

**TITLE**

Stress Responses to Repeated Exposure to a Combined Physical and Social Evaluative Laboratory Stressor in Young Healthy Males

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1           **Stress Responses to Repeated Exposure to a Combined Physical and Social**  
2                           **Evaluative Laboratory Stressor in Young Healthy Males**

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12           **Stress Responses to Repeated Exposure to a Combined Physical and Social**  
13                           **Evaluative Laboratory Stressor in Young Healthy Males**

14   **Abstract**

15   Repeated exposure to homotypic laboratory psychosocial stressors typically instigates rapid  
16   habituation in hypothalamic - pituitary - adrenal (HPA) axis-mediated stress responses in  
17   humans. However, emerging evidence suggests the combination of physical stress and  
18   social evaluative threat may be sufficient to attenuate this response habituation.

19   Neuroendocrine, cardiovascular and subjective stress responses following repeated  
20   exposure to a combined physical and social evaluative stress protocol were assessed to  
21   examine the habituation response dynamic in this context.

22   The speech task of the Trier social stress test (TSST; Kirschbaum et al. 1993) and the  
23   socially evaluated cold pressor task (SECPT; Schwabe et al, 2008) were administered in a  
24   combined stressor protocol. Salivary cortisol, cardiovascular and subjective stress  
25   responses to a non-stress control and repeat stressor exposure separated by six weeks  
26   were examined in males ( $N = 24$ ) in a crossover manner.

27   Stressor exposure resulted in significant elevations in all stress parameters. In contrast to  
28   the commonly reported habituation in cortisol response, a comparable post-stress response  
29   was demonstrated. Cortisol, heart rate and subjective stress responses were also  
30   characterised by a heightened response in anticipation to repeated stress exposure. Blood  
31   pressure responses were comparatively uniform across repeated exposures. Findings  
32   suggest a combined physical and social evaluative stressor is a potentially useful method for  
33   study designs that require repeated presentation of a homotypic stressor.

34   **Keywords:** Cortisol; Habituation, Social evaluative threat; Stress induction; Laboratory  
35   stress

36

37

## 1. Introduction

38 Rapid habituation of response to stress is a frequently reported characteristic of the HPA  
39 axis. The cortisol response in humans has been shown to rapidly habituate in a number of  
40 stress contexts including repeated parachute jumps (Deinzer et al., 1997) and following  
41 repeated exposure to psychosocial stress protocols (Federenko et al., 2004; Gerra et al.,  
42 2001; Jonsson et al., 2010; Kirschbaum et al., 1995; Schommer et al., 2003). Response  
43 habituation to psychosocial stress is often specific to the HPA axis. Biomarkers of  
44 sympathetic activation (e.g., epinephrine [EPI], norepinephrine [NE], blood pressure [BP])  
45 tend to show comparatively uniform activation patterns across repeated stress exposures  
46 (Gerra et al., 2001; Mischler et al., 2005; Schommer et al., 2003; von Kanel et al., 2006; von  
47 Kanel et al., 2004).

48

49 Rodent models suggest the HPA axis predominantly habituates to processive  
50 (psychological) stressors. Comparatively less habituation to physiological stressors  
51 comprising a proximate physical threat is demonstrated (Grissom and Bhatnagar, 2009).  
52 Indeed, different neural pathways may underpin the HPA axis response to processive and  
53 physiological stressors. Processive stressors primarily activate the paraventricular nucleus  
54 (PVN) of the hypothalamus via limbic pathways. Conversely, rapid activation of the PVN via  
55 the brainstem nuclei, without significant activation of limbic circuitry, has been demonstrated  
56 to underpin responses to physical stressors (Emmert and Herman, 1999).

57

58 A combined physical (cold pressor task) and social evaluative (speech task) stressor has  
59 been employed without significant habituation in cortisol response (Prof. Sheila West;  
60 personal communication). A lack of significant habituation in cortisol response following  
61 repeated exposure to a physical stressor combined with elements of social evaluative threat  
62 (the socially evaluated cold pressor test [SECPT]) has also recently been reported (Minkley

63 et al., 2014). Thus the combination of social evaluation and a physical stressor may be a  
64 promising method for reducing habituation to repeated stress induction. A stress protocol  
65 suitable for repeated application without significant habituation in HPA axis activation would  
66 be a useful methodological tool. Significant habituation results in difficulties separating the  
67 effect of stress from response habituation when interventions are assessed under repeated  
68 exposures.

