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Altered feeding differentially regulates circadian rhythms and energy metabolism in liver and muscle of rats

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ABSTRACT

Energy metabolism follows a diurnal pattern responding to the light/dark cycle and food availability. This study investigated the impact of restricting feeding to the daylight hours and feeding a high fat diet on circadian clock (*bmal1*, *dbp*, *tef* and *e4bp4*) and metabolic (*pepck*, *fas*, *ucp3*, *pdk4*) gene expression and markers of energy metabolism in muscle and liver of rats. The results show that in chow-fed rats switched to daylight feeding, the peak diurnal expression of genes in liver was shifted by 6–12 h while expression of these genes in muscle remained in a similar phase to rats feeding *ad libitum*. High fat feeding during the daylight hours had limited effect on clock gene expression in liver or muscle but shifted the peak expression of metabolic genes (*pepck*, *fas*) in liver by 6–12 h. The differential effects of daylight feeding on gene and protein expression in muscle and liver were accompanied by an 8% reduction in whole body energy expenditure, a 20–30% increased glycogen content during the light phase in muscle of day-fed rats and increased adipose tissue deposition per gram food consumed. These data demonstrate that a mismatch of feeding and light/dark cycle disrupts tissue metabolism in muscle with significant consequences for whole body energy homeostasis.

1. Introduction

Many aspects of the physiology and behavior of an animal are coordinated or aligned with the light/dark cycle by the circadian clock [1–3]. One role of the circadian oscillator is involvement in the regulation of energy metabolism through influencing processes such as food intake and the expression, secretion and activity of hormones and many metabolic enzymes [4–6]. Disruption of the temporal organization of energy metabolism through, for example, the misiming of food intake as occurs with rotational shift work and jetlag, may carry major implications for human health and has been suggested to contribute to the recent rise in obesity and other metabolic disorders [7,8].

The molecular machinery underlying circadian rhythms is a transcriptional/translational autoregulatory feedback loop with a period of approximately 24 h [9–11]. These intracellular clocks, to a large extent orchestrate rhythmic mRNA expression, which has recently been estimated to account for up to 15% of the entire transcriptome of a cell [12–15]. The molecular clock in the suprachiasmatic nucleus (SCN) is recognized as a central pacemaker thought to entrain molecular oscillators in the periphery through neural and endocrine signals [13]. Light is believed to be the principle cue, however other stimuli such as food-borne nutrients are capable of resetting the peripheral clocks and have the potential to uncouple clocks in the peripheral tissues from the master clock in the SCN [16–19]. The extent to which circadian physiology in each tissue is governed by local clocks, the central clock or nutrient-borne stimuli is still uncertain [15,20–22], however it is clear that disruptions in circadian rhythms may have severe consequences to metabolism as suggested by several knockout models of components of the molecular clock [21,23,24]. These animals showed a range of abnormalities in various metabolic parameters suggestive of metabolic syndrome and highlight the importance of intact circadian rhythms to energy homeostasis.

Most studies investigating the effects of altering the timing of food-intake have previously been performed in mouse models [16–19], and in these studies limited consideration has been given to the impact of restricted feeding in skeletal muscle [9]. Considering that the skeletal muscle is a key site for glucose utilization and fatty acid oxidation and accounts for about 20–30% of an animal's resting energy expenditure [25,26], it is likely that any disruptions to metabolism in muscle would compromise whole-body energy homeostasis. There is also a possibility that different nutrients signal to circadian clocks in specific ways and this was supported by studies which showed disruptions to several cycling parameters in a tissue-specific manner in a mouse model of diet-induced obesity [27,28]. Surprisingly, there has been little attention devoted to studying the impact of altered feeding pattern on the circadian metabolism in the context of a high fat diet.

In this study we investigated the effect of high fat feeding and restricting feeding to the daylight hours on diurnal metabolic parameters by examining circulating hormone concentrations and metabolite levels along with gene and protein expression in the liver and muscle of rats.

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The overall metabolic state of animals was also evaluated via measurements of energy expenditure and substrate utilization under conditions of altered feeding cues.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Animal Resources Centre, Perth, Australia) weighing between 200 and 250 g at the start of the experiment were used. Animals were communally housed at 22 ± 1 °C with a controlled 12:12 hour light/dark cycle (lights on 0700 to 1900) and free access to water. All experimental procedures performed were approved by the Animal Ethics Committee (Garvan Institute of Medical Research/St Vincent's Hospital, Darlinghurst, Australia) and were in accordance with the National Health and Medical Research Council of Australia's guidelines on animal experimentation.

2.2. Experimental procedure

Animals were randomly divided into four groups; chow-ad lib, chow-day, HF-ad lib and HF-day. Animals were left to acclimatize for one week. The chow-ad lib and HF-ad lib animals were given diet *ad libitum*, day-fed animals were given food between zT0 (lights on) and zT12 (lights off) with free access to water across 24 h. Chow-fed animals received a standard chow diet (Rat maintenance Diet, Gordon's Specialty Feeds, Sydney Australia) containing 8% fat, 21% protein and 71% carbohydrate (as calories) plus fiber, vitamins and minerals. HF-fed animals received a high fat diet composed of 60% calories as fat, 21% protein and 19% carbohydrate.

Preliminary observations established that ad lib, chow-fed rats consumed an average of 28.9 ± 0.9 g of food per day and approximately 6% of this daily intake was consumed during the light period. Similarly, ad lib fat-fed rats consumed an average of 22.1 ± 0.8 g of food per 24 h and less than 10% of the daily total was eaten during the light phase. The pattern of food intake in ad lib fed rats indicated that 20–30% of total food intake was consumed in each 3 h period across the 12 h dark phase. When animals were only allowed access to food during the light phase, 60% of the total food intake occurred within the first 3 h, 20% in the next 3 h, 5% in the third 3 h and 15% in the last 3 h of the light phase. In a separate study a group of night-fed chow rats were pair-fed to a group of day-fed chow rats by presenting the night-fed rats with the same amount of food (in grams) consumed by the day-fed rats in the previous 24 h.

2.3. Surgical insertion of cannula for blood and tissue collection

The duration of the feeding regime was three weeks after which the animals were anesthetized with an injection of xylazine (10 mg/kg)/ketalar (60 mg/kg) and a single cannula was inserted in the left jugular vein and exteriorized to the back of the neck. After recovering from surgery and regaining their pre-operative weight rats were assigned to groups to be sacrificed at 3-hour intervals over the 24 h period. At zeitgeber time (zT) 0, 3, 6, 9, 12, 15, 18 and 21, rats were removed from the holding room and a blood sample was taken via the jugular cannula before infusion of an overdose of pentobarbitone (60 mg/kg) to euthanize the animal. Liver, muscle and epididymal fat were rapidly dissected and freeze-clamped in liquid nitrogen. The time from removing the rats from the holding room to tissue collection was never longer than 10 min. Each timepoint comprises 4–8 animals per feeding group.

2.4. Plasma and tissue analysis

Serum insulin, leptin and adiponectin levels were assayed using radioimmunoassay kits from Linco Research Inc. (St Louis MO., USA). Non-esterified fatty acids (NEFA) were analyzed by an enzymatic colorimetric method (NEFA-C kit, Wako Pure Chemical Industries, Osaka, Japan). Tissue glycogen content was measured using a colorimetric assay as described previously [29]. Plasma, muscle, and liver triglyceride contents were determined using a colorimetric assay kit (Triglycerides GPO-PAP; Roche Diagnostics, Indianapolis, IN) as previously described [30].

