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► To cite this version:

N. Gharibeh, Catherine C. Chaumontet, N. Darcel, Gilles Fromentin, I. Denis, et al.. Intermittent sucrose solution intake and its schedule of access modulate energy intake and weight gain in response to chronic variable stress in mice. *Appetite*, 2022, 176, pp. 106123. 10.1016/j.appet.2022.106123 . hal-04151706

HAL Id: hal-04151706

<https://hal.science/hal-04151706>

Submitted on 5 Jul 2023

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Intermittent sucrose solution intake and its schedule of access modulate energy intake and weight gain in response to chronic variable stress in mice

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Running Head: sucrose solution intake and chronic stress

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1 **Abstract**

2
3 There is a strong relationship between stress and the intake of calorically-dense palatable food.
4 Additionally, intake of sodas is an important contributory factor to obesity, and is often
5 associated with palatable food consumption. We studied the effects of 2-hour intermittent access
6 to sucrose-sweetened water (SSW, 12.3%, soda-like) and its schedule of administration on the
7 response to chronic variable stress in mice fed a high-fat, high-sugar diet. C57BL/6 mice (n=64)
8 had access to water or to both water and 2-hour SSW during 5 weeks, in addition to their diet.
9 After the first two weeks, half of the animals from each group were stressed daily using a chronic
10 variable stress (CVS) paradigm, while the other half were kept undisturbed. During the CVS
11 exposure period, 2-hour SSW access was either scheduled randomly, right before the stressors or
12 right after the stressors. The effects of SSW and its schedule of administration on dietary intake,
13 stress hormones and adiposity were analyzed. Results showed a larger consumption of SSW and
14 higher bodyweight gain in mice receiving SSW after the stressor. In addition, SSW consumption
15 was shown to affect appetite regulation by reducing CCK sensitivity. The present study suggests
16 that SSW leads to overconsumption and weight gain only if provided after exposure to stress.
17 These findings may implicate a relation between exposure to stress, binge-drinking behaviors of
18 sugar sweetened beverages that ensues, and weight gain in humans consuming a western diet.

19
20 Keywords: sucrose solution, intermittent access, chronic variable stress, binge drinking, energy
21 balance.

32 1. INTRODUCTION

33 Exposure to chronic stress results in a sustained allostatic load to the body which increases
34 susceptibility to a wide variety of diseases and may instigate behavioral changes including
35 modifications in eating behavior (Yau & Potenza, 2013). These alterations in feeding behavior
36 are the sequelae of the action of different hormones and neuropeptides including corticotropin-
37 releasing hormone, glucocorticoids, and neuropeptide Y which are released as part of the stress
38 response (Mastorakos & Zapanti, 2004). Stress may also affect eating behavior by inducing
39 changes in gastrointestinal signalling (Taché et al., 2001). For instance, it may amplify the
40 anorexic effects of cholecystokinin (CCK), a peptide hormone secreted by the small intestine,
41 and which is known to regulate gastrointestinal motility and to delay gastric emptying in
42 response to luminal nutrients (Yamaguchi et al., 2020; Warrilow et al., 2022). In rodents, just
43 like in humans, bidirectional changes in food intake may occur in response to stress depending
44 on the type and intensity of the stressor applied (Adam & Epel, 2007). Predominantly, food
45 consumption decreases in stressed rodents unless highly palatable foods are available (Dallman
46 et al., 2003; Dallman, Pecoraro, & la Fleur, 2005).

47
48 Palatable foods are known to be one of the most powerful rewards in animals (Berridge, 2009).
49 Their increased intake in response to stress is attributed to their hedonic properties (Pecoraro, et
50 al., 2004; Tomiyama, Dallman, & Epel, 2011; Zellner et al., 2006) and exerts negative feedback
51 on the Hypothalamus-Pituitary-Adrenal (HPA) axis, leading to dampened emotional and
52 physiological reactions to chronic stress (Foster et al., 2009; Pecoraro et al., 2004; Ulrich-Lai et
53 al., 2007). For instance, chronically stressed rats, provided with *ad libitum* access to chow and
54 comfort food, are known to experience a lower HPA axis response (lower corticosterone and
55 adrenocorticotrophic hormone (ACTH) levels) compared to rats given free access to chow only
56 (Pecoraro et al., 2004). In addition, it has been demonstrated that highly palatable foods improve
57 mood-related phenotypes like anxiety and depression in rodents (Maniam & Morris, 2010).
58 Sugar sweetened beverages are one commonly consumed comfort food which effect on the stress
59 response has been widely explored in animal models.

60
61 Given its high caloric content and palatability, sugar sweetened beverage consumption results in
62 activation of reward centers in the brain, increases in central opioidergic tone (Kendig, 2014),

63 and an overall attenuation of the stress response (Kendig, 2014). When provided intermittently,
64 sugar sweetened beverages have been shown to result in a binge-drinking behavior (Avena,
65 Rada, & Hoebel, 2008; Colantuoni et al., 2001; Rada, Avena, & Hoebel, 2005) and withdrawal
66 symptoms. This addictive effect of sugar is not observed in rats provided with continuous access
67 to sugar sweetened beverages and may therefore be linked to their schedule of administration
68 (Corsica & Pelchat, 2010; Corwin & Grigson, 2009). In other words, current evidence from
69 rodent models suggests that consumption of sugar sweetened beverages attenuates the response
70 to stress and that reward-driven behaviors may vary according to schedule of sugar sweetened
71 beverage administration.

