1  **Altered cellular redox status, sirtuin abundance and clock gene expression in a**

2  **mouse model of developmentally primed NASH**

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20  Abreviations:

21  Non-alcoholic fatty liver disease (**NAFLD**), Non-alcoholic steatohepatits (**NASH**), High Fat

22  (**HF**), Control (**C**), Sirtuin 1 (**sirt1**), Sirtuin 3 (**sirt3**), Circadian Locomoter Output Cycles

23  Kaput (**Clock**), Bmal1 (also called Arntl1), Period 2 (**Per2**), Crytochrome 2 (**Cry2**), retinoic

24  acid receptor-related orphan receptors alpha (**RORα**), and sterol regulatory element binding

25  protein-1c (**Srebp1c**), Nicotinamide adenine dinucleotide (**NAD**), Osteopontin (**OPN**).

28

**Abstract**

**Background:** We have previously shown that high fat (HF) feeding during pregnancy primes the development of non-alcoholic steatohepatits (NASH) in the adult offspring. However, the underlying mechanisms are unclear.

**Aims:** Since the endogenous molecular clock can regulate hepatic lipid metabolism, we investigated whether exposure to a HF diet during development could alter hepatic clock gene expression and contribute to NASH onset in later life.

**Methods:** Female mice were fed either a control (C, 7% kcal fat) or HF (45% kcal fat) diet. Offspring were fed either a C or HF diet resulting in four offspring groups: C/C, C/HF, HF/C and HF/HF. NAFLD progression, cellular redox status, Sirtuin expression (*Sirt1*, *Sirt3*), and the expression of core clock genes (*Clock*, *Bmal1*, *Per2*, *Cry2*) and clock-controlled genes involved in lipid metabolism (*Rev-Erbα*, *Rev-Erbβ*, *RORα*, and *Srebp1c*) were measured in offspring livers.

**Results:** Offspring fed a HF diet developed NAFLD. However HF fed offspring of mothers fed a HF diet developed NASH, coupled with significantly reduced NAD+/NADH ($p < 0.05$, HF/HF vs C/C), Sirt1 ($p < 0.001$, HF/HF vs C/C), Sirt3 ($p < 0.01$, HF/HF vs C/C), perturbed clock gene expression, and elevated expression of genes involved lipid metabolism, such as Srebp1c ($p < 0.05$, C/HF and HF/HF vs C/C).

**Conclusion:** Our results suggest that exposure to excess dietary fat during early and post-natal life increases the susceptibility to develop NASH in adulthood, involving altered cellular redox status, reduced Sirtuin abundance, and desynchronised clock gene expression.


Key words - High fat, maternal diet, fatty liver, development, aging, circadian

**INTRODUCTION**

Non-alcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease worldwide, and is present in a third of the general population and the majority of individuals with obesity and type 2 diabetes [1;2]. In its milder form, NAFLD is characterised by excessive intra-hepatocyte triglyceride (TG) accumulation (hepatic steatosis). In advanced stages steatosis is coupled with inflammation and termed non-alcoholic steatohepatits (NASH) [3], which can eventually result in liver failure. NAFLD is commonly associated with older individuals; however, recent evidence suggests that NAFLD is increasingly common in young adults, children and adolescents [4-6]. The precise mechanisms underlying the premature onset of liver disease are unclear.

A substantial body of evidence suggests that early life is a critical period of plasticity, in which the developing organism physiologically adapts to its surrounding environment. In some cases this may be advantageous [7;8], however, imbalanced nutrition during early life may also have deleterious effects on the development of key metabolic organs. This is particularly true of the liver, which undergoes important maturation stages during late gestation and early postnatal life. Hence, the liver is highly susceptible to chronic maternal high fat (HF) diets, which can lead to a NAFLD phenotype in offspring independent of maternal and offspring obesity [9]. In addition, we have previously shown in mice, that exposure to a high fat (HF) diet during gestation and lactation primes the development of the severe form of fatty liver (NASH) in adult offspring, involving impaired mitochondrial function and increased expression of genes involved in lipogenesis [10]. Further studies support the notion that early HF exposure can prime the onset of NAFLD in adulthood. In a rat model of high fat feeding during pregnancy only, obese mothers gave rise to offspring with altered transcriptional and epigenetic regulation of key regulators of fatty acid oxidation such as peroxisome proliferator-activated receptor (PPAR)α [11]. Similarly offspring of HF fed dams have shown increased expression of the lipogenic transcription factor, *Srebp1c* [12;13].

Nutritional status is constantly being assessed by transcriptional networks that control lipid homeostasis [14]. In mammals, one such network is the endogenous molecular clock network, which mediates transcriptional changes controlling a number of metabolic outputs [15]. While the suprachiasmatic nucleus (SCN) of the hypothalamus is the central pacemaker, molecular clocks also exist in peripheral tissues such as the liver where they can be entrained by local nutrient availability [16]. The core components of the clock network *Clock, Bmal1, Per1*, *Per2*, *Cry1 and Cry2*, self-regulate through transcriptional feedback loops resulting in 24 hours oscillatory patterns of gene expression [17;18]. Interestingly, genetic disruption of the clock genes and changes in the canonical 24 hour oscillatory patterns can lead to fatty liver disease [19;20]. This is likely due to the fact that the core

89     clock genes transcriptionally regulate downstream clock controlled genes (CCGs) with

90     important roles in hepatic lipid metabolism. For example, *Bmal1* has been shown to directly

91     regulate CCGs involved in hepatic carbohydrate and lipid metabolism [21]. In addition, the

92     *Rev-Erb and ROR* nuclear transcription factors are able to activate and repress *Bmal1*

93     expression respectively, thus fine tuning the circadian regulation of metabolic genes [22].

94     The *Rev-Erb* isoforms, previously considered as accessory components of the clock system,

95     have been shown to coordinate a number of genes involved in lipid metabolism [23]. Recent

96     studies using *Rev-Erb* agonists have revealed alterations in the nycthemeral (day vs night)

97     patterns in both behaviour and hepatic gene expression [24]. Importantly, the maternal

98     environment has the capacity to alter clock gene expression in the developing liver, causing

99     changes that persist postpartum [25]. For example, in non-human primate liver, exposure to

100    a maternal high-fat diet significantly alters expression of fetal hepatic Npas2, a paralog of the

101    clock transcription factor [26]. In rodents, offspring of obese dams show reduced hepatic

102    expression of both circadian and metabolic genes, which is associated with altered mRNA

103    dynamics [11]. More recently it has been shown that maternal obesity interacts with an

104    obesogenic post-weaning diet to disrupt the canonical rhythmicity of gene expression in the

105    liver, and alters the DNA methylation levels at both the *Bmal1* and *Per2* promoters [27].