2.5. Real-time quantitative PCR

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Mulgrave, Vic., Australia), according to the manufacturer's instructions. After DNase digest, 0.65 μg of total RNA was reverse transcribed with an Omniscript RT kit (Qiagen, Clifton Hill, Vic., Australia) and analyzed using TaqMan® Gene Expression Assays (bmal1: Rn00577590_m1, dbp: Rn00497539_m1, e4bp4: Rn01434874_s1, fas: Rn00569117_m1, pdk4: Rn00585577_m1, pepck: Rn01529014_m1, tef: Rn00569911_m1, ucp3: Rn00565874_m1) on the ABI 7900 system (Applied Biosystems, Foster City, CA, USA). The standard curve method was used to quantitate mRNA transcripts. This was achieved by extrapolating a value by comparing unknowns to the standard curve of known amounts of transcripts.

2.6. Western blot analysis

Liver and muscle tissue (~50 mg) was homogenized in ice-cold RIPA buffer with protease and phosphatase inhibitors (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Nonidet NP-40, 0.5% sodium Deoxy-cholate, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 100 mg/ml phenylmethylsulfonyl fluoride, and 2 mg/ml aprotinin). Lysates were resolved by SDS-PAGE, transferred onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Sydney, Australia) and immunoblotted with antibodies against phosphoenolpyruvate carboxykinase (PEPCK; Cayman Chemical Company, Ann Arbor, MI), fatty acid synthase (FAS; Cell Signaling, Qld, Australia), pyruvate dehydrogenase kinase 4 (PDK4; kind gift of Robert A. Harris, Indiana University, IN), and uncoupling protein 3 (UCP3; Thermo Scientific, Wilmington, DE). Immunolabeled bands were quantified by densitometry with ImageJ 1.42q software (freeware, Wayne Rasband, NIH, USA).

2.7. Indirect calorimetry

A Columbus oxymax indirect calorimetry system (Columbus Instruments, Columbus, OH, USA) was used to determine the oxygen consumption (VO_2) and carbon dioxide production (VCO_2) of animals. Rats were placed individually in airtight metabolic chambers with *ad libitum* access to water. Food was available in the chambers according to the feeding group of the animal. The airflow through each chamber was 1.2 l/min and O_2 and CO_2 measurements were taken every 15 min across 24 h. The respiratory exchange ratio (RER) of each animal was calculated as the quotient of VCO_2/VO_2. Activity was measured in the chambers using an OPTO-M3 sensor system (Columbus Instruments), where counts were a record of photo-beam breaks.

2.8. Statistical analysis

All results are expressed as means ± SEM. Statistical analyses were performed with the Graphpad Prism5 (Graphpad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to assess whether a certain parameter had a circadian profile. Peak and trough of expression were determined by a Bonferroni post-hoc test. Two-way analysis of variance (ANOVA) was conducted to investigate the main effects of group (diet or phase of feeding) and time. A Bonferonni's post-hoc test was applied for statistical differences at each timepoint between two groups. One disadvantage of this type of analysis is that when a transcript cycles in a different phase but similar amplitude between two groups, two-way ANOVA will not regard the difference as significant. Therefore, the post-hoc test is important as it highlights the

significant difference between groups at each timepoint. $P<0.05$ was considered significantly different. Repeated measures analysis was used for calorimetry measures. Repeated measures analysis was not considered appropriate for other measures as the samples at each time point were from different animals.

3. Results

3.1. Effect of HF feeding and daylight feeding on plasma parameters

High fat feeding induced a pronounced increase in the plasma levels of leptin and FFA across the entire 24 h in both *ad lib* and daylight fed groups (Fig. 1; $p<0.01$ two-way ANOVA for effect of diet). Daylight feeding shifted the diurnal profile of insulin, leptin and FFAs but not corticosterone. Insulin levels were altered by daylight feeding ($p<0.001$ two-way ANOVA for effect of feeding phase) with higher circulating insulin correlating with the time of food intake for each feeding group (Fig. 1A and B). The effect of daylight feeding was pronounced for leptin and FFA in the HF-fed animals (Fig. 1F and H). The peak in leptin ($p<0.05$) and FFA ($p<0.001$) in the HF-day animals appeared in the opposite phase relative to the HF-ad lib animals. Plasma corticosterone exhibited a similar profile in the ad lib-fed and day-fed animals but an ancillary peak was also present in the day-fed animals at zT0, (Fig. 1C and D). There was no significant circadian variation in adiponectin levels in plasma in the chow-ad lib group (Fig. S1). Day-feeding raised the adiponectin levels significantly in chow-day ($p<0.001$ by two-way ANOVA) and HF-day animals ($p<0.01$ by two-way ANOVA) compared to the ad lib-fed animals and introduced a diurnal rhythm in the chow-day animals.

3.2. Effect of HF feeding and daylight feeding on clock gene expression

To assess the effects of high-fat diet and daylight feeding on clock gene expression the amount of mRNA of the core clock and output genes *bmal1* and *dbp* was examined in the liver and skeletal muscle of rats from the four different feeding groups. The mRNA of *bmal1* and *dbp* varied in a circadian fashion in both tissues (Fig. 2A, C, E and G) however the amplitude of expression was about 10 fold higher in the liver relative to skeletal muscle. HF-ad lib feeding blunted *bmal1* expression in the liver (Fig. 2A and B) but did not affect the rhythm of clock genes in either tissue (compare Fig. 2A, C, E, G with Fig. 2B, D, F, H). Daylight feeding of a chow diet shifted the temporal expression of *bmal1* and *dbp* to the opposite phase in the liver ($p<0.001$ two-way ANOVA, effect of feeding phase) (Fig. 2A and C). In contrast, the amplitude of expression of these two genes was strongly attenuated in the muscle with daylight feeding and the rhythmicity was either lost in the case of *bmal1* (ns by one-way ANOVA) (Fig. 2E) or maintained in the same phase as the chow-ad lib animals (*dbp*) (Fig. 2G). Surprisingly, although daylight feeding reversed liver clock gene expression in the chow-day animals (Fig. 2A and C), a similar change in the phase of expression of *bmal1* and *dbp* was not observed in the liver of HF-day animals (Fig. 2B and D). The temporal expression was similar to the HF-ad lib animals and for *dbp*, the amplitude of expression was markedly dampened ($p<0.001$ two-way ANOVA, effect of feeding phase). HF-day feeding in the muscle only attenuated the amplitude of *dbp* transcript at the peak of its expression at zT9 and 12 (Fig. 2H) but had no effect on the temporal pattern for either *bmal1* or *dbp* (Fig. 2F and H). Interestingly, the same tissue-specific and feeding-group specific patterns of gene expression were observed for other clock genes *tef* and *e4bp4* (Fig. S2).

3.3. Expression of metabolic genes is shifted to reflect food availability in the liver but not in muscle

The mRNA of *pepck*, coding for phosphoenol pyruvate carboxykinase, a rate-limiting enzyme in gluconeogenesis and *fas* coding for fatty acid synthase, a key enzyme in *de novo* lipogenesis was measured across 24 h. These genes were expressed in a circadian fashion in normal animals (Fig. 3A and C). Regardless of the diet consumed, the phase of expression of *pepck* and *fas* was reversed (i.e. ~12 h apart) in animals feeding in daylight hours compared to ad lib-fed animals ($p<0.001$ two-way ANOVA, effect of feeding phase) (Fig. 3A, B, C, D). There was a significant blunting of *fas* expression ($p<0.01$ two-way ANOVA, effect of diet) in both HF ad lib- and HF day-fed animals (Fig. 3D).

