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Effects of two different acute and subchronic stressors on gastrointestinal transit in the rat: A radiographic analysis

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Abstract

Background: The reaction to stress is an adaptive response necessary for survival. When stressors are repeated, the organism adapts, although these adaptive responses can become dysregulated and result in disease, causing gastrointestinal (GI) disorders. Radiographic methods allow the non-invasive study of how a given factor affects GI transit in the same animal at different time points. These methods have never been applied to study the consequences of stress on GI motor function and their dependency on time and stimulus. Therefore, our aim was to characterize, using radiographic techniques, the effect on GI transit of cold-restraint (CR) and forced swim (FS) stress applied acutely and subchronically in the rat.

Methods: Male Wistar rats (260–330 g) were submitted to FS or CR stress, during 1 (acute) or 4 (subchronic) consecutive days. To study GI transit, radiographic methods were used. Radiographs were taken 0–24 h after barium intragastric administration on the 1st or 4th day of stress, which was applied 1 h after contrast.

Results: Acute FS or CR slowed down gastric and small intestinal emptying but had opposite effects in the caecum: CR tended to accelerate barium transit and feces formation while FS tended to slow these parameters down. When the stimuli were applied subchronically, GI transit was not completely normalized in most of the studied parameters.

Conclusion and Inferences: Mild stress alters GI transit differently depending on the nature of the stressor and its duration. Exposure to mild stressors should be considered as contributing factors to different functional GI disorders.

KEYWORDS
cold restraint, forced swim stress, gastrointestinal transit, radiographic analysis, rat, stress, stress adaptation

1 | INTRODUCTION

Stressors are imminent or perceived challenges to homeostasis. The stress response is an innate stereotypic adaptive response to stressors, which induces cognitive, behavioral, and physiologic phenomena with the purpose of restoring homeostasis. Importantly, habituation to repeated stressors is an adaptive mechanism which can limit the occurrence/severity of stress-related symptoms, including stress-induced alterations of gastrointestinal (GI) transit.[1,2] However, these adaptive responses can become dysregulated and result in disease.[3] That is, 77% of Americans regularly experienced physical symptoms caused by stress in 2017.[4] Nowadays, and due to the COVID-19 pandemic, GI diseases such as functional dyspepsia or irritable bowel syndrome have shown to worsen the symptoms associated with an increase in psychological stress.[5]

The control of the digestive functions is complex; it is regulated by an intrinsic, the enteric nervous system (ENS),[6] and an extrinsic innervation. During the stress response, GI transit can be affected through the release of different hormones from the central nervous system, the modulation of the autonomic nervous system, but also through the ENS, resulting in different alterations depending on the type and duration of the stressor and organ studied.[7,8]

Because of ethical reasons, the study of many aspects on how stress affects the pathogenesis of different GI diseases cannot be performed in humans, therefore reliable animal models are needed. Many different animal models have been proposed to study the effects of stress on GI function, including water avoidance, restraint stress, forced swimming, or the communicating box (for review see Lopez-Gomez et al.[9]).

Of all animal models, one of the most frequently used is stress induced by restraint, introduced in 1988,[8] which is performed by wrapping the upper forelimbs, shoulder, and trunk of rats in cloth tape to induce mild restraint stress. Variations of this model have been used; for example, animals are restraint within a plastic cylinder,[10] or adding a second stressor such as cold temperature, which has been shown to have additive stressful effects.[11] Another model, whose effects on GI transit have been less studied, is the forced swim stress model.[12] It is based on the aversion that rats have to water. Both models have shown to increase stress-related hormone blood levels.[13,14] Importantly, repetition of the stressor (homotypic stress) reduces the intensity of GI and hormonal effects, demonstrating the existence of adaptive mechanisms also in animal models.[2]

So far, most studies of the effect of acute or repeated stress on GI transit have been performed through methods which require the sacrifice of the animals (i.e., to measure gastric emptying) or provide relatively little information (defecation rate, feces wetness/consistency). Radiographic methods allow the non-invasive study of GI transit in the same animal at different time points with the advantage that changes in size/content density of the GI organs may be quantitatively evaluated.[15,16] To the best of our knowledge, these methods have never been used to study the effects of acute or repeated stress in preclinical models.

Therefore, our aims were to characterize, using radiographic techniques, the effect on GI transit of cold-restraint (CR) and forced swim stress applied acutely and to evaluate modifications of these alterations when these same stressors are applied subchronically.

2 | MATERIALS AND METHODS

The experiments were designed and performed in accordance with the European and Spanish legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and were approved by the Ethics Committee of Universidad Rey Juan Carlos (URJC) and Comunidad de Madrid (PROEX 280/19).

2.1 | Animals

A total of 48 male Wistar rats (260–330 g) were obtained from the veterinary Unit of URJC (Madrid, Spain) and housed (3–4/cage) in standard transparent cages (60 × 40 × 20 cm), under environmentally controlled conditions (temperature =20°C; humidity =60%), with a 12-h light/12-h dark cycle (lights on: 8–20 h). Animals had free access to standard laboratory rat chow (Harlan Laboratories Inc) and sterile tap water.

2.2 | Stress models

Rats were randomly divided into five different groups (n = 8–10 in each group), which were submitted to four different stress protocols, or no stress (control).

2.2.1 | Acute cold-restraint stress (ACRS)

Rats were placed in rigid plexiglass tubes, which can be adapted to the animal size, and maintained at 4°C environment for 40 min.

2.2.2 | Acute forced swim stress (AFSS)

Rats were individually placed for 20 min in a plastic cylinder (40 cm diameter, 50 cm height) filled with fresh tap water (24–26°C) to a height of 30 cm. After each session, rats were dried with a paper towel and put back into their cages.

2.2.3 | Subchronic cold-restraint stress (SCRS) and subchronic forced swim stress (SFSS)

Rats were submitted to the same stressor as in the corresponding acute protocol but for four consecutive days.

2.2.4 | Control group

Rats were left undisturbed in their cages.

2.3 | Gastrointestinal transit evaluation

GI motor function was studied by radiographic methods as previously described.[15,16] All efforts were made to minimize stress associated with the procedure. Thus, barium sulfate (Barigraf® AD; Juste SAQF) suspended in distilled water (2.5 mL, 2 g/ml, $t = 22°C$) was administered by gavage by an experimented technician, and the procedure lasted less than one minute. X-rays were obtained at different times (immediately and 1, 2, 3, 4, 6, 8, and 24 h: T0-T24) after administration of the contrast medium with a CS2100 (Carestream Dental) digital X-ray apparatus (60 kV, 7 mA), recorded on Carestream Dental T-MAT G/RA film (15 × 30 cm) housed in a cassette provided with regular intensifying screen and developed using a Kodak X-omat 2000 automatic processor. Exposure time was adjusted to 20–60 ms, and focus distance was manually fixed to 50 ± 1 cm. Immobilization of the rats in prone position was achieved by placing them inside adjustable handmade transparent plastic tubes, so that they could not move. To reduce stress, rats were released immediately after each shot (immobilization lasted less than 2 min). Previously, we have demonstrated that the insertion of animals in the plastic tubes does not alter GI transit.[15]

A rectangular metallic block (3 × 1 × 1 cm) was positioned aside the plastic tube in which the rat was placed so that it could serve as a reference for morphometric and densitometric analyses (see below). While taking the radiographs, a qualified investigator remained behind a leaded wall, where radioactivity while shooting was not different from environmental readings.

A trained investigator blind to the stressor performed the X-ray analysis. Alterations in gut transit were semiquantitatively determined from the images by assigning a compounded value to each region of the GI tract considering the following parameters[15]: Percentage of the GI region filled with contrast (0–4); intensity of contrast (0–4); homogeneity of contrast (0–2); and sharpness of the GI region profile (0–2). Each of these parameters was scored, and a sum (0–12 points) was made. The X-ray images were also digitized and analyzed with the aid of an image analysis system (Image J 1.38 for Windows; National Institute of Health, free software: http://rsb.info.nih.gov/ij/), and the alterations in size and barium density within the stomach, cecum, and fecal pellets were quantitatively analyzed, using the metallic block as reference (considering its length for morphometry; its color intensity as 100% density). These alterations could indicate wall mechanical changes (that could alter mainly size) or modifications in hydration of luminal contents (that could alter mainly density of contrast). For fecal pellet analysis, the number within the colon at each time point was first counted; then, the size, diameter, and density of the stained fecal pellets found in each rat at each time point were averaged and further used to determine the possible changes (only X-rays showing fecal pellets were used to determine the changes in morphometric and densitometric pellet characteristics due to treatment).

