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Food Entrainment of Circadian Gene Expression Altered in PPARα-/- Brown Fat and Heart

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TABLE OF CONTENTS

Figure Legends

Figure 1. Circadian Gene Expression Profiles in Metabolic Tissues of Wild Type and PPARα-null Mice. The mRNA expression profile of "positive" (*Npas2, Bmal1*) and "negative" (*Per1*, *Per3*) circadian transcriptional regulators and their immediate downstream targets (*DBP*, *Rev-erbα*, *Rev-erbβ*) are displayed over a 24-hour period in brown adipose tissue (BAT), liver, and epididymal white adipose tissue (eWAT) mRNAs pooled from groups of n = 3 mice per time point. In this and all subsequent figures, the 24-hour expression profile has been duplicated to visually display evidence of circadian rhythmicity. The qRT-PCR values (mean ± S.D. of triplicate determinations) were normalized relative to *Cyclophilin B*. Cosinor analysis documented p values < 0.05 for all measurements in all tissues with the exception of *Per1* (wild type and PPARα-null BAT and wild type eWAT) and *Per3* (wild type BAT).

Figure 2. (A) Serum Levels of Cholesterol and Triglyceride in Wild Type and PPARα-null Mice under *Ad Libitum* and Temporally Restricted Food Access. The cholesterol and triglyceride levels in mg/dl (mean ± S.D. of n = 3 animals per time point) were determined in serum isolated from wild type (solid line) and PPARα-null (dotted line) mice with either *ad libitum* (ad lib) or temporally restricted (RF) food access. **(B) Expression of PPARα mRNA Levels in Tissues of Wild Type Mice under *Ad Libitum* and Temporally Restricted Food Access.** The PPARα mRNA expression levels are displayed in brown adipose tissue (BAT), heart, liver, and epididymal white adipose tissue (eWAT) isolated from wild type mice as a function of time under ad libitum (ad lib, solid line) and temporally restricted food access (RF dotted line). Values are the mean ± S.D. of triplicate qRT-PCR assays conducted using mRNA pools from n=3 animals per time point and normalized relative to *Cyclophilin B*. Cosinor

analysis documented p values < 0.05 for all measurements in all tissues with the exception of ad lib liver (p = 0.273) and RF heart (p = 0.244).

Figure 3. Circadian Gene Expression in Brown Adipose and Cardiac Tissue of Wild Type and PPARα-null Mice under *Ad Libitum* and Temporally Restricted Food Access. The mRNA expression of *Bmal1*, *Per3*, and *Rev-erbα* was determined in brown adipose tissue (BAT) and heart isolated from wild type (solid line) and PPARα-null (dotted line) mice with either *ad libitum* (ad lib) or temporally restricted (RF) food access. Values are the mean ± S.D. of triplicate qRT-PCR assays conducted using mRNA pools from n=3 animals per time point and normalized relative to *Cyclophilin B*. Cosinor analysis documented p values < 0.05 for all measurements in both tissues.

Figure 4. Circadian Gene Expression in Liver and Epididymal White Adipose Tissue of Wild Type and PPARα-null Mice under *Ad Libitum* and Temporally Restricted Food Access. The mRNA expression of *Bmal1*, *Per3*, and *Rev-erbα* was determined in liver and epididymal white adipose tissue (eWAT) isolated from wild type (solid line) and PPARα-null (dotted line) mice with either *ad libitum* (ad lib) or temporally restricted (RF) food access. Values are the mean ± S.D. of triplicate qRT-PCR assays conducted using mRNA pools from n=3 animals per time point and normalized relative to *Cyclophilin B*. Cosinor analysis documented p values < 0.05 for all measurements in both tissues with the exception of *Rev-erbα* in wild type liver RF (p = 0.112) and wild type eWAT ad lib (p = 0.081) and RF (p = 0.072).

Figure 5. Primer Oligonucleotide Sequences. These primer sequences were used to perform quantitative Real-Time PCR.