In muscle, PDK4 is important in regulating the switch from glucose to lipid metabolism and UCP3 is involved in fatty acid metabolism. The expression of both these genes exhibits circadian variation and is increased by high fat diets [31]. *Pdk4* ($p<0.05$ one-way ANOVA) and *ucp3* ($p<0.001$ one way ANOVA) transcripts cycled in the muscle with higher expression in the light-phase compared to the dark-phase (Fig. 3E and G). The temporal expression of these two genes was shifted slightly in chow-day animals, with increased transcript levels occurring in the late-dark phase instead of the light-phase as observed in the chow-ad lib animals. HF-feeding had a profound effect on the expression of both *pdk4* and *ucp3*, increasing the expression at some timepoints greater than 10-fold relative to the chow-fed animals (Fig. 3F and H). The most marked elevation in *pdk4* and *ucp3* transcript in HF-fed rats occurred in the late dark-phase creating a pronounced diurnal rhythm for both genes ($p<0.001$ one way ANOVA).

3.4. Effect of HF feeding and daylight feeding on protein expression

In order to investigate whether the changes seen at the gene expression level were translated into similar changes at the protein level, we examined the content of the proteins encoded by the four metabolic genes measured above. Fig. 4A shows PEPCK protein had a diurnal variation ($p<0.001$ one-way ANOVA) similar to its mRNA transcript (Fig. 3A) in the chow-ad lib animal. In chow-day animals the rhythmic expression was reversed compared to the chow-ad lib animals (Fig. 4A). HF-diet blunted the amplitude of PEPCK protein compared to the chow-fed groups (Fig. 4B). The variation in diurnal expression of FAS protein was less than that observed for its mRNA transcript (Fig. 4C). HF feeding decreased the amount of FAS and abolished any temporal variation in FAS protein ($p<0.001$ two-way ANOVA, effect of diet) (Fig. 4D). Interestingly, day-fed animals had increased FAS protein levels ($p<0.001$ two-way ANOVA, effect of feeding phase) compared to ad lib-fed animals on either diet (Fig. 4C and D).

In chow-ad lib and chow-day animals there was no difference between PDK4 and UCP3 protein in muscle and these proteins did not display a significant diurnal variation (Fig. 4E, G). Under conditions of HF feeding, the protein content of PDK4 and UCP3 was elevated at least 2 fold ($p<0.001$ two-way ANOVA, effect of diet) (Fig. 4F, H). HF-day animals had an altered phase of PDK4 expression compared to HF-ad lib animals ($p<0.001$ two-way ANOVA, effect of feeding phase) (Fig. 4F) but time of feeding had no effect on UCP3 protein content on either diet (Fig. 4H).

3.5. Fuel metabolism is disturbed in animals feeding in the light-phase

In order to investigate whether the changes at the gene and protein level of the molecular clock and metabolic proteins correlated with changes in fuel metabolism at the whole body level, we examined energy balance and markers of energy metabolism in liver and skeletal muscle in a subset of rats. Limiting rats to daytime feeding resulted in a decrease in food intake for the first 3–5 days after which day-fed animals ate a similar amount of food as *ad libitum* fed rats. This resulted in a significant deficit in the cumulative food intake in day-fed animals over the 3 weeks of study compared to ad lib-feeding rats on both the chow and high-fat diet (Table 1). Despite this deficit in food intake there was no significant difference in body weight or the weight of epididymal fat pads of *ad libitum* and day-fed rats (Table 1). This suggested that changing the feeding paradigm may alter energy expenditure in day fed rats.

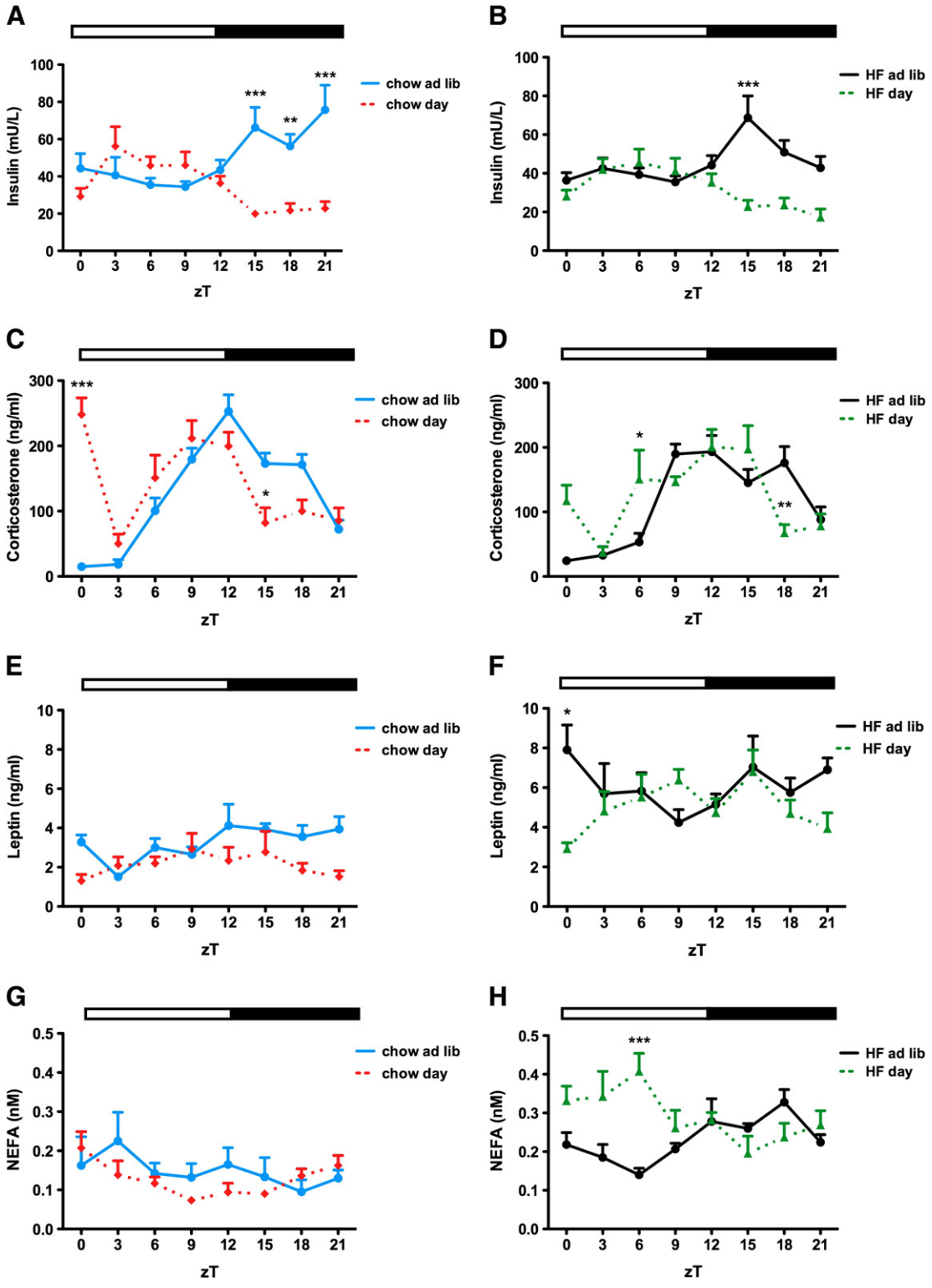

Fig. 1. Effect of alternate feeding schedules on the 24 h plasma profile. Insulin (A and B), corticosterone (C and D), leptin (E and F) and FFA (G and H) of chow-ad lib (blue), chow-day (red broken line), HF-ad lib (black) and HF-day (green broken line) fed animals collected at 8 time points across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, *p<0.05, **p<0.01, ***p<0.001 with Bonferroni post-hoc test for a significant difference between the treatment groups at specific timepoints, n = 4–8.

