1   **Sex differences in pain-related behaviors and clinical progression of disease in**

2   **mouse models of visceral pain**

3   Abbreviated title: Sex differences in visceral pain in mice

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ABSTRACT

Previous studies have reported sex differences in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) patients, including differences in visceral pain perception. Despite this, sex differences in behavioral manifestations of visceral pain and underlying pathology of the gastrointestinal tract have been largely understudied in preclinical research. In this study, we evaluated potential sex differences in spontaneous visceral nociceptive responses, referred abdominal hypersensitivity, disease progression and bowel pathology in mouse models of acute and persistent colon inflammation. Our experiments show that females exhibit more visceral nociceptive responses and referred abdominal hypersensitivity than males in the context of acute but not persistent colon inflammation. We further demonstrate that, following acute and persistent colon inflammation, visceral pain-related behavioral responses in females and males are distinct, with increases in licking of the abdomen only observed in females and increases in abdominal contractions only seen in males. During persistent colon inflammation, males exhibit worse disease progression than females, which is manifested as worse physical appearance and higher weight loss. However, no measurable sex differences were observed in persistent inflammation-induced bowel pathology, stool consistency or fecal blood. Overall, our findings demonstrate that visceral pain-related behaviors and disease progression in the context of acute and persistent colon inflammation are sex-dependent, highlighting the importance of considering sex as a biological variable in future mechanistic studies of visceral pain as well as in the development of diagnostics and therapeutic options for chronic gastrointestinal diseases.

INTRODUCTION

Patients with irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and other chronic gastrointestinal diseases often manifest altered visceral pain perception that has been related to the development of visceral hypersensitivity [9,34,47]. Importantly, clinical studies have shown that women with IBS exhibit higher sensitivity to repetitive rectal distention and report more severe abdominal pain or discomfort than men, suggesting sex differences in visceral pain perception in IBS [3,4,44]. Sex differences have also been reported in the clinical manifestation, disease course, complications, psychiatric comorbidities, central processing of visceral pain and pathophysiology of IBS and other chronic gastrointestinal disorders [13,26,37,43]. In contrast, a recent study found no sex differences in visceral pain thresholds to rectal balloon distensions in healthy young women and men [19], suggesting that baseline visceral sensitivity is not dependent on sex. Evaluation of the mechanisms underlying sex differences in visceral sensitivity in human subjects is limited by methodological and experimental challenges and are, thus, not completely understood [1,11,16]. Preclinical studies in rodents offer a valuable alternative to evaluate sex-specific factors in chronic gastrointestinal diseases in a more controlled setting, providing insights towards the development of more effective diagnostic and treatment options for patients.

Preclinical studies evaluating sex differences in visceral sensitivity in rodents have predominantly measured visceromotor responses to colorectal distension in rats. These

69   studies have shown that visceral sensitivity in rodents is sex-dependent, with females

70   exhibiting higher visceromotor responses to colonic distension than males at baseline and

71   after acute inflammation of the colon [23,45]. Sex differences in visceromotor responses

72   to colonic distention have also been reported in the context of stress, where female rats

73   exhibit a stronger relationship between early life adversity and the development of stress-

74   induced visceral hypersensitivity [41]. Separate studies further show that sex hormones

75   contribute to sex differences in stress-induced visceral hypersensitivity, with estradiol

76   facilitating and testosterone decreasing stress effects on visceral responses [21]. Despite

77   this knowledge, sex differences in visceral sensitivity after acute and persistent

78   inflammation of the bowel remains poorly understood. Evaluating and characterizing

79   potential sex differences in visceral pain-related responses in mice under pathological

80   conditions is essential for the evaluation of mechanisms driving sex differences in visceral

81   pain.

82   In the present study, we systematically evaluated potential sex differences in

83   visceral pain-related responses using two well-characterized mouse models of chemically

84   induced visceral hypersensitivity: intracolonic capsaicin, which elicits transient

85   neurogenic inflammation [28], spontaneous nociceptive responses and referred

86   abdominal hyperalgesia [29,42]; and the Dextran Sodium Sulfate (DSS) model of colitis,

87   which elicits prolonged inflammation in the bowel as well as visceral and referred

88   abdominal hypersensitivity [2,10] (**Figure 1**). Parallel experiments evaluated potential sex

89   differences in disease progression of DSS-induced colitis and associated bowel

90   pathology. Our experiments revealed sex differences in pain-related behaviors after acute

91   colonic irritation as well as in the clinical progression of colitis. Visceral pain-related

92   behaviors and colon pathology after persistent inflammation of the bowel, however, were

93   similar in males and females. Collectively, these findings set a foundation for future

94   mechanistic studies of sex differences in visceral pain.

95

96   **RESULTS**

97   **Female mice display more capsaicin-induced spontaneous pain-related behaviors**

98   **than male mice**

99   To begin evaluating potential sex differences in visceral pain-related behaviors, we

100 used the intracolonic capsaicin model and measured spontaneous nociceptive responses

101 in both male and female mice (**Figure 2A**). Spontaneous capsaicin-induced nociceptive

102 behaviors were defined as licking, stretching, and dragging of the abdomen as well as

103 abdominal contractions. Consistent with previous reports [28,29], intracolonic application

104 of capsaicin (0.01%) elicited robust pain-related behaviors that were significantly ($p <$

105 0.0001) higher than those observed following an intracolonic injection of vehicle in female

106 mice (**Figure 2B**). In marked contrast, however, spontaneous pain-related behaviors

107 were indistinguishable in male mice following the intracolonic administration of 0.01%

108 capsaicin or vehicle control (**Figure 2B**). To determine if male mice can display capsaicin-

109 induced hypersensitivity, we next injected mice with a higher dose of capsaicin (0.03%).

110 As illustrated in **Figure 2C**, intracolonic administration of 0.03% capsaicin elicited

111 significant ($p = 0.0010$) increases in spontaneous pain-related behaviors in males,

112 compared to vehicle-injected mice. These results demonstrate that capsaicin-induced

113 spontaneous nociceptive responses are sex-dependent, with higher doses of capsaicin

114 required in males than in females to elicit comparable nociceptive behavioral responses.

115   The individual components contributing to the total number of capsaicin-induced

116   nociceptive behaviors were analyzed individually to identify which specific behaviors

117   contribute to the observed sex differences (**Figure 2D-K**). Consistent with the results

118   observed in the cumulative analysis, females injected with 0.01% capsaicin displayed

119   significant increases in most behavioral parameters when compared to control vehicle-

120   injected females. Thus, female mice injected with 0.01% capsaicin displayed a significant

121   ($p < 0.01$) increase in the number of licking bouts (**Figure 2D**), dragging bouts (**Figure**

122   **2F**) and stretching bouts (**Figure 2H**), compared to vehicle-injected females. The number

123   of contraction bouts was the only parameter in females that was indistinguishable

124   between capsaicin- and vehicle-injected mice (**Figure 2J**). In marked contrast and

125   consistent with the results observed in the cumulative behavioral analysis, all the

126   parameters measured were indistinguishable between vehicle- and capsaicin-injected

127   male mice (**Figure 2D, 2F, 2H, 2J**), confirming the lack of measurable behavioral

128   hypersensitivity in male mice following intracolonic injection of 0.01% capsaicin.

129   Evaluation of the individual behavioral components in male mice injected with a

130   higher dose of capsaicin (0.03%) revealed that the capsaicin-induced increases in

131   behavioral responses observed at this concentration were mainly driven by significant (p

132   < 0.01) increases in abdominal dragging and contraction bouts (**Figure 2G, 2K**) but not

133   by changes in licking or stretching of the abdomen (**Figure 2E, 2I**). Together, these results

134   highlight that the behavioral manifestation of capsaicin-induced visceral hypersensitivity

135   is sexually dimorphic, with increases in licking and stretching of the abdomen observed

136   in females only and increases in abdominal contractions seen solely in male mice. The

137   common behavioral manifestation observed between sexes following intracolonic

138   capsaicin administration was increases in dragging of the abdomen.

139   Previous studies have indicated that intracolonic capsaicin elicits freezing

140   behaviors in a dose-dependent manner [6]. Consistent with these findings, the time spent

141   freezing was significantly ($p < 0.05$) higher in females following intracolonic injection of

142   0.01% capsaicin than in females after the injection of vehicle control (**Figure 2L**). In line

143   with the sex differences presented above, no measurable differences were observed in

144   the time spent freezing in male mice injected with 0.01% capsaicin when compared to

145   vehicle control (**Figure 2L**). Time spent freezing, however, was significantly ($p < 0.01$)

146   higher in males when a higher dose of capsaicin (0.03%) was used, compared with

147   vehicle control (**Figure 2M**). These results demonstrate that while both males and

148   females exhibit capsaicin-induced freezing, males require a higher dose of capsaicin than

149   females to display freezing behaviors, further confirming that the manifestation of

150   capsaicin-induced nociceptive responses is sex-dependent and that females are more

151   hypersensitive.

152

153   **Clinical progression of disease in a DSS-induced mouse model of colitis is sexually**

154   **dimorphic**

155   The DSS model of colitis was used to evaluate sex differences in clinical

156   progression of disease as well as visceral pain-related responses and referred abdominal

157   sensitivity in mice. Male and female mice were treated with either 2.5% DSS or water, ad

158   libitum for seven days (**Figure 3A**). Disease Activity Index (DAI), defined as a cumulative

159   score based on the percentage of weight loss, stool consistency, presence of fecal blood

160   and physical appearance (**Table 1**), was calculated daily to monitor the clinical

161   progression of colitis. As illustrated in **Figure 3B**, the DAI score was dependent on the

162   dose of DSS administered, with increasing percent of DSS resulting in higher DAI score.

163   The rest of the experiments in this study were performed using 2.5% DSS which

164   consistently elicited disease in all mice but was not at ceiling level.

165   Consistent with previous studies [2,10,39], 2.5% DSS treatment in drinking water

166   resulted in increases in DAI scores in both males and females in a time-dependent

167   manner, with higher DAI scores as the days of treatment progressed. As expected, DAI

168   scores in DSS-treated male and female mice were significantly ($p < 0.0001$) higher than

169   scores in their respective water controls, validating that DSS treatment induces a

170   symptom profile consistent with colitis in both sexes (**Figure 3C**). Notably, DSS-treated

171   male mice displayed significantly ($p = 0.0031$) higher DAI scores when compared to

172   female mice, demonstrating that male mice have worse clinical progression of colitis than

173   female mice.

