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Daily Rhythms of Reproduction, Early Development and Lipid Metabolism in Teleost Fish: Influence of Light and Feeding Cycles.

Ritmos Diarios de Reproducción, Desarrollo Temprano y Metabolismo Lipídico en Peces Teleósteos: Influencia de los Ciclos de Luz y de Alimentación.

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Thesis submitted by Juan Fernando Paredes Salas

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Index

1. Introduction

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1.1. The biological clock in fish

1.1.1. Circadian rhythms

Life on our planet constantly confronts cyclic environmental changes of light/darkness, tides and seasons due to the Earth rotational and translational movements. These predictable cycles fostered organisms to develop Biological Clocks to keep track of time anticipating dependable cyclic events by means of physiological and behavioral adaptations (Panda et al., 2002). Exogenous synchronizing cues (or *zeitgebers*, ZT) as light-dark, temperature and feeding cycles synchronize the molecular clocks which in turn, generate the circadian rhythms (Paredes et al., 2019a) (Fig. 1). Animals adjust their rhythms to these external stimuli giving rise to a wide range of adaptive strategies such as locomotor activity, feeding, reproduction and spawning to occur at specific times of the day/year increasing animal survival and minimizing energy expenditure (Decoursey, 2004).

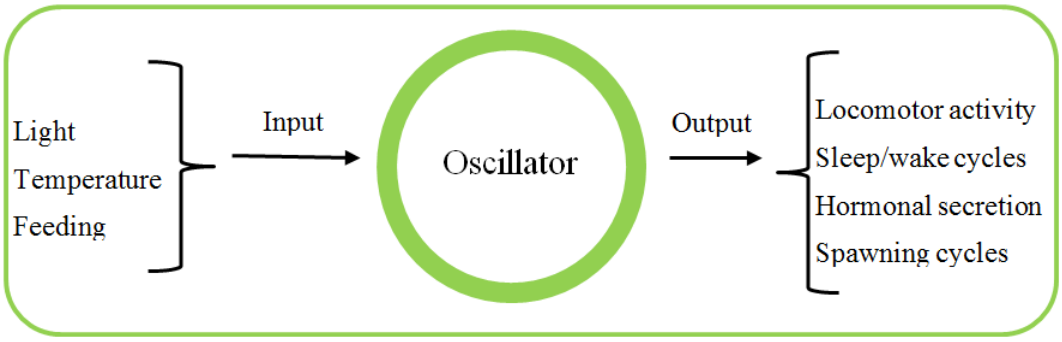

Figure 1. Schematic diagram of a time-measuring system of an organism.

The main characteristic of biological rhythms is their persistence under constant environmental conditions. Endogenous rhythm is the term for rhythms when they appear in absence of external stimuli. Their periodicity differs (shorter or larger, depending on the

specie) respect to that of a solar day, but it is approximately around 24 h. For this reason, the time of the internal clock is referred to as *circadian time* (CT).

In vertebrates, the pineal organ transduces seasonal changes (photoperiod and temperature) into melatonin rhythms. The melatonin appears at night and its secretion varies in inverse relation to the day length working as a clock/calendar signal (Lincoln, 2002; Reiter, 1980). This hormonal signal informs the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. Clocks in other tissues of the body acquire the name of peripheral oscillators and its degree of autonomy depends on the specie. In fish, the rhythmicity of a biological process comprehends the existence of a central self-sustained pacemaker at the pineal organ that directly informs about environmental light conditions (Ekström and Meissl, 2003).

Circadian regulation controls most physiological activities during a day. It covers multiple activities such as lipogenesis, xenobiotic detoxification, cholesterol synthesis, ribosome biogenesis, mitochondrial respiration, sleep-wake rhythms, hormone secretion or cognitive tasks. Thus such a clock-dependent regulation should be of importance when studying fish rhythms (Paredes et al., 2015).

1.1.2. The molecular clock

The molecular mechanism beneath the self-sustained circadian oscillators consists of interlocked transcriptional/translational feedback loops of circadian clock genes and proteins. The core components of the biological clock are *Clock* (*Circadian locomotor output cycles kaput*), *Bmal* (*Brain and muscle ARNT-like protein*), *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. The positive elements of this loop are the *CLOCK* and *BMAL1* heterodimers that activates the transcription of genes *Per*, *Cry* and the orphan nuclear receptor (*Rev-erbα*) binging to the E-box. When PER and CRY proteins achieve critical levels in the cytoplasm, they create a big negative complex that translocates back towards the nucleus inhibiting *per*, *cry* and *rev-erbα* transcription via interference with CLOCK-BMAL1 complex, thus closing the negative feedback loop (Fig. 2). Simultaneously, the positive loop continues when the *CLOCK* and *BMAL1* heterodimers activate transcription of the rev-erbα. The REV-ERBα protein represses *bmal1* transcription by acting through the response elements in its promoter.

As a result, BMAL1 products fall while PER and CRY increase giving stability and robustness to the clock core loop system (Reppert and Weaver, 2002).

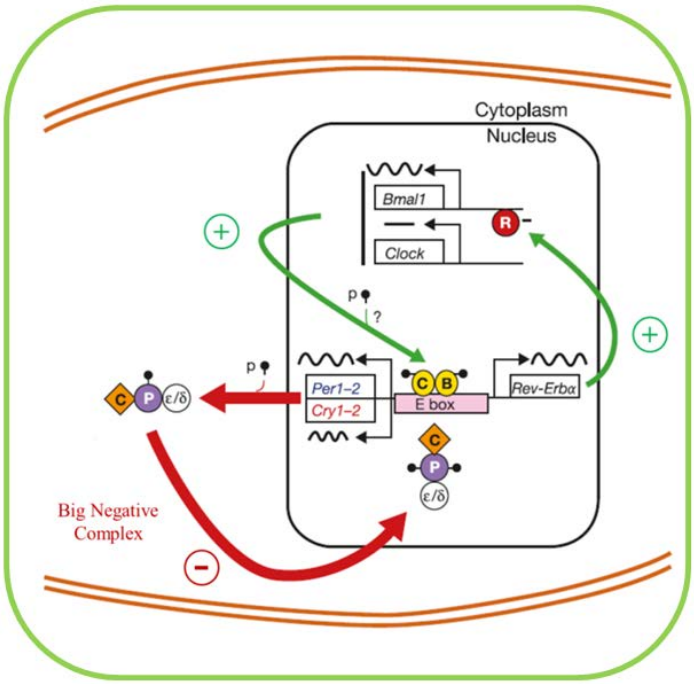

Figure 2. Molecular clock model. The clock includes an interactive positive (green) and negative (red) feedback loops. CLOCK (C, yellow oval) and BMAL1 (B, yellow oval) form the heterodimers complex that activates transcription of *per*, *cry* and *rev-erbα* genes. Protein products of PER (P, purple circle) and CRY (C, orange diamond) constitute a negative complex that binds the CLOCK-BMAL1 complex shutting down transcription while the heterodimer remains bound to the DNA. The positive loop continues with the REV-ERBα protein increment (R, red circle) repressing *bmal1* transcription (Figure modified from Reppert and Weaver, 2002).

1.2. Synchronizing cycles

Geophysical cycles promoted the evolution of biological clocks. The most powerful abiotic factors entraining biological rhythms in animals are light-dark, temperature and food availability (Mistlberger, R. E., 2009; Panda et al., 2002; Rensing, L. and Rouff, P., 2002). Additionally, in the aquatic environment, cycles of light and water temperature generate daily photo- and thermo-cycles.

1.2.1. Photoperiod

Solar light displays daily changes in irradiance, wavelength, polarization and composition. In the aquatic habitat, light changes its spectral quality at different depth levels as water column acts as a chromatic filter. The shortest (below violet, $\lambda < 390$ nm) and longest (beyond red, $\lambda > 390$ nm) wavelengths scatter near the surface generating coastal waters. Blue wavelengths ($\lambda \approx 390$ nm) penetrate deep water oceans reaching depths up to 150 meters (Fig. 3).

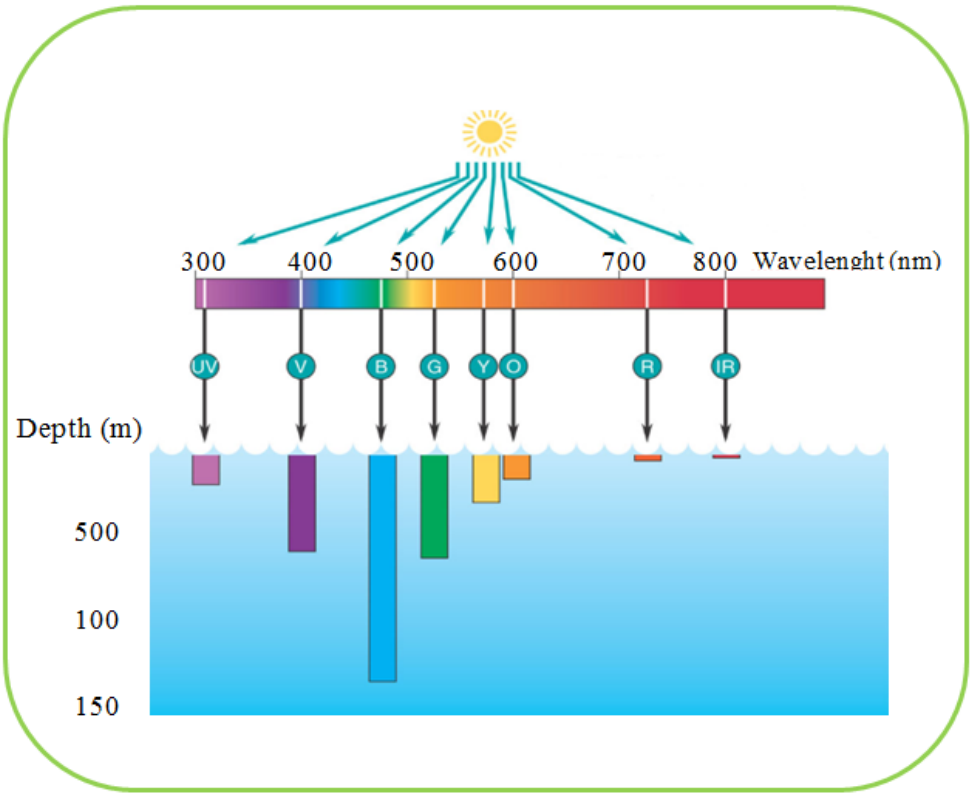

Figure 3. Profile of light spectrum in the water column of the ocean.
All colors are separated for visualization.

Fish have colonized nearly all habitats in this dynamic landscape adapting to cope with the environmental changes. Recent research reveals that in teleost fish, the whole life cycle is

influenced by light to such an extent of displaying a self-sustained pacemaker called light-entrainable oscillator (LEO) at the SCN of the hypothalamus that regulates light entrainment. These light conditions determine embryo development, hatching rates, larval development, growth performance, sexual maturation, spawning and reproduction. Thus light turns to be the predominant signal for entraining the circadian oscillators in fish (Aranda et al., 2001; Blanco-Vives and Sánchez-Vázquez, 2009; Mañanós, 2008; Paredes et al., 2019a; Villamizar et al., 2009).

Nowadays, several investigations highlight the influence of photoperiod in the establishment of daily rhythms in an animal life during a 24 h cycle. Additionally, we bring forward that organisms also use absolute measures of day length and the direction of day length changes as cues for regulating seasonal cycles in physiology and behavior (Goldman, 2001). For instance, medaka is a small egg laying freshwater fish with a seasonal reproduction critically dependent on summer conditions. Long photoperiods and warm temperature trigger reproduction while short days and cold waters conditions produce egg lying stop and gonad regression (Sakai, N. et al., 1987).

1.2.2. Temperature

Natural alternations between night and day generate cyclic changes in daily temperature (thermocycles). Water cools during the night (cryophase) and warms during the day (thermophase) (Fig. 4). These temperature fluctuations are strong enough to entrain rhythms such as hatching, growth and gene expression even in presence of light oscillators (Boothroyd et al., 2007; Villamizar et al., 2012). In aquatic ectotherms, this dynamic landscape fosters a challenging adaptive response to avoid thermal stress. In fish, temperature influences virtually most features of their behavior, foraging ability, physiology, growth and sex differentiation. Temperature cycles maybe critical to such an extent that in complete absence of light, zebrafish embryos are able to develop and entrain their circadian rhythm of their clock gene expression (Bennett, W.A. and Beitinger, T.L., 1997; Boothroyd et al., 2007; Villamizar et al., 2012).

Water temperature may induce irreversible changes in the course of sensitive periods at early developments in fish. Recent investigations display interesting data regarding the effects of temperature on growth, jaw malformations, yolk sack absorption, eye migration/complete

metamorphosis and sex ratio. Literature suggests that sex determination in fish involves certain degree of temperature-dependence thus conditioning fish to become female or male. Additionally, epigenetic mechanisms point out to the bridge linking high temperatures and masculinization via hypermethylation of the promoter of the gonadal aromatase gene (*cyp19a*) (Blanco-Vives et al., 2010; Navarro-Martín et al., 2011)

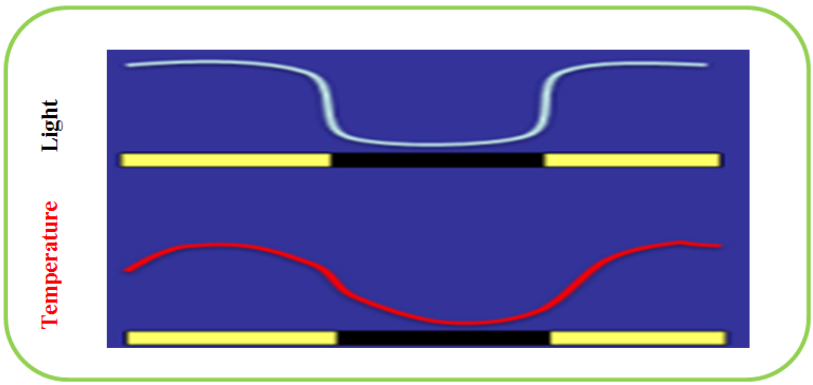

Figure 4. Environmental cycle of light and temperature. Yellow and black bars indicate day and night phases, correspondently.

1.2.3.Food availability

Food availability is not a constant factor in natural environment. On the contrary, it displays a cyclic accessibility that appears to be the highest during certain windows of a day. These maximum food occurrences arise with the maximum appearance of predators. Under these trading off circumstances, animals display a time keeping mechanism to predict feeding time. Hence, mammals present a food-entrainable oscillator (FEO) at the SCN of the hypothalamus. FEO works as a self-sustained pacemaker and explains the existence of feeding entrainment. This clock mechanism allows animals to activate in advance physiological process as locomotor or enzyme activity so that food digestion and absorption may display their maximum efficiency. Indeed, food availability exhibits seasonal variations that coincide with another specie reproductive timing thus ensuring critical periods of food for the survival of the offspring. For instance, short gestational animals (e.g., hamsters and small mammals) begin reproductive processes in spring while long gestational ones (e.g., ruminants) do it late

in summer so that parturition occurs at the highest food availability (Aranda et al., 2001; López-Olmeda, J. F and Sánchez-Vázquez, F.J., 2010).

1.3. Reproduction and early development rhythms

Reproduction is a seasonal phenomenon in most fish species. However, daily and lunar rhythms play important roles in fish reproduction. Fish covers almost every aquatic habitat on the plant, adapting to cyclic variations of light, temperature, food, salinity, oxygen or any other chemical/physical water properties (Fig. 5).

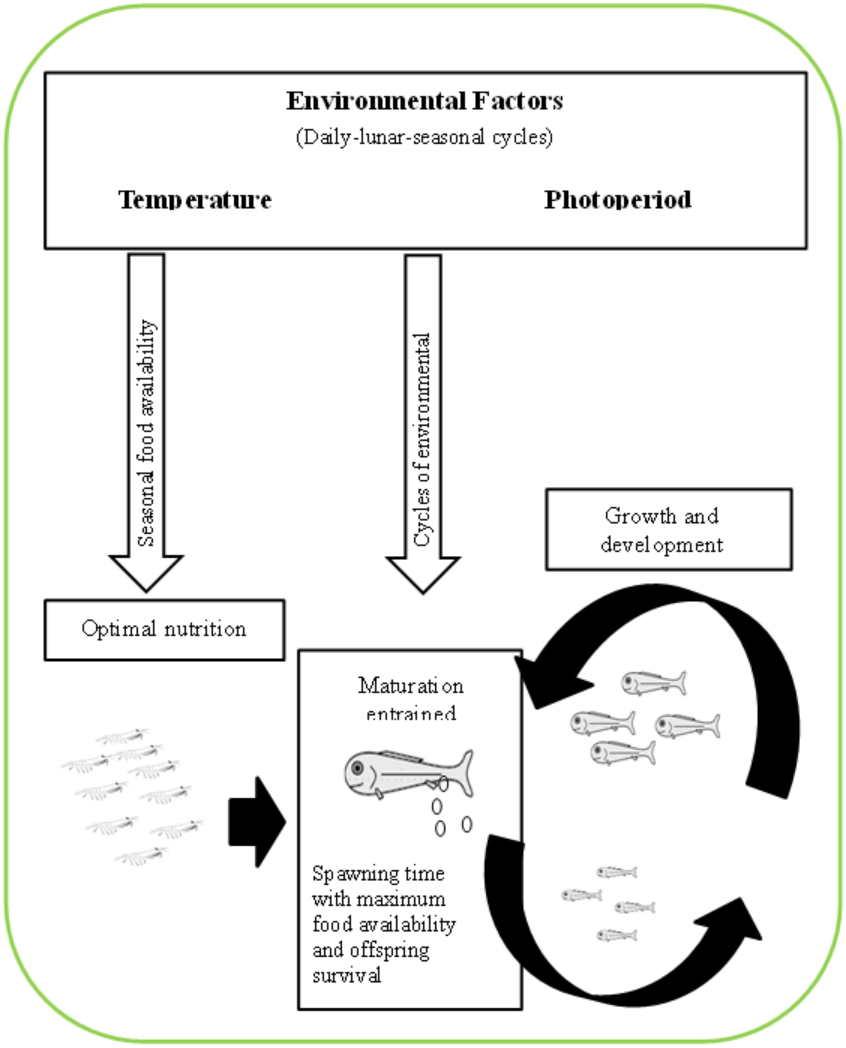

Figure 5. Diagram of environmental factors influencing reproduction. Modified from Mañanós, 2008.

