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Central and peripheral clocks in Atlantic bluefin tuna (*Thunnus thynnus*, L.): Daily rhythmicity of hepatic lipid metabolism and digestive genes

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Abstract

Atlantic bluefin tuna (ABT; *Thunnus thynnus*) is a highly regarded and consumed species, but farming is still in its infancy. Currently, nothing is known about the presence of circadian rhythmicity at central or peripheral tissues, or if there are daily rhythms in expression of genes involved in lipid metabolism. In order to elucidate clock gene regulation of genes of lipid metabolism in ABT, six clock genes (*bmal1*, *clock*, *cry1*, *cry2*, *per1* and *per2*) were sequenced and 24 h expression of these genes determined in brain and liver of fish acclimated to a light:dark (L:D) photoperiod. Additionally, the daily expression of lipid metabolism and digestive enzyme genes in ABT was also determined in liver. All six clock genes displayed rhythmicity in the brain and liver, other than *cry2*, which did not show an acrophase in liver. In liver, all the transcription factors analysed other than *srebp1* and *srebp2* displayed rhythmicity, with *lxr* and *pparα* displaying diurnal expression, whereas *pparγ* was highly expressed at the end of the scotophase. Some of the target genes of *lxr* such as *elovl5* and *lpl* also oscillated rhythmically, with acrophases during the photophase. In contrast, only three of the eight digestive enzyme genes studied displayed rhythmicity, at different times of the day, suggesting that either ABT display different feeding periods or the digestion of some nutrients (e.g. lipids) is prioritized over others. The present study showed that clock and lipid metabolism genes displayed strong daily rhythmicity in ABT brain and liver, which could be an area of considerable interest for the establishment of efficient feeding protocols in this new aquaculture species.

Key words: bluefin tuna; clock genes; brain; liver; lipid metabolism; digestive enzyme genes.

Abbreviations: ABT, Atlantic bluefin tuna; *alp*, alkaline phosphatase; *amy*, amylase; *anpep*, amino peptidase; *bal1*, bile salt activated lipase 1; *bal2*, bile salt activated lipase 2; *bmal1*, Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; *clock*, Circadian Locomotor Output Cycles Kaput; *cry1*, cryptochrome 1; *cry2*, cryptochrome 2; *ef1α*, elongation factor 1 alpha; *elovl5*, fatty acyl elongase 5; *fads2d6*, delta-6 fatty acyl desaturase; L:D, light:dark; *lpl*, lipoprotein lipase; *lxr*, liver X receptor; *per1*, period 1; *per2*, period 2; *pla2*, phospholipase A2; *pparα*, peroxisome proliferator-activated receptor alpha; *pparγ*, peroxisome proliferator-activated receptor gamma; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2; *tryp*, trypsin.

Highlights

- Daily rhythmicity was demonstrated in central and peripheral tissues in tuna
- Clock and lipid metabolism genes showed strong daily rhythmicity in brain and liver
- Several digestive enzyme genes studied also displayed rhythmicity
- The novel results could facilitate the development of efficient feeding protocols
- The knowledge will further support the aquaculture of this iconic species

1. Introduction

Organisms have developed biological clocks that regulate physiological processes in response to cyclic environmental conditions that vary in a regular manner (Panda et al., 2002). These clocks need to be adjusted daily by exogenous cues (*zeitgebers*, ZT) such as light-dark cycles in order to synchronize the molecular clocks that, in turn, generate the circadian rhythms (DeCoursy, 2004). In addition to this central circadian clock mechanism, there are peripheral clock systems that provide targeted rhythmic control to specific processes. The liver peripheral clock appears to play a key role in the entrainment of the circadian timing system in mammals (Yang et al., 2006; Le Martelot et al., 2009), with some recent studies focussing on teleosts (Vera et al., 2013; Betancor et al., 2014; Paredes et al., 2014, 2015).

The molecular mechanisms of the circadian clock is composed of intracellular transcriptional-translational feedback loops that are, in turn, regulated by clock genes and corresponding proteins (Vatine et al., 2011). The positive arm of the circadian clock consists of two genes: the Circadian Locomotor Output Cycles Kaput (Clock) and aryl hydrocarbon receptor nuclear translocator-like protein-1 (also known as Brain and Muscle ARNT-Like Bmal1) that operate as a dimer, whereas the negative arm is controlled by period (Per) and cryptochrome (Cry) genes (Cahill, 2002). In mammals, an interplay between clock genes and the regulation of lipid and cholesterol metabolism has been described (Le Martelot et al., 2009) involving the activation of nuclear receptors such as sterol regulatory element binding proteins (Srebp; Sato, 2010), liver X receptor (Lxr; Evans et al., 2004) and peroxisome proliferator – activated receptors (Pparα and Pparγ; Cruz-García et al., 2009) and their respective target genes.

Fish in the wild display feeding rhythms that are related to food availability or the presence of predators (López-Olmeda and Sánchez-Vázquez, 2010a). Under farming conditions when they are provided with a continuous supply of feed, fish continue to display feeding rhythms (López-Olmeda and Sánchez-Vázquez, 2011). Given that the main operating cost in intensive aquaculture farms is feed, it is pivotal to know the feeding habits of the different fish species in order to minimise this cost, as the feeding regime applied could greatly impact on feed efficiency and waste. In this respect, the domestication and farming of Atlantic bluefin tuna (ABT; *Thunnus thynnus*) is in its infancy and little is known in this iconic species about their feeding habits or metabolic patterns throughout the day, including lipid metabolism or digestion. Given that this species is a top predator, it is not appropriate to simply extrapolate feeding protocols used for other species such as European sea bass (*Dicentrarchus labrax*) or gilthead sea bream (*Sparus aurata*).

A fuller understanding of all the physiological mechanisms is essential for the successful domestication of new fish species. The overall objective of the present study was to evaluate the relationships between the daily hepatic expression patterns of genes of the circadian clock, and genes involved in lipid metabolism and digestion in ABT. In this respect, daily patterns of expression of transcription factors and some of their target genes were studied. In addition, the daily expression pattern of the main components of the molecular clock were studied in the brain of ABT in a 24 h cycle. As this is the first study to report the expression patterns of the clock genes in ABT, firstly the complete or partial open reading frames of the six genes (*bmal1*, *clock*, *per1*, *per2*, *cry1* and *cry2*) were identified and their expression determined during a daily cycle (14L:10D) by real time quantitative PCR (qPCR). By evaluating the rhythmic daily regulation of lipid metabolism and digestive genes in ABT liver, deeper insight into the mechanisms involved in the regulation of absorption, transport and utilization of lipids and other nutrients will be achieved.

2. Materials and methods:

2.1. Isolation of clock genes

Sequences of genes encoding for clock mechanism genes (*bmal1*, *clock*, *per1*, *per2*, *cry1* and *cry2*) were obtained by identifying the sequences from Sequence Read Archives (SRA) specific to *T. thynnus* (SRX2255758, ERX555873 and ERX555874). Once the contiguous sequences for each gene were obtained, these were assembled using CAP3 (Huang and Madan, 1999), confirming the identity of the deduced amino acid (aa) sequences by BLASTp sequence analysis service of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Next, primers were designed in order to sequence in full or partially the open reading frames (ORF) of each gene (Table 1) using a pool of cDNA from liver as template (see below). PCR products obtained were purified using the Illustra GFX PCR DNA or Gel Band Purification kit (GE Healthcare, Little Chalfont, UK) and sequenced to confirm identity (Sanger ABI3730xl, Eurofins Genomics, Konstanz, Germany).

2.2. Experimental animals and sampling procedures

All experimental procedures followed the Spanish legislation on Animal welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/EU) and Spanish Legislation (RD 1201/2005 and Law 32/2007) for the use of laboratory animals. Additionally, the experimental protocol was subjected to ethical review at the University of Stirling by the Animal Welfare and Ethical Review Board (AWERB/1718/172).

