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Night-time feeding of *Bmal1*$^{-/-}$ mice restores SCFA rhythms and their effect on ghrelin

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Abstract

The known crosstalk between short-chain fatty acids (SCFAs) and the circadian clock is tightly intertwined with feeding time. We aimed to investigate the role of the core clock gene *Bmal1* and feeding time in the diurnal rhythms in plasma and caecal SCFAs levels and in their effect on the release of the hunger hormone ghrelin in the stomach and colon. WT, *Bmal1*$^{-/-}$ (*ad libitum* fed) and night-time-restricted-fed (RF)-*Bmal1*$^{-/-}$ littermates were sacrificed at *Zeitgeber* time (ZT) 4 and 16. SCFA concentrations were measured by gas chromatography. To investigate the effect of SCFAs on ghrelin release, ~~S~~stomach and colonic full-thickness strips were incubated with Krebs or a SCFA mix mimicking plasma ~~(360 µM)~~ or caecal concentrations ~~(124 mM)~~ ~~and~~ after which octanoyl ghrelin release was measured by radioimmunoassay. Diurnal rhythms in caecal and plasma SCFAs oscillated in phase but rhythmic changes were abolished in *Bmal1*$^{-/-}$ mice. RF of *Bmal1*$^{-/-}$ mice restored fluctuations in caecal SCFAs. Plasma SCFA concentrations failed to affect gastric ghrelin release. The effect of caecal SCFA concentrations on colonic ghrelin release was rhythmic (inhibition at ZT 4, no effect at ZT 16). In *Bmal1*$^{-/-}$ mice, the inhibitory effect of SCFAs at ZT 4 was abolished. RF *Bmal1*$^{-/-}$ mice restored the inhibitory effect and increased colonic *Clock* expression. To conclude, ~~D~~diurnal fluctuations in caecal SCFAs and the effect of SCFAs on colonic ghrelin release are regulated by feeding time, independent of the core clock gene BMAL1. However, local entrainment of other clock genes might contribute to the observed effects.

Introduction

Ghrelin, a 28-amino acid peptide, is the only known circulating hormone that stimulates food intake (Kojima, et al. 1999). Ghrelin is mainly produced in the stomach and is acylated by ghrelin O-acyltransferase (GOAT) on Ser^3, which is essential for its physiological functions (Gutierrez, et al. 2008; Yang, et al. 2008). Besides activation of orexigenic neural circuits, ghrelin has numerous other physiological effects, including regulation of glucose metabolism, stimulation of gut motility and gastric acid secretion, and modulation of sleep, taste sensation and reward seeking behaviour (Avau, et al. 2013; Müller, et al. 2015).

In humans, plasma ghrelin levels increase before a regularly timed meal and decrease to trough levels within 1 hour after eating (Cummings, et al. 2001). Furthermore, plasma ghrelin levels show a 24-hour diurnal rhythm in both humans and rodents, reaching peak values during their resting phase (Bodosi, et al. 2004; Laermans, et al. 2015; Yildiz, et al. 2004). Diurnal rhythms in plasma ghrelin levels and gastric ghrelin expression are abolished in mice that lack the core clock gene *Bmal1*, indicating that ghrelin levels are regulated by the circadian clock (Laermans et al. 2015).

The circadian clock aligns behavioural patterns with the solar day, anticipating the body's metabolic needs (Reinke and Asher 2019). The master clock, located in the suprachiasmatic nucleus, is synchronized or entrained by the light-dark cycle. Circadian rhythms are maintained by a set of core clock genes that form transcription-translation feedback loops in which they not only regulate their own transcription but that of many clock-controlled genes. The CLOCK-BMAL1 heterodimer forms the positive loop, while period (PER1, PER2, and PER3) and cryptochrome (CRY1 and CRY2) genes form the negative loop (Partch, et al. 2014). Animals with mutations or ablations of these core clock genes have altered food intake patterns, body weight and metabolism (Laermans and Depoortere 2016).

The master clock synchronizes the rhythm of peripheral clocks present in the liver, gastrointestinal tract, adipose tissue and numerous other tissues and organs (Mohawk, et al. 2012). However, peripheral circadian rhythms are also regulated by local entrainment signals or *Zeitgebers* ~~(ZTs)~~, such as ~~quality, quantity and~~the timing of ~~food~~ meals (Panda 2016). Desynchronizing these ~~*ZTs*~~ *Zeitgebers* by e.g. rotating shift work~~,~~ or frequent flying ~~and/or a Western diet~~ misaligns master and peripheral clocks, causing chronodisruption (Dibner 2019). Night work and obesity are associated with loss of diurnal rhythmicity of plasma ghrelin levels, altered ghrelin cell sensitivity and blunted post-meal suppression of ghrelin (Crispim, et al. 2011; Schiavo-Cardozo, et al. 2013; Vancleef, et al. 2018; Wang, et al. 2019; Yildiz et al. 2004). This might play a role in the development of metabolic diseases, which are prevalent in chronodisrupted individuals (Karlsson, et al. 2001; Laermans and Depoortere 2016; Pan, et al. 2011).

The post-meal suppression of ghrelin levels is dependent on the caloric content and type of ingested nutrients (Callahan, et al. 2004; Foster-Schubert, et al. 2008). The sensing of these nutrients is likely mediated by different chemosensory receptors that are present on ghrelin cells (Hass, et al. 2007; Janssen, et al. 2012; Janssen, et al. 2011; Steensels and Depoortere 2018; Vancleef, et al. 2015; Vancleef et al. 2018). Surprisingly, the short-chain fatty acid (SCFA) receptor free fatty acid receptor 2 (FFAR2) is highly expressed on gastric ghrelin cells. Engelstoft *et al.* showed that SCFAs inhibited ghrelin secretion in mouse primary gastric mucosal cells, mainly through FFAR2 and a $G\alpha_{i/o}$ mechanism. However, other studies using isolated gastric ghrelin cells and a ghrelinoma cell line failed to show an effect of SCFAs on ghrelin release (Engelstoft, et al. 2013; Koyama, et al. 2016; Lu, et al. 2012). It is currently unknown whether plasma SCFA concentrations are high enough to activate FFAR2 on gastric ghrelin cells. Nevertheless, ghrelin is also expressed in more distal regions such as the colon where bacterial fermentation of dietary carbohydrates in the caecum and colon takes place and results in higher concentrations of SCFAs. Therefore, the effects of SCFAs on ghrelin release might be more pronounced in the colon. SCFAs are an important energy source for

the host, but are also key signalling molecules that propagate beneficial metabolic effects (Koh, et al. 2016).

Additionally, SCFAs can entrain peripheral circadian rhythms in the host (e.g. liver) by adjusting peripheral clock gene expression (Leone, et al. 2015; Tahara, et al. 2018). In turn, the circadian clock can regulate the diurnal rhythm in the levels of fecal SCFAs, SCFA receptor expression and their functional effects (Mukherji, et al. 2013; Segers, et al. 2018).

In the present study, we aimed to investigate whether 1) plasma and caecal SCFAs levels ~~are~~ show a diurnal rhythm and affect ghrelin release in the stomach and colon in a circadian manner, respectively. 2) the diurnal fluctuations of SCFAs and their rhythmic effects on ghrelin release are dampened by deletion of the core clock gene BMAL1 3) the loss in the day/night food intake pattern induced by deletion of BMAL1 are the trigger for the loss in rhythmicity and can be restored by two weeks of night-time restricted feeding.

Material and Methods

Mice studies

Mice

C57BL/6J mice were purchased from Janvier Labs (Le Genest Saint Isle, France). *Bmal1*$^{+/-}$ mice (kindly provided by R. Lijnen, KU Leuven, Leuven, Belgium) (Hemmeryckx, et al. 2011) were bred in the animal facility of the KU Leuven to generate *Bmal1*$^{-/-}$ and wild type (WT) littermates and were genotyped by PCR on total genomic DNA from the ear. Mice had *ad libitum* access to chow and drinking water unless otherwise specified and were housed in a temperature-controlled environment under a 12h/12h light/dark-cycle (*Zeitgeber* time (ZT) 0 = lights on). All experiments were approved by the Ethical committee for Animal Experiments of the KU Leuven and carried out in accordance with the approved guidelines.

Experimental design

C57BL/6J mice (male, age 12-15 weeks) were sacrificed over the course of 24 hours at 4-hour intervals. *Ad libitum* fed *Bmal1*$^{-/-}$ and WT littermates (male~~, age 12-16 weeks~~) were sacrificed at ZT 4 (resting phase) and 16 (active feeding phase) at age 12 – 16 weeks, as *Bmal1*$^{-/-}$ mice older than 16 weeks show growth retardation (Kondratov, et al. 2006). To study the effect of restoration of diurnal rhythmicity in food intake, a group of *Bmal1*$^{-/-}$ mice was only fed (*ad libitum*) during the night for 2 weeks (From ZT 12 to ZT 24; *Bmal1*$^{-/-}$ RF) prior to the day of sacrifice. On the day of sacrifice, *Bmal1*$^{-/-}$ RF mice were sacrificed at ZT 4 (fasted from ZT 0 - 4) and 16 (fed from ZT 12 - 16).

Luminal content of the caecum and stomach and/or plasma were collected to measure SCFA concentrations. The stomach and proximal colon were removed for ghrelin release experiments. The mucosa of the proximal colon was stored in RNAlater (Qiagen, Hilden, Germany) and processed for quantitative real-time PCR (qRT-PCR).

Analysis of total SCFA concentrations in chyme, faeces and plasma

Total SCFA concentrations in luminal content of the caecum and stomach were determined as previously described.[35] Plasma samples were acidified using HCl after addition of a mixture of [$^2H_3$]-acetate, [$^2H_5$]-propionate and [$^2H_7$]-butyrate as internal standard. SCFAs were converted to their respective 2,4-difluoroanilides by addition of 2,4-difluoroaniline and 1,3-dicyclohexylcarbodiimide and extracted to ethyl acetate in the presence of $NaHCO_3$. The supernatant was added to a micro-insert (VWR International, Radnor, PA) and analysed on a TRACE 1300 Gas Chromatograph coupled to a DSQ II Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA), equipped with an analytical column (Restek Stabilwax-DA column, Restek, Bellefonte, PA, USA) of 30 m x 0.25 mm i.d. and 0.25 µm film thickness.