Hence fish develop a wide range of rhythmic adaptive strategies such as reproduction to occur at specific times of the day and/or year, increasing animal survival. Indeed, reproduction success in fish relies on choosing optimal timing for mating and offspring release. Therefore, cyclic environmental changes of photoperiod, temperature and food availability trigger reproduction rhythms. Long photoperiod, warmer temperatures and high food availability catalyze maturational processes of reproduction, activate mating activity and constitute critical periods for larvae and juvenile feeding (Mañanós, 2008). However, in this dynamic environment it is photoperiod, hormonal rhythms of melatonin/ thyroid-stimulating hormone (TSH) and the Brain-Pituitary-Gonadal axis (BPG) oscillations the critical players that trigger reproductive rhythms (Cowan et al., 2017; Paredes et al., 2019).

1.3.1. The Brain-Pituitary-Gonad axis (BPG)

A cascade of hormones along the BPG axis regulates the reproductive cycle. In this cascade the key players in the endocrine control of reproduction are the pituitary gonadotropins (Gths): follicle-stimulating hormone (Fsh) and the luteinizing hormone (Lh). The brain controls pituitary Fsh and Lh secretion via the stimulatory/inhibitory actions of preoptic/hypothalamic gonadotropin-releasing hormones (Gnrh), kisspeptins (Kiss) and gonadotropin-inhibitory hormone (Gnih). These three neuropeptides constitute the first system that regulates the reproductive axis by integrating environmental cues and transducing neuroendocrine inputs for the regulation of the BPG axis. In the gonad, the interplay among Fsh, Lh, androgens and estrogens triggers processes as vitellogenesis, spermatogenesis, maturation, ovulation and spermiation. Gonadal steroids promote feedback actions in the pituitary and in the brain that modulate gonadotropins and neuropeptides secretion and reproductive behavior. Therefore, the BPG axis regulates the activation of all the neuroendocrine machinery that triggers the harmonious progression of the reproductive rhythm in both sexes: from gametogenesis to spawning, leading to egg fertilization (Mañanós, 2008; Muñoz-Cueto et al., 2017; Paredes et al., 2019; Weltzien et al., 2004) .

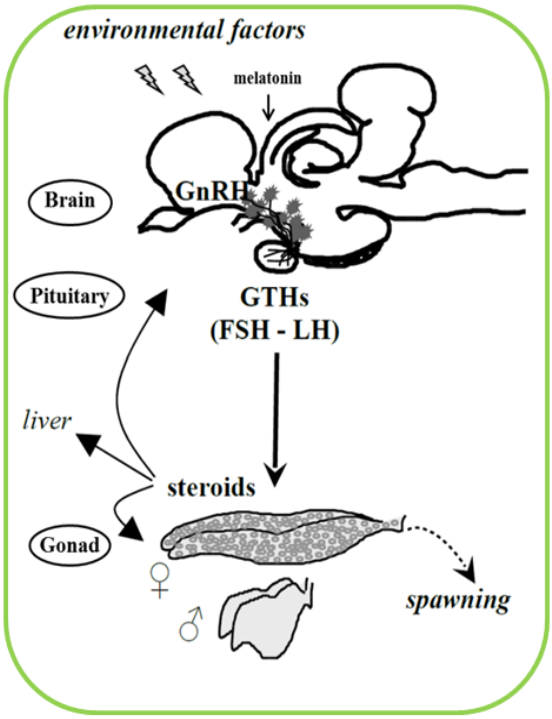

Figure 6. Schematic diagram of the Bran-pituitary-gonadal (BPG) axis displaying the main hormones regulating fish reproduction. Apparently, melatonin acts on the SCN and PT of the adenohypophysis synchronizing reproduction. The brain initiates reproduction releasing GnRH. The GnRH triggers synthesis and release of GTHs at the pituitary. The GTHS stimulate synthesis of sex steroids in the gonads. The sex steroids perform feedback actions on the brain/pituitary and on the female liver promote vitellogenesis for the oocyte production (modified from Mañanós, 2008).

1.3.2. Influence of photoperiod in reproduction: melatonin/TSH

Reproduction is a physiological process that works in parallel with environmental fluctuations depending on the specie. Thus in nature we observe specie with a seasonal fertility timetable, lunar timing or daily spawners. Fish reproductive functioning depends on its reproductive strategy which in turn response to photoperiodic conditions. For instance, Atlantic salmon, Nile tilapia, Atlantic cod or Atlantic halibut exhibit seasonal rhythms of reproduction. Senegal sole with its kin perception of natural moon light is able to synchronize its biological rhythms adjusting reproductive activities and spawning to the lunar cycles. Zebrafish, gilthead sea bream and European sea bass exhibit daily reproduction rhythms. All of these different reproductive rhythms imply the activation of physiological processes as steroidogenesis, gonad maturation, spermgenesis, oogenesis, spawning and spermiation which in turn response to the brain-pituitary-gonad axis. So, it seems reasonable to assume that the neuroendocrine system driving these rhythms would also oscillate according to the light-dark cycles. The extent in which these environmental cues influence or determine the

activation/inhibition of the reproductive rhythms is a question yet to answer (Cowan et al., 2017a; Mañanós, 2008).

Light influences physiological activities in fish to such a point that day length variations activate or inhibit reproduction in fish. Photoperiodism is the phenomenon by which animals use day length cues to track time-of-year and anticipate predictable annual environmental changes. The BPG axis in response to ecological oscillations (diurnal, annual and lunar) triggers all the neuroendocrine machinery of reproduction according to the fish reproductive strategy. Thus long photocycles of 14 hours light: 10 hours dark (14L:10D) activate the reproductive machinery and short photocycles inhibit it (≤ 10 light/day). Now, the degree in which these photocycle variations influence the BPG axis is a question yet to reveal (Goldman, 2001).

The pineal organ of fish transduces seasonal changes (photoperiod and temperature) into a hormonal melatonin signal. This hormone rises during the night and becomes almost undetectable during the day. Cyclic oscillations of light and darkness play the key role on regulating melatonin rhythms. The duration of the nocturnal melatonin increment varies in inverse relation to the day length. Thus short photoperiods (long nights) foster longer melatonin secretion than long photoperiods (short nights). Besides, the pineal organ of fish is so sensitive that is able to detect and transduce a very dim light during lunar phases into melatonin rhythms or even inhibit its production. These findings suggest that melatonin rhythms contribute with extremely precise information about the time of the day, the season of the year and the lunar cycle (Lincoln, 2002; Reiter, 1980).

Temperature also influences melatonin production. Wherein, temperature determines the amplitude of the rhythm while photoperiod regulates the length of the melatonin increment. Hence, the shape of the melatonin profile displays differently depending on the season. For instance, in Senegal sole, melatonin values increase with temperature but those values disappear in constant temperature treatments. In the same way, melatonin values exhibit larger peaks under high temperature than under low temperature exposition in the goldfish (Falcón et al., 2010; Iido, M. and Aida, K., 1995; Oliveira et al., 2007).

Melatonin rhythms enable the synchronization of several physiological processes including reproduction. Apparently, the pineal organ via melatonin production transduces environmental cues activating the neuroendocrine machinery for reproduction (BPG axis).

However, pinealectomy and melatonin administration studies conclude that melatonin is not the only factor triggering the BPG axis to star reproduction. Recent data suggest that the *pars tuberalis* (PT) region of the anterior pituitary gland is also a seasonal controller producing thyroid-stimulating hormone (TSH) at levels that rise by long day exposure and drop by short days. Interestingly, the melatonin signaling controls TSH secretion via interaction with high density melatonin receptors at the PT. In turn, the overall result produces changes in summer phenotypes, growth, food intake and reproductive status (Cowan et al., 2017; Dardente et al., 2010; Nakao et al., 2008; Ono et al., 2008; Paredes et al., 2019).

To sum up, we highlight that the main role of the pineal organ is to transduce environmental time cues into hormonal outputs (melatonin) that ultimately trigger reproduction.

1.3.3. In vitro fertilization in fish (IVF)

Wild conditions foster natural breeding, spawning, fertilization and offspring production in fish. On the contrary, captivity environments induce some degree of reproductive dysfunctions at levels of vitellogenesis, maturation, ovulation and significant reductions of quantity/quality of eggs and sperm. So, as long as reproductive problems persist, assisted reproduction (e.g., IVF) turns to be a useful solution in fish farming.

The success of reproduction falls on the harmonious neuroendocrine progression. Starting from immature germ cells and good qualify gametes production to the synchronized process of spawning/spermiation and viable fertilized egg formation. Additionally, recent literature highlights the existence of rhythms in the BPG axis driving spawning and spermiation to coincide in the optimal time for offspring release. Thus, it seems logical to think that the same rhythmic pattern would appear in assisted reproduction trials answer (Cowan et al., 2017a; Mañanós, 2008).

1.3.4. Epigenetic mechanisms in gonads

Genomic information displays not only in the DNA sequence but also in the imprinting of epigenetic modifications. Hereby, epigenetic phenomena stand for post-genetic molecular mechanisms that give rise to different phenotypes without alteration of the underlying genome. Epigenetic marks include cytosine methylation, histone modifications (via lysine

and arginine methylation, lysine acetylation/ubiquitination or serine phosphorylation) and chromatin structure organization. Methylation and demethylation of cytosines at CpG-rich regions, enhancers and histones appear to be the basic epigenetic mechanism that inactivates or activates genes, respectively. The methylation of specific regions directly interferes with the binding of transcriptional regulators or indirectly inactivates a gene by starting the formation of a heterochromatic state (Xia et al., 2015).

In mammals, recent investigations highlight the existence of several DNA methylation sites oscillating in synchrony with the cell cycle which in turn attends a circadian clock regulation. Therefore, the date points out that epigenetic marking may also oscillate with a daily rhythm. Additionally, studies in the zebrafish gonads confirm that epigenetic machinery of DNA methylation and demethylation displays circadian oscillations under photoperiod of 12 hours light/12 hours darkness or even in conditions of constant darkness (Brown et al., 2007; Nagoshi, E. et al., 2004; Paredes et al., 2018).

Epigenetic modifications display a dynamic landscape on gene regulation responding to environmental stimuli as temperature variations, nutrition alterations or compound expositions. The epigenetic changes lead to a wide opened variety of beneficial or detrimental phenotypical outcomes that probable transmit over generations. Importantly, DNA and histone modifications, which transfer during cell division even over generations, may be the responsible for this transgenerational phenotypical endurance. Some effects may include aberrant DNA methylation patterns, disease-related properties, biological/developmental processes alterations and X-chromosome inactivation.

In fish, epigenetic marking exhibit a critical role in several physiological processes including cardiovascular performance, hypoxic tolerance, feeding, morphology, behavior, reproduction, sex determination and gonadal differentiation. Sex determination and sex differentiation exhibit a complicated mechanism depending on the interplay between genetic and environmental cues ranging from genotypic sex determination (GSD) to environmental temperature-dependent sex determination (TSD). Indeed, water temperature may produce unchangeable effects during early fish development. For instance, in the European see bass (*Dicentrarchus labrax*) an epigenetic mechanism links high temperature with masculinization via hypermethylation of the gonadal aromatase gene (*cyp19a*) promoter. Additionally, recent literature also highlights the connection between temperature effects on

sex ratios and the inhibition of the *cyp19a* expression, hence proving the key role of the conserved aromatase in female sex differentiation. Consequently, temperature-sex-dependent effects occur in species with true TSD (as in pejerrey), in species with a combinations between genetic and environmental sex determination (as olive flounder) and even in species with a strong basis for genotypic sex determination (as Atlantic Halibut, Hippoglossus hippoglossus) (Piferrer et al., 2005, 2012; Villamizar et al., 2012).

Fish plasticity provides the possibility of colonizing a vast range of ecological niches. Environmental conditions that fish experience at early stages lead to noticeable changes in size, growth rate, metabolism and age at reproduction. Identifying the factors that foster such a phenotypic plasticity would help understanding the way organism adapt to the environment. The pluripotent and environmental exposed egg initiates the dialogue with the external and internal factors that prolong during ontogeny until the next generation. Developmental larval stage is also crucial in interpreting environmental cues and transducing them into epigenetic information as DNA methylation patterns, imprinting or gene silencing. Therefore, as the individual develops the epigenetic pressure activates or silences genes in an epigenetically heritable fashion. The primordial germ cells (PGCs) of the gonads are also susceptible of epigenetic actions. Thus the development of the next generation is partially also a result of the imprinting of gene expression patterns on the PGC of the parent during its own embryonic stage (Pittman et al., 2013).

1.4. Lipid metabolic rhythms

In fish, lipids play a critical role as a source of energy and as basis for essential fatty acids vital for reproductive processes. Female egg production and male breeding activities require large amounts of lipids for enhanced swimming activity, competition, courtship, sexual hormone synthesis, parental care and nesting (Sutharshiny et al., 2013).

Fish covers a wide-open variety of reproductive strategies depending on the specie. For instance, the salmoniform presents a seasonal periodicity in the gonadal maturation, while *Blennius pholishas* displays ripe ovaries throughout all the year; most *Oncorhynchus* spp. spawns once a year while *Anguilla* spp. spawns once in a lifetime. Interestingly, fluctuations of lipid contend in gonads, muscle and liver determine all the maturation and spawning cycle independently of the reproductive strategy of the species. Thus, it is possible to imagine that

the lipid metabolic cycle entrains regarding the specie reproductive strategy to cope with its requirements. In addition, recent investigations about lipid nutritional importance, among other topics, also deal with lipid dietary supplementation, lipid diet energy increments, effects of lipids in body composition or influence of lipids in the immune response (Kim et al., 2012; Sutharshiny, S. et al., 2013).

There is evidence for lipid metabolism driven by a circadian clock. Actually, the lipid enzyme activity and the endocrine system work together orchesting a harmonious progression for lipid metabolic processes. Transcriptome, metabolome and proteome comparative analysis also reveals the existence of a clock-dependent regulation for several metabolic pathways in the mouse liver. In fish, recent research pointed to such a clock-dependence regulates multiple activities as lipogenesis, lipolysis, cholesterol synthesis or hormone secretion (Paredes et al., 2014, 2015).

Evidence suggests that the central pacemaker (brain) determines daily rhythms of peripheral tissues via systemic signal outputs orchestrating the clock system as a whole. The liver behaves as a peripheral oscillator displaying circadian machinery susceptible to factors other than light-dark cycles, as variations in the nutrient composition like a high-fat diet and food intake timing. This is so to such a point that antagonistic feeding times (mid-light food intake vs. mid-dark food intake) reset the liver peripheral clock almost completely, producing variations in rhythmic gene expressions and circadian phase changes. Interestingly, these alterations display at the liver clock gene expression (peripheral oscillator) that synchronizes to the feeding schedule; but not at the brain (central oscillator) that synchronizes to the light-dark cycle. This suggests that food intake scheduling plays a critical role not only in the regulation of lipid metabolic rhythms (Vera et al., 2013). For instance, research reports that restricted feeding influences circadian periodicity of rat hepatic *per1* expression, and that starvation interval and food amount also affect circadian hepatic *per2* gene expression in mice. Likewise, other experiments show that diurnal high-fat feeding foster greater weight gain than natural nocturnal feeding in mice (Eckel-Mahan et al., 2012).

These findings suggest that food intake timing itself is related to weight gain due to alterations of the peripheral clock and metabolism. In the liver, clock-controlled systems and metabolic pathways interact with each other in a positive and negative feedback loop. For instance, hepatocytes display lipolytic and lipogenic rhythms with acrophases at different

times of day; otherwise their activity upon storage or fatty acid utilization would be inappropriate leading to obesity and diabetes (Huang et al., 2011).

5. Species in focus

In this Thesis, three teleost fish species were employed according to their unique characteristics. The rationale for using them follows. In one hand, a well-known basic model such as zebrafish was used to test basic reproductive mechanisms. On the other hand, another basic fish model such as medaka was used because of its seasonal reproduction and photoperiodic responses. Finally, a fish species with great interest in European aquaculture such as gilthead sea bream was used to test lipidic metabolic gene variations.

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

Zebrafish is a teleost fresh water fish habiting around slow-moving or quite waters around Ganges, Brahmaputra, Bangladesh, Nepal, Pakistan Myanmar and Sri Lanka. This area exhibits a typical monsoon with specific seasonal rain variations and a wide daily and/or seasonal temperature changes. Ranging temperatures are around 14.2° C and 33° C. Zebrafish diet includes zooplankton, phytoplankton, insects and any other organic material present in its habitat (Spence et al., 2007). Zebrafish taxonomical hierarchy corresponds as fallows (Fang, 2003).

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

Figure 7. Zebrafish hierarchy

The zebrafish in wild conditions exhibits a seasonal reproduction during the monsoon period. In laboratory conditions zebrafish is able to breed during all year, one or two times per week. Zebrafish is an ideal study model for displaying several features as small size, short generation/development time, abundant spawning, rapid embryonic development and egg transparency. These characteristics turn this specie with a great value for our reproduction related investigations.

5.2. Medaka (*Oryzias latipes*, Temminck and Schlegel,1846)

Medaka is a small fresh water fish habiting places around south-east of Asia and tolerating a wide range of temperatures from 0° C to 40° C. It presents a life time of approximately one year reaching its sexual maturity two months after hatching. Medaka is an ideal model for genetic and developmental biology investigations for displaying advantageous features as small size, high fecundity, transparent embryos and rapid embryo development. Additionally, when comparing with zebrafish, Medaka presents a smaller genome with a higher quality annotation tolerating wide inbreeding. Medaka taxonomical hierarchy corresponds as fallows (Shima, A. and Mitani, H., 2004).

Phylum: Chordata
Subphylum: Vertebrata
Class: Teleostei
Order: Beloniformes
Family: Adrianichthyidae
Genus: Oryzias
Species: *Oryzias latipes* (Temminck and Schlegel, 1846)

Figure 8. Medaka hierarchy

Medaka presents a seasonal reproduction critically dependent on summer light conditions. Long photoperiods of 14h light: 10h darkness (14L:10D) and high temperatures trigger reproduction while short day conditions and low temperatures produce egg lying stop and gonad regression. These features conveyed Medaka with the requirements we were looking for to study the influence of photoperiodism on the BPG axis (Shima, A. and Mitani, H., 2004).

5.3. Gilthead Sea Bream (*Sparus aurata,* Linnaeus, 1758)

Gilthead Sea bream is a teleost fish inhabiting marine and brackish-water (costal lagoons and estuarine areas) environments all along the Mediterranean Sea and the Eastern Atlantic coasts from Great Britain to Senegal. Their usual habitat includes rocky landscapes, sea grass meadows of *Posidonia oceanica* or sandy grounds. Gilthead Sea bream breeds in the late autumn in the open sea. At early spring, the juveniles migrate towards sheltered coastal waters searching for trophic resources and milder temperatures. Gilthead Sea bream is protandrous hermaphrodite. Sexual maturity displays at 2 years in male (20-30 cm) and at 2-3 years in female (33-40 cm). Gilthead Sea bream taxonomical hierarchy corresponds as fallows.