174   To dissect out the components within the cumulative DAI scores that contribute to

175   the observed sex differences in the progression of colitis, we analyzed and compared

176   each disease parameter individually in both male and female mice (**Figure 3D-G**). As

177   predicted, DSS-treated male and female mice showed higher scores than their respective

178   water controls in all the individual DAI components evaluated. Similar to the composite

179   DAI scores, the progression of the individual disease components was also time-

180   dependent, with higher scores observed with treatment progression in both males and

181   females. While DSS-induced changes in body weight, physical appearance, and stool

182   consistency were observed within a few days of treatment (**Figure 3D, E and F**), the

8

183   presence of blood in the stool was not observed until days 4-6 after treatment (**Figure**

184   **3G**).

185   Comparison of the individual disease components in males and females further

186   revealed that the sex differences observed in the composite DAI scores in DSS-treated

187   mice arise from differences in the percentage of body weight loss and physical

188   appearance scores but not from differences in the scores for stool consistency or fecal

189   blood. Thus, as shown in **Figure 3D**, while both DSS-treated male and female mice lost

190   weight during the days of treatment, the percentage of weight loss over time was

191   significantly ($p = 0.0193$) higher in male than in female mice. Male mice also displayed

192   worse DSS-induced changes in physical appearance than female mice, with a

193   significantly ($p = 0.0002$) higher appearance score measured in male than in female mice

194   (**Figure 3E**). In contrast, stool consistency and fecal blood scores were comparable in

195   male and female mice for the duration of the DSS treatment (**Figure 3F-G**), demonstrating

196   that these two disease components are not sex-dependent.

197   Evaluation of the time-course for DSS-induced disease progression further

198   revealed that the onset of symptoms associated with colitis is also sex-dependent (**Table**

199   **2**). DSS-treated males, for example, exhibited significant ($p = 0.0042$) changes in

200   cumulative DAI scores at treatment day 1 whereas female mice did not exhibit significant

201   ($p = 0.0157$) changes until treatment day 2. Sex differences in symptom onset consistent

202   with colitis were most pronounced in DSS-induced changes in body weight where males

203   started to lose significant ($p = 0.0124$) weight from treatment day 1 while females did not

204   display significant ($p = 0.0098$) DSS-induced weight loss until treatment day 7. Similarly,

205   males start to exhibit significantly ($p < 0.0001$) worse appearance scores on day 2 of DSS

9

206 treatment while the onset for significant (p = 0.0165) changes in appearance scores in

207 females is on day 4. Consistent with the lack of sex differences in DSS-induced changes

208 in stool consistency and presence of blood in the stool, analysis of the onset of changes

209 in these two parameters was comparable in both sexes. Thus, significant (p < 0.05) DSS-

210 induced changes in stool consistency scores were first observed on treatment day 2 and

211 significant (p < 0.05) changes in fecal blood scores were observed on treatment day 6 in

212 both male and female mice.

213   Altogether, these results demonstrate that while both male and female mice

214 treated with 2.5% DSS develop a clinical profile consistent with colitis, marked sex

215 differences are observed in both the severity and onset of symptoms with males

216 consistently exhibiting worse presentation of symptoms compared to females.

217

218 **Motivation to groom is comparable in both sexes after DSS-induced colitis**

219 The experiments described above revealed worse physical appearance in DSS-treated

220 males compared to females (**Figure 3E**). To assess if differential motivation in self-

221 grooming behavior contributes to sex differences in DSS-induced changes in physical

222 appearance, we performed the splash test in control and DSS-treated male and female

223 mice while simultaneously scoring coat states to monitor changes in physical appearance

224 as a function of time (**Figure 4A**). Immediately after sucrose solution application, all

225 animals had a maximum coat state score independently of sex or DSS/water treatment

226 (**Figure 4B**). The coat state in control male and female mice returned to baseline 50

227 minutes after sucrose solution application. Consistent with the appearance results shown

228 in **Figure 3E**, the coat state of DSS-treated male and female mice did not return to

10

229 baseline, displaying a scruffy and humid physical appearance for the duration of the

230 experiment. Analysis of the total time spent grooming revealed, however, that control and

231 DSS-treated male and female mice spend comparable time grooming (**Figure 4C**),

232 suggesting that self-grooming motivation does not contribute to worse physical

233 appearance in DSS-treated animals independently of sex.

234

235 **Capsaicin-induced hypersensitivity is comparable in male and female mice after**

236 **DSS-induced colitis, but the behavioral manifestation is sex-dependent**

237   Altered pain perception in patients with Irritable Bowel Syndrome (IBS) and other

238 functional gastrointestinal disorders has been associated with the development of visceral

239 pain hypersensitivity [9,36]. Thus, the model of intracolonic capsaicin was used to

240 evaluate spontaneous visceral pain-related behaviors in male and female mice treated

241 with 2.5% DSS as a model of IBS, IBD and other gastrointestinal disorders (**Figure 5A**).

242 Consistent with the results observed in naïve female mice (**Figure 2**), DSS-treated

243 females displayed a significant ($p < 0.0001$) increase in the total number of spontaneous

244 nociceptive behaviors upon intracolonic administration of 0.01% capsaicin compared to

245 respective control mice injected with vehicle (**Figure 5B**). Interestingly, unlike naïve male

246 mice (**Figure 2**), DSS-treated males showed a significant ($p < 0.001$) increase in the total

247 number of spontaneous nociceptive behaviors upon intracolonic administration of 0.01%

248 capsaicin compared to those injected with vehicle control (**Figure 5B**).

249   To identify the specific pain-related behaviors contributing to capsaicin-induced

250 visceral hypersensitivity observed in both sexes, the individual components that comprise

251 the total number of pain-related behaviors were analyzed (**Figure 5C-F**). Consistent with

11

252 the results observed in naïve female mice (**Figure 2**), DSS-treated females showed a

253 significant (p < 0.01) increase in the number of capsaicin-induced licking (**Figure 5C**),

254 dragging (**Figure 5D**) and freezing responses (**Figure 5G**) compared to vehicle-treated

255 females while the number of contraction bouts (**Figure 5F**) were indistinguishable

256 between capsaicin- and vehicle-treated females. In contrast, unlike the capsaicin-induced

257 increases in stretching bouts seen in naïve female mice (**Figure 2**), capsaicin and vehicle-

258 injected females displayed comparable stretching bouts after DSS treatment (**Figure 5E**).

259 Our experiments in naïve male mice showed that both the cumulative and

260 individual spontaneous pain-related behaviors where comparable in mice injected with

261 0.01% capsaicin or vehicle control (**Figure 2**). In contrast with these results, evaluation

262 of the individual capsaicin-induced pain-related behaviors after DSS-induced colitis

263 showed significant (p < 0.05) increases in the number of dragging (**Figure 5D**) and

264 stretching bouts (**Figure 5E**) after intracolonic injection of 0.01% capsaicin, compared to

265 intracolonic vehicle control injections. Consistent with the results observed in naïve male

266 mice, however, the number of licking (**Figure 5C**) and contraction bouts (**Figure 5F**) as

267 well as time spent freezing (**Figure 5G**) were indistinguishable in capsaicin and vehicle-

268 injected DSS-treated males. Collectively, these results demonstrate that both sexes

269 develop capsaicin-induced hypersensitivity to intracolonic application of 0.01% capsaicin

270 after DSS-induced colitis. The manifestation of hypersensitivity, however, was sex-

271 dependent, with both sexes displaying increases in dragging behavior (**Figure 5D**) but

272 only females showing increases in licking and freezing behaviors (**Figure 5C and 5G**)

273 and only males displaying increases in stretching behaviors (**Figure 5E**).

274

275 **Referred abdominal hypersensitivity is sex-dependent after acute but not**

276 **persistent colon inflammation**

277 Previous studies have shown that following intracolonic administration of capsaicin or

278 DSS-induced colitis, mice display referred abdominal hypersensitivity, manifested as an

279 increase in the frequency of pain-related responses to tactile stimulation of the abdomen

280 during the von Frey Test (Laird, Martinez-Caro et al. 2001, Jain, Hassan et al. 2015). The

281 next set of experiments aimed at evaluating potential sex differences in baseline

282 responses to tactile stimulation of the abdomen using a 0.16g von Frey filament as well

283 as in referred abdominal hypersensitivity after acute and persistent colon inflammation,

284 induced by intracolonic administration of 0.01% capsaicin or DSS in drinking water,

285 respectively (**Figure 6A**). Response frequency was indistinguishable between males and

286 females in control conditions (**Figure 6B**), demonstrating that baseline responses to

287 tactile stimulation of the abdomen are not sex dependent. Consistent with previous

288 reports [29], intracolonic injection of 0.01 % capsaicin significantly ($p < 0.001$) increased

289 response frequency to tactile stimulation of the abdomen in female mice compared to

290 intracolonic vehicle control injections (**Figure 6B**). In contrast, response frequencies were

291 indistinguishable in male mice following intracolonic administration of 0.01% capsaicin or

292 vehicle control. These findings are consistent with the higher capsaicin-induced

293 spontaneous nociceptive visceral responses we observe in females (**Figure 2**), further

294 demonstrating that referred abdominal hypersensitivity is also sex-dependent in the

295 context of acute colon inflammation.

296 Evaluation of DSS-induced referred abdominal hypersensitivity in males and

297 females revealed that both male and female mice displayed significant ($p < 0.0001$)

298 increases in response frequencies to tactile stimulation of the abdomen using a 0.16g von

299 Frey filament after intracolonic administration of 0.01% capsaicin when compared to

300 animals injected with intracolonic vehicle control (**Figure 6B**). Further analyses showed

301 that, as expected, capsaicin-induced hypersensitivity to tactile stimulation is potentiated

302 by DSS-induced persistent colon inflammation, with both sexes showing a significant (p

303 < 0.05) increase in response frequencies to tactile stimulation of the abdomen after

304 intracolonic administration of 0.01% capsaicin in the context of DSS-induced colitis when

305 compared to control mice injected with 0.01% capsaicin. These combined results are

306 consistent with our findings showing that spontaneous capsaicin-induced visceral

307 responses in the context of DSS-induced colitis is not sex-dependent (**Figure 5**) and

308 further demonstrate that referred abdominal hypersensitivity in the context of persistent

309 colon inflammation is also not sex-dependent.

