

Bryn Mawr College

## Scholarship, Research, and Creative Work at Bryn Mawr College

---

Psychology Faculty Research and Scholarship

Psychology

---

2020

### Assessment of Stress Effects on Cognitive Flexibility using an Operant Strategy Shifting Paradigm

Laura A. Grafe

*Bryn Mawr College*, lgrafe@brynmawr.edu

Andrew T. Gargiulo

*Bryn Mawr College*, agargiulo1@brynmawr.edu

Xinyue Li

*Bryn Mawr College*, xli5@brynmawr.edu

Follow this and additional works at: [https://repository.brynmawr.edu/psych\\_pubs](https://repository.brynmawr.edu/psych_pubs)



Part of the Behavioral Disciplines and Activities Commons

[Let us know how access to this document benefits you.](#)

---

#### Custom Citation

Gargiulo, Andrew T., Xinyue Li and Laura A. Grafe. 2020. "Assessment of Stress Effects on Cognitive Flexibility using an Operant Strategy Shifting Paradigm." *Journal of Visualized Experiments* 159: e61228.

This paper is posted at Scholarship, Research, and Creative Work at Bryn Mawr College.  
[https://repository.brynmawr.edu/psych\\_pubs/83](https://repository.brynmawr.edu/psych_pubs/83)

For more information, please contact [repository@brynmawr.edu](mailto:repository@brynmawr.edu).

1 **TITLE:**  
2 Assessment of Stress Effects on Cognitive Flexibility Using an Operant Strategy Shifting  
3 Paradigm  
4

5 **AUTHORS AND AFFILIATIONS:**  
6 Andrew Gargiulo<sup>1</sup>, Ariel Li<sup>1</sup>, Laura A. Grafe<sup>1</sup>  
7

8 <sup>1</sup>Department of Psychology, Bryn Mawr College, Bryn Mawr, PA, USA  
9

10 **Corresponding Author:**  
11 Laura A. Grafe (Lgrafe@brynmawr.edu)  
12

13 **Email Addresses of Co-Authors:**  
14 Andrew Gargiulo (Agargiulo1@brynmawr.edu)  
15 Ariel Li (xli5@brynmawr.edu)  
16

17 **KEYWORDS:**  
18 sex differences, cognitive flexibility, stress, prefrontal cortex, attention, perseverative errors  
19

20 **SUMMARY:**  
21 Stressful life events impair cognitive function, increasing the risk of psychiatric disorders. This  
22 protocol illustrates how stress affects cognitive flexibility using an automated operant strategy  
23 shifting paradigm in male and female Sprague Dawley rats. Specific brain areas underlying  
24 particular behaviors are discussed, and translational relevance of results are explored.  
25

26 **ABSTRACT:**  
27 Stress affects cognitive function. Whether stress enhances or impairs cognitive function  
28 depends on several factors, including the 1) type, intensity, and duration of the stressor; 2) type  
29 of cognitive function under study; and 3) timing of the stressor in relation to learning or  
30 executing the cognitive task. Furthermore, sex differences among the effects of stress on  
31 cognitive function have been widely documented. Described here is an adaptation of an  
32 automated operant strategy shifting paradigm to assess how variations in stress affect cognitive  
33 flexibility in male and female Sprague Dawley rats. Specifically, restraint stress is used before or  
34 after training in this operant-based task to examine how stress affects cognitive performance in  
35 both sexes. Particular brain areas associated with each task in this automated paradigm have  
36 been well-established (i.e., the medial prefrontal cortex and orbitofrontal cortex). This allows  
37 for targeted manipulations during the experiment or the assessment of particular genes and  
38 proteins in these regions upon completion of the paradigm. This paradigm also allows for the  
39 detection of different types of performance errors that occur after stress, each of which has  
40 defined neural substrates. Also identified are distinct sex differences in perseverative errors  
41 after a repeated restraint stress paradigm. The use of these techniques in a preclinical model  
42 may reveal how stress affects the brain and impairs cognition in psychiatric disorders, such as  
43 post-traumatic stress disorder (PTSD) and major depressive disorder (MDD), which display  
44 marked sex differences in prevalence.

45

46 **INTRODUCTION:**

47

48 In humans, stressful life events can impair cognitive function (i.e, cognitive flexibility<sup>1</sup>), which  
49 denotes the ability to adapt cognitive processing strategies to face new conditions in the  
50 environment<sup>2</sup>. Impairment in cognition precipitates and exacerbates many psychiatric  
51 disorders, such as Post Traumatic Stress Disorder (PTSD) and Major Depressive Disorder  
52 (MDD)<sup>3,4</sup>. These disorders are twice as prevalent in females<sup>5-8</sup>, yet the biological basis for this  
53 disparity remains unknown. Aspects of executive functioning in humans can be assessed using  
54 the Wisconsin Card Sorting Task, a demonstration of cognitive flexibility<sup>2</sup>. Performance in this  
55 task is impaired in patients with PTSD<sup>9</sup> and MDD<sup>10</sup>, but the neural basis of this change can only  
56 be examined by brain imaging<sup>11</sup>.

57

58 Advances in understanding how stress affects the brain have been made through the use of  
59 animal models, particularly rodents. As cognitive flexibility is affected in stress-related diseases,  
60 it is an exceptionally relevant phenotype to examine in rodents. To date, most stress  
61 neurobiology literature has used an alternative cognitive flexibility paradigm (sometimes  
62 referred to as the digging task)<sup>12-15</sup>. While this task has been extensively vetted, it requires  
63 more time and effort by the experimenter to train rodents. Adapted and described here is a  
64 well-established automated set-shifting protocol<sup>16</sup> to assess cognitive flexibility in male and  
65 female Sprague Dawley rats using various stress models<sup>17,18</sup>. The procedure requires minimal  
66 oversight by the experimenter and allows multiple rats to be tested simultaneously. In addition,  
67 unlike other versions of this automated task<sup>19</sup>, the adaptation of this paradigm only requires 3  
68 days of training and includes an efficient programmed data analysis.

