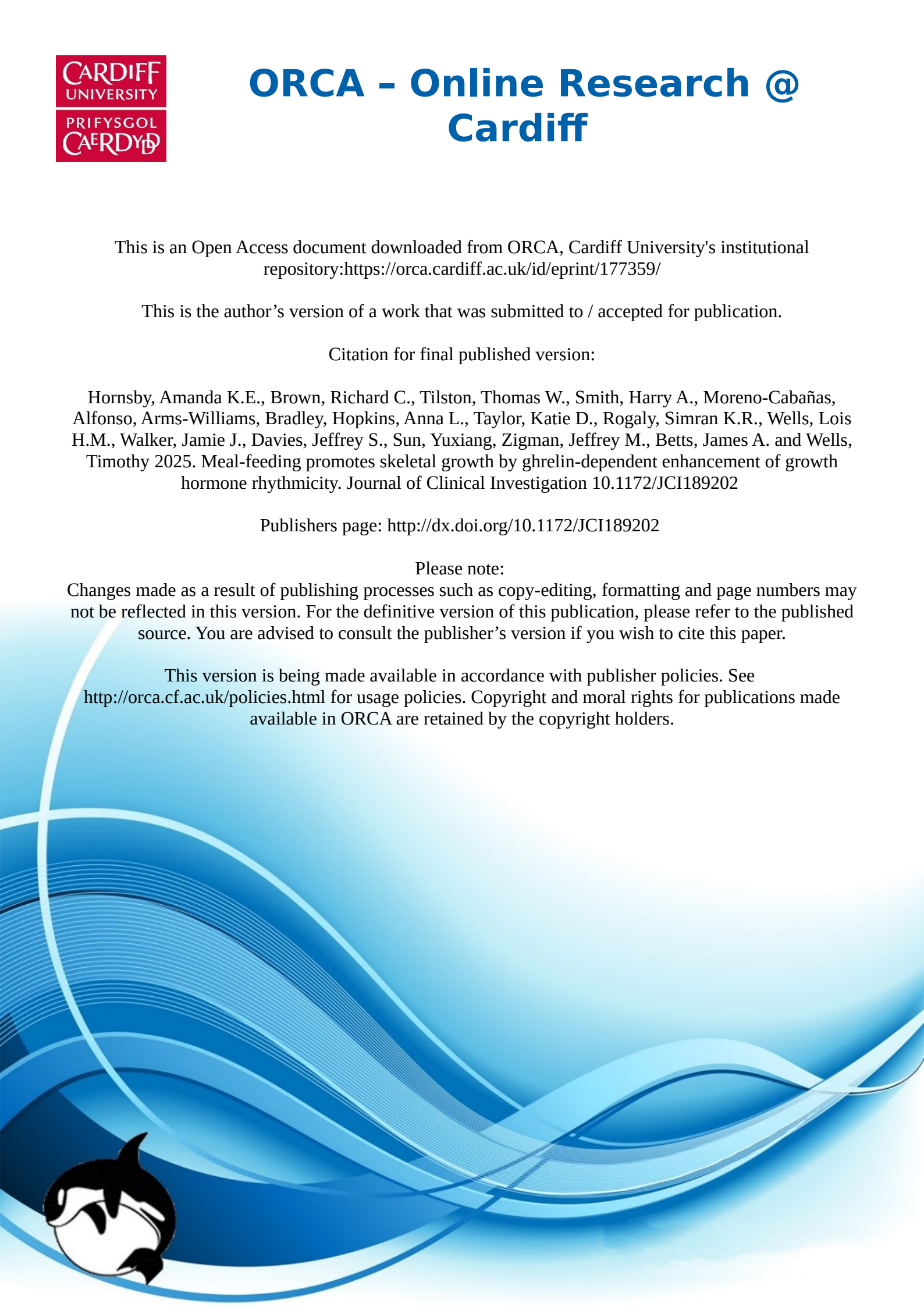


JCI The Journal of Clinical Investigation

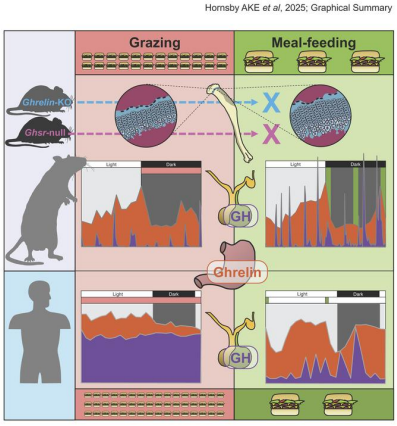
Meal-feeding promotes skeletal growth by ghrelin-dependent enhancement of growth hormone rhythmicity

Amanda K.E. Hornsby, … , James A. Betts, Timothy Wells

J Clin Invest. 2025. https://doi.org/10.1172/JCI189202.

Research In-Press Preview **Endocrinology** **Metabolism**

Graphical abstract



Meal-feeding promotes skeletal growth by ghrelin-dependent enhancement of growth hormone rhythmicity

Amanda KE Hornsby[1,2], Richard C Brown[1], Thomas W Tilston[1], Harry A Smith [3], Alfonso Moreno-Cabañas [3], Bradley Arms-Williams[1], Anna L Hopkins[1], Katie D Taylor[1], Simran KR Rogaly[1], Lois HM Wells[1], Jamie J Walker[4], Jeffrey S Davies[2], Yuxiang Sun[5], Jeffrey M Zigman[6], James A Betts[3] and Timothy Wells[1*]

[1]School of Biosciences, Cardiff University, Museum Avenue, Cardiff, CF10 3AX, United Kingdom

[2]Institute of Life Sciences, School of Medicine, Swansea University, Swansea, SA2 8PP, United Kingdom

[3]Centre for Nutrition, Exercise & Metabolism, Department for Health, University of Bath, BA2 7AY, United Kingdom

[4]Department of Mathematics and Statistics, University of Exeter, North Park Road, Exeter, EX4 4QF, United Kingdom

[5]Department of Nutrition, Texas A&M University, College Station, Texas, TX 77843, USA

[6]UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

Running head: Feeding patterns and growth

***Author for correspondence:**

Dr Timothy Wells

School of Biosciences, Cardiff University, Cardiff. CF10 3US, UK

Tel: 0044 (0)2920 874977

E-mail: wellst@cardiff.ac.uk

Abstract

The physiological impact of ultradian temporal feeding patterns remains a major unanswered question in nutritional science. We have employed automated and nasogastric feeding to address this question in male rodents and human volunteers. While grazing and meal-feeding reduced food intake in parallel (compared to *ad libitum*-fed rodents), body length and tibial epiphysial plate width were maintained in meal-fed rodents via the action of ghrelin and its receptor, GHS-R. Grazing and meal-feeding initially suppressed elevated pre-prandial ghrelin levels in rats, followed by either a sustained elevation in ghrelin in grazing rats or pre-prandial ghrelin surges in meal-fed rats. Episodic growth hormone (GH) secretion was largely unaffected in grazing rats, but meal-feeding tripled GH secretion, with burst height augmented and two additional bursts of GH per day. Continuous nasogastric infusion of enteral feed in humans failed to suppress circulating ghrelin, producing continuously elevated circulating GH with minimal rhythmicity. In contrast, bolus enteral infusion elicited post-prandial ghrelin troughs accompanied by reduced circulating GH, with enhanced ultradian rhythmicity.

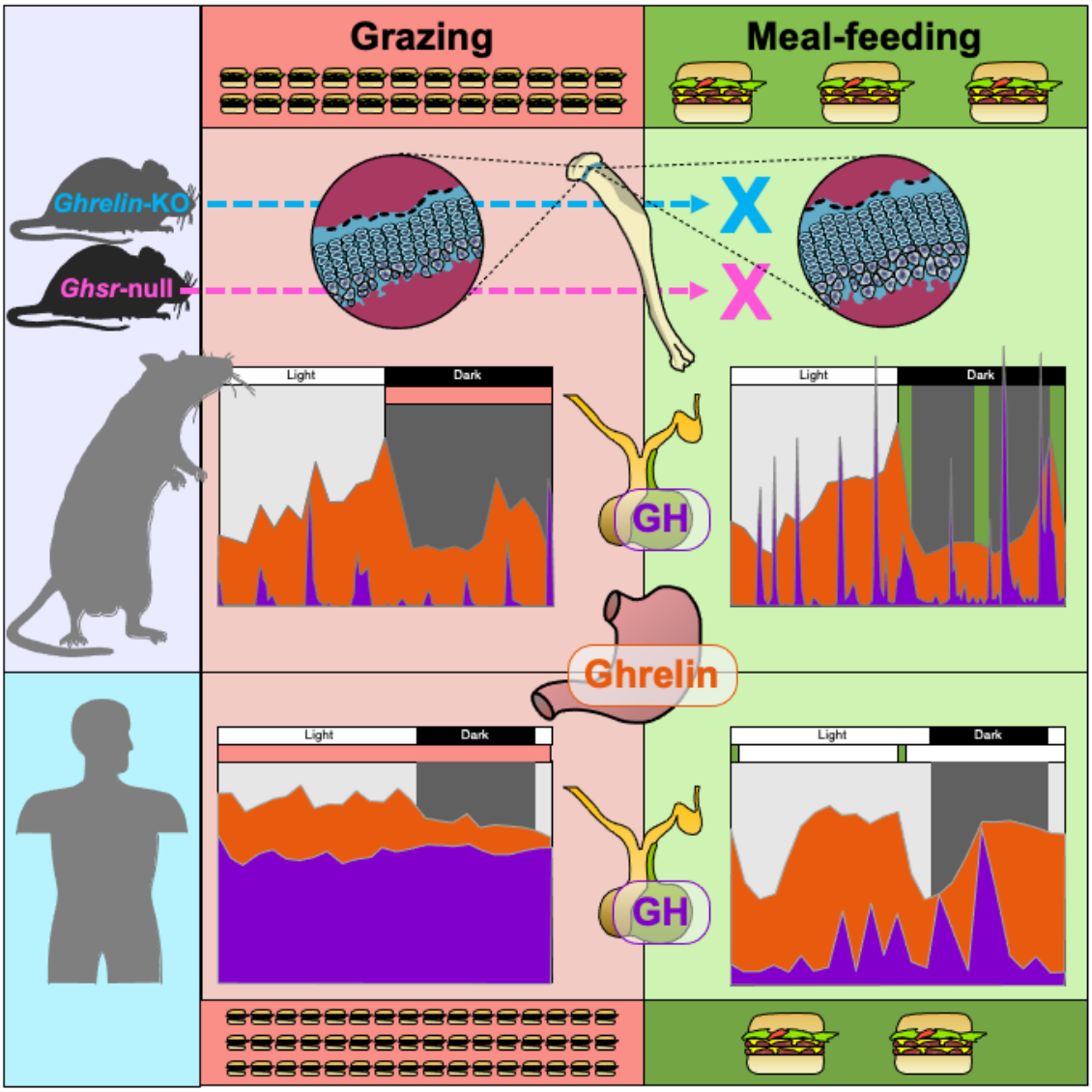
Taken together, our data imply that the contemporary shift from regular meals to snacking behaviour may be detrimental to optimal skeletal growth outcomes by sustaining circulating GH at levels associated with undernourishment and diminishing GH pulsatility.

Keywords:

Feeding pattern, enteral feeding, grazing, meal-feeding, ghrelin, growth hormone, skeletal growth

Graphical Summary

Hornsby AKE *et al*, 2025; Graphical Summary



Introduction

It is well established that circadian feeding patterns influence a wide range of physiological outcomes, but while epidemiological evidence is emerging for a potential impact of more frequent, ultradian feeding rhythms (1, 2), this aspect of chrononutrition has not been systematically explored (3, 4).