Measurement of ghrelin release from intestinal full-thickness strips

Eight full-thickness strips of the corpus of the stomach (± 5 x 5 mm) and two full-thickness strips of the whole proximal colon were dissected and incubated with 1 mL Krebs-buffer or Krebs-buffer containing a SCFA mix mimicking peak plasma (acetate: 348 µM, propionate: 9 µM, butyrate: 3 µM) or peak caecal (acetate: 85 mM, propionate: 8 mM, butyrate: 31 mM) concentrations. Strips were incubated for 2 hours at 37°C, 5% CO_2, and 10 µL of 57 mM protease inhibitor phenylmethylsulfonyl fluoride was added at the start and after 1 hour of incubation. The release of octanoyl ghrelin in the medium was measured by radioimmunoassay. Tissue strips were dried to correct the ghrelin release for dry tissue weight of the strip.

Cell line studies

Cell culture

The MGN3-1 ghrelinoma cell line was kindly provided by Prof. Hiroshi Iwakura (Iwakura, et al. 2010). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C in 5% CO_2.

SCFA-stimulated ghrelin release

Cells were incubated for 1 hour (37 °C, 5% CO_2) with Krebs-buffer or Krebs-buffer containing SCFA mixes mimicking concentrations in plasma, stomach or caecum. Acetate, propionate and butyrate concentrations used were 348-9-3 µM (plasma), 12-4-0.2 mM (stomach) and 85-8-31 mM (caecum), respectively. The release of octanoyl ghrelin in the medium was measured by radioimmunoassay.

Ghrelin radioimmunoassay

Cell and tissue culture supernatants were acidified (10% 1 M HCl), extracted on a Sep-Pak C18 column (Waters Corporation, Milford, MA) and vacuum-dried. The radioimmunoassay for octanoyl ghrelin was performed as previously described (Janssen et al. 2011).

Quantitative real-time PCR (qRT-PCR)

RNA was isolated using the RNeasy Mini kit (Qiagen, Venlo, the Netherlands). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). qRT-PCR was performed using the Lightcycler 480 with the Lightcycler 480 Sybr Green I Master mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Results were corrected for interrun variability and expressed relative to the geometric mean of the normalized expression of three stable housekeeping genes (*β-actin*, *Ppib* and *Hmbs*) that do not show a circadian rhythm (Vandesompele, et al. 2002). Primer sequences are listed in table 1.

Statistics

~~Results are presented as mean ± SEM.~~ Diurnal rhythms were analysed using the free Cosinor software (version 3.1, R. Refinetti, Boise State University, Boise, ID), which determines the best-fitting cosine curve (diurnal: period = 24 hours; ultradian: period = 12 hours) for a data set using the cosinor procedure as described by Nelson *et al*. (Nelson, et al. 1979). Since all data collected in *Bmal1*$^{-/-}$ mice and their WT littermates and MGN3-1 ghrelin release experiments was distributed in a non-normal and/or non-homogeneous manner, log-transformed data was used for all further analyses. Student's t-test was performed to detect differences in SCFA concentrations in stomach chyme of WT mice between ZT 4 and 16. Differences in SCFA concentrations, in ghrelin release from MGN3-1 cells and from stomach and colon strips, and in clock gene expression between different time points and groups were analysed using a mixed model analysis (SAS Studio University Edition 9.4, SAS Institute Inc., Cary, NC, USA). Significance was accepted at the 5% level. Results are presented as back-transformed mean ± SEM.

Results

Diurnal fluctuations in plasma, caecal and stomach SCFA concentrations

The diurnal rhythm of SCFA concentrations was investigated in the plasma and luminal content of the caecum of C57BL/6J mice, sacrificed over the course of 24 hours (4 hours interval). Plasma acetate, propionate and butyrate concentrations all showed a diurnal rhythm of 24 hours ($P < 0.05$), peaking at ZT 21h02, ZT 19h09 and ZT 20h20, respectively (Figure 1 A). Caecal acetate and butyrate concentrations peaked at ZT 19h43 and ZT 19h52 ($P < 0.01$), respectively, while propionate concentrations showed an ultradian rhythm (period of 12 hours, $P < 0.001$), peaking at ZT 3h52 and 15h52 (Figure 1 B).

Total SCFA concentrations in plasma peaked at ZT 20h51, in phase with the rhythms of total SCFA concentrations in the caecum (peak at ZT 19h40), but not with the distal colon, where in a previous study(Segers et al. 2018) we have shown that total SCFA concentrations peaked at ZT 5h57 (Figure 1 C).

Average SCFA concentrations in plasma were 294-fold lower than in the caecum (341 ± 20 µM vs. 101 ± 4 mM). SCFAs were also detected in the luminal content of the stomach of WT mice (21 ± 4 mM) and were 60-fold higher than in the plasma (341 ± 20 µM). Furthermore, stomach SCFA concentrations were 1.6 fold higher in the resting phase (ZT 4) compared to the active feeding phase (ZT 16) ($P < 0.05$; Figure 1 D).

Diurnal effects of SCFAs on ghrelin release

Concentration-dependent effects of SCFAs on ghrelin release from a ghrelinoma cell line (MGN3-1)

The effect of SCFA mixes mimicking plasma, stomach and caecal concentrations on octanoyl ghrelin release from MGN3-1 gastric ghrelinoma cells was measured. While plasma (360 µM)

and stomach (16 mM) SCFA concentrations did not affect octanoyl ghrelin release, caecal concentrations (124 mM) stimulated octanoyl ghrelin release ($P < 0.001$, Figure 2 A).

Time- and concentration-dependent effects of SCFAs on ghrelin release from stomach and colon strips

The effect of SCFAs on octanoyl ghrelin release from stomach full-thickness strips from WT mice sacrificed in the resting phase (ZT 4) and active feeding phase (ZT 16) was investigated. The SCFA mix mimicking plasma concentrations (360 µM) had no effect on octanoyl ghrelin release from stomach strips at both ZTs. However, the SCFA mix mimicking caecal concentrations (124 mM) stimulated octanoyl ghrelin release at ZT 4 ($P < 0.01$) and had no effect at ZT 16, resulting in a significant ($P < 0.01$) interaction effect (Figure 2 B).
In the proximal colon, the SCFA mix mimicking caecal concentrations affected octanoyl ghrelin release at ZT 4 and not at ZT 16 (Figure 2 C). However, in contrast to the stomach, the SCFA mix inhibited octanoyl ghrelin release from the proximal colon ($P < 0.01$).

Role of the clock gene BMAL1 in the rhythm of SCFAs and their diurnal effects on ghrelin release

To study the role of the circadian clock in the diurnal fluctuations of SCFA concentrations, plasma and caecal SCFA levels were measured in arrhythmic *Bmal1*$^{-/-}$ mice and their WT littermates at ZT 4 and 16. Similar to C57BL/6J mice, total plasma SCFA concentrations were significantly ($P < 0.01$) higher in WT littermates at ZT 16 compared to ZT 4, total caecal concentrations showed a similar trend ($P = 0.12$). These differences were abolished in *Bmal1*$^{-/-}$ mice. (Figure 3 A-B).
The caecal SCFA mix stimulated octanoyl ghrelin release from the stomach of WT mice at ZT 4. In contrast, in *Bmal1*$^{-/-}$ mice, this SCFA mix inhibited octanoyl ghrelin release ($P < 0.05$) (Figure 3 C). In the proximal colon of WT mice, the SCFA mix inhibited octanoyl ghrelin release at ZT 4. In *Bmal1*$^{-/-}$ mice, the SCFA mix had no effect on octanoyl ghrelin release (Figure 3 D).

Restoration of diurnal fluctuations in SCFAs and of SCFA-stimulated ghrelin release in *Bmal1*$^{-/-}$ RF

Since *Bmal1*$^{-/-}$ mice eat continuously and hence do not show the typical day/night feeding rhythm (Laermans et al. 2015), we aimed to investigate whether the loss of rhythms in SCFA concentrations and of the effects of SCFAs on ghrelin release was due to their altered food intake pattern. A group of *Bmal1*$^{-/-}$ mice was only fed during the dark phase for two weeks (*Bmal1*$^{-/-}$ RF). Food intake was measured daily and body weight was measured every three days. Food intake and body weight were stable during night-time restricted feeding (Figure S1 A-B). Body weight at sacrifice (ZT 4) was higher in *Bmal1*$^{-/-}$ RF compared to *ad libitum* fed *Bmal1*$^{-/-}$ mice (Figure S1 C). both *ad libitum* and RF fed *Bmal1*$^{-/-}$ mice weighed less than WT mice at sacrifice.

Restricting the access of *Bmal1*$^{-/-}$ mice to food to the dark phase (*Bmal1*$^{-/-}$ RF) restored fluctuations in SCFA concentrations in the caecum ($P < 0.05$), but not in the plasma (Figure 3 A-B).

The inhibitory effect of SCFAs on octanoyl ghrelin release from the stomach of *ad libitum* fed *Bmal1*$^{-/-}$ mice was abolished by RF (Figure 3 C), while in the proximal colon RF restored the inhibitory effect observed in WT mice ($P < 0.01$; Figure 3 D).

Effect of night-time restricted feeding of Bmal1$^{-/-}$ *mice on colonic clock gene expression*

Finally, we investigated whether night-time restricted feeding of *Bmal1*$^{-/-}$ mice could affect circadian clock gene expression in the colonic mucosa at ZT 4, despite absence of the core clock component BMAL1. While *Clock* mRNA expression was not different between WT and *Bmal1*$^{-/-}$ mice, it was significantly upregulated in RF *Bmal1*$^{-/-}$ mice ($P < 0.001$). *Per2* and *Reverbα* mRNA expression was higher and lower in *Bmal1*$^{-/-}$ mice compared to WT mice, respectively ($P < 0.001$), but was not affected by RF of *Bmal1*$^{-/-}$ mice (Figure 4).