69

70 Whether stress enhances or impairs cognitive function depends on the type, intensity, and  
71 duration of the stressor, as well as the timing of the stressor in relation to learning or executing  
72 a cognitive task<sup>20,21</sup>. Thus, the protocol incorporates stress procedures both before and after  
73 the operant training. It also examines representative results from stress studies. In addition, the  
74 brain regions underlying particular aspects of set-shifting have been well-established<sup>2,16,22</sup>; thus,  
75 the report also describes how to target and assess particular brain regions during or after the  
76 stress and strategy shifting procedures.

77

78 There has been limited research on directly examining sex differences in cognitive flexibility<sup>18,23</sup>.  
79 The protocol describes how to 1) incorporate both male and female rats into the experimental  
80 paradigm, then 2) track estrous cycles before and during the procedures in freely cycling  
81 females. Prior studies have indicated that stress before operant training can lead to sex-specific  
82 deficits in cognitive flexibility in rats<sup>17</sup>. Particularly, female rats exhibit disruptions in cognitive  
83 flexibility after stress, whereas cognitive flexibility improves in male rats after stress<sup>17</sup>.

84 Interestingly, a major hallmark of stress-related psychiatric disorders, which have a sex-biased  
85 incidence in humans, is cognitive inflexibility. These results suggest that females may be more  
86 vulnerable to this type of cognitive impairment than males. The use of these techniques in  
87 animal models will shed light on the effects of stress on the brain and how it impairs cognition  
88 in psychiatric disorders in humans.

89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132

**PROTOCOL:**

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Bryn Mawr College.

**1. Animal preparation**

1.1. Acquire male and female adult Sprague Dawley rats.

NOTE: The rats can be delivered before 65 days of age, but do not begin procedures until after this point to ensure that both males and females are fully mature.

1.2. Pair-house same-sex rats for as long as possible, as long-term isolation is a stressor<sup>24</sup>. For food restriction, singly house rats just prior to the operant strategy shifting protocol.

1.3. After 1 week of acclimation, gently begin to handle rats for 3–5 min per day. Collect the body weight of each rat. Additionally, if interested in assessing how gonadal hormones may affect the results, collect vaginal lavage for female rats (described in section 2).

1.4. Before food restriction procedures begin, obtain approval from the institutional IACUC or other regulatory body. Restrict (from food) animals that will be run in the operant strategy shifting paradigm at least 3 days before the training begins so that they successfully learn the task. Ensure that water is always freely available.

1.4.1. If employing a stress procedure for more than 3 days before training, adjust the food restriction to match the number of days of stress (e.g., 5 days of restraint plus food restriction<sup>25</sup>).

1.4.2. Each day, deliver 80% of the normal daily food intake (i.e., 4 g of food per 100 g of body weight)<sup>26</sup>. Use the daily weight collection for the rat to calculate how much food to give each day.

1.4.3. Continue the food restriction through the training and testing days. However, do not place food in the home cage until after the rat has completed training or testing for the day, or else they will not be motivated to perform the tasks for a food pellet reward. Ensure that the timing of food delivery to rats upon completion of the task is fairly unpredictable since this helps to avoid reduced motivation to perform in the operant chamber (in favor of simply waiting for food in the home cage afterwards).

NOTE: Animals undergoing the restraint stress paradigm do not exhibit significantly greater weight loss than control, unstressed subjects. However, various stress procedures may themselves induce weight loss, resulting in rats receiving less food than unstressed counterparts during body weight-based food restriction. This may present an additional,

133 confounding stressor. If this appears to be the case, alternatively use a fixed amount of food  
134 given to each subject, regardless of weight<sup>27</sup>.

135

## 136 **2. Vaginal lavage**

137

138 NOTE: Gonadal hormones (i.e., estrogen and progesterone) are known to affect the stress  
139 response and cognition<sup>28–30</sup>. These hormones fluctuate over the estrous cycle of female rats<sup>31</sup>.  
140 If interested in tracking the estrous cycle of freely cycling female rodents to correlate with  
141 stress or cognitive flexibility data, collect vaginal lavage as described below. Representative  
142 data considering estrous cycle stage are not provided.

143

144 2.1. To obtain vaginal lavage samples from females, gather warm water in a clean beaker, a  
145 glass eyedropper, a “lavage” slide (microscope slide with acrylic paint circles to hold the lavage  
146 sample), and one empty beaker.

147

148 2.2. Fill the eyedropper with a small amount of warm water (~0.5 mL), then insert the tip into  
149 the vagina of the female rat (by lifting by its tail). Expel the sterile water 2x–3x and expel the  
150 collected fluid onto a microscopic slide. Do not overflow the lavage slide circle.

151

152 2.3. Expel any excess liquid into the empty beaker. Label the lavage slide with rat numbers and  
153 put the samples from each rat in that order so it is clear which sample belongs to each rat.

154

155 2.4. Thoroughly rinse the eyedropper by pipetting clean warm water and dispensing it into the  
156 “excess” beaker several times before filling the eyedropper to sample the next rat.

157

158 2.5. Carefully carry the lavage slide to a brightfield microscope to image the lavage sample and  
159 classify the day within the estrous cycle as described in Becker et al<sup>31</sup>.

160

161 NOTE: Ideally, lavaging should be done for a few weeks to properly track a female’s cycle and  
162 should be performed at a very similar time each day to control for circadian rhythms.

163 Preferably, this procedure should be performed before stress and operant strategy shifting  
164 procedures. Data for female rats can be analyzed post-hoc according to estrous cycle day  
165 (consider days of cycle when stress is performed and/or day of cycle when testing occurs).

166

## 167 **3. Equipment and software**

168

169 **3.1. Use operant chambers for behavioral training and testing.**

170

171 **3.1.1. Ensure that the chambers contain at least two retractable levers with two stimulus lights**  
172 **above, a house light, and a dispenser for reinforcement for these tasks.**

173

174 **3.1.2. Check that the levers are on the either side of the central reinforcement delivery area**  
175 **with one stimulus light above each lever.**

176

177 3.1.3. Use the house light to illuminate the chamber without interfering with detection of the  
178 light stimulus (it is best if the house light is on the back wall of the chamber, opposite to the  
179 levers and stimulus lights).

