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TECHNICAL NOTE

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An optimization and refinement of the whole-gut transit assay in mice

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Abstract

Background: Gastrointestinal motility measurements in mice are currently performed under suboptimal conditions, as these nocturnal animals are measured during light conditions. In addition, other stressors, like individual housing, placement in a new cage during observation, and lack of bedding and cage enrichment cause animal discomfort and might contribute to higher variability. Here we aimed to develop a refined method of the widely-used whole-gut transit assay.

Methods: Wildtype mice ($N = 24$) were subjected to the standard or refined whole-gut transit assay, either with or without a standardized slowing in gastrointestinal motility induced by loperamide. The standard assay consisted of a gavage with carmine red, observation during the light period and individual housing in a new cage without cage enrichment. For the refined whole-gut transit assay, mice were gavaged with UV-fluorescent DETEX®, observed during the dark period, while pairwise housed in their home cage with cage enrichment. Time until excretion of the first colored fecal pellet was assessed, and pellets were collected to assess number, weight, and water content.

Key Results: The DETEX®-containing pellets were UV-detectable, allowing to measure the mice in their active period in the dark. The refined method caused less variation (20.8% and 16.0%) compared to the standard method (29.0% and 21.7%). Fecal pellet number, weight, and water content was significantly different between the standard and refined method.

Conclusions & Inferences: This refined whole-gut transit assay provides a reliable approach to measure whole-gut transit time in mice in a more physiological context, with reduced variability compared to the standard method.

KEYWORDS
carmine red, DETEX, gastrointestinal transit, intestinal motility, loperamide

Simone L. Schonkeren and Saskia Seeldrayers contributed equally to this work.

1 | INTRODUCTION

Measurement of gastrointestinal motility in laboratory animals is necessary to assess drug dosing and efficacy of new therapeutics, and to aid in the basic understanding of gastrointestinal physiology and pathology.[1]

In preclinical studies, terminal methods are available to determine regional motility, by measuring the distance a tracer has traveled since oral gavage.[2–4] Alternatively in an ex vivo setup, intestinal segments can be isolated from the animal and motor patterns can be assessed in an organ bath.[5–7] However, these methods require killing of the experimental animals. Non-terminal approaches to study gastrointestinal motility often require anesthesia and advanced imaging modalities like scintigraphy, ultrasound, or radiography.[1,8,9] Therefore, the most commonly used in vivo assessment of gastrointestinal motility remains the whole-gut transit assay. In this method, a non-absorbable dye such as carmine red is administered orally and the time until observation of the first dye-containing fecal pellet expelled by the animal is defined as the whole-gut transit time (WGTT).[1,10–14] Although this is considered the gold standard,[1] there are several limitations to this approach.

Due to the necessity to visually observe the dye-containing fecal pellet, the experiment is usually carried out during the light period, the resting period for nocturnal animals, and thus a physiologically less appropriate period to assess gastrointestinal transit. Another limitation is that the whole-gut transit assay is usually not performed in the home cage, for example, by individually housing in a new cage devoid of bedding. This environmental change induces stress and might affect thermoregulation.[15–17] Stress is known to influence gastrointestinal motility,[18] and might confound results even more. In addition, to refine animal experimentation, procedures should be performed aiming to minimize harm and distress. Therefore, we here evaluate the potential of a refined and optimized method for examining whole-gut transit time. We use a UV-fluorescent dye that is administered to mice on a reversed light–dark schedule, enabling observation during their active period. In addition, mice are socially housed in their home cage to reduce stress. To validate this approach, the refined and standard whole-gut transit method are compared in presence and absence of loperamide to induce a standardized delay of whole-gut transit.

Key Points

- Whole-gut transit time in mice is currently assessed in suboptimal conditions that limit physiologically-relevant measurements.
- We designed a method that enables measuring whole-gut transit in mice during their active period in the dark, and that reduces stress by socially housing mice in their home cage.
- The refined method results in less within-group variation, allowing for a reduction in animal use.

2 | MATERIALS AND METHODS

2.1 | Animals

Twelve male and twelve female wildtype C57Bl/6J mice (4 weeks old) were obtained from Charles River (Charles River Laboratories Germany) and maintained under specific pathogen-free conditions on a 12 h:12 h light–dark schedule until experiments started. Mice had ad libitum access to food and water and were housed pairwise in individually ventilated cages with cage enrichment (polycarbonate mouse cottage, cardboard roll, Diamond Twists, Sizzle-Nest). All procedures were conducted with approval from the Animal Welfare Body of Maastricht University and were performed according to Dutch regulations under project license AVD1070020174264.