To investigate possible disruptions to energy utilization, the 24 h energy expenditure (oxygen consumption), substrate utilization and activity of rats under the four different feeding regimes was determined. As expected, there was a significant circadian variation in oxygen consumption between the light and dark phases of chow-ad lib and HF-ad lib animals ($p<0.001$ one-way ANOVA) correlating with the active/feeding and resting periods of these animals (Fig. 5A). HF feeding had no significant effect on oxygen consumption. Chow-day animals

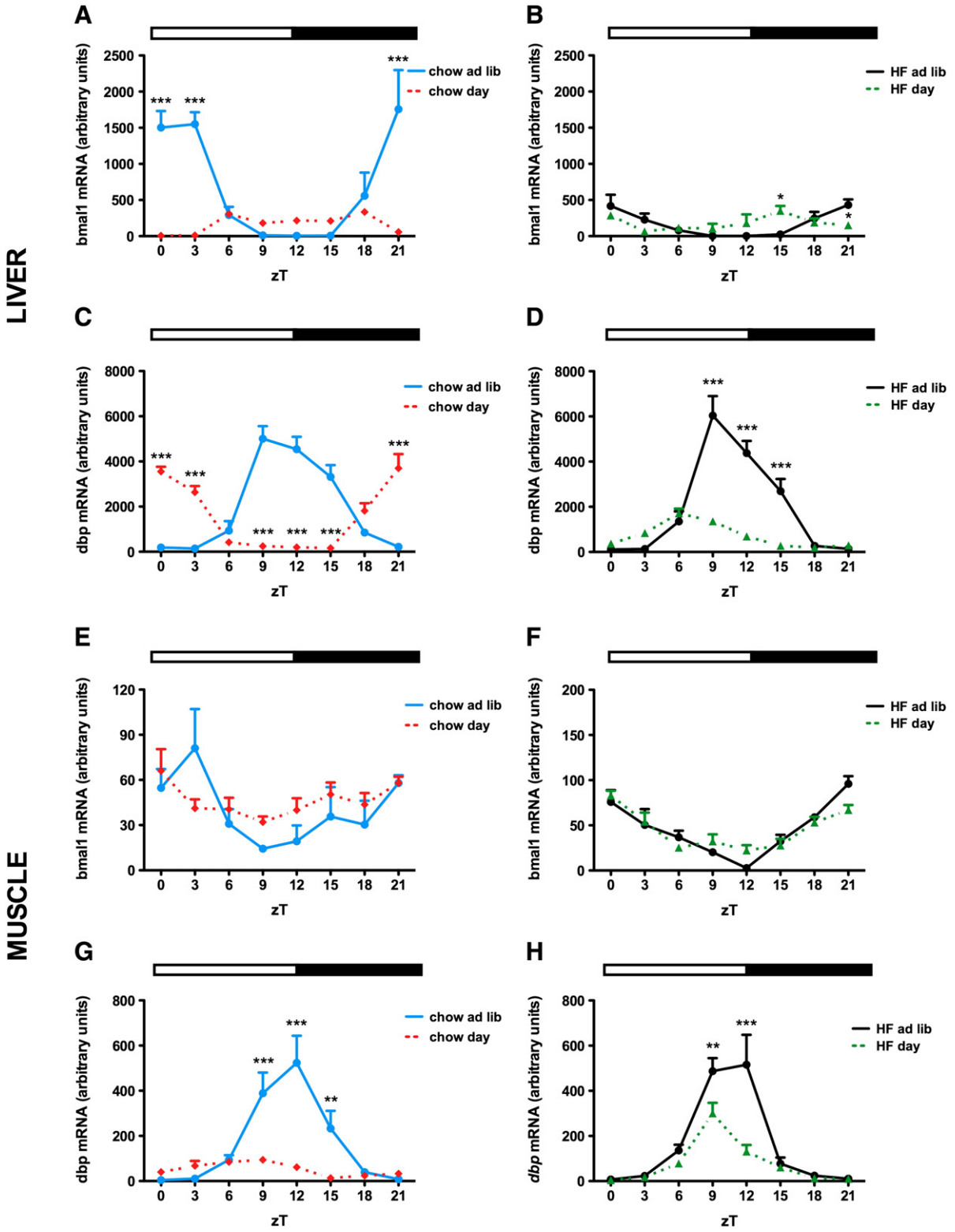

Fig. 2. Effect of alternate feeding schedules on clock gene expression in liver and muscle. Liver *bmal1* (A and B), liver *dbp* (C and D), muscle *bmal1* (E and F) and muscle *dbp* (G and H) of chow-ad lib (blue), chow-day (red broken line), HF-ad lib (black) and HF-day (green broken line) fed animals collected at 8 time points across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$ with Bonferroni post-hoc test for a significant difference between the treatment groups at specific timepoints, n = 4–8.

showed circadian variation in the pattern of oxygen consumption by one-way ANOVA ($p<0.001$), that was largely due to a spike in energy expenditure at the beginning of the light-phase corresponding to the presentation of food and increased feeding at this time. In contrast to the ad lib-fed animals which had a prolonged phase of energy expenditure which lasted about 12 h, the day fed group displayed only a short (2 h) period of increased oxygen consumption after which oxygen consumption declined and remained relatively constant for the remainder