69

70 Whilst Minkley et al. (2014) have demonstrated no cortisol habituation to the SECPT, a more  
71 complete measurement of the habituation response is required since Minkley et al. collected  
72 only two salivary cortisol samples. Further, the short and variable duration of the SECPT,  
73 determined by the length of time the hand is retained in an ice bath (0 – 3 min), results in  
74 cardiovascular and subjective stress responses that are limited to the duration of stressor  
75 (Giles et al., 2014), and are fully extinguished by the cortisol response peak (21 – 40 min  
76 post-stress onset; Dickerson and Kemeny, 2002). In comparison to the SECPT, more  
77 prolonged stress responses are elicited by social-evaluative speech tasks (TSST; Giles et  
78 al., 2014). Therefore, the addition of a speech task to the SECPT may ensure a more  
79 sustained cardiovascular and subjective stress response. A stressor capable of sustaining  
80 concurrent responses post-stress exposure has greater utility for studies examining the  
81 impact of stress on dependent variables. For example, the effects of stress on cognitive  
82 performance are often only observed during synergistic cortisol and sympathetic activation  
83 (Elzinga and Roelofs, 2005; Kuhlmann and Wolf, 2006); a relationship that would be difficult  
84 to examine using the SECPT. This paper reports the neuroendocrine, cardiovascular and  
85 subjective responses following repeated exposure to a combined physical and social  
86 evaluative laboratory stressor. The combination of a social-evaluative speech task and the  
87 SECPT was expected to elicit robust and enduring cortisol stress response over repeated  
88 exposures.

89

90

## 2. METHODS

### 2.1 Sample

92 Twenty-five medication-free, non-smoking males aged 19 – 32 years ( $\bar{x}$  = 21.83, SD = 3.55)

93 with a normal body mass index ( $\bar{x}$  = 22.36, SD = 1.79 kg/m<sup>2</sup>) were recruited via email and

94 poster advertisements around the University campus and local community. Exclusion criteria

95 included endocrine, cardiovascular, or other chronic diseases (ascertained using a health

96 screening questionnaire), smokers, BMI > 30 kg/m<sup>2</sup>, current psychological affective/mood

97 disorders (assessed by the Hospital Anxiety and Depression Scale [HADS]; Zigmond and

98 Snaith, (1983); score on either scale > 8 excluded as potential ‘caseness’; Bjelland, Dahl,

99 Haug, and Neckelmann, 2002), and night shift work. Previous experience of a stress

100 induction protocol was also an exclusion criterion.

### 2.2. Design

102 The study conformed to a repeated measures, crossover design comprising an initial

103 counterbalanced control and stress visit in week one (separated by no more than three

104 days), and a repeat stress visit after a six weeks delay. Stress visit 1 and the non-stress

105 control day were counterbalanced to account for potential practise and order effects

106 influencing performance on cognitive tasks. Participants completed three short, low demand

107 cognitive tasks (2 back, Ospan, and an attention-switching task) post-stress in between

108 measurement collection time points + 20 and + 40 (not reported here). Stress visit 2 was

109 completed six weeks ( $\pm$  2 days) after completion of stress visit 1. The study was approved by

110 the University of Leeds’ School of Psychology Research Ethics Committee and undertaken

111 in accordance with the principles expressed in the Declaration of Helsinki (World Medical,

112 2013). An honorarium of £40 was paid upon completion of the study. All participants

113 provided written informed consent prior to participation.

114

### 115 **2.3 Procedure**

116 All participants were exposed to the protocol between 1200h and 1600h to account for  
117 diurnal variation in endogenous cortisol levels. A procedural timeline is shown in Figure 1.  
118 Participants were asked to refrain from exhaustive exercise, consuming large meals or  
119 caffeinated/low pH drinks, and brushing their teeth at least 1 h prior to testing. Upon arrival a  
120 standardised meal and glass of water were consumed. Following a 1 h relaxation period,  
121 cardiovascular, endocrine and subjective measures were taken at regular intervals pre-, mid-  
122 and post-stress exposure (see Figure 1 for measurement timings). Measures collected  
123 during the control visit were time-matched to those collected during stress visits. For the  
124 control visit, participants were instructed to walk to the stress induction room and back to  
125 match the physical exertion of stress sessions and relaxed in the test cubicle for thereafter.

126 <FIGURE 1>

127 A partial debrief was given to participants following the completion of stress visit 1 explaining  
128 that none of the 'recorded' data would be analysed until completion of stress visit 2. A full  
129 debrief was provided at study completion. All visits were matched within 1 h within  
130 participants to control for time of day effects.

131

### 132 **2.4 Stress Protocol**

133 The combined physical and social evaluative threat stress induction protocol comprised the  
134 public speech task from the TSST (Kirschbaum et al., 1993) and a SECPT (Schwabe et al.,  
135 2008). Speech tasks have been previously demonstrated to elicit larger and more consistent  
136 endocrine (ACTH and cortisol) and cardiovascular responses than mental arithmetic tasks  
137 (AIAbsi et al., 1997). Hence, the TSST speech task was retained rather than the maths task.