Epidemiological studies suggest associations between ultradian feeding patterns and multiple physiological variables, such as food choice (5), energy intake (6) and metabolic outcome (7, 8). Not everyone agrees (9), and this controversy arises in part from the well-recognised inaccuracy of self-reported food intake (10, 11) and the potential distortion of participant attrition (12). While the development of mobile Apps may improve reporting accuracy (13, 14), compelling evidence will only emerge from laboratory-based studies in which patterned food intake is fully controlled.

Despite their predominantly nocturnal feeding behaviour, a number of approaches have been developed to study contemporary human feeding patterns in rodents (15), including the ClockLab (16), BioDAQ (17) and SnackClock (18) systems, which deliver pelleted food in user-defined patterns. However, these systems are unable to deliver the smoothed access required to study snacking/grazing behaviour and are compromised by the propensity of laboratory rodents, especially female mice, to hoard food in the home cage (19, 20), thereby thwarting researcher-imposed control.

We have taken two approaches to overcome these limitations. Firstly, we have utilised a modified CLAMS-based system to deliver crushed diet in consistent, reproducible temporal patterns in rats and mice (21), the combined use of automated serial blood sampling in rats enabling us to characterise the impact of these patterns on spontaneous hormone rhythmicity.

Secondly, we have complemented this approach in human volunteers with patterned nasogastric delivery of enteral feed coupled with serial blood sampling (22).

We report here the impact of two specific feeding patterns, nocturnal grazing and nocturnal meal-feeding on skeletal growth in male rats and mice, including those with a null allele for ghrelin (23) or transcriptional blockade of the growth hormone secretagogue receptor (GHSR; 24), the cognate receptor for ghrelin. We also report the effect of these feeding patterns on the temporal secretion of ghrelin and growth hormone (GH) in rats alongside the impact of continuous or bolus infusions of enteral feed on ghrelin and GH in men.

Results

Study 1: Meal-feeding protects skeletal growth in male rats

To characterise the impact of temporal feeding patterns, male rats received standard chow in a grazing (permitted access to set small amounts every 30 mins during the dark phase (18:00-06:00h)) or meal-feeding (three 1-h periods of *ad libitum* access at the beginning (18:00h) middle (23:30h) and end (05:00h) of the dark phase) pattern for 6 weeks. Both patterns reduced cumulative caloric intake by 20% compared to *ad libitum*-fed rats (Figure 1A; P=0.0001 and 0.0002 respectively). Since caloric intake did not differ between grazing and meal-fed rats at any timepoint (Day 42 cumulative caloric intake: 3560±346 kcal (grazing); 3460±75 kcal (meal-fed); P>0.999), differences in physiological endpoints result from these patterns of feeding.

Body weight gain was reduced by 16% and 12% (*vs ad libitum*-fed rats) in grazing (P=0.0014) and meal-fed (P=0.0236) rats respectively (Figure 1B) but was not different between grazing and meal-fed cohorts. Body length was reduced by 3% in grazing rats (P=0.0108 *vs ad libitum*-fed rats), but not in meal-fed animals (Figure 1C). Tibial lengths showed a similar pattern, with mean length in grazing rats being 97% of that in *ad libitum*-fed animals, but this was not significantly different (Figure 1D; P=0.1129). However, tibial epiphyseal plate width (EPW; an accurate index of skeletal growth rate) was reduced by 17% in grazing rats (Figure 1H; P=0.0001 *vs ad libitum*-fed), whereas EPW in meal-fed rats was unaffected (P=0.3299 vs *ad-libitum*-fed; P=0.0064 vs grazing). This reduction in EPW in grazing rats was largely due to 18% and 17% reductions in proliferative (Figure 1J; P=0.0010) and hypertrophic zone widths (Figure 1K; P=0.0034), germinal zone width being unaffected (Figure 1I).

Study 2: Grazing reduces the rate of chondrocyte migration

To determine whether these changes in skeletal growth are reflected in chondrocyte migration, three cohorts of rats were treated as in study 1, with BrdU given to “birth-date” dividing cells 5 days prior to 3 weeks of grazing or meal-feeding. Caloric intake and weight gain paralleled those seen after 3 weeks in study 1 (Supplementary Table 1). While tibial length was not significantly affected after 3 weeks of patterned feeding, tibial EPW was reduced in grazing rats by 18% (*P*=0.003 vs *ad libitum*-fed), and this was reflected in 20% and 18% reductions in the width of the proliferative and hypertrophic zones (*P*=0.010; *P*=0.041; Supplementary Table 1). Longitudinal growth rate (distance from the proximal surface of the germinal zone to the first BrdU+ve nucleus/days since BrdU injection (Figure 1, L-N)) was reduced by 16% in grazing rats (Figure 1O; *P*=0.0099 *vs ad libitum*-fed). Neither total EPW, zone widths, nor longitudinal growth rate were affected in meal-fed rats (Supplementary Table 1; Figure 1O).

Study 3: The growth effects of meal-feeding and grazing are reversed in ghrelin-KO mice

Given that ghrelin secretion is regulated by feeding events (25, 26, 27) and its biological activity is pattern-dependent (28, 29), we investigated the role of ghrelin in these feeding pattern-induced changes in *ghrelin*-KO mice. Since our CLAMS system is designed for rats, we were constrained to use larger (6-month old) mice in this study and provided a more generous grazing allowance (see methods). In this context, grazing mice consumed 30% more calories than meal-fed mice (*P*=0.0004; Figure 2A), the latter consuming 14% fewer calories than *ad libitum*-fed mice (*P*=0.032; Figure 2A). These effects were abolished in *ghrelin*-KO mice, due largely to the increased consumption in the meal-fed animals (Figure 2B). As expected for adult mice, these feeding patterns had little effect on weight gain (Figure 2, C and D), body length, or any of the organ weights measured (Supplementary Table 2). Nevertheless, despite the less organised appearance of epiphyseal plates in older animals (Figure 2, E-J) and the increased caloric intake in grazing WT mice, tibial EPW was still reduced by 8% (*P*=0.030; Figure 2K), with EPW not significantly affected in meal-fed mice. Remarkably, this response was not only abolished in

ghrelin-KO mice, but was reversed, grazing *ghrelin*-KO mice showing no reduction in EPW (*P*=0.909), and EPW in meal-fed *ghrelin*-KO being 12% (*P*=0.012) and 16% (*P*=0.002) lower than that in *ad libitum*-fed and grazing mice respectively (Figure 2K). Although mean plasma IGF-1 in grazing WT males was only 70% of that in *ad libitum*-fed mice, mean IGF-1 values were not significantly different (Supplementary Table 2).

Among the technical challenges occurring during this study (discussed in reference 21), the ability of mice to stand on the food hopper, theoretically permitted grazing animals to consume up to their own body weight in each feeding episode before anything is registered by the system. To overcome this potential drawback and permit the study of younger mice, the diameter of the food access aperture was reduced (Supplementary Figure 1).

Study 4: The growth effects of meal-feeding and grazing are abolished in GHSR-null mice

Deletion of *ghrelin* removes ghrelin, des-acyl ghrelin and obestatin, while leaving the action of liver-enriched antimicrobial peptide 2 (LEAP2) intact. To delineate the role of this system further, we characterised the impact of these feeding patterns in juvenile mice in which transcription of the receptor for ghrelin, GHSR, is blocked. With a more tightly controlled grazing allowance, younger WT mice showed only transient hyperphagia (Figure 3A), with no impact on weight gain (Figure 3C). In contrast, meal-fed younger mice displayed a transient reduction in caloric intake on days 1-3 (*vs ad libitum*-fed mice) (Figure 3A), with body weight gain only significantly reduced on days 2-4 (Figure 3C). These effects on caloric intake and weight gain were largely replicated in GHSR-null animals (Figure 3, B and D), with the exception that final cumulative caloric intake was reduced by 15% in meal-fed GHSR-null mice (*P*=0.0036 *vs ad libitum*-fed; *P*=0.057 *vs* grazing). Neither feeding pattern affected body length, tibial length or organ size (Supplementary Table 3). However, although skeletal growth rate was unaffected in grazing WT males (Figure 3, F and K), meal feeding elevated tibial EPW by 14% (*P*=0.0166 *vs ad libitum*-fed, *P*=0.0009 vs

grazing; Figure 3, G and K), mean proliferative zone width and mean hypertrophic zone width in meal-fed WT mice being 116% (*P*=0.1051; Figure 3M) and 117% (*P*=0.2281; Figure 3N) of that in *ad libitum*-fed mice. These growth rate effects were entirely abolished in the absence of GHSR (Figure 3, H-N). Although not significantly different, the profile of IGF-1 concentrations was broadly similar to the growth rate (Supplementary Table 3).

Study 5: Meal-feeding and grazing produce different circulating ghrelin profiles

Since these growth-promoting effects of grazing and meal-feeding are ghrelin/GHSR-dependent, we characterised the impact of these feeding patterns on the dynamics of ghrelin secretion in chronically-catheterised pattern-fed rats. Catheterisation did not alter the impact of these feeding patterns on caloric intake, which remained similar to that observed in study 1 (cumulative caloric intake reduced by 15% (*P*=0.0001) and 12% (*P*=0.0008) in grazing and meal-fed rats respectively; Supplementary Table 4).

Although total ghrelin secretion (AUC) in grazing and meal-fed rats was 150% of that in *ad libitum*-fed animals (Figure 4G), these means were not significantly different (*P*=0.238 and *P*=0.246 *vs ad libitum*-fed respectively). Mean peak ghrelin levels in grazing and meal-fed rats were 153% and 148% of that in *ad libitum*-fed rats (Figure 4J; *P*=0.101 and *P*=0.154 respectively), with neither baseline (Figure 4H) nor median (Figure 4I) secretion being significantly different. Circulating ghrelin in *ad libitum*-fed rats showed the expected circadian rhythm (30), with peak concentration occurring at 11:00h (Figure 4D), immediately prior to the first major spontaneous feeding event (Figure 4A). Thereafter, circulating ghrelin declined, reaching a nadir at 24:00/00:00h (Figure 4D). In contrast, plasma ghrelin concentrations increased across the light phase in grazing (Figure 4E) and meal-fed (Figure 4F) rats prior to the commencement of nocturnal feeding (Figure 4, B and C). Although commencement of feeding produced a precipitous decline in circulating ghrelin in grazing and meal-fed rats (Figure 4, E and F), ghrelin

levels remained higher in grazing rats at 19:00h (Figure 4E). Despite constant food intake throughout the dark phase (Figure 4B), grazing was accompanied by a sustained doubling in mean circulating ghrelin in the second half of the dark phase (from 02:00-05:00h; Figure 4E; *P*=0.09 vs *ad libitum*-fed). Although meal-fed rats failed to display a pre-prandial rise in ghrelin before the second (midnight) meal, a trebling of circulating ghrelin occurred between 01:00h and 05:00h prior to the end-dark phase meal, declining sharply on the commencement of feeding (Figure 4, F and C). Thus, while grazing failed to maintain suppressed circulating ghrelin, meal feeding produced a rapid reduction in ghrelin secretion.