Phylum: Chordata
Subphylum: Vertebrata
Class: Teleostei
Order: Perciformes
Family: Sparidae
Genus: Sparus
Species: *Sparus aurata* (Linnaeus, 1758)

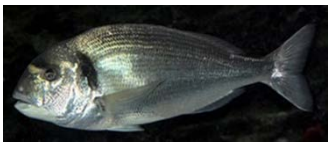

Figure 9. Gilthead Sea bream hierarchy

Gilthead Sea bream is an aquaculture example for hatchery production, artificial breeding and fish farming at Spain, Italy and Greece. Gilthead presents a high adaptability to intense rearing conditions in ponds and cages. These qualities still grand Gilthead Sea bream one of the highest ranking in the aquaculture business (FAO, 2019).

5.4. Senegalese sole (Solea senegalensis, Kaup, 1858)

Senegalese sole is a marine teleost flatfish inhabiting sandy and muddy bottoms around brackish lagoons and shallow waters to coastal regions up to 100 m depth. Sole occurs at the coastal Atlantic from Senegal, Canary Islands up towards Brittany-France. It also appears in the western Mediterranean Sea as far as Tunis (Rodríguez and Rodríguez, 1980).

High market demands include Senegalese sole in the first rankings for aquaculture production. However, reproductive problems hinder the consolidation of sole fish farming. Those problems involve the cycle of reproduction: first generation born in captivity displays poor quality spawning batch and in some cases no spawning at all. Probably, captivity conditions foster the male functioning in the synthesis o realize of gonadotropins and reproductive hormones (Dinis et al., 1999; Matsuyama et al., 1991).

Phylum: Chordata
Subphylum: Vertebrata
Class: Actinopterygii
Order: Pleuronectiform
Family: Soleidae
Genus: Solea
Species: *Solea senegalensis* (Kaup, 1858)

Figure 10. Senegalese sole hierarchy

Objetives

2. Objectives

The overall aim of this doctoral thesis was to elucidate the impact of time cues such as light, temperature and meal timing, on the synchronization of daily reproduction rhythms in fish, considering gamete production, fertilization, embryo/larvae development, and lipid metabolism. On this basis we highlighted the importance of biological rhythms in the establishment of the harmonious progression of the reproductive and early development processes, including Brain-Pituitary-Gonad (BPG) axis activation, gamete daily variations, *in vitro* fertilization rates, gonad epigenetic mechanism rhythms and lipidic gene variations in liver. Specific objectives follow:

1. Reveal the existence of daily rhythms in the expression of key genes in the BPG-liver axis of zebrafish.
2. Investigate the influence of seasonal variations (light and temperature) as favorable or inhibitory reproductive conditions on key genes involved on the BPG axis of medaka.
3. Describe daily rhythms of *in vitro* fertilization in zebrafish to improve protocols considering daily gamete variations.
4. Search for cross-talk mechanisms transducing environmental factors into physiological responses looking at daily rhythms in epigenetic mechanism of methylation and demethylation in the gonads of zebrafish.
5. Evaluate daily rhythms in lipid metabolic gene expression in response to light-dark and feeding cycles in gilthead seabream and in zebrafish liver.
6. Summarize key environmental cues as light and temperature cycles during early development in Senegal solea.

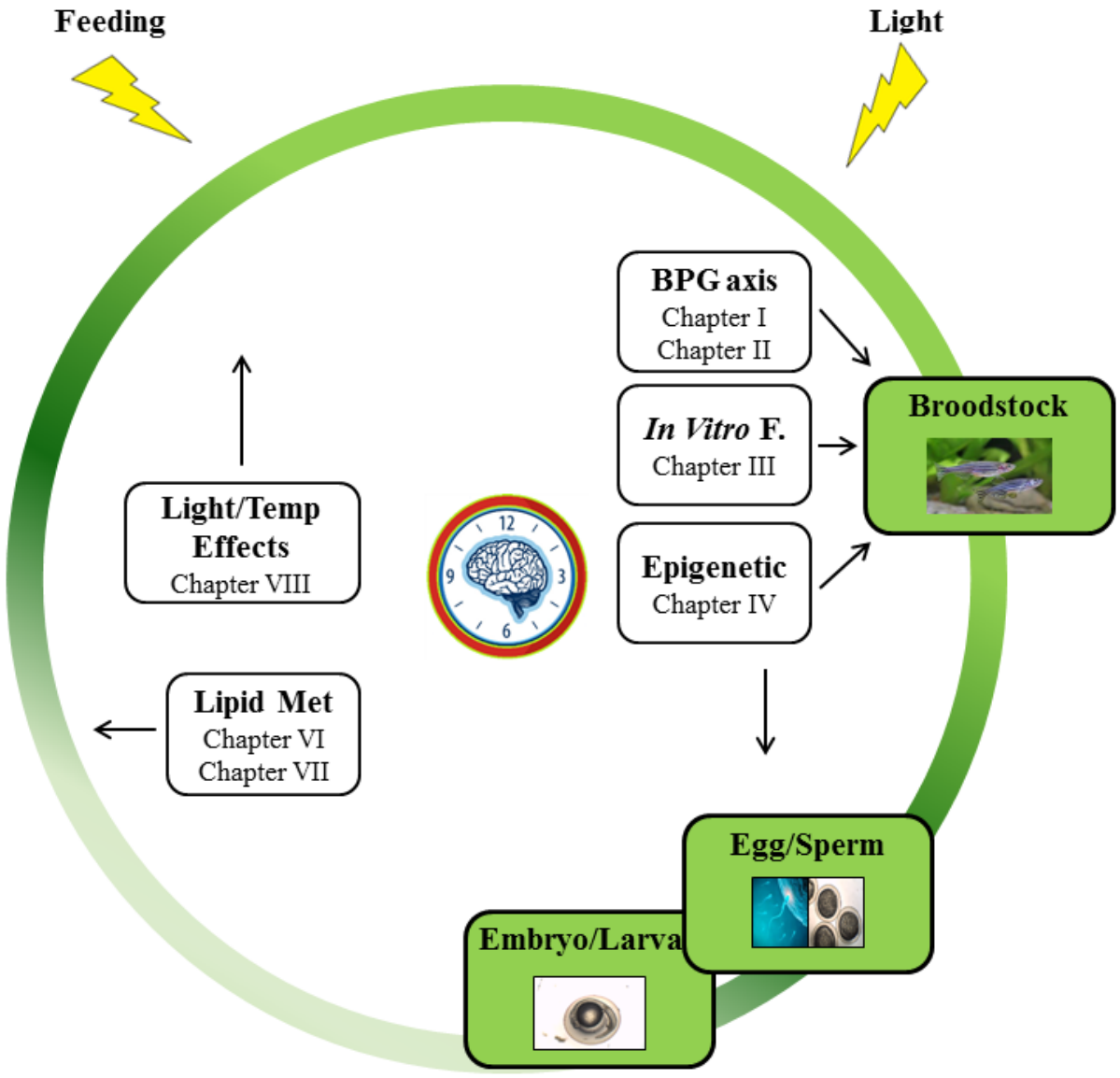

Figure 10. Schematic overview of the thesis

Experimental Chapters

Experimental Chapter I

Daily rhythms of expression in reproductive genes along the brain-pituitary-gonad axis and liver of zebrafish

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Summery

The brain-pituitary-gonadal (BPG) axis controls the activation of all the endocrine machinery that triggers the reproductive process. For this reason, in this chapter we have focused on describing the daily expression rhythms of key reproductive genes involved in the BPG axis of zebrafish. In the experiment, male and female zebrafish were submitted to an extended reproduction stimulating photoperiod of 14 h light:10 h dark (LD) cycle. Brain, pituitary, gonad and liver samples were taken every 4 hours during a 24 h cycle. In the results we unveil that most of the genes presented statistically significant daily rhythms. Central reproductive genes in the brain (*gnrh2, gnrh3, kiss1, kiss2* and *gnrhr3*) displayed a daily rhythm of expression with a nocturnal acrophase (between ZT14:34 and ZT18:34 h). Male *kiss2* gene presented non-significant results. Male *gnrh3* and female *kiss2* genes presented diurnal peaks of expression at ZT06:34 h and ZT04:34 h, respectively. The genes at the pituitary (*fshβ, lhβ, gnrhr2, gnrhr3*) showed daily rhythms of expression, with an acrophase during the light period (between ZT02:10 and ZT10:35 h). Male *gnrhr3* gene presented non-significant results. The genes at the gonads (*star, cyp17a1, 20βhsd, lhr, fshr, cyp19a1a, foxl2, amh, dmrt1* and *11βhsd*) revealed statically significant daily rhythms with an acrophase during the dark phase, except for *cyp17a1a* (ZT06:21) and *20βhsd* (ZT05:19) female genes. Lastly, the genes in the female liver (*erα* and *vtg2*) presented daily rhythms with a maximum peak of expression around the transition phase from darkness to light. The *erα* gene at ZT01:00 h and the *vtg2* gene at ZT23:09 h. In this chapter we described, for the first time in zebrafish, the daily expression patterns of key genes belonging to the reproductive axis and their synchronization to environmental photoperiod.

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Experimental Chapter II

Daily Rhythms in the Brain-Pituitary-Gonad Axis of Medaka (Oryzias latipes) at Long and Short Photoperiod

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Introduction

Animals that are constantly confronted to environmental cycles have developed rhythmic physiological and behavioral processes that are driven by an internal time-keeping system. This biological clock provides animals with a temporal organization to cope with cyclic events (e.g., day/night or season) [1] (Panda et al., 2002). Such a timing mechanism has fostered the development of wide range of reproductive strategies to occur at specific times of the season increasing animal survival [2] (De Coursey et al., 2004).

Seasonal changes in physiology and behavior are timed by the use of day length cues in a phenomenon called photoperiodism [3] (Goldman, 2001). This mechanism is driven by circadian oscillators that allow animals to track time-of-year and anticipate predictable annual environmental changes. The brain-pituitary-gonad axis (BPG) harmoniously triggers all the neuroendocrine machinery involved in reproduction responding to environmental and ecological oscillations (diurnal, annual and lunar) according to the animal reproductive strategy [4,5] (Cowan et al., 2017; Paredes et al., 2019). Thus, long photocycles of 14 hours light: 10 hours dark (14L:10D) activate the reproductive machinery [6,7] (Lee et al., 1986; Freeman et al., 2000) and short photocycles inhibit it (≤ 10 light/day) [8,9] (Tavolaro et al., 2015; Francisco et al., 2004). Consequently, we observe species with a seasonal fertility timetable: short gestational animals (e.g., hamsters and small mammals) begin in spring [10] (Reiter, 1980), while long gestational ones (e.g., ruminants) do so late in summer [11] (Lincoln, 2002). In most fish species from temperate areas, the seasonal daylenght changes drive reproduction rhythms (Oliveira et al., 2009). For instance, in salmonids the most important environmental cue that drives reproduction is the photoperiod [12] (Bromage et al., 2001), however in cyprinids the predominant environmental cue that drives reproduction is a combination of temperature and photoperiod [13] (Hontela and Stacy, 1990). Therefore, to which extent do these seasonal oscillations (photoperiod and temperature) influence reproductive rhythms in the brain-pituitary-gonad axis (BPG) is still unsolved.

The pineal organ transduces seasonal changes (photoperiod and temperature) into melatonin rhythms [10,11] (Reiter, 1993; Lincoln, 2006). The melatonin is secreted at night and its secretion varies in inverse relation to the day length working as a clock/calendar signal. Recent data suggest that the *pars tuberalis* (PT) region of the anterior pituitary gland is also a seasonal controller producing thyroid-stimulating hormone (TSH) at levels that are increased

by long day exposure and suppressed by short days [14] (Dardente et al., 2010). Interestingly, the melatonin is a circadian signal that controls TSH secretion via a high density of melatonin receptors localized in the PT [15–18] (Hanon et al., 2008; Ono et al., 2008; Nakao et al., 2007; Hazlerigg and Louden, 2008). In turn, the overall result produces changes in summer phenotypes, growth, food intake and reproductive status. In the last decade, several studies of seasonal species have helped us understand the effects of photoperiodism on the neuroendocrine hypothalamus [19] (Dardente et al., 2003). For example, observations in hamsters, sheep and rats have confirmed that seasonal cycles in reproduction are critically dependent on TSH regulation [14] (Dardente et al., 2010). Now, to which extent do these photoperiodic variations influence the BPG that is the regulator of the activation of all the neuroendocrine machinery that triggers reproduction in both sexes, is steel unknown [5] (Paredes et al., 2019).

Medaka is a small egg laying freshwater fish with a seasonal reproduction critically dependent on summer light conditions. Long photoperiods trigger reproduction while short day conditions produce egg lying stop and gonad regression [20] (Sakai et al., 1987). Investigations shortening light conditions and lowering water temperature have also proved to down-regulate/inhibit egg production (Thesis Ines). Additionally, histological techniques have shown that photoperiodism is key signaling the process of activation/inhibition of reproduction mainly in females [21] (Koger et al., 1999).

The objective of this research was investigating the influence of seasonal variations on key genes pattern in the BPG axis of medaka. For this purpose, we analyzed the daily expression rhythm of genes in the brain (*gnrhI, gnrhII and kissI*), pituitary (*fsh and lh*) and gonad (*fsh, lhr, star, cyp11a, cyp17, cyp19a1a and amh*) in both female and male under activating or inhibitory reproductive conditions

MATERIALS AND METHODS

Ethic statement

The present investigation was carried out in the laboratories of the University of Murcia (Spain). Fish were reared and manipulated following Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 1201/2005 and Law 32/2007)

for the use of laboratory animals, and were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare (A13150103).

Animal rearing

Medaka (Cab Strain) (N=144) (72 Females and 72 males) 0.3 ± 0.1 g (mean±SD) of body weight were reared in the facilities. Fish were divided into two groups. One group consisted of 36 females and 36 males divided into six 9-L glass aquaria (6 female- 6 male per aquaria) with a photoperiod of 14 hours light and 10 hours dark (14L:10D) at a constant 26±0.5ºC . The other group was equally divided holding an shorten light photoperiod of 10L:14D at a constant 20±0.5ºC . The time of lights on was designated as *Zeitgeber* Time 0h (ZT0h). Light was provided by LED strips (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain), with a light intensity on the water surface of 0.84 $W \cdot m^{-2}$ (~200 lx). Commercial feed (Tropical fish flakes, Casone, Parma, Italy) was delivered through an automatic timer-feeder (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany) placed in each aquarium. The feeder delivered feed 3 times a day (ZTxxh, ZTxxh and ZTxxh) at 1.5% of the fish body weight per time.

Experimental procedure

To investigate the daily rhythms of the BPG-liver genes, the two experimental groups were submitted to XXX months to holding conditions until reproductive maturity was reached. Fish were sampled at ZT1, ZT5, ZT9, ZT12, ZT17 and ZT21 h. and each experimental tank was sampled at only one sampling time to avoid the effects of sampling stress on subsequent samplings. Fish were anesthetized by submersion in icy water (5 parts ice/1 part water, 0-4°C) and sacrificed by decapitation. The brain, pituitary and gonad samples were collected from each female and male. Liver samples were collected only from the female fish. All the samples were frozen immediately in dry ice and stored at -80 °C until processing. Sampling during the dark phase was performed under a dim red light (λ>600 nm).

Molecular analysis

Samples were homogenized using Trizol reagent (Invitrogen, Carlsbad, CA, USA) with a tissue homogenizer (POLYTRON®, PT1200, Kinematica, Lucerne, Switzerland) to obtain total RNA. The RNA purity was determined by spectrometry (Nanodrop® ND-1000, Thermo Fisher Scientific Inc., Wilmington, DE, USA). 1 µg RNA was treated with DNase I amplification grade (1 unit/µg RNA, ThermoFisher Scientific, Massachusetts, USA) for 30

minutes at 37°C to prevent genomic DNA contamination. A Reverse Transcriptase SensiFAST Kit (Bioline, London, UK) was used to produce first-strand cDNA according to the manufacturer's instructions.

The qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA). All the samples were run in duplicate. Primers of each gene (Table 1) were tested to verify their efficiency by means of a standard curve. Elongation factor 1 alpha (*ef1α*) was selected as housekeeping gene after assessing that its coefficient of variation (CV) was lower than 5% and that not daily rhythmic pattern was displayed within each tissue and sex. Each PCR well had a final 20 µl volume: 5 µl of cDNA, 10 µl of the qPCR Master Mix and 5 µl of each forward and reverse specific primer concentration (Table 1). The thermal cycling conditions were as follows: holding stage of polymerase activation (10 min at 95°C); cycling stage (40 cycles of 95°C for 15sec and 60°C for 1min). The specificity of the reaction was validated by analysis of the melting curve. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Cosinor analysis (software CSR 3.0.2) was accomplished to determine whether daily expression of the studied genes fitted the cosine function $Y = M + A * [\text{Cos}\ (\Omega\tau + \Phi)]$, hence revealing the existence of statistically significant daily rhythms. *M* is mesor, *A* is amplitude, Ω is angular frequency (360°/24h for the circadian rhythms), τ is time period (24h) and Φ is acrophase. In addition, cosinor analysis also provided statistical value for null hypothesis of zero amplitude, so that for a statistical significance of $p < 0.05$ the null hypothesis was rejected and the amplitude was considered different from zero (significant rhythm). Statistical differences between different sampling times for each gene analyzed were also tested by means of one-way ANOVA, followed by Tukey's *post hoc* test (SPSS v.19 software IBM, Armonk, NY, USA). Significance level was fixed at $p < 0.05$ for all the statistical analyses.

RESULTS

Brain gene expression: *gnrhI, gnrhII and kissI*

The cosinor analysis revealed that nearly all genes investigated (*gnrhI, gnrhII and kissI*) displayed statistically significant daily rhythms ($p<0.05$). Females under long photoperiod

(14L:10D) presented significant daily rhythm in the *gnrhI* and *kissI* genes except for *gnrhII* (Table 2). Their maximum expression peaks were located around the last hours of the dark phase (between ZT19:52h and ZT20:15h). For short photoperiod in females, only the *gnrhII* gene displayed a daily expression rhythm (Cosinor, $p<0.05$). The *gnrhI, gnrhII and kissI* genes presented a similar daily pattern with maximum expression peak around the last hour of the light phase (~ZT09:00 h) (Figure 1). Male exhibited daily expression rhythm in all genes for both long and short photoperiod (Table 2). One-way ANOVA revealed statistically significant differences between sampling points ($p<0.05$) (Figure 1) for most female and male genes analyzed.

