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RADIOGRAPHIC ASSESSMENT OF THE IMPACT OF SEX AND THE CIRCADIAN RHYTHM-DEPENDENT BEHAVIOR ON GASTROINTESTINAL TRANSIT IN THE RAT

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RADIOGRAPHIC ASSESSMENT OF THE IMPACT OF SEX AND THE CIRCADIAN RHYTHM-DEPENDENT BEHAVIOR ON GASTROINTESTINAL TRANSIT IN THE RAT

Sex, circadian rhythm & GI transit

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ABSTRACT

Relatively little is known about the influence of sex and the circadian rhythm on gastrointestinal transit. However, these factors could have an important impact on aspects such as digestion, oral absorption of drugs or the clinical manifestation of gastrointestinal diseases, among others. Remarkably, preclinical models have scarcely taken these factors into consideration. In this study, we assessed the gastrointestinal transit of young adult Wistar Han rats of both sexes, under normal and inverted light cycle. To do this, serial radiographs were taken for 24h (T0-T24) after intragastric barium administration and subsequently analyzed to construct transit curves for each gastrointestinal region. Under a normal light cycle, transit curves were similar, except for a slower transit in females compared with males from T8 to T24. Under the inverted cycle, there was a significant acceleration in stomach emptying (similar in both sexes), emptying of the small intestine (even faster in females) and filling of the caecum and colon (which was also even faster in females). This study confirms, using X-ray non-invasive methods for the first time, that both, sex and circadian rhythm (probably through its effect on behavior) influence gastrointestinal transit in laboratory animals.

KEYWORDS: circadian rhythm, gastrointestinal transit, radiographic methods, rat, sex.

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INTRODUCTION

Gastrointestinal transit may be influenced by many factors that cause relevant inter- and intra-subject variability, both in human and animal models. Amid these factors, the role of sex and the circadian rhythm on gastrointestinal transit has been scarcely studied although they could be important factors in processes such as digestion, oral absorption of drugs or gastrointestinal pathologies, among others.

In relation to the impact of sex, early human studies showed a shorter gastrointestinal transit time in healthy men compared to women[1-4]. Recent data further support the concept that men have faster gastric emptying and intestinal transit than women[5]. Sex hormones, on the one hand, and the phases of the menstrual cycle, on the other, are important variables to consider.[6] With respect to the circadian rhythm, most life forms engage a 24-hour cycle of feeding and fasting.[7] However, relatively little attention has been paid to the investigation of the relationship between the circadian rhythm and the functions of the alimentary tract.[8,9] For example, one early study compared colonic transit in healthy patients using 24-hour ambulatory colonic manometry, and showed significant less pressure activity in the colon during daylight hours in women when compared to men.[10] Similarly, in a recent investigation in mice, both sex and time of the day when the experiments were carried out significantly influenced intestinal transit.[11]

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In general, preclinical studies have used invasive techniques to evaluate the effects of sex and/or circadian rhythm on gastrointestinal motor function.[11] An attractive and non-invasive alternative is the use of radiographic techniques, which allow the study of gastrointestinal transit and changes in size and density of the gastrointestinal regions using radiopaque contrast.[12] Until now, we and others have used these techniques to evaluate the impact on gastrointestinal transit of different drugs as well as to analyze gastrointestinal transit in aged or stressed animals, or in those exposed to different dietary modifications (see supplementary Table I for references). However, these studies were carried out with rodents (mainly male or both sexes, without comparison) under normal light cycle (lights on during the day: animals are studied in their low-activity circadian phase) (supplementary Table I). To our knowledge, radiographic studies which compare gastrointestinal transit in male and female laboratory animals under both normal and inverted light cycles are lacking.

Therefore, the aim of this study was to evaluate the effect of sex and the circadian cycle on gastrointestinal transit, using radiographic techniques.

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METHODS AND MATERIALS

Animals

The experiments were designed and performed in accordance with the EU Directive for the Protection of Animals Used for Scientific Purposes (2010/63/EU) and Spanish regulations (Law 32/2007, RD 53/2013 and order ECC/566/2015) and were approved by the Ethical Committee at Universidad Rey Juan Carlos (URJC) and Comunidad Autónoma de Madrid (PROEX 063/18, PROEX 023/19). The health and welfare of the animals used for the study was supervised by the personnel of the URJC Veterinary Unit where the study was performed. All experiments were designed to minimize the number of animals used and their suffering.

Male (N=24; weight= 342-520 g) and female (N=24; weight= 191-270 g) sexually-mature, young adult (3-4 months old) Wistar HAN healthy rats were obtained from the Veterinary Unit of URJC and housed (2-4/cage), after simple randomization, in standard transparent cages (60 x 40 x 20 cm) in a temperature (20ºC) and humidity-controlled room (60%), with a 12 h light/12 h dark cycle (lights off between 20:00 and 08.00 hours for animals with normal light cycle conditions or between 8.00 and 20:00 hours for animals with inverted light-dark cycle). Animals were divided in 4 groups (N=12/group): Males, Normal Cycle (M-N) (this was considered the control or reference group); Males,

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Inverted Cycle (M-I); Females, Normal Cycle (F-N); Females, Inverted Cycle (F-I). Animals had free access to standard laboratory rat chow (LASQ diet® Rod 14-A www.altromin.de) and tap water until sacrifice.

Gastrointestinal transit

Gastrointestinal motor function was evaluated once in the URJC animal facility, radiographically, as described.[12] Prior to the X-ray assay, the experimental animals were not fasted, due to the long duration of the X-ray study (24 h), but all of them were weighed, and the estrous cycle phase of females was analyzed by vaginal cytology.[13,14] In addition, their health conditions were observed before and during the experimental procedures, i.e. the appearance and color of the hair coat, legs, eyes and nose and also their behavior and movement. For the radiographic evaluation, barium sulfate suspension (Barigraph® AD, Juste SAQF, Madrid, Spain; 2 g mL−1 in tap water, temperature=22ºC, 2.5 mL) was administered by gavage at 9 am and serial radiographs were obtained at 0, 1, 2, 4, 6, 8 and 24 h (T0-T24) after contrast administration. Plain facial radiographs of the gastrointestinal tract were obtained using a CS2100 (Carestream Dental, Madrid, Spain) digital X-ray apparatus (60 kV, 7 mA), and X-rays were recorded on Carestream Dental T-MAT G/RA film (15×30 cm) housed in a cassette provided with regular intensifying screen. Exposure time for X-ray shots was set to 0.02 seconds and focus distance was manually fixed to 50±1 cm. Immobilization of the rats

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in prone position was achieved by placing them inside hand-made transparent plastic tubes (recording chamber), which were adjusted to the size of the rat so they could not move, scape or turn around (Fig. S1). Moreover, training was not necessary, because, as shown before, this procedure does not cause stress-induced alterations in gastrointestinal transit.[12] Radiographs were then developed using a Kodak X-OMAT 2000 automated processor (Kodak AG, Stuttgart, Germany). For each animal, radiographs were taken in the same order at each time point, so that time intervals between shots were of the same duration for all animals.