Abstract

The circadian clock is subject to food entrainment. Since PPARα exhibits a circadian expression profile, I hypothesized that a PPARα deficiency would alter the food entrainable response of adipose, cardiac and liver tissues. Wild type and PPARα-null mice were compared under *ad libitum* or restricted food access to the lights on period for the expression of circadian regulatory factor-encoding mRNAs. Temporally restricted food access caused a 4-8 hour phase shift in the expression profiles of the circadian genes *Bmal1*, *Per3,* and *Rev-erbα* in all tissues of control mice. These conditions phase shifted the circadian genes in brown adipose and cardiac tissues of PPARα-null mice by 8-16 hr with amplitude attenuation. The prolonged phase shift observed in the brown adipose and cardiac tissue circadian regulatory factors in the food entrained PPARα-null mice may contribute to their increased susceptibility to hypoglycemia and hypothermia during periods of fasting.

1. Introduction

Circadian rhythms play a major role in many, if not all, physiological processes, including energy consumption and expenditure [1]. Emerging evidence speculates that circadian dysregulation could even contribute to metabolic syndrome but there are limited findings that mechanistically link the two. The circadian clock, comprised of a highly conserved group of regulatory factors (*Clock*, *BMAL1*, *Period* (*Per*), and *Cryptochrome* (*Cry*)), regulates the oscillatory expression profile of downstream targets including albumin D binding protein (*DBP*) and the orphan nuclear hormone receptors, *Rev-erbα* and *Rev-erbβ* [1, 2]. While the suprachiasmatic nucleus (SCN) within the brain acts as the core circadian oscillator, recent findings indicate that autonomous circadian clocks exist within peripheral tissues [3, 4]. When organ cultures were prepared with peripheral tissues isolated from Per2 promoter/luciferase reporter transgenic mice, their luciferase expression profile displayed circadian rhythmicity for over 20 days *ex vivo* [4]. Moreover, the peripheral tissues maintained their circadian rhythmicity even after SCN ablation *in vivo*. Thus, the circadian mechanisms throughout the body can display some degree of independence.

The circadian clock is entrained by food exposure and access in addition to photic stimuli [1, 2]. Temporal restriction of food access has been found to alter animal behavioral activities, leading to the hypothesis that a "food entrainable oscillator (FEO)" exists within the brain or some other site within the organism [5-7, 29]. Restricted food access influences the expression profile of genes encoding the circadian transcription factors in the fat, liver, and other peripheral tissues [8-14]. When food access was restricted for AKR/J mice to the 12 hour lights on period for seven days, a phase shift was observed in the expression profile of multiple circadian

transcription factors (*Bmal1*, *Clock*, *Cry1-2*, *Npas2*, *Per1-3*) and their immediate downstream targets (*DBP*, *E4bp4*, *Id2*, *Rev-erbα*, *Rev-erbβ*) in brown and white adipose tissues and liver [8]. This correlated with both a phase shift and amplitude attenuation in serum corticosterone levels [8].

Previous research has shown that the alpha isoform of peroxisome proliferators-activated receptors (PPARα) exhibits circadian rhythmicity and plays a significant role in lipid metabolism [15-17]. PPARα is known to be a major transcriptional regulator in fatty acid oxidation (FAO) through several processes such as fatty acid transport and activation of acyl-CoA esters [15, 17]. Additionally, PPARα has been shown to display patterns of expression that are in coordination with the core circadian genes' profiles [16, 18, 19]. PPARα plays a critical metabolic role as evidenced by the hypothermia and hypoglycemia displayed by PPARα-null mice relative to wild type controls under conditions of prolonged fasting [20, 21]. Based on these findings, I postulated that the circadian response to food entrainment will differ in PPARα-null mice relative to their wild type controls. The current study tests this hypothesis by examining the impact of PPARα deficiency on the expression of circadian genes in peripheral tissues following temporally restricted food access.