Study 6: Meal feeding enhances growth hormone (GH) pulsatility in rats

Given that ghrelin promotes GH secretion in a pattern-dependent manner (28), we characterised GH secretory dynamics in grazing and meal-fed rats. *Ad libitum*-fed animals showed an episodic GH secretion characteristic of male rats (31, 32), with 8-9 bursts of GH occurring in each 24h period, separated by troughs in which GH was virtually undetectable (Figure 5, D, G and J; Supplementary Figure 2A). These bursts of GH secretion were unsynchronised between individual animals (Figure 5, D and G). Despite showing a similar reduction in cumulative caloric intake to that reported in study 1 and 2 (Figure 5B; Supplementary Table 5), grazing had no impact on total (Figure 6A) or baseline (OC_5; Figure 6B) GH secretion, or the parameters of secretory dynamics (Figure 6, C-N), but induced inter-animal burst synchronisation (Figure 5, E and H; Supplementary Figure 2B). In contrast, despite inducing the same reduction in caloric intake (Figure 5C; Supplementary Table 5), meal feeding almost tripled total GH secretion (Figure 5, F, I and L; Supplementary Figure 2C; Figure 6A; *P*=0.013 *vs ad libitum*-fed, *P*=0.047 *vs* grazing), inducing a degree of synchronisation (Figure 5F) without significantly influencing baseline secretion (Figure 6B). Fourier analysis revealed that while the dominant period in all three feeding patterns remained in the 150-200 min range (7.2-9.6 bursts per day; Figure 5, J-N), meal-feeding was accompanied by the presence of numerous peaks in the higher frequency

range (Figure 6L), without influencing the dominant period or frequency significantly (Figure 6, M and N). A simple "burst" metric revealed that meal-feeding elicited two additional secretory bursts per day (Figure 6C; *P*=0.0057 *vs ad libitum*-fed, *P*=0.0006 *vs* grazing), which coincided with the second pre-prandial ghrelin surge in the second half of the dark phase (Figure 6G). A tripling of mean burst height (Figure 6D; *P*=0.0054 *vs ad libitum*-fed, 0.0176 *vs* grazing), was most prominent in the second half of the light phase and first half of the dark phase (Figure 6H). Given that mean burst duration in meal-fed rats was 79% of that in *ad-libitum*-fed animals (Figure 6E; *P*=0.1868 *vs ad libitum*-fed), burst mass was not significantly increased (data not shown; *P*=0.3496 vs *ad libitum*-fed). Thus, meal-feeding in rats was accompanied by an increase in the frequency and magnitude of spontaneous GH secretory bursts.

Study 7: Meal feeding enhances ghrelin and GH pulsatility in humans

To determine whether these feeding pattern-induced changes in ghrelin and GH dynamics are replicated in humans, healthy male volunteers received enteral liquid formula through a naso-gastric tube in either two 30 min bolus infusions (at 08:00h and 20:00h; Figure 7B) or an equicaloric continuous infusion for 24 h (Figure 7A). Analysis of hourly blood samples revealed that bolus-infused volunteers displayed a 4hr suppression of circulating ghrelin after each infusion (Figure 7C). In contrast, circulating ghrelin remained at pre-prandial levels in continuously-infused volunteers (Figure 7C). In addition, continuous nasogastric infusion produced consistently high circulating hGH (Figure 7D). Since the lower sampling frequency did not permit rigorous pulse analysis, normalising the values to the 24-h profile mean for each individual revealed that continuously-infused volunteers displayed minimal ultradian rhythmicity (Figure 7E). In contrast, a post-prandial fall in hGH in bolus-infused volunteers (60% lower after the first bolus than in continuously-infused participants; *P*<0.05; Figure 7D) was followed by the emergence of marked individual ultradian rhythmicity in all six individuals (Figure 7F). This was especially prominent following the second bolus infusion.

Thus, while grazing was insufficient to maintain post-prandial suppression of ghrelin secretion and was accompanied by elevated hGH exposure, meal-feeding induced intermittent ghrelin exposure and enhanced hGH pulsatility.

Discussion

Direct mechanistic evidence that ultradian feeding patterns influence physiological outcomes has been lacking. To address this deficit, we have exploited the flexibility and reliability of the CLAMS system to determine the impact of grazing and "three meals a night" on the endocrine regulation of growth in laboratory rodents. When combined with our evidence of parallel acute responses in humans, our study presents the first direct evidence that temporal feeding patterns regulate indices of hormone secretory dynamics to influence developmental endpoints.

It is clear from of our rodent studies that grazing slowed the rate of longitudinal growth in the tibial epiphyseal plate. We initially assumed from study 1 that this was due to the noticeable reduction in caloric intake, but when this phenomenon was repeated in older mice in the context of maintained, or even partially elevated food intake (study 3), it was clear that nutritional restriction was not the underlying cause. However, the abolition of the grazing-induced reduction in growth rate in *ghrelin*-KO mice clearly implies a contribution for this gastric hormone, or potentially one of its co-products. Our analysis of ghrelin profiles indicates that nocturnal grazing magnified the amplitude of the daily ghrelin rhythm seen in *ad libitum*-fed rats, with the addition of a large anticipatory surge (27) before the commencement of dark phase feeding. This pattern of ghrelin exposure was insufficient to reduce total GH output in rats or alter the indices of GH burst dynamics that determine its biological effectiveness (28, 29). The observed alignment of the GH bursts between individual rats was intriguing and deserves comment. The commencement of the light phase is a powerful entraining signal for the GH axis (33), but drift in individual periodicity enables progressive misalignment between individuals. The large daily pre-prandial ghrelin surge immediately prior to the commencement of the dark phase (i.e. in 3-hourly phase with the lights-on entrainment) acts as an additional entraining signal at the obverse side of the light-dark cycle to reinforce GH burst alignment.

It remains unclear at present how these changes in ghrelin secretion could influence skeletal growth in the absence of altered GH secretion. One possibility is a direct action of ghrelin in the growth plate. It has been reported that ghrelin (34), GHSR (35, 36) and the activating enzyme ghrelin O-acyl transferase (37, 38) are expressed in chondrocytes, especially in the proliferative and hypertrophic zones where the effects of grazing are most prominent (Figure 1), but whether expression of these components is modified by feeding patterns remains to be determined. A potential paracrine or autocrine stimulation of chondrocyte GHSR is supported by the 7% reduction in body weight observed in *ghrelin*-KO mice (P=0.0003; data not shown) at the start of the study.

Our human study indicates an additional mechanism. Slow continuous nasogastric infusion of enteral feed for 24 h failed to suppress circulating ghrelin, which remained at pre-prandial levels throughout the feeding period. In the short term, this was accompanied by a sustained elevation in circulating hGH. The difference between this result and our rat study is likely to reflect the period of feeding (24 h infusion in human *vs* 12 h grazing in rats) and the shorter duration of the human study. Indeed, we have previously shown in rats that a week-long continuous infusion ghrelin or a GHSR agonist reduces skeletal growth (29) by suppressing GH secretion (28). Thus, the sustained starvation signal that is represented by continuously elevated ghrelin is most likely to result in reduced GH secretion and impaired growth outcomes in the long term, even in the context of maintained nutrient supply.

In contrast to grazing, nocturnal meal feeding defends skeletal growth in the context of caloric restriction, even accelerating growth rate in younger mice. To see this effect reflected in measurable changes in tibial length is likely to require longer studies, but the lack of meal-induced growth rate enhancement in the absence of GHSR expression and the reversal of the effect in *ghrelin*-KO mice clearly imply a role for the acylated form of ghrelin. At first glance,

however, there appears little difference in circulating ghrelin profiles between grazing and meal-fed rats, overall, median and peak secretion being entirely comparable. This serves to emphasize the importance of timing in eliciting the observed effects, meal-fed rats displaying transient pre-prandial peaks before the first and third meals. Our evidence that twice-daily bolus nasogastric infusion of enteral feed elicited matched suppressions of ghrelin secretion in humans not only concurs with early evidence of pre-prandial surges of ghrelin in humans (26), but confirms that meal-feeding results in intermittent ghrelin exposure.

While we cannot exclude the possibility of a direct action of ghrelin in the growth plate, the enlargement of the proliferative and hypertrophic zones and the increased chondrocyte migration rate imply augmentation of GH-IGF-1 axis activity. Thus, the trebling of GH secretion in rats, resulting from a combination of doubled pulse height and increased burst frequency, appears the most likely mechanism. We have reported a similar impact on GH pulse height and skeletal growth in response to intermittent intravenous infusion of ghrelin (28, 29), but the change in burst frequency is more unusual. While meal-fed rodents are subjected to the same triggering influences of the dark/light interface and the large pre-dark phase surge in ghrelin as grazing animals, they also receive two additional cues, the meals commencing at 23:30h and 05:00h, the latter with an accompanying pre-prandial ghrelin surge. Since these are not separated by multiples of 3 hours, but by multiples of 2.75 hours, this appears to have a “concertinaring” effect, shortening the refractory period between individual GH bursts, thereby permitting two additional bursts per day. Spontaneous bursts of GH secretion in male rodents are thought to occur when peaks of growth hormone-releasing hormone (GHRH) secretion coincide with a trough in somatostatin secretion (39). The lack of a shift in the period of the peak frequency in the Fourier profiles (Figure 6), suggests that the mechanism giving rise to this dominant frequency is largely unaffected by these feeding patterns. However, the emergence of additional bursts in meal-fed

rats suggests additional somatostatin troughs, especially in the dark phase, while the elevation is burst height is most likely due to larger GHRH bursts.

At first glance, these findings do not appear to be replicated in our human data, acute bolus nasogastric infusions being accompanied by lower overall circulating GH compared to “grazing” humans. However, the growth-promoting action of GH is not determined solely by the level of exposure or total exposure time, pulsed infusions of GH being more effective in promoting growth in rats (40) and elevating bone formation markers in humans (41). In this context the emergence of pulsatile GH secretion in all six bolus-infused volunteers is significant, and corroborates evidence that prominent GH pulsatility emerges in male volunteers after midnight (42). Taken together, our data indicate that meal-feeding augments GH pulsaility, increasing the number of GH bursts in rats into the optimal range for promoting axial growth.

Our data have a number of important implications. From a narrow perspective, our human study indicates that in addition to content and total delivery rate (43), the physiological effectiveness of enteral feeding is determined by the impact of the delivery pattern on hormone profiles. Secondly, while our study has focused on the impact of feeding patterns on the growth axis, it is clear that the impact of ghrelin and GH on a wide range of physiological endpoints, including the regulation of fat mass, insulin sensitivity, epigenetic mechanisms and drug metabolism is pattern-dependent (44, 45, 46) and therefore potentially susceptible to changes in feeding pattern. Indeed, it is possible that the effect of manipulating feeding patterns to enhance GH pulsatility in females will be more dramatic. Taken together, our data imply that the contemporary shift from regular meals to snacking behaviour (47, 48) may be detrimental to optimal skeletal growth outcomes, particularly in the context of undernourishment.

Acknowledgements

The authors gratefully acknowledge the financial support of the Bill & Melinda Gates Foundation (Grant: OPP1061040), the Rosetrees Trust (Grant: A2248), the Waterloo Foundation (Grants: 1403/3689, 1403/3758, 1403/4120) and Cardiff University's School of Biosciences Equipment Fund, Research Contingency Fund and Neuroscience and Mental Health Research Institute Seedcorn Fund and Future Minds Programme. Enteral formula for the human feeding study was supplied by Nestlé Health Sciences. J.J.W acknowledges financial support from the Medical Research Council (Grants MR/N008936/1 and MR/T032480/1).