2.2 | Reagents

The gavage solution was 300 µL 6% (w/v) carmine red (Sigma C1022) in 0.5% (w/v) methylcellulose (Sigma M0512) in sterile water with or without 3 mg/kg loperamide hydrochloride (Sigma L4762) in PBS, or 300 µL 0.24 g/mL DETEX® Soft Bait (Bell Laboratories Inc. 230-000037 Killgerm) in sterile water with or without 3 mg/kg loperamide. DETEX® contains Lumitrack®, which fluoresces under UV-light, and contains 6.45 ± 0.40 g crude protein, 24.80 ± 0.40 g crude fat, 6.16 ± 0.45 g crude fiber, 4.39 ± 0.26 g crude ash, and 51.94 ± 0.80 g nitrogen-free extractive per 100 g, tested by Merieux NutriSciences.

2.3 | Experimental design

Mice were 8–10 weeks old at the start of experiments. Four experimental conditions were compared (Table 1): RED, RED + LOP (standard method), DETEX, and DETEX + LOP (refined method) in a crossover design with eight experimental groups, in which conditions were tested in different order (Table S1). Animals were randomly allocated to each group and experimenters were blinded for intervention (with/without loperamide).

The light–dark schedule was gradually reversed by shifting the light period by 1 h every day (phase delay) for 14 days until the light–dark schedule was completely reversed (Figure 1A), thereby reducing "jet lag".[19] For groups starting with DETEX/DETEX + LOP, the light–dark schedule was first reversed, and later reversed back to normal for RED/RED + LOP conditions (Figure 1B). Experiments were started after at least 2 days acclimatization.

Conditions RED/RED + LOP were measured during the light period, DETEX/DETEX + LOP were measured in the dark on a reversed light–dark schedule, starting at 9:15 ± 15 min a.m. Food

TABLE 1 Summary of the four different conditions.

Condition	Dye	Intervention	Light/dark schedule	Housing	Social housing	Cage enrichment	Time of measurements
RED	Carmine red	Vehicle	Light (normal)	Experimental cage	Individual	No	Daytime
RED + LOP	Carmine red	Loperamide	Light (normal)	Experimental cage	Individual	No	Daytime
DETEX	Detex with lumitrack®	Vehicle	Dark (reversed)	Home cage	Pairwise	Yes	Daytime
DETEX + LOP	Detex with lumitrack®	Loperamide	Dark (reversed)	Home cage	Pairwise	Yes	Daytime

Note: For the RED and RED + LOP conditions, the light/dark schedule is normal and experiments are performed during daytime. During the experiment, animals are housed individually, in a new empty cage lined with diaper cloth to absorb moisture and no cage enrichment (experimental cage). For the DETEX and DETEX + LOP conditions, the light/dark schedule is reversed and experiments are performed during daytime in the dark. During the experiment, animals are housed pairwise in their home cage, with a cardboard roll as cage enrichment.

Abbreviations: DETEX + LOP, DETEX and loperamide; RED, carmine red; RED + LOP, carmine red and loperamide.