2. Methods and Materials

2.1 Experimental Design

Protocols were reviewed by the Oklahoma Christian University and the Pennington Biomedical Research Center Institutional Animal Care and Use Committees. Founders of the breeding colony were provided by Dr. F. Gonzales (NCI, NIH) [15]. ***Experiment 1*** investigated the differences in gene expression between *ad libitum* fed wild type and PPARα-null mice. Eight to ten week old C57BL/6N x Sv/129 wild type (WT) male mice were compared against 8-10 week old C57BL/6N x Sv/129 homozygous PPARα-null male mice. Animals were acclimatized for 2 weeks to a regular chow diet (Purina 5015) with *ad libitum* access under a strict 12h light/ 12h dark cycle. After this period, groups of 3 animals from each genotype were sacrificed at 4 hour intervals over a 24-hour period. Liver, brown adipose tissue (BAT), epididymal adipose tissue (eWAT), and plasma were harvested, flash frozen in liquid nitrogen, and subsequently processed for total RNA isolation or cholesterol/triglyceride assay. ***Experiment 2*** examined the effect of temporally restricted feeding (RF). The study was conducted with groups of 40 wild type and 40 PPARα-null male mice (age ranges, 7-25 weeks) acclimatized to a regular chow diet (Purina 5015) and maintained on a strict 12h light/ 12h dark cycle. The wild type and PPARα-null mice were then sub-divided into two cohorts; an *ad libitum* cohort that retained free access to food and an experimental cohort whose feeding time was restricted to the 12-hour lights on period. This feeding schedule was maintained for 8 days until the time of harvest. Body weights and food intake were monitored daily for both cohorts. Animals from each cohort were then sacrificed as described in ***Experiment 1***.

2.2 Quantitative Real-Time PCR (RT-PCR)

Total RNA from the selected tissues was isolated and purified using TriReagent and 1-bromo-3-chloropropane, BCP (Molecular Research Center). 2μg of RNA was then reversely transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Promega) with Oligo dT at 42˚C for 1 hour in a 20μl working volume. The cDNA was then diluted into 1:100 for the liver and 1:25 solutions for the heart and fat depots. Primers for the selected circadian and lipid metabolism genes were designed using Primer Express (Applied Biosystems) (**Figure 5**). Real-Time PCR was then performed using the SYBR® Green PCR Master Mix (Applied Biosystems) on the 7900 Real Time PCR system (Applied Biosystems). Universal cycling conditions of 95°C for 10 min and 40 two step cycles consisting of 95°C for 15 sec followed by 60°C for 1 min were used. Genes of interest were normalized against *Cyclophilin B*, which has been shown to maintain the consistency of expression required for a "housekeeping" control in a manner similar to *actin* and *GAPDH* [8, 22]. All RT-PCR assays were performed in triplicate and the *Cyclophilin B*-normalized ratio data presented in the figures represents the mean ± standard deviation.

2.3 Cholesterol and Triglyceride Assays

Plasma cholesterol and triglyceride levels were determined using assays from Wako Chemicals (Richmond, VA) and Sigma Chemical (St. Louis, MO), respectively, and the replicates were averaged.

2.4 Statistical Analysis

Oscillation patterns and periodicity of the qRT-PCR and serum data were evaluated using Time Series Analysis-Single Cosinor v. 6.0 software (Expert Soft Technologie) [23]. Analyses with p values <0.05 were considered significant.

