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

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Running title: Rhythms in the BPG axis of zebrafish

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ABSTRACT

The brain-pituitary-gonadal (BPG) axis regulates the activation of the endocrine machinery that triggers reproduction, which is a typical rhythmic process. In this research we focused on investigating the daily expression rhythms of the key reproductive genes involved in the BPG axis and the liver of zebrafish. To this end, male and female zebrafish were subjected to a stimulating photoperiod with a 14h light:10h dark cycle. Brain, pituitary and gonads, as well as female liver samples, were taken every 4 hours during a 24 h cycle. The results revealed that most genes exhibited statistically significant daily rhythms. Most of the brain reproductive genes (*gnrh2, gnrh3, kiss1, kiss2* and *gnrhr3*) displayed a daily rhythm of expression with a nocturnal acrophase (between Zeitgeber Time [ZT] 14:34h and ZT18:34h, lights off at ZT=14h). The male *kiss2* gene presented neither significant rhythms nor daily variations, while the male *gnrh3* and female *kiss2* genes exhibited diurnal peaks of expression at ZT06:34h and ZT04:34h, respectively. In contrast, the pituitary genes (*fshβ, lhβ, gnrhr2*) showed daily rhythms of expression with an acrophase during the light phase (between ZT02:10h and ZT10:35h). The female *gnrhr3* gene exhibited neither significant rhythms nor daily variations. The male *gnrhr3* gene presented a nocturnal acrophase (ZT14:32h). The gonad genes (*star, cyp17a1, 20βhsd, lhr, fshr*, *cyp19a1a, foxl2*, *amh, dmrt1* and *11βhsd*) revealed statistically significant daily rhythms with nocturnal acrophases, except for female *cyp17a1a* (ZT06:21h) and *20βhsd* (ZT05:19h). Lastly, the female liver genes presented daily rhythms with a maximum peak of expression around the transition phase from darkness to light (ZT01:00h for *erα* and at ZT23:09h for *vtg2*). These findings are consistent with the daily reproduction rhythms displayed by zebrafish, which are timed by the reproductive axis. Considering that reproductive success is critical for survival of the species, the knowledge of the rhythms of the endocrine BPG machinery

provides useful information to understand the reproduction process and to establish optimal protocols and conditions for reproductive treatments.

Key words: reproduction, rhythms, neuropeptides, gonadotropins, steroids, vitellogenin, light synchronization

INTRODUCTION

Life on Earth is constantly confronted with cyclic environmental conditions. Thus, organisms have developed a biological clock to keep track of time and anticipate periodic environmental changes, such as day/night alternations, lunar phases, tides or seasons (Panda et al., 2002). These clocks require daily adjustment entrained by external synchronizers (*zeitgebers*, ZT) that include light and feeding cycles (Panda et al., 2002; López-Olmeda, 2017). In mammals, there is a light-sensitive central (master) clock, located at the suprachiasmatic nucleus (SCN) of the hypothalamus, which controls the peripheral clocks located at other parts of the body (Meijer and Rietveld, 1989). In the zebrafish, the existence of a hierarchical organization of the circadian system is controversial as most peripheral clocks, and even cell lines, are entrainable by direct exposure to light, thus displaying an autonomous rhythm (Vatine et al., 2011; Carr et al., 2006; Whitemore et al., 2000). The disruption of such rhythms is related to diseases such as metabolic syndrome, obesity, diabetes, inflammatory traits, cancer, drug efficacy/toxicity and sterility (Paredes et al., 2018; Mañanós, 2008).

The presence of such a timing mechanism have fostered the development of a wide range of rhythmic adaptive strategies such as reproduction to occur at specific times of the day and/or year, increasing animal survival (De Coursey et al., 2004). Actually, reproduction success in fish relies on choosing optimal timing for mating and offspring release (Cowan et al., 2017). In fish, it has been reported that the pineal organ and melatonin rhythms act on the brain-pituitary-gonad (BPG) axis and transduce environmental information by activating the neuroendocrine machinery for reproduction (Falcón et al., 2007, 2010).

Reproduction rhythms are triggered by environmental time cues (i.e. "*zeitgebers"*), such as light, temperature or food availability cycles (Boden and Kennaway, 2006). Day length and feeding play the most important role in the timing of gonad maturation and reproduction (Bronson, 1985; Mañanós, 2008, Sumpter, 1990). On the one hand, as seasonal reproduction rhythms are well-known, fertility timetables are established: short gestational animals (e.g., hamsters and small mammals) begin in spring (Reiter, 1980), while long gestational ones (e.g., ruminants) do so late in summer (Lincoln, 2002). The seasonal ovulation rhythm comprehends a multi-oscillatory system that includes a central and a peripheral (ovarian) clock responsible for the timing of ovulation (Ball, 2007; Sellix and Menaker, 2010).

On the other hand, daily reproduction rhythms are less understood despite examples, such as domestic hens (*Gallus domesticus*) (Sharp et al., 1984; Silver, 1986) and the Japanese quail (*Coturnix Japonica*), which show daily rhythms of ovulation-oviposition (Ball, 2007; Nakao et al., 2007; Underwood et al., 1997) so that egg-laying occurs early in the morning or mid-afternoon for hens and quails, respectively. In fish, reproduction also exhibits well-known seasonal rhythms, as reported in Atlantic salmon (*Salmo salar*) (Thorpe et al., 1990), European sea bass (*Dicentrarchus labrax*) (Carrillo et al., 1995), gilthead sea bream (*Sparus aurata*) (Zohar et al., 1995), Atlantic cod (*Gadus morhua*) (Davie et al., 2007), Atlantic halibut (*Hippoglossus hippoglossus*) (Smith et al., 1991), Senegalese sole (*Solea solea*) (Oliveira et al., 2008), Nile tilapia (*Oreochromus niloticus*) (Campos-Mendoza et al., 2004), catfish (*Heteropneustes fossilis*) (Sundararaj and Sehgal, 1970) and common carp (*Cyprinus carpio*) (Davies et al., 1986abc). Daily reproduction rhythms have been reported only in a few species, such as zebrafish, gilthead sea bream, European sea bass and Senegal sole (Blanco-Vives and Sánchez-Vázquez, 2009; Bayarri et

al., 2004; Meseguer et al., 2008; Oliveira et al., 2009). As gonad maturation, gametogenesis and spawning rhythms appear species-specific depending on the fish rhythmic reproductive strategy (Blanco-Vives and Sánchez-Vázquez, 2009; Mañanós, 2008), it seems reasonable to assume that the neuroendocrine system controlling these processes would also oscillate in a rhythmic fashion.