180

181 3.2. Use dustless food pellets (here, 45 mg pellets are used: 18.7% protein, 5.6% fat, and 4.7%  
182 fiber) for reinforcement in food-restricted rats. Do not use pellets high in sucrose or fat (unless  
183 there is interest in how stress affects palatable food intake).

184

185 3.3. Control the presentation of stimuli, lever operation, and data collection from a computer  
186 with software capable of operating the chamber (**Table of Materials**).

187

188 NOTE: For information related to coding of programs using this software, contact the authors.  
189 MED-PC scripts are included as supplemental files. This software collects information about the  
190 animal's responses for each trial (which lever is pressed, whether it is correct/incorrect/no  
191 response, and latency to make the choice). From this information, users can calculate various  
192 measures in the behavioral paradigm, as described in the behavioral analysis section.

193

194 3.4. Perform training/testing at the same time each day to control for circadian rhythms in  
195 stress hormones<sup>32</sup> (and other relevant measures).

196

197 3.5. Fill the bottom tray of each operant box with fresh bedding to collect feces/waste.  
198 Following each session, dump each tray, clean trays with alcohol wipes, and replace with fresh  
199 bedding before placing a new animal in the chamber.

200

## 201 **4. Stress procedures**

202

203 4.1. Decide whether the stress procedure should be performed before, during, and/or after  
204 training on the operant strategy shifting paradigm (e.g., 5 days of restraint stress prior to 3 days  
205 of operant training vs. 3 days of operant training followed by a single restraint and testing).

206

207 4.2. Execute the stress procedure at the same time daily with respect to operant training. (e.g.,  
208 30 min of restraint stress starting at 9 A.M., followed by placement in the operant chamber).

209

210 4.3. Perform the stress procedures in a separate room from both the colony room and strategy  
211 shifting paradigm rooms (to ensure there are no confounding factors associated with witness  
212 stress)<sup>33</sup>. Briefly, place the rat in a Broome-style transparent restraint tube and seal the  
213 opening, taking care not to pinch the limbs or tail.

214

215 NOTE: Estimate how long the first group of rats will spend in the operant chambers. This will  
216 vary depending on training vs. test day; however, after running several cohorts, an average  
217 time to complete each task to estimate future tasks can be calculated.

218

219 4.4. Depending on how many operant chambers are available, stagger the stress procedure for  
220 subjects. For example, four rats undergo restraint stress and are placed in four operant

221 chambers. One hour later, four more animals undergo stress procedures to be followed by the  
222 operant chamber.

223

## 224 **5. Training**

225

226 NOTE: This paradigm is modified from the operant set-shifting procedure developed by  
227 Floresco et al. such that it can be completed in 3 days<sup>19</sup>. Training procedures for rats require 3  
228 days (1 day to learn each task as described below). It is rare that a rat does not learn these  
229 tasks. If a rat fails to learn each task, it should be excluded from the final study. See **Figure 1A**  
230 for a visual depiction of the training paradigm described below.

231

232 5.1. Before placing the rat in the chamber, ensure that there are enough food pellets in the  
233 dispenser and that the operant boxes are properly functioning. To accomplish this, load and  
234 initiate a training or test day program in an empty chamber, manually testing that the correct  
235 lever appropriately delivers one reward per lever press.

236

### 237 5.2. Training the rat to press each lever

238

239 5.2.1. Before placing the rat in the box for the first day of training, manually set one food pellet  
240 reward on the correct lever, as designated upon loading the training procedure within each  
241 chamber.

242

243 5.2.2. Train the rat using a fixed ratio (FR-1) schedule, such that each correct lever press is  
244 rewarded with one reinforcement. Counterbalance the correct lever per day across subjects  
245 and/or experimental conditions (shaping only one lever at a time) by designating the correct  
246 lever upon loading the training procedure on the computer operating the chambers.

247

248 5.2.3. Allow the rat to press the lever until it reaches the criterion by pressing the correct lever  
249 50x, usually completing the task between 30–45 min.

250

251 5.2.4. The following day force the rat to perform this task on the opposite lever using the same  
252 program as the first day of training, but designate the opposite lever as the correct one. There  
253 is no need to “shape” the lever with a food pellet on this day of training. Typically, this criterion  
254 is quickly acquired after rats have learned to press the first lever.

255

### 256 5.3. Training the rat to respond to the light cue

257

258 5.3.1. On the third day of training, illuminate the light above both levers for 15 s trials, during  
259 which the rat may press one of lever to potentially receive a food pellet reward. During the light  
260 discrimination task, this program will randomly select which lever is correct on a trial-by-trial  
261 basis.

262

263 5.3.2. If the rat presses the correct lever, ensure that the lights remain illuminated for 3 s and  
264 the reward is delivered, followed by a 5 s period, during which the lights are shut off preceding

265 the next trial. If the rat presses the incorrect lever, ensure that no reward is delivered and that  
266 lights are shut off for 10 s preceding the next trial.

267  
268 5.3.3. Following this last day of training, calculate “side bias” to determine if the rat has a  
269 preference for the left or right lever by dividing the number of presses of one lever divided by  
270 the total number of lever presses. On the test day, the rat will start on its least preferred side to  
271 ensure that it is learning the specific response-reward contingency, rather than responding to a  
272 preferred lever.

273

## 274 **6. Testing**

275

276 NOTE: See **Figure 1B** for a visual depiction of the testing paradigm described below.

277

278 6.1. On day 4 (test day), place the rat in the operant chamber following stress procedures and  
279 test them in side discrimination, side reversal, and light discrimination tasks serially. Ensure that  
280 the light discrimination task only illuminates the light above the “correct” lever. In each task,  
281 rats must consecutively achieve eight correct trials to complete each discrimination without  
282 pressing the unrewarded, incorrect lever. An incorrect lever press will reset this chain of trials.

283

284 6.1.1. Test rats using the side discrimination task. Using the side discrimination program,  
285 reward the rat for pressing the lever on its least preferred side as determined from the third  
286 day of training, regardless of the light cue. The task ends upon pressing the correct lever 8x  
287 consecutively (excluding omissions).