3. Results

3.1 Circadian core oscillator gene expression in wild type and PPARα-null mice with ad libitum food access

I first examined the RNA expression patterns in peripheral tissues harvested from wild type and PPARα-null mice with *ad libitum* food access. **Figure 1** displays duplicated 24-hour profiles of liver, BAT, and eWAT gene expression as a function of Zeitgeber Time (ZT) where ZT0 corresponds to the initiation of the 12-hour lights on period. These 24-hour profiles have been double plotted to a 48-hour time span to visually demonstrate circadian rhythmicity. The positive circadian transcriptional regulators, *Bmal1* and *Npas2*, displayed similar expression profiles and amplitudes in all three tissues of wild type and PPARα-null mice. Total mRNA levels, normalized to *Cyclophilin B*, reached their zenith between ZT0-ZT-4 and their nadir between ZT8-ZT12. The expression profile of the negative circadian transcriptional regulators, *Per1* and *Per3*, was temporally out of phase relative to the positive elements, with a zenith between ZT8-ZT16 and a nadir between ZT20-ZT0. The amplitudes of *Per1* and *Per3* were similar between wild type and PPARα-null mice in all tissues except eWAT. *DBP*, *Rev-erbα*, and *Rev-erbβ* are downstream transcriptional targets of *Bmal1* and *Npas2*. While *DBP* and *Rev-erbα* oscillated with a zenith between ZT4-ZT8 and a nadir between ZT16-ZT20, *Rev-erbβ* displayed a zenith of ZT8-ZT12 and a nadir of ZT20. While the amplitude of *DBP* was dampened in all tissues from PPARα-null mice relative to wild type mice, the differences for *Rev-erbα* and *Rev-erbβ* were less pronounced. The circadian rhythmicity of all genes in all tissues was statistically significant ($p < 0.05$) based on Cosinor analysis.

3.2 Effect of temporally restricted food access on serum cholesterol and triglyceride profiles in wild type and PPARα-null mice

In ***Experiment 2***, all cohorts displayed comparable body weights at the initiation of the study. During the first two days, the wild type and PPARα-null mice with temporally restricted food access exhibited reduced food intake relative to their *ad libitum* fed counterparts; however, by day 3, all cohorts displayed comparable food intake and body weights in all cohorts were comparable at the conclusion of the study. Serum cholesterol and triglyceride levels were measured in wild type and PPARα-null mice with access to food *ad libitum* or restricted only to the 12-hour lights-on period (**Figure 2 A**). Consistent with prior observations [24, 25], the PPARα-null mice displayed elevated serum levels of cholesterol as compared to the wild type mice in both the *ad libitum*. With restricted food access, the amplitude and maximal serum cholesterol levels were reduced in both wild type and PPARα-null mice. The circadian rhythmicity of the triglyceride levels was statistically significant in the *ad libitum* state for both wild type and PPARα-null mice; with restricted food access, rhythmicity was lost ($p = 0.5222$ and 0.1492 in wild type and PPARα-null mice) and the amplitude and absolute value of the triglyceride levels were reduced in both wild type and PPARα-null mice.

3.3 Tissue expression profile of PPARα in wild type mice

The PPARα mRNA levels displayed a statistically significant circadian profile with standard deviations in all tissues in wild type mice under *ad libitum* conditions (**Figure 2 B**). With temporally restricted food access, the zenith of *PPARα* expression shifted by 4 to 8 hrs. With the exception of cardiac tissue, the *PPARα* circadian expression profile remained statistically significant with restricted food access.

3.4 Effect of temporally restricted food access on the circadian gene expression profiles in wild type and PPARα-null mice

Under conditions of *ad libitum* food access, the amplitudes and timing of the zeniths and nadirs for representative circadian gene markers (*Bmal1*, *Per3*, *Rev-erbα*) were similar in the BAT and cardiac tissue of wild type and PPARα-null mice (**Figure 3**). When food access was temporally restricted, the wild type mice phase shifted the zenith and nadir of circadian gene expression by ~4 to 8 hrs. In contrast, with temporally restricted food access, the PPARα-null mice phase shifted gene expression by ~8 to 16 hrs relative to the *ad libitum* state. In BAT, temporally restricted food access reduced the amplitude and zenith of circadian gene expression in both wild type and PPARα-null mice (**Figure 3**). In the cardiac tissue, temporally restricted food access reduced the amplitude of wild type *Bmal1* and PPARα-null *Rev-erbα* expression and only increased this parameter for PPARα-null *Bmal1* (**Figure 3**). In eWAT, temporal restriction of food access shifted the zenith and nadir of circadian gene expression by 4-8 hrs in wild type mice and by 8 hrs in PPARα-null mice; this was associated with a change in amplitude in some but not all genes. (**Figure 4**) In liver, temporal restriction of food access shifted the zenith and nadir of circadian gene expression by 8-12 hrs in wild type mice and 12-16 hrs in PPARα-null mice (**Figure 4**). The amplitude of gene expression under temporal restricted food access was not significantly changed relative to the *ad libitum* state for either wild type or PPARα-null mice. In all tissues, the period of circadian gene expression ranged between 23.0 to 25.1 hours and achieved statistically significant rhythmicity in nearly all instances. An exception was *Rev-erbα* expression in the liver of wild type mice with temporally restricted food access; this displayed a statistically significant recurring period of 12.2 hours.