72

73 Since sugar sweetened beverages are often consumed alongside fatty foods and high sugar/high
74 fats foods are major contributors to the obesity epidemic, it is important to further examine
75 whether this combination would have a synergistic action on dietary behavior, appetite
76 mechanisms and the response to chronic stress. The aim of the present study was therefore to
77 assess the effects of intermittent access to sucrose-sweetened water (SSW, 12.3% wt/v sucrose,
78 soda-like) and its schedule of administration (random, before or after stressor) on appetite
79 regulation and the response to chronic variable stress (CVS) in mice fed a palatable high-fat high
80 sugar (HFHS) diet.

81 We hypothesized that the schedule of administration of SSW would influence its comforting
82 effect in stressed mice.

83

84 2. MATERIALS AND METHODS

85

86 A summary of the experimental protocol can be found in Figure 1.

87

88 2.1 Animals and SSW access

89 Sixty-four C57BL6/J male mice (Lebanese American University stock, Lebanon) initially 7
90 weeks old were housed in groups of four in a temperature and humidity-controlled room under a
91 12:12 light/dark cycle (lights on at 8 a.m.). Mice were housed in groups of 4 in cages with cage
92 dividers. The animals were fed a powdered HFHS diet enriched in lard (4.5kcal/g, 14% protein,

93 45% fat and 41% carbohydrates including 20% sugar) *ad libitum* during the whole length of the
94 experiment (5 weeks).
95 During the first two weeks (the baseline period), mice were given access to water only (one
96 group, n=16), or access to water as well as intermittent access to sucrose-sweetened water (SSW)
97 for a 2-hour period on 5 random days per week (three groups, n=16/group). After the first two
98 weeks had elapsed, mice from each of the four groups were again divided into two weight-
99 matched groups (n=8/group): control (n=8) and stressed (n=8). For a period of 3 weeks, stressed
100 groups were exposed to a daily stressor using a CVS model while controls were kept undisturbed
101 in their home cages.

102

103 **2.2 Chronic variable stress procedure**

104 The CVS model applied consists of a 21- day variable stressor paradigm during which a single
105 individual stressor is applied per day for varying amounts of time daily and at different times of
106 the day in order to minimize predictability. Stressors varied from one day to the other and
107 consisted of: (a) cage tilt to 30 degrees from the horizontal; (b) space reduction whereby mice in
108 groups of 8 were placed in a collective cage usually designed for 4 to 5 mice; (c) restraint
109 whereby mice were placed into a perforated plastic tube in which they cannot turn over for 1 to 3
110 hours; (d) forced swimming in cold water (18°C) or in (e) warm water (26°C); (f) flashing light;
111 (g) placement in unclean unoccupied neighboring cages (Zeeni et al., 2013; Zeeni et al., 2015).
112 Control and stressed mice were housed in two separate sections of a large animal room to
113 minimize their excitement from smells and noise exchange. Table 1 reports the detailed stressor
114 schedule.

115 The three stressed groups receiving SSW differed by the timing of access to SSW in reference to
116 the stressor: access to SSW was either scheduled **randomly at varying times of day, during the**
117 **light period, independently of when the stressor event occurred** (Stressed-SSW-random), or right
118 before the stressor (Stressed-SSW-before) or right after the stressor (Stressed-SSW-after). Each
119 control group received SSW at the same time as its corresponding stressed group.

120 All experimental protocols were approved by the Animal Ethical Committee of the Lebanese
121 American University (Protocol # LAU.ACUC.SAS.NZ1), which complies with the Guide for the
122 Care and Use of Laboratory Animals (National Research Council of the United States, 2011).

123

124 **2.3 Food and SSW intake**

125 Food and SSW intakes were monitored daily throughout the study. **Individual** food intake,
126 corrected for spillage, was recorded at 8 a.m. by measuring the difference in food cup weight
127 before and after presentation to the mice. **Individual** SSW intake was measured every time it was
128 presented to the animals **by measuring the difference in cup weight before and after presentation.**
129 Average food intake and SSW intake **per mouse** were calculated for each of the baseline period
130 and the CVS exposure period.

131

132 **2.4 Body weight**

133 The mice were weighed individually and daily during the whole study. An average daily body
134 weight gain was calculated during each of the baseline and the CVS exposure period.

135

136 **2.5 Blood collection and dissection**

137 The mice were fasted overnight at the end of the CVS exposure period. The following morning,
138 they were euthanized by decapitation. Trunk blood was collected and centrifuged at 2000 g for
139 15 min. The collected serum was then stored at -80 °C for subsequent analysis. Intra-abdominal
140 fat (epididymal, mesenteric and retroperitoneal) and adrenal glands were collected and weighed.

141

142 **2.6 CCK sensitivity.**

143 During the 5th week, food deprived mice (**fasted overnight**) were weighed and received 2µg/kg
144 cholecystokinin (CCK) i.p. injections or 50µl i.p. injections of a vehicle solution. Five minutes
145 after CCK or saline injections, mice were given their respective diets, and **individual** food intake
146 was measured at 60 min, 120 min and 180 min. CCK and vehicle injections were randomly
147 administered (adapted from Soto et al., 2015).

148

149 **2.7 Hormones and Metabolites**

150 **2.7.1 Circulating hormones and metabolites.** RIA or ELISA kits were used to measure serum
151 corticosterone (Double Antibody 125I Radioimmunoassay Kit for rats and mice, MP
152 Biomedicals, LLC, Solon, OH, USA) and insulin (DRG diagnostics, Germany). Spinreact kits
153 (Spinreact, Spain) were used to measure blood glucose levels.