2.4 | Body weight and food and water intakes during the X-ray session

Body weight, food and water intake were recorded at the beginning of the experiments and 4, 8, and 24 h after barium administration. The percentage of body weight loss was calculated for each of these time points. The amount of water and food intake was calculated as the amount eaten per rat and hour during the first 8 h of radiographic analysis (9–17 h), which corresponds with most part of the circadian inactivity phase, and the amount consumed from T8 to T24 (17–9 h), which includes the whole activity phase of the circadian cycle.

2.5 | Macroscopic evaluation of GI organs

After sacrifice, GI organs were removed en bloc, the intestines were straightened out on graph paper, and pictures of the GI tract placed over it were taken. To analyze the area of the stomach and caecum and the length of the small intestine and colon, the pictures were opened using Image J 1.38 and the graph paper was used to set the scale for each picture.

After the photograph was taken, the stomach, small intestine, caecum, and colorectum were separated and weighed. Before weighing, the stomach was placed in a beaker with abundant physiologic solution, to register if it floated, as an indirect measurement of gas accumulation.[17] Once small intestine and colon were weighed, both were emptied as previously described. The small intestine was cut at the ileocecal junction and its contents were deposited on a small tray by milking the whole length of the small intestine with the fingers,[18] the colorectum was separated from the caecum and physiological solution was gently infused through the lumen, with a 10 ml syringe, until all fecal pellets were removed.[19] Empty small intestine and colon, as well as "milked" small intestinal contents, were weighed.

2.6 | Protocol

On the day of the experiment, barium was administered by gavage and radiographs corresponding to T0 and T1 were taken. Immediately after T1, rats were exposed to cold-restraint stress (CRS, 40 min) or forced swim stress (FSS, 20 min) in a different room. Afterward, animals were taken back to the X-ray room, and the radiographic protocol was continued: Radiographs were taken 2, 3, 4, 6, 8, and 24 h after barium administration. After T24, animals were sacrificed by decapitation and pictures of the GI organs and weights were recorded as previously described.

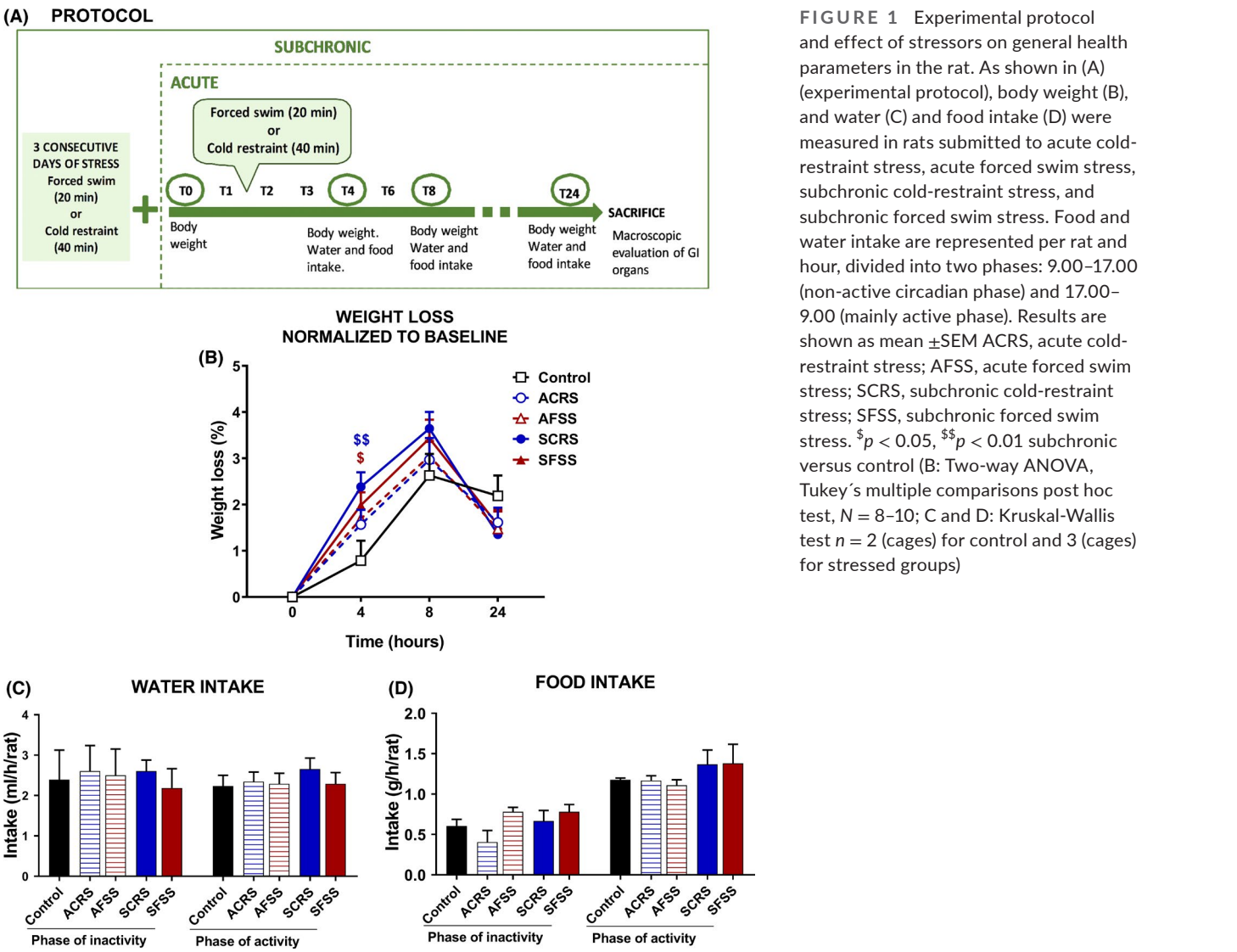

FIGURE 1 Experimental protocol and effect of stressors on general health parameters in the rat. As shown in (A) (experimental protocol), body weight (B), and water (C) and food intake (D) were measured in rats submitted to acute cold-restraint stress, acute forced swim stress, subchronic cold-restraint stress, and subchronic forced swim stress. Food and water intake are represented per rat and hour, divided into two phases: 9.00–17.00 (non-active circadian phase) and 17.00–9.00 (mainly active phase). Results are shown as mean ±SEM ACRS, acute cold-restraint stress; AFSS, acute forced swim stress; SCRS, subchronic cold-restraint stress; SFSS, subchronic forced swim stress. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ subchronic versus control (B: Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8–10$; C and D: Kruskal-Wallis test $n = 2$ (cages) for control and 3 (cages) for stressed groups)

In the subchronic stress protocol, animals were submitted for 3 consecutive days to the same stressful stimulus and on the fourth day the same protocol was followed as in the acute stress studies; thus, these groups were submitted to 4 consecutive days to the same stressor at approximately the same time every day (between 10 a.m. and noon) (Figure 1A).

2.7 | Statistical analysis

Statistical analyses were performed using Prism 7.0 (GraphPad Software Inc.). Most data passed the D'Agostino and Pearson's normality test, therefore one or two-way ANOVA followed by Tukey´s multiple comparison post hoc test was used for analyses, and Student´s *t* test (with Welch´s correction if necessary) was used when only two groups were compared. When data did not pass normality test, the Kruskal-Wallis test was applied. To compare the filling rate of the colorectum in the different stress paradigms, the slope of each curve corresponding to the points between 20% and 80% of the maximal possible content was calculated (between T3 and T6 for control and the FSS groups, and T2–4 for the CRS groups). The results are expressed as means ±SEM. Differences between the means were considered significant when $p < 0.05$.

3 | RESULTS

3.1 | Body weight

As seen in Figure 1B, all rats lost weight during the first 8 h of the radiographic session, which correspond to the hours in which most of it was carried out. Though, while control (and acutely stressed) rats lost approximately 3% of their weight by T8, subchronically stressed rats lost more weight during the first 4 hours, which was most noticeable, compared to controls (SFSS $p < 0.05$; SCRS $p < 0.01$). After T8, rats were left undisturbed in their cages until the next morning, during this period of time animals recovered most of their body weight, so the percentage of body weight loss was similar (around 1.5%–2%) across groups at this time ($p > 0.05$).