The analysis of the radiographs was performed by a trained investigator who was blinded to the experimental groups. Transit curves were constructed for each gastrointestinal region (stomach, small intestine, caecum and colorectum) using a semi-quantitative score, assigning a range of values to each region considering the following parameters (Fig. S2): percentage of the region filled with contrast (0–4); contrast intensity (0–4); contrast homogeneity (0–2); and sharpness of the profile of the gut region (0–2). Each of these parameters was scored and summed (0–12 points). In addition, the size and density of the barium contrast were analyzed for stomach, caecum, and fecal pellets, with the aid of an image analysis system (Image J 1.38 for Windows, National Institute of Health, USA, free software: http://rsb.info.nih.gov/ij/).

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The number of fecal pellets within the colorectum was also determined for each rat at each time point.

Moreover, at T0, right after the administration of barium, the animals were placed in new cages with fresh bedding and the feces present in the cage at each time point of the radiographic session (T1-T24) were collected. The following parameters were measured: the % of labeled feces and their radiopacity; the weight of the feces at collection and after drying them in an oven (70 ºC, 24-48 h); their moisture (dry vs. wet fecal material, as difference).

Statistical analysis

Sample size for each experiment was estimated using G*power assuming $\alpha = 0.05$ and power = 0.8 and 2-tailed tests. Mean and SD for the variables of the control group in the gastrointestinal transit experiments were based on those obtained in our previous study.[12]

Data were analyzed using Graph PadPrism, v. 7.0.®. Data are presented as the mean values ± SEM. All the data obtained during the experiments were included in the statistical analysis, and no animal was excluded from the analysis. Each animal was considered as an experimental unit when analyzing the differences related to transit, whilst the cages were considered as the experimental unit when analyzing the data

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related to feces. All data passed the D'Agostino and Pearson's normality test, thus differences between groups were analyzed using unpaired Student's t-test, with Welch's correction when appropriate, or one- or two-way ANOVA followed by Tukey post-hoc multiple comparison tests. The differences between female groups regarding the distribution of the estrous cycle phases were analyzed with the Chi-square test. Values of $P < 0.05$ were regarded as being significantly different.

RESULTS

Animal characteristics at T0

Body weight was significantly lower in females when compared to males. Additionally, the average weight of M-I was significantly higher than that of M-N (Fig. 1A).

As seen in Fig. 1B, just before the X-ray scan, all phases of the estrous cycle (Fig. 1C) were represented in F-N whereas only three of them were represented in F-I. However, these differences were not statistically significant ($p= 0.3$).

Radiographic analysis of gastrointestinal motor function

Semiquantitative analysis

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Gastric emptying in animals under normal cycle (M-N, F-N) was progressive from barium administration (T0) until the end of the study (T24) without statistically significant differences between sexes (Fig. 2A, 2F). Likewise, gastric emptying of the animals under inverted-cycle (M-I, F-I) was similar between males and females, but significantly faster compared to their sex-matched group under normal cycle. (Fig. 2A, 2F).

In the small intestine, as in the stomach, the inverted cycle groups showed a significantly faster emptying of the small intestine than the normal-cycle ones (Fig. 2B, 2F). Additionally, no significant sex-dependent differences were found in animals with the same cycle, except for a faster intestinal emptying in F-I compared to M-I at T2, and a higher barium content in F-N compared to M-N at T24 (Fig. 2B).

In the normal-cycle groups, barium reached the caecum at T2 after administration and completely filled this organ by T4 (Fig. 2C, 2F). Caecum emptying only started after T8, and at T24 it was almost empty in M-N but not in F-N (Fig. 2C, 2F). In the inverted-cycle groups, caecum filling was slightly but significantly faster at T2 and its emptying was also slightly faster in the inverted-cycle animals, although at T24 M-I was significantly slower than M-N and F-I was significantly faster than F-N and M-I, and similar to M-N (Fig. 2C, 2F).

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Finally, in the normal-cycle groups barium reached the colorectum at T4 after administration and completely filled this organ by T8, with no significant differences between sexes. Nevertheless, at T24, whilst in M-N the colorectum was almost completely empty again, in F-N it showed significantly more barium (Fig. 2D, 2F). Again, colorectum filling was, in general, faster in the inverted-cycle groups, particularly in F-I, which reached the colorectum already at T2 (Fig. 2D, 2F). At T24 all groups showed more barium than M-N in the colorectum (Fig. 2D).

Fecal pellet number in the colorectum

The number of fecal pellets counted in the colorectum followed the same trend as the semiquantitative score in this organ, with no differences found in the amount of feces observed between F-N and M-N, except at T24 (Fig. 2D, 2E). Likewise, the occurrence of fecal pellets was accelerated in the animals under inverted cycle, particularly in females, although males presented a much larger amount of feces than females, with the maximum number occurring at T6 in both sexes, whereas it was at T8 in the normal cycle groups (Fig. 2E).

Morphometric and densitometric analysis

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The morphometric (size) and densitometric (contrast density) analysis of stomach, caecum and fecal pellets showed similar changes throughout the experiment, as those found in the semiquantitative study. Thus, here we will focus on the maximum values obtained for size and contrast density of these items.

The maximum size of the stomach at T0, was around 480-550 mm^2, except for F-I, which was significantly smaller, around 376 mm^2 (Fig. 3A). The maximum gastric density, also obtained at T0, was close to 100% for all groups (Fig. 3B).

In contrast, the maximum size of the caecum was slightly, but significantly, smaller in females than in males, regardless of the type of light cycle (Fig 3C). When analyzing the density, all groups reached similar maximum values at T2-T4, without statistically significant differences at these time points (Fig. 3D).

Finally, the fecal pellet area and density values were averaged between T4 and T8 (when these values reached their maximum). The maximum size was similar for all groups, around 70-85 $mm^{2,}$ except for F-I which was significantly smaller, around 53 mm^2 (Fig. 3E). With respect to barium density, the fecal pellets of the M-I group had a lower density than the M-N group, whilst no differences were observed due to the cycle in

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females. Furthermore, the density in the F-I group was higher when compared to M-I (Fig. 3F).

Characteristics of the feces collected during the X-ray session

Figure 4A shows representative images of barium-stained and non-stained fecal pellets at T24. Rats expelled 0-4 fecal pellets per hour, without significant differences among groups (Fig. S3A). The percentage of expelled stained fecal pellets increased in all groups in a time-dependent manner, with the F-I group being significantly faster than the other groups, followed by M-I, M-N and F-N, in that order (Fig. 4B). The radiopacity pattern was similar to that of the % of stained fecal pellets, but interestingly M-I practically overlapped with M-N throughout the whole study, whilst significant differences in the radiopacity along time were found between F-N and F-I (Fig. 4C).