A total of 42 Atlantic bluefin tuna juveniles (55 days after hatching; 4.3 ± 1.1 g initial weight; 6.8 ± 0.6 cm initial length) were maintained in a single cylindro-conical 5 m^3 tank with incoming seawater filtered at 10 µm and UV sterilized (water temperature = 29.3 ±1.1 ºC). Experimental fish were adapted to a long day photoperiod cycle (L:D 14:10) with lights switched on at ZT0. The fish were manually fed a commercial tuna feed (Magokoro®, Marubeni Nisshin Feed Co., Japan) in excess during photophase (ZT0-ZT14). After 1-week of acclimation, fish were fasted for 24 h and sacrificed by an overdose of anaesthetic MS222 every 4 h over a 24 h period (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT24). At ZT0 samples were collected in the light, while at ZT24 samples were collected in the dark, just before the lights were on. At each sampling point, liver and brain were collected from 4 individuals, stabilized in RNA later® (Sigma -Aldrich, Dorset, UK) and stored at 20 °C until RNA extraction. A dim red light was used for nocturnal sampling

2.3. RNA extraction and cDNA synthesis

Samples of approximately 100 mg of liver and whole brain were homogenized in 1 mL of TRI Reagent (Sigma-Aldrich, Dorset, UK) by a bead tissue disruptor (BioSpec, Bartlesville, OK, USA). The tubes were centrifuged to remove genomic DNA and the content transferred into a fresh tube before being mixed with 100 µL BCP (1–bromo–3–chloropropane, phase separation reagent; Sigma-Aldrich). Next, the upper aqueous phase was transferred to a clean tube and RNA was precipitated by mixing with RNA precipitation solution (sodium chloride/sodium citrate sesquihydrate, Sigma-Aldrich) and isopropanol. After centrifugation, the RNA pellet could be visualized and was then washed twice with ethanol. Finally, the RNA was resuspended in molecular biology grade water and quantity and quality determined by spectrophotometry using a NanoDrop ND-1000 (Labtech Int., East Sussex, UK). The RNA integrity was determined by electrophoresis using 200 ng of total RNA in 1 % agarose gel. Finally, cDNA was synthesized using 2 µg of total RNA and random primers in 20 µL reactions and the high capacity reverse transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington, UK). The obtained cDNA was diluted 1 in 20 in order to perform the qPCR reactions.

2.4. Quantitative PCR (qPCR) analysis of gene expression

Primers for qPCR were designed on the obtained PCR fragments for the six Clock genes using the online software Primer3 (Untergasser et al., 2012), and were available for genes of digestive enzymes, lipid metabolism, and housekeeping from previous studies on ABT (Betancor et al., 2017a,b) (Table 1). From the three tested housekeeping genes (*elongation factor 1α*, *ef1α*; *ubiquitin*, *ubiq*; and *β-actin*, *bactin*), *ef1α* was selected as being most stable according to geNorm (M stability value = 0.165;

Vandesompele et al., 2002). The efficiency of primers for each gene was evaluated by serial dilutions of cDNA pooled from the samples to confirm it was > 85 % for all primer pairs. Quantitative PCR was carried out using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany). Ninety-six well plates were used, each sample in duplicate 20 µL reaction volumes containing 10 µL of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µL of each primer corresponding to the analyzed gene (10 pmol concentration; Eurofins Genomics), 3 µL of molecular biology grade water, and 5 µL of cDNA. In the case of housekeeping genes, only 2 µL of cDNA were used increasing the molecular biology grade water to 6 µL. Additionally, amplifications were carried out with a systematic negative control (NTC, no template control) containing molecular biology grade water instead of cDNA. Standard amplification parameters contained a UDG pre-treatment at 50 °C for 2 min and an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the corresponding annealing temperature (as indicated in Table 1) and 30 s at 72 °C. A melt curve of 0.5 °C increments from 75 °C to 90 °C was performed at the end of the qPCR run, enabling confirmation of the amplification of a single product in each reaction and the lack of contamination with genomic DNA. The relative expression levels (gene expression fold-change) of the target genes were calculated following the method described by Pfaffl (2001).

2.5. Statistical Analysis

The software Ritme (University of Barcelona, Spain) was used to perform the cosinor analysis. This type of analysis indicates if the daily expression of the genes of interest fitted a cosine function. In addition, statistical differences between different sampling times for each gene tested was also analysed by one way-ANOVA followed by a Tukey post-hoc test ($p \leq 0.05$; SPSS v 23.0 software IBM, NY, USA). All data are presented as mean ± SEM (standard error of the mean) with n = 4.

3. Results

3.1. Brain gene expression

In the brain, all the clock genes studied displayed statistically significant daily rhythms in ABT subjected to a photoperiod of 14L:10D (Fig. 1; Table 2). Three of the genes (*bmal1*, *clock* and *cry1*) peaked in the second half of the photophase (ZT12:44 h, ZT11:32 h and ZT12:29 h, respectively; Fig. 2). The acrophase of *per1* was observed 20 min after lights were switched on (ZT0:20 h), whereas the acrophase of *per2* was delayed more than three hours (ZT3:50 h; Fig. 1). In the case of *cry2*, the acrophase was observed at the end of the scotophase, some minutes before lights were switched off again (ZT23:10 h; Fig. 1).

In addition, most of the genes presented statistically significant differences between sampling points, except for *clock* (Fig. 1). In this respect, the highest levels of expression were found at ZT12 for *bmal1* and *cry1*, although these were not different to the levels at ZT8 and ZT16 in the case of *bmal1* (Fig. 1). For both *per1* and *cry2*, peak expression was observed at ZT24, which was not different to the expression levels at ZT0. In the case of *per2*, the highest expression level was observed at ZT0, with a steady decrease in the mRNA copy number after this.

3.2. Liver gene expression

3.2.1. Hepatic daily rhythms of expression of Clock genes

Cosinor analysis revealed the existence of statistically significant daily rhythms in liver expression of all the Clock genes analysed except for *cry2* (Fig. 2; Table 2). The acrophases of *bmal1*, *clock* and *cry1* were located at dusk (between ZT12 and ZT14), whereas for *per1* and *per2*, the acrophase occurred at the beginning of the photophase (ZT2 and ZT7, respectively; Fig. 2). Furthermore, the levels of expression of *bmal1*, *clock*, per2 and *cry1* decreased steadily from the beginning of the scotophase. In addition, one-way ANOVA also revealed statistically significant differences in gene expression between time points for all genes with the exception of *cry2* (Fig. 2).

3.2.2. Daily rhythms of expression of transcription factor genes

Daily rhythms of expression were found in three out of the five transcription factors studied, with the exceptions being *srebp1* and *srebp2* that did not show any significant rhythmicity (Fig 3; Table 2). With the expression of both *lxr* and *pparα*, acrophase was attained soon after the lights were switched on (ZT2 and ZT5, respectively), whereas for *pparγ* the acrophase was observed at the end of the dark phase (ZT24; Fig. 3). The levels of expression between time points was significant in the case of *lxr* and *pparγ,* with the lowest copy numbers of mRNA at ZT12 for *lxr* and between ZT8 and ZT16 for *pparγ*.

3.2.3. Daily rhythms of expression of lipid metabolism genes

Within the lipogenic genes (*fadsd2* and *elovl5*), only *elovl5* displayed a significant rhythmic pattern of expression, with the acrophase at ZT6 (Fig. 4; Table 2). One-way ANOVA also showed statistically significant differences in expression in *elovl5* between time points.

Among the lipolytic genes (*pla2*, *bal1*, *bal2* and *lpl*), only *pla2* and *lpl* displayed rhythmicity, both showing their acrophase in the second half of the photophase (ZT15 and ZT9, respectively; Fig. 5; Table 2). The bile-activated lipase (*bal1* and *bal2*) genes did not show any rhythmic expression, although both genes displayed a similar pattern of expression, peaking at ZT8. None of the lipolytic genes showed significant differences in expression between time points.

3.2.4. Daily rhythms of expression of protein and carbohydrates digestive enzymes

Of the four protein and carbohydrate digestive enzymes tested, only *alp* exhibited a significant daily rhythm, with acrophase occurring at the beginning of the scotophase (ZT16; Fig. 6; Table 2). One-way ANOVA indicated there were no significant differences in expression among the sampling points for any of the protein and carbohydrate digestive enzymes genes analyzed.

An acrophase map was plotted, including the genes that displayed rhythmicity in brain and liver (Fig. 7). It can be observed how the acrophases of the clock genes (*clock*, *bmal1*, *cry1*, *per1* and *per2*) were coupled in brain and liver, achieving the acrophases at similar times. The expression of the transcription factors *pparα* and *lxr* peaked during the first half of the photophase, whereas *pparγ* showed an acrophase in the dark phase. Similarly, the digestive enzymes *pla2* and *alp* showed the highest expression level in the scotophase, whereas the lipase *lpl* peaked towards the end of the photophase.

4. Discussion

The present study described for the first time the comparison between the central and peripheral (liver) clock mechanisms in ABT. Moreover, daily rhythmic expression patterns of specific genes of lipid metabolism and digestion in liver of ABT were demonstrated, which indicated which molecular control mechanisms were involved. Here, partial cDNA sequences of six core clock genes (*bmal1*, *clock*, *per1*, *per2*, *cry1* and *cry2*) were obtained in ABT. The results showed that, other than *cry2* in liver, all these clock genes were synchronized in both central (brain) and peripheral (liver) oscillators. These data, are in agreement with those reported in other teleost species where a daily rhythm of expression was also observed in brain (Davie et al., 2009; Velarde et al., 2009; López-Patiño et al., 2011, Vera et al., 2013). Several external entrainers, such as photoperiod or feeding regime, are known to impact the rhythmicity of the core clock genes at both central and peripheral levels (Vera et al., 2013, Betancor et al., 2014; Paredes et al., 2014; Paredes et al., 2015). Indeed, light has been identified as the strongest synchronizer in the brain of zebrafish (*Danio rerio*) (López-Olmeda and Sánchez-Vázquez, 2010b) and sea bream (Vera et al., 2013). The expression of genes belonging to the positive limb of the clock transcriptional loop, *clock* and *bmal1*, peaked at the end of the photophase (~ ZT11 and ZT12, respectively) in ABT kept under a 14L:10D photoperiod. Similarly, the expression of *bmal1* peaked at dusk in the brain of sea bream regardless of the feeding regime (Vera et al., 2013) whereas, in other teleosts, the expression of both genes was found to be maximal at the L:D transition (Whitmore et al., 1998; López-Patiño et al., 2011). The different acrophases displayed by different species could be due to different feeding habits.