Rats in the subchronic stressed groups had a similar body weight at the beginning of the experiment, without statistically significant differences (SCRS =298 ± 4; SFSS =302 ± 2, $p > 0.05$), and did not lose weight during the 3 days of application of the stress stimuli. However, the rats in the SCRS group gained less weight than the SFSS group, the difference being statistically significant (SCRS: 9 ± 1.3 g, 3.08% weight gain; SFSS: 15.4 ± 1.9 g, 5.08% weight gain; $p < 0.05$).

On the other hand, during the first 8 h of radiographic analysis (performed during the inactivity phase), rats ate less amount of food than during the time that elapsed between T8 and T24 (which included the 12 h of activity phase), although the differences did not reach statistical significance. No statistically significant differences were found either between stress protocols, although in the phase of activity (T8–T24), subchronically stressed rats tended to eat more (Figure 1C,D).

3.2 | Water and food intake

All animals drank approximately the same amount of water, independently of the stress protocol and phase of activity, without statistically significant differences among the experimental groups (Figure 1C).

3.3 | Effect of acute stress on GI motor function

As mentioned above, stress was applied between T1 and T2 and therefore GI transit curves between T0 and T1 were overlapping for all groups and differences started to be visible only after T1 (Figure 2).

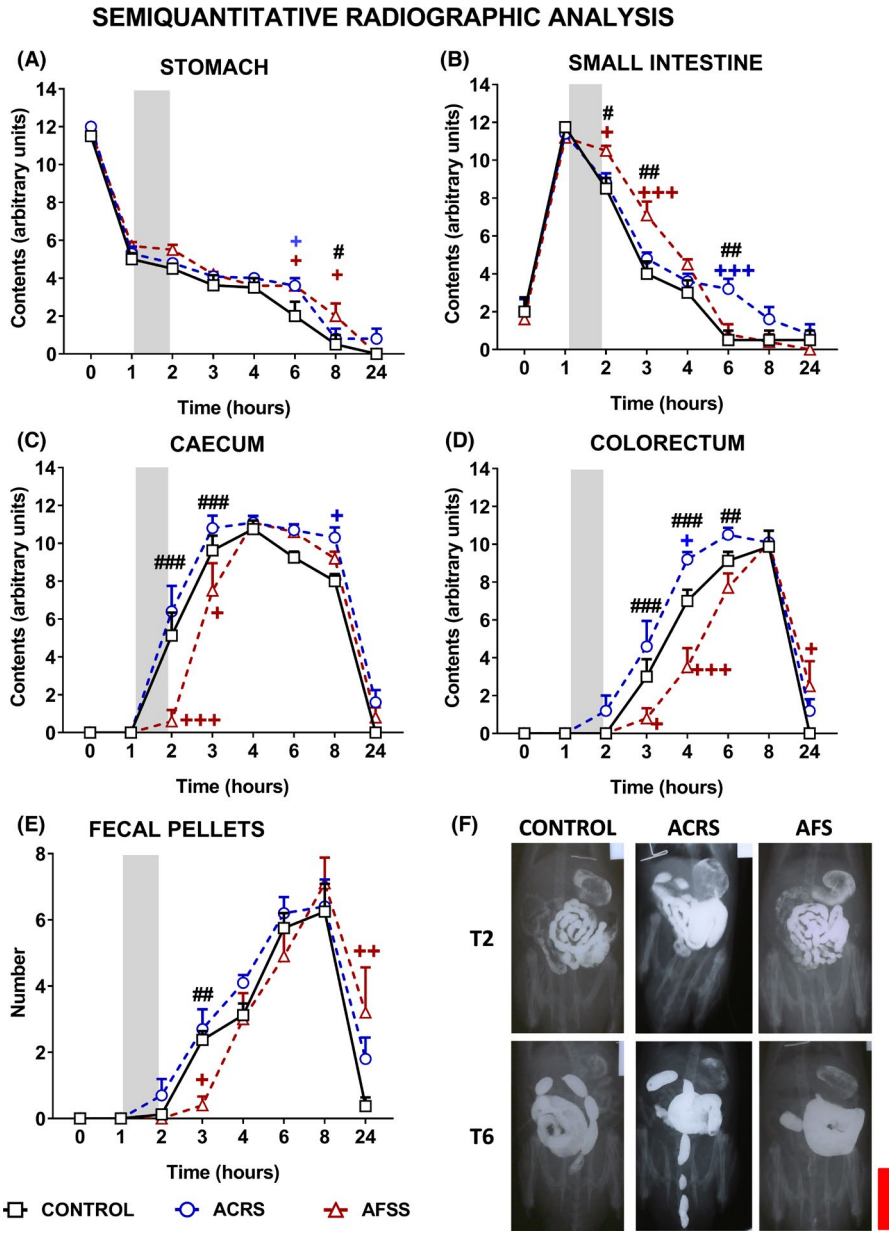

FIGURE 2 Radiographic analysis of the effect of acute forced swim stress and acute cold-restraint stress on gastrointestinal motor function—semiquantitative study. Curves show barium transit in the stomach (A), small intestine (B), caecum (C), and colorectum (D), using a semiquantitative score (see text for details), number of fecal pellets at each time point (E) and representative X-rays (F). Gray area shows the interval of time in which the stressful stimulus was applied. Results are shown as mean ±SEM ACRS, acute cold-restraint stress; AFSS, acute forced swim stress. $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ acute versus control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ ACRS versus AFSS (Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8–10$)

As seen in Figure 2A, stomach transit curves were similar across all groups during the first hours post-stress, but at T6, gastric emptying was slightly delayed in both stressed groups ($p < 0.05$) and this effect was maintained in the AFSS group at T8 ($p < 0.05$). Consequently, AFSS and ACRS groups were different at T8 ($p < 0.05$).

For the small intestine, the transit curves were clearly different during their emptying phase. Thus, while AFSS delayed intestinal emptying during the first experimental hours when compared to control (T2: $p < 0.05$; T3: $p < 0.001$), ACRS delayed it slightly at T6 ($p < 0.001$ vs. control group). At T24, there were no differences across groups. These different patterns made the curves for AFSS and ACRS different at T2 ($p < 0.05$), T3 and T6 ($p < 0.01$) (Figure 2B).

The filling of the caecum in the ACRS group was similar to that in the control group although slightly faster, with no statistically significant differences ($p > 0.05$). However, its emptying was delayed after T4, the difference with control reaching statistical significance at T8 ($p < 0.05$). On the contrary, AFSS delayed caecum filling from the beginning (T2: $p < 0.001$; T3: $p < 0.05$), and, although not statistically significant, the AFSS group presented a delay in the emptying of the caecum similar to that found for the ACRS group. At T24, there was hardly any barium left in the caecum of the stressed groups and no statistically significant differences were found across groups (Figure 2C). The different effect of the stressors on the GI tract made the curves for ACRS and AFSS different at T2 and T3 ($p < 0.001$).

The patterns of the curves in the colorectum were similar to those in the caecum (Figure 2D). ACRS accelerated the appearance of barium in the colorectum, which could already be seen at T2, and presented statistically significant differences at T4 ($p < 0.05$) versus the control group. On the contrary, AFSS delayed the arrival of barium, with statistically significant differences at T3 ($p < 0.01$) and T4 ($p < 0.001$) versus control. These different patterns made the curves for the stressed groups statistically different at T3, T4 ($p < 0.001$) and T6 ($p < 0.01$). At T24, there was still some barium in the colorectum, and the difference with the control was statistically significant for the AFSS group ($p < 0.05$). To analyze the filling of colorectum with barium, the slope of the 3 curves were analyzed and compared; the slope for ACRS tended to be faster than that of the other groups, but the differences did not reach statistical significance (control: 1.9 ± 0.7 a.u./h, ACRS 4 ± 0.4 a.u./h, AFSS 2.3 ± 0.2 a.u./h; a.u. = arbitrary units, for GI barium content).

The quantitative analysis of fecal pellets in the colorectum supported the findings of the semiquantitative analysis in that region. Thus, when compared to control, the occurrence of fecal pellets was slightly faster in the ACRS group, though without statistically significant differences. In contrast, this was slightly slower in the AFSS group; thus, the number of fecal pellets was reduced in the AFSS group at T3 compared with both control and ACRS groups. At T24, there were more fecal pellets in both stressed groups than in the control one, the difference being statistically significant for the AFSS group ($p < 0.01$) (Figure 2E). Representative images of radiographs corresponding to control and acutely stressed animals can be seen in Figure 2F.