288

289 6.1.2. Perform the side reversal test by running rats using the side discrimination program  
290 again, but this time designating the lever opposite to the correct one from the side  
291 discrimination task as correct. Ensure that the rat is rewarded for pressing this lever, regardless  
292 of the light cue. The task ends upon pressing the correct lever 8x consecutively (excluding  
293 omissions).

294

295 6.1.3. Perform the light discrimination task, which rewards the rat for pressing the lever with  
296 the light illuminated above. Each operant testing is complete upon pressing the correct lever 8x  
297 consecutively (excluding omissions).

298

299 NOTE: Based on previous studies, these tasks encode a minimum of 30 trials, regardless of  
300 consecutive presses, to ensure that rats have sufficient time to learn the rules of each task<sup>18</sup>.  
301 Thus, if the rat consecutively achieves eight correct trials before 30 trials have occurred, the  
302 task will remain engaged until 30 trials are completed.

303

## 304 **7. Behavioral analysis**

305

306 NOTE: The data acquired for each animal on the test day are automatically recorded and saved  
307 by the computer, as long as a MED-PC script for each task been initiated and allowed to  
308 complete (see supplementary materials for MED-PC scripts).



309

310 7.1 Open the data for each test day task (side discrimination, side reversal, and light  
311 discrimination) using the computer program. The main measures recorded by the program are  
312 trials to criterion, errors in criterion, and time to criterion. These measures are described in  
313 detail below.

314

315 NOTE: The authors have generated a MATLAB script that allows for automation of the analysis  
316 process as well as analysis of perseverative vs. regressive errors (contact authors for code  
317 information to streamline data analysis).

318

319 7.1.1. Use trials to criterion (which refers to the total number of trials [not including omissions]  
320 necessary for the rat to consecutively complete eight correct trials, including those eight trials)  
321 as the main indicator of accuracy. This data is located in the first column in array B in a data file  
322 generated by the MED-PC script for any of the tasks on test day.

323

324 7.1.2. Examine the total errors made during each task. This data is located in the third column  
325 of array B in a data file generated by the MED-PC script for any of the tasks on test day. These  
326 errors are also categorized into perseverative or regressive errors. Perseverative errors are  
327 committed when the rat continues to follow the earlier rule from the previous task. Regressive  
328 errors are committed after it has disengaged from the previous rule but continues to try to  
329 acquire the new rule (for more details on how these types of errors are calculated, refer to the  
330 published method<sup>18</sup>).

331

332 7.1.3. If the rat did not respond to a light cue within 15 s, the trial is categorized as an omission,  
333 not counting it towards the total number of trials to criterion. Calculate this by first adding  
334 together the number of correct responses (located in the second column of array B in data file)  
335 and number of errors (located in the third column of array B in data file). Next, subtract this  
336 number from the total number of trials to criterion (this is the last number in the first column of  
337 array B in a data file, different from the trials to criterion).

338

339 7.1.4. Use start and finish times recorded by the program (located at the top of a data file  
340 generated by the MED-PC script for any of the tasks on test day) to calculate time to criterion.  
341 Latency to the first lever press can also be calculated from the data file by subtracting the  
342 variable K (elapsed time in seconds from the first lever press) from the time to criterion.

343

344 7.1.5. Average the data for each behavioral measure for rats within the same treatment group.  
345 Perform appropriate statistical analyses (depending on how many variables are being  
346 examined).

347

## 348 **8. Brain substrates**

349

350 8.1. Determine an interested brain area and/or aspect of cognitive flexibility. For example, if  
351 stress increases perseverative errors in the side reversal task, the orbitofrontal cortex (OFC)  
352 may be of particular interest, as previous lesion studies have indicated this brain region plays a

353 role in many forms of reversal learning (i.e., spatial reversal tested in the side reversal task)<sup>34–36</sup>.  
354 In this example, sacrifice rats after the strategy shifting paradigm is completed and examine c-  
355 fos (measure of neural activation<sup>37</sup>) in the OFC using described immunohistochemical  
356 methods<sup>25</sup> and described briefly here.

357

358 8.1.1. First, extract brains from animals and cut into 40 µm slices.

359

360 8.1.2. Wash the tissue in phosphate-buffered saline (PBS) 4x for 5 min each, then incubate in  
361 0.3% hydrogen peroxide for 10 min to quench endogenous peroxidases.

362

363 8.1.3. Wash tissue in PBS 2x for 5 min each, then incubate in mouse anti-c-fos primary antibody  
364 (1:500), 3% normal donkey serum (NDS), and 0.3% Triton X overnight.

365

366 8.1.4. The next day, wash tissue in PBS 3x for 5 min each, then incubate in biotin-SP-conjugated  
367 donkey anti-mouse sary antibody (1:500) for 2 h.

368

369 8.1.5. Wash tissue in PBS 3x for 5 min each, then incubate in avidin-streptavidin AB complex for  
370 1 h.

371

372 8.1.6. Wash tissue in PBS 3x for 5 min each, then incubate in DAB solution for up to 10 min as  
373 tissue undergoes an oxidation chromogenic reaction.

374

375 8.1.7. Wash tissue in PBS 3x for 5 min each, then mount the brain slices on glass microscope  
376 slides.

377

378 8.1.8. Coverslip the tissue using toluene based mounting medium and image using a brightfield  
379 microscope.

380

381 NOTE: Here, as reflected in the representative results, rats are sacrificed 30 min after the  
382 strategy shifting paradigm ends, roughly 60–90 min after the reversal task has been completed  
383 (depending on each rat's performance in the light task). This should represent optimal timing  
384 for c-fos expression<sup>38</sup>, reflecting performance in the reversal task.

385

386 8.2. Alternatively, cannulate a specific brain area for drug injection or viral injection prior to the  
387 execution of stress or the operant strategy shifting paradigm.

388

389 NOTE: Researchers may want to examine how manipulating neural substrates alters the effects  
390 of stress on cognitive flexibility. For example, researchers can block a particular  
391 neurotransmitter receptor in the prefrontal cortex prior to testing.

