Adipokines and adipocyte function in *Clock* mutant mice that retain melatonin rhythmicity

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Running head: Adipokines and adipocyte function in Clock mutants

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Abstract

Clock$^{\Delta 19}$+MEL mutant mice, which retain melatonin rhythmicity, but lack peripheral tissue rhythmicity have impaired glucose tolerance, but reduced plasma free fatty acids, increased plasma adiponectin and improved insulin sensitivity. Here, we report their response to a high fat diet and adipocyte rhythmicity and function. The diet increased epigonadal fat weight similarly (2 fold) in both wild-type and *Clock*$^{\Delta 19}$+MEL mice. The *Clock*$^{\Delta 19}$ mutation abolished rhythmicity of *Per2*, *Rev erb α* and *Pparγ* mRNA in epigonadal fat, but not *Bmal1* mRNA, and reduced *Rev erb α* mRNA by 59% and 70% compared to the wild type mice on the control and high fat diets respectively. The mutants had increased *Adipoq* mRNA expression in epigonadal fat (22%; $P < 0.05$) on a control diet, but showed no further change on a high fat diet, and no change in *Lep*, *Nampt* or *Retn* mRNA on either diet. The *Clock*$^{\Delta 19}$ mutation abolished rhythmicity of genes in epigonadal fat that contribute to plasma free fatty acids for mice on both diets, and increased *Lipe* mRNA expression in those on the high fat diet. The persistent melatonin rhythm and reduced plasma free fatty acids in *Clock*$^{\Delta 19}$+MEL mutants may contribute to their enhanced insulin sensitivity, ameliorate the extent of impaired glucose homeostasis and protect against the adverse effects of a high fat diet.

Introduction

The importance of daily rhythms in metabolic homeostasis and the consequences of its disruption are increasingly evident, with a two-fold higher prevalence of diabetes reported in shiftworkers compared to day workers, which is exacerbated with increasing exposure to shift work (1, 2). People engaged in shift work long term, are forced to regularly shift their work/rest period by up to 12 hours for prolonged periods and this lifestyle disrupts sleep/wake rhythmicity, eating patterns and light exposure (3), melatonin secretion (4) and presumably rhythms in gene expression in key organ systems (5).

Up to 10 % of the transcriptome is rhythmically expressed across 24 hours, with many of the rhythmic genes in the liver (6), muscle (7), pancreas (8) and adipose tissue (9) involved in glucose and lipid metabolism. Animals with genetically disrupted rhythmicity are providing insight into the impact of circadian rhythms in physiology. For example mice carrying the *Clock* $^{\Delta 19}$ mutation are entrained to a 12L:12D photoperiod, exhibit slightly increased daytime activity and feeding (10). There have been few studies examining the physiological consequences of this rhythm disruption, but initial reports suggested that *Clock* $^{\Delta 19}$ (C57Bl/6) the mice were obese, hyperglycaemic, hyper lipidaemic, hyperleptinaemic and had hepatic steatosis (11). Rudic et al (12) further showed that *Clock* $^{\Delta 19}$ (C57Bl/6) mice had impaired glucose tolerance, despite having enhanced insulin sensitivity.

We identified a potential confounding influence with the original *Clock* $^{\Delta 19}$ (C57Bl/6) mutant mice since the parent strain lacks the capacity to synthesise melatonin (13-15) and carries a further mutation in a critical mitochondrial enzyme Nicotinamide nucleotide transhydrogenase (*Nnt*) (16). We bred the *Clock* $^{\Delta 19}$ mutation into mice of the melatonin proficient CBA strain to produce *Clock*

$^{\Delta 19}$+MEL mutant mice (17). These mice produce melatonin rhythmically each night (although the peak is delayed by several hours) and the rhythm persists in constant darkness. Together with rhythmic *Per2* mRNA expression in the suprachiasmatic nucleus (SCN) this is compelling evidence for retained central rhythmicity. Importantly *Clock* $^{\Delta 19}$+MEL mutant mice lack cellular rhythmicity in the key metabolic tissues, liver and skeletal muscle (18). We have shown previously that the *Clock* $^{\Delta 19}$+MEL mutant mice have impaired glucose tolerance, reduced hepatic expression and loss of rhythmicity of *gck*, *pfkb3* and *pepck* mRNA and reduced muscle *Glut4* mRNA (19). Furthermore this also occurred despite enhanced whole body insulin tolerance in these *Clock* $^{\Delta 19}$+MEL mutants. Unexpectedly, the mutants had reduced plasma free fatty acids, normal plasma leptin and elevated plasma adiponectin, suggesting that loss of peripheral tissue rhythmicity may alter adipocyte function.

The maintenance of melatonin secretion in the *Clock* $^{\Delta 19}$+MEL mutants is emerging as an important issue since the pineal hormone is increasingly being associated with glucose metabolism (20) and a series of association studies have implicated the melatonin MT2 receptor with poor glucose tolerance (21). The preservation of the melatonin rhythm and wild-type Nicotinamide nucleotide transhydrogenase allows us to address more specifically the impact of loss of tissue rhythmicity on metabolic function

In the current study we have examined the impact of loss of peripheral rhythmicity on adiposity, plasma adipokines and expression of genes involved in adipogenesis, insulin sensitivity, adipokine secretion and lipolysis in adipose tissue of *Clock* $^{\Delta 19}$+MEL mice fed either normal chow or a high fat diet.

Methods

Adult male and female *Clock* $^{\Delta 19}$ *+MEL* mutants (17) previously selectively bred from *Clock* $^{\Delta 19}$ (Balb/c) mutants (10) and CBA/6CaH mice, together with wild type control mice were maintained in 12L:12D with lights off at 2000h. The control diet was standard mouse chow (7 % fat) available *ad libitum*. The study was approved by the Animal Ethics Committee of the University of Adelaide.

Clock $^{\Delta 19}$ *+MEL* mutant and wild type mice (3 – 7 mice of each sex per time point) were killed by decapitation at 2 months of age every four hours across 24 hours at 0800h, 1200h, 1600h, 2000h, 2400h and 0400h (19). Epigonadal fat depots were rapidly dissected and immediately placed in RNAlater® (Ambion, Austin, TX). Plasma hormone and metabolite concentrations, glucose and insulin tolerance tests and gene expression analyses in the SCN, liver and muscle have been reported for these animals previously (18, 19).

To determine the effects of a high fat diet on plasma hormones and metabolites and tissue gene expression, *Clock* $^{\Delta 19}$ *+MEL* mutant and wild type mice were fed a high fat diet (22 % fat (w/w), 4.37 kcal/Kg, SF01-025, Specialty Feeds, Glen Forrest, Western Australia) from 3 to 8 weeks of age. Groups of 3 males and 3 females were killed at 4 hour intervals over 24 hours and blood and tissues collected as above.

For body composition studies, a separate cohort of male and female *Clock* $^{\Delta 19}$ *+MEL* mutant and wild type mice (n = 3 - 5 mice of each sex per genotype) were fed the control diet or the high fat diet from 3 to 8 weeks of age and then weighed and killed during the mid light period and the epigonadal fat pads dissected and weighed.

Intraperitoneal Glucose Tolerance Test (IPGTT)

Wild type and *Clock* $^{\Delta 19}$ *+MEL* mutant male mice were maintained on a high fat diet from weaning (3 weeks) to 2 months of age (n = 6-7), fasted overnight and injected with glucose (1 mg/g body

weight; Sigma Chemical Company, St Louis, MO) starting 2 hours after the lights were turned on as previously described (19).

Intraperitoneal Insulin Tolerance Test (IPITT)

Wild type and *Clock* $^{\Delta 19}$ *+MEL* mutant male mice were maintained on a high fat diet from weaning (3 weeks) to 2 months of age (n = 6). Food was withheld from 2 hours before the mice were injected with insulin (0.75 IU/kg body weight; Actrapid) 2 – 3 hours after lights on as previously described (19).

Hormone and metabolite assays

Plasma glucose and free fatty acids were measured enzymatically (19). Plasma triglycerides were measured with a Hitachi 912 automated sample system using a kit from Roche Diagnostics, NSW, Australia. Plasma insulin, adiponectin and leptin were assayed by RIA (19).

Real Time RT-PCR

To investigate gene expression in adipose tissue total RNA from approximately 100 mg of the epigonadal fat was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). See (18) and Table 1 for primers used. Amplification of cDNA and the expression of each gene within each sample determined as previously described for liver and muscle (18).

