Food entrainment in mice leads to sex- and organ-specific responses in nutrient metabolism.

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Abstract

One of the main zeitgebers in the digestive system is food intake, however little is known about sex-specific differences in food-driven circadian regulation. Thus, we placed male and female C57Bl/6 mice on time-restricted feeding (TRF), limiting food intake to 8 hours per 24-hour period. TRF was started either at dark (ZT12) or light (ZT0) onset and continued for 28 days, with or without additional 4h forward shift on day 15 of entrainment. Changes in weight gain, fasting blood glucose, intestinal transepithelial resistance (TEER) and nutrient metabolism-related gene expression were assessed in multiple digestive organs at various timepoints.

Long-term time-restricted night feeding led to the highest weight gain in females, but the lowest weight gain in males, while improving TEER in both sexes. However, it also led to a disappearance of diurnal rhythmicity in expression of several hepatic genes. Physiological responses in shifted night-fed females were largely indistinguishable from the night-fed group, whilst in males shifted night feeding led to an increase in weight gain and a decrease of fasting plasma glucose. Food intake during late dark phase also acted as a powerful zeitgeber for genes encoding regulators of nutrient intake and metabolism in liver and duodenum. Surprisingly, food intake during lights-on phase caused only minor changes in physiological responses. However, it did lead to an overall downregulation of gene expression in liver and an upregulation in stomach and duodenum, and to flattening of the diurnal responses. Placing food intake in the late lights-on phase negatively affected glucose metabolism and the integrity of the intestinal epithelium, causing an increased fasting glucose level in both sexes and low ileal TEER in male mice. It had similar effects on overall gene expression in liver and stomach as day feeding, however in duodenum it acted as a potent zeitgeber.

These results demonstrate that adjustment to food intake time in mice is highly sex- and organ-specific. In our model, TRF was not able to achieve full diurnal rhythm synchronization across the digestive system.

Instead, we observed that the same food intake time might be a strong zeitgeber in one organ, and a rhythm disruptor in another.

Introduction

Our daily schedule and meal times cause rhythmic variations in environmental and nutritional stimuli, leading to generation of circadian rhythms to help our body to anticipate recurring events (1). Early work on the existence of circadian rhythms in mammals revealed diurnal oscillations in body temperature (2-4), food intake (5) and specific organ functions, e.g. rhythmic renal excretion of water and salts (6, 7). It was also observed that humans placed in an environment without any indication of the time of day, preserved their previous circadian activity rhythm (8). In humans the circadian period is approximately 24h long and seems to be influenced by age, race, geographical location, and, possibly, sex (9-12). The term "zeitgeber" (time-giver) was introduced in the year 1945 to describe stimuli that affect the timing of the daily rhythm of any biological process within an organism (13). At present the daily light-dark cycle is the best researched zeitgeber; other known zeitgebers include food (content and intake time), temperature, exercise and drugs of abuse (14-17).

Simplistically, the mammalian molecular circadian clock is a transcriptional feedback loop, expressed in almost every cell, that drives expression patterns of multiple core clock genes with approximately 24-hour rhythmicity. The positive limb of this feedback loop consists of two master transcription factors - CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein), which together form a heterodimer. The CLOCK-BMAL1 heterodimer binds to the promoters of its target genes, including those comprising the negative limb of the feedback loop – *Per1*, *Per2*, *Per3* (further: PERs – mammalian analogues of *Drosophila period* gene) and *Cry1*, *Cry2* (further: CRYs). PERs and CRYs then dimerize, translocate back to nucleus and inhibit the activity of CLOCK-BMAL1 heterodimer, subsequently inhibiting their own expression (for extensive reviews on core clock function and mechanism see: (1, 18-22)). There are multiple secondary loops that modulate the output of the core clock mechanism, such as the orphan nuclear receptor families REV-ERBs and RORs. Both of these families have been suggested to provide a link between metabolism and the circadian clock, especially towards the regulation of lipid metabolism (23, 24). In addition to the translational regulation, the circadian clock is also regulated post-translationally, with multiple kinases, phosphatases, microRNAs and RNA-binding protein complexes modulating various aspects and the output of core clock (25).

A central circadian oscillator is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, receiving an input from environmental signals (e.g., light) and conveying the generated rhythms to the periphery directly via neural and hormonal signalling and indirectly via regulation of body temperature. However, peripheral tissues such as the gastrointestinal (further: GI) tract and liver also possess self-sustaining, entrainable circadian rhythms, with food entrainment being the most potent zeitgeber (26, 27). It has been shown that peripheral zeitgebers are even able to override the central, light-entrained rhythms (26, 28),

however the overall knowledge of peripheral clock entrainment is fragmented as research has been skewed towards specific organs. The food-entrained circadian clock has been extensively studied in the liver, but somewhat neglected in the digestive system, despite the gut being vital for food intake, breakdown and absorption of nutrients. To investigate the role of food intake in circadian rhythm generation, time-restricted feeding (further: TRF) is commonly used over prolonged period of time, as it has been shown that food-intake after a long fast (≥12h) is a strong determinant of the peripheral clock phase (29).

Another poorly investigated area in circadian rhythm research is sex-specific differences. Research in rodents has shown that the morphology and neuropeptide expression in SCN differs between males and females. Research in humans has been mostly focused on sex differences in sleep rhythms, with women exhibiting earlier sleep phase and longer overall circadian period. Both, rodent and human studies have shown sex differences in corticosteroid secretion (for reviews on sex differences see (30, 31)). In response to food entrainment, male mice have been shown to develop an earlier and higher amplitude increase in physical activity during food anticipation when compared to female mice (32). Despite this evidence, sex-specific differences in circadian rhythmicity at the molecular level have been poorly studied, especially regarding core clock components, as well as nutrient handling and metabolism.

Circadian rhythms coordinate physiological responses that depend on the anticipated stimuli; thus, they can be disturbed when stimuli are changed, e.g., by changes in caloric intake, during shift work, travel between time zones, or eating during the resting phase. Disruption of the clock has been shown to play such a major role in development and comorbidities of metabolic syndrome and obesity, that there has even been a suggestion to rename the metabolic syndrome as "circadian syndrome" (33). Consequently, synchronization of diurnal rhythms seem to lead to the opposite effect - TRF in coordination with the body's daily rhythm has beneficial effects on general health (34, 35), although organ-specific effects of particular feeding rhythms have not been studied in detail.

We used 8h TRF placed either in dark or light phase to investigate clock-dependent regulation of nutrient metabolism in the digestive system of male and female mice. Our investigation focused on weight gain, fasting glucose values, intestinal transepithelial resistance (further: TEER) and clock- and metabolism-related gene expression. In addition, we investigated the effects of a 4h food intake time shift within the same light-dark cycle period.

Materials and Methods

Ethical approval

All experimental procedures and handling involving mice were approved by University of Melbourne Animal Ethics Committee (application #1914983) and performed in compliance with the Australian code for the care and use of animals for scientific purposes.

Experimental animal origin and housing

In total 180 male and 180 female C57Bl/6 mice (RRID:IMSR_JAX:000664) from Animal Resources Centre (Canning Vale, WA, Australia) were used in the study. All mice arrived in the local animal facility aged 5-7 weeks and were kept under 12:12 h light-dark (further: LD) cycle, with standard chow (Barastoc 102108, Ridley Corporation, Melbourne, Australia) and water provided *ad libitum*. After three days of post-arrival adjustment to the local facility, mice were transferred to either LEDDY cages (black cages with individual lighting system) (Cat# GM500) or to the Aria ventilated cabinet (Cat# 9BIOC44R4Y1, both from Tecniplast, Buguggiate, Italy), and the light onset of 12:12 h LD cycle was adjusted in such way that the sample collection from day-fed and night-fed mice could be done in parallel. Mice were adjusted to the new 12:12 h LD cycle and housing conditions for 1 week, with chow and water provided *ad libitum*. All mice were weighed at the end of the dark phase (ZT24). Obtained values were recorded as pre-time-restricted feeding (further: pre-TRF) weights and used as a baseline for weight gain calculations.

Food-entrainment of mice

After 1 week adjustment to the new LD cycle, mice were subjected to time-restricted feeding (further: TRF) for 14 days (Fig. 1, Pre-shift). During this time, mice were fed standard chow for 8 hours either starting from the dark onset at ZT12 (Fig. 1, night-fed, grey bar) or the light onset at ZT0 (Fig. 1, day-fed, yellow bar), followed by a 16 h fasting period. To ensure that mice did not feed on chow debris during fasting, TRF was done by using two cages: chow-containing cage ("food cage") and cage that did not contain any chow ("empty cage"). Mice were placed in the "food cage" during the feeding period and in the "empty cage" during the fasting period. Water was always provided *ad libitum* for the whole duration of the experiment. Mice were weighed weekly, at the end of the feeding period (Fig. 1, blue arrows).