Discussion

In the present study, we showed that plasma, stomach and caecal SCFA concentrations fluctuated diurnally. For the SCFAs in the plasma and luminal content of the caecum, the peak of the rhythm occurred at the end of the active phase while for the distal colon SCFAs peaked in the resting phase. Plasma SCFA concentrations did not affect gastric octanoyl ghrelin release, while caecal concentrations of SCFAs only affected octanoyl ghrelin release from stomach and colon in the resting phase (ZT 4) and had no effect in the active phase (ZT 16). Finally, we showed that night-time restricted feeding restored diurnal fluctuations in caecal SCFA concentrations and the effect of SCFAs on colonic octanoyl ghrelin release ~~were regulated by feeding time, independent of the core clock gene BMAL1~~in *Bmal1*$^{-/-}$ mice. However, night-time restricted feeding enhanced the expression of other clock genes (*Clock*) expressed in the colonic mucosa, which might contribute to the restoration of the rhythm.

In WT mice, total plasma SCFA concentrations showed a 24-hour rhythm with a peak in the active phase, in phase with the rhythms of caecal SCFA concentrations. SCFAs in the distal colon peaked 10h17 after the acrophase of SCFAs in the caecum. This delay might partially be caused by the transit time from the caecum to the distal colon, which has previously been shown to be 5-6 hours (Padmanabhan, et al. 2013). Consequently, SCFAs in the plasma likely mainly originate from the caecum, the main site of microbial SCFA production (den Besten, et al. 2013). ~~Peak values of caecal SCFA concentrations (124 ± 9 mM) were 2-3-fold higher than previously reported caecal concentrations in mice (Olguín-Calderón, et al. 2019; Pan, et al. 2009; Tamura, et al. 1999), possibly because these studies measured caecal SCFAs in the resting phase (daytime), when caecal SCFA concentrations are minimal.~~

Interestingly, average SCFA concentrations in the stomach content of WT mice reached 21 ± 4 mM. The measured acetate and propionate in the chyme might have originated from the chow, since we measured 25 mmol/kg acetate and 30 mmol/kg propionate in the chow

(butyrate under detection limit; mouse maintenance diet (Ssniff Spezialdiäten GmbH, Soest, Germany)). However, the presence of butyrate in the luminal content of the stomach indicates that coprophagic behaviour might have also contributed to the measured SCFA concentrations. Coprophagic behaviour has been shown to peak at the start of the resting phase (ZT 0 – 4) (Ebino 1993). ~~which~~This corresponds to the increased SCFA concentrations at ZT 4 compared to ZT 16. In addition, local bacterial production of SCFAs cannot be excluded since the anterior part of the stomach of rodents is known to harbour a large population of microorganisms at a pH that permits bacterial multiplication (Smith 1965).

Although SCFA concentrations in the stomach were 60 times higher than in the plasma, neither concentrations affected octanoyl ghrelin release from gastric ghrelinoma cells. Only caecal levels of SCFAs (124 mM) could stimulate octanoyl ghrelin release. It is unlikely that these concentrations reach gastric ghrelin cells *in vivo*. Previous studies have shown that FFAR2 and FFAR3 are highly expressed on gastric brush cells around the limiting ridge. This chemosensory cluster has been suggested to convey signals to closely associated ghrelin cells and might play a role in the effect of SCFAs on gastric ghrelin release *in vivo* (Eberle, et al. 2014; Hass et al. 2007). However, we found that the SCFA mix mimicking plasma concentrations also failed to affect octanoyl ghrelin release from stomach full-thickness strips, which contain brush cells ~~at both ZT 4 and 16~~. The SCFA mix mimicking caecal concentrations stimulated octanoyl ghrelin release at ZT 4 and had no effect at ZT 16, indicating that the effect of SCFAs on ghrelin release is time-dependent~~, although the physiological relevance of these results with high concentrations of SCFAs is questionable~~.

It remains to be determined whether these findings are representative for possible effects of SCFAs on human gastric ghrelin cells. ~~Although~~ SCFAs can be present in food (e.g. in vinegar and other fermented food, as preservatives and in dairy products) (EFSA 2014; Ho, et al. 2017; McNabney and Henagan 2017). However, their concentrations in the human stomach are likely lower and much more variable (both intra- and inter-individual).

Although ghrelin is mainly produced in the stomach (Kojima et al. 1999), we found that at ZT 4, basal octanoyl ghrelin release from the proximal colon was only 3 times lower compared to release from the gastric corpus ~~and~~. Colonic ghrelin release might therefore significantly contribute to plasma octanoyl ghrelin levels. ~~Since w~~We previously showed that SCFAs had maximal effect on proximal colon contractility at ZT 4h17, in phase with diurnal SCFA concentrations in the distal colon (Segers et al. 2018). Accordingly, we hypothesized that the effect of SCFAs on octanoyl ghrelin release from the proximal colon would also peak around ZT 4. Indeed, the SCFA mix only affected octanoyl ghrelin release at ZT 4 and not at ZT 16. However, in contrast to the stomach, the SCFA mix inhibited octanoyl ghrelin release from the proximal colon. The opposite findings of the effect of SCFAs on ghrelin release between stomach and colon might be due to activation of different signalling pathways by activation of FFAR2, as FFAR2 can be coupled both to the stimulatory $G\alpha_q$ or the inhibitory $G\alpha_i$ pathway (Le Poul, et al. 2003). Additionally, SCFAs might affect release of gastric- or colon-specific hormones (Koh et al. 2016), that in turn influence local ghrelin release.

Increasing microbial SCFA production by increasing fibre intake has been linked with decreased plasma ghrelin concentrations in humans and rats (Cani, et al. 2004; Rahat-Rozenbloom, et al. 2017; Tarini and Wolever 2010). ~~,~~ ~~Bwhich, b~~ased on our results, this might be caused by inhibition of colonic octanoyl ghrelin release. Other studies failed to see any effects on plasma ghrelin levels after increased dietary fibre intake (Cluny, et al. 2015; Steensels, et al. 2017). However, future studies should consider measuring luminal, fecal and/or plasma SCFAs and their functional effects at different time points, since differences might only be evident at a certain time of day.

We showed that the circadian clock dictates rhythms in SCFAs and plays a role in SCFA-stimulated ghrelin release~~, as. d~~Deletion of the core clock gene *Bmal1* abolished fluctuations in plasma and caecal SCFA concentrations and abolished the effects of SCFAs on gastric and colonic octanoyl ghrelin release. To investigate whether this was due to the arrhythmic food intake pattern in *Bmal1*$^{-/-}$ mice, we restricted *Bmal1*$^{-/-}$ mice's access to food to the dark phase.

It has been shown that giving access to food only during the dark phase restored rhythms in the microbiota of arrhythmic *Per1/2*$^{-/-}$ mice (Thaiss, et al. 2014). Night-time restricted feeding in *Bmal1*$^{-/-}$ mice restored SCFA fluctuations in the caecum, but did not restore them in the plasma, indicating that BMAL1 might play a role in SCFA transport from the lumen of the gastrointestinal tract to the plasma.

Furthermore, night-time restricted feeding abolished the inhibitory effect of SCFAs on gastric octanoyl ghrelin release observed in *ad libitum* fed *Bmal1*$^{-/-}$ mice at ZT 4, although it did not restore the stimulation observed in WT mice. In contrast, RF of *Bmal1*$^{-/-}$ mice completely restored the inhibitory effect of SCFAs on colonic octanoyl ghrelin release at ZT 4, to the same extent as the inhibition observed in WT mice. ~~. Previous studies have shown that restricted feeding can entrain the timing of food anticipatory locomotor activity even in the absence of the core clock gene *Bmal1* (Mistlberger, et al. 2008; Pendergast and Yamazaki 2018; Pendergast, et al. 2009; Storch and Weitz 2009). Crosby *et al.* showed that entrainment by feeding time is dependent on the correct temporal relationship between hormonal cues (glucocorticoids and insulin and IGF-1) and upregulation of PER2 via activation of insulin and IGF-1 receptors (Crosby, et al. 2019). We found that night-time restricted feeding of *Bmal1*$^{-/-}$ mice did not affect *Per2* mRNA expression in colonic mucosa at ZT 4 compared to *ad libitum* fed *Bmal1*$^{-/-}$ mice. However,~~ Additionally, *Clock* mRNA expression was upregulated, compared to both WT and *Bmal1*$^{-/-}$ mice, indicating that night-time restricted feeding could affect the circadian system present in the colonic mucosa. Further studies should elucidate whether this could explain the restoration of the effect of SCFAs on octanoyl ghrelin release. A limitation of the study is that we did not include a WT RF group. We can therefore not conclude that night-time restricted feeding, normalized the effects in Bmal1$^{-/-}$ mice but only that they counteracted the clock disrupting effects of the absence of Bmal1$^{-/-}$. In addition, tissue was not obtained under constant conditions (i.e. fasting for two 24h cycles, constant darkness) for ethical reasons. Therefore, the true circadian nature of these diurnal findings remains to be determined. However, our results already showed that RF can be used to counteract the

consequences of clock-disruption due to the absence of BMAL1. We cannot make any statements whether this also resulted in a normalization of the effects observed in *Bmal1*$^{-/-}$ mice.

Night-time restricted feeding has been shown to keep clock-disrupted mice healthy, preventing obesity and metabolic syndrome, even when fed a high-fat diet (Chaix, et al. 2019b). Although time-restricted eating (TRE) in humans is less well studied, the first reports indicate that restricting food intake~~, especially to the early time of day,~~ induces beneficial metabolic effects (improved insulin sensitivity, decreased blood pressure, decreased ghrelin and appetite), both in lean and obese individuals (Chaix, et al. 2019a; Ravussin, et al. 2019). Further studies are needed to investigate the potential role of the microbiota and SCFAs in these effects and to elucidate whether time-restricted eating could be used to counteract the deleterious effects of clock disruption by e.g. shift work.

