UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Impact of Lighting Conditions and Feeding Cycles
Rhythms of Early Development, Behaviour and Sexual
Differentiation in Zebrafish *Danio rerio*

Impacto de las Condiciones de Iluminación y Ciclo
de Comida en los Ritmos de Desarrollo Temprano,
Comportamiento y Diferénciación Sexual en
Pez Cebra *Dario rerio*

Dª. Viviana Di Rosa

2015

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Facultad de Biologia
Departamento de Fisiologia
Unidad de Fisiologia Animal

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TESIS DOCTORAL

Viviana Di Rosa

Murcia, 2015

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with the University of Ferrara

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Faculty of Biologia
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DOCTORAL THESIS

Viviana Di Rosa

Murcia, 2015

Dissertation submitted by Viviana Di Rosa to obtain the PhD Degree by the University of Murcia in Co-Direction with the University of Ferrara.

Directors: Javier F.Sanchez Vazquez
Cristiano Bertolucci
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Universidad de Murcia

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Introduction

1. Introduction

1.1. Biological clock in fish

1.1.1. The Circadian System

In this planet life is subjected to periodically changes due to the rotation of the heart on its axis and around the sun, and the revolution of the moon around the earth (Kumar, 2002). Through the curse of evolution, organisms developed ability to orientate in time and in space thanks to an internal clock mechanism, which allows animals and plants to keep track of time in order to anticipate environmental changes, by physiological and behavioural adaptations. Marine organisms have been subjected not only to daily variation, represented by the alternation of day and night, but also to tidal and lunar cycle. The external conditions, light and temperature, abiotic nature cues, and biotic cue like food availability are able to act as potent synchronizers (Zeitgeber) ("time givers" in german) of the endogenous oscillator.

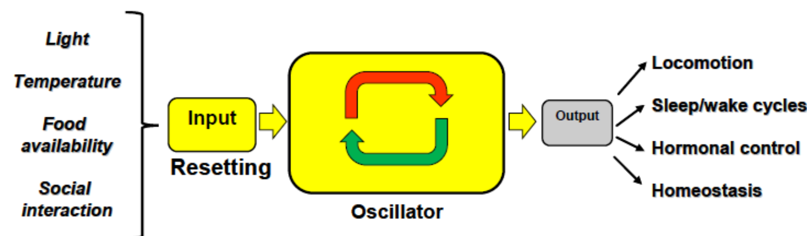

Figure 1. Schematic presentation of biological timekeeping systems. The core oscillator autonomously generates circadian rhythm. Daily resetting by environmental signals, via the input pathway, ensures that it remains synchronized with the natural 24-hour cycle. The pacemaker then drives the expression of output involved in many aspect of physiology.

Animals adjust its own endogenous rhythm to the external stimuli to predict the environmental changes, and to be able to perform various biological functions at the right time of the day. Important events in the life of the animals such as feeding, reproduction, spawning happen in the best condition and moment, in order to ensure the surviving of the specie (DeCoursey 2004).

In absence of these cycles the animals free-run, revealing its natural periodicity (free running rhythms) that is always close to the 24 hours (Roenneberg et al., 2003). The

majority of animal presents daily and annual rhythms of activity. Organisms that live in the oceans depth and underground caves, which are not subjected to periodicity, developing strategy to counteract violent variation in their extreme habitat, represent the exception. The circadian system is responsible for transducing environmental cues into hormal signal, its major component is the pineal organ in vertebrate (Reiter, 1993; Falcón et al 2007). In mammals, this organ has lost the photosensitive capacity so the circadian clock is located in the suprachiasmatic nucleus SCN, which receives photic information from the lateral eyes (Yu and Reiter, 1992). In fish, the circadian clock is located in the pineal organ that has maintained the ability to perceive the light, presenting also a self-sustained oscillator (Iigo et al., 1994; Ekström & Meissl, 1997).

1.1.2. The molecular clock

The circadian clock mechanism that regulates the rhythmicity in vertebrates consists of interacting positive and negative transcriptional/translational feedback loops. Positive elements, such as CLOCK and BMAL, bind to E-box elements located in the regulatory regions of negative elements (*per*s and *cry*s).After translation, dimerization and translocation to the nucleus, CRY and PER proteins physically interact with and thereby down-regulate their own expression by inhibiting CLOCK-BMAL complex (Reppert

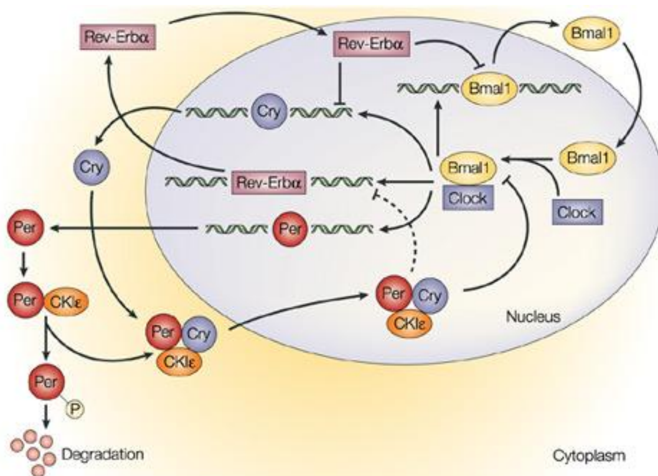

Figure. 2: Molecular clock. The clock is composed of two interconnecting loop: In the core loop heterodimers of CLK and BMAL1, positive limb, activate transcription of Period, Cryptocrome and clock-controlled genes. Heterodimers of PER and CRY, negative limb bind to CLK-BMAL1 inhibiting their own transcription. In the stabilizing loop CLK and BMAL1 induce the expression of REV-ERBα and RORα which subsequently regulate transcription of Bmal1.

and Weaver, 2001), allowing the feedback loop to exist. The robustness and stability of of the core loop is due to the existence of on additional loop that directs the rhythmic expression of the *bmal* transcript (Emery and Reppert, 2004). The transduction of

circadian information is achieved by rhythmic activation of clock-controlled output genes that regulate downstream processes (Reppert and Weaver, 2002). For instance, DBP is a D-box binding protein whose rhythmic expression is driven by CLOCK-BMAL through an E-Box-mediated activation (Mueller et al., 1990; Ripperger 2000). These transcription factors controlled by the clock confer circadian expression on downstream genes, modulating various physiological processes (Gachon et al., 2007). In zebrafish, the existence of multiple forms of the key clock genes *cry*, *per, clock* and *bmal* has been reported. Three *clock* genes (*clock1*, *2 3*) and three *bmal* genes (*bmal1a*, *1b* and *2*), four *per* genes (*per1a*, *1b*, *2* and *3*) and six *cry* genes (*cry1a*, *1b*, *2a*, *2b*, *3* and *4*) (Vatine et al., 2011; Idda et al., 2012). *Per1* is a clock-controlled gene that is present with two homologues (*per1a* and *per1b*), whereas *per2* is a light-driven gene necessary for the ontogeny of the clock (Ziv et al 2005).

1.2. Environmental cycles

1.2.1. Photoperiod and light spectrum

Light is characterized by daily changes in irradiance, wavelength, polarization composition and direction (Björn, 2002.). The water column acts as a potent chromatic filter, instead as light penetrate the water column, water molecules, dissolved substances and suspended particles not only attenuate the intensity of light but also alter the spectral quality of light. As light enter the water columns, the shortest and longest wavelength are absorbed quite near the surface: the below violet (λ <390 nm) and beyond red (λ > 600 nm). Blue wavelength can penetrate in the deep water, reaching about 150 m in the ocean (Lalli & Parsons, 1995; Fig. 3). Solar light represents a complex environmental signal that influences the evolution of most

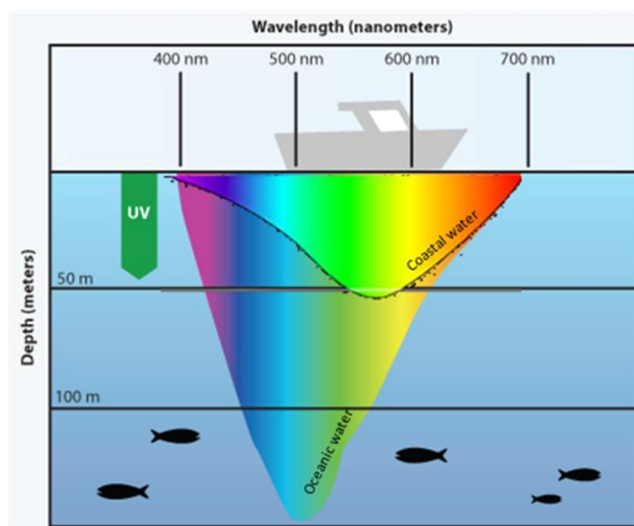

Figure 3. Spectral profile of light incidents in the water column of ocean

biological process in the earth, instead in teleost fish, the whole life cycle is directly correlated to light, controlling the embryo development, the hatching and sexual maturation (Downing & Litwak, 2001; Villamizar et al., 2012; Migaud et al., 2010). Light influences the entire cycle of life, from embryo development to sexual maturation, being the predominant signal for entrainment of circadian oscillators in fish (Bromage et al., 2001; Aranda et al., 2001). Previous studies highlight the importance of light for the onset of the feeding activity through the visual system (Puvanendran & Brown, 2002) and for larval development acting on retinal photoreception (in the pineal and deep brain). Light stimuli perceived by non-retinal photoreceptors are transduced through rhythmic melatonin secretion, a time-keeping hormone (Amano et al., 2003; Falcón et al., 2010., Migaud et al 2010.).

In the last years many investigations are taking into account a significant role of the light and different wavelengths. Dekens et al. demonstrated the effect of LD cycle during the zebrafish development, promoting the rhythm establishment. The same phenomenon was revealed also in cell line (Dekens et al 2003). Photoperiod 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 and Sanchez Vazquez., 2001). Some fishes can shift their activity phase along their life, presenting diurnal o nocturnal behavioral pattern in the same species. This phenomena is known as dualism, due to high plasticity of the circadian system and seem to be more frequent in diurnal species ((Lopez-Olmeda and Sanchez-Vazquez, 2010a; Reebs, 2002) such as goldfish and European sea bass (Lopez-Olmeda and Sanchez-Vazquez, 2010). Recently, investigations have been reported in seabass and zebrafish the effects of light spectrum on genetic expression of opsins (Temple, 2011), early development (Villamizar *et al.*, 2011) and behaviour (Li *et al.*, 2012).

1.2.2. Photoreception

In vertebrates like reptiles, birds and fish photoreception occurred by several specialized photosensitive organs. Light sensitive structures are present in different sides of the animal, retina, pineal gland, skin. In the retina the presence of rods and cones of

different sensitivities permit to fish to catch the photic signal from the environment. Opsin are G proteins coupled receptor characterized by their ability to bind the 11 cis-retinal via a Schiff base linkage using a lysine residues in the 7th transmembrana α-helix, transforming it in all-trans-retinal (Burns et al., 2001).

Exist five groups of rod visual opsin including rhodopsin and four types of cone opsin. organized into a repeating, two-dimensional mosaic that extends across the entire retina: long wavelength-sensitive (L) (red), medium wavelength-sensitive (M) (green), short wavelength-sensitive (S) (blue), and UV wavelength-sensitive (UV) cones, each of which expresses L-, M-, S-, or UV-opsin, respectively (Vihtelic 1999, Figure 4). Zebrafish have two L-opsin (*opn1lw1*, *opn1lw2*) and four M-opsin genes (*opn1mw1*, *-2*, *-3*, *-4*) in contrast to S- and UV-opsins, which are each encoded by a single gene.

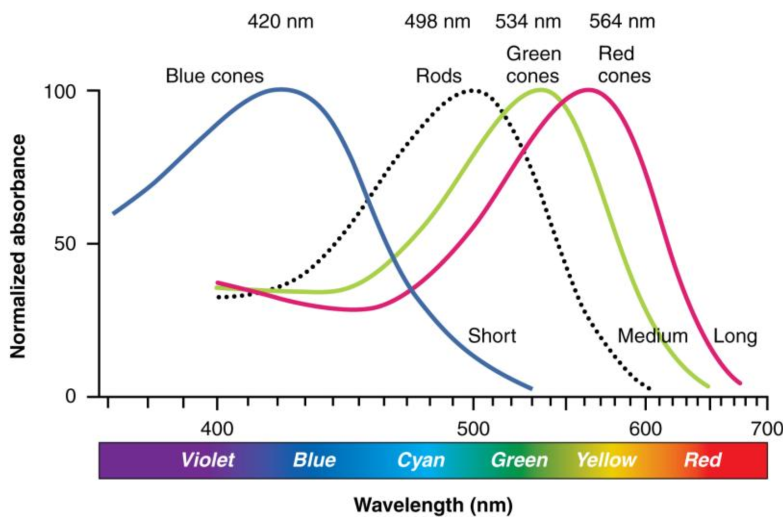

Figure 4. Normalized visual photoreceptor absorbances for different wavelengths of light

(Chinen et al., 2003). Interestingly these opsins are not confined only in the retina, whereas the new opsins, like exorhodopsin, Teleost Multiple Tissue (TMT) opsin and melanopsin (Opn4m2) are present in many other different structures such as brain, heart, liver, kidney (Bellingham et al., 2002; Mano et al., 1999; Moutsaki et al., 2003). In the skin, the dermal melanophores contain photoreceptors which control skin colour depending on the ambient light level (Weber, 1983). Various studies in zebrafish have highlighted the capacity of organs, tissues and cells to entrain to photic stimuli displaying circadian oscillators and sensitive mechanisms, without the involvement of the retinal and pineal photoreceptors. (Whitmore et al., 1998; 2000).

1.3. Ontogeny of the rhythm

1.3.1. Ontogeny of biological clock

Several investigations have been carried out to investigate to the ontogeny of the clock in vertebrates. Zebrafish represents the specie more extensively studied to explore the origin and early functions of the circadian clock through molecular genetic analysis (Vallone et al., 2005; Nusslein –Volhard and Daham., 2002). Exposure to LD cycles or temperature is sufficient to initiate circadian rhythms of clock gene expression (Lahiri et al., 2005). In the presence of LD cycle a significant nocturnal rhythm of melatonin was detected the second night post-fertilization, confirmed by the mRNA relative expression pattern of *aanat2* in the pineal gland. (Gothilf et al., 1999; Kazimi and Cahill 1999). *Per1b* rhythmic expression is detected even during the first day under LD cycle (Dekens and whitmore 2008) and furthermore, the presence of a single light-to-dark or dark-to-light transition is sufficient to establish and set the phase of rhythmic *aanat2* expression and melatonin synthesis (Ziv et al., 2005; Vuilleumier et al., 2006). In contrast embryos raised under condition of constant temperature and constant darkness lack of circadian rhythms. Theoretically, the lack of any rhythmicity in embryos raised under constant conditions could be explained by the presence of clock in the cell, which are asynchronous with respect to each other (Vatine et al 2011). Delaunay *et al*. concluded that a functional circadian clock was maternally inherited with the phase of the rhythm being inherited from the mother (Delaunay et al 2003). This conclusion resulted inconsistent with earlier results (Kazimi and Cahill 1999; Hurd and Cahill 2002), and subsequent studies that confirmed that the transcript of numerous clock genes are maternally inherited, with levels of RNA that decline rapidly between 3 and 9 hpf. *Clock* and *bmal* levels remain elevated and constant until the fourth day of development when oscillation start to be detected in embryos subjected to LD cycles (Dekens and Whitmore 2008; Dekens et al., 2003; Kaneko and Cahill 2005; Lahiri et al., 2005). A functional circadian clock is present in the embryo as the pineal gland and the retina become functional (20-24 hours and 3 days respectively) (Wilson a Easter, 1991; Easter and Nicola, 1996 Gothilf et al., 1999) but far before the differentiation of specialized light receptive structure is completed.

1.3.2. Ontogeny of behaviour

Behavioural circadian rhythms are regulated by cellular circadian pacemakers. The generation of the cellular circadian rhythms doesn't require the neuronal differentiation; however, pacemaking systems are located in central neuronal and endocrine structures and may require differentiation of specific cell types or intracellular signalling system (Hurd and Cahill. 2002). Several studies investigated circadian rhythm development in zebrafish and the effect of light on the locomotor activity of adults and larvae, especially submitted under light cycle and daylengths (Cahill et al., 1998; Hurd et al., 1998; Hurd & Cahill, 2002; Colwill and Creton, 2011; Ben-Moshe at al., 2014; Vignet et al., 2013). Also temperature plays a key role, showing significant effect on the locomotor activity rhythms in adult zebrafish reared at 21ºC (Hurd et al., 1998). Hurd and Cahill manipulated light exposure during the first days of zebrafish development demonstrating that the behavioural rhythms are driven by endogenous, entrainable circadian clock. During the development, zebrafish starts to display behavioural rhythms depending on the environmental cues. Considering that larvae hatch during the third day post fertilization, they generally remain inactive through the fourth day post fertilization. At the fifth day the swim bladders is inflated and larvae begin to swim and feed. Zebrafish clock develops gradually over the first four days post-fertilization requiring LD cycle to initiate circadian rhythmicity. LD cycles not only set the phase of the clock that controls larval zebrafish behaviour, they also increase the amplitude of the locomotor rhythm. (Hurd and Cahill. 2002).Adult and larvae are mostly active (64 %) during the light phase and the circadian rhythmicity persist under constant dark condition. Curiously the onset of darkness induce in larvae a behavioural increment of activity, but after the onset of illumination, larvae rapidly increase baseline activity levels. This behaviour confers advantages to the fish, because of their hyperactivity aimed at finding shelter prior to night, maybe to maximize time spent in well-lit environments suitable for feeding (Burgess and Granato, 2007)

1.4.Feeding rhythms in fish

In natural environment food is not constant available but restricted in a particular time of the day, so fish have evolved mechanisms to predict feeding time activating behavioural, physiological and genetic processes in advance. This capacity permits fish to avoid any risk and to allow the best exploitation of the feeding sources. (Madrid et

al., 2001). The fish are not active during the 24 hours constantly, but concentrate its higher activity during the light or the dark phase of the day. The most studied feeding rhythms were the circadian rhythms long 24 hours, but also tidal, lunar and seasonal rhythms have been observed. In most species the diurnal o nocturnal feeding behaviour has been fixed genetically. Some fish species can display diurnal and nocturnal behaviours, shifting from one phase to the other along their life. This ability common in fish is known as diurnalism (Eriksson 1978), a behaviour suggested to be related to the highly flexible circadian system, that allow fish to quickly adapt to environmental changes (Sanchez Vazquez et al 1996; Lopez Olkmeda and Sanchez Vazquez 2009).
Recent studies have highlighted the increasing of locomotor activity just before mealtime when fish are fed on a single daily meal, provided at fixed time (Mistlberger, 1994). This behaviour is known as food-anticipatory activity (FAA), and it has been reported in a number of fish species (Davis & Bardach, 1965; Sánchez-Vázquez et al., 1995a; Bolliet et al., 2001; López-Olmeda and Sánchez-Vázquez, 2010; López-Olmeda et al., 2010). FAA characteristic is its gradual occurrence, its development influenced also by the energy quantity and the size of the food, for instance, smaller sizes of food induce stronger anticipatory response (Sánchez-Vázquez et al., 2001). Feeding entrainment represents an important adaptive advantage to fish permitting them to maximize the food utilization by activation of all digestive process hours before the time to of feeding (Herrero et al., 2005). Recent studies in sea bream , sea bass and goldfish have showed the increment of amylase activity just before the mealtime, in fish with scheduled feeding (Montoya et al 2010; del Pozo et al., 2011, Vera et al.,2007), confirming the capacity of entrainment to induce not only behavioral response but also metabolic and digestive functions.

1.4.1. LEO vs. FEO

In mammals, two different oscillators have been described, one driven by the light, called light-entrainable oscillator (LEO), which is located in the suprachiasmatic nuclei of the hypothalamus (Meijer & Rietveld, 1989), and one driven by food, called food-entrainable oscillator (FEO), which is independent from the LEO and whose anatomical location is still unknown (Stephan, 2002; Davidson, 2006). Recently, a model for the mammalian FEO has been proposed, which is based on interconnected structures in the brain able to entrain humoral signals deriving from periodic feeding (Carneiro & Arujo,

2009). In fish, the presence of both a LEO and FEO has been suggested, although contrary to the situation in mammals, in fish both oscillators seem to be coupled, which can be clearly observed when light and food cycles are present with different phases (Sánchez-Vázquez et al., 1995b; López-Olmeda et al., 2010). The anatomical location of either LEO or FEO in fish has not yet been identified. Therefore in fish, two possible mechanisms can be suggested: 1) the existence of separate by tightly coupled light –and food entrainable oscillators, or 2) a single oscillator entrainable by both light and food, one synchronizer being stronger than the other (Sánchez-Vázquez et al., 1997; Aranda et al., 2001). The feeding rhythms in fish has been demonstrated to have endogenous origin in several species such as the European sea bass, goldfish, rainbow trout and zebrafish (Sánchez-Vázquez et al., 1995a; 1996; Sánchez-Vázquez and Tabata, 1998; del Pozo et al., 2011).

1.5.Sexual differentiation

Many teleost are gonochorists, where individuals develop only as males or females and remain the same sex throughout their life spans. It is possible to divide this species in two species depending on the gonadal development strategies (Yamamoto 1969). Differentiated gonochoristics species, such as coho salmon (*Oncorhynchus kistuch*) (Piferrer and Donaldson, 1989),*Abramis brama* (Talikina, 1995), *E. masquinongy* (Lin et al., 1997), S. schlegeli (Lee et al., 1996), *Dicentrarchus labrax* (Blázquez et al., 1998), and *C. carpio* (Komen et al., 1992b)] where early gonad development proceeds from an undifferentiated gonad The second strategies is the undifferentiated species where all individuals initially develop ovarian tissues. In zebrafish all gonads initially develops as ovaries but after the ovarian tissues can degenerate and invaded as additional somatic cells ready for masculinization((Takahashi, 1977; Takahashi & Shimizu, 1983). Most individual mature as only one sex (gonochorists) rare individuals can have undergone sex change (Sadovy & Colin, 1995), for example European and Japonese eels (*Anguilla anguilla* and *A. japonica*) , initially possess a bipotential intersexual gonad that can develop directly on to an ovary or testis (Tesch 1977; Colombo and Grandi,1989, 1990, 1995, 1996, Beullens et al., 1997). Steroids are the factors implicated in sexual differentiation and maturation in fish. The developmental pathway leading to steroid production requires complex regulation of various genes, (Swain & Lovell-Badge, 1977) involved in the initial differentiation of steroid-

producing cells. If steroids are critical for directing initial sex differentiation rather than being a consequence of it, then the appearance of steroid-producing cells and differences in steroid production between the sexes should be apparent prior to morphological differentiation of the gonad. Differentiation of steroid producing cells in the ovary of amago salmon (Nakamura & Nagahama,1993) and Tilapia (Nakamura & Nagahama, 1985) occurred in the same time of stromal aggregation, formation of ovarian cavity and meiotic activity of oocytes, very early in gonadal differentiation. In zebrafish aromatase mRNA is detectable early in development and levels of the brain form is distributed in both sexes, suggesting an important role for this gene in sex differentiation (Trant at al., 2001). However, these observations clearly indicate that if steroid production is not responsible for directing early sex differentiation, then the two events are otherwise very closely coordinated.

In gonochoristic vertebrates, sex determining mechanisms can broadly be classified as genotypic (GSD) or temperature-dependent (TSD) (Bull, 1983; Valenzuela et al., 2003). In species with TSD, there are no consistent genetic differences between sexes. The earliest ontogenetic difference between sexes is an environmental one because the ambient temperature during sensitive periods of early development irreversibly determines phenotypic sex and, therefore, the sex ratio (Bull, 1983; Valenzuela et al., 2003). In any case, the presence of TSD in a given species is not incompatible with the existence of genotype x environment interactions, which are common in fish, including Menidia (Devlin and Nagahama, 2002; Conver and Heins, 1987a; Conver and Heins, 1987b). However, too often assignment of TSD in many fish species has proceeded regardless of evidence such as the presence of sex chromosomes, which is strongly indicative of GSD (Bull, 1983; Valenzuela et al., 2003; Devlin and Nagahama, 2002) has been pointed out that observed sex ratio shifts under these circumstances might be the consequence of thermal effects on GSD (GSD+TE) rather than proof of the presence of TSD (Valenzuela et al., 2003; Conver, 2004). Further, species with TSD exhibit only one general sex ratio response pattern to temperature (Álvares and Piferrer, 2008)

1.5.1. Steroid production

Once sex has been determined, gonadal differentiation usually may be influenced by fluctuation in intrinsic factors such as growth or behaviour or by extrinsic environmental factors such as temperature, endocrine hormones or pollution. Endocrine control of sex differentiation involves a complex interaction between brain and gonad, through the production of gonadotropin and steroid produced in brain and gonad Bieniarz & Epler, 1992; Nagahama, 1994), for example Estradiol 17 β is found at higher levels in female than in male, and is believed to be major steroid involved in ovarian development (Yamamoto, 1969). Testosterone and 11-ketotestosterone are found in male, representing the major androgen responsible for testicular development (Jiang et al., 1996; Miura et al., 1996; Nagahama, 1999). Enzymes are involved in the biosynthesis of sex steroid in fish (Nagahama, 1994 Figure 5). Specific genes responsible of steroid biosynthesis are expressed differentially in the somatic cells of testis and ovary (Omura & Morohashi, 1995; Nakamura et al., 1998) producing a variety of sex steroids.

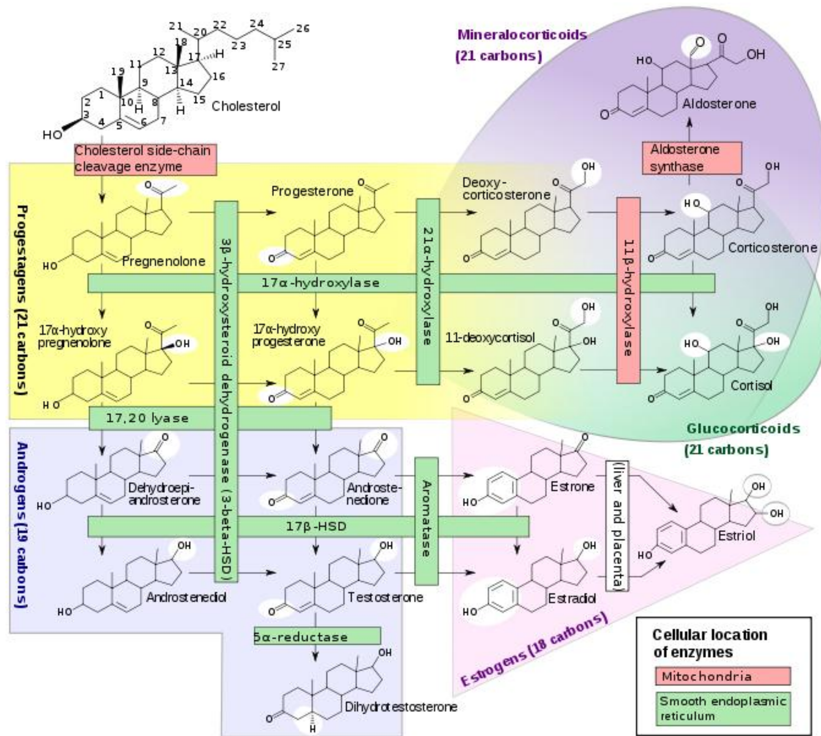

Figure 5. Steroidogenesis, the biosynthesis pathway: Estradiol and and Aldosterone are generated from cholesterol, throught the catalytic action of Aromatase and Aldosterone synthase.

The pathway initiates with the synthesis of the pregnenolone via cholesterol by *P4550scc*, which is followed by production of progesterone. Testicular tissues present

as dominant circulating hormone the 11-ketotestosterone (Cavaco et al., 1997). In ovary the enzyme aromatase (P450aro), the product of the *cyp19a* gene, is expressed, resulting in the irreversible conversion of testosterone to estradiol-17β, necessary for oocytes growth. Multiple aromatase genes has been identified in goldfish and zebrafish (Callard & Tchoudakova, 1997) in two distinct tissues, brain and gonad. Recent studies have revealed the different levels expression of the brain aromatase in distinct individual zebrafish embryos during sex determination (Trant at al., 2001) supposing a possible role of this gene in sex differentiation or that it responds differentially to gonadal development in males and females. In turn, *cyp19a* negatively regulate the levels of the *amh* (Anti-Müllerian hormone) gene (and visceversa) which has been shown to have a dimorphic expression as it plays a key role in the proliferation and differentiation of spermatogonia of fish species such as the Nile tilapia (*O. niloticus*), the rainbow trout (*Oncorhynchus mykiss*), the Japanese flounder *Paralichthys olivaceus* and the zebrafish (Ijiri et al., 2008; Vizziano et al., 2007; Yoshinaga et al., 2004; Rodríguez-Marí et al., 2005).

1.5.2. Reproduction rhythms

Reproduction in fish is known as a seasonal phenomenon. The existence of multiple environmental cycles, (daily, tidal, lunar, seasonal) have effect on the reproduction time. Depending on the environmental conditions, most fishes start its reproductive behavior in a specific season, the most suitable to ensure the offspring survival. The seasonality is entrained by the cyclic changes that occur during the year, as it is photoperiod, temperature, climate conditions and food availability. However, the main responsible for the cueing and timing of reproduction is the seasonally daylength for the temperate latitude fish. Teleost fish can be classified according to their reproductive behavior as long of short day-breeders, for example Senegal sole presents notable seasonality in its reproduction, and it is classified as long day breeder (from March until June)(Cabral and Costa, 1999; Cabral, 2000). Sex steroids rhythms presented higher concentration values at pre-spawning, being in phase with each other (Garcia-Lopez et al., 2006; Guzmán et al., 2008). Other species such as gilthead sea bream and the European sea bass presented reproductive behavior during the winter. The first specie starts spawning in November with high level of E2 and testosterone (Zohar & Gordin 1979; Zohar et al., 1984). The European sea bass reproduces in the late winter, with high levels of steroids

just before and during the spawning (Asturiano et al., 2000). Salmonids are a group of fish inhabiting cold water habitats, and present a peculiar reproductive behavior, they spend most of their life at sea 1- 4 years and then migrate to the rivers during summer only to reproduce during the autumn (Hansen et al., 1993; Thorpe et al., 1995; Holm et al., 2000). Some species in absence of any changes of environmental cues presented annual rhythms of reproduction, suggesting the endogenous control of these rhythms (Bromage et al., 2001). In tropical and subtropical fish the photoperiod play a minor rule, for the reduced annual alteration in daylength. These fish use other environmental cues to entrain its reproductive rhythm, such as temperature or increased productivity.

These species are known to poses lunar reproduction rhythms due to a relative stable environment, with little season photoperiod and temperature changes. In rabbitfish during the reproductive season, the spawning occurs during the night in the first quarter of the moon (during the newmoon), corresponding of high levels of melatonin concentration in plasma (Takemura et al., 2004). The perception of the moon cues maybe by the lighting cycle or the changes in earth-moon- sun gravitational forces, which are repeated with interval of 2 weeks (Leatherland et al., 1992). The lunar entrained reproduction rhythms can be of different periodicity: once a month or twice a month, named respectively: lunar rhythms and semi-lunar rhythms. During the reproductive season fish don’t continually release gametes during all the day. Fish synchronize reproduction to the environmental factors, selecting the best moment of the day to achieve the highest survival rate of the progeny. In zebrafish has been already showed the existence of daily spawning rhythms which permits to fish to improve the egg survival, selecting the most appropriate moment of the day. The diurnal spawning rhythms can be suppressed with a dark pulse of 1 hour after the light on (Blanco-Vives and Sánchéz 2009). In sole, sea bass and zebrafish the spawning rhythms coincide with their behavioral rhythms (Oliveira et al., 2009; Villamizar et al., 2012; Blanco-Vives and Sánchéz 2009). Sole showed to be very sensitive to light and able to perceive the moonlight and synchronize to the lunar cycle. Since this species showed nocturnal activity rhythms (Bayarri et al., 2004), reproduction occurred when the night were darkest (Oliveira et al., 2009). Seabass revealed to syncrinize their spawning rhytym depending on the daily patterns of locomotor activity (nocturnal/diurnal) that during reproduction switch to nocturnal.Finally, zebrafish spawn during the light phase, time that coincide with its major locomotor activity (Blanco-Vives and Sánchez-Vázquez,

2009). All these findings suggest that in each species, reproduction and behavioral rhythms are strongly related with each other.

1.6. Species in focus: *D. rerio* and *P. andruzzii*

1.6.1. Zebrafish Danio rerio

The name *Danio* derives from the Bengali name "dhani" meaning "of the rice field" (Talwar and Jhingran, 1991). This freshwater fish common name originates from the five uniform, pigmented and horizontal blue stripes which extend to the end of the caudal fin.