Author contributions:

Conceptualization, J.A.B., T.W.; Methodology, J.A.B., T.W.; Validation, T.W.; Formal Analysis, A.K.E.H., H.A.S., A.M-C., L.H.M.W., J.J.W. and T.W.; Investigation, A.K.E.H., R.D.B., T.W.T., B.A.-W., A.L.H., K.D.T., S.K.R.R., L.H.M.W. and T.W.; Resources, Y.S. and J.M.Z.; Writing Original Draft, T.W.; Review and Editing, A.K.E.H., H.A.S., J.A.B., J.S.D., Y.S. and T.W.; Visualization, T.W.; Supervision, J.A.B., J.S.D. and T.W.; Project Administration, T.W.; Funding Acquisition, J.S.D. and T.W.

Declaration of interests:

J.A.B. is an investigator on research grants funded by BBSRC, MRC, NIHR, British Heart Foundation, Rare Disease Foundation, EU Hydration Institute, GlaxoSmithKline, Nestlé, Lucozade Ribena Suntory, ARLA foods, Cosun Nutrition Center, American Academy of Sleep Medicine Foundation and Salus Optima (L3M Technologies Ltd); has completed paid consultancy for PepsiCo, Kellogg's, SVGC and Salus Optima (L3M Technologies Ltd); is Company Director of Metabolic Solutions Ltd; receives an annual honorarium as a member of the academic advisory board for the International Olympic Committee Diploma in Sports Nutrition; and receives an

annual stipend as Editor-in Chief of International Journal of Sport Nutrition & Exercise Metabolism. The remaining authors have no competing interests to declare.

The authors have not used generative AI or AI-assisted technologies in the preparation of this paper.

Materials and Methods

Sex as a biological variable

Our study examined the effects in male rodents and humans because the GH secretory profile is more amenable to the quantification of changes in the variables of pulsatility. It is unclear whether the findings we report will be applicable in females.

Animals

Male Sprague-Dawley rats (Studies 1, 2, 5 & 6) were purchased from Charles River (Margate, UK) and housed upon receipt as described below. Male WT mice (C57/Bl6J) and their homozygous *ghrelin*-KO (Study 3) and GHSR-null (loxTB-GHSR ; Study 4) littermates were obtained from heterozygous x heterozygous matings of breeding stock derived from embryos (*ghrelin*-KO) or mice (GHSR-null) imported from the vivaria at Baylor College of Medicine (Houston, TX, USA) and the University of Texas Southwestern (Dallas, TX, USA) respectively. Genotype identification for was performed by PCR analysis of DNA extracted from ear punches, as previously described (23, 24).

All experimental animals were individually housed in the metabolic room of the BIOSV animal facility, Cardiff University, under conditions of 12h light/12h dark (lights on at 06:00h), with water available *ad libitum* and diet supplied in one of three patterns as previously described (21) and summarised briefly below:

a. Nocturnal grazing: Grazing animals were permitted to eat one 24th of the mean total daily food consumption of a concurrent cohort of three age-matched *ad-libitum*-fed control animals every 30 min during the dark phase, the first access period coinciding with lights out (18:00h).

This allowance increased in parallel with the daily food intake of the growing *ad libitum*-fed control animals. Thus, grazing rats were denied large meals.

b. Nocturnal meal-feeding: Meal-fed animals were permitted three 1-hour periods of *ad libitum* dietary access at the beginning (18:00h) middle (23:30h) and end (05:00h) of the dark phase, the access lid remaining closed at all other times. Thus, meal-fed animals were not permitted to graze between meals.

c. Ad libitum-feeding*:* In order to calculate the food intake allowance for grazing animals, cohorts of age- and weight-matched animals were housed in either standard transparent cages (Rats; Cat # 2154, Tecniplast UK Ltd, Kettering, Northamptonshire, UK) or metabolic cages (mice; Cat # 3700M061; Tecniplast UK Ltd) and permitted *ad libitum* access to the same crushed diet (see dietary information below). Food consumption was quantified daily between 09:00-10:00h. The effectiveness of this approach and a more detailed description of procedural considerations have been published previously (21).

Human volunteers

Sixteen healthy male volunteers (Study 7; Supplementary Table 6) were recruited via local advertisement. General health and validated chronotype questionnaires were used to screen participants and assess habitual sleep patterns and diurnal preferences (49, 50, 51).

Study 1: Meal-feeding protects skeletal growth in male rats

Three groups of 4-week old male Sprague-Dawley rats (weighing 83.8 - 118.8g) were fed a standard non-purified rodent chow (SRC; SDS RM3; Special Diet Services Ltd, UK; containing 4.2% crude fat (AFE 13.9% fat); 22.4% crude protein; 4.2% crude fibre; 7.6% crude ash (see Ref

21 for full dietary components)) in either *ad libitum*, grazing or meal-fed patterns for 6 weeks. Food intake and body weight were quantified daily. After weighing on day 42, each rat was anaesthetized with isoflurane, nose-anus length measured, and decapitated. Right tibiae were dissected, and length measured with a hand-held micrometer. Tibiae were fixed in buffered formal saline for 48hrs at 4°C and decalcified in 0.5M EDTA (pH7.6) for >3 weeks, before being stored in 70% ethanol at 4°C for subsequent quantification of epiphyseal plate width (EPW). Note: Two animals were omitted from the *ad libitum*-fed group, one showing cumulative food intake >2x standard deviations (SD) from the mean and one showing body weight gain >2x standard deviations from the mean.

Study 2: Grazing reduces the rate of chondrocyte migration

Three groups of 4-week old male Sprague-Dawley rats (weighing 81.3-127.5g) received BrdU (1mg/kg; i.p.) on three consecutive days and were fed with SRC *ad libitum*. After five days they continued to receive SRC in either *ad libitum*, grazing or meal-fed patterns for 3 weeks. At the end of this period, rats were anaesthetized (Dolethal (200mg/kg, i.p.); Vetoquinol UK Ltd, Towcester, UK) and killed by transcardial perfusion-fixation. Tibiae were excised, the length measured and processed as above for quantification of total EPW and zonal widths and the migration of BrdU+ cells by immunohistochemistry (IHC; see below). Note: one animal was omitted from the grazing and meal-fed groups as >2 variables differed from the mean by >2x SD, and another from the meal-fed group because tibial EP was sheared.

Study 3: The growth effects of meal-feeding and grazing are reversed in ghrelin-KO mice

Three groups of 6-month old male ghrelin-KO mice (BW: 28.4-33.9g; 30.9±0.5g (n=18)) and male WT littermates (BW: 29.4-37.4g; 33.1±0.5g (n=18); $P<0.01$) were permitted to consume SRC in either *ad libitum*, grazing or meal-fed patterns. Older mice were used because they were big enough to be housed in the unmodified rat CLAMS system cages. Meal feeding was applied as

above, but grazing mice were permitted to consume 0.5g (approximately 11% of total *ad libitum* food intake) every 30 mins during the dark phase throughout the study. After 3 weeks of exposure to these dietary patterns, during which body weight and daily food consumption were monitored daily, mice were anaesthetized with isoflurane and killed by decapitation. Plasma separated from trunk blood samples was stored at -80°C prior to quantification of circulating IGF-1, with pituitary, liver, kidney and adrenal glands dissected and weighed. Tibiae were collected as in study 1.

Study 4: The growth effects of meal-feeding and grazing are abolished in GHSR-null mice

Three groups of 6-week old male GHSR-null mice (BW: 18.18-20.72g; 18.56±0.28g (n=23)) and three groups of male WT littermates (BW: 12.99-21.59g; 19.25±0.38g (n=24); P=0.047) were permitted to consume SRC in either *ad libitum*, grazing or meal-fed patterns. Meal feeding was applied as above, but grazing mice were permitted to consume 0.2g every 30 mins during the dark phase throughout the study. After 3 weeks mice were anaesthetized with Dolethal (as above) and killed by decapitation, with tissues collected as in study 3.

Study 5: Meal-feeding and grazing produce different circulating ghrelin profiles

Three groups of 4-week old male SD rats were fed with SRC in either *ad libitum*, grazing and meal-fed patterns for 3 weeks. On day 18, rats were anaesthetized with isoflurane and prepared with a single-bore right jugular vein catheter, as previously described (21, 33). After recovery from surgery, catheters were connected to an automated blood sampling system and patency maintained by an hourly flushing protocol in which blood was drawn to the top of the catheter and returned to the rat with the infusion of a 20µl bolus of sterile heparinized saline (10IU/ml). After a further 48hrs, automated serial blood sampling was commenced, in which 100µl of 1:2 blood (50µl blood in 50µl heparinized saline) was collected every hour for 24 hrs, beginning at 06:00h. Blood samples were collected on a refrigerated fraction collector bed, vortexed, and centrifuged

at 4000rpm for 5 mins, before 100µl of 1:2 plasma was removed and stored at -20°C for subsequent determination of circulating ghrelin by radioimmunoassay (RIA; see below). On day 21, rats were re-anaesthetised, nose-anus length measured and decapitated, with tissues being collected as above.

Study 6: Meal feeding enhances GH pulsatility in rats

Three groups of 4-week old male SD rats were fed with SRC in either *ad libitum*, grazing and meal-fed patterns for 3 weeks. On day 18 rats were prepared with a single bore right jugular vein catheter and patency maintained as above. After 48hrs, automated serial blood sampling was commenced, in which 100µl of 1:5 blood (20µl blood in 80µl heparinized saline) was collected every 10 mins for 24 hrs, beginning at 06:00h. Blood samples were collected on a refrigerated fraction collector bed, vortexed, and centrifuged at 4000rpm for 5 mins, before 20µl of 1:5 plasma was removed and stored at -20°C for subsequent determination of circulating GH by RIA (see below).

Study 7: Meal feeding enhances ghrelin and GH pulsatility in humans

Sixteen male volunteers (18-42 y) fitted with a naso-gastric tube received liquid feed (Nestlé Peptamen: 100kcal (7.6 g carbohydrate; 3.8 g fat; 9.2 g protein; vitamins and minerals)/100 mL standardised to individual resting metabolic rate (Supplementary Table 6) in either two 30-min bolus infusions (08:00h and 20:00h; 1875±117 kcal/day) or as a continuous infusion for 24 h (1910±218 kcal/day) (lights on (800 lux) 07:00; lights off (0 lux) 22:00h). To negate the potential confounding influence of gastric filling, bolus- and continuously-infused volunteers received continuous (82±10 mL/hr for 24 h) or bolus (two 30-min infusions at 08:00h and 20:00h) naso-gastric water infusions respectively (Figure 7A & B). Hourly blood samples (10 mL) were withdrawn manually from an indwelling median cubital vein catheter into tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately centrifuged at 3466 x *g* at 4°C for 10

mins. Separated plasma was aliquoted and stored at -80°C prior to quantification of circulating ghrelin and hGH by ELISA.

Tissue Processing

Quantification of tibial growth rates

Tibiae were fixed in buffered formal saline for 48hrs, decalcified (in 10% EDTA for 3 weeks) and embedded in paraffin wax, with 7µm longitudinal anterior-posterior sections collected and stained with Masson's trichrome. Epiphyseal plate and individual zonal widths were measured under light microscopy (mean of 3 measurements per section, 3 sections per bone) using Leica Q-win software (v3). BrdU-positive nuclei were visualised by IHC (primary antibody: Rat anti-BrdU (BioRad MCA2060); secondary antibody: Goat ant-rat IgG (Vector Labs ImmPRESS-AP MP-5404)). The distance between the closest BrdU-positive nuclei in each column (to the geminal zone) and the top of the germinal zone (Figure 1L-N) was divided by the number of days between the last injection and the day of termination to obtain an index of actual growth rate.