106         Recent findings suggest that the *Clock* and *Bmal1* heterodimer sense the metabolic

107    status of the cell via a functional interaction with the NAD+ dependant deacetylase SIRT1

108    [28;29]. SIRT1 belongs to a class of Sirtuins proteins with roles in circadian rhythms,

109    mitochondrial metabolism, and aging [30-32]. Moreover, recent findings have shown that

110    SIRT3, a mitochondrial protein deacetylase, is down-regulated in response to maternal fat

111    exposure and is involved in the development of fatty liver disease [33;34]. Thus, it is possible

112    that SIRT1 and SIRT3 functionally link mitochondrial metabolism, lipid homeostasis,

113    circadian biology and age-associated metabolic decline.

114         In light of these findings we tested the hypothesis that HF feeding during development

115    could contribute to the priming of severe fatty liver disease, through altered circadian

116    biology, clock gene expression, and altered regulation of CCGs with key roles in lipid

117    homeostasis.

118    **METHODS**

119    **Animal Model**

120    All animal procedures were carried out in accordance with the United Kingdom Animals

121    (Scientific Procedures) Act 1986 and were approved by the local ethics review committee.

122    Female C57/BL6J mice were maintained under a 12h light/dark cycle (lights on at 07.00h),

123    and at a constant temperature of 22±2°C with food and water available *ad libitum*. Dams

124    were randomly allocated to one of two diets: control (C; 7% kcal fat; Special Dietary Services

UK), or a high fat diet (HF; 45% kcal fat; Special Dietary Services UK). We have previously used this HF diet to bring about an obese phenotype in both the pregnant dams and their offspring [10]. Dams were fed the designated diet 6 weeks pre-pregnancy, through to pregnancy and lactation. Pregnant dams were allowed to deliver their pups, and litter size was standardized to six pups (three males and three females, whenever possible) to ensure that no litter was nutritionally biased. Offspring were randomly allocated to either the C or HF diet at weaning at 3 weeks of age resulting in 4 offspring groups; C/C, C/HF, HF/C and HF/HF. These offspring were fed the diets for the next 12 weeks. At 15 weeks of age, male offspring were killed by cervical dislocation at 2 time points during the 24h light-dark period, at 3 pm (Zeitgeber time 8 or ZT8; eight hours into the light or day period) and at 3am (ZT20; eight hours into the dark or night period). Liver tissue was immediately frozen in liquid nitrogen and stored at -80°C or paraffin embedded for histological analysis.

**Day-night changes in food intake and energy expenditure**

Food intake and energy expenditure were recorded over a 24h light-dark cycle in the four offspring groups (C/C, C/HF, HF/C and HF/HF, n = 6-10 per offspring group) using a closed modular indirect calorimetric system at 12 weeks of age (Oxylet, Panlab SLU, Spain). Oxygen consumption ($V$O$_2$) and carbon dioxide production ($V$CO$_2$) were recorded at 5-min intervals using a computer-assisted data acquisition program (Metabolism; Panlab SLU, Spain) over a 24-h period. From this, the animal's energy expenditure (EE; in kcal/day/body weight$^{0.75}$) was calculated. Food intake was continuously measured using an extensiometric food weight transducer device (Panlab SLU, Spain). To determine day and night differences in food intake and energy expenditure between the offspring groups, the mean values were calculated during the light and dark phases of the cycle for each mouse in each treatment groups.

**Glucose Tolerance Test**

Glucose tolerance test was performed after an overnight fast at 14 weeks of age. Fasting glucose concentration was measured in whole blood obtained from the tail vein before the fasted mice were intraperitoneally injected with D-glucose (2 g per kg mouse body weight), and blood glucose concentration was measured using a glucometer (Aviva Accu-Chek, Roche Diagnostics Ltd UK) at 15, 30, 60 and 120 min.

**Evaluation of steatosis by point counting**

Evaluation of steatosis was done by point counting. Previous studies have shown that the point counting method of evaluating steatosis had a strong and significant correlation to hepatic triglyceride levels (Catta-Preta et al 2011). Briefly, captured digital images of liver

159 sections stained with hematoxylin and eosin (H&E) were projected on an LCD Monitor. For
160 each H&E stained liver sections, sequential images were taken at 20x magnification across
161 a section (avoiding large vessels). A total of 3 stained sections were scanned per animal
162 (n=4 animals per treatment group). Using a computer software FIJI (http://fiji.sc/Fiji), a
163 10X10 grid system of 100 test points (PT) was superimposed on the image field. The
164 percentage volume density of hepatic steatosis (Vv[steatosis, liver]%) was then estimated as
165 the ratio of the points hitting the vesicles of fat (Pp) compared to the number of test points:
166 Vv[steatosis, liver]%=(Pp[steatosis]/PT)x100.

## Histological analysis

168 The Kleiner scoring system was used to assess the severity of NAFLD [35].  An activity
169 score (AF) was generated by adding the individual score for necroinflammatory features;
170 steatosis (<5% = 0, 5-33% = 1, 33-66% = 2, >66% = 3); ballooning (none = 0, few = 1,
171 prominent = 2); and lobular inflammation (none = 0, <2 foci = 1, 2-4 foci = 2, >4 foci = 3).  A
172 score of <3 correlates with mild NAFL, a score of 3-4 correlates with moderate NAFL and a
173 score of 5 or more correlates with NASH. The average score for each histological
174 characteristic in each group was used as previously [10].

## NAD/NADH Assay

176 NAD+ and NADH were extracted from left lobe liver samples and quantified using an
177 enzymatic calorimetric method (Enzychrom, BioAssays Systems, CA, USA) according to
178 manufacturer's instructions. Both absolute levels and NAD+/NADH ratios are presented.

## Plasma lipid assay

180 Day (ZT8) and night (ZT20) triglyceride levels in plasma samples (n=5 per group, per time
181 point) was measured with a commercial enzymatic colorimetric method (cholesterol 50 kit;
182 Sigma, Poole, UK), and plasma NEFA was measured by an enzymatic method (Alpha
183 Laboratories, Eastleigh, UK).