Statistics

Hormone and gene expression data were analysed by univariate ANOVA (SPSS v17), using sex, genotype and time of day as the dependent variables. For the body composition, hormone and gene expression data collected across 24 hours, the Estimated Marginal Means are reported in the text for the various comparisons. To determine whether hormone and gene expression data were rhythmic, i.e. fitted a sine curve, the data was analysed using CircWaveBatch v3.3 (22) http://www.rug.nl/biologie/onderzoek/onderzoekgroepen/chronobiologie/downloads/index

Results

Body composition

Genotype altered body weight in males (30.4 ± 0.3 g in wild-type vs. 26.8 ± 0.3g in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001; Fig. 1). Diet altered body weight (26.7 ± 0.3 g in chow fed mice vs. 30.5 ± 0.3 g in mice fed the high fat diet; P = 0.001) and similarly in each genotype (P > 0.05). Epigonadal fat weight did not vary with genotype, but varied with diet (310 ± 25 mg/ 20 g body weight in mice fed chow vs. 671 ± 29 mg/ 20 g body weight in mice fed the high fat diet; P = 0.001).

Genotype altered body weight in females (22.3 ± 0.4 g in wild-type vs. 20.1 ± 0.5g in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.003; Fig. 1). Diet altered body weight (19.3 ± 0.4 g in chow fed mice vs. 23.1 ± 0.4 g in mice fed the high fat diet; P = 0.001) and similarly in each genotype (P > 0.05). Genotype altered epigonadal fat weight (445 ± 6 mg/ 20 g body weight in wild-type vs. 319 ± 19 mg/ 20 g body weight in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001). Diet altered epigonadal fat weight (258 ± 18 mg/ 20 g body weight in mice fed chow vs. 506 ± 17 mg/ 20 g body weight in mice fed the high fat diet; P = 0.001) and differently in females (diet X genotype; P = 0.022). Epigonadal fat weight in wild-type females was 290 ± 23 mg/ 20 g body weight and 600 ± 21 mg/ 20 g body weight for control and high fat diet fed mice respectively (107% increase), while for *Clock* $^{\Delta 19}$ *+MEL* mice, epigonadal fat weights were 227 ± 26 mg/ 20 g body weight and 412 ± 26 mg/ 20 g body weight respectively (81% increase).

24 hour profile of plasma metabolites and hormones

<u>*Control diet*</u>*:* In our previous report of the effect of the *Clock* $^{\Delta 19}$ *+MEL* mutation in the 2 month old mice fed a control diet (19), plasma glucose, insulin and leptin were unchanged, plasma free fatty acids were reduced (24%) and plasma adiponectin was increased (40%) across 24 hours. In a

separate cohort of 2 month old male wild-type and *Clock* $^{\Delta 19}$ +*MEL* mutants there was no effect of genotype (P > 0.05) on plasma triglyceride in 2 month old mice (wild-type 3.0 ± 0.7 mM vs. *Clock* $^{\Delta 19}$ +*MEL* mutants 2.4 ± 0.2 mM).

<u>*High fat diet*</u>: Plasma glucose varied with genotype (11.3 ± 0.2 mM in wild-type vs. 10.1 ± 0.2 mM in *Clock* $^{\Delta 19}$ +*MEL* mutants; P = 0.001, Fig. 2), time (P = 0.03) and sex (9.9 ± 0.2 mM in females vs. 11.5 ± 0.2 mM in males; P = 0.001). Plasma free fatty acid varied with genotype (1.83 ± 0.07 meq/L in wild-type vs.1.06 ± 0.07 meq/L in *Clock* $^{\Delta 19}$ +*MEL* mutants; P = 0.001; Fig. 2), time (P = 0.02), but not with sex (P > 0.05). Plasma insulin did not vary with genotype, sex or time (P > 0.05; Fig. 2). Plasma adiponectin varied with sex (5.5 ± 0.2 μg/ml in females vs. 3.0 ± 0.2 μg/ml in males; P = 0.001; Fig. 2), but not with genotype or time (P > 0.05). Plasma leptin varied with genotype (14.1 ± 1.1 ng/ml in wild-type vs. 11.0 ± 1 ng/ml in *Clock* $^{\Delta 19}$ +*MEL* mutants; P = 0.045; Fig. 2) and sex (14.6 ± 1.2 ng/ml in females vs. 10.5 ± 1 ng/ml in males; P = 0.009), but not with time (P > 0.05).

High fat diet: Glucose tolerance

Clock $^{\Delta 19}$ +*MEL* mutant and wild type mice fed a high fat diet had similar fasting plasma glucose (P > 0.05). The plasma glucose response to a glucose challenge (area under the curve from 0 to 60 minutes) varied with genotype (wild type; 237 ± 30 units vs. *Clock* $^{\Delta 19}$ +*MEL*; 342 ± 32 units; P = 0.028; Fig. 3).

High fat diet: Insulin tolerance

Clock $^{\Delta 19}$ +*MEL* mutant and wild type mice fed a high fat diet had similar plasma glucose prior to insulin (P > 0.05). Insulin administration reduced plasma glucose maximally within 30 minutes of injection in both strains (figure 3). In wild type mice, plasma glucose reached a nadir of 15% of

fasting plasma glucose, returned to baseline by 60 minutes and increased further over the next 60 minutes (figure 3). In contrast, in *Clock* $^{\Delta 19}$ *+MEL* mice, plasma glucose reached a nadir of approximately 60% of fasting plasma glucose 30 - 60 minutes after injection and remained low for a further 60 minutes (figure 3). The plasma glucose response to an insulin challenge (area under the curve from 0 to 60 minutes) varied with genotype (wild type; 835 ± 55 units vs. *Clock* $^{\Delta 19}$ *+MEL*; 1192 ± 53 units; P = 0.001; Fig. 3).

Clock gene expression in epigonadal fat

<u>*Control diet*</u>*:* Expression of *Per2* mRNA in epigonadal fat varied with genotype (0.41 ± 0.03 units in wild-type vs. 0.16 ± 0.03 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001; Fig. 4) and time (P = 0.001), but not with sex (P > 0.05). *Per2* expression varied with genotype differently with time (P = 0.001), such that expression varied across 24 hours in wild type (P = 0.001), but not in *Clock* $^{\Delta 19}$ *+MEL* mutants (P > 0.05). This was confirmed by Cosinor analysis, indicating rhythmic expression of *Per2* mRNA occurred in the wild-type mice, but not the mutants (P = 0.001 and P = 0.156 respectively).

Bmal1 mRNA expression varied with genotype (0.53 ± 0.04 units in wild-type vs. 0.76 ± 0.04 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001; Fig. 4) and time (P = 0.001) but not with sex (P > 0.05). *Bmal1* expression varied with genotype differently with time (P = 0.001), such that expression varied across 24 hours in wild type (P = 0.001) and in *Clock* $^{\Delta 19}$ *+MEL* mutants (P = 0.022). This was confirmed by Cosinor analysis indicating rhythmic expression occurred in the wild-type mice and the mutants (P = 0.001 and P = 0.027 respectively).

Rev erbα mRNA expression varied with genotype (0.37 ± 0.05 units in wild-type vs. 0.15 ± 0.05 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.002; Fig. 5) and time (P = 0.001), but not with sex (P > 0.05). Expression was rhythmic in wild-type mice but not *Clock* $^{\Delta 19}$ *+MEL* mutants (Cosinor

analysis; P = 0.023 and P = 0.094 respectively), with maximal *Rev erbα* mRNA expression in wild-type mice at 1600h, 4 hours before dark.

<u>*High fat diet*</u>: Expression of *Per2* mRNA in epigonadal fat varied with genotype (0.48 ± 0.04 units in wild-type vs. 0.27 ± 0.04 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001; Fig. 4) and time (P = 0.001) but not with sex (P > 0.05). *Per2* expression varied with genotype differently with time (P = 0.001) such that expression varied with time across 24 hours in wild type (P = 0.001), but not in *Clock* $^{\Delta 19}$ *+MEL* mutants (P > 0.05). This was confirmed by Cosinor analysis indicating rhythmic expression occurred in the wild-type mice but not the mutants (P = 0.001 and P = 0.165 respectively).

Bmal1 mRNA expression varied with genotype (0.43 ± 0.04 units in wild-type vs. 0.71 ± 0.04 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001; Fig. 4) and time (P = 0.001), but not with sex (P > 0.05). *Bmal1* expression varied with genotype differently with time (P = 0.001) such that expression varied across 24 hours in wild type (P = 0.001) and in *Clock* $^{\Delta 19}$ *+MEL* mutants (P = 0.003). *Bmal1* expression in *Clock* $^{\Delta 19}$ *+MEL* mutants was higher than in wild-type mice between 1600h and 2400h (P < 0.05) Rhythmic *Bmal1* mRNA expression was confirmed by Cosinor analysis in the wild-type mice and the mutants (P = 0.001 and P = 0.001 respectively).