Its taxonomic hierarchy is as follows (Fang, 2003):

Phylum: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: Danio
Species: *Danio rerio* (Hamilton, 1822)

In the wild zebrafish live in the Ganges and Brahmaputra basins in north eastern India, Bangladesh and Nepal. In addition has been also reported in rivers of Pakistan Mayanmar, Sri Lanka (Engeszer et al., 2007; Spence et al.,2008) In this area the climate is typical monsoon with marked seasonal variation, with wide temperature variation of the water with both daily and seasonal variations (Payne & Temple; 1996). The lowest water temperature reported was 14.2ºC and the highest was 33ºC, furthermore some authors have reported water temperature as 6ºC in winter and 38ºC in summer (Spence et al., 2008). Its diet consists primarily of zooplankton and insects as well as phytoplankton and other biological material found in its habitat (Spence *et al*., 2007). Zebrafish in wild tend to reproduce seasonally meanly during the monsoon season, maybe due to the water temperature, while in laboratory breeding can be performed all year, once or twice a week, certainly with no special condition of light and temperature (Spence et al., 2008). In laboratory usually the temperature for breeding is maintained around 26 and 28ºC and the photoperiod of 14:14 LD. Reproductive behaviour and spawning is strongly dependent from the photoperiod and the temperature, starting at few minute of light exposition and continuing for approximately 1-2 hours (Darrow &

Harris, 2004). Fertilized eggs are demersal and hatching takes place within 48–72 hours depending on temperature, chorion thickness and muscular activity of the embryo (Kimmel et al., 1995). Zebrafish egg are large relative to other fish (0.7 mm) in diameter and optically transparent. Fertilisation is external so live embryos are accessible to manipulation and van be monitored through all development stages under microscope (Lawrence, 2007; Kimmel et al 1995). Development is rapid, with precursors to all major organs developing within 36 h and larvae displaying food seeking and active avoidance behaviours within five days post fertilisation, i.e. 2-3 days after hatching (Westerfield, 2000, Kimmel et al., 1995). The species has been described as diurnal. Furthermore, has been reported the dualism of zebrafish under different conditions, displaying nocturnal activity when submitted to LD cycle 12:12 and temperature of 20ºC (López-Olmeda and Sánchez-Vázquez, 2009). When a scheduled feeding is delivered during the night phase, zebrafish displayed a splitting of locomotor activity into two components: a night component synchronized to the feeding time and a day component synchronized to thelight phase of the LD cycle. (López-Olmeda et al., 2010). However, when fish was submitted to self feeding, was able to maintain the locomotor activity during the day, displaying a strictly nocturnal feeding activity (del Pozo et al., 2011). During larval development, it has been observed that a minimum number of LD cycles are essential for the establishment of its clock rhythmicity (Dekens and Whitmore, 2008; Cavallari et al., 2011). The zebrafish is increasingly important in biomedical research (Dooley & Zon, 2000; Shin & Fishman, 2002), particularly as model of human disease (Berghmans et al,, 2005) and for the screening of therapeutic drugs (Rubinstein, 2003, 2006). Zebrafish results more tractable to genetic and embryological manipulation than mammalian model species such as mice. Over the past thirty years zebrafish become of increasing interest for its use as a model for understanding the genetic basis of behavior (Gerlai, 2003; Guo, 2004, Miklosi & Andrew, 2006). The greatest advantage of the zebrafish as a model system comes from its well-characterised genetics, genetic and developmental techniques and tools, and the availability of well-characterized mutants.

1.6.2. Cavefish *Phreatichtys andruzzii*

P. andruzzii is a phreaticolous fish from Somalia (Ercolini et al., 1982), a tropical cyprinid that inhabits the subterranean waters under the central Somalian desert. Its taxonomic hierarchy is as follows:

Phylum: Chordata
 Class: Actinopterygii
  Order: Cypriniformes
   Family: Cyprinidae
    Genus: Phreatichthys
      Species: P. andruzzii (Vinciguerra, 1924).

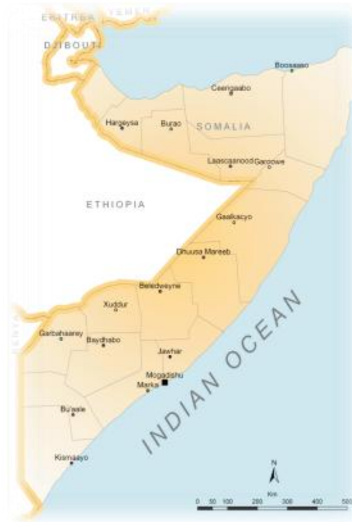

Figure. 5. Phreatichthys andruzzii is a species of ray-finned fish in the Cyprinidae family, and the only species of the genus Phreatichthys. Ancestors of *P.andruzzii* entered the large phreatic layers of the Somalian desert, that developed in Eocene horizontal limestone formations, at the end of the Pliocene (1.4-2.6 million years ago) and became isolated with the extinction of epigean sister species as the result of extreme climatic changes. The Adult cavefish were collected in the wild, in the oasis of Bud-Bud in the centre of the Somalian desert during several expeditions to Africa (1960-1980).

The different localities at witch is found, have different levels of salinity, within what probably is the same underground, karstic hydrographic system. This specie has evolved convergent "regressive" pheno types including many aspects of behaviour, such as negative phototaxis (a kind of taxis which occurs when a whole organism moves in response to a light stimulus in the opposite direction) and changes in anatomy, such as loss of the eyes and pigmentation, which appear to represent key adaptations to life in these environments. In embryo stage, P. andruzzii develops eye in a morphologically normal way, reaching its maximal development by 36 hpf after which, rapid

degeneration starts as a result of a decrease in neuroblastic cell proliferation (Berti *et al.*, 2001). *P. andruzzii* still shows clear behavioural responses to light stimulation.
Specifically, it shows a notable photic sensitivity in behavioural tests where it tends to avoid illuminated areas in preference for completely dark areas (Tarttelin et al., 2012). Furthermore, the fish seem capable of perceiving not only the presence of light but are also able to discriminate between different wavelengths and quantity, instead photophobic response is enhanced under blue light (wavelength of 480 nm), but it is also present under green (539 nm), orange (615 nm) and red (692 nm) light of different irradiance (Tarttelin et al., 2012) (Ercolini and Berti, 1975).

Objectives

2. Objectives

The aim of the present thesis was to investigate the effect of light of different wavelength and photoperiod on early development, hatching rhythms and locomotor activity of zebrafish larvae. Morover, the study of an additional zeitgeber (food) permitted to evaluate the feeding rhythms synchronized to different feeding period in adult zebrafish and cavefish blind specie. Finally we aims to describe in adult zebrafish, the daily rhythms of genes involved on sexual differentiation.

In the present Doctoral thesis the specific objectives were established:

1. To determine the onset of clock genes (*clock1, bmal1, per1b, per2, dbp*) expression during the early developmental stage in zebrafish larvae submitted to a 12:12 light-dark cycle, and how it changes depending on different light wavelengths (LDW, LDB, LDR, DD).
2. To investigate the influence of different light conditions of different wavelengths on the ontogeny of zebrafish larval locomotor activity, as results of circadian behavioural output of clock gene expression.
3. To determine the effect of illumination changes of different spectrum (LDW, LDB, LDR) on the locomotor activity of zebrafish larvae, and the presence of opsin photoreceptors at 3 day post fertilization (dpf) capable to mediated the light response.
4. To describe the existence of hatching rhythm and synchronization of embryos to different light-dark cycle (LD, DL2, DL, LL, DD).
5. To evaluate, the existence of food anticipatory activity, and the influence of different feeding period on it, in two fish species: zebrafish and cavefish under total darkness.
6. To investigate the effect of a deleyed feeding time on fish, evaluation of, the capacity of resynchronization and the presence of the transient.
7. To describe, the daily variation of genes expression (*cyp19a, amh, cyp19b, dmrt1, cyp11b and foxl2*) involved on sexual differentiation in zebrafish adult female and male gonads and brain.

Experimental Chapters

Chapter I

The light wavelength affects the ontogeny of clock gene expression and activity rhythms in zebrafish larvae

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Introduction

Circadian rhythms are regulated by an endogenous system of circadian oscillators that act in harmony with the environmental cycles. They represent an adaptive advantage that allows organisms to predict and anticipate cyclic environmental changes (Pittendrigh, 1993; Devlin and Kay, 2011). Light and temperature are the predominant signals for the entrainment of circadian oscillators (Pittendrigh, 1993). Light acts through changes in intensity (day/night variations) and photoperiod (seasonal variations), while temperature influences biological rhythms through natural oscillations of the thermo-cycle (both daily and seasonal) (Hut, et al., 2013). In the aquatic environment, light has an additional feature since the spectral composition of incident light changes with depth: longer wavelengths (reddish) are absorbed rapidly, while shorter wavelengths (bluish) become predominant (Jerlov, 1968; Loew and McFarland, 1990). Thus, in fish, photic sensitivity depends on adaptation of their photoreceptor system (lateral eyes, pineal gland, deep brain and dermal photoreceptors) to the different properties of the light in the water column, which change depending on factors such as chemical composition, organic substances and depth (Loew and McFarland, 1990; Peirson et al., 2009). Zebrafish is a small cyprinid teleost fish, native of the Ganges River in eastern India, traditionally considered a diurnal species in both its adult and larval stages (Cahill et al., 1998; Hurd et al., 1998; López-Olmeda et al., 2006). It has become a common model species used to investigate the vertebrate circadian clock, embryology and developmental biology (Idda et al., 2012; Haffter et al., 1996; Mullins et al., 1994), and it is an ideal candidate model for studying the effect of light on the development and early emergence of light-responsive structures (Tamai et al., 2004; Ziv and Golthilf, 2006; Dekens and Whitmore, 2008; Hurd and Cahill, 2002).

Several studies have investigated the effect of light on the locomotor activity of zebrafish adults and larvae, focusing on light cycles and daylengths [Cahill et al., 1998; Hurd et al., 1998, Hurd and Cahill, 2002; Colwill and Creton 2011; Ben-Moshe at al., 2014; Vignet et al., 2013). Depending on water temperature, zebrafish larvae hatch at 2-3 day post-fertilization (dpf) and remain generally inactive prior to inflation of the swim bladder and the start of feeding (4-5 dpf). Adult and larvae zebrafish are mostly active (>65%) during the light phase and circadian rhythmicity persists in constant darkness [Cahill et al., 1998; Hurd et al., 1998; Hurd and Cahill, 2002). Interestingly, previous investigation pointed out the importance of light during early development because only

20% of larvae reared under constant darkness showed circadian rhythmicity, further stressing the importance of entraining signals (i.e. light) to initiate circadian rhythmicity, which is regulated by a pacemaker sensitive from 2 dpf (Colwill and Creton, 2011). Furthermore, a recent paper on light composition also showed that different light spectra have different effects on growth performance in zebrafish (Villamizar et al., 2013). Similar investigations in marine fish, such as sole (*Solea senegalensis*) and sea bass (*Dicentrarchus labrax*) confirmed the importance of light characteristics for embryo development, hatching rhythms and larval growth (Blanco-Vives et al., 2010; Villamizar et al., 2009).

The circadian clock mechanism that regulates the rhythmicity in vertebrates consists of interacting positive and negative transcriptional/translational feedback loops. Positive elements such as CLOCK and BMAL bind to E-box elements located in the regulatory regions of negative elements (*per*s and *cry*s). CRY and PER proteins down-regulate their own expression by inhibiting CLOCK-BMAL (Reppert and Weaver, 2001), allowing the feedback loop to exist. The transduction of circadian information is achieved by rhythmic activation of clock-controlled output genes that regulate downstream processes (Reppert and Weaver, 2002). For instance, DBP is a D-box binding protein whose rhythmic expression is driven by CLOCK-BMAL through an E-Box-mediated activation (Muller et al., 1990; Ripperger et al., 2000). These transcription factors controlled by the clock confer circadian expression on downstream genes, modulating various physiological processes (Gachon, 2007). In zebrafish, the existence of multiple forms of the key clock genes *cry*, *per, clock* and *bmal* has been reported (Vatine et al., 2011; Idda et al., 2012). *Per1* is a clock-controlled gene that is present with two homologues (*per1a* and *per1b*), whereas *per2* is a light-driven gene necessary for the ontogeny of the clock (Ziv et al., 2005).

Different investigations have described the role of LD cycles on the expression of different light-responsive genes involved in the molecular clock (Carr and Whitmore, 2005; Ben-Moshe et al., 2010; Mracek et al., 2012; Kaneko and Cahill, 2005; Vallone et al., 2005; Weger et al., 2011). Dekens and Whitmore (2008) observed the light-independent initiation of zygotic *per1* transcription in the first day of development, whose oscillations were asynchronous (Dekens and Whitmore, 2008). Moreover, during the first 3 days of development, *clock* and *bmal1* were not rhythmic , and the onset of their rhythmic expression coincided with the appearance of several circadian clock

output processes, such as locomotor activity and DNA replication (Dekens and Whitmore, 2008; Hurd and Cahill, 2002; Dekens et al., 2003). However, to date, the effects of different light wavelengths on the ontogeny of molecular clock genes remain unknown.

The objectives of the present research were to investigate the influence of 12:12 light-dark (LD) cycles with a different light spectrum (white, LDW; blue, LDB; red, LDR) on the ontogeny of clock gene and behavioural rhythms in zebrafish. For this purpose, we first looked at the locomotor activity, a well-known circadian behavioural output, on larvae reared under different light conditions during the first 7 days of life, in order to ascertain whether rhythmic patterns depend on photic conditions. Next, we focused on a subset of core-clock (*per1b*, *clock1*, *bmal1*), light-regulated (*per2*), and clock-output (*dbp*) genes to study how changes in the lighting conditions might alter the onset of daily and circadian gene expression.

Materials and Methods

Ethics Statement

The present research was carried out in the Chronobiology laboratories of the University of Murcia (Spain) and of the University of Ferrara (Italy). All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). The experimental protocol was previously authorized by the Spanish National Committee on Animal Welfare (Law 32/2007) and the Bioethical Committee of the University of Murcia (Spain) and by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health.

Animal rearing

Wild-type adult fish were obtained from commercial provider (Alimar Pets S.L., Murcia, Spain) and housed for 1 year in 9 L glass aquaria (1 fish L^{-1}) according to standard method (Nüsslein-Volhard et al., 2002). The reproductive fishes were fed 2 and 6 hours after lights on with dry food (Tropical fish flakes; PRODAC, Italy). For spontaneous spawning, the sexually mature fish were separated in groups of 5 fish (3 females and 2 males) and transferred into a 2.5 L breeding net cage during the afternoon. Spawning took place the next morning, approximately 2 hours after lights on.

Eggs were collected, pooled and distributed into 85x10 mm plastic Petri dishes with cover (20 eggs per Petri dish) filled with embryo medium (Nüsslein-Volhard et al., 2002). The Petri dishes were placed to float in a 12 L aquarium at 27ºC.

Experimental procedure

The experimental groups were exposed to four different lighting conditions: LDW (white), LDB (blue), LDR (red) and DD (constant darkness). The light-dark (LD) cycle was of 12 hours light and 12 hours darkness. Illumination was provided by means of neutral red, blue, and white LED light lamps (Superlight Technology Co. Ltd., China). Irradiance was measured with a spectro-radiometer (FieldSpec ASD, Colorado, USA) set at 1.62 E+18 photons m^{-2} s^{-1}. The λ_{max} of the red and blue LED light lamps were 639 nm and 465 nm, respectively. The temperature was held constant (27 ºC) by means of water heaters (50 W, Sera GmbH, Germany) and recorded every 10 minutes with data loggers (Hobo Pendant, Onset Computer Corporation, Massachusetts, USA).

Behavioural recording

The embryos were collected immediately after spawning, incubated in 12-well clear bottom plastic plates filled with 5 ml of embryo medium and exposed to a specific light condition. Each multi-well plate was placed on the water surface of a 10 L aquarium. The plate was fixed on the base of the aquarium to avoid any change of position during the recording. The larvae were fed at 5 dpf and the embryo medium was partially changed every 2-3 days. Swimming activity patterns were recorded from 2 to 7 dpf, for each light condition. Larvae were recorded by means of a webcam adapted for infrared recording by removing the UV filter in front of the lens placed on the top of the aquarium and connected to a computer. An infrared LED (monocolor diode, model L-53F3BT, 5 mm) covered with a blurred white panel was placed under the aquarium to permit the video recording during the dark phase of experiment. These IR lamps emitted at 940 nm, which is not detected by zebrafish (Lythgoe, 1988). Two specialized software packages, Multiviewer and FishTracker (Computer System Department, University of Murcia), were used. The Multiviewer allowed simultaneous webcam recording. Every minute, 60 images (1 frame/s) were stored. The FishTracker quantified the larvae movements and has already been validated in sea bream (Vera et al., 2010) and zebrafish (Sánchez-Vázquez et al., 2011).

Molecular analysis

Embryos and larvae were maintained under different light conditions in Petri dishes and sampled at five different time points (ZT/CT 3, ZT/CT 9, ZT/CT 15, ZT/CT 21; ZT 0=lights on, ZT 12=lights off), during day 0, 3 and 7 post fertilization (dpf). For each ZT/CT, 20 embryos at 0 dpf and 10 larvae at 3 and 7 dpf were sampled and pooled. Four pooled samples per ZT/CT were collected (n=4). Total RNA was isolated from zebrafish embryos and larvae using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The amount, quality and composition of isolated RNA were analysed by BioSpec-nano (Shimadzu, Kyoto, Japan). One microgram of total RNA was incubated with DNase I (Invitrogen) at room temperature for 30 min and then at 85°C for 15 min to inactivate the enzyme. DNase-treated RNA was used to perform cDNA synthesis in a final volume of 20 µl, using iScript cDNA Synthesis Kit (Biorad, Milan, Italy). The reaction was performed at 42°C for 30 min, followed by an inactivation step of 5 min at 85°C. Three microliters of 1:10 diluted first-strand cDNA was PCR amplified with a Chromo4 Real-Time PCR Detection System (Bio-Rad, Milan, Italy) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling conditions were as follows: 3 min denaturation at 95°C, followed by 40 cycles of a 5 s denaturation step at 95°C and an annealing-elongation step for 20 s at 60°C. After amplification, a melting curve analysis to confirm the specificity of the amplicon was performed from 60 to 95°C, with increments of 0.5°C 10 s^{-1}. Gene-specific primers for *clock1, bmal1, per1b, per2* and *dbp* have been previously described (Idda et al., 2012; Cavallari et al., 2011). We verified the efficiency of the primers by constructing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. The relative levels of each sample were calculated by the $2^{-\Delta\Delta CT}$ method (where CT is the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen et al., 2001). As housekeeping genes we used *gapdh*, because of it is frequently used in zebrafish expression studies, and *loopern4*, an expressed repetitive elements recently showed as stable reference target for qPCR normalization (Vanhauwaert et al., 2014). Nearly identical results were observed with both housekeeping genes. Each CT value used for these calculations is the mean of three replicates of the same reaction.

Data analysis

The videos were analysed by FishTracker, a software developed by the Computer Vision Research Group of the University of Murcia (Vera et al., 2010; Sánchez-Vázquez et al., 2011). The program tracks the movement of the larvae and provides the spatial coordinates, corresponding to the X:Y position in the well. The distance between two consecutive points (X_1:Y_1; X_2:Y_2) was calculated using the distance formula derived from the Pythagoras´ theorem and data were arranged in 10 minutes batches for a total of 144 data per day. The locomotor activities were analysed using the chronobiology software "El Temps" (v. 275, Prof. Díez-Noguera, University of Barcelona) which allows actograms to be drawn and calculates the daily meanwave of the locomotor activity.

Statistical analysis

All the results are expressed as means ± SEM. Data were normally distributed (D'Agostino-Pearson normality test, $p<0.05$) and all populations had the same variance (Bartlett's test for equal variances, $p<0.05$). One-way and two-way analysis of variance (ANOVA) were used to determine differences in the locomotor activity among ZT/CT, dpf and lighting conditions. Two-way ANOVA tests were also carried out to determine statistical differences in gene expression between ZT/CTs and lighting conditions. Tukey´s HSD post-hoc test was used for the multiple comparison among groups ($p<0.05$). ANOVAs were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). To evaluate the presence of a rhythmic gene expression over a defined period of 24 hours, Cosinor analyses were performed (El Temps, v. 275, Prof. A. Díez-Noguera, University of Barcelona, Spain).

The daily acrophase of the locomotor activity rhythm for each larvae was calculated and the average acrophase for each day and for each group was determined by vector addition (El temps). The Rayleigh test was used to test whether the acrophases deviated from uniform ($p<0.05$). Uniform scores test was applied to test for differences between the acrophases among days either intra- or inter-group ($p<0.05$) (Fisher, 1993).

Results

Locomotor activity

All larvae hatched between 2 and 3 dpf. At 4 dpf, larvae from LDB group started to display a daily rhythm of locomotor activity (Fig. 1B; Cosinor, $p<0.001$). Daily rhythms of activity became significant at 5 dpf in LDW and LDR groups (Figs. 1A and C; Cosinor, $p<0.001$ and $p<0.01$, respectively).

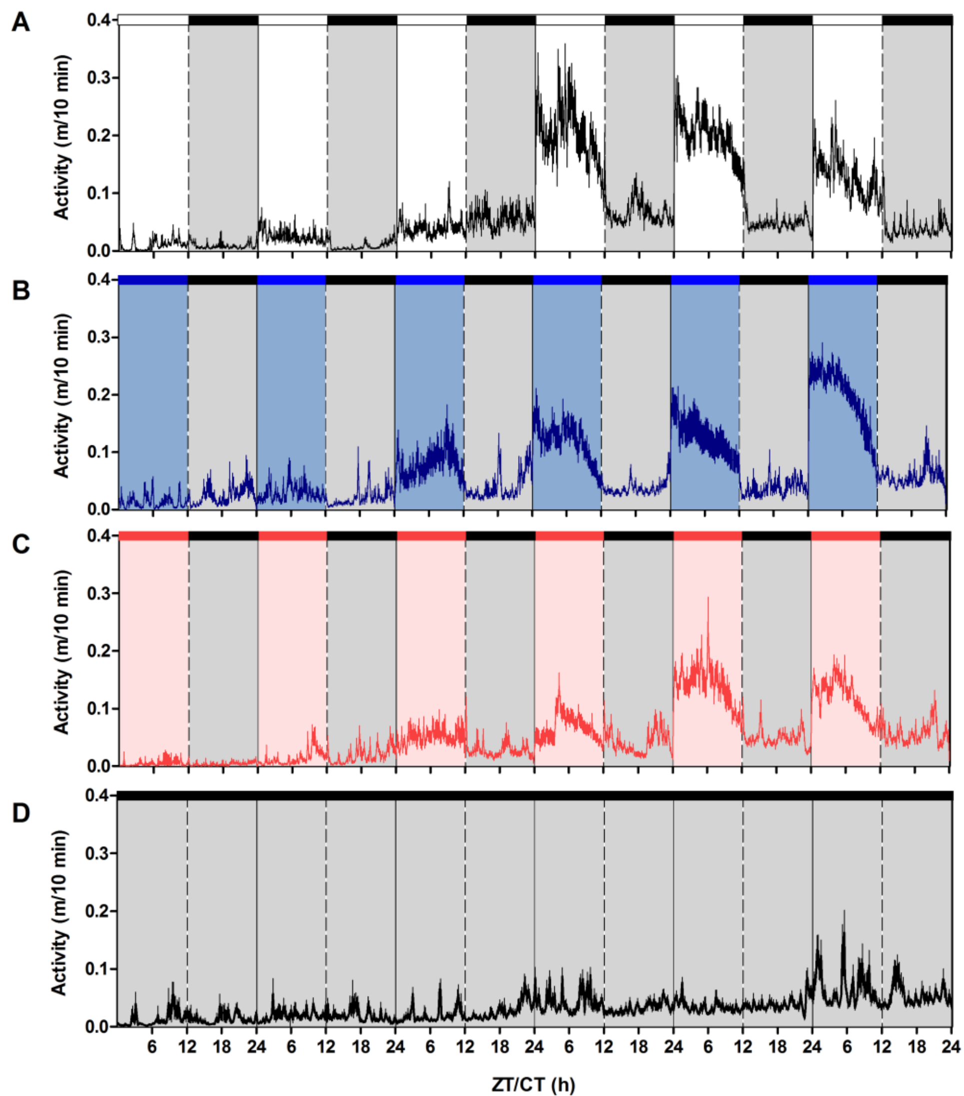

Figure. 1. Larvae reared under different light conditions (**A**: LDW, n=35, **B**: LDB, n=30, **C**: LDR, n=30, **D**: DD, n=45). Vertical axis represents activity (metres/10 min) and horizontal axis zeitgeber/circadian time (ZT/CT). Bars above each panel indicate the lighting conditions [black bars indicate darkness, white bars indicate white light (LDW), blue bars indicate blue light (LDB), and red bars indicate red light (LDR)] and the day post-fertilization (dpf). Data are expressed as mean ± SEM.

Larvae reared under DD were arrhythmic during all the days recorded (Fig. 1D; Cosinor, $p>0.1$). Larvae from all LD groups display the typical diurnal pattern of zebrafish, with higher activity (>65%) during the light phase.To verify the accuracy of the entrained rhythm we estimated the time of acrophases respect to the lights on (ZT0) in all groups from 5 to 7 dpf. Using a circular statistic approach, we showed that the distribution of acrophases deviated from uniform in LDW, LDB and LDR groups (Fig. 2; Rayleigh test, $0.05<p<0.001$), and the mean acrophases fell between ZT 2 and 7 (Fig. 2).The distribution of acrophases from 5 to 7 dpf differed only between LDB and LDR groups (Mardia-Watson-Wheeler Test: LDW: $W_4=5.4$, $p<0.05$; LDB: $W_4=38.9$, $p<0.0001$; LDR: $W_4=45.3$, $p<0.0001$). The distribution among groups did not differ at 7 dpf (Mardia-Watson-Wheeler Test: $W_4=4.5$, $p>0.3$), and the mean acrophases fell at ZT 03:18-04:36 (Fig. 2).All groups showed an increase in the total daily locomotor activity throughout development (two-way ANOVA, $p<0.05$) (Fig. 3). Larvae kept under LDB and LDR showed an increase in the total daily activity from 4 dpf, whereas a significant increase in activity under LDW and DD occurred at 5 dpf (Figs. 1 and 3).

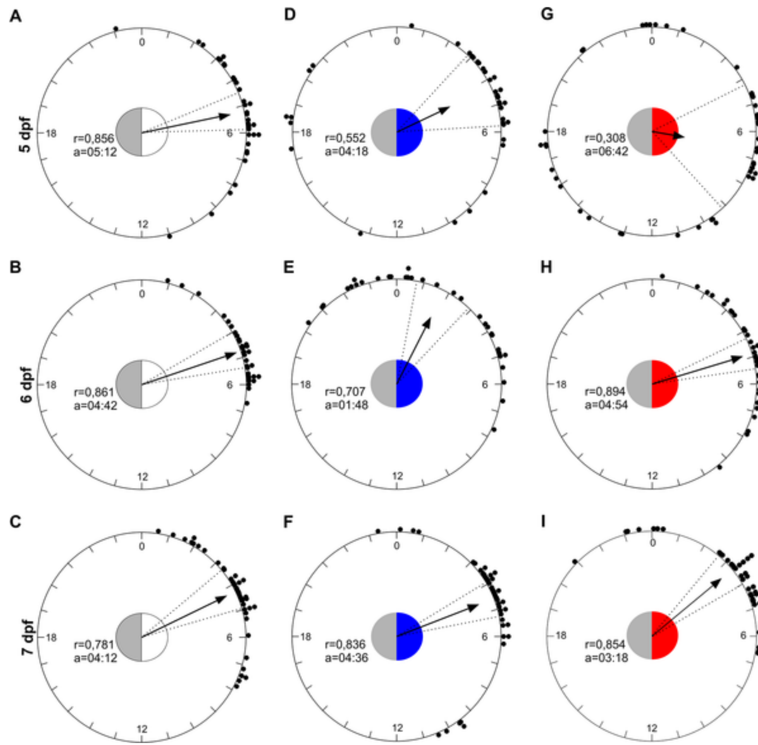

Figure 2. A circular representation of the phases of zebrafish activity across the 24 hours from 5 to 7 dpf under LDW, LDB and LDR. The dots represent the acrophase of each zebrafish larvae. The arrows indicate the average phases represented as vector and in each circle the mean vector length (r) and the mean acrophases (a) in ZT are reported. The circle inside each panel represents critical values of the Rayleigh test ($p<0.05$) and the coloured part show the duration of light phase (ZT 0-12). The dotted lines represent the confidence intervals.

Considering the whole period of recording (5 days, from 2 to 7 dpf), larvae reared under LDW and LDB displayed significantly higher overall activity than larvae under LDR

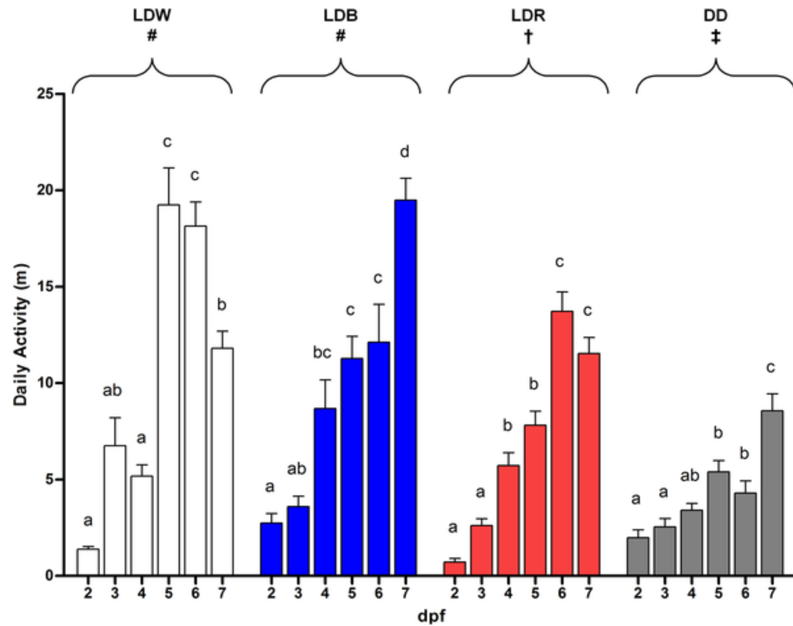

Figure 3. Daily activity under different lighting conditions from 2 to 7 dpf. Data are expressed as mean ± SEM. Letters indicate statistical differences between the different days for each lighting condition (one-way ANOVA; Tukey's post-hoc test, p<0.05). Symbols indicate statistical differences between the lighting conditions (two-way ANOVA; Tukey's post-hoc test, p<0.05).

and DD (LDW: 60.17 m; LDB: 56.51 m; LDR: 42.05 m; two-way ANOVA, p<0.05). Furthermore, larvae reared in DD showed lower total activity with respect larvae reared in the other lighting conditions (DD: 27.36 m; two-way ANOVA, p<0.05).

Clock gene expression

Embryos during the first 24 hours of life (0 dpf) showed variation of expression levels in all genes investigated (one-way ANOVA, p<0.05; Fig. 4A-E) and these variations were not affected by lighting conditions (two-way ANOVA, p>0.3; Fig. 4A-E). For the positive loop of the molecular clock, Cosinor analysis showed a significant rhythmicity (p<0.05) for *bmal1* under LDW, LDR and DD, whereas *clock1* expression levels were in all lighting conditions arrhythmic (p >0.05, Fig. 4A-B). For the negative elements, *per1b* and *per2* rhythmicity appeared only in some lighting conditions (Cosinor, p<0.05; *per1b*: LDW and LDR; *per2*: LDB; Fig. 4C-D). The clock-controlled gene *dbp* was rhythmic in LDB, but not under the other lighting conditions (Fig. 4E).

At 0 dpf all rhythmic genes displayed their acrophases during the light phase, with the exception of *per1b* under LDW (ZT 13:13 h) (Table 1).