~~Since SCFA fluctuations were restored in the caecal and distal colonic (data not shown) content after night-time restricted feeding, and SCFAs have been shown to affect clock gene expression (Leone et al. 2015; Tahara et al. 2018), it would be interesting to investigate whether the correct timing of the peak of fecal SCFA concentrations is another entrainment cue of feeding time. The correct timing of ghrelin inhibition by SCFAs, but also of their effect on release of insulin, glucagon and other hormones (Canfora, et al. 2015; Pingitore, et al. 2019) might also contribute to their potential role as an entrainment factor of peripheral clocks (Mukherji, et al. 2015a; Mukherji, et al. 2015b; Verhagen, et al. 2011). Future dietary intervention studies where SCFAs or dietary fibre are administered should take into account these possible time-, concentration- and location-dependent effects of SCFAs.~~

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Declaration of Interest

None declared.

Author Contribution

AS, LD, KV, JT and ID conceived and designed the experiments. AS, LD and SS performed the experiments. AS and ID analysed the results and wrote the manuscript. All authors reviewed the manuscript.

Ethical approval

Ethical Committee for Animal Experimentation of the KU Leuven.

References

Avau B, Carbone F, Tack J & Depoortere I 2013 Ghrelin signaling in the gut, its physiological properties, and therapeutic potential. *Neurogastroenterol Motil* **25** 720-732.

Bodosi B, Gardi J, Hajdu I, Szentirmai E, Obal F, Jr. & Krueger JM 2004 Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *Am J Physiol Regul Integr Comp Physiol* **287** R1071-1079.

Callahan HS, Cummings DE, Pepe MS, Breen PA, Matthys CC & Weigle DS 2004 Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans. *J Clin Endocrinol Metab* **89** 1319-1324.

Canfora EE, Jocken JW & Blaak EE 2015 Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat Rev Endocrinol* **11** 577-591.

Cani PD, Dewever C & Delzenne NM 2004 Inulin-type fructans modulate gastrointestinal peptides involved in appetite regulation (glucagon-like peptide-1 and ghrelin) in rats. *Br J Nutr* **92** 521-526.

Chaix A, Manoogian ENC, Melkani GC & Panda S 2019a Time-Restricted Eating to Prevent and Manage Chronic Metabolic Diseases. *Annu Rev Nutr*.

Chaix A, Lin T, Le HD, Chang MW & Panda S 2019b Time-Restricted Feeding Prevents Obesity and Metabolic Syndrome in Mice Lacking a Circadian Clock. *Cell Metab* **29** 303-319.e304.

Cluny NL, Eller LK, Keenan CM, Reimer RA & Sharkey KA 2015 Interactive effects of oligofructose and obesity predisposition on gut hormones and microbiota in diet-induced obese rats. *Obesity (Silver Spring)* **23** 769-778.

Crispim CA, Waterhouse J, Damaso AR, Zimberg IZ, Padilha HG, Oyama LM, Tufik S & de Mello MT 2011 Hormonal appetite control is altered by shift work: a preliminary study. *Metabolism* **60** 1726-1735.

Crosby P, Hamnett R, Putker M, Hoyle NP, Reed M, Karam CJ, Maywood ES, Stangherlin A, Chesham JE, Hayter EA, et al. 2019 Insulin/IGF-1 Drives PERIOD Synthesis to Entrain Circadian Rhythms with Feeding Time. *Cell* **177** 896-909.e820.

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE & Weigle DS 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50** 1714-1719.

den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ & Bakker BM 2013 The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* **54** 2325-2340.

Dibner C 2019 The importance of being rhythmic: Living in harmony with your body clocks. *Acta Physiol (Oxf)* e13281.

Eberle JA, Widmayer P & Breer H 2014 Receptors for short-chain fatty acids in brush cells at the "gastric groove". *Front Physiol* **5** 152.

Ebino KY 1993 Studies on coprophagy in experimental animals. *Jikken Dobutsu* **42** 1-9.

EFSA 2014 EFSA Panel on Food additives and Nutrient Sources added to Food (ANS). Scientific Opinion on the re-evaluation of propionic acid (E 280), sodium propionate (E 281), calcium propionate (E 282) and potassium propionate (E 283) as food additives. *EFSA Journal* **12** 3779.

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, et al. 2013 Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Mol Metab* **2** 376-392.

Foster-Schubert KE, Overduin J, Prudom CE, Liu J, Callahan HS, Gaylinn BD, Thorner MO & Cummings DE 2008 Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates. *J Clin Endocrinol Metab* **93** 1971-1979.

Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE & Hale JE 2008 Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* **105** 6320-6325.

Hass N, Schwarzenbacher K & Breer H 2007 A cluster of gustducin-expressing cells in the mouse stomach associated with two distinct populations of enteroendocrine cells. *Histochem Cell Biol* **128** 457-471.

Hemmeryckx B, Himmelreich U, Hoylaerts MF & Lijnen HR 2011 Impact of clock gene Bmal1 deficiency on nutritionally induced obesity in mice. *Obesity (Silver Spring)* **19** 659-661.

Ho CW, Lazim AM, Fazry S, Zaki U & Lim SJ 2017 Varieties, production, composition and health benefits of vinegars: A review. *Food Chem* **221** 1621-1630.

Iwakura H, Li Y, Ariyasu H, Hosoda H, Kanamoto N, Bando M, Yamada G, Hosoda K, Nakao K, Kangawa K, et al. 2010 Establishment of a novel ghrelin-producing cell line. *Endocrinology* **151** 2940-2945.

Janssen S, Laermans J, Iwakura H, Tack J & Depoortere I 2012 Sensing of fatty acids for octanoylation of ghrelin involves a gustatory G-protein. *PLoS One* **7** e40168.

Janssen S, Laermans J, Verhulst PJ, Thijs T, Tack J & Depoortere I 2011 Bitter taste receptors and alpha-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. *Proc Natl Acad Sci U S A* **108** 2094-2099.

Karlsson B, Knutsson A & Lindahl B 2001 Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occup Environ Med* **58** 747-752.

Koh A, De Vadder F, Kovatcheva-Datchary P & Backhed F 2016 From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* **165** 1332-1345.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656-660.

Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV & Antoch MP 2006 Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* **20** 1868-1873.

Koyama H, Iwakura H, Dote K, Bando M, Hosoda H, Ariyasu H, Kusakabe T, Son C, Hosoda K, Akamizu T, et al. 2016 Comprehensive Profiling of GPCR Expression in Ghrelin-Producing Cells. *Endocrinology* **157** 692-704.

Laermans J & Depoortere I 2016 Chronobesity: role of the circadian system in the obesity epidemic. *Obes Rev* **17** 108-125.

Laermans J, Vancleef L, Tack J & Depoortere I 2015 Role of the clock gene Bmal1 and the gastric ghrelin-secreting cell in the circadian regulation of the ghrelin-GOAT system. *Sci Rep* **5** 16748.

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, et al. 2003 Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem* **278** 25481-25489.

Leone V, Gibbons SM, Martinez K, Hutchison AL, Huang EY, Cham CM, Pierre JF, Heneghan AF, Nadimpalli A, Hubert N, et al. 2015 Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism. *Cell Host Microbe* **17** 681-689.

Lu X, Zhao X, Feng J, Liou AP, Anthony S, Pechhold S, Sun Y, Lu H & Wank S 2012 Postprandial inhibition of gastric ghrelin secretion by long-chain fatty acid through GPR120 in isolated gastric ghrelin cells and mice. *Am J Physiol Gastrointest Liver Physiol* **303** G367-376.

McNabney SM & Henagan TM 2017 Short Chain Fatty Acids in the Colon and Peripheral Tissues: A Focus on Butyrate, Colon Cancer, Obesity and Insulin Resistance. *Nutrients* **9**.

Mistlberger RE, Yamazaki S, Pendergast JS, Landry GJ, Takumi T & Nakamura W 2008 Comment on "Differential rescue of light- and food-entrainable circadian rhythms". *Science* **322** 675; author reply 675.

Mohawk JA, Green CB & Takahashi JS 2012 Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci* **35** 445-462.

Mukherji A, Kobiita A & Chambon P 2015a Shifting the feeding of mice to the rest phase creates metabolic alterations, which, on their own, shift the peripheral circadian clocks by 12 hours. *Proc Natl Acad Sci U S A* **112** E6683-6690.

Mukherji A, Kobiita A, Ye T & Chambon P 2013 Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. *Cell* **153** 812-827.

Mukherji A, Kobiita A, Damara M, Misra N, Meziane H, Champy MF & Chambon P 2015b Shifting eating to the circadian rest phase misaligns the peripheral clocks with the master SCN clock and leads to a metabolic syndrome. *Proc Natl Acad Sci U S A* **112** E6691-6698.

Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, Batterham RL, Benoit SC, Bowers CY, Broglio F, et al. 2015 Ghrelin. *Mol Metab* **4** 437-460.

Nelson W, Tong YL, Lee JK & Halberg F 1979 Methods for cosinor-rhythmometry. *Chronobiologia* **6** 305-323.

512 Olguín-Calderón D, González-Escobar JL, Ríos-Villa R, Dibildox-Alvarado E, De León-
513 Rodríguez A & Barba de la Rosa AP 2019 Modulation of Caecal Microbiome in Obese Mice
514 Associated with Administration of Amaranth or Soybean Protein Isolates. *Polish Journal of*
515 *Food and Nutrition Sciences* **69** 35-44.

516 Padmanabhan P, Grosse J, Asad AB, Radda GK & Golay X 2013 Gastrointestinal transit
517 measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT.
518 *EJNMMI Res* **3** 60.

519 Pan A, Schernhammer ES, Sun Q & Hu FB 2011 Rotating night shift work and risk of type 2
520 diabetes: two prospective cohort studies in women. *PLoS Med* **8** e1001141.

521 Pan X, Chen F, Wu T, Tang H & Zhao Z 2009 Prebiotic oligosaccharides change the
522 concentrations of short-chain fatty acids and the microbial population of mouse bowel*. *J*
523 *Zhejiang Univ Sci B* **10** 258-263.

524 Panda S 2016 Circadian physiology of metabolism. *Science* **354** 1008-1015.

525 Partch CL, Green CB & Takahashi JS 2014 Molecular Architecture of the Mammalian
526 Circadian Clock. *Trends Cell Biol* **24** 90-99.