## Relative Gene expression

185 Total RNA was isolated from 0.025g of left lobe of the liver from 15 week-old offspring (n=5
186 per time point and dietary group) using Trifast reagent (Peqlab, Germany) according to
187 manufacturer's instructions. RNA quantification, quality, and integrity was determined via
188 (260/230 and 260/280 ratios and concentrations) a NanoDrop spectrophotometer (Thermo
189 Fisher Scientific, UK) and agarose gel electrophoresis. Total RNA (1$\mu$g) was reverse
190 transcribed to cDNA using reverse M-MLV transcriptase (Promega, UK). Samples were then
191 incubated at 37$°$C for one hour, 42$°$C for ten minutes, followed by an enzyme activation step
192 at 75$°$C for ten minutes. The cDNA synthesised was then diluted to a concentration of

193 5ng/µL before amplification. Expression of the *Sirt1, Sirt3*, the core clock genes (*Clock,*

194 *Bmal1, Cry1, Cry2, Per2*), and the lipogenic transcription factors *Rev-Erbα*, *Rev-Erbβ*,

195 *RORα, and Srebp1c* were determined at ZT8 and ZT20, otherwise referred to as day and

196 night, by quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR;

197 Applied Biosystems 7500 Fast Real-Time PCR Thermal Cycler). Expression of *OPN* was

198 determined at ZT8 only. We used the housekeeping genes (HKG) *YWHAZ* and *βactin* to

199 determine relative gene expression, since they were the most stably expressed gene

200 following challenge with HF diets and at different circadian time points [36]. PCR

201 amplification was performed for 50 cycles. Following an initial enzyme activation step for 10

202 minutes at 95°C, each cycle consisted of denaturation for 15 seconds at 95°C, annealing for

203 30 seconds at 50°C and 15 seconds at 72°C. Analysis of duplicated samples determined

204 that the intra-assay coefficients of variability (CV) was >5%.

205 **Statistical analysis.**

206 Data are expressed as mean±SEM, and a significant difference was accepted at p< 0.05.

207 For 24 hour metabolic phenotype measurements, day (light period) or night (dark period)

208 area under the curve (AUC) analysis was performed on individual animals. A one way

209 analysis of variance (ANOVA) was performed between offspring groups with Bonferroni

210 *post-hoc* analysis for multiple comparisons, to determine the groups which significantly

211 differed. For blood analysis, mean day (ZT8) and night (ZT20) values were analysed by

212 ANOVA and Bonferroni *post-hoc* tests. For gene expression analysis, mean delta delta CT

213 values for each offspring group at either day (ZT8) or night (ZT20) were tested for significant

214 difference using ANOVA followed by Bonferroni *post-hoc* analysis.

215 **RESULTS**

216 **Food intake and energy expenditure is perturbed in 15 week old male offspring**

217 **exposed to high fat diets during development.**

218 To determine the effect of increased fat exposure on circadian physiology, we measured

219 nycthemeral patterns of food intake and energy expenditure (EE) in adult offspring. Control

220 offspring showed a nocturnal phenotype and consumed food mainly during the subjective

221 night (Figure 1a). The canonical night-time feeding behaviour was also observed in the HF/C

222 offspring. In contrast, both offspring groups fed a HF diet during adulthood ate significantly

223 more during the day than the control offspring (Figure 1a; p<0.01, C/HF or HF/HF vs C/C).

224 Although total food intake was greatest in the C/HF offspring, these offspring also had the

225 significantly higher EE in both the day and the night than other offspring groups (Figure 1b;

226 p<0.05, C/HF vs C/C). It is plausible to suggest that such as increase in EE offsets the

227 increased caloric intake observed in the C/HF offspring, and limits further weight gain. This

228 may partially explain why the HF/HF offspring, which lack increased EE, are significantly

229 heavier than other offspring groups (Figure 1c; $p < 0.001$, C/HF vs C/C; $p < 0.0001$, HF/HF vs

230 C/C). Although speculative, the maternal HF challenge may alter metabolic capacity in the

231 offspring. In the HF/HF offspring this prevents an adaptive increase in EE that would

232 otherwise limit further weight increase, while in the HF/C this manifests as significantly

233 higher body weight in the absence of increased food intake.

**Offspring exposed to HF diets during development are primed to develop severe fatty liver in adulthood.**

236 We have previously shown that female offspring exposed to high fat diets during

237 development are primed to develop severe fatty liver disease, characterised by micro and

238 macro vesicular steatosis, ballooning degeneration, and inflammation [10]. In the present

239 study, we asked whether male offspring developed a similar condition. Steatosis was directly

240 quantified by point counting to show that while HF/C offspring had increased fat

241 accumulation compared to control, fat accumulation was further increased in the C/HF

242 offspring, and further still in their HF/HF counterparts (Figure 1d). Histological analysis and

243 Kleiner scoring methods were used to compare the severity of liver disease between

244 offspring groups. Histological analysis confirmed that while offspring exposed to a HF diet

245 during adulthood (C/HF) displayed marked hepatic lipid accumulation, offspring exposed to

246 HF diets during development and later life (HF/HF) displayed a severe liver histopathology

247 akin to human NASH (Figure 1e). In confirmation, histological scoring of liver sections

248 revealed that livers from the HF/HF offspring had scores of 5 or greater, which were

249 consistent with the features of NASH (Figure 1f).

250 Glucose tolerance tests (GTT) were performed to determine the effect of HF feeding on

251 glucose homeostasis. GTT revealed that baseline fasting glucose levels were significantly

252 higher in the HF/HF offspring compared to the other offspring groups vs C/C (Figure 1g

253 $p < 0.01$). Following (intraperitoneal) IP injection of glucose, circulating glucose levels

254 remained significantly elevated, even at after 2h post-IP in both groups of offspring exposed

255 to a HF diet during development ($p < 0.05$ in HF/C and $p < 0.01$ in HF/HF vs C/C) and those

256 fed a HF diet in later life ($p < 0.05$, C/HF vs C/C). Since this elevated blood glucose and

257 reduced glucose clearance is associated with insulin resistance we measured day and night

258 circulating insulin levels. (Figure 1h). Insulin was significantly higher in the C/HF offspring

259 during the night ($p < 0.001$, C/HF vs C/C). However, HF/HF offspring were hyperinsulinemic

260 during both the day and night, and the nycthemeral pattern of insulin production was

261 seemingly abolished ($p < 0.001$, HF/HF vs C/C).

262 Since murine models readily develop NAFLD, but not NASH, we set out to measure

263 *OPN* expression, a marker of fibrosis and severe fatty liver disease in offspring liver (Figure

264    2a). We found that OPN expression was significantly increased the livers of HF/HF offspring

265    (p<0.001 in HF/HF vs C/HF), but not in any other offspring groups. This suggest that the

266    combination of high fat feeding during both early and post-natal life, predisposes to the

267    development of a more severe fatty liver disease akin to human NASH.