154

155 **2.7.2. Fecal corticosterone metabolites (FCM).** Fecal pellets were collected weekly and stored
156 at -20°C. They were then dried, homogenized and 0.05 g of the dry weight was extracted with 1
157 ml of 80% methanol. Concentrations of FCM were then analyzed for immunoreactive fecal
158 corticosterone metabolites using a 5 α -pregnane-3 β ,11 β ,21-triol-20-one enzyme-immunoassay
159 (EIA) (Touma, Palme, & Sachser, 2004; Touma, Sachser, Möstl, & Palme, 2003).

160

161 **2.8 Statistical analysis**

162 Descriptive data are reported as mean \pm standard deviation (SD). Hypotheses and analyses were
163 specified a priori. Differences between stress treatments (absence or presence of stressor) and
164 drinking treatments (type and time of presentation of the drink) in energy intake and bodyweight
165 gain during the CVS exposure period as well as in endpoints of body composition, plasma
166 hormones, and blood glucose were tested using linear mixed effect model. The fixed effects
167 accounted for included stress treatment, drinking treatment, and their interaction. The random
168 effect accounted for was cage number nested within the interaction of stress and drinking
169 treatments. Differences between stress treatments and drinking treatments in fecal corticosterone
170 metabolites (at the end of each week) and between CCK and saline treatments in food intake (at
171 60, 120, 180 min) were tested over time using linear mixed effect model. In the model testing for
172 differences between stress treatments and drinking treatments in fecal corticosterone metabolites,
173 the fixed effects accounted for included time-point, drinking treatment, stress, and their
174 interaction. The random effect accounted for was mouse ID nested within the interaction of cage,
175 stress, and drinking treatments. In the model testing for differences between CCK and saline
176 treatments in food intake (at 60, 120, 180 min), the fixed effects accounted for included time-
177 point, drinking treatment, stress, and CCK. The random effect accounted for was mouse ID
178 nested within the interaction of stress, drinking, and CCK/saline treatment. Visual assessment of
179 the generated residuals indicated no substantial significant outliers and Tukey's test was used for
180 post-hoc adjustments. All data were analyzed using SAS University Edition (SAS Institute Inc.,
181 Cary, N.C.) with statistical significance set at $p < 0.05$.

182

183 **3. RESULTS**

184

185 **3.1 Food, sucrose-sweetened water, and overall energy intake**

186 During the CVS period (weeks 3-5), food intake **per mouse** did not vary amongst groups
187 ($p=0.88$), according to stress treatment ($p = 0.48$), and drinking treatment ($p = 1.00$). SSW intake
188 **per mouse** was different amongst groups ($p = 0.03$). Amongst the groups receiving SSW,
189 Stressed SSW-after mice (8.62 ± 0.50 kJ) had higher SSW intake **on average** compared to all
190 other groups: Non-stressed SSW-random mice (5.51 ± 0.81 kJ), $p < 0.0001$), Stressed SSW-
191 random mice (6.93 ± 0.85 kJ, $p = 0.01$), Non-stressed SSW-before mice (5.90 ± 1.44 kJ, $p <$
192 0.0001), Stressed-SSW-before mice (6.86 ± 1.13 kJ, $p = 0.01$), Non-stressed SSW-after mice
193 (6.69 ± 0.96 kJ, $p = 0.003$) (Fig 2A). When accounting only for the fixed effects of stress
194 treatment and drinking treatment, stressed mice had a higher SSW intake (5.35 ± 3.50 kJ) **on**
195 **average** compared to non-stressed mice (4.49 ± 2.88 kJ, $p = 0.003$). In addition, amongst mice
196 receiving SSW, SSW-after mice (7.51 ± 1.26) had a higher SSW intake **on average** compared to
197 SSW-random mice (6.27 ± 1.08 , $p = 0.02$) and SSW-before mice (6.35 ± 1.35 , $p = 0.04$).
198 Energy intake **from food and SSW combined**, during the CVS period, was smaller **on average** in
199 the groups receiving water (46.63 ± 2.37 kJ) compared to those receiving SSW (SSW-Random:
200 52.71 ± 4.54 kJ, $p = 0.01$; SSW-before: 52.88 ± 2.93 kJ, $p = 0.01$; SSW-after: 54.21 ± 3.54 kJ,
201 $p = 0.003$).

202

203 **3.2 Bodyweight gain**

204 There was a significant effect of drinking on bodyweight gain of mice ($p = 0.0002$) (Fig 2B)
205 while stress ($p = 0.29$) and drinking x stress ($p = 0.58$) had no significant effect on bodyweight
206 gain. During the CVS period, SSW-random mice gained more weight (0.11 ± 0.06 g) compared
207 to H₂O-mice (0.07 ± 0.04 g, $p = 0.049$). In addition, SSW-after mice gained more weight (0.16
208 ± 0.03 g) compared to H₂O-mice (0.07 ± 0.04 g, $p < 0.0001$) and compared to SSW-before mice
209 (0.11 ± 0.04 g, $p = 0.03$).