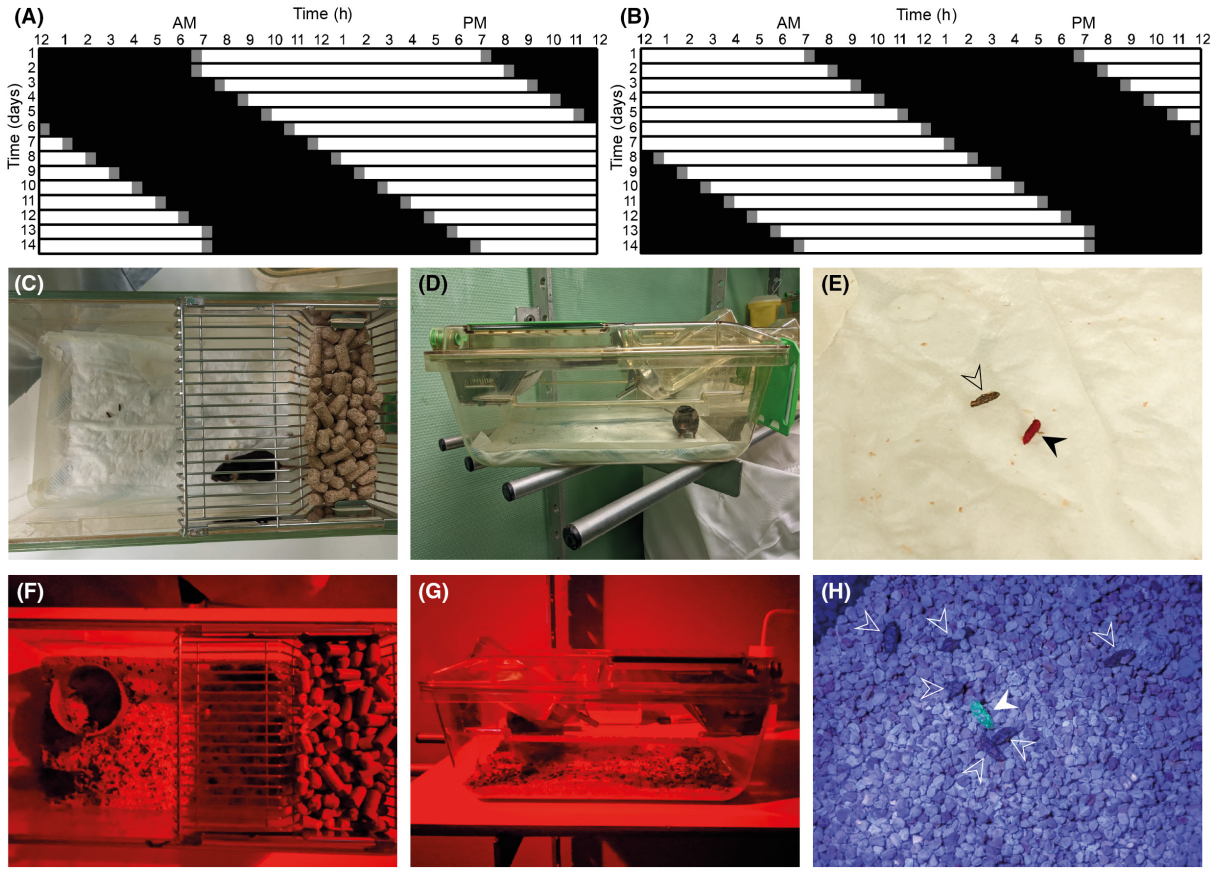

FIGURE 1 (A) Shifting the light–dark schedule with 1 h every day (phase delay) to enable experiments during the day in the dark, without causing a "jet lag". Lights off-hours are shown in black, lights on-hours are shown in white. A period of half an hour before turning the lights on and after turning the lights off is shown in gray; during this time there was a dim light on to soften the transition between lights on and off (twilight zone). (B) Shifting the light–dark schedule back to the regular schedule (7:00 a.m. lights on; 7:00 p.m. lights off) in the same way as in (A). (C) For the RED and RED + LOP conditions, cages are lined with diaper cloth to absorb urine and enable visual inspection of dye-containing fecal pellets. (D) Cages are placed outside of the ventilation unit to enable visual observation. (E) Red fecal pellets (closed arrowhead) can be readily distinguished from normal fecal pellets (open arrowhead). (F) For the DETEX and DETEX + LOP conditions, mice are housed pairwise in their home cage with a cardboard roll as cage enrichment. (G) Cages are placed in the animal room, outside of the ventilation unit to enable visual observation. (H) Green fluorescent fecal pellets (closed arrowhead) can be readily distinguished from normal fecal pellets (open arrowhead) with a UV flashlight.

was omitted for 1 h before oral gavage and available ad libitum throughout the procedure in all conditions. For RED/RED + LOP, mice were transferred to individual cages lined with a diaper cloth to absorb urine during the observation period (Figure 1C). For DETEX/DETEX + LOP, cage changes were not performed later than 24 h preceding the experiment to reduce stress. The oral gavage was given to one of the pairwise housed mice, after which it was returned to its home cage for observation. Part of the cage

enrichment was removed (cottage, paper strands) to enable observation of fecal pellets (Figure 1F).