310

311 **DSS-induced bowel pathology is comparable in males and females**

312 The results presented above show that clinical progression of disease and

313 behavioral manifestation of hypersensitivity following DSS treatment are sexually

314 dimorphic. To gain insight into whether DSS-induced bowel pathology contributes to the

315 observed sex differences, colon lengths and histopathological analysis of the bowels of

316 control and DSS-treated mice of both sexes were evaluated (**Figure 7A**). Bowel length is

317 commonly used to evaluate macroscopic manifestations of gastrointestinal disease in

318 rodents, with shortening of the bowel reported in many gastrointestinal conditions,

319 including DSS-induced colitis [2,39]. Consistent with previous reports, analysis of our

320 experiments showed that both sexes exhibit significant ($p < 0.0001$) shortening of the

14

321   bowel after DSS treatment when compared to their respective water controls (**Figure 7B-**

322   **C**). Further analysis revealed that female bowels are significantly ($p < 0.05$) shorter than

323   male bowels in both control and DSS-treated mice. Comparison of the change in average

324   bowel length in control and DSS-treated mice reveals, however, that both sexes display

325   a bowel shortening of ~19% after DSS treatment, when compared to their respective

326   water controls (**Figure 7C**). These results demonstrate that the manifestation of DSS-

327   induced colitis is comparable in males and female mice at the gross pathological level.

328   Histopathological analysis of the colon of both control and DSS-treated male and

329   female mice was performed to assess bowel pathology at the microscopic level (**Figure**

330   **7D-H**). Analyses of the cumulative histology score, composed of glands-mucosa,

331   inflammation and % of colonic tissue affected scores, revealed that DSS induces

332   increases in histology scores in bowels from both male and female mice in a dose-

333   dependent manner, with higher histology scores associated with increasing dose of DSS

334   administered (**Figure 7E**). Consistent with our analysis of gross bowel pathology,

335   analyses of histology scores showed comparable pathology at the microscopic level

336   between DSS-treated male and female mice. Analyses of the individual components

337   further demonstrated comparable damage of the colonic crypts and mucosa (**Figure 7F**),

338   inflammatory infiltration of leucocytes (**Figure 7G**) and % of colonic tissue affected

339   (**Figure 7H**) in DSS-treated males and females. Altogether, these results show no

340   measurable sex difference in DSS-induced gross and microscopic colon pathology.

341

342   **DISCUSSION**

343   Chronic visceral pain is predominantly reported in women and higher pain

344   sensitivity has been shown in women patients [46]. Despite this, the majority of preclinical

345   pain studies have focused only on males [35]. The limited inclusion of sex as a biological

346   variable in preclinical studies results in an incomplete understanding of the biological

347   processes underlying pain [35]. In this study, we evaluated and characterized potential

348   sex differences in visceral pain-related responses, disease progression of colitis and

349   colitis-induced colon pathology using the intracolonic capsaicin and DSS-induced colitis

350   models of chemically induced visceral hypersensitivity in mice (**Figure 8**). Our behavioral

351   experiments show sex-dependent differences in visceral pain-related behaviors, with

352   females exhibiting more pain-related responses and referred abdominal hypersensitivity

353   than males in the context of acute colon inflammation induced by intracolonic capsaicin

354   (**Figure 8A-B**). We further show that colitis-induced visceral hypersensitivity and referred

355   abdominal hypersensitivity are similar between males and females, but the manifestation

356   of capsaicin-induced behavioral responses is sex-dependent. While both sexes exhibited

357   dragging, stretching, and freezing in response to intracolonic capsaicin, increases in

358   licking of the abdomen were only observed in females and increases in abdominal

359   contractions were only displayed by males (**Figure 8A**). Consistent with other studies

360   [2,10,39], we also show that progression of DSS-induced colitis is sex-dependent, with

361   males exhibiting worse clinical progression, manifested as worse appearance and higher

362   weight loss, than in females (**Figure 8C**). No measurable sex differences were observed,

363   however, in colitis-induced bowel pathology, stool consistency or fecal blood (**Figure 8D**).

364   Together, these findings stress the importance of incorporating both sexes when studying

365   mechanisms that drive clinical and behavioral manifestations of visceral hypersensitivity.

366

Behavioral manifestation of visceral pain-related behaviors is sex-dependent

367   While previous studies consistently show that the model of DSS-induced colitis and intracolonic capsaicin produce visceral hypersensitivity and referred abdominal hyperalgesia in both sexes [20,29], potential sex differences in pain-related behaviors in these two models of visceral pain have not been evaluated. In the present study, we demonstrated that visceral pain-related behaviors in freely behaving mice is sex-dependent. We specifically showed that females display higher capsaicin-induced spontaneous nociceptive responses than males and that the behavioral manifestation of pain-related responses to intracolonic capsaicin is also dependent on sex, with increases in licking of the abdomen observed only in females and increases in abdominal contractions only displayed by males (**Figures 2 and 5**).

378   Sex differences in behavioral outputs have been described previously in other contexts such as fear and escape behaviors [12,14,27], highlighting the importance of characterizing behavioral assays in both males and females. Previous studies have further shown that neural circuits driving distinct pain-related responses, such as paw withdrawal or licking in response to evoked tactile stimulation, are unique [17]. Our behavioral experiments showing that male and female mice exhibit distinct behavioral responses to intracolonic capsaicin suggest that recruitment of neural circuits in responses to a particular noxious stimulus might be sex-dependent.

386

Clinical progression of disease in a mouse model of colitis are sex-dependent

17

388   Our experiments showed that DSS treatment induces a clinical profile consistent

389   with colitis in both male and female mice and that males exhibit a worse clinical

390   progression of disease (**Figure 3**). The disease activity index (DAI) used to measure

391   clinical progression of colitis is typically presented as a cumulative score of the following

392   components: appearance, weight loss, stool consistency and fecal blood

393   [2,6,10,25,32,39]. Analysis of the individual DAI components in the present study

394   revealed that higher DAI scores in males are driven by greater weight loss and worse

395   appearance but that DSS-induced changes in stool consistency and fecal blood are

396   similar between sexes. The lack of measurable sex differences in stool consistency and

397   fecal blood, together with the comparable DSS-induced shortening of the bowel (**Figure**

398   **7**) suggests that gross pathology of the bowel is not the driving force behind the sex

399   differences in weight loss. Weight loss is a hallmark in animal models of experimental

400   colitis and has been associated with decreases in food intake and/or changes in

401   metabolic rates, locomotor activity or body fat content [33]. Similarly, weight loss in IBD

402   patients has been related with decreased appetite, alterations in metabolic hormones

403   and/or malabsorption of nutrients [7]. Future studies to explore whether DSS induces sex-

404   dependent changes in food consumption and/or metabolic alterations that contribute to

405   colitis-induced weight loss are needed.

406   Despite the worse physical appearance seen in DSS-treated males (**Figure 3E**),

407   coat state and time spent grooming after sucrose solution application in the splash test

408   was similar between males and females (**Figure 4**). These results are consistent with

409   findings from previous studies in males [20] and suggest that motivation to self-groom is

410   not affected after DSS-induced colitis and it is not sex-dependent. When interpreting

411 these findings, it is important to consider that sucrose solution application creates an

412 acute challenge that experimentally evokes grooming. Motivation to groom when

413 presented with an acute challenge like sucrose application might be different to motivation

414 to self-groom for daily upkeeping. Previous studies have shown that self-grooming is an

415 evolutionary conserved and innate behavior in mice that involves a complex sequencing

416 pattern and contributes to hygiene maintenance, thermoregulation, social

417 communication, and other biological processes [24]. The worse physical appearance in

418 DSS-treated males, combined with the lack of sex or treatment-dependent effect on time

419 spent grooming in response to sucrose application, suggest that males are less efficient

420 at grooming than females after the induction of colitis.

421   An important caveat of the experiments presented here is that the same

422 percentage of DSS was used in males and females, despite males having greater initial

423 body weight than females. Consequently, the dose of DSS per body weight is most likely

424 lower in males than it is in females. Whether sex differences in metabolism and excretion

425 rates of DSS influence the observed DSS-induced phenotypes is also unknown. Despite

426 potential differences in the absolute DSS dose intake, however, males exhibit worse

427 disease progression than females, further emphasizing sexual dimorphism in disease

428 progression in the context of colitis.

429

430 **Comparison with other studies and potential translational application**

431   Our behavioral experiments show that female mice display more capsaicin-

432 induced spontaneous visceral pain-related responses than males (**Figure 2**). These

433 results are consistent with previous studies showing that female rats have higher

434 viceromotor responses to noxious colorectal distension at baseline and after intracolonic

435 injection of mustard oil than male rats [15,22,23]. Measurements of visceromotor

436 responses to colorectal distension are typically performed in lightly anesthetized or

437 loosely restrained rats, which may confound the behavioral outcomes measured and limit

438 the interpretation of findings. The consistencies between our behavioral findings in freely

439 behaving mice and those in previous studies using lightly anesthetized or loosely

440 restrained rats demonstrate that sex differences in visceral pain-related behaviors are

441 comparable and can be equally studied in freely behaving mice or in rats under light

442 anesthesia or loosely restrained. Our results showing that female rodents display higher

443 visceral sensitivity than males are also consistent with clinical studies in humans that also

444 show higher visceral sensitivity in women than men [3,4,44], validating the use of rodent

445 preclinical models to mechanistically study sex differences in visceral pain. Lastly, the

446 demonstration that sex differences in visceral pain-related responses are recapitulated in

447 mice is important as they offer a more enriched repertoire of molecular genetic tools to

448 study behavior at circuit and mechanistic levels.