Rev erbα mRNA expression varied with genotype (0.40 ± 0.03 units in wild-type vs. 0.12 ± 0.03 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.001; Fig. 5) and time (P = 0.001), but not with sex (P > 0.05). Expression was rhythmic in wild-type mice but not *Clock* $^{\Delta 19}$ *+MEL* mutants (Cosinor analysis; P = 0.001 and P = 0.103 respectively), with maximal *Rev erbα* mRNA expression in wild-type mice at 1600h, 4 hours before dark.

Adipokine gene expression in epigonadal fat depots

<u>*Control diet*</u>*: Pparγ* mRNA expression varied with time (P = 0.026) and sex (0.54 ± 0.05 units in females vs. 0.74 ± 0.05 units in males; P = 0.003; Fig. 5) but not with genotype (P > 0.05). Expression was rhythmic in wild-type mice but not *Clock* $^{\Delta 19}$ *+MEL* mutants (Cosinor analysis; P =

0.018 and P = 0.430 respectively), with maximal *Pparγ* mRNA expression in wild-type mice at 2000h, at the onset of dark.

Adiponectin (Adipoq) mRNA expression varied with genotype (0.88 ± 0.05 units in wild-type vs. 1.07 ± 0.05 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.008; Fig. 6) and sex (0.84 ± 0.05 units in females vs. 1.11 ± 0.05 units in males; P = 0.001; Fig. 5), but not with time (P > 0.05).

Leptin (Lep) mRNA expression varied with sex (0.49 ± 0.06 units in females vs. 1.02 ± 0.06 units in males; P = 0.001; Fig. 6), but not with genotype or time (P > 0.05).

Resistin (Retn) mRNA expression did not vary with genotype, sex or time (P > 0.05; Fig. 6).

Nicotinamide phosphoribosyltransferase /visfatin (Nampt) mRNA expression varied with sex (0.60 ± 0.045 units in females vs. 0.74 ± 0.045 units in males; P = 0.036; Fig. 6) and time (P = 0.01), but not genotype (P > 0.05). *Nampt* expression varied with time differently with sex (P = 0.003) with a multiphasic pattern observed in males and a monophasic pattern in females, peaking in darkness (data not shown).

<u>*High fat diet*</u>*: Pparγ* mRNA expression varied with time (P = 0.009; Fig. 5), but not with genotype or sex (P > 0.05). Expression was rhythmic in wild-type mice but not *Clock* $^{\Delta 19}$ *+MEL* mutants (Cosinor analysis; P = 0.001 and P = 0.233 respectively), with maximal *Pparγ* mRNA expression in wild-type mice at 2000h (onset of dark).

Adipoq mRNA and *Lep* mRNA expression did not vary with genotype, sex or time (P > 0.05; Fig. 6).

Retn mRNA expression varied with time (P = 0.048; Fig. 6), but not with genotype or sex (P > 0.05). Cosinor analysis, however, indicated that the expression did not fit a sine curve (P = 0.281 and P = 0.149, for wild-type and *Clock* $^{\Delta 19}$ *+MEL* mutants respectively).

Nampt mRNA expression did not vary with genotype, sex or time (P > 0.05; Fig. 6). Cosinor analysis, however, indicated that the *Nampt* expression fitted a sine curve in wild-type mice (P = 0.001), but not *Clock* $^{\Delta 19}$ *+MEL* mutants (P = 0.850).

Lipase gene expression in fat depots

<u>Control diet</u>*: Hormone sensitive lipase (Lipe)* mRNA expression varied with time (P = 0.033; Fig. 7), but not with genotype or sex (P > 0.05). The pattern of expression across 24 hours was complex and did not fit a sine curve (P = 0.338 and P = 0.325 for wild-type and *Clock* $^{\Delta 19}$ *+MEL* mutants respectively).

Desnutrin (Pnpla2) mRNA expression did not vary with genotype, sex or time (P > 0.05; Fig. 7). Cosinor analysis indicated, however, that the expression pattern of *Pnpla2* mRNA in wild-type mice was rhythmic (P = 0.013) with peak expression at 0400h, but not in *Clock* $^{\Delta 19}$ *+MEL* mutants (P = 0.439)

Adiponutrin (Pnpla3) mRNA expression did not vary with genotype, sex or time (P > 0.05; Fig. 7). Cosinor analysis indicated, however, that the expression pattern of *Pnpla3* mRNA in wild-type mice fitted a sine curve (P = 0.014) with peak expression at 2400h, but not in *Clock* $^{\Delta 19}$ *+MEL* mutants (P = 0.132).

<u>High fat diet</u>: *Lipe* mRNA expression varied with genotype (0.58 ± 0.07 units in wild-type vs. 0.70 ± 0.07 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.030; Fig. 7) but not with time or sex (P > 0.05). Cosinor analysis, however, indicated that the *Lipe* expression fitted a sine curve in wild-type mice (P = 0.014) with peak expression at 2000h, but not *Clock* $^{\Delta 19}$ *+MEL* mutants (P = 0.121).

Pnpla2 mRNA expression did not vary with genotype, sex or time (P > 0.05; Fig. 7).

Pnpla3 mRNA expression varied with sex (0.61 ± 0.08 units in wild-type vs. 0.88 ± 0.07 units in *Clock* $^{\Delta 19}$ *+MEL* mutants; P = 0.011; Fig. 7), but not with genotype or time (P > 0.05).

Discussion

In this study we show that the mutation of *Clock* in the *Clock* $^{\Delta 19}$ *+MEL* mice impacted substantially on adipocyte function, enhancing adiponectin mRNA expression and abolishing rhythmicity of some, but not all key clock genes in adipose tissue. Maintenance on a diet containing 22% fat (18.3 MJ/Kg) from weaning until 2 months of age increased the body weight of both wild-type and *Clock* $^{\Delta 19}$ *+MEL* mutant mice to the same degree (15 - 22%). This was accompanied by an approximate doubling of the weight of the epigonadal fat pads relative to body weight regardless of genotype. Food intake was not measured in the current study. This contrasts with the original *Clock* $^{\Delta 19}$ (C57Bl/6J) mutant mice maintained on a normal diet which were characterised by an obese phenotype (11). As well as possibly being related to the lack of melatonin synthesis in the *Clock* $^{\Delta 19}$ (C57Bl/6) strain (13) the different response to a high fat diet may reflect a strong tendency to develop impaired glucose control and obesity due to a mutation in mitochondrial nicotinamide nucleotide transhydrogenase (*Nnt*) (16). It is interesting to speculate that the preservation of rhythmic melatonin secretion in the *Clock* $^{\Delta 19}$ *+MEL* mutants (17) may be responsible for the residual rhythmicity in the adipose tissue especially since functional *MT2* receptors have been demonstrated in adipocytes (23).

Clock $^{\Delta 19}$ *+MEL* mutant mice fed the high fat diet had overall lower plasma glucose, no change in insulin or adiponectin, but lower free fatty acids and leptin across 24 hours than wild type mice. This contrasts the profile of *Clock* $^{\Delta 19}$ *+MEL* mutant mice kept on the control diet, which were characterised by low free fatty acids, low insulin and elevated adiponectin (19), but normal plasma triglycerides (current study) compared to wild type.

Enhanced adipose tissue expression of *Adipoq* mRNA in the *Clock* $^{\Delta 19}$ *+MEL* mutant mice fed normal chow corresponded with elevated circulating plasma adiponectin levels (19) which we suggested were contributing to their increased insulin sensitivity. This not only may limit the extent of metabolic impairment resulting from disrupted peripheral rhythm disruption in these mice, but protect against further metabolic impairment upon exposure to a high fat diet. This was confirmed by the observation that *Clock* $^{\Delta 19}$ *+MEL* mutant mice fed a high fat diet had lower plasma glucose, free fatty acids and leptin, than did wild type mice on the diet.

In the current study we confirm that CBA mice express *Bmal1* and *Per2* rhythmically in epigonadal fat along with Svlmj (24), AKR/J (25), C57Bl/6J (26) and C57BL/6N x Sv/129 (27) mouse strains. The timing of peak expression of the genes at 0800h and 2000h, respectively, is consistent with their roles in generating cellular rhythmicity. The *Clock* $^{\Delta 19}$ mutation abolished this rhythmicity and reduced the expression of *Per2* mRNA, whereas there was increased expression and maintenance of rhythmicity of *Bmal1* mRNA. This pattern was not observed in liver or skeletal muscle in these animals (18) although there was relatively high constitutive expression of *Bmal1* in these tissues. One possible explanation for elevated *Bmal1* mRNA expression in epigonadal fat in the *Clock* $^{\Delta 19}$ *+MEL* mutants is the low and arrhythmic expression of the repressor, *Rev erba*.