To evaluate the moisture of the feces, the difference between wet and dry weight (wet weight – dry weight; Fig. S3B and C show these parameters individualized) was calculated. All the groups had similar values throughout the experiment except M-N group at T1, when the difference was significantly greater compared to the rest of the groups. The other groups had a value of about half of that found in M-N at T1 (Fig. 4C).

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DISCUSSION

Although many different techniques have been used to analyze gastrointestinal transit in laboratory animals (for example, see Table S1 in [15]), non-invasive techniques are preferable for both ethical reasons and translatability. In the present study we have demonstrated, for the first time using non-invasive radiographic techniques, the effects of the circadian rhythm and its related behavior and that of sex on gastrointestinal transit. Importantly, our results agree with those of other researchers using other invasive or indirect techniques,[11,16] with the advantages of including a relatively low number of animals and obtaining more detailed information from the different gastrointestinal organs along time.

X-ray study of gastrointestinal transit in male rats under normal light cycle

In this 24-hour study, we used the M-N group as a reference, in the same way as in most rodent X-ray studies, including those carried out by our research group in rats (Supplementary Table I), since the transit patterns are well established in these animals. As expected, in this study the transit pattern in M-N group was similar to that previously found by other authors and also by our group.[12,17-19]

The present study benefits from the performance of a comprehensive analysis of the fecal pellets collected during the radiographic session. The percentage of stained fecal

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pellets showed a progressive increase from T4 to T24. Similar to the number of stained fecal pellets within the colorectum, radiopacity increased up to T8 and decreased afterwards. Since radiopacity is measured using the average of all fecal pellets, the decrease at T24 is a reflection of the production of new pellets (without staining) during the night, when animals are more active and also eat more.[20-22]

The increased moisture (associated with highest wet fecal matter expulsion, Fig. S3B) of the fecal pellets collected at T1 (Fig. 4D), probably reflects some level of psychological stress, since increased fecal moisture and fecal production are generally considered as indirect markers of stress in male rats.[23]

X-ray study of gastrointestinal transit in female rats under normal light cycle

To our knowledge, no previous study has specifically evaluated the influence of sex on gastrointestinal transit using radiographic methods in rodents. In the few radiographic studies in which females were used, results from animals of both sexes were either combined[24-28] or evaluated separately without a specific comparison[29] and methodological differences (including animal species) preclude proper comparison with our results. In the present study, the F-N transit curves were similar to those of M-N from the moment of barium administration (T0) until T8 for all regions, but from this point till T24 gastrointestinal transit was delayed in F-N. Although early human studies

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showed shorter gastrointestinal transit times in healthy men compared to healthy women,[1-5] our results suggest that, under normal light conditions, gastrointestinal transit is equivalent in rats of both sexes for the first 8 h, when the animals are relatively inactive, eat less and, consequently, their gastrointestinal motility is less stimulated (which could be somehow similar to fasting in humans). Afterwards, during the activity phase, transit of the large intestine appears to be delayed in females compared to males, with a certain degree of retention of barium-stained content in both the caecum and colorectum. The reduction in the maximum size of the caecum found in females, is probably related to its sexual dimorphism in body weight.[30,31] However, these morphometric differences would have favored a faster transit in the large intestine. Thus, they do not seem to contribute to the transit differences between the two sexes under normal cycle.

In F-N, the curve for the percentage of stained fecal pellets showed a similar pattern to those of M-N, except for the fact that at T8 no stained fecal pellet was recovered from the cage. Interestingly, the absence of stained fecal pellets in the cage at T8 was followed by a slight increase in stomach size and small intestine staining at T24 in this group of animals, maybe due to coprophagia, which is a common behavior in rats.[32,33]

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A difference between sexes, unlikely related with their body weight, was the fact that at T1 females produced less fecal matter with significantly lower moisture. Interestingly, in a previous study also performed in male and female mice under normal light cycle, we found similar results.[34] In that study, mice were isolated in cages without bedding for 4 hours after intragastric administration of barium and the fecal pellets produced were radiographically analyzed. Despite the evident methodological differences, in both species, males produced more feces and with more moisture at the beginning of the study than at later moments, reflecting a certain level of initial stress, perhaps associated with the manipulation (barium administration) and the new conditions (new cage). This phenomenon may reflect some important dimorphism in rodent biology that deserves further investigation regarding its mechanisms and function and could be attributed to differences in the gastrocolic response to mechanical stimulation of the stomach by barium administration and/or psychological stress associated with the initial handling and exposure to the new environment, aforementioned.[23,35]

Influence of the circadian rhythm on the gastrointestinal transit of male and female rats

Although the impact of the circadian rhythm on gastrointestinal transit has been evaluated in different species, including humans,[36-38] to the best of our knowledge, it has never been addressed in laboratory animals using radiographic methods.

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Compared with M-N, M-I showed much faster transit in the upper gastrointestinal tract (stomach and small intestine) and faster filling and emptying of the caecum and the colorectum during the first 8 h of the study. However, emptying of caecum and colorectum was delayed at T24. These results were expected, since in the M-I group the experiments performed from T0-T8 occur during their activity phase, when animals move, eat, and defecate more. [20-22,39]

Interestingly, the moisture of the fecal pellets did not increase at T1 in M-I as seen for M-N, suggesting that during their activity phase the males might be less sensitive to the stress produced by the new experimental conditions (transport to the X-ray room, barium gavage, brief restraint…) than during their inactivity phase.

Finally, in F-I, gastric emptying was similar to that of M-I but emptying of the small intestine and caecum was much faster in F-I than in any other group, including F-N, leading to much faster colorectum filling which was also reflected in a higher percentage of expelled stained pellets at earlier times. Furthermore, female groups were not significantly different in terms of their body weight or their distribution among estrous phases, suggesting that these factors had little contribution to our transit results. In the morphometric analysis, F-I animals showed smaller stomach (at T0) and fecal pellets (at T4-T8), but their maximum caecum size (at T4-T6) was not significantly different from

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that of F-N group. Thus, although we did not measure the small and large intestine lengths at sacrifice, which would have helped to ascertain this issue, it is unlikely that the morphometric differences found in the X-rays explain such a fast gastrointestinal transit in F-I group. Furthermore, a higher level of stress at the beginning of the study does not seem to underlie the faster transit either, since at T1 the moisture parameters of fecal pellets were as in M-I and F-N. Nevertheless, our results agree with a recent invasive study in mice, in which Soni et al[11] compared the transit of males and females at different phases of the day and with different fasting times. They found that females analysed in the morning had a slower gastrointestinal transit than those analysed in the afternoon and concluded that females are more sensitive than males to the phase of the circadian rhythm. Moreover, an indirect study, based on the analysis of the microbiota, also found differences between the sexes associated with the circadian rhythm.[16]

Although other activities, such as locomotor activity, may affect gastrointestinal transit, the impact of food ingestion is a relevant driving force leading to its acceleration. In this sense, food ingestion increases during the phase of activity, which corresponds to the lights off period[20]. Although fasting is usually imposed in gastrointestinal transit studies and its duration has an impact on the results,[11] in the present study we did not fast the animals before the experiments for ethical reasons (fasting duration would have been much longer than 24 h). Therefore, manipulation, which was the same for all animals,

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was only limited to the unavoidable handling of the animals needed to take the X-rays. Thus, in this study, the animal activities that normally take place during the different moments of the day were only minimally altered.