449 It is important to note that our experiments showed that visceral hypersensitivity in

450 the context of DSS-induced colitis is not sex-dependent (**Figure 5**). These findings

451 contrast with clinical studies that report that female IBS patients exhibit higher visceral

452 sensitivity and report more severe abdominal pain or discomfort than men (Chang, Mayer

453 et al. 2006, Tang, Yang et al. 2012, Camilleri 2020). As discussed above, however, our

454 experiments also demonstrated that the behavioral manifestation of visceral pain-related

455 responses in mice is sex-dependent (**Figure 2 and 5**), suggesting that pain perception is

456 differentially experienced by females and males. Given that pain is a multidimensional

20

457 experience that comprises affective, cognitive, and somatosensory components, studies

458 that evaluate these components in humans are needed to understand the mechanisms

459 driving sex differences in visceral pain. Based on these results, improved diagnostic and

460 therapeutic options for pain relief can be developed.

461 At a pathological level, consumption of DSS in drinking water has been shown to

462 disturb the colonic lumen structure, eliciting an inflammatory response that emulates

463 inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease

464 (CD) [30]. Consistent with previous reports [39], we also show that DSS induces

465 shortening and histopathological changes in the colon (**Figure 7**) that resemble the

466 shortening of the small bowel and histopathological features observed in patients with

467 ulcerative colitis (UC) and Crohn's disease (CD) [38], further validating the applicability of

468 this model to study IBDs. Notably, however, while previous studies have reported sex

469 differences in DSS-induced bowel shortening and histopathology [2,10,31], our

470 experiments showed that bowel pathology is comparable between sexes. These

471 discrepancies could be due to differences in strain used, DSS supplier or animal facility-

472 specific factors that have been previously shown to strongly influence DSS experiments

473 [5,31].

474 The experiments in the present study provide evidence for sex differences in

475 visceral pain-related behaviors and clinical progression of colitis in preclinical models of

476 acute and persistent colonic irritation. Together, these results highlight the importance of

477 considering sex as a biological variable in both preclinical and clinical pain studies.

478

479 **MATERIALS AND METHODS**

21

Subjects

480

481 All animal procedures were performed in accordance with the guidelines of the National

482 Institutes of Health (NIH) and were approved by the Animal Care and Use Committee of

483 the National Institute of Neurological Disorders and Stroke and the National Institute on

484 Deafness and other Communication Disorders. Adult male and female Swiss-Webster

485 mice (Taconic Farms) between 8-13 weeks old were used for all experiments. Littermates

486 were randomly assigned to experimental groups and group housed (up to 5 mice per

487 cage) under reversed 12 h light/dark cycle (9 pm to 9 am light). One week prior to

488 experiments, mice were housed in pairs in clean home cages separated by a perforated

489 Plexiglas divider. Food and water were provided ad libitum. Prior to all behavioral

490 experiments, mice were handled as previously described for at least 5 days to minimize

491 potential stress effects associated with handling [18]. Male and female mice were never

492 tested simultaneously in the same behavior room. All experimental procedures were

493 performed by an observer blind to experimental treatment and replicated at least 3 times.

494

DSS-induced Model of Colitis

496 Male and female mice were randomly assigned to drinking water (control group) or 2.5%

497 (wt/vol) Dextran Sulfate Sodium (DSS) salt (reagent-grade, mol. wt. 36 to 50 kDa; MP

498 Biomedicals, #16011050) dissolved in drinking water to induce colitis (experimental

499 group), ad libitum for seven days. On day 7, mice were switched to regular water ad

500 libitum for the duration of the experiment. Intracolonic capsaicin, referred abdominal

501 sensitivity and bowel sample collection experiments were performed on day 8 (**Figure**

502 **1A**). Each day, along with the handling of the mouse, the disease activity index (DAI) was

22

503 logged by an investigator blind to experimental treatment to evaluate and score disease

504 progression as previously described (Kim et al., 2012). The DAI is a composite of scores

505 for weight loss, appearance, stool consistency, and blood presence in the stool using a

506 Hemoccult card (Beckman Coulter). See **Table 1**.

507

508 **Intracolonic Capsaicin**

509 On experiment day 8 (see full experimental timeline above and in **Figure 1A**), male or

510 female mice were habituated (for 1hr) and tested on an elevated mesh platform in

511 individual 11 × 11 × 13 cm ventilated opaque white Plexiglas boxes. A mirror was placed

512 at 45-degree angle under the mesh platform to allow full visualization of the animals.

513 Intracolonic injections were performed as previously described [40]. Briefly, mice were

514 anesthetized with 1% isoflurane in an induction chamber, and then kept lightly

515 anesthetized with 0.5%–1% isoflurane at a flow rate of 0.5 L/min. A light layer of petroleum

516 jelly (Vaseline) was applied to the peri-anal area and tubing to avoid the stimulation of

517 somatic areas and ease tube insertion. Capsaicin (0.01% or 0.03%) was prepared using

518 a stock solution of capsaicin (Sigma Aldrich) diluted in ethanol, Tween80, and saline

519 (10/10/80, respectively). 50µl of capsaicin (0.01% or 0.03%) or vehicle control (10%

520 ethanol, 10% Tween 80, and 80% saline) was slowly injected via PE-10 non-toxic, sterile

521 polyethylene tubing (0.28 mm ID / 0.61 mm OD; Daigger Scientific) with a rounded tip

522 connected to a blunted 30G x 1/2 needle (BD PrecisionGlide) and 1 cc syringe (Terumo)

523 that was gently introduced 4 cm into the colon via the anus. Spontaneous nociceptive

524 responses to intracolonic capsaicin (or vehicle control) were measured for 20 minutes

525 immediately following intracolonic injections. Spontaneous nociceptive responses were

23

526 defined as licking of abdomen, stretching of abdomen, dragging and abdominal

527 contractions. The time spent freezing following the injection was also measured during

528 the 20-minute test period.

529

530 **Referred Abdominal Hypersensitivity**

531 Referred abdominal hypersensitivity was evaluated 45 minutes after capsaicin (or vehicle

532 control) intracolonic injection as described previously [40]. The abdominal hair of all test

533 mice was removed (24 h) before testing with a depilatory (Nair). A 0.16g von Frey filament

534 (North Coast Medical, Inc. San Jose, CA) was applied to the abdomen for approximately

535 1–2 s, with a stimulus interval of 15 s. Positive responses were defined as a rapid

536 withdrawal, jumping, licking or abdominal contractions immediately following application

537 of the von Frey filament to the abdomen. A total of 10 trials were performed and the

538 number of positive responses per animal were recorded and reported as response

539 frequency.

540

541 **Splash Test**

542 On experiment day 8 (see full experimental timeline above and in **Figure 1A**), control and

543 DSS-treated male and female mice were individually transferred to a new home cage with

544 regular bedding and were habituated in them for at least 1hr. Each mouse received two

545 sprays of 10% sucrose solution on their dorsal coat. Immediately following sucrose

546 solution application, the coat state of the animals was scored every 10 min for 1hr and

547 every 20 min starting at the 1-hour timepoint until the 2-hours timepoint from the sucrose

548 solution application. The coat state score was obtained using the following system: 8 =

24

549   wet, soaked dorsal coat; 4 = scruffy and humid dorsal coat; 2 = dry and smooth lower

550   back coat but upper back coat is still scruffy and/or humid; 1 = mostly dry and smooth

551   dorsal coat with a few scruffy patches and 0 = completely dry and smooth dorsal coat.

552   The time spent in grooming behavior was measured during a five-minute period

553   immediately after sucrose solution application as well as 35 and 65 minutes after sucrose

554   solution application. The observer was always blind to experimental treatment.

555

556   **Bowel Sample Collection and Histopathological Analysis**

557   On experiment day 8 (see full experimental timeline above and in **Figure 1A**), control and

558   DSS-treated males and females were anesthetized with 1% isoflurane in an induction

559   chamber and euthanized by cervical dislocation. Bowels were dissected and the colon

560   was carefully removed to measure its length. The colon was subsequently divided in

561   proximal, medial, medial/distal, and distal 1 cm sections, fixed in 10% Neutral Buffered

562   Formaldehyde (Azer Scientific) for 24hrs and stored in 70% ethanol until processing.

563   Tissue samples were embedded in paraffin blocks, cut in 5μm sections, fixed to glass

564   slides, and stained with hematoxylin and eosin (H&E) by Histoserv (Germantown, MD).

565   Histopathological analysis of the tissue samples was performed as previously described

566   [8,25]. Tissue damage was assessed using a cumulative histology score of glands-

567   mucosa, inflammation and % of colonic tissue affected. The score for glands-mucosa was

568   as follows: 0 = no changes in glands-mucosa structure; 1 = loss of up to 1/3 of gland,

569   crypts lifted off muscularis mucosae; 2 = loss of 2/3 of gland, crypts lifted off muscularis

570   mucosae, little inflammation, thinning and loss of epithelial cells in the *intact* glands,

571   cryptitis; 3 = loss of all the glands but the superficial epithelium is intact, mild infiltrate; 4

572 = erosion, ulceration, mild - moderate infiltrate. Inflammation score was defined as 0 =

573 none to few leucocytes; 1 = mild, some increase in leucocytes at tips of crypts; 2 =

574 moderate; 3 = severe, dense infiltrate throughout, transmural. Percent of colonic tissue

575 affected was scored as 1 = 1 – 25%; 2 = 25 – 50%; 3 = 50 – 75%; 4 = 75 – 100 %.

576 Analyses of the histology score and its individual components were initially performed

577 separately per anatomical section of the colon collected (i.e. proximal, medial,

578 medial/distal, and distal). This analysis showed that DSS-induced histopathology is

579 comparable between all colon sections evaluated within bowels from mice of the same

580 sex and dose of DSS administered. Based on these results, we calculated the average

581 score of all 4 colonic sections per mouse for each parameter and used these values for

582 subsequent graphs and analyses.

583

584 **Statistics**

585 Results are expressed as mean ± standard errors of the mean (SEM). Analysis was

586 performed using either Unpaired t-tests (without Welch's correction for variance), Mann-

587 Whitney U tests, two-way analyses of variance (ANOVA) or two-way analyses of variance

588 (ANOVA) followed by Tukey's multiple comparison tests or by Šidák's multiple

589 comparison. All analyses were performed using GraphPad Prism (v. 9) and p values lower

590 than 0.05 were considered significant and are reported in figure legends.