268

269    **Offspring exposed to a HF diet during development and adulthood have imbalanced**

270    **cellular redox status and altered metabolic state.**

271    To determine whether HF feeding during development resulted in alterations in cellular redox

272    status, we measured the availability of NAD+ and NADH in offspring liver. Offspring exposed

273    to a HF diet during both development and adulthood (HF/HF) had significantly reduced

274    hepatic NAD+ (Figure 2b; p<0.01, HF/HF vs C/C). In contrast, hepatic NADH concentrations

275    were similar in all offspring groups (Figure 2c). Consequently, the NAD+/NADH ratio was

276    significantly lower the in HF/HF offspring compared to all other offspring groups (Figure 2d;

277    p<0.05, HF/HF vs C/C).

278    **Offspring exposed to HF diets have altered lipid homeostasis**

279    To determine the effect of HF feeding on circadian lipid homeostasis, we measured

280    circulating non-esterified fatty acids (NEFA) and Triglycerides (TG) in each offspring group at

281    a specific time during the day (ZT8), or night (ZT20). Offspring exposed to a HF diet at any

282    life stage, had increased daytime circulating NEFA compared to C/C offspring (Figure 2e;

283    p<0.001, C/HF vs C/C; p<0.01, HF/C or HF/HF vs C/C). Similarly, daytime TG levels were

284    significantly higher in all offspring fed a HF diet (Figure 2f; p<0.01, C/HF vs C/C; p<0.05,

285    HF/C or HF/HF vs C/C). Moreover, offspring fed a HF diet in the post-natal period showed

286    an increase in nighttime TGs compared to control offspring (p<0.05, C/HF or HF/HF vs C/C).

287    **Hepatic Sirtuin and core clock protein expression is perturbed in offspring exposed to**

288    **a HF diet during early life and adulthood.**

289    We had previously shown that mitochondrial function is impaired in offspring exposed to a

290    HF diet during development [10]. Hence we determined the gene expression of

291    mitochondrial *Sirt3* following HF feeding. *Sirt3* was significantly reduced in livers of offspring

292    that had been fed a HF diet during adulthood (Figure 3a; p<0.01, C/HF vs C/C),

293    corresponding to a -3.8 fold decrease (Table 1). Nighttime expression of *Sirt3* was further

294    reduced (-12.5 fold, Table 1) in offspring that had been exposed to a HF diet during both

295    development and adulthood (p<0.01, HF/HF vs C/C). However, expression of *Sirt3* was not

296    reduced in the livers of offspring that had been exposed to a HF diet during development

297    only (i.e. the HF/C group). In contrast, *Sirt1* expression was reduced by -1.9 (Table 1) during

298    the day in the livers of offspring exposed to a HF diet during the developmental period

(Figure 3b; p<0.05, HF/C vs C/C.), a decline that was exacerbated by post-natal HF feeding, showing 5.7 and 8 fold reduction (Table 1) in the C/HF and HF/HF offspring, respectively (Figure 3b; p<0.001, C/HF or HF/HF vs C/C.). In addition, to validate the reduction in *Sirt1* and *Sirt3* gene expression, we measured protein levels of SIRT1 and SIRT1 (Supplemental Figure 1a and 1b, respectively) to show that HF diets reduce significantly reduce Sirtuin abundance, with the greatest effect being observed in the HF/HF offspring (P<0.001 HF/HF vs C/C).

Since SIRT1 interacts with CLOCK in an NAD+ dependant manner [18;28], we measured the expression of core clock genes and CCGs with key roles in lipid homeostasis. Here, we show that *Clock* transcript levels were robustly expressed in livers of all offspring (Figure 3c). In contrast, hepatic daytime gene expression of *Bmal1* (Figure 3d) showed a 22 fold increase (Table 1) in offspring that have been fed a HF diet during development and adulthood, compared to control offspring (p<0.01; HF/HF vs C/C). In addition, *Bmal1* expression, which showed a 13.75, 6 and 8.35 fold increase in the day in the C/C, C/HF and HF/HF offspring respectively, was reversed in the HF/HF offspring (Table 2; -2.2 fold decrease in HF/HF).

CRY2 forms a functional heterodimer with PER2 which has an inhibitory effect on CLOCK/BMAL1. Interestingly, while the hepatic expression of *Per2* remains unperturbed following HF feeding (Figure 3e), daytime expression of *Cry2* was significantly altered in offspring exposed to a HF diet (Figure 3f; p<0.05, HF/C or HF/HF vs C/C). The nycthemeral pattern was also altered in the HF/C and HF/HF offspring, and showed higher expression in the night (Table 2; 5.18 and 2.4 fold respectively), compared to higher expression in the day on both the C/C and HF/HF offspring.

We next determined the expression of CCGs with important roles in lipid metabolism. Specifically, the nighttime expression of both *Rev-Erbα* and *Rev-Erbβ* was significantly increased in the HF/HF offspring compared to control (Figure 3g and Figure 3h, respectively; p<0.05, HF/HF vs C/C). In addition, the daytime expression of *Rev-Erbα* was also significantly increased compared to control (Figure 3g; p<0.05, HF/HF vs C/C). The expression *Rev-Erbβ* is normally much higher in the day than in the night (Table 2; -6.73 in C/C). However, with increasing exposure to a HF diet, the nycthemeral pattern of *Rev-Erbβ* expression is lost (Table 2; -3.22, -1.59, -1.23 fold in C/HF, HF/C and HF/HF, respectively). The orphan nuclear receptor *RORα*, also a key modulator of lipid metabolism, was similarly perturbed in the HF/C offspring in the day (Figure 3i; p<0.05, HF/C vs C/C) and night (Figure 3i; p<0.001, HF/C vs C/C). Both *Rev-Erb and RORα* may regulate lipid metabolism through the lipogenic transcription factor *Srebp1c.* Expression of this gene was increased in livers

334  from both the C/HF and HF/HF offspring (Figure 3j; $p<0.05$, C/HF and HF/HF vs C/C),

335  consistent with increased hepatic fat accumulation in these groups.

336  **DISCUSSION**

337  Our findings suggest that the developmental priming of severe fatty liver involves a complex

338  molecular pathology, involving alterations in circadian biology and lipogenesis. Specifically,

339  we show that increased fat intake during development is associated with disruptions in

340  circadian physiology, cellular redox status, Sirtuin abundance, and transcription of core clock

341  genes and down-stream lipogenic transcription factors (a schematic representation of this is

342  illustrated in Figure 4). These molecular changes appear to persist until adulthood, and may

343  have deleterious effects on hepatic lipid homeostasis, increasing the likelihood of developing

344  severe fatty liver disease in adulthood.

345      Since a growing number of young individuals are exposed to diets that are rich in fat

346  during critical periods of development, it is likely that excess fat intake in early life contributes

347  to the "primed" onset of NAFLD in young adults [37]. In this study we aimed to further

348  understand the mechanisms underlying developmentally primed severe fatty liver disease.