Imposition of a high fat diet did not have a major impact on the rhythmic expression of *Bmal1* and *Per2* mRNA in wild type mice, although peak expression did occur at lights on and lights off respectively, compared to approximately 2 hours before lights on and approximately 4 hours after lights off in animals on the control diet. This contrasts with a recent report of reduced amplitudes but not time of peak expression of *Clock*, *Bmal1* and *Per2* mRNA expression in fat of C57Bl/6 mice fed a similar high fat diet for 6 weeks (28). Interestingly, here we found that *Clock* $^{\Delta 19}$ *+MEL* mutants on a high fat diet exhibited rhythmic *Bmal1* mRNA expression with a low amplitude, but

high basal level of expression, while *Per2* mRNA expression was arrhythmic and low across 24 hours, similar to what was found when the mice were maintained on the control diet (19). The nature of the signal(s) driving the daily *Bmal1* changes is not known, but clearly the changes are not sufficient to allow rescue of cellular rhythmicity by recruitment of *Npas2*.

Another aim of this study was to investigate the functional significance of any loss of adipose tissue rhythmicity. Of particular interest is the nuclear receptor REV ERBα, which is reported to have a critical role in adipogenesis (29, 30), potentiation of adipocyte differentiation (31) and lipid metabolism (32). *Rev erb*α deficient mice have increased plasma triglycerides and VLDL particles (33). Since the expression of *Rev erb*α in epigonadal fat was constitutively low in *Clock* $^{\Delta 19}$ *+MEL* mutant mice, we predicted that this aberrant level and pattern of expression of *Rev erb*α, would have inhibited adipocyte development. *Rev erb*α mRNA expression was not affected by the high fat diet in the wild type mice and was constitutively low in the *Clock* $^{\Delta 19}$ *+MEL* mutant mice compared to the wild type mice. There was no evidence that *Clock* $^{\Delta 19}$ *+MEL* mutants fed either a control or a high fat diet had major alterations in fat pad weight compared to wild type mice.

Peroxisome proliferator-activated receptor gamma (PPARγ) is a regulator of adipocyte and macrophage function in adipose tissue, with effects on lipid metabolism and endocrine function. It is an important mediator in the maintenance of whole body insulin sensitivity, protecting tissues against the effects of excess lipid and controlling the production of adiponectin and leptin. Since there is significantly improved insulin sensitivity in *Clock* $^{\Delta 19}$ *+MEL* mutant mice (19), we investigated the impact of the mutation on expression of *Ppar*γ mRNA in epigonadal fat. *Ppar*γ mRNA was rhythmically expressed in wild-type mice, but constitutively high and arrhythmic in *Clock* $^{\Delta 19}$ *+MEL* mutant mice on both control and high fat diets. This suggests that the enhanced

insulin sensitivity of *Clock* $^{\Delta 19}$ *+MEL* mutant mice and their elevated plasma levels of insulin sensitising adipokines, may in part relate to this elevated expression of *Pparγ.*

Clock $^{\Delta 19}$ *+MEL* mutant mice have increased whole body insulin sensitivity of glucose metabolism (19) and it is known that adipocytes secrete a range of proteins that influence glucose action in target tissues. We showed that *Adipoq* mRNA was elevated in the *Clock* $^{\Delta 19}$ *+MEL* mutant mice, which is consistent with our previous observation of elevated plasma levels of this adipokine (19). Unlike a previous report using C57Bl/6 mice (26) we did not detect a circadian rhythm in *Adipoq* mRNA expression in epigonadal fat. *Lep* mRNA expression was not rhythmic in wild-type mice nor was it altered in *Clock* $^{\Delta 19}$ *+MEL* mutant mice, which is consistent with the plasma leptin levels in these mice, but different from the *Clock* $^{\Delta 19}$ (C57Bl/6) mutants, which were reported to be hyperleptinaemic (11). Visfatin (*Nampt*) mRNA was rhythmically expressed in wild type mice with maximal expression during darkness, while the rhythm was abolished in the *Clock* $^{\Delta 19}$ *+MEL* mutant mice as previously reported (34) and overall levels did not differ between wild-type and mutant mice. Finally, adipose tissue expression of the putative insulin de-sensitising hormone, resistin (*Retn*) was not rhythmic in wild-type mice and wild-type and *Clock* $^{\Delta 19}$ *+MEL* mutant mice had similar levels of expression. *Retn* and *Nampt* mRNA expression were also similar in wild type and *Clock* $^{\Delta 19}$ *+MEL* mutants fed the high fat diet, suggesting that neither adipokine is likely to be involved in the altered insulin sensitivity of the *Clock* $^{\Delta 19}$ *+MEL* mutants. A high fat diet normalised *Adipoq* and *Lep* mRNA expression in epigonadal fat of *Clock* $^{\Delta 19}$ *+MEL* mutants compared to the wild type mice. This paralleled the changes in plasma adiponectin and suggests that excess caloric intake abolishes this aspect of the insulin sensitising phenotype of adipose tissue in the *Clock* $^{\Delta 19}$ mutants.

Clock $^{\Delta 19}$ (C57Bl/6) mutant mice are hyperlipidaemic (triglycerides and cholesterol were 20% and 16% higher respectively in mutant mice compared to the wild-type) (11). However *Clock* $^{\Delta 19}$ *+MEL* mutant mice have low plasma free fatty acids (19). In an attempt to determine the cause of their lower circulating free fatty acids, we investigated the expression of 3 key enzymes involved in the release of free fatty acids from stores, hormone sensitive lipase (*Lipe*), adiponutrin (*Pnpla3*) and desnutrin (*Pnpla2*). *Lipe* mRNA exhibited a biphasic expression pattern in both wild-type and *Clock* $^{\Delta 19}$ *+MEL* mutant mice, with peak expression during the mid-light period and at the onset of light in both strains. Small *increases* in *Lipe* mRNA were detected in adipose tissue of *Clock* $^{\Delta 19}$ *+MEL* mutant mice fed either the control or high fat diets. Both *Pnpla2* and *Pnpla3* mRNA were expressed rhythmically in wild-type mice with peak expression during darkness, but there was no difference in overall expression between the strains on either the control or high fat diets. Thus changes at the transcription level for these enzymes cannot account for the altered free fatty acid levels in the *Clock* $^{\Delta 19}$ *+MEL* mutant mice.

Clock $^{\Delta 19}$ *+MEL* mutants fed either a normal chow or a high fat diet fail to develop a severe metabolic disturbance. On the contrary, chow fed *Clock* $^{\Delta 19}$ *+MEL* mutants have low plasma free fatty acids and elevated plasma adiponectin, which are normally associated with enhanced insulin action and good metabolic control. Similarly *Clock* $^{\Delta 19}$ *+MEL* mutants fed a high fat diet were protected from elevated plasma glucose and had lower plasma free fatty acids and leptin. Another perspective is that the phenomena that we observe in the 2 month old mutant mice may represent a life-long physiological adaptation to maintain normal metabolic homeostasis perhaps facilitated by the persistence of melatonin rhythmicity. If so, the *Clock* $^{\Delta 19}$ *+MEL* mutants may be reflective of normal interrelationships between physiological systems in mice, whereas the same *Clock* mutation in other more vulnerable strains simply amplifies underlying pathologies, such as the *Nnt* mutation (16). Nevertheless these findings and others add further support to the major role that circadian

rhythms play in normal metabolic homeostasis. Importantly, understanding how *Clock* mutation enhances the metabolic state of adipocytes, producing a profile that promotes metabolic homeostasis, may lead to novel ways to improve the health of people who have their circadian rhythms disrupted by shift work.

Acknowledgements

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Figure legends

Figure 1

The body weight and relative epigonadal fat pad weights of wild type (WT) and *Clock* $^{\Delta 19}$ + *MEL* (Clock) mice fed chow or a high fat diet. The data are the actual means ± SEM (n = 3 - 5 mice per group). (a, c) males, (b, d) females. The symbol * indicates that there was a significant difference ($P < 0.05$) within a genotype due to the high fat diet.