Regarding the core negative elements of the transcriptional loop, *per1* and *per2*, both showed maximum expression during the first part of the photophase (~ ZT0 and ZT3) in brain samples, which is in agreement with studies in other teleosts (Vatine et al., 2011; Vera et al., 2013). Conversely, the other two genes from the negative limb, *cry1* and *cry2*, displayed their acrophase either at the end of the photophase (~ ZT12) or at the end of the scotophase (~ ZT23), respectively. Similar results for *per2* were observed in sea bream kept in a L:D photoperiod and fed in the middle of the light phase (Vera et al., 2013) and in Atlantic salmon maintained on a short-day photoperiod (Davie et al., 2009). In agreement with previous studies, expression of *per1*, *per2* and *cry2* was in antiphase with those from the positive arm of the regulatory loop, a common finding in brain and other peripheral entrainer tissues of teleosts (Davie et al., 2009; Vera et al., 2013; Lazado et al., 2014), which reflects the feedback loop between the positive and negative arms of the core clock. It should be noted that the expression levels of *per2* differed between ZT0 and ZT24 in both brain and liver, which may reflect the fact that the samples were collected in light (ZT0) and darkness (ZT24). A previous study in zebrafish showed that the promoter of *per2* contained a light responsive module with D-box enhancer element that directs light driven expression and is independent of the BMAL/CLOCK activity (Vatine et al., 2009).

In the present study, the daily expression patterns of the clock genes were evaluated in ABT liver for the first time. Out of the six studied genes, only *cry2* failed to show significant rhythmicity. The circadian clock system in fish is more complex than it is in mammals due to the existence of several paralogs per mammalian ortholog (Vatine et al., 2011; Lazado et al., 2014). This is probably a consequence of the teleost-specific third round of whole genome duplication (Woods et al., 2005) and, therefore, differences in daily expression might be related to functional diversification, as has been suggested in Atlantic cod (*Gadus morhua*) (Lazado et al., 2014). Indeed, *cry* and *per* genes are known to present different acrophases depending on the isoform (Vatine et al., 2011) which, in the light of the present results, suggests that *cry2* plays a tissue-specific role in ABT, and is not functional in liver. In agreement, *cry2* and *cry3* did not present daily rhythmicity in fast muscle of *Siniperca chuatsi*, whereas daily rhythms for these genes were observed in slow muscle (Wu et al., 2016). In contrast, the acrophases for the five clock genes that displayed rhythmicity in liver were generally the same as observed in brain, which was expected given the experimental design as the fasting period probably allowed the liver clock to come into synchrony with the brain central clock as observed previously in a teleost (Feliciano et al., 2011).

In the present trial, the link between the daily expression of key components of the core clock and genes of lipid metabolism, including the main transcription factors and their target genes, was investigated in ABT liver. As the specific feeding regime employed can affect hepatic expression of

clock core genes in some teleost species (Vera et al., 2013; Paredes et al., 2014, 2015), it is reasonable to suggest that the expression of these genes is linked with the metabolic functions of the liver. In addition to being a peripheral synchronizer, liver is the main tissue regulating lipid metabolism and it is well-known that lipid metabolism follows a circadian rhythm in mammals (Piccione et al., 2003; Alila-Johansson et al., 2004; Bertolucci et al., 2008). In particular, Pparα and Pparγ have direct interactions with the core clock genes (Inoue et al., 2005; Wang et al., 2008) that, in turn, suggests that these transcription factors may act as molecular links between daily rhythm and energy metabolism. The expression of *pparα* displayed a diurnal rhythm in ABT liver (~ ZT5) in agreement with mouse (Yang et al., 2006) and Atlantic salmon (*Salmo salar*) (Betancor et al., 2014), which was in contrast to previous studies in zebrafish and sea bream (Paredes et al., 2014, 2015), where a nocturnal rhythm was observed regardless of the feeding protocol employed.

In contrast, the peak of *pparγ* expression was observed at the end of the scotophase (~ ZT23), and was still high during the first hours of the photophase. One function of Pparγ is to act as a lipid regulator playing an important role in lipid accumulation (Fajas et al., 2001). In this respect, different rat tissues displayed an increase in *pparγ* mRNA levels when the fast was broken (Escher et al., 2001). The increase in *pparγ* at the very end of the dark phase appears to suggest that ABT liver anticipates the forthcoming meal, potentially increasing the capacity of the liver to better utilize high fat diets. The capacity to anticipate an upcoming meal is a valuable adaptation as it promotes behavioural and physiological changes to improve the the efficiency of how nutrients are utilized, as has been previously described in several teleost species (López-Olmeda & Sánchez-Vázquez, 2010a).

Unexpectedly, neither *srebp* analyzed nor the Srebp target gene *fads2* displayed a rhythmic pattern of expression, in contrast to other studies in teleosts (Betancor et al., 2014; Paredes et al., 2014, 2015). Nonetheless, another Srebp target gene involved in LC-PUFA biosynthesis, *elovl5*, showed a rhythmic expression pattern (acrophase ~ ZT6). It should be noted that, in teleosts, the expression levels of *fads2* or *elovl5* are affected by dietary lipid content and/or fatty acid profiles (Thomassen et al., 2012; Betancor et al., 2015, 2019) and, therefore, the presence/lack of rhythmicity observed in the different studies might be related to variations in dietary levels of these essential nutrients. In the case of ABT, this might be key given that, firstly, requirements of LC-PUFA for this species have not yet been accurately determined and, secondly, ABT are top predators known to possess very limited *elovl5* activity (Morais et al., 2011; Betancor et al., 2019). On the other hand, Lxr, a nuclear receptor that regulates the metabolism of several important lipids and is known to activate the transcription of *srebp* genes (Schultz et al., 2000) exhibited a diurnal rhythm, achieving its zenith at ~ ZT3. The uncoupling of *srebp* and *lxr* expression levels could be related to the role that Lxr plays in cholesterol metabolism. In mammals, Lxrα is activated by binding oxysterols (cholesterol

metabolites) that are a consequence of increased intracellular cholesterol (Repa et al., 2000). Therefore, as feeding starts when lights switch on, enhanced circulating cholesterol levels lead to enhanced *lxr* mRNA copy numbers in ABT. In a previous study in Atlantic salmon, the acrophase of *lxr* was observed at dusk (Betancor et al., 2014) but, in this case, fish were fed continuously during the light and the dark phase and it is known that feeding time can shift the acrophase of this gene in zebrafish liver (Paredes et al., 2015).

It has been demonstrated that when the feeding protocol mimics the natural feeding pattern, enhanced growth and use of nutrients is attained in several teleost species (Azzaydi et al., 2000; Hossain et al., 2001), which could indicate that animal can be adapted to effectively utilize the nutrients provided. Limited information exists about the feeding behaviour of ABT, although surveys of wild specimens have indicated that guts are empty in fish captured at night, speculating that ABT is a diurnal feeder (Butler et al., 2009), and this is why experimental ABT were only fed during the photophase. Despite constant feeding during the photophase, only three of the eight digestive enzymes investigated in the present study displayed rhythmicity, two of them showing acrophase at dusk (*pla2* and *alp*, ~ ZT16) and *lpl* during the light phase (~ ZT9). The main role of lpl, a lipase showing high mRNA copy numbers in liver of ABT (Betancor et al., 2017b), is to hydrolyze TAG in plasma lipoproteins and, consequently, to supply free fatty acids for either storage in adipose tissue or for oxidation as an energy source in other tissues (Nilsson-Ehle et al., 1980; Kersten, 2014; Ayisi et al., 2018). Therefore, the peak of expression of this gene is expected to occur in the period of greater feeding activity, which should be at the photophase in the case of ABT. In agreement, Senegalese sole (*Solea senegalensis*) post-larvae, nocturnal feeders, showed an increase in overall lipase activities during the night, matching their highest locomotor activity phase (Navarro-Guillén et al. 2015). Indeed, it would have been very interesting to study feeding activity of captive ABT in order to determine exactly their feeding periods as the existence of more than one feeding period could explain why the acrophases for the other two digestive enzymes (*pla1* and *alp*) occurred at dusk and not during the light phase. Another possible explanation for this could be a preference to use some nutrients more than others for energy and growth. Intermediary metabolism in carnivorous fish, such as ABT, is dominated by lipid and protein pathways given that carbohydrate is only a minor component of their natural diet (Tocher, 2003; Mourente and Tocher, 2003, 2009). This could explain the lack of rhythmicity in enzymes related to carbohydrate digestion such as amylase, whereas the lack of rhythmicity in protein digestive enzymes such as *tryp* and *anpep* could be due to the preference for lipid rather than protein as a substrate for energy.