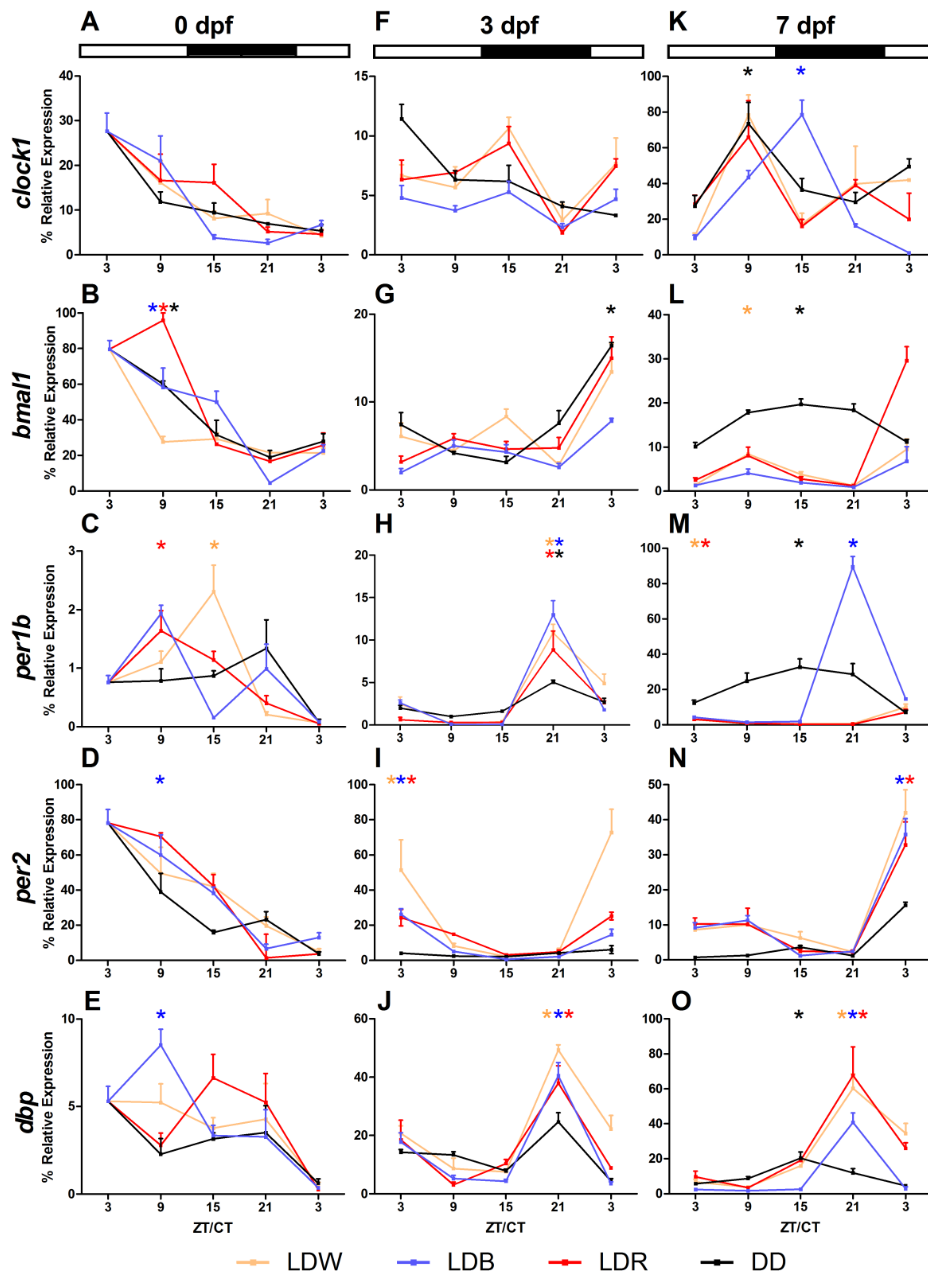

Figure. 4. Larvae reared under different light conditions (LDW, LDB, LDR, DD) were sampled every 6 hours at 0 (**A**, **D, G, L, O**), 3 (**B**, **E, H, M, P**) and 7 (**C**, **F, I, N, Q**) dpf. Data are expressed in % (100% is the maximum level detected for each gene in all dpf) and each value represents mean ± SEM (n=4). The bars above each group indicate the daily LD cycle. White bars represent the light phase and black bars represent phases of darkness. The DD group was kept under constant darkness. Asterisks indicate significant rhythms identified by Cosinor analysis (p<0.05).

At 3 dpf larvae had hatched, but the swim bladder was not developed and the yolk sac was the only source of energy. At this developmental stage, the negative element *per1b* was rhythmically expressed (one-way ANOVA, p<0.001; Cosinor, p<0.005; Fig. 4H; Table 1), and its expression was mainly affected by LDB and DD conditions (two-way ANOVA, p<0.01; Fig. 4H). *Per2*, other negative element of the loop and a light-inducible gene, also showed a significant rhythmic variation of the expression levels across the day under all wavelengths (one-way ANOVA, p<0.001; Cosinor, p<0.04; Fig. 4I; Table 1), but not in DD (one-way ANOVA, p>0.09; Fig. 4I). The highest induction of *per2* by light was under LDW (two-way ANOVA, p<0.001; Fig. 4I). *Per1b* and *per2* rhythms were not in phase: *per1b* showed the acrophase ranging between ZT/CT 20:34-22:07, *per2* at ZT/CT 3:06-4:24 (Table 1).

		0 dpf			3 dpf			7 dpf		
		Mesor (r.e.)	Amplitude (r.e.)	Acrophase ZT/CT	Mesor (r.e.)	Amplitude (r.e.)	Acrophase ZT/CT	Mesor (r.e.)	Amplitude (r.e.)	Acrophase ZT/CT
clock1	LDW	-	-	-	-	-	-	-	-	-
	LDB	-	-	-	-	-	-	7.5	7.82	13:21
	LDR	-	-	-	-	-	-	-	-	-
	DD	-	-	-	-	-	-	0.65	0.32	09:04
bmal1	LDW	-	-	-	-	-	-	0.61	0.55	08:33
	LDB	5.81	4.13	07:19	-	-	-	-	-	-
	LDR	6.56	5.37	07:36	-	-	-	-	-	-
	DD	5.95	3.9	06:35	0.95	0.6	01:18	2.35	0.71	15:07
per1b	LDW	1.31	1.17	13:13	4.47	6.54	22:07	2.83	4.29	02:32
	LDB	-	-	-	4.44	7.39	21:05	29.6	53.89	21:03
	LDR	1.07	0.92	11:03	3.08	5.24	21:04	2.17	3.11	03:12
	DD	-	-	-	3.13	2.48	20:34	28.8	15.2	15:21
per2	LDW	-	-	-	2.73	4.39	03:06	-	-	-
	LDB	5.27	4.58	08:26	0.93	1.43	03:24	1.29	1.54	04:22
	LDR	-	-	-	1.5	1.58	04:24	1.15	1.28	04:24
	DD	-	-	-	-	-	-	-	-	-
dbp	LDW	-	-	-	7.45	8.11	21:33	10.1	10.94	20:28
	LDB	1.61	1.13	09:58	5.6	6.85	21:13	4.79	7.77	20:04
	LDR	-	-	-	6.23	6.76	21:09	10.27	12.67	20:01
	DD	-	-	-	-	-	-	4.56	2.9	15:32

Table 1. Mesor, Amplitude defining clock gene expression rhythms at 0, 3 and 7 dpf, given as relative expression values (r.e.) and Acrophase as ZT. Rhythms are considered significant when p<0.05. Only statistically significant values (p<0.05) are reported.

Although ANOVAs revealed a significant variation during the 3 dpf (one-way ANOVA, p<0.05) and an effect of lighting conditions (two-way ANOVA, p<0.03), both positive elements *clock1* and *bmal1* did not show rhythmic variations of expression levels (Cosinor, p>0.1; Fig. 4 F; Table 1), except for *bmal1* under DD (Cosinor, p<0.02; Fig. 4 G; Table 1). *Dbp* was rhythmic (one-way ANOVA, p<0.003; Cosinor, p<0.001;

Fig. 4 J; Table 1) and affected by lighting conditions (two-way ANOVA, p<0.001). *Dbp* rhythms had acrophases between 21:09-21:33 ZT/CT (Fig. 4 J; Table 1).

At 7 dpf larvae started exogenous feeding and were able to swim freely. In contrast to the other developmental stages analysed, *clock1* expression levels at 7 dpf showed daily variations during the day (one-way ANOVA, p<0.04) and oscillated rhythmically under LDB and DD (Cosinor, p<0.008) with acrophases at ZT 13:21 and CT 09:04, respectively (Fig. 4 K). The other positive element investigated, *bmal1*, showed a similar daily pattern: rhythmic in DD and LDW (one-way ANOVA, p<0.04; Cosinor, p<0.03; Fig. 4 L; Table 1) with acrophases at CT 15:07 and ZT 8:33, respectively. The negative elements *per1b* and *per2* showed temporal variation in expression levels (one-way ANOVA, p<0.002). *Per1b* was rhythmic under all lighting conditions (Cosinor. p<0.01, Table 1, Fig. 4 M) with acrophases at ZT 2:32-3:12 under LDW and LDR respectively and ZT/CT 15:21-21:03 under DD and LDB, respectively. *Per2* was rhythmic only under LDB and LDR (Cosinor, p<0.01, Table 1) with acrophases at ZT 4:24 under LDB and 4:24 under LDR (Fig. 4N). *Per1b* and *Per2* rhythms were strongly influenced by lighting conditions (two-way ANOVA, p<0.001). *Dbp* expression levels changed during the 7 dpf (one-way ANOVA, p<0.001) and showed rhythmic oscillations depending on the lighting conditions (two-way ANOVA, p<0.001). *Dbp* expression showed acrophase during the dark phase: ZT 20:28 under LDW, 20:04 under LDB and 20:01 LDB and CT 15:32 under DD (Cosinor, p<0.003; Fig. 4O; Table 1).

The comparison of mean expression levels of each gene showed differences depending on the experimental conditions (Fig. 5). For instance, the highest levels of *clock1*, *bmal1* and *per2* expression levels were at 0 dpf (one-way ANOVA, p<0.01; Fig. 5A-B, D). Differently, *per1b* and *dbp* showing the highest values at 3 and 7 dpf (one-way ANOVA, p<0.05; Fig. 5C, E). The daily mean expression level of *dbp* was higher at 3 and 7 dpf respect to 0 dpf in LDW, LDR and DD (two-way ANOVA, p<0.001; Fig. 5E), whereas in LDB the mean levels did not change during the first week of life (Tukey's post-hoc, p<0.05; Fig. 5E). *Clock1* relative expression was mainly influenced by LDB (Tukey's post-hoc, p<0.008, Fig. 5A). No statistical differences were detectable for both *bmal* and *dbp* among the lighting conditions (two-way ANOVA, p<0.2, Fig.5B, E). LDB and DD influenced the *per1b* mean expression differently from LDW and LDR (Tukey's post-hoc, p<0.03; Fig. 5C). The effect LDW on *Per2*

expression is different from DD but not from LDB and LDR (Tukey's post-hoc, LDW vs DD: p<0.01, LDW vs LDB-LDR: p<0.6 , LDB-LDR vs DD: p<0.3; Fig. 5D).

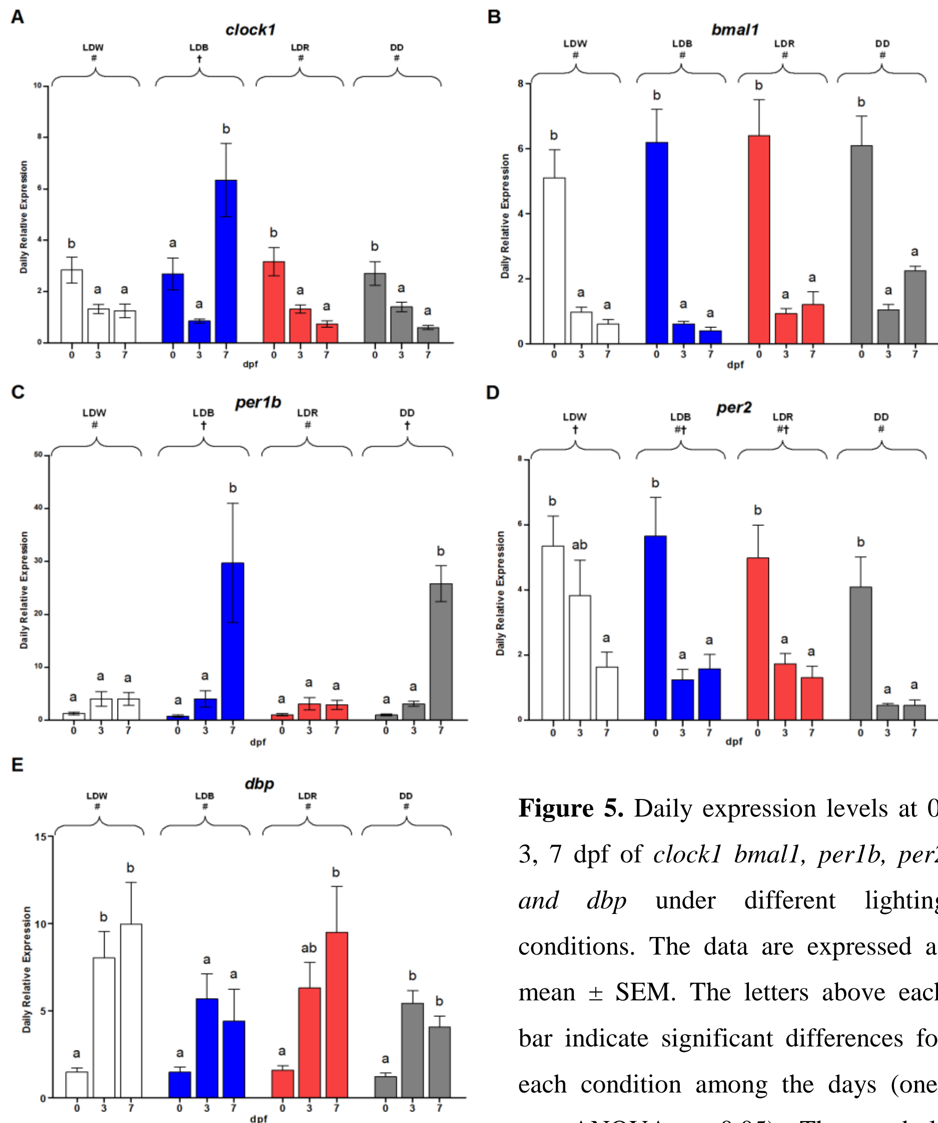

Figure 5. Daily expression levels at 0, 3, 7 dpf of *clock1 bmal1, per1b, per2 and dbp* under different lighting conditions. The data are expressed as mean ± SEM. The letters above each bar indicate significant differences for each condition among the days (one-way-ANOVA, p<0.05). The symbols on the top indicate statistically differences between the lighting conditions (two-way-ANOVA, Tukeys post-hoc test, p<0.05).

Discussion

Solar light is a complex environmental signal that influences the evolution of most biological processes on the Earth. Light is characterized by daily changes in irradiance, wavelength composition, direction and polarization (Björn, 2002). In the last years many investigations are taking into account a significant role of the different wavelengths (Villamizar et al., 2013; Blanco-Vives et al., 2010; Villamizar et al., 2009).

Larvae were arrhythmic under DD, while they developed daily activity rhythms under LD cycles, which appeared earlier in LDB (4 dpf) than in LDW or LDR (5 dpf). Furthermore, larvae reared under LDW and LDB displayed significantly higher overall activity than larvae under LDR. At 7 dpf the phase of the rhythm in all lighting conditions is identical and the acrophases fell in the early day (ZT 3-4). Previous investigations showed that the rise of swimming behaviour in zebrafish larvae is linked to the maturation of serotoninergic neurons (Brustein et al., 2003). We cannot exclude that the anticipation of 1 day (4 dpf respect to 5 dpf) in the onset of the daily rhythm of locomotor activity and the high overall activity under blue light depends to a stimulation of the serotoninergic system by this lighting conditions.

Previous studies have shown that zebrafish behaviour under constant lighting conditions is regulated by an endogenous clock and that the LD cycle sets the phase of this clock (Cahill et al., 1998; Hurd and Cahill, 2002). For instance, LD cycles are required for the correct onset of behavioural rhythmicity in zebrafish larvae (Hurd and Cahill, 2002). The amplitude of activity rhythms is directly correlated with the number of LD cycles to which embryos are subjected before they are transferred to DD conditions. Subjecting the zebrafish embryos to only one or two LD cycles after fertilization has been seen to significantly reduce the number of animals displaying circadian rhythmicity (Hurd and Cahill, 2002). Our results agree with this study, since larvae reared under DD conditions, which only received 3 hours of light after fertilization, did not develop locomotor rhythmicity and displayed lower activity levels than fish reared under LD cycles. A recent investigation also showed the effects of DD on zebrafish larval development, growth and survival (Villamizar et al., 2013). Zebrafish larvae raised in DD died before 18 days post-hatching (dph). Interestingly, larvae transferred to an LD cycle at 5 and 10 dph showed an improved survival rate compared with the larvae maintained in DD (Villamizar et al., 2013), which further underlines the importance of LD cycles to sustain normal development during early larval stages.

The analysis of clock gene ontogeny revealed different results in the animals under DD and LD conditions. For instance, under DD *per2* rhythms failed to establish during the development, which has been observed in other fish species such as the medaka (*Oryzias latipes*) and the Senegalese sole (*S. senegalensis*) (Dekens and Whitmore, 2008; Martin-Robles et al., 2012; Cuesta et al., 2014). This effect of DD conditions

would be explained by the fact that *per2* is a light-inducible gene, and thus requires the presence of light for its daily rhythmicity to develop correctly (Vatine et al., 2009). Interestingly, the presence of constant light does not make for the regular expression of clock genes. In the rainbow trout, *Oncorhynchus mykiss,* the clock genes *per1* and *clock* showed persistent rhythmicity in the larvae reared under LD conditions from 0 to 58 dpf, but not under constant lighting, when the rhythmicity is lost or developed later respect to the LD conditions (Davie et al., 2011). The other negative element investigated, *per1b*, showed rhythmicity in DD from 3 dpf, whereas *clock1* and *dbp* were rhythmic from 7 dpf. Only the positive element *bmal1* had a rhythmic expression under DD from the embryo stage (0 dpf).
After 3 days of exposition to LD cycles, *per1b*, *per2* and *dbp* showed a significant variation in the daily gene expression. On the contrary, genes from the positive loop of the clock, *clock1* and *bmal1*, needed a longer time to start oscillating and after 7 days in LD cycles only in two conditions they were rhythmic (*clock1* in LDB and *bmal1* in LDW). These discrepancies among the times of occurrence of rhythmicity among key components of the circadian clock has been suggested in other studies in fish (Dekens and Whitmore, 2008; Martin-Robles et al., 2012; Cuesta et al., 2014).

Interestingly, we observed a daily rhythm at 0 dpf under all lighting conditions for some genes, with the acrophase located during the first hours after fertilization and expression levels falling during the rest of the day. A similar response has been observed previously in zebrafish and in Senegalese sole (Martin-Robles et al., 2012, Dekens and Whitmore, 2008). Rather than a real rhythmicity driven by an endogenous clock, this result might depend on a direct light induction of the first hours of light after fertilization (Dekens and Whitmore, 2008), since in the present experiment even embryos reared in DD received 3 hours of light, while eggs were being collected, or alternatively, to the presence of maternal RNA (*clock1*, *bmal1* and *per2* at ZT 3 of 0 dpf) (Harvey et al., 2013).

Clock genes from the negative loop (*per1b* and *per2*) displayed rhythmic expression early during development compared with *clock1* and *bmal1*, results that did not depend on the photoreceptive system in zebrafish larvae. By 5 dpf, the larval retina is differentiated and functional, displaying responses evoked by visual stimuli. It expresses different opsins including melanopsin, a photopigment involved in circadian photoreception. Recent investigation in *Danio rerio* ZEM-2S cells points to melanopsin

as the photopigment that mediating the photoresponse increasing *per2* and *cry1a* and slightly modulating *per1b* and *cry1b* expression (Matos-Cruz et al., 2011; Fadool and Dowling, 2008; Ramos et al., 2014). The pineal gland is formed around 18-22 hours post-fertilization and starts displaying rhythmicity under LD cycles at 1 dpf (Ziv et al., 2005; Vuilleumier et al., 2006). Photoreceptive cells expressing TMT-opsin and melanopsin are present in the brain of 3-6 day old zebrafish larvae Tassmar-Raible et al., 2007; Fernandes et al., 2012). To date, it is unknown which requirements or processes are involved in delaying the appearance of *clock1* and *bmal1* rhythms compared with *per*s. However, in medaka too, the rhythmic expression of *per* is detected very early during development and rhythmic *clock* and *bmal* expression occurs later (Cuesta et al., 2014).

In the case of light wavelength, its effects on the ontogeny of fish are scarcely understood to date. Recent papers on this topic have focused on wavelength effects on larval performance, survival and the occurrence of malformations, finding that, in general, short (blue) wavelengths are better for fish development than long (red) wavelengths (Villamizar et al., 2013; 2009). A drastic effect of short wavelengths on larval behaviour has also been found in the Senegalese sole (Blanco-Vives et al., 2012). The blue light condition was able to generate a switch in locomotor activity, changing the active phase from diurnal to nocturnal during larval metamorphosis onset. Conversely, long (red) wavelengths did not show any effect on the locomotor activity (Blanco-Vives et al., 2012). Also zebrafish PAC-2 cells seem to be more sensitive to short wavelengths: the expression level of light-inducible genes, *cry1a, cry5* and *per2*, was higher when submitted to blue than to red lighting conditions (Mracek et al., 2013). Our results agree with those obtained in the other fish species reported above, since both behavioural and clock gene rhythms appeared earlier in the larvae reared under short wavelengths (LDB) than in those reared under long wavelengths (LDR).

In summary, our study provides novel insights into the ontogeny and the effects of lighting conditions on molecular daily rhythms, and how these rhythms are reflected in the behavioural rhythmicity of zebrafish larvae. The present results also underline the relevance of lighting conditions on fish development. The LD cycle and specific wavelength is essential for normal development of the circadian system. These conditions should be carefully considered when fish embryos and larvae of zebrafish or

other model fish species reared in laboratory facilities and in fish hatcheries for aquaculture companies.

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Author Contributions

Conceived and designed the experiments: VDR, FJSV, CB. Performed the experiments: VDR, EF, CB. Analysed the data: VDR, EF, JFLO, FJSV, CB. Contributed reagents/materials/analysis tools: FJSV, CB. Wrote the paper: VDR, EF, JFLO, FJSV, CB.

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Chapter II

Light transition induces locomotor response in zebrafish larvae: effect of different wavelengths

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Introduction

Light plays an essential role on the modulation of the behavior and regulation of physiological processes. Vertebrates detect light with the lateral eyes, and other extra-retinal photoreceptors. Mammals have lost their capacity to detect light with other organs due to evolutionary morphological changes. However, other forms of photoreception, not involved in the image-forming system show the capacity to detect irradiance, direction of illumination and light polarization. These alternative forms of light detections in vertebrates influence behavior (Underwood and Gross, 1982).

In the water ecosystem the light is absorbed and scattered by particle present in the water column which thus affect irradiance, polarization, wavelength, direction and propagation of the light (Smith, 1974).The water column acts as a potent chromatic filter, quickly absorbing violet and red wavelengths. However, blue wavelengths ($\lambda \approx$ 450 nm) penetrate deeper in the water. Fish living in different underwater environment have maximized their sensitivity by developing and adapting their photopigment at the surrounding environment (Kusmic and Gultieri, 2000).

The first step of photic transduction is represented by retinal photoreceptors, formed by cones and rod of different sensitive and kinetics. Four groups of visual opsin has been already identified: rod opsin (rh1) and 4 cone opsin (lws/mws, rh2; sws1 and sws2) (Vithelic et al., 1999; Bilotta and Saszik, 2001; Allison et al., 2010). Fish presents multiple subtypes of cone photoreceptors, differing for their sensitivity as results of different expression of opsin genes (Allison et al., 2010). In non-mammalian vertebrates interestingly are present homologous functional counterpart of these proteins, the extraretinal-photoreceptors, located within the pineal complex (pineal and parapineal organs, frontal organ and parietal eye), the deep brain and the dermal melanophores (Shand and Foster, 1999). These non-visual opsins have been differentiated in: VA opsin, and ERrod-like opsin in the pineal, melanopsin (different isoforms), VA opsin (different isoforms); rhodopsin, encephalopsin and tmt-opsin in the deep brain (Philp et al., 2000; Drivenes et al., 2003; Kojima et al., 2000; Masuda et al., 2003; Moutsaki et al.; 2003). Each one of these opsins have a different spectral sensitivity that allows animals to perceive all light spectrum.

In zebrafish the effect of the light cycle has been largely studied, although only recently has been studied the crucial effect of different light spectrum on the development of zebrafish larvae. The different wavelengths of light influence many physiological

processes, such as hatching, growth, survival in zebrafish (Villamizar et al., 2014). Similar results has been revealed on sea bass and zebrafish larvae which showed that in blue and white LD cycle larvae display a grater development and total length (Villamizar et al., 2009) and were mostly active in contrast than larvae reared in constant darkness or red LD cycle conditions (Villamizar et al., 2011). In sole the same light conditions modified the onset of daily rhythms, promoting a switch from diurnal to nocturnal activity (Blanco-Vives et al., 2012). Also a pulse of different wavelength at mid night affects the behavior of adult zebrafish, showing an increase of swimming activity and photophobic response in a wavelength dependent manner (Del Pozo et al., 2015). In sea bass a light pulse at night, provoke physiological changes, by inhibiting the melatonin release. In addition, blue light was more effective on inhibiting the hormone, then red light (Bayarri et al., 2002) as occurs also in sole (Oliveira et al., 2007). Light plays a key role on the behavior of animals that adjust their own activity in response of its information. Fish are classified as diurnal, nocturnal or crepuscolar depending if their greatest activity occurs during the photophase, the scotophase or during the dawn or dusk, respectively (Madrid et al., 2001). Some individual can switch from diurnal to nocturnal 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 (Eriksson, 1978).

The exposure of entrainment signals are essential for the initiation of behavioral rhythmicity of zebrafish larvae, which remain generally inactive until 4-5 day post fertilization. The early exposure to light cycle set the phase of the clock that controls larvae zebrafish behavior and increase the amplitude of the locomotor rhythms (Hurd and Cahill, 2002). The light cycles of different wavelengths, induce the initiation of locomotor rhythmicity in different way, instead larvae submitted at blue light start their locomotor activity at 4 dpf whereas in white and red light at 5 dpf (Di Rosa et al., 2015). The light transition from the light phase to the dark one and *vice versa* produces behavioral responses in different manner. For example in loss of illumination, larvae are transiently hyperactive before adopting a quiescent state, possibly in order to avoid looming predators, after 5-10 minutes the locomotor activity decline, reaching baseline levels (Burgess and Granato, 2007). During light adaptation, the locomotor activity increases reaching its maximum after 15 minutes, resulting in higher baseline activity. This behavior may be due to the need of light to feed and to avoid the risk of predation.

(Clark, 1981; Gahtan et al., 2005; McElligot and O'Malley, 2005). Fernandes et al., displayed that the light responses of larvae with increment of locomotor activity is mediated by deep brain photoreceptors and not by retina or pineal gland (Fernandes et al., 2012).

Our data show the effect of different wavelengths light transition on the locomotor activity of zebrafish larvae from 3 to 10 dpf, confirming, as previous described, the increment of activity during both light/dark-adaptation and revealing for the first time the onset of this behavior under different light spectra.

Materials and Methods

Ethics Statement

The present research was carried out in the Chronobiology laboratories of the University of Murcia (Spain) and of the University of Ferrara (Italy). All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). The experimental protocol was previously authorized by the Spanish National Committee on Animal Welfare (Law 32/2007) and the Bioethical Committee of the University of Murcia (Spain) and by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health.

Animal rearing

Wild-type adult fish were obtained from commercial provider (Alimar Pets S.L., Murcia, Spain) and housed for 1 year in 9 L glass aquaria (1 fish L^{-1}) according to standard method (Nüsslein-Volhard et al., 2002). The reproductive fishes were fed 2 and 6 hours after lights on with dry food (Tropical fish flakes; PRODAC, Italy). For spontaneous spawning, the sexually mature fish were separated in groups of 5 fish (3 females and 2 males) and transferred into a 2.5 L breeding net cage during the afternoon. Spawning took place the next morning, approximately 2 hours after lights on. Eggs were collected, pooled and distributed into 85x10 mm plastic Petri dishes with cover (20 eggs per Petri dish) filled with embryo medium (Nüsslein-Volhard et al., 2002). The Petri dishes were placed to float in a 12 L aquarium at 27ºC.

Experimental procedure

The experimental groups were exposed to three different lighting conditions: LDW (white), LDB (blue) and LDR (red). The light-dark (LD) cycle was of 12 hours light and 12 hours darkness. Illumination was provided by means of neutral red, blue, and white LED light lamps (Superlight Technology Co. Ltd., China). Irradiance was measured with a spectro-radiometer (FieldSpec ASD, Colorado, USA) set at 1.62 E+18 photons m^{-2} s^{-1}. The λ_{max} of the red and blue LED light lamps were 639 nm and 465 nm, respectively. The temperature was held constant (27 ºC) by means of water heaters (50 W, Sera GmbH, Germany) and recorded every 10 minutes with data loggers (Hobo Pendant, Onset Computer Corporation, Massachusetts, USA).

Behavioural recording

The embryos were collected immediately after spawning, incubated in 12-well clear bottom plastic plates filled with 5 ml of embryo medium and exposed to a specific light condition. Each multi-well plate was placed on the water surface of a 10 L aquarium. The plate was fixed on the base of the aquarium to avoid any change of position during the recording. The larvae were fed at 5 dpf and the embryo medium was partially changed every 2-3 days. Swimming activity patterns were recorded from 2 to 11 dpf, for each light condition. Larvae were recorded by means of a webcam adapted for infrared recording by removing the UV filter in front of the lens placed on the top of the aquarium and connected to a computer. An infrared LED (monocolor diode, model L-53F3BT, 5 mm) covered with a blurred white panel was placed under the aquarium to permit the video recording during the dark phase of experiment. These IR lamps emitted at 940 nm, which is not detected by zebrafish (Lythgoe, 1988). Two specialized software packages, Multiviewer and FishTracker (Computer System Department, University of Murcia), were used. The Multiviewer allowed simultaneous webcam recording. Every minute, 60 images (1 frame/s) were stored. The FishTracker quantified the larvae movements and has already been validated in sea bream (Vera et al., 2010) and zebrafish (Sánchez-Vázquez et al., 2011).

Molecular analysis

After fertilization, embryos and larvae were maintained under different light conditions in Petri dishes and sampled during day 3 (dpf). Samples were formed by pooled larvae

of four different time points during the day to avoid any differences of expression during the day. For each time point, 10 larvae were sampled and pooled. Total RNA was isolated from zebrafish embryos and larvae using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The amount, quality and composition of isolated RNA were analysed by BioSpec-nano (Shimadzu, Kyoto, Japan). One microgram of total RNA was incubated with DNase I (Invitrogen) at room temperature for 30 min and then at 85°C for 15 min to inactivate the enzyme. DNase-treated RNA was used to perform cDNA synthesis in a final volume of 20 μl, using iScript cDNA Synthesis Kit (Biorad, Milan, Italy). The reaction was performed at 42°C for 30 min, followed by an inactivation step of 5 min at 85°C. Zebrafish genes were amplified by PCR using GoTaq DNA Polymerase (Promega, Madison, WI). Gene-specific primers for *tmt opsin, opn4m1, opnlw1, exorhodopsin,* have been designed by Primer3 software. Thermal cycling conditions were as follows: 2 min denaturation at 94°C, followed by 40 cycles of a 30 s denaturation step at 94°C and an annealing step for 30 s at 58°C and an elongation step for 30 at 72°C. Negative control reactions containing water or RNA instead of cDNA were included in the PCR reactions. As housekeeping gene was amplified the 18s, using specific primer obtained by primer3. To revealing the amplification products, an electrophoresis test of 2% agarose gel was performed.

Data analysis

The videos were analysed by FishTracker, a software developed by the Computer Vision Research Group of the University of Murcia (Vera et al., 2010; Sánchez-Vázquez et al., 2011). The program tracks the movement of the larvae and provides the spatial coordinates, corresponding to the X:Y position in the well. The distance between two consecutive points (X_1:Y_1; X_2:Y_2) was calculated using the distance formula derived from the Pythagoras´ theorem and data were arranged in 10 minutes batches for a total of 144 data per day.

Statistical analysis

All the results are expressed as means ± SEM. Data were normally distributed (D'Agostino-Pearson normality test, $p<0.05$) and all populations had the same variance (Bartlett's test for equal variances, $p<0.05$). To determine differences of locomotor

activity before and after the lightening change, was used a Student t-student test at two tailed, dependent sample. One-way analysis of variance (ANOVA) was used to determine differences in the increment of locomotor activity among ZT, dpf and lighting conditions. Tukey´s HSD post-hoc test was used for the multiple comparison among groups ($p<0.05$). ANOVAs were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

We studied the visual motor response of zebrafish larvae from 3 to 10 dpf, after light and dark adaptation. We misured the % of activity of larvae during the light and dark phase revealing that larvae reared under LDW showed to be diurnal from 3 dpf, whereas red from 4 dpf and blue from 5 dpf (Fig. 1).