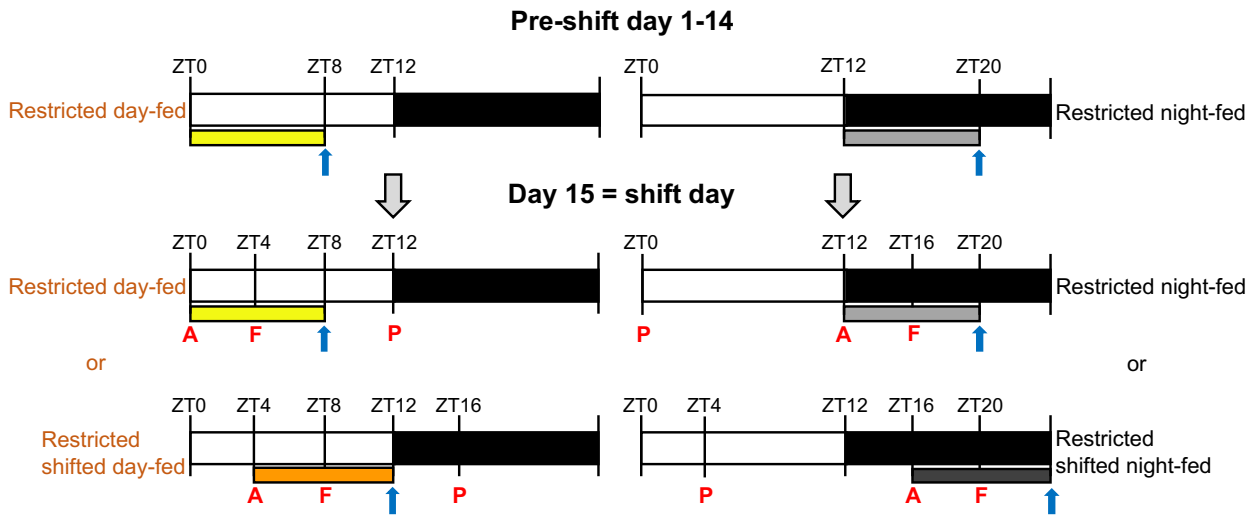

Figure 1. Food-entrainment groups. Yellow bars show day feeding period, grey bars – night feeding period. Shifted feeding period is shown with the orange bar (shifted day feeding) or the dark grey bar (shifted night feeding). Shifted and non-shifted groups were run in parallel. n = 45 male and 45 female mice per group. Blue arrows – weekly weight measurement timepoints. Mice were sacrificed at 3-, 7- or 14-days post-shift. Letters in red show sacrifice timepoints on each day: A – food anticipation, F – food intake, P – postprandial, n = 5 per sex.

After 14 days of the initial entrainment, the 8 h feeding period was shifted 4 h forward for half of the mice (Fig. 1, shift day). The new feeding groups were labelled as 'restricted shifted night-fed' (Fig. 1, ZT16-ZT0, dark grey bar) and 'restricted shifted day-fed' (Fig. 1, ZT4-ZT12, orange bar). The other half of the mice continued with their initial feeding regimen. Weekly weight gain measurements were continued as previously stated (Fig. 1, blue arrows,). On 3rd (D3), 7th (D7) and 14th day (D14) after the shift, mice were sacrificed for organ collection. Three timepoints were chosen to assess food-driven diurnal responses on each sacrifice day: 1) food anticipation – after 16h of fasting, before the anticipated transfer to "food cage" (Fig. 1, "A"); 2) food intake – 4 h after the start of feeding period (Fig. 1, "F"); 3) postprandial period – 4 h after the end of feeding period (Fig. 1, "P"). On each sacrifice day n=5 female and n=5 male mice were sacrificed per timepoint.

Mice were anaesthetised with isoflurane (Cat# FGISO0250, Pharmachem, Eagle Farm, Australia) followed by a cervical dislocation. Upon sacrifice, their body weight was measured, and the following organs/tissue were collected and snap frozen using liquid nitrogen: the right lobe of the liver, the dorsal half of the stomach and duodenal mucosa (first 2 cm of duodenum opened and scraped with surgical scalpel at 45° angle). Ileum (5 cm proximal from cecum) was collected only during the food intake timepoint (Fig. 1, "F"), and used to measure intestinal permeability and transepithelial electrical resistance (further: TEER).

Blood glucose measurements

Blood glucose was measured at the food anticipation timepoint after 16 h fasting (Fig. 1, "A"). A tail vein blood sample was applied on Accu-Chek® Performa Test strip (Cat# 06454038020) and glucose concentration

was measured with Accu-Chek® Performa (Cat# 05894964014) blood glucose meter (both from Roche Diagnostics, Manheim, Germany).

Intestinal electrical resistance measurements

Ileum collected during food intake timepoint (Fig. 1, "F") was cut in half. Both pieces were placed in Krebs-Henseleit buffer (11.1 mM glucose, 118 mM NaCl, 4.8 mM KCl, 1.0 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, pH 7.4), opened along the mesenteric border and pinned (full-thickness) onto Ussing chamber sliders (Cat# P2311, 0.3 cm^2 apertures, Physiological Instruments, San Diego, USA). Sliders were inserted in the middle of two-part Ussing chambers (EasyMount Diffusion Chambers, Physiologic Instruments, San Diego, USA) and 5 ml Krebs-Henseleit buffer solution was added to the serosal side of tissue. The mucosal side of tissue received 5 ml of modified Krebs-Henseleit buffer where glucose was substituted with 11.1 mM mannitol. This was done to avoid apical uptake of glucose, while still maintaining an osmotic balance. Buffers in both chambers were kept at 37°C and bubbled with carbogen (5% CO_2, 95% O_2) to maintain an optimal pH level. A multichannel voltage-current clamp (VCC MC6, Physiologic Instruments, San Diego, USA) was applied to each chamber through a set of four electrodes (2 voltage sensing and 2 current passing electrodes) and agar bridges (3% agarose/3 M KCl in the tip and backfilled with 3 M KCl), installed on opposite sides of the tissue. Tissue was left to equilibrate for 20 min before clamping the voltage to 0 V.
To calculate TEER ($\Omega \cdot cm^2$), 2 sec pulses of 2 mV were administered to tissue every 60 secs for 1 h, and the measured net resistance was multiplied by the surface area. Voltage and short circuit current (Isc) measurements were recorded using a PowerLab amplifier and software LabChart® 5 (RRID:SCR_017551, both ADInstruments, Sydney, Australia).

RNA extraction

Total RNA from approx. 20 mg of the right lobe of liver, dorsal half of stomach and scraped duodenal mucosa was extracted using ISOLATE II RNA Mini Kit (Cat# BIO-52073, Meridian Bioscience, Cincinnati, USA) with following adjustments of manufacturer's instructions. First, tissue pieces after collection were snap frozen in 2 ml screw cap tubes containing Lysing Matrix D (Cat# 6540-434, Lot# 99999, MP Biomedicals, Irvine, USA). Second, the volume of lysis buffer was increased to 500 µl per sample, adding 5 µl of β-mercaptoethanol. Lysis buffer was applied while the samples were still frozen and immediately followed by lysis at 6000 rpm for 2 x 30 sec in a Precellys 24 homogenizer (RRID:SCR_022979, Cat# 03119-200-RD010, Bertin Technologies SAS, Montigny-le-Bretonneux, France). Due to a higher volume of lysis buffer, lysate filtration was done in two consecutive steps and thus the volume of RNAse free Ethanol (Cat# EA043, Chem-supply, Gillman, Australia), used for RNA binding, was increased to 500 µl. The centrifugation time for first and second washing steps was increased to 1 min. Samples were eluted in 100 µl (liver) or 60 µl (stomach, duodenal mucosa) of RNase free water (Cat# 10977-015, Lot# 2186758, Invitrogen by Life technologies, Grand Island, USA). The elution step was repeated using the initial eluate to increase the RNA yield. The quality and quantity of the extracted RNA were assessed using 2200 Tape Station (RRID:SCR_014994, Agilent Technologies, Santa Clara,

USA) and a Nanodrop ND-1000 UV spectrophotometer (RRID:SCR_016517, NanoDrop Technologies, Wilmington, USA), respectively. The isolation was randomized and done in small batches (24 samples), containing samples from both sexes collected at various sacrifice days and timepoints.

The synthesis of cDNA was done from 100 ng RNA in 20 µl total reaction volume using iScript Reverse Transcription Supermix kit (Cat# 1708841, Bio-Rad, Hercules, USA), without any changes from themanufacturer's instructions. Samples were split across 96-well plates and each plate included 6 negative controls (-RT) – randomly chosen RNA samples where no cDNA synthesizing enzyme was added. Synthesis reaction was carried out using a thermal cycler - PCRExpress (Cat# 630-003, Thermo Hybaid, Franklin, USA) or Bio-Rad T100™ (RRID:SCR_021921, Bio-Rad, Hercules, USA).

Determination of gene expression

After cDNA synthesis, 10 µl of each cDNA sample were delivered to Translational Research Facility within Monash Health Translational Precinct for further processing using The Fluidigm Digital Array Integrated Fluidic Circuits (36) (further: IFCs). First, all samples underwent quality control, where expression of either *Gapdh* (forward: TGACCTCAACTACATGGTCTACA, reverse: CTTCCCATTCTCGGCCTTG) or *β-actin* (forward: GGCTGTATTCCCCTCCATCG, reverse: CCAGTTGGTAACAATGCCATGT) was tested as SYBR assays using QuantStudio 6 Flex RealTime PCR reader (RRID:SCR_020239, Thermo Fisher Scientific, Waltham, USA). Afterwards, samples underwent pre-amplification according to manufacturer's instructions (37). All 24 TaqMan assays for genes of interest (Table 1) were first pooled and diluted in Tris-EDTA buffer (pH 8.0) to final concentration of 180 nM per assay. Afterwards 3.75 µl of gene assay mix was added to 1.25 µl of cDNA sample and pre-amplified for 14 cycles using Veriti™ 96-well Thermal Cycler (RRID:SCR_021097, Cat# 9902, Thermo Fisher Scientific, Waltham, USA). Pre-amplified cDNA samples were further diluted 1:5 with Tris-EDTA buffer (pH 8.0) and loaded on the 192.24 Dynamic array IFC (Cat#100-6266, multiple lots used, Fluidigm, San Francisco, USA) together with gene assays in accordance to Fluidigm® 192.24 Real-Time PCR Workflow Quick Reference PN 100-6170 (37). Used gene assays are shown in Table 1. The qPCR reaction was performed using Biomark™ HD system (RRID:SCR_022658, Cat# BMKHD, Fluidigm, San Francisco, USA).

