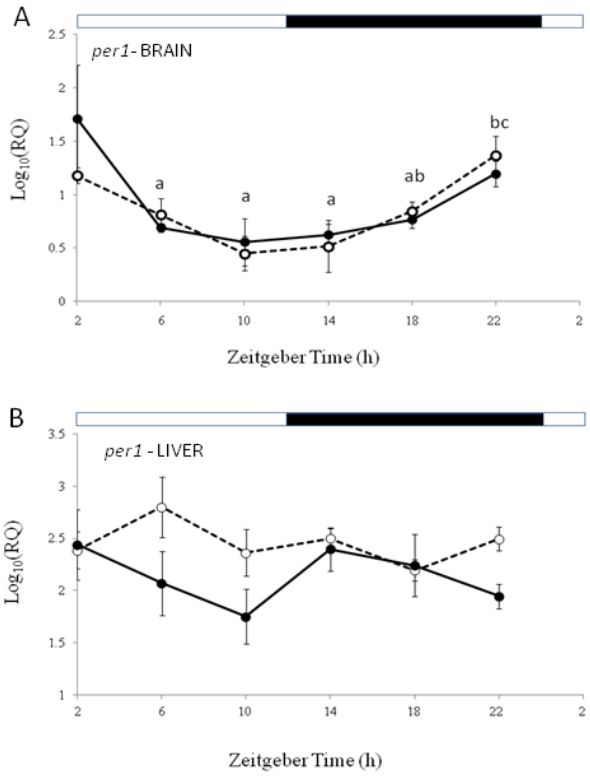

Figure 6. Per1 relative daily expression in brain (A) and liver (B) of diurnal and nocturnal seabass. Each point represents the mean ± SEM of 7 diurnal fish (dotted line) and nocturnal (continuous line). Letters denote significant differences between time points. Black and white bars at the top indicate the dark and light phase. The horizontal axis shows the zeitgeber time (in hours). Modified from Del Pozo et al. (2012a).

1.4.4. Mechanisms controlling dualism

The exact mechanisms which control the switch from one type of phasing to another remain unknown. Nevertheless, it is known that some synchronisers affect the dualism in different ways, and ongoing research on the characterization of seabass clock-controlled genes and reproduction, and their role during the phase inversions are shedding light to understand the cause/effect of dualism.

a) *Zeitgebers*

While changes in photoperiod along the year could explain by themselves the seasonal inversions in high latitude fish, other abiotic (e.g. temperature) and biotic factors (e.g. food availability), together with the photoperiod, could lead seasonal inversions in temperate fish like seabass (Lopez-Olmeda and Sanchez-Vazquez 2010). The following external factors which may drive the seasonal rhythms of feeding behaviour in seabass have been studied:

- The **food availability** restriction seems to be successful to lead the feeding phase inversion in seabass, since some individual seabass or groups (but not all) adapted their food demands to "feeding windows" during photophase and/or darkphase, even when these windows were contrary to their previous feeding phase (Sanchez-Vazquez *et al.* 1995b). This evidence together with a partial coupling between food-entrained and light-entrained activity under conflicting *zeitgebers* (LD 13:13 h and restricted-feeding (RF) 4:20 h), suggesting the existence of a feeding entrainable oscillator (FEO) in addition to the master light entrainable oscillator (LEO). The FEO hypothesis was supported by the higher feeding anticipatory activity (FAA) when the period of both *zeitgebers* (LD cycles and RF) was the same, instead of conflicting periods (LD 26 h and RF 24 h). However, food availability alone is not the only factor involved in the control of dualism, since some seabass did not invert their feeding phase following the food-restriction, and moreover, the food provided in the middle of the food availability phase did not facilitate the inversion (Aranda *et al.* 1997).

- **Photoperiod**: There are two ways to study the photoperiod effect on the animal rhythms: (i) modifying the photoperiod length or (ii) providing the animal with information regarding dark-light transitions in skeleton photoperiods. This latter way let differentiate masking and total darkness effects (Pittendringh 1981). In seabass, photoperiod length strongly synchronized the feeding activity phase, since fish confined their food demands following the contraction and expansion of daytime until extremely

short (2:22 h LD) and long (22:2 h LD) photoperiods (Aranda *et al.* 1999). However, the photoperiod by itself did not control the phasing of the rhythm, thus some individual showed phase inversions (even "double inversions") regardless photoperiod changes. On the other hand, either 15 min or 1 h light pulses separated by 12 h synchronized feeding rhythms in nocturnal seabass, while only the 1 h light pulses does in diurnal seabass. Later, under constant darkness two free-running components appeared, supporting the multioscillatory circadian system.

- **Temperature**: Although seasonal inversions under natural conditions occur with decreasing temperatures around 17º C (in winter) and the highest percentage of nocturnalism occurred at 13.2º C (Sanchez-Vazquez *et al.* 1998). However, in other study about the manipulating water temperature did not drive the seabass feeding inversions. Cool water (16º C) did not manage to shift the feeding phase from diurnal to nocturnal (Aranda *et al.* 1999). Moreover, two fish of a high temperature group remained nocturnal when the temperature rose.

- **Photoperiod/Temperature**: Variations in photoperiod and water temperature are connected in nature; therefore, both factors should be manipulated together. Nevertheless, when a short photoperiod was combined with low water temperatures, and a long photoperiod with high water temperatures, seabass failed to change their feeding phase, which remained (photophase for diurnal and darkphase for nocturnal fish).