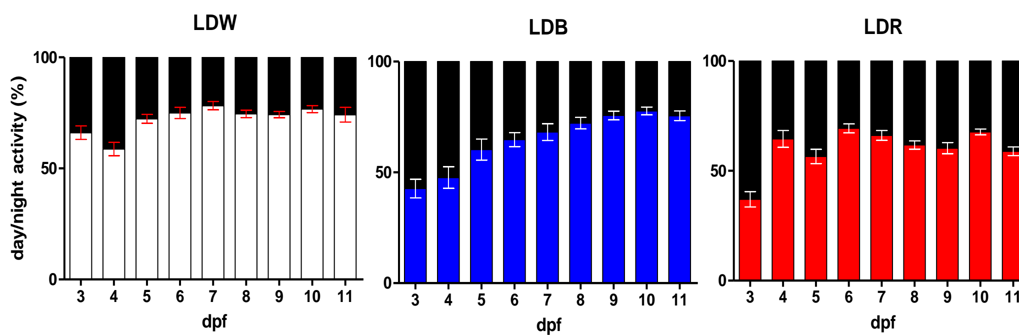

Figure 1. Diurnal and nocturnl activity of larvae from 3 to 11 dpf under different light wavelengths. Data are expressed as mean ± SEM. (n=18). White, blue and red bars represent the light phase, black bars represent the dark phase.

To check the existence of differences on locomotor activity of larvae before and after the lights on and off, we compared the total activity 10 minutes before and after the change of illumination and found statistical differences in term of activity quantity (Student's t test, $p< 0.05$). At 3 dpf larvae did not display significant differences in terms of increment of locootor activity, among light conditions in both light transitions (One way-ANOVA $p<0.001$; Fig.2 A, B).

Conversely, at 8 dpf larvae responded differently during dark adaptation, showing higher activity response under LDW than the other light conditions (one way-ANOVA; Tukey's post hoc test. Fig. 2 A).

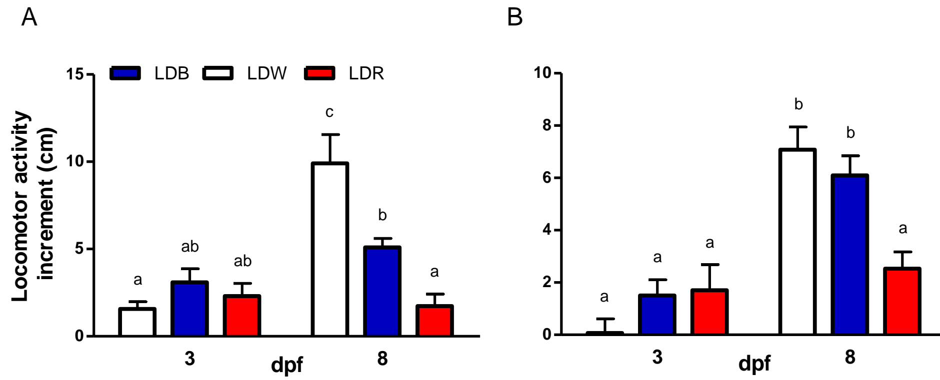

Figure 2. Locomotor increment activity after the illumination change in larvae of 3 and 8 dpf. The light applied was of different light spectrum. Data are represented as mean ± SEM. A: Illumination change: light off. B illumination change Light on

Morover, also larvae during the light-on responded in different manner depending on the different light wavelength, showing higher increment of activity under LDW and LDB rather LDR (One way- ANOVA, p<0.001. fig 2 B).

Regarding the meanwave profiles of adaptation, zebrafish displayed, during dark adaptation at 8 dpf, hyperactivity just 1-2 minutes after the transition, reaching after 5 minutes the same levels of pre-transition activity (Fig. 5).

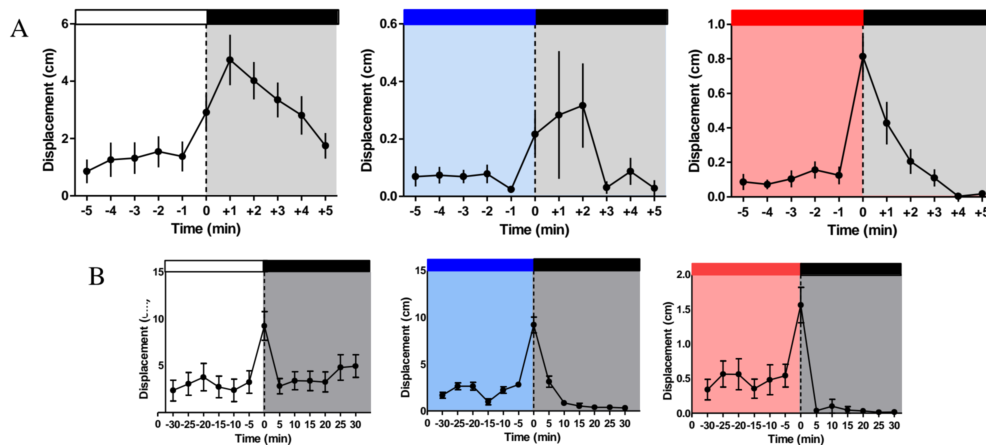

Figure 3. Light off behavioural response of larvae at 3 dpf reared under LDW, LDB and LDR. Meanwave profiles of light response 5 minutes before and after illumination change (A) and 30 minutes before and after the light change (B). Data are expressed as mean ± SEM.

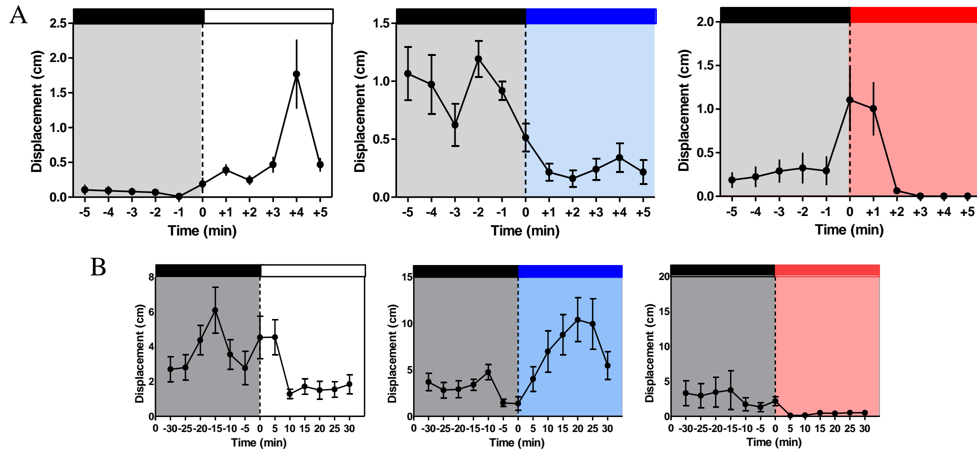

Figure 4. Light on- behavioural response of larvae at 3 dpf reared under LDW, LDB and LDR. Meanwave profiles of light response 5 minutes before and after illumination change (A) and 30 minutes before and after the light change (B). Data are expressed as mean ± SEM.

During light adaptation, the profile appears different because zebrafish increased its activity that persists reaching higher activity levels respect to that in previous dark condition (Fig.6).

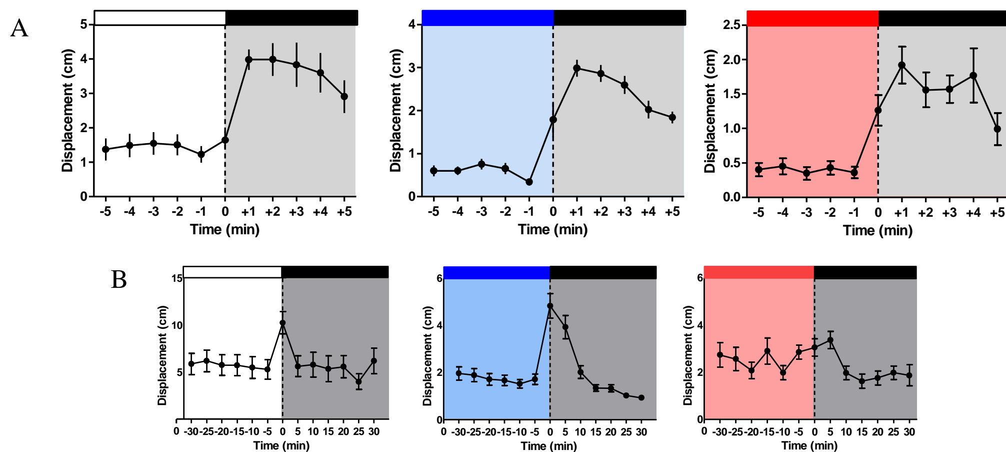

Figure 5. Light off behavioural response of larvae at 8 dpf reared under LDW, LDB and LDR. Meanwave profiles of light response 5 minutes before and after illumination change (A) and 30 minutes before and after the light change (B). Data are expressed as mean ± SEM.

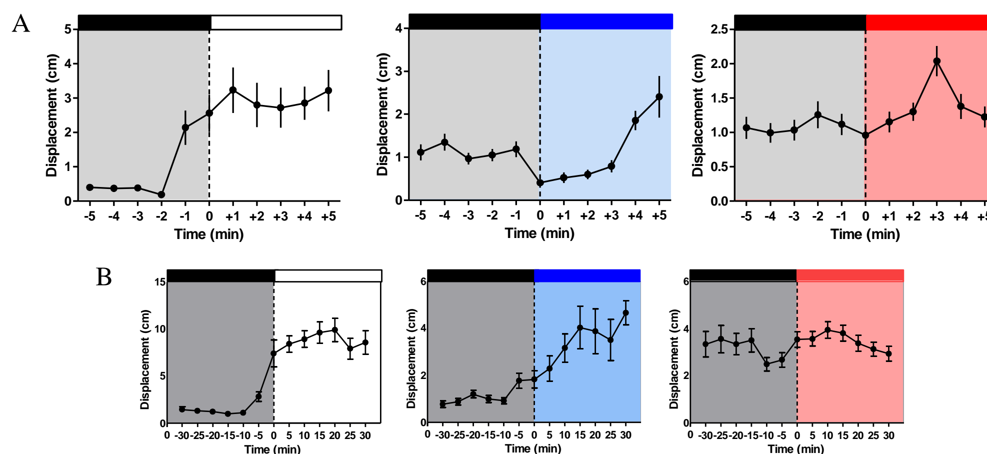

Figure 6. Light on- behavioural response of larvae at 8 dpf reared under LDW, LDB and LDR. Meanwave profile of light response 5 minutes before and after illumination change (A) and 30 minutes before and after the light change (B). Data are expressed as mean ± SEM.

The molecular analysis of opsins at 3 dpf revealed that all larvae reared under different light condition express the investigated opsins (Fig. 7)

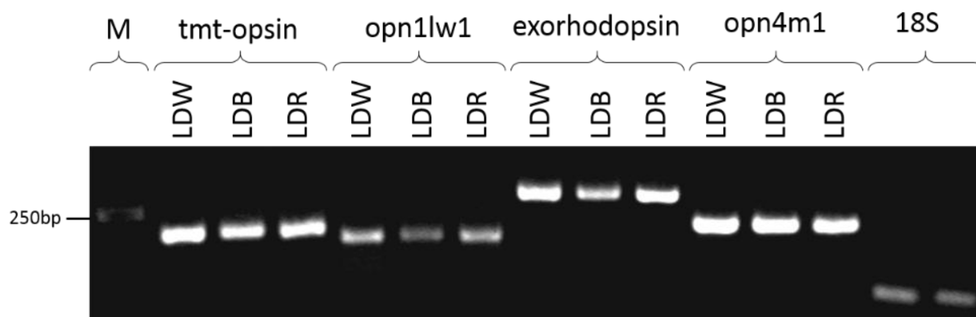

Figure 7. Expression analysis opsin's genes in zebrafish larvae of 3 dpf reared under different lightening conditions by means of qualitative

Discussion

In the present study, we show that an increment of activity of larvae during the lightening transition is present from the 3rd dpf. These increments have different profiles depending on the initial state of the larvae, whether they proceed from light or dark condition. We revealed that zebrafish hyperactivity, induced by the lightening transition, is already present at day 3, when the retinal photoreceptors are not still functional. It is reasonable to think that the stimulus the drives the behaviour is

mediated by deep brain photoreceptors. Our findings are in accordance with a previous study on which the visual motor response persisted also when the pineal was ablated, suggesting that this behavior was mediated by deep brain photoreceptors. Melanopsin *opn4m1* was the candidate for mediating deep brain photoreception, and its expression was proposed to be involved in the dark photokinesis (Fernandes et al., 2012).

A previous investigation highlighted the different behavior response, in terms of scoots and turns, of zebrafish larvae during light-dark and dark-light transition (Burgess and Granato, 2007). We revealed the same behavioral response in zebrafish even where the pre-transition state is 12 hours longer, reinforcing the strong effect of the light transition on the locomotor activity and reactive response during development. This behavior can represents an ancient mechanism that enables larvae to move out of dark environment, where there is insufficient light for retinal cone photoreceptors to control rapid visual behaviors such as hunting or predator avoidance (Fernandes et al., 2013).

A different locomotor response of larvae reared under different wavelengths light is revealed at 8 dpf, whereas a 3 dpf the lighting conditions did not affect differently the activity. White light is able to induce the main change in terms of activity in larvae dark or light adapted at 8 dpf, whereas blue light induces a different response respect to white. It seems to be strictly dependent by the switch on or off the light; instead blue light differs only when the light is turned off. The high sensitivity for the white and blue light has been already revealed in zebrafish larvae instead a previous study highlighted the effect of this light condition on the ontogeny of the behavior and onset of biological clock (Di Rosa et al., 2015). We can speculate that this higher sensitivity for the blue (low wavelengths) and white (full spectrum) despite for the red light could be due to adaptation of animal to rounded environment, considering that blue light has the capacity to penetrate deeper in the water. Zebrafish present deep brain photoreceptors sensitive to different wavelength, we investigated *tmt-opsin*, *exorhodopsin* and *opn4m1*, genes that express opsins sensitive for blue and green wavelengths. *Opnl1w1* is a visual opsin that perceives the red wavelength. We have revealed the expression of these photoreceptors from day 3, in accordance with the onset of behavioral activity. The hyperactivity of zebrafish was attributed, in a previous work, to the expression of melanopsin, OPN4M1, instead in animals *otpa* mutants (orthopedia transcriptor factor of *opn4M1*) are severely affected in the visual motor response (Fernandes et al., 2012). Our results coincide with the previous, instead under LDB and LDW zebrafish

displayed higher activity respect to LDR, already expressing melanopsin and other photoreceptors capable to perceive the blue light (Fernandes et al., 2012). Curiously, also in larvae under red light, has been reported differences on locomotor activity, so the perception is not attributable to melanopsin rather to OPNLW1. Considering that OPNLW1 is a visual opsin, and that, for its normal function, requires a complete visual system, we can suppose that the gene expression does not contribute to the red light perception because at 3 dpf the visual system has not already completed its own development. We can speculate that perception of red light could depend by another opsin present in the brain, sensitive to the long wavelength as was demonstrated by a recent study on tropical damselfish, showing a new opsin in brain capable to perceive long wavelengths (Takeuchi et al., 2011). Our findings strengthen the notion on the onset of locomotor activity, highlighting the different effect of wavelength on the perception of the stimulus. Furthermore, more studies are needed, to explain perception at different wavelength and associate it to specific photoreceptor, to date the onset of each protein functionally active.

Aknoweledgents

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Chapter III

Do different Lighting conditions affect the time of hatching in zebrafish?

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Introduction

The rotation and translation movements of the Earth and moon produce daily, lunar, and annual light cycles that characterize the environment on which animals live (López-Olmeda and Sánchez-Vázquez, 2009). Animals have adapted to periodic changes in their environment by evolving a circadian system or biological clock able to measure internal time. This biological clock allows them to anticipate external events such as sunrise and sunset and to entrain rhythmic physiology and behavior (Duguay and Cermakian, 2009; Farhat et al., 2009). The fish biological clock has been linked with the daily rhythms of cell division as most cell types are not only influenced by the response of the fish central and peripheral pacemakers to environmental signals, but also by the cells own circadian clock (Schibler and Sassone-Corsi, 2002). Interestingly, fish embryonic cells are capable to detect light as early as gastrula stage (5 hours post fertilization, hpf), well ahead of non-visual (pineal organ develops by 24 hpf) and visual (retina by 2-3 days post fertilization, dpf) photoreception (Burrill and Easter, 1994; Kazimi and Cahill, 1999; Tamai et al., 2004).

The reproduction rhythm in fish is controlled by the pineal organ and melatonin action on the hypothalamus-pituitary-gonadal axis (Falcón J, 2007). Daily changes in the maturity of oocyte and the secretion of sexual steroid or gonadotropins have been already reported on many species (Matsuyama M et al., 1998; Carragher and Pankhurst, 1993; Kobayashi M et al., 1988; Bayarri MJ et L., 2004.). All this evidence point to the existence of daily rhythms in the reproduction of fish, as observed in the Senegal sole *Solea senegalensis* and gilthead sea bream *Sparus aurata* (Meseguer C et al., 2008).In aquatic invertebrates, exist evidence that hatching is promoted by light (e.g., Hempel-Zawitkowska, 1970; Mitchell, 1990; Murugan and Dumont, 1995; Takahashi, 1977). In the Atlantic halibut (*Hippoglossus hippoglossus*), hatching appears to be a photoregulated event because exposure to light inhibited hatching, whereas a return to dark conditions before 18 days post fertilization resulted in the synchronized hatching of all embryos within 90 to 140 min (Helvik and Bernt, 1993). This can occurs thanks to the pineal organ, which has the capacity to perceive and mediate photic information before hatching (Forsell et al., 1997). In zebrafish and Senegalese sole the light cycle play a key role on the hatching event showing different effect depending on the wavelength of the light during the light dark cycle (Blanco Vives et al., 2011; Villamizar et al., 2013).

The zebrafish (*Danio rerio*) is a model species for developmental research due to favorable characteristics such as high fertility, early light responsiveness, fast embryogenesis and embryo transparency. Regarding light, the zebrafish embryo's tissues and cells are directly light sensitive as early as 5 hpf and their exposure to LD cycles causes a rise in cellular proliferation and expression of clock genes which are synchronized to the light phase (Tamai et al., 2004; Di Rosa et al., 2015). Furthermore has been revealed the existence of hatching rhythms dependent by temperature and strongly synchronized by light cycles (Villamizar et al., 2013).

The purpose of this work was to investigate the hatching rhythms of embryo reared under different LD cycle, LD, DL, DL2, LL and DD at constant temperature. By observing the hatching of embryos for each condition we evaluated the We observed embryo hatching and evaluated the time on which occurred for each light cycle.

Materials and Methods

Animals and housing

The present study takes place in the chronobiology laboratory of the University of Murcia and of Ferrara. Wild-type adult fishes are obtained from local commercial provider (Jumipez S.A., Murcia, Spain) and housed in 9 L glass aquarium for 5 months. Adult sexually matured fishes are used for reproduction; in the afternoon groups of 5 fishes (3 females-2 males) were divided in breeding net cages(SERA GmbH, Germany). The next morning spontaneous spawning occurs 2 hours after sunset and soon after being fertilized the eggs were collected, washed and pooled. Each embryo were individually distributed in a single well of a sterile 48 well cell culture plates filled with 1 ml of embryo-medium (Nüsslein-Volhard and Dahm, 2002) and placed carefully at the water surface of a 9,2 L glass aquarium.

Experimental procedure

From 0 to 75 hpf, four multiwell plates for each condition were carefully placed at the water surface (water bath) of two 9L thermostat-controlled glass aquaria (N=192). The embryos were divided in five groups and submitted to LD cycle (12 hours light: 12 hours darkness); DL1 (12 hours darkness: 12 hours light with the first light phase of 3 hours); DL2 (12 hours darkness: 12 hours light with the first light phase of 24 hours); DD (24 hours darkness); LL (24 hours light)(Fig. 1.).

The temperatures applied were 28ºC by means of water heaters (100 Watt, Prodac, Italy. Temperature was recorded every 10 minutes by means of an underwater data logger

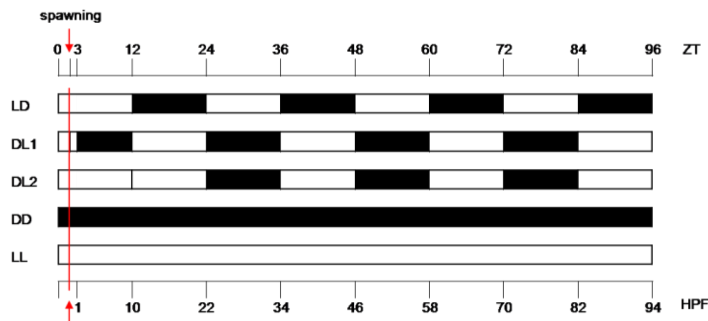

Fig.1 Schematic representation of photoperiods applied.

(Hobo Pendant ®Onset Computer Corporation, Massachussets, Usa). Illumination was provided using neutral white LED light (flexible LED stripes, Superlight technology Co., Ltd, China which irradiance (1.7 E^{+18} photons $m^{-2}s^{-1}$) was measured with a spectroradiometer (FieldSpec® ASD, Colorado, USA).

Data Collection and analysis

The influence of light on the hatching rhythms was evaluated by observing hatching times and calculating the hatching rate (final percentage of hatched larvae with respect to the initial number of fertilized eggs of each treatment). Embryos were observed every 2 hours and the number of newly hatched larvae was registered until 75. At the end of the experiment, the total number of hatched larvae was recorded in order to calculate the hatching rate in the five different light conditions.

All data were first tested for normality with the Kolmogorov-Smirnov's test. After normality confirmation, data were arcsine transformed before being examined by a one-way analysis of variance (ANOVA) to determine significant differences between light regimes. To assess statistical differences in hatching rate between light condition, all percentage data were normalized and arcsin transformed before statistical analysis. Then, a one-way analysis of variance (ANOVA) was used to analyze all treatments. All statistical analyses were carried out using the software SPSS 15.0 (SPSS Inc.). *P* values < 0.05 were considered statistically significant. All data are expressed as mean ± S.E.M.

Results

Embryos submitted to LD cycle start hatching at 47 hpf rising its higher peak at 53 hpf, and ending at 61 hpf. The event happens clearly during the light phase, whereas a minor

percentage of larvae hatched during the 3 hours after the light off. A rhythmic pattern of hatching was clearly observed under LD. Larvae submitted to DL2 hatched from 45 to 61 hpf, almost half hatched at at 47 hpf corresponding to the subjective light phase.

Embryos subjected to DL1 are affected differently; instead, their hatching is delayed,

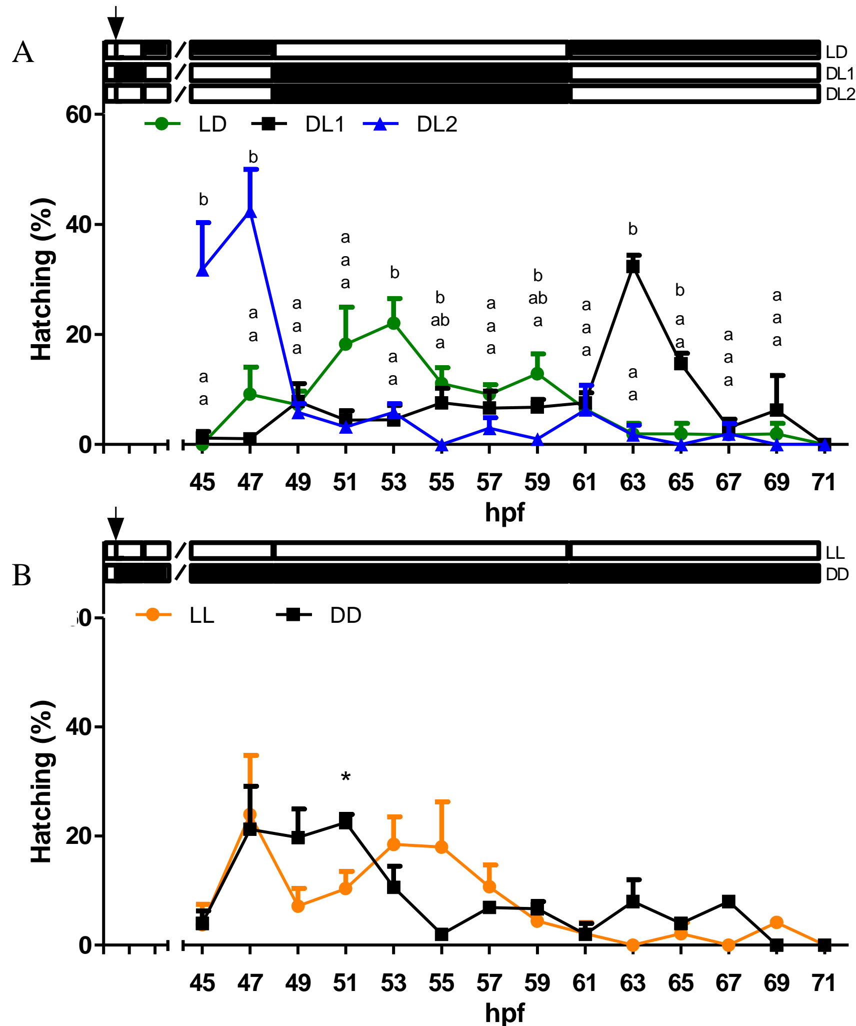

Figure 2. Hatching rhythms of zebrafish when reared under different light conditions. Values are expressed as percentage of newly hatched embryos every 2 h ± SEM. Different letters: Significant differences found at each sampling time among treatments (Light) (ANOVA, P<0.05). Asterisk represents statistical difference found between conditions in the same time point (Student's T test; p<0.05). Horizontal black/white bars represent the night and day periods of the 12:12LD cycle and the white and black bars represent the constant light (LL) and constant darkness (DD) regimes. The figure legend corresponds to each experimental group of light (LD, DL2, DL1; LL and DD) combined with the temperature regimes

showing its peak during the subjective light phase at 63hpf. The whole hatching event

occurs from 49 to 69 hpf (Fig.2 A). Under LL and DD conditions, a hatching rhythm was still observed although LL presented two peaks at 47 and 53-57 hpf, whereas DD maintained an high percentage of hatching during a longer period, from 47
to 51 hpf (Fig.2 B)

The hatching rate of eggs incubated in the LD treatment was higher (56.77% ± 0.9%) than that of eggs kept in DD (53.12% ± 2.08%), whereas the treatments causing the lowest hatching rate were DL1 (46.87% ± 4.9%), DL2 (46.87% ± 7.31) and and LL (46.35% ± 4.2 %) (Fig 3).

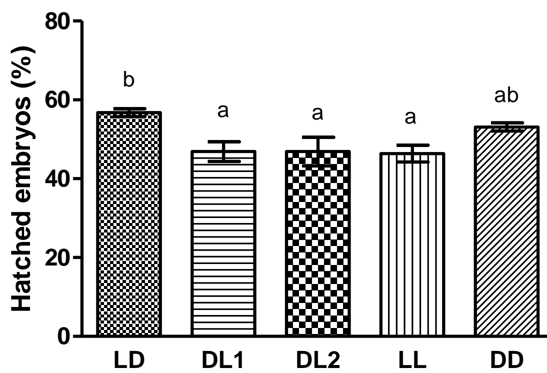

Figure 3. Hatching rate of zebrafish eggs under different LD cycles. Data are expressed as mean ± SEM. Experimental conditions were compared by one-way ANOVA followed by Tukey's post hoc test. Different letters indicate statistical differences between groups ($p < 0.02$).

Regarding the phase of the day, larvae under LD, DL1 and DL2 hatched during the light phase. Statistical differences are displayed in DL1 respect to LD and DL2. A minor percentage of larvae hatched during the dark phase (one-way ANOVA $p<0.05$, Tukey's post hoc test, Fig. 4).

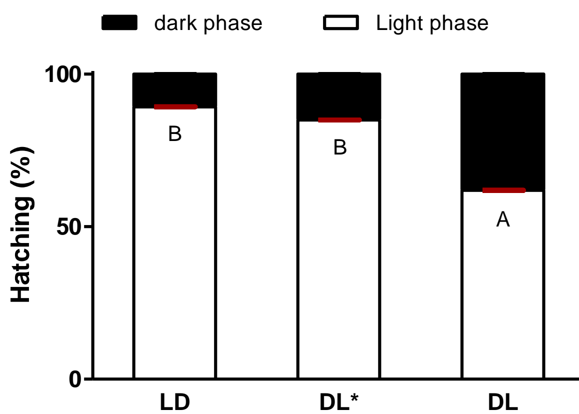

Figure 4. Hatching phase of embryos under the light phase or dark phase. Data are expressed as mean ± SEM. Experimental conditions were compared by one-way ANOVA followed by Tukey's post hoc test. Different letters indicate statistical differences between groups ($p < 0.02$).

Discussion

In this study, the existence of a daily rhythmic process during the development was observed in zebrafish. Hatching rhythms were observed in zebrafish, where under LD conditions brought out a clear rhythmic activity synchronized by light, occurring during the light phase. Under DL2 the rhythm developed also during the light phase whereas under DL it occurred hours later respect to the other conditions coinciding with the light phase. Is reasonable to think that the quantity of light after fertilization is essential to determine the hatching time, instead embryos under DL group which received only 3 hours of light after fertilization, delayed the hatching time, whereas the DL2 group that received 24 consecutive hours of light, advanced the hatching. However, under constant conditions (LL and DD) the hatching rhythm persisted, suggesting that time in embryos is kept according to an endogenous periodic oscillation mechanism.

The presence of an endogenous clock in the zebrafish has been also observed to control the spawning rhythm as under LD, spawning takes place within 2 h of lights on (Selman et al., 1993). However, pulses of darkness applied during the light phase caused a delay in the spawning peaks (Blanco-Vives and Sánchez-Vázquez, 2009). In the present study, the hatching rhythms of zebrafish coincided with those previously found when the hatching rhythm of this species was firstly described (Villamizar et al., 2013).

The hatching rate was highest under LD cycle and significant lower under LL. Under DL2, DL and DD the percentage is less than 50 % which suggests the importance of light cues on the early development whereas in constant darkness DD the percentage is little higher than 50 % but not really different from DL2 and DL group. The constant condition of light and darkness have been suggested as impair the normal development of embryos and larvae of several teleost fish (Blanco-Vives et al., *2010*; Villamizar et al., 2009; Liu et al., 1994).

On the other hand, LD cycles are thought to influence a broad range of functional and morphological aspects during the early stages of fish, such as the regulation of cell proliferation, the activation of UV protective systems (DNA repair enzymes), pigmentation through visual and non-visual photoreceptors and the expression of diverse light responsive genes (Dekens et al., 2003; Tamai et al., 2004; Shiraki et al., 2010; Vatine et al., 2011). In conclusion, our findings reveal for the first time the effect of different light cycles on the circadian hatching rhythms in embryos, confirming that rhythms during early development are strongly synchronized to light in a specific way.

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Chapter IV

Entrainment of food anticipatory activity (FAA) under different feeding periods in zebrafish (*Danio rerio*) and the Somalian cavefish (*Phreatichtys andruzzii*)

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Introduction

The existence of circadian clocks allow animals to synchronize their rhythms with the external stimuli, providing them with an adaptive advantage as they can anticipate environmental changes and time physiological processes to occur in optimal moments of the day. The circadian clock is synchronized by environmental cues such as light (Panda et al., 2002), temperature (Rensing and Ruoff, 2002) or food availability (Mendoza, 2006; Mendoza and Challet, 2009; Stephan, 2002). When food is provided in a cyclic manner, most fish species can synchronize their behavioral rhythms to feeding time (López-Olmeda and Sánchez-Vázquez, 2010). These rhythms are controlled by an endogenous pacemaker or clock since they persist when the food signal is removed (constant conditions) (Aranda et al., 2001). In this case, the overt rhythm of behavior free-runs with the same period of the pacemaker (Aschoff, 1981).The endogenous origin of the feeding rhythms has been demonstrated in different fish species such as European sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) (Sánchez-Vázquez et al., 1995a, 1996; Sánchez-Vázquez and Tabata, 1998; López-Olmeda et al., 2010).In mammals, two different oscillators have been described, one driven by the light, called light-entrainable oscillator (LEO), which is located in the suprachiasmatic nuclei of the hypothalamus (Meijer and Rietveld, 1989), and one driven by food, called food-entrainable oscillator (FEO), which is independent from the LEO and whose anatomical location is still unknown (Stephan et al., 2002; 2006). Recently, a model for the mammalian FEO has been proposed, which is based on interconnected structures in the brain able to entrain humoral signals deriving from periodic feeding (Carneiro and Arujo, 2009). In fish, the presence of both a LEO andFEO has been suggested, although contrary to the situation in mammals, in fish both oscillators seem to be coupled, which can be clearly observed when light and food cycles are present with different phases (Sanchez-Vazquez et al., 1995b; López-Olmeda et al., 2010). The anatomical location of either LEO or FEO in fish has not yet been identified.