To conclude, the present study has provided the first evidence for the daily expression of clock genes in both the brain and the liver of ABT under a 14L:10D cycle, which suggests the presence of a

peripheral clock in liver. Additionally, rhythmic expression of genes involved in lipid metabolism in the liver of ABT was also demonstrated for the first time, with transcription factors showing a strong rhythmic expression that reinforces the theory that these are potentially coupled to the core transcription and translation feedback loop to maintain oscillation in the organism. Given that only one photoperiod and feeding regime was employed, it is not possible to identify the main entrainer for each tissue but, with these experimental conditions, most of the studied genes were synchronized. In future it would be useful to evaluate the locomotor and feeding activity of ABT in order to obtain further insight, particularly regarding the expression patterns of digestive enzymes. Taken together, the results show clear differences in lipid metabolism and digestive physiology in ABT compared to other marine carnivorous/omnivorous teleost species, which suggests the need for the development of specific zootechnical/feeding protocols for this iconic and highly valued species.

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References

Alila-Johansson, A., Eriksson, L., Soveri, T., Laakso, M.L., 2004. Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (*Capra hircus*) under different photoperiods. Comp. Biochem. Physiol. 138A, 119-131.

Ayisi, C.L., Yamei, C., Zhao, J.-L., 2018. Genes, transcription factors and enzymes involved in lipid metabolism in fin fish. Agri. Gene 7, 7–14.

Azzaydi, M., Martínez, F.J., Zamora, S., Sánchez-Vázquez, F.J., Madrid, J.A., 2000. The influence of nocturnal vs. diurnal feeding under winter conditions on growth and feed conversion of European sea bass (*Dicentrarchus labrax*, L.). Aquaculture 182, 329–338.

Bertolucci, C.F., Fazio, F., Piccione, G., 2008. Daily rhythms of serum lipids in dogs: Influences of lighting and fasting cycles. Comp. Med. 58, 485-489.

Betancor, M.B., McStay, E., Minghetti, M., Migaud, H., Tocher, D.R., Davie, A., 2014. Daily rhythms in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). PLoS ONE 9, e106739.

Betancor, M.B., Sprague, M., Usher, S., Sayanova, O., Campbell, P.J., Napier, J.A., Tocher, D.R., 2015. A nutritionally – enhanced oil from transgenic Camelina sativa effectively replaces fish oil as a source of eicosapentaenoic acid for fish. Sci. Rep. 5, 8104.

Betancor, M.B., Ortega, A., De la Gandera, F., Tocher, D.R., Mourente, G., 2017a. Lipid metabolism-related gene expression pattern of Atlantic bluefin tuna (*Thunnus thynnus*, L.) larvae fed on live prey. Fish. Physiol. Biochem. 43, 493-516.

Betancor, M.B., Ortega, A., De la Gandera, F., Tocher, D.R., Mourente, G., 2017b. Molecular aspects of lipid metabolism, digestibility and antioxidant status of Atlantic bluefin tuna (*T. thynnus* L.) larvae during first feeding. Aquaculture 479, 357-369.

Betancor, M.B., Ortega, A., de la Gándara, F., Tocher, D.R., Mourente, G., 2019. Performance, feed utilization and hepatic metabolic response of weaned juvenile Atlantic Bluefin tuna (*Thunnus thynnus* L.): Effects of dietary lipid level and source. Fish Physiol. Biochem. 45, 697-718.

Butler, C.M., Rudershausen, P.J., Buckel, J.A., 2009. Feeding ecology of Atlantic Bluefin Tuna (*Thunnus thynnus*) in North Carolina: diet, daily ration, and consumption of Atlantic menhaden (*Brevoortia tyrannus*). Fish. Bull. 108, 56-59.

Cahill, G.M., 2002. Clock mechanisms in zebrafish. Cell Tissue Res. 309, 27-34.

Cruz-García, L., Minghetti, M., Navarro, I., Tocher, D.R., 2009. Molecular cloning, tissue expression and regulation of Liver X Receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. 153B, 81–88.

Davie, A., Minghetti, M., Migaud, H., 2009. Seasonal variations in clock gene-expression in Atlantic salmon (*Salmo salar*). Chronobiol. Int. 26, 379-395.

DeCoursey, P.J., 2004. Overview of biological timing from unicells to humans. In: Dunlap JC, Loros JJ, DeCoursey PJ, editors. Chronobiology: Biological Timekeeping. Sunderland, MA: Sinauer; 2004. pp. 3–24.

Escher, P., Braissant, O., Basu-Modak, S., Michalik, L., Wahli, W., Desvergne, B., 2001. Rat PPARs: Quantitative analysis in adult rat tissues and regulation in fasting and refeeding. Endocrinology 142, 4195-4202.

Evans, R., Barish, G.D., Wang, Y.X., 2004. PPARs and the complex journey to obesity. Nat. Med. 20, 355–361.

Fajas, L., Debril, M.B., Auwerx, J., 2001. Peroxisome proliferator-activated receptor gamma: from adipogenesis to carcinogenesis. J. Mol. Endocrinol. 27, 1-9.

Feliciano, A., Vivas, Y., de Pedro, N., Delgado, M.J., Velarde, E., Isorna, E., 2011. Feeding time synchronizes clock gene rhythmic expression in brain and liver of goldfish (*Carassius auratus*). J. Biol. Rhythms 26, 24-33.

Hossain, M.A.R., Haylor, G.S., Beveridge, M.C.M., 2001. Effect of feeding time and frequency on the growth and feed utilization of African catfish *Clarias gariepinus* (Burchell 1822) fingerlings. Aquac. Res. 32, 999–1004.

Inoue, I., Shinoda, Y., Ikeda, M., Hayashi, K., Kanazawa, K., Nomura, M., Matsunaga, T., Xu, H., Kawai, S., Awata, T., Komoda, T., Katayama, S., 2005. CLOCK/BMAL1 is involved in lipid metabolism via transactivation of the Peroxisome Proliferator – Activated Receptor (PPAR) response element. J. Atheroscler. Thromb. 12, 169-174.

Kersten, S., 2014. Physiological regulation of lipoprotein lipase. BBA- Mol. Cell Biol. Lipids. 1841, 919–933.

Lazado, C.C., Kumaratunga, H.P.S., Nagasawa, K., Babiak, I., Giannetto, A., Fernandes, J.M.O., 2014. Daily rhythmicity of clock gene transcripts in Atlantic cod fast skeletal muscle. PLoS ONE 9, e99172.

Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B., Lo Sasso, G., Moschetta, A., Schibler, U., 2009. REV-ERB alpha participates in circadian SREBP signalling and bile acid homeostasis. PLoS Biol 7.

López-Olmeda, J.F., Sánchez- Vázquez, F.J., 2010a. Feeding rhythms in fish: from behavioural to molecular approach. In: Biological Clock in Fish. Edited By Ewa Kulczykowska, Wlodzimierz Popek, B.G. Kapoor (300 pages, Chapter 8). Published by CRC Press, Boca Raton.

López-Olmeda, J.F., Sánchez-Vázquez, F.J., 2010b. Feeding entrainment of food-anticipatory activity and per1 expression in the brain and liver of zebrafish under different lighting and feeding conditions. Chronobiol. Int. 27, 1380-1400.

Lopez-Olmeda, J.F., Sánchez-Vázquez, F.J., 2011. Thermal biology of zebrafish (*Danio rerio*). J. Therm. Biol. 36, 91-104.

López-Patiño, M.A., Rodríguez-Illamola, A., Conde-Sieira, M., Soengas, J.L., Míguez, J.M., 2011. Daily rhythmic expression patterns of Clock1a, Bmal1 and Per1 genes in retina and hypothalamus of the rainbow trout, *Oncorhynchus mykiss*. Chronobiol. Int. 28, 381–389.

Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R., 2011. Expression of fatty acyl desaturase and elongase genes, and evolution of DHA/EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 313, 129-139.

Mourente, G., Tocher, D.R., 2003. An approach to study the nutritional requirements of the bluefin tuna (*Thunnus thynnus* L.). Cah. Options Mediterr. 60, 143-150.

Mourente, G., Tocher, D.R., 2009. Tuna nutrition and feeds: Current status and future perspectives. Rev. Fish. Sci. Aquac. 17, 373-390.

Navarro-Guillén, C., Moyano, F.J., Yúfera, M., 2015. Diel food intake and digestive enzyme production patterns in *Solea senegalensis* larvae. Aquaculture 435, 33–42.

Nilsson-Ehle, P., Grafinkel, A.S., Schotz, M.C., 1980. Lipolytic enzymes and plasma lipoprotein metabolism. Annu. Rev. Biochem. 49, 667-693.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Kay, S.A., Takahashi, J.S., Hogonesch, J.B., 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307-320.