All mice were observed in their cage on a table outside of the ventilation unit with a filter cap to maintain SPF conditions (Figure 1D,G). Pellet production was monitored every 5–10 min until observation of a dye-containing pellet for each mouse or up to 480 min (Figure 1E,H). For DETEX/DETEX+LOP conditions, a UV flashlight was used to enable visualization of the Lumitrack® dye. After expulsion of the first dye-containing fecal pellet, the pellets were collected for 1 h and the cage was returned to the ventilation unit. All fecal pellets were weighed and dried overnight at 75°C to determine their dry weight. Fecal water content was calculated as the difference between wet and dry weight as a percentage of wet weight.

2.4 | Statistical analysis

Two-way mixed ANOVA was used to compare the four different conditions in all groups for whole-gut transit time, after passing Mauchly's test for sphericity and Levene's test of equality of error variances (based on median). For fecal pellet number, weight, and water content, a Shapiro–Wilk test was used to assess normality followed by a paired Student's *t*-test, or the non-parametric equivalent Wilcoxon test. SPSS v.28.0.0.0 was used for statistical analysis with an α-significance level of 0.05. Mean and standard deviation were reported. Graphs were created using GraphPad Prism 5.

3 | RESULTS

The UV-fluorescent DETEX allowed to study the mice in their active period, in the dark. The DETEX provided an excellent alternative to carmine red, as it was clearly visible with a UV flashlight (Figure 1H). There were no adverse effects on body weight nor observations of discomfort (e.g., altered activity, behavior, or facial expression) after the procedures. To assess the effects of the four conditions (RED, RED+LOP, DETEX, and DETEX+LOP; Table 1) and the eight different groups (cross-over study design) and possible interaction (group*condition) on WGTT, a two-way mixed ANOVA was used. This showed a significant effect of condition on WGTT ($F(3, 48) = 61.736$, $p = 1.68 \times 10^{-16}$), while there was no significant group effect ($F(7, 16) = 1.183$, $p = 0.366$), nor interaction effect ($F(21, 48) = 1.255$, $p = 0.253$). Therefore, datapoints from all groups were plotted per condition for ease of interpretation (Figure 2). Pairwise comparison with Bonferroni correction showed that the WGTT in conditions RED (159.8 ± 46.4 min) and RED+LOP (280.6 ± 60.8 min) were significantly different ($p = 1.75 \times 10^{-7}$), and in the conditions DETEX (140.4 ± 29.2 min) and DETEX+LOP (210.5 ± 33.6 min; $p = 3.55 \times 10^{-7}$), showing that the refined method performs equally well as the standard method in measuring the loperamide-induced transit delay (Figure 2A). The WGTT in the RED and DETEX condition did not significantly differ. Interestingly, the coefficients of variation were larger for the RED and RED+LOP conditions (29.0% and 21.7%) compared to the DETEX and DETEX+LOP conditions (20.8% and 16.0%).

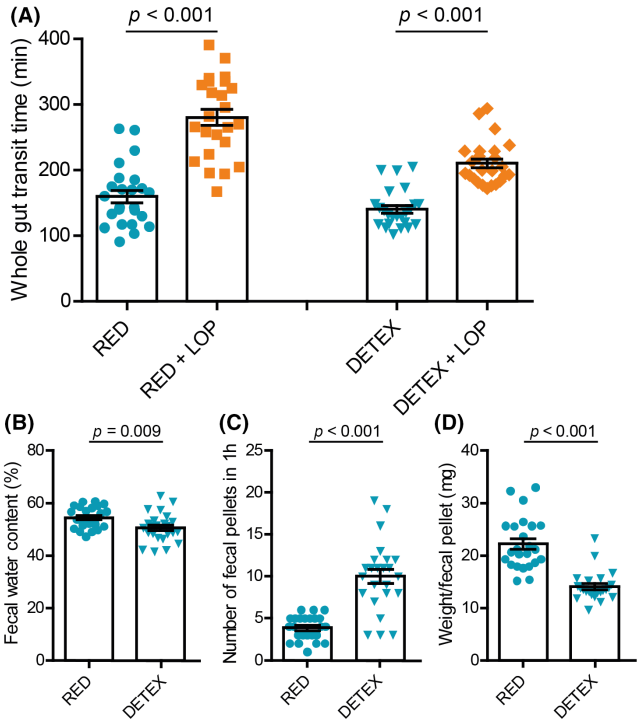

FIGURE 2 Comparison of whole-gut transit time (WGTT) and fecal pellet characteristics between the standard (RED) and refined (DETEX) method. Animals received oral gavage with carmine red (RED) or DETEX® Soft bait (DETEX) in absence or presence of loperamide (LOP, 3 mg/kg), and were subsequently monitored while housed under corresponding conditions to determine WGTT ($N = 24$; A). All dye-containing fecal pellets expelled within 1 h following the first pellet were collected, counted ($N = 24$; B), weighed ($N = 24$; C) and assessed for water content ($N = 24$; D).