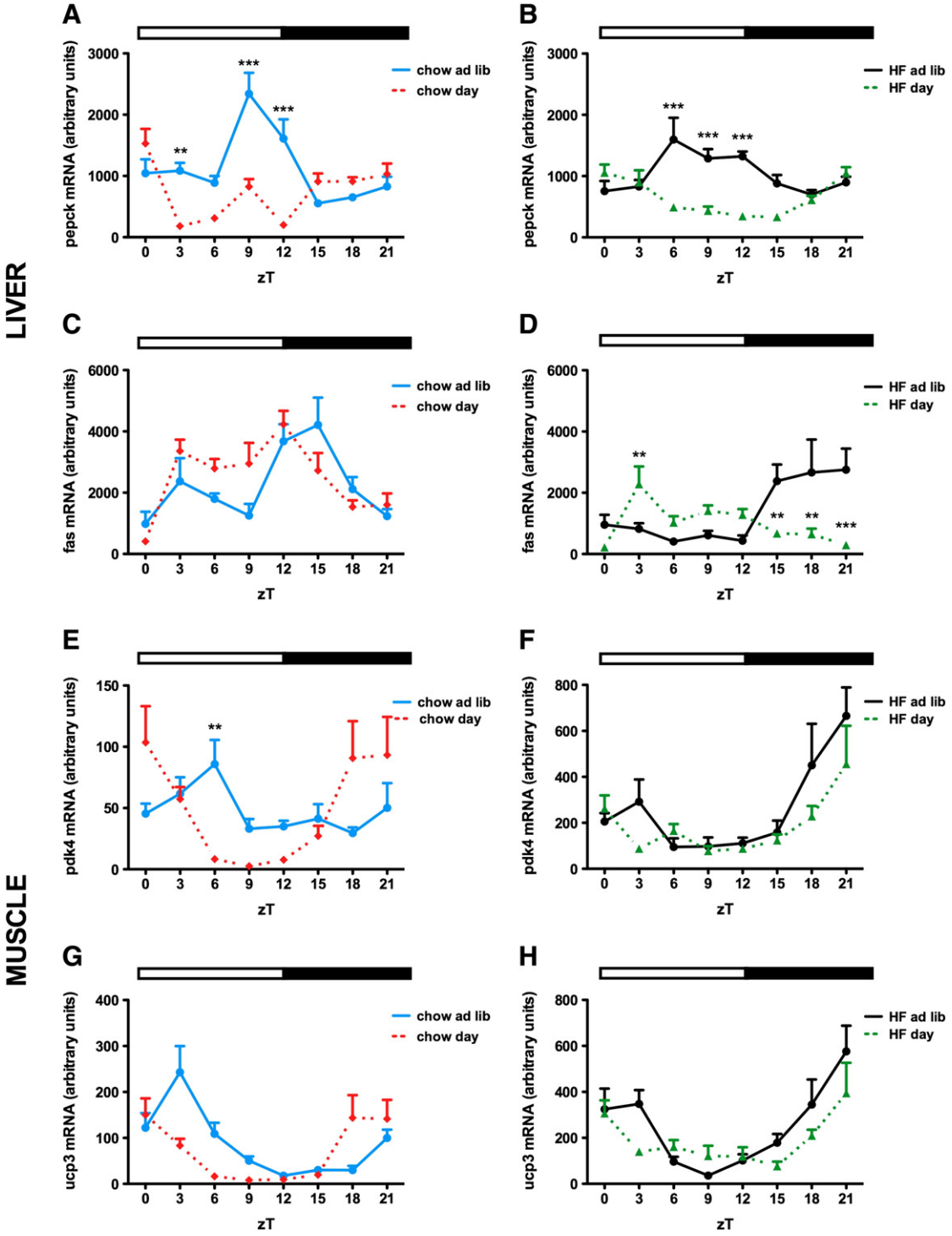

Fig. 3. Effect of alternate feeding schedules on metabolic gene expression in the liver and muscle. *Pepck* (A and B), *fas* (C and D), *pdk4* (E and F) and *ucp3* (G and H) of chow-ad lib (blue), chow-day (red broken line), HF-ad lib (black) and HF-day (green broken line) fed animals collected at 8 time points across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, *p<0.05, **p<0.01, ***p<0.001 with Bonferroni post-hoc test for a significant difference between the treatment groups at specific timepoints, n = 4–8.

of the day. The HF-day animals maintained some diurnal variation in their energy expenditure ($p<0.001$ by one-way ANOVA) however, the difference between oxygen consumption in the day and night phase was considerably dampened compared to the ad lib-fed animals. Overall, day-fed animals had a significantly reduced rate of energy expenditure (VO_2 (ml/kg/24 h): 39,096 chow-ad lib versus 36,398 chow-day and 41,057 HF-ad lib versus 37189 HF-day ($p<0.01$ two-way ANOVA, effect of feeding phase)). Since increased adiposity has previously been reported in restricted feeding paradigms [32–34] we further examined the possibility of altered energy balance in this study. Food intake of a group of night-fed rats was restricted (pair-fed) to the amount of food eaten by a day-fed group in the previous 24 h. Food was presented to

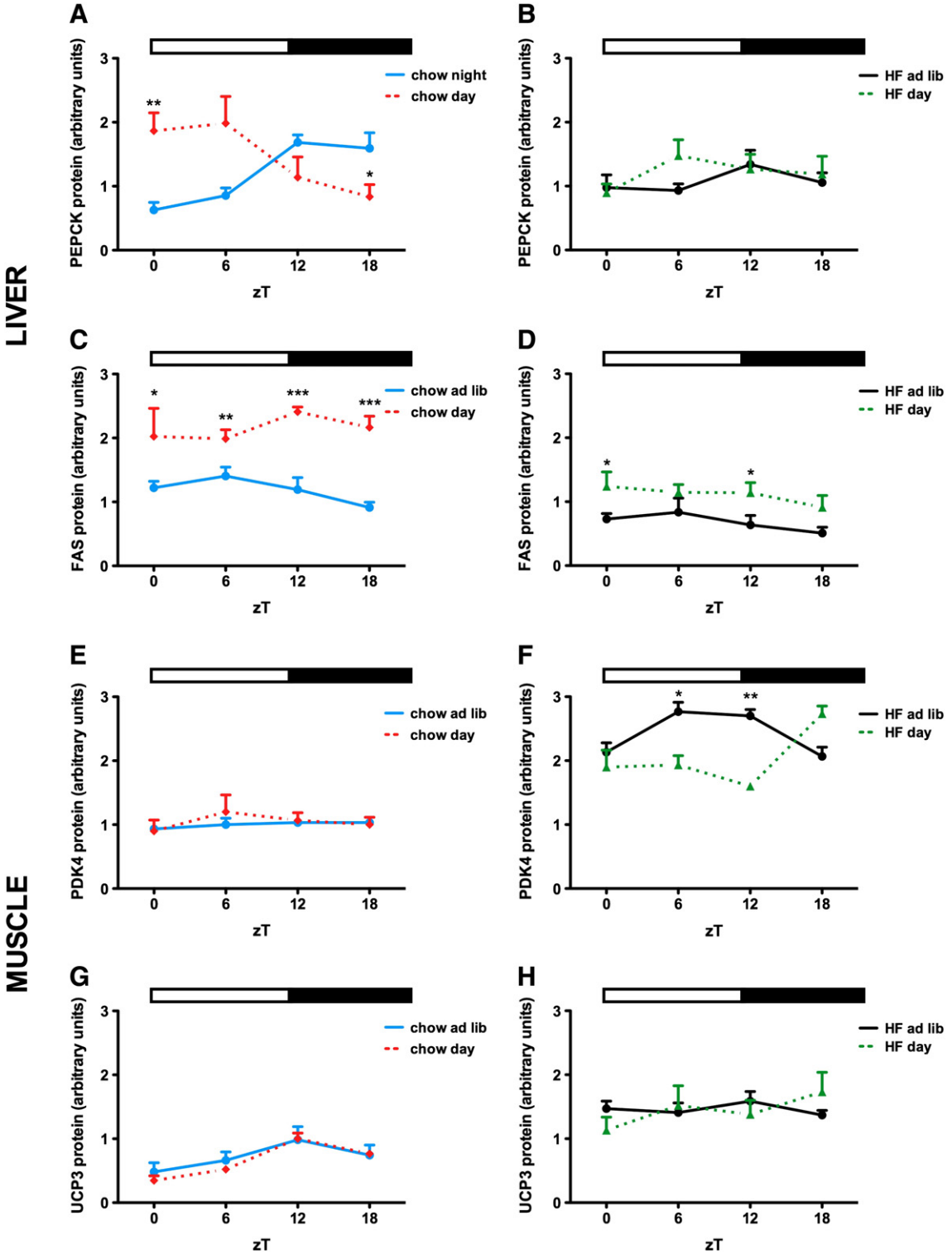

Fig. 4. Effect of alternate feeding schedules on metabolic protein expression in the liver and muscle. PEPCK (A and B), FAS (C and D), PDK4 (E and F) and UCP3 (G and H) of chow-ad lib (blue), chow-day (red broken line), HF-ad lib (black) and HF-day (green broken line) fed animals collected at 8 time points across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$ with Bonferroni post-hoc test for a significant difference between the treatment groups at specific timepoints, n = 4–8.

the pair-fed group at the start of the dark period. The comparison of energy expenditure and fat pad weight in the night, pair-fed and the day-fed group confirmed reduced energy expenditure in the day-fed group and when food intake was controlled, day-fed rats had increased epididymal fat pads compared to night, pair-fed controls (Fig. S3).