Finally, it could seem that our results were mainly due to the difference in body weight displayed by male and female rats (which ranged from 72 to 329 g). In agreement, M-N tended to produce more wet and dry fecal matter than F-N, particularly at T6-T24 (Fig. S3B, C). However, the amount of fecal matter collected from the cage of the animals under the inverted cycle, was practically the same up to T8, regardless of their sex (Fig. S3B, C). Thus, the differences in body weight alone do not suffice to explain our results on fecal matter production and gastrointestinal transit.

CONCLUSIONS

In the present study, the influence on gastrointestinal transit of sex and the circadian rhythm and its related behavior was evaluated in the rat using radiographic methods for the first time. When the study was performed under normal light cycle, i.e., during the inactivity phase of the animals, males and females had similar transit times despite their different body weight and slightly different defecation. Under an inverse light/dark cycle, animals of both sexes showed an accelerated gastrointestinal transit compared to animals under a normal light cycle, but females displayed an even more accelerated

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transit when compared to males, although fecal matter production was similar. Thus, both sex and the circadian rhythm (or its associated feeding and locomotor activities) have a paramount influence on gastrointestinal transit.

Our results highlight the need for more detailed studies to precisely define the influence of sex on the gastrointestinal and other physiological functions, and how these functions change throughout the day.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

RA designed the study and provided financial support. CGR, LLG, YLT y AB performed the experiments. CGR analyzed the data. CGR and RA wrote the manuscript. MLSM provided

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essential intellectual input. All authors critically reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author (Raquel Abalo) upon reasonable request. Contact email: Raquel.abalo@urjc.es

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FIGURE LEGENDS

Figure 1. Animal characteristics at T0. (A) Body weight, values represent the mean ± SEM. (B) Estrous cycle phase, values represent the % of females in each phase. *$^{\#}$ p<0.05, $^{\#\#\#\#}$ p<0.0001 vs M-N; $^{\$\$\$\$}$ p<0.0001 vs M-I* (One-way ANOVA followed by Tukey post-hoc test). (C) Representative images of the estrous phases.

Figure 2. Radiographic study of the differences in gastrointestinal transit by sex and circadian rhythm: semiquantitative analysis. Data represent mean ± SEM for motor function in stomach (A), small intestine (B), caecum (C) and colorectum (D). (E) Number of fecal pellets stained within the colon at each time point of the X-ray session. *$^{\#}$ p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#\#}$ p<0.0001 vs M-N; $^{\$}$ p<0.05, $^{\$\$\$\$}$ p<0.0001 vs M-I; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs F-N* (Two-way ANOVA followed by Tukey post-hoc test). (F) Representative X-rays of rats.

Figure 3. Radiographic study of the differences in gastrointestinal transit by sex and circadian rhythm: morphometric and densitometric analysis. (A), (C), (E) Changes in the size of stomach, caecum and fecal pellets, respectively. (B), (D), (F) Changes in density of barium within the same stained organs. Values represent the mean ± SEM. *$^{\#}$ p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#\#}$ p<0.0001 vs M-N; $^{\$}$ p<0.05, $^{\$\$}$ p<0.01, $^{\$\$\$}$ p<0.001 vs M-I; *p<0.05, **p<0.01,*

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$^{****}p<0.0001$ *vs F-N* (A-D, Two-way ANOVA followed by Tukey post-hoc test; E-F, One-way ANOVA followed by Tukey post-hoc test). Abbreviations: M-N, male-normal cycle; F-N, female-normal cycle; M-I, male-inverted cycle; F-I, female-inverted cycle.

Figure 4. Characteristics of the feces collected during the X-ray session: staining and moisture. (A) Representative images showing a photograph of the feces collected at T24 in one cage (left) and their radiographic appearance (right). Barium-stained, residually-stained and non-stained fecal pellets are shown. (B) % of stained fecal pellets. (C) Radiopacity. (D) Fecal pellet moisture measured as difference (wet-dry fecal matter). Data represent the mean ± SEM. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ *vs M-N;* $^{\$\$\$}p<0.001$, $^{\$\$\$\$}p<0.0001$ *vs M-I;* $^{*}p<0.05$, $^{****}p<0.0001$ *vs F-N* (Two-way ANOVA followed by Tukey post-hoc test). Abbreviations: M-N, male-normal cycle; F-N, female-normal cycle; M-I, male-inverted cycle; F-I, female-inverted cycle.

Figure S1. Restraining device for the radiographic study. The restraining device is a hand-made flexible transparent plastic tube with two hind flaps and one front flap that allow the non-stressful insertion and release of the animal, respectively (A). To limit the movement of the animals during the X-ray procedures, they are inserted in the restraining tube. Once the animal has entered the trap, the base is closed with two

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Velcro tabs located at the base of the tube itself (hind flaps); after the X-ray has been taken, the animal is allowed to exit the tube by opening the front flap (B).

Figure S2. Characteristic transit pattern for the stomach, small intestine, caecum and colorectum obtained from male rats during normal cycle. A single dose of barium sulfate (2.5 mL, 2 g mL−1) was intragastrically administered at time 0 and X-rays were taken immediately at 0, 1, 2, 4, 6, 8 and 24 h after administration. In (A), (B), (C) and (D) the data for each parameter analyzed at each time point for each organ are shown: Percentage of the organ filled with contrast (P, up to 4 points); Intensity of contrast (I, up to 4 points); Sharpness of the profile of the organ (S, up to 2 points); Homogeneity of contrast (H, up to 2 points). In (E) the sum of each of the analyzed parameters for each organ is shown at each of the experimental time points. Data represent the mean±SEM. (F) Representative X-rays obtained from the normal cycle male rats at 1, 4, 8 and 24 h after administration of barium sulfate. Abbreviations in F: St, stomach; SI, small intestine; C, caecum; FP, fecal pellets in the colorectum.