In all experimental groups, the stomach size (i.e., the area of the stomach stained with barium) decreased progressively from the start point of the experiment to 24 h after barium administration in a similar way, without any statistically significant differences among groups (Figure 3A). However, when analyzing the density, both stressors increased the density in the stomach from T2 until T8 when compared to the control group, the difference being more significant for the ACRS group (T2: $p < 0.01$, T3–6 $p < 0.05$; T8 $p < 0.001$ vs. control), than in the AFSS group ($p < 0.05$ at T2 and T8 vs. control) (Figure 2B).

The curves for the size (Figure 3C) and density (Figure 3D) of the caecum further supported the findings of the semiquantitative analysis. Thus, ACRS accelerated and AFSS delayed filling, with statistically significant differences among them in size and density at T2 ($p < 0.001$) and in size at T3 ($p < 0.01$) (Figure 3D). Afterward, both groups remained similar to the control group, although density tended to be higher in the acute stress models and was statistically significantly different at T24 ACRS group when compared with control ($p < 0.001$).

Fecal pellet size, diameter, and density were averaged for pellets found in the X-rays from T3 to T8, because at T3, there started to be pellets in all groups and reached a maximum at T8. No differences were found in these parameters across groups ($p > 0.05$), although density tended to be a bit higher in the acute stress models.

3.4 | Effect of subchronic cold-restraint stress on GI transit

As seen in Figure 4A,B, when animals were exposed to 4 consecutive sessions of cold stress, the small differences found in the ACRS groups were normalized, and no differences were found when compared to the control group ($p > 0.05$). Therefore, the scores at T6 were significantly lower than the acute group in the stomach ($p < 0.01$) and small intestine ($p < 0.05$).

The curves for the filling of the caecum between the stress and control groups were very similar, though the emptying was slightly slowed in both of the stressed groups, only reaching statistical differences in the ACRS at T8, but not in the SCRS group (Figure 4C).

The arrival of barium to the colorectum was slightly accelerated in the subchronic group, just as in the acute one, though after T4 the curve was very similar as that for the control group. However, no differences were found between the stressed groups nor with control (Figure 4D). When the slopes of the filling curves were compared, that for SCRS was intermediate to control and ACRS, although the differences did not reach statistical significance (control: 1.9 ± 0.7, ACRS 4 ± 0.4, SCRS 3 ± 0.1 contents/h).

The number of fecal pellets was similar in the subchronic stress and control groups, with only a slight increment at T4 versus control ($p < 0.01$). There were more fecal pellets stained with barium at T24 in the acute group when compared to the subchronic group

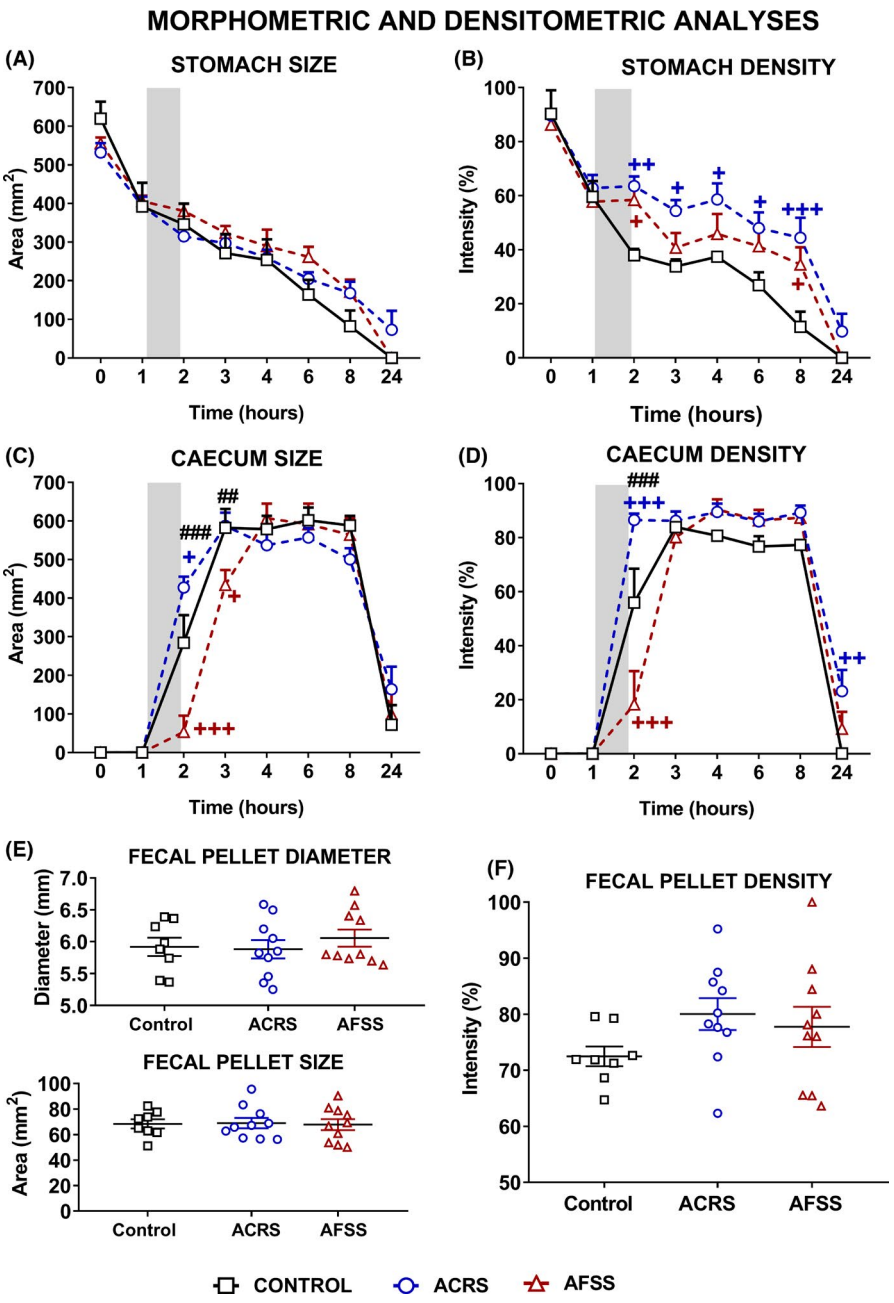

FIGURE 3 Radiographic analysis of the effect of acute forced swim stress and acute cold-restraint stress on gastrointestinal motor function—morphometric and densitometric studies. Morphometric and densitometry analyses of the stomach (A), (B), caecum (C), (D) and fecal pellet diameter, size (E) and density (F). X-ray images were taken immediately and after 1, 2, 3, 4, 6, 8, and 24 h from the time of contrast administration. Fecal pellet parameters were averaged from data obtained at T3 to T8. The analysis was performed using ImageJ 1.38 for Windows (National Institute of Health, USA; free software: https://rsb.info.nih.gov/ij/). Gray area shows the interval of time in which the stressful stimulus was applied. Results are shown as the mean ±SEM ACRS, acute cold-restraint stress; AFSS, acute forced swim stress. $^{+}p < 0.05$; $^{++}p < 0.01$, and $^{+++}p < 0.001$ acute versus control; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ ACRS versus AFSS (Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8$–10)

(Figure 4E). Representative images of radiographs corresponding to control and ACRS and SCRS animals can be seen in Figure 4F.

In relation to the size of the stomach, there were no differences across groups. The density was increased in the SCRS and lay between the acute and control groups (T2: $p < 0.05$ vs. control; T8: $p < 0.05$ vs. ACRS) (Figure 5A,B).

The curves for the density and size of the caecum were similar to the semiquantitative analysis: Though the size of the caecum was reduced when compared to the acute stress group at T2 and T24 ($p < 0.05$), both size and density remained similar in the subchronic and acute groups from T3 to T8 group and no differences were found with the control group (Figure 5C,D).

No differences were found in the fecal pellet size or diameter among the three groups. Though, as in the acute stressed group, density was slightly increased in the SCRS group, the difference with the control one, being statistically significant ($p < 0.05$) (Figure 5E,F).