210

211 **3.3 Adrenal glands and body composition**

212 There was a significant effect of stress ($p = 0.003$) on the adrenal gland weight of the mice, with
213 stressed groups having significantly higher adrenal gland weight (6.73 ± 1.66 mg) compared to
214 non-stressed groups (4.91 ± 1.65 mg) (Fig 3A and Table 2). There was no significant effect of
215 stress ($p = 0.45$) or drinking ($p = 0.34$) on abdominal fat weight (Table 2).

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3.4 Corticosterone and fecal corticosterone metabolites

3.4.1 Corticosterone

There was a significant effect of stress on serum corticosterone levels of the mice ($p = 0.006$). Stressed animals showed increased serum corticosterone (68.33 ± 13.29 ng/ml) levels compared to non-stressed animals (54.57 ± 9.63 ng/ml). However, there was no significant difference of the different drinking treatments on the corticosterone levels ($p = 0.37$) and no drinking x stress interaction ($p = 0.42$) (Fig 3B and Table 2).

3.4.2 FCM

There were significant effects of stress ($p = 0.0006$) and time ($p < 0.0001$) on the FCM levels of the mice. Stressed animals showed increased FCM levels (92.71 ± 30.56 ng/ml) compared to non-stressed animals (78.90 ± 25.06 ng/ml). However, there was no significant difference between the FCM levels of mice on different drinking treatments ($p = 0.35$) (Table 2).

3.5 CCK sensitivity

There was a significant effect of CCK ($p = 0.02$), drinking treatment ($p < 0.0001$), and time ($p < 0.0001$) but not of stress ($p=1.00$) on food intake. **On average**, mice injected with CCK had a lower food intake post-injection compared to mice injected with saline (CCK: 1.87 ± 0.98 g, saline: 2.12 ± 1.05 g, $p= 0.02$). Moreover, **on average**, mice drinking water only had significantly lower food intake (1.54 ± 0.88 g) in response to CCK compared to mice on SSW-random (2.13 ± 1.11 g, $p = 0.001$), SSW-before (2.16 ± 0.99 g, $p = 0.0007$), and SSW- after (2.20 ± 0.97 g, $p = 0.0003$) (Fig 4).

3.6 Serum glucose and insulin

There was a borderline significant interaction effect of drinking and stress on the serum glucose levels of the animals ($p=0.0503$) (Table 2). There were no significant effects of stress ($p = 0.72$), drinking ($p = 0.57$), or their interaction ($p = 0.30$) on mice insulin levels (Table 2).

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250

251 4. DISCUSSION

252

253 The present study compared the response to intermittent access to SSW (12.3% wt/v sucrose,
254 soda-like) according to its schedule of administration in respect to chronic variable stress (CVS)
255 (random, before or after stressor) in mice fed a palatable high-fat high sugar diet. The applied
256 CVS protocol induced a significant stress-response and resulted in an increased SSW intake
257 compared to non-stressed mice. In addition, SSW schedule of administration influenced SSW
258 consumption: SSW consumption and weight gain were found to be higher in mice receiving
259 SSW after the stressor compared to prior to the stressor or at random times. Last but not least,
260 SSW consumption was found to reduce CCK sensitivity and thus affect appetite regulation.

261

262 Stressed mice showed increased serum and fecal corticosterone levels as well as higher adrenal
263 gland weights compared to non-stressed mice. The HPA axis stress response in rodents results in
264 the release of stress hormones from the adrenal glands including corticosterone (Gong et al.,
265 2015). In addition, adrenal hypertrophy is a commonly observed sequel of chronic stress in the
266 rat model (Ostrander et al., 2006; Ulrich-Lai et al., 2007) that occurs due to an increased adrenal
267 responsiveness to ACTH in chronic stress. Our results corroborate previous findings from stress-
268 exposed rodent models (Lucas et al., 2007; Wu et al., 2012) and indicate a persistent stimulation
269 of the HPA axis in response to chronic exposure to unpredictable and variable stress. These
270 measures therefore indicate that the CVS protocol induced a significant stress-response in our
271 mice. However, CVS did not alter mice bodyweight gain, contrary to other findings in rodents
272 showing stress-induced weight loss and weight gain depending on the type of stressor and the
273 diet (Harris, 2015; Adam & Epel, 2007; Dallman et al., 2003; Dallman et al., 2005). Weight loss
274 is a typical physiological response to stress in rodents likely because of the acute and variable
275 experimental stressors applied and the lack of access to palatable foods (Harris, 2015).
276 Decreased food intake and body weight in response to chronic stress may have been
277 compensated for in our study due to sucrose access and comfort food which are known to induce
278 changes in the corticosterone/insulin ratio (Harris, 2015). The lack of bodyweight change in our

279 stressed-H₂O mice suggests that the intensity of the stress response was moderate and that the
280 palatable HFHS diet sustained food intake and bodyweight.

281

282 Stressed mice had a higher SSW intake compared to non-stressed mice. Furthermore, the SSW
283 schedule of administration influenced SSW consumption as mice offered SSW after the stressors
284 had an increased consumption of SSW compared to mice offered SSW prior to or at random
285 times in relation to the stressors. This higher SSW intake after the stressor is probably not due to
286 a stress-induced increase in energy need because these mice also had a greater weight gain
287 compared to the other groups. Then this increased SSW drinking indicates that the rewarding
288 effect of sugar consumption (Kendig, 2014) is more wanted or liked when consumed after the
289 stressor. This suggests that post-stress access to SSW can exacerbate binge-drinking behavior,
290 which is commonly observed in rodents given limited and intermittent access to SSB (Avena et
291 al., 2008; Colantuoni et al., 2001; Rada et al., 2005). Overall, these findings show the potential
292 deleterious association of chronic stress and SSBs on the risk of obesity.