Figure S3. Characteristics of feces collected during the X-ray session. Motor function was measured by radiological methods (see text). Four groups of animals were used, according to sex (males, M; females, F) and the exposure to normal (lights on 8 am to 8 pm, N) or inverted

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(lights on 8 pm to 8 am, I) light cycle: M-N, M-I, F-N, F-I. The fecal pellets were collected from the cages along the X-ray session and weighted both before and after drying in an oven (see text). Data represent the mean ± SEM for the number of fecal pellets (A), as well as the wet (B) and dry (C) weight of fecal pellets. *# p<0.05 vs M-N; * p<0.05 vs F-N* (Two-way ANOVA followed by Tukey post-hoc test).

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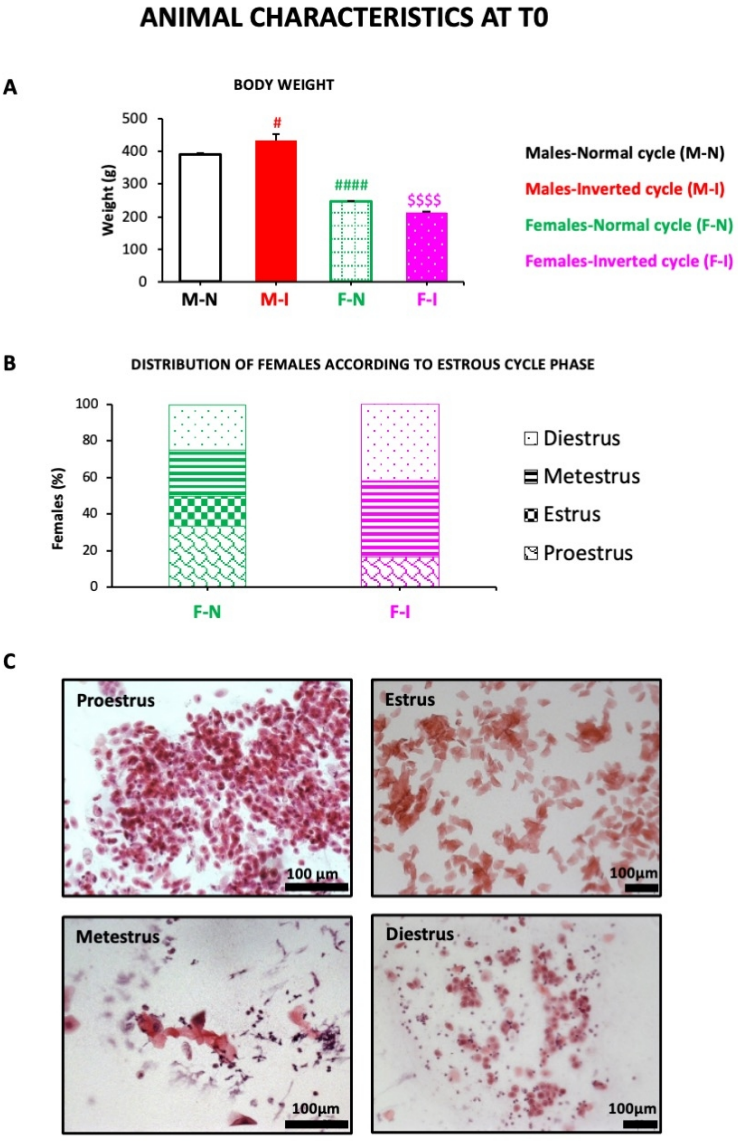

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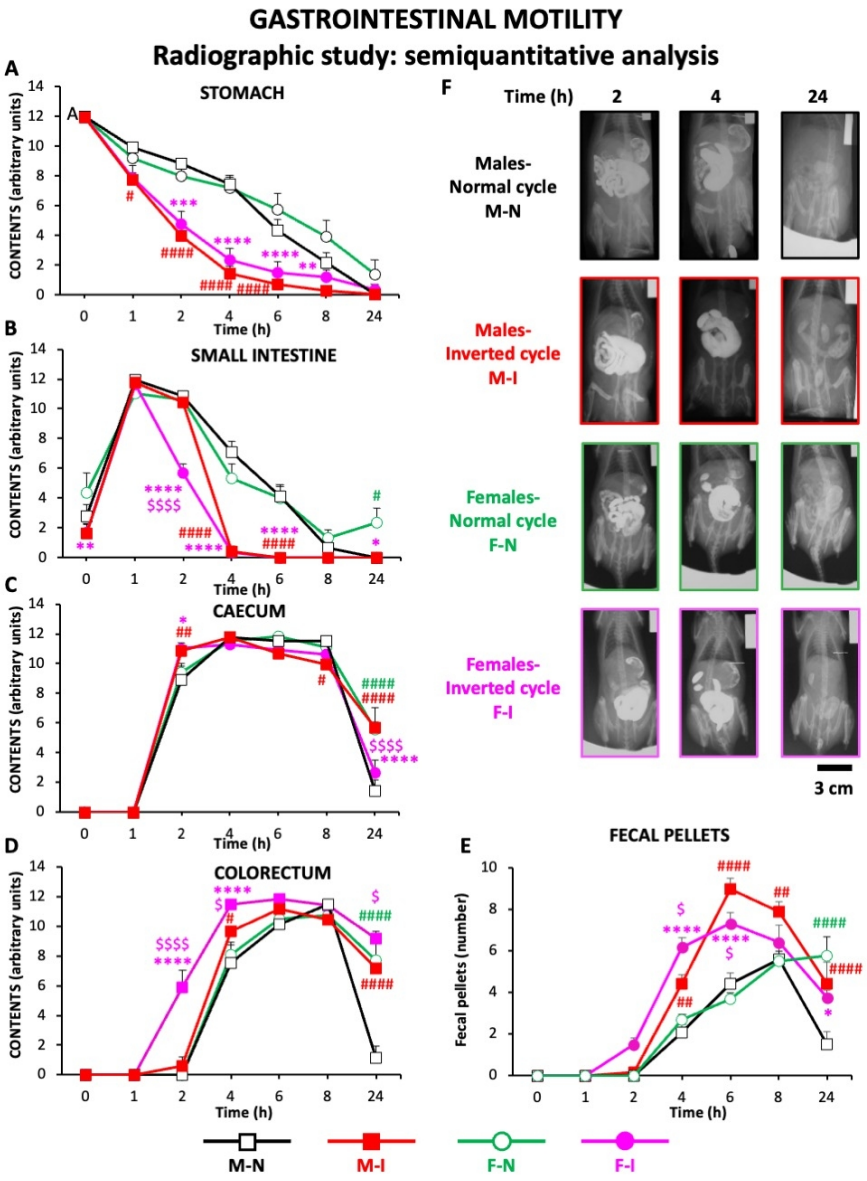