Table 1. TaqMan Gene assays used to determine relative gene expression levels

Assigned group by function	**Gene**	**Gene accession number**	**Assay ID**	**Tested in samples from:**
Reference gene	*18s (Rn18s)*	NR_003278.3	Mm04277571_s1	Liver, stomach, duodenum
Core clock genes	*Clock*	NM_001289826.1	Mm00455950_m1	
	Bmal1 (Arntl)	NM_001243048.1	Mm00500226_m1	
Glucose sensing, transport and metabolism	*Tas1r2*	NM_031873.1	Mm00499716_m1	
	Sglt1 (Slc5a1)	NM_019810.4	Mm00451203_m1	
	Glut2 (Slc2a2)	NM_031197.2	Mm00446229_m1	
	Pepck (Pck1)	NM_011044.2	Mm01247058_m1	
	Gys2	NM_145572.2	Mm01267381_g1	Liver

Amino acid and peptide transport	*Pept1 (Slc15a1)*	NM_053079.2	Mm04209483_m1	Liver, stomach, duodenum
	B^0at1 (Slc6a19)	NM_028878.3	Mm01352157_m1	
Lipid metabolism	*Mttp*	NM_001163457.1	Mm00435015_m1	
	Fasn	NM_007988.3	Mm00662319_m1	
	Cyp7a1	NM_007824.2	Mm00484152_m1	
	Pparα	NM_001113418.1	Mm00440939_m1	
	Srebf1	NM_011480.3	Mm00550338_m1	Liver
Biosynthesis of NAD+	*Nampt*	NM_021524.2	Mm00451938_m1	
Hormone signalling	*Ffg21*	NM_020013.4	Mm00840165_g1	
	Igf-1	NM_001111274.1	Mm00439560_m1	
	Tph1	NM_001136084.2	Mm01202614_m1	Stomach and duodenum
	Sst	NM_009215.1	Mm00436671_m1	
	Ghrl	NM_001286404.1	Mm00612524_m1	
	Gast	NM_010257.3	Mm00439059_g1	Stomach
	Lepr	NM_001122899.1	Mm00440181_m1	
	Pga5	NM_021453.4	Mm01208256_m1	
	Gip	NM_008119.2	Mm00433601_m1	Duodenum
	Gcg	AF276754.1	Mm00801714_m1	
	Cck	NM_031161.4	Mm00446170_m1	
Pro-/Anti-inflammatory response	*TNFα*	NM_013693.3	Mm99999068_m1	Liver, stomach, duodenum
	Il-6	NM_031168.1	Mm00446190_m1	
	Il-17a	NM_010552.3	Mm00439619_m1	
	Crp	NM_007768.4	Mm00432680_g1	Liver
	Gpx1	NM_008160.6	Mm00656767_g1	
	Gpx2	NM_030677.2	Mm00850074_g1	Stomach and duodenum

18s was used as a reference gene for calculation of relative expression ratios. The relative expression ratios were calculated as $R = 2^{-(Ct(test\ RNA)-Ct(18S\ RNA))}$. All relative expression ratios were further normalized against night fed males on 3rd day after feeding shift (D3) at food anticipation timepoint (Fig. 1, point "A"), to assess sex-specific diurnal changes across feeding conditions.

Data analysis and statistics

Experimental data analysis was performed using GraphPad Prism v8.2 (RRID:SCR_002798, GraphPad software, San Diego, CA, USA). To demonstrate weight gain (Fig. 2A), data from each individual mouse within the same feeding group and sex (n=90 for pre-TRF, week 1 and week 2; n = 30 for week 3, n = 15 for week 4) were pooled and shown as a mean group value and 95% Confidence Interval, CI. Differences in weight gain between night-fed group (controls) and other feeding groups were assessed by Two-way analysis of variance (ANOVA) with Šidák's multiple comparison test.

Measured TEER values were submitted to an outlier test and all values outside of mean +/- 2x standard deviation interval were excluded from further analysis. This was done to exclude tissue pieces that might have been damaged and thus not intact during the resistance measurements.

Weight at the time of sacrifice (Fig. 2B), fasting blood glucose values (Fig. 2C), and TEER values (Fig. 2D) were shown as a separate data point per each individual mouse, together with mean group value and standard deviation (Further: SD) indicated as a bar and lines. Two-way ANOVA was used together with Dunnett's multiple comparison test to show differences between feeding groups (night-fed as a control) and sacrifice days (D3 as control).

To show gene expression differences across timepoints and feeding conditions normalized relative expression ratios from each individual mouse representing the same feeding group, sacrifice timepoint and sex were pooled together and shown as a tile within a heatmap. Fold changes in gene expression were shown as Log2 values. Value ranges were adjusted to include most of the genes within the same organ. Nonparametric Kruskal-Wallis test together with Dunn's multiple comparison test was used to assess the diurnal differences in gene expression (=diurnal change), using food anticipation group as a control (Figs. 3-6). The p-values were shown as: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{***}p \leq 0.0001$. Differences in gene expression at a single timepoint introduced by 4 h feeding shift (=baseline change) were assessed with the same statistical test, comparing values from each timepoint with values from its shifted counterpart (e.g., night-fed postprandial vs shifted-night fed postprandial) (Figs. 3-6). The p-values were shown as: $^{£}p \leq 0.05$, $^{££}p \leq 0.01$, $^{£££}p \leq 0.001$, $^{££££}p \leq 0.0001$.

Results

Effects of food-entrainment on weight gain, plasma glucose values and intestinal permeability

Weight gain shows sex-specific response to food-entrainment

Male and female postprandial weights (Fig. 1, blue arrows) were recorded before (pre-TRF). All mice started time restricted feeding (further: TRF) as either day-fed (further: DF) or night-fed (further: NF) and remained on this regime for 2 weeks. Half of the mice then underwent additional 4 h feeding shift, creating additional shifted night-fed (further: SNF) and shifted day-fed (further: SDF) groups. The other half of mice remained on their initial TRF regime. Weight gain was assessed weekly for the whole duration of the experiment. Afterwards, measured weights were related to the individual pre-TRF values and expressed as a change in percentage (%) over time.

We observed striking sex-specific differences in weight gain between the feeding groups. At the end of the 2nd entrainment week, DF males showed an 2.9% increase in weight, when compared to their night-fed counterparts (Fig. 2A, in yellow vs in grey). No such differences were seen in female mice, where both night and day feeding led to an almost identical weight gain.

However, by the end of the 3rd week of food-entrainment, DF and SDF female mice showed a significant delay in weight gain when compared to the NF group (-4.4% and -4.7%, respectively) (Fig. 2A in yellow and orange vs grey). This delay seemed to partially resolve by the end of the 4th entrainment week, with only SDF mice still showing slowed (-4.3%) weight gain. No differences in weight gain were observed between NF and SNF females. These results suggest that in female mice the food intake during the lights-on phase could be detrimental for weight gain. The opposite was seen in male mice. With longer entrainment, the initially increased weight gain in DF male mice seemed to plateau and was no longer different from NF controls (Fig. 2A, yellow vs grey). At the same time, both 4 h feeding shifts led to approximately 2.8% increase in weight gain by the end of week 3 (Fig. 2A, in black and orange vs grey). This response was sustained in SDF mice, leading to an 3.9% weight gain increase by the end of week 4 (Fig. 2A, orange vs grey).

Overall, day feeding led to an early increase in weight gain in males, and to a delayed weight gain in females over time. In addition, the weight gain in male mice seemed to be more sensitive to the 4 h feeding shift than in females, demonstrating sex-specific weight gain adaptation to the food intake time.