In summary, experimental manipulation of these two environmental factors (photoperiod and water temperature) failed to produce consistent changes in phasing. One explanation could be the need for gradual changes, similar to those that occur in nature along the seasons.

b) Reproduction rhythms and the dual oscillator hypothesis

In the wild teleost fish from temperate waters synchronize their reproductive rhythms to the natural environmental cycles in order to ensure the best environmental conditions for the offspring (Oliveira and Sanchez-Vazquez, 2010). Thus, fish have developed time-keeping systems that use cyclic oscillations of photoperiod as a reliable environmental cue to anticipate and activate gametogenesis long before spawning. Melatonin, the "time-keeping" hormone secreted by the pineal, acts as a phototransducing signal on the hypothalamic-pituitary-gonad axis timing the production of gonadotropins, sex steroids and growth factors in the fish gonad

(Amano *et al.* 2000, Bromage *et al.* 2001, Bayarri *et al.* 2004, Falcon *et al.* 2007). Actually, manipulating light and thus melatonin rhythms, has an impact on the fish biological clock and reproduction rhythms, which can be used for aquaculture purposes. Increasing/decreasing photoperiods or continuous light regimes can be used to fully inhibit reproduction and prevent precocity, although the results are species-dependent (Falcon *et al.* 2010). In European seabass light affects the daily rhythm of luteinizing hormone (LH) and also the daily melatonin rhythm, which oscillates faithfully to the seasonal changes of daylength (García-Allegue et. al. 2001, Bayarri *et al.* 2004). Furthermore, in seabass a given photoperiod is needed to sustain circadian oscillations in reproductive hormones and normal reproduction, because continuous light suppresses daily rhythms of key hormones such as melatonin and LH in fish, fully arresting gonad development and maturation (Bayarri *et al.* 2009). These issues will be further discussed in different chapters in this book.

In seabass, maturation starts in September/October and post-vitellogenic oocytes are first observed in December. Ovulation lasts from January to mid-March coinciding with the spawning period (Asturiano *et al.* 2000). Other seasonal changes have been observed regarding plasma concentration of vitellogenin and sex esteroids (E2 and T) which present a peak between December and February (Prat *et al.* 1990). Most interestingly, the observed seasonal phase shifts of feeding rhythms of European seabass (nocturnal during winter, diurnal in spring-summer) (Sanchez-Vazquez *et al.* 1998) coincides with the spawning and resting season of the adults. This finding strongly suggests that the seasonal change of behaviour is linked to reproduction and it may be based on the particular reproductive strategy of this species. Indeed in a recent study, European seabass broodstock showed nocturnal locomotor activity during the spawning season (winter and early spring) and diurnal through the resting period (Villamizar *et al.* 2012). Although the role of environmental cues on the synchronization of reproduction in seabass is clear, previous studies have also found that when this species is kept under constant photoperiod it is able to maintain the annual spawning rhythm, which suggests a strong endogenous mechanism that regulates the reproductive process (Prat *et al.* 1999).

Since fish synchronize reproduction to environmental factors in order to select the best season to reproduce, it is reasonable to think that the egg release and their subsequent fertilization also occur at a specific moment of the day or night. In most fish species the discovery of daily modifications in oocyte maturation and the secretion of sexual steroids or gonadotropins have led to the study of the daily reproduction rhythms. Indeed species such as the red snapper *Lutjanus campechanus* (Jackson *et al.* 2006), gilthead seabream *Sparus aurata*

(Meseguer *et al.* 2008) and the zebrafish *Danio rerio* (Blanco-Vives and Sanchez-Vazquez 2009) spawn at specific times of the day/night. In seabass, the daily spawning rhythm has a nocturnal acrophase, so that eggs start to be released 4 h after lights off, with two spawning peaks, at 6 and 11 hours after lights off. Further observations found that the egg viability was highest in the batches released when spawning peaked and the reproductive activity of the broodstock was positively correlated with its locomotor activity (Villamizar *et al.* 2012). This finding further links the dualism of seabass with the timing of reproduction.

It should be also noted that although seasonal phase inversions occur in parallel with reproduction, juvenile seabass also become nocturnal in winter and stay diurnal along the rest of the year (Sanchez-Vazquez *et al.* 1998, Del Pozo *et al.* 2012b). The reproductive period take place in winter, driven by annual changes in the mRNA expression and hormone levels of key elements of the reproductive axis, such as gonadotropin-releasing hormone (GnRH), three gonadotropin (GtH) subunits, namely glycoprotein α (GPα), follicle-stimulating hormone β (FSHβ) and luteinizing hormone β (LHβ), which alter their levels from the first year of seabass life (Moles *et al.* 2007) in immature fish. Possibly these genetic, physiological and behavioural annual variations are controlled by a clock system, which provides seabass with daily and seasonal information.

Finally, Aranda *et al.* (1999) hypothesized that feeding rhythms are driven by an endogenous multioscillatory circadian system, by means of several oscillators interact to form a diurnal or nocturnal "configuration". This hypothesis is supported by: (i) the low frequency of free-running rhythms, (ii) the phase instability, (iii) two free-running components appeared under continuous darkness, and (iv) the phase inversions do not occurred at the same time in all fish. Expanding this hypothesis based on our current knowledge, a **dual oscillator** circadian system could lead the seasonal appearance of seabass dualism. In this way, two oscillators: "**Daytime**" and "**Nocturnal Seasonal Reproduction (NSR)**" are coupled during almost whole year (from spring to winter), showing a diurnal configuration held by the "Daytime", which also is suppressing the "NSR" nocturnal configuration (Figure 7). However, during the reproductive period (from winter to spring) some external or more probably internal signal (i.e. a neuroendocrine compound from the reproductive axis) decouples both oscillators, allowing the "NSR" expression switch to a nocturnal configuration. A similar hypothesis has been reported to explain the "*zugunrhue*" behaviour in songbirds (Bartell and Gwinner 2005). Zugunrhue is the nocturnal migratory restlessness, which otherwise strictly diurnal birds show during spring and autumn migratory periods. This zugunrhue is endogenous circadian and circannual controlled and strongly coordinated with others processes such as reproduction,

moult and feeding (in according to the nutritional status) (Coppack and Bairlein 2011, Kumar *et al.* 2010, Gwinner 2003). Therefore, environmental factors would act on circannual rhythms that be closely involved in the seasonal organization of animal behaviour.