Paredes, J.F., Vera, L.M., Navarro, I., Martínez-López, F.J., Sánchez-Vázquez, F.J., 2014. Circadian rhythms of gene expression of lipid metabolism in Gilthead sea bream liver: synchronization to light and feeding time. Chronobiol. Int. 31, 613-626.

Paredes, J.F., López-Olmeda, J.F., Martínez, F.J., Sánchez-Vázquez, F.J., 2015. Daily rhythms of lipid metabolic gene expression in zebrafish liver: Response to light/dark and feeding cycles. Chronobiol. Int. 32, 1438-1448.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. 678 Nucleic Acid Res. 29, e45.

Piccione, G., Caola, G., Refinetti, R., 2003. Circadian rhythms of body temperature and liver function in fed and food-deprived goats. Comp. Biochem. Phys. 134 A, 563-572.

Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M.A., 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. Genes Dev. 14, 2819-2830.

Sato, R., 2010. Sterol metabolism and SREBP activation. Arch Biochem Biophys 501: 177-181.

Schibler, U., Sassone-Corsi, P., 2002. A web of circadian pacemakers. Cell 111, 919-922.

Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolem, M., Mangelsdorf, D.J., Lustig, K.D., Shan, B., 2000. Role of LXRs in control of lipogenesis.

Thomassen, M.S., Berge, R.D., Gerd, M., Østbye, T.K., Ruyter, B., 2012. High dietary EPA does not inhibit Δ5 and Δ6 desaturases in Atlantic salmon (*Salmo salar* L.) fed rapeseed oil diets. Aquaculture 360-361, 78-85.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. Aquac. 11, 107-184.

Untergasser, A., Cutcutache, I., Koressaar, T, Ye J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40e115.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3: RESEARCH 0034.

Vatine, G., Vallone, D., Appelbaum, L., Mracek, P., Ben-Moshe, Z., Lahiri, K., Gothilf, Y., Foulkes, N.S., 2009. Light directs zebrafish period2 expression via conserved D and E boxes. PLoS Biol. 7, e1000223.

Vatine, G., Vallone, D., Gothilf, Y., Foulkes, N.S., 2011. It's time to swim! Zebrafish and the circadian clock. FEBS Letters 585, 1485-1494.

Velarde, E., Haque, R., Iuvone, P.M., Azpeleta, C., Alonso-Gómez, A.L., Delgado, M.J., 2009. Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of Period and Cryptochrome transcripts in retina, liver and gut. J. Biol. Rhythms 24, 104-113.

Vera, L.M., Negrini, P., Zagatti, C., Frigato, E., Sánchez-Vázquez, F.J., Bertolucci, C., 2013. Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*). Chronobiol. Int. 30, 649-661.

Wang, N., Yang, G., Jia, Z., Zhang, H., Aoyagi, T., Soodvilai, S., Symons, J.D., Schnermann, J.B., Gonzalez, F.J., Litwin, S.E., Yang, T., 2008. Vascular PPARγ controls circadian variation in blood pressure and heart rate through Bmal1. Cell Metabolism 8, 482-491.

Welsh, D.K., Logothetis, D.E., Meister, M., Reppert, S.M., 1995. Individuals neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14, 697-706.

Whitmore, D., Foulkes, N.S., Strahle, U., Sassone-Corsi, P., 1998. Zebrafish clock rhythmic expression reveals independent peripheral circadian oscillators. Nat. Neurosci. 1, 701-707.

Woods, I.G., Wilson, C., Friedlander, B., Chang, P., Reyes, D.K., Nix, R., Kelly, P.D., Chu, F., Postlethwait, J.H., Talbot, W.S., 2005. The zebrafish gene map defines ancestral vertebrate chromosomes. Genome Res. 15, 1307-1314.

Wu, P., Li, Y.L., Cheng, J., Zhu, X., Feng, Z.G., Zhang, J.S., Chu, W.Y., (2016) Daily rhythmicity of clock gene transcript levels in fast and slow muscle fibers from Chinese perch (*Siniperca chuatsi*). BMC Gen 17, 1008.

Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Traume, M., Mangelsdorf, D.J., Evans, R.M., 2006. Nuclear receptor expression links the circadian clock to metabolism. Cell 126, 801-810.

Figure legends

Figure 1. Twenty-four hour expression profiles of clock genes in the brain of Atlantic bluefin tuna juveniles acclimated to LD photoperiod. White and black bars on the bottom indicate the light and dark periods, respectively. Results are displayed in relation to Zeitgeiber time (ZT), where ZT0 is the onset of light. Data are shown as the mean ± SE (n = 4). Superscript letters denote statistically significant differences between sampling points according to one-way ANOVA and Tukey's post-hoc test. The presence of a cosine wave denotes a significant circadian rhythm by acro analysis.

Figure 2. Twenty-four hour expression profiles of clock genes in the liver of Atlantic bluefin tuna juveniles acclimated to LD photoperiod. White and black bars on the bottom indicate the light and dark periods, respectively. Results are displayed in relation to Zeitgeiber time (ZT), where ZT0 is the onset of light. Data are shown as the mean ± SE (n = 4). Superscript letters denote statistically significant differences between sampling points according to one-way ANOVA and Tukey's post-hoc test. The presence of a cosine wave denotes a significant circadian rhythm by acro analysis.

Figure 3. Twenty-four hour expression profiles of transcription factor genes in the liver of Atlantic bluefin tuna juveniles acclimated to LD photoperiod. White and black bars on the bottom indicate the light and dark periods, respectively. Results are displayed in relation to Zeitgeiber time (ZT), where ZT0 is the onset of light. Data are shown as the mean ± SE (n = 4). Superscript letters denote statistically significant differences between sampling points according to one-way ANOVA and Tukey's post-hoc test. The presence of a cosine wave denotes a significant circadian rhythm by acro analysis.

Figure 4. Twenty-four hour expression profiles of lipogenic genes in the liver of Atlantic bluefin tuna juveniles acclimated to LD photoperiod. White and black bars on the bottom indicate the light and dark periods, respectively. Results are displayed in relation to Zeitgeiber time (ZT), where ZT0 is the onset of light. Data are shown as the mean ± SE (n = 4). Superscript letters denote statistically significant differences between sampling points according to one-way ANOVA and Tukey's post-hoc test. The presence of a cosine wave denotes a significant circadian rhythm by acro analysis.

Figure 5. Twenty-four hour expression profiles of lipid digestive enzyme genes in the liver of Atlantic bluefin tuna juveniles acclimated to LD photoperiod. White and black bars on the bottom indicate the light and dark periods, respectively. Results are displayed in relation to Zeitgeiber time (ZT), where ZT0 is the onset of light. Data are shown as the mean ± SE (n = 4). Superscript letters denote statistically significant differences between sampling points according to one-way ANOVA and Tukey's post-hoc test. The presence of a cosine wave denotes a significant circadian rhythm by acro analysis.

Figure 6. Twenty-four hour expression profiles of carbohydrate and protein digestive genes in the liver of Atlantic bluefin tuna juveniles acclimated to LD photoperiod. White and black bars on the bottom indicate the light and dark periods, respectively. Results are displayed in relation to Zeitgeiber time (ZT), where ZT0 is the onset of light. Data are shown as the mean ± SE (n = 4). Superscript letters denote statistically significant differences between sampling points according to one-way ANOVA and Tukey's post-hoc test. The presence of a cosine wave denotes a significant circadian rhythm by acro analysis.

Figure 7. Map of the acrophases of the clock (*clock*, *bmal1*, *per1*, *per2*, *cry1* and *cry2*), transcription factor (*lxr*, *pparα* and *pparγ*), lipogenic (*elovl5*) and digestive enzyme (*pla2*, *alp*, *lpl*) genes analysed

in liver and/or brain of Atlantic bluefin tuna subjected to 14L:10D photoperiod. Data are shown as the mean acrophase ± SE (n = 4).

Table 1. Sequence, annealing temperature (Tm) and fragment size produced by the primer pairs used for quantitative PCR (qPCR).