138

139 Following a 5 min anticipation period, participants were required to give an extemporaneous  
140 5 min speech (standing) presenting themselves as a job candidate to two non-responsive,

141 evaluative female confederates. Upon completion of the speech participants completed a  
142 CPT in front of the social-evaluative panel. The SECPT required the submersion of the hand  
143 above the wrist in ice cold water (0 – 4 °C) for as long as possible (a maximum of three  
144 minutes) whilst maintaining eye contact with the panel (seated). Participants were falsely  
145 informed that performance on both tasks would be video and audio recorded for further  
146 analysis. An opposite sex (female) evaluative panel was selected to increase the level of  
147 social-evaluative threat. Opposite sex panels have been demonstrated to be more  
148 efficacious in the elicitation of cortisol stress responses compared to single sex panels  
149 (Duchesne et al., 2012). The stress protocol, including stress response measures taken mid-  
150 stress induction, lasted approximately 15 min dependent upon the time taken to complete  
151 the SECPT.

152

153 Novelty, lack of control, unpredictability, and social-evaluative threat have been identified as  
154 primary psychological determinants of cortisol responsivity to acute psychosocial stress  
155 (Dickerson and Kemeny, 2002; Mason, 1968; Rose, 1984). Repeated exposure to a  
156 homotypic stressor reduces the moderating influence of these psychological characteristics  
157 on the engendered response as the contextual and psychological elements of the stressor  
158 are perceived as more familiar, predictable and controllable (Harl et al., 2006; Schommer et  
159 al., 2003; Voigt et al., 1990). Increased familiarity, control and predictability may also reduce  
160 the impact of perceived social evaluation experienced during exposure to a social evaluative  
161 threat. Therefore, a number of contextual changes were made to the stress induction  
162 protocol across stress visits 1 and 2. The primary researcher, panel members, stress  
163 induction room and speech task were changed between visits. For stress visit 2 participants  
164 were asked to present their character and personality to the panel including at least one  
165 negative and one positive aspect about themselves. Participants were not explicitly told what  
166 stress visit 2 would entail, only that they would complete two challenging tasks

167



168 **2.5 Study Measures**

169 **2.5.1 Cortisol assessment**

170 Salivary cortisol samples were collected using a Salivette® device (Sarstedt, Numbrecht,  
171 Germany). Saliva was extracted from cotton wool swabs by centrifugation (2500 rpm, 5 min)  
172 and frozen at - 20°C until assay. Salivary-free cortisol concentrations were determined using  
173 a Salivary cortisol enzyme immunoassay kit (EIA; Sarstedt; Nümbrecht, Germany). Intra-  
174 and inter-assay variability were below 4.5 and 10.4% respectively.

175

176 **2.5.2 Cardiovascular data**

177 Systolic (SBP), diastolic BP (DBP), and HR were measured using a Spacelab ambulatory  
178 BP monitor (ABP, model 90207, Spacelabs Burdick, USA). This monitor has been widely  
179 validated for ambulatory cardiovascular measurement (Amoore and Geake, 1997; Marquez  
180 Contreras et al., 1998; O'Brien et al., 1991). The ABP was fitted on the upper non-dominant  
181 arm of each participant and worn throughout the study protocol (un-inflated between  
182 measures). All measures were taken when the participant was seated. To account for  
183 potential variability in blood pressure monitor reading, two consecutive measurements were  
184 taken at each time point and the average of the readings employed in all analyses.

185

186 **2.5.3 Subjective stress measures**

187 The Stress and Arousal Checklist (SACL; Mackay , Cox, Burrows, and Lazzerini, 1978) is a  
188 30-item adjective list of self-reported feelings of stress (18 items) and arousal (12 items).  
189 Respondents rate the extent to which each adjective (e.g., stimulated, apprehensive, up  
190 tight) describes how they are feeling at this moment in time. Responses are made with  
191 reference to a four-point Likert scale.

192

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193 The Perceived Stress Scale (PSS; Cohen, Kamarck, and Mermelstein, 1983) is a 10-item  
194 self-report global measure of perceived stress that assesses how frequently respondents  
195 have experienced an uncontrollable, unpredictable or overloading situation during the last  
196 month, and the perceived effectiveness of individual ability and confidence to cope with this  
197 stress. Responses are made in reference to a five-point Likert scale. Participants completed  
198 the PSS at screening  $\leq$  5 days prior to stress visit 1. A second PSS was completed at stress  
199 visit 2 to explore for potential differences in chronic stress levels between the repeated  
200 stress visits.

201

## 202 **2.6 Nutritional status**

203 To reduce the potential of variability of glucose load moderating cortisol response to stress  
204 exposure (Kirschbaum et al., 1997), participants were given a standardised tomato risotto  
205 meal prior to each test session to standardise nutritional status (providing 224 kcal/125g;  
206 carbohydrates: 39.1g; protein: 4.6g; fat: 5.1g). A fingerprick lancet was used to collect  
207 capillary blood samples to measure glucose response. Blood glucose levels (mmol/L) were  
208 assayed using a Glucomen LX meter (A. Menarini Diagnostics, UK). One capillary blood  
209 glucose sample was taken pre (+ 10) and post (+ 35) stress induction (time-matched on  
210 control day).