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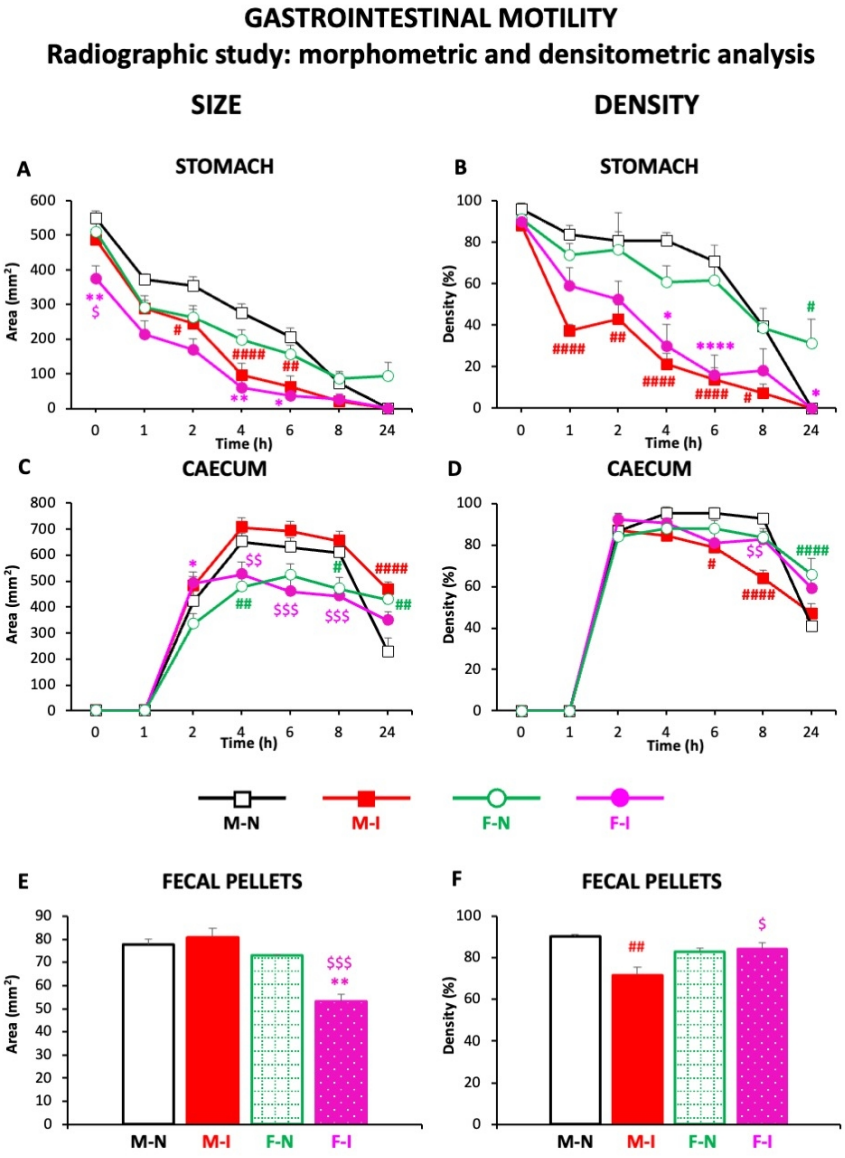

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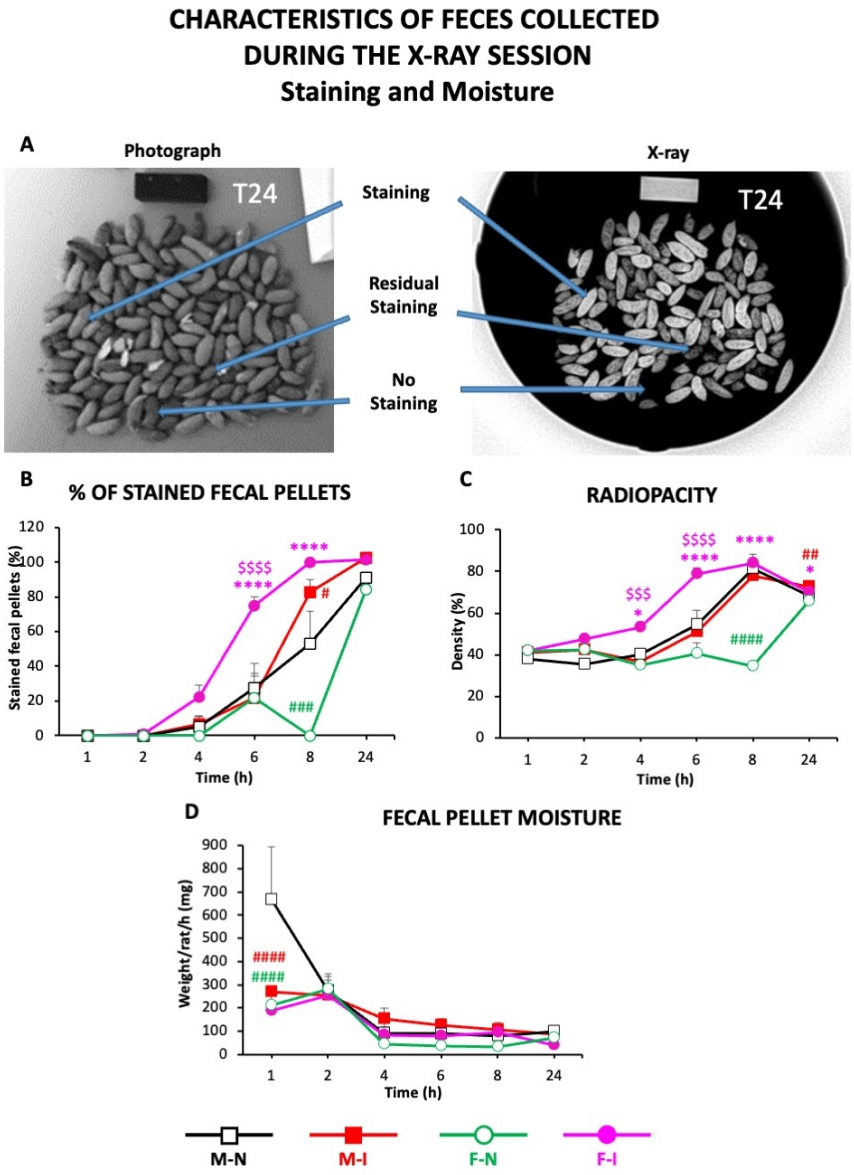

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Table I. Studies using non-invasive radiographic methods to evaluate gastrointestinal motility in small experimental animals (mainly rodents).