293

294 Interestingly, our results indicate that SSW consumption affects appetite regulation. Indeed,
295 SSW access was associated with a decrease in CCK sensitivity, with mice offered SSW having
296 higher food intake in response to CCK injection compared to mice receiving water only. This
297 decrease in the response to one of the main gastro-intestinal short-term signals of satiety may
298 explain why SSW-drinking mice did not reduce their food intake compared to mice receiving
299 water only despite the significant increase of energy intake associated to SSW. This led to
300 significantly higher bodyweight gain in SSW-After mice consuming more SSW. This result is in
301 line with a study showing that high-fat fed mice given 2h-intermittent SSW access were
302 hyperphagic with an increased energy intake leading to a greater body weight gain as compared
303 to mice drinking water (Soto et al., 2015). Our study is the first to our knowledge to show that
304 intermittent SSW intake may cause a desensitization to satiety signals such as CCK. This may be
305 due to an impairment in dopaminergic and opioidergic pathways that regulate reward sensitivity
306 and food intake (Soto et al., 2015).

307

308 This study presents with strengths and limitations. It explores the impact of intermittent sucrose
309 solution intake scheduled either randomly, right before or right after the stressors on appetite

310 regulation and the response to CVS; a research question which to our knowledge has not been
311 previously addressed. One of its strengths is its design as it combines a HFHS diet with SSW in
312 mice exposed to CVS. This may be comparable to the consumption of SSBs in response to
313 variable life stressors in individuals who are on an unhealthy western diet. Most animal studies
314 on the effect of SSBs have employed healthy diets, e.g., well-balanced chow. The combination
315 of SSW with a HFHS diet is not commonly reported in the literature and is a strength of the
316 current experiment. Our findings suggest that mice receiving SSW-after mice drink more sucrose
317 solution and gain more weight than mice in various control conditions; a novel finding.
318 Nonetheless, the differences are relatively small and their clinical significance remains to be
319 explored further using larger groups given that individual response to stress may vary. While the
320 present study does not provide evidence about the mechanisms behind the results, the larger
321 consumption of SSW and higher bodyweight gain observed in mice receiving SSW after the
322 stressor as well as the reduced CCK sensitivity associated with SSW consumption could be
323 attributed to the reward system stimulation. In fact, consumption of a sucrose solution was found
324 to trigger opioid and dopamine release in the nucleus accumbens of rats, and to lead to an
325 activation of the central nucleus of the amygdala (Pomonis et al., 2000; Levine, Kotz, & Gosnell,
326 2003). In the present experiment, mice provided with SSW after being stressed may have a
327 higher tendency to consume it given their increased need for reward or comfort. Overall, the
328 study does not thoroughly explore the mechanisms underlying the impact of SSW intake and its
329 schedule of administration on appetite regulation and the response to CVS. Measurement of
330 additional metabolites including cortisol, triglycerides, and cholesterol would have provided
331 additional insight on these relations. Further research elucidating the mechanisms involved is
332 required.

333

334 In summary, the present study showed that intermittent access to sucrose-sweetened water (SSW,
335 12.3% wt/v sucrose, soda-like) and its schedule of administration (random, before or after
336 stressor) leads to changes in feeding behavior and energy intake in mice fed a palatable high-fat
337 high sugar diet and under chronic variable stress. A larger consumption of SSW was observed in
338 mice receiving SSW after the stressor compared to prior to the stressor or at random times. In
339 addition, SSW consumption was shown to affect appetite regulation by reducing CCK
340 sensitivity. These findings may have significant implications for the study of human obesity as

341 they shed light on the relationship between exposure to stress and binge-drinking behaviors
342 which may exacerbate weight gain. This is especially important given that individuals
343 consuming SSBs also tend to ingest calorie-dense solid foods. Future experiments should focus
344 on the mechanisms and neuronal signalling pathways that are involved in this relationship.

345

346 **Acknowledgements**

347 This research did not receive any specific grant from funding agencies in the public, commercial,
348 or not-for-profit sectors.

349 The authors would like to thank Joumana Aouf & Hussin Ashour for their help in handling the
350 animals and collecting data.

351 The authors report no conflict of interest.

352 Authors' contributions: NZ, ND, GF and CC designed the study. NZ supervised the data
353 collection process. NZ and NG analyzed the data. NZ, NG, CC and ID wrote the manuscript.