Pituitary gene expression: *fsh and lh*

In both female and male under long and short photoperiod, the cosinor analysis revealed the existence of statistically significant daily rhythms ($p<0.05$) in most genes studied (Table 2). The one-way ANOVA also revealed the existence of statistically significant differences between sampling points for most of the genes investigated in female and male fish ($p<0.05$) (Figure 2). Only the female *lh* gene under short photoperiod failed to exhibit significant rhythmicity (Cosinor, $p>0.05$) (Figure 2). For long photoperiod females, the acrophases of *fsh* and *lh* were located at ZT18:24h and ZT18:47h, respectively; while for short photoperiod *fsh* the acrophase exhibited 3 hours later (ZT20:59h). For long photoperiod males, the acrophases of *fsh* and *lh* displayed at ZT08:45h and ZT08:49h, respectively; while for short photoperiod the acrophases were also located 3 hours later at ZT12:27h and ZT11:57h, respectively (Figure2).

Gonadal receptors gene expression: *fshr and lhr*

For both genes investigated in female under long and short photoperiod, neither the cosinor nor the ANOVA analysis detected statistical significant daily rhythms or differences between sampling points ($p>0.05$) (Table2). For both genes in male under long and short photoperiod, the analysis of the cosinor disclosed statistically significant daily rhythms ($p<0.05$) (Table 2). The one-way ANOVA also revealed the existence of statistically significant differences between sampling points in both genes ($p<0.05$) (Figure 3). The *fshr and lhr* acrophases for long photoperiod males were located during the second half of the light phase at ZT13:39h and ZT12:28h, respectively (Figure3). On the other hand, the *fshr and lhr* acrophases for short

photoperiod males were located during the first half of the light phase at ZT02:40h and ZT04:05h, respectively (Figure 3).

Gonadal steroid expression: *star, cyp11a, cyp17, cyp19a1a and amh*

The cosinor revealed that all genes for females under long photoperiod exhibited statistically significant daily rhythms ($p<0.05$). All genes presented similar pattern with the acrophases were located during the transition phase between ZT22:33h and ZT05:15h (Table2) (Figure 4). The one-way ANOVA revealed the existence of statistically significant differences between sampling points in all long photoperiod female genes investigated ($p<0.05$) (Figure 4). For short photoperiod female genes no statistical significant rhythms were found ($p>0.05$). Only the *star* and the *cyp11a* genes presented significant differences between sampling points (Figure 4). For all genes in male under long and short photoperiod, the cosinor analysis confirmed the existence of a statistically significant daily rhythm ($p<0.05$) (Table 2) except for the *cyp11* gene (14:10). The one-way ANOVA revealed the existence of statistically significant differences between sampling points in all male genes under long photoperiod ($p<0.05$) (Figure 4).

DISCUSSION

The presence investigation revealed that seasonal variations (long/short photoperiod and high/low temperature) altered daily rhythms in key reproductive genes in the brain, pituitary and gonadal axis of medaka. Most females under long photoperiod/high temperature exhibited a daily rhythmic gene pattern. However, most females under short photoperiod/low temperature presented non-significant daily gene expression pattern. On the other hand, both male under long-short photoperiod/ high-low temperature presented similar daily expression rhythms.

The brain is the starting activator of the reproductive axis that integrates internal (melatonin and TSH) and external (environmental cycles) inputs and consequently responds with a neuroendocrine signal, via Gnrh synthesis [5,22–24] (Ekstrom and Meissl, 2003; Falcón et al., 2007; Paredes et al., 2019). In our study, *gnrhI, gnrhII* and *kissI* genes for females under long photoperiod presented similar daily expression pattern (in phase) (Figure 1) with the acrophases located at the end of the night phase (between ZT19:32h and 20:15h). Daily variations on the *gnrh* forms have been reported in the European sea bass [25] (Servili et

al., 2013), gilthead sea bream [26] (Gothilf et al., 1997), orange-spotted grouper [27] (Chai et al., 2013) and medaka [28] (Karigo et al., 2012). In our results, the maximum values for *gnrhI* (ZT19:52h) suggest its implication inducing a parallel surge in the pituitary LH [5] (Paredes et al., 2019). Kisspeptin is a key neuroendocrine factor that regulates gonadotropin release via Gnrh secretion [29–31] (De Roux et al., 2003; Seminara et al., 2003; Messager et al., 2005) and contributes in the seasonal control of reproduction in teleosts [32–36] (Shi et al., 2010; Mylonas and Zohar et al., 2010; Migaud et al., 2012; Alvarado et al., 2013; Espigares et al., 2015). Thus, our results showed that long photoperiod female for *kissI* and *gnrhI* genes synchronously peak in phase (ZT19:52h and ZT20:15h, respectively) hence remarking the influence of the seasonal conditions in driving the reproductive gene rhythms in the brain. On the other hand, for females under short photoperiod, the *gnrhI* and *kissI* genes rhythmicity was lost and the acrophase for *gnrhII* gene was shifted to the light phase (ZT06:57h). Probably the loss of rhythmicity and the phase-shift indicate non-reproductive stage in medaka due to the inhibitory seasonal conditions (short photoperiod and low water temperature). For males under long and short photoperiod the gene expression presented a similar pattern (in phase) expect for *gnrhI* (Figure 1). However, the time of maximum value differs between long and short photoperiod in the *gnrhI* and *kissI* genes (Table 2). Here, it is remarkable to notice that for long photoperiod males the *gnrhI* and *kissI* acrophases synchronously appear in time (ZT23:35h and ZT01:26h, respectively). However for short photoperiod males, there is a 3 hours difference between the *gnrhI* and the *kissI* genes (ZT08:17h and ZT05:16h, respectively) suggesting that such a displacement could block reproductive functionality in males during winter conditions.

In the pituitary, Gnrh is the principal regulator of gonadotropins synthesis and release [37] (Kim et al., 2011). Reports showed that *gnrhr* transcript levels in the pituitary exhibit a seasonal pattern which correspond with a seasonal responsiveness to Gnrh stimulation, so that highest levels of *gnrhr* entail highest responsiveness of the pituitary coinciding with the spawning seasonal cycles [24] (Mañanós, 2008). In our results, the *fsh* and the *lh* genes for long photoperiod females presented an acrophase located at the end of the dark phase (ZT18:24h and ZT18:47h, respectively). Accordingly, the *gnrhI* in the brain displayed an acrophase at ZT19:32h for females under long photoperiod. Thus, the coinciding acrophase times of *gnrhI/fsh/lh* for long photoperiod females suggests that in medaka the *gnrhI* is

directly involved in catalyzing the synthesis of Fsh/Lh gonadotropins responding synchronously to an environmental seasonal cue. On the other hand, for short photoperiod females, the rhythmicity of the *gnrhI* and the *lh* genes is lost and the *fsh* acrophase appeared later in time (ZT20:59h). Probably, this miss coordination between the brain and the pituitary determine the cease in reproductive capabilities during winter season in medaka. The *fsh* and *lh* genes for long and short photoperiod males exhibited daily expression rhythm but differed in the acrophase regarding photoperiod exposure. The *fsh* and *lh* acrophase under long photoperiod was located at ZT08:45h and 08:49h, respectively; meanwhile for short photoperiod was at ZT12:27h and 11:57h correspondingly.

In the gonad, the gonadotropins (Fsh/Lh) trigger the last step in the reproductive axis fostering steroidogenesis and promoting ovulation/spermiation [24] (Mañanós. 2008). In our investigation, we have studied key factors involved in the synthesis of steroids (*star*, *cyp19a*, *cyp17* and *cyp19a1a*). Long photoperiod females, displayed a similar daily expression pattern (Figure 5) with an acrophase located around the beginning of the light phase (between ZT22:33h and 05:15h) (Table 2). The acrophases of fsh/lh (ZT18:24 and ZT18:47 respectively) suggest a time line sequence synchronization that points to a direct relationship between gonadotropins gene expression and the beginning of steroid synthesis.

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DECLARATION OF INTEREST

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Figure Legends

Table 1. Gene IDs and primer sequences used for real-time PCR (in preparation).

Table 2. Cosinor analysis results for BPG axis genes in medaka.

Figure 1. Relative expression values in percentage of brain genes in the BPG axis of medaka under long photoperiod/ high temperature (14L:10D/26° C) and short photoperiod/ low temperature (10L:14D/22° C) cycle. Open and full bars on the top of each graph indicate the light and dark periods, respectively. The sinusoidal line on the graphs represent the data adjustment to the cosine function for statistically significant daily rhythm ($p<0.05$). Statistical differences between sampling points in one gene are indicated by capital and lowercase letters for 14L:10D/26° C and 10L:14D/22° C, respectively (one-way ANOVA, $p<0.05$).

Figure 2. Acrophase map of BPG axis genes studied in this research. Open and full bars on the top and button of the graph indicate the light and dark periods, respectively. …..

Figure 3. Relative expression values in percentage of pituitary genes in the BPG axis of medaka under 14L:10D/26° C and 10L:14D/22° C cycle. Open and full bars on the top of each graph indicate the light and dark periods, respectively. The sinusoidal line represents the

adjustment to a sinusoidal rhythm calculated by Cosinor analysis in the cases where this analysis was statistically significant ($p<0.05$). Different letters indicate statistically significant differences among different sampling points within each group and gene (one-way ANOVA, $p<0.05$).

Figure 4. Relative expression values in percentage of gonadal receptors genes involved in the BPG axis of medaka. White and black bars on the top mean day and night, respectively. The sinusoidal line on the graphs represent the data adjustment to the cosine function for statistically significant daily rhythm ($p<0.05$). Statistical differences between sampling points in one gene are indicated by capital and lowercase letters (one-way ANOVA, $p<0.05$).

Figure 5. Relative expression values in percentage of gonadal steroids involved in the process of reproduction in medaka. White and black bars on the top mean day and night, respectively. The analysis of the cosinor ($p<0.05$) is represented by a sinusoidal line in the graphs. Statistical differences between sampling points in one gene are indicated by capital and lowercase letters (one-way ANOVA, $p<0.05$).

Table 2.

Tissue	Gene	Sex	Photoperiod	Significance	Acrophase (ZT h)
Brain	*gnrhI*	Female	14:10	**	19:52 ± 3:16
		Female	10:14	-	-
		Male	14:10	**	23:35 ± 3:24
		Male	10:14	*	08:17 ± 3:46
	gnrhII	Female	14:10	-	-
		Female	10:14	**	06:57 ± 3:33
		Male	14:10	**	06:47 ± 3:47
		Male	10:14	*	06:42 ± 4:23
	kissI	Female	14:10	*	20:15 ± 4:50
		Female	10:14	-	-
		Male	14:10	*	01:26 ± 4:14
		Male	10:14	*	05:16 ± 3:15
Pituitary	*fsh*	Female	14:10	**	18:24 ± 2:32
		Female	10:14	*	20:59± 4:17
		Male	14:10	*	08:45± 4:20
		Male	10:14	*	12:27± 4:23
	lh	Female	14:10	*	18:47± 3:24
		Female	10:14	-	-
		Male	14:10	*	08:49± 3:37
		Male	10:14	*	11:57± 3:59
Gonadal Receptors	*fshr*	Female	14:10	-	-
		Female	10:14	-	-
		Male	14:10	*	13:39± 4:13
		Male	10:14	*	02:40± 4:07
	lhr	Female	14:10	-	-
		Female	10:14	-	-
		Male	14:10	**	12:28± 2:07
		Male	10:14	**	04:05± 1:93
Gonadal Steroids	*star*	Female	14:10	*	02:35± 4:53
		Female	10:14	-	-
		Male	14:10	**	17:05± 1:27
		Male	10:14	**	16:27± 1:10
	cyp11a	Female	14:10	*	23:26± 5:23
		Female	10:14	-	-
		Male	14:10	-	-
		Male	10:14	*	23:20± 4:06
	cyp17	Female	14:10	**	05:15± 1:49
		Female	10:14	-	-
		Male	14:10	*	04:09± 4:00
		Male	10:14	*	02:35± 2:00
	cyp19a1a	Female	14:10	**	22:33± 2:31
		Female	10:14	-	-
	amh	Male	14:10	**	15:25± 3:00
		Male	10:14	-	-

Figure 1.

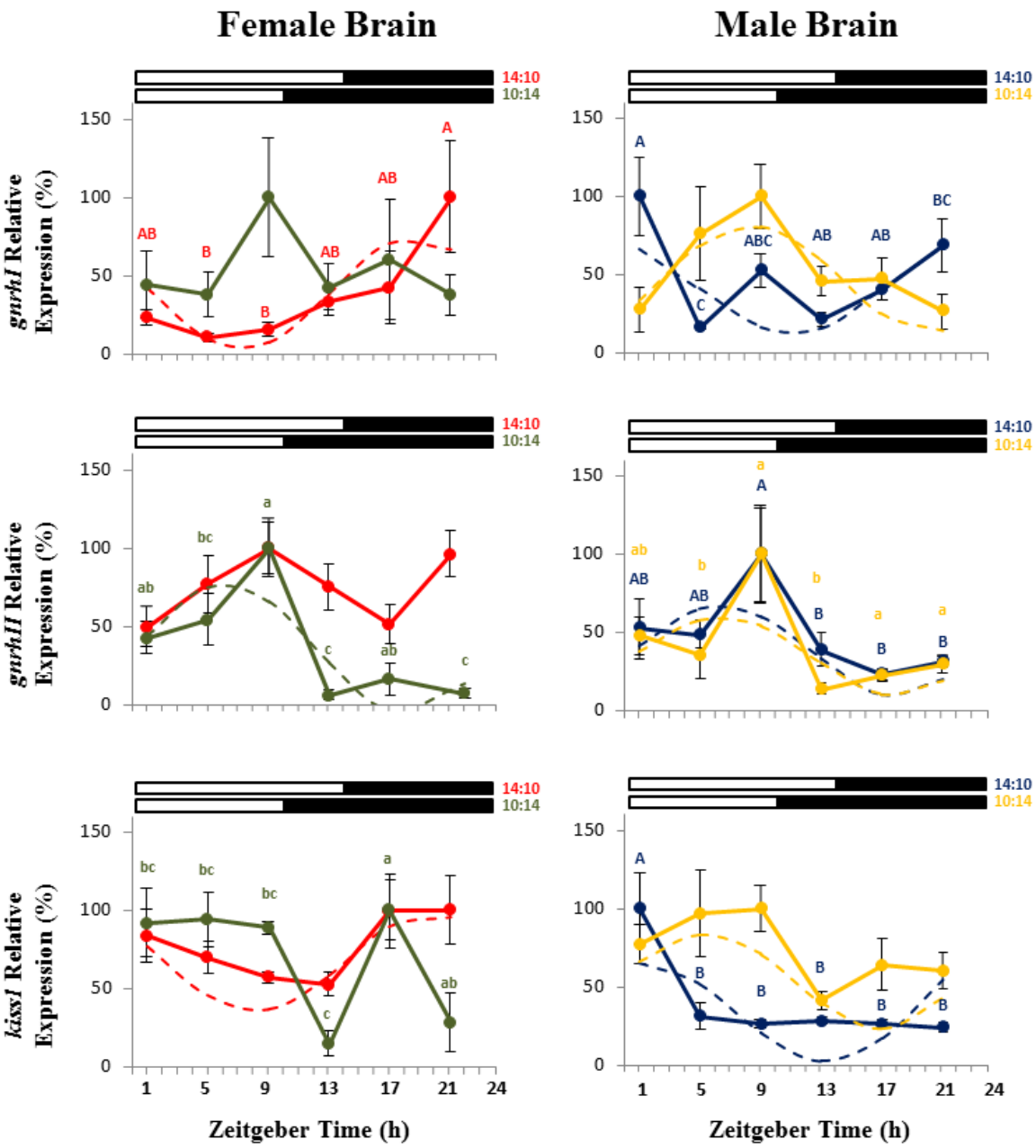

Female Brain
Male Brain
14:10
10:14
gnrh1 Relative Expression (%)
gnrh2 Relative Expression (%)
kiss1 Relative Expression (%)
150
100
50
0
AB
B
AB
B
A
AB
A
C
ABC
AB
AB
BC
ab
bc
a
c
ab
c
ab
AB
b
A
B
b
B
a
a
B
bc
bc
bc
a
c
ab
A
B
B
B
B
B
1
5
9
13
17
21
24
Zeitgeber Time (h)
Zeitgeber Time (h)

Figure 2.

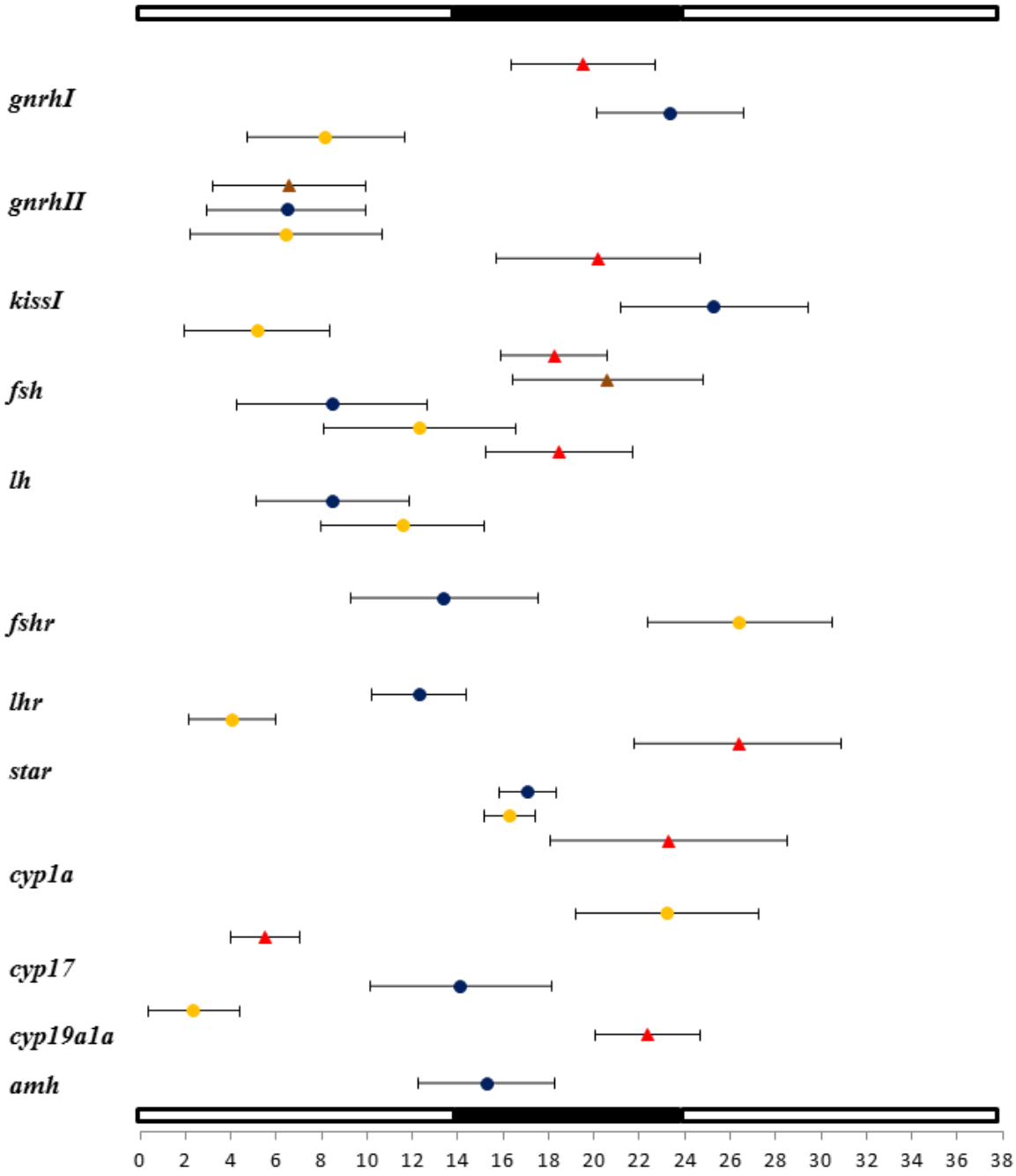

gnrhI
gnrhII
kissI
fsh
lh
fshr
lhr
star
cyp1a
cyp17
cyp19a1a
amh
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38

Figure 3.