The respiratory exchange ratio (RER) provides an indication of the type of substrate being oxidized [35]. In chow-ad lib rats the RER declined throughout the light-period (as animals became more dependent on stored fat) to its lowest point 0.87 at zT11 and then increased and remained high for the duration of the dark-period when animals were feeding and relying for energy on the carbohydrate in the diet ($p<0.001$ one-way ANOVA, effect of time) (Fig. 5B). In HF-ad lib animals, the RER was approximately 0.85 across 24 h (ns one-way ANOVA). The RER profile for the chow-day animals was shifted by approximately

Table 1
Body weight, food intake and adipose tissue weight of rats fed chow and high-fat diets either ab libitum or only during the light phase.

	Chow-ad lib	Chow-day	HF-ad lib	HF-day	Effect of diet	Effect of feeding phase	Interaction
Body weight							
Initial (g)	234 ± 3^a	242 ± 2^a	243 ± 4^a	237 ± 3^a	ns	ns	ns
Final (g)	323 ± 9^a	327 ± 4^a	348 ± 6^b	340 ± 4^b	***	ns	ns
Weight gain (g)	89 ± 9^a	84 ± 4^a	105 ± 4^b	103 ± 7^b	***	ns	ns
Cumulative food intake (g)	378 ± 19^a	300 ± 4^b	318 ± 29^b	260 ± 6^c	***	***	ns
Adipose tissue							
WATepi (g)	0.94 ± 0.1^a	0.81 ± 0.04^a	1.86 ± 0.18^b	1.63 ± 0.12^b	***	ns	ns
WATepi (g/100 g BW)	0.55 ± 0.05^a	0.48 ± 0.03^a	0.99 ± 0.09^b	0.91 ± 0.06^b	***	ns	ns

Mean body weights, adipose tissue weights and cumulative food intake ± SEM. Different letters indicate significant differences among groups ($p<0.05$), ***$p<0.001$ indicates a significant difference by two-way ANOVA for effect of diet or feeding phase, n = 12 (ad lib-fed animals) and n = 33–35 (day-fed animals).

12 h compared to the chow-ad lib animals, which reflected the change in feeding and fasting phases. Interestingly, in the middle of the light-phase the RER values remained above 1.0 for a number of hours, indicating the possibility of significant lipogenesis in response to the chow diet fed during the day [35] that was not present in ad lib chow-fed rats. In HF-day animals RER was significantly lower for all timepoints compared to chow-day animals ($p<0.001$ two-way ANOVA, effect of diet), however the RER values did exhibit a significant variation between 0.8 and 0.9 across the 24 h ($p<0.001$ one-way ANOVA).

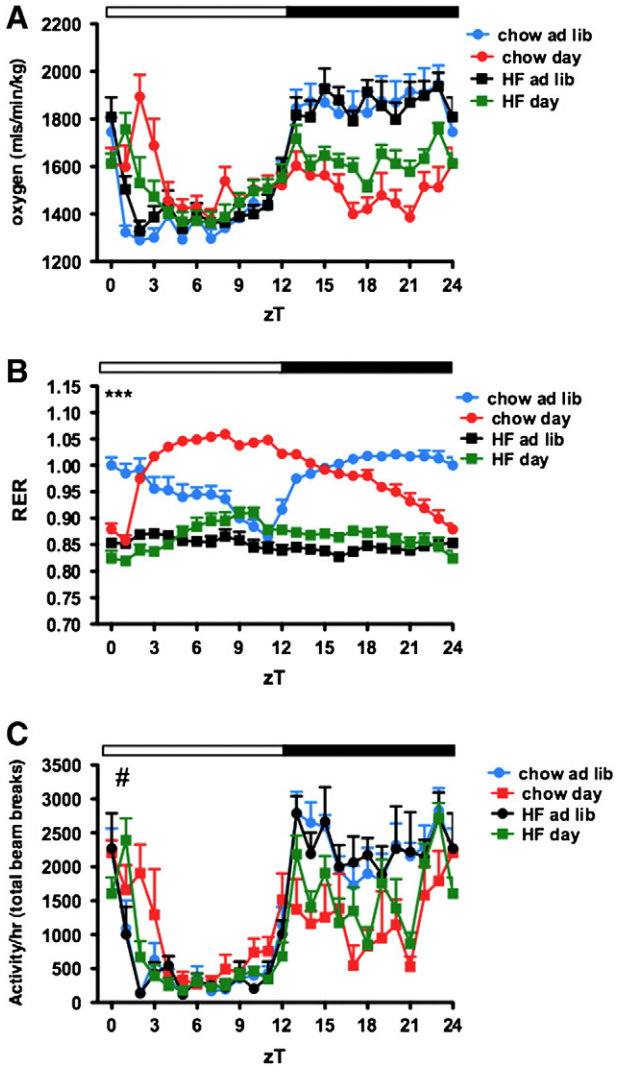

Fig. 5. Twenty-four hour measurements of energy utilization and activity under different feeding schedules. Oxygen consumption (A), RER (B) and activity (C) measurements in chow-ad lib (blue), chow-day (red), HF-ad lib (black) and HF-day (green) fed animals collected every hour across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, ***$p<0.001$ for significant effect of diet using repeated measures two-way ANOVA and #$p<0.05$ for a significant effect of feeding phase using repeated measures two-way ANOVA, n = 4–5.

The activity of animals during the period they were being investigated for energy expenditure and substrate utilization was assessed as the total number of beam breaks (X + Y + Z) per hour (Fig. 5C). Chow-ad lib and HF-ad lib animals displayed essentially identical activity patterns which were lower in the day than in the night ($p<0.001$ unpaired Student's t-test), therefore reflecting the normal nocturnal activity of rodents. Chow-day animals displayed a slightly higher activity in the day period and lower activity in the night period compared to chow-ad lib animals, reflecting a dampening in the normal difference between the light and dark phases of the day. Nevertheless, the activity was still greater in the dark-phase ($p<0.001$ unpaired Student's t-test). HF-day animals displayed similar activity in the day period as the ad lib-fed animals, however in the dark-phase their activity was significantly lower than the ad lib-fed animals ($p<0.01$ two-way ANOVA, effect of feeding-phase). The total activity in the day-fed animals was reduced compared to ad lib-fed animals as assessed by total beam breaks across 24 h ($p<0.05$ unpaired Student's t-test).