Figure 2

Plasma glucose (a, b), free fatty acids (c,d), insulin (e, f), adiponectin (g, h) and leptin (i, j) levels in female (left panels) and male (right panels) wild type and *Clock* $^{\Delta 19}$ + *MEL* mice fed a high fat diet from 3 weeks of age until 8 weeks of age and killed at 4 hour intervals across 24 hours. Data are the mean ± SEM for n = 3 females and 3 males for each genotype at each time point, wild type mice (○ — ○) and *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●). The accompanying histograms represent the estimated marginal means ± SEM of the individual gene expression as calculated from the ANOVA for the combined male and female data. The horizontal bars represent the period of darkness.

Figure 3

The plasma glucose response to the intra peritoneal administration of glucose (1mg/kg) or insulin (0.75IU/Kg) to wild type and *Clock* $^{\Delta 19}$ + *MEL* mice fed a high fat diet from 3 weeks of age. (a) the mean ± SEM plasma glucose (n = 6 – 7; wild type mice (○ — ○), *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●)) following glucose administration. (b) the mean area under the curve (± SEM) of the plasma glucose profile from baseline to 60 minutes post glucose injection. (c) the mean ± SEM plasma glucose (n = 6; wild type mice (○ — ○), *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●)) following insulin

administration. (d) the mean area under the curve (± SEM) of the plasma glucose profile from baseline to 120 minutes post insulin injection.

Figure 4

The relative gene expression across 24 hours of *Bmal1* and *Per2* mRNA in the epigonadal fat of wild type and *Clock* $^{\Delta 19}$ + *MEL* mice fed a normal (a, c) or high fat diet (b, d). The data are the relative expression for each gene compared to actin mRNA (mean ± SEM, 3 females and 3 males for each genotype), wild type mice (○ — ○) and *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●). The highest expression of each gene for wild type mice was set at 1. (a, b) *Bmal1* mRNA, (c, d) *Per2* mRNA. The accompanying histograms represent the estimated marginal means ± SEM of the individual gene expression as calculated from the ANOVA. The absence of an SEM bar indicates that it is obscured by the symbol. The horizontal bars represent the period of darkness. The symbol * indicates that there was a significant effect of genotype on the gene expression ($P < 0.05$).

Figure 5

The relative gene expression of *Rev erb* and *Ppar*γ mRNA across 24 hours in the epigonadal fat of wild type and *Clock* $^{\Delta 19}$ + *MEL* mice fed a normal diet (a, c) or a high fat diet (b, d) from 3 weeks of age until 8 weeks of age. The data are the relative expression for each gene compared to actin mRNA (mean ± SEM, n = 3 females and 3 males for each genotype at each time point), wild type mice (○ — ○) and *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●). The highest expression of each gene for wild type mice was set at 1. (a, b) *Rev erb* α and (c, d) *Ppar*γ mRNA. The accompanying histograms represent the estimated marginal means ± SEM of the individual gene expression as calculated from the ANOVA. The absence of a SEM bar indicates that it is obscured by the symbol. The horizontal

bars represent the period of darkness. The symbol * indicates that there was a significant effect of genotype on the gene expression ($P < 0.05$).

Figure 6

The relative gene expression of *Adipoq*, *Lep, Retn* and *Nampt* mRNA in the epigonadal fat of wild type and *Clock* $^{\Delta 19}$ + *MEL* mice fed a normal diet (a, c, e, g) or a high fat diet (b, d, f, h) from 3 weeks of age until 8 weeks of age. across 24 hours. The data are the relative expression for each gene compared to actin mRNA (mean ± SEM, 3 females and 3 males for each genotype), wild type mice (○ — ○) and *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●). The highest expression of each gene for wild type mice was set at 1. (a, b) *Adipoq* mRNA, (c, d) *Lep* mRNA (e, f) *Retn* mRNA and (g, h) *Nampt* mRNA. The accompanying histograms represent the estimated marginal means ± SEM of the individual gene expression as calculated from the ANOVA. The absence of an SEM bar indicates that it is obscured by the symbol. The horizontal bars represent the period of darkness. The symbol * indicates that there was a significant effect of genotype on the gene expression ($P < 0.05$).

Figure 7

The relative gene expression of *Lipe*, *Pnpla2* and *Pnpla3* mRNA in the epigonadal fat of wild type and *Clock* $^{\Delta 19}$ + *MEL* mice fed a normal diet (a, c, e) or a high fat diet (b, d, f) from 3 weeks of age until 8 weeks of age. The data are the relative expression for each gene compared to actin mRNA (mean ± SEM, 3 females and 3 males for each genotype), wild type mice (○ — ○) and *Clock* $^{\Delta 19}$ + *MEL* mice (● — ●). The highest expression of each gene for wild type mice was set at 1. (a, b) *Lipe* mRNA, (c, d) *Pnpla2* mRNA and (e, f) *Pnpla3* mRNA. The accompanying histograms represent the estimated marginal means ± SEM of the individual gene expression as calculated from the ANOVA. The absence of an SEM bar indicates that it is obscured by the symbol. The

horizontal bars represent the period of darkness. The symbol * indicates that there was a significant effect of genotype on the gene expression ($P < 0.05$).

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Figure 1

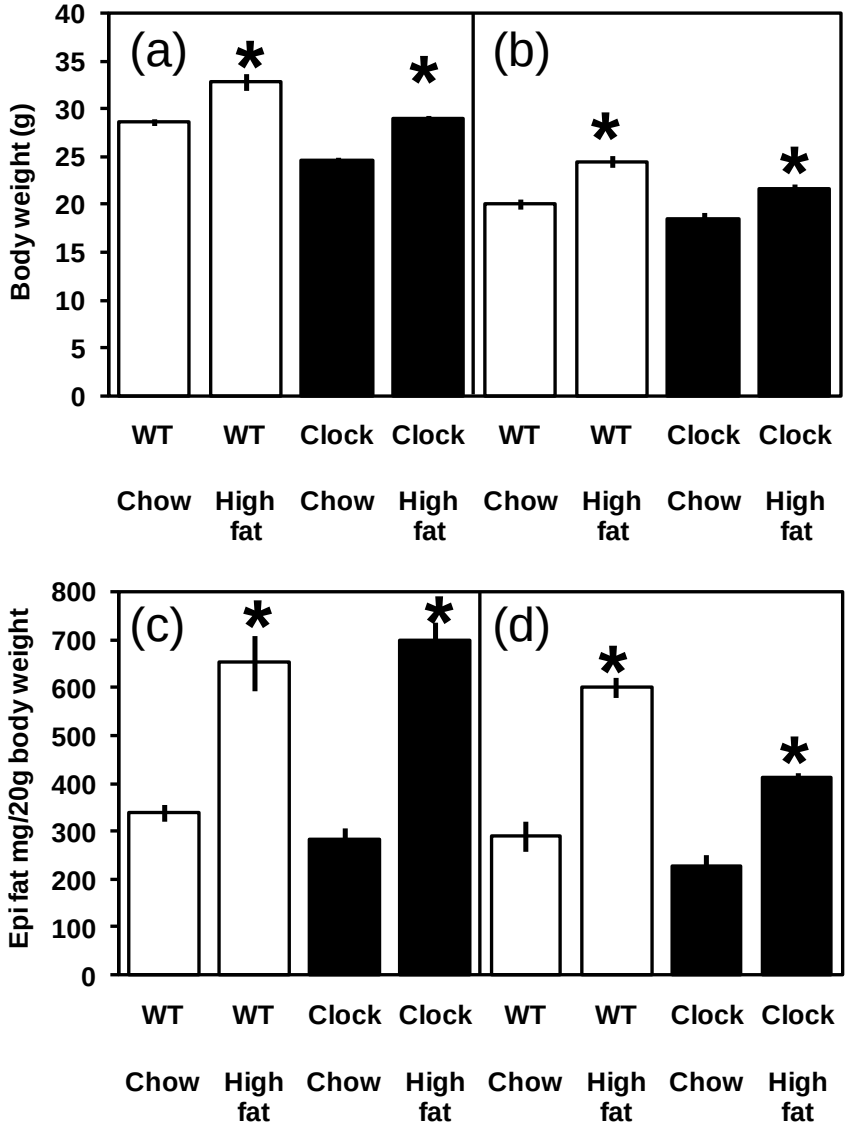
(a)
(b)
(c)
(d)
Body weight (g)
Epi fat mg/20g body weight
0
5
10
15
20
25
30
35
40
100
200
300
400
500
600
700
800
WT
Clock
Chow
High fat