392

### 393 **REPRESENTATIVE RESULTS:**

394

395 The adapted automated operant strategy shifting paradigm outlined above was used to  
396 determine if repeated restraint stress affects cognition in male and female Sprague Dawley

397 rats. Representative behavioral data are described in **Figure 2** below. In short, control and  
398 repeatedly restrained rats performed this operant strategy shifting test, which consisted of a  
399 series of tasks: side discrimination, side reversal, and light discrimination.

400  
401 Trials to criterion for each task are depicted in **Figure 2A**. Typically, better performance on each  
402 task was represented by a reduced number of trials to criterion. These data indicate that,  
403 following acute restraint, males completed the side reversal task in significantly fewer trials  
404 than unstressed, control males. Conversely, stressed females required a significantly greater  
405 number of trials to complete the side reversal task. These results suggest that males exhibited  
406 improved performance following stress, whereas females exhibited impaired performance. In  
407 the light discrimination task, stress increased the number of trials to criterion compared to  
408 control females, thereby impairing performance in females but not males in this task.

409  
410 The total number of errors made for each attention task is depicted in **Figure 2B**. Consistent  
411 with the number of trials to criterion, stressed males made significantly fewer errors than  
412 control males, whereas stressed females made more errors in the side reversal task.  
413 Furthermore, in the light discrimination task, females also made significantly more errors. In  
414 sum, these data suggest that repeated stress improves cognitive performance in males but  
415 impairs cognitive performance in females.

416  
417 Total errors were further categorized into perseverative or regressive errors in **Figure 2C** (for a  
418 distinction between these two types of errors, refer to section 7 of the protocol). Interestingly,  
419 stressed males made fewer perseverative errors in the side reversal task than control males. On  
420 the other hand, in both the side reversal and light discrimination tasks, stressed females made a  
421 greater number of perseverative errors than control females. There were no differences  
422 between the treatment groups in the number of regressive errors made during either task.

423  
424 Omissions in each trial and time to reach criterion are shown in **Figure 2D** (for more  
425 information on how these were calculated, refer to section 7 of the protocol). These measures  
426 were evaluated in the side reversal task only, as this task exhibited the largest sex differences.  
427 Stressed females made a higher percentage of omissions compared to all other treatment  
428 groups. In addition, while stress appeared to decrease the time to complete the side reversal  
429 task in males, stress prolonged completion of the task in females. In sum, repeated stress  
430 impaired cognitive flexibility in females but not males.

431  
432 Brain substrates underlying cognitive flexibility are depicted in **Figure 3**. As stark sex differences  
433 were observed in the side reversal task, the brain areas underlying this task were examined to  
434 determine whether they displayed similar sex differences in neural activity. As previously  
435 discussed, lesion studies have indicated that the orbitofrontal cortex (OFC) mediates the side  
436 reversal task<sup>34</sup>. Thus, c-fos, a measure of neural activation<sup>37</sup>, was labeled in the OFC at 30 min  
437 after the completion of strategy shifting, which should have reflected performance in the side  
438 reversal task<sup>38</sup>. However, it is possible that OFC may also play a role in the extradimensional  
439 strategy shifting component of this task<sup>39</sup>. Thus, it is important to perform the sacrifice at the  
440 appropriate time to reflect brain activity during a particular task within the operant strategy

441 shifting paradigm. Here, stress induced a significant increase in neuronal activation in the OFC  
442 of males compared to controls. However, stress induced a significant decrease in neuronal  
443 activation in the OFC of females compared to controls. Furthermore, in males, OFC activation  
444 and trials to criterion were negatively correlated; specifically, higher OFC activation was  
445 associated with fewer trials to criterion. In contrast, there was no correlation between OFC  
446 activation and performance in females, suggesting that the OFC was disengaged during these  
447 performances.

448

#### 449 **FIGURE LEGENDS:**

450

451 **Figure 1: Schematic of the operant strategy shifting paradigm during training and test days.**

452

453 **Figure 2: Representative behavioral data from operant strategy shifting paradigm. (A)** Trials to  
454 criterion for each task on test day. In the side reversal task, stress improved performance in  
455 males but impaired performance in females. In the light discrimination task, stress weakened  
456 performance in females, while it did not affect males. **(B)** Number of errors for each task on test  
457 day. Stress reduced the number of errors made in males but increased errors in females in both  
458 side reversal and light discrimination tasks. **(C)** Perseverative and regressive error  
459 categorization. Stress decreased perseverative errors made in males but increased  
460 perseverative errors made in females in both side reversal and light discrimination tasks. **(D)**  
461 Percent trials omitted and time to criterion in the side reversal task. Stress increased the  
462 percent omissions in female rats. Stress decreased the time required by males but increased  
463 the time required by females to complete the task. Statistics were calculated using two-way  
464 ANOVA followed by Tukey's t-test ( $n = 12$  rats per group; error bars represent SEM;  $\#p \leq 0.10$ ,  
465  $*p < 0.05$ ). This figure has been modified from a previous publication<sup>17</sup>.

466

467 **Figure 3: Representative neural activation after operant strategy shifting paradigm. (A)** OFC  
468 activation after strategy shifting task. Representative images of immunohistochemical 3,3'-  
469 diaminobenzidine (DAB) staining using an antibody against c-fos in the OFC visualized using  
470 brightfield microscopy, then quantified. Stress significantly increased activation (demonstrated  
471 by the number of c-fos-expressing cells) in the OFC of males, while it decreased activation in  
472 females. Scale bar in bottom-right image panel represents 200  $\mu\text{m}$ . Statistics were calculated  
473 using two-way ANOVA followed by Tukey's t-test ( $n = 12$  rats per group, 6–8 sections of OFC  
474 analyzed per rat; error bars represent SEM;  $*p < 0.05$ ). **(B)** Trials to criterion in the side reversal  
475 task correlated with OFC activation. Males displayed a significant negative correlation, whereas  
476 females did not.