Year	Drug or condition studied	Species	Sex of animals	Light cycle phase	Reference
1979	No drug administration. Diabetic animals.	Chinese hamsters	Both, without comparison	N.S.	Diani et al., 1979
1983	Different drugs (dopamine antagonists, apomorphine, atropine, eserine, prazosin, propranolol).	Guinea pig	Both, without comparison	N.S.	Costall et al., 1983
1993	No drug administration. Groups with different ages at the point of X-ray study.	Rat	Female	N.S.	Perry et al., 1993
1994	No drug administration. 1,000 eggs of *Taenia taeniaformis* dosed orally.	Rat	Female	N.S.	Perry et al., 1994
1995	No drug administration. Dietary fiber (wheat bran) at 0, 20 or 40% (% weight).	Rat	N.S.	N.S.	Munakata et al., 1995
2005	Ethosuximide (150 mg/kg) oral administration for 15 days. One single neostigmine (4 ml kg^{-1} 0.25%, i.m.) or metoclopramide (10 mg kg^{-1}, i.p.) administration.	Rat	Male	N.S.	Sirakov et al., 2005
2008	One single cisplatin (3 or 6 mg kg^{-1}, i.p.) administration.	Rat	Male	Normal	Cabezos et al., 2008
2009	WIN 55,212-2 (0.5 or 5 mg kg^{-1}, i.p) administration, once a day for 14 consecutive days.	Rat	Male	Normal	Abalo et al., 2009
2010	One single WIN 55,212-2 (0.5, 1, 2 and 5 mg kg^{-1}, i.p.) administration. One single CB1 antagonist AM251 (1 mg kg^{-1}, i.p.) and/or the CB2 antagonist SR144528 (1 mg kg^{-1}, i.p.) administration.	Rat	Male	Normal	Abalo et al., 2010
2010	Cisplatin (at 1, 2, or 3 mg kg^{-1}, i.p.) administration once a week for four weeks	Rat	Male	Normal	Cabezos et al., 2010

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2011	WIN 55,212-2 (0.5 or 5 mg kg^{-1}, i.p.) administration alone or after CB1 antagonist/ inverse agonist AM251 (1 mg kg^{-1}, i.p.) administration, once a week for four weeks.	Rat	Male	Normal	Abalo et al., 2011
2013	One single loperamide (5 or 10 mg kg^{-1}, i.p.) administration.	Mice	Female	Inverted	Myagmarjalbuu et al., 2013
2013	WIN 55,212-2 (0.5 or 1 mg kg^{-1}, i.p.) and cisplatin (2 mg kg^{-1}, i.p.) administrations, once a week for four weeks.	Rat	Male	Normal	Abalo et al., 2013
2014	Loperamide (5 mg kg^{-1}, s.c.), metoclopramide (10 mg kg^{-1}, i.p.) or milk of magnesia (0.2 mL, gavage) administration.	Mice	Both, without comparison	Normal	Reed et al., 2014
2014	Granisetron (1 mg kg^{-1}, i.p.) and, 30 minutes after, cisplatin (2 mg kg^{-1}, i.p.), once per week for 4 weeks.	Rat	Male	Normal	Vera et al., 2014
2014	One single 6-OHDA stereotaxic administration in the medial forebrain bundle.	Rat	Male	Normal	Vegezzi et al., 2014
2015	MSG (4 g L^{-1}) in the drinking water for 6 weeks.	Rat	Male	Normal	López-Miranda et al., 2015
2015	One single AM841 (0.1 or 1 mg kg^{-1}, i.p.) or WIN 55,212-2 (5 mg kg^{-1}, i.p.) administration. One single CB1 (AM251, 1 mg kg^{-1}, i.p.) or CB2 (AM630, 1 mg kg^{-1}, i.p.) antagonist administration prior to the agonists.	Rat	Male	Normal	Abalo et al., 2015
2016	One single morphine (5 or 10 mg/kg, i.p.) administration.	Rat /mice	Male	Normal	Girón et al., 2016
2016	Prucalopride or loperamide (1, 2, or 4 mg kg^{-1}, s.c.) continuous (osmotic mini-pump) administration, for seven days, in aged animals (18 months-old).	Rat	Male	Normal	Dalziel et al., 2016
2016	5-FU (23 mg kg^{-1}, i.p.) administration three times a week for two weeks.	Mice	Male	Normal	McQuade et al., 2016
2017	No drug administration. Genetic model of IBD (Winnie mice).	Mice	Both, without comparison	Normal	Robinson et al., 2017

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2017	One single vincristine (0.1 or 0.5 mg kg^{-1}, i.p.) administration, alone or with CB1 (AM251) or CB2 (AM630) administered 1-3 times (1 mg kg^{-1}, i.p.; 20 min before, 12 h after, 24 after vincristine).	Rat	Male	Normal	Vera et al., 2017
2017	5-FU (150 mg kg^{-1}, i.p.) once a day for two consecutive days alone or with WIN 55,212-2 (0.5 mg kg^{-1}, i.p.) administration once a day for four days.	Rat	Male	Normal	Abalo et al., 2017
2017	Basal conditions. WKY (stress-prone) rats	Rat	Male	Normal	Dalziel et al., 2017
2017	Prucalopride or loperamide (1, 2, or 4 mg kg^{-1}, s.c.) continuous (osmotic mini-pump) administration, for seven days, in aged animals (18 months-old) fed a control or test diet (enriched in milk proteins, whey or casein, hydroized or not).	Rat	Male	Normal	Dalziel et al., 2017
2018	Oxaliplatin (3 mg kg^{-1}, i.p.) with or without BGP-15 (15 mg kg^{-1}, i.p.) administration three times a week for two weeks.	Mice	Male	Normal	McQuade et al., 2018
2018	Vincristine (0.1 mg kg^{-1}, i.p.) administration once daily in 2 cycles of 5 days each.	Rat	Male	Normal	López Gomez et al., 2018
2018	Normal rats at different ages (2-3, 12, 18, 24 months) Streptozotocin (60 mg kg^{-1}, i.p.) administration and X-ray evaluation 4 weeks after.	Rat	Male	Normal	Abalo et al., 2018
2019	Coffee silverskin melanoidins in drinking water for 4 weeks.	Rat	Male	Normal	Tores de la Cruz et al., 2019
2019	Granisetron (1 mg kg^{-1}, i.p.) followed by cisplatin (6 mg kg^{-1}, i.p.) administration 30 minutes after.	Rat	Male	Normal	Martín-Ruíz et al., 2019
2019	5-FU (23 mg kg^{-1}, i.p.) administration with or without BGP-15 (15 mg kg^{-1}, i.p.) three times a week.	Mice	Male	Normal	McQuade et al., 2019
2019	Spent coffee grounds (1 g kg^{-1}, gavage) administration by oral gavage every day for 4 weeks.	Rat	Male	Normal	Iriondo-DeHond et al., 2019