Figure 7

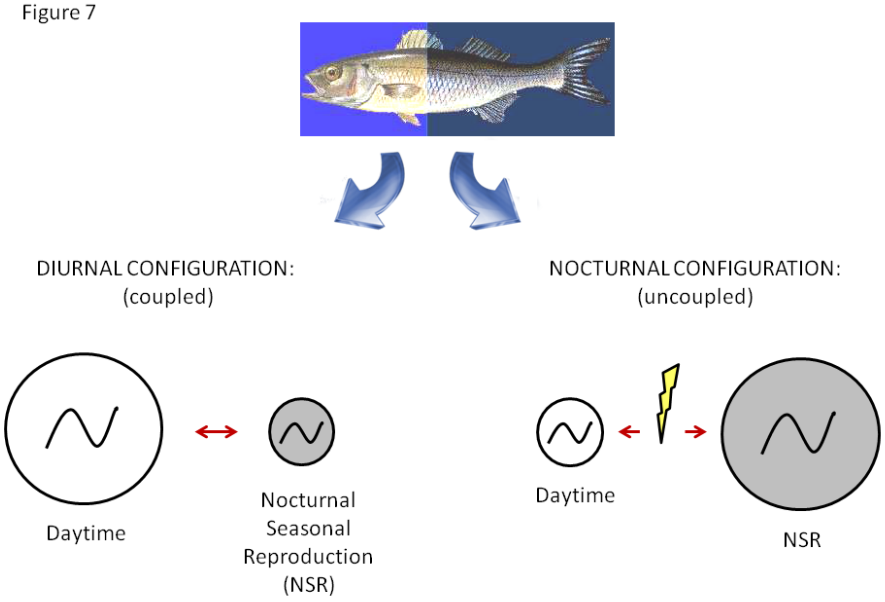

Figure 7. Diagram of dual oscillatory system, which could regulated the seabass seasonal behavior inversions, linking to other annual phenomena, such as reproduction. Two oscillators, "daytime" and "Nocturnal Season reproduction (NSR)", would be normally (from winter to spring) coupled (as indicate the double headed narrow), so "daytime" would suppress the "NSR" nocturnal configuration and provide a diurnal configuration. Nevertheless, "NSR" would gain relevance when some external or internal signal decouples both oscillators, displaying the nocturnal configuration in winter. A reproduction signal may act as decoupler to modify the behavior configuration to nocturnal, which has place during the reproductive period, in winter.

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7.2. ANNEX II: *Curriculum vitae*

PERSONAL DATA

Forename: Ana

Family name: del Pozo Cano

ID no: 48503807G Date of birth : 27/02/1985 Gender: Female

Nationality: Spanish

PRESENT PROFESIONAL POSITION

Institution: University of Murcia

Faculty, School or Institute: Faculty of Biology

Department: Department of Physiology

Address: Campus de Espinardo, C.P. 30100 - Murcia

Country: Spain

Telephone: +34 868 884931

Fax: +34 868 883963

E-mail: adelpozo@um.es

Field of study (UNESCO codes): 240102, 240705, 240108, 240113, 240991, 249001, 310502.

Professional status: Unemployed Start date: 01/01/2013

Administrative status

☐ Permanent Staff ☐ Hired on contracts ☐ Acting ☐
Fellowship holder X Others specify: PhD Student

☐ Full-time ☐ Part-time

PRESENT RESEARCH AREA

Brief summary (key words).

Biological rhythms in fish: behavioural, endocrine, enzymatic. Influence of light intensity and spectrum on biological rhytms in teleosts species. Light-induced cell proliferation in fish. Clock genes in fish. Food entrainment of fish rhythms. Influence of spectrum and feeding schedule on the early development of fish. Fish welfare.

ACADEMIC BACKGROUND

Bachelor	Centre	Date
Bachelor of Biology	University of Murcia	June 2008

MSc	Centre	Date
Aquaculture: "Both basic and applied aspects"	University of Murcia	June 2009

MSc Thesis title: "Ritmos diarios de actividad locomotora y alimentación a demanda en pez cebra: Sincronización a la luz y a la hora de alimentación". **Defence**: 15/07/2009 **Mark**: 9.8 (Outstanding)

PAST SCIENTIFIC EXPERIENCE (*)

Position	R&D Centre	Institution (**)	Start date	End date
Collaborating student for research	Dep. of Physiology	University of Murcia	09/2006	06/2008
Collaborating researcher (MEC)	Dep. of Physiology	University of Murcia	09/2007	06/2008
Starting researcher (UMU)	Dep. of Physiology	University of Murcia	09/2008	12/2008
Predoctoral Fellowship (UMU)	Dep. of Physiology	University of Murcia	01/01/2009	31/12/2010
Predoctoral Contract (UMU)	Dep. of Physiology	University of Murcia	01/01/2011	31/12/2012

(*) The information contained in this chart will be used to verify the placement requirements, according to the section twenty-fourth.3 of the Call. (**) If the Institution is a “joint centre”, you should specify all the centres involved in running it.

LANGUAGES (N = NORMAL, G = GOOD, P = PERFECTLY)

Language	Speaking	Reading	Writing
Spanish	P	P	P
English	N	G	N

PARTICIPATION IN RESEARCH PROJECTS

PROJECT TITLE: Influencia del ciclo de fotoperiodo, temperatura y alimentación en el desarrollo del sistema circadiano: implicaciones en el establecimiento de ritmos de actividad alimentaria y reproducción en peces.
FINANCIAL ENTITY: Spanish Ministry of Education and Science (MEC, AGL2007 66507 C02 02).
LENGHT FROM: 2008 TO: 2010
PRINCIPAL INVESTIGATOR: Dr. Francisco Javier Sánchez Vázquez

PROJECT TITLE: Ontogeny of the sea bass biological clock: light entrainment during early development and influence on the diurnal/nocturnal feeding rhythms.
FINANCIAL ENTITY: Fundación Séneca (08743/PI/08).
LENGHT FROM: 2009 TO: 2011
PRINCIPAL INVESTIGATOR: Dr. Francisco Javier Sánchez Vázquez

PROJECT TITLE: Aquagenomics: mejora de la producción en acuicultura mediante herramientas de biotecnología.
FINANCIAL ENTITY: MEC (Programa Consolider-Ingenio 2010, proy. Nº 28502).
LENGHT FROM: 2007 TO: 2012.
PRINCIPAL INVESTIGATOR: Dr. F.J. Sánchez-Vázquez. (Coordinator: A. Figueras)

PROJECT TITLE: Fish chronobiology network: ontogeny of the biological clock and reproduction rhythms.
FINANCIAL ENTITY: Spanish Ministry of Science and Innovation (MICINN) (Acciones Integradas, IT2009-0057 y DE2009-0045).
LENGHT FROM: 2010 TO: 2012.
PRINCIPAL INVESTIGATOR: Dr. F.J. Sánchez-Vázquez.