4. Discussion

4.1 Alteration of Circadian Food Entrainment in PPARα-null mice

Temporally restricted food access entrains a phase shift in circadian gene expression profiles in peripheral tissues of wild type mice [8-14]. The current work reports that PPARα deficiency significantly modulates this phenomenon in BAT and cardiac muscle, shifting the zenith and nadir of circadian gene expression by an additional 4 to 8 hrs relative to the wild type controls. While eWAT displayed a prolonged food entrained phase shift and/or attenuation in the circadian expression of some genes (*Bmal1, Rev-erbα*) in the PPARα-null mice, it was less than that of BAT and cardiac tissue. In contrast, liver showed evidence of a prolonged food entrained phase shift only in the expression of *Bmal1* in the PPARα-null mice relative to controls and little or no attenuation.

The majority of my results are consistent with those of Canaple et al. [18]. Both studies demonstrate the food entrainment of circadian transcription factor gene expression in the liver of wild type and PPARα-null mice and of the PPARα mRNA level in the liver of wild type mice **(Figure 2 B)**. Both studies demonstrate a comparable shift in the hepatic expression of *Per 3* and *Rev-erbα* between daytime and nighttime fed PPARα-null and wild type mice [18]. The study findings differ in some respects [18]. Under *ad libitum* feeding conditions, Canaple et al. [18] observed a reduction of *Bmal1* mRNA and an elevation of *Per 3* mRNA in the liver of PPARα-null mice relative to *ad libitum* fed wild type controls while I observed comparable levels of *Bmal1* and a non-significant elevation in PPARα-null *Per 3*. These differences in outcome may be due to the following variables between study designs: Canaple's use of constant

darkness during the final 24 hours prior to sacrifice, the frequency and number of data collection points (6 collections at 4 hr intervals in the current study vs. 4 collections at 7 hr intervals by Canaple), and the length of the diet acclimatization period (8 vs. 14 days in the current study) [18]. However, the latter point is unlikely since previous experiments have recorded circadian shifts in serum cortisol within the 8 day acclimatization period [8]. Likewise, independent studies by Satoh et al. have demonstrated that murine energy metabolism and hepatic circadian mRNA expression profiles acclimated within six days to a similar time-restricted feeding regimens [26].

Mice deficient in PPARα display altered cardiac tissue metabolism, relying primarily on glycolysis rather than fatty acid oxidation, resulting in decreased energy reserves and reduced contractile function under stress [20, 27, 28]. With prolonged fasting, PPARα-null mice are prone to hypothermia, consistent with altered BAT function [20, 21]. These findings may be linked to the current observation that BAT and cardiac tissue of PPARα-null mice show altered food entrainable circadian gene expression.

4.2 Significance of Results

Establishing and defining the relationship between the circadian core oscillatory genes and the machinery responsible for metabolism, particularly fatty acid oxidation, is crucial in supporting the hypothesis that circadian dysregulation contributes to metabolic disease. My investigation into PPARα's role in the food entrainable circadian response in peripheral tissues demonstrates an intimate link between a major metabolic gene and the core circadian clock. These

observations further the understanding that the core circadian oscillatory genes significantly influence the regulation of energy flux and metabolism.

4.3 Future directions

Because this study provides strictly observational data into the effects of the absence of PPARα, further studies will be necessary to define the precise mechanism connecting the circadian regulatory factors and PPARα in these peripheral tissues. This process could possibly be achieved through various techniques such as ChIP assays.

This study's findings have been reviewed and published in *Biochemical and Biophysical Research Communications* 360 (2007) 828-833.