The BPG axis regulates the activation of the neuroendocrine machinery that triggers the harmonious progression of the reproductive rhythm in both sexes: from gametogenesis to spawning, leading to egg fertilization (Weltzien et al., 2003). In the BPG axis, the key players are 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), which are neuropeptides that also integrate environmental information (Mañanós, 2008; Kim et al., 2011; Zohar et al., 2010; Muñoz-Cueto et al., 2017). At the gonadal level, the interplay among Fsh, Lh, androgens and estrogens controls processes such as vitellogenesis, spermatogenesis, maturation, ovulation and spermiation. Gonadal steroids exert feedback actions in the pituitary and the brain that modulate gonadotropins and neuropeptides secretion and reproductive behavior (Weltzien et al., 2004; Tokarz et al., 2015; Bittman 2016; Cowan et al., 2017). The steroidogenic function of gonadal cells begins with the uptake of cholesterol via the steroidogenic acute regulatory protein (Star) (Stocco and Clark, 1996). Then pregnolone and progesterone are synthesized and follow the route toward dehydroepiandrosterone (DHEA) and androstenedione formation, respectively, via 17 α-hydroxylase (Cyp17a1), plus Lh positive feedback. The 17 α-hydroxy-progesterone, precursor of androstenedione can also follow the route toward 17α, 20β dihydroxy-4-

pregnen-3-one (DHP) formation via 20 β-hydroxysteroid dehydrogenase (20β-Hsd) (Simpson, 1979; Simard et al., 2005). In many teleosts, DHP plays a role as the maturation-inducing steroid (MIS) (Cowan et al., 2017). Androstenedione is the precursor of androgens (testosterone) and estrogens (β-estradiol). Gonadal aromatase (Cyp19a1a) initiates estrogens synthesis, plus forkhead box L2 (Foxl2) and Fsh positive feedback (Wang et al., 2007). 11 β-hydroxysteroid dehydrogenase 2 (11β-Hsd2) transforms testosterone (T) into 11-ketotestosterone (11-KT), which is the most potent androgen in teleosts (Ijiri et al., 2008). In male, the antimüllerian hormone (Amh) and doublesex and Mab-3 related transcription factor 1 (Dmrt1) are key players in sex differentiation and testis development, respectively (Guo et al., 2005; Di Rosa et al., 2016). During vitellogenesis, theca cells synthesize T, which later turns into estradiol (E2) in granulosa cells via Cyp19a1a. E2 travels and binds to liver cells via estrogen receptors (Er), which leads to the physiological response of vitellogenesis by increasing the synthesis and secretion of vitellogenins (Vtg) and other yolk-related proteins (Levi et al., 2009; Mañanós, 2008).

Despite all the above data, a clear-cut description of the actual rhythms on the BPG-liver axis, which are responsible for the daily spawning rhythms described in zebrafish (Blanco-Vives and Sánchez-Vázquez, 2009), remains unknown. For this reason, in this paper we investigated the existence of daily rhythms of expression of 21 key genes involved in the BPG-liver axis of zebrafish. To perform this task, we looked at: (a) five brain genes (*gnrh2, gnrh3, kiss1, kiss2 and gnrhr3*); (b) four pituitary genes (*lhβ, fshβ, gnrhr2* and *gnrhr3)*; (c) ten gonadal genes in both female and male (*star*, *cyp17a1, 20βhsd*, *lhr, fshr, cyp19a1a, foxl2, amh*, mab-3, *dmrt1* and *11βhsd2*); and (d) two genes from female liver (*erα* and *vtg2*).

MATERIALS AND METHODS

Animals and housing

Wild-type zebrafish (*Danio rerio*, age ~2 months) (N=144) of 0.3 ± 0.1 g (mean±SD) of body weight were obtained from a local provider (Alimar S.A., Murcia, Spain). Fish were reared in 9-L glass aquaria at the Chronobiology Laboratory (a light-tight isolated room with a strictly controlled environment) at the Faculty of Biology, University of Murcia (Spain). The photoperiod was set at a 14h:10h light:dark (LD) cycle, with the time of lights on 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). The water temperature was held constant at 28±0.5ºC throughout the acclimation and experimental period with a water heater (200W Magictherm, Prodac, Italy). 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 tank. The feeder delivered feed 3 times a day (ZT2h, ZT6h and ZT10h) at 1.5% of the fish body weight per time. Supplementary artemia pellets (Prodac International, Cittadella, Italy) made of freeze-dried artemia (47% protein content) were given to the fish in each tank at ZT4 and ZT9 *ad libitum* every day.

Experimental design

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).

To investigate the daily rhythms of the BPG-liver genes, fish (N=144, 72 females and 72 males) were divided into six tanks (12 females and 12 males per tank). The sex of each fish was determined as described elsewhere (Westerfield, 2007). After 2 months under holding conditions, reproductive maturity was assessed by natural mating: females were verified to naturally spawn and males to successfully fertilize oocytes. At 6 hours post fertilization (6hpf) embryos were confirmed to be fertilized (Kimmel et al., 1995). Two weeks later, the adult zebrafish broodstocks fasted for 24 h and were sampled at ZT2, ZT7, ZT12, ZT16, ZT19 and ZT22 h (ZT0 was the beginning of the light phase, and ZT14 the beginning of the dark phase). 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 and gonad samples were collected from each female and male. Pituitary samples were pooled (n=3) to provide four replicates for each female and male sampling time. 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).

RNA extraction and cDNA synthesis

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

follows: *gnrh2, gnrh3, kiss1, kiss2,* and *gnrhr3* in the brain; *fshβ, lhβ, gnrhr2* and *gnrhr3* in the pituitary; *star, cyp17a1, 20βhsd, lhr, fshr* for both female and male gonads, *cyp19a1a* and *foxl2* only for ovary, and *amh, dmrt1* and *11βhsd2* only for testis; and *erα* and *vtg* in female liver. A screening test for Cycle threshold (C_T value) was assessed for all brain and pituitary genes at ZT6. The qPCR was performed for those genes with C_T values between 16 and 29 (brain: *kiss1* and *kiss2*; pituitary: *fshβ* and *lhβ*; all genes from gonads and liver). Genes with low expression levels ($C_T \geq 30$) were analyzed with ddPCR (brain: *gnrh2, gnrh3* and *gnrhr3*; pituitary: *gnrhr2* and *gnrhr3*).

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 (Cosinor, $p>0.05$). 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).

For ddPCR analysis, reaction mixtures of 20 µl were prepared using 10 µl of QX200 EvaGreen ddPCR Supermix (Bio-Rad, Hercules, USA), 100 nM of forward and reverse primers, and 11.4-50 ng cDNA. All samples were run in duplicate. Each 20 µl reaction was loaded into a sample well of a DG8 cartridge (Bio-Rad), after which 70 µl of QX200 droplet generation oil for EvaGreen (Bio-Rad) was added to oil wells. A DG8 gasket (Bio-Rad) was then secured over the cartridge and the cartridge placed in a QX200 droplet generator (Bio-Rad). After droplet generation, droplets were transferred to a semi-skirted 96 well PCR plate (Eppendorf, Hamburg, Germany) and the plate sealed with a pierceable foil heat seal (Bio-Rad) using a heat sealer (Eppendorf). Plates were then transferred to a C1000 Touch thermal cycler (Bio-Rad) and cycled under the following conditions: enzyme activation (5 min at 95 °C); cycling stage (40 cycles of 95°C for 30 s then 60 °C for 1 min); signal stabilization (5 min at 4 °C, then 5 min at 90°C). Following amplification, droplets were analyzed in a QX200 droplet reader (Bio-Rad) and the absolute quantification of target DNA was calculated by QuantaSoft software (version 1.7.4, Bio-Rad) using automatic threshold settings to assign PCR-positive and PCR-negative droplets.