GRANTS AND SCHOLARSHIPS OBTAINED

NAME OF THE PROGRAM: Aid for predoctoral stay in a foreign institution
FINANCIAL ENTITY: University of Murcia
DURATION: 3 months (2011)
NAME OF ORGANISATION: Institut für Toxikologie und Genetik (Karlsruhe Institute of Technology)
LOCATION: Karlsruhe, Germany
TOTAL AMOUNT: 4.360 €

PROJECT TITLE: Influencia de los ritmos de alimentación diurnos/nocturnos sobre el comportamiento y el reloj molecular del Pez cebra y la Lubina.
NAME OF THE PROGRAM: Predoctoral Fellowships UMU
FINANCIAL ENTITY: University of Murcia
DURATION: 4 years (01/01/2009 - 31/12/2012)
NAME OF ORGANISATION: University of Murcia
LOCATION: Murcia, Spain
TOTAL AMOUNT: 62400 €

NAME OF THE PROGRAM: Aid for integration into research
FINANCIAL ENTITY: University of Murcia
DURATION: 4 months (September - December 2008)
NAME OF ORGANISATION: University of Murcia
LOCATION: Murcia, Spain
TOTAL AMOUNT:

NAME OF THE PROGRAM: Collaborating researcher
FINANCIAL ENTITY: Spanish Ministry of Education and Science (MEC)
DURATION: 7 months (December 2007 – September 2008)
NAME OF ORGANISATION: University of Murcia
LOCATION: Murcia, Spain
TOTAL AMOUNT: 3000 €

PUBLICATIONS

Key: B= full book, CB.= chapter of book, A= article, R= review, E= editor
(*) Those publications in process and not yet published, just specify publication status.
(**)When applicable/available

AUTHORS: **Ana del Pozo**, Jose Antonio Sánchez-Férez, Francisco Javier Sánchez-Vázquez
TITLE: Circadian rhythms of self-feeding and locomotor activity in zebrafish (*Danio rerio*)
JOURNAL TITLE: Chronobiology International KEY: A
VOLUME: 28(1) FIRST AND LAST PAGE: 39–47
DATE OF PUBLICATION (*):2011
JOURNAL QUARTILE (**): Q1 (Biology); Q1 (Physiology)
IMPACT FACTOR (**): 4.028 (2011).
TOTAL NUMBER OF TIMES CITED: 4

AUTHORS: **Ana del Pozo,** Ander Montoya, Luisa María Vera, Francisco Javier Sánchez-Vázquez
TITLE: Daily rhythms of clock gene expression, glycaemia and digestive physiology in diurnal/nocturnal European seabass.
JOURNAL TITLE: Physiology & Behavior KEY: A
VOLUME: 106(4) FIRST AND LAST PAGE: 446-450
DATE OF PUBLICATION (*): 2012
JOURNAL QUARTILE (**): Q2 (Behavioural sciences)
IMPACT FACTOR (**): 2.869 (2011).
TOTAL NUMBER OF TIMES CITED: 1

AUTHORS: **Ana del Pozo,** Luisa María Vera, Jose Antonio Sánchez, Francisco Javier Sánchez-Vázquez
TITLE: Molecular cloning, tissue distribution and daily expression of cry1 and cry2 clock genes in European seabass (*Dicentrarchus labrax*).
JOURNAL TITLE: Comparative biochemistry and physiology A-Molecular & Integrative physiology VOLUME: 163 KEY: A
DATE OF PUBLICATION (*): 2012 FIRST AND LAST PAGE: 364-371
JOURNAL QUARTILE (**): Q1 (Zoology); Q2 (Physiology)
IMPACT FACTOR (**): 2.235 (2011).
TOTAL NUMBER OF TIMES CITED: 0

AUTHORS: **Ana del Pozo,** Luisa María Vera, Ander Montoya, Francisco Javier Sánchez-Vázquez
TITLE: Daily rhythms of blood glucose differ in diurnal and nocturnal European sea bass (*Dicentrarchus labrax* L.) undergoing seasonal phase inversions.
JOURNAL TITLE: Fish Physiology and Biochemistry
DOI: 10.1007/s10695-012-9732-z KEY: A
VOLUME: FIRST AND LAST PAGE:
DATE OF PUBLICATION (*): 2012
JOURNAL QUARTILE (**): Q2 (Fisheries); Q4 (Physiology)
IMPACT FACTOR (**): 1.528 (2011)
TOTAL NUMBER OF TIMES CITED: 0

AUTHORS: **Ana del Pozo,** Jack Falcon, Francisco Javier Sánchez-Vázquez
TITLE: The biological clock and dualism.
BOOK TITLE: The biology of sea bass. (Eds.: F.J. Sánchez-Vázquez and J.A. Muñoz-Cueto)
KEY: CB
DATE OF PUBLICATION (*): In preparation.

STAYS IN INTERNATIONALLY RECOGNIZED CENTRES

KEY: D=Ph.D student, P=postdoctoral. G= guest, S=staff, O=others (specify)

CENTRE: Institut für Toxikologie und Genetik (Karlsruhe Institute of Technology)
PLACE: Karlsruhe COUNTRY: Germany YEAR: 2011 LENGHT: 5 months
KEY: D
TOPIC: Influence of self *vs.* schedule feeding in both zebrafish growth and spawning, and effect of light spectrum on cell proliferation in zebrafish early development.