211

## 212 **2.7 Statistical Analysis**

213 Statistical analyses were performed using SAS (Statistical Analysis System, Version 9.2;  
214 SAS Institute, Inc., Cary, NC). The data from twenty four participants were analysed as one  
215 participant was removed from the study entirely after failing to attend the second study visit.  
216 All data were screened and residual outlying variables were removed ( $\pm$  2.58 SD) and  
217 residual plots inspected for deviations from normality. Cortisol data were positively skewed  
218 and normalised using a logarithmic transformation. Paired t-tests were employed to compare

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219 participant characteristics (reported chronic stress [PSS] and SECPT hand submersion time)  
220 across stress visits. The SAS-mixed models procedure (PROC MIXED) was employed to  
221 examine the within-subjects change in stress response outcome variables and capillary  
222 glucose within and between control and stress visits. Participant ID was entered as a  
223 random factor; visit (control, stress visit 1, and stress visit 2) and measurement time points  
224 were entered as fixed factors. The order in which participants were exposed to the initial  
225 stress visit 1 and control visit was entered as a covariate in all models, but removed due to  
226 non-significance.

227

228 The delta increase in cortisol response was calculated by subtracting the baseline cortisol  
229 value from the peak post-stress induction level. Area under the curve with respect to ground  
230 (AUCg), and area under the curve with respect to increase (AUCi) were calculated using the  
231 trapezoid method (Pruessner et al., 2003): For all analyses, the significance level was set at  
232  $\alpha = 5\%$ . The nominal  $\alpha$  level was adjusted for multiple post-hoc least squares mean  
233 comparisons using the Tukey-Kramer correction (Tukey, 1951). All results (including figures  
234 and tables) are presented as mean and standard error of the mean (*SEM*).

235

236

### 3. RESULTS

#### 237 3.1 Sample characteristics

238 Paired t-tests revealed a significantly higher mean chronic stress (PSS) score the month  
239 prior to stress visit 2 ( $\bar{x} = 12.13 \pm 1.07$ ) compared to stress visit 1 ( $\bar{x} = 8.63 \pm 0.81$ ),  $t(22) = -$   
240 4.58,  $p < .001$ . Perceived stress scores at both time points were included as covariates in all  
241 analyses of stress response parameters but did not significantly account for any variance in  
242 outcome measures and were subsequently removed from all models. Hand submersion time  
243 (SECPT) did not differ significantly between stress visit 1 ( $\bar{x} = 146.08 \pm 11.68$  secs) and 2

244 ( $\bar{x} = 145.74 \pm 11.77$  secs),  $t(22) = 0.10$ ,  $p = .92$ . Participants reported no use of any  
245 medication known to affect HPA axis function between stress visits.

246

### 247 **3.2 Salivary Cortisol Response**

248 A significant time $\times$ visit interaction,  $F(10,215) = 11.93$ ,  $p < .001$ , and main effects of time,  
249  $F(5,115) = 8.65$ ,  $p < .001$ , and visit,  $F(2,43) = 94.89$ ,  $p < .001$ , were revealed for salivary  
250 cortisol response. Significant salivary cortisol responses were demonstrated across both  
251 stress visits (Figure 2). This response elevation was significantly higher than corresponding  
252 control measures from mid-stress induction (+10 min) onwards for both stress visits (all  
253 significant at  $p < .001$ ). Cortisol responses to stress exposure were also significantly  
254 elevated from baseline levels within the respective stress visit response profiles. This was  
255 more consistent during stress visit 1 with cortisol levels from + 10 min onwards significantly  
256 higher than pre-stress baseline measures (- 20 and - 10 min; all significant at  $p < .05$ ).  
257 Cortisol levels were not significantly in excess of baseline levels (- 20 and - 10 min) until +  
258 20 and + 30 min ( $p < .03$ ) during stress visit 2.

259 <FIGURE 2 >

260 Baseline salivary cortisol levels in anticipation of repeated stress exposure at stress visit 2  
261 were sufficiently elevated such that levels at - 20 and - 10 min were significantly higher than  
262 corresponding stress visit 1 ( $p < .01$ ) and control ( $p < .03$ ) levels. However, no significant  
263 differences between cortisol levels were revealed between stress visits 1 and 2 from mid-  
264 stress induction (+ 10 min) onwards suggesting a comparable post-stress induction  
265 response to the stress protocol.

266 <FIGURE 3>

267 Aggregated measures of cortisol response are shown in Figure 3. A significant main effect of  
268 visit was revealed for AUCi,  $F(2,40) = 11.50$ ,  $p < .001$ , AUCg,  $F(2,40) = 14.01$ ,  $p < .001$  (left  
269 axis), and delta increase,  $F(2,40) = 12.06$ ,  $p < .001$  (right axis). Stress visits 1 and 2

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270 provoked significantly higher cortisol responses than the control visit across all aggregated  
271 measures (all significant at  $p < .003$ ). No significant differences in aggregated measures of  
272 cortisol between stress visits 1 and 2 were revealed.