Additionally, fecal pellet number, weight, and water content were compared between conditions. Fecal pellet number was significantly different between RED and DETEX (3.8 ± 1.5 and 10.0 ± 4.2, $t = -7.319$ and $p = 1.907 \times 10^{-7}$), as was fecal pellet weight (22.3 ± 5.0 g and 14.0 ± 2.9 g, $Z = -4.136$, $p = 3.5 \times 10^{-5}$) and fecal water content (54.5 ± 4.2% and 51.0 ± 5.2%, $t = 2.861$, $p = 0.009$; Figure 2B–D), indicating that the refined method results in altered fecal pellet characteristics compared to the standard method.

4 | DISCUSSION AND CONCLUSION

In this study, we show that oral administration of UV-fluorescent DETEX® allows for a refined method of whole-gut transit assessment in mice, by allowing observation during their active period

in the dark while socially housed in their home cage. This refined method of whole-gut transit assessment detects loperamide-induced delay of transit time equally well and results in less variation compared to the standard method, allowing the detection of alterations in transit time with lower numbers of experimental animals. Therefore, our refined method contributes to two of the 3Rs (Refinement and Reduction), a guiding principle to improve animal experimentation.[20,21]

As the refined method differs from the standard method in more than one way, that is, dye (nutrient) composition, light/dark schedule, and housing conditions, it is difficult to attribute the reduced variation and differences in fecal properties to a single factor. The possible disturbance of mice due to exposure to UV light in the dark phase cannot be excluded; however, the refined method allows social interaction, more activity and better thermoregulation, thereby reducing stress and avoiding impact on physiological parameters and disturbance of circadian rhythm. Moreover, biological rhythms, food intake and stress significantly affect gastrointestinal motility,[22–26] affirming the importance of the timing of testing and considerations for experimental housing conditions. Although other optimizations of the whole-gut transit have been described by others,[27,28] our method is, as far as we know, the first to include the influence of housing conditions and social interaction in the experimental design to improve welfare.

In conclusion, we developed an optimized and refined approach to measure WGTT in mice, that allows for reducing experimental animal numbers due to reduced variability within groups.

AUTHOR CONTRIBUTIONS

SS, RCJL, VM, WB, and SLS were involved in the study design. SLS, SS, and MST performed the experiments. SLS performed data analysis and created the figures. All authors edited and approved the manuscript versions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no commercial interest in any of the described materials, their providers, or used methods.