CENTRE: Laboratoire Ressources Halieutiques de La Rochelle
PLACE: La Rochelle COUNTRY: France YEAR: 2009 LENGHT: 1 week
KEY: D
TOPIC: Performance a sampling of European seabass with diurnal/nocturnal self-feeding.

PRESENTATIONS IN CONGRESSES

AUTHORS**:** J.A. Sánchez, B.Blanco-Vives, N. Villamizar, A. Del Pozo y F.J. Sánchez-Vázquez
TITLE: Ontogenia de mecanismos de genes reloj en Lubina Europea (Dicentrarchus labrax) durante estadíos tempranos de desarrollo.
TYPE OF PRESENTATION: Oral
CONGRESS: II Workshop Hispano-Brasileño de Acuicultura
MEETING PLACE: Murcia (Spain)
DATE: October 2009

AUTHORS**:** J.A. Sánchez, A. Del Pozo y F.J. Sánchez-Vázquez
TITLE: Clonación y distribución de la expresión de dos *cryptochromos* de lubina.
TYPE OF PRESENTATION: Poster
CONGRESS: XII Congreso Nacional de Acuicultura
MEETING PLACE: Madrid (Spain)
DATE: 24-11-2009 / 27-11-2009

AUTHORS**:** A. Del Pozo, J.A. Sánchez y F.J. Sánchez-Vázquez
TITLE: Ritmos diarios de actividad locomotora y alimentación a demanda en pez cebra.
TYPE OF PRESENTATION: Poster
CONGRESS: XII Congreso Nacional de Acuicultura
MEETING PLACE: Madrid (Spain)
DATE: 24-11-2009 / 27-11-2009

AUTHORS**:** J.A. Sánchez, A. Del Pozo y F.J. Sánchez-Vázquez
TITLE: The clock genes: molecular cloning and characterization.
TYPE OF PRESENTATION: Oral
CONGRESS: XIII Congreso de la Sociedad Española de Biología Celular (SEBC)
MEETING PLACE: Murcia (Spain)
DATE: 16-12-2009/18-12-2009

AUTHORS**:** F.J. Sánchez-Vázquez, A. del Pozo
TITLE: Dual-phasing rhythms in fish
TYPE OF PRESENTATION: Oral
CONGRESS: 26th Conference of the International Chronobiology Society
MEETING PLACE: Vigo (Spain)
DATE: 05-07-2010/09-07-2010

AUTHORS**:** Ana del Pozo, Ander Montoya, Luisa María Vera, Francisco Javier Sánchez-Vázquez.
TITLE: Daily patterns of clock gene expression and gut physiology in European sea bass under diurnal and nocturnal restricted feeding.
TYPE OF PRESENTATION: Poster
CONGRESS: XII Congress of European Biological Rhythms Society (ESBR)
MEETING PLACE: Oxford (UK)

DATE: 20-08-2011/26-08-2011

TEACHING EXPERIENCE

ACADEMIC YEAR: 2010/2011
SUBJECT: Animal Physiology
UNIVERSITY: University of Murcia DEGREE: Bachelor of Biology
HOURS: 54

ACADEMIC YEAR: 2010/2011
SUBJECT: Nutrition and Feeding in Aquaculture
UNIVERSITY: University of Murcia DEGREE: Master of Fisheries and Aquaculture Management
HOURS: 6

ACADEMIC YEAR: 2011/2012
SUBJECT: Animal Ecophysiology
UNIVERSITY: University of Murcia DEGREE: Bachelor of Biology
HOURS: 20

ACADEMIC YEAR: 2011/2012
SUBJECT: Human Physiopathology
UNIVERSITY: University of Murcia DEGREE: Bachelor of Biology
HOURS: 5

ACADEMIC YEAR: 2011/2012
SUBJECT: Animal Physiology
UNIVERSITY: University of Murcia DEGREE: Bachelor of Biology
HOURS: 30

ACADEMIC YEAR: 2011/2012
SUBJECT: Functional Biology of Animals
UNIVERSITY: University of Murcia DEGREE: Bachelor of Biotechnology
HOURS: 30

OTHER ACHIEVEMENTS
(in one DIN A4 page only)

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1. Reviewer in the scientific journal “Comparative biochemistry and physiology”.

2. Member of the Board of Directors in the “Association of Young Researchers of the Murcia University” as Secretary (December 2012 - Nowadays).

3. Certificado de Aptitud Pedagógica (CAP). 300h. Date: 6/03/2009. Instituto de Ciencias de la Educación de la Universidad de Murcia.

4. I Jornadas de Prevención en Laboratorios y talleres. 10 Crts. Date: 17/11/2008. Servicio de Prevención de la Universidad de Murcia.

5. Curso: “Análisis Nitrógeno-Proteína, Grasa y Fibra”. 4h. Date: 24/09/2008. Tecnoquim S.L.

6. IV Jornadas de Alimentación y Salud. 15h. Date: 9/11/2007. Depto. Tecnología de Alimentos, Nutrición y Bromatología, University of Murcia.

7. Curso: “El Reloj Biológico del Envejecimiento” 40h. Date: September-2006. Universidad Internacional de Mar, University of Murcia.

8. Curso: “IV Curso de Cría, Mantenimiento y Patología de Animales Exóticos”. 40h. Date: 22/05/2006. University of Murcia.