Figure 2

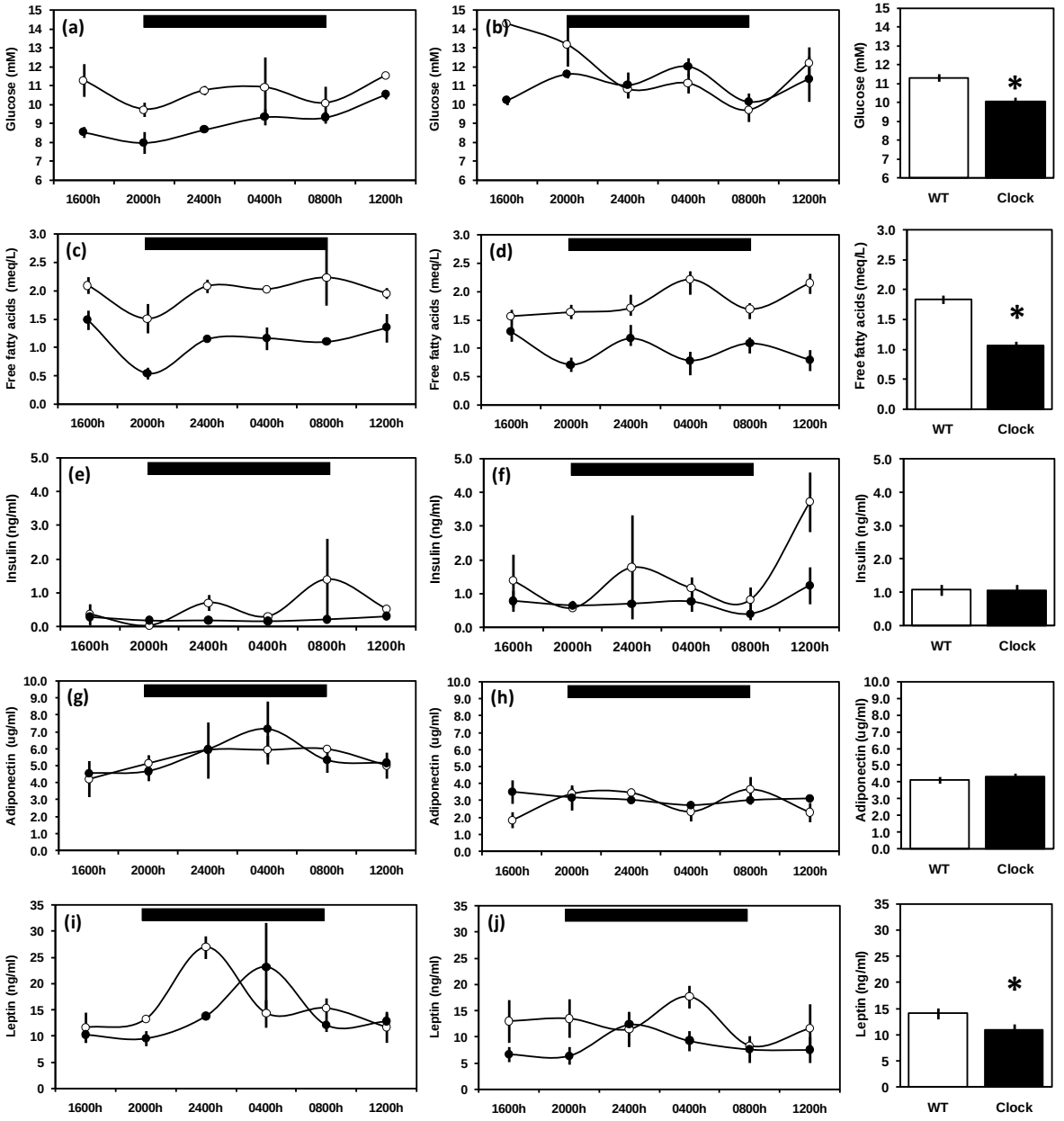
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
Glucose (mM)
Free fatty acids (meq/L)
Insulin (ng/ml)
Adiponectin (ug/ml)
Leptin (ng/ml)
1600h
2000h
2400h
0400h
0800h
1200h
WT
Clock
*

Figure 3

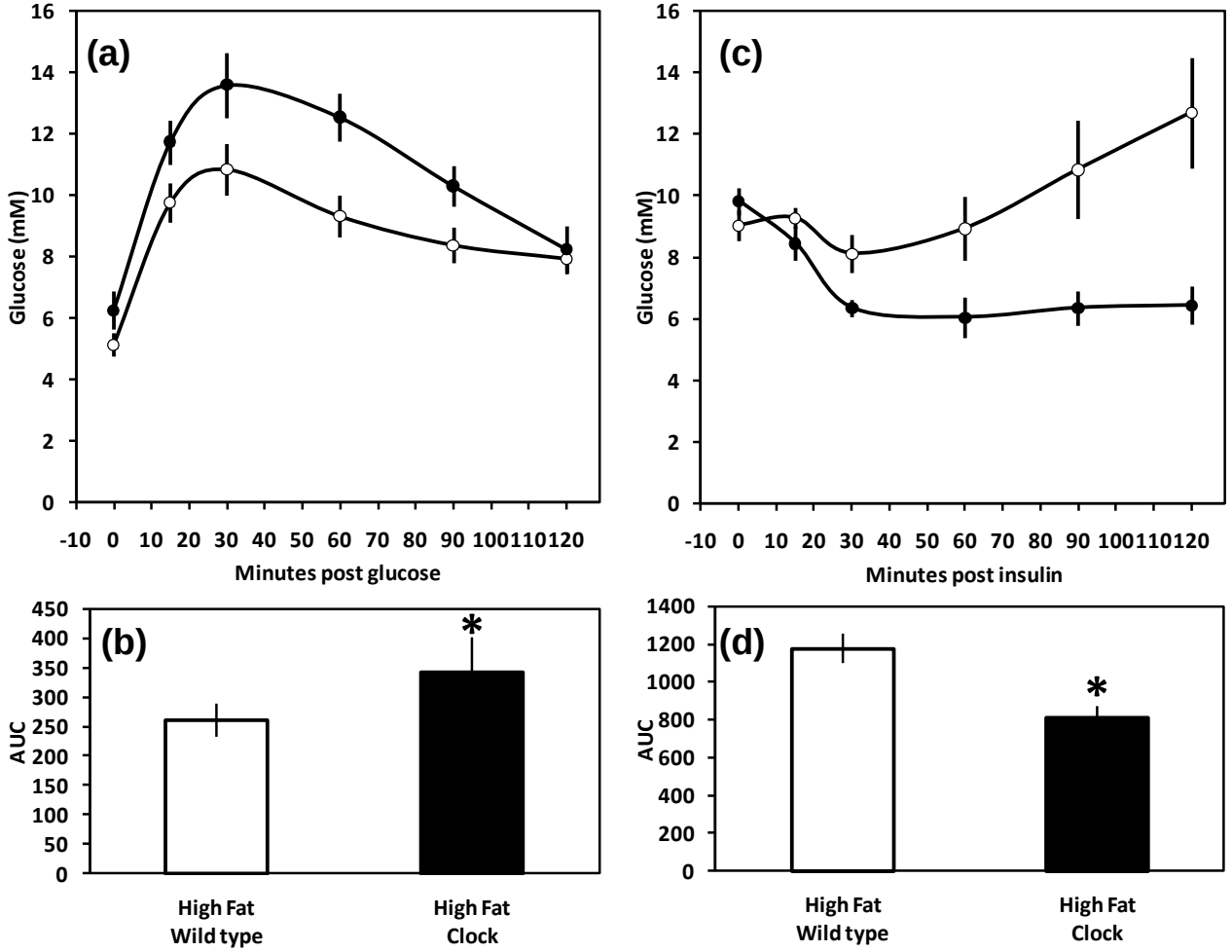
(a)
Glucose (mM)
Minutes post glucose
(c)
Glucose (mM)
Minutes post insulin
(b)
AUC
High Fat
Wild type
High Fat
Clock
(d)
AUC
High Fat
Wild type
High Fat
Clock