Data 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 reproductive genes: *gnrh2, gnrh3, kiss1, kiss2* and *gnrhr3*

The cosinor analysis revealed that the expression of nearly all genes investigated (*gnrh2, gnrh3, kiss1, kiss2* and *gnrhr3*) displayed statistically significant daily rhythms ($p<0.05$) in both female and male (Table 2). In addition, one-way ANOVA revealed statistically significant differences between sampling points ($p<0.05$) (Figure 1) for most female and male genes analyzed. Only male *kiss2* gene failed to exhibit significant daily rhythms and variations, as revealed by cosinor and ANOVA ($p>0.05$), respectively. The acrophases of most of these genes were located in the first half of the dark phase (between ZT14:34h and ZT18:34h) (Figure 2). Only female/male *gnrh3* and female *kiss2* and *gnrhr3* genes presented diurnal expression acrophases at ZT13:10h/ZT06:34h, ZT04:34h and ZT13:12h, respectively (Figure 2).

Pituitary genes: *fshβ, lhβ, gnrhr2* and *gnrhr3*

In both female and male, cosinor analysis revealed the existence of statistically significant daily rhythms (p<0.05) in most genes investigated (Table 2). 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 3). Only the female *gnrhr3* gene failed to exhibit significant rhythmicity (Cosinor, p>0.05) or statistically significant differences between sampling times (one-way ANOVA, p>0.05) (Figure 3). The acrophases of expression of these genes in both female and male fish were located along the light phase (between ZT02:10h and ZT10:35h), while the acrophase of expression of the male *gnrhr3* gene was shifted toward the beginning of the dark phase at ZT14:32h (Figure 2).

Gonadal genes: *star, cyp17a1, 20βhsd, lhr, fshr*, *cyp19a1a, foxl2*, *amh, dmrt1* and *11βhsd*

The cosinor analysis revealed statically significant daily rhythms (p<0.05) in most genes of both female and male zebrafish (Table 2). The acrophases of most female and male gonadal genes analyzed were located in the dark phase, in many cases at the end of the night (~ZT23-24h, female *lhr*, *cyp19a1a, foxl2* and male *dmrt1*) (Table 2, Figure 2). In contrast, female *cyp17a1a* and *20βhsd* genes had their acrophases during the light phase (ZT06:21h and ZT05:19h, respectively) (Figure 2). Male *cyp17a1a* and female/male *fshr* did not present significant daily rhythms (p>0.05). The one-way ANOVA revealed the existence of statistically significant differences between sampling points for most genes of both female and male fish (p<0.05) (Figure 4).

Female liver genes: *erα* and *vtg2*

Both genes investigated exhibited significant daily rhythms as detected by the cosinor analysis ($p<0.05$) (Table 2). The analysis of the one-way ANOVA ($p<0.05$) also revealed statistically significant differences between sampling points (Figure 5). The acrophases were located around the transition phase from darkness to light (ZT01:00h for *erα* and ZT23:09h for *vtg2*).

DISCUSSION

In mammals, reproductive rhythms and the BPG axis have been the focus of investigation in the last decade (Cowan et al., 2017; Haus, 2007). However, the daily rhythmic nature of the BPG-liver axis has not yet been fully described, especially in fish. In the present research, we revealed the existence of daily rhythms of expression in nearly all key genes involved in the BPG-liver axis in both female and male zebrafish. The sound orchestration of the Gnrhs-Gths-sex steroids systems is required for the harmonious time-functioning of the whole reproductive process, from early gametogenic stages to spawning (Mañanós, 2008). This synchronized process warrants the final spawning window possibly occurring simultaneously in both mates, coinciding with favorable conditions for offspring survival (Sumpter, 1990; Blanco-Vives and Sánchez-Vázquez, 2009).

The brain is the main regulator of the reproductive axis integrating internal (e.g., melatonin, energy storages, steroid feedback) and external (e.g., environmental cycles) inputs thus responding with neuroendocrine signals, via Gnrh synthesis (Ekstrom and Meissl, 2003; Falcón et al., 2007). This neuroendocrine regulator acts as the primary neuronal system coordinating the entire reproductive rhythm (Mañanós, 2008). The existence of daily variations in the expression of hypophysiotropic forms of *gnrh* in the

European sea bass (Servili et al., 2013), gilthead sea bream (Gothilf et al., 1997), orange-spotted grouper (Chai et al., 2013) and medaka (Karigo et al., 2012) has been reported. Our findings described the existence of daily rhythms in *gnrh* forms, with acrophases located 7-8 h before spawning/spermiation in both female and male zebrafish (Blanco-Vives et al., 2009). Accordingly, Gothilf et al. (1997) revealed that the maximum *gnrh* transcript levels in gilthead sea bream occurred 8 h before spawning. Karigo et al. (2012) also described a *gnrh1* neuronal firing activity increment in the afternoon before spawning. Considering the above date plus the diurnal spawning activity in zebrafish (Blanco-Vives et al., 2009), we suggest that the nocturnal Gnrh synthesis in zebrafish may induce Lh surge triggering spawning and spermiation the following morning.

Another key player in the rhythmicity and timing of reproduction in vertebrates is Kisspeptin (Ando et al., 2014; Shahjahan et al., 2011). This oscillating upstream neuroendocrine factor regulates gonadotropin release via stimulation of Gnrh secretion (De Roux et al., 2003; Seminara et al., 2003; Messager et al., 2005) and it has been suggested to play a role in the seasonal control of reproduction in teleosts (Shi et al., 2010; Zohar et al., 2010; Migaud et al., 2012; Alvarado et al., 2013; Espigares et al., 2015). In our study, *kiss1, gnrh2* and *gnrh3* presented a similar daily expression pattern (in phase) in both female and male zebrafish. This synchrony of expression of *kiss1/gnrh2/gnrh3* suggests that these genes might provide a timed triggering cue synchronized to the LD cycle. Accordingly, Chai et al. (2013) suggested that *kiss1* is the main modulator of Gnrh neuron activity in tilapia. In the grass puffer (*Takifugu niphobles*), the *kiss2* gene is also responsible for driving *gnrh* expression pattern (Ando et al., 2014). Thus, the species-specific function of kisspeptins suggests that the expression pattern of *kiss2* could differ from *kiss1* probably because *kiss2* would interact with *gnrh1* (Chai et al., 2013), mediating processes such as

reproductive capacity, growth hormone control or prolactin secretion (Chai et al., 2013; Klausen et al., 2002; Marchant et al., 1989; Weber et al., 1997). However more recent knockout studies of *kiss 1* and *kiss 2*, along with their receptors, have revealed that kisspeptinergic systems are dispensable for reproduction in small model teleost species and may probably be involved in general processes of endocrine regulation rather than in the specific control of gonadotropin release (Nakajo et al., 2018).