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REFERENCES

1. Camilleri M, Linden DR. Measurement of gastrointestinal and colonic motor functions in humans and animals. *Cell Mol Gastroenterol Hepatol*. 2016;2(4):412-428.
2. Nagakura Y, Naitoh Y, Kamato T, Yamano M, Miyata K. Compounds possessing 5-HT3 receptor antagonistic activity inhibit intestinal propulsion in mice. *Eur J Pharmacol*. 1996;311(1):67-72.
3. Miller MS, Galligan JJ, Burks TF. Accurate measurement of intestinal transit in the rat. *J Pharmacol Methods*. 1981;6(3):211-217.
4. De Winter BY, De Man JG, Seerden TC, et al. Effect of ghrelin and growth hormone-releasing peptide 6 on septic ileus in mice. *Neurogastroenterol Motil*. 2004;16(4):439-446.
5. Hennig GW, Costa M, Chen BN, Brookes SJ. Quantitative analysis of peristalsis in the Guinea-pig small intestine using spatio-temporal maps. *J Physiol*. 1999;517(Pt 2):575-590.
6. Roberts RR, Murphy JF, Young HM, Bornstein JC. Development of colonic motility in the neonatal mouse-studies using spatiotemporal maps. *Am J Physiol Gastrointest Liver Physiol*. 2007;292(3):G930-G938.
7. Fida R, Lyster DJ, Bywater RA, Taylor GS. Colonic migrating motor complexes (CMMCs) in the isolated mouse colon. *Neurogastroenterol Motil*. 1997;9(2):99-107.
8. Padmanabhan P, Grosse J, Asad ABMA, Radda GK, Golay X. Gastrointestinal transit measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT. *EJNMMI Res*. 2013;3(1):1-8.
9. Szarka LA, Camilleri M. Methods for measurement of gastric motility. *Am J Physiol Gastrointest Liver Physiol*. 2009;296(3):G461-G475.
10. Sasselli V, Boesmans W, Vanden Berghe P, Tissir F, Goffinet AM, Pachnis V. Planar cell polarity genes control the connectivity of enteric neurons. *J Clin Invest*. 2013;123(4):1763-1772.
11. De Schepper S, Verheijden S, Aguilera-Lizarraga J, et al. Self-maintaining gut macrophages are essential for intestinal homeostasis. *Cell*. 2018;175(2):400-415.e413.
12. Becker L, Spear ET, Sinha SR, Haileselassie Y, Habtezion A. Age-related changes in gut microbiota alter phenotype of muscularis macrophages and disrupt gastrointestinal motility. *Cell Mol Gastroenterol Hepatol*. 2019;7(1):243-245.e242.
13. Spear ET, Holt EA, Joyce EJ, et al. Altered gastrointestinal motility involving autoantibodies in the experimental autoimmune encephalomyelitis model of multiple sclerosis. *Neurogastroenterol Motil*. 2018;30(9):e13349.
14. Obata Y, Castaño Á, Boeing S, et al. Neuronal programming by microbiota regulates intestinal physiology. *Nature*. 2020;578(7794):284-289.
15. Gordon CJ. Thermal physiology of laboratory mice: defining thermoneutrality. *J Therm Biol*. 2012;37(8):654-685.
16. Ulfhake B, Lerat H, Honetschlager J, et al. A multicentre study on spontaneous in-cage activity and micro-environmental conditions of IVC housed C57BL/6J mice during consecutive cycles of bi-weekly cage-change. *PLoS One*. 2022;17(5):e0267281.
17. Olsson IAS, Westlund K. More than numbers matter: the effect of social factors on behaviour and welfare of laboratory rodents and non-human primates. *Appl Anim Behav Sci*. 2007;103(3):229-254.
18. Martinez V, Wang L, Million M, Rivier J, Taché Y. Urocortins and the regulation of gastrointestinal motor function and visceral pain. *Peptides*. 2004;25(10):1733-1744.
19. Jud C, Schmutz I, Hampp G, Oster H, Albrecht U. A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. *Biol Proced Online*. 2005;7:101-116.
20. Russell WMS, Burch RL. *The Principals of Humane Experimental Technique*. Universities Federation for Animal Welfare; 1959 (as reprinted 1992).
21. Guhad F. Introduction to the 3Rs (refinement, reduction and replacement). *J Am Assoc Lab Anim Sci*. 2005;44(2):58-59.
22. Hoogerwerf WA. Role of clock genes in gastrointestinal motility. *Am J Physiol Gastrointest Liver Physiol*. 2010;299(3):G549-G555.

23. Hoogerwerf WA, Shahinian VB, Cornélissen G, et al. Rhythmic changes in colonic motility are regulated by period genes. *Am J Physiol Gastrointest Liver Physiol*. 2010;298(2):G143-G150.
24. Martínez V, Wang L, Rivier J, Grigoriadis D, Taché Y. Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *J Physiol*. 2004;556(1):221-234.
25. Xie X, Kukino A, Calcagno HE, Berman AM, Garner JP, Butler MP. Natural food intake patterns have little synchronizing effect on peripheral circadian clocks. *BMC Biol*. 2020;18(1):1-11.
26. Francois M, Canal Delgado I, Shargorodsky N, Leu C-S, Zeltser L. Assessing the effects of stress on feeding behaviors in laboratory mice. *Elife*. 2022;11:e70271.
27. Baker KL, Izydorczak M, Jackson R, Verhagen JV. An automated sensitive approach for measuring whole gut transit time. *Neurogastroenterol Motil*. 2020;32(9):e13894.
28. Kacmaz H, Alto A, Knutson K, et al. A simple automated approach to measure mouse whole gut transit. *Neurogastroenterol Motil*. 2021;33(2):e13994.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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