7.2. ANNEX III: Certificate of stay

CERTIFICADO DE ESTANCIA EN INSTITUCIÓN EXTRANJERA O ESPAÑOLA
CERTIFICATE OF STAY IN A FOREING INSTITUTION

Datos personales/Personal particulars:

Nombre/Name Ana del Pozo Cano
DNI/National Identity Card 48503807G
Organismo de origen/Home Institution Department of Physiology, University of Murcia

Centro receptor/Host Institution:

Nombre/Name Institut für toxikologie und genetik (Karlsruhe institute of technology)
Dirección/Address Hermann-Von-Helmoltz-Platz 1,76344, Eggestenn-Leopoldshafen
País/Country Germany

Persona responsable del centro receptor/Responsable person in the host institution:

Nombre/Name Nicholas Foulkes
Dirección/Post Universität Heidelberg, Grabengasse 1, D-69117 Heidelberg

La presente es para certificar que la persona mencionada anteriormente ha cumplido su estancia en esta institución en las fechas:/This is to certify that the above mentioned person has performed a stay in this institution in the following dates:

Principio/From 13th February 2011
Final/To 23th June 2011

Lugar y Fecha:
City and date: KARLSRUHE, 6/2/13

Firma y sello / Signature & stamp

KIT
Karlsruher Institut für Technologie
Institut für Toxikologie und Genetik
Prof. Dr. Nicholas Foulkes
Postfach 3640, 76021 Karlsruhe

During my stay in the Karlsruhe Institute of Technology, some studies, which are not included in the present Thesis, were performed and they will be part of futher papers in collaboration with that institution. These works have been detailed below:

1. Analyze the influence of spectrum (red and blue) on cellular cycle during the early zebrafish development.

2. Study the growth (weitgh and length) and quality of spawning (number of egg per cross and egg survival) in self-feeded zebrafish.

RESUMEN EN CASTELLANO

8. Resumen en castellano:

El objetivo de la presente tesis doctoral es investigar los efectos de la luz (fotoperiodo y espectro) y los patrones diarios de alimentación (diurnos *vs.* nocturnos) sobre el reloj biológico de dos especies de peces teleósteos: lubina Europea (*Dicentrarchus labrax*) y el pez cebra (*Danio rerio*). Esta investigación fue desarrollada a través de tres niveles organizativos: reloj molecular, fisiología y comportamiento.

Objetivos específicos:

1. Clonar dos genes de *cryptocromo* (*cry1* y *cry2*) en la lubina Europea y describir su distribución por tejidos y su ritmo de expresión diaria en osciladores centrales (cerebro) y periféricos (corazón e hígado).

2. Evaluar las diferencias en el reloj molecular (expresión del gen *period1*) y fisiología digestiva (glucosa sanguínea y actividad amilasa) en lubinas con comportamiento alimentario a demanda diurno y nocturno.

3. Investigar los ritmos diarios de glucosa sanguínea en lubinas durante las inversiones de fase estacionales (cambio en el comportamiento alimentario a demanda desde diurno a nocturno y *viceversa*) en dos momentos diferentes del año (invierno y primavera).

4. Diseñar y testar un comedero a demanda adecuado para pez cebra e investigar sus ritmos diarios de alimentación a demanda y locomoción, así como la naturaleza endógena de estos ritmos bajo condiciones constantes.

5. Estudiar los efectos del espectro de luz sobre las respuestas comportamentales (actividad natatoria, velocidad máxima de natación, tiempo de descanso y distribución vertical) en pez cebra, usando un sistema de seguimiento por video.

Dichas cuestiones científicas se han abordado a través de los 5 siguientes capítulos, los cuales componen la tesis doctoral y están interconectados. A nivel molecular, dos genes de *criptocromo* (*cry1* y *cry2*) han sido clonados y su expresión diaria caracterizada (**Capítulo 1**), así como el ritmo de expresión del gen *period1* (*per1*) en lubinas diurnas y nocturnas (**Capítulo 3**). En lo que concierne al nivel fisiológico, la

influencia de los patrones de alimentación sobre los ritmos diarios de actividad amilasa y glucosa sanguínea ha sido evaluada en lubina (**Capítulo 3**); mientras la conexión entre el ritmo de glucosa y comportamiento diurno/nocturno ha sido estudiado durante las inversiones de alimentación estacionales naturales que ocurren en lubina (**Capítulo 2**). A nivel comportamental, la capacidad de peces pequeños para usar un nuevo comedero a demanda por aproximación ha sido probada en lubinas de estadíos muy tempranos (**Capítulo 2**) y pez cebra adultos (**Capítulo 4**), y sus ritmos de alimentación fueron caracterizados. Además, la capacidad de la lubina para cambiar su fase de alimentación cuando son sometidas a restricciones de recompensa de alimento ha sido investigada en el **Capítulo 3**. Finalmente, los efectos de la restricción horaria de recompensa de alimento y los efectos de la longitud de onda de la luz sobre la actividad locomotora del pez cebra han sido estudiados en el **Capítulo 4** y **5**, respectivamente.

Capítulo experimental 1: CLONACIÓN MOLECULAR, DISTRIBUCIÓN POR TEJIDOS Y EXPRESIÓN DIARIA DE LOS GENES RELOJ *cry1* Y *cry2* EN LA LUBINA EUROPEA (*Dicentrarchus labrax*).

Los ritmos biológicos son conducidos por osciladores circadianos, los cuales son controlados en último lugar por la expresión cíclica de genes reloj. Los cryptocromos (CRY), fotoreceptores para la luz azul, pertenecen a los elementos negativos del bucle de retroalimentación transcripcional dentro del reloj molecular. Este artículo describe la clonación y caracterización de dos *criptocromos* (*cry1* y *cry2*) en lubina Europea, la cual es considerada un modelo de interés cronobiológico debido a su comportamiento dual (diurno/nocturno). Los fragmentos de cDNA clonados codificaron dos proteínas de 567 y 668 amino ácidos, los cuales incluyeron los dominios de unión-FAD y fotoliasa-DNA. Además, ambas proteínas tuvieron alta homología con las proteínas de cryptocromos (Cry) de otros peces telósteos. Estos genes cry1 y cry2 fueron expresados en varios tejidos de lubina (cerebro, hígado, corazón, retina, músculo, vesícula, agallas e intestino). Al mismo tiempo, la expresión diaria de *cry1* fue rítmica en cerebro, corazón e hígado con acrofases alrededor de ZT 03:15 h (después del encendido de las luces). Similarmente, la expresión diaria de *cry2* fue rítmica en hígado, mostrando su pico en ZT 03:28 h, mientras en cerebro la

acrofase tuve lugar en ZT 11:08 h (poco antes del apagado de las luces). Estos hallazgos aportan nuevos elementos para ayudar a comprender el funcionamiento del reloj molecular de la lubina.