Hormone quantification

In the absence of protease inhibitor use to protect the acyl sidechain, we were only able to quantify total ghrelin in rat and human samples. Plasma ghrelin (total) concentrations in rat samples were determined by radioimmunoassay (Millipore RIA kit GHRT-89HK), Millipore St Charles MI, USA) according to the manufacturer's instructions [quality control (QC) values fell within the specified ranges QC1 = 0.54ng/ml (range 0.39-0.91ng/ml); QC2 = 1.43ng/ml (range 0.95-1.97ng/ml); intra-assay variation (IAV) 3.97%; sensitivity 0.13ng/ml]. Ghrelin (total) concentrations in human plasma samples were quantified by ELISA (Invitrogen^{TM} ghrelin human kit BMS2192) according to the manufacturer's instructions.

Circulating GH concentrations were determined in rat plasma samples by RIA, with the results expressed in terms of the reference preparation RP-2 (rGH), using reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases and I^{125}-labelled rGH (IRC-105, Institute of Isotopes Co Ltd, Budapest, Hungary) (intra-assay variation 2.62%; sensitivity 0.12ng/ml). GH concentrations in human plasma samples were quantified by ELISA (R&D Systems human growth hormone DuoSet DY1067) according to manufacturer's instructions.

Plasma IGF-1 concentrations were determined in rodent samples by ELISA (R&D Systems Mouse/Rat IGF-1 DuoSet DY791) according to manufacturer's instructions.

Statistics

Feeding profiles for individual animals are presented as individual feeding events with the superimposition of corresponding cumulative food intake data or corresponding hormone profiles. Total hormone secretory output was determined by calculating the area under the curve (using MS Excel version 16.15 for Mac). Given the episodic nature of GH secretion several approaches were taken to characterising the parameters of secretion. Distribution analysis was used to estimate baseline secretion (OC_5 (the cut off value below which 5% of the samples fall when ranked in ascending order of concentration)) (21). Using the distribution analysis output, secretory "bursts" were identified where the value exceeded OC_{80}, but returned to OC_{30}, before the next "burst". Burst duration represented the period in which GH concentration in consecutive samples was $>OC_{30}$. Values were determined for the total 24hr period and for four 6hr periods representing the first and second halves of the light and dark phases. To analyse burst frequency in the rat GH data, missing data points were linearly interpolated and the data detrended using the smoothness priors approach (SPA) with the smoothing parameter set at 300 (52). The power spectrum of the detrended data was then computed using the discrete Fourier transform (DFT) applied to a 24-h period time window. The dominant frequency was taken as the frequency value

corresponding to the maximum spectral power of the discrete transform, which was calculated using a quadratic interpolation. These approaches were not applicable to the human data due to the lower sampling frequency. To visualise ultradian hGH variation, individual values were normalised to the profile mean for each volunteer and expressed as %-mean.

Apart from representative profiles, all data are presented as either mean (±SEM), or in box and whisker plots (showing median line, mean (+), upper and lower quartile range (bars), data range (whiskers) and individual data points). Comparisons were made by 1-way ANOVA and Bonferroni selected pairs *post hoc* test (GraphPad Prism, version 7.0d for Mac OS X) or Student's t-test (MS Excel version 18.86 for Mac), as indicated in the Figure and table legends, with $P<0.05$ being considered significantly different.

Study Approval

All animal procedures (including those in genetically modified mice) were performed under the authority of the Animals (Scientific Procedures) Act, 1986 (UK) in accordance with the ARRIVE guidelines and were specifically approved by the Cardiff University Animal Welfare Ethical Review Body. Human volunteers were fully briefed on the study requirements prior to provision of written informed consent. Procedures were conducted in accordance with the latest version of the Declaration of Helsinki, authorised by the NHS research ethics committee (reference: 18/SW/0176) and the trial registered on clinicaltrials.gov (NCT03906409).

Data Availability

Underlying data for this publication are accessible in the Supporting Data Values xlsx file.

Figures

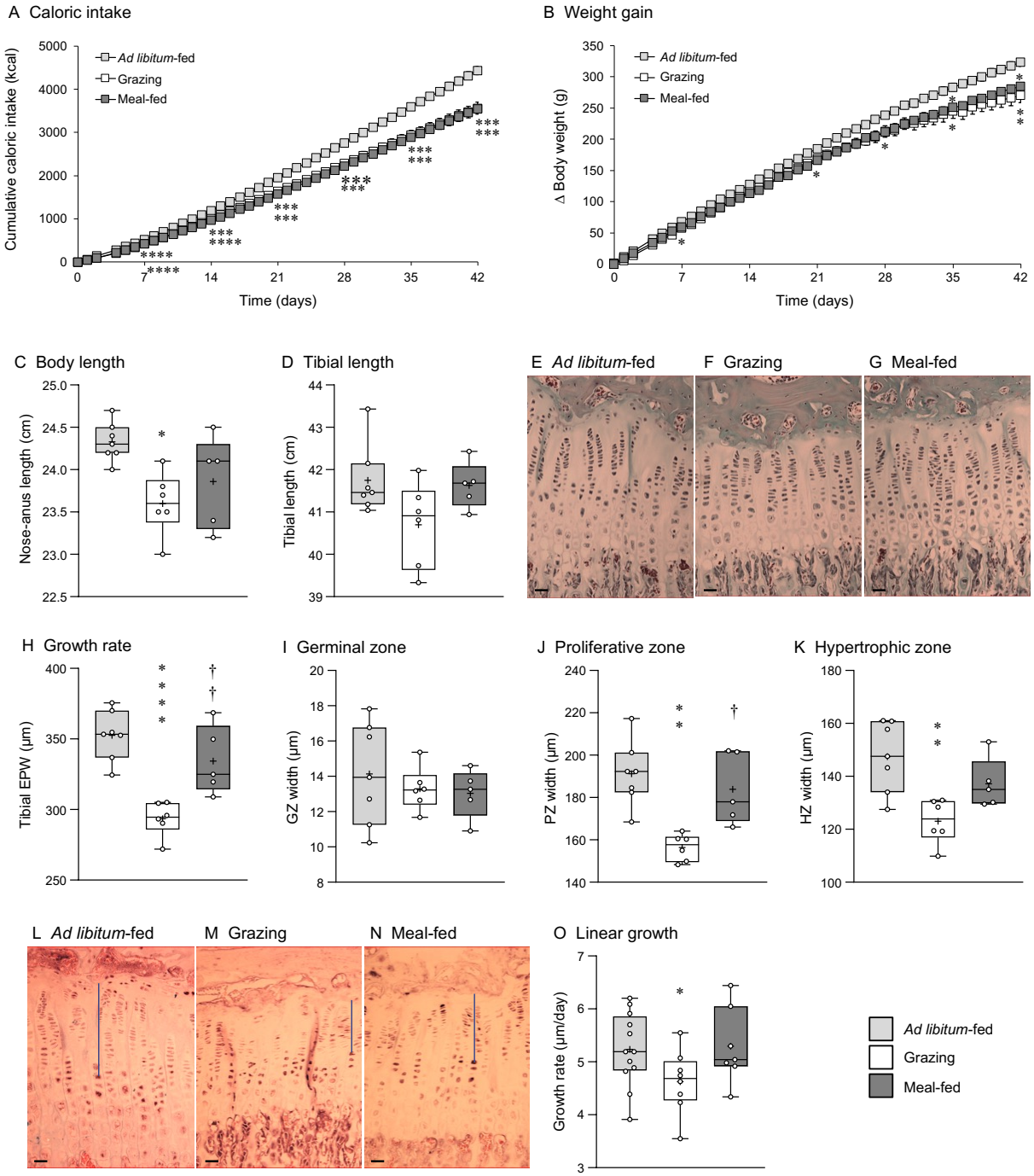


Figure 1: Meal-feeding protects skeletal growth (Studies 1 and 2). Cumulative caloric intake (**A**) and body weight gain (**B**), body length (**C**), tibial length (**D**), tibial epiphysial plate width (EPW; **H**), germinal (GZ; **I**), proliferative (PZ; **J**) and hypertrophic (HZ; **K**) zone widths (in Masson's

trichrome-stained sections **E-G** (scale bar 20μm)) from male rats receiving a standard chow in either *ad libitum* (light grey symbols/bars), grazing (white symbols/bars) or meal-feeding (dark grey symbols/bars) patterns for 6 weeks. In addition, linear growth rate (**O**) was measured in tibial sections (**L-N**) stained for BrdU (dark nuclei; scale bars 20μm; blue bars = distance from GZ to first BrdU-labelled nucleus in column) in a separate cohort of rats receiving these feeding patterns for 3 weeks. Data shown are mean ± SEM (A,B), box and whisker plots (**C-D**, **H-K**, **O**) showing median line, mean (+), upper and lower quartile range (bars), data range (whiskers) and individual data points (*n*=7 (*ad libitum* (**A-K**)), 6 (grazing (**A-K**)), 5 (meal-fed (**A-K**)), 12 (*ad libitum* (**O**)) and 8 (grazing and meal-fed (**O**))) with statistical comparisons performed by 1-way ANOVA and Bonferroni's selected pairs *post hoc* test (**P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 *vs ad libitum*-fed; †*P*<0.05; ††*P*<0.01vs grazing).

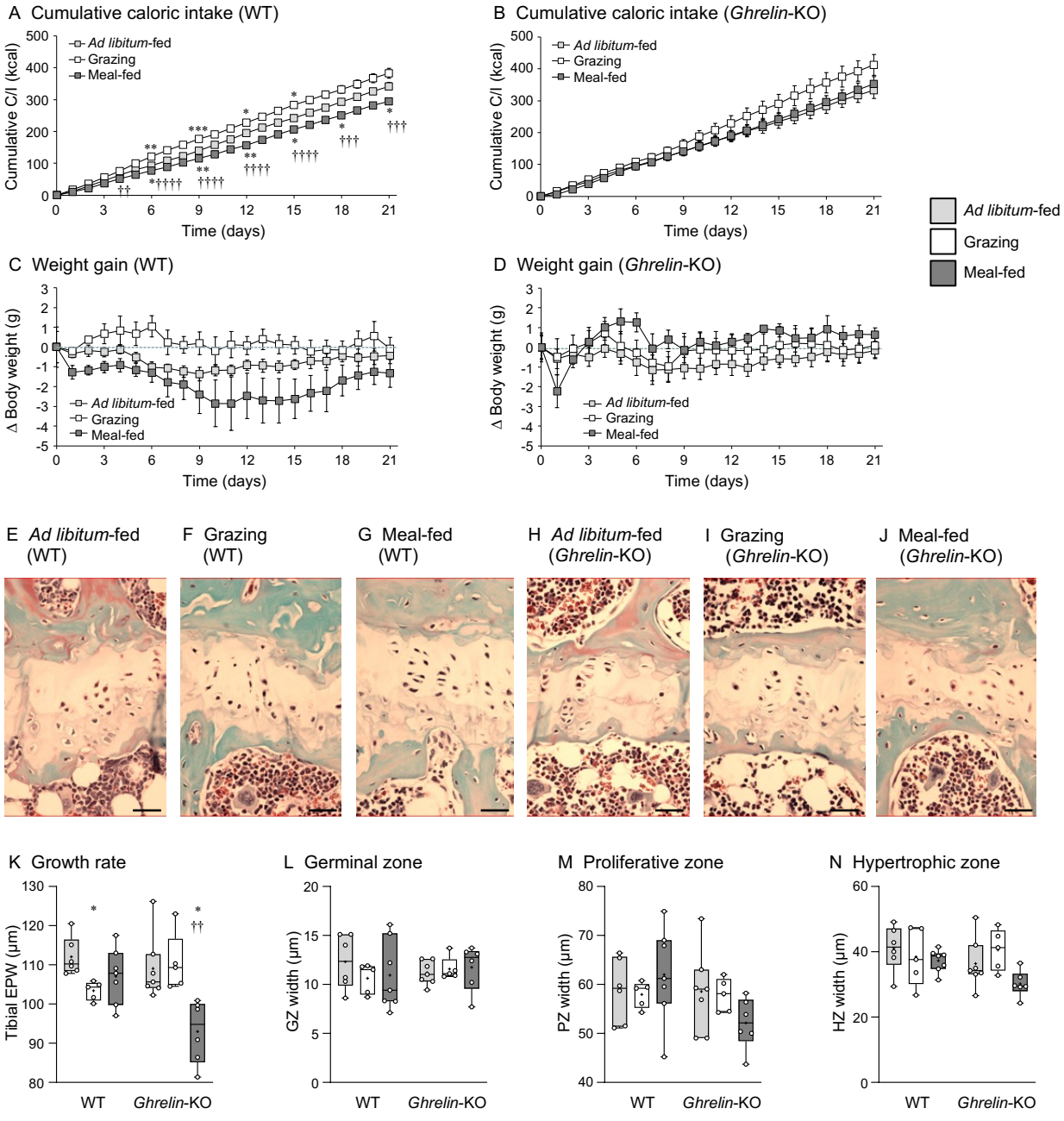


Figure 2: Meal-feeding promotes skeletal growth via a ghrelin-dependent mechanism.