527 Pendergast JS & Yamazaki S 2018 The Mysterious Food-Entrainable Oscillator: Insights from
528 Mutant and Engineered Mouse Models. *J Biol Rhythms* **33** 458-474.

529 Pendergast JS, Nakamura W, Friday RC, Hatanaka F, Takumi T & Yamazaki S 2009 Robust
530 food anticipatory activity in BMAL1-deficient mice. *PLoS One* **4** e4860.

531 Pingitore A, Gonzalez-Abuin N, Ruz-Maldonado I, Huang GC, Frost G & Persaud SJ 2019
532 Short chain fatty acids stimulate insulin secretion and reduce apoptosis in mouse and human
533 islets in vitro: Role of free fatty acid receptor 2. *Diabetes Obes Metab* **21** 330-339.

534 Rahat-Rozenbloom S, Fernandes J, Cheng J & Wolever TMS 2017 Acute increases in serum
535 colonic short-chain fatty acids elicited by inulin do not increase GLP-1 or PYY responses but
536 may reduce ghrelin in lean and overweight humans. *Eur J Clin Nutr* **71** 953-958.

537 Ravussin E, Beyl RA, Poggiogalle E, Hsia DS & Peterson CM 2019 Early Time-Restricted
538 Feeding Reduces Appetite and Increases Fat Oxidation But Does Not Affect Energy
539 Expenditure in Humans. *Obesity (Silver Spring)* **27** 1244-1254.

Reinke H & Asher G 2019 Crosstalk between metabolism and circadian clocks. *Nat Rev Mol Cell Biol* **20** 227-241.

Schiavo-Cardozo D, Lima MM, Pareja JC & Geloneze B 2013 Appetite-regulating hormones from the upper gut: disrupted control of xenin and ghrelin in night workers. *Clin Endocrinol (Oxf)* **79** 807-811.

Segers A, Desmet L, Thijs T, Verbeke K, Tack J & Depoortere I 2018 The circadian clock regulates the diurnal levels of microbial short-chain fatty acids and their rhythmic effects on colon contractility in mice. *Acta Physiol (Oxf)* e13193.

Smith HW 1965 The development of the flora of the alimentary tract in young animals. *J Pathol Bacteriol* **90** 495-513.

Steensels S & Depoortere I 2018 Chemoreceptors in the Gut. *Annu Rev Physiol* **80** 117-141.

Steensels S, Cools L, Avau B, Vancleef L, Farre R, Verbeke K & Depoortere I 2017 Supplementation of oligofructose, but not sucralose, decreases high-fat diet induced body weight gain in mice independent of gustducin-mediated gut hormone release. *Mol Nutr Food Res* **61**.

Storch KF & Weitz CJ 2009 Daily rhythms of food-anticipatory behavioral activity do not require the known circadian clock. *Proc Natl Acad Sci U S A* **106** 6808-6813.

Tahara Y, Yamazaki M, Sukigara H, Motohashi H, Sasaki H, Miyakawa H, Haraguchi A, Ikeda Y, Fukuda S & Shibata S 2018 Gut Microbiota-Derived Short Chain Fatty Acids Induce Circadian Clock Entrainment in Mouse Peripheral Tissue. *Scientific Reports* **8** 1395.

Tamura M, Hirayama K & Itoh K 1999 Effects of guar gum and cellulose on cecal enzyme activity and cecal short-chain fatty acids in young and aged mice. *Ann Nutr Metab* **43** 60-65.

Tarini J & Wolever TM 2010 The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Appl Physiol Nutr Metab* **35** 9-16.

Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, Abramson L, Katz MN, Korem T, Zmora N, et al. 2014 Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell* **159** 514-529.

568 Vancleef L, Van Den Broeck T, Thijs T, Steensels S, Briand L, Tack J & Depoortere I 2015
569 Chemosensory signalling pathways involved in sensing of amino acids by the ghrelin cell. *Sci*
570 *Rep* **5** 15725.

571 Vancleef L, Thijs T, Baert F, Ceulemans LJ, Canovai E, Wang Q, Steensels S, Segers A, Farre
572 R, Pirenne J, et al. 2018 Obesity Impairs Oligopeptide/Amino Acid-Induced Ghrelin Release
573 and Smooth Muscle Contractions in the Human Proximal Stomach. *Mol Nutr Food Res* **62**.

574 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F
575 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of
576 multiple internal control genes. *Genome Biol* **3** research/034.

577 Verhagen LA, Egecioglu E, Luijendijk MC, Hillebrand JJ, Adan RA & Dickson SL 2011 Acute
578 and chronic suppression of the central ghrelin signaling system reveals a role in food
579 anticipatory activity. *Eur Neuropsychopharmacol* **21** 384-392.

580 Wang Q, Liszt KI, Deloose E, Canovai E, Thijs T, Farre R, Ceulemans LJ, Lannoo M, Tack J
581 & Depoortere I 2019 Obesity alters adrenergic and chemosensory signaling pathways that
582 regulate ghrelin secretion in the human gut. *Faseb j* fj201801661RR.

583 Yang J, Brown MS, Liang G, Grishin NV & Goldstein JL 2008 Identification of the
584 acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* **132**
585 387-396.

586 Yildiz BO, Suchard MA, Wong ML, McCann SM & Licinio J 2004 Alterations in the dynamics
587 of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc Natl Acad Sci U S A* **101**
588 10434-10439.

589

Figure legends

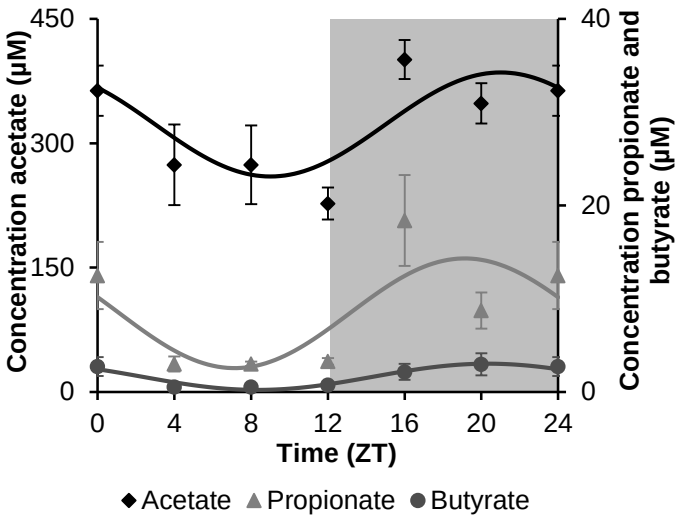
Figure 1: Diurnal fluctuations in plasma, caecal and stomach SCFA concentrations. (a-b) Diurnal fluctuations in SCFA concentrations in plasma (a) and in the luminal content of the caecum (b) of C57BL/6J mice. The fitted cosine curves determined by cosinor analysis are shown. Light and dark phases are shaded in white and grey respectively (N = 7-8 mice/time point). (c) Acrophase comparison of SCFA concentrations in plasma and in the luminal content of caecum and distal colon(Segers et al. 2018) of C57BL/6J mice. (d) SCFA concentrations in the luminal content of the stomach of WT mice ($N_{ZT\,4}$ = 5 mice, N_{ZT16} = 8 mice).

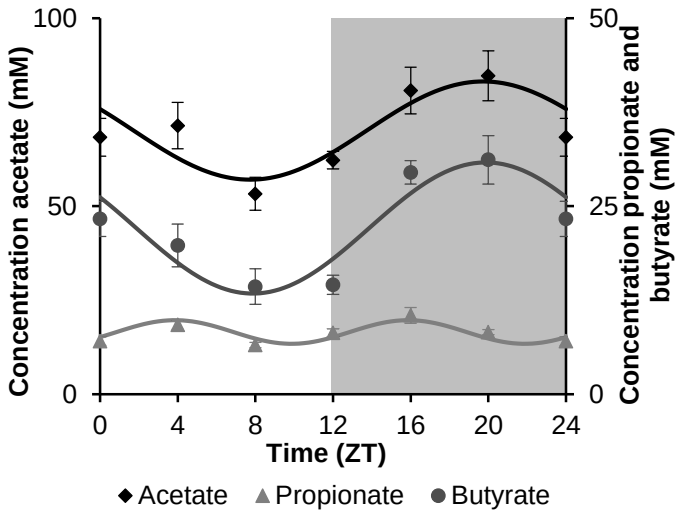
Figure 2: Diurnal fluctuations in SCFA-stimulated octanoyl ghrelin release. (a) Octanoyl ghrelin release of MGN3-1 cells after 1-hour stimulation with SCFA mixes mimicking plasma (360 µM), stomach (16 mM) and caecal (124 mM) SCFA concentrations. *** = $P < 0.001$ compared to Krebs (N = 3-7 independent experiments, n = 24-35 wells/stimulus). (b-c) Octanoyl ghrelin release of full-thickness strips from the stomach (b) and proximal colon (c) after a 2-hour stimulation with Krebs or a SCFA mix mimicking plasma (360 µM) and caecal (124 mM) concentrations at ZT 4 and 16. **: $P < 0.01$ compared to Krebs. $$: $P < 0.01$, interaction effect (treatment*ZT) (N = 8 mice/time point, $n_{stomach}$ = 14-31 strips/stimulus/time point, n_{colon} = 8 strips/stimulus/time point).