349  Here, we show that feeding a HF diet leads to NAFLD, however, the livers of male offspring

350  of HF-fed mothers who are also given a HF diet in postnatal life (HF/HF offspring) show

351  features pertaining to a more severe NASH-like phenotype. These results recapitulate our

352  previous findings in female offspring [10], and moreover, demonstrate that the

353  developmental priming of severe fatty liver is not sex-specific.

354      To further understand the pathogenesis of developmentally primed metabolic disorder, it

355  is useful to examine differences in circadian physiology between offspring groups. Both

356  groups of offspring fed a HF diet in the post-natal period (HF/HF and C/HF) show changes in

357  metabolism akin to pre-clinical metabolic disorder, such as hyperglycaemia, hyperlipidaemia,

358  increased food intake, and fatty liver disease. However, there are a number of interesting

359  phenotypic differences between these two offspring groups. Firstly, the C/HF mice display

360  increased insulin levels at night, when food intake and insulin levels are expected to

361  concomitantly rise, albeit elevated due to the macronutrient composition of the HF diet. In

362  contrast, the HF/HF mice show hyperinsulinemia in both the day and the night, highlighting

363  marked changes in circadian physiology following developmental HF exposure, and

364  suggesting that these offspring are insulin resistant, a known factor in the pathogenesis of

365  fatty liver disease. In addition, the C/HF offspring also show increased energy expenditure

366  and remain glucose tolerant. It is plausible that this is an adaptive response to increased

367  energy intake and fat consumption in a heavier, yet healthy organism. These adaptations,

368  namely increased EE may offer protection against increased ectopic lipid accumulation and

369  severe fatty liver disease. In contrast the HF/HF offspring fail to increase energy expenditure

370 in response to increased energy intake, and also exhibited pronounced hyperglycaemia, at

371 both baseline and post glucose bolus, which may be indicative of a developmentally

372 programmed inability to respond to nutritional excess. Thus the HF/HF are less able to

373 response to increased fat consumption, and unlike their C/HF counterparts, who were fed

374 the same diet but did not experience a developmental HF challenge, are not protected

375 against increased ectopic lipid accumulation and continue to develop severe fatty liver

376 disease

377      Reduced metabolic capacity in the HF/HF offspring is consistent with our previous work,

378 which showed that developmental fat exposure can impair mitochondrial electron transport

379 chain (ETC) function and metabolic capacity in offspring livers [10]. In further support, in the

380 present study we observe impaired redox status and significantly reduced *Sirt1* and *Sirt3*

381 gene expression in the HF/HF offspring. Importantly, SIRT3 is thought to interact with

382 subunits of complexes I and II of the electron transport chain (ETC) directly, leading to

383 increased mitochondrial efficiency [38]. Thus, it is plausible to suggest that prolonged

384 exposure to diets containing excessive amounts of fat could alter nutrient sensing via

385 mitochondrial Sirtuins and directly influence mitochondrial capacity and disease

386 susceptibility.

387      Our findings are consistent with recent studies implicating SIRT3 as a key player in fatty

388 liver disease pathogenesis [39]. Interestingly, SIRT3 mediated lipotoxicity is thought to

389 involve hyperacteylation of mitochondrial proteins and dysregulation of the electron transport

390 chain (ETC) enzymes, leading to protein oxidation and impaired mitochondrial function [40].

391 Since ETC enzyme function is impaired in offspring exposed to a HF diet during

392 development, we suggest that SIRT3 reduction plays an important role in developmentally

393 primed fatty liver. How SIRT3 becomes reduced is unclear, however, SIRT3 reduction is

394 often coupled to reduced NAD+ levels [39], and supplementation with the NAD+ precursor

395 nicotinamide ribose activates SIRT3 and enhances oxidative metabolism to protect against

396 HF-induced metabolic abnormalities [41]. Here, we show that NAD+ is markedly reduced in

397 the livers of offspring from obese mothers that were also fed a HF diet in later life,

398 suggesting that depleted NAD+ levels in these HF/HF offspring contributes to reduced

399 SIRT3 abundance. Whether increased fat intake activates NAD+ consuming enzymes to

400 deplete the cellular pool of NAD+, altering redox balance and reducing sirtuin abundance

401 remains to be tested. Nonetheless, in support of this premise *Sirt1* gene expression, for

402 which NAD+ is also a rate-limiting co-substrate, is also down regulated in the livers of HF/HF

403 offspring.

404      Multiple cellular processes play in important role in maintaining the NAD+/NADH ratio.

405 For example, during glycolysis, beta-oxidation, and the citric acid cycle NAD+ is reduced to

406  NADH. Once in the mitochondria NADH is oxidized by the electron transport chain enzymes
407  during oxidative phosphorylation. Since we have previously shown that the HF/HF offspring
408  exhibit impaired oxidative metabolism through reduced electron transport chain activity
409  (Bruce et al., 2009), it is possible to suggest that the HF/HF offspring are in a state of redox
410  imbalance, and although their livers are able to readily reduce NADH during catabolic redox
411  reactions (in response to increased fat intake), their ability to replenish NAD+ reserves is
412  lacking due to impaired oxidative capacity. Conversely, the C/HF offspring, which do not
413  have an impaired oxidative metabolism, are able to maintain redox balance even during
414  increased food intake. Similarly, the HF/C offspring, which may also have developmentally
415  impaired electron transport chain enzymes, are not burdened with the post-natal challenge
416  of a high fat diet, and thus are also able to maintain a favorable NAD+/NADH ratio.

417      As the liver ages it is more prone to metabolic changes such as ectopic lipid
418  accumulation [42]. This is consistent with growing evidence suggesting that fatty liver
419  disease is more prevalent in older individuals [43;44]. Here, we show that HF feeding during
420  early and later life is associated with depleted NAD+ reserves and reduced sirtuin
421  abundance, both established hallmarks of metabolic aging [31]. Importantly, offspring
422  exhibiting these hallmarks also display serve fatty liver disease at a fairly young age (15
423  weeks old). Therefore, we suggest that HF exposure during development may impose
424  chronic metabolic stress, culminating in accelerated organ decline and a propensity to
425  develop fatty liver disease at relatively early stages of life. Our findings begin to shed light on
426  the mechanisms underlying HF-induced developmental programming of fatty liver disease
427  and premature organ decline.