477

#### 478 **DISCUSSION:**

479

480 The protocol demonstrates how to measure the effects of stress on cognitive function.  
481 Specifically, a modified operant strategy shifting paradigm is used in rodents, which measures  
482 cognitive flexibility (analogous to the Wisconsin Card Sorting Task in humans)<sup>1</sup>. Cognitive  
483 flexibility denotes the ability to adapt cognitive processing strategies to face new conditions in  
484 the environment, and it is crucial for normal daily functioning<sup>2</sup>. As human studies on cognitive

485 flexibility are mostly limited to brain imaging<sup>11</sup>, the use of this paradigm in animals will greatly  
486 advance the understanding of effects of stress on the brain and cognition.

487

488 Stress can impair cognitive function<sup>40</sup>. In fact, this is one of the most common phenotypes in  
489 stress-related illnesses such as PTSD and MDD<sup>3,41</sup>. Moreover, there are stark sex differences in  
490 the occurrence of stress-related psychiatric illnesses<sup>5-7</sup>, yet there is little understanding of the  
491 neurobiology behind these biased incidences. Thus, use of this operant strategy shifting  
492 paradigm in animals of both sexes may help advance the current understanding of sex  
493 differences in psychiatry.

494

495 This operant strategy shifting task allows researchers to examine key aspects of cognition  
496 relevant to psychiatric disorders. For example, perseverative errors after experimental  
497 manipulation are calculated in this paradigm. Perseveration is observed in stress-related  
498 psychiatric disorders such as PTSD, and it impairs the ability of one to learn a new set of rules,  
499 ultimately impairing working memory<sup>3</sup>. Thus, the measure of perseverative errors is  
500 translationally relevant. Moreover, omissions in attention tasks have been noted in patients  
501 with PTSD, indicating slower cortical processing<sup>3</sup>. Accordingly, omission data from this paradigm  
502 may have clinical counterparts. In sum, cognitive flexibility measured as by this experimental  
503 paradigm models key phenotypes that are observed in psychiatric disorders.

504

505 This experimental paradigm also allows for precision in targeting neural substrates underlying  
506 cognitive flexibility. For example, the literature has indicated that the prefrontal cortex (PFC) is  
507 a crucial brain region for cognitive flexibility<sup>3</sup>, including the medial prefrontal (mPFC) and  
508 orbitofrontal cortex (OFC). Of these subregions in the PFC, the OFC is important for  
509 performance in the side reversal task<sup>34,35</sup>. These brain areas are also a key targets for stress-  
510 induced functional alterations<sup>42,43</sup>. Interestingly, the model of stress used here does appear to  
511 play a role in the subsequent performance of rodents in tests of cognitive flexibility; thus, it  
512 should be considered in the design of future experiments. These varying responses to stress  
513 point to potentially novel mechanisms by which cognition is impacted by stress. Thus, targeting  
514 specific neurotransmitters, proteins, or activation of these brain regions may shed light on how  
515 stress affects cognition in male and female rodents. Researchers can choose to manipulate  
516 these neural substrates at different timepoints in conjunction with stress or strategy shifting, or  
517 alternatively measure neural substrates after exposure to these behavioral paradigms.

518

519 This modified operant strategy shifting task has clear advantages over other cognitive flexibility  
520 paradigms used in the stress literature (i.e., the digging task<sup>12-15</sup>), which require more time and  
521 effort by the experimenter to train rodents. This procedure requires minimal oversight by the  
522 experimenter and allows multiple rats to be tested simultaneously. In addition, unlike other  
523 versions of this automated task<sup>19</sup>, the paradigm only requires 3 days of training and includes an  
524 efficient programmed data analysis.

525

526 The operant strategy shifting paradigm does have certain limitations. One limitation is that it  
527 can only test two stimulus dimensions (e.g., left or right lever vs. light cue), whereas the digging  
528 task<sup>12-15</sup> can test a third stimulus dimension (e.g., digging media vs. odor vs. texture). However,

529 the task described in this protocol still allows for testing of the rat's ability to shift to different  
530 rules, which allows testing of the cognitive flexibility constructs. In addition, it is possible to add  
531 other parameters to the operant chambers to allow for a third stimulus (e.g., an odor), but this  
532 may prolong the training required for the task.  
533

534 The primary advantage of this task is its simplicity and ability to pair it with stressful or  
535 pharmacological manipulations to further understand how stress affects the brain. It should be  
536 noted that this simplicity comes with an increased difficulty that subjects face while learning to  
537 lever press, compared to the ecologically relevant digging task. While this operant task is far  
538 less labor-intensive, rodents will generally require more trials to acquire this task. However,  
539 both the digging task and this paradigm engage similar neurobiological mechanisms and thus  
540 represent valid options for the examination of cognitive flexibility<sup>16,44</sup>. While there have been  
541 varied results in the literature regarding the effects of stress on cognitive flexibility using the  
542 digging task and this operant procedure<sup>23,25,27,45,46</sup>, the presented method reflects the complex  
543 effects that the type, intensity, and duration of a stressor can have on cognitive function<sup>20,21</sup>.  
544

545 Another limitation of the task is that rodents are housed in closed opaque boxes; thus,  
546 behaviors other than those that are collected via the computer interface cannot be coded. For  
547 example, a high number of omissions by a rat may be due to behavioral inhibition inflicted by  
548 stress, or because the rat is asleep. Moreover, other stereotypical behaviors, such as grooming  
549 (which is particularly relevant in studying stress), may be interesting to analyze during the task.  
550 Mounting cameras in operant chambers may allow for this type of behavioral precision.  
551

552 Overall, this report details the use of stress procedures in conjunction with an operant strategy  
553 shifting paradigm to further understand how stress affects the brain. It should be noted that, in  
554 addition to stress procedures and cognitive assessment in adults, research on different  
555 developmental stages may provide crucial information about the etiology of cognitive  
556 inflexibility. In addition to studying the effects of stress on cognitive flexibility, this simple and  
557 efficient operant strategy shifting paradigm can be paired with many experimental  
558 manipulations to investigate how the brain adapts to changing environments. Moreover,  
559 alternate experimental approaches can be used to study the neural basis of cognitive flexibility,  
560 including lesions, pharmacology, gene editing, and electrophysiology. As cognitive inflexibility is  
561 one of the key phenotypes in psychiatric disease, more research must be conducted to further  
562 understand its neurobiological substrates.  
563