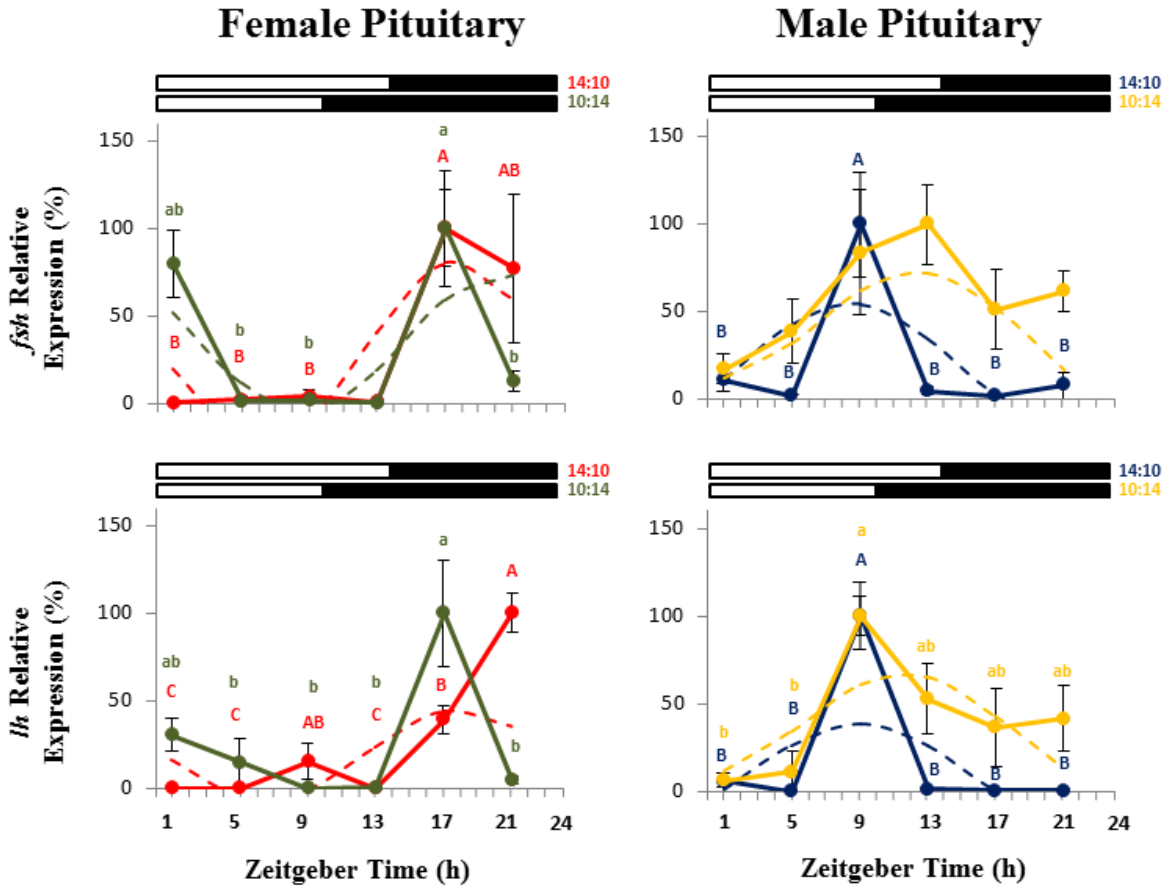

Female Pituitary
Male Pituitary
14:10
10:14
fsh Relative Expression (%)
lh Relative Expression (%)
Zeitgeber Time (h)
0
50
100
150
1
5
9
13
17
21
24

Figure 4.

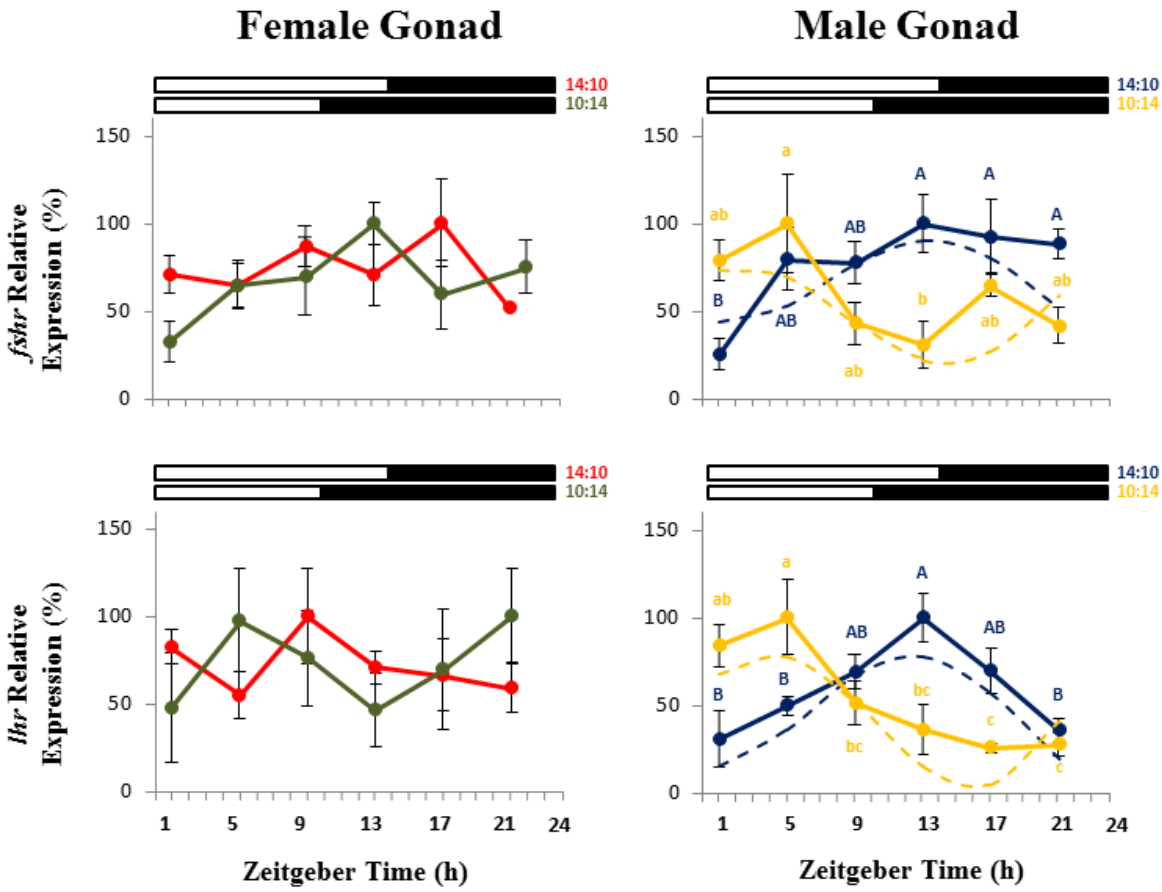
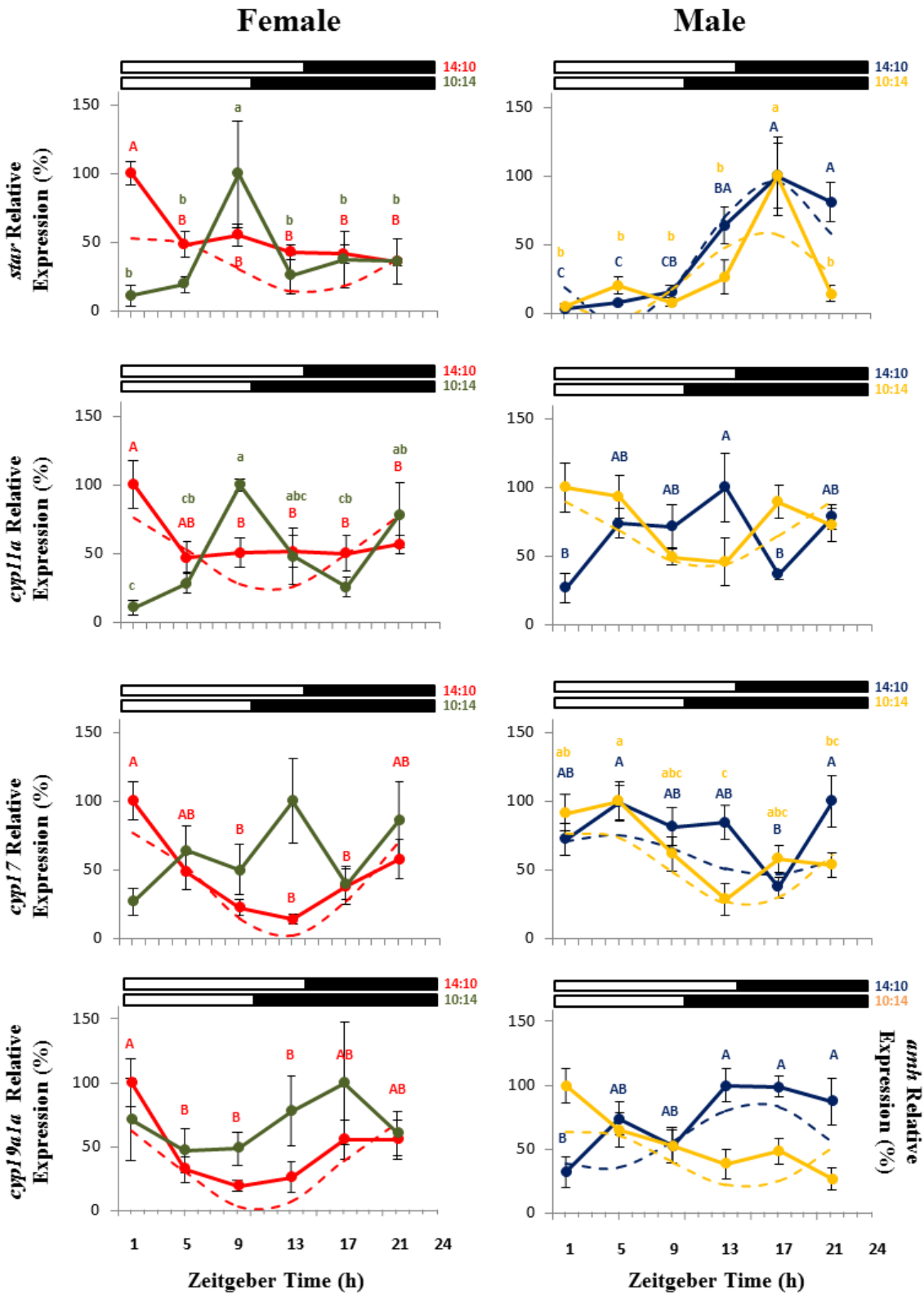

Female Gonad
Male Gonad
14:10
10:14
fshr Relative Expression (%)
lhr Relative Expression (%)
150
100
50
0
1
5
9
13
17
21
24
Zeitgeber Time (h)
a
ab
b
ab
ab
A
A
A
AB
AB
B
a
ab
A
AB
AB
B
B
bc
bc
c
c

Figure 5.


Female
Male
14:10
10:14
star Relative Expression (%)
cyp11a Relative Expression (%)
cyp17 Relative Expression (%)
cyp19a1a Relative Expression (%)
amh Relative Expression (%)
Zeitgeber Time (h)
Zeitgeber Time (h)

Experimental Chapter III

Daily Rhythms of *In Vitro* Fertilization in Fish are Driven by Oocyte Rhythmicity

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Manuscript

Introduction

Cyclic environmental changes (time-givers or "*zeitgebers"*) such as light-dark oscillations, temperature variations or food availability, have fostered organisms to develop a wide range of rhythmic adaptive strategies. Thus, reproductive rhythms occur at specific times of the day, moon phase or season increasing offspring survival [1] (De Coursey et al., 2004). Rhythms in the fish brain-pituitary-gonad axis (BPG) trigger the activation of all the neuroendocrine machinery in response to environmental and ecological oscillations (diurnal, annual and lunar) [2,3] (Cowan et al., 2017; Paredes et al., 2018). This promotes a harmonious progression of the reproductive rhythm in both sexes: from gametogenesis to spawning, leading to egg fertilization [4–6] (Falcón et al., 2007, 2010; Weltzien et al., 2003). There are species with a seasonal fertility timetable: short gestational animals (e.g., hamsters and small mammals) begin in spring [7] (Reiter, 1980), while long gestational ones (e.g., ruminants) do it late in summer [8] (Lincoln, 2002). In addition, several observations have described the existence of spawning rhythms related to the daily activity pattern of the species. For instance, zebrafish is a diurnal species which spawns after dawn [10] (Blanco-Vives and Sánchez-Vázquez, 2009). Senegal sole and flounder, however, are nocturnal flatfishes which spawn at night [11] (Oliveira et al., 2009)[12] (Nichols, 1989), while gilthead seabream spawn around sunset [13] (Velázquez et al., 2004). Since gametogenesis, maturation, ovulation, spermiation and spawning display species-specific rhythmicity [14] (Mañanós 2008), it seems reasonable to assume that such oscillations would strongly influence artificial reproductive technologies such as assisted fertilization trials.

Zebrafish (*Danio rerio*) is a widely used model for biomedical research in vertebrates [15] (Fishman, 2001) and its unique characteristics (high fertility rate, short reproductive cycle and embryo transparency) have made zebrafish a popular specie in reproductive biology [15] (Fishman, 2001). In the medical field, fertility investigations have contributed with impactful discoveries in assisted reproductive technologies (*in vitro* fertilization, IVF), endocrine dysfunction medications and hormonal treatment techniques [16–18] (Hoo et al., 2016; Homburg et al., 1990; Guzick et al., 1999). Unfortunately, infertility problems still persist worldwide due to poor oocyte/sperm qualities/quantities, abnormal ovulation, hormonal imbalance and/or detrimental environmental conditions [19–27] (Saleh et al., 2003; Parikh et al., 1997; Laven et al., 2002; Jasper et al., 2006; Chidrawar et al., 2011; Lynch et al., 20014;

Snijder et al., 2012; Zafar et al., 2015; Al-Griw et al., 2015) (141--149). The alarming amount of 20% of couples is diagnosed infertile [28] (Turchi et al., 2015). Whether the detrimental qualities come from the oocyte or from the sperm is difficult to diagnose because both cells express an immense array of biological properties to achieve fertilization [16,29] (Davis et al., 2015; hoo et al., 2016). In the cattle livestock production, infertility is also one of the major bottlenecks affecting farm profitability due to unaccomplished breeding processes, low calving rates and reduced milk yielding [15] (Fitsum, 2017). All farm fish also undergo some degree of reproductive dysfunctions varying from total absence of spawning to significant reductions of quantity/quality of eggs and sperm [14] (Mañanós, 2008). The complexity of factors influencing the reproductive cycle to achieve good quality gametes is still unknown for most species and even if they were not, it would be difficult to apply them all for culture farming conditions [30] (Brooks et al., 1997). However, the most factors we control, the less problems our breeders will exhibit. In any case, when reproductive problems persist, hormonal treatments and assisted reproduction become useful [15] (Fitsum, 2017).

The success of the reproductive cycle relies upon the harmonious neuroendocrine progression from immature germ cells to the production of good quality gametes with the final purpose of obtaining viable fertilized eggs [14] (Mañanós 2008). Nowadays there is not a clear cut definition determining gamete quality [30] (Brooks et al., 1997). However, broodstock diet/endocrine status, nutrient oocyte deposition, parental gene heritage and oocyte *zona pellucida* (ZP) distribution are some of the major contributory factors defining gamete quality [30] (Brooks et al., 1997). In view of the great importance of zebrafish for the development of reproductive sciences plus the close similarity in the reproductive regulation to most vertebrates, in this paper we have focused on investigating daily *in vitro* fertilization rhythms, gamete importance, oocyte daily phase variations and oocyte differential expresses genes during a day period. This data may be of great importance to improve assisted reproduction protocols in vertebrates.

Results

Daily Rhythms of *In Vitro* Fertilization (IVF)

To determine whether *in vitro* fertilization presented a daily rhythmic pattern we performed IVF trials at different *Zeitgeber* times (ZT) (ZT0, ZT1, ZT2, ZT3, ZT4, ZT7, ZT15,

ZT21 and ZT23) during a 24 h cycle under light: dark (14L:10D) conditions. We showed for the first time that IVF in zebrafish displays a daily rhythm synchronized to the LD cycle (Figure 1). The highest fertilization rates displayed around the first hours of the light phase (ZT23:10 h) and diminished along the day. To reveal significant differences between IVF times, we examined our data using an ANOVA (p <0.05). The cosinor analysis revealed the existence of a statistically significant daily IVF rhythm (p <0.05).

In some species, daily reproductive rhythms persist in absence of an external synchronizer disclosing the existence of an endogenous control [32–36] (Sharp et al., 1984; Silver, 1986; Ball, 2007; Nakao et al., 2007; Underwood et al., 1997). To investigate whether such an endogenous control persist for the IVF in the zebrafish we performed fertilization trials in conditions of constant darkness (DD) at different hours of a subjective day (Circadian time, CT) (CT1, CT2, CT3, CT4 and CT7). Our results revealed that under DD conditions the IVF rates still displayed a daily rhythm with the highest fertilization values during the first hours of the subjective day (CT00:10h) (Circadian time, CT) diminishing toward the end of the day (Figure 1). This revealed the existence of an endogenous control of the rhythm. Again the cosinor (p <0.05) analysis confirmed the results with a statistically significant rhythm. In DD conditions, sampling times showed no significant differences (ANOVA p >0.05). The rhythm in DD conditions is delayed respect to the LD conditions, probably indicating that an endogenous clock is taking over the rhythm, thus showing its true endogenous nature different from the cyclic LD synchronization.