273

### 274 **3.3 Cardiovascular response**

#### 275 **3.3.1 Blood pressure**

276 A significant time $\times$ visit interaction, SBP:  $F(14,314) = 11.06$ ,  $p < .001$ ; DBP:  $F(14,315) = 6.32$ ,  
277  $p < .001$ , and main effects of time, SBP:  $F(8,161) = 58.44$ ,  $p < .001$ ; DBP:  $F(8,161) = 27.51$ ,  
278  $p < .001$ , and visit, SBP:  $F(2,45) = 97.83$ ,  $p < .001$ ; DBP:  $F(2,45) = 45.23$ ,  $p < .001$ , were  
279 revealed for BP response. Blood pressure increased significantly across both stress visits,  
280 whilst only a minor excursion from baseline level (corresponding with the time point  
281 participants were asked to walk to the stress test room [+ 5 min]) was demonstrated under  
282 control conditions (Figure 4). Stress exposure significantly elevated SBP and DBP above  
283 corresponding control measures between + 5 (speech anticipation) and + 20 min across  
284 both stress visits (all significant at  $p < .001$ ). Blood pressure responses to stress exposure  
285 were also significantly elevated from baseline levels within the respective stress visit  
286 response profiles. Systolic BP and DBP between + 5 and + 20 min were significantly higher  
287 than baseline measures across both stress visits (- 20 and - 10 min; all significant at  $p <$   
288  $.03$ ).

289 No significant differences in SBP or DBP response between stress visits 1 and 2 were  
290 revealed.

291 <FIGURE 4>

#### 292 **3.3.2 Heart Rate**

293 A significant time $\times$ visit interaction,  $F(14,315) = 2.40$ ,  $p < .001$ , and significant main effects of  
294 time,  $F(7,161) = 3.40$ ,  $p < .001$ , and visit,  $F(2,45) = 32.19$ ,  $p < .001$ , were revealed for HR  
295 response. Comparison of HR responses between visits revealed significantly higher HR at +

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296 5 and + 10 min during stress visits compared to control ( $p < .03$ ). Within the HR response  
297 profiles, a pre-stress increase in HR was evident at stress visit 1 but no significant elevations  
298 in HR were demonstrated across the profile (Figure 4). At stress visit 2, an anticipatory pre-  
299 stress HR response peak was significantly higher at + 5 compared to post-stress levels at +  
300 15 and + 20 min ( $p < .04$ ). Significantly higher HR response at + 30 min during stress visit 2  
301 compared to the corresponding control measure was also recorded ( $p < .04$ ). No significant  
302 differences in HR between stress visits 1 and 2 were revealed.

303 <FIGURE 5>

### 304 **3.4 Subjective Response**

#### 305 **3.4.1 Stress**

306 A significant timexvisit interaction,  $F(8,177) = 3.81, p < .001$ , and significant main effects of  
307 time,  $F(4,92) = 2.41, p = .05$ , and visit,  $F(2,45) = 20.13, p < .001$ , were revealed for subjective  
308 stress ratings (Figure 5). No significant subjective stress response was reported under  
309 control conditions. During stress visit 1 subjective stress rating was significantly higher post-  
310 stress at + 20 min compared to pre-stress ratings (- 20 and - 10 min;  $p < .01$ ). An anticipatory  
311 baseline subjective stress response at stress visit 2 resulted in no significant increases in  
312 stress ratings across the profile. However, stress ratings across both stress visits were  
313 significantly higher at + 20 min compared to the control visit. An anticipatory peak subjective  
314 stress rating at - 10 min during stress visit 2 was significantly higher than both corresponding  
315 ratings at control and stress visit 1 (both significant at  $p < .001$ ).

#### 316 **3.4.2 Arousal**

317 A significant timexvisit interaction,  $F(8,177) = 3.42, p < .001$ , and a main effect of time,  
318  $F(4,92) = 2.84, p < .03$ , were revealed for subjective arousal (Figure 5). No significant  
319 differences were revealed across the stress visit 2 and control visit profile. Subjective  
320 arousal ratings during stress visit 1 peaked significantly post-stress at + 20 min ( $> - 20, -10,$   
321 + 30 and + 40 min;  $p < .01$ ) and was also significantly higher than the corresponding arousal

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322 rating during the control visit ( $p < .001$ ). No differences between arousal rating at stress visit  
323 2 and control reached significance.

324

### 325 **3.5 Nutritional Status**

326 No significant differences were revealed between pre- and post-stress capillary blood  
327 glucose levels across study visits suggesting the standardised meals ensured a stable  
328 nutritional state (Table 1). Pearson's product moment (two-tailed) correlations revealed  
329 glucose levels were not significantly related to salivary cortisol response.