591

592 **Data Availability**

593 All data in this study is available from the corresponding author.

594

595 **AUTHOR CONTRIBUTIONS**

596 Conceptualization, C.P., S.M.G., Y.C., A.M.F.M; Investigation, S.M.G., T.D.W., L.G.R.,

597 A.M.F.M; Data Analysis, A.M.F.M, S.M.G., T.D.W., L.G.R., L.R.B., Y.C.; Writing –

598 Original Draft, A.M.F.M, Y.C.; Writing – Review & Editing,; Supervision, Y.C.; Funding

599 Acquisition, Y.C.

600

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609

610 **COMPETING INTERESTS**

611 The authors declare no conflict of interests.

612

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FIGURE LEGENDS

Figure 1. Experimental timeline for behavioral and histological experiments in a model of DSS-induced colitis. Male and female mice were treated with either 2.5% DSS or water and clinical progression of disease was monitored for 7 days. Spontaneous responses to intracolonic capsaicin (0.01% or 0.03%) or vehicle control and referred abdominal sensitivity to tactile stimulation were measured 1d after the end of DSS treatment. Bowels were dissected and measured and histologically processed for pathology assessment.

Figure 2. Capsaicin-induced nociceptive behaviors are higher in female than male mice and the behavioral manifestation is sex-dependent. (A) Timeline of behavioral experiments. Male and female mice were injected with intracolonic capsaicin (0.01% or 0.03%) and capsaicin-evoked nociceptive responses, defined as licking, stretching, dragging, contractions of the abdomen, and freezing behaviors were recorded. (B-M) Total number of pain-related behaviors (B-C), licking bouts (D-E), dragging bouts (F-G), stretching bouts (H-I), contraction bouts (J-K) and time spent in freezing behavior (L-M) in vehicle control, 0.01% capsaicin and 0.03% capsaicin. Data is presented as mean ± SEM. n = 6-10 females and 5-9 males. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: veh vs 0.01% cap; two-way ANOVA followed by Šídák's multiple comparisons test; ##$p < 0.01$: veh vs 0.03% cap; unpaired t-test; §§$p < 0.01$: veh vs 0.03% cap; Mann Whitney test.

Figure 3. Clinical progression of disease in a DSS-induced mouse model of colitis is sexually dimorphic. (A) Timeline for the induction of experimental colitis. Male and female mice were treated with either 2.5% DSS or water, ad libitum for seven days. Disease Activity Index (DAI) cumulative score was obtained daily to monitor the

35

783   progression of DSS-induced colitis. (B) DAI score was dependent on the dose of DSS

784   administered. (C-G) Cumulative DAI scores (C) and its individual components, defined as

785   percentage of weight change (D), appearance (E), stool consistency (F) and fecal blood

786   (G) were analyzed. Data is presented as mean ± SEM. n = 6-12 females and 6-12 males.

787   *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: male vs female; two-way ANOVA.

788   **Figure 4. Motivation to groom is comparable in both sexes after DSS-induced**

789   **colitis.** (A) Timeline for splash test experiment. 10% sucrose solution was applied to the

790   dorsal coat of control and DSS-treated male and female mice. (B-C) Coat state score (B),

791   and total time spent grooming (C) were recorded. Data is presented as mean ± SEM. n =

792   5 females and 5 males. **$p < 0.01$: control vs 5% DSS; two-way ANOVA.

793   **Figure 5. Capsaicin-induced hypersensitivity is comparable in male and female**

794   **mice after DSS-induced colitis, but the behavioral manifestation is sex-dependent.**

795   (A) Timeline of behavioral experiments. DSS-treated male and female mice were injected

796   with intracolonic 0.01% capsaicin and capsaicin-evoked nociceptive responses, defined

797   as licking, stretching, dragging, and contractions of the abdomen, and freezing behaviors

798   were recorded. Total number of pain-related behaviors (B), licking bouts (C), dragging

799   bouts (D), stretching bouts (E), contraction bouts (F) and time spent in freezing behavior

800   (G) in vehicle control and 0.01% capsaicin. Data is presented as mean ± SEM. n = 6-9

801   females and 6-13 males. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$: veh vs 0.01%

802   cap; two-way ANOVA followed by Šídák's multiple comparisons test.

803   **Figure 6. Referred abdominal hypersensitivity is sex dependent after acute but not**

804   **persistent colon inflammation.** (A) Timeline for von Frey behavioral test in the context

805   of acute and persistent colon irritation. Control and DSS-treated male and female mice

806 were injected with intracolonic 0.01% capsaicin or vehicle control and abdominal

807 sensitivity to tactile stimulation was measured. (B) Quantification of response frequency

808 to tactile stimulation of the abdomen in control and DSS-treated male and female mice

809 after intracolonic injection of 0.01% capsaicin or vehicle control. Data is presented as

810 mean ± SEM. n = 6-13 males and 6-10 females. *p < 0.05: control-0.01% cap vs DSS-

811 0.01% cap, **p < 0.01: control-0.01% cap vs DSS-0.01% cap, ***p < 0.001: control-veh

812 vs control-0.01% cap, and ****p < 0.0001: DSS-veh vs DSS-0.01% cap; two-way ANOVA

813 followed by Tukey's multiple comparisons test.

814 **Figure 7. DSS-induced bowel pathology is comparable in males and females.** (A)

815 Timeline for bowel pathology analysis. Bowels of control and DSS-treated male and

816 female mice were dissected, colon length was measured 1d after the end of DSS

817 treatment and colonic samples were collected for histopathological analysis. (B)

818 Representative images of control (top) and DSS-treated (bottom) colon samples. (C)

819 Colon length in control and DSS-treated male and female mice. (D) Timeline for

820 histological analysis of DSS-induced colon pathology. (E) Cumulative histology score in

821 bowels from control and DSS-treated male and female mice and its individual

822 components, defined as glands/mucosa score (F), inflammation score (G) and % colonic

823 tissue affected (H). Data is presented as mean ± SEM. n = 6-13 females and 5-21 males.

824 *p<0.05: males vs females and ****p<0.0001: control vs 2.5% DSS; two-way ANOVA

825 followed by Tukey's multiple comparisons test.

826 **Figure 8. Summary of main findings.** (A) Behavioral manifestation of capsaicin-induced

827 spontaneous visceral nociceptive responses is sex dependent after acute and persistent

828 colon inflammation. After intracolonic capsaicin, females exhibit more capsaicin-induced

37

829   nociceptive behaviors than males. In the context of DSS-induced colitis, visceral

830   hypersensitivity is comparable in both sexes, but the manifestation is distinct. (B) Referred

831   abdominal hypersensitivity is higher in females after intracolonic capsaicin but is similar

832   between sexes after persistent colon inflammation or at baseline. (C) Progression of DSS-

833   induced colitis is sex dependent. Worse physical appearance and greater weight loss is

834   observed in males compared to females. (D) No measurable sex difference was observed