Gamete Daily Phase Determines *In Vitro* Fertilization Success

Qualities that make an oocyte or a spermatozoon to be optimal for fertilization are questions long studied but still unsolved [30–36] (Bobe and Labbé 2010, 2015; Zarski et al., 2012, 2017; Migaud et al., 2013; Brooks et al., 1997; Özlem et al., 2007). In order to investigate the role of the gametes daily phase regarding IVF rates (oocyte *vs*. spermatozoon, as limiting factors), we fertilized gametes from the first hour of the light phase (ZT1) with gametes from the first hour of the dark phase (ZT15) at the same time. On the one hand, results revealed that oocytes from ZT1 independently fertilized with sperm from ZT1 and ZT15 presented fertilization rates of (98 ± 0.9) and (95.8 ± 1.4) percent, respectively. On the other hand, oocytes from ZT15 were neither capable of being fertilized by sperm from ZT1 nor by sperm from ZT15 (Figure 2). Thus, these results revealed that the limiting factor for

the success of IVF in zebrafish is the oocyte daily phase. Our results confirmed that daily spawners present oocyte quality variations during a day cycle resulting in daily variations in the IVF rates (Supplementary Figure 1).

Functional Assessment of *Zona Pellucida* Solubility and Oviduct-specific glycoprotein (*ovpg1*) gene expression

In mammals, *zona pellucida* (ZP) hardens the oocyte structure conferring resistance to pronase digestion, preventing from polyspermy, promoting oocyte-sperm interaction and warranting normal embryo development [37] (Coy et al., 2008). To investigate whether ZP undergoes daily changes that affect the egg structure we submitted zebrafish oocytes from different hours of the day (ZT1, ZT7, ZT15 and ZT21) to pronase solution. Digestion time with pronase was registered using a recording system, as the time from the beginning of the exposure until the oocyte inner content departed from the chorion. Our results revealed that ZP resistance displayed a daily expression rhythm (cosinor, $p < 0.05$) with a maximum digestion time at the first hour of the light phase (ZT1=72.93 ± 2.17 min), diminishing toward the night and again increasing toward the end of the dark phase (ZT21=34.72 ± 7.07 min) (Figure 3a). Sampling times also presented significant differences (ANOVA, $p < 0.05$).

In mammals, the ovpg1 participates in the functional modification of the ZP providing more resistance to enzymatic digestion and to sperm penetration, contributing to the control of polyspermy [38] (Coy et al., 2008). To investigate whether gonad *ovpg1* gene expression changes during a 24h cycle altering the oocyte resistance, we examined the gene pattern at different hours during a day cycle (ZT2, ZT7, ZT12, ZT16, ZT19 and ZT22). *Ovpg1* gonad gene expression displayed a daily rhythmic pattern with a maximum expression peak during the first hours of the light phase (cosinor, $p < 0.05$) (ZT6:11h) (Figure 3b). Sampling times also presented significant differences (ANOVA, $p < 0.05$).

Oocyte Daily Expression of *Zona Pellucida* (*zp2*) and Calcium Wave (*fyn*) Genes

ZP layer is solely synthesized by growing oocytes and consists of glycoproteins that assemble into a matrix around the oocyte plasma membrane [39] (Wassarman et al., 2008). The fusion between the oocyte and the spermatozoon produces a calcium wave reaction that surrounds the egg and marks the activation of embryo development [40,41] (Sharma and Kinsey, 2012; Heindryckx et al., 2013). Prompted by the IVF rates and the different pronase

digestion times on the results above we hypothesize that those variations may be due to *zp2* and *fyn* genes daily oscillations. To investigate about the possible existence of a daily rhythmic expression of the zona pellucida (*zp2*) and calcium wave (*fyn*) genes we performed quantitative real time PCR at different hours during a 24 h cycle (ZT0, ZT1, ZT2, ZT3, ZT4, ZT7, ZT15, ZT21 and ZT23). The *zp2* and *fyn* genes statistically showed daily expression pattern (cosinor, $p < 0.05$) with an acrophase at the first hour of the dark phase (ZT14:08h) for *zp2* and at the first hour of the light phase (ZT01:24h) for *fyn*. *Zeitgeber* times presented significant differences (ANOVA, $p < 0.05$) (Figure 4).

Discussion

Daily Rhythms of *In Vitro* Fertilization (IVF)

Natural spawning time in zebrafish occurs at the first hours of the light phase triggered by environmental cues (*zeitgebers*) as photoperiod (Light: Dark cycle, LD) [10] (Blanco-Vives and Sánchez-Vázquez, 2009). This spawning time is related to the locomotor activity pattern of the specie so that, diurnal species spawn during the light phase and nocturnal ones spawn during the dark phase [10]. Such a correlation has also been described alike in Senegal sole, flounder and gilthead seabream [11–13] (Oliveira et al., 2009; Nichols, 1989; Velázquez et al., 2004). Despite these clear spawning timetables, the influence of the time of day for *in vitro* fertilization trials has not been fully investigated. Understanding the synchronous spawning and spermiation timetable which ensures offspring survival is therefore important to optimize IVF protocols. Here, we have shown that the highest IVF rates displayed around the first hours of the light phase and diminished along the day. Our results match with those described by Blanco-Vives and Sánchez-Vázquez (2009) who showed that zebrafish possessed a naturally spawning window around the first hours of the light phase. In addition, previous studies have shown that spawning times are most probably driven by specie-specific rhythms in the BPG axis that simultaneously answer to environmental cues [42] (Boden and Kennaway, 2006). So, one possibility is that the harmonious progression of the BPG axis fosters the display of both spawning/spermiation and IVF rhythms. Rhythmicity and increments in specific sexual steroids in the BPG axis have well been described to anticipate hours previous to spawning/spermiation [2] (Cowan et al., 2017). Therefore, prompted by this information we suggest that the IVF timetable may also be determined by the BPG axis daily rhythm.

In some species, daily reproductive rhythms persist in absence of an external synchronizer disclosing the existence of an endogenous control [32–36] (Sharp et al., 1984; Silver, 1986; Ball, 2007; Nakao et al., 2007; Underwood et al., 1997). Here, we have shown that IVF rates still displayed a daily rhythm in conditions of complete darkness (DD) with the highest fertilization values around the first hours of the day thus revealing the existence of an endogenous clock. The existence of such a clock has also been described in the domestic hens (*Gallus domesticus*) [43,44] (Sharp et al., 1984; Silver, 1986) and in the Japanese quail (*Coturnix Japonica*) whose oviposition-ovulation persisted when submitted to conditions of complete darkness (DD).

Gamete Daily Phase Determines *In Vitro* Fertilization Success

One of the biggest obstacles in assisted reproduction is determining gamete quality which is defined as the ability to fertilize or to be fertilized and subsequently develop into a viable embryo [32] (Bobe and Labbé 2010). Here we have shown that the oocyte daily phase determines IVF success rates in the zebrafish. We acknowledge that the quality of both female and male gametes are important, but this especially comes true for the female gamete which displays several features that make its production and availability more difficult to control and optimize than the male gamete [40] (Migaud et al., 2013). Seasonal rhythms in fish reproduction is a phenomenon well described at the neuroendocrine, maturational, ovulation/spermiation and spawning level [2] (Cowan et al., 2017). In addition, seasonal spawners present variations in egg production during the season (Kjesbu et al., 1996). In the same way, our results confirmed that daily spawners also presented oocyte quality variations during a day cycle (Supplementary Figure 1) resulting in daily variations in the IVF rates. We suggest that those oocyte quality variations become limiting factors for the success of fertilization at the dark phase of the daily cycle (ZT15). Thus we consider the necessity of paying attention regarding daily cycle when dealing with assisted reproduction.

Functional Assessment of *Zona Pellucida* (ZP) Solubility and Oviduct-specific Glycoprotein (*ovpg1*) Gene Expression.

Identifying a predictive estimator of good quality oocyte is crucial for major applications in research and industry. The hardening characteristic conferred by the ZP warrants oocyte resistance to pronase digestion and normal embryo development [37] (Coy et

al., 2008). Interestingly, in our results the digestion times parallel IVF rates previously described above (Supplementary Figure 2). The maximum digestion time coincides with the highest fertilization rate (ZT1); similarly the lowest digestion times also matched with the minimum fertilization rates (ZT7 & ZT15). Thus one possibility could be that daily variations in the ZP digestion time may imply changes in the ZP structure that consequently influence IVF rates. Additionally, changes in the ZP resistance due to structural modifications performed during the oocyte transit along the oviduct have been demonstrated to increase resistance to enzymatic digestion and to sperm penetration [37] (Coy et al., 2008). Accordingly, in our results *ovpg1* daily gene expression also presented high values at the first hours of the light phase, decrease along the day and increased at the end the night (Figure 3b). To which degree the ZP and *ovpg1* are determining oocyte quality is unknown. Currently there are not effective predictive markers of oocyte quality apart from the extremely low quality markers as buoyance and appearance [32] (Bobe and Labbé 2010). We suggest that assisted reproduction techniques should consider daily/seasonal variations in oocyte resistance for optimizing assisted reproduction protocols.

Oocyte Daily Expression of *Zona Pellucida* (*zp2*) and Calcium Wave Genes (*fyn*)

ZP layer is solely synthesized by growing oocytes [39] (Wassarman et al., 2008) and *fyn* gene is involved in the calcium wave that marks the starting point of development of the embryo after fertilization [45] (Swann et al., 1994). Therefore, it is logical to think that daily oscillations in the *zp2* and *fyn* gene expression may cause variations in the IVF rates and egg quality. The *zp2* gene expression is tissue-specific (ovary) and temporally confined to the earliest phases of developing oocytes [46] (Mold et al., 2009). Additionally, we know that zebrafish produces oocytes in a daily manner thus, we suggest that this temporal restriction could explain that the maximum gene expression peak in our results also displayed only at one specific time during the 24-h cycle (ZT14:08h). The *zp2* acrophase appeared 11 hours before maximum natural spawning, maximum IVF success rates and maximum digestion time, probably it is the time needed to confer the oocyte optimal qualities for the success of fertilization. In addition, these results revealed that the *zp2* gene expression also appeared in the oocyte, most probably in response to zebrafish reproductive strategy, dependent on daily production of large number of eggs, thus the zebrafish *zp2* gene copy could not be limited to the ovary but expanded to the oocyte. Zebrafish oocyte cortex is highly specialized to produce

a rapid and high intensity calcium wave in the cortex [45] (Sharma et al., 2008). Our interpretation of maximum *fyn* gene expression at ZT01:24h is that the zebrafish oocyte calcium wave requires Fyn kinase activity to allow maximal calcium release from the cortical endoplasmic reticulum. Thus the concentration of Fyn kinase at the animal pole at the first hour of the light phase is critical to amplify the initial signal from the oocyte-spermatozoon fusion that temporally matches the maximum fertilization window.

Concluding Remarks

The *in vitro* fertilization presents a daily rhythmic pattern that is driven by the oocyte daily phase. The functional test (pronase digestion time) revealed to be an efficient predictive estimator of good quality oocyte. The differential gene expression displayed key family genes involved in the success of IVF.

Experimental Procedures

Fish Maintenance

Wild-type zebrafish (*Danio rerio*, age ~ 2 months) (N=1808) of 0.30 ± 0.10 g (mean±SD) of body weight were obtained from a local provider (Alimar S.A., Murcia, Spain). Fish were equally distributed and reared in 9-L glass aquaria at the chronobiology laboratory (a completely isolated room where light and temperature are strictly controlled) of the University of Murcia (Spain). Photoperiod was set at a 14h:10h light:dark (LD) cycle, with the time of lights on designated as *Zeitgeber* Time 0h (ZT0). Light was provided by LED stripes (SOLBRIGHT®, LED Flex Strip 1043-W, Rayte, S.L., Murcia, Spain), with a light intensity at the water surface of 0.84 $W \cdot m^{-2}$ (200 lx)). Water temperature was held constant at 28 ± 0.5ºC throughout the acclimation and experimental period with a water heater (200 W Magictherm, Prodac, Italy). Fish were fed with commercial feed (Tropical fish flakes, Casone, Parma, Italy) and supplementary artemia pellets (Prodac Artemia, Italy). To deliver food an automatic feeder (Eheim GmbH & Co. KG, model 3581, Deizisau, Germany) was place in each tank. The feeder delivered food three times a day (ZT2, ZT6 and ZT10) at 1.5% of the fish body weight per time. Supplementary artemia pellets were given to fish of each tank at ZT4 and ZT9 *ad libitum* every day. After three months under these conditions, reproductive maturity was assessed by natural mating: females were verified to naturally spawn and males to

successfully fertilize the oocyte. Six hours after natural spawning, embryos were confirmed fertilized.

Ethics Statement

All experimental procedures, rearing and fish manipulation followed the Spanish Legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals and were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

Experiment 1: Daily Rhythms of *In Vitro* Fertilization

Experiment 1 was designed to describe *in vitr*o fertilization rhythms under light: dark (14L:10D) conditions and to unveil the existence of an endogenous control in constant darkness (DD). For this porpoise, *in vitro* fertilization procedures were performed according to chapter two of the Zebrafish Book (Walker and Streisinger, 2007). Trials were performed in triplicates for each fertilization time (ZT0, ZT1, ZT2, ZT3, ZT4, ZT7, ZT15, ZT21 and ZT23) and (CT1, CT2, CT3, CT4 and CT7). For each trial, *in vitro* fertilization was performed in a 12-multi-well plate (FALCON®, Multiwell Plate, Thomas Scientific, Swedesboro, NJ, USA). For every each three wells, a pool of eggs was obtained from two mature female zebrafish. Then, 50 eggs were placed in each of the three wells and immediately activated with 1ml water-tank. Each single well was fertilized with a sperm pool of two male zebrafish. Same procedure was performed for each set of three wells of the 12-multi-well plate. After fertilization, the multi-well plate was held in constant water temperature (28 ± 0.5ºC). One hour later, *in vitro* fertilization was assessed with a magnifying glass (Leica®, EZ4 HD, Leica Microsystems, Wetzlar, Germany) according to Kimmel, (1995). In total, for one trial, 8 female and 24 male zebrafish were used per each multi-well plate. *In vitro* fertilization trials during the dark phase was performed under a dim red light (λ>600nm).

Experiment 2: Gamete Daily Phase Determines *In Vitro* Fertilization Success

Experiment 2 was designed to determine the role of the gamete (oocyte and spermatozoon) daily phase for *in vitro* fertilization success rates. To this end, two chronobiology laboratories (a light-tight isolated room with a strictly controlled environment) at the Faculty of Biology,

University of Murcia (Spain) were set with opposite phase starting daily cycles. One lab with a daily cycle of Light: Darkness (14L:10D) and a second lab with a daily cycle of darkness and light (10D:14L). Thus, gametes (oocytes and spermatozoa) from LD-lab would be at ZT1 and gametes from DL-lab at ZT15 at the same sampling time. *In vitro* fertilization trials were performed as in experiment 1 in duplicates.

Experiment 3: Functional Assessment of *Zona Pellucida* Solubility

Experiment 3 was designed to determine the oocyte resistance to enzymatic pronase digestion at different times of the day (ZT1, ZT7, ZT15 and ZT21). For this porpoise, oocytes from four females were pooled; 15 activated oocytes were transferred into PBS and place into 70 µl of 0.5% (wt/vol) pronase solution (28 ± 0.5° C). Oocytes were continuously observed under a Leica® recording system. The dissolution time of the *zona* of each oocyte was register as the time between placement of samples in the pronase and the time at which the inner content depart from the chorion. This time was referred to as "ZP Digestion Time". Trials were run in duplicates.

Experiment 4: Daily Expression of *Zona Pellucida* and Calcium Wave Genes

Experiment 4 was designed to describe relative gene expression at different hours during a day (ZT0, ZT1, ZT2, ZT3, ZT4, ZT7, ZT15, ZT21 and ZT23). To this end, the qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA). All the samples were run in duplicates. Primers of each gene were tested to verify its efficiency by means of a standard curve. Elongation factor 1 alpha (*ef1α*) was selected as housekeeping gene after assessing its coefficient of variation (CV) within each tissue lower than 5%. Each PCR well had a final 20 µl volume: 5 µl of cDNA, 10 µl of the qPCR Master Mix and 5 µl of each forward and reverse specific primer concentration (Table 1). The thermal cycling conditions were as follows: holding stage of polymerase activation (10 min at 95°C); cycling stage (40 cycles of 95°C for 15sec and 60°C for 1min). The specificity of the reaction was validated by analysis of the melting curve. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

DECLARATION OF INTEREST

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Figure legends

Figure 1. Daily rhythms of *in vitro* fertilization in percentage under light/dark (LD) and complete darkness (DD) conditions. Open and full bars on the top indicate the light and dark periods, respectively. Black and grey bars on the top stand for conditions of complete darkness. Statistical differences between sampling points are indicated by lowercase letters on the top of the sampling time.

Figure 2. Percentage of *in vitro* fertilization depending on gamete daily phase.

Figure 3. a. Digestion time at different hours of the day. Open and full bars on the top indicate the light and dark periods, respectively. Statistical differences between sampling points are indicated by lowercase letters on the top of the sampling time. **b.** Daily expression rhythm of *ovpg1* gene. Statistical differences between sampling points are indicated by lowercase letters on the top of the sampling time. The sinusoidal line represents the adjustment to a sinusoidal rhythm calculated by Cosinor analysis ($p<0.05$). Lowercase letters indicate statistically significant differences among different sampling points gene (one-way ANOVA, $p<0.05$).

Figure 4. Daily expression rhythm of *ZP* and *fyn* gene.

Supplementary figure 1. Morphological oocyte variation.

Supplementary figure 2. Comparison between *in vitro* fertilization and digestion time.