428      Here, we show clock gene expression is deregulated in offspring fed a HF diet. Since
429  SIRT1 functionally interacts with the core clock genes, it is plausible to suggest that our
430  observed changes in NAD+ and sirtuin abundance play a role in deregulated clock genes
431  expression. We show that hepatic *Bmal1* and *Cry2* gene expression is profoundly altered in
432  offspring of HF fed mothers. It is important to highlight that the simultaneous reduction of
433  *Cry2* and increase in *Bmal1* observed in the HF/HF offspring are directional changes that are
434  consistent with the current model of the core-clock transcriptional feedback loops. Our
435  findings suggest that *Bmal1* expression is influenced by nutritional challenges, and that
436  offspring of dams fed a high fat diet, that are fed a HF diet in post-natal life have significantly
437  up-regulated *Bmal1* expression in liver. Importantly, resent studies suggests that *Bmal1* is a
438  novel metabolic regulator that is able to couple circadian rhythm and lipid metabolism. *Bmal1*
439  knockout mice show both impaired insulin signalling and lipid homeostasis [45]. Recent
440  studies have shown that mice with either a global or hepatic *Bmal1* deficiency have reduced
441  lipogenic gene expression following re-feeding [46]. Conversely, *Bmal1* over expression in

the liver is sufficient to elevate the expression of genes involved in *de novo* lipogenesis, such as *Srebp1c* and *Fasn* (Zhang et al., 2014). Again, this is consistent with our observations showing the concurrent increase in CCGs (*Rev-Erb, RORα* and *Srebp1c*) with key roles in lipid lipogenesis. Specifically, *Rev-Erbα* is known to regulate the *Srebp1c* promoter and mRNA expression [47]. Here we show that in the C/HF and HF/HF offspring we see a subtly different pattern of hepatic "circadian" *Srebp1c* gene expression, but overall the expression of *Srepb1c* was elevated compared to the control offspring, as expected in steatotic livers. Nonetheless, *Srebp1c* levels were the highest the HF/HF group. Interestingly, while there was a trend towards increased *Rev-Erbα* expression in the C/HF group, only the HF/HF group show significantly elevated expression. Thus, we hypothesize that the elevated *Rev-Erbα* may be associated with increased *Srepb1c* expression observed in the HF/HF offspring. However, we cannot rule out other possible pathways that may be contributing to the increased expression of both of these genes, following differential dietary exposures between the offspring groups. Taken together our results suggest that a deregulated core clock gene-lipogenic transcription factor axis culminates in elevated hepatic fat accumulation and disease progression (Figure 4).

The impact of HF diets on clock gene expression and lipid homeostasis in the liver has been the subject of intense research of late. Nonetheless, controversy remains as to whether HF diets can significantly affect hepatic clock gene expression. Relatively short term HF feeding in mice produced a metabolic syndrome phenotype, but did not alter hepatic clock gene expression [48]. In contrast, recent studies have shown that HF feeding leads to changes in circadian locomotor activity, food intake, and hepatic clock gene expression [49;50]. In addition, long term HF feeding can have a major impact on clock gene expression in the liver [51]. It is therefore possible that the length or timing of HF exposure may have variable effects on the liver. Importantly, a recent study showed that developmental HF exposure disrupts circadian rhythmicity and contributes to metabolic syndrome including hepatic steatosis in a rat model of maternal obesity [11]. Maternal obesity has also been shown to program offspring NALFD through disruption of circadian rhythms and clock gene expression in offspring livers in mice [27]. Our data are both consistent with, and extend these recent observations, which show that hyper-calorific diets *in utero* can permanently disrupt the chronobiological network [27]. Collectively these findings highlight the importance of the developmental period for liver development, and that nutritional exposures during this time can persistently alter clock genes to permanently alter liver metabolism and increase disease susceptibility in later life.

In conclusion, during the last decade fatty liver disease has become the most common chronic liver disease in young individuals [52]. Therapies tend to focus on treating the co-

478  presenting metabolic imbalances such as obesity and increased BMI, and often have limited

479  efficacy treating liver disease itself. Pharmacological interventions that target the underlying

480  mechanism have considerable potential to prevent the onset of severe liver disease and

481  additional features of the metabolic syndrome. Our findings highlight the importance of

482  nutrition during early life as a promising area for intervention. Although further research in

483  this area is warranted to fully understand the multifaceted nature of developmentally primed

484  liver disease, our mechanistic insights suggest that supplementation with factors able to

485  reverse the effects of depleted NAD+ reserves, and/or SIRT1 and SIRT3 abundance may

486  also have the potential to alleviate the effects of developmental HF exposure, and rescue the

487  increased susceptibility to develop severe fatty liver disease in later life. Our model provides

488  a platform to further our understanding of the molecular pathogenesis of developmentally

489  primed fatty liver disease, and to identify novel interventions.

490

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**FIGURE LEGENDS**

**Figure 1.** 15 week old male offspring exposed to high fat diets during development and adulthood have perturbed nycthemeral (day vs night) rhythms of food intake and energy expenditure, and also develop severe fatty liver and imbalanced hepatic cellular redox status. (**a.**) Total amount of food intake of male offspring from each dietary group, during the 12h light (daytime, open bars) and 12h dark (nighttime, closed bars) periods. (**b.**) Energy expenditure levels in male offspring from each dietary group, during the 12h light (open bars) and 12h dark periods (closed bars). (**c.**) Body weight (recorded at ZT8) of the offspring at 15 weeks of age. (**d.**) Percentage of steatosis in liver sections determined by point counting (**e.**) Haematoxylin and eosin stained liver sections from 15 week old male offspring. C/C and HF/C offspring show normal liver architecture. C/HF offspring livers exhibit lipid accumulation. HF/HF livers contain significant lipid accumulation and ballooning degeneration. (**f.**) Kleiner scores for offspring liver (n=4 for each offspring group), show that C/C and HF/C are histologically normal, C/HF show a histological phenotype similar to human non-alcoholic fatty liver (NAFL), and HF/HF offspring liver have the appearance of non-alcoholic steatohepatitis (NASH). (**g.**) Fasting blood glucose over 2h following bolus intraperitoneal (IP) injection of glucose (inverted arrow). Inset histogram depicts the area under the curve (AUC) levels over the 2h monitoring period. (**h.**) Circulating insulin levels during the 12h light (daytime, open bars) and 12h dark (nighttime, closed bars) periods. For each analysis offspring groups were compared against C/C, where *$p<0.05$, **$p<0.01$, ***$p<0.001$ or ****$p<0.0001$ vs C/C

**Figure 2.** As a measure of fibrosis (**a.**) OPN gene expression was measured in offspring liver. Concentration of pyridine nucleotides (**b.**) NAD+, (**c.**) NADH in offspring liver (left lobe), and (**d.**) the NAD+/NADH ratio in offspring liver. Circulating levels of (**e.**) non-esterified fatty acids (NEFAs) and (**f.**) triglycerides were determined from mouse plasma taken during the light (daytime at ZT8, open bars) or dark (nighttime at ZT20, closed bars) periods, (For each analysis offspring groups were compared against C/C, where *$p<0.05$, **$p<0.01$ or ***$p<0.001$ vs C/C.