3.5 | Effect of subchronic forced swim stress on GI transit

In animals submitted to forced swimming stress, the radiographic analysis of the subchronic group was similar to that of the acute group in the stomach and small intestine, that is, in the stomach the curve was very similar to that of the control group, although there was a slightly higher score at T1 ($p < 0.05$) (Figure 6A). In the small intestine, the emptying phase of the curve was shifted to the

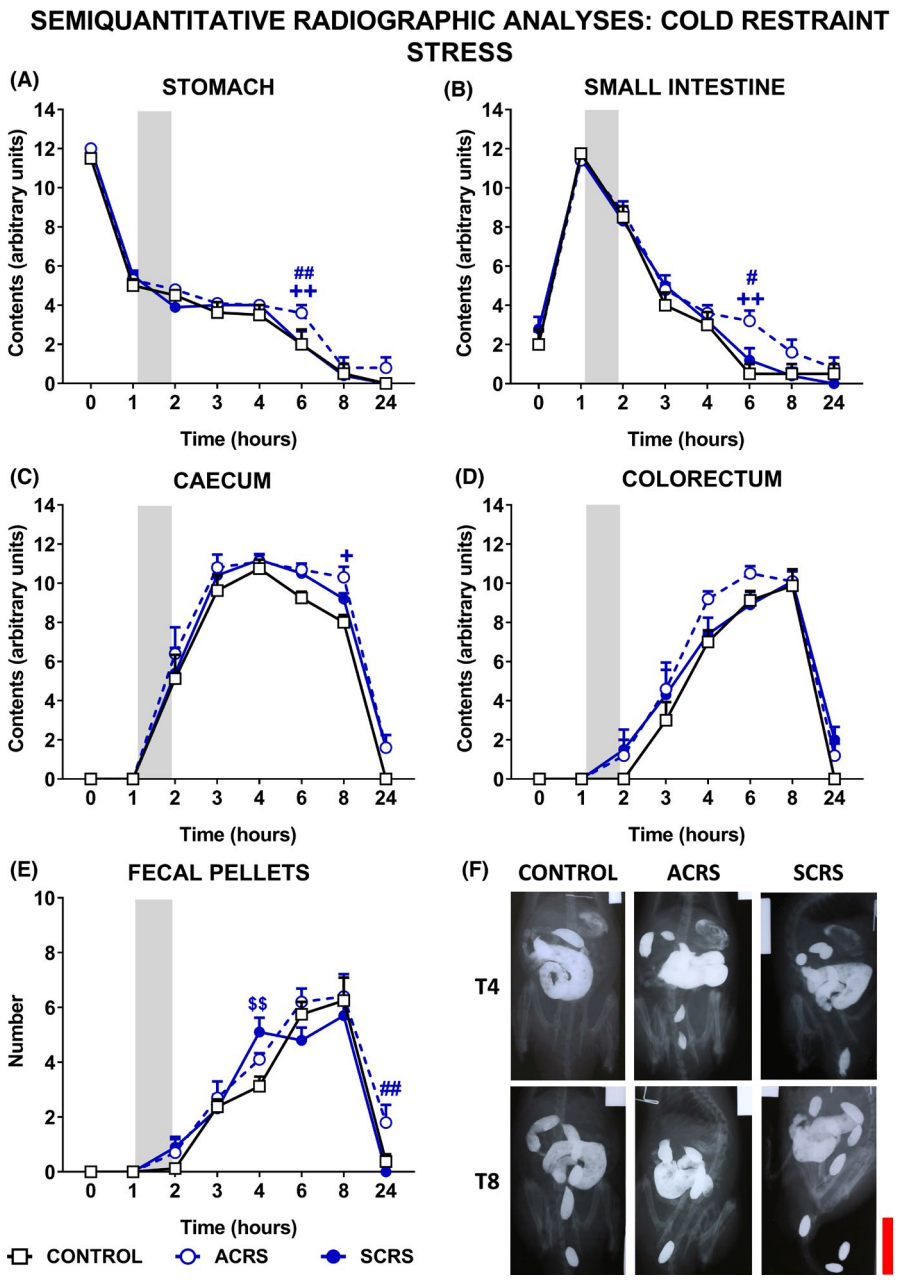

FIGURE 4 Radiographic analysis of the effect of acute and subchronic cold-restraint stress on gastrointestinal motor function—semiquantitative study. Curves show barium transit in the stomach (A), small intestine (B), caecum (C), and colorectum (D), using a semiquantitative score (see text for details), number of fecal pellets at each time point (E) and representative X-rays (F). Gray area shows the interval of time in which the stressful stimulus was applied. Results are shown as mean ±SEM ACRS, analysis of the effect of acute; SCRS, subchronic cold-restraint stress. $^{+}p < 0.05$; $^{++}p < 0.01$ acute versus control; $^{\$\$}p < 0.01$ subchronic versus control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, acute versus subchronic cold restraint (Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8–10$)

right, just as in the AFSS group, and differences were found at T2 ($p < 0.01$) and T3 ($p < 0.001$) when compared to the control group (Figure 6B).

The arrival of barium to the caecum in the SFSS was still slightly delayed when compared to the control group ($p < 0.01$), although it reached the maximum sooner than the AFSS group ($p < 0.001$). No differences were found among the groups during the rest of the radiographic analysis although the curves were slightly delayed for the stressed groups compared with control group (Figure 6C).

The curve obtained for the colorectum in the SFSS group was shifted to the left when compared to the AFSS group, the difference being statistically significant at time points T4 ($p < 0.05$) and T6 ($p < 0.001$), but similar to control group (Figure 6D).

There were less fecal pellets in both stressed groups than in control at T3, although the difference with control did not reach statistical significance in the subchronic group. The number of fecal pellets was similar in all groups from T4 to T8, whereas at T24 there were more fecal pellets in the stressed groups, although the difference with control was not statistically significant for the SFSS group (Figure 6E). Representative images of radiographs corresponding to control and ACRS and SCRS groups can be seen in Figure 6F.

The size of the stomach was similar in all groups (Figure 7A) and density was increased in the subchronic group immediately after FS, at T2 and then at T8 ($p < 0.05$) (Figure 7B).

The curves obtained in the acute and subchronic groups for both caecum size (Figure 7C) and density (Figure 7D) were very similar between both stressed groups. When compared to the control group, the size of the caecum ($p > 0.05$) and density ($p < 0.001$) were reduced at T2 for both stressed groups, with no further statistically