When animals are fed on a single daily meal, provided at fixed time, they show an increasing of locomotor activity just before mealtime (Mistlberger, 1994; Sunuma et al., 2009). This physiological process is known as food-anticipatory activity (FAA), and it has been reported in a number of fish species such as mummichog (*Fundulus heterocritus*), European sea bass, rainbow trout (*Oncorhynchus mykiss*), European

catfish (*Silurus glanis*), goldfish and zebrafish (Davis and Bardach, 1965; Sánchez-Vázquez et al., 1995a; Bolliet et al., 2001; López-Olmeda and Sánchez-Vázquez, 2010; López-Olmeda et al., 2010). An important characteristic of FAA is its gradual occurrence, its development influenced also by the energy quantity and the size of the food, for instance, smaller sizes of food induces stronger anticipatory response Sánchez-Vázquez et al., 2001). Moreover, when food access is shifted, the FAA is progressively shifted to the new feeding time (Stephan, 2002). When the feeding sugnal is removed, under constant conditions, FAA presents free-running rhythms, which indicates that this process is controlled by an endogenous pacemaker (Aranda et al., 2001).Fishes are also able to anticipate the place of the aquaria where food is delivered. (Sánchez-Vázquez et al., 2001). Feeding entrainment confers adaptive advantages to fish because, when food availability is predictable, the animals may use this information to anticipate and maximize the food utilization. A constant activity state of the animal produces an energy expense too big, while the synchronization and the anticipation reduces the animal activity to an exact time of the day , and also prepares the animal's physiology, improving its food consumption and nutrient use (Sánchez-Vázquez et al., 2001).
Cavefish represent an important model for its extreme habitat, its lack of eyes and its ability to entrain to external cues. Cavallari et al. reveals that *P. andruzzii* possess a clock able to entrain to periodic feeding. Experiment with cell culture reveled rhythmic expression of a clock regulated reporter gene, which surprisingly exhibited a 47-h free-running period. Cavefish clock is not entrainable by light. Putative circadian photopigments able to regulate the photic entrainment of biological clocks are mutated and not functional. Although this fish species still presents some type of photoreception since it is able to present a photophobic response, directly correleted with the intensity of the light and the wavelength (Tarttelin et al., 2012). This model results very interestingly for the comparative studies on development of the biological clock in vertebrates (Cavallari et al., 2011).
In this research, we investigated the effects of periodic feeding provided with different periods (T=24, 36, 44, 48, 72 and 96 h) on the occurrence of food-anticipatory activity and the endogenous origin of the anticipation of two fish species, zebrafish and Somalian cavefish, in the absence of light signals (constant darkness, DD). In a second experiment, fish were submitted to 24 and 96 h feeding periods, which were later

shifted by 8 h to check for the capacity of resynchronization and the appearance of transients.

Materials and Methods

Ethics Statement

The present research was carried out in the Chronobiology laboratories of the University of Murcia (Spain) and of the University of Ferrara (Italy). All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). The experimental protocol was previously authorized by the Spanish National Committee on Animal Welfare (Law 32/2007) and the Bioethical Committee of the University of Murcia (Spain) and by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health.

Animal rearing

Adult wild-type zebrafish (*D. rerio*) of mixed sexes were obtained from a local provider (Jumipez S.A., Murcia, Spain). Zebrafish were housed in the Chronobiology laboratory of the University of Murcia, where all the experiments using zebrafish were performed. Adult Somalian cavefish (*P. andruzzii*) belonged to the colony maintained at the Chronobiology laboratory located at the University of Ferrara, where all the experiments using cavefish were performed.

A total of 180 zebrafish and 88 cavefish were used in the experiments. Zebrafish were divided into 18 groups of 10 fish placed in 9.2 L aquaria; cavefish were divided into 11 groups of 8 cavefish placed in 55 L aquaria. All aquaria had a close water system equipped with mechanic and biological filters. In all experiments, the aquaria were kept in chronobiology chambers, completely isolated, where light and temperature could be controlled. The temperature was maintained at 27 ° C by means of water heaters (100 W Askoll, Italy) and as recorded every 10 minutes by means of an underwater data logger (HOBO PENDANT® Onset Computer Corporation, Massachussetts, USA). Food was provided by means of a automatic feeders (Eheim 3581, Germany) located in the upper part of the aquaria and controlled by an electronic timer (Data log 2, Orbis, Spain). Food provided in all experiments (Tropical fish flakes, Prodac, Italy) was 1 % of fish body weight.

Locomotor activity was measured throughout all the experimental period by means of infrared photocells (Omron, mod E3S-AD62, Japan) placed against the aquarium wall. The number of light beam interruptions was counted and stored every ten minutes by a computer. Zebrafish aquaria were equipped with one photocell located in the middle of the tank, at 4 cm under the water level and at 11.5 cm from the lateral. Cavefish aquaria were equipped with 2 photocells, one in the upper side, 4 cm under the water level and one close to the bottom of the aquaria at 24 cm under the water level.

Experimental Design

Experiment 1: FAA entrainment to feeding cycles of different periods
The animals have been submitted at different feeding periods to investigate the feeding activity rhythms in absence of any synchronizer. Fish were maintained in darkness for 1 month to permit the acclimation and fed randomly to avoid any synchronization. We used 5 different experimental feeding periods (T) for both species: 24, 36, 44, 48, 72 and 96 h.Six groups of 10 zebrafish and from 3-6 groups of 8 cavefish were used for each feeding period.
We perfomed the experiment in two steps: the first applying a feeding period (24 hours group 1; 36 hours group 2 and 48 hours group 3), follow by a fasting period. After verifying the absence of any entreinment signal we applied the second feeding period (44 hours in group 1; 48 hours in group 2 and 72 hours group 3) follow by a fasting period. Zebrafish and cavefish after respectively 21 and 30 days, under each feeding conditions, were fasted for 15 and 20 days, in order to check for the existence of free-running rhythms that would confirm the existence of entrainment and the value of the endogenous period (tau).

Experiment 2: Resynchronisation to a mealtime shift
The experiment 2 was carried out in both species to improve the strength of the entrainment and the quick adaptation at the new stimulus. Fish were maintained in darkness for 3 weeks to permit the acclimation and fed randomly to elude any synchronization. 60 zebrafish divided in 6 groups and 16 cavefish divided in 2 groups were submitted to 24 hours feeding period. Only cavefish were submitted to 96 hours feeding period (16 fish divided in 2 groups). When the fish were entrained to the feeding period the mealtime were shifted 8 hours later from ZT 0 to ZT 8 in order to

check the existence of a transient and the ability of the fish to readjust its rhythm at the new feeding time.

Data Analysis

Analysis of locomotor activity records, representation of actograms and wave forms, and periodogram and center of gravity analyses were performed using the chronobiology software *El Temps* (version 1,275; Prof. Díez-Noguera, University of Barcelona). The length of endogenous period (tau) was determined by means of the chi-square periodogram analysis at a confidence level of 95% (Sokolove and Bushell, 1978), whereas the center of gravity represents the mean daily clock time of all recorded activity impulses being a meaningful representation of the central tendency of circadian activity(Kenagy 1980). The duration of FAA was determined as the time elapsed between feeding time and the rise of anticipatory activity, which was defined as a 2.5-fold increase over baseline activity sustained for at least 30 min and not followed by any inflection for more than 1 h, as described elsewhere (Stephan, 1997). The baseline activity was defined as the median of the daily locomotor activity.

Results

Experiment 1: FAA entrainment to feeding cycles of different periods. Fish kept in constant darkness conditions and fed under different feeding periods showed significant differences in their behavior. When fish were fed every 24 hours, some days after the start of periodic feeding, fish concentrated their daily activity around feeding time, with an increment hours before the food delivery that corresponds to the food-anticipatory activity (FAA) (Figs. 1A-B).This FAA is clearly visible in the meanwaves, were the onset of FAA was 6 hours before feeding in the zebrafish and 4 hours before the feeding time in the cavefish. During the fasting phase, the FAA rhythms persisted in free-running in both species, confirming its endogenous origin (Table 1). The first days of fasting both species displayed clearly a rhythm but during the following days it started to weaken, disappearing completely along more days (Figs.1A-B). The periodogram performed during the fasting phase showed in zebrafish and cavefish the existence of a significant rhythm with periods of about 24 hours (circadian) (Table 1). We observed significant endogenous periods (tau) in all experimental fish groups (6 groups of

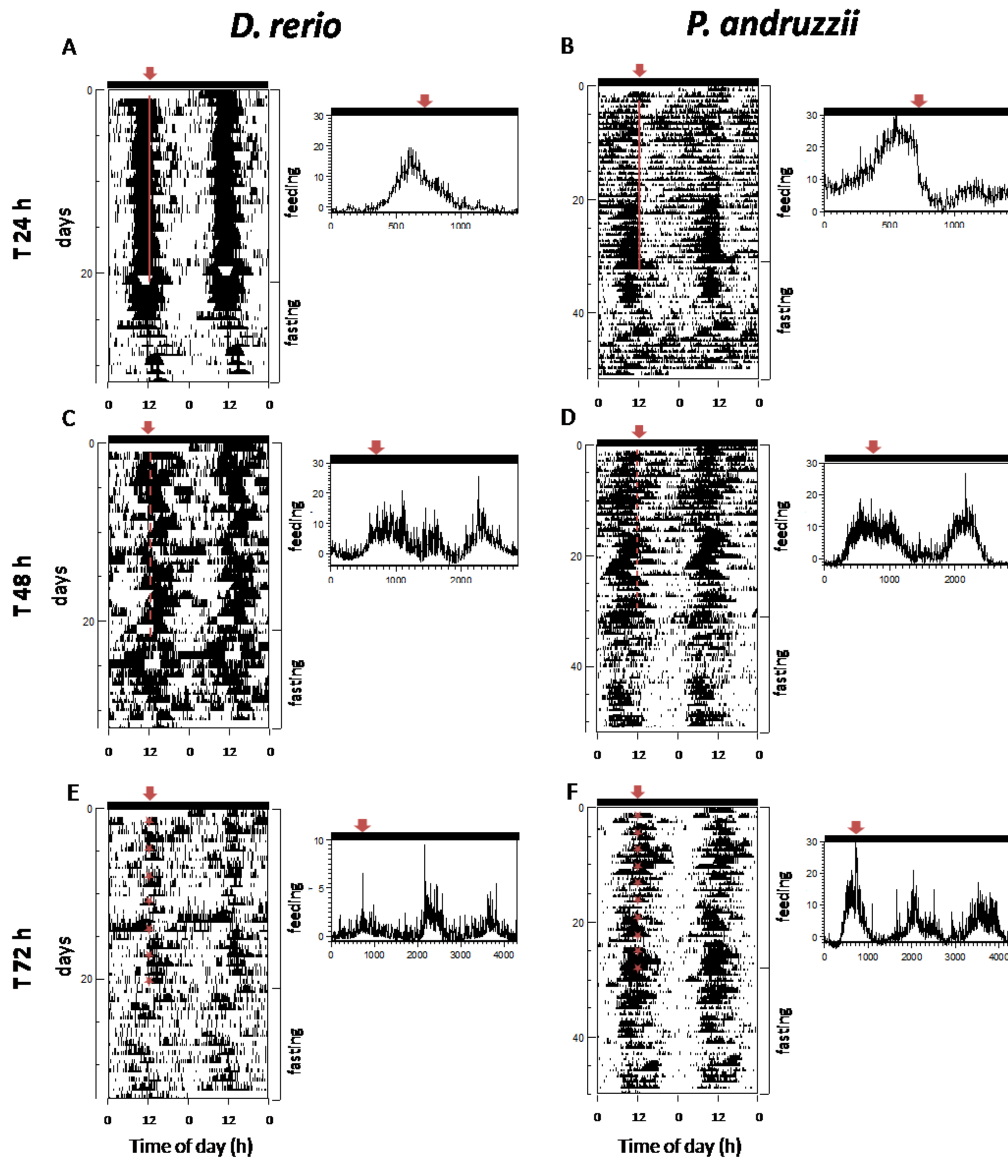

Figure 1. Representative actograms and mean waveforms of locomotor activity of groups of zebrafish (A, C and E) and cavefish (B, D and F) subjected to periodic feeding of T=24 h (A and B), T=48 h (C and D) and T=72 h (E and F). Actograms have been doble plotted (time scale 48 h), the height of each point representing the number of infrared light-beam interruptions/10 min. Each horizontal line shows one experimental day along the vertical axis, and the hours of the day are represented in the x axis. The black bar on the top of each actogram represents the constant darkness (DD) conditions. Arrow on the top of the actogram indicate the start of scheduled feeding, the continuous lines indicate feeding time in the T=24 h groups, dashed lines indicate feeding time in the T=48 h groups, and the starts indicate feeding time in the T=72 h groups. In the meanwaves, each point has been calculated as the mean ± S.D. from 10-min binned data across all the experimental days. Each waveform is represented using the period length of the feeding period, and feeding time is indicated by the arrow at the top of the

zebrafish and 5 groups of cavefish), with tau values ranging between 22.67- 24.17 h for zebrafish and 23.92 -24.50 h for cavefish.

The average tau values for all zebrafish groups was 23.8 ± 0.6 hours (mean ± S.D.) and 24.1± 0.2 hours for cavefish .

When food was provided with a T=48 hours, zebrafish concentrated most of their activity after mealtime and FAA displayed was only of 30 minutes (Fig 1 C). During the fasting phase, we observed significant free running rhythm in two groups out of 6, with tau values of 23.17 and 24.17 h (Table 1). Cavefish fed every 48 hours showed FAA of 4:16 hoursthat persisted in fasting conditions(Fig. 1 D). All cavefish groups showed significant free-running rhythms with mean values of 24.9 ± 0.8 hours (mean ± S.D.).

When food was provided every 72 hours, zebrafish presented a FAA of duration of 1 hour before the feeding time. In contrast with the drop observed under other feeding periods, the increase in activitycontinued after food delivery (Fig.1 E). The periodogram analysis showed significant values of free-running rhythms for two out of six replicates, confirming the low percentage of entrainment at this period (Table 1).

		Aquarium Number					
		#1	#2	#3	#4	#5	#6
T=24h	CF	24.00	23.92	24.00	24.50	24.00	-
	ZF	22.67	23.33	24.17	24.17	24.17	24.17
T=36h	CF	ns	24.00	22.67	24.25	-	-
	ZF	ns	ns	ns	25.33	23.67	ns
T=44h	CF	23.75	-	-	-	-	-
	ZF	ns	ns	ns	ns	25.17	21.83
T=48h	CF	25.67	25.50	24.00	24.50	-	-
	ZF	ns	23.17	ns	ns	ns	24.17
T=72h	CF	23.17	24.00	24.17	ns	ns	24.17
	ZF	23.17	ns	ns	ns	21.00	ns
T=96h	CF	26.00	-	-	-	-	-
	ZF	-	-	-	-	-	-

Table I: The period length of the free running feeding rhythms for each aquarium
ns = the data analysis was not significant by the

Conversely, cavefish anticipated the feeding time increasing its activity 5 hours before the mealtime (Fig 1 F). An endogenous origin of the rhythm for 66% of the replicates (4 out of 6) was detected during the fasting phase, with an average tau value of 23.9 ± 0.5 h (Table 1).

Zebrafish groups that received the meal with a period of 36 and 44 h did not display any anticipation but only a post-feeding increment of activity in the groups fed with T=44 h (Fig 2 A,C). Under both periods, 2 out of 6 of the replicates presented free-running rhythms in fasting, with tau average values of 24.5 ± 1.2 h and 23.5 ± 2.4 h for the groups fed with a period of 36 and 44 h, respectively (Table 1). In the case of cavefish, these groups presented a different pattern than zebrafish since no activity increment was observed before and after mealtime (Fig.2 B, D). The periodogram detected significant

free-running rhythms in the only replicate fed with T=44 h and in 3 out of 4 replicates fed with T=36 h, with tau values located in the circadian range, between 22.67 and 24.25 h (Table 1). Cavefish that fed every 96 hours did not display anticipation but periodogram detected free running period in fasting with a tau value of 26.00 hours.

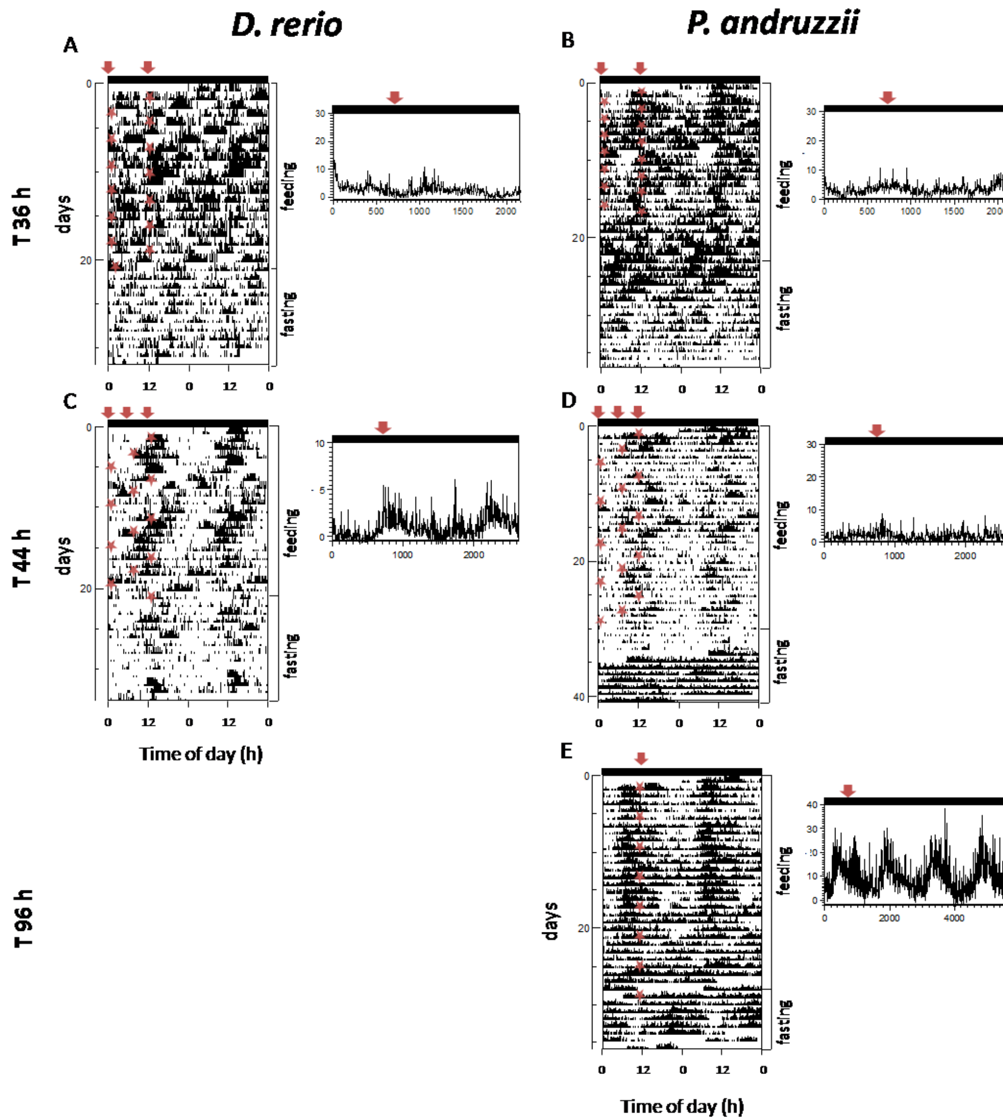

Figure 2. Representative actograms and mean waveforms of locomotor activity of groups of zebrafish (A and C) and cavefish (B and D) subjected to periodic feeding of T=36 h (A and B) and T=44 h (C and D) and T= 96 (E). For more details on actogram and waveform representation see Figure 1.

Experiment 2: Resynchronisation to a mealtime shift. The group of zebrafish that was submitted to a feeding period of 24 hours displayed FAA as observed in the groups from Experiment 1, with an increment of locomotor activity that occurs 2:16 h before the feeding time. When mealtime was shifted by a delay of 8 h, fish showed a transient displacement of the duration of 3 days in term of activity. The meanwave displayed the FAA during the first phase of feeding. Days after the shift, fishes presented an increment of activity just after the mealtime (Fig 3 A).

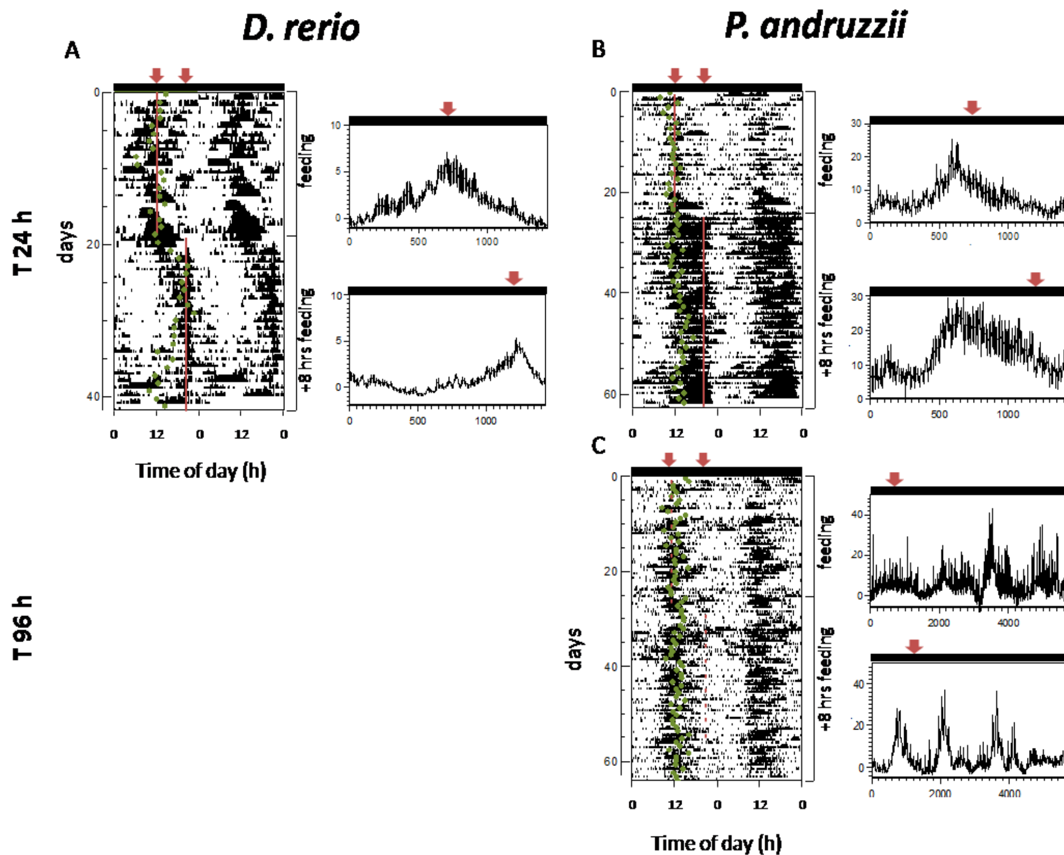

Figure 3. Representative actograms and mean waveforms of locomotor activity of groups of zebrafish (A) and cavefish (B) subjected to a periodic mealtime of T=24 h, which later was shifted (delayed) by 8 h. In addition, cavefish were also subjected to periodic feeding of T=96 h that was also shifted with an 8 h delay. The green dots indicate the center of gravity which represents the mean time of the activity events. Continuous lines indicate mealtime in the T=24 h groups and the dashed line indicates mealtime in the T=96 h group. For more details on actogram and waveform representation see Figure 1.

In cavefish appeared the same results because the fishes increase their activity 2.15 hours before the time of the food delivery and after the shift presented a very high post-

feeding activity (Fig.3 B). The change of mealtime affects the cavefish that rest in phase with the previous mealtime,
whereas zebrafish can shift its activity showing a transient phase that follow the shift. During the first phase cavefish showed anticipation of about 4 hours to respect the mealtime, and it persisted also after the shift. It's clear the lack of a transient days after the shift, instead the center of gravity indicate values that persists between the same range of the first phase.

Discussion

In the present study, the two fish species (zebrafish and Somalian cavefish) displayed FAA. The length of the increase of anticipation varied depending on the fish species and the feeding period used. This anticipation had an endogenous origin, as demonstrated by the persistence of FAA under fasting conditions in many groups of fish, and it was generated by a circadian pacemaker since the endogenous periods were around 24 h regardless of the feeding period. Cavefish showed a higher entrainment than zebrafish to feeding periods longer than 24 h, which points to a higher capacity of food synchronization in this species. In a second experiment, zebrafish resynchronized gradually to a shift in mealtime, in contrast to cavefish that did not display this transient resynchronization.
The circadian feeding rhythms have been observed in a great variety of vertebrates (Stephan, 2002), among them different species of fish such as goldfish (*Carassius auratus*), European sea bass (*Dicentrarchus labrax*) and tench (*Tinca tinca*) (Azzaydi et al., 2007; Herrero et al., 2005; Sánchez and Sánchez-Vázquez, 2009). Many studies in fish have also shown that FAA persists during food deprivation, confirming the existence of a biological oscillator that controls food anticipation (López-Olmeda et al., 2010; López-Olmeda and Sánchez-Vázquez, 2010). The biological advantages for a circadian system to entrain to periodic feeding have been widely discussed (Sánchez-Vázquez et al., 2001). In both mammals and fish, a daily periodic feeding can entrain the circadian rhythms in the organism, not only to induce a behavioral response but also to synchronize metabolic and digestive functions (Vera et al., 2007; Scheving et al., 1983). The physiological processes can be activated before an external periodic event, allowing the organism to exploit the available food much more efficiently (Herrero et al. 2005; Montoya et al., 2010). The present research confirmed the endogenous origin of

the feeding anticipatory activity in the two fish species studied, as revealed by the occurrence of free running activity from both zebrafish and cavefish feeding activity when all environmental cues were removed. In zebrafish, this endogenous origin had been reported previously (López-Olmeda et al., 2010). However, this experiment represents the first study on the endogenous origin of feeding rhythms in the Somalian cavefish, till now only the clock genes rhythms have been studied, (Cavallari et al., 2011). Moreover, relevant importance assumes the study of feeding rhythm in specie which has loss the capacity to entrain to light and food represents the only cue able to entrain the clock.

When feeding periods longer than 24 h were applied, cavefish showed more flexibility to entrain to these different periods. A smaller percentage of zebrafish showed rhythmicity with periods of 48 and 72 hours, a multiple of 24 h that can be explained considering that the endogenous oscillators can entrain to periods that differ respect to its own, but staying between a fixed limit. The limits of entrainment depend on the animal species, age and synchronizer strength (Madrid et al., 1998). In mammals, several experiments have been carried out to study the range of feeding entrainment. For instance, rats submitted to different time cycles (from T=22 h to T= 28 h) did not displayed any entrainment rhythms at T 22, 27 and 28 h. Thus, the entrainment in mammals seems to be constrained to the range in which the circadian rhythm can exist, ranging between 23 and 26 hours (Madrid et al., 1998). However, fish seem to have a higher plasticity for the entrainment to periods quite out of the circadian range, as demonstrated by the present study. On the other hand, cavefish can entrain better to different periods compared with zebrafish. We can hypothesize that the extreme subterranean habitat has contributed to the development of this ability in cavefish, remaining this fish species completely isolated from the day-night cycle for millions of years (Colli et al., 2009) and showing an extreme troglomorphic phenotype. Upon exposure to alternative zeitgebers cavefish clock oscillates with remarkably long infradian period, reflecting the loss of mechanisms that are not essential for animals that live under constant condition of darkness and temperature. Due to strong selective pressure, cavefish lost the capacity to entrain by light, due to this extreme habitat in constant darkness, and the only advantage is represented by entrain to the food the only cue available (Cavallari et al., 2011).

When periodic food delivery is advanced or delayed, many species respond to the shift in feeding time with a gradual re-entrainment of the FAA, which is known as transient (Stephan, 1984; 1992). Some fish species under constant lighting conditions are able to show transients after a shift of feeding time, which start the first days at the previous feeding time and progressively adapt to the new feeding time (Davis and Bardach, 1965). This was the case observed for zebrafish in our experiments, which took 3 days to progressively resynchronize to the new feeding time. On the other hand, some fish such as the goldfish did not show transients after a 9-h delay of mealtime (Sánchez-Vázquez et al., 1997). As observed in the cavefish in the present study, some goldfish resynchronized immediately to the new feeding time by keeping constant the onset of activity and lengthening the activity phase until feeding (Sánchez-Vázquez et al., 1997). Therefore, fish can respond in different ways to a delay in the mealtime, but whether this is a species-dependent response remains to be elucidated.

In conclusion, our results reveal that FAA is driven by an endogenous timing system in the two fish species studied. In addition, the endogenous period was always within the circadian range (around 24 h) regardless the previous feeding period, proving that it is generated by a circadian pacemaker. When feeding periods longer than 24 h were applied, cavefish presented a better entraining capacity than zebrafish as a higher number of cavefish groups displayed free-running rhythms. Taken together, these results are especially relevant in the case of the Somalian cavefish since it is the first time that this endogenous origin of behavioral feeding rhythms is described in this species. Cavefish represents a unique model for studies on chronobiology, mainly those related with the food-entrainable oscillators as this species lacks the light entrainment, making it a natural mutant for the light-entrainable oscillator.

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Chapter V

Daily rhythms of expression of genes involved in sex differentiation in zebrafish.

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Introduction

Most animals are active during the day or the night but not during the 24 hours. Animals living under environmental cycles developed physiological and behavioral processes that occur in a rhythmic manner driven by a time-keeping system. Biological clocks allow animals to anticipate these cyclic events (e.g. day/night or seasons) and to cope better with them. Light and temperature represent the main time-cues able to entrain the biological clocks, allowing setting the phase of the endogenous rhythms with the phase of the cyclic events in the environment. The alternation of light and darkness (the 24 hr LD cycle) plays a major role in the synchronization of daily rhythms (Dunlap et al., 2004). In ectotherms, temperature assumes a relevant role as the water dynamics create an ecosystem in which animals have to adapt to live (Schaefer and Ryan, 2006).

Reproduction in fish presents both daily and seasonal rhythms in most fish species. In zebrafish, the existence of daily spawning rhythms has been reported so that fish select the most appropriate moment of the day to ensure egg and larvae survival (Blanco-Vives and Sánchez-Vázquez, 2009). A number of investigations reported daily variations of sex steroids such as estradiol (E2), testosterone, 11-ketotestosterone (11-KT) and progesterone in different fish species like Japanese Charr (*Salvelinus leucomaenis*) (Yamada et al., 2002), wrasse (*Pseudolabrus sieboldii*) (Ohta et al., 2008), catfish (*Heteropneustes fossilis*) (Lamba et al., 1983) and Senegalese sole (*Solea senegalensis*) (Oliveira et al., 2009). Daily changes in oocytes maturation and secretion of sex steroids have been reported in seabream (*Pagrus major*) (Matsuyama et al., 1998), snapper (*Pagrus auratus*) (Carragher et al., 1993), kisu (*Sillago japonica*) (Kobayashi et al., 1988) and in the European sea bass (*Dicentrarchus Labrax*) (Bayarri et al., 2004). In accordance with the seasonality of fish reproduction, annual changes in sex steroids have been reported in common dentex (*Dentex dentex*) (Pavlidis et al., 2000), sea bass (Prat et al., 1990; Mañanos et al., 1997), rainbow trout (*Oncorhynchus mykiss*) (Baynes and Scott, 1984.) and Senegalese sole (Oliveira et al., 2009). Previous investigations have also highlighted the effect of photoperiod and temperature as determinant factors changes in the sex ratio (Bromage, 1987; Aida and Amano, 1995; Tranger et al., 1995; 1998; Blázquez et al., 1998; Colombo et al., 1998; Pavlidis et al., 2000; Blázquez et al., 2009). In addition, daily cycles of environmental factors have been reported to act differently during sex differentiation. For instance, thermo-cycles induce a high proportion of female in zebrafish, whereas constant temperature led to more males (Villamizar et al., 2012).