**Figure 3.** Mean relative hepatic mRNA expression of the Sirtuins (**a.**) *Sirt1* and (**b.**) *Sirt3*; of genes involved in co-ordinating circadian rhythms (**c.**) *Clock*, (**d.**) *Bmal1*, (**e.**) *Per2*, and (**f.**) *Cry2*; and downstream lipogenic transcription factors (**g.**) *Rev-Erbα*, (**h.**) *Rev-Erbβ*, (**i.**) *RORα,* and (**j.**) *Srebp1c* during during the light (daytime at ZT8, open bars) or dark (nighttime at ZT20, closed bars) periods. For each analysis offspring groups were compared against C/C, where *$p<0.05$, **$p<0.01$ or ***$p<0.001$ vs C/C.

687 **Figure 4.** Hypothetical schematic representation of how maternal high fat and post-natal
688 high fat (HF) diet alters Sirtuin and clock gene expression leading to severe hepatic steatosis
689 in offspring liver. A HF diet during both early and post-natal life reduces Sirtuin levels and
690 alters cellular energy status (reduction in NAD+ levels and NAD/NADH ratio). This is
691 associated with desynchronises circadian clock gene activity and upregulates downstream
692 lipogenic transcription factors such as *Srebp1c* to developmentally prime the lipogenic
693 pathway, resulting in further up-regulation of fat accumulation.

694 **Supplemental Figure 1**. Abundance of **a**. SIRT1 and **b**. SIRT3 was determined by western
695 blotting. 20µg of total protein from (ZT8) 15 week old male offspring (N=4) was separated by
696 12% SDS-PAGE. After electrophoresis protein was transferred to polyvinylidene difluoride
697 (PVDF) membrane in 75V wet transfer tank (Bio-rad, UK) for 90 min depending on protein
698 size. Incubation with SIRT1 and SIRT3 primary antibodies (Cell Signalling Technology, The
699 Netherlands) was carried overnight in 4°C in the presence of 3% non-fat milk/TBST buffer.
700 Membrane was washed in TBST buffer and incubated 2 hours with HRP-conjugated
701 secondary antibodies dissolved in 3% milk in TBS-T buffer. Signals were detected using
702 Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).
703 Western blots were visualized and analyzed using Versa Doc and QuantityOne 1-D analysis
704 software (Bio-Rad, USA).

705

706

Figure 1

# Figure 2

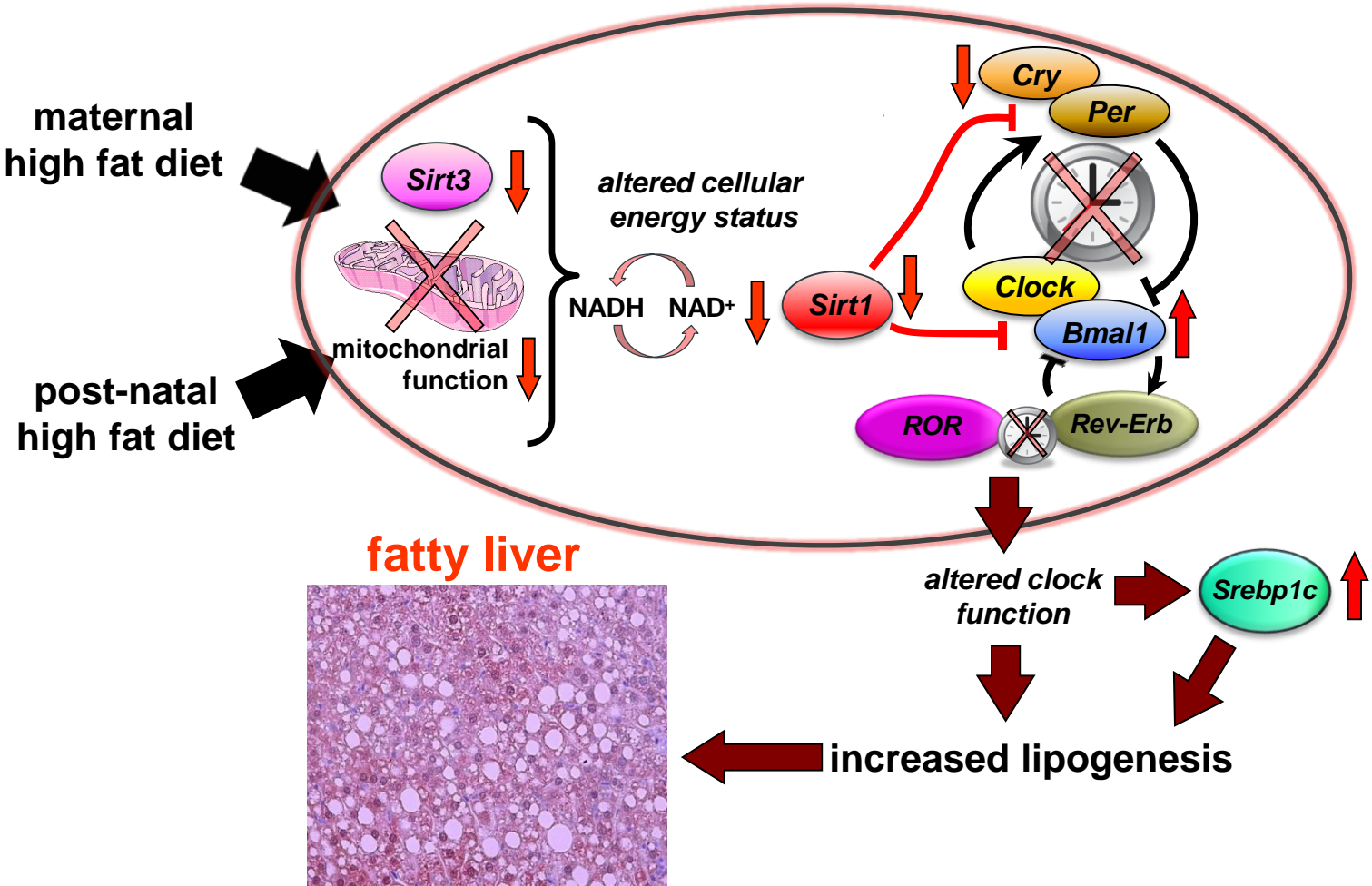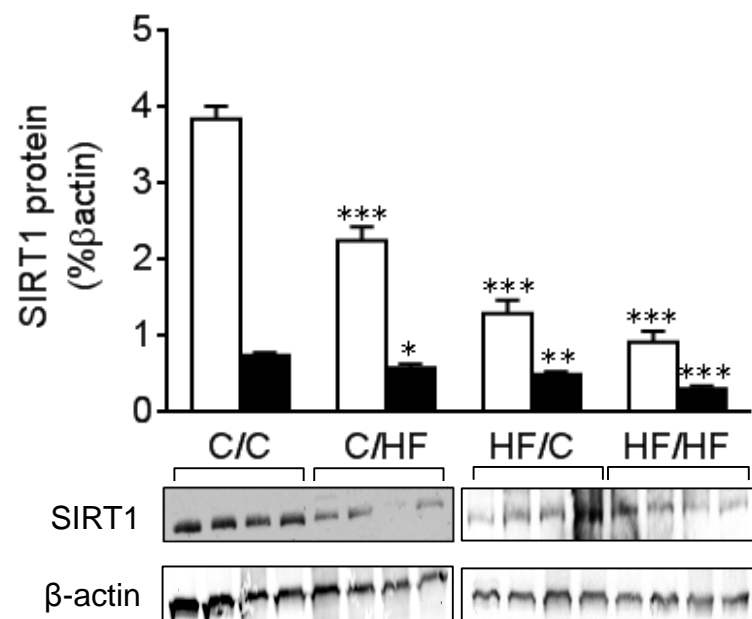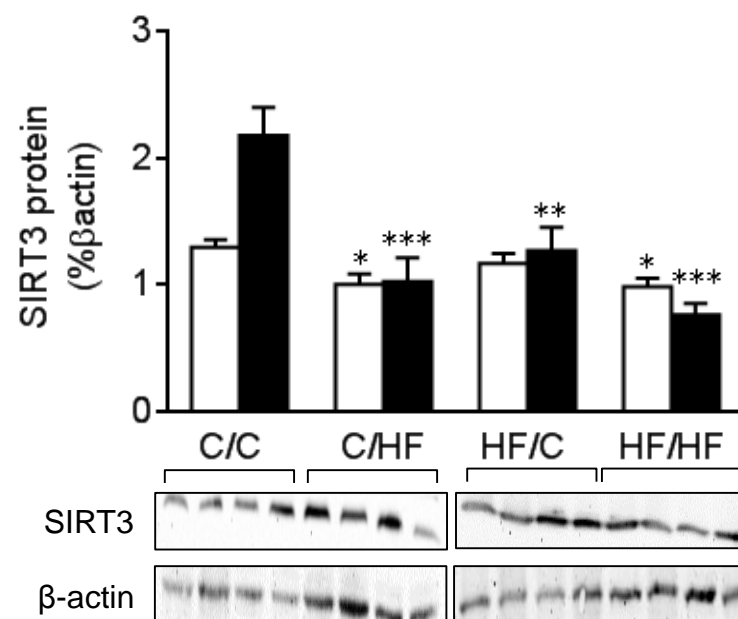# Figure 3