<u>Capítulo experimental 2</u>: RITMOS DIARIOS DE EXPRESIÓN DE GENES RELOJ, GLUCEMIA Y FISIOLOGÍA DIGESTIVA EN LUBINAS DIURNAS/NOCTURNAS.

La lubina es una especia de pez con comportamiento alimentario dual (diurno/nocturno), aunque poco se conoce sobre los cambios en su reloj molecular, fisiología y metabolismo, en relación con este comportamiento dual. En la investigación descrita aquí, fueron estudiadas las posibles diferencias en la expresión de genes reloj en osciladores centrales (cerebro) y periféricos (hígado), y en la fisiología (glucosa sanguínea y actividad amilasa en intestino medio), en lubinas con patrones de autoalimentación diurno y nocturno bajo condiciones de LD 12:12 h (luz:oscuridad) (encendido de las luces = *Zeitgeber* Time (ZT) 00:00 h). Los resultados revelaron que la expresión de *per1* en cerebro mostró ritmicidad diaria con la acrofase (Φ) alrededor del apagado de las luces (ZT 12:00, Cosinor, $p < 0.01$) en ambos, lubinas diurnas y nocturnas. En hígado, los niveles diarios de expresión de *per1* fueron mayores en peces diurnos (Modelo Lineal General Univariante, $p < 0.02$). Variaciones de glucosa sanguínea fueron observadas en ambos grupos (ANOVA I, $p < 0.01$), con mayores niveles de glucosa durante la noche en peces nocturnos que diurnos, aunque solo las lubinas diurnas mostraron un ritmos diario significativo (Φ = ZT 16:52 h, Cosinor, $p < 0.02$). Los mayores valores de actividad amilasa coincidieron con la fase de alimentación de los peces; esto es, el máximo se alcanzó en ZT 18:00 h (ANOVA I, $p < 0.01$) en peces nocturnos, mientras que la Φ fue en ZT 03:39 h (Cosinor, $p < 0.02$) para peces diurnos. En resumen, nuestros hallazgos indicaron que los ritmos de alimentación (diurno *vs.* nocturno) influenciaron fuertemente a los patrones de funciones digestivas y expresión de genes reloj en el hígado (reloj encarrilado por la alimentación), pero no en el cerebro (reloj encarrilado por la luz).

<u>Capítulo experimental 3</u>: LOS RITMOS DIARIOS DE GLUCOSA SANGUÍNEA DIFIEREN EN LUBINAS EUROPEAS DIURNAS Y NOCTURNAS (*Dicentrarchus labrax* L.) GUIADAS POR LAS INVERSIONES DE FASE ESTACIONALES.

La lubina cambia sus ritmos de alimentación de diurna a nocturna en invierno, volviendo a diurna en primavera. A pesar de los datos sobre comportamiento, los cambios fisiológicos que tienen lugar durante tales cambios permanecen inexplorados. En este artículo, los ritmos de glucosa sanguínea de lubinas Europeas con ritmos de auto-alimentación diurnos/nocturnos fueron investigados durante las inversiones de fase de su comportamiento alimentario (en invierno y primavera), cuando ambos peces, diurnos y nocturnos, coexisten. La glucosa sanguínea mostró variaciones diarias en ambas estaciones (ANOVA, $p < 0.03$), ajustándose a una función coseno (COSINOR, $p<0.05$) en todos los casos, excepto en peces diurnos en primavera. Los niveles medios de glucosa sanguínea de peces nocturnos en invierno (2.67 ± 0.09 mmol/l, mean ± SEM) fueron significativamente mayores (t-test, $p < 0.01$) que en primavera (2.20 ± 0.08 mmol/l), mientras que estos fueron similares (~2.25 mmol/l) en peces diurnos de ambas estaciones. Estos hallazgos revelaron por primera vez señales de cambios fisiológicos estacionales que acompañan los cambios en los ritmos de comportamiento de lubinas diurnas y nocturnas.

<u>Capítulo experimental 4</u>: RITMOS CIRCADIANOS DE ACTIVIDAD AUTO-ALIMENTARIA Y LOCOMOTORA EN PEZ CEBRA (*Danio rerio*).

Para investigar los ritmos diarios de alimentación en pez cebra, los autores han desarrollado un nuevo sistema de auto-alimentación, donde una fotocélula infrarroja actúa como sensor de demanda de alimento, el cual permite a peces de pequeño tamaño, tales como el pez cebra, activar un comedero a demanda. En este artículo, los autores usaron ocho grupos de 20 peces. Los ritmos de actividad locomotora fueron también estudiados por medio de sensores infrarrojos. Bajo un ciclo de 12 h luz (L): 12 h oscuridad (D), los peces cebras mostraron un claro patrón de alimentación nocturno (88.0% de las demandas de alimento diarias totales ocurrieron durante la fase de luz). Además, ambos ritmos, de alimentación y locomoción, fueron

conducidos endógenamente, ya que ellos persistieron bajo condiciones de ritmo-libre. La longitud del periodo (τ) media de los ritmos de locomoción y alimentación fueron menores (τ = 22.9 h) y mayores (τ = 24.6 h) de 24 h, respectivamente. Mientras las horas de disponibilidad de alimento fueron restringidas, los peces podían alimentarse solo durante ZT0-ZT12 o ZT12-ZT16. Esto resultó en que la actividad alimentaria se vio significativamente modificada de acuerdo con la hora de alimentación, mientras que los patrones de actividad locomotora permanecieron sincronizados a los ciclos LD y no cambiaron durante este ensayo. Estos hallazgos revelaron la existencia de una independencia de fase entre el ritmo de las actividades locomotora y alimentaria (las cuales son mayormente nocturna y diurna, respectivamente), lo cual apoya el concepto de control multioscilatorio de la ritmicidad circadiana del pez cebra.