All Gnrh receptors seem to participate in reproductive functions (Volkoff and Peter, 1999) and have a high sensitivity to Gnrh2 (Bogerd et al., 2002; Illing et al., 1999; Kah et al., 2007; Lethimonier et al., 2004; Moncaut et al., 2005; Okubo et al., 2003; Servili et al., 2010). In our results, *gnrhr3* displays a similar expression pattern as *gnrh2*; most likely as a response to couple with *gnrh2* that, in addition, presents the most potent bioactivity among the *gnrh* isoforms as described in goldfish (Chang et al., 1990; Khakoo et al., 1994), catfish (Rebers et al., 2000; Schulz et al., 1993) and sea bream (Zohar et al., 1995). At the pituitary level, Gnrh is the pivotal regulator of gonadotropins synthesis and release (Kim et al., 2011). Recent literature reports that *gnrhr* gene expression levels in the pituitary display a seasonal pattern which also reflects a seasonal responsiveness to Gnrh stimulation (Mañanós, 2008), so that highest levels of *gnrhr* entail highest responsiveness of the pituitary coinciding with the pre-spawning period (Mañanós, 2008). In our results, *gnrhr2* acrophases were located at the beginning of the light phase (~ZT03h) in both female and male. Accordingly, *fshβ* and *lhβ* gonadotropin subunits presented a daily expression pattern with an acrophase during the middle hours of the light phase (between ZT06:54h and ZT10:35h). Thus, the time line expression pattern of *gnrhr2* (~ZT03h) and *fshβ/lhβ* (~ZT07) suggest that Gnrhr2 might have a direct implication in Fshβ/Lhβ synthesis around the spawning window in zebrafish. Knowing these data is critical, not only to determine the

reproductive stage but also to establish Gnrh-based hormonal therapy timetables as its efficiency varies depending on the responsiveness of the Gnrh receptors along the day.

The gonad is the ultimate effector of the reproductive axis. Here, the Fsh/Lh pair stimulates synthesis of sex steroids and promotes the ovulation/spermiation process (Mañanós, 2008). The Lh surge has been involved in the stimulation of *star* gene expression (Stocco et al., 2001). On the steroidogenic pathway, Star is the first key rate-limiting step shuttling cholesterol from the outer to the inner mitochondrial membrane (Stocco et al., 2005). In our results, both female and male *star* genes presented a daily expression pattern with an acrophase during the dark phase (ZT18:00h and ZT21:01h, respectively). This probably marked the beginning of sex steroid biosynthesis in the zebrafish gonads. In this research, the female *lhr* acrophase was located at the end of the dark phase (ZT24:00h), probably to couple with a previous Lh surge in the pituitary (ZT08:26h) that may also trigger subsequent steps of the sex steroid synthesis (*cyp17a1* and *20βhsd*) (Ptak et al., 2017). The *cyp19a1a* (ZT23:34h) and *foxl2* (ZT23:00h) acrophases appeared later in time, most probably because their biosynthetic activities and functions are more relevant in the last steps of the steroidogenic pathway in response to Fsh positive regulation via Fshr (Ptak et al., 2017). In male zebrafish, these genes displayed a similar expression pattern (*star, 20βhsd, lhr, amh, dmrt1* and *11βhsd2*), with a peak during the dark phase. We suggest that these synchronous molecular events occur almost simultaneously as a tightly-linked pathway leading to steroid production and, ultimately, to spermiation. The sex steroids also play a crucial role in non-gonadal tissues with feedback actions on the brain/pituitary and in female liver to promote vitellogenin synthesis (Mañanós, 2008). Here, both *erα* and *vtg2* displayed daily expression rhythms with a maximum peak during the dark-light transition phase (~ZT24h). The *erα* expression correlated to estrogen increase

promoting vitellogenin synthesis in the liver. Thus, daily expression pattern of ovarian *cyp19a1a* (responsible of estradiol synthesis) and liver *erα* also appeared to be synchronized in time, which once again proves the harmonious sequence on the reproductive BPG-liver axis in the zebrafish.

In conclusion, our results revealed coordinated rhythms in the expression of key reproductive genes along the BPG-liver axis of zebrafish, which start in the brain, and trigger gonadotropin synthesis (and probably, release) in the pituitary that, in turn, promotes gonadal steroid synthesis. Moreover, our results have shown that the phase of rhythms in sex steroid machinery precedes/matches the spawning rhythm reported in zebrafish, thus explaining the diurnal spawning activity previously described by Blanco-Vives and Sánchez-Vázquez (2009) in this species. These findings revealed that the BPG axis regulates the reproductive performance and rhythms on a daily basis, which depends on the harmonically synchronized functioning of all the components of this axis (Figure 6). The coordinated secretion of the Gnrhs, Gths and sex steroids is crucial for successful spawning and fertilization (Cowan et al., 2017). Yet we acknowledge that there may appear species-specific differences in the phasing of the BPG axis related to diverse reproductive strategies (e.g. diurnal *vs.* nocturnal spawners). Considering that reproductive disruption in captivity constitutes a major problem in the aquaculture of many fish species (Dubois et al., 2002; Mylonas et al., 2001; Mylonas et al., 2007; Somoza et al., 2002), our present results may provide useful information to understand these failures and optimize daily hormone therapies and *in vitro* fertilization schedules.

DECLARATION OF INTEREST

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Figure Legends

Table 1. Gene IDs and primer sequences used for real-time PCR.

Table 2. Cosinor analysis results for BPG-Liver axis genes in zebrafish.

Figure 1. Relative and absolute expression values in percentage of brain genes in the BPG axis of zebrafish in a 14L:10D cycle. Open and full bars the top of each graph indicate the light and dark periods, respectively. The sinusoidal line on the graphs represents 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 lowercase letters (one-way ANOVA, $p<0.05$).

Figure 2. Acrophase map of BPG-liver axis genes studied in this research. Open and full bars on the top and bottom of the graph indicate the light and dark periods, respectively. The female (triangle) and male (circle) symbols indicate the day time at which the gene expression is maximum. The fiducial limits (set at 95%) are indicated by the lateral bars at the side of each symbol. The name of each gene is indicated at the left of the graph.

Figure 3. Relative and absolute expression values in percentage of pituitary genes on the BPG axis of zebrafish in a 14L:10D 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 genes involved on the BPG axis of zebrafish. White and black bars on the top mean day and night, respectively. The sinusoidal line on the graphs represents 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 lowercase letters (one-way ANOVA, $p<0.05$).

Figure 5. Relative expression values in percentage of liver involved in the process of vitellogenesis in zebrafish. 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 lowercase letters (one-way ANOVA, $p<0.05$).

Figure 6. Conceptual diagram representing the time line functioning of the BPG and liver axis. White and black bars on the top and bottom mean day and night, respectively. Positive signs on the diagram mean positive feedback. Functioning time for each tissue is indicated at the end of the dotted line as ZT.