Cumulative caloric intake (C/I; **A**,**B**), body weight gain (**C**,**D**), tibial epiphyseal plate width (EPW; **K**) and germinal (GZ; **L**) proliferative (PZ; **M**) and hypertrophic zone (HZ; **N**) widths (measured in Masson's trichrome-stained sections (**E-J**) (scale bar 20μm)), from 6-month old male *ghrelin*-KO mice (*Ghr*-KO; **B**,**D**,**H-J**) and their wild-type (WT) male littermates (**A**,**C**,**E-G**) fed a standard non-

purified rodent diet (13.9% AFE fat) in either *ad libitum* (light grey symbols/bars), grazing (white symbols/bars) or meal-feeding (dark grey symbols/bars) patterns for 3 weeks. Data shown are mean ± SEM (**A-D**), with box and whisker plots (**K-N**) showing median line, mean (+), upper and lower quartile range (bars), data range (whiskers) and individual data points (n=5 (WT grazing, *ghrelin*-KO grazing), 6 (WT *ad libitum*-fed, *ghrelin*-KO meal-fed), 7 (WT meal-fed, *ghrelin*-KO *ad libitum*-fed). Statistical comparisons were performed by 1-way ANOVA and Bonferroni's selected pairs *post hoc* test (**P*<0.05; ***P*<0.01; ****P*<0.001 *vs ad libitum*-fed males (same genotype); ††*P*<0.01 †††*P*<0.001 ††††*P*<0.0001vs grazing males (same genotype)).

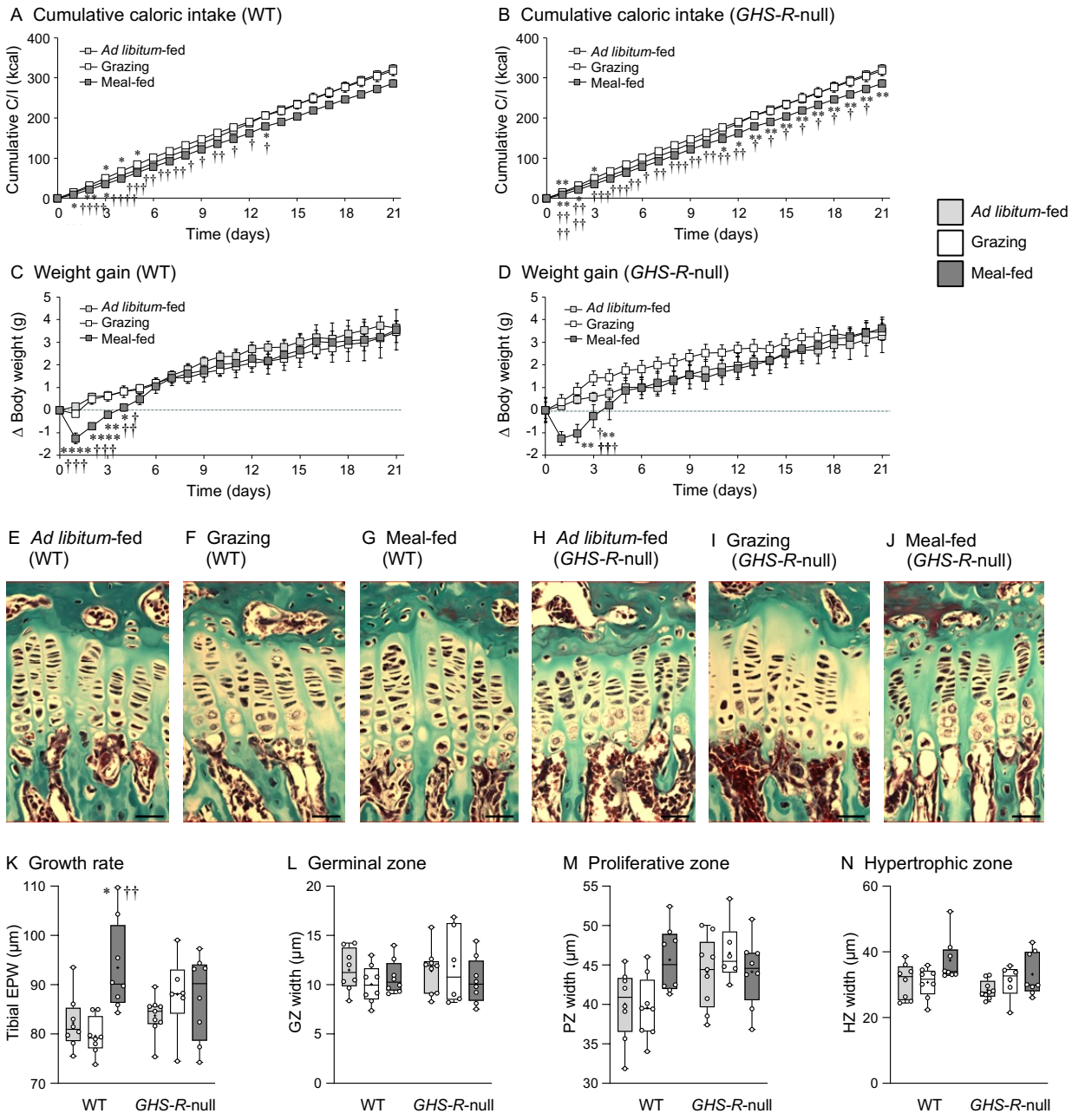


Figure 3: Meal-feeding promotes skeletal growth via a *GHS-R*-dependent mechanism. Cumulative caloric intake (C/I; **A**,**B**), body weight gain (**C**,**D**), tibial epiphyseal plate width (EPW; **K**) and germinal (GZ; **L**) proliferative (PZ; **M**) and hypertrophic zone (HZ; **N**) widths (measured in Masson's trichrome-stained sections (**E-J**) (scale bar 20μm)), from 6-week old male *GHS-R*-null mice (**B**,**D**,**H-J**) and their wild-type (WT) male littermates (**A**,**C**,**E-G**) fed a standard non-purified rodent diet (13.9% AFE fat) in either *ad libitum* (light grey symbols/bars), grazing (white symbols/bars) or meal-feeding (dark grey symbols/bars) patterns for 3 weeks. Data shown are

mean ± SEM (**A-D**), with box and whisker plots (**K-N**) showing median line, mean (+), upper and lower quartile range (bars), data range (whiskers) and individual data points (n=9 (*ad libitum*-fed GHSR-null) and 8 (all other groups)). Statistical comparisons were performed by 1-way ANOVA and Bonferroni's selected pairs *post hoc* test (**P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 *vs ad libitum*-fed males (same genotype); †*P*<0.05; ††*P*<0.01 †††*P*<0.001 ††††*P*<0.0001vs grazing males (same genotype)).

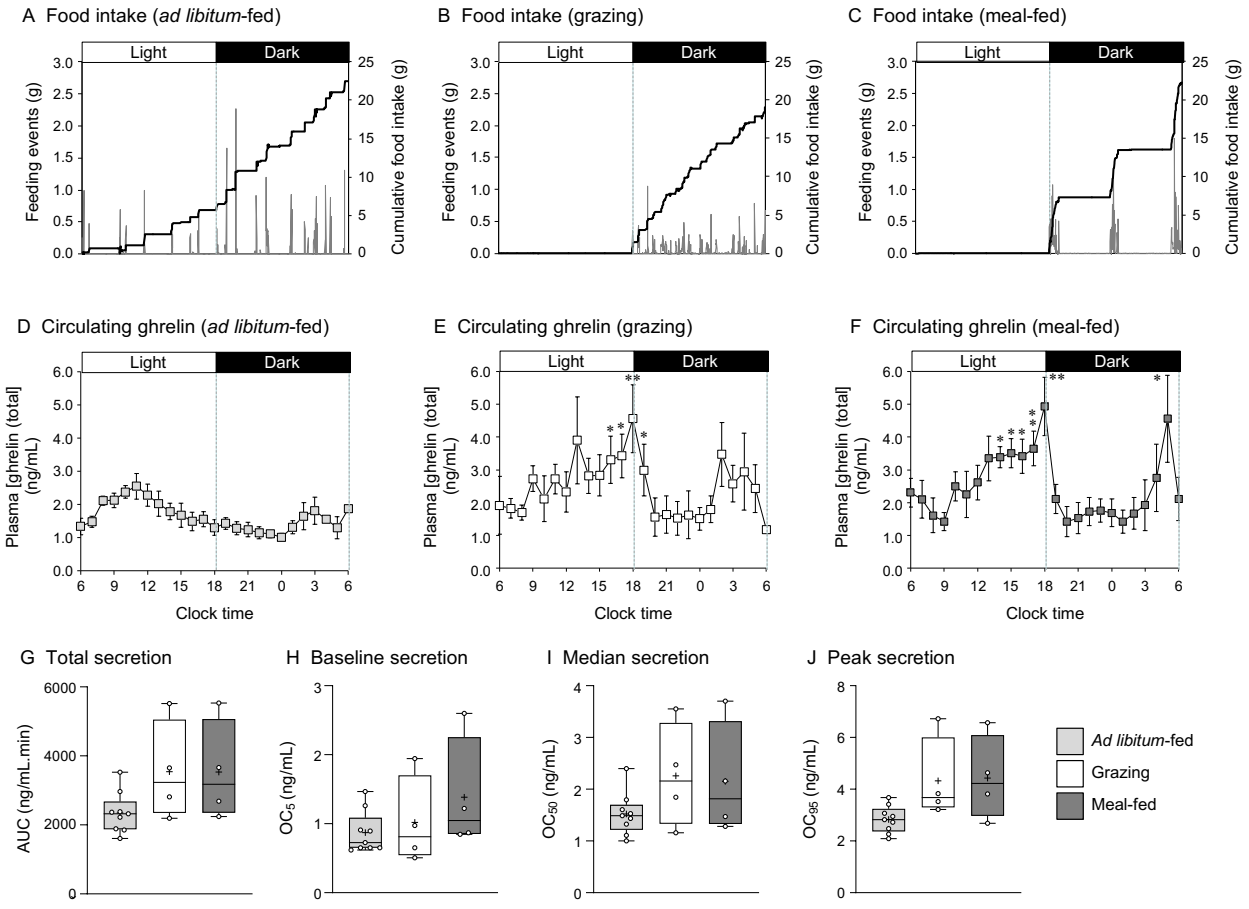


Figure 4: Grazing and meal-feeding modify circulating ghrelin profiles. Mean food intake profiles (**A-C**) and circulating ghrelin (total) profiles (**D-F**) in male rats fed a standard non-purified rodent diet in either *ad libitum* (**A**,**D**), grazing (**B**,**E**) or meal-feeding (**C**,**F**) patterns. Food intake profiles show individual feeding events (vertical grey bars) and cumulative intake (solid black line). Total ghrelin secretion (area under curve (AUC); **G**), baseline secretion (observed concentration at 5% (OC_5); **H**), median secretion (OC at 50% (OC_{50}); **I**) and peak ghrelin secretion (OC at 95% (OC_{95}); **J**) are also shown. Ghrelin data shown are mean ± SEM (**D-F**), box and whisker plots (**G-J**) showing median line, mean (+), upper and lower quartile range (bars), data range (whiskers) and individual data points (n=9 (*ad libitum*) and 4 (grazing and meal-fed rats)), with statistical comparisons performed by 1-way ANOVA and Bonferroni's selected pairs *post hoc* test (rats study; *P<0.05; **P<0.01 *vs ad libitum*-fed; †P<0.05 *vs* grazing).