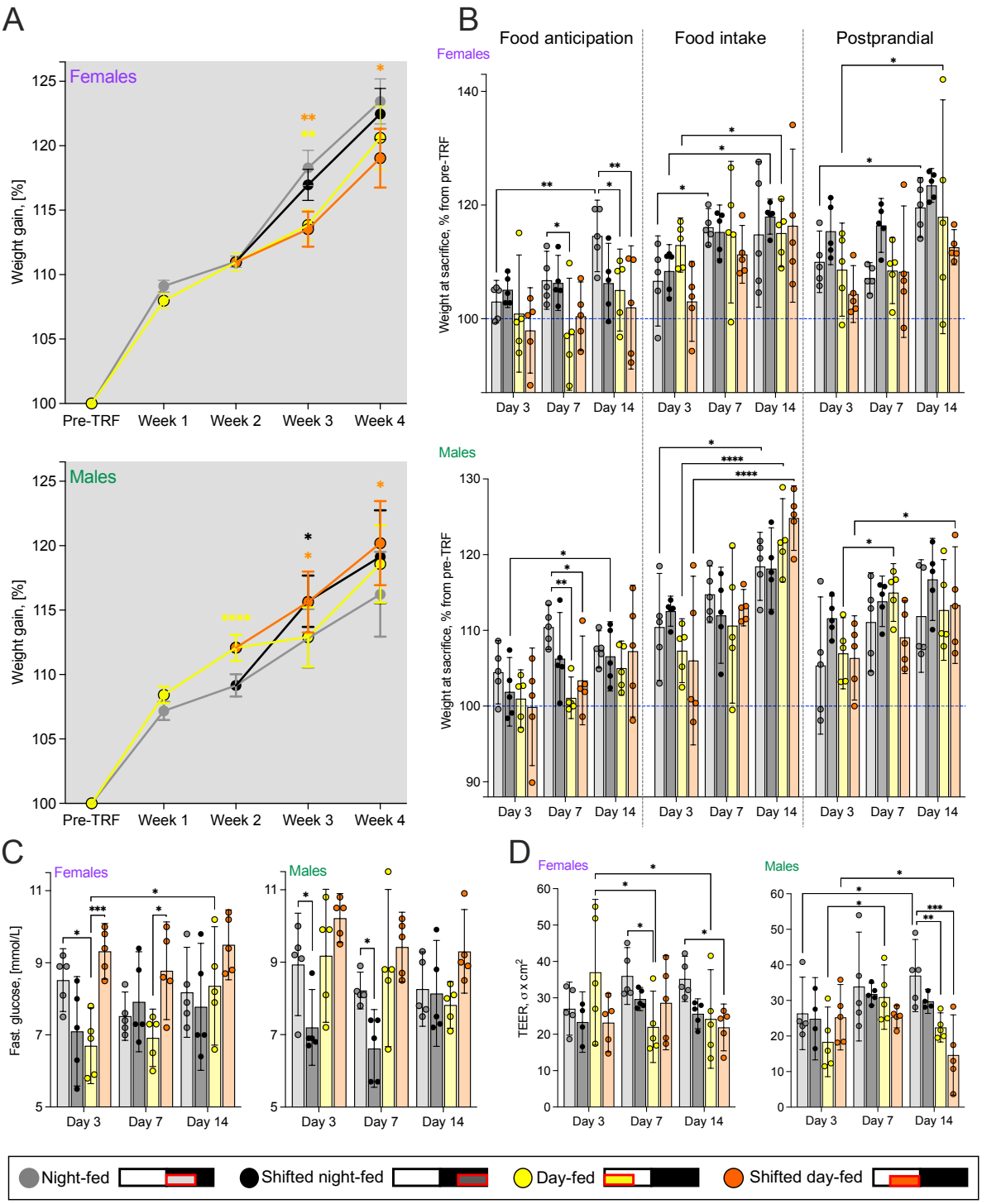

Figure 2. Physiological effects of time-restricted feeding regimes. **A**: Post-feeding weights were normalized to individual pre-food entrainment (pre-TRF) values. Mean (95% CI) shown, n = 90 mice per sex at pre-TRF, week 1 and week 2; week 3 n = 30 mice per sex; week 4 n = 15 mice per sex. Two-way ANOVA with Šidák's multiple comparison test (vs night-fed group), *p<0.05, **p<0.01, ****p<0.0001. **B**: Differences in recorded sacrifice weights. Values normalized to individual pre-food-entrainment (pre-TRF) values. **C**: Fasting glucose values recorded during food anticipation. **D**: Transepithelial electrical resistance in ileum, recorded during food intake. **B to D:** Mean (SD) shown, n = 5 mice per sex per timepoint. Two-way ANOVA with Dunnett's multiple comparison test, showing differences between feeding groups (night-fed as a control) and sacrifice days (Day 3 as a control), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Feeding during lights-on phase leads to severe weight loss after prolonged fasting

We also recorded the bodyweight at each sacrifice timepoint – food anticipation (after 16 h fast, when mice expect to be fed, Fig. 1, point "A"), food intake (4 h after food was added, Fig. 1, point "F") and postprandial period (4 h after food was removed, Fig. 1, point "P") on all sacrifice days. The earliest sacrifice was done 3 days after the feeding shift (day 3), followed by 7 (day 7) and 14 days (day 14) post-shift.

We then compared these sacrifice weights to pre-TRF values (Fig. 2B, blue dotted line). Surprisingly, on sacrifice days 3 and 7, DF and SDF female mice showed a severe weight loss after 16 h fasting, reaching or even dipping below the weight that was recorded pre-TRF (Fig. 2B, upper panel, Food anticipation). Similar weight loss was also observed in DF male mice (Fig. 2B, lower panel, Food anticipation), meaning that during a single 16 h fasting period these mice lost almost all of their weight gained over past 2-3 weeks of TRF. However, the fasting weight did become more stable over time, especially in the male mice. This was also observed at the postprandial timepoint (4 h fasting) (Fig. 2B, Postprandial).

Weights at the food intake timepoint (Fig. 2B, Food intake) also tended to increase over time, especially in male mice. This supports our observation that adjustment to the new TRF regime is not immediate. However, mice from all TRF groups seem to be consuming similar amounts of food, as there were no significant weight differences between the various feeding groups.

Fasting blood glucose increases in female mice fed during late lights-on phase

Fasting blood glucose was measured at food anticipation timepoint (Fig. 2, point "A"). Again, female mice struggled to quickly adjust to day feeding. On the post-shift day 3, DF mice showed significantly lower fasting blood glucose levels, while SDF mice showed a trend for an increased blood glucose ($p=0.06$), when compared to NF controls (Fig. 2C). A similar trend was observed on post-shift day 7, however by post-shift day 14, DF mice were no longer different from NF controls (Fig. 2C, in yellow). Fasting glucose levels in SDF mice, however, did not show any change over time and remained slightly elevated ($p=0.056$) (Fig. 2C, in orange).

Male mice did not exhibit the exact same fasting glucose pattern. Shifting the food intake to late lights-off phase significantly lowered fasting blood glucose levels for at least 7 days post-shift (Fig. 2C, right panel, black). Similar to observations in female mice, shifted day feeding slightly raised the blood glucose concentration($p=0.06$), however it tended to decrease over time, reaching NF-like levels by day 14 (Fig. 2C, right, in orange).

It appears that food intake during the late lights-on phase (ZT4-ZT12) can lead to a slight increase in fasting blood glucose levels in both sexes, while food intake during late lights-off phase (ZT16-ZT0) can lower glucose levels in male mice.

Food intake during passive phase leads to lower transepithelial electrical resistance of ileal epithelia

Ileum from mice sacrificed during the food intake timepoint (Fig. 1, "F") was used to determine transepithelial electrical resistance (further: TEER). We did not observe an early response to the food intake shift, as all feeding groups showed similar TEER values on post-shift day 3 (Fig. 2D, Day 3). However, we did observe changes under prolonged TRF. Night feeding led to a slight increase in TEER in both sexes, with males showing significant improvement in ileal resistance by day 14 (Fig. 2D, in grey). Interestingly, SNF group was the only one showing no changes in TEER over time in any of the sexes (Fig. 2D, in black). Under day or shifted day feeding TEER seemed to worsen by day 14 post-shift (Fig. 2D, in yellow and orange, respectively), with stronger reduction in male mice. Overall, similar to observations in blood glucose values and weight gain, the most detrimental effects were observed under shifted day feeding, that places the food intake in the last 8 h of lights-on period.

Food-entrained gene expression in the digestive system

Gene expression in the digestive system shows three distinct response types to food entrainment

Expression of genes involved in molecular clock, nutrient metabolism and immune response was determined in liver, stomach corpus and duodenal mucosa. Tissue samples were collected from all experimental animals on the 3rd, 7th and 14th day post-shift at food anticipation, food intake and postprandial timepoints (Fig. 1, collection timepoints indicated with the red letters). Gene expression was normalised against housekeeping gene *18s* and further related to expression level in NF male mice at food anticipation timepoint on post-shift day 3.

In general, we observed 3 distinct gene expression responses to food intake time, summarized in Table 2 (ignoring sex- and timepoint-specific differences). First, where multiple tested genes did not show any difference in expression, we classified these as "non-responsive". Second, where we observed a rhythmic food-entrained response – changes in expression between food anticipation and food intake or postprandial period. These were classified as "diurnal response" genes. And last, where significant gene population did show differences in expression due to the 4 h food intake shift. This difference could be observed in both – diurnally unresponsive and responsive genes. Thus, in some cases we would observe diurnal response together with additional up- or down-regulation of gene expression due to the feeding shift, suggesting that food entrainment could lead to a multi-tier regulation. All genes that showed changed expression in response to the feeding shift, independently to their diurnal response, were classified as "baseline change" genes. Interestingly, in stomach all diurnally responsive genes also showed a baseline change.

Table 2. Summary of gene expression responses to food-entrainment. Data from all sacrifice days pooled, ignoring sex-specific differences.

Organ	Non-responsive genes	Diurnal response	Baseline change
Liver	*Tas1r2* *Pept1, B^0at1* *Il-17a, Il-6, Crp, Igf-1*	*Bmal1, Clock* *Glut2, Gys2, Pepck* *Pparα, Fasn, Cyp7a1, Srebf1, Mttp* *Nampt* *Fgf21* *TNFα*	*Bmal1, Clock* *Glut2, Sglt1, Gys2, Pepck* *Pparα, Mttp, Fasn, Srebf1* *Nampt* *TNFα, Gpx1*
Stomach	*Tas1r2, Sglt1, Glut2* *Pept1, B^0at1* *Mttp, Fasn, Cyp7a1* *Il-17a*	*Bmal1, Clock* *Pepck* *Pparα* *Tph1, Gast, Sst, Lepr, Pga5, Ghrl* *Il-6, Gpx2, TNFα*	
Duodenum	*Cyp7a1, Mttp, Fasn* *Sst, Ghrl* *Il-6, Il-17a*	*Bmal1, Clock* *Tas1r2, Sglt1, Glut2, Pepck* *Pept1, B^0at1* *Pparα* *Gip, Gcg, Cck, Tph1* *Gpx2, TNFα*	*Bmal1, Clock* *Tas1r2, Glut2, Sglt1, Pepck* *Pept1, B^0at1* *Pparα* *Gip, Gcg, Cck, Tph1* *TNFα*

Colours mark different assigned groups by gene product function: light blue – core clock genes; purple – glucose sensing, transport, and metabolism; orange – lipid metabolism; pink – biosynthesis of NAD+; red – amino acid and peptide transport, green – hormone signalling; grey – pro-/anti-inflammatory response.