330

331 Table 1 Mean capillary blood glucose (mmol/L) response (mean  $\pm$  SEM) pre (- 10) and post  
332 (+ 20) standardized meal intake on control and repeated stress protocol exposure visits

Study Visit	Capillary blood glucose (mmol/L)	
	- 10 min (pre)	+ 20 min (post)
Control	6.54 $\pm$ 0.21	6.40 $\pm$ 0.24
Stress visit 1	6.13 $\pm$ 0.81	6.75 $\pm$ 0.24
Stress visit 2	6.16 $\pm$ 0.89	6.49 $\pm$ 0.19

333

334

## 4. Discussion

335 An overall pattern of distinct habituation of the cortisol response is reported following  
336 repeated exposure to laboratory psychosocial stress protocols (e.g., Epel et al., 2000; Engert  
337 et al., 2010; Gerra et al., 2001; Jonsson et al., 2010; Kirschbaum et al., 1995; von Kanel et  
338 al., 2006; Schommer et al., 2003; Wust et al., 2005). Here, the cortisol response to a  
339 combined physical and social evaluative stressor was heightened in anticipation of, and  
340 demonstrated a comparable response dynamic during and after, stress induction. This  
341 modified TSST/SECPT protocol therefore appears sufficient to attenuate the commonly  
342 demonstrated habituation in HPA axis responsivity to a repeated homotypic laboratory  
343 stressor in males. A stress induction technique characterised by comparable, robust cortisol

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344 response profiles over repeated exposures has utility for crossover study designs in which  
345 outcomes (e.g., cognitive performance) are measured following stress induction in the same  
346 individuals over repeated exposures. This reduces the difficulties comparing performance  
347 across non-response equivalent repeated stress exposures associated with the use of  
348 laboratory protocols which are prone to rapid habituation.

349

350 A higher salivary cortisol response in anticipation of stress induction at visit 2 resulted in  
351 cortisol levels not significantly exceeding baseline levels until + 20 min; compared to + 10  
352 min at stress visit 1. This may also be interpreted as evidence of response habituation;  
353 namely, a small reduction in the magnitude of response from baseline (mean delta increase  
354 difference of 1.09 nmol/L). However, inspection of aggregated measures of cortisol revealed  
355 no significant differences in absolute levels (AUC) or responsiveness (delta increase, AUCi)  
356 suggesting the overall response, and response reactivity, were comparable. Moreover, Wust  
357 et al. (2005) have previously demonstrated that habituating salivary cortisol responses to  
358 repeated stress exposure are characterised by significantly reduced levels across the entire  
359 response profile (both in anticipation of, and reactive responses to, stress induction).  
360 Therefore, the pattern of sensitised anticipatory and comparable reactive responses  
361 demonstrated here is different to the habituation pattern previously reported in the literature  
362 for this hormone. Indeed, the response pattern reported is analogous to that of persistent  
363 non-habituating high responders reported by Kirschbaum et al. (1995).

364

365 The lack of significant habituation in cortisol response is comparable to the findings reported  
366 by Minkley et al. (2014) following repeated exposure to the SECPT. Whilst Minkley et al.  
367 reported no habituation to SECPT exposure, cortisol levels were only measured twice, at – 6  
368 and + 18 min relative to stress onset. Measurement of the salivary cortisol response profile  
369 over a longer period, as reported here, would be required to fully disconfirm response  
370 habituation. Further, cardiovascular and subjective stress responses to the SECPT had



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371 significantly reduced by + 3 min post-stress onset in the Minkley et al. study. This supports  
372 the findings of Giles et al. (2014) that cardiovascular and subjective responses to the SECPT  
373 are limited to the duration of the stressor, reducing the utility of the SECPT in studies of  
374 sustained stress responses.

375

376 Here, the addition of the speech task resulted in sustained BP and subjective stress  
377 responses in excess of + 20 min post-stress onset across repeated exposures. This  
378 represents a consistent and sustained response of at least 5 min post-stress cessation; a  
379 more prolonged response than to the SECPT alone and a longer response window in which  
380 to administer interventions or tasks (e.g., cognitive tests). Moreover, cardiovascular and  
381 subjective responses to the SECPT in isolation are extinguished by + 18 min post-stress  
382 onset (Minkley et al., 2014), a time when cortisol levels approach peak amplitude. The short,  
383 variable duration of the SECPT, when administered alone, exposes participants to the  
384 stressor for minimally seconds to maximally three minutes. The inclusion of the 5 minute  
385 speech task (and 5 minute anticipation period) ensures a level of standardisation in terms of  
386 initial stress exposure and activation of a sustained stress response cascade. This resulted  
387 in cardiovascular and subjective responses being sufficiently sustained to concur with the  
388 peak cortisol response (+ 20 min relative to stress onset). Therefore, this protocol has  
389 increased utility for experimental designs which examine the effect of stress on outcome  
390 variables during the post-stress period due to responses being sustained both beyond  
391 cessation of the stressor, and to coincide with peak cortisol activation. Accordingly, the  
392 addition of the speech task to the SECPT may have specific utility that outweighs the added  
393 resource load.