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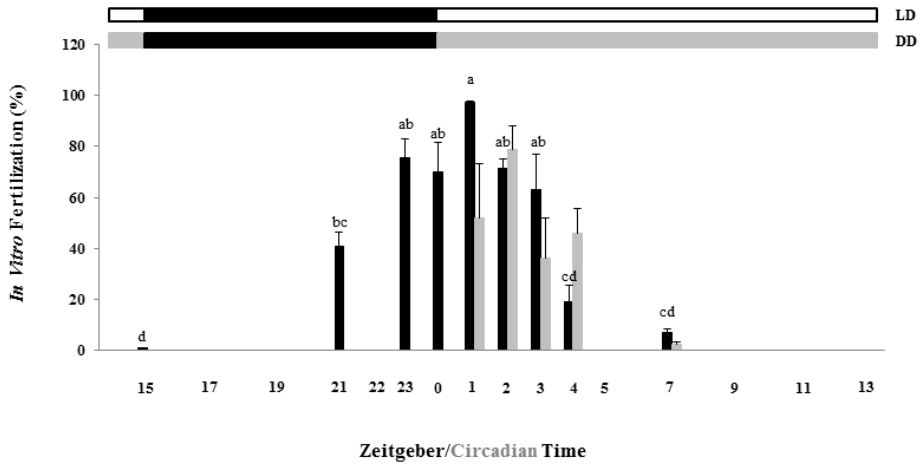
Figure 1


LD
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In Vitro Fertilization (%)
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11
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Zeitgeber/Circadian Time

Figure 2

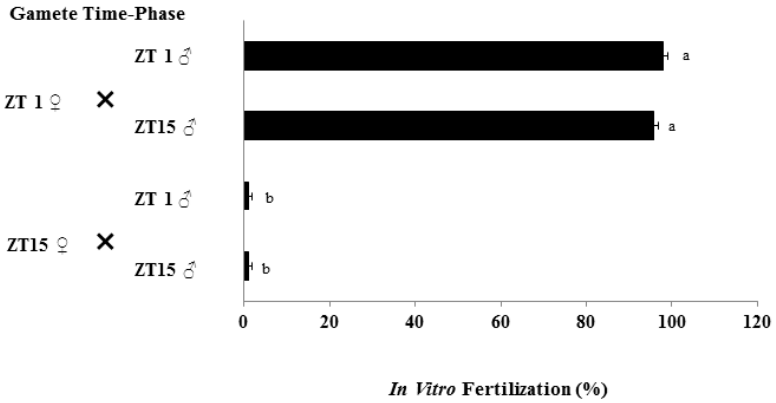


Figure 3a

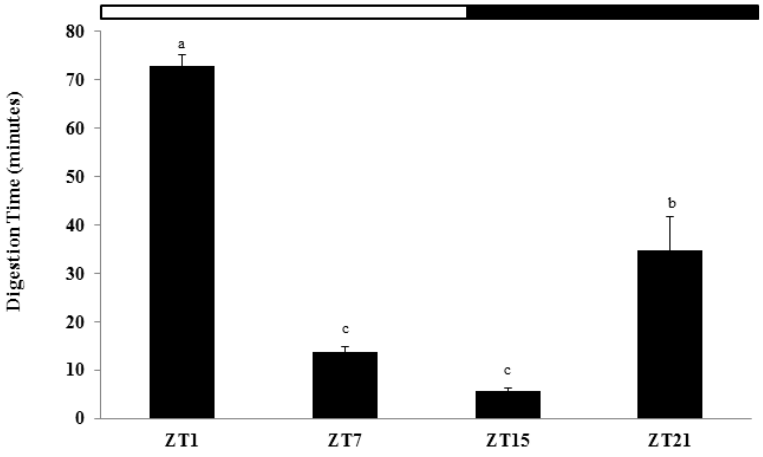
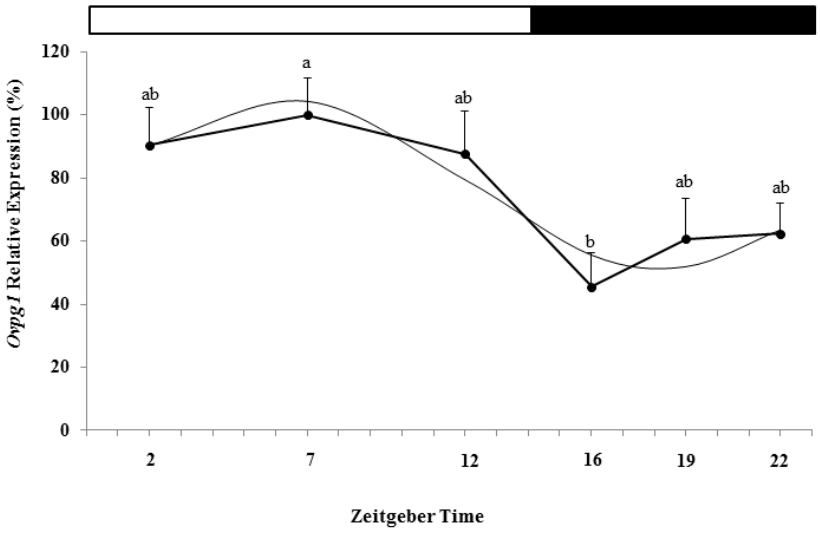


Figure 3b


120
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Oygg1 Relative Expression (%)
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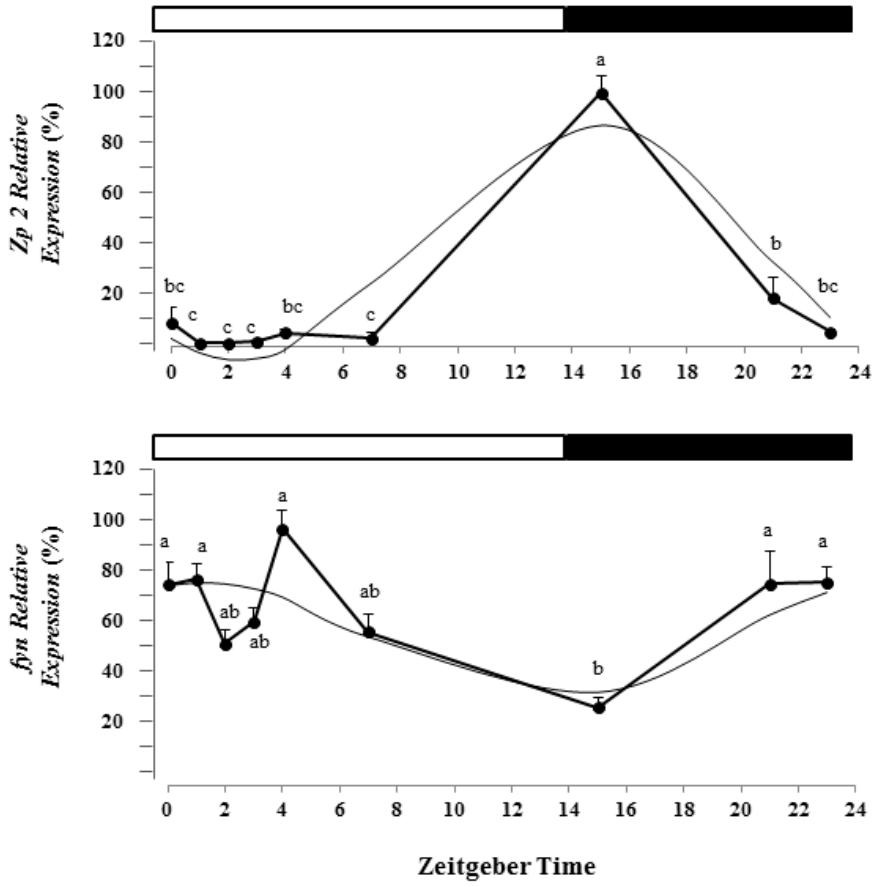
Zeitgeber Time

Figure 4


Zp 2 Relative Expression (%)
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fyn Relative Expression (%)
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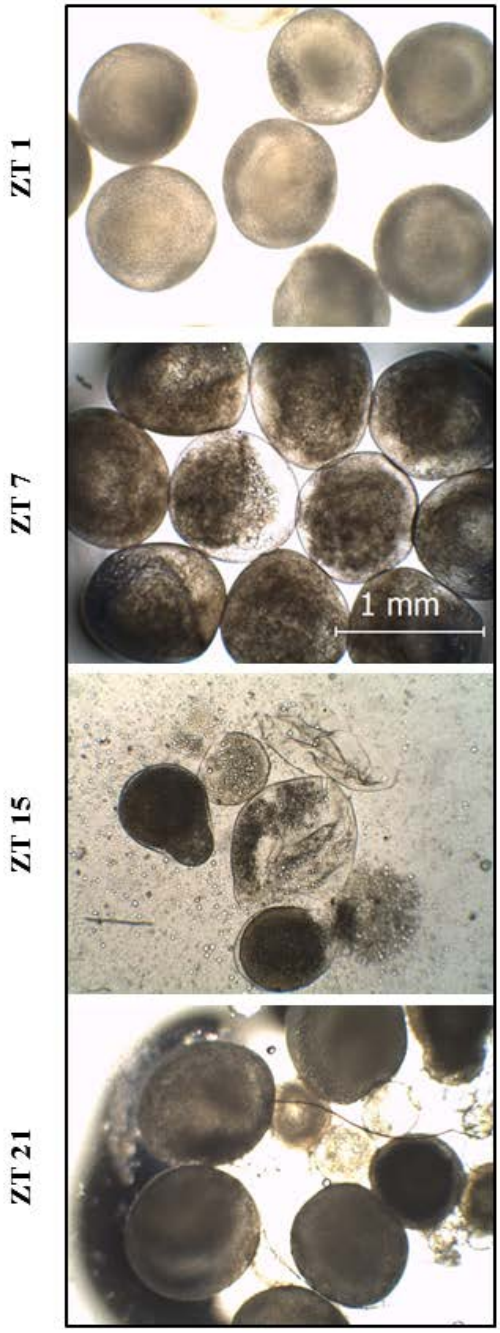
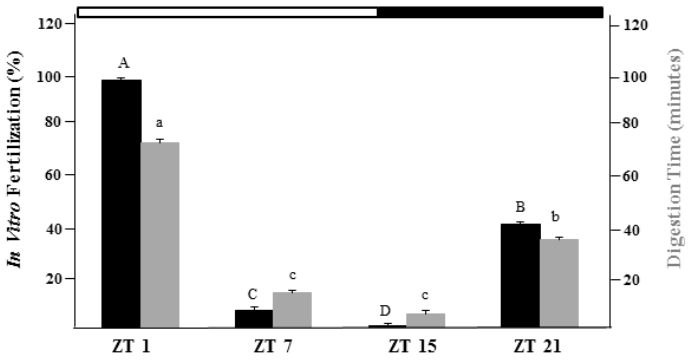
Zeitgeber Time

Supplementary Figure 1



Supplementary Figure 2



Experimental Chapter IV

Circadian expression of DNA methylation and demethylation genes in zebrafish gonads

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Summery

The purpose of this chapter was to investigate daily expression rhythms of key genes involved in epigenetic mechanisms of DNA methylation and demethylation in zebrafish gonads. The results revealed that most of the epigenetic genes investigated exhibited a well-defined daily rhythm. Both DNA methylation and demethylation genes presented a nocturnal peak in female and male gonads. In addition, DNA methylation and demethylation gene rhythms persisted in conditions of complete darkness. These findings suggest that the epigenetic mechanisms of DNA methylation and demethylation in the gonads obeys to an endogenous clock, that functions as a player of the translation network bridging the environmental cues into somatic responses in fish gonads

PMID: 29509082

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Experimental Chapter V

Circadian rhythms of gene expression of lipid metabolism in Gilthead Sea bream liver: synchronization to light and feeding time

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Summery

The purpose of this chapter was to evaluate daily rhythms in lipid metabolic gene expression in response to light-dark and feeding cycles in gilthead seabream liver. We aimed to describe the daily pattern of lipogenic and lipolytic genes and their synchronisation to different light and feeding cycles. The outcome of our investigation unveiled that all genes exhibited well defined daily rhythms. Lipolysis genes presented nocturnal maximum expression peak. Contrasting, lipogenesis genes showed a diurnal rhythm. These findings point out that lipid utilisation in the liver follows a rhythmic pattern, separated in time for lipogenic/lipolytic processes and that is strongly synchronised to the LD cycle regardless feeding time

DOI: 10.3109/07420528.2014.881837

Experimental Chapter VI

Daily rhythms of lipid metabolic gene expression in zebrafish liver: Response to light/dark and feeding cycles

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Publish in "Chronobiology International" (2015).

Summery

The purpose of chapter six was to determine daily rhythms in lipid related genes in response to light-dark and feeding cycles in a fish model as zebrafish. We aimed to reveal lipogenesis and lipolysis daily behavior in zebrafish liver. Our results showed that most of the genes investigated displayed a significant daily rhythm with a maximum expression peak during the dark phase. The time of feeding scarcely affected daily expression rhythm. Our results revealed that lipid related genes are synchronized to the LD cycle.

PMID: 26595085

DOI: 10.31109/07420528.2015.1104327

Experimental Chapter VII

(Book chapter)

Effects of light, temperature and feeding cycles during early development

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Published in The Biology of Sole (2019). CRC Press Taylor & Francis Group. "Effects of light and temperature cycles during early development". Pp: 253-262.

Summery

The purpose of chapter seven was to review the state of art knowledge regarding the influence of light and temperature cycles on growth, development, sexual maturation, reproduction and hatching times in *Solea senegalensis.* In this chapter we summarized the effects of light spectrum and photoperiod on growth, yolk sac absorption, jaw malformation, eye migration and completed metamorphosis. We also described the effect of temperature cycles on growth, jaw malformation, yolk sac absorption, sex ratio and hatching rhythms.

The Biology of Sole (2019). CRC Press Taylor & Francis Group. “Effects of light and temperature cycles during early development”. Pp: 253-262.

General Discussion

4. **General discussion**

The present PhD thesis reveals the existence of an environmentally synchronized harmonious progression in both sexes of all the neuroendocrine machinery controlling reproduction: from the BPG axis activation, gametogenesis, spawning to conclude with the egg fertilization. On chapter 1-2 we found rhythms in the brain-pituitary-gonadal (BPG) axis and the influence of light and temperature on activating/inhibiting this neuroendocrine axis of reproduction. On the chapter 3, we revealed for the first time *in vitro* fertilization rhythms which may help improving fertilization protocols by considering the time of day for best fertilization rates according to an up/down gene regulation of the oocyte. On chapter 4, we revealed daily rhythms in the mechanism by which environmental factors transduce into a physiological response in the gonads: epigenetic mechanisms of methylation and demethylation. On chapter 5-6, we reported the light/feeding time importance on lipid metabolism rhythms, which may act as critical factors influencing reproduction. Finally, on chapter 7, we reviewed the influence of light and temperature cycles on growth, development, sexual maturation, reproduction and hatching times.

4.1. **Reproduction rhythms**

Fish reproduction is a seasonal/daily rhythmic process that requires a precise interpretation of environmental cues (light and temperature). Hence, this information transduces into a hormonal cascade triggering, synchronizing and orchesting all reproductive activities. Thus organisms display a wide range of rhythmic adaptive strategies choosing reproduction to occur at specific time of the day and/or year with an optimal timing for mating and offspring release. It is the BPG axis that controls all reproductive processes and it is this axis the one on which the environmental factors work upon. So, when trying to understand about reproductive problems, dysfunctions or alterations, it is logical to have an insight on the BPG axis (Mañanós, 2008).

In the first chapters of the thesis, we demonstrated that environmental factors such as light and temperature activate or inhibit the neuroendocrine reproductive machinery promoting or blocking the appearance of rhythms on the BPG axis. In the zebrafish we describe a harmonious and daily rhythmic time line activation/progression of the reproductive axis. The

activation starts in the brain via the stimulatory/inhibitory signaling of the preoptic/hypothalamic gonadotropin-releasing hormone (Gnrh), kisspeptins (kiss) and gonadotropin-inhibitory hormone (Gnih); neuropeptides that integrate environmental information. Those signals arrive at the pituitary and promote the synthesis and most probably the release of the follicle-stimulating hormone (Fsh) and the luteinizing hormone (LH). Then, the FSH and LH travel to the gonads triggering steroid synthesis that together with androgens and estrogens regulates spermatogenesis, vitellogenesis, maturation, ovulation, reproductive behavior, spermiation and spawning. Thus, our results put forward the existence of a coordinated expression rhythms in key genes along the BPG axis in the zebrafish. Additionally, we also reveal that seasonal variations such as long/short photoperiod in combination with high/low temperature alter daily rhythms in key reproductive genes on the BPG axis of medaka. Most females under favorable reproductive conditions (long photoperiod and high temperature) display a daily rhythmic gene expression. However, most females under inhibitory reproductive conditions (short photoperiod and low temperature) exhibit a non-significant daily gene expression pattern or a shift in the gene phase (nocturnal/diurnal). Curiously, male show similar daily gene rhythms no matter the condition. Hence, our results remark the influence of seasonal conditions in driving the reproductive rhythms. It is possible that the loss of rhythmicity and the phase-shift indicate non-reproductive stage in medaka due to the inhibitory seasonal conditions (Cowan et al., 2017; Paredes et al., 2019).

4.2. ***In vitro*** **fertilization rhythms and oocyte transcriptome**

The success of reproduction relies on the coordinated functioning of reproductive activities. These processes culminate with the production of good quality gametes with the final purpose of fertilizing and obtaining viable eggs. In addition, considering that gametogenesis, maturation, ovulation, spermiation and spawning display with rhythmicity depending on the specie, it seems reasonable to assume that such oscillations would persist in related reproductive processes as assisted reproduction and gamete quality. However, nowadays there is neither a clear-cut timing schedule for *in vitro* fertilization practices nor for determining good quality gametes. In our results we demonstrate that the success for IVF rates depends on the time of the day. We also reveal that those rate variations correspond to the up/down oscillation of the oocyte transcript content which explains the quality of the oocyte

during a day. Those transcript contents exhibit precise up/down regulation of family genes involve in biological processes as positive regulation of reproduction, binding of sperm to z*ona pellucida*, single fertilization, sperm-egg recognition, acrosome reaction, positive regulation of fertilization or syngamy. Possibly, these functions convey the oocyte optimal requirements for being a viable egg during the first hours of the light phase (ZT23-ZT2) thus matching high IVF rates. In view of the great importance of zebrafish for the development of reproductive sciences plus the close similarity in the reproductive regulation to most vertebrates, we consider our results to be of great importance to improve assisted reproduction protocols in fish as much as in higher vertebrates (Coy et al., 2008; Mañanós, 2008).