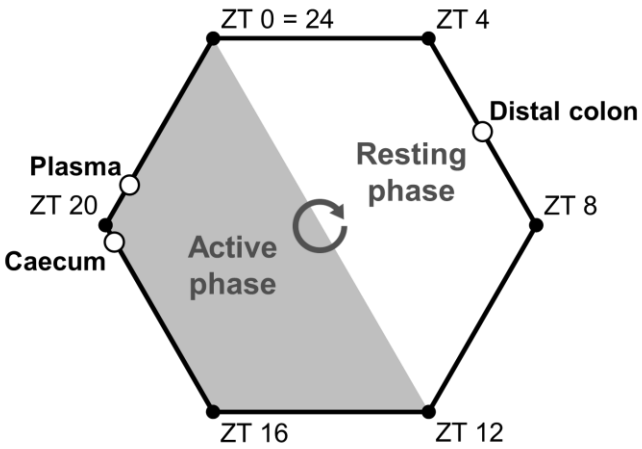
Figure 3: Effect of the clock gene *Bmal1* and of night-time restricted feeding of *Bmal1*$^{-/-}$ mice (*Bmal1*$^{-/-}$ RF) on diurnal fluctuations in SCFA concentrations and of SCFA-stimulated octanoyl ghrelin release. (a-b) SCFA concentrations in plasma (a) and luminal content of the caecum (b) of WT, *Bmal1*$^{-/-}$ and *Bmal1*$^{-/-}$ RF mice at ZT 4 and 16. *: $P < 0.05$, **: $P < 0.01$ compared to ZT 4 (N = 8-10 mice/group/time point). (c-d) Octanoyl ghrelin release of full-thickness strips from the stomach (c) and proximal colon (d) of WT, *Bmal1*$^{-/-}$ and *Bmal1*$^{-/-}$ RF mice after a 2-hour stimulation with Krebs or SCFAs (124 mM) at ZT 4. *: $P < 0.05$, **: $P < 0.01$ compared to Krebs. $: $P < 0.05$, $$: $P < 0.01$, interaction effect (treatment*group) (N = 7-10 mice/group, $n_{stomach}$ = 14-38 strips/stimulus/group, n_{colon} = 6-8 strip/stimulus/group).

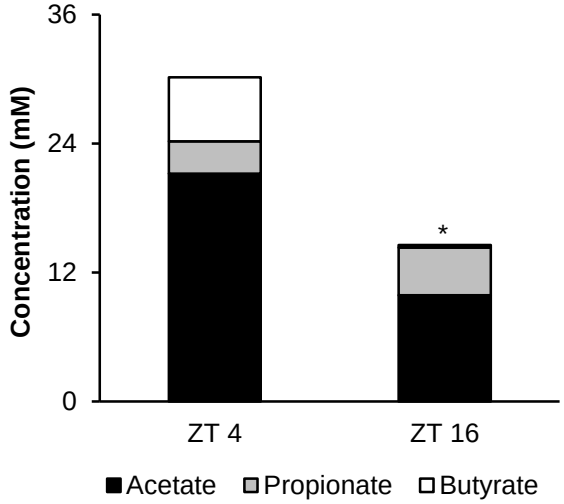
Figure 4: Effect of night-time restricted feeding of *Bmal1*$^{-/-}$ mice (*Bmal1*$^{-/-}$ RF) on clock gene expression in colonic mucosa. *Clock* (a), *Per2* (b) and *Reverbα* (c) mRNA expression in the mucosa

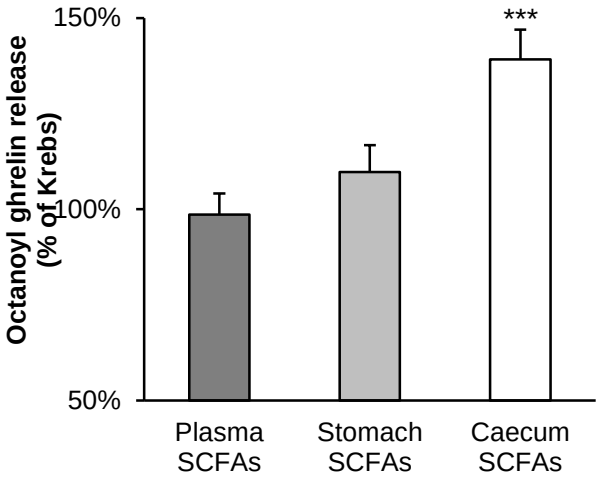
618 from the proximal colon of WT, *Bmal1*$^{-/-}$ and *Bmal1*$^{-/-}$ RF mice at ZT 4. ***: $P < 0.001$ (N = 6-10
619 mice/group).

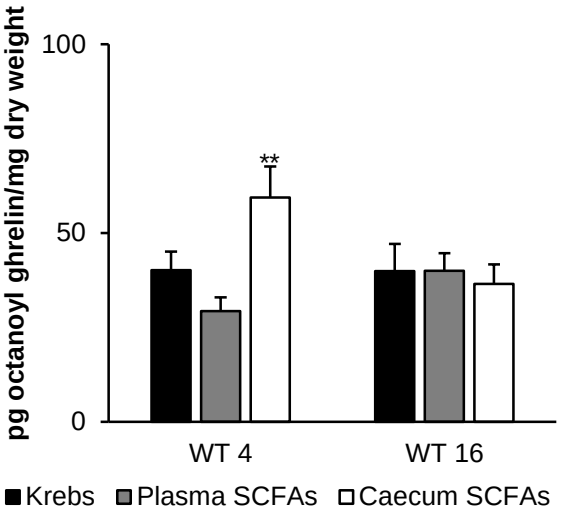

a
Concentration acetate (μM)
Concentration propionate and butyrate (μM)
450
300
150
0
40
20
0
0 4 8 12 16 20 24
Time (ZT)
Acetate Propionate Butyrate

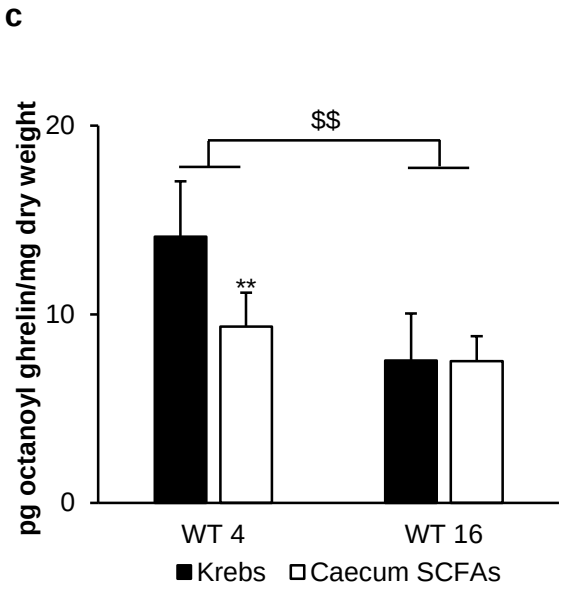

b
Concentration acetate (mM)
Concentration propionate and butyrate (mM)
100
50
0
50
25
0
0 4 8 12 16 20 24
Time (ZT)
Acetate Propionate Butyrate

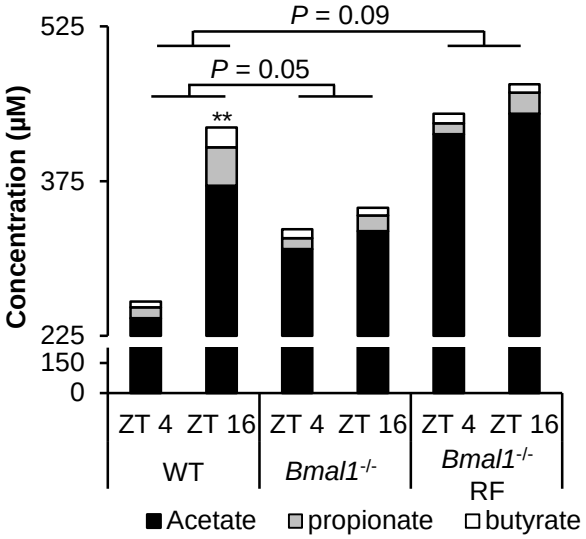

c
ZT 0 = 24
ZT 4
Distal colon
Plasma
ZT 20
Caecum
Resting phase
Active phase
ZT 8
ZT 16
ZT 12

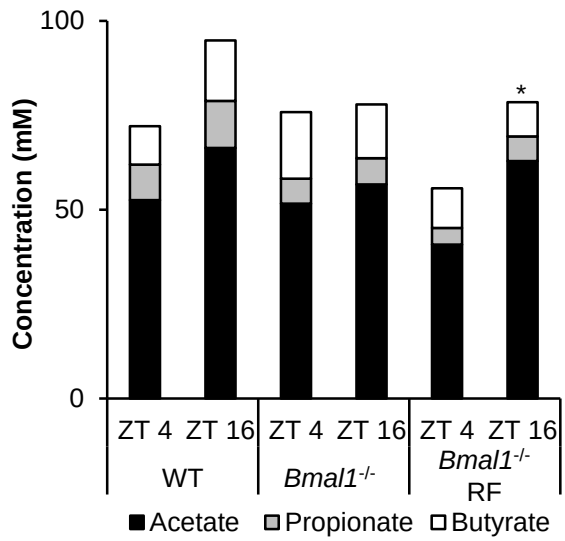

d
Concentration (mM)
36
24
12
0
*
ZT 4
ZT 16
Acetate Propionate Butyrate

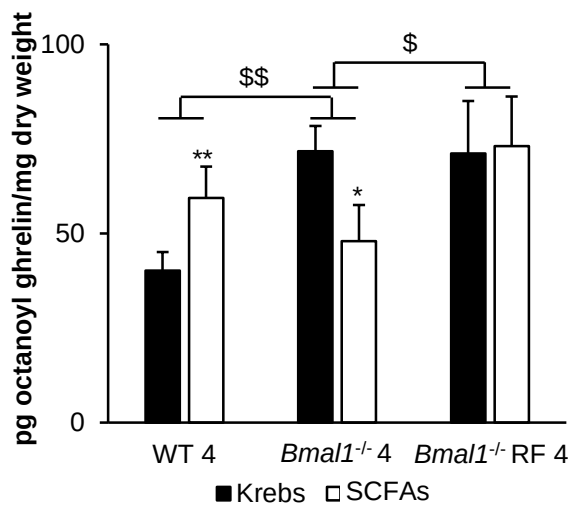

a
Octanoyl ghrelin release (% of Krebs)
150%
100%
50%
***
Plasma SCFAs
Stomach SCFAs
Caecum SCFAs