354 NG, CC, ND, GF, ID, and NZ approved the final article.

355

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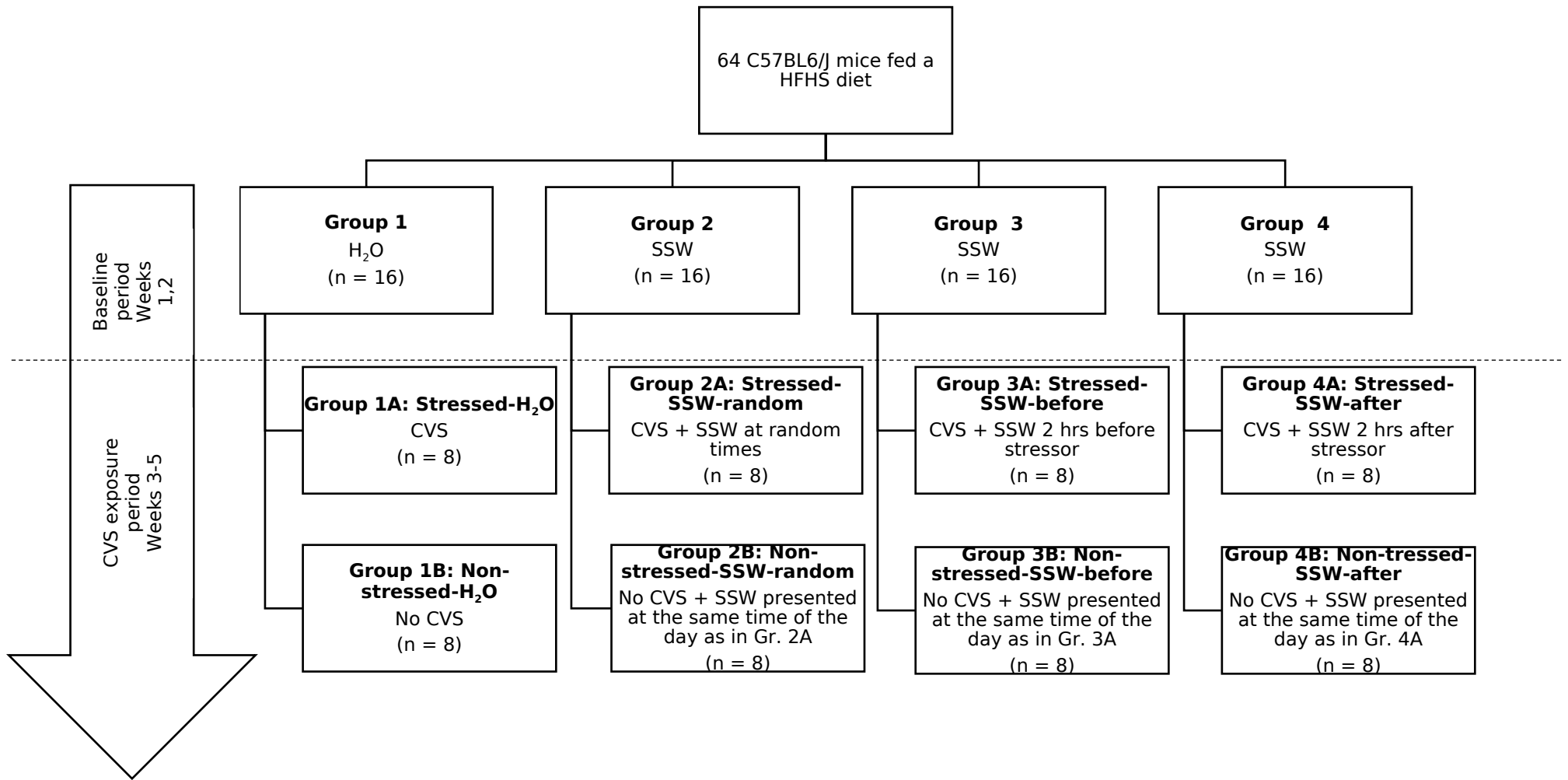


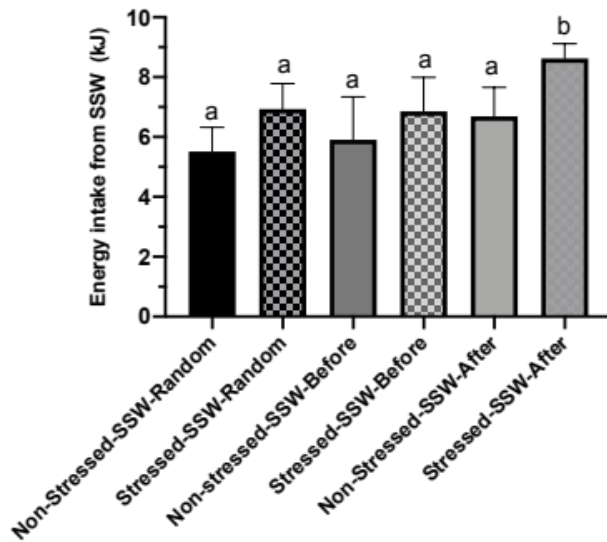
Figure 1. Experimental protocol. Sixty-four male C57BL6/J mice were fed a high fat, high sugar diet and assigned to one of four drinking groups, one group given access to water only, and three groups given access to water as well as intermittent access to sucrose-sweetened water (SSW) for a 2 hour period on 5 random days per week. After the first 2 weeks (baseline period), mice from each group were divided in two groups: control and stressed. Controls (no CVS) were kept undisturbed in their home cages during the following 3-weeks of treatment (CVS exposure period), and the others were stressed daily for the remaining period of the study using a chronic variable stress (CVS) model. In the stressed groups, access to SSW was either scheduled randomly (Stressed-SSW-random), right before the stressors (Stressed-SSW-before) or right after the stressors (Stressed-SSW-after).