Food intake time entrains digestive clocks in an organ-specific manner

To determine whether the chosen TRF regimes were able to uncouple and reset peripheral clocks, we looked at expression of clock genes *Bmal1* and *Clock* in liver, stomach and duodenum of male and female mice. In all tested organs, *Bmal1* showed stronger diurnal response than *Clock*, indicating possible uncoupling of *Bmal1-Clock* dimer (Fig. 3). Even in NF mice, *Clock* showed low relative expression and barely any rhythmicity (Fig. 3, NF). This was a very surprising result, as night feeding mimics the natural food intake period in mice and aligns with the central clock, and thus is not expected to disrupt diurnal rhythmicity of peripheral clock genes. We also observed a highly organ-specific *Clock* and *Bmal1* response to TRF. Livers of both DF and SDF mice had overall lower expression of both of these genes (Fig. 3, top panel). In stomach and duodenum, we observed the opposite – upregulation of *Clock* and *Bmal1* under DF and SDF (Fig. 3 mid- and bottom panels), accompanied by loss of diurnal rhythmicity of *Bmal1*.

In addition, we observed strong sex-specific differences. In both NF and DF female mice hepatic *Bmal1* showed loss of diurnal response over time, however this could be rescued by the 4 h feeding shift (Fig. 3, top left panel). In male mice hepatic *Bmal1* diurnal response strengthened in NF and SNF mice, but disappeared in DF mice over prolonged TRF, and remained unchanged under shifted day feeding (Fig. 3, top right panel). Similar diurnal response pattern with females showing strong early response, but loss over time and males

showing the opposite, was also observed for hepatic *Clock* and duodenal *Bmal1* expression. Interestingly, in stomach *Bmal1* diurnal expression showed the opposite sex-specific pattern – rhythmicity weakened in males, but not in females. It appears, that food intake did not become the dominant zeitgeber for clock genes in the tested digestive organs, as evident by these conflicting responses.

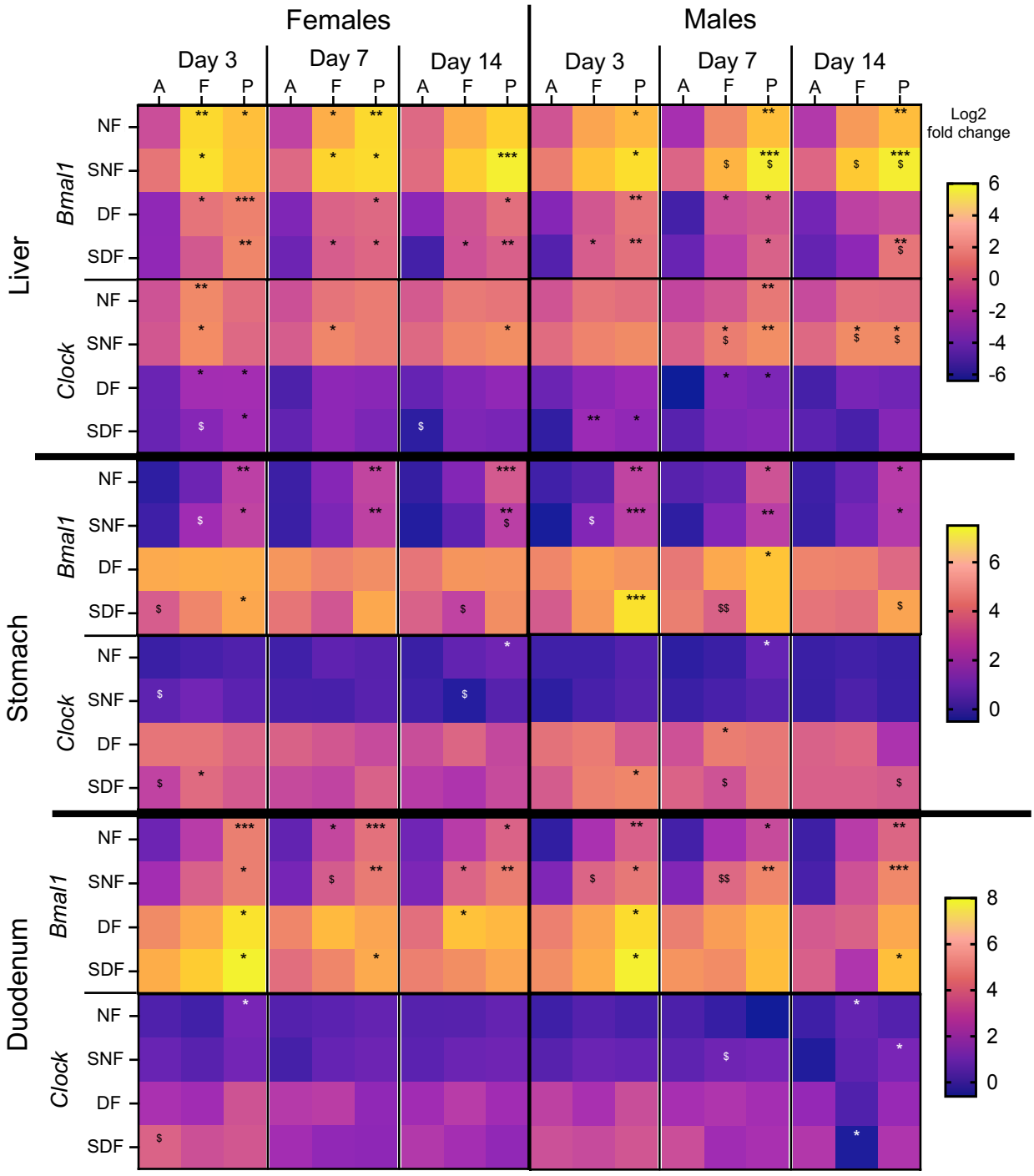

Figure 3. Changes in clock gene expression in liver, stomach and duodenum in response to food intake time. Sample collection timepoints shown at the top: A = food anticipation, F = food intake, P = postprandial period. Feeding conditions shown on the left: NF = night-fed (ZT12 – ZT20), SNF = shifted night-fed (ZT16 – ZT24), DF = day-fed (ZT0 – ZT8), SDF = shifted day-fed (ZT4 – ZT12). Relative gene expression values (Log2) normalized to NF male mice on sacrifice day 3 at food anticipation timepoint and shown as means, n= 4-5 mice. Non-parametric Kruskal-Wallis test with Dunn's

multiple comparison test used to show diurnal (vs food anticipation) expression differences, *p<0.05, **p<0.01, ***p<0.001. Same test used to assess baseline differences (non-shifted vs shifted) at each timepoint, $^{\$}$p<0.05, $^{\$\$}$p<0.01.

Food intake during late lights-off phase is a strong zeitgeber in liver, while daytime feeding downregulates gene expression

Similar to our observations regarding the clock genes, also hepatic nutrient metabolism- and inflammatory response-related genes showed a lower expression in DF and SDF mice of both sexes (Fig. 4). Food intake during the lights-on phase also led to a poor diurnal response, suggesting that TRF during daytime was not able to fully uncouple the local hepatic clocks from the central clock-driven regulation.

Even food intake during the lights-off phase was not able to induce strong and persistent diurnal responses in some of the tested genes. For example, *Pparα* and *Srebf1*, that are the principal transcription factors regulating hepatic lipid metabolism, showed almost no diurnal rhythmicity in their gene expression (Fig. 4). The only condition leading to an appearance of a diurnal rhythm in *Pparα* was SNF. In male mice we observed that SNF was also inducing diurnal rhythmicity in hepatic *Srebf1,* and a baseline upregulation of both of these genes. Poor rhythmicity in *Pparα* and *Srebf1* expression could negatively affect the diurnal response of the whole lipid metabolism, and thus could potentially explain the lack of stable diurnal and baseline expression changes in genes involved in hepatic lipid secretion (*Mttp*) and cholesterol catabolism (*Cyp7α1*). However, not every gene associated with lipid metabolism was unresponsive to TRF. Gene encoding fatty acid synthase (*Fasn*) showed a strong diurnal response in both sexes under SNF, with rhythm being present also in NF and DF females and NF males. Similar to our observations with Clock genes, females were again more likely to show diurnal response early (day 3 post-shift) and then loose it over time, while males showed the opposite. A gene encoding nicotinamide adenine dinucleotide (NAD^+) biosynthetic enzyme (*Nampt*), that through its regulation of NAD synthesis is involved in cellular energy metabolism, including fatty acid oxidation (38), was responsive to food intake time as well, especially in NF males, DF females and in both sexes under SNF.

Interestingly, the most robust diurnal rhythmicity in liver was shown by a gene encoding fibroblast growth factor 21 (*Fgf21*), that is involved in regulation of nutrient and energy homeostasis and secreted during fasting (39). Food intake time seemed to affect the dip in *Fgf21* expression, shifting it from the food intake period in NF mice into postprandial period in almost all other mice groups.