The timing of reproduction and the production of sex steroids are controlled by the hypothalamic-pituitary-gonad axis (BPGa), necessary for multiple processes such as sex differentiation, gonad maturation and spawning (Bayarri et al., 2004; Falcón et al., 2007). Aromatase has a key role during sex differentiation, presenting two different genes: *cyp19a* and *cyp19b*. The former is also called “ovarian aromatase”, as this gene is mainly expressed in the differentiating and adult gonad of teleost fish. It is an enzymatic complex that facilitates the estrogen synthesis from testosterone (Conley and Hinshelwood, 2001). *Cyp19b is* also called “brain aromatase” because this gene is highly expressed in teleost brain in both female and male (Patil and Gunasekera, 2008). The antimüllerian hormone (*amh*) initiates in testis the regression of the Müllerian ducts and inhibits the expression of aromatase (*cyp19a*), therefore avoiding the transformation of androgens into estrogens. *Foxl2* is a transcription factor, which is known to be a potent transcriptional activator of *cyp19a* (Wang et al., 2007), playing an important role in species where temperature is able to determine sex ratio (Yamaguchi et al., 2007). High temperature during the thermosensitive period suppresses *cyp19a* gene expression, resulting in a low aromatase activity and E2 levels (Guiguen et al., 2010). *Dmrt1* has been found to be expressed in Japanese medaka (*Oryzias latipes*) and it is an inhibitor of germ cell proliferation, only expressed during testicular differentiation, whereas in Nile tilapia (*Oreochromis niloticus*) this factor down-regulates *cyp19a* during testicular differentiation (Wang and Nagahama 2008). In zebrafish, *dmrt1* is not only associated with testis development but it may also be important in ovary differentiation (Guo et al., 2005). *Cyp11b* gene contributes to the synthesis of 11-KT from testosterone, which represents the most potent androgen in teleost fish, with higher expression in male gonads than in female (Ijiri et al., 2008).

Zebrafish is a mostly a diurnal species (Hurd et al., 2011), although it is capable of displaying either diurnal or nocturnal behavioral rhythms (i.e., nocturnal self-feeding) (del Pozo et al., 2011). Daily thermocycles can also drive behavioral rhythms in zebrafish (López-Olmeda et al., 2006; López-Olmeda and Sánchez-Vázquez, 2009). When zebrafish is submitted to a long photoperiod (LD 14:10 hours) fish spawn during the light phase, coinciding with the peaks in locomotor activity. The diurnal spawning rhythm is maintained during the light phase even when zebrafish is fed during the night, even though its locomotor activity becomes nocturnal. The zebrafish ability to maintain diurnal spawning rhythm confirms the strong influence of the LD-cycle on the

entrainment of the spawning rhythms (Blanco–Vives and Sánchez-Vázquez, 2009). However, little is known about daily rhythms in sex steroids and their synchronization to light.

The aim of this research was to investigate the expression of six specific genes, involved on the sex differentiation, in two different tissues: gonads and brain. In ovary we tested the expression of *cyp19a,* whereas in testis the *amh* gene. In brain were analyzed the *dmrt1*, *foxl2*, *cyp19b* and *cyp11β*. We investigated the existence of a daily rhythmic pattern on the expression of these genes in adult zebrafish male and female..

Materials and Methods

Ethics Statement

The present research was carried out in the Chronobiology laboratories of the University of Murcia (Spain). All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). The experimental protocol was previously authorized by the Spanish National Committee on Animal Welfare (RD 1201/2005 and law 32/2007) and the Bioethical Committee of the University of Murcia (Spain).

Animal rearing

Adult wild-type zebrafish (*D. rerio*) of mixed sexes were obtained from a local provider (Jumipez S.A., Murcia, Spain). Fish had an approximate body weight of 0.75 g and 4 cm of total length and were housed for 6 months in our laboratories.

Fish (N=160) were classified and divided according to the sex in 2 aquaria of 60 L (60x30x32 cm), males were placed in one tank and females in the other tank. Each aquarium had a closed water circulation system provided with aeration and with mechanical and biological filters. The aquaria were kept in a clock room, completely isolated, where light and temperature were controlled. The temperature was maintained at 27 ± 0.5 °C by means of a water heater (100 W, Askoll, Italy). Temperature was recorded every 10 minutes by means of an underwater data logger (HOBO PENDANT® Onset Computer Corporation, Massachussetts, USA). The lighting conditions were set at a 12:12 h LD cycle, consisting of 12 hours of light and 12 hours darkness, with light onset at 8:00 a.m. (*Zeitgeber* Time 0, ZT 0). Fish were fed by means of an automatic feeder (Eheim, Germany) located in the upper part of the aquaria, that

released the food (Tropical fish flakes, Prodac, Italy) at quantity daily rate of 1 % of the total biomass in each aquaria, at 12 a.m..

Data sampling and analysis

When fish were acclimated to the artificial environmental conditions, samples were collected along a 24 h at six different time points (ZT 2, 6, 10, 14, 18 and 22 h). In each time point, five fish from each sex were anesthetized on ice and sacrificed by decapitation. The brain and the gonads (ovaries from females and testis from males) were extracted from each fish. Total RNA was isolated from each sample, using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The

Gene	Direction	Primer sequence 5'-3'	Accession number
cyp19a	F	TGCTGGCCATCAGACACCAT	AF183906
	R	CAGATGAACCGACAGTAGGAGACAA	
cyp19b	F	TCGGCACGGCGTGCAACTAC	AY780257
	R	CATACCTATGCATTGCAGACC	
amh	F	GGGTGTGCATGCTACAGAAGAT	AY677080
	R	CTCAGAAATGCAAACAGTCTGTGT	
cyp11b	F	CCTCGGGCCCATATACAGAGA	NM_001080204
	R	CGTCCCGTTCTTGAGGAAGA	
dmrt1	F	ATGGCAGAGCAGAACGATTT	NM_205628
	R	TAGTCCCACAACAGCATGGA	
foxl2	F	AAACACTGGGAAGGTTTGCGTGC	NM_205628
	R	TTTGTCCGGCCCCTTCTCTGG	

Table 1. Primer sequences used for quantitative PCR analyses.

amount, quality and composition of isolated RNA were analyzed by Nanodrop ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA). Total RNA (1 μg) was incubated with DNase I (Invitrogen) at room temperature for 30 min and then at 85°C for 15 min to inactivate the enzyme. DNase-treated RNA was used to perform cDNA synthesis in a final volume of 20 μl, using QuantiTect Reverse Transcription Kit (Quiagen, USA). The reaction was performed at 42°C for 30 min, followed by an inactivation step of 5 min at 85°C. Then, cDNA was PCR amplified with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR-green primer master mix according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were as follows: 15 min of denaturation at 95°C, followed by 40 cycles of a 15 s denaturation step at 95°C

and an annealing–elongation step for 30 s at 60°C. After amplification, a melting curve analysis was performed to confirm the specificity of the amplicon. All samples were run in triplicate. Gene-specific primers for *cyp19a*; *amh*; *cyp19b*; *cyp11β*; *dmrt1*; and *foxl2* were designed with primer Express software (Applied Biosystems) (Table1). The primer sequences for *amh* were retrieved from the literature (Wang et al., 2007). The efficiency of the primers was verified by constructing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. Levels of relative expression of each sample were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). As housekeeping genes, *βactin* and *loopern4* were used (Vanhauwaert et al., 2014). Nearly identical results were observed with both housekeeping genes.

Statistical analysis

All the results are expressed as mean ± SEM The significance threshold (α) was set at 0.05 in all statistical tests performed.. Data were normally distributed (D'Agostino-Pearson normality test, $p<0.05$) and all populations had the same variance (Bartlett's test for equal variances, $p<0.05$). Data from each gene was subjected to one-way analysis of variance (ANOVA) to determine statistical differences between time points. Tukey´s HSD *post hoc* test was used for the multiple comparison among groups. ANOVAs were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). In order to evaluate the presence of a rhythmic gene expression over a defined period of 24 hours, Cosinor analyses were performed using El Temps (v. 275, Prof. A. Díez-Noguera, University of Barcelona, Spain).

Results

All genes analyzed showed either daily rhythmicity (Cosinor, $p<0.05$), significant differences between time points (one-way ANOVA, $p<0.05$) or both. *Cyp19a* was expressed in the ovary with a significant peak in the middle of the light phase (ML, ZT 6 h) (one-way ANOVA $p<0.001$; Fig. 1A).

The analysis of this gene also revealed a sinusoidal rhythmic pattern with the acrophase at ZT 5:13 h (Cosinor, $p<0.003$; Table 2).

Amh also showed a significant peak of expression in testis, but it occurred at the beginning of the dark phase (ZT 14 h) in contrast with the peak of *cyp19a* during the

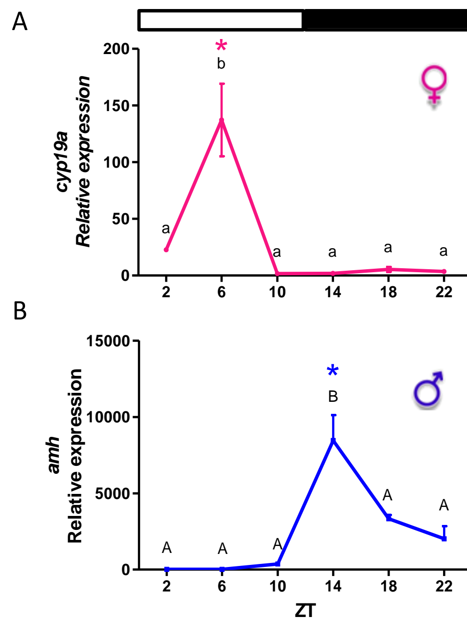

Figure 1. Relative gene expression in ovary (A) and in testis (B). Letters indicate statistical differences of each gene during the 24 hours in Female (small letters) and in male (Capitle letters) (1-way ANOVA, p<0.05). Asterisk indicates rhythmicity of gene expression during the 24 hours (Cosinor, p<0.05).

light phase (one-way ANOVA, p<0.001; Fig. 1B). In addition, amh also presented significant sinusoidal rhythms, with the acrophase located at ZT 15:39 h (Cosinor, p<0.05; Table 2). In brain, the expression of *dmrt1* presented statistically significant differences of expression in both males and females, with the highest expression at ML (ZT 6 h) in both sexes (one-way ANOVA, p<0.0002; Fig. 2A).

	Female			Male		
	mesor (r.e)	amplitude (r.e.)	acrophase ZT	mesor (r.e)	amplitude (r.e.)	acrophase ZT
cyp19a	30.82	50.16	5:13	-	-	-
amh	-	-	-	2367	3405	15:39
cyp19b	ns	ns	ns	ns	ns	ns
foxl2	ns	ns	ns	1.3	0.78	22:27
cyp11b	ns	ns	ns	ns	ns	ns
dmrt1	0.7	0.35	6:43	1.04	1.15	06:56

Table 2. Mesor, Amplitude and Acrophase values of all genes in female and male. Mesor and Amplitude are given as relative expression values (r.e.) and acrophase as ZT. Rhythms are considered significant when p<0.05. Only statistically significant values (p<0.05) are reported, NS are not significant sample values for cosinor analysis, the dash means no sample investigated.

This gene displayed also significant daily rhythms in both males and females, with the acrophases around ML (ZT 6:56 and 6:43 h for males and females, respectively) (Cosinor, p<0.007; Table 2).

Foxl2 presented similar profiles in both sexes, with highest expression at ZT 22 h (one-way ANOVA p<0.002; Fig. 2B), but presented daily rhythmicity only in males, with the acrophase located at ZT 22:27 h (Cosinor, p<0.003; Table 2). Regarding the expression of *cyp19b*, both females and males showed statistically significant differences during the day. In males the peak occurred during the dark phase (ZT 22 h), whereas in females the values were rather constant and only a decrease in the expression at ZT 10 h was detected (one-way ANOVA, p<0.005; Fig. 2C). *Cyp11β* presented differences in expression during the day both in males and in females. The highest expression in female occurred during the day at ZT 10 h, just before the light offset (one-way ANOVA, p<0.0001; Fig. 2D), whereas in males it occurred at ZT 18 h, at the middle of the night (MD) (one-way ANOVA, p<0.006; Fig. 2D).

In both cyp19b and cyp11b, no significant daily rhythms were detected (Cosinor, p>0.05; Table 2).

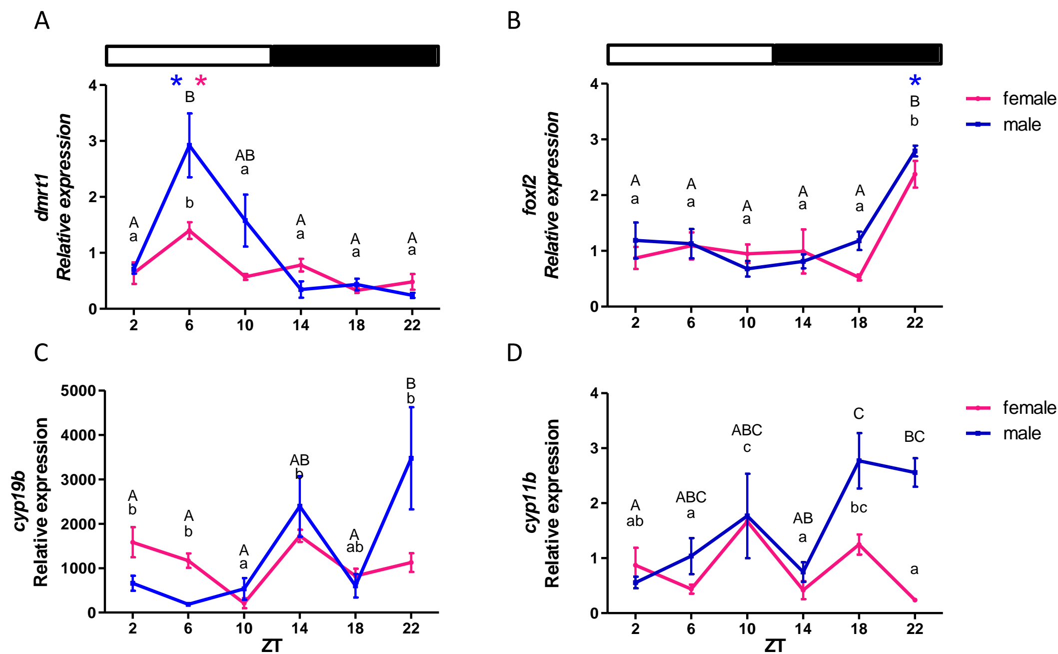

Figure 2. Brain relative gene expression in *dmrt1* (A), *foxl2* (B), *cyp19b* (C) and *cyp11b* (D). Letters indicate statistical differences of each gene during the 24 hours in male (Capital letters) and in female (small letters) (1-way ANOVA, p<0.05). Asterisk indicates rhythmicity of gene expression during the 24 hours (blue:male; pink: female)(Cosinor, p<0.05).

Discussion

Although the characterization, localization and expression of the genes involved in sexual differentiation have been studied in many fish species, their daily rhythms of expression had never been explored. The present findings revealed that the expression profiles of these genes are not constant but change during the day, in some cases displaying daily rhythms such as brain *dmrt1* in both sexes and brain *foxl2* in males. Moreover, differences in these rhythmic profiles can be found depending on the sex, as was observed in the ovarian *cyp19a* that presented a diurnal peak and the expression of *amh* in the male testis that displayed a peak located at the beginning of the dark phase.

Estrogens are essential hormones for ovarian differentiation, and aromatase is the key enzyme involved in their conversion from androgens (Piferrer, 2001). *Cyp19a* is present only in the ovarian of females and its expression follows a rhythmic pattern. The gonadal aromatase, presented its maximum expression level during the light phase at MD, showing a a diurnal pattern. The gene expression levels of *cyp19a* in female did not occur in phase with them of *amh* expression in male gonad. *Amh* in testis, presented a peak during the night, three hours after light off.

While the mechanisms in gonads are largely studied, in brain many questions remain to resolve. In brain, we analyzed *cyp19b*, the “neural aromatase”, which presented similar profiles in both sexes, in accordance with previous studies, which displayed the same level range expression in female and male. (Sawyer et al., 2006; Jørgensen et al., 2008).

There is evidence for a role of aromatase in the sexual differentiation of the brain (Tomy et al., 2007), and the different activity depending on the sex and on the phase of life. In one-year-old juvenile European sea bass, brain aromatase activity was lower than in first time spawners and similar in both sexes. In contrast, in mature animals aromatase activity was much higher in spawners (González and Piferrer; 2003). Regarding the other genes present in the brain, in our study has been revealed rhythmicity only in two genes *foxl2* (male) and *dmrt1* (both sexes) in different time of the day, *foxl2* during the night, two hours before the light on, whereas *dmrt1* at ML. *Cyp19b* and *cyp11b*, presented statistically difference of expression during the 24 hours, with maximum peak during the night in phase with *amh* and *foxl2* genes.

Recent studies of steroids rhythmic levels have been carried out in other fish species. In Senegalese sole for example the testosterone (T) and estradiol (E_2) are both secreted in

higher quantity during the dark phase of the day, because of the nocturnal nature of the sole (Oliveira et al., 2009). According to Bayarri et al., (2004) in sea bass, in addition to daily rhythms of plasma T in males, also plasma LH, pituitary LH and GnRH were expressed rhythmically during the day. In snapper (Pagrus auratus) plasma E_2 and T changed according to the time of the day (Carragher and Pankhurst, 1993). The japanase whiting (*sillago japonica*) also presented a diurnal pattern of plasma E_2 (Matsuyama et al., 1990). The gene and hormone rhythmicity also correspond to rhythmicity on physiological processes, instead previous investigation displayed the presence of spawning rhythm, that coincides with the highest moment of locomotor activity period. The spawning took place at dawn (Blanco-Vives et al., 2009), although this has not been related to their circadian control (Ziv and Gothilf, 2006). In Senegal sole, nocturnal specie, the spawning took place at the beginning of the night revealing a correlation with the locomotor activity increment (Oliveira et al., 2009). In gilthead sea bream, the spawning starts in the afternoon continuing till few hours after the beginning of the night (Meseguer et al., 2008). Several studies have revealed the existence of daily spawning rhythms in many other fish species such as: coral fishes that showed a daily spawning rhythms (Sancho et al., 2000), flounder (Pleuronectes platessa L.) spawns during the night (Nichols, 1989).

The molecular and cellular, as the physiological processes, drive to a reproductive behavior, which in fish is a seasonal phenomenon, even if exist as lunar and daily reproduction rhythm. Most species reproduce during the spring, to increase the survival of the larvae making the most of food availability and the higher water temperature, other during the winter such as European sea bass and the sea bream. Sex steroid rhythms were expressed in higher concentration before the spawning period, being expressed in phase each other (García-López et al., 2006; Guzmán et al., 2008). Since fish synchronize reproduction to environmental factors to select the best time of the day to spawn their eggs, it's reasonable to think that they select the best moment of the year in term of conditions to the offspring. This suggests that in each species, reproduction and behavioral rhythms are strongly related each other, strongly dependent to the LD cycle, in accordance with our results that reveals the relation between gene expression and time of the day.

Althought the role of temperature was not evaluated in the present study, the results obtained may be of importance for future studies on processes of sex

differentiation regulated by temperature. In fish, water temperature seems to have a high influence on sex differentiation in many species belonging to very divergent orders (Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Ospina-Álvarez and Piferrer, 2008). In zebrafish, temperature was reported to affect the sex ratio (Penman and Piferrer 2008). Moreover, thermocycles also affect sex differentiation compared with constant water temperatures (Villamizar et al., 2012), thus sex ratio being affected not only by the temperature but also by the time of the day at which the temperature acts, suggesting the existence of windows of sensitivity at specific times of the day. The present results would support this hypothesis since both *cyp19a* and *amh* displayed daily rhythms with acrophases located at different times of the day. Considering these recent findings, we can speculate that our results can help to better understand the complex mechanisms by which temperature is able to induce these relevant changes. In summary, our findings revealed for the first time the importance of the time of the day on the daily expressions of genes involved on sex differentiation, being essential to consider when sampling for steroids, resulting necessary optimize protocols to take account onto the real existence of fluctuation of gene expression during the day.

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General Discussion

General discussion

The present PhD thesis confirms the importance of external cues, especially the light during embryogenesis to set the biological clock, physiological processes that reflect to behavioural responses. Morover this thesis point to elucidate the different effect of light wavelength and to highlight the strength of an additional synchronizer: the periodic feeding.

Solar light is a complex environmental signal that influences the evolution of most biological processes on the Earth. Light is characterized by daily changes in irradiance, wavelength composition, direction and polarization (Björn, 2002). In the last years many investigations are taking into account a significant role of the different wavelengths (Villamizar et al., 2013; Blanco-Vives et al., 2010; Villamizar et al., 2009). The chapter 1 present the study performed to determine the ontogeny of clock genes and locomotor activity in larvae submitted from the first hours of life to different light wavelengths. At the behavioural level, light induces different locomotor responses depending on the light wavelengths. The illumination conditions of LDW and LDB have the major influence on the locomotory activity, promoting a higher overall activity than larvae reared under LDR. Previous investigations showed that the rise of swimming behaviour in zebrafish larvae is linked to the maturation of serotoninergic neurons (Brustein et al., 2003). Previous studies have shown that zebrafish behaviour under constant lighting conditions is regulated by an endogenous clock and that the LD cycle sets the phase of this clock (Cahill et al., 1998; Hurd and Cahill, 2002). For instance, LD cycles are required for the correct onset of behavioural rhythmicity in zebrafish larvae (Hurd and Cahill, 2002).

The analysis of clock genes ontogeny revealed different results in the animals under DD and LD conditions. For instance, under DD *per2* rhythms failed to establish during the development, which has been observed in other fish species such as the medaka (*Oryzias latipes*) and the Senegalese sole (*S. senegalensis*) (Dekens and Whitmore, 2008; Martin-Robles et al., 2012; Cuesta et al., 2014). This effect of DD conditions would be explained by the fact that *per2* is a light-inducible gene, and thus requires the presence of light for its daily rhythmicity to develop correctly (Vatine et al., 2009). Interestingly, the presence of constant light does not make for the regular expression of clock genes. In the rainbow trout, *Oncorhynchus mykiss,* the clock genes

per1 and *clock* showed persistent rhythmicity in the larvae reared under LD conditions from 0 to 58 dpf, but not under constant lighting (Davie et al., 2011). These discrepancies among the times of occurrence of rhythmicity among key components of the circadian clock has been suggested in other studies in fish (Dekens and Whitmore, 2008; Martin-Robles et al., 2012; Cuesta et al., 2014).

In the case of light wavelength, its effects on the ontogeny of fish are scarcely understood to date. Recent papers on this topic have focused on wavelength effects on larval performance, survival and the occurrence of malformations, finding that, in general, short (blue) wavelengths are better for fish development than long (red) wavelengths (Villamizar et al., 2013; 2009). A drastic effect of short wavelengths on larval behaviour has also been found in the Senegalese sole (Blanco-Vives et al., 2012). The blue light condition was able to generate a switch in locomotor activity, changing the active phase from diurnal to nocturnal during larval metamorphosis onset. Conversely, long (red) wavelengths did not show any effect on the locomotor activity (Blanco-Vives et al., 2012). Our results agree with those obtained in the other fish species, since both behavioural and clock gene rhythms appeared earlier in the larvae reared under short wavelengths (LDB) than in those reared under long wavelengths (LDR).

In order to determine the effect of illumination changes of different spectrum (LDW, LDB, LDR) on the locomotor activity of zebrafish larvae in the chapter 2 we revealed that zebrafish hyperactivity, induced by the lighting transition, is already present at day 3, when the retinal photoreceptors are not still functional. It is reasonable to think that the stimulus the drives the behaviour is mediated by deep brain photoreceptors. Our findings are in accordance with a previous study on which the visual motor response persisted also when the pineal was ablated, suggesting that this behavior was mediated by deep brain photoreceptors. Melanopsin OPN4M1 was the candidate for mediating photoreception, and its expression was proposed to be involved in the dark photokinesis (Fernandes et al., 2012). White light is able to induce the main change in terms of activity in larvae dark or light adapted at 8 dpf, whereas blue light induces a different response respect to white. The high sensitivity for the white and blue light has been already revealed in zebrafish larvae indeed a previous study highlighted the effect of this light condition on the ontogeny of the behavior and onset of biological clock (Di

Rosa et al., 2015). We can speculate that this higher sensitivity for the blue (low wavelengths) and white (full spectrum) despite for the red light could be due to adaptation of animal to rounded environment, considering that blue light has the capacity to penetrate deeper in the water. We have revealed the expression of deep brain photoreceptors sensitive to different wavelength: *tmt-opsin*, *exorhodopsin*, *opn4m1 and opnl1w1*, from day 3, in accordance with the onset of behavioral activity. The hyperactivity of zebrafish was attributed, in a previous work, to the expression of melanopsin, OPN4M1, indeed in animals *otpa* mutants (orthopedia transcriptor factor of *opn4M1*) are severely affected in the visual motor response (Fernandes et al., 2012). Curiously, also in larvae under red light, has been reported differences on locomotor activity, so the perception is not attributable to melanopsin rather to OPNLW1. We can suppose that perception of red light could depend by another opsin present in the brain, sensitive to the long wavelength as was demonstrated by a recent study on tropical damselfish, showing a new opsin in brain capable to perceive long wavelengths (Takeuchi et al., 2011). Furthermore, more studies are needed, to explain perception at different wavelength and associate it to specific photoreceptors, to date the onset of each protein functionally active.

The existence of hatching rhythms has been studied in chapter 3, and the different rhythm pattern described in zebrafish embryos reared under different light-dark cycles. Hatching occurs in all light conditions, but presenting differences Furthermore, the different effect of DL cycle and DL2 on hatching can depends by different quantity of light and light cycles reciveid during the first hours of development. This hypothesis is reinforced by a previous study on which the number of LD cycles to which embryos are subjected before they are transferred to DD conditions, directly influence the amplitude of activity rhythms. Subjecting the zebrafish embryos to only one or two LD cycles after fertilization has been seen to significantly reduce the number of animals displaying circadian rhythmicity (Hurd and Cahill, 2002).
The presence of an endogenous clock in the zebrafish has been also observed to control the spawning rhythm as under LD, spawning takes place within 2 h of lights on (Selman et al., 1993). However, pulses of darkness applied during the light phase caused a delay in the spawning peaks (Blanco-Vives and Sánchez-Vázquez, 2009). In the present study, the hatching rhythms of zebrafish coincided with those previously found when

the hatching rhythm of this species was firstly described (Villamizar et al., 2013). The constant condition of light and darkness have been suggested as impair the normal development of embryos and larvae of several teleost fish (Blanco-Vives et al., *2010*; Villamizar et al., 2009; Liu et al., 1994). On the other hand, LD cycles are thought to influence a broad range of functional and morphological aspects during the early stages of fish, such as the regulation of cell proliferation, the activation of UV protective systems (DNA repair enzymes), pigmentation through visual and non-visual photoreceptors and the expression of diverse light responsive genes (Dekens et al., 2003; Tamai et al., 2004; Shiraki et al., 2010; Vatine et al., 2011).

Regarding food anticipatory activity, chapter 4 confirms the presence of this behavior in response of periodic feeding, in both zebrafish and cavefish. Our results highlight the length of anticipation that varied depending on the fish species and the feeding period used. This anticipation had an endogenous origin, as demonstrated by the persistence of FAA under fasting conditions in many groups of fish, and it was generated by a circadian pacemaker since the endogenous periods were around 24 h regardless of the feeding period. Cavefish showed a higher entrainment than zebrafish to feeding periods longer than 24 h, which points to a higher capacity of food synchronization in this species. The circadian feeding rhythms have been observed in a great variety of vertebrates (Stephan, 2002), among them different species of fish such as goldfish (*Carassius auratus*), European sea bass (*Dicentrarchus labrax*) and tench (*Tinca tinca*) (Azzaydi et al., 2007; Herrero et al., 2005; Sánchez and Sánchez-Vázquez, 2009). Many studies in fish have also shown that FAA persists during food deprivation, confirming the existence of a biological oscillator that controls food anticipation (López-Olmeda et al., 2010; López-Olmeda and Sánchez-Vázquez, 2010). The biological advantages for a circadian system to entrain to periodic feeding have been widely discussed (Sánchez- Vázquez et al., 2001). The present research confirmed the endogenous origin of the feeding anticipatory activity in the two fish species studied, as revealed by the occurrence of free running activity from both zebrafish and cavefish feeding activity when all environmental cues were removed. In zebrafish, this endogenous origin had been reported previously (López-Olmeda et al., 2010). The limits of entrainment depend on the animal species, age and synchronizer strength (Madrid et al., 1998). However, fish seem to have a higher plasticity for the entrainment

to periods quite out of the circadian range, as demonstrated by the present study. On the other hand, cavefish can entrain better to different periods compared with zebrafish. We can suppose that the extreme subterranean habitat has contributed to the development of this ability in cavefish, remaining this fish species completely isolated from the day-night cycle for millions of years (Colli et al., 2009) and showing an extreme troglomorphic phenotype. When periodic food delivery is advanced or delayed, many species respond to the shift in feeding time with a gradual re-entrainment of the FAA, which is known as transient (Stephan, 1984; 1992). Zebrafish resynchronized gradually to a shift in mealtime, in contrast to cavefish that did not display this transient resynchronization. As observed in the cavefish in the present study, some goldfish resynchronized immediately to the new feeding time by keeping constant the onset of activity and lengthening the activity phase until feeding (Sánchez-Vázquez et al., 1997). Therefore, fish can respond in different ways to a delay in the mealtime, but whether this is a species-dependent response remains to be elucidated.

Regarding the daily expression of genes involved in sex differentiation in adult zebrafish, the chapter 5 revealed that the expression profiles of these genes are not constant but change during the day, in some cases displaying daily rhythms such as brain *dmrt1* in both sexes and brain *foxl2* in males. Moreover, differences in these rhythmic profiles can be found depending on the sex, as was observed in the ovarian *cyp19a* that presented a diurnal peak and the expression of *amh* in the male testis that displayed a peak located at the beginning of the dark phase.

Estrogens are essential hormones for ovarian differentiation, and aromatase is the key enzyme involved in their conversion from androgens (Piferrer, 2001). While the mechanisms in gonads are largely studied, in brain many questions remain to resolve. Recent studies of steroids rhythmic levels have been carried out in other fish species. In Senegalese sole for example the testosterone (T) and estradiol (E_2) are both secreted in higher quantity during the dark phase of the day, because of the nocturnal nature of the sole (Oliveira et al., 2009). Several studies have revealed the existence of daily spawning rhythms in many other fish species such as: coral fishes that showed a daily spawning rhythms (Sancho et al., 2000), flounder (Pleuronectes platessa L.) spawns during the night (Nichols, 1989). Since fish synchronize reproduction to environmental factors to select the best time of the day to spawn their eggs, it’s reasonable to think that

they select the best moment of the year in term of conditions to the offspring. Although the role of temperature was not evaluated in the present study, the results obtained may be of importance for future studies on processes of sex differentiation regulated by temperature. In fish, water temperature seems to have a high influence on sex differentiation in many species belonging to very divergent orders (Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Ospina-Álvarez and Piferrer, 2008). In zebrafish, temperature was reported to affect the sex ratio (Penman and Piferrer 2008). Moreover, thermocycles also affect sex differentiation compared with constant water temperatures (Villamizar et al., 2012), thus sex ratio being affected not only by the temperature but also by the time of the day at which the temperature acts, suggesting the existence of windows of sensitivity at specific times of the day. Our results can help to better understand the complex mechanisms by which temperature is able to induce these relevant changes.

Conclusions

Conclusions.

1. The ontogeny of clock genes expression develops differently depending on the light cycles and the wavelength of the light. The genes of the negative loop (*per1b* and *per2*) develop rhythmicity before, during development, respect those positive loop (*clock* and *bmal*).

2. The locomotor activity is strongly influenced by light cycles, showing no rhythmicity under continuous darkness. The different spectrum of light induces the asynchronous start of locomotor rhythmicity in larvae.

3. The locomotor activity increment as response of illumination change of different spectrum of the light cycles is related to the presence of several non visual photoreceptors already at 3 dpf.

4. Zebrafish hatch rhythmically during the light phase, appearing strongly synchronized to light-dark cycles. The phase of this rhythm at constant temperature depends on the lighting cycle.

5. The feeding anticipatory activity has endogenous origin in both species. The oscillator appears to posses: high plasticity in the synchronization of its rhythm to feeding cycle different of 24-hour and ability to re-synchronize when feeding time is delayed.

6. The expression of genes involved in sexual differentiation in adults varies during the day and the phase depends on the light cycle and sex.

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Annexes

7. Annexes

7.1. Annex I: Scientific production resulting from the present PhD Thesis

Scientific Pubblications:

Di Rosa V, Frigato E, López-Olmeda JF, Sánchez-Vázquez FJ, Bertolucci C. The Light Wavelength Affects the Ontogeny of Clock Gene Expression and Activity Rhythms in Zebrafish Larvae. PLoS One. 2015; 10(7): e0132235.