Name	Sequence (5'-3')	Amplicon size (bp)	Tm ºC
bmal1	F: AAAGACTTCCCCTCCACCTG	238	60
	R: AGACGTACTCTGTGGGCTTC		
clock	F: AGACACATCCAAGAGCAGCT	187	60
	R: TGAGCTGCTGAGTTCCTGAA		
per1	F: AGATGCGCTACTACCCCTTC	183	60
	R: GGAAAAGGCAGTTGGGAGTG		
per2	F: TCCTCTGTTGGGTTTCCTCC	250	60
	R: GACTTTGTGCCTGCCAATGA		
cry1	F: AGACCACGGAGACAAGTACG	178	60
	R: AGTTGGCATTCATCCTGCGA		
cry2	F: GTAAGGAGAGGGATGGAGCC	180	60
	R: TCCAGTCTGCTCACAATGGT		
srebp1	F: CCAGCTACACATGACAGGGA	153	59
	R: GCTTTGACCCTTAGAGCTGC		
srebp2	F: AGATCCAGTGAGTCGTTGGT	212	60
	R: CTACAGCCCCTTCTCCCTTC		
lxr	F: CACACTGGATCCACAACAGC	192	58
	R: ATCTCCTGCACCGACATGAT		
pparα	F: TGGTCATGGAGGTGGAAGAC	152	60
	R: ATGGATGACGAAAGGAGGGG		
pparγ	F: ACCTGACCAACATGGACTAC	118	60
	R: GAGAAAACAGGACTGTCAGC		
fads2d6	F: CCGTGCACTGTGTGAGAAAC	152	60
	R: CAGTGTAAGCGATAAAATCAGCTG		
elovl5	F: CCACGCTAGCATGCTGAATA	236	60
	R: ATGGCCATATGACTGCACAC		
lpl	F: CCGAAAGAACCGCTGCAATA	212	59
	R: GATCCTCCTTCTCTCCGTGG		
pla2	F: GGATGATCTGGACAGGTGCT	217	59
	R: TCTGGCAAAACACTCAACGG		
bal1	F: CATGGATGGACACCTCTTTACTGGT	126	59
	R: AAACCAGCCTGGCCCTTCTCTTTAG		
bal2	F:GGATGGGCACCTCTTCACATCACAG	120	59
	R: CCAGCTTGGCCCTTCTCTTTGGTAT		
alp	F: ACTCTGACAACGAGATGCCA	189	60
	R: TTCCGTCTTTTCTTGTGCCG		
anpep	F: CCTGAGGTGGTGGAATGACT	82	60
	R: GGGTTCAGCTTTGTCTGCTC		
amy	F: TCATGTGGAAGCTGGTTCAG	105	60
	R: AATATTGCCACTGCCAGTCC		
tryp	F: CCCCAACTACAACCCCTACA	145	60
	R: CCAGCCAGAGACAAGACACA		
ef1α	F: CCCCTGGACACAGAGACTTC	119	60
	R: GCCGTTCTTGGAGATACCAG		

bmal1, Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; *clock*, Circadian Locomotor Output Cycles Kaput; *per1*, period 1; *per2*, period 2; *cry1*, cryptochrome 1; *cry2*, cryptochrome 2; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2; *lxr*, liver X receptor; *pparα*, peroxisome proliferator-activated receptor alpha; *pparγ*, peroxisome proliferator-activated receptor gamma; *fads2d6*, delta-6 fatty acyl desaturase; *elovl5*, fatty acyl elongase 5; *lpl*, lipoprotein lipase; *pla2*, phospholipase A2; *bal1*, bile salt

activated lipase 1; *bal2*, bile salt activated lipase 2; *alp*, alkaline phosphatase; *anpep*, amino peptidase; *amy*, amylase; *tryp*, trypsin; *ef1α*, elongation factor 1 alpha

Table 2. P value of 24 h profiles of gene expression Acro and ANOVA analysis and acrophase where significant rhythm is present.

Gene	Acro (p value)	Acrophase ZT	ANOVA (p value)
Liver			
bmal1	**	12:27 (11:01~13:52)	**
clock	**	13:58 (12:24~15:30)	**
per1	**	2:00 (23:57~3:59)	**
per2	**	7:12 (4:54~9:34)	**
cry1	**	12:50 (11:11~14:16)	**
cry2	n.s.	-	n.s.
lxr	**	2:50 (0:17~5:07)	*
srebp1	n.s.	-	n.s.
srebp2	n.s.	-	n.s.
pparα	**	4:58 (0:13~9:18)	n.s.
pparg	**	23:05 (19:28~6:55)	**
fads2d6	n.s.	-	n.s.
elovl5	**	6:34 (5:10~8:01)	**
pla2	*	15:37 (10:14~20:18)	n.s.
bal1	n.s.	-	n.s.
bal2	n.s.	-	n.s.
alp	**	16:38 (15:07~18:03)	**
amy	n.s.	-	n.s.
tryp	n.s.	-	n.s.
anpep	n.s.	-	n.s.
lpl	*	9:13 (5:38~13:29)	**
Brain			
bmal1	**	12:44 (11:03~14:23)	**
clock	*	11:32 (7:12~15:53)	n.s.
per1	**	0:20 (22:32~2:05)	**
per2	**	3:50 (0:33~6:40)	**
cry1	*	12:29 (8:13~16:36)	**
cry2	*	23:10 (19:20~27:08)	*