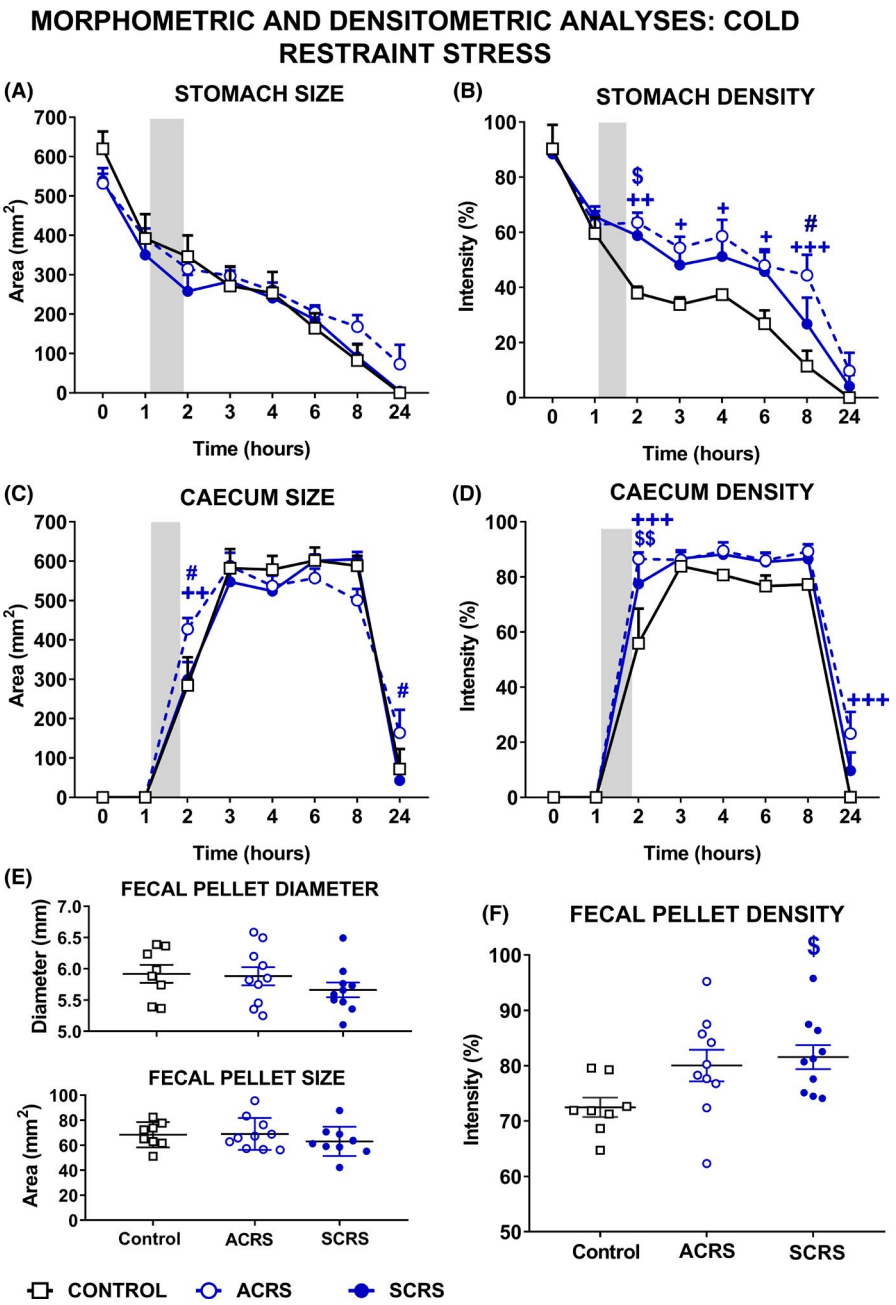

FIGURE 5 Radiographic analysis of the effect of acute and subchronic cold-restraint stress on gastrointestinal motor function—morphometric and densitometric studies. Morphometry and densitometry analyses of the stomach (A), (B), caecum (C), (D) and fecal pellet diameter, size (E) and density (F). X-ray images were taken immediately and after 1, 2, 3, 4, 6, 8, and 24 h from the time of contrast administration. Fecal pellet parameters were averaged from data obtained at T3 to T8. The analysis was performed using ImageJ 1.38 for Windows (National Institute of Health, USA; free software: https://rsb.info.nih.gov/ij/). Gray area shows the interval of time in which the stressful stimulus was applied. Results are shown as the mean ±SEM. $^{+}p < 0.05$; $^{++}p < 0.01$ and $^{+++}p < 0.001$ acute versus control; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$ subchronic versus control; $^{\#}p < 0.05$ acute versus subchronic forced swim stress (Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8–10$)

significant differences between the subchronic and acute or control groups, although in the stressed groups density reached slightly higher values than control between T4 and T8.

The fecal pellet size was not significantly different across groups (Figure 7E), whereas density tended to be higher in both of the stressed groups, almost reaching statistical significance in the subchronic when compared to control group ($p = 0.05$) (Figure 7F).

3.6 | Macroscopic evaluation of GI organs

At sacrifice, no statistically significant differences were found in body weight among the groups, although values were slightly higher in the SFSS group (Table 1).

The weights of the organs were normalized to the weight of each animal for an easier comparison. No statistically significant differences were found in normalized gastric weight across groups. In contrast, the normalized weight of the full small intestine was reduced in all stress protocols, although statistically significant differences were reached only for both CR stress groups. This could be due to a reduction in the weight of the empty small intestine and a reduction of its content, because both measures were reduced in all stressed groups, the difference with control being statistically significant for weight of the empty small intestine in the ACRS group and for weight of its contents in the AFSS ($p < 0.05$) and SCRS ($p < 0.01$) groups. On the contrary, the caecum of the subchronically stressed groups was more affected, displaying a statistically significantly lower normalized weight than the control group in the SCRS ($p < 0.05$) and

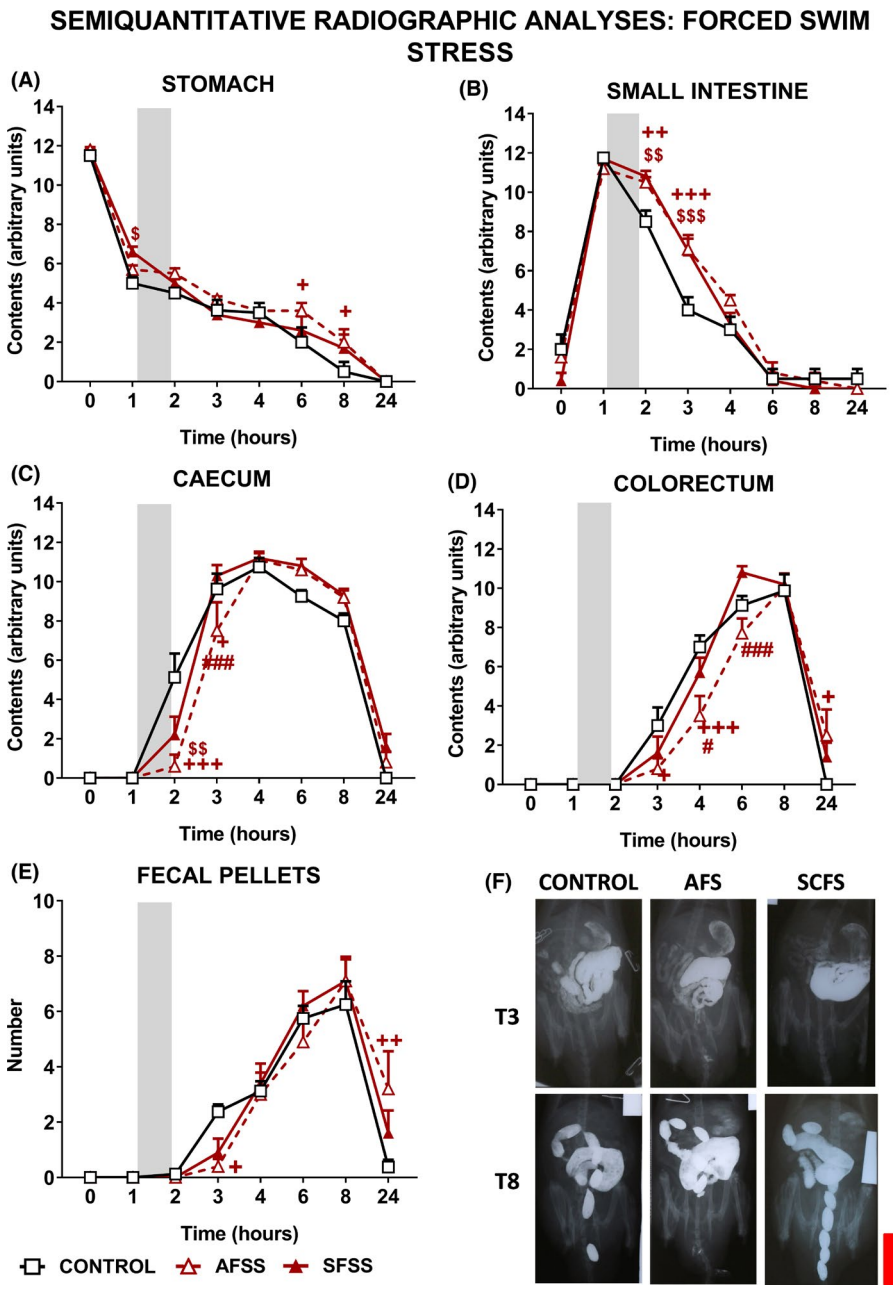

FIGURE 6 Radiographic analysis of the effect of acute and subchronic forced swim stress on gastrointestinal motor function—semiquantitative study. Curves show barium transit in the stomach (A), small intestine (B), caecum (C), and colorectum (D), using a semiquantitative score (see text for details), number of fecal pellets at each time point (E) and representative X-rays (F). Gray area shows the interval of time in which the stressful stimulus was applied. Results are shown as mean ±SEM. $+p<0.05$, $++p<0.01$, $+++p<0.001$ acute *vs* control; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ subchronic versus control; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ acute versus subchronic cold restraint (Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8–10$)

in an almost significant manner in the SFSS ($p = 0.06$). There were also significant differences between the acute and subchronic group submitted to CR stress ($p < 0.05$). Stress did not significantly affect the weight of the colorectum, either full or empty (Table 1).

None of the stress protocols modified the area nor the length of the different organs in a statistically significant manner, although the acute forms of stress tended to reduce the area of the stomach, and ACRS tended to reduce the length of the small intestine (Table 1).

4 | DISCUSSION

In the present study, we evaluated, using radiographic means for the first time, how two different stressors, applied acutely or subchronically, affect GI transit in the rat. The stress protocols were mild and did not importantly alter food or water intakes or weight. When applied acutely, both stressors slightly delayed gastric emptying and FSS delayed small intestinal emptying, while in the large intestine, FSS and CRS delayed and accelerated their filling, respectively. When both stressors were applied subchronically, a slight habituation seemed to occur, but parameters did not return to control values.