As liver plays a major role in glucose metabolism and storage, we also looked at the gene expression of key players in these processes. No robust diurnal responses were observed for genes encoding glucose transporters (*Glut2, Sglt1*), nevertheless a strong rhythm was present for genes involved in regulation of glycogen synthesis (*Gys2*) and gluconeogenesis (*Pepck*), especially in SNF female mice.

Not much is known about diurnal regulation of peripheral inflammation. Unfortunately, we only observed poor, inconsistent diurnal responses in the tested genes, with male mice likely to show an upregulated expression of *TNFα* during food intake under several TRF regimes. Overall, SNF (ZT16-ZT20) appeared to be the most effective zeitgeber in the liver, leading to diurnal responses in multiple tested genes in both sexes.

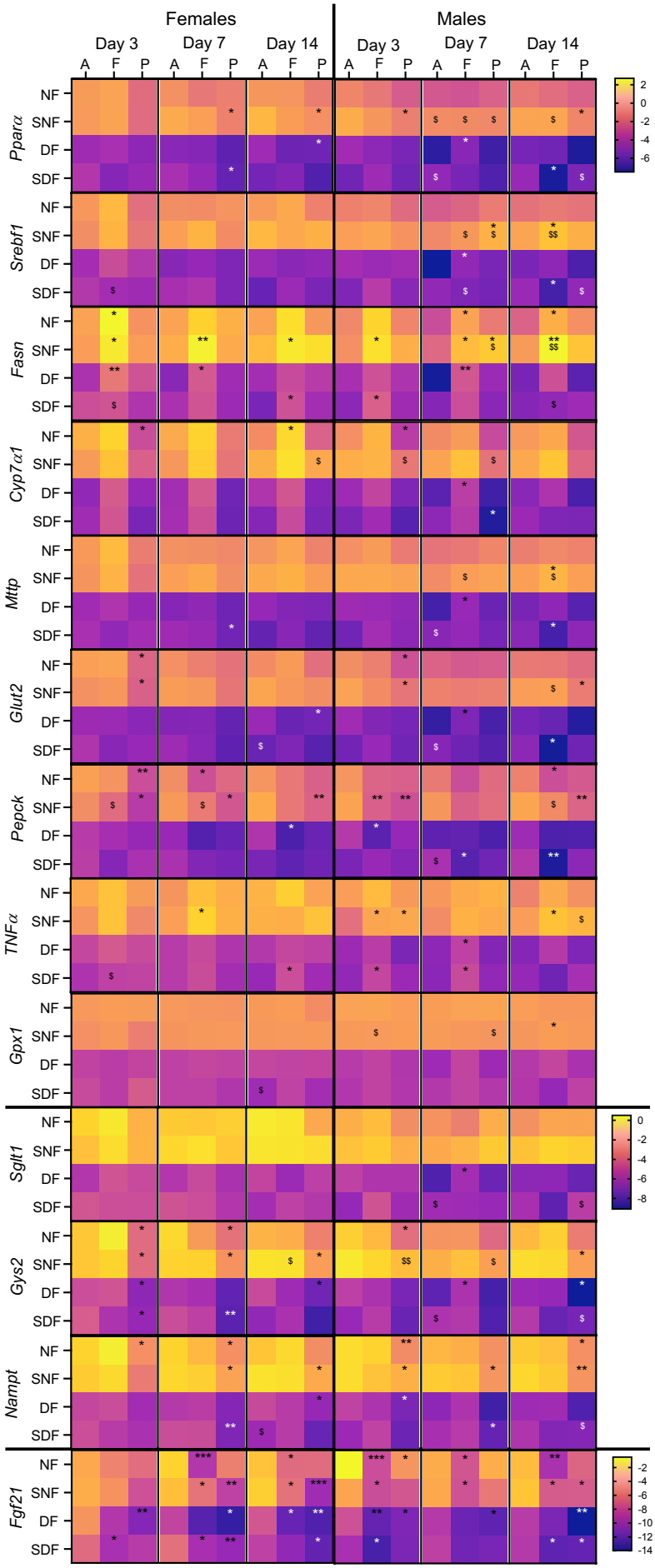

Figure 4. Hepatic diurnal and baseline gene expression changes in response to the food intake time. Sample collection timepoints shown at the top: A = food anticipation, F = food intake, P = postprandial period. Feeding conditions shown on the left: NF = night-fed (ZT12 – ZT20), SNF = shifted night-fed (ZT16 – ZT24), DF = day-fed (ZT0 – ZT8), SDF = shifted day-fed (ZT4 – ZT12). Relative gene expression values (Log2) normalized to NF male mice on sacrifice day 3 at food anticipation and shown as means, n= 4-5 mice. Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test used to show diurnal (vs food anticipation) expression differences, *p<0.05, **p<0.01, ***p<0.001. Same test used to assess baseline differences (non-shifted vs shifted) at each timepoint, $p<0.05, $$p<0.01.

TRF is a poor zeitgeber in stomach, with daytime feeding leading to an overall gene upregulation

The gastrointestinal (further: GI) tract, including stomach and duodenum, is directly exposed to the ingested food, thus one would expect its function being highly adaptive to nutrient-derived signals. Surprisingly, in our study stomach was the least responsive to TRF. Most of the tested genes did not show any diurnal response in their expression, especially in female mice (Fig. 5). The food intake during lights-on phase caused an overall gene upregulation (Fig. 5, DF, SDF), following the pattern seen for the gastric clock genes (Fig. 3).

Genes encoding major GI hormones or their precursors, such as *Ghrl, Sst, Pga5* and gene *Lepr* encoding leptin receptor, all showed fairly similar expression patterns with an appearance of weak diurnal rhythm in female mice after prolonged NF. In contrary, in male mice, with an exception of *Sst*, diurnal response was able to form after prolonged DF, although *Ghrl* and *LepR* were responsive also to NF and SNF, respectively. In general, it seems that long-term TRF might be needed to entrain these hormone encoding genes.

Intriguingly, one of the strongest diurnal patterns was observed for gene encoding gastrin (*Gast*) – a hormone stimulating gastric acid release and GI motility. However, this rhythmicity seemed chaotic, with an appearance only in DF males and females and SDF females on the post-shift day 7 and in NF and SNF males on the post-shift day 3. Some studies have suggested that *Ppar*α could regulate *Gast* expression and/or translation, indicating that they might belong to the same regulatory pathway (40, 41). However, we observed no similarities between their diurnal patterns. A similar appearance and disappearance of diurnal response was also seen for *Tph1 (*encodes rate limiting protein in serotonin synthesis) in male mice, while females developed *Tph1* rhythmicity only under prolonged NF and DF.

Strong sex-specificity in diurnal responses and slow rhythm formation was also present for genes encoding (anti-)inflammatory markers *Gxp2*, *Il-6* and *TNF*α. Whilst in female mice the changes in gene expression were negligible, prolonged DF was able to induce (*Gxp2, TNF*α) or sustain (*Il-6*) diurnal responses in male mice.

Similar to our observations in the liver, also in the stomach the gene showing the strongest diurnal rhythmicity was a surprising find. *Pepck*, a gene encoding rate limiting enzyme for gluconeogenesis, showed a robust rhythm in SNF mice of both sexes. In addition, also NF females and DF and SDF males showed a strong *Pepck* diurnal rhythmicity, with peak expression during the food anticipation. Apart from the liver, gluconeogenesis has been shown to occur in the small intestine (42), especially after gastric bypass surgery (43), but currently there is no evidence for glucose production in stomach. Perhaps, a strong diurnal regulation of gastric *Pepck* could be connected with its suggested role in Krebs cycle (44). Also, as we observed a robust *Pepck* rhythm in the liver (Fig. 4), we cannot exclude an inter-organ communication leading to systemic regulation.