Congress Contribution

V. Di Rosa, E. Frigato, C. Turcato, M. Cavallini, J.F. López-Olmeda, C. Bertolucci, F.J. Sánchez-Vázquez. 2014. Light wavelength differentially affects the ontogeny of behavioral rhythms in zebrafish larvae. Oral Communication in the "11 th International Congress on the Biology of Fish" Edinburgh, Scotland.

V. Di Rosa, E. Frigato, J.F. López-Olmeda, F.J. Sánchez-Vázquez, C. Bertolucci. 2014. Effects of different light wavelengths on the ontogeny of clock gene expression in zebrafish larvae. Poster in the "11 th International Congress on the Biology of Fish" Edinburgh, Scotland.

V. Di Rosa, J.F. López-Olmeda, A. Burguillo1, F. Piferrer, F.J. Sánchez-Vázquez. 2015. Daily rhythms of expression of genes involved in sex differentiation in zebrafish. Poster in the "9th International Congress of Comparative Physiology and Biochemistry" Krakow, Poland

V. Di Rosa, J.F. López-Olmeda, A. Burguillo1, F. Piferrer, F.J. Sánchez-Vázquez. 2015. Ritmos diarios de expresión de genes involucrados en la differenciacion sexual en zebrafish. Poster in the "XV Congreso Nacional y I Congreso Ibérico de Acuicultura" Huelva, Spain.

7.2. Annex II: Scientific production resulting from collaborations with colleagues

Villamizar N, Blanco-Vives B, Oliveira C, Dinis MT, **Di Rosa V**, Negrini P, Bertolucci C, Sánchez-Vázquez FJ. Circadian rhythms of embryonic development and hatching in fish: a comparative study of zebrafish (diurnal), Senegalese sole (nocturnal), and Somalian cavefish (blind). Chronobiol Int. 2013; 7: 889-900.

Tarttelin EE, Frigato E, Bellingham J, **Di Rosa V**, Berti R, Foulkes NS, Lucas RJ, Bertolucci C.Encephalic photoreception and phototactic response in the troglobiont Somalian blind cavefish Phreatichthys andruzzii. J Exp Biol. 2012; 215(Pt 16): 2898-903.

7.3. Annex III: Project supporting the present PhD thesis

-Project Title: Reproductive rhythms in Senegalese sole: neuroendocrine regulation and role of thermo- and photo-cycles during early development on their establishment and maturation (Cronosolea).

Financer Organization: MINECO (AGL-2010-22139-CO03-01)

Coordinator: Dr F. J. Sánchez Vázquez

-Project Title: Ritmos embrionarios y larvarios: papel de los ciclos ambientales en el desarrollo, determinacion/diferenciacion sexual y reproduccion del lenguado. (Solembryo)

Financer Organization: MICINN (AGL2013-49027-C3-1-R)

Coordinator: Dr F. J. Sánchez Vázquez

-Financer Organization: MIUR Minstero Italiano dell’Istruzione, Universitá e Ricerca

Coordinator: Dr. C. Bertolucci

Summary in English

Chapter 1. The light wavelength affects the ontogeny of clock gene expression and activity rhythms in zebrafish larvae.

Light plays a key role in synchronizing rhythms and setting the phase of early development. However, to date, little is known about the impact of light wavelengths during the ontogeny of the molecular clock and the behavioural rhythmicity. The aim of this research was to determine the effect of light of different wavelengths (white, blue and red) on the onset of locomotor activity and clock gene (*per1b, per2, clock1, bmal1* and *dbp*) expression rhythms. For this purpose, 4 groups of zebrafish embryo/larvae were raised from 0 to 7 days post-fertilization (dpf) under the following lighting conditions: three groups maintained under light:dark (LD) cycles with white (full visible spectrum, LDW), blue (LDB), or red light (LDR), and one group raised under constant darkness (DD). The results showed that lighting conditions influenced activity rhythms. Larvae were arrhythmic under DD, while under LD cycles they developed wavelength-dependent daily activity rhythms which appeared earlier under LDB (4 dpf) than under LDW or LDR (5 dpf). The results also revealed that development and lighting conditions influenced clock gene expression. While *clock1* rhythmic expression appeared in all lighting conditions at 7 dpf, *per1b*, *per2* and *dbp* showed daily variations already at 3 dpf. Curiously, *bmal1* showed consistent rhythmic expression from embryonic stage (0 dpf). Summarizing, the data revealed that daily rhythms appeared earlier in the larvae reared under LDB than in those reared under LDW and LDR. These results emphasize the importance of lighting conditions and wavelengths during early development for the ontogeny of daily rhythms of gene expression and how these rhythms are reflected on the behavioural rhythmicity of zebrafish larvae.

Chapter 2. Light transition induces locomotor response in zebrafish larvae: effect of different wavelengths

Light represents an essential stimulus capable to induce and modulate behavioural activity The light is detected by several receptors, visual (retina rod and cone) and non visual (opsins) that differs each other from sensitivity as results of different expression of opsin gene. In zebrafish the effect of the light cycle has been largely studied, although only recently has been studied the crucial effect of different light spectrum on the development of zebrafish larvae. Our data show the effect of different wavelengths

light transition on the locomotor activity of zebrafish larvae from 3 to 11 dpf, revealing for the first time that the hyperactivity of zebrafish larvae induced by lightening transition is already present at 3 dpf. The profiles of locomotor response differ depending whether larvae proceed from light or darkness and on the different wavelength of the light. Is reasonable to think that the early light response of zebrafish can be mediated by deep brain photoreceptors, considering the absence of a functional retinal pathways at 3 dpf.

Chapter 3. Do different LD cycles affect the time of hatching in zebrafish?

During early development, animals display rhythmic physiological processes that are shaped by the daily changes of their surrounding environment (i.e. light and temperature cycles). In fish, the effects of daily photo-cycles during their first developmental stages remain largely unexplored. Here we investigated the existence of circadian rhythms in embryos during the hatching. For this purpose, fertilized eggs were exposed to five light regimes: 12 hours of light: 12 hours of darkness cycle (LD), 12 hours darkness: 12 hours light with the first light phase of 24 hours (DL2); 12 hours darkness: 12 hours light with the first light phase of 3 hours (DL); continuous light (LL), or continuous darkness (DD); at the temperature treatment of 28ºC. Zebrafish under LD hatched during the light phase as expected, from 47 to 61 hpf. Also under inverted light cycle the fish showed the hatching peak during the subjective light phase, differently in DL that embryos deleyed their hatching. These results highlight the importance of the light cycle and the light duration, during the first hours of development. In DD and LL conditions, a hatching rhythm was still observed although its period was longer in contrast with the LD groups. These findings revealed for the first time that early developmental rhythms in fish are endogenously driven, strongly synchronized to light, with the phasing being determined by the number of light cycle received at the beginning of development.

Chapter 4. Entrainment of food anticipatory activity (FAA) under different feeding periods in zebrafish (Danio rerio) and the Somalian cavefish (Phreatichtys andruzzii)

In vertebrates, periodic feeding represents a potent stimulus for the entrainment of circadian rhythms. This entrainment can be detected using the occurrence of food-anticipatory activity (FAA), which consists in an increment on the locomotor activity several hours before mealtime. In the present research, we investigated the effect of

different feeding periods on the entrainment of behavioral rhythms of fish in constant darkness (DD). We compared two fish species, zebrafish and the Somalian cavefish, a blind fish that possesses a food-entrainable circadian system but not a light-entrainable one. We submitted fishes to feeding cycles of 24, 36, 44, 48, 72 and 96 h of period (T) and analyzed the presence of FAA and its endogenous origin under fasting conditions. In a second experiment, we analyzed the resynchronization of FAA to an 8-h shift of feeding time, using feeding cycles of T=24 and 96 h. Zebrafish displayed food anticipatory activity when submitted to 24, 48 and 72 h periods, all 24 multiple periods. Cavefish displayed a stronger anticipation not only when fed with T=24, 48, 72 and 96 h. This study reveals for the first time that scheduled feeding can entrain the oscillators of both fish species depending on the periodicity of the food availability and the presence of transients after feeding mealtime delay. In addition, we showed that food is a stronger synchroniser in the cavefish than in zebrafish, which can be an evolutive advantage for this fish species since it lives in constant darkness in the wild and has lost the light-entrainment capacity.

Chapter 5. Daily rhythms of expression of genes involved in sex differentiation in zebrafish.

Sex steroids play an important role in fish sex differentiation and their production depends on the environmental cycles (i.e. light and temperature) and the biological clock. However, daily rhythms in the endocrine mechanisms responsible for sex ratio control in fish remain unexplored to date. Here, we investigated the daily rhythmic expression patterns of genes involved in the production of sex steroids in zebrafish maintained under a 12:12 h light-dark cycle at a constant temperature of 27°C. We analysed the expression of key genes in gonads and brain of female and male individuals. In gonads, the expression of aromatase (*cyp19a*, ovary) and antimüllerian hormone (*amh,* testis) was rhythmic with opposite acrophases: ZT 5:13 h (in the day) for *cyp19a* and ZT 15:39 h (at night) for *amh*. In brain, the expression of *dmrt1* was also rhythmic in both sexes, in female the acrophase being located at ZT 6:43 h, in phase with ovarian aromatase expression. The expression of *foxl2* presented a peak in both sexes during the last hours of the night. Finally, *cyp19b* (neuronal aromatase) and *cyp11b* presented daily differences, especially in males where the gene expression peaked during the night. These results provide the first evidence for marked time-

dependent differences in the expression of genes involved in the production of sex steroids and sex differentiation in fish.

Resumen en Castellano

8. Resumen en castellano

El objetivo de la presente tesis fue investigar el efecto de la luz de diferente longitud de onda y el fotoperiodo durante el desarrollo temprano, el ritmo de eclosión y la actividad locomotora de larvas de pez cebra. Además, el estudio de un sincronizador adicional (alimento) con diferente periodo permitió evaluar los ritmos de alimentación en dos especies. Finalmente, se describió en el pez cebra adulto los ritmos diarios de genes implicados en la diferenciación sexual.

En la presente tesis doctoral se establecieron los objetivos específicos:

1. Determinar la aparición de la expresión de los genes del reloj (*clock1, bmal1, per1b, per2, dbp*) durante el desarrollo temprano en larvas de pez cebra sometidas a un ciclo de luz-oscuridad de 12:12 horas, y cómo cambian dependiendo de diferentes longitudes de onda de luz (LDW, LDB, LDR, DD).
2. Investigar la influencia de diferentes condiciones de luz y diferentes longitudes de onda en la ontogenia de la actividad locomotora de larvas de pez cebra.
3. Determinar el efecto de los cambios de iluminación de diferente espectro (LDW, LDB, LDR) sobre la actividad locomotora de las larvas de pez cebra y la presencia de opsinas fotorreceptoras capaces de mediar la respuesta a la luz al día 3 después de la fertilización (dpf).
4. Describir la existencia de un ritmo de eclosión y la sincronización de los embriones a diferentes ciclos de luz-oscuridad (LD, DL *, DL, LL, DD).
5. Evaluar la existencia de la actividad anticipatoria a una alimentación periódica, proporcionada con diferentes períodos, en dos especies de peces: pez cebra y pez cavernícola de Somalia en oscuridad continua.
6. Investigar el efecto del cambio de la hora de alimentación en el pez cebra y evaluar la capacidad de resincronización y la presencia de desplazamientos progresivos de la fase.
7. Describir la variación diaria de la expresión de los genes (*cyp19a, amh, cyp19b, dmrt1, cyp11b y foxl2*) que participan en la diferenciación sexual en macho y hembra de pez cebra.

<u>Capitulo 1.</u> La luz de diferente longitud de onda afecta a la ontogenia de la expresión de genes reloj y el ritmo de actividad en larvas de pez cebra

La luz juega un papel clave en la sincronización de los ritmos y el ajuste de la fase del desarrollo temprano. Sin embargo, hasta la fecha, se sabe poco sobre el impacto de las longitudes de onda de luz durante la ontogenia del reloj molecular y la ritmicidad del comportamiento. El objetivo de esta investigación fue determinar el efecto de la luz de diferentes longitudes de onda (blanco, azul y rojo) en la aparición de la actividad locomotora y los ritmos de expresión de genes reloj (*per1b, per2, clock1, bmal* y *dbp*). Para este propósito, 4 grupos de embriones/larvas de pez cebra se mantuvieron desde 0 a 7 días después de la fertilización (dpf) bajo las siguientes condiciones de iluminación: tres grupos mantenidos bajo ciclo de luz:oscuridad (LD) con luz blanca (espectro visible completo, LDW), azul (LDB) o roja (LDR), y un grupo mantenidos bajo oscuridad constante (DD). Los resultados mostraron que las condiciones de iluminación influyeron en los ritmos de actividad. Las larvas fueron arrítmicas en DD, mientras que en ciclos LD desarrollaron ritmos de actividad diaria dependiendo de la longitud de onda. El ritmo apareció antes en condiciones de LDB (4 dpf) respecto a LDW o LDR (5 dpf). Los resultados también revelaron que el desarrollo y las condiciones de iluminación influenciaron la expresión de genes reloj. Si bien la expresión rítmica de *clock1* apareció en todas las condiciones de iluminación a 7 dpf; *per1b*, *per2* y *dbp* mostraron variaciones diarias a partir de 3 dpf. Curiosamente, *bmal1* mostró expresión rítmica desde la etapa embrionaria (0 dpf). En resumen, los datos revelaron que los ritmos diarios aparecieron antes en larvas mantenidas en LDB que en aquellas en LDW y LDR. Estos resultados enfatizan la importancia de las condiciones de iluminación y longitudes de onda durante el desarrollo temprano para la ontogenia de los ritmos diarios de la expresión génica y la forma en que estos ritmos se reflejan en la ritmicidad del comportamiento de las larvas de pez cebra.

<u>Capítulo 2.</u> La transición de luz induce una respuesta locomotora en larvas de pez cebra: efecto de diferentes longitudes de onda

La luz representa un estímulo esencial capaz de inducir y modular el comportamiento. La luz es detectada por varios receptores, tanto visuales (conos y bastones) como no visuales (opsinas), que difieren uno de otros por su sensibilidad dando como resultado diferentes expresiones genéticas. En el pez cebra el efecto de los ciclos de luz se ha

estudiado ampliamente, aunque sólo recientemente se ha estudiado el efecto crucial del diferente espectro de luz sobre el desarrollo de las larvas de pez cebra. Nuestros datos mostraron el efecto de la transición de diferentes longitudes de onda de luz en la actividad locomotora de larvas de pez cebra de 3 a 10 dpf, revelando por primera vez que la hiperactividad de larvas de pez cebra inducida por la transición de la luz ya está presente en 3 dpf. Los perfiles de respuesta locomotora difieren dependiendo de si las larvas proceden de la luz o de la oscuridad y de la longitud de onda de la luz. Es razonable pensar que la respuesta temprana a la luz en pez cebra podría estar mediada por fotorreceptores cerebrales profundos, teniendo en cuenta de la ausencia de unas vías de retina funcionales a 3 dpf.

Capitulo 3. **¿Los distintos ciclos de luz-oscuridad (LD) afectan al momento de eclosión en el pez cebra?**

Durante el desarrollo temprano, los animales muestran procesos fisiológicos rítmicos debido a los cambios diarios de su entorno (ciclos de luz y temperatura). En los peces, los efectos de los foto-ciclos diarios durante sus primeras etapas de desarrollo han sido poco estudiados. En este trabajo se investigó la existencia de los ritmos circadianos en la eclosión de los embriones. Para este propósito, los huevos fertilizados se expusieron a cinco regímenes de luz: 12 h de luz:12 h de oscuridad (LD); 12 h de oscuridad:12 h de luz con la primera fase de oscuridad de 24 horas (DL1); 12 h de oscuridad:12 h de luz con la primera fase de luz de 24 horas (DL2); luz continua (LL); y oscuridad continua (DD). Los embriones de pez cebra en LD eclosionaron durante la fase de luz como se esperaba, entre 47-61 hpf. También los grupos del ciclo de luz invertida los peces mostraron el pico de eclosión durante la fase de luz, aunque de manera diferente en DL ya que los embriones retrasaron su eclosión. Estos resultados ponen de manifiesto la importancia del ciclo de la luz y la duración de la luz durante las primeras horas de desarrollo. En condiciones de DD y LL, un ritmo de eclosión se observó aunque su período fue mayor en comparación con los grupos de LD. Estos resultados revelan por primera vez que los ritmos del desarrollo temprano en los peces son conducidos de forma endógena, fuertemente sincronizados a la luz, y que la fase del ritmo está determinada por la cantidad de luz y por el número de ciclo de luz recibidos en el inicio del desarrollo.

<u>Capítulo 4.</u> Encarrilamiento de la actividad anticipatoria al alimento (FAA) con diferentes periodos de alimentación en el pez cebra (*Danio rerio*) y el pez cavernícola somalí (*Phreatichtys andruzzii*)

La alimentación periódica representa en vertebrados un estímulo potente para el encarrilamiento de los ritmos circadianos. Este encarrilamiento se puede detectar usando la presencia de actividad anticipatoria al alimento (FAA), que consiste en un incremento en la actividad locomotora varias horas antes de la hora de la comida. En la presente investigación se determinó el efecto de diferentes periodos de alimentación en el encarrilamiento de los ritmos de comportamiento de peces en oscuridad constante (DD). Se compararon dos especies de peces, el pez cebra y pez cavernícola de Somalia, un pez ciego que posee un sistema circadiano encarrilable a la comida pero no a la luz. Los peces se sometieron a ciclos de alimentación de 24, 36, 44, 48, 72 y 96 h de periodo (T) y se analizó la presencia de FAA y su origen endógeno en condiciones de ayuno. En un segundo experimento se analizó la resincronización de la FAA después de un desplazamiento de la hora de comida de 8 h, con ciclos de alimentación de T = 24 y 96 h. El pez cebra mostró actividad anticipatoria a los alimentos cuando fue sometido a periodos de alimentación de 24, 48 y 72 h, que son periodos múltiplos de 24 h. El pez cavernícola mostró una anticipación más fuerte cuando fue alimentado con periodos de 24, 48, 72 y 96 h. Este estudio revela por primera vez que la alimentación programada puede encarrilar los osciladores de dos especies de peces en función de la periodicidad de la disponibilidad de alimento y la presencia de desplazamientos de fase después de modificar la hora de la comida. Además, hemos demostrado que la alimentación es un sincronizador más fuerte en el pez cavernícola que en el pez cebra, lo que se traduce en una ventaja evolutiva para esta especie de pez, ya que vive en la oscuridad constante en la naturaleza y ha perdido su capacidad de sincronizarse a la luz.

<u>Capítulo 5.</u> Ritmos diarios de expresión de genes implicados en la diferenciación sexual en el pez cebra

Los esteroides sexuales juegan un papel importante en la diferenciación sexual de los peces y su producción depende de los ciclos ambientales (luz y temperatura) y del reloj biológico. Sin embargo, los ritmos diarios en los mecanismos endocrinos responsables del control de la relación sexual en peces permanecen inexplorados hasta la fecha. En este trabajo hemos investigado los patrones de expresión rítmica diaria de genes

implicados en la producción de esteroides de peces cebra mantenidos bajo un ciclo de luz-oscuridad de 12:12 horas a una temperatura constante de 27 ° C. Analizamos la expresión de genes clave en gónadas y cerebro de los individuos femeninos y masculinos por separado. En las gónadas, la expresión de aromatasa (*cyp19a*, ovario) y la hormona antimülleriana (*amh*, testículo) fue rítmica pero con acrofases opuestas: ZT 05:13 h (durante el día) para *cyp19a* y ZT 15:39 h (durante la noche) para *amh*. En el cerebro, la expresión de *dmrt1* fue rítmica en ambos sexos, en hembras la acrofase se encuentra a ZT 06:43 h, en fase con la expresión de aromatasa en ovario. La expresión de *foxl2* presentó un pico en ambos sexos durante las últimas horas de la noche. Finalmente, *cyp19b* (aromatasa neuronal) y *cyp11b* presentaron diferencias diarias, especialmente en los machos, donde la expresión del gen alcanzó su punto máximo durante la noche. Estos resultados proporcionan la primera evidencia de diferencias en función de la hora del día en la expresión de genes implicados en la producción de esteroides sexuales y la diferenciación sexual en peces.

Discusión General

La presente tesis doctoral confirma la importancia de las señales externas durante la embriogénesis, especialmente la luz, para ajustar el reloj biológico y los procesos fisiológicos que se reflejan en las respuestas de comportamiento. Además, esta tesis profundiza en los diferentes efectos de la longitud de onda de la luz y destaca la importancia de un sincronizador adicional: la alimentación periódica.

La luz solar es una señal ambiental compleja que influye en la evolución de la mayoría de los procesos biológicos en la Tierra. La luz se caracteriza por los cambios diarios en la radiación de longitud de onda, la composición, la dirección y la polarización (Björn, 2002). En los últimos años muchas investigaciones han tenido en cuenta el importante papel de las diferentes longitudes de onda (Villamizar et al, 2013;.. Blanco-Vives et al, 2010;. Villamizar et al, 2009). El capítulo 1 presenta el estudio realizado para determinar la ontogenia de los genes del reloj y la actividad locomotora en larvas desde las primeras horas de vida a diferentes longitudes de onda de luz. A nivel comportamental, la luz induce diferentes respuestas del aparato locomotor en función de la longitud de onda. Las condiciones de iluminación de LDW y LDB presentan la mayor influencia sobre la actividad locomotora, promoviendo una mayor actividad global respecto a las larvas criadas en LDR. Estudios anteriores han

demostrado que el comportamiento del pez cebra en condiciones de iluminación constante es regulado por un reloj endógeno y que el ciclo LD establece la fase de reloj (Cahill et al., 1998; Hurd y Cahill, 2002). Por ejemplo, se requieren ciclos LD para el correcto inicio de la ritmicidad del comportamiento en larvas de pez cebra (Hurd y Cahill, 2002). El análisis de la ontogenia de los genes reloj reveló resultados diferentes en los animales en condiciones DD y LD. Por ejemplo, en DD, el ritmo de *per2* no aparece durante el desarrollo, como se ha observado en otras especies de peces como el medaka (*Oryzias latipes*) y el lenguado senegalés (*S. senegalensis*) (Dekens y Whitmore, 2008; Martin-Robles et al, 2012;.. Cuesta et al, 2014). Este efecto de las condiciones DD se explicaría por el hecho de que *per2* es un gen inducible por la luz y por lo tanto requiere la presencia de luz para desarrollar correctamente su ritmicidad diaria (Vatine et al., 2009). Curiosamente, la presencia de luz constante no induce la expresión rítmica de genes del reloj. En la trucha arcoíris, *Oncorhynchus mykiss*, los genes reloj *per1* y *clock* se expresan rítmicamente en larvas criadas bajo condiciones LD de 0 a 58 dpf, pero no en iluminación constante (Davie et al., 2011). Estas discrepancias entre los tiempos de comienzo de la ritmicidad entre los componentes clave del reloj circadiano se ha sugerido en otros estudios en los peces (Dekens y Whitmore, 2008; Martin-Robles et al, 2012;. Cuesta et al, 2014).

En el caso de la longitud de onda de la luz, sus efectos en la ontogenia de peces apenas han sido considerados hasta hoy. Algunas trabajos recientes se han centrado en los efectos de la longitud de onda en el rendimiento de las larvas, la supervivencia y la aparición de malformaciones encontrando que, en general, longitudes cortas de onda (azules) son mejores para el desarrollo de los peces en comparación con las de onda de largas longitudes (rojo) (Villamizar et al., 2013 ; 2009). También se ha observado un efecto drástico de longitudes de onda cortas sobre el comportamiento de las larvas en el lenguado senegalés (Blanco-Vives et al., 2012). La luz azul es capaz de generar un cambio en la fase activa de diurna a nocturna durante el inicio de la metamorfosis larvaria. Por el contrario, las largas longitudes de onda (rojo) no mostraron ningún efecto sobre la actividad locomotora (Blanco-Vives et al., 2012). Nuestros resultados coinciden con los obtenidos en las otras especies de peces, ya que tanto los ritmos de genes reloj como de comportamiento aparecieron antes en las larvas criadas con longitudes de onda cortas (LDB) que en aquellas criadas con longitudes de onda largas (LDR).

Con el fin de determinar el efecto de los cambios de iluminación de diferente espectro (LDW, LDB, LDR) en la actividad locomotora de las larvas de pez cebra, el capítulo 2 muestra que la hiperactividad de pez cebra inducida por la transición de iluminación ya está presente en el día 3, cuando los fotorreceptores de la retina no son todavía funcionales. Es razonable pensar que el estímulo que induce a este comportamiento está mediado por los fotorreceptores profundos del cerebro. Nuestros resultados concuerdan con un estudio previo en el que la respuesta motora visual persistió después la ablación de la glándula pineal, lo que sugiere que este comportamiento fue mediado por fotorreceptores profundos del cerebro. La melanopsina, OPN4M1, era el candidato para mediar la fotorrecepción, de modo que se ha propuesto que su expresión estaría involucrada en la respuesta de fotocinesis a la oscuridad (Fernandes et al., 2012). La luz blanca es capaz de inducir el cambio principal en términos de actividad en larvas que se hayan adaptado previamente a un ambiente con luz u oscuridad a 8 dpf, mientras que la luz azul induce una respuesta diferente respecto al blanco. La alta sensibilidad para la luz blanca y azul ha sido revelada en larvas de pez cebra en otro estudio previo donde se puso de relieve el efecto de esta condición de la luz en la ontogenia del comportamiento y la aparición de ritmicidad del reloj biológico (Di Rosa et al., 2015). Podemos suponer que esta mayor sensibilidad para el azul (longitudes de onda cortas) y blanco (espectro completo) comparada con la luz roja podría ser debida a la adaptación del animal al medio ambiente, teniendo en cuenta que la luz azul tiene la capacidad de penetrar a más profundidad en el agua. En esta Tesis además hemos revelado la expresión de los fotorreceptores cerebrales profundos sensibles a diferentes longitudes de onda: TMT-opsina, exorhodopsina, opn4m1 y opnl1w1, a partir de 3 días, de acuerdo con el inicio de la actividad comportamental. La hiperactividad del pez cebra se atribuyó, en un trabajo previo, a la expresión de la melanopsina, OPN4M1. De hecho, animales mutantes para *otpa* (factor de transcriptor ortopedia de opn4M1) se ven muy afectados en la respuesta motora (Fernandes et al., 2012). Curiosamente, se han descrito diferencias en la actividad locomotora en larvas bajo la luz roja, por lo que la percepción no es atribuible a la melanopsina sino más bien a OPNLW1. Podemos suponer que la percepción de la luz roja podría depender de otra opsina presente en el cerebro, sensible a la longitud de onda larga, como demostró un reciente estudio sobre damisela tropical, mostrando una nueva opsina en el cerebro capaz de percibir longitudes de onda largas (Takeuchi et al. ,

2011). Además, se necesitan más estudios para explicar la percepción de diferentes longitudes de onda y asociarlo a fotorreceptores específicos, para identificar el momento en el que cada proteína empieza a ser funcionalmente activa.

La existencia de ritmos de eclosión ha sido estudiada en el capítulo 3, donde se describen los diferentes patrones rítmicos presentes en embriones de pez cebra criados en diferentes ciclos de luz-oscuridad. La eclosión se produce en todas las condiciones de luz, pero existen diferencias debidas al diferente efecto del ciclo de DL y DL*. La eclosión depende de la cantidad de luz y los ciclos de luz recibida durante las primeras horas de desarrollo. Esta hipótesis se ve reforzada por un estudio previo en el que el número de ciclos LD al que se someten los embriones antes de ser transferidos a las condiciones DD influyen directamente en la amplitud de los ritmos de actividad. Someter los embriones de pez cebra a sólo uno o dos ciclos LD, después de la fertilización, produce una reducción significativa del número de animales que presentan ritmicidad circadiana (Hurd y Cahill, 2002). También se ha observado la presencia de un reloj endógeno en el pez cebra que controla el ritmo de desove viendo como, en LD, el desove ocurre dentro de 2 h después del encendido de la luces (Selman et al., 1993). Sin embargo, los pulsos de oscuridad aplicada durante la fase de luz causan un retraso en los picos de desove (Blanco-Vives y Sánchez-Vázquez, 2009). En el presente estudio, los ritmos de eclosión de pez cebra coincidieron con los encontrados previamente cuando se estudió por primera vez el ritmo de eclosión de esta especie (Villamizar et al., 2013). La condición constante de luz y oscuridad han sido consideradas perjudiciales para el desarrollo normal de los embriones y larvas de varios peces teleósteos (Blanco-Vives et al, 2010; Villamizar et al, 2009;. Liu et al., 1994). Por otro lado, se cree que los ciclos LD pueden influir en una amplia gama de aspectos funcionales y morfológicos durante las primeras etapas de peces, tales como la regulación de la proliferación celular, la activación de sistemas de protección UV (enzimas de reparación del ADN), la pigmentación a través de fotorreceptores visuales y no visuales y la expresión de genes de respuesta a la distina luz (Dekens et al, 2003;Tamai y col., 2004; Shiraki et al, 2010;. Vatine et al, 2011).

En cuanto a la actividad anticipatoria al alimento, el capítulo 4 confirma la presencia de este comportamiento en respuesta a la alimentación periódica, tanto en el pez cebra como en el pez cavernícola. Nuestros resultados resaltan la longitud del tiempo de anticipación, que varía dependiendo de las especies de peces y el período de

alimentación. Esta anticipación tuvo un origen endógeno, como lo demuestra la persistencia de la FAA en condiciones de ayuno en muchos grupos de peces, y se genera por un marcapasos circadiano ya que los períodos endógenos fueron de alrededor de 24 h, independientemente del período de alimentación. El pez cavernícola mostró un encarrilamiento mayor que el pez cebra a la alimentación de los períodos de más de 24 h, lo que apunta a una mayor capacidad de sincronización al alimento en esta especie que sólo presenta sincronización a la comida. Los ritmos circadianos de alimentación se han descrito en una gran variedad de vertebrados (Stephan, 2002), entre ellos diferentes especies de peces como el pez dorado (*Carassius auratus*), lubina (*Dicentrarchus labrax*) y la tenca (*Tinca tinca*) (Azzaydi et al ., 2007;. Herrero et al, 2005; Sánchez y Sánchez-Vázquez, 2009). Muchos estudios en peces han demostrado también que la FAA persiste durante la privación de alimento, lo que confirma la existencia de un oscilador biológico que controla la anticipación al alimento (López-Olmeda et al, 2010; López-Olmeda y Sánchez-Vázquez, 2010). Las ventajas biológicas para un sistema circadiano que se encarrila a la alimentación periódica se han discutido ampliamente (Sánchez-Vázquez et al., 2001). La presente investigación confirmó el origen endógeno de la actividad anticipatoria al alimento en las dos especies de peces estudiadas, revelado por la presencia de actividad en curso libre tanto de pez cebra como del pez cavernícola cuando se eliminaron todas las señales ambientales. En el pez cebra, este origen endógeno se había estudiado anteriormente (López-Olmeda et al., 2010). Los límites de encarrilamiento dependen de la especie animal, la edad y la fuerza del sincronizador (Madrid et al., 1998). Sin embargo, los peces parecen tener una plasticidad mayor para el encarrilamiento a períodos bastante fuera del rango circadiano, como lo demuestra el presente estudio. Por otro lado, el pez cavernícola puede encarrilar mejor su ritmo a diferentes periodos comparado con el pez cebra. Podemos suponer que el hábitat subterráneo extremo ha contribuido al desarrollo de esta habilidad en este pez ya que ha permanecido completamente aislado del ciclo día-noche durante millones de años (Colli et al., 2009) y que muestra un fenotipo troglomórfico extremo. Cuando la hora de alimentación periódica se avanzada o se retrasada, muchas especies responden al cambio en el tiempo de alimentación con una resincronización gradual de la FAA, que se conoce como transitorio (Stephan, 1984; 1992). El pez cebra se sincronizó gradualmente a un cambio en la hora de la comida, en contraste con el pez cavernícola que no mostró esta resincronización transitoria. Como

se observa en el pez cavernícola en el presente estudio, algunos peces vuelven a sincronizar inmediatamente a la nueva hora de alimentación, manteniendo constante el inicio de la actividad y el alargamiento de la fase de actividad hasta la alimentación (Sánchez-Vázquez et al., 1997). Por lo tanto, los peces pueden responder de diferentes maneras a un retraso en la hora de la comida, lo que no ha sido estudiado hasta el momento es si esta respuesta es dependiente de la especie.