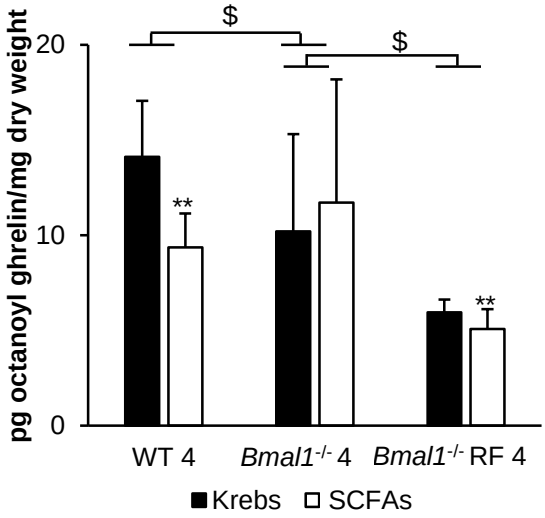

b
pg octanoyl ghrelin/mg dry weight
100
50
0
**
WT 4
WT 16
Krebs
Plasma SCFAs
Caecum SCFAs

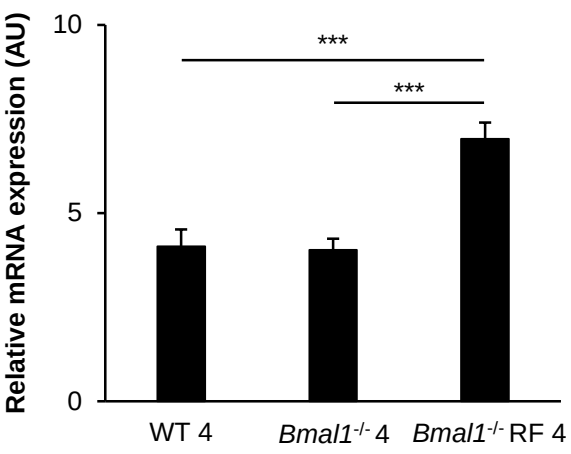

c
pg octanoyl ghrelin/mg dry weight
20
10
0
$$
**
WT 4
WT 16
Krebs
Caecum SCFAs

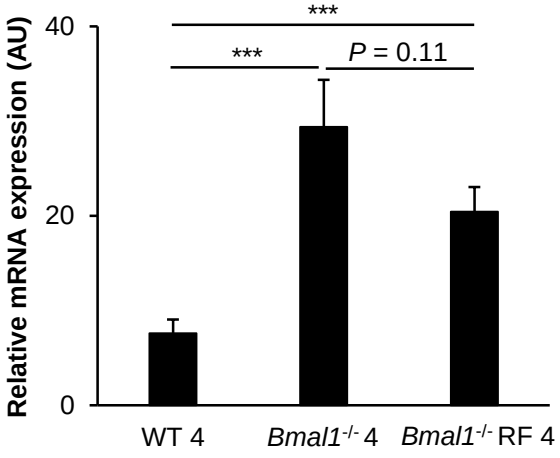

a
P = 0.09
P = 0.05
**
Concentration (μM)
525
375
225
150
0
ZT 4 ZT 16 ZT 4 ZT 16 ZT 4 ZT 16
WT
Bmal1-/-
Bmal1-/- RF
Acetate
propionate
butyrate


b
*
Concentration (mM)
100
50
0
ZT 4 ZT 16 ZT 4 ZT 16 ZT 4 ZT 16
WT
Bmal1-/-
Bmal1-/- RF
Acetate
Propionate
Butyrate


c
$$
$
**
*
pg octanoyl ghrelin/mg dry weight
100
50
0
WT 4
Bmal1-/- 4
Bmal1-/- RF 4
Krebs
SCFAs


d
$
$
**
**
pg octanoyl ghrelin/mg dry weight
20
10
0
WT 4
Bmal1-/- 4
Bmal1-/- RF 4
Krebs
SCFAs


a
Relative mRNA expression (AU)
10
5
0
***
***
WT 4
Bmal1-/- 4
Bmal1-/- RF 4


b
Relative mRNA expression (AU)
40
20
0
***
***
P = 0.11
WT 4
Bmal1-/- 4
Bmal1-/- RF 4

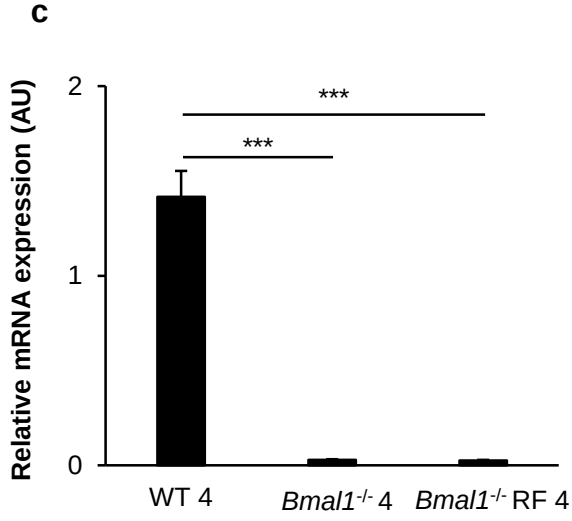

c
Relative mRNA expression (AU)
2
1
0
***
***
WT 4
Bmal1-/- 4
Bmal1-/- RF 4

Table 1. Primer sequences

Gene	Forward primer	Reverse primer
β-actin	GATCTGGCACCACACCTTCTAC	TGGATGGCTACGTACATGGCTG
Clock	TCTACAGAAGAGCATTGATTTTTTGC	TCATTACTAAGGAATGTGGGTTTCC
Hmbs	CTGAAGGATGTGCCTACCATAC	AAGGTTTCCAGGGTCTTTCC
Per2	GATGACAGAGGCAGAGCACAAC	TTTGTGTGCGTCAGCTTTGG
Ppib	GGAGATGGCACAGGAGGAAA	CCCGTAGTGCTTCAGCTTGAA
Reverbα	CCCTGGACTCCAATAACAACACA	GCCATTGGAGCTGTCACTGTAG

Answer to Reviewers' Comments to Author

Reviewer: 1

1. Tissues were sampled at two time points, ZT4 and ZT16, in adlib fed and nocturnal food restricted WT and bmal1 KO mice. The 2-week nocturnal food restriction schedule restored day-night differences in caecal SCFAs, and the authors note that this could be clock gene mediated, given that expression of *Clock* was increased by the feeding schedule. My question is whether the mice were fed at ZT12 prior to sacrifice at ZT16. To establish that a rhythm is 'clock-controlled' (a true rhythm), as opposed to reflecting time since the last feeding (an 'hourglass' process), it is necessary to establish that the rhythm persists in 'constant conditions' (in this case, fasting) for at least two 24h cycles. So, the best protocol would have been to fast the mice after the last nocturnal feeding, and sample at ZT4, ZT16 and again ZT4 (and better still, also a second ZT16 time point). The details of the feeding schedule prior to tissue sampling should be added to the methods section, and the discussion should include note on the limitations of the data for interpreting the restoration of time of day fluctuations by nocturnal restricted feeding.

Mice were fed at ZT 12 prior to sacrifice at ZT 16. We agree with the reviewer that in order to determine whether a rhythm is truly clock-controlled or circadian, this rhythm needs to be present under constant conditions, i.e. fasting. However, as Bmal1$^{-/-}$ mice show a severe phenotype (symptoms include early aging and body weight loss at a later age), we did not get ethical permission to fast these animals for more than 24 hours. However, to our knowledge there are no studies investigating the effect of night-time restricted feeding of Bmal1$^{-/-}$ mice on plasma and caecal SCFAs levels and in their effect on ghrelin release. Therefore, in our study we aimed to determine whether diurnal (thus 24-hour, not necessarily clock-controlled) rhythms were present under non-constant conditions. This is why we also did not use the term circadian but diurnal throughout our manuscript. Future studies using constant conditions in different clock-disruption models, with a less severe phenotype, could investigate whether the rhythms are truly clock-controlled.

Additional details have been added to the methods section, line 132:

"On the day of sacrifice, Bmal1$^{-/-}$ RF mice were sacrificed at ZT 4 (fasted from ZT 0 - 4) and 16 (fed from ZT 12 - 16)."

The following section has been added to the discussion noting the limitations of our study (line 380):

" A limitation of the study is that we did not include a WT RF group. We can therefore not conclude that night-time restricted feeding, normalized the effects in Bmal1-/- mice but only that they counteracted the clock disrupting effects of the absence of Bmal1-/-. In addition, tissue was not obtained under constant conditions (i.e. fasting for two 24h cycles, constant darkness) for ethical reasons. Therefore, the true circadian nature of these diurnal findings remains to be determined. However, our results already showed that RF can be used to counteract the consequences of clock-disruption due to the absence of BMAL1. We cannot make any statements whether this also resulted in a normalization of the effects observed in Bmal1-/- mice."

2. My second question concerns the ability of bmal1 KO mice to adapt to the 12h nocturnal restricted feeding schedule. Were the KO mice able to maintain body weight, or was this also a caloric restriction schedule? If there was caloric restriction, could observed effects (or lack of effects) of the feeding schedule be related to caloric restriction rather than meal timing?

We have included a supplemental figure showing food intake (measured daily) and body weight (measured every three days) of Bmal1$^{-/-}$ RF mice to the manuscript (Figure S1 A-B). As mentioned in the methods section mice on the nocturnal feeding schedule were fed ad libitum, thus no caloric restriction. Bmal1$^{-/-}$ RF mice adapted quickly to the restricted feeding schedule and as such did not lose weight during night-time restricted feeding. At sacrifice, Bmal1$^{-/-}$ RF even weighed more than ad libitum fed Bmal1$^{-/-}$ mice (although they still weighed less than WT mice; Figure S1 C).

Reviewer: 2

-Why was there not an RF WT group? This seems an important control group within the study. Mice typically eat ~30% of their food in the day, and therefore the night-restricted feeding will impact on gut physiology, microbiome activity and SCFA production in WT mice.