Glycogen is the major storage form of glucose in tissues and in liver, glycogen levels oscillate throughout the day to replenish blood glucose levels at times when glucose is not being provided by ingestion of food (Fig. 6A). In skeletal muscle glycogen levels do not vary significantly over the 24 h period (Fig. 6C). HF feeding resulted in lower glycogen levels in both liver and muscle ($p<0.05$ two-way ANOVA, effect of diet) but produced no change in the temporal profile. Daylight feeding shifted the profile of glycogen in the liver to the opposite phase ($p<0.001$ two-way ANOVA, effect of feeding phase). Interestingly, there was an increase in muscle glycogen in the light-phase in day-fed animals (particularly in the chow-day rats). This increase introduced a diurnal profile in muscle glycogen ($p<0.001$ one-way ANOVA) in the animals that consumed food only in the light phase (Fig. 6C).

Triacylglyceride (TAG) content was also investigated in the liver and skeletal muscle. High fat feeding increased the amount of TAGs in both liver and skeletal muscle ($p<0.01$ two-way ANOVA, effect of diet) (Fig. 7B and D). Day-feeding only had an effect in the liver of chow-fed animals ($p<0.05$ two-way ANOVA, effect of feeding phase) where the diurnal increase of liver TAGs at zT6 and 9 in the chow-ad lib animals was no longer present in the chow-day fed rats (Fig. 7A).

4. Discussion

Analysis of clock and metabolic gene expression in rats with different feeding schedules and dietary composition exposed a differential response to nutrient signals in liver and muscle. The liver rapidly adjusted its physiology to daylight food availability which contrasted with skeletal muscle, where clock and metabolic gene expression were dampened in amplitude but remained in a similar phase to muscle in the ad lib-feeding animal. This loss of synchrony between the liver and muscle

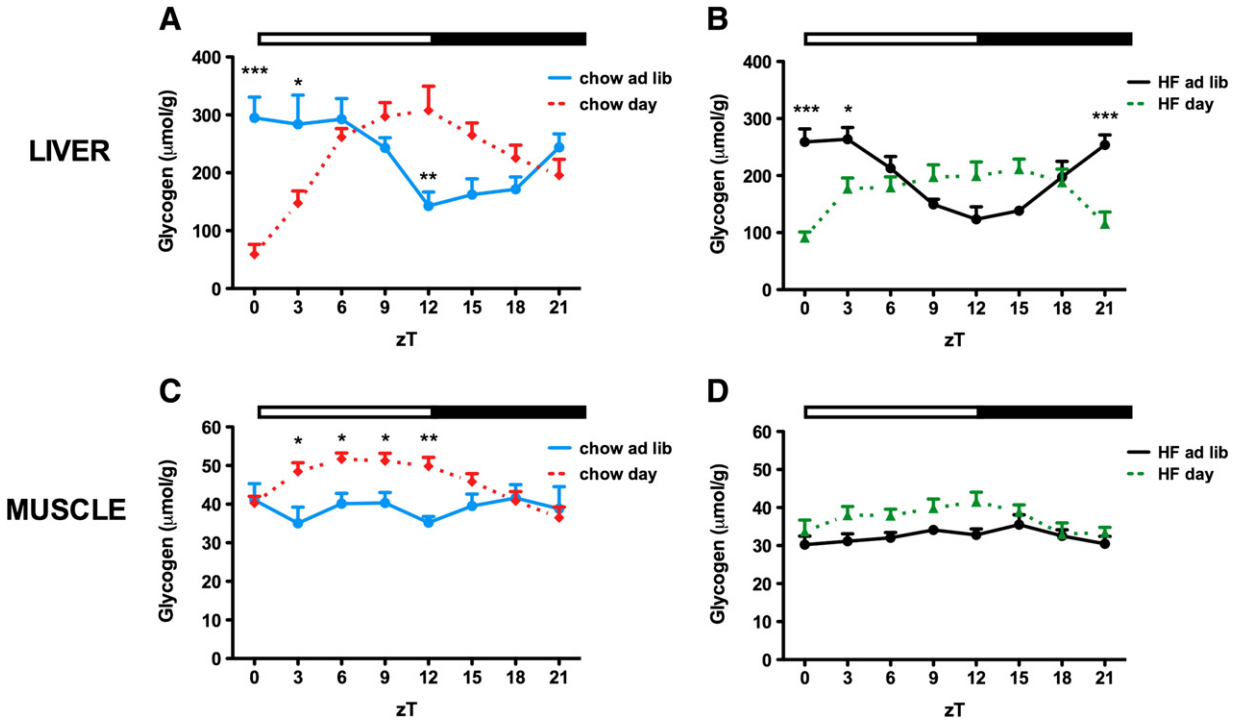

Fig. 6. Effect of alternate feeding schedules on glycogen profile in liver and skeletal muscle. Glycogen in liver (A and B) and muscle (C and D) of chow-ad lib (blue), chow-day (red broken line), HF-ad lib (black) and HF-day (green broken line) fed animals collected at 8 time points across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ with Bonferroni post-hoc test for a significant difference between the treatment groups at specific timepoints, n = 4–8.

resulted in disrupted energy homeostasis reflected in day-fed rats by a lower energy expenditure and altered glycogen accumulation in muscle but not liver. Because skeletal muscle is a major contributor to energy utilization, and total activity was also reduced in day-fed rats, it appears that increased food efficiency in the day-fed rats resulted in excess energy being stored as glycogen in muscle and under isocaloric conditions, fat in adipose tissue. These data have significant implications for our understanding of the interaction between feeding cues and light/dark cues and the possible causes of weight gain and metabolic disorders in people with altered feeding and sleeping patterns such as shift workers.

Distinct differences in tissue response to food cues as observed in the present study suggest discrete routes of entrainment in the liver and muscle. It is becoming evident that in the liver, food-borne cues act as dominant zeitgebers [15,36]. In fact, in the absence of feeding but presence of a functional molecular clock in liver, 80% of normally cycling hepatic transcripts cease to cycle [15]. Previous reports have shown that misalignment of food intake with the light/dark cycle, switches the liver expression profile to match the new schedule imposed by nutrient availability, thereby uncoupling liver from the SCN [17,19]. Further evidence supporting different modes of entrainment in liver and muscle is supplied by Guo et al. [20] who demonstrated that SCN-lesioned mice were able to re-instate rhythmic expression of clock genes in the liver by integrating rhythmic metabolites, neuronal and endocrine outputs as a result of rhythmic feeding whereas skeletal muscle remained arrhythmic. Although the liver has been shown to re-adjust the rhythms of its clock genes within two days of the introduction of a new feeding schedule [16], we show that three weeks of reverse feeding was not enough to re-align the molecular clock in skeletal muscle. Other non-photic zeitgebers such as scheduled bouts of activity have been shown to entrain behavior and assist in phase shifting in both humans and rodents [9]. Although activity in the current study showed dampened amplitude in day-fed rats, the diurnal variation in activity was maintained in the same phase as ad lib-fed rats. If activity is an important cue for controlling the muscle clock, it is possible that these rats did not switch clock gene expression because activity rhythms did not switch despite the altered feeding schedule.