n.s., non-significant; * $p \leq 0.05$; ** $p \leq 0.01$

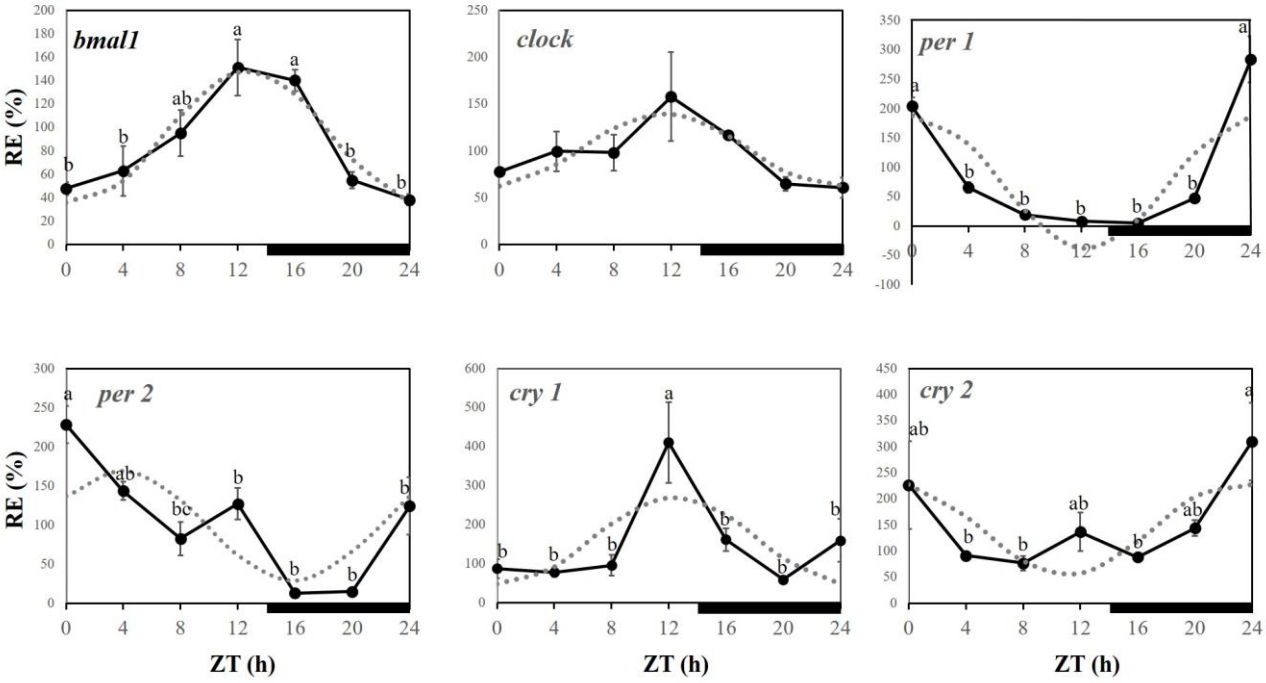

Figure 1

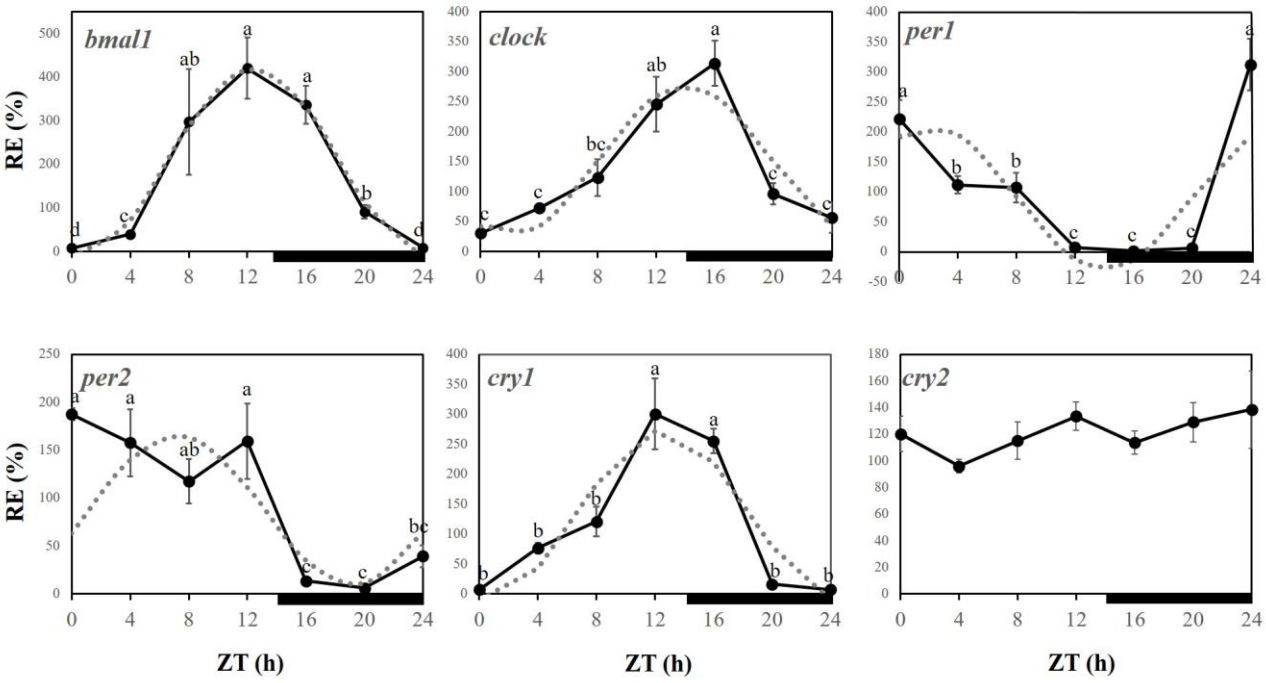

Figure 2

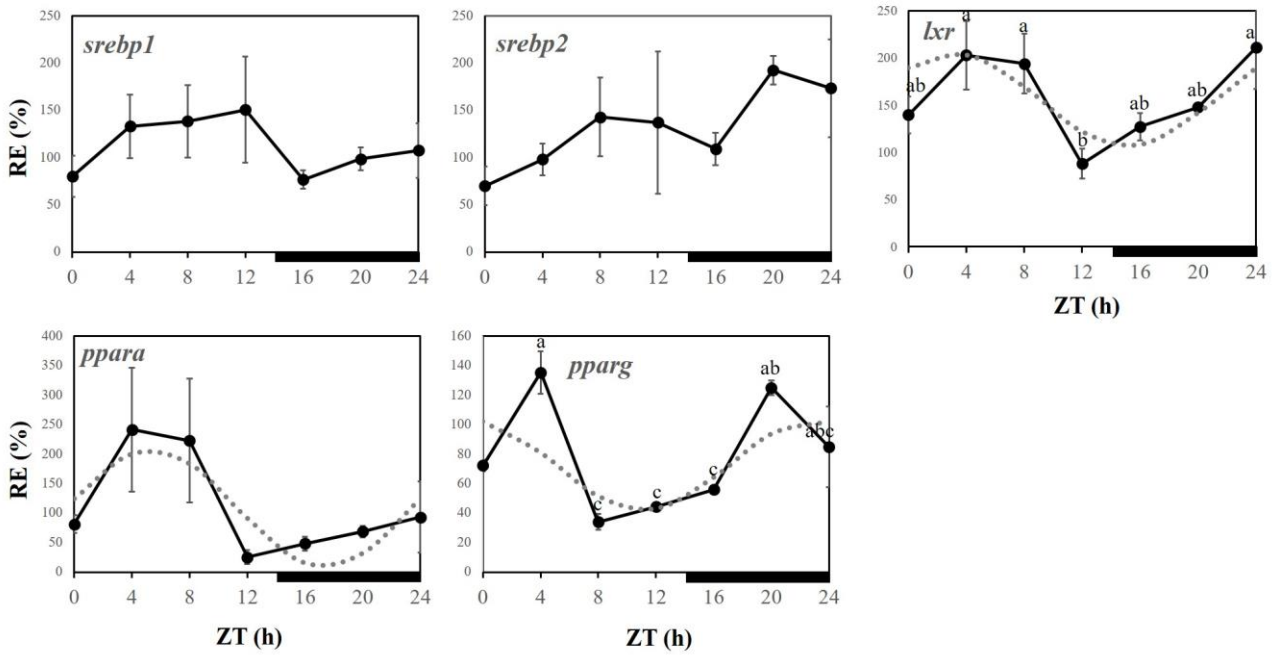

Figure 3

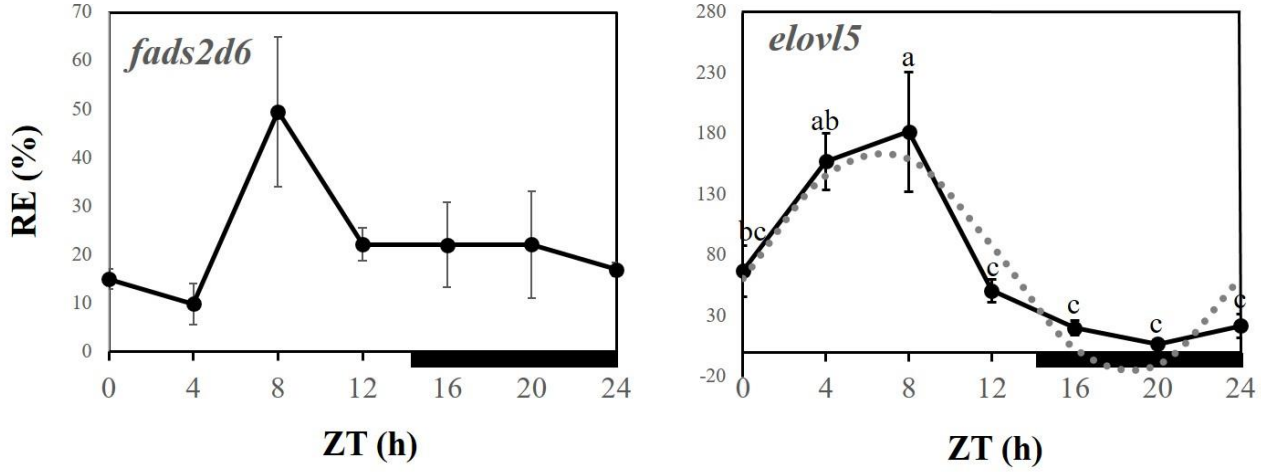

Figure 4

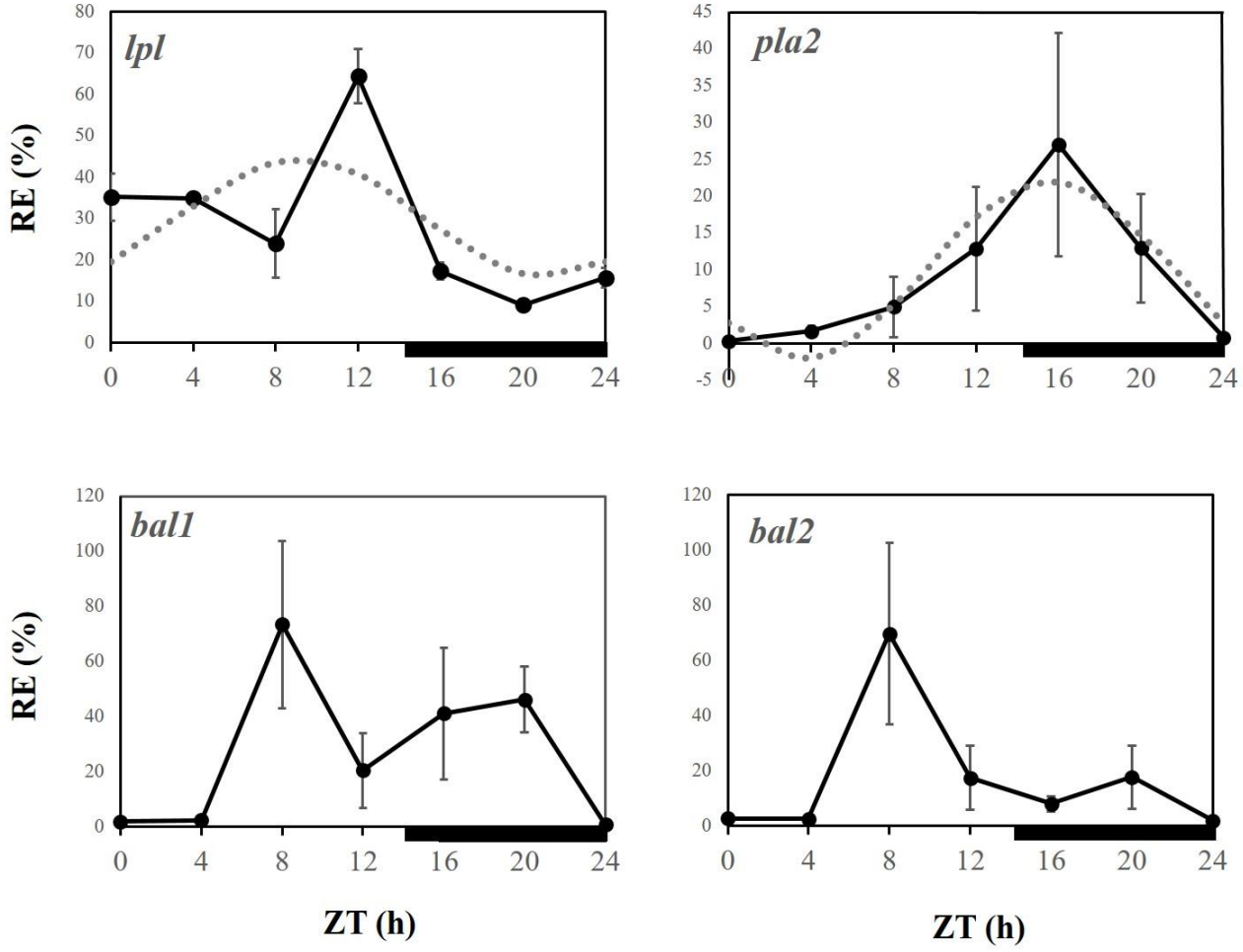

Figure 5

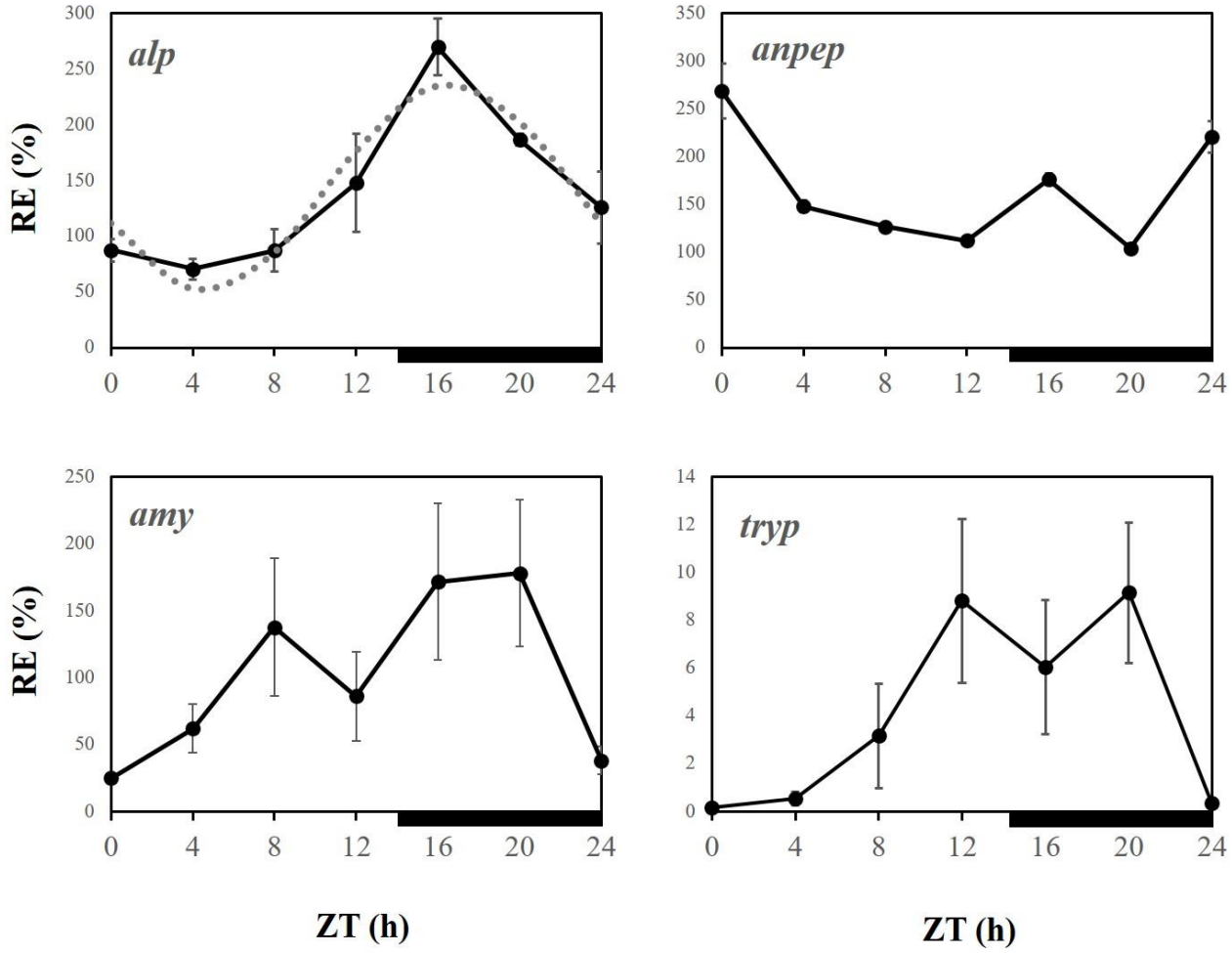