| Day of treatment | Stressor Used | Duration | Time of administration |
|-------------------------|-----------------------|-----------------|-------------------------------|
| 1 | Flashing light | 4h | 12:00 - 16:00 |
| 2 | Neighbor cage | 14h | 17:00-07:00 |
| 3 | Cage tilt | 3h | 10:00- 13:00 |
| 4 | Space reduction | 4h | 08:00-12:00 |
| 5 | No stressor | x | |
| 6 | Flashing light | 4h | 14:00-18:00 |
| 7 | Forced swimming-warm | 10min | 12:00-12:10 |
| 8 | Restraint | 1h | 09:00-10:00 |
| 9 | Cage tilt | 4h | 08:00-12:00 |
| 10 | Flashing light | 3h | 12:00 - 15:00 |
| 11 | Neighbor cage | 14h | 17:30-07:30 |
| 12 | No stressor | x | |
| 13 | Cage tilt | 3h | 11:00-14:00 |
| 14 | Restraint | 3h | 10:00- 13:00 |
| 15 | Flashing light | 4h | 14:00-18:00 |
| 16 | Forced swimming- cold | 10min | 15:00-15:10 |
| 17 | Restraint | 2h | 12:00-14:00 |
| 18 | Space reduction | 5h | 08:00-13:00 |
| 19 | No stressor | x | |
| 20 | Restraint | 2h | 09:00-11:00 |
| 21 | Forced swimming-warm | 10min | 16:00-16:10 |

Table 1. Schedule of stressor agents used during the chronic variable stress treatment

A.

Drinking: $p < 0.0001$, stress: $p < 0.0001$, drinking*stress: $p = 0.03$



B.

Drinking: $p = 0.0002$, stress: $p = 0.29$, drinking*stress: $p = 0.58$

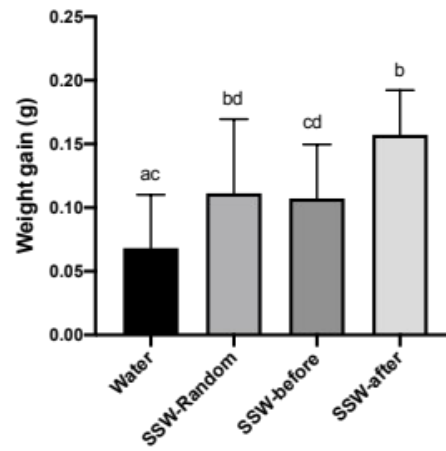
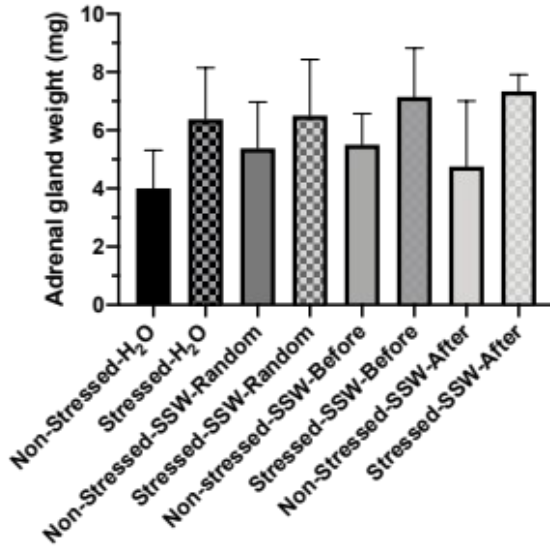


Figure 2. Mean energy intake from SSW (kJ) and mean weight gain Data are presented as means \pm SD of $n=8$ /group (2A) and $n=16$ /group (2B) and are analyzed using a linear fixed effect model adjusted for the effects of drinking treatment, stress, and their interaction. Distinct letter superscripts indicate statistically differences between groups after adjustment for multiple comparisons ($P < 0.05$).

A. Drinking: $p = 0.35$, stress: $p = 0.003$,
drinking*stress: $p = 0.67$



B. Drinking: $p = 0.37$, stress: $p = 0.006$,
drinking*stress: $p = 0.42$

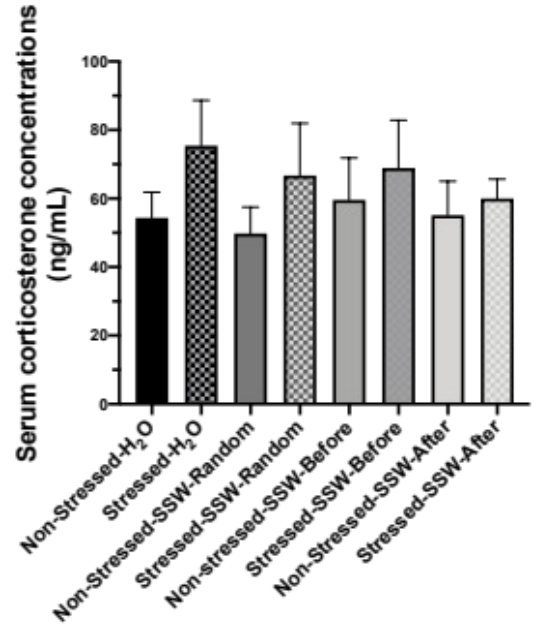


Figure 3. Mean adrenal gland weight (mg) (A) and serum corticosterone concentrations (ng/mL) (B) in mice fed a high fat high sugar diet and either submitted to a chronic variable stress paradigm or not. Results are presented as means \pm SD of $n = 8$ /group and are analyzed using linear fixed effect model adjusted for the effects of drinking treatment, stress, and their interaction.