835   in DSS-induced bowel shortening.
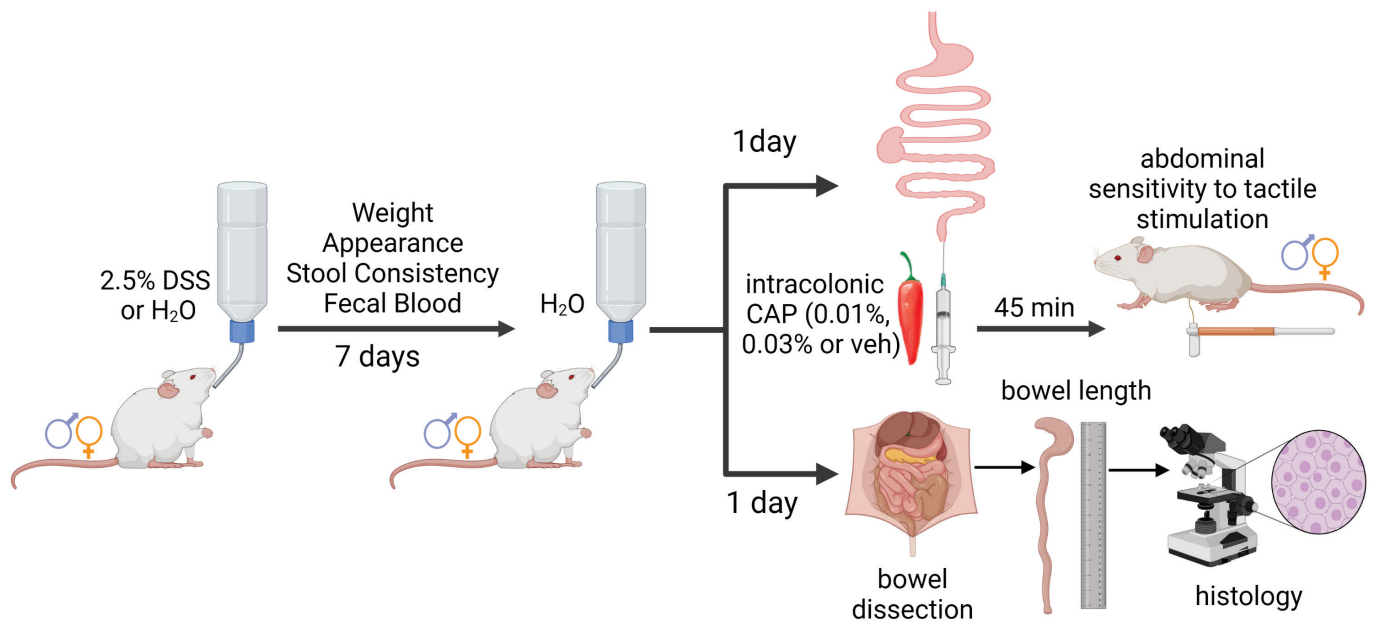
Figure 1.
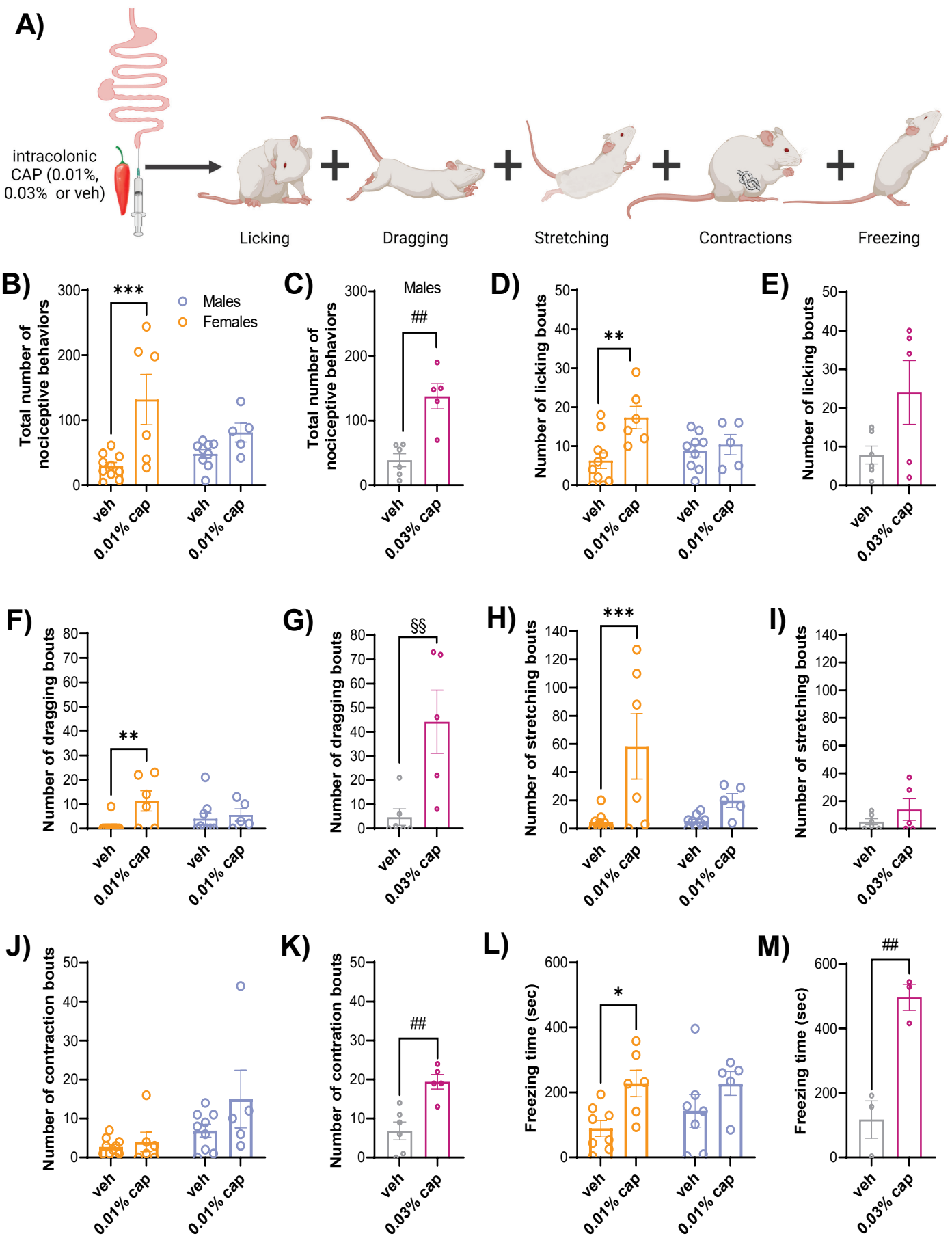
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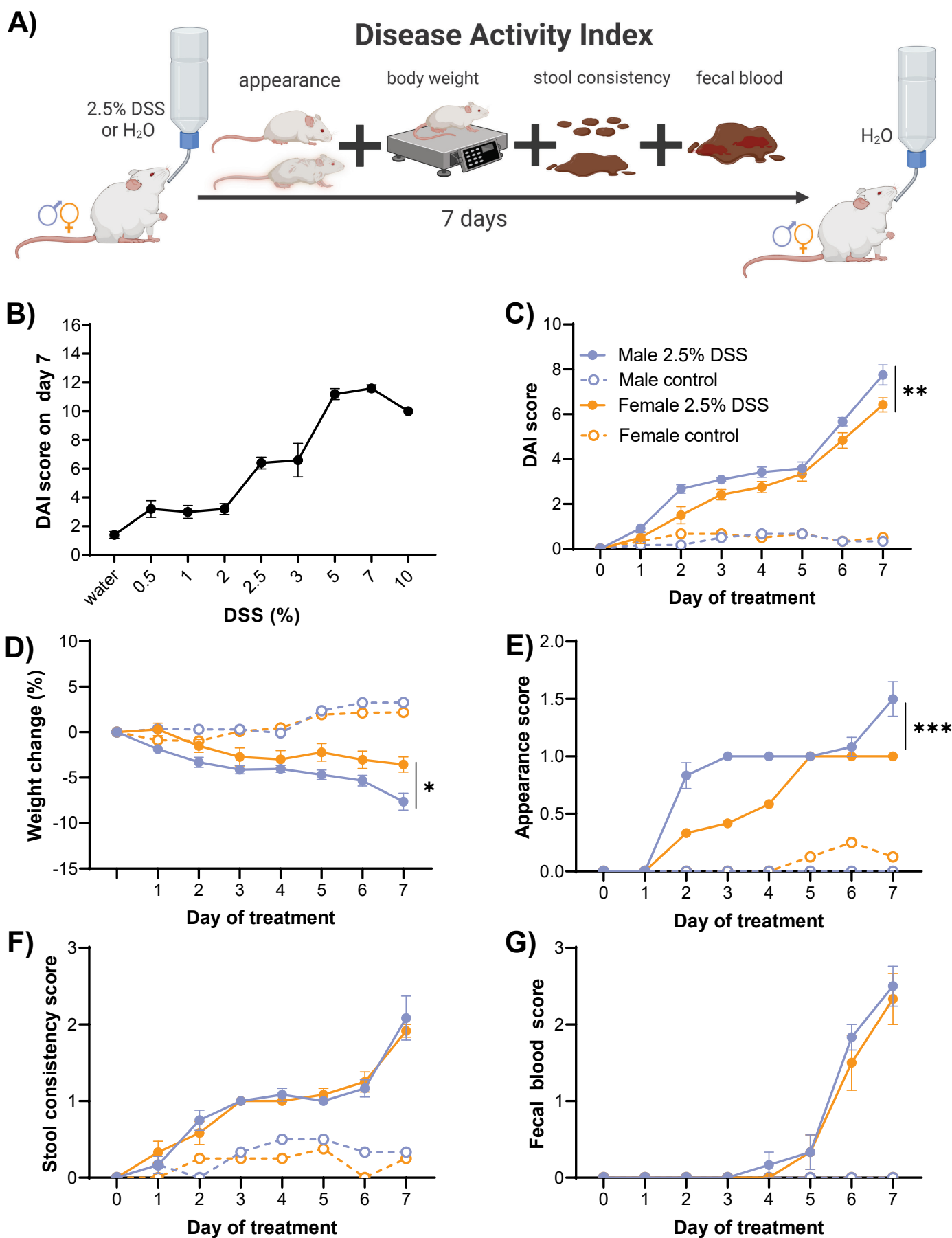
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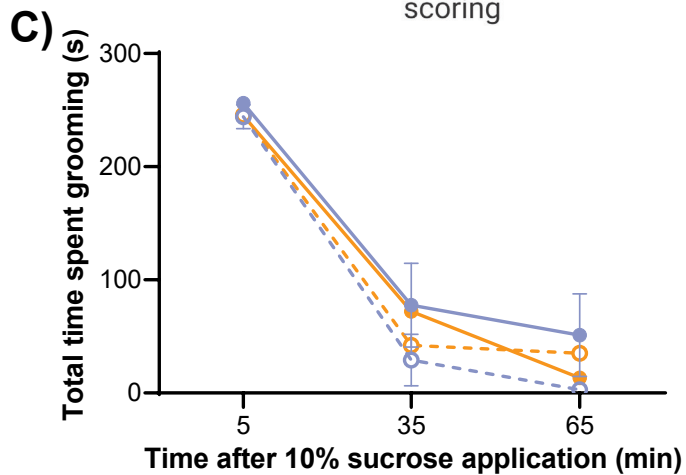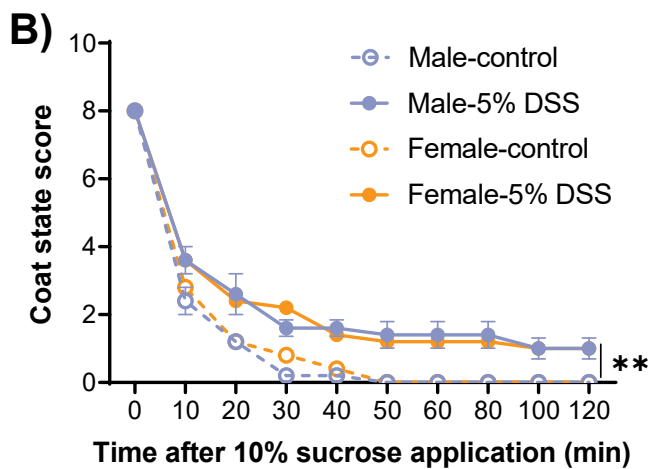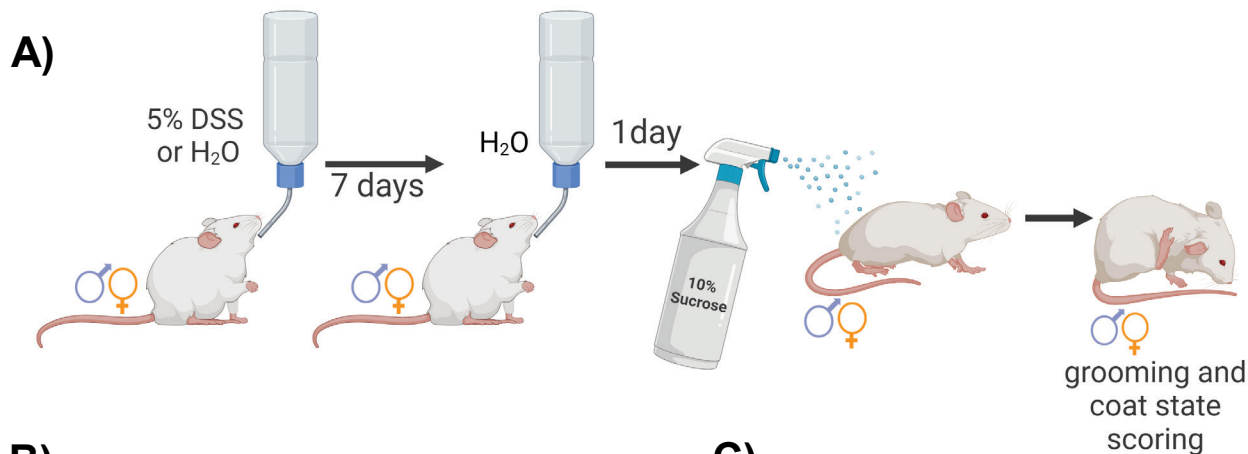
Figure 4.
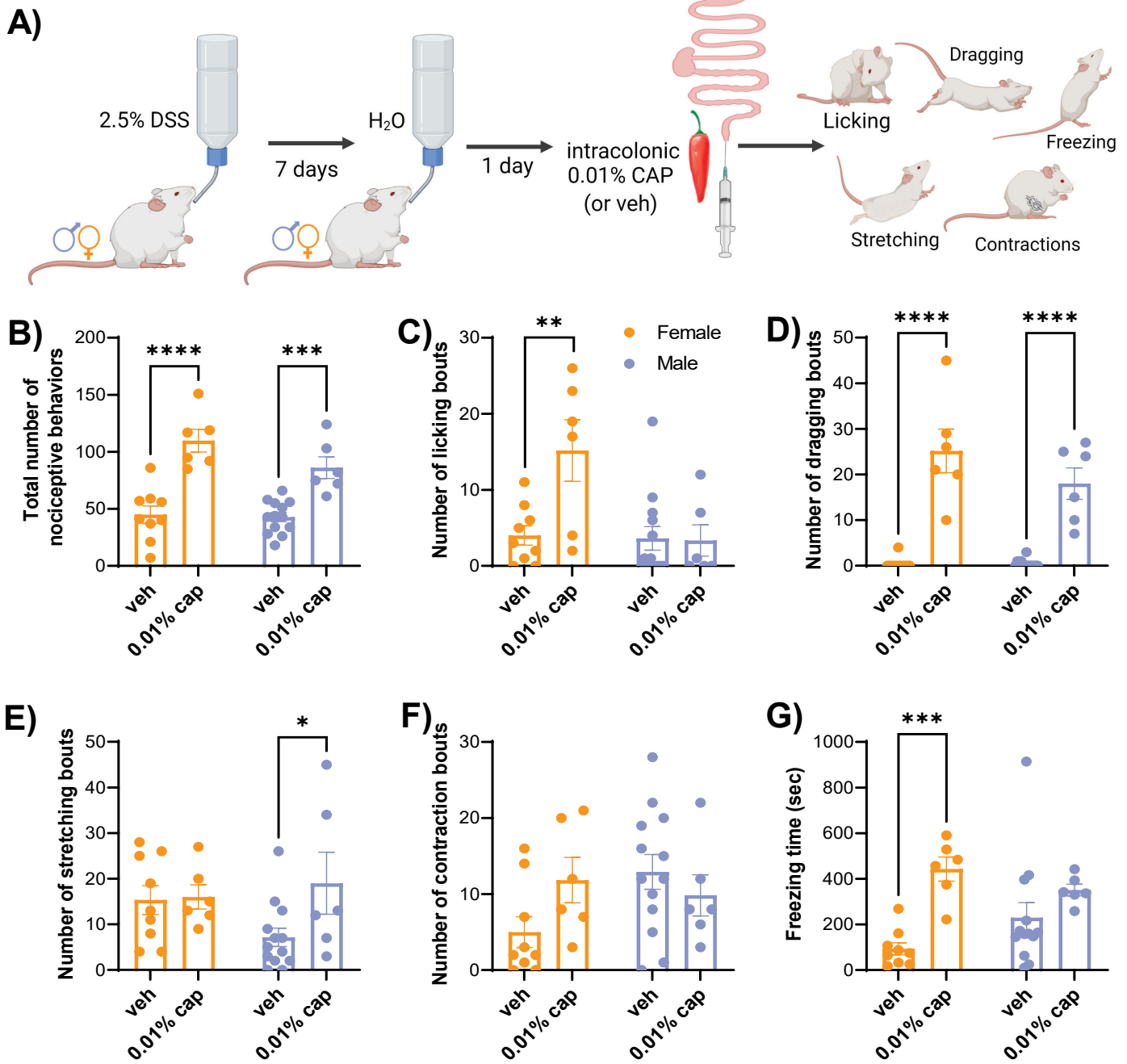
Figure 5.