Figure 6

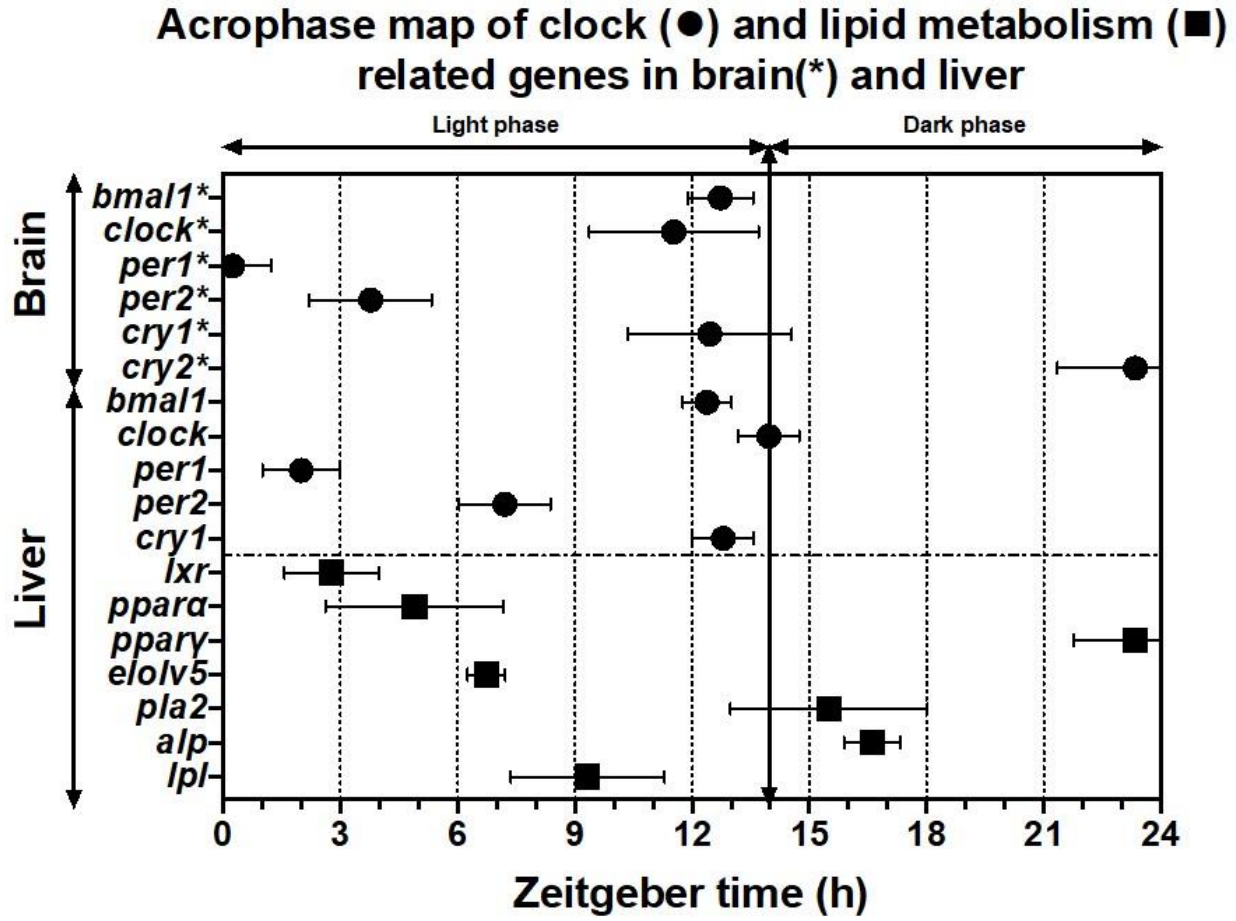
Acrophase map of clock (●) and lipid metabolism (■) related genes in brain(*) and liver
Light phase
Dark phase
Brain
Liver
bmal1*
clock*
per1*
per2*
cry1*
cry2*
bmal1
clock
per1
per2
cry1
lxr
pparα
pparγ
elolv5
pla2
alp
lpl
0
3
6
9
12
15
18
21
24
Zeitgeber time (h)

Figure 7