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Figure 1

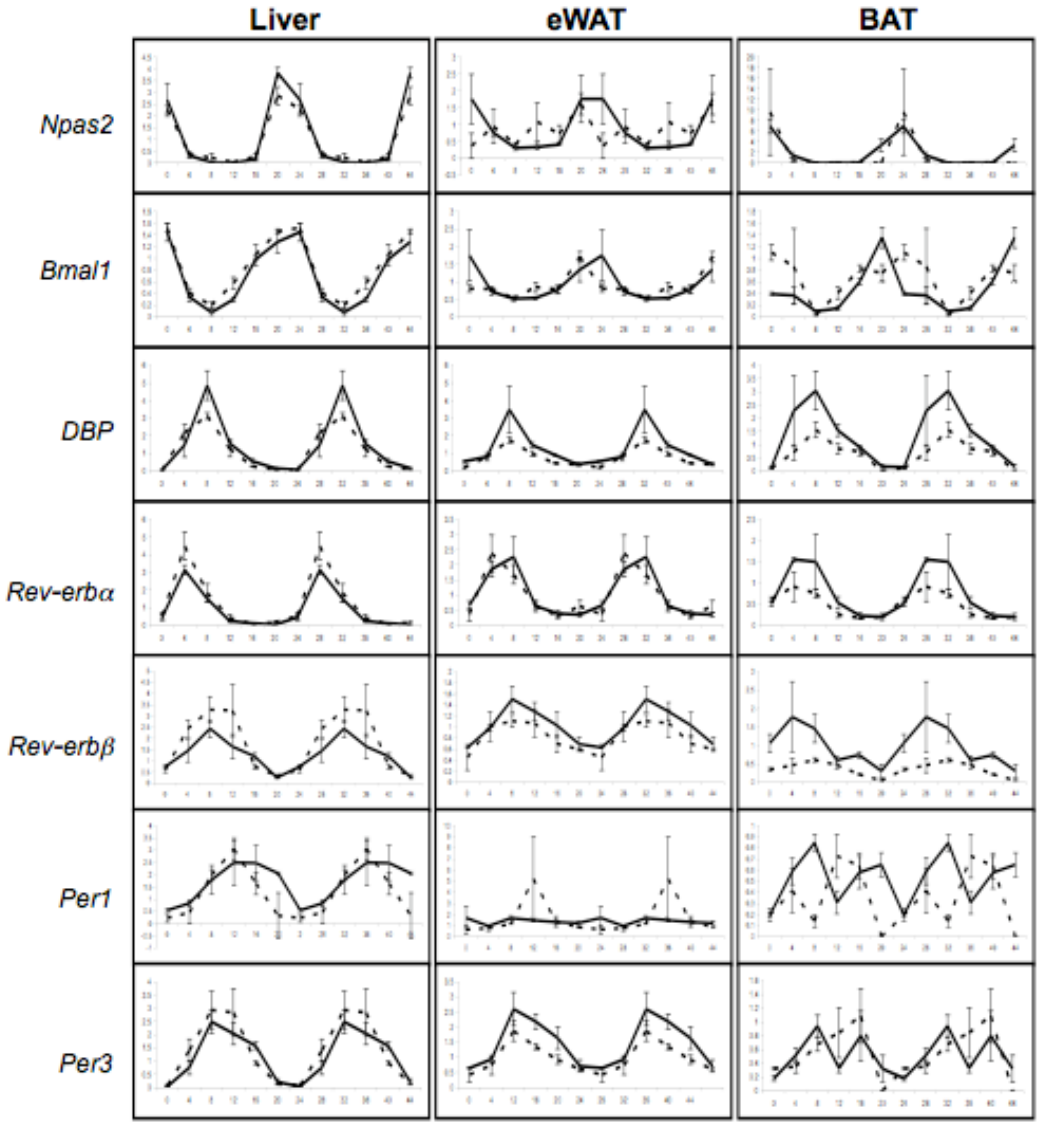
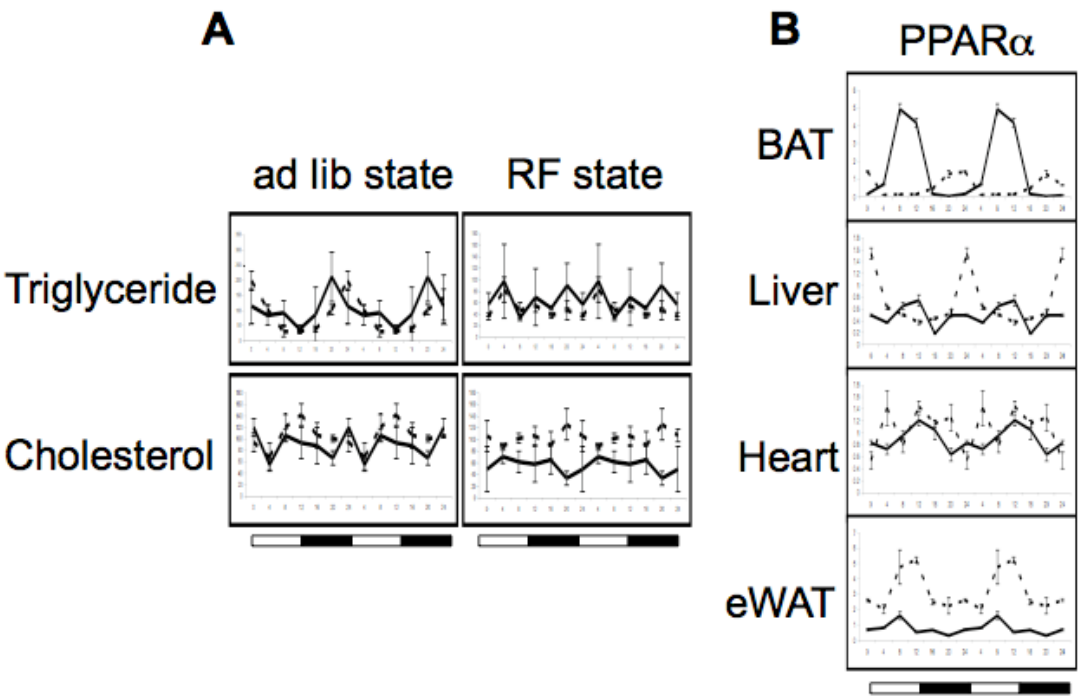