A)
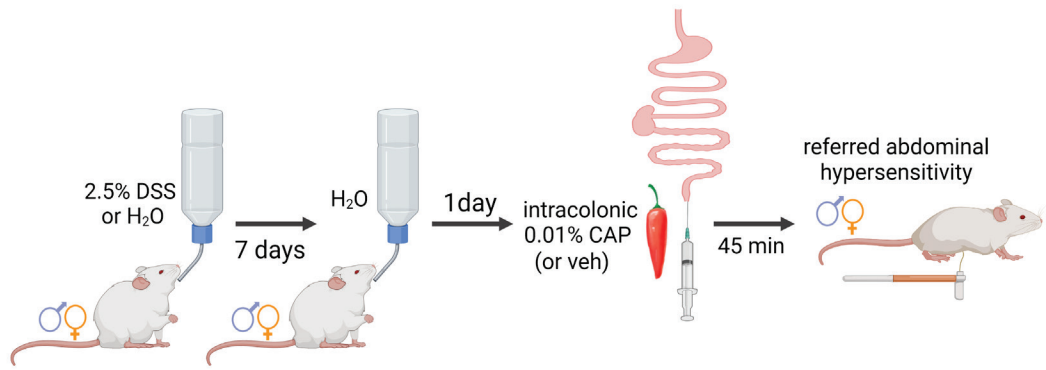
B)
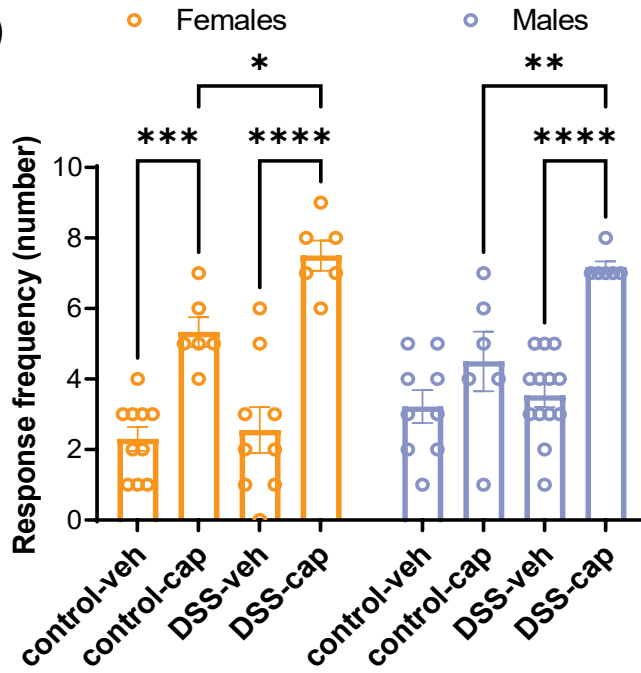
Figure 6.
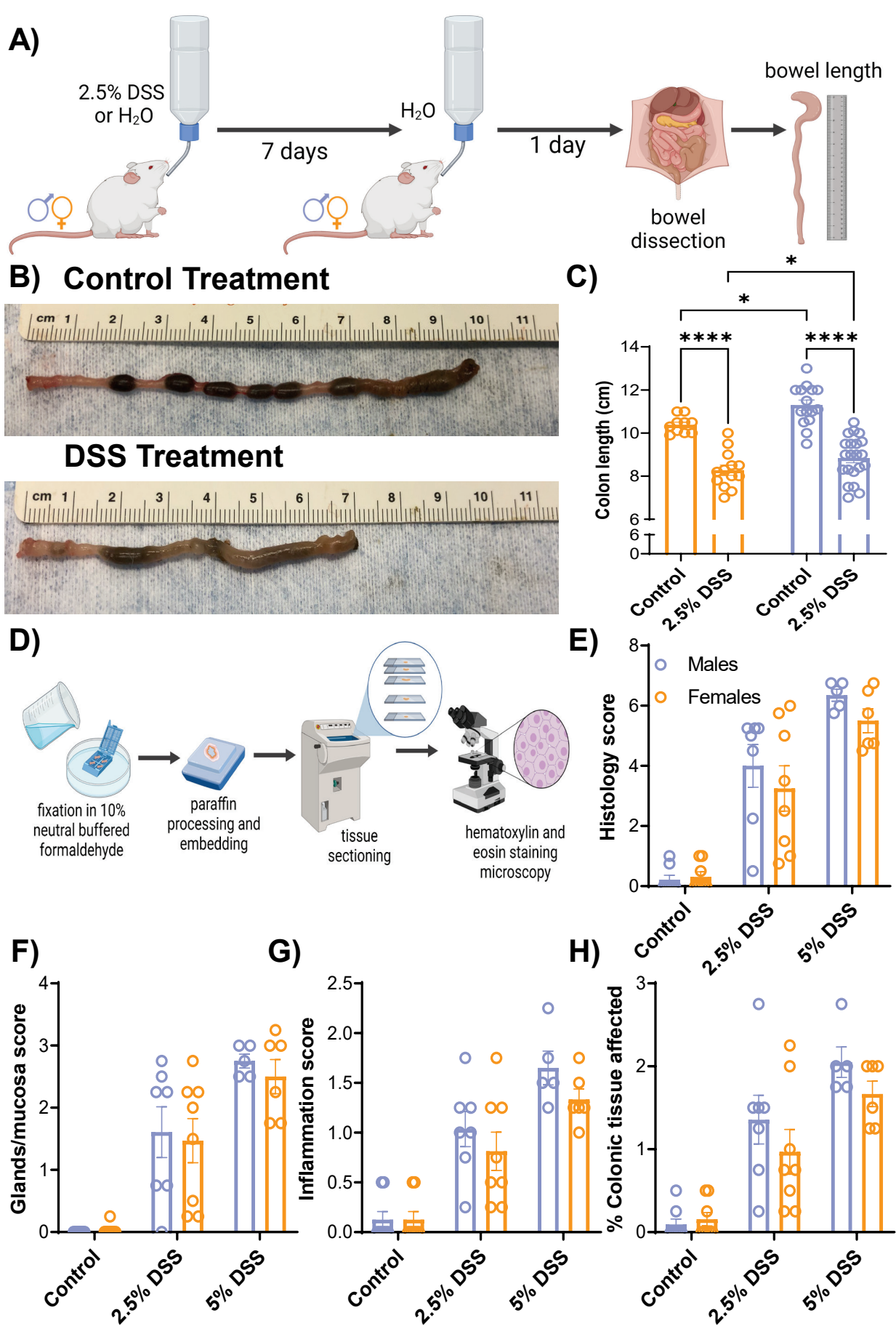
Figure 7.