394

395 A shift toward anticipatory responses was demonstrated in HR and subjective stress.  
396 Conversely, BP responses remained stable across the stress visits. Stability in BP response  
397 to laboratory stress has been previously demonstrated (Sherwood et al., 1997). Subjective

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398 arousal ratings were indicative of response habituation by stress visit 2. This appears  
399 contradictory to evidence of significant and consistent sympathetic arousal demonstrated  
400 across both stress visits. However, correspondence between subjective appraisal and  
401 physiological stress response parameters is often weak (Campbell and Ehlert, 2012). It is  
402 likely that the significant increase in subjective arousal at stress visit 1 was associated with  
403 the initial novelty of the stress protocol which had diminished at stress visit 2.

404

405 The addition of the physical stressor to the social evaluative component of the speech task  
406 may underpin the lack of significant cortisol habituation observed, as was proposed to  
407 explain the lack of habituation reported by Minkley et al. (2014). Animal models demonstrate  
408 divergent neural processing of physical and psychological stressors, and less pronounced  
409 HPA axis habituation to physical stressors comprising a proximate physical threat (Grissom  
410 and Bhatnagar, 2009). However, proximate physical threat in this context refers to serious  
411 threats to the organism's homeostasis (e.g., hypothermia and hypoglycaemia) rather than  
412 the mild physical pain elicited by the CPT (Emmert and Herman, 1999; Herman and  
413 Cullinan, 1997; Lovallo, 1975). Therefore, it is unlikely that the level of physical threat  
414 associated with the SECPT alone would be sufficient to represent a significant proximate  
415 stressor akin to this level of physical threat.

416

417 The psychological component of performing a physical task in a social-evaluative setting  
418 may amplify the stress-provoking nature of the stressor. The combination of a physical  
419 stressor (ice water CPT) and social-evaluation, rather than social-evaluation alone (warm  
420 water CPT), is necessary for the SECPT to elicit a cortisol response (Schwabe et al., 2008).  
421 This suggests increased HPA axis activation under conditions in which individuals are  
422 particularly concerned about self-presentation (i.e., demonstration of capacity to endure  
423 physical pain). The social self-preservation theory states that threats to the social self may,  
424 under certain circumstances, represent a fundamental drive akin to threats to the physical

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425 self (Gruenewald et al., 2004). Rohleder et al. (2007) suggest this threat to the social self  
426 may be significant enough to ensure that cortisol responses do not readily habituate. Whilst  
427 the threat to the social-self encountered in laboratory psychosocial contexts (e.g., TSST)  
428 may not be sufficient to sustain significant cortisol responses over repeated exposures in  
429 most individuals, the addition of an evaluated physical stress component may act to increase  
430 the level of threat experienced and thus maintain cortisol responsiveness.

431

432 The capacity of this combined physical and social evaluative stressor to elicit a high level of  
433 threat may be reflected in the heightened stress responses prior to the second stress  
434 induction. Cortisol, subjective stress and HR responses to repeated exposure to the stress  
435 protocol demonstrated a significant response sensitization in anticipation of stress induction.  
436 Whilst the exact nature of the stress tasks at the second stress visit was withheld,  
437 participants would have had an idea of the imminent challenge. Heightened anticipatory  
438 responses are likely initiated by individual appraisal of the demands of a forthcoming  
439 challenge and perceived coping potential (Lazarus and Folkman, 1984). The heightened  
440 subjective stress rating prior to repeat stress induction suggests the protocol constituted a  
441 significant psychological threat even after previous exposure. Moreover, rather than  
442 habituating, elevated subjective pre-stress ratings were maintained post-stress at a  
443 comparable level to that demonstrated after the first stressor exposure. However, it is  
444 possible that a mid-stress peak was missed so significant habituation in peak response  
445 cannot be fully ruled out (Hellhammer and Schubert, 2012). Furthermore, a heightened  
446 cortisol response solely in anticipation of (Kirschbaum et al., 1992), and after repeated  
447 exposure to (Kirschbaum et al., 1995a), the TSST has been reported. Physically challenging  
448 stressors in particular are associated with increased anticipatory cortisol response (Mason et  
449 al., 1973; Salvador et al., 2003; Sutton and Casey, 1975). Increased HR response in  
450 anticipation of psychosocial stress has also been previously reported (Preston et al., 2007).

451 Therefore, such heightened stress responses in anticipation of induction are not specific to  
452 this stress protocol.

453

454 The effect of manipulating contextual variables of a stressor (e.g., changing the  
455 experimenter, stress induction location, and panel) upon response habituation may largely  
456 be determined by the extent to which habituation to a repeated stimulus can be considered  
457 an associative process. Contextual cues associated with a repeated stimulus may eventually  
458 themselves prime the retrieval of the stimulus from memory. In turn, this primed memory of  
459 the stimulus may act to inhibit the normal prepotent responses to that stimulus, eventually  
460 leading to response habituation (Wagner, 1979, 1981). Whilst manipulation of contextual  
461 variables may have contributed to the capacity of this stress protocol to elicit significant  
462 cortisol responses across repeated exposures, contextual manipulations have been  
463 employed for repeated administration of the TSST without significant attenuation of  
464 habituated response (e.g., Hellhammer et al., 2012; Schommer et al., 2003; von Kanel et al.,  
465 2006).