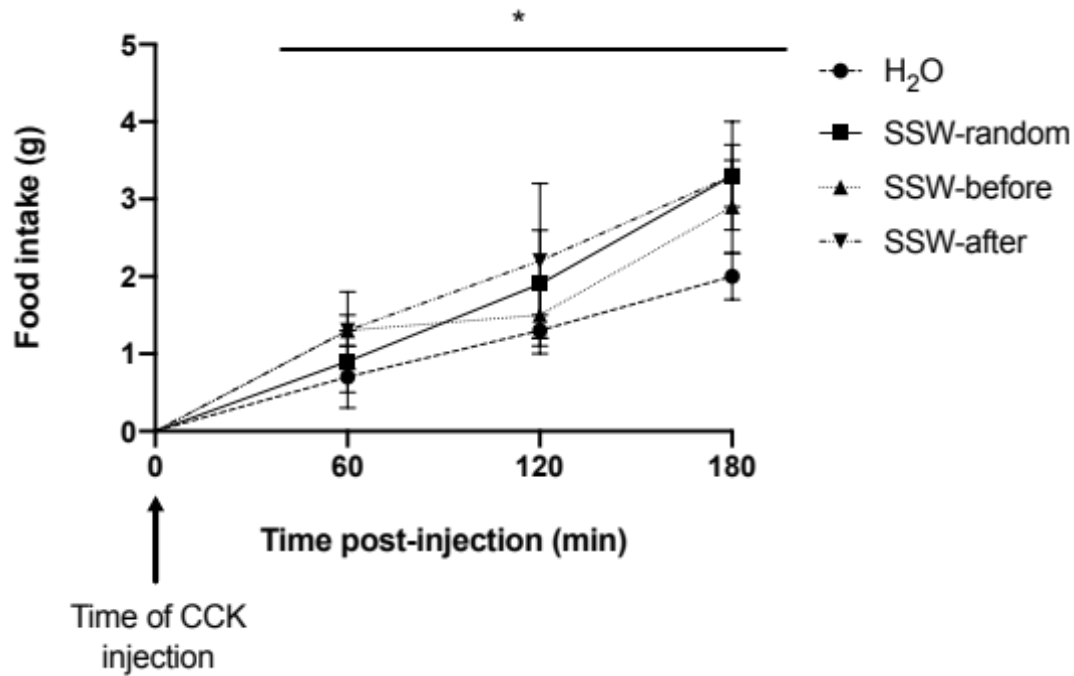


Figure 4. Food intake at 60, 120, and 180 min post-injection of CCK according to drinking treatment: H₂O (Gr. 1), SSW-random (Gr. 2), SSW-before (Gr. 3), and SSW-after (Gr. 4). Each drinking treatment group includes both stressed and non-stressed mice. Results are presented as means \pm SD of n = 16/group and are analyzed over time using linear mixed effects model adjusted for the effects of drinking treatment, stress, CCK injection, and time. * indicates statistical difference with regard to the H₂O group (P < 0.05).

| | High fat high sugar diet | | | | | | | | Drinking* stress effect | Drinking effect | Stress effect |
|---|----------------------------|--------------------|--------------------------|--------------------|----------------------|--------------------|---------------------|--------------------|-------------------------------|--------------------|------------------|
| | H ₂ O (n=16) | | SSW- Random (n=16) | | SSW-before (n=16) | | SSW-after (n=16) | | | | |
| | CVS (n=8) | No CVS (n=8) | CVS (n=8) | No CVS (n=8) | CVS (n=8) | No CVS (n=8) | CVS (n=8) | No CVS (n=8) | | | |
| Glucose (mmol/L) | 115.3 ± 25.2 | 147.3 ± 33.1 | 122.0 ± 32.4 | 115.8 ± 17.7 | 97.3 ± 30.7 | 129.0 ± 32.8 | 217.7 ± 32.9 | 130.8 ± 33.4 | P = 0.0503 | P = 0.057 | P = 0.55 |
| Insulin (ng/mL) | 1.6 ± 0.3 | 1.4 ± 0.2 | 1.3 ± 0.2 | 1.6 ± 0.4 | 1.8 ± 0.4 | 1.5 ± 0.2 | 1.4 ± 0.3 | 1.7 ± 0.3 | P = 0.30 | P = 0.57 | P = 0.72 |
| Corticosteron e (ng/mL) | 75.4 ± 13.2 | 54.4 ± 7.5 | 66.7 ± 15.2 | 49.8 ± 7.7 | 68.9 ± 13.9 | 59.6 ± 12.3 | 60.0 ± 5.6 | 55.1 ± 9.9 | P = 0.42 | P = 0.37 | P = 0.006 |
| Fecal corticosterone metabolites (ng/mL) | 95.3 ± 32.4 | 79.1 ± 24.5 | 93.4 ± 31.3 | 75.9 ± 23.8 | 96.5 ± 32.3 | 83.8 ± 27.4 | 84.0 ± 24.1 | 77.2 ± 24.9 | P = 0.75 | P = 0.35 | P = 0.0006 |
| Adrenal gland weight (mg) | 6.4 ± 1.8 | 4.0 ± 1.3 | 6.5 ± 1.9 | 5.4 ± 1.6 | 7.1 ± 1.7 | 5.5 ± 1.1 | 7.3 ± 0.6 | 4.8 ± 2.3 | P = 0.67 | P = 0.35 | P = 0.003 |
| Abdominal fat (g) | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | P = 0.75 | P = 0.34 | P = 0.45 |

Table 2. Effect of CVS, SSW consumption and its time of administration in relation to the stressor on serum levels of glucose, insulin, corticosterone, fecal corticosterone metabolites, adrenal gland weight, and abdominal fat. Values are expressed as the mean ± SD.

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