Figure 4

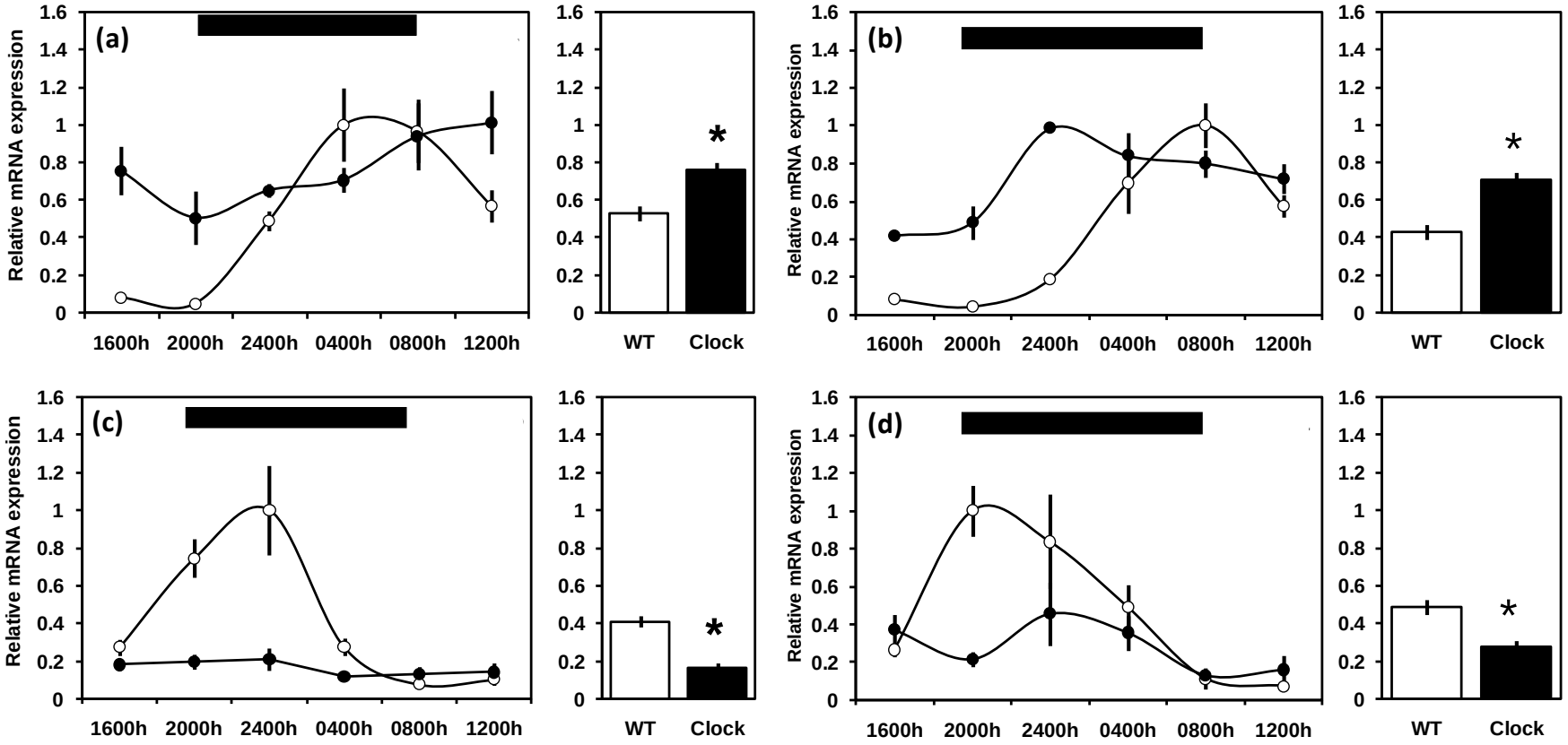
(a)
(b)
(c)
(d)
Relative mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1600h
2000h
2400h
0400h
0800h
1200h
WT
Clock
*

Figure 5

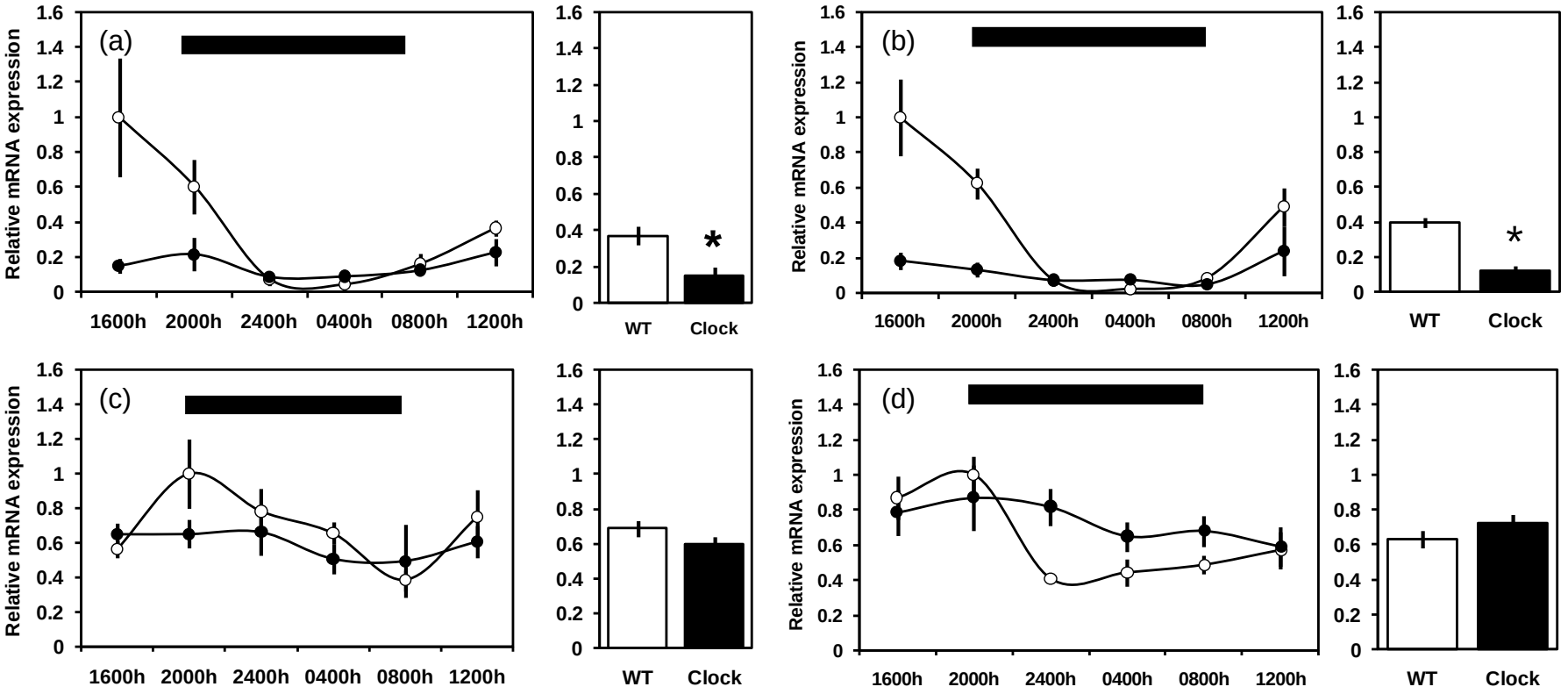
(a)
(b)
(c)
(d)
Relative mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1600h
2000h
2400h
0400h
0800h
1200h
WT
Clock
*