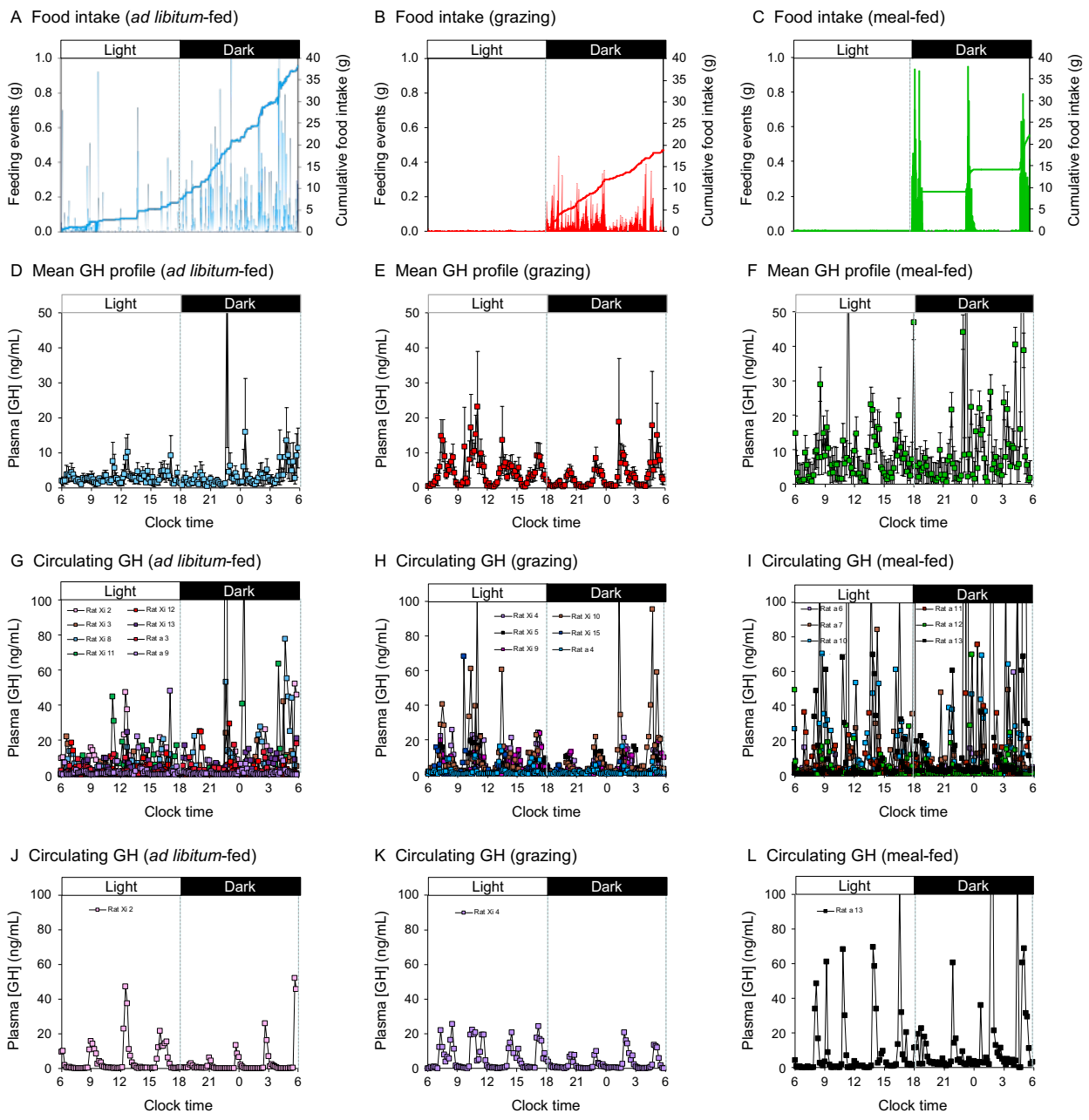


Figure 5: Meal-feeding amplifies GH rhythmicity. Mean food intake profiles (**A-C**), mean GH profiles (± SEM; **D-F**), superimposed individual GH profiles (**G-I**) and representative individual GH profiles (**J-L**) in male rats fed a standard non-purified rodent diet in either *ad libitum* (**A**,**D**,**G**,**J**; n=8), grazing (**B**,**E**,**H**,**K**; n=6) or meal-feeding (**C**,**F**,**I**,**L**; n=6) patterns. Food intake profiles (**A-C**) show individual feeding events (vertical bars) and cumulative intake (solid line).

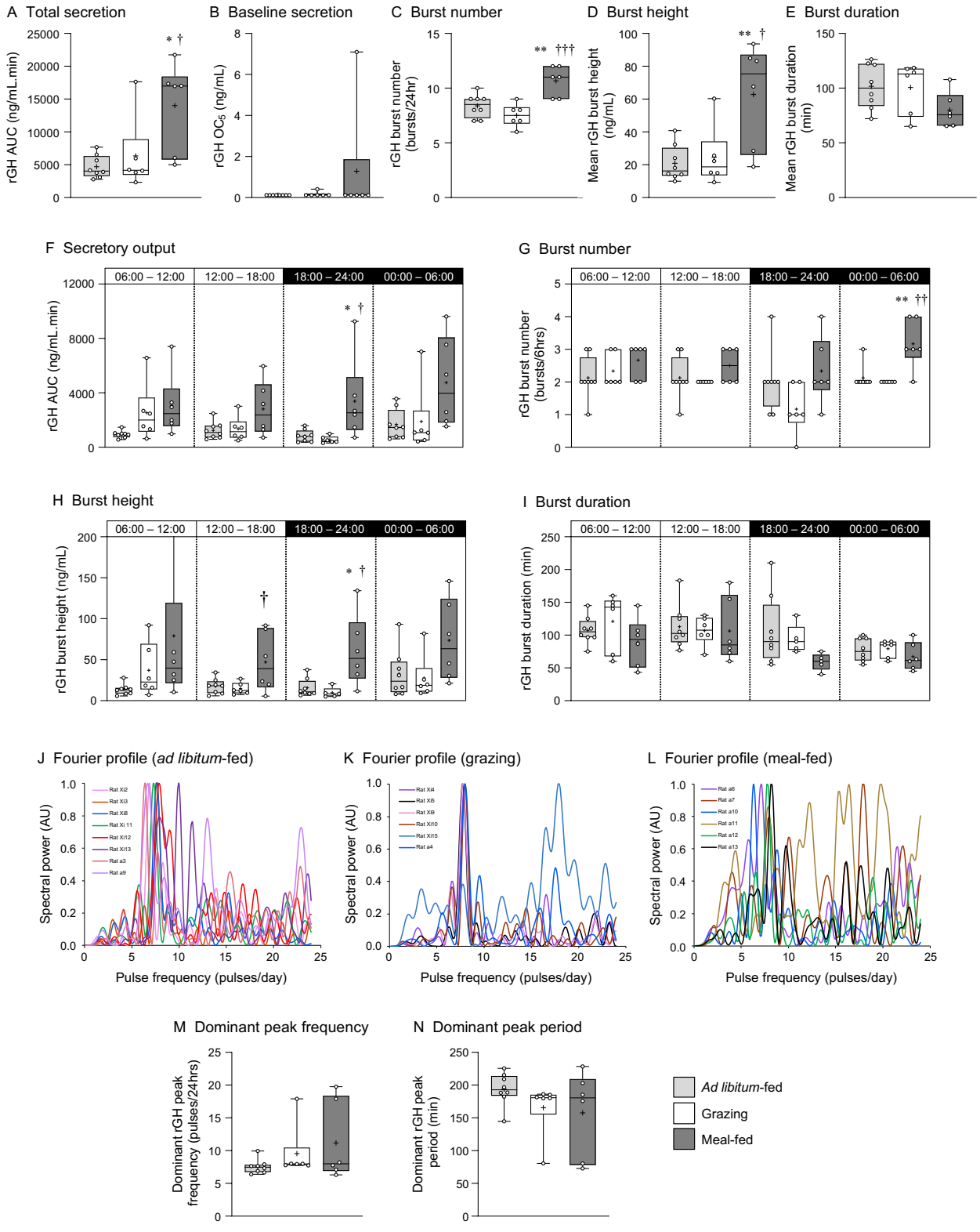


Figure 6: Meal-feeding enhances GH pulsatility predominantly in the dark phase. GH secretory output (area under curve (AUC); **A**,**F**), baseline secretion (OC_5; **B**), burst number (**C**,**G**),

burst height (**D**,**H**) and burst duration (**E**,**I**), for the full 24hr period (**A-E**) or subdivided into the 6hr periods representing the first or second half of the light and dark phases (**F-I**) in male rats fed a standard non-purified rodent diet in either *ad libitum* (**A**; n=8), grazing (**B**; n=6) or meal-feeding (**C**; n=6) patterns. Fourier analysis of GH frequency spectra (normalised spectral power in arbitrary units (AU); **J-L**) enabled derivation of dominant GH peak frequency (**M**) and period (**N**). Data shown are individual spectral power profiles (**J-L**), box and whisker plots (**A-I**, **M-N**) showing median line, mean (+), upper and lower quartile range (bars), data range (whiskers) and individual data points, with statistical comparisons performed by 1-way ANOVA and Bonferroni's *post hoc* test (**P*<0.05; ***P*<0.01 *vs ad libitum*-fed; †*P*<0.05; ††*P*<0.01; †††*P*<0.001 *vs* grazing).