#### 564 **ACKNOWLEDGMENTS:**

565 The authors would like to thank Hannah Zamore, Emily Saks, and Josh Searle for their help in  
566 establishing this operant strategy shifting paradigm in the Grafe lab. They would also like to  
567 thank Kevin Snyder for his help with the MATLAB code for analysis.  
568

#### 569 **DISCLOSURES:**

570 The authors have nothing to disclose.  
571

#### 572 **REFERENCES**

- 573 1. Hurtubise, J. L., Howland, J. G. Effects of stress on behavioral flexibility in rodents.  
574 *Neuroscience*. **345**, 176-192 (2016).
- 575 2. Bissonette, G. B., Powell, E. M., Roesch, M. R. Neural structures underlying set-shifting:  
576 Roles of medial prefrontal cortex and anterior cingulate cortex. *Behavioural Brain*  
577 *Research*. **250**, 91–101 (2013).
- 578 3. Vasterling, J. J., Brailey, K., Constans, J. I., Sutker, P. B. Attention and memory dysfunction  
579 in posttraumatic stress disorder. *Neuropsychology*. **12** (1), 125–33 (1998).
- 580 4. Bangasser, D. A., Kawasumi, Y. Cognitive disruptions in stress-related psychiatric  
581 disorders: A role for corticotropin releasing factor (CRF). *Hormones and Behavior*. **76**,  
582 125–135 (2015).
- 583 5. Nestler, E. J. et al. Neurobiology of depression. *Neuron*. **34** (1), 13–25 (2002).
- 584 6. Keane, T. M., Marshall, A. D., Taft, C. T. Posttraumatic stress disorder: etiology,  
585 epidemiology, and treatment outcome. *Annual Review of Clinical Psychology*. **2**, 161–  
586 1697 (2006).
- 587 7. Seeman, M. V. Psychopathology in women and men: focus on female hormones. *The*  
588 *American Journal of Psychiatry*. **154** (12), 1641–1647 (1997).
- 589 8. Hodes, G. E., Epperson, C. N. Sex Differences in Vulnerability and Resilience to Stress  
590 Across the Life Span. *Biological Psychiatry*. **86** (6), 421–432 (2019).
- 591 9. Monika, T.-B., Antoni, F., Piotr, G., Marian, M., Krzysztof, Z. Wisconsin Card Sorting Test  
592 in psychological examination of patients with psychiatric disorders. *Polski merkurusz*  
593 *lekarski: organ Polskiego Towarzystwa Lekarskiego*. **25 Suppl 1**, 51–2 (2008).
- 594 10. Merriam, E. P., Thase, M. E., Haas, G. L., Keshavan, M. S., Sweeney, J.A. Prefrontal cortical  
595 dysfunction in depression determined by Wisconsin Card Sorting Test performance. *The*  
596 *American Journal of Psychiatry*. **156** (5), 780–782 (1999).
- 597 11. Monchi, O., Petrides, M., Petre, V., Worsley, K., Dagher, A. Wisconsin Card Sorting  
598 revisited: distinct neural circuits participating in different stages of the task identified by  
599 event-related functional magnetic resonance imaging. *The Journal of Neuroscience: the*  
600 *Official Journal of the Society for Neuroscience*. **21** (19), 7733–7741 (2001).
- 601 12. Bulin, S. E., Hohl, K. M., Paredes, D., Silva, J. D., Morilak, D. A. Bidirectional  
602 optogenetically-induced plasticity of evoked responses in the rat medial prefrontal  
603 cortex can impair or enhance cognitive set-shifting. *eNeuro*. **7** (1) ENEURO.0363-19  
604 (2019).
- 605 13. Chaby, L. E., Karavidha, K., Lisieski, M. J., Perrine, S. A., Liberzon, I. Cognitive Flexibility  
606 Training Improves Extinction Retention Memory and Enhances Cortical Dopamine With  
607 and Without Traumatic Stress Exposure. *Frontiers in Behavioral Neuroscience*. **13**, 24  
608 (2019).
- 609 14. Drozd, R., Rojek-Sito, K., Rygula, R. The trait ‘pessimism’ does not interact with cognitive  
610 flexibility but makes rats more vulnerable to stress-induced motivational deficits: Results  
611 from the attentional set-shifting task. *Behavioural Brain Research*. **335**, 199–207 (2017).
- 612 15. Birrell, J. M., Brown, V. J. Medial frontal cortex mediates perceptual attentional set-  
613 shifting in the rat. *The Journal of Neuroscience: the Official Journal of the Society for*  
614 *Neuroscience*. **20** (11), 4320–4 (2000).
- 615 16. Floresco, S. B., Block, A. E., Tse, M. T. L. Inactivation of the medial prefrontal cortex of the  
616 rat impairs strategy set-shifting, but not reversal learning, using a novel, automated