<u>Capítulo experimental 5</u>: Pulsos de luz por la noche provocan respuestas comportamentales dependientes de la longitud de onda en el pez cebra (*Danio rerio*).

La columna de agua actúa como un filtro cromático con diferente tramitancia dependiendo de las longitudes de onda. En este trabajo, se investigaron las respuestas comportamentales del pez cebra tras la exposición nocturna a un pulso de luz de 1h de varias longitudes de onda (violeta, azul, verde, naranja, roja y blanca y oscuridad como controles). Para este fin, 42 peces fueron grabados en video 1h antes, durante y después de los pulsos. Un software para el seguimiento de los peces analizó las grabaciones, localizando los peces en cada segundo. Los resultados revelaron que (i) la actividad natatoria aumentó ante todas las longitudes de onda (3,3 a 6,5 veces que cambia) con respecto a su actividad antes del pulso, aunque la blanca y las longitudes de onda cortas causaron los mayores incrementos, permaneciendo elevados durante la hora después del pulso; (ii) la máxima velocidad y los tiempos en reposo fueron también mayores y menores a longitudes de onda, respectivamente; and (iii) durante el pulso de luz los peces tendieron a descender en la columna (0,7 veces que cambia), recuperando sus posiciones originales durante la hora posterior al pulso (excepto para la violeta). No aparecieron cambios en la posición vertical bajo luz

verde o roja, solo mostrando fototaxis positiva tras el pulso rojo. Estos profundos efectos sobre el comportamiento del pez cebra causados por la luz (incluso después del estimulo luminoso) sugieren que señales internas diferentes son activadas por diferentes longitudes de onda.

Conclusiones generales:

1. Las secuencias génicas completas de dos criptocromos, *cry1* y *cry2*, han sido clonadas en la lubina Europea, aumentando nuestro conocimiento sobre genes reloj en esta especie. Ambas proteínas presentaron los dos dominios típicos de las proteínas CRY: ADN-fotoliasa y *FAD-binding*. El análisis estructural y filogenético de estas proteínas Cry han revelado una alta homología con proteínas Cry de otros peces teleósteos, agrupándose juntos todos los Cry de teleósteos, y separados los Cry1 de los Cry2.

2. Los genes *cry1* y *cry2* se expresaron en todos los tejidos de lubina analizados (cerebro, hígado, corazón, retina, músculo, vesícula, agallas e intestino), apoyando la existencia de múltiples relojes en esta especie. Además, el ritmo de expresión diario de *cry1* fue similar en hígado, corazón y cerebro de lubina, muestran un pico poco después del encendido de las luces. La expresión de *cry2* fue rítmica también en hígado y cerebro, aunque no en corazón. En hígado, la acrofase de *cry1* y *cry2*, coincidieron, mientras que en cerebro la acrofase de *cry2* ocurrió al final de la fase de luz. Por tanto, diferentes sincronizadores podrían encarrilar la expresión de genes *cry* en diferentes tejidos.

3. La lubina ajustó sus demandas de alimento a la fase donde la recompensa de alimento estaba disponible, aunque el ritmo diario de expresión de *per1* en cerebro no se vio afectado, con los niveles máximos alrededor del encendido de las luces, tanto en lubinas diurnas como nocturnas. Sin embargo, en hígado, la expresión de *per1* difirió entre lubinas diurnas y nocturnas, obteniendo mayores niveles en peces diurnos. Por tanto, la hora de alimentación parece afectar a la expresión de *per1* en osciladores periféricos de lubina (hígado), pero no en el central (cerebro).

4. La fase de alimentación a demanda influyó de forma diferente sobre las variaciones de parámetros de la fisiología digestiva, como glucosa sanguínea y actividad amilasa en el intestino medio. Por un lado, lubinas diurnas y nocturnas sometidas a alimentación a demanda restringida a una fase, mostraron los mayores valores de actividad amilasa durante su fase de alimentación, aunque los niveles de glucosa sanguínea mostraron un pico por la noche en los dos grupos. Por otro lado, lubinas diurnas o nocturnas durante las inversiones naturales de comportamiento mostraron los mayores niveles de glucosa sanguínea durante su fase de alimentación. Además, los ritmos diarios de glucosa sanguínea presentaron variaciones estacionales: en invierno, la amplitud fue mayor y la acrofase anterior, y las lubinas nocturnas mostraron los mayores niveles medios.

5. Un nuevo comedero a demanda por aproximación fue diseñado y desarrollado para peces de pequeño tamaño. Tanto el pez cebra como juveniles de lubina aprendieron satisfactoriamente a utilizarlo, ajustando finalmente su ingesta.

6. A pesar de que el pez cebra ha sido considerado una especie diurna, los ritmos de alimentación a demanda tuvieron lugar por la noche, con la mayoría de las demandas de alimento concentradas en las últimas horas de la fase de oscuridad. Por tanto, el pez cebra mostró independencia de fase entre los ritmos de alimentación y locomoción, el cual permaneció diurno. Ambos ritmos estuvieron bajo control endógeno y entraron en curso libre bajo condiciones constantes, con un periodo menor de 24 h para locomoción (22,9 h) y mayor para alimentación (24,6 h).

7. La exposición nocturna a luz blanca y longitudes de onda cortas (i.e. violeta y azul) indujo las mayores respuestas comportamentales en pez cebra, incrementando la actividad natatoria durante la duración del pulso de luz. Tales cambios persistieron durante la hora posterior al apagado del pulso, excepto después de la exposición a luz roja. Además las luces blanca, violeta, azul y naranja provocaron fototaxis negativa durante el pulso de luz, aunque los peces recobraron su posición vertical previa durante la hora siguiente al apagado del puso (excepto para la violeta). Por el

contrario, la luz verde no causó fototaxis negativa y la roja que produjo fototaxis positiva.