En cuanto a la expresión diaria de genes implicados en la diferenciación sexual en el pez cebra adulto, el capítulo 5 reveló que los perfiles de expresión de estos genes no son constantes sino que cambian durante el día. En algunos casos muestran ritmos diarios tales como *dmrt1* en el cerebro de ambos sexos y *foxl2* en el cerebro en los machos. Por otra parte, las diferencias en estos perfiles rítmicos se pueden encontrar en función del sexo, como se observó en el *cyp19a* de ovario que presenta un pico diurno y la expresión de *amh* en el testículo masculino, que muestra un pico situado al inicio de la fase de oscuridad. Los estrógenos son hormonas esenciales para la diferenciación de ovario, y la aromatasa es la enzima clave implicada en su conversión a partir de los andrógenos (Piferrer, 2001). Mientras los mecanismos en las gónadas han sido ampliamente estudiados, en el cerebro muchas preguntas están sin resolver. Recientemente se han llevado a cabo estudios de los niveles rítmicos de esteroides em otras especies de peces. En lenguado senegalés, por ejemplo, la testosterona (T) y el estradiol (E2) son secretados a la vez en mayor cantidad durante la fase oscura del día, que se correlaciona con el comportamiento natural nocturno del lenguado (Oliveira et al., 2009). Varios estudios han resaltado la existencia de ritmos diarios de desove de muchas otras especies de peces tales como los peces de coral, que mostraron unos ritmos de desove diarios (Sancho et al, 2000), o la platija (*Pleuronectes platessa L.*), que desova durante la noche (Nichols, 1989). Dado que los peces sincronizan su reproducción a los factores ambientales para seleccionar el mejor momento del día para desovar sus huevos, es razonable pensar que también seleccionarán el mejor momento del año en términos de condiciones favorables para la descendencia. Aunque el papel de la temperatura no se evaluó en el presente estudio, los resultados obtenidos pueden ser de importancia para los futuros estudios sobre los procesos de diferenciación sexual regulados por la temperatura. En los peces, la temperatura del agua parece tener una alta influencia en la diferenciación sexual en muchas especies que pertenecen a órdenes muy divergentes (Baroiller et al., 1999; Baroiller y D'Cotta, 2001; Devlin y Nagahama, 2002;

Ospina-Álvarez y Piferrer, 2008). En el pez cebra se han reportado los efectos de la temperatura sobre la proporción de sexos (Penman y Piferrer 2008). Por otra parte, los termociclos también afectan a la diferenciación sexual en comparación con las temperaturas constantes del agua (Villamizar et al., 2012), por lo que la proporción de sexos se verá afectada no sólo por la temperatura sino también por la hora del día en la cual la temperatura actúa, lo que sugiere la existencia de ventanas de la sensibilidad a determinadas horas del día. Nuestros resultados pueden ayudar a comprender mejor los complejos mecanismos por los cuales la temperatura es capaz de inducir estos importantes cambios.

Conclusiones.

1 La ontogenia de la expresión de los genes reloj se desarrolla de manera diferente dependiendo de los ciclos de luz y también de la longitud de onda. Los genes del bucle negativo (*per1b* and *per2*) parecen desarrollar antes la ritmicidad, durante el desarrollo, respeto a aquellos del bucle positivo (*clock* and *bmal*) que no resulta dependiente de la luz.

2 La actividad locomotora está influenciada por los ciclos de luz, no mostrando ninguna ritmicidad bajo oscuridad continua. El diferente espectro de la luz influye en el inicio de la ritmicidad de las larvas a diferentes momentos.

3 El incremento de la actividad locomotora debido al cambio de iluminación de los ciclos de luz y del diferente espectro está relacionado con la presencia de varios fotorreceptores no visuales presentes desde el día 3 dpf.

4 La eclosión del pez cebra tiene un ritmo diario y está sincronizada a los ciclos de luz-oscuridad. La fase de este ritmo depende del tipo de ciclo de iluminación.

5 La actividad de anticipación al alimento es de origen endógeno en las dos especies. El pez cebra tiene una elevada plasticidad en el encarrilamiento de su ritmo a ciclos de comida de periodo diferente de 24 horas y presenta resincronización gradual tras retrasar la hora de alimentación.

6 La expresión de genes involucrados en la diferenciación sexual en adultos varia durante el día y la fase depende del ciclo de luz y del sexo.

Riassunto in Italiano

9. Riassunto in Italiano

Lo scopo della presente tesi è stato studiare l'effetto della luce di diversa lunghezza d'onda e fotoperiodo sullo sviluppo precoce, ritmi di schiusa e attività locomotoria delle larve di zebrafish. Inoltre lo studio di un "zeitgeber" aggiuntivo (alimento) ha permesso di studiare i ritmi di alimentazione sincronizzati a diversi periodi di alimentazione in zebrafish adulto e cavefish (specie cieca). Infine si propone di descrivere in zebrafish adulto, i ritmi di espressione giornaliera di geni coinvolti nella differenziazione sessuale.

Nella presente tesi di dottorato sono stati stabiliti i seguenti obiettivi specifici:

1. Determinare l'insorgenza dell'espressione di geni orologio (*clock1, bmal1, per1b, per2, dbp*) durante la fase di sviluppo precoce nelle larve zebrafish sottoposte ad un ciclo luce-buio 12:12, e come puo' subire modifiche a seconda delle diverse lunghezze d'onda della luce (LDW, LDB, LDR, DD).
2. Studiare l'influenza delle diverse condizioni di luce di diverse lunghezze d'onda sulla ontogenesi dell'attività locomotoria in larve di pesce zebra, come risultato di una risposta comportamentale all'espressione genica dell'orologio biologico.
3. Determinare l'effetto delle variazioni di illuminazione di diverso spettro (LDW, LDB, LDR) sull'attività locomotoria delle larve di zebrafish, e la presenza di fotorecettori tre giorni dopo la fecondazione (dpf) capace di mediare la risposta alla luce.
4. Descrivere l'esistenza di un ritmo di schiusa e la sincronizzazione di embrioni a ciclo diversi di luce-buio (LD, DL *, DL, LL, DD).
5. Valutare l'esistenza attività anticipatoria all'alimentazione, e l'influenza del diverso periodo di alimentazione su di esso, in due specie di pesci: zebrafish e cavefish in buio totale.
6 Studiare l'effetto della posticipazione dell'ora di alimentazione nel pesce zebra; valutare la capacità di risincronizzazione e la presenza del transiente.
7 Descrivere la variazione giornaliera dell' espressione di geni (*cyp19a, amh, cyp19b, dmrt1, cyp11b* e *foxl2*) coinvolti nella differenziamento sessuale un nelle gonadi e cervello di femmina e maschio adulto.

Capitolo 1. La lunghezza d'onda della luce influisce sull'ontogenesi di espressione dei geni orologio e sul ritmo di attivitá in larvae di pesce zebra

La luce riveste un ruolo fondamentale nel sincronizzare i ritmi ed impostare la fase del primo sviluppo. Tuttavia, ad oggi, poco si sa circa l'impatto di lunghezze d'onda di luce durante l'ontogenesi dell'orologio molecolare e la ritmicità comportamentale. L'obiettivo della ricerca è stato quello di determinare l'effetto di diverse lunghezze d'onda della luce (bianco, blu e rosso) sulla comparsa di attività locomotoria e espressione ritminca dei geni orologio (per1b*, per2, clock1, bmal1* e *dbp*). A questo scopo, 4 gruppi embrioni/ larve di pesce zebra sono stati allevati da 0 a 7 giorni dopo la fecondazione (dpf), alle seguenti condizioni di luce: tre gruppi mantenuti sotto la luce: (LD) cicli di luce-buio con il bianco (spettro visibile completo, LDW), blu (LDB) o luce rossa (LDR), e un gruppo cresciuto sotto buio costante (DD). I risultati hanno mostrato che le condizioni di illuminazione hanno influenzato i ritmi di attivitá. Le larve mantenute in DD sono risultate aritmiche, mentre larve mantenute sotto cicli LD hanno sviluppato ritmi di attività giornaliera dipendente dalla lunghezza d'onda, il primo ad apparire è stato il gruppo mantenuto in LDB (4 DPF) rispetto a gruppo in LDW o LDR (5 dpf).Inoltre i risultati hanno rivelato che lo sviluppo e le condizioni di illuminazione influenzano l'espressione dei geni orologio. Mentre l'espressione ritmica di *clock1* é apparsa in tutte le condizioni di illuminazione a 7 dpf, l'espressione ritmica di *per1b*, *per2* e *dbp* é apparsa gia a 3 dpf. Curiosamente, *bmal1* ha mostrato espressione ritmica costante sin dallo stadio embrionario (0 DPF). Riassumendo, i dati hanno rivelato che il primo ritmo giornaliero si è riscontrato in larvae mantenute in LDB rispetto a quelle allevate in LDW and LDR. Questi risultati sottolineano l'importanza della condizione di illuminazione e lunghezza d'onda durante le prime tappe di sviluppo per l'ontogenesi dei ritmi diari di espressione genica e di come questi ritmi si riflettano sulla ritmicità del comportamento delle larvae di zebrafish.

Capitolo 2. La risposta locomotoria in larve di zebrafish indotta dalla transizione di luce: effetto delle diverse lunghezze d'onda

Luce rappresenta uno stimolo essenziale capace di indurre e modulare l'attività comportamentale. La luce viene rilevata da diversi recettori, visivi (coni e bastoncelli retinici) e non visivi (opsine) che si differenziano tra loro per la sensibilità, e sono il risultato della diversa espressione di geni per le opsine. In zebrafish l'effetto de cicli

luce-buioi è stato largamente studiato, anche se solo recentemente è stato studiato l'effetto cruciale dello spettro luce diversa sullo sviluppo di larve zebrafish. I nostri dati mostrano l'effetto della transizione di luce a differenti lunghezze d'onda sull'attività locomotoria di larve di zebrafish da 3 a 11 dpf, rivelando per la prima volta che l'iperattività di larve di zebrafish indotta dalla transizione di luce è già presente a 3 dpf. I profili di risposta locomotoria differiscono a seconda se le larve procedono da una condizione di luce o buio e dalla lunghezza d'onda. È ragionevole pensare che la risposta precoce alla luce di zebrafish può essere mediato da fotorecettori cerebrali profondi, considerando la non funzionalità della retina a 3 dpf.

Capitolo 3. I diversi cicli di LD influenzano il tempo della schiusa in zebrafish?

Durante lo sviluppo precoce, gli animali mostrano processi fisiologici ritmici che dipendono dai cambiamenti giornalieri del loro ambiente circostante (ad esempio cicli di luce e temperatura). Nei pesci, gli effetti dei cicli giornalieri della luce durante il primo stadio di sviluppo rimangono in gran parte inesplorati. Qui abbiamo studiato l'esistenza di ritmi circadiani negli embrioni durante la schiusa. A tal fine, uova fecondate sono state esposte a cinque regimi di luce: ciclo di 12 ore di luce: 12 ore di buio (LD), 12 ore di buio: 12 ore di luce con la prima fase luce di 24 ore (DL *); 12 ore buio: 12 ore di luce con la prima fase luce di 3 ore (DL); luce continua (LL), o oscurità continua (DD); alla temperatura di 28 ° C. Zebrafish sotto LD ha schiuso durante la fase di luce come previsto, 47-61 hpf. Anche sotto ciclo di luce invertito zebrafish ha mostrato il picco di schiusa durante la fase di luce soggettivo, diversamente in DL dove gli embrioni hanno ritardato la loro schiusa. Questi risultati sottolineano l'importanza del ciclo di luce e la durata della luce, durante le prime ore di sviluppo. In condizioni DD e LL, un ritmo di schiusa è stata ugualmente osservata anche se il periodo risulta essere più lungo rispetto ai gruppi LD. Questi risultati hanno rivelato per la prima volta che i primi ritmi di sviluppo nei pesci sono di natura endogena, fortemente sincronizzati alla luce, con la fase fortemente dipendente dal numero di cicli di luce ricevuta all'inizio dello sviluppo.

Capitolo 4. Sincronizzazione dell'attivitá anticipatoria all'alimento (FAA) durante diversi periodi di alimentazione nel pesce zebra (*Danio rerio*) e il pesce Somalo (*Phreatichtys andruzzii*).

Nei vertebrati, l'alimentazione periodica rappresenta un potente stimolo per la sincronizzazione dei ritmi circadiani. Questa sincronizzazione può essere rilevata verificando la presenza di attività anticipatoria all'alimento (FAA), che consiste in un incremento nella attività locomotoria diverse ore prima dell'orario di alimentazione. Nella presente ricerca, abbiamo studiato l'effetto di diversi periodi di alimentazione sulla sincronizzazione dei ritmi comportamentali dei pesci in buio costante (DD). Abbiamo confrontato due specie di pesci, pesce zebra e la specie somala, cavefish, un pesce cieco che possiede un sistema circadiano in grado di sincronizzarsi all'alimento ma non alla luce. Abbiamo esposto i pesci a cicli di alimentazione dal periodo di 24, 36, 44, 48, 72 e 96 ore (T) ed é stata analizzata la presenza di FAA e la sua origine endogena in condizioni di digiuno. In un secondo esperimento, abbiamo analizzato la risincronizzazione di FAA dopo un variazione di otto ore nell'orario di alimentazione, con cicli di alimentazione di T = 24 e 96 ore. Zebrafish mostró attività anticipatoria quando sottoposto a perioodi di alimentazione di 24, 48 e 72 h, tutti i multipli di 24 ore. Cavefish presentó una forte anticipazione non solo quando alimentato con T = 24, 48, 72 e 96 h. Questo studio rivela per la prima volta che,l'alimentazione programmata puó sincronizzare gli oscillatori di entrambe i pesci, dipendendo dalla periodicitá della disponibilitá di cibo e la presenza di un transiente dopo un ritardo dell'orario solito di alimentazione. Inoltre, abbiamo dimostrato che il cibo è un sincronizzatore in cavefish più forte che in zebrafish, il che rappresenta un vantaggio evolutivo per questa specie in quanto vive in natura in buio costante perdendo cosií la capacitá di sincronizzarsi alla luce.

<u>Capitolo 5.</u> **Ritmo giornaliero d'espressione di geni coinvolti nella differenziazione sessuale in zebrafish.**

Gli steroidi sessuali svolgono un ruolo importante nella differenziazione sessuale e la loro produzione dipende dai cicli ambientali (ad esempio luce e temperatura) e dall'orologio biologico. Tuttavia, ritmi giornaliero dei meccanismi endocrini responsabili del controllo del sesso nei pesci rimangono inesplorato fino ad oggi. Qui, abbiamo studiatoil l'espressione ritmica giornaliera di geni coinvolti nella produzione di steroidi sessuali in zebrafish adultli mantenuti in un ciclo12:12 h di luce-buio, a temperatura costante di 27 ° C. Abbiamo analizzato l'espressione di geni chiave nelle gonadi e cervello di individui femminili e maschili. Nelle gonadi, l'espressione

dell'aromatasi (*cyp19a*, ovaio) e l'ormone antimüllerian (*amh*, testicoli) è stata rítmica con acrofase opposta: ZT 05:13 (di giorno) per cyp19a e ZT 15:39 h (di notte) per *amh* . Nel cervello, l'espressione di *dmrt1* era ritmica in entrambi i sessi, nella femmina l'acrofase era situata a ZT 6:43, in fase con l'espressione di aromatasa nell'ovaio. L'espressione di *foxl2* presentó un picco in entrambi i sessi durante le ultime ore della notte. Infine, *cyp19b* (aromatasi neuronale) e *cyp11b* presentarono differenze nell arco delle 24 ore, soprattutto nei maschi, in cui l'espressione genica culmina durante la notte. Questi risultati hanno fornito per la prima volta prove sulle differenze, tempo dipendenti, di espressione di geni coinvolti nella produzione di steroidi sessuali e differenziazione sessuale nei pesci.

Discussione Generale

La presente tesi di dottorato conferma l'importanza degli stimoli esterni durante l'embriogenesi,, soprattutto la luce per regolare l'orologio biologico, processi fisiologici che si riflettono in risposte comportamentali. Inoltre, la tesi ha lo scopo di chiarire il diverso effetto della lunghezza d'onda della luce e di mettere in risalto la forza di un ulteriore sincronizzatore in più: l'alimentazione periodica.

La luce solare è un segnale ambientale complesso che influenza l'evoluzione della maggior parte dei processi biologici sulla Terra. La luce è caratterizzata da variazioni giornaliere di irraggiamento, la composizione delle lunghezze d'onda, la direzione e la polarizzazione (Björn, 2002). Negli ultimi anni molte indagini stanno prendendo in considerazione il ruolo significativo delle diverse lunghezze d'onda (Villamizar et al, 2013; Blanco-Vives et al, 2010;. Villamizar et al, 2009). Il capitolo 1 presenta lo studio effettuato per determinare l'ontogenesi di geni orologio e dell'attività locomotoria in larve sottoposte sin dalle prime ore di vita a diverse lunghezze d'onda della luce. A livello comportamentale, la luce induce risposte locomotorie diverse a seconda delle lunghezze d'onda di quest'ultima. Le condizioni d' illuminazione di LDW e LDB hanno una maggiore influenza sull'attività locomotoria, promuovendo un'attività complessiva superiore rispetto alle larve allevate sotto LDR. Studi precedenti hanno dimostrato che, l'aumento del comportamento di nuoto in larve zebrafish è legata alla maturazione dei neuroni serotoninergici (Brustein et al., 2003). Precedenti studi hanno dimostrato che il comportamento di zebrafish in condizioni di illuminazione costanti è regolato da un

orologio endogeno e che il ciclo LD regola la fase di questo orologio (Cahill et al, 1998;. Hurd e Cahill, 2002). Infatti, i cicli LD sono necessari per il corretto inizio della ritmicità comportamentale nelle larve di zebrafish (Hurd e Cahill, 2002).
L'analisi dell'ontogenia dei geni orologio ontogenesi ha rivelato risultati diversi negli animali in condizioni DD e LD. Per esempio, in condizione di DD, *per2* non risulta ritmico durante lo sviluppo, cosa che è stata osservata in altre specie di pesci, come il Medaka (Oryzias latipes) e la sogliola senegalese (S. senegalensis) (Dekens e Whitmore, 2008; Martin-Robles et al, 2012;.. Cuesta et al, 2014). Questo effetto dovuto dalla condizione DD sarebbe spiegato dal fatto che *per2* è un gene inducibile dalla luce, e quindi richiede la presenza di luce per sviluppare correttamente la propria ritmicità giornaliera (Vatine et al., 2009). È interessante notare che la presenza di luce costante non induce una regolare espressione di geni orologio. Nella trota iridea, *Oncorhynchus mykiss*, i geni orologio *per1* e *clock* segnarono ritmicità persistente in larve allevate in condizioni di LD da 0 a 58 dpf, ma non in condizioni di illuminazione costante (Davie et al., 2011). Queste discrepanze tra i tempi di insorgenza di ritmicità tra i componenti chiave del orologio circadiano è stato suggerito in altri studi nei pesci (Dekens e Whitmore, 2008; Martin-Robles et al, 2012;. Cuesta et al, 2014).
In caso di lunghezza d'onda, i suoi effetti sulla ontogenesi di pesci sono scarsamente conosciuti ad oggi. Documenti recenti su questo argomento si sono concentrati sugli effetti di lunghezza d'onda sulle prestazioni larvale, la sopravvivenza e la comparsa di malformazioni, scoprendo che, in generale, lunghezze d'onda corte (blu) sono migliori per lo sviluppo dei pesci rispetto a lunghezze d'onda lunghe (rosso) (Villamizar et al., 2013 ; 2009). Un effetto drastico delle lunghezze d'onda corte è stata trovata anche sul comportamento larvale della sogliola senegalese (Blanco-Vives et al., 2012). La condizione di luce blu era in grado di generare una viariazione nell'attività locomotoria, cambiando la fase attiva da diurna a notturna durante l'insorgenza della metamorfosi larvale. Al contrario, le lunghezze d'onda lunghe (rosso) non hanno mostrato alcun effetto sull'attività locomotoria (Blanco-Vives et al., 2012). I nostri risultati concordano con quelli ottenuti nelle altre specie di pesci, dal momento che entrambi i ritmi comportamentali e dei geni orologio appaiano prima in larve allevati sotto le lunghezze d'onda corte (LDB) rispetto a quelli allevate con lunghezze d'onda lunghe (LDR).

Per determinare l'effetto di variazioni di illuminazione di differente spettro (LDW, LDB, LDR) sull'attività locomotoria di larve di zebrafish, nel capitolo 2 abbiamo rivelato che l'iperattività di zebrafish, indotta dalla transizione di illuminazione, è già presente al giorno 3, quando fotorecettori retinici non sono ancora funzionanti. E' ragionevole pensare che lo stimolo che induce il comportamento sia mediato da fotorecettori cerebrali profondi. I nostri risultati sono in accordo con uno studio precedente in cui la risposta motoria visiva persisteva anche quando la pineale veniva asportata, suggerendo che questo comportamento è mediato da fotorecettori cerebrali profondi. Melanopsina OPN4M1 era il candidato di per la mediazione della fotorecezione, e la sua espressione è stato proposta essere coinvolta nella photokinesis all'oscurità. (Fernandes et al., 2012). La luce bianca è in grado di indurre un forte cambiamento in termini di attività in larve adattate alla luce o al buio all' 8 dpf, mentre la luce blu induce una risposta diversa rispetto al bianco. L'elevata sensibilità per la luce bianca e blu è stato già rivelata nelle larve zebrafish infatti un precedente studio ha evidenziato l'effetto di questa condizione di luce sulla ontogenesi del comportamento e insorgenza dell orologio biologico (Di Rosa et al., 2015). Possiamo ipotizzare che questa maggiore sensibilità per il blu (basse lunghezze d'onda) e bianco (spettro completo), invece che la luce rossa, potrebbe essere dovuto all'adattamento degli animali all'ambiente circostante, se si considera che la luce blu ha la capacità di penetrare in profondità nell'acqua. Il nostro studio rivela l'espressione dei fotorecettori cerebrali profondi sensibili a diverse lunghezza d'onda: tmt-opsin, exorhodopsin, opn4m1 e opnl1w1, dal giorno 3, in concomitanza con l'inizio dell'attività comportamentale. L'iperattività di zebrafish è stato attribuita, in un precedente lavoro, all'espressione di melanopsina, OPN4M1, infatti animali mutanti per *otpa* (fattore ortopedia transcriptor di opn4M1) sono gravemente colpiti nella risposta motoria visiva (Fernandes et al., 2012). Curiosamente, anche nelle larve alleva con luce rossa, sono state riportato differenze sull'attività locomotoria, in modo che la percezione non sia imputabile a melanopsina, ma a OPNLW1. Possiamo supporre che la percezione di luce rossa potrebbe dipendere da un altro opsina presente nel cervello, sensibile alla lunghezza d'onda specifica, come è stato dimostrato in un recente studio sulla damigella blu, mostrando una nuova opsina nel cervello in grado di percepire lunghezze d'onda lunghe (Takeuchi et al. , 2011). Inoltre, sono necessari ulteriori studi, per approfondire

sulla percezione a diverse lunghezze d'onda e associarla a fotorecettori specifici, e datare l'inizio dell'attività di ciascuna proteina.

L'esistenza di ritmi di schiusa è stato studiato nel capitolo 3, dove è stato descritto diverso pattern ritmico in embrioni di zebrafish allevati sotto diversi cicli di luce-buio. La schiusa avviene in ogni condizione di luce, ma presenta delle differenze. Inoltre, il diverso effetto di ciclo DL e DL *, sulla schiusa può dipende dalla diversa quantità di luce e cicli di luce ricevuti durante le prime ore di sviluppo. Questa ipotesi è rafforzata da uno studio precedente in cui il numero di cicli LD a cui gli embrioni sono sottoposti, prima di essere trasferiti in condizioni DD, influenzano direttamente l'ampiezza dei ritmi di attività. Sottoponendo gli embrioni di zebrafish a uno o due cicli di LD dopo la fecondazione, è stato riscontrata una sensibile riduzione del numero di animali che mostrano ritmicità circadiana (Hurd e Cahill, 2002).
In zebrafish è stato osservato che l' orologio endogeno controlla anche il ritmo di schiusa sotto cicli LD, schiusa che avviene entro 2 ore dall'accensione della luce (Selman et al., 1993). Tuttavia, un pulso di buio applicata durante la fase di luce ha causato un ritardo nei picchi di deposizione delle uova (Blanco-Vives e Sánchez-Vázquez, 2009). Nel presente studio, i ritmi da schiusa di zebrafish coincidono con quelli precedentemente trovati quando il ritmo di schiusa di questa specie è stata descritta per la prima volta (Villamizar et al., 2013). La condizione costanti di luce o buio influenzano negativamente il normale sviluppo degli embrioni e larve di diversi pesci teleostei (Blanco-Vives et al, 2010;. Villamizar et al, 2009;. Liu et al., 1994). D'altra parte, cicli LD influenzano una vasta gamma di aspetti funzionali e morfologici durante le prime fasi di sviluppo, come la regolazione della proliferazione cellulare, l'attivazione dei sistemi di protezione UV (enzimi di riparazione del DNA), pigmentazione attraverso fotorecettori visivi e non visuali e l'espressione dei geni che rispondono a diverse luci (Dekens et al, 2003;.. Tamai et al, 2004;. Shiraki et al, 2010;. Vatine et al, 2011).

Per quanto riguarda l'attività anticipatoria all'alimentazione(FAA), il capitolo 4 conferma la presenza di questo comportamento in risposta all' alimentazione periodica, sia in zebrafish e cavefish. I nostri risultati evidenziano la lunghezza di anticipazione che varia a seconda della specie e il periodo di alimentazione utilizzato. Questa

anticipazione presentò origine endogena, come dimostra la persistenza di FAA a digiuno in molti gruppi di pesci, ed è stato generato da un pacemaker circadiano quando i periodi endogeni erano circa 24 h indipendentemente dal periodo di alimentazione. Cavefish ha mostrato una sincronizzazione superiore rispetto a zebrafish a periodi di alimentazione più lunghi di 24 ore, il quale punta a una maggiore capacità di sincronizzazione al cibo in questa specie. I ritmi di alimentazione circadiani sono stati osservati in una grande varietà di vertebrati (Stephan 2002), tra i quali diverse specie di pesci come il pesce rosso (Carassius auratus), spigole (Dicentrarchus labrax) e tinca (Tinca tinca) (Azzaydi et al ., 2007;. Herrero et al, 2005; Sánchez e Sánchez-Vázquez, 2009). Molti studi nei pesci hanno inoltre dimostrato che FAA persiste durante la privazione di cibo, confermando l'esistenza di un oscillatore biologico che controlla l'anticipazione al cibo (López-Olmeda et al, 2010;. López-Olmeda e Sánchez-Vázquez, 2010). I vantaggi di un sistema biologico circadiano che si sincronizza all'alimentazione periodica sono stati ampiamente discussi (Sánchez Vázquez et al., 2001). La presente ricerca ha confermato l'origine endogena dell' attività anticipatoria all'alimento nelle due specie di pesci studiate, come rivela la presenza di attività di free running sia in zebrafish che in cavefish, quando tutti i segnali ambientali sono stati rimossi. In zebrafish, questa origine endogena era stata segnalata in precedenza (López-Olmeda et al., 2010). I limiti di sincronizzazione dipendono dalla specie animali, età e forza del sincronizzatore (Madrid et al., 1998). Tuttavia, i pesci sembrano avere una plasticità più elevata per la sincronizzazione a periodi un poco fuori il range circadiano, come dimostra il presente studio. D'altra parte, cavefish può sincronizzarsi meglio a periodi diversi rispetto a zebrafish. Si può supporre che l'habitat sotterranea estremo di cavefish ha contribuito allo sviluppo di questa capacità, rimanendo completamente isolati dal ciclo giorno-notte per milioni di anni (Colli et al., 2009),essendo un fenotipo troglomorfico estremo. Quando la somminiztrazione periodica di cibo è anticipata o ritardata, molte specie rispondono allo spostamento nel tempo dell' alimentazione con una graduale re-sincronizzazione della FAA, che è noto come transiente (Stephan, 1984; 1992). Zebrafish si re-sincronizza gradualmente ad un cambiamento dell'ora di alimentazione, in contrasto con cavefish che non mostra questa re-sincronizzazione. Come osservato nel cavefish nel presente studio, alcuni pesci rossi si re-sincronizzano immediatamente al nuovo tempo di alimentazione mantenendo costante l'insorgenza di attività e prolungando la fase di attività fino alimentazione (Sanchez-Vazquez et al.,

1997). Pertanto, i pesci possono rispondere in modi diversi in seguito allo spostamento dell’ora del cibo, ma se questa è una risposta specie-dipendente rimane ancora da chiarire.

Per quanto riguarda l'espressione quotidiana di geni coinvolti nella differenziazione sessuale in adulti di zebrafish, il capitolo 5 ha rivelato che i profili di espressione di questi geni non sono costanti, ma cambiano durante il giorno, in alcuni casi, che mostrano ritmi quotidiani, come *dmrt1* nel cervello in entrambi i sessi e *foxl2* nel cervello dei maschi. Inoltre, delle differenze di questi profili ritmici sono riscontrabili a seconda del sesso, come osservato nella *cyp19a* ovarico che presenta un picco diurno e l'espressione di *amh* nel testicolo maschile il cui picco d’espressione appare situato all'inizio della fase di buio.
Gli estrogeni sono ormoni essenziali per la differenziazione delle ovaie, e l’aromatasi è l'enzima chiave coinvolto nella loro conversione a partire dagli androgeni (Piferrer, 2001). Mentre i meccanismi nelle gonadi sono ampiamente studiati, tuttavia rimangono molte domande da risolvere sui meccanismi a livello cerebrale.
Recenti studi dei livelli ritmici degli steroidi sono state effettuate in altre specie. Nella sogliola Senegalese, per esempio, il testosterone (T) ed estradiolo (E2) sono entrambi secreti in quantità superiore durante la fase di buio, a causa della natura notturna della sogliola (Oliveira et al., 2009). Diversi studi hanno rivelato l'esistenza di ritmi di riproduzione quotidiani in molte altre specie di pesci come: pesci corallo che mostrano un ritmo di deposizione delle uova giornaliero, la platessa (Pleuronectes platessa L.) che depone durante la notte (Nichols 1989). Dal momento che i pesci sincronizzano la riproduzione a fattori ambientali, scegliendo il momento migliore della giornata per deporre le uova, è ragionevole pensare che selezionino il migliore momento dell'anno in termini di condizioni alla prole. Sebbene il ruolo della temperatura non è stato valutato in questo studio, i risultati ottenuti possono essere di importanza per i futuri studi sui processi di differenziazione sessuale regolati dalla temperatura. Nei pesci, la temperatura dell'acqua sembra avere una grande influenza sulla differenziazione sessuale in molte specie appartenenti a ordini molto divergenti (Baroiller et al, 1999;. Baroiller e D'Cotta, 2001; Devlin e Nagahama, 2002; Ospina-Álvarez e Piferrer, 2008). In zebrafish, la temperatura è stata indicate, influenzare il rapporto tra i sessi (Penman e Piferrer 2008). Inoltre, I termocicli influenzano anche alla differenziazione sessuale

rispetto rispetto a temperature costanti dell'acqua (Villamizar et al., 2012), in tal modo il rapporto tra I sessi sembra essere influenzato non solo dalla temperatura, ma anche dal tempo del giorno in cui la temperatura attui, suggerendo l'esistenza di finestre di sensibilità in momenti specifici della giornata. I nostri risultati possono aiutare a comprendere meglio i complessi meccanismi con cui la temperatura è in grado di indurre questi cambiamenti rilevanti.

Conclusioni

1. L'ontogenesi dell'espressione dei geni orologio sviluppa in modo diverso a seconda dei cicli di luce e la lunghezza d'onda della luce. I geni del loop negativo (*per1b* e *per2*) sviluppano ritmicità prima, durante lo sviluppo, rispetto quelli del loop positivo (*clock* e *bmal*).
2. L'attività locomotoria è fortemente influenzato dai cicli di luce, infatti non mostrare ritmicità in condizioni di buio continuo. La luce di diverso spettro induce l'inizio asincrono della ritmicità dell'attività locomotoria delle larvae.
3. L'incremento dell' attività locomotoria come risposta al cambiamento di illuminazione di diverso spettro dei cicli di luce è legato alla presenza di numerosi fotorecettori non visivi già a 3 dpf.
4. Zebrafish schiude ritmicamente durante la fase di luce, che apparendo questo processo fortemente sincronizzato al ciclo di luce-buio. La fase di questo ritmo a temperatura costante dipende dal ciclo di illuminazione.

5 L'attività anticipatoria all'alimentazione ha origine endogena in entrambe le specie. L'oscillatore sembra possedere: elevata plasticità nella sincronizzazione del ritmo a cicli di alimentazione diversi di 24 ore e la capacità di re-sincronizzazione quando l'orario di alimentazione viene ritardato.

6 L'espressione dei geni coinvolti nella differenziazione sessuale negli adulti varia durante il giorno e la fase dipende dal ciclo di luce e dal sesso.