We agree with the reviewer that addition of a WT RF group to our study would have been interesting to compare whether night-time restricted feeding could normalize the effects in Bmal1$^{-/-}$ mice. Our main focus was to investigate whether RF can be used to counteract the consequences of clock-disruption due to the absence of BMAL1. Therefore, ad libitum fed Bmal1$^{-/-}$ mice were only compared with night-time restricted fed Bmal1$^{-/-}$ mice. We have added the following section to the discussion noting the limitations of our study (line 380):

"A limitation of the study is that we did not include a WT RF group. We can therefore not conclude that night-time restricted feeding, normalized the effects in Bmal1$^{-/-}$ mice but only that they counteracted the clock disrupting effects of the absence of Bmal1$^{-/-}$. In addition, tissue was not obtained under constant conditions (i.e. fasting for two 24h cycles, constant darkness) for ethical reasons. Therefore, the true circadian nature of these diurnal findings remains to be determined. However, our results already showed that RF can be used to counteract the consequences of clock-disruption due to the absence of BMAL1. We cannot make any statements whether this also resulted in a normalization of the effects observed in Bmal1$^{-/-}$ mice."

-What is the reason for the increased serum SCFA in the RF Bmal1-/- mice at ZT 4? This may be linked to an interesting difference in microbiota, and/or gut permeability etc in these mice.

This is indeed an interesting and unexpected finding that could be explained by differences in microbiota or gut permeability, which could be explored in future studies. However, measuring microbiota composition and gut permeability were beyond the scope of our study.

-The difference in ghrelin release from the colon in the Bmal1-/-RF4 (fig 3d) does not look convincing despite having a reported p value<0.01.

For these experiments, the colon was cut lengthwise, creating two segments. In three mice, the left segment was incubated with Krebs and the right segment with SCFAs, and vice versa in the

other three mice. In each mouse, an inhibition by SCFAs was observed. Our mixed model analysis showed a P-value of 0.0075 based on paired data of these six mice.

-Basal ghrelin release from the Bmal1-/- stomach is higher than WT (krebs responses in fig3c). Is this reflected in circulating levels of the hormone in vivo? Unfortunately, this basal increase complicates interpretation of the results (related to SCFA-induced ghrelin release between the three test groups). A RF-WT condition would have helped here.

*As basal gastric ghrelin release was indeed different between the groups, we looked at interaction effects (treatment*group) between the different groups to decide whether the effect of SCFAs differed between groups. Based on previous results from our group, this basal increase is not reflected in the circulating ghrelin levels. Bmal1$^{-/-}$ mice lose the rhythm in plasma octanoyl ghrelin levels, leading to lower plasma ghrelin levels during the day (Laermans et al., Scientific Reports 2015). This might be caused by, among others, decreased ghrelin release from the lower GI tract.*

-I don't really see the point of clock gene assessment in Fig 4. Without a larger time-course, this data does little beyond confirming the genotype of the mice (by absent REVERBα expression).

To make definitive conclusions about the effects on the local circadian clock, the expression of the different clock genes would indeed need to be measured at several time points over the course of 24 hours. However, our analysis at one time point already shows that clock gene expression (Clock) was affected by RF. While it's impossible to identify the cause of this change, e.g. due to an overall increase, a shift of the rhythm or due to other adaptations, we believe it is still relevant to show that there is a change. This indicates that the local clock machinery is affected, and might give future RF studies an incentive to study these local clock genes using several time points over a 24-hour cycle.

-Bmal1-/- mice have a complex and age-related health deterioration. The authors should comment about the general condition of the animals at the experimental age used. Also, some comment on food intake and body weight would be appropriate.

We sacrificed animals at age 12 – 16 weeks, as previous studies reported that Bmal1$^{-/-}$ mice start to show growth retardation at age 16 – 18 weeks (Kondratov, et al. 2006). We have included a supplemental figure showing food intake (measured daily) and body weight (measured every three days) of Bmal1$^{-/-}$ RF mice to the manuscript (Figure S1 A-B). Bmal1$^{-/-}$ RF mice adapted quickly to the restricted feeding schedule and did not lose weight during the period of night-time

restricted feeding. At sacrifice, Bmal1$^{-/-}$ RF weighed more than ad libitum fed Bmal1$^{-/-}$ mice (although they still weighed less than WT mice; Figure S1 C). This might indicate that RF Bmal1$^{-/-}$ mice are healthier than their ad libitum counterparts. However, since we had to control the light-dark schedule of these animals we could not confirm this hypothesis using metabolic cages.

Additional details have been added to the results section, line 253:

"A group of Bmal1$^{-/-}$ mice was only fed during the dark phase for two weeks (Bmal1$^{-/-}$ RF). Food intake was measured daily and body weight was measured every three days. Food intake and body weight were stable during night-time restricted feeding (Figure S1 A-B). Body weight at sacrifice (ZT 4) was higher in Bmal1$^{-/-}$ RF compared to ad libitum fed Bmal1$^{-/-}$ mice (Figure S1 C). both ad libitum and RF fed Bmal1$^{-/-}$ mice weighed less than WT mice at sacrifice."

-Under a 12:12 LD cycle, the Bmal1-/- mice will still show rhythmic behavior (due to light masking of activity). Do the authors know the relative light/dark phase food intake in their mice under ad libitum feeding conditions? A previous paper was cited (laermans et al 2015), but this data should have been confirmed in your mice and lab/lighting conditions.

As we could not perform these experiments in metabolic cages due to the necessity to control the light-dark cycle, all food intake measurements needed to be done manually. As such, we decided not to measure food intake of the ad libitum fed animals and cited the previous study from our group (Laermans et al., Scientific Reports 2015). We used mice from the same colony, same age and in the same lab and lighting conditions (the same ventilated cabinet with controlled light-dark cycle).

-Statistical methods – if the data was non-normal or non-homogeneous as suggested, then it is not really appropriate to show mean+/-sem.

All data analysis was performed on log-transformed data. However, for ease of interpretation of the graphs, all data was back-transformed. We have clarified this in the statistics section line 196: Results are presented as back-transformed mean ± SEM.

-Line 251-255. This is too strong of a conclusion based on the results. Firstly, the lack of RF-WT limits interpretation of the impact of feeding time, and secondly, the basal effect of Bmal1 deletion (increased release of ghrelin from the stomach (c) and decreased release from colon(d)) make it difficult to interpret the SCFA-induced release.

*For the ghrelin release studies, we determined the interaction effect (treatment (i.e. Krebs vs. SCAs) * group) and did not compare absolute values of SCFA-stimulated ghrelin release strips between different groups, thereby accounting for the differences in basal ghrelin release.*

We have weakened the conclusion. We have changed: "Finally, we showed that diurnal fluctuations in caecal SCFA concentrations and the effect of SCFAs on colonic octanoyl ghrelin release were regulated by feeding time, independent of the core clock gene BMAL1."

to the following: "Finally, we showed that night-time restricted feeding restored diurnal fluctuations in caecal SCFA concentrations and the effect of SCFAs on colonic octanoyl ghrelin release in Bmal1$^{-/-}$ mice."

-The discussion is overly long for the scope of the data. Writing could be improved (e.g. increase the use of commas, and shorter sentence structure to increase clarity)

The discussion has been corrected for long sentences to improve readability and several paragraphs have been shortened or removed.

-Define short chain fatty acids (SCFA) in the abstract (currently this does not happen until line 79).
Lines 25/109 – add "time" after zeitgeber. Also ZT is not an abbreviation for zeitgeber (lines 67, 68) it refers to zeitgeber time. There is a subtle but important difference.

We have defined short chain fatty acids (SCFAs) in the abstract and have adjusted Zeitgeber to Zeitgeber time (line 26, 123) and ZT to Zeitgeber (line 73), according to the correct definitions. We have adjusted the definition for ZT from "Zeitgeber" to "Zeitgeber time".

-Abstract methods section has an odd level of detail/description. For example, "Stomach and colonic full-thickness strips were incubated with Krebs or a SCFA mix mimicking plasma (360 μM) or caecal concentrations (124 mM) and octanoyl ghrelin release was measured by radioimmunoassay" The concentrations etc are less important here than getting across that this to assess SCFA driven release of ghrelin.

We have clarified the methods section of the abstract (line 27):

"To investigate the effect of SCFAs on ghrelin release, stomach and colonic full-thickness strips were incubated with Krebs or a SCFA mix mimicking plasma or caecal concentrations after which octanoyl ghrelin release was measured by radioimmunoassay."

-Line 67-69. The authors need to be careful with the clarity and meaning of there statements. For example, the quality and quantity of food are not zeitgebers, and 'western diet' per se does not cause internal misalignment.

These oversimplifications have been removed, line 73-75 now state:

"However, peripheral circadian rhythms are also regulated by local entrainment signals or Zeitgebers, such as the timing of meals (Panda 2016). Desynchronizing these Zeitgebers by e.g. rotating shift work or frequent flying misaligns master and peripheral clocks, causing chronodisruption (Dibner 2019)."

-Lines 91-94. could use more detail.

We have added the following sentence (line 94): "Therefore, the effects of SCFAs on ghrelin release might be more pronounced in the colon."

-Line 95-96. SCFAs are not diurnal, but they follow a diurnal rhythm. (as in line 97)

We have adjusted the incorrect formulation of "diurnal SCFAs" to diurnal rhythm or diurnal fluctuations (line 106, 109 and 643):

"…regulate diurnal levels of fecal SCFAs…" changed to "…regulate the diurnal rhythm in the levels of fecal SCFAs…"

"…plasma and caecal SCFAs levels are diurnal…" changed to "…plasma and caecal SCFAs levels show a diurnal rhythm…"

"…Diurnal SCFA concentrations…" changed to "…Diurnal fluctuations in SCFA concentrations…"

-Lines 272-276. Does coprophagic behaviour increase during night-restricted feeding, and if so how might this have affected results?

We have not found any data about coprophagic behaviour in night-time-restricted fed mice, but it seems plausible that an imposed fasting period could increase coprophagic behaviour when food is absent. This might restore diurnal rhythms in SCFA concentrations in the stomach, which in turn could contribute to the changes observed in SCFA-stimulated ghrelin release.