Table 1

Gene	Accession number	F/R	Primer sequence (5´- 3´)
gnrh2	NM_181439.4	F	ACATCCTCAAGACAATACTGCTGGA
		R	GAAAAGGCAGGCCAAATGTG
gnrh3	NM_182887.2	F	ATGGAGGCAACATTCAGGATGT
		R	CCTTTCAGAGGCAAACCTTCA
kiss1	NM_001113489	F	ACAAGCTCCATACCTGCAAGTG
		R	AATACTGAAAATGCCCAGAGGG
kiss2	NM_001142585	F	GCCTATGCCAGACCCCAAA
		R	TTTACTGCGTGCTAGTCGATGTTT
fsh	AY424303	F	CAGATGAGGATGCGTGTGC
		R	ACCCCTGCAGGACAGCC
lhβ	AY424304/AY424305	F	ATGTTATTGGCTGGAAATGG
		R	CTAGTATGCGGGGAAATCC
gnrhR2	NM_001144979.1	F	TGGACCATGAGTGTCGTGTTG
		R	GCACTGGACAAACTGCTTTGG
gnrhR3	NM_001177450.1	F	CACAACAGCAACAAAGGTGATTC
		R	CCAGATGCCCAGCAGGTAAT
star	ENSDARG00000006137	F	TCAAATTGTGTGCTGGCATT
		R	CCAAGTGCTAGCTCCAGGTC
cyp17a1	ENSDARG00000033566	F	AGGTGGCATTGAAGGATCTG
		R	TGAGTGCTTCAGCCATTGTC
20 β-hsd	AF298898	F	TGCGCACCAACTTCTGGGGAACGCTG
		R	GCGTTCAATAGAATCCCATCACCTGGC
fshR	ENSDARG00000071494	F	ATGTGGCAGGATTCTTCACC
		R	CTGCATGGCATAAGTGATGG
lhR	ENSDARG00000026081	F	ATCACTCACGCTCTCCGACT
		R	GCTGCTGACGCCTATTAAGG
cyp19a1a	AF183906	F	TGCTGGCCATCAGACACCAT
		R	CAGATGAACCGACAGTAGGAGACAA
foxl2	NM_001045252	F	AAACACTGGGAAGGTTTGCGTGC
		R	TTTGTCCGGCCCCTTCTCTGG
amh	AY677080	F	GGGTGTGCATGCTACAGAAGAT
		R	CTCAGAAATGCAAACAGTCTGTGT
dmrt1	NM_205628	F	ATGGCAGAGCAGAACGATTT
		R	TAGTCCCACAACAGCATGGA
11β-hsd2	ENSDARG00000001975	F	TGCTGCTGGCTGTACTTCAC
		R	TGCATCCAACTTCTTTGCTG
erα	AF349412	F	GATACATCAGTGAGAGAGAGAAAGCATCC
		R	TCGCTGCCTGGCACCAA
vtg2	AY729645	F	GGTGACTGGAAGATCCAAG
		R	TCATGCGGCATTGGCTGG

Tissue	Gene	Sex	Significance	Acrophase (ZT hours)
Brain	*gnrh2*	female	*	16:16 ± 4:03
		male	*	18:18 ± 3:49
	gnrh3	female	*	13:10 ± 5:10
		male	*	06:34 ± 4:37
	kiss1	female	*	14:34 ± 3:29
		male	*	16:16 ± 5:32
	kiss2	female	*	04:34 ± 2:53
		male	NS	-
	gnrhr3	female	*	13:12 ± 3:30
		male	*	18:34 ± 4:30
Pituitary	*fshβ*	female	**	06:51 ± 3:25
		male	**	07:04 ± 2:04
	lhβ	female	*	08:26 ± 2:07
		male	*	10:35 ± 5:00
	gnrhr2	female	**	04:22 ± 2:30
		male	*	02:10 ± 3:22
	gnrhr3	female	NS	-
		male	*	14:32 ± 4:28
Gonad	*star*	female	*	18:00 ± 2:34
		male	*	21:01 ± 5:26
	cyp17a1	female	*	06:21 ± 3:06
		male	NS	-
	20βhsd	female	*	05:19 ± 4:00
		male	*	18:00 ± 5:00
	fshr	female	NS	-
		male	NS	-
	lhr	female	**	24:00 ± 2:40
		male	*	16:16 ± 2:09
	cyp19a1a	female	*	23:34 ± 4:00
	foxl2	female	*	23:00 ± 3:50
	amh	male	*	17:35 ± 3:28
	dmrt1	male	*	24:00 ± 4:05
	11βhsd	male	*	21:32 ± 4:47
Liver	*erα*	female	*	01:00 ± 4:08
	vtg2	female	*	23:09 ± 5:11