- 617 procedure. *Behavioural Brain Research*. **190** (1), 85–96 (2008).
- 618 17. Grafe, L. A., Cornfeld, A., Luz, S., Valentino, R., Bhatnagar, S. Orexins Mediate Sex  
619 Differences in the Stress Response and in Cognitive Flexibility. *Biological Psychiatry*. **81**  
620 (8), 683–692 (2017).
- 621 18. Snyder, K. P., Barry, M., Valentino, R. J. Cognitive impact of social stress and coping  
622 strategy throughout development. *Psychopharmacology*. **232**(1),185-9 (2014).
- 623 19. Brady, A. M., Floresco, S. B. Operant procedures for assessing behavioral flexibility in  
624 rats. *Journal of Visualized Experiments*. (96), e52387 (2015).
- 625 20. Sandi, C., Pinelo-Nava, M. T. Stress and Memory: Behavioral Effects and Neurobiological  
626 Mechanisms. *Neural Plasticity*. 1–20 (2007).
- 627 21. Shansky, R. M., Lipps, J. Stress-induced cognitive dysfunction: hormone-neurotransmitter  
628 interactions in the prefrontal cortex. *Frontiers in Human Neuroscience*. **7**, 123 (2013).
- 629 22. Ragozzino, M. E., Detrick, S., Kesner, R. P. Involvement of the prelimbic-infralimbic areas  
630 of the rodent prefrontal cortex in behavioral flexibility for place and response learning.  
631 *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*. **19** (11),  
632 4585–94 (1999).
- 633 23. Liston, C. et al. Stress-induced alterations in prefrontal cortical dendritic morphology  
634 predict selective impairments in perceptual attentional set-shifting. *The Journal of*  
635 *Neuroscience: the Official Journal of the Society for Neuroscience*. **26** (30), 7870–4 (2006).
- 636 24. Hatch, A., Wiberg, G. S., Balazs, T., Grice, H. C. Long-Term Isolation Stress in Rats. *Science*.  
637 **142** (3591), 507–507 (1963).
- 638 25. Grafe, L. A., Cornfeld, A., Luz, S., Valentino, R., Bhatnagar, S. Orexins Mediate Sex  
639 Differences in the Stress Response and in Cognitive Flexibility. *Biological Psychiatry*. **81**  
640 (8), 683–692 (2017).
- 641 26. Animal Care and Use Committee, T. J. H. U. Species Specific Information: Rat. at  
642 <<http://web.jhu.edu/animalcare/procedures/rat.html>>.
- 643 27. Lapiz-Bluhm, M. D. S. et al. Behavioural assays to model cognitive and affective  
644 dimensions of depression and anxiety in rats. *Journal of Neuroendocrinology*. **20** (10),  
645 1115–37 (2008).
- 646 28. McEwen, B. S. Permanence of brain sex differences and structural plasticity of the adult  
647 brain. *Proceedings of the National Academy of Sciences*. **96** (13), 7128–7130 (1999).
- 648 29. Manber, R., Armitage, R. Sex, steroids, and sleep: a review. *Sleep*. **22** (5), 540–55 (1999).
- 649 30. Sherwin, B. B. Estrogen and Cognitive Functioning in Women. *Endocrine Reviews*. **24** (2),  
650 133–151 (2003).
- 651 31. Becker, J. B. et al. Strategies and methods for research on sex differences in brain and  
652 behavior. *Endocrinology*. **146** (4), 1650–73 (2005).
- 653 32. Koch, C. E., Leinweber, B., Drenberg, B. C., Blaum, C., Oster, H. Interaction between  
654 circadian rhythms and stress. *Neurobiology of Stress*. **6**, 57–67 (2017).
- 655 33. Warren, B. L. et al. Neurobiological sequelae of witnessing stressful events in adult mice.  
656 *Biological Psychiatry*. **73** (1), 7–14 (2013).
- 657 34. McAlonan, K., Brown, V. J. Orbital prefrontal cortex mediates reversal learning and not  
658 attentional set-shifting in the rat. *Behavioural Brain Research*. **146** (1–2), 97–103 (2003).
- 659 35. Schoenbaum, G., Saddoris, M. P., Stalnaker, T. A. Reconciling the roles of orbitofrontal  
660 cortex in reversal learning and the encoding of outcome expectancies. *Annals of the New*



- 661 *York Academy of Sciences*. **1121** (1), 320–35 (2007).
- 662 36. Meunier, M. Effects of orbital frontal and anterior cingulate lesions on object and spatial  
663 memory in rhesus monkeys. *Neuropsychologia*. **35** (7), 999–1015 (1997).
- 664 37. Zappulla, R. A., Wang, W., Friedrich, V. L., Grabel, J., Nieves, J. CNS activation patterns  
665 underlying motor evoked potentials as demonstrated by c-fos immunoreactivity.  
666 *Electroencephalography and Clinical Neurophysiology*. **43**, 155–69 (1991).
- 667 38. Schoenenberger, P., Gerosa, D., Oertner, T. G. Temporal Control of Immediate Early Gene  
668 Induction by Light. *PLoS ONE*. **4** (12), e8185 (2009).
- 669 39. Chase, E. A., Tait, D. S., Brown, V. J. Lesions of the orbital prefrontal cortex impair the  
670 formation of attentional set in rats. *The European Journal of Neuroscience*. **36** (3), 2368–  
671 75 (2012).
- 672 40. Hancock, P. A., Warm, J. S. A dynamic model of stress and sustained attention. *Human*  
673 *Performance in Extreme Environments*. **7** (1), 15–28 (2003).
- 674 41. Johnson, P. L., Molosh, A., Fitz, S. D., Truitt, W. A., Shekhar, A. Orexin, stress, and  
675 anxiety/panic states. *Progress in Brain Research*. **198**, 133–61 (2012).
- 676 42. Leuner, B., Shors, T. J. Stress, anxiety, and dendritic spines: what are the connections?  
677 *Neuroscience*. **251**, 108–19 (2013).
- 678 43. Holmes, A., Wellman, C. L. Stress-induced prefrontal reorganization and executive  
679 dysfunction in rodents. *Neuroscience and Biobehavioral Reviews*. **33** (6), 773–83 (2009).
- 680 44. Placek, K., Dippel, W. C., Jones, S., Brady, A. M. Impairments in set-shifting but not  
681 reversal learning in the neonatal ventral hippocampal lesion model of schizophrenia:  
682 further evidence for medial prefrontal deficits. *Behavioural Brain Research*. **256**, 405–13  
683 (2013).
- 684 45. Nikiforuk, A., Popik, P. Long-lasting cognitive deficit induced by stress is alleviated by  
685 acute administration of antidepressants. *Psychoneuroendocrinology*. **36** (1), 28–39  
686 (2011).
- 687 46. Bondi, C. O., Rodriguez, G., Gould, G. G., Frazer, A., Morilak, D. A. Chronic unpredictable  
688 stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by  
689 chronic antidepressant drug treatment. *Neuropsychopharmacology*. **33** (2), 320–31  
690 (2008).
- 691