Figure 6

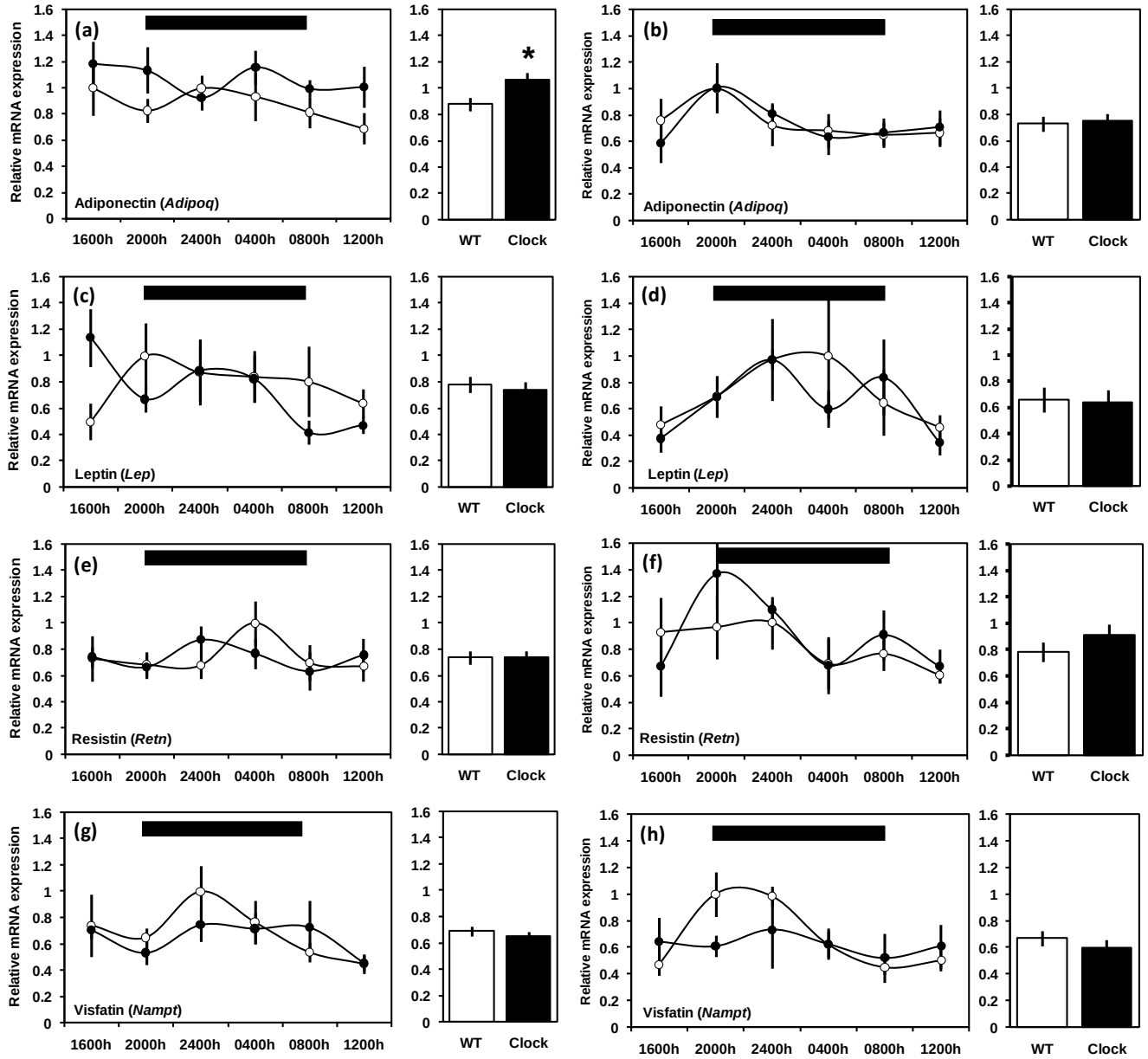
(a)
Adiponectin (Adipoq)
(b)
Adiponectin (Adipoq)
(c)
Leptin (Lep)
(d)
Leptin (Lep)
(e)
Resistin (Retn)
(f)
Resistin (Retn)
(g)
Visfatin (Nampt)
(h)
Visfatin (Nampt)
Relative mRNA expression
1600h 2000h 2400h 0400h 0800h 1200h
WT
Clock
*

Figure 7

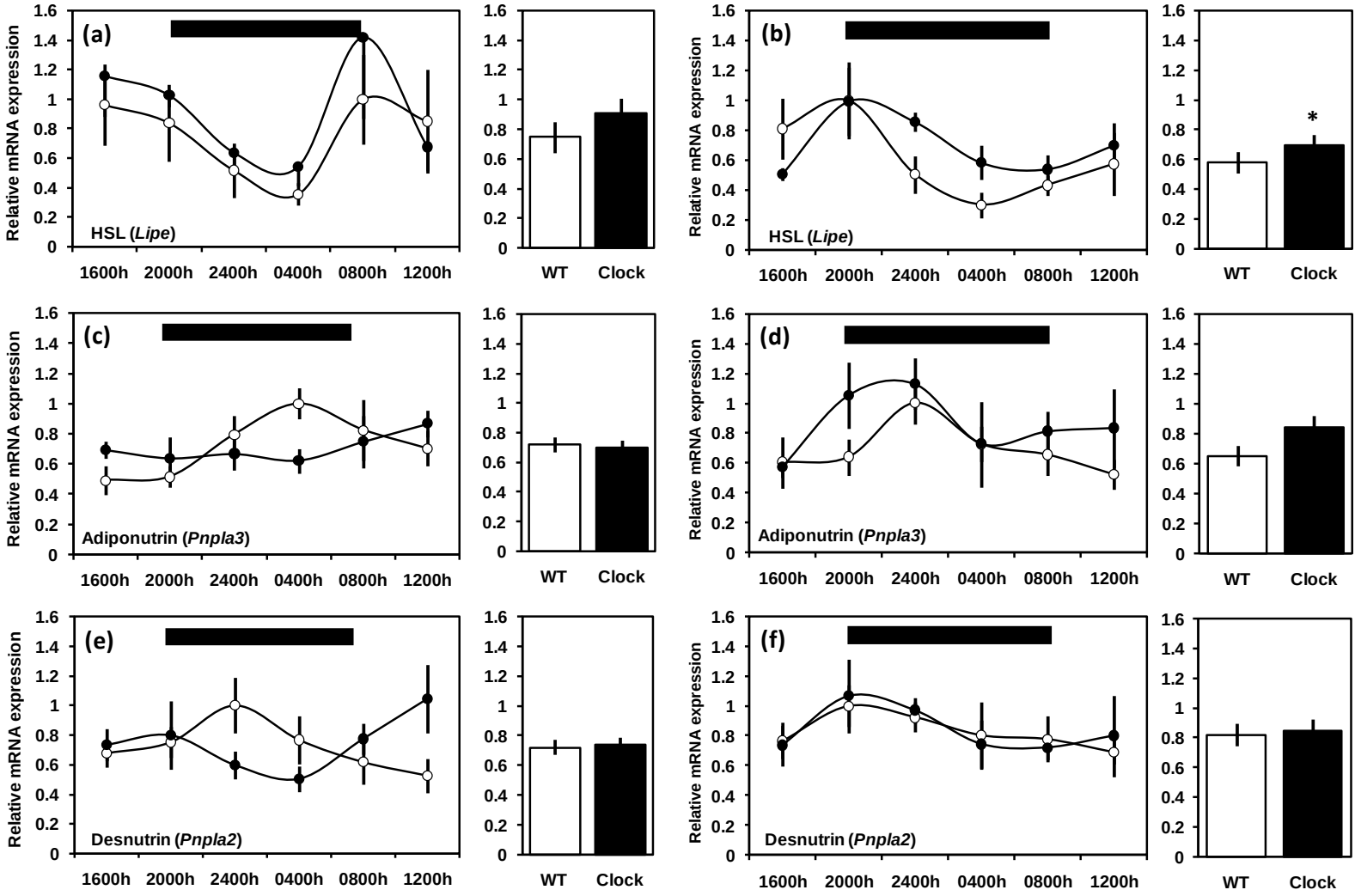
(a)
HSL (*Lipe*)
(b)
HSL (*Lipe*)
(c)
Adiponutrin (*Pnpla3*)
(d)
Adiponutrin (*Pnpla3*)
(e)
Desnutrin (*Pnpla2*)
(f)
Desnutrin (*Pnpla2*)
Relative mRNA expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1600h
2000h
2400h
0400h
0800h
1200h
WT
Clock
*

Table 1

Primer sequences for the Real Time RT-PCR analyses

Gene		Accession Number		Primers	Amplicon Length
Adiponectin	*Adipoq*	NM_009605	F	TGTTGGAATGACAGGAGCTGAA	104bp
			R	CACTGAACGCTGAGCGATACA	
Leptin	*Lep*	NM_008493	F	CAGCCTGCCTTCCCAAAA	137bp
			R	CATCCAGGCTCTCTGGCTTCT	
Resistin	*Retn*	NM_022984	F	CCTTTTCTTCCTTGTCCCTGAA	101bp
			R	ACAGGGAGTTGAAGTCTTGTTTGAT	
Visfatin	*Nampt*	NM_021524	F	TTTTGAACACATAGTAACACAGTTCTCATC	101bp
			R	GGTCTTCACCCCATATTTTCTCA	
Pparγ	*Pparγ*	NM_011146	F	CGCTGATGCACTGCCTATGA	101bp
			R	AGAGGTCCACAGAGCTGATTCC	
HSL	*Lipe*	U08188	F	AGAGACACCAGCCAACGGATA	101bp
			R	TTTTGCGGTTAGAAGCCACA	
Adiponutrin	*Pnpla3*	AY037763	F	CAGACAATGTCCACCAGGTCAT	104bp
			R	TTCGTCTTTGGAATGGAACTCA	
Desnutrin	*Pnpla2*	AY731699	F	TTGCAGCCTTATAGAAAAGATCGAA	101bp
			R	GTGGTCATCAGGTCCTTTGGTT	