Figure 2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Heart
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Figure 3

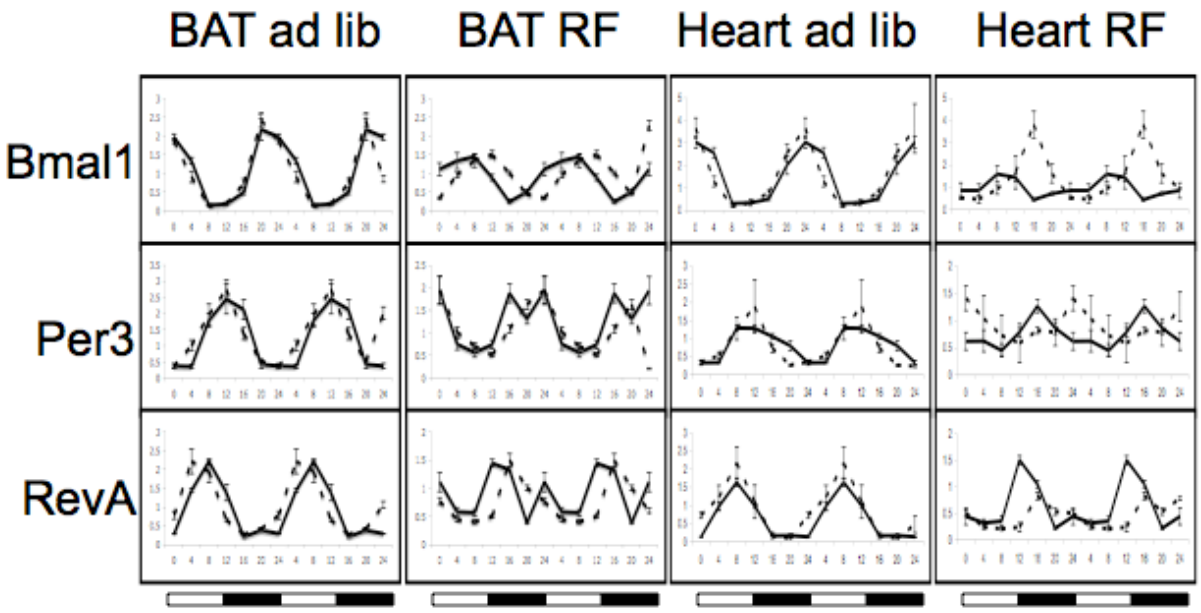

Figure 4

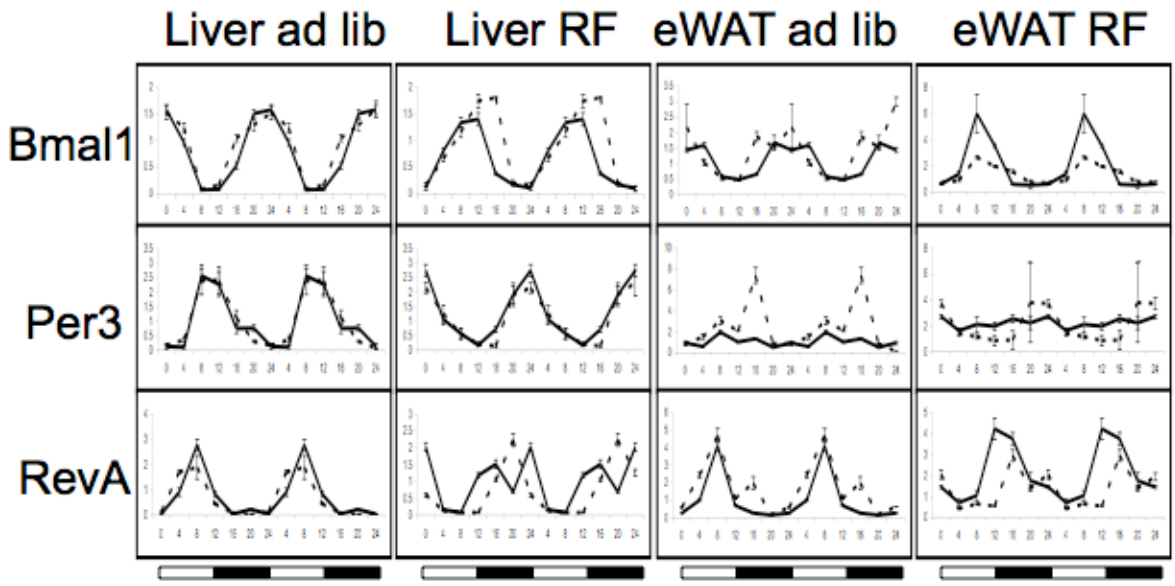

Figure 5

Bmal1 forward	AACCTTCCCGCAGCTAACAG
Bmal1 reverse	AGTCCTCTTTGGGCCACCTT
Npas2 forward	ACGCAGATGTTCGAGTGGAAA
Npas2 reverse	CGCCCATGTCAAGTGCATT
DBP forward	GGAACTGAAGCCTCAACCAAT
DBP reverse	CTCCGGCTCCAGTACTTCTCA
Rev-erbα forward	CCCTGGACTCCAATAACAACACA
Rev-erbα reverse	GCCATTGGAGCTGTCACTGTAG
Rev-erbβ forward	GGAACGGACCGTCACCTTT
Rev-erbβ reverse	TCCCCTGCTCCCATTGAGT
Per1 forward	CCAGATTGGTGGAGGTTACTGAGT
Per1 reverse	GCGAGAGTCTTCTTGGAGCAGTAG
Per3 forward	CCGCCCCTACAGTCAGAAAG
Per3 reverse	GCCCCACGTGCTTAAATCCT
PPARα forward	AGCTCCACCTGCAGAGCAA
PPARα reverse	TTTGAAGGAGCTTTGGGAAGAG