4.3. **Rhythms in epigenetic mechanisms of methylation/demethylation in gonads**

Now, in view of the importance of the environmental factors we wonder how these external cues transduce into a physiological response. We know that environmental changes and the circadian system co-working with epigenetic mechanisms influence the endocrine reproductive system in gonads and thus ultimately affecting reproduction. However, little we know about the daily rhythmic nature of the epigenetic mechanisms in fish and how this regulation coordinates gene activation/inhibition in gonads. Methylation and demethylation are the basic mechanism working via epigenetics. In our results we describe that most methylation/demethylation genes display a daily rhythmic pattern with a maximum expression peak during the dark phase. Interestingly, this gene peak occurs during the resting phase of the fish. Similar studies in mouse also highlight that methylation activity works during the resting phase of the animal (zebrafish being diurnal and mouse nocturnal). This data probably suggests the existence of a common regulatory methylation mechanism dependent on the sleep-wake rhythm. Furthermore, most methylation/demethylation gene expressions persist with a circadian rhythmicity in conditions of constant darkness during two consecutive days. This implies the existence of an endogenous control of the epigenetic rhythms probably necessary for a continuous genome imprinting. Thus, we suggest that these methylation/demethylation mechanisms would act rhythmically upon the genome up/down regulating gene expression of particular biological processes (Paredes et al., 2018; Piferrer et al., 2005, 2012).

4.4. **Light/feeding time importance on lipid metabolic rhythms**

Feeding cycle-food availability become of great importance regarding development, sexual maturation and reproduction. Its biological implications go as far as evolutionary pressure the development of a food-entrainable oscillator (FEO) probably at the suprachiasmatic nucleus (SCN) of the hypothalamus in fish. Additionally, food availability not only marks optimal conditions for offspring release but also triggers previous reproductive processes as steroidogenesis, sexual behavior, spermatogenesis or oogenesis. Thus, in this part of the thesis we focus on understanding the implications of feeding and light cycles regarding lipid metabolism. This metabolism is the starting trigger for several reproductive responses that use lipids as basic for steroid synthesis. In our results, we highlight that lipid metabolism fallows a daily rhythmic pattern for lipogenesis and lipolysis genes. This anabolic and catabolic functions display during the light phase or during the dark phase, correspondently. Thus, suggesting that in fish liver anabolic and catabolic pathways display separately in time performing their corresponding function efficiently upon storage or fatty acid utilization. Interestingly, we also reveal that this rhythmic pattern obeys to the light-dark but not to the feeding cycle. This suggests that the brain master clock, in response to light-dark cues, regulates daily expression of lipid metabolic genes, regardless of the feeding time. Possibly, lipid metabolism includes Clock genes dependence. Clock genes synchronize to the light-dark cycle in the brain but in the liver their rhythmicity depends on the feeding time. Possibly, this suggests that Clock gene oscillations may involve certain degree of connection with lipid gene rhythms. Thus, lipid metabolism responses to light-dark environmental cues instead of obeying meal times (López-Olmeda, J. F and Sánchez-Vázquez, F.J., 2010; Paredes et al., 2014, 2015b; Vera et al., 2013).

4.5. **Influence of light and temperature cycles**

To sum up, regarding the importance of light and temperature as key factor for influencing the existence of rhythms in fish, we conclude with a chapter book reviewing the effects of these environmental cues during early development in fish. Photoperiod and light spectrum create a dynamic photo-environment constantly challenging aquatic life. Thus, interesting experiments put forward the effects of those environmental cues on growth, yolk sac absorption, jaw formation, spawning, hatching rhythms, larval development, eye migration

and completed metamorphosis. Additionally, temperature cycle by itself is strong enough influencing hatching rhythms, gene expression, behavioral activity, physiology, foraging ability, growth and sex determination/differentiation. Thus, we consider of great importance the effects of light and temperature on most physiological processes during the fish life. For this reasons, developmental biology and fish farming industry should consider it when establishing fish developmental and reproductive protocols (Blanco-Vives et al., 2010; Paredes et al., 2019; Villamizar et al., 2012).

Conclusions

5. Conclusions

1. In both female and male zebrafish, nearly all key genes involved in the BPG-liver axis display daily rhythms. Those genes follow a harmonious time-line progression during the reproductive period, which warrants the spawning window and fertilization occur coinciding with favorable conditions for maximum offspring survival.

2. In medaka, seasonal variations (long/short photoperiod and high/low temperature) altered daily rhythms in key reproductive genes in the BPG axis. Most females under long photoperiod/high temperature (summer conditions) exhibited a daily rhythmic gene pattern, while those under short photoperiod/low temperature (winter conditions) failed to present significant rhythms.

3. *In vitro* fertilization displays a strong daily rhythm in zebrafish determined by the oocyte daily phase. Maximum fertilization rates occur around lights on, and circadian rhythmicity persisted under constant darkness. The transcriptome oocyte analysis at different times of the day revealed differential expressed genes (DEGs) grouped in reproductive-related families that may explain the daily variations in the fertilization rates.

4. Epigenetic mechanisms **(**DNA methylation and demethylation) show daily rhythms of expression synchronized to the light/dark cycle in zebrafish gonads. In constant darkness such rhythmicity persisted for two consecutive days, suggesting its endogenous control.

5. Lipid metabolism in both zebrafish and gilthead seabream liver shows daily rhythms strongly synchronized to the light/dark cycle, regardless feeding time. Lipogenesis genes presented a diurnal acrophase, while lipolysis genes were nocturnal.

6. In Senegal sole, light and temperature cycles condition growth, yolk sac development, metamorphosis, survival, fin development and jaw malformations.

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Annexes

7. Annexes

7.1. Scientific publications

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Summary

8. **Summery in Spanish**

El objetivo general de esta tesis doctoral fue dilucidar el impacto de las señales de ambientales, como la luz, la temperatura y el tiempo de las alimentación, en la sincronización de los ritmos diarios de reproducción en los peces, considerando la producción de gametos, la fertilización, el desarrollo de embriones / larvas y el metabolismo de los lípidos. Sobre esta base, destacamos la importancia de los ritmos biológicos en el establecimiento de la progresión armoniosa de los procesos reproductivos y de desarrollo temprano, incluida la activación del eje Brain-Pituitary-Gonad (BPG), las variaciones diarias de los gametos, las tasas de fertilización in vitro, los ritmos del mecanismo epigenético de las gónadas y las variaciones de genes lipídicos en el hígado. Los objetivos específicos son:

1. Revelar la existencia de ritmos diarios en la expresión de genes clave en el eje BPG-hígado del pez cebra.
2. Investigar la influencia de las variaciones estacionales (luz y temperatura) como condiciones reproductivas favorables o inhibitorias en genes clave involucrados en el eje BPG de medaka.
3. Describir los ritmos diarios de fertilización *in vitro* en el pez cebra para mejorar protocolos de reproducción considerando las variaciones diarias de los gametos.
4. Descubrir mecanismos epigenéticos encargados de transducir factores ambientales en respuestas fisiológicas observando los ritmos diarios en el mecanismo epigenético de metilación y desmetilación en las gónadas del pez cebra.

5. Evaluar los ritmos diarios en la expresión de genes del metabolismo lipídico en respuesta a los ciclos de luz y oscuridad y alimentación en el hígado de dorada y de pez cebra.
6. Resumir las claves ambientales clave como los ciclos de luz y temperatura durante el desarrollo temprano de la Solea de Senegal.

Discusión general

La presente tesis doctoral revela la existencia de una progresión armoniosa ambientalmente sincronizada en ambos sexos en toda la maquinaria neuroendocrina que controla la reproducción: desde la activación del eje BPG, gametogénesis, desove, concluyendo en la fertilización del huevo. En el capítulo 1-2 encontramos ritmos en el eje

cerebro-pituitario-gonadal (BPG) y la influencia de la luz y la temperatura en la activación/inhibición de este eje de reproducción neuroendocrino. En el capítulo 3, revelamos por primera vez los ritmos de fertilización *in vitro* que pueden ayudar a mejorar los protocolos de fertilización considerando la hora del día para obtener las mejores tasas de fertilización respecto a una baja/alta regulación del transcripto en el ovocito. En el capítulo 4, revelamos ritmos diarios en el mecanismo por el cual los factores ambientales se transducen en una respuesta fisiológica en las gónadas: mecanismos epigenéticos de metilación y desmetilación. En el capítulo 5-6, informamos la importancia del tiempo de luz/alimentación en los ritmos de metabolismo de los lípidos, que pueden actuar como factores críticos que influyen en la reproducción. Finalmente, en el capítulo 7, revisamos la influencia de los ciclos de luz y temperatura en el crecimiento, el desarrollo, la maduración sexual, la reproducción y los tiempos de eclosión.

Ritmos de reproducción

En el primer capítulo de la tesis, demostramos que los factores ambientales como la luz y la temperatura activan o inhiben la maquinaria reproductiva neuroendocrina, promoviendo o bloqueando la aparición de ritmos en el eje BPG. En el pez cebra describimos una activación/progresión temporal, rítmica y armoniosa del eje reproductivo. La activación comienza en el cerebro mediante la señalización estimuladora/inhibidora de la hormona liberadora de gonadotropina preóptica/hipotalámica (Gnrh), kisspeptinas (kiss) y la hormona inhibidora de gonadotropina (Gnih); que son neuropéptidos que integran información ambiental. Esas señales llegan a la pituitaria y promueven la síntesis y muy probablemente la liberación de la hormona folículo estimulante (Fsh) y la hormona luteinizante (LH). Luego, la FSH y la LH viajan a las gónadas, lo que desencadena la síntesis de esteroides que, junto con los andrógenos y los estrógenos, regula la espermatogénesis, la vitelogénesis, la maduración, la ovulación, el comportamiento reproductivo, la espermiación y el desove.

Ritmos de fertilización *in vitro* (FIV) y transcriptoma del ovocito

El éxito de la reproducción depende del funcionamiento coordinado de las actividades reproductivas. Estos procesos culminan con la producción de gametos de buena calidad con el

propósito final de fertilizar y obtener huevos viables. Además, teniendo en cuenta que la gametogénesis, la maduración, la ovulación, la espermiación y la reproducción aparecen con ritmicidad dependiendo de la especie, parece razonable suponer que tales oscilaciones persistirían en procesos reproductivos relacionados como la reproducción asistida y la calidad de los gametos. Sin embargo, hoy en día no existe un horario claro para las prácticas de fertilización *in vitro,* ni para la determinación gametos de buena calidad. En los resultados demostramos que el éxito de las tasas de FIV depende de la hora del día. También revelamos que tales variaciones en el ratio de FIV se corresponden con oscilaciones en el transcrito del ovocito, lo que explica la calidad del ovocito a lo largo día. Esos contenidos de la transcripción exhiben una regulación precisa en la alta/baja expresión de familia de genes involucrados en procesos biológicos como la regulación positiva de la reproducción, la unión de los espermatozoides a la zona pellucida, la fertilización individual, el reconocimiento de óvulos, reacción acrosómica, regulación positiva de la fertilización o singamia. Posiblemente, estas funciones transmiten los requisitos óptimos a los ovocitos para ser un huevo viable durante las primeras horas de la fase de luz (ZT23-ZT2), igualando así las altas tasas de FIV. En vista de la gran importancia del pez cebra para el desarrollo de las ciencias reproductivas más la estrecha similitud en la regulación reproductiva con la mayoría de los vertebrados, consideramos que nuestros resultados son de gran importancia para mejorar los protocolos de reproducción asistida en peces tanto como en los vertebrados superiores (Coy et al., 2008; Mañanós, 2008).

Ritmos en mecanismos epigenéticos de metilación / desmetilación en gónadas

En vista de la importancia de los factores ambientales, nos preguntamos cómo estas señales externas se transduce en una respuesta fisiológica. Sabemos que los cambios ambientales y el sistema circadiano, en cooperación con los mecanismos epigenéticos influyen en el sistema reproductivo endocrino en las gónadas, y en consecuencia, también afectan a todo el proceso reproductivo. Sin embargo, poco sabemos sobre la naturaleza rítmica diaria de los mecanismos epigenéticos en los peces y cómo esta regulación coordina la activación/inhibición de genes en las gónadas. La metilación y la desmetilación son el mecanismo básico que funciona a través de la epigenética. En nuestros resultados, describimos que la mayoría de los genes de metilación/desmetilación muestran un patrón rítmico diario con un pico de expresión máximo durante la fase de oscuridad. Curiosamente, este pico en los genes ocurre durante la fase de

reposo de los peces. Estudios similares en ratones también destacan que la actividad de metilación funciona durante la fase de reposo del animal (el pez cebra es diurno y el ratón nocturno). Estos datos probablemente sugieren la existencia de un mecanismo de metilación regulatorio común que depende del ritmo de sueño-vigilia. Además, la mayoría de las expresiones en genes de metilación/desmetilación persisten con una ritmicidad circadiana en condiciones de oscuridad constante durante dos días consecutivos. Esto implica la existencia de un control endógeno de los ritmos epigenéticos probablemente necesarios para una impresión continua del genoma. Por lo tanto, sugerimos que estos mecanismos de metilación/desmetilación actuarían rítmicamente sobre el genoma regulando la expresión de genes implicados en procesos biológicos específicos (Paredes et al., 2018; Piferrer et al., 2005, 2012).

Importancia del tiempo de luz / alimentación en los ritmos metabólicos lipídicos

La disponibilidad de alimento y el ciclo de alimentación adquieren una gran importancia con respecto al desarrollo, la maduración sexual y la reproducción. Sus implicaciones biológicas van tan lejos hasta el punto que la presión evolutiva ha dado lugar al desarrollo de un oscilador encarrilado por la alimentación (FEO) situado probablemente en el núcleo supraquiasmático (SCN) del hipotálamo en los peces. Además, la disponibilidad de alimentos no solo marca las condiciones óptimas para la liberación de la descendencia, sino que también desencadena procesos reproductivos previos como esteroidogénesis, comportamiento sexual, espermatogénesis u oogénesis. Por lo tanto, en esta parte de la tesis nos centramos en comprender las implicaciones de la alimentación y los ciclos de luz con respecto al metabolismo de los lípidos. Este metabolismo es el desencadenante inicial de varias respuestas reproductivas que usan lípidos como base para la síntesis de esteroides. En nuestros resultados, destacamos que el metabolismo de los lípidos sigue un patrón rítmico diario para los genes de lipogénesis y lipólisis. Estas funciones anabólicas y catabólicas se muestran durante la fase de luz o durante la fase de oscuridad, correspondientemente. Por lo tanto, lo que sugiere que en el hígado de peces, las vías anabólicas y catabólicas se muestran por separado en el tiempo, realizando su función correspondiente de manera eficiente tras el almacenamiento o la utilización de ácidos grasos. Curiosamente, también revelamos que este patrón rítmico obedece a los ciclos de luz-oscuridad pero no al ciclo de alimentación. Esto sugiere que el reloj principal del cerebro, en respuesta a señales de luz y de oscuridad, regula

la expresión diaria de genes metabólicos lipídicos, independientemente del tiempo de alimentación. Posiblemente, el metabolismo de los lípidos incluye la dependencia de Genes Reloj. Los Genes del Reloj se sincronizan con el ciclo luz-oscuridad en el cerebro, pero en el hígado su ritmo depende del tiempo de alimentación. Posiblemente, esto sugiere que las oscilaciones del gen Clock pueden implicar cierto grado de conexión con los ritmos de los genes lipídicos. Por lo tanto, el metabolismo de los lípidos responde a las señales ambientales de luz y de oscuridad en lugar de obedecer las horas de alimentación (López-Olmeda, J. F y Sánchez-Vázquez, F.J., 2010; Paredes et al., 2014, 2015b; Vera et al., 2013).

Influencia de los ciclos de luz y temperatura.

En resumen, con respecto a la importancia de la luz y la temperatura como factor clave para influir en la existencia de ritmos en peces, concluimos con un capítulo de libro que resume los efectos de las señales ambientales durante el desarrollo temprano en peces. El fotoperiodo y el espectro de luz crean un entorno fotográfico dinámico que desafía constantemente la vida acuática. Por lo tanto, interesantes experimentos presentan los efectos de esas señales ambientales sobre el crecimiento, la absorción del saco vitelino, la formación de la mandíbula, el desove, los ritmos de eclosión, el desarrollo larval, la migración ocular y la metamorfosis completa. Además, el ciclo de temperatura en sí mismo es lo suficientemente fuerte como para influir en los ritmos de eclosión, la expresión de genes, la actividad conductual, la fisiología, la capacidad de búsqueda de alimento, el crecimiento y la determinación/diferenciación sexual. Por lo tanto, consideramos de gran importancia los efectos de la luz y la temperatura en la mayoría de los procesos fisiológicos durante la vida de los peces. Por estas razones, los estudios de biología reproductiva y la piscicultura deben tener en cuenta estos factores ambientales al establecer protocolos de desarrollo y reproducción de peces (Blanco-Vives et al., 2010; Paredes et al., 2019; Villamizar et al., 2012).

Conclusiones

1. Tanto en el pez cebra hembra como en el macho, casi todos los genes claves involucrados en el eje BPG-hígado muestran un ritmo diario. Estos genes siguen una progresión temporal armoniosa durante el período reproductivo, lo que garantiza que la ventana de desove y de fertilización se produzcan coincidiendo con condiciones favorables para la máxima supervivencia de la descendencia.

2. En Medaka, las variaciones estacionales (fotoperiodo largo/corto y temperatura alta/baja) alteraran los ritmos diarios en genes reproductivos clave en el eje BPG. La mayoría de las hembras bajo fotoperiodo largo/temperatura alta (condiciones de verano) exhibieron un patrón rítmico de genes a lo largo del día, mientras que aquellas hembras bajo fotoperiodo/temperatura baja (condiciones de invierno) no presentaron ritmos significativos.

3. La fertilización *in vitro* en pez cebra muestra un fuerte ritmo diario determinado por la fase de luz de los ovocitos. Las tasas máximas de fertilización se dan alrededor de las primeras horas de luz. Además, tal ritmicidad persistió bajo condiciones de oscuridad constante. El análisis del transcriptoma en ovocitos a diferentes momentos del día reveló genes expresados diferencialmente (DEG) dando lugar a familias de genes relacionadas con la reproducción lo cual puede explicar las variaciones diarias en las tasas de fertilización.

4. Los mecanismos epigenéticos (metilación y desmetilación del ADN) muestran ritmos diarios de expresión sincronizados con el ciclo de luz/oscuridad en las gónadas de pez cebra. En condiciones de oscuridad constante, dicha ritmicidad persistió durante dos días consecutivos, lo que sugiere la existencia de un control endógeno.

5. El metabolismo de los lípidos tanto en el pez cebra como en el hígado de dorada muestra ritmos diarios fuertemente sincronizados con el ciclo luz/oscuridad, independientemente del tiempo de alimentación. Los genes de lipogénesis presentaron una acrofase diurna, mientras que los genes de lipólisis fueron nocturnos.

6. En el lenguado de Senegal, los ciclos de luz y temperatura condicionan el crecimiento, el desarrollo del saco vitelino, la metamorfosis, la supervivencia, el desarrollo de las aletas y las malformaciones de la mandíbula.