Figure 4

Table 1. Nighttime and daytime fold changes versus corresponding C/C levels

| | Daytime values vs C/C daytime values | | | Nighttime values vs C/C nighttime values | | |
|---|---|---|---|---|---|---|
| | C/HF | HF/C | HF/HF | C/HF | HF/C | HF/HF |
| *Sirt3* | -3.81±0.66** | -1.22±0.32 | -2.19±0.58 | -2.31±0.38 | 1.41±0.52 | -12.51±2.06 |
| *Sirt1* | -5.71±0.66**** | -1.97±0.25** | -8.08±1.30**** | 1.19±0.27 | 1.23±0.51 | -6.22±2.03 |
| *Clock* | -1.16±0.42 | -1.11±0.21 | -1.05±0.20 | 1.14±0.22 | -1.59±0.76 | 1.36±0.12 |
| *Bmal1* | 3.30±1.67 | -1.89±0.75 | 22.20±10.08* | 1.45±0.32 | -3.12±0.86 | -1.25±0.34 |
| *Per2* | 1.12±0.15 | -1.28±0.25 | 1.23±0.32 | 1.82±0.41 | 2.16±0.48 | 2.25±0.56 |
| *Cry2* | 1.29±0.39 | -2.38±0.32 | -3.65±1.82 | -2.16±0.61 | 3.65±1.82 | 1.10±0.24 |
| *Rev-Erbα* | 1.62±0.37 | 1.46±0.19 | 1.51±0.19 | 1.74±0.28 | 1.74±0.60 | 3.37±1.03 |
| *Rev-Erbβ* | -1.27±0.14 | -1.73±0.19 | -1.93±0.21 | 1.65±0.24 | 2.44±0.68* | 2.83±0.38** |
| *RORα* | -1.71±0.19* | -1.75±0.20* | 1.02±0.14 | -1.54±0.55 | 1.92±0.87 | 1.25±0.21 |
| *Srebp1c* | 1.14±0.28 | 1.73±0.39 | 1.97±0.31* | 3.32±0.69** | 1.83±0.92 | 3.16±0.49* |

*p<0.05, **p<0.01, ****p<0.0001 vs corresponding daytime or nighttime C/C levels

Table 2. Nighttime fold changes versus daytime levels

|  | C/C | C/HF | HF/C | HF/HF |
|---|---|---|---|---|
| *Sirt3* | -1.52±0.40 | 1.08±0.35 | 1.13±0.42 | -8.68±2.51** |
| *Sirt1* | -2.77±0.35** | 2.44±0.58* | -1.14±0.29 | -2.13±0.52 |
| *Clock* | 1.95±0.53 | 1.93±0.34 | 1.38±0.32 | 2.15±0.19** |
| *Bmal1* | 13.75±3.78*** | 6.06±1.34*** | 8.35±2.89** | -2.02±0.92 |
| *Per2* | -1.78±0.18 | -1.13±0.18 | 1.56±0.42 | 1.03±0.25 |
| *Cry2* | -1.68±0.22 | -4.66±1.30* | 5.18±1.91** | 2.40±0.53* |
| *Rev-Erbα* | -11.54±2.29*** | -10.80±2.46*** | -9.82±1.26*** | -5.18±0.64** |
| *Rev-Erbβ* | -6.73±0.73**** | -3.22±0.84* | -1.59±0.44 | -1.23±0.15 |
| *RORα* | -1.20±0.14 | -1.08±0.17 | 2.79±0.80** | 1.01±0.17 |
| *Srebp1c* | -2.48±0.56 | 1.17±0.24 | 1.28±0.64 | -1.55±0.24 |

*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs corresponding daytime or nighttime C/C levels