NS. Non-significant
* $p < 0.05$
** $p < 0.01$

Figure 1

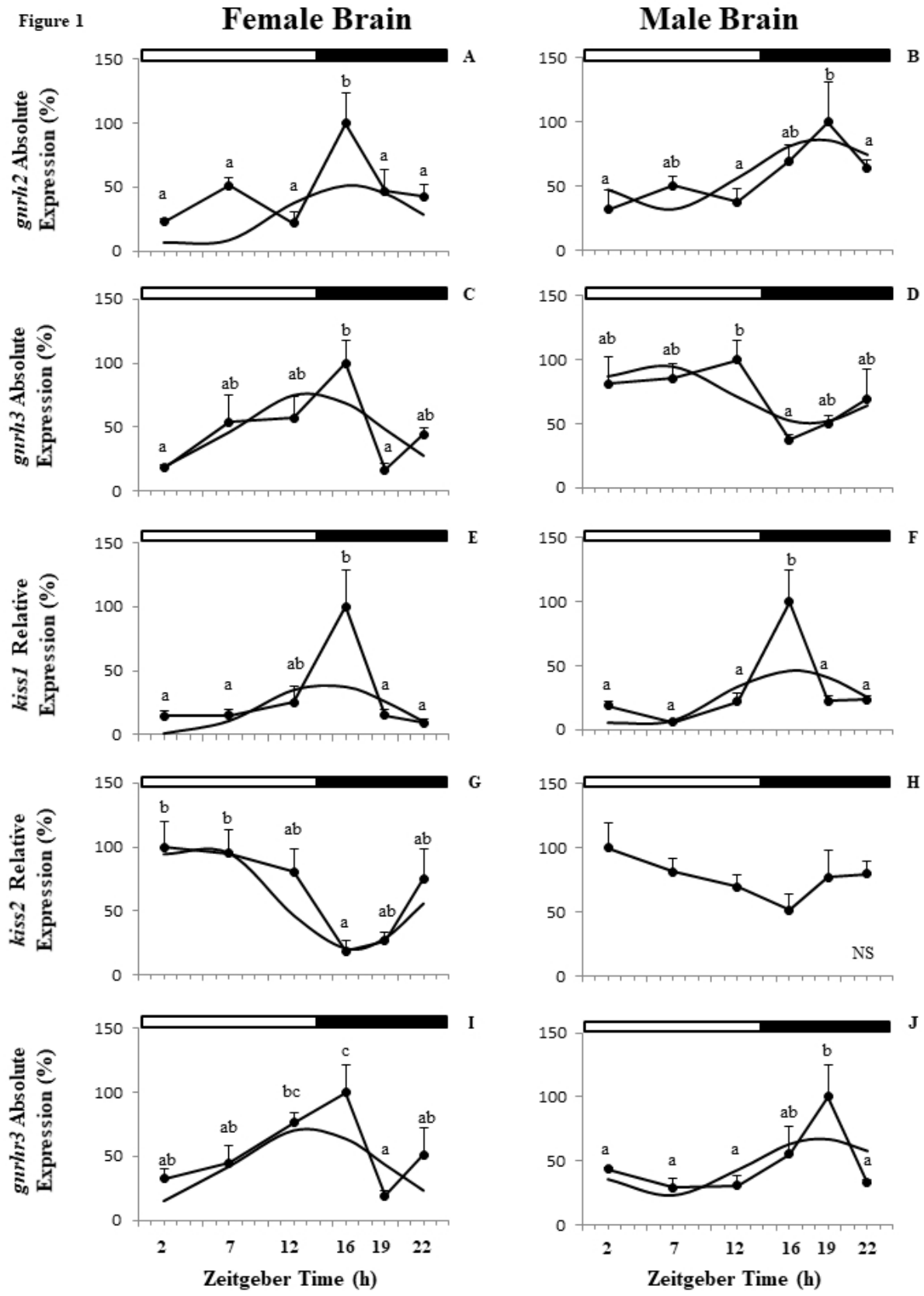

Female Brain
Male Brain
gnrh2 Absolute Expression (%)
gnrh3 Absolute Expression (%)
kiss1 Relative Expression (%)
kiss2 Relative Expression (%)
gnrhr3 Absolute Expression (%)
A
B
C
D
E
F
G
H
I
J
NS