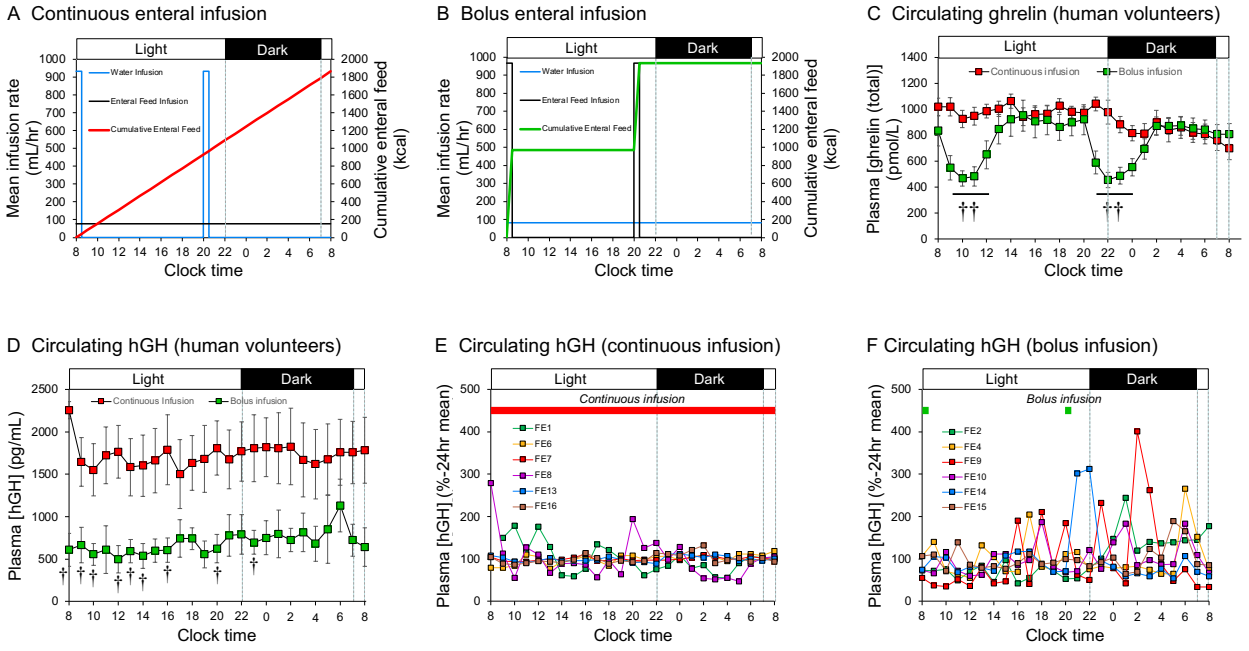


Figure 7: Meal-feeding enhances ghrelin and GH rhythmicity in human males. Male human volunteers received infusions of water (blue lines) and enteral feed via a naso-gastric tube, with enteral feed given as a continuous ("Grazing"; Red line; **A**) or bolus ("Meal-fed"; Green line; **B**) infusion, water being administered in the opposite profile. Circulating ghrelin (**C**) and GH (**D**) data presented are mean ± SEM (n=8 (both infusion patterns)), with statistical comparisons performed by unpaired Student's t-test ($^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$ *vs* continuously-infused). In addition, individual circulating hGH profiles normalised (to each individual profile mean) in continuously-infused (**E**) and bolus-infused (**F**) male human volunteers are presented.

List of Supplementary Material:

Supplementary Table 1: *Post-mortem* analysis of growth in pattern-fed rats (Study 2)

Supplementary Table 2: *Post-mortem* analysis of growth end points in pattern-fed 6-month old WT and *ghrelin*-KO mice (Study 3)

Supplementary Figure S1: CLAMS Food access port adapters improve patterned food delivery profiles in mice (Study 4)

Supplementary Table 3: *Post-mortem* analysis of growth end points in pattern-fed 6-week old WT and GHS-R-null mice

Supplementary Table 4: *Post-mortem* analysis of growth endpoints in pattern-fed rats (Study 5)

Supplementary Figure S2: Meal-feeding enhances GH secretion and rhythmicity in rats (Study 6)

Supplementary Table 5: *Post-mortem* analysis of growth endpoints in pattern-fed rats (Study 6)

Supplementary Table 6: Human participant characteristics (Study 7)

References

1 Zarrinpar A, et al. Daily eating patterns and their impact on health and disease. *Trends Endocrinol Metab* 2016; 27:69-83.

2 Paoli A, et al. The influence of meal frequency and timing on health in humans: the role of fasting. *Nutrients* 2019; 11:719.

3 Abbasi J. NIH charts a path for nutritional science. *JAMA* 2017; 317:243-244.

4 Allison DB, et al. Goals in Nutrition Science 2015-2020. *Front Nutr* 2015; 2:26.

5 Laska MN, et al. How we eat what we eat: identifying meal routines and practices most strongly associated with healthy and unhealthy dietary factors among young adults. *Public Health Nutr* 2014; 18:2135-2145.

6 Duffey KJ, Popkin BM. Energy intake, portion size, and eating occasions: contributions to increased energy intake in the United States, 1977-2006. *PLoS Med* 2011; 8:e1001050.

7 Ritchie LD. Less frequent eating predicts greater BMI and waist circumference in female adolescents. *Am J Clin Nutr* 2012; 95:290-296.

8 Jääskeläinen A, et al. Associations of meal frequency and breakfast with obesity and metabolic syndrome traits in adolescents of North Finland Birth Cohort 1986. *Nutr Metab Cardiovasc Dis* 2012; 23:1002-1009.

9 Tumin R, Anderson SE. Television, home-cooked meals, and family meal frequency: Associations with adult obesity. *J Acad Nutr Diet* 2017; 117:937-945.

10 Pryer JA, et al. Who are the 'low energy reporters' in the Dietary and Nutritional Survey of British Adults? *Int J Epidemiol* 1997; 26:146-154.

11 Poppitt SD, et al. Assessment of selective under-reporting of food intake by both obese and non-obese women in a metabolic facility. *Int J Obes* 1998; 22:303-311.

12 Ware JH. Interpreting incomplete data in studies of diet and weight loss. *N Engl J Med* 2003; 348:2136-2137.

13 Carter MC, et al. Adherence to a smartphone application for weight loss compared to website and paper diary: pilot randomized controlled trial. *J Med Internet Res* 2013; 15:e32.

14 Gill S, Panda S. A smartphone app reveals erratic diurnal eating patterns in humans that can be modulated for health benefits. *Cell Metab* 2015; 22:1-10.

15 Acosta-Rodríguez VA, et al. Mice under caloric restriction self-impose a temporal restriction of food intake as revealed by an automated feeder system. *Cell Metab* 2017; 26:267-277.

16 http://www.actimetrics.com/products/clocklab/clocklab-feeder-control/

17 http://www.researchdiets.com/biodaq/applications/food-liquid-restriction

18 https://kravitzlab.github.io/SnackClock/

19 Keen-Rinehart E, et al. Physiological mechanisms for food-hoarding motivation in animals. *Philos Trans R Soc Lond B Biol Sci* 2010; 365:961-975.

20 Golding DM, et al. Paradoxical leanness in the imprinting-centre deletion mouse model for Prader-Willi syndrome. *J Endocrinol* 2017; 232:123-135.

21 Tilston TW, et al. A novel automated system yields reproducible temporal feeding patterns in laboratory rodents. *J Nutr* 2019; 149:1674-1684.

22 Loizides-Mangold U, et al. Lipidomics reveals diurnal lipid oscillations in human skeletal muscle persisting in cellular myotubes cultured in vitro. *Proc Natl Acad Sci (USA)* 2017; 114:E8565-E8574.

23 Sun Y, et al. Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* 2003; 23:7973-3981.

24 Zigman JM, et al. Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J Clin Invest* 2005; 115:3564-3572.

25 Tschöp M, et al. Ghrelin induces adiposity in rodents. *Nature* 2000; 407:908-913.

26 Cummings DE, et al. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001; 50:1714-1719.

27 Drazen DL, et al. Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology* 2006; 147:23-30.

28 Thompson NM, et al. Pattern-dependent suppression of growth hormone (GH) by ghrelin and GH-releasing peptide-6 in moderately GH-deficient rats. *Endocrinology* 2003; 144:4859-4867.

29 Thompson NM, et al. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 2004; 145:234-242.

30 Bodosi B, et al. 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* 2004; 287:R1071-R1079.

31 Tannenbaum GS, Martin JB. Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* 1976; 98:562-570.

32 Clark RG, et al. Automated repetitive microsampling of blood: growth hormone profiles in conscious male rats. *J Endocrinol* 1986; 111:27-35.

33 Davies JS, et al. Photic stimulation inhibits growth hormone secretion in rats: a hypothalamic mechanism for transient entrainment. *Endocrinology* 2004; 145: 2950-2958.

34 Caminos JE, et al. The endogenous growth hormone secretagogue (ghrelin) is synthesized and secreted by chondrocytes. Endocrinology 2005 146:1285-1292.

35 Gaun XM, et al. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* 1997; 48:23-29.

36 Liang Z-T, et al. Ghrelin up-regulates cartilage-specific genes via the ERK/STAT3 pathway in chondrocytes of patients with adolescent idiopathic scoliosis. *Biochem Biophys Res Commun* 2019; 518:259-265.

37 Hopkins AL, et al. Unacylated ghrelin promotes adipogenesis in rodent bone marrow via ghrelin O-acyl transferase and GHSR1a activity: evidence for target cell-induced acylation. *Sci Rep* 2017; 7:45541.

38 Gómez R, et al. Expression and modulation of ghrelin O-acyltransferase in cultured chondrocytes. *Arthritis Rheum* 2009; 60:1704-1709.

39 MacGregor DJ, Leng G. Modelling the hypothalamic control of growth hormone secretion. *J Neuroendocrinol* 2005; 17:788-803.

40 Clark RG, et al. Intravenous growth hormone: growth responses to patterned infusions in hypophysectomised rats. *J Endocrinol* 1985; 104:53-61.

41 Jaffe CA, et al. Growth hormone secretion is an independent regulator of growth hormone actions in humans. *Am J Physiol Endocrinol Metab* 2002; 283:E1008-E1015.

42 Ribeiro-Oliveira A Jr, et al. Complex rhythmicity and age-dependence of growth hormone secretion are preserved in patients with acromegaly: further evidence for a present hypothalamic control of pituitary somatotropinomas. *J Clin Endocrinol Metab* 2013; 98:2959-2966.

43 Kreymann KG, et al. ESPEN guidelines on enteral nutrition: intensive care. *Clin Nutr* 2006; 25:210-223.

44 Laursen T, et al. Long-term effects of continuous subcutaneous infusion *versus* daily subcutaneous injections of growth hormone (GH) on the insulin-like growth factor system, insulin sensitivity, body composition, and bone and lipoprotein metabolism in GH-deficient adults. *J Clin Endocrinol Metab* 2001; 86:1222-1228.

45 Rampersaud A, et al. Plasma growth hormone pulses induce male-biased pulsatile chromatin opening and epigenetic regulation in adult mouse liver. *eLife* 2023; 13:RP91367.

46 Legraverend C, et al. Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J* 1992; 6:711-718.

47 Zizza C, et al. Significant increase in young adults' snacking between 1977-1978 and 1994-1996 represents a cause for concern! *Prev Med* 2001; 32:303-310.

48 Kant AK, Graubard BI. 40-year trends in meal and snack eating behaviors of American adults. *J Acad Nutr Diet* 2015; 115:50-63.

49 Horne JA, Ostberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* 1976; 4:97-110. PMID: 1027738

50 Buysse DJ et al. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989; 28:193-213.

51 Roenneberg T, et al. Life between clocks: daily temporal patterns of human chronotypes. *J Biol Rhythms* 2003; 18:80-90.

52 Tarvainen MP, et al. An advanced detrending method with application to HRV analysis. *IEEE Trans Biomed Eng* 2002; 49:172-175.