Overall, the health parameters obtained from the analysis of weight loss and water and food intake were not altered in an important manner. When the stressors were applied for 4 consecutive days, animals submitted to cold restraint tended to gain weight at a slower rate than the SFSS group, which is in line with previous studies, that show that weight gain is dependent on the frequency of stress application, without affecting it when FS or CR are applied acutely.[20-23] Thus, the conditions in which the stressors were applied in these experiments were mild and well tolerated by the animals.

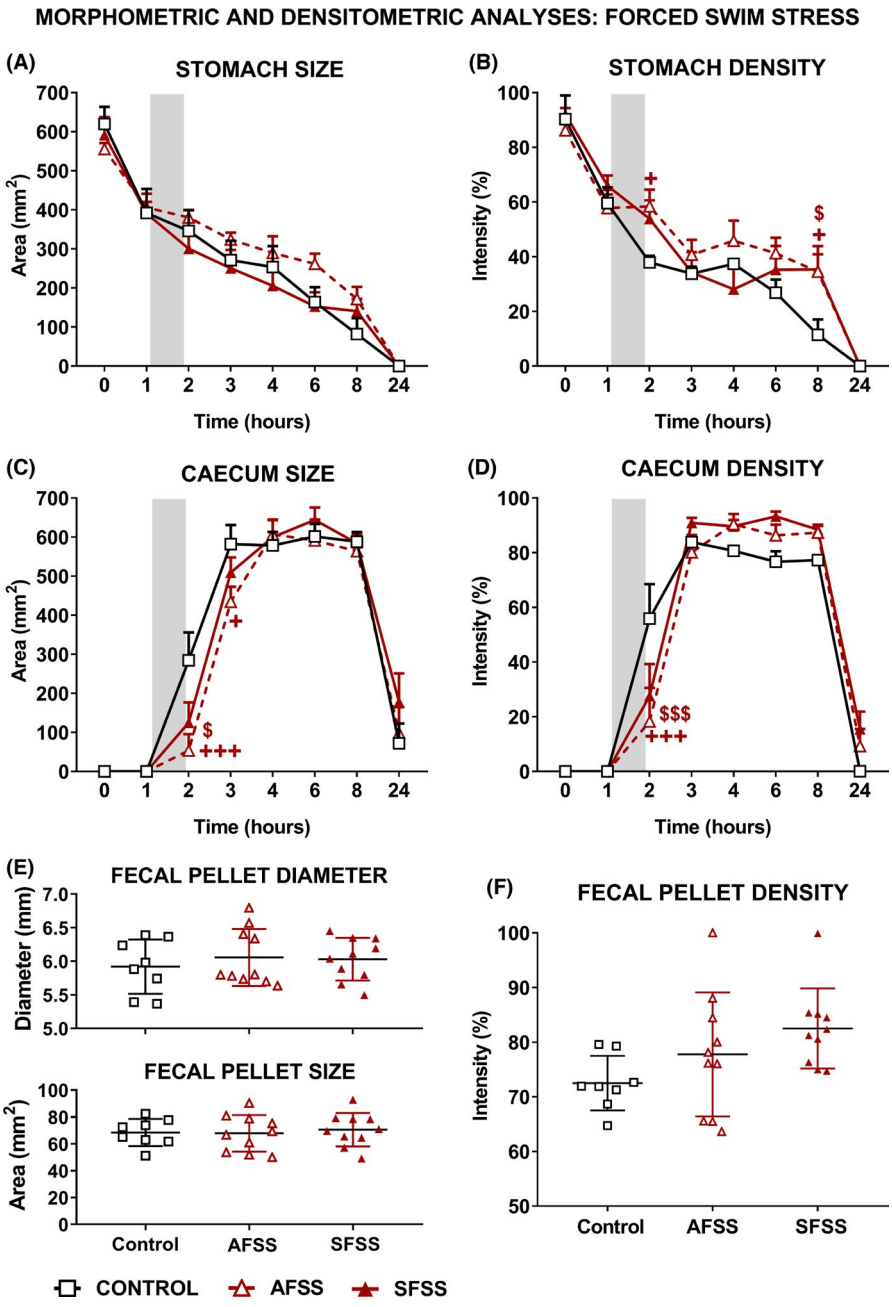

FIGURE 7 Effect of acute and subchronic forced swim on gastrointestinal motor function in rats—morphometric and densitometric studies. Morphometry and densitometry analyses of the stomach (A), (B), caecum (C), (D), and fecal pellets (E), (F). X-ray images were taken immediately and after 1, 2, 3, 4, 6, 8, and 24 h from the time of contrast administration. Fecal pellet parameters were averaged from data obtained at T3 to T8. The analysis was performed using ImageJ 1.38 for Windows (National Institute of Health, USA; free software: https://rsb.info.nih.gov/ij/). Gray area shows the interval of time in which the stressful stimulus was applied. Results are shown as the mean ±SEM $^{+}p < 0.05$, and $^{+++}p < 0.001$ acute versus control; $^{\$}p < 0.05$; $^{\$\$\$}p < 0.001$ subchronic versus controlsss. (Two-way ANOVA, Tukey´s multiple comparisons post hoc test, $N = 8$–10)

For radiographic analysis, we needed to administer barium through gavage. Although we have not performed a specific analysis whether it can affect GI transit, it is a very fast procedure (it takes less than one minute) and was applied only once and one hour before stress application (which lasted for 20–40 min). Furthermore, all animals (control and submitted to acute or subchronic stress) were exposed to barium gavage in the same way and by the same (very experienced) technician. Thus, we believe we were able to minimize the possible stressful effect of gavage, particularly in comparison with the experimental stressors used in the study.

When evaluating the effect that ACRS had on transit, we did not find important alterations in semiquantitative analysis of stomach throughout the experimental procedure. On the contrary, an important increase in the density values was obtained when compared to control animals, which suggests that there is a decrease in the proportion of water. This finding could be due to an increase in water absorption or to a relative retention of the solid content (that remains stained with barium: Barium is a highly dense heavy metal quite insoluble in water[24]). The latter explanation seems more plausible, because the literature indicates that the overall absorption of water from the stomach is low and most water is absorbed from the small intestine and colon.[25,26] Additionally, liquids are expelled much faster from the stomach than solids; thus, at the moment of stress induction (1 h after barium administration), most water could have already left the stomach leaving the solid content behind. Thus, our findings would be in agreement with the literature, where there seems no contradiction on the delaying effect of acute stressors on gastric emptying.[2,27-29]

TABLE 1 Effects of the stress on weight, area, and length of the gastrointestinal organs

		Control	ACRS	AFSS	SCRS	SFSS
Weight of animals at sacrifice		292.1 ± 6.7	292.5 ± 6.6	290.7 ± 9	302.8 ± 3.9	312.7 ± 4
Weight of organs at sacrifice (% of body weight)	Stomach	1.7 ± 0.09	1.5 ± 0.1	1.4 ± 0.09	1.6 ± 0.14	1.7 ± 0.2
	Full small intestine	4.03 ± 0.11	3.51 ± 0.15^{+}	3.66 ± 0.09	3.49 ± 0.11^{+}	3.71 ± 0.15
	Empty small intestine	2.81 ± 0.05	2.46 ± 0.08^{++}	2.64 ± 0.07	2.6 ± 0.08	2.66 ± 0.07
	Milking	1.21 ± 0.09	1.01 ± 0.08	0.97 ± 0.04+	0.86 ± 0.07^{++}	0.99 ± 0.08
	Caecum	1.81 ± 0.1	1.75 ± 0.07	1.89 ± 0.07	1.46 ± 0.12$^{+\#}$	1.63 ± 0.06
	Full colorectum	1.08 ± 0.09	1.1 ± 0.07	1.03 ± 0.06	1.2 ± 0.14	1.1 ± 0.06
	Empty colorectum	0.69 ± 0.03	0.7 ± 0.04	0.66 ± 0.03	0.65 ± 0.03	0.7 ± 0.05
Area and length of organs at sacrifice	Stomach (cm^2)	5.3 ± 0.28	4.8 ± 0.26	4.8 ± 0.19	5.2 ± 0.42	5.5 ± 0.49
	Caecum (cm^2)	6.4 ± 0.36	6.5 ± 0.23	7.3 ± 0.23	5.8 ± 0.4	6.2 ± 0.3
	Small intestine (cm)	57.9 ± 2.85	53.6 ± 1.39	57.9 ± 1.12	56.9 ± 1.75	56.4 ± 2.35
	Colorectum (cm)	11.59 ± 0.44	11.54 ± 0.47	11.52 ± 0.28	12.2±0.38	12.05±0.5

Abbreviations:: ACRS, acute cold-restraint stress; AFSS, acute forced swim stress; SCRS, subchronic cold-restraint stress; SFSS, subchronic forced swim stress.