Zeitgeber Time (h)
Zeitgeber Time (h)

Figure 2


gnrh2
gnrh3
kiss1
kiss2
gnrhr3
fshβ
lhβ
gnrhr2
gnrhr3
star
cyp17a1
20βhsd
lhr
cyp19a1a
foxl2
amh
dmrt1
11βhsd
erα
vtg2
0
7
14
24
7
14
Zeitgeber Time (h)

Figure 3

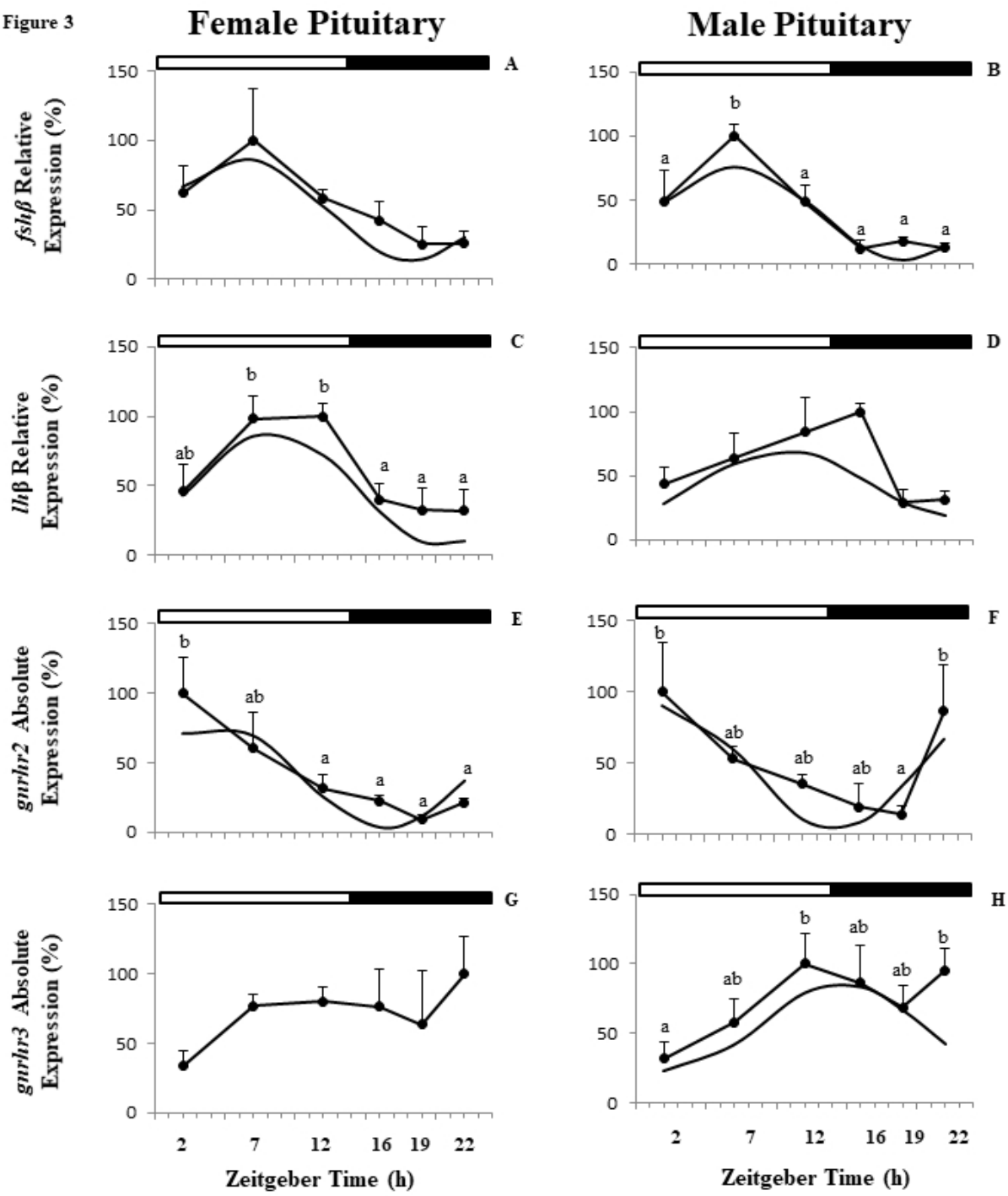
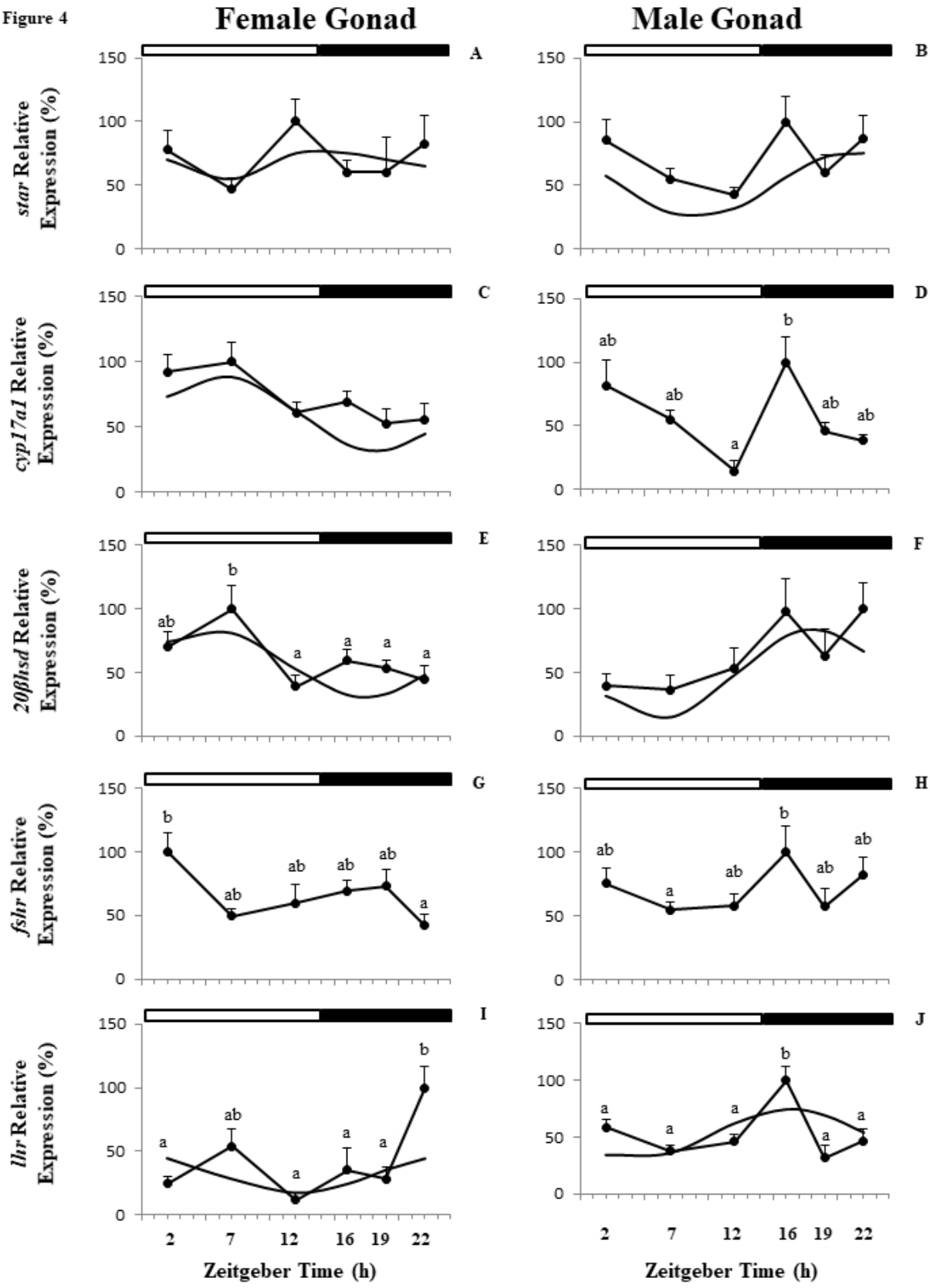