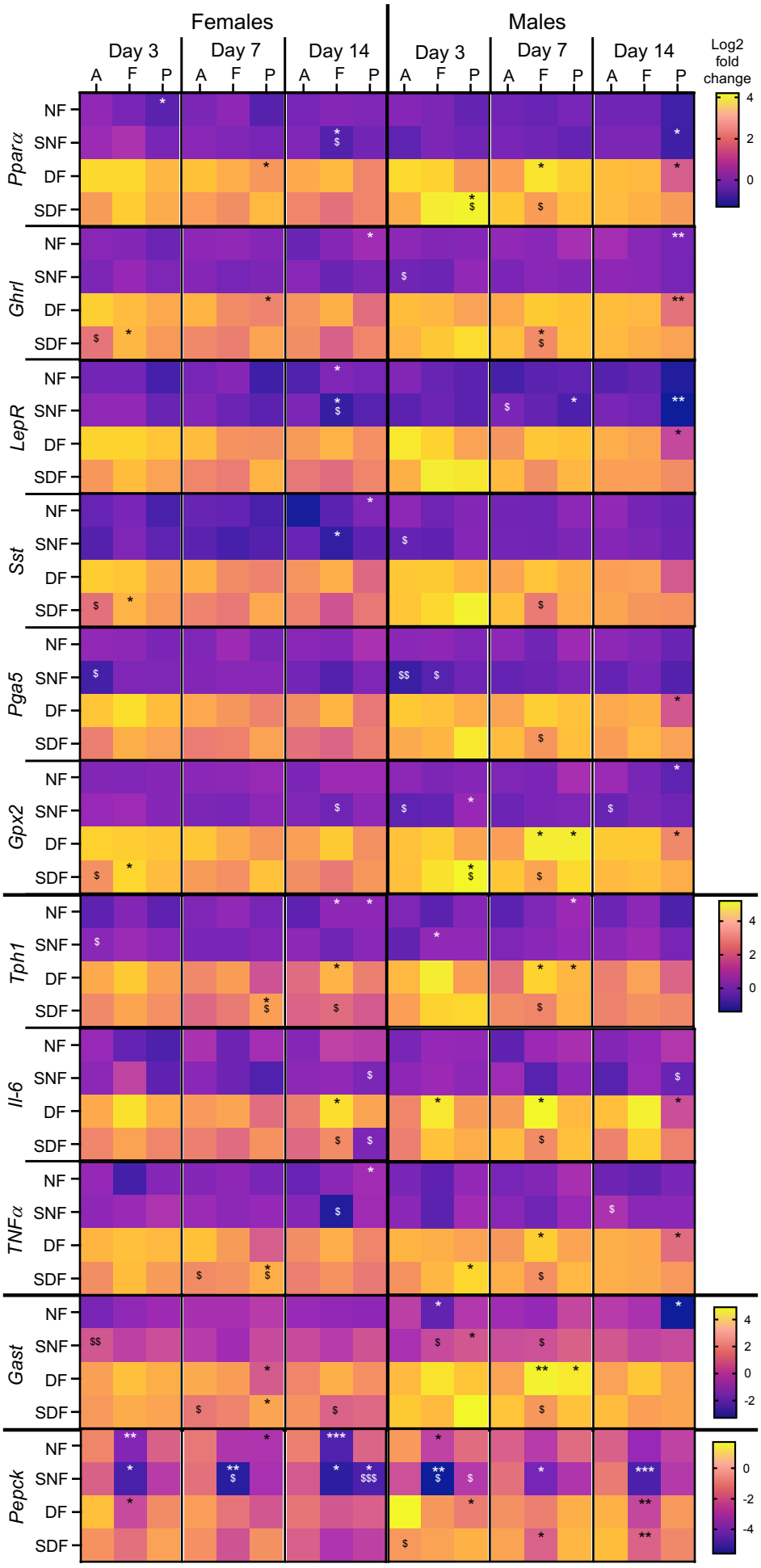

Figure 5. Diurnal and baseline gene expression changes in stomach in response to TRF. Sample collection timepoints shown at the top: A = food anticipation, F = food intake, P = postprandial period. Feeding conditions shown on the left: NF = night-fed (ZT12 – ZT20), SNF = shifted night-fed (ZT16 – ZT24), DF = day-fed (ZT0 – ZT8), SDF = shifted day-fed (ZT4 – ZT12). Relative gene expression values (Log2) normalized to NF male mice on sacrifice day 3 at food anticipation and shown as means, n= 4-5 mice. Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test used to show diurnal (vs food anticipation) expression differences, *p<0.05, **p<0.01, ***p<0.001. Same test used to assess baseline differences (non-shifted vs shifted) at each timepoint, $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001

In duodenum TRF acts as a strong zeitgeber for genes involved in nutrient detection and transport

The duodenum is responsible for further digestion and absorption of nutrients, and thus food intake and nutrient-related signals would be expected to override the central clock. We observed an overall gene upregulation under daytime TRF (Fig. 6, DF, SDF), similar to our observations in stomach, albeit to a lesser extent. Another similarity with the stomach was the poor diurnal rhythmicity in genes encoding GI hormones or their precursors (*Cck, Gip, Ccg, Tph1*). On the other hand, while DF was able to prompt diurnal response in stomach, in duodenum the best entrainment was achieved by NF.

Robust diurnal rhythm was seen for genes encoding large amino acid (*B^0at1*) and peptide (*Pept1*) transporters under NF and SDF. In addition, male mice were able to develop diurnal response in both of these genes when placed under prolonged DF, and female mice showed *Pept1* rhythm also under SNF. The same rhythmicity pattern was seen for *Sglt1*, that encodes a major glucose transporter. Despite this, another gene encoding glucose transporter - *Glut2-*, showed poor diurnal response, with only prolonged SNF leading to rhythmic expression in both sexes, and prolonged DF – in male mice. Distinct sex-specific responses were also observed for *Pparα*, with females preserving or developing rhythms over time in all tested conditions, but males losing it when fed during the lights-off period. Gene encoding the sweet taste receptor (*Tas1r2*) showed very similar diurnal pattern to *Pparα*, just with a larger amplitude.

In addition to metabolism-related genes, also genes encoding (anti-)inflammatory markers showed sex-specific responses towards TRF. Anti-inflammatory marker *Gxp2* showed a good rhythm adaptation to food intake time in females, but not in males. On contrary, *TNFα* exhibited no rhythmicity in female duodenum, but was able to develop a diurnal response in male mice under prolonged NF or SDF.

Gene *Pepck*, that had a strong diurnal response under SNF in both liver (Fig. 4) and stomach (Fig. 5), showed a great adaptation to TRF in male duodenum as well. In female mice it developed robust rhythmicity only under NF. It is intriguing though, that *Pepck* showed strong food entrainment in all tested organs, something not seen even for *Clock* gene. This implies that a disruption of cellular *Bmal1-Clock* function does not automatically result in the loss of all local circadian rhythms and also that food entrainment is highly gene-specific.

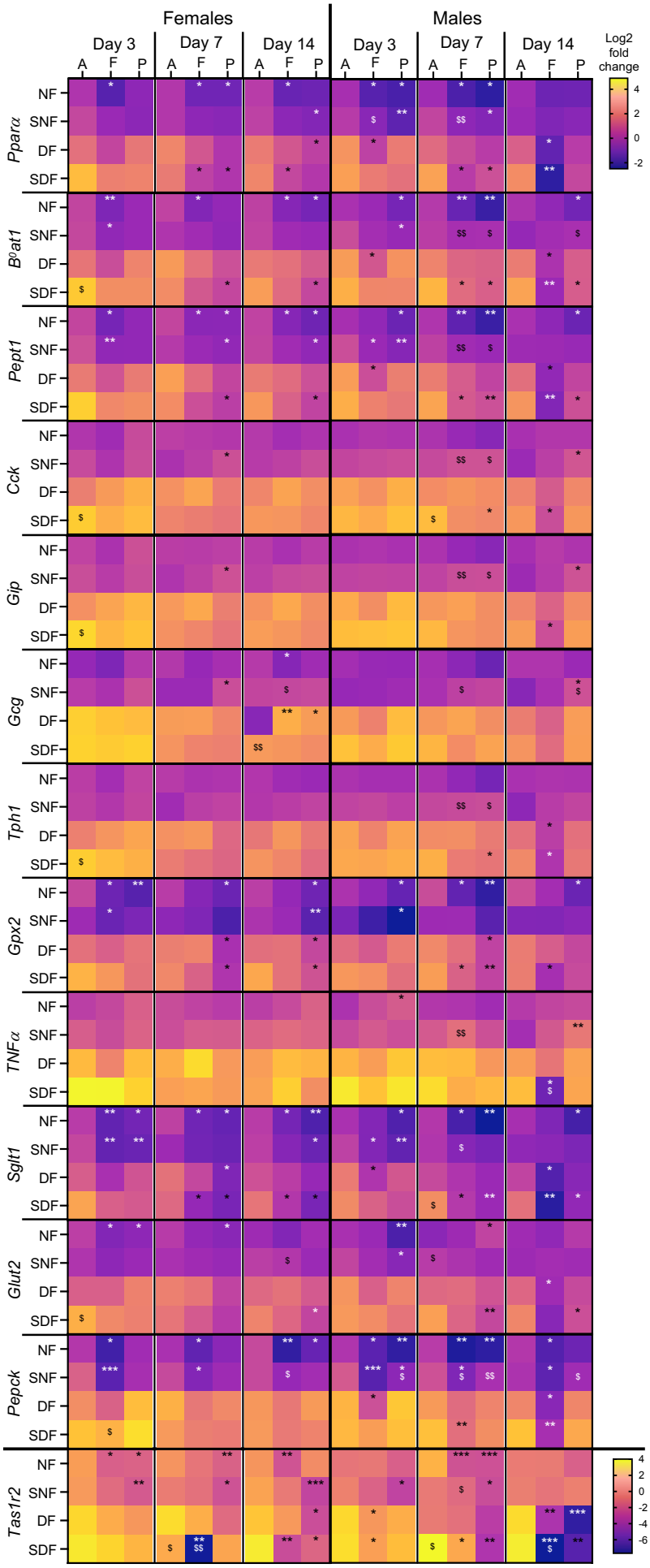

Figure 6. Diurnal and baseline gene expression changes in duodenum in response to TRF. Sample collection timepoints shown at the top: A = food anticipation, F = food intake, P = postprandial period. Feeding conditions shown on the left: NF = night-fed (ZT12 – ZT20), SNF = shifted night-fed (ZT16 – ZT24), DF = day-fed (ZT0 – ZT8), SDF = shifted day-fed (ZT4 – ZT12). Relative gene expression values (Log2) normalized to NF male mice on sacrifice day 3 at food anticipation and shown as means, n= 4-5 mice. Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test used to show diurnal (vs food anticipation) expression differences, *p<0.05, **p<0.01, ***p<0.001. Same test used to assess baseline differences (non-shifted vs shifted) at each timepoint, $^{\$}$p<0.05, $^{\$\$}$p<0.01

Discussion

This study describes how the food intake time affects weight gain, fasting glucose levels, intestinal permeability and an expression of peripheral clock and nutrient metabolism-related genes in the digestive system of male and female mice. We utilized TRF model to limit the food consumption to 8 h in 24 h period, with initial experimental groups staring their feeding period either at ZT12 (NF) or ZT0 (DF). After 14 days of entrainment, food intake time was shifted 4 h forwards in half of the mice (Fig. 1). Observed changes in physiological parameters (relative to NF controls) after 3-4 weeks of food entrainment were summarized in Table 3.