A) Spontaneous Visceral Responses

Intracolonic 0.01% CAP
Intracolonic 0.03% CAP
2.5% DSS + Intracolonic 0.01% CAP

♀
Licking
Stretching
Dragging
Freezing

NOT TESTED

Licking
Dragging
Freezing

♂
NO RESPONSE

Dragging
Freezing
Contractions

Stretching
Dragging

B) Referred Abdominal Sensitivity

Baseline
Intracolonic 0.01% CAP
2.5% DSS + Intracolonic 0.01% CAP

♀
♂
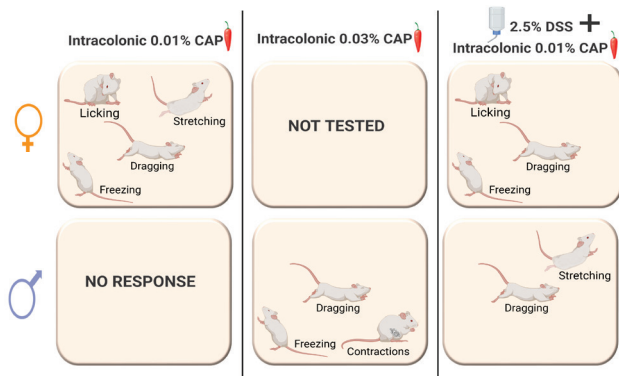
less sensitivity
more sensitivity

C) Disease Progression

♀
2.5% DSS
7 days
less changes in apperance and weight loss

♂
2.5% DSS
7 days
worse apperance and more weight loss
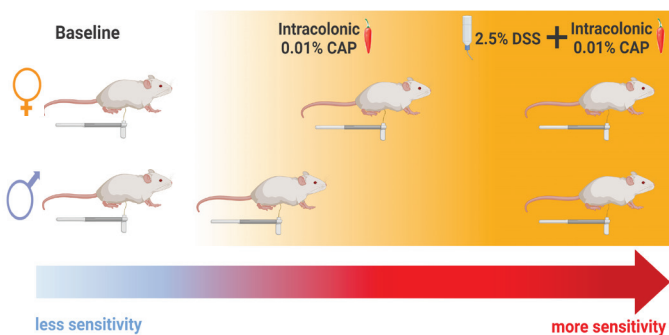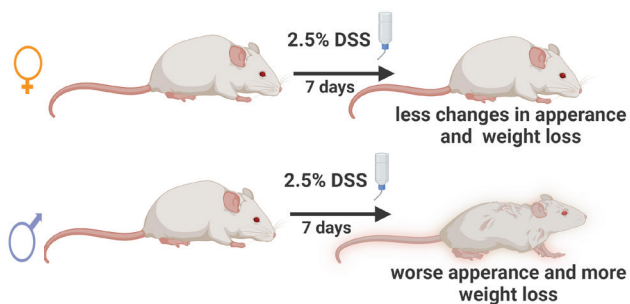
D) Gross Colon Pathology

♀
feces
colon length
2.5% DSS
feces
colon length

♂
feces
colon length
2.5% DSS
feces
colon length
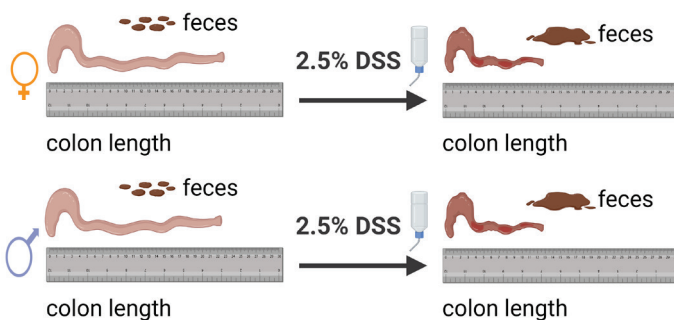
Figure 8.

Score	Weight Loss (%)	Stool Consistency	Fecal Blood	Appearance
0	None or gain	Formed and hard	Absence	"Normal"
1	1-5	Formed and soft		Scruffy
2	5-10	Loose stools/semiformed	Presence/he moccult positive	Hunched and scruffy
3	10-15	Mild diarrhea (watery)		Non-motile, hunched, and scruffy
4	15 or more	Gross diarrhea	Gross bleeding	

Table 1. Disease Activity Index (DAI). DAI score is the sum of the following components: the percentage of weight loss, stool consistency, fecal blood and appearance. Each individual component was scored according to the descriptions in the table.

	Males		Females	
	Water	2.5% DSS	Water	2.5% DSS
Disease Activity Index (DAI)	(n= 6)	(n= 12)	(n=6)	(n=12)
Day 1	-0.2 ± 0.2	-0.9 ± 0.2**	-0.3 ± 0.2	-0.5 ± 0.3
Day 2	-0.2 ± 0.2	-2.7 ± 0.2****	-0.7 ± 0.3	-1.5 ± 0.4*
Day 3	-0.5± 0.3	-3.1 ± 0.1****	-0.7 ± 0.3	-2.4 ± 0.2****
Day 4	-0.7 ± 0.3	-3.4 ± 0.2****	-0.5 ± 0.2	-2.8 ± 0.3****
Day 5	-0.7 ± 0.3	-3.6 ± 0.3****	-0.7 ± 0.5	-3.3 ± 0.3****
Day 6	-0.3 ± 0.2	-5.7 ± 0.2****	-0.3 ± 0.2	-4.8 ± 0.3****
Day 7	-0.3 ± 0.2	-7.8 ± 0.4****	-0.5 ± 0.3	-6.4 ± 0.3****
Body Weight Loss (%)				
Day 1	-0.4 ± 0.4	1.9 ± 0.5*	0.9 ± 0.6	-0.3 ± 0.7
Day 2	-0.3 ± 0.9	3.3 ± 0.6***	1.0 ± 1.1	1.5 ± 0.7
Day 3	-0.3 ± 0.9	4.1 ± 0.5****	-0.1 ± 1.6	2.7 ± 1.0
Day 4	0.1 ± 1.2	4.0 ± 0.4****	-0.5 ± 1.5	3.0 ± 1.0
Day 5	-2.4 ± 1.1	4.7 ± 0.5****	-1.9 ± 1.3	2.2 ± 1.0
Day 6	-3.2 ± 0.8	5.3 ± 0.6****	-2.1 ± 1.0	3.0 ± 1.0
Day 7	-3.3 ± 1.0	7.7 ± 0.9****	-2.2 ± 1.3	3.6 ± 0.8**
Appearance Score				
Day 1	0	0	0	0
Day 2	0	-0.8 ± 0.1****	0	-0.3 ± 0.1
Day 3	0	-1.0 ± 0.0****	0	-0.4 ±0.1
Day 4	0	-1.0 ± 0.0****	0	-0.6 ± 0.1*
Day 5	0	-1.0 ± 0.0****	-0.1 ± 0.1	-1.0 ± 0.0****
Day 6	0	-1.1± 0.1****	-0.3 ± 0.2	-1.0 ± 0.0****
Day 7	0	-1.5 ± 0.2****	-0.1 ± 0.1	-1.0 ± 0.0****
Stool Consistency Score				
Day 1	-0.2 ± 0.2	-0.2 ± 0.1	0	-0.3 ± 0.1
Day 2	0	-0.8 ± 0.1***	-0.3 ± 0.2	-0.6 ± 0.1*
Day 3	-0.3 ± 0.2	-1.0 ± 0.0****	-0.3 ± 0.2	-1.0 ± 0.0****
Day 4	-0.5 ± 0.2	-1.1 ± 0.1****	-0.3 ± 0.2	-1.0 ± 0.0****
Day 5	-0.5 ± 0.2	-1.0 ± 0.0****	-0.4 ± 0.2	-1.1 ± 0.1****
Day 6	-0.3± 0.2	-1.2 ± 0.1****	0	-1.3 ± 0.1****
Day 7	-0.3 ± 0.2	-2.1 ± 0.3***	-0.3 ± 0.2	-1.9 ± 0.1****
Fecal Blood Score				
Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0
Day 4	0	-0.2 ± 0.2	0	0
Day 5	0	-0.3 ± 0.2	0	-0.3 ± 0.2
Day 6	0	-1.8 ± 0.2*	0	-1.5 ± 0.4****
Day 7	0	-2.5 ± 0.3***	0	-2.3 ± 0.3****

Table 2. Onset of individual disease activity index (DAI) components following DSS treatment in male and female mice. Values are presented as the mean ± SEM difference from day 0 within the same treatment. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$; Repeated Measures Two-Way ANOVA followed by Šídák's multiple comparisons test compared to day 0.