Another interesting observation was the difference in the response to daylight feeding of the expression of molecular clock genes and metabolic genes. The expression profiles of *pepck* and *fas* switched in liver to match the feeding schedule, irrespective of whether the diet was chow or high fat, and these changes were not related to changes in clock gene expression. This supports the idea that there is a hierarchy of circadian regulation where rhythmic hormonal and metabolic signals can override the endogenous clocks, perhaps to allow the tissue to quickly adapt to sudden changes in the environment [15,21]. In contrast to the liver, the temporal expression of the skeletal muscle metabolic genes *pdk4* and *ucp3* did not change significantly when feeding was restricted to the 12 h of daylight. In high-fat fed rats however, *pdk4* and *ucp3* expression was higher throughout the 24 h most probably via a fatty acid activated PPAR transcriptional program which is known to regulate these two genes [37,38]. In the HF-day animals however, the peak of expression of *pdk4* and *ucp3* did not shift to the light-phase despite a marked shift to elevated fatty acids in the light-phase. This apparent inability of increased fatty acids to activate the PPAR system in the daytime [31] could be because the muscle has not altered gene expression of other components of the PPAR system to match the new time of feeding. Further studies examining a potential shift in PPAR gene or protein expression with food entrainment or change in diet would be necessary to determine the exact role of PPARs in the observed effects although previous studies using knockout mice and food entrainment have demonstrated the importance of these transcription factors in diurnal energy metabolism in mice [39].

Another important observation is that the protein levels of PDK4 and UCP3 are higher in high fat-fed muscle, but do not follow the diurnal variation in mRNA. This reflects the fact that individual proteins have unique turnover rates that depend on many factors other than the availability of mRNA for translation [40,41].

A possible explanation for the differences in the effects of chow and fat diets on clock gene expression in liver may be that specific nutrients exert entraining effects on different modulators of the molecular clock in a specific manner. For example nuclear receptors such PPARα, RORα and Rev-erbα influence the molecular clock and their activity is regulated

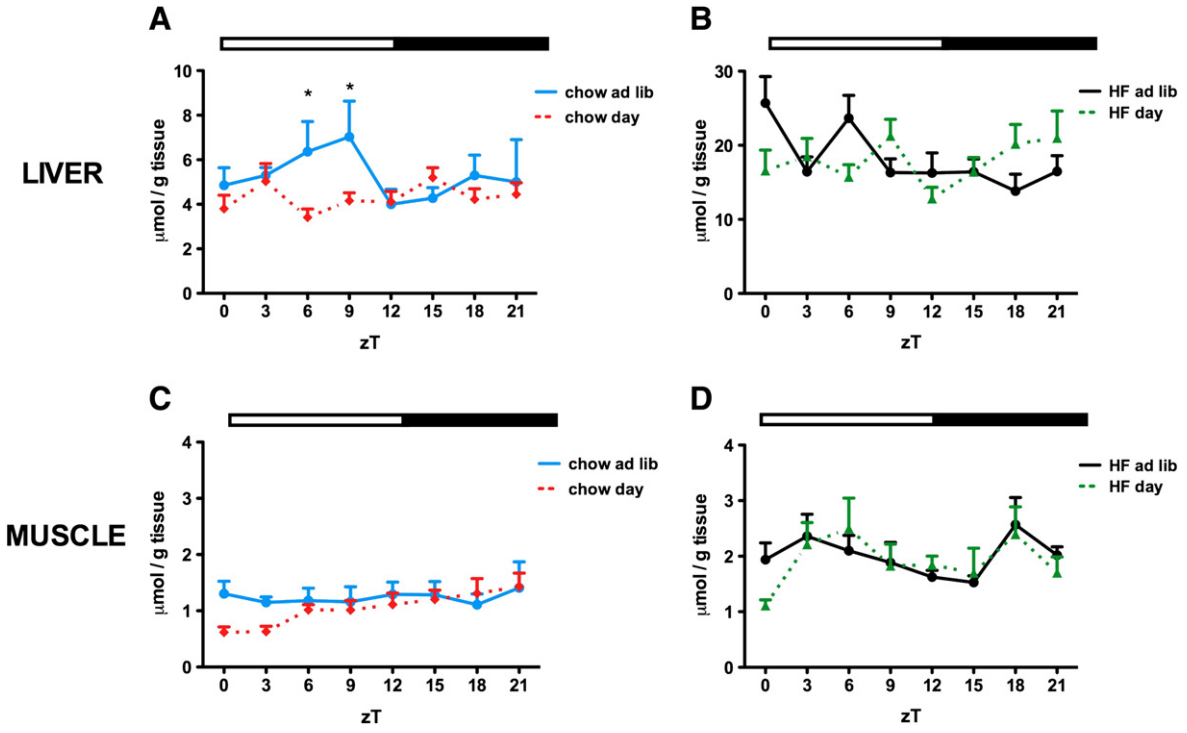

Fig. 7. Effect of alternate feeding schedules on triglyceride profile in liver and skeletal muscle. Triglyceride content in liver (A and B) and muscle (C and D) of chow-ad lib (blue), chow-day (red broken line), HF-ad lib (black) and HF-day (green broken line) fed animals collected at 8 time points across 24 h. The clear and colored bar denotes the light and dark phase respectively. Data are means ± SEM, *p<0.05, with Bonferroni post-hoc test for a significant difference between the treatment groups at specific timepoints, n = 4–8.

through ligands including fatty acids [42,43]. SIRT1 has also been shown to influence the molecular clock and the activity of SIRT1 is modulated via the redox state of the cell [44,45], which may be different in the context of a high fat diet. Furthermore, as reported previously and observed in the present study, high fat diets affect the levels of adipokines such as leptin and adiponectin which have also been implicated in signaling to molecular clocks [27,46]. Further work will be necessary to elucidate the exact mechanisms involved in specific nutrients adjusting the expression of the molecular clock.

The data presented above reveal a de-synchronization of metabolic events in different tissues when feeding cues are altered, which has significant implications for whole-body metabolism in an organism. Evolutionary alignment of light/dark phases with feeding patterns, activity and metabolic processing of nutrients promotes efficient utilization of energy. Therefore aberrant nutrient cues that signal in different ways in different tissues disrupt otherwise coordinated processes and alter the balance of overall homeostasis. Other recent studies investigating mis-aligned feeding paradigms have also found that feeding outside the active-phase promotes accumulation of adipose stores and weight gain [32–34]. This idea is supported by our current data and studies in human populations which indicate that shifting food intake to the night-time produces a greater risk of obesity [47]. Disruptions in normal circadian metabolism in skeletal muscle may also contribute to the increased fat accumulation considering that this tissue accounts for 20–30% of energy utilization in an organism. We observed an inability of skeletal muscle to respond to nutrient cues under altered feeding conditions and suggest that this drives changes in energy metabolism in this tissue such as abnormal accumulation of glycogen in the day-phase and decreased energy expenditure.

In conclusion, this study highlights the sensitivity of circadian rhythms and energy fluxes to nutrients and the distinct ways different metabolic parameters are modulated through these nutrient signals. Our data also illustrate how deviations from the evolutionary-derived organization where endogenous rhythms are synchronized to external light/dark cycles can lead to disruptions in metabolic pathways and altered energy homeostasis.

Author contributions

G.J.C. and N.T. who conceived and supervised the project, were involved in feeding and monitoring the animals and edited the manuscript. J.R. was involved in feeding and monitoring the animals, designed and performed experiments, analyzed data and wrote the manuscript. E.P. and D.L.W. were involved in feeding and monitoring the animals, sample collection, performed experiments and analyzed data. S.B. performed experiments and analyzed data.

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