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2019	Loperamide (0.1, 1, or 10 mg kg^{-1}, i.p.) administration.	Rat	Male	Normal	Vera et al., 2019
2019	Four different diets (standard, AIN-93G and AIN-93G enriched in coconut or evening primrose oil) with a different amount and composition of fatty acids for 4 weeks.	Rat	Male	Normal	Mosinska et al., 2019
2019	LPS (0.1, 1 or 5 mg kg^{-1}, i.p.) administration.	Rat	Male	Normal	Abalo et al., 2019
2019	Viral antigen Poly I:C i.p. administration on gestational day 15. X-rays were taken at young adult age of the male offspring.	Rat	Male	Normal	Gálvez et al., 2019
2021	Genetic model of IBD (Winnie mice) treated with APX3330 (25 mg kg^{-1}, i.p.) administration, twice daily for 2 weeks	Mice	Both, without comparison	Normal	Sahakian et al., 2021
2021	Three different diets (AIN-93G and AIN-93G enriched in coconut or evening primrose oil) with a different amount and composition of fatty acids for 6 weeks.	Rat	Both, with comparison	Normal	Jacenik et al., 2021
2021	MSG (4 g L^{-1}) in drinking water for 6 weeks (0-5). Cisplatin (2 mg kg^{-1}, i.p.) administration on the first day of weeks 1-5.	Rat	Male	Normal	López-Tofiño et al., 2021
2021	No drug administration. Acute and subchronic stress through forced swim or restrain at 4ºC.	Rat	Male	Normal	Bagués et al., 2021

Abbreviations: 5-FU, 5-fluorouracil; 6-OHDA, 6-hydroxydopamine; i.m., intramuscular; ip., intraperitoneal; LPS, lipopolysaccharide; MSG, monosodium glutamate; N.S., not specified; s.c., subcutaneous.

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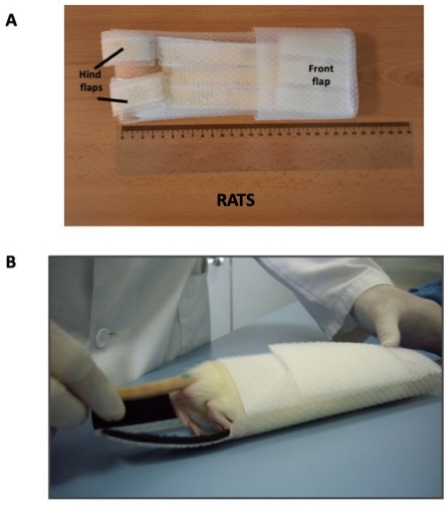

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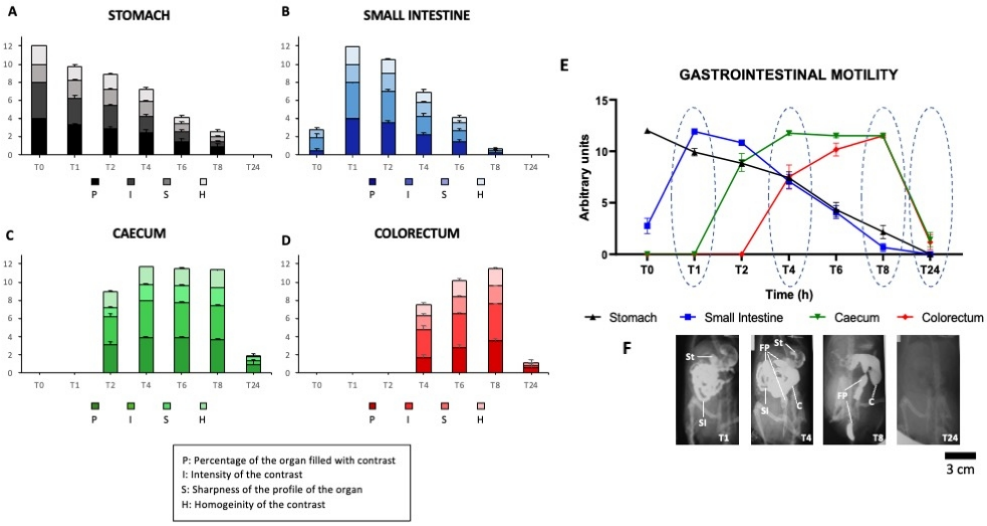

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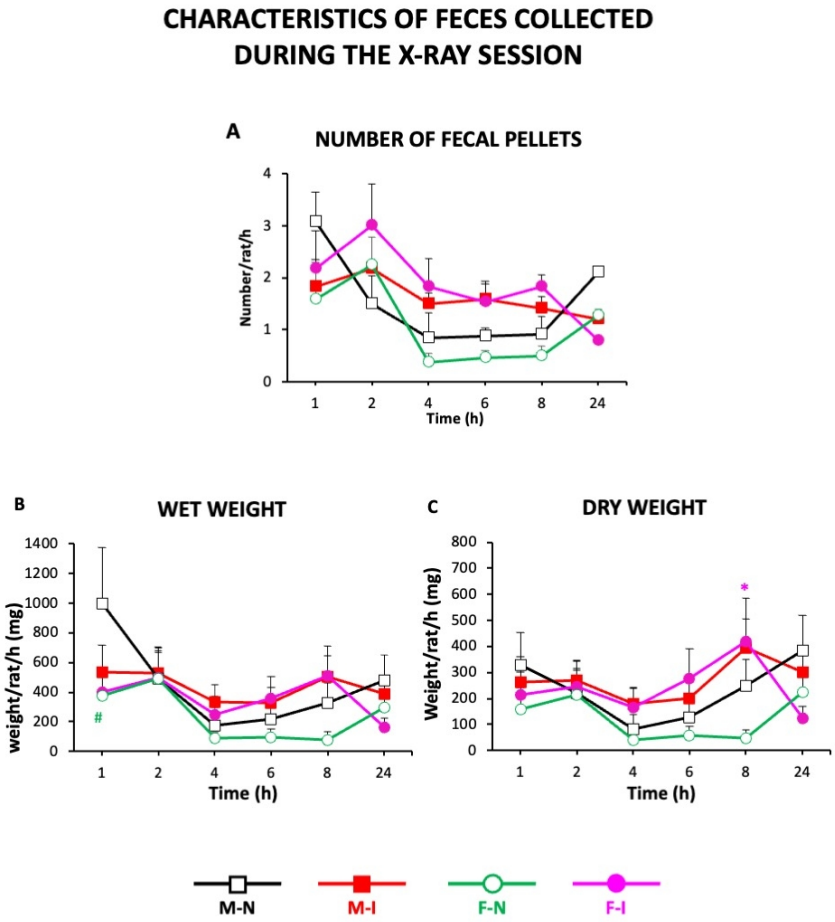

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