Table 3. Changes in physiological parameters due to prolonged food-entrainment, relative to NF controls

Feeding groups	**Weight gain**		**Fasting blood glucose**		**TEER**	
	Females	*Males*	*Females*	*Males*	*Females*	*Males*
Night-fed (NF)	highest	Lowest	No change over time		Increases over time	
Shifted night-fed (SNF)	-	-/↑	-	↓	-	-
Day-fed (DF)	-/↓	-	-	-	-/↓	↓
Shifted day-fed (SDF)	↓	↑	↑	-	↓	↓↓

The observed sex-specific differences in weight gain in this study were remarkable, with males and females showing almost the opposite effects (Fig. 2A). Food intake during the lights-on phase led to an overall lower weight gain in females, with a little to no impact of the 4 h feeding shift. Males had their lowest weight gain under NF and an increase in weight gain due to any of the 4 h feeding shifts. Lack of significant difference in weight gain in male mice under NF or DF regime has been observed before (45, 46), however, to our knowledge, there are no studies that have focused on weight gain in female mice under TRF.

We also observed that DF and SDF mice, especially females, lost a significant amount of weight during a single 16 h fasting period, even after prolonged entrainment (Fig. 2B). We hypothesise that these mice struggle to quickly adjust their activity to the new food intake time, staying active in the dark period, during which they are not able to replenish the lost calories. There have been some indications that regulation of activity is fairly complicated, involving both the central clock and feedback from food-entrained oscillators (47). Therefore, additional measurements of locomotion and central clock activity would be needed to assess their sex-specificity and role in weight gain under various TRF regimes.

Regulation of blood glucose levels showed less sex-specificity. Both sexes exhibited hyperglycaemia when food intake was shifted to the late lights-on phase (ZT4 – ZT12) (Fig. 2C). Higher fasting blood glucose levels in DF mice have been detected before (48), and studies with rats have observed poor glucose tolerance under daytime feeding (49), suggesting that the food intake during lights-on phase might be detrimental for glucose

clearance. However, it has also been shown that mice on standard 12 h LD cycle show the peak of blood glucose concentration during the first half of lights-on phase (ZT0-ZT4) (50), that coincides with the glucose measurement time in SDF mice (ZT4). Thus, we have to take into the account, that the observed fasting glucose levels in SDF mice might have also reflected the light-driven rhythm of glucose metabolism.

The disruption of the intestinal barrier has been understudied in the context of food entrainment. Thus, we measured TEER to determine whether the epithelial resistance is affected by the food intake time. Studies using colitis models in mice have shown that TRF during the dark phase leads to high expression levels of tight junction proteins, preserving intestinal integrity (51). Our data align with these observations, demonstrating an increase in TEER due to prolonged NF in both sexes (Fig. 2D). It has also been shown that the intestinal permeability increases during constant 24 h lights-on period (52) or under LD cycle shifts (53), again matching our observed reduction in TEER in DF and SDF mice, implying intestinal leakiness.

Overall, our data suggest that both glucose handling and intestinal permeability show poor adjustment to daytime feeding. At the same time, if TRF is applied during dark phase, these physiological functions improve. Therefore, we propose that an alignment between light-driven regulation and peripheral zeitgebers is necessary for glucose tolerance and intestinal barrier function.

Regarding the nutrient-metabolism related gene expression, we observed two, seemingly independent types of regulation, that were highly sex- and organ-specific: 1) a change in expression levels due to the 4 h feeding shift (=baseline change); and 2) differences between the timepoints within the same feeding condition (=diurnal response). There hasn't been much focus on non-diurnal responses to TRF, but organ- and sex-specificity has already been observed in multiple studies (for reviews see (54, 55), raising an interest in a systems-level circadian regulation. In this study, organ and sex-specificity was especially evident in the expression of circadian clock encoding genes *Clock* and *Bmal1* (Fig. 3). Daytime feeding led to a downregulation of these genes in the liver, but to an upregulation in the stomach and duodenum. Females were more likely to show an early rhythmicity that dissipated over time, while males showed improved rhythm under prolonged TRF. These observations suggest, that in the upper GI tract: a) food intake time is not able to fully uncouple peripheral digestive clocks; b) daytime feeding might lead to a significant upregulation of *Bmal1-Clock* dimer, potentially disrupting downstream gene transcription, c) female mice show faster, but less stable food-entrainment than males. Expression patterns observed for nutrient metabolism- and inflammatory response-related genes also generally aligned with these suggestions (Figs. 4-6).

Taken together, our observations have shown that peripheral diurnal rhythms in gene expression are highly organ-specific, as not a single TRF regime led to a strong entrainment and synchronization across the whole digestive system. However, our results do not imply a total absence of an inter-organ communication. We did observe some alignment in diurnal rhythms between the organs, for example, gene *Pepck*, encoding phosphoenolpyruvate carboxykinase, showed robust rhythms in all tested organs, especially under SNF (Figs. 4-6). Gene-specific responses were in general not uncommon, even if the genes belonged to the same

metabolic pathway, e.g., *Fgf21* had strong diurnal rhythm in the liver, despite its upstream regulator *Ppar*α (56) being largely unresponsive to TRF (Fig. 4). This suggests that zeitgeber signals might be integrated on a gene-level, questioning the existence of a single food-entrainable oscillator and the strict master-peripheral clock hierarchy.

The suprachiasmatic nucleus (SCN) is thought to be the main coordinator of the light-entrained circadian rhythms and thus has been given a role of the master circadian regulator. The SCN-set rhythms are believed to then initiate and/or to synchronize oscillations in peripheral clock genes (21, 57, 58). However, we are not alone in suggesting a more complicated interaction between central and peripheral clocks that could not happen without an assessment and integration of all zeitgeber signals, independently of their origin. The essential master clock role of the SCN has been questioned before, as studies have shown persistent circadian rhythms in explants of adipose tissue, lung, liver and kidney in the complete absence of SCN signalling (59, 60). In addition, in rats and mice under DF, neuronal activity in SCN was observed to be reduced, allowing food anticipatory activity to occur during the passive, lights-on phase (47, 61). On the gene expression level, clock genes *Rev-erbα* and *Bmal1* in the SCN have been shown to respond to food-entrainment in rats kept in a constant light (62). Thus, it appears that peripheral zeitgebers might exert at least a modulatory effect on SCN, despite being seen as 'lesser' zeitgebers. In addition, it has also been shown that application of various zeitgebers at once might lead to a system-level disruption of circadian rhythms, leading to a conflicting regulation – something our observations would also support. For example, studies with mice on alternating light-schedules have shown that whilst TRF during the dark phase led to an adjustment in activity and body temperature, it did not affect the plasma triglyceride levels and energy expenditure (63). Another study observed that 4 weeks of TRF seemed to reduce the overall levels of various disease and inflammatory markers in jejunum, however, an additional supplementation of an anti-inflammatory vitamin A analog ATRA (all-*trans* retinoic acid) surprisingly led to an increase in several pro-inflammatory and disease markers (64). ATRA itself seems to act as a zeitgeber and its generated rhythm did not match that of the TRF used (65), explaining the lack on synergistic effects, and instead leading to a circadian incoherence and disruption on systemic level. This, of course, raises a question how diurnal rhythmicity can emerge at all, if conflicting zeitgeber signals are present. It has been suggested that circadian clocks can be divided into weak and strong oscillators. Based on tests with explants of lung tissue and SCN, peripheral tissue seems to act as a weak oscillator, being able to achieve larger phase shifts and easily aligning their amplitude to zeitgeber signals. SCN seems to be a strong oscillator, entraining within narrower ranges, and its amplitude seems to be unaffected by the period of zeitgeber (66). However, if multiple weak oscillators (e.g. various organs/tissues) are coupled, they act as strong oscillator as well (66-68). This means that peripheral organs are more sensitive to entrainment, however, systemic rhythms will only emerge when multiple zeitgebers align.

In conclusion, the existence of weak and strong clocks and the complex interaction of various zeitgebers could explain our detected organ-and gene-specific TRF responses. We did observe that in the digestive system diurnal rhythmicity was most likely to appear under SNF (Figs. 3-6) suggesting that specific food intake

time might be beneficial in synchronizing the rhythms between digestive organs. However, TRF will not achieve systemic synchronization alone. Circadian regulation will instead happen by integration of all zeitgeber signals. Untangling the level at which this regulation happens (e.g at a single gene or gene groups) would require a large-scale peripheral circadian rhythm study, looking at genes that belong to well characterized pathways across various organs and using aligning vs conflicting zeitgebers. In addition, sex-specific diurnal regulation seems to be broadly present in periphery as well. Thus, the future circadian studies assessing the impact of shift-work, sleep disturbances, time zone changes and chronotherapies should utilize experimental models representing both sexes.

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Competing interests

The authors of this study confirm there are no competing interests.

Author contributions

All experiments were carried out at the Department of Anatomy and Physiology, The University of Melbourne and the Florey Institute of Neuroscience and Mental Health. L.O-R., M.N. and J.B.F designed the study and necessary experiments. L.O-R., M.N., R.M., B.H., A.K., M.R., T.C. and L.J.F carried out the experiments. L.O-R., M.N. and J.B.F. analysed and interpreted the data. M.N., R.M., L.O-R. and J.B.F. wrote and critically revised the manuscript. All authors approved the final version of the manuscript. All persons qualifying for authorship are listed as such.

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