Data are expressed as the mean ±SEM $^{+}p < 0.05$, $^{++}p < 0.01$, versus control; $^{\#}p < 0.05$ versus acute stressor (One way ANOVA followed by Tukey´s multiple comparison test and unpaired *t* test for acute vs. subchronic stressor; $n \geq 8$–10).

The gastric effect of AFSS was similar to that of ACRS, though slightly milder. The effects of stress on GI transit depend on the type of stressor, the frequency, and duration it is applied.[11] Indeed, FSS and CRS were applied for 40 min and 20 min, respectively.

On the other hand, we did not observe any important alterations in the ACRS group in the small intestine when compared with control except for T6. Our results are similar to those obtained by Barone et al.,[11] who found a very slight reduction in intestinal emptying, when applying cold restraint for 35 min. Other authors have found a reduction in intestinal transit when submitting rats to restraint stress, although they have used much longer stress duration than in the present study.[30,31]

On the contrary, AFSS did reduce the emptying of the small intestine. Similarly, other authors have shown that AFSS reduces intestinal emptying.[32] Vigorous exercise causes inhibition of GI transit, which is thought to be mediated through the potent activation of the sympathetic nervous system, since reducing sympathetic activity is associated with an increase in gastric and intestinal emptying.[33] Interestingly, previous studies have analyzed the contractile properties of the ileum, in vitro, after different stress paradigms; while after swimming there was a reduced contractile response to carbachol,[34] in a model where rats were submitted to centrifuge (and no exercise was performed), no statistical differences were found between control and stressed rats to the response to acetylcholine, although it tended to be slightly increased.[35]

With regard to the caecum and the colorectum, their filling was faster in ACRS and slower in AFSS, compared with that in the control group. This might be due to different factors: In the ACRS group, there was a 5 cm shortening of the small intestine; additionally, a slightly accelerated emptying of the small intestine could be possible (although not clearly appreciated radiographically due to intestinal folding, this acceleration would be in line with previously discussed studies). In contrast, the notorious delay seen in the case of the AFSS group might be partly due to the delayed gastric and intestinal emptying. Indeed, AFSS delayed transit in all GI regions.

Many studies have demonstrated that stress increases colonic transit in multiple stress paradigms, for review see Lopez-Gomez et al.[9] probably mediated through stress-induced release of corticotropin-releasing factor (CRF), which may act on both central and peripheral CRF1 receptors.[36] In our study, we could not see the immediate effect of the stressor on colonic transit because this was applied only 1 h after barium administration and at that time point, barium had not reached the colorectum yet. CRF peptides have been shown to remain increased in the colon even after 8 h after partial restraint application.[37] When comparing the slopes of the filling of the colorectum, we did not find statistically significant differences between the stressed and control group, and although the fecal pellets were collected at the different time points in the radiographic sessions after stress application, no differences were recorded across control and stressed groups (data not shown). Possibly, small differences could be better observed by using metabolic cages (although this would introduce additional stress), or delaying the moment of stress application and visualization of the effects through X-rays or, even better, fluoroscopy.[38]

When the stressors were applied for 4 consecutive days, we found that the density of content in the stomach remained higher than in the control group; thus, we did not see a clear habituation to the stressful stimuli. The effect of repeated stressful stimuli on stomach emptying is somewhat contradictory in the literature; that is, psychological stress induced by the communicating box decreased gastric emptying when applied chronically,[39] gastric emptying was accelerated when rats were kept in cages filled with 2 cm of water for 5 consecutive days,[40] and habituation to partial restraint stress was found after 5 days of this same stressful stimulus,[2,41] though this

adaptation was not seen after 5 consecutive days of CRF administration.[41] Possibly, and taking into account the tendency to habituation that we have observed, more days would be needed to finally observe a total habituation. Also, another possible explanation for the differences across studies is that we have used Wistar rats in the present study, while in the studies where habituation to restraint stress was found, Sprague-Dawley rats were used.[2,41] Indeed, important differences have been previously described regarding the response to stress across different strains. Thus, Sprague-Dawley rats display a lower anxiety level when compared to other strains, such as Wistar Kyoto or Fischer 344.[42,43] In this line, they have shown to have less susceptibility to GI disturbances than Lewis rats[44] and are less affected by aging with regard to neuronal loss of the myenteric neurons when compared to Wistar rats.[45] Thus, although there are no studies which directly compare the effects of stress on GI transit of Wistar and Sprague-Daley rats, it is very plausible that differences among strains can also account for the differences among studies.

On the other hand, caecum filling in the SCRS was similar to the control group, which correlates with the normalization in the length of the small intestine when compared to the ACRS group and the partial habituation in the stomach. Similarly, in the SFSS group, the filling of the caecum was slightly accelerated when compared to the AFSS group, which could be due to the slight habituation observed in the stomach.

In the colorectum, the curves for both subchronically stressed groups seem to be similar to that in the control group. Although in this particular study, we could not properly analyze fecal pellet output (number of fecal pellets, weight, consistency, and moisture) during stress application, we did observe that fecal output tended to decrease on the last day of subchronic stress compared with the first day (data not shown). Thus, the obtained results seem to suggest an adaptation in the lower GI organs, while not in the upper ones, as previous studies have demonstrated.[2,46]

After the last radiographic session (T24), animals were sacrificed and their GI organs weighed and measured. The stress models did not modify the areas or lengths of stomach or colorectum in a significant manner (similar to the maximum sizes found in the morphometric analyses during the X-rays sessions). On the other hand, the weight of the small intestine was reduced in a significant manner in both the acute and subchronic CR models, though different factors seemed to contribute to this: While in the acute model there was a slight reduction in the length of the small intestine and the reduction of the milking (small intestinal content) was not too important, in the subchronic model it seemed more related to a reduction in the intestinal content, which is in line with the reduced weight gain found in these animals, when comparing the SCRS to the SFSS group. In line with the reduction of the milking, the weight of the caecum was reduced in both subchronic stress models, although the SCRS group seemed to be more affected. This reduction in the weight of the milking and caecum could be in line with previous studies in which CR and FS stress have shown to reduce body weight and food intake.[21,47] Additionally, other factors such as an increase in nutrient absorption[48] and augmented metabolic efficiency due to cold temperature,[49] could possibly also contribute to the decrease in weight gain and reduced milking.

Previous studies have demonstrated that both acute and subchronic stress models can induce histological modifications of the small intestine. That is, Mazzon et al.[50] in 2002 saw that one session of restraint stress induced an increase in the percentage of open tight junctions with an alteration in the distribution of different proteins in the ileum. Kpodo et al.[51] in 2020 found that 3 h of heat stress caused a reduction in villus height and villus height-to-crypt depth ratio in the ileum and jejunum of pigs. Wan et al.[52] studied the histology of the jejunum after 3 days of a simulated transport stress and found severe damage to the intestinal villi with desquamation and exposure of the lamina propia. Although we did not perform a histological analysis in this study, the reduced weight of the empty small intestine in all the studied groups might be, at least partially, due to structural changes induced by stress similar to those reported by others, especially in the CR groups. Additionally, the reduction in the length of the small intestine has been also observed in cold reared piglets,[53] although the reasons for these changes are, up to date, unknown.

To conclude, we have shown here, for the first time by means of a radiographic, non-invasive, and highly translatable to the clinic technique, that different types of stressors, applied acutely or subchronically, affect transit in a different manner, and this is associated with some macroscopic changes in the different GI organs. The differences found across studies may be related to the activation of different neurotransmitters and hormones which, in turn, facilitate or not an adaptation to the stressful stimulus.[54] Specific studies will be needed to determine the exact mechanisms involved in the different GI consequences observed when animals are exposed to the different stressful stimuli. Furthermore, future studies using female rats will be of great interest to determine differences between both sexes.

Accurately determining how stressors alter the whole GI function is essential for the understanding of the different pathologies in which stress is an important etiological factor, and may help develop new, better targeted therapies for these complex diseases.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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