Female Pituitary
Male Pituitary
fshβ Relative Expression (%)
lhβ Relative Expression (%)
gnrhr2 Absolute Expression (%)
gnrhr3 Absolute Expression (%)
A
B
C
D
E
F
G
H
150
100
50
0
a
ab
b
2
7
12
16
19
22
Zeitgeber Time (h)

Figure 4


Female Gonad
Male Gonad
star Relative Expression (%)
cyp17a1 Relative Expression (%)
20βhsd Relative Expression (%)
fshr Relative Expression (%)
lhr Relative Expression (%)
A
B
C
D
E
F
G
H
I
J
150
100
50
0
ab
b
a
2
7
12
16
19
22
Zeitgeber Time (h)

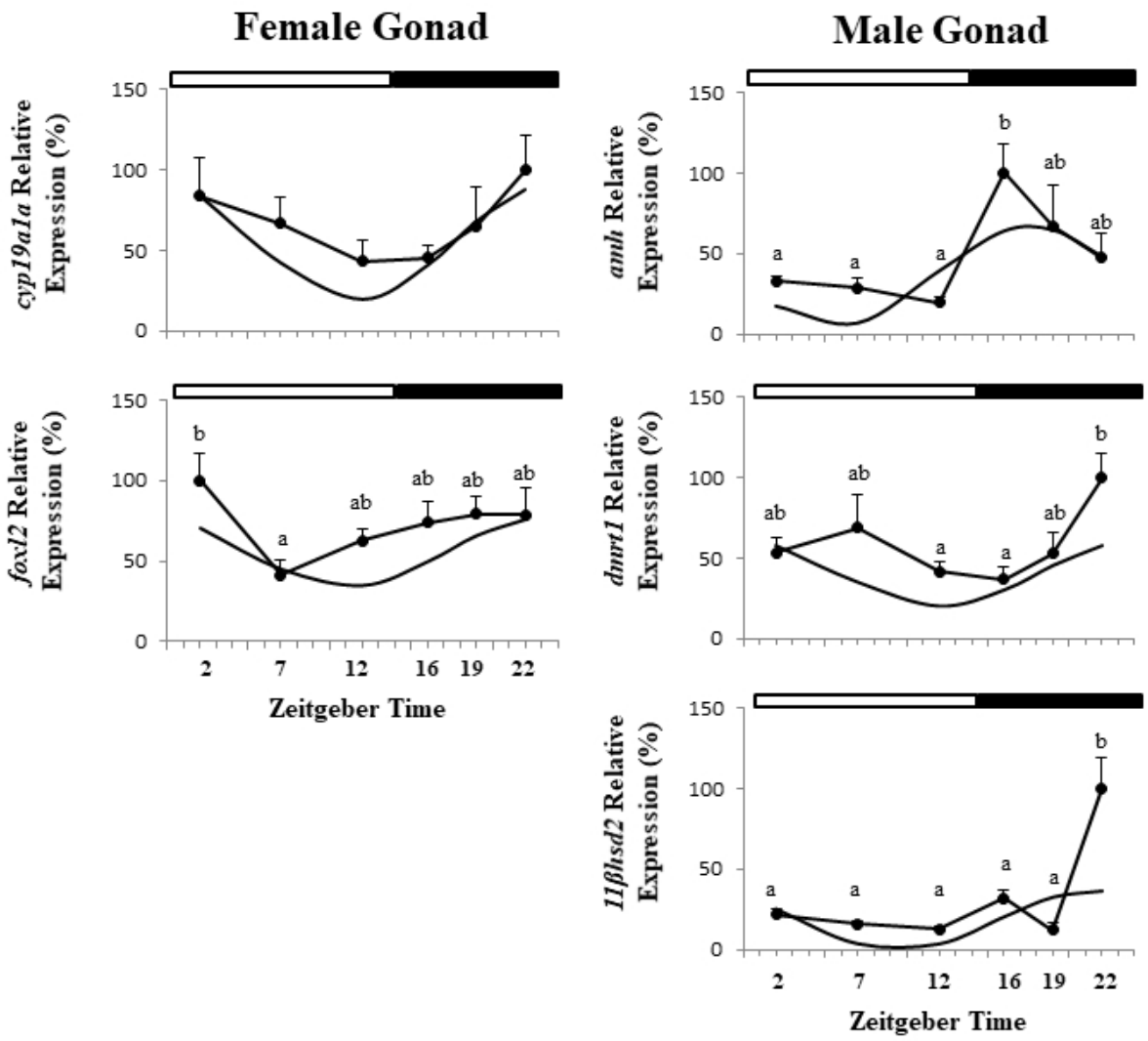
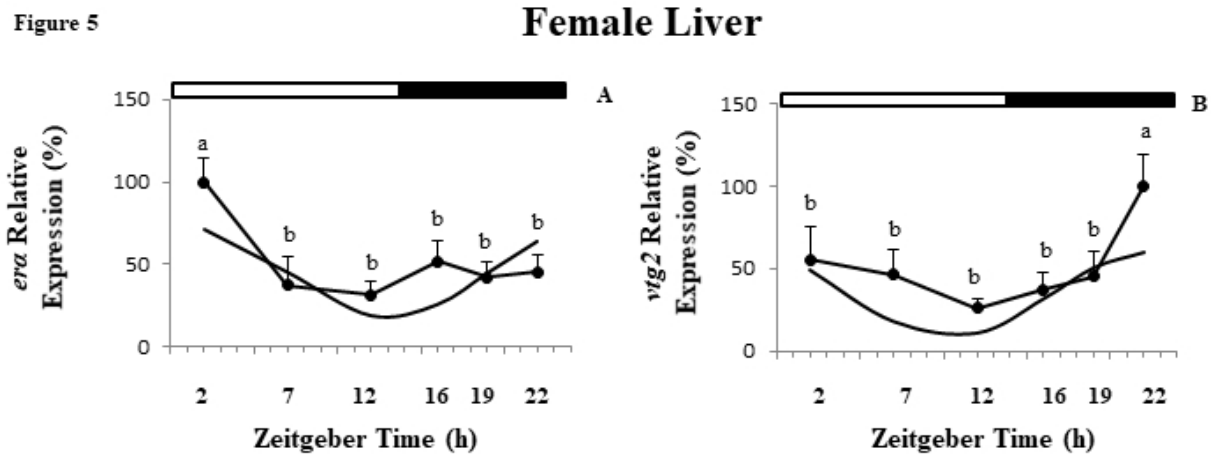
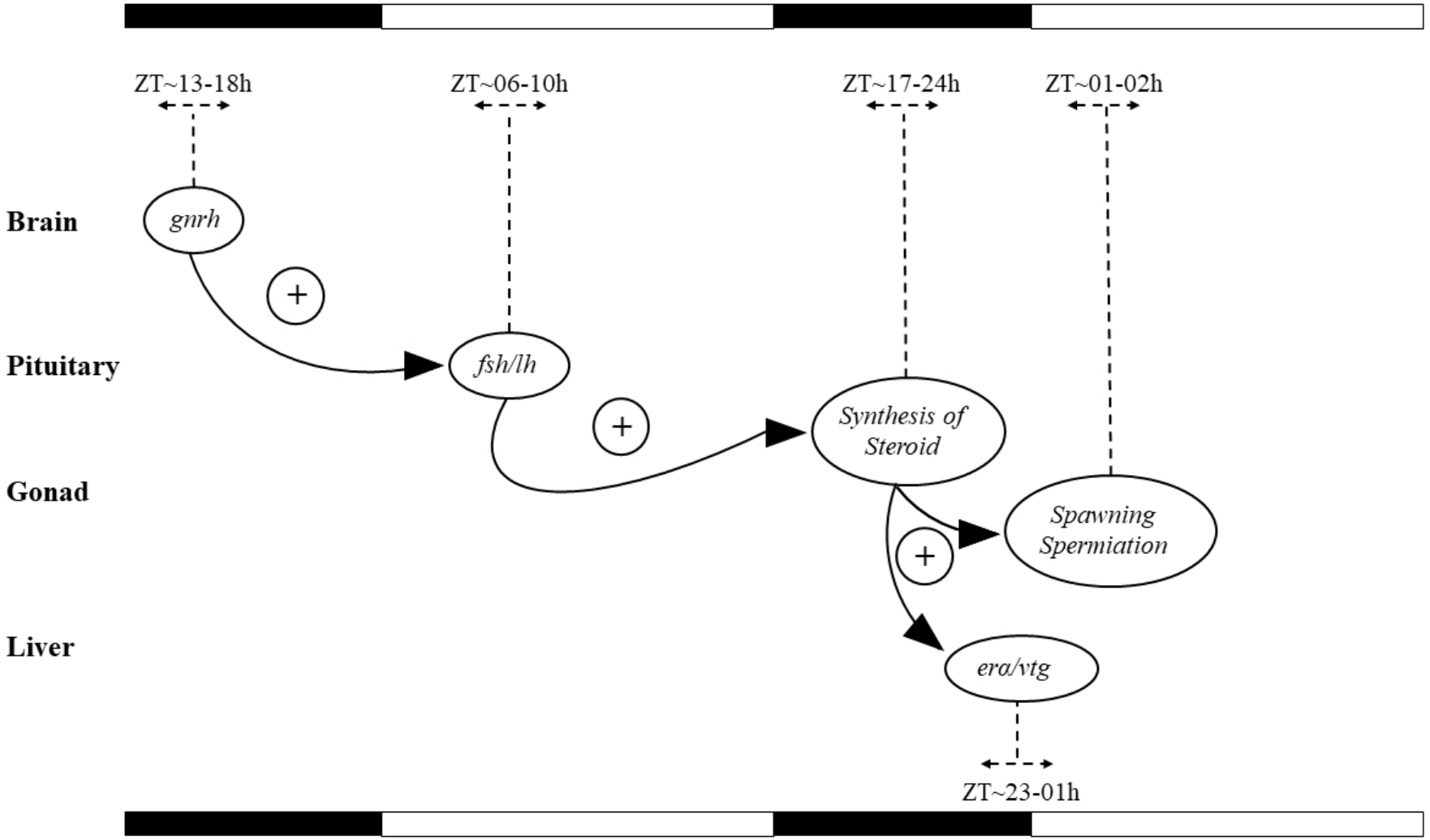

Female Gonad
Male Gonad
cyp19a1a Relative Expression (%)
amh Relative Expression (%)
foxl2 Relative Expression (%)
dmrt1 Relative Expression (%)
11βhsd2 Relative Expression (%)
150
100
50
0
a
a
a
b
ab
ab
b
ab
ab
ab
ab
ab
ab
a
a
a
a
a
a
a
b
b
2
7
12
16
19
22
Zeitgeber Time

Figure 5


Female Liver
A
B
erα Relative Expression (%)
vtg2 Relative Expression (%)
150
100
50
0
a
b
2
7
12
16
19
22
Zeitgeber Time (h)


ZT~13-18h
ZT~06-10h
ZT~17-24h
ZT~01-02h
Brain
Pituitary
Gonad
Liver
gnrh
+
fsh/lh
+
Synthesis of Steroid
Spawning Spermiation
+
erα/vtg
ZT~23-01h