466

467 Frequency of stressor exposure is a relevant factor for HPA axis habituation. A number of  
468 studies have exposed participants to repeated TSSTs separated by intervals of 24 h (Epel et  
469 al., 2000; Jonsson et al., 2010; Kirschbaum et al., 1995a), seven days (Engert et al., 2010;  
470 Gerra et al., 2001; von Kanel et al., 2006; Wust et al., 2005), and four weeks (Schommer et  
471 al., 2003). Despite evidence of individual response variability (e.g., Gerra et al., 2001;  
472 Kirschbaum, et al., 1995), overall, habituation in cortisol response was demonstrated over  
473 repeated exposures. Here, no significant habituation after an inter-stressor delay of six  
474 weeks was shown. Conversely, Minkley et al (2014) report a lack of significant cortisol  
475 habituation after a delay of 24 h using a combined physical and social evaluative stressor.  
476 Thus, the type of stressor employed, rather than the temporal delay between exposures,  
477 contributes more to the observed lack of habituation. The nature of the debrief given

478 between repeated stress exposures may also be of relevance. Here, participants were told  
479 only that they would complete two challenging tasks at both visits. Minkley et al. (2014) only  
480 informed participants of the presence of the camera over repeated exposures. The majority  
481 of studies examining cortisol habituation do not state what, if any, debrief was given between  
482 visits (e.g., Gerra et al., 2001; Kirschbaum et al., 1995; Pruessner et al., 1997; Wust et al.,  
483 2015). Therefore, the effect of stress task expectancy over repeated exposures on  
484 responses is not yet known but should be considered in future studies.

485

486 Increasing evidence suggests that opposite sex effects are important in elicitation of  
487 endocrine, sympathetic, and subjective stress responses to psychosocial stress (Duchesne  
488 et al., 2012; Larkin et al., 1998; Martinso and Zerface, 1970; Roney et al., 2007; Roney and  
489 Simmons, 2008). Significant cortisol stress responses have been demonstrated when  
490 evaluated by the opposite, but not same, sex (Duchesne et al., 2012). Therefore, the  
491 presence of female social-evaluative panel members here, may have heightened perceived  
492 social evaluation and threat in this male sample. However, the specific contribution of panel  
493 sex on cortisol responsivity over repeated exposures has yet to be systematically examined  
494 and would require counterbalancing of male vs. female only social-evaluative panels over  
495 repeated exposures.

496

497 The strengths of this study lie in the robust methodology adopted. For example,  
498 standardisation of nutritional status prior to stress induction is lacking in the stress literature,  
499 despite evidence of nutritional state moderating cortisol response (Kirschbaum et al., 1997).  
500 Further, the cortisol response to repeated stress exposure was repeatedly measured over a  
501 period of 1 h capturing a more enduring temporal response dynamic than that previously  
502 published (e.g., two time points (pre and post); Gerra et al., 2001; Minkley et al., 2014).  
503 However, a number of limitations of the present study are acknowledged. A male only

504 sample was recruited owing to sexual dimorphism in acute cortisol response to stress  
505 (Kirschbaum et al., 1999). Evidence of the modulatory impact of the menstrual cycle and oral  
506 contraceptive (OC) use suggests the gold standard study design for examining HPA axis-  
507 mediated responses in mixed samples would be to test women in the luteal phase (Kudielka  
508 et al., 2009). However, the prevalence of OC use in young women creates difficulties in  
509 recruiting such a sample; especially if age-matching is required. The inter-stressor delay in  
510 excess of four weeks also creates difficulties matching female participants for menstrual  
511 cycle phase pre- and post-intervention, compounded by variability in cycle length and  
512 regularity between and within female participants (Chiazze et al., 1968). It is acknowledged  
513 that further examination of response habituation to repeated stressor exposure in women is  
514 essential.

515

516 Whilst a stress protocol characterised by comparatively stable stress responses over  
517 repeated exposures has utility in many contexts, it is acknowledged that a combined  
518 physical and mental stressor is not suitable for all study designs. In some contexts, it may be  
519 advantageous to be able to distinguish between the effects of an intervention on responses  
520 to mental and physical stress independently. For example, myocardial ischemic responses  
521 to mental stress induction offer superior prognostic capacity to predict future cardiac events  
522 compared to responses to physical stress (such as exercise; Jiang et al., 1996).

523 Interventions have also been demonstrated to differentially attenuate myocardial ischemia to  
524 mental but not exercise induced stress (Jiang et al., 2013). Finally, without demonstration of  
525 response to the SECPT over a longer time period, it is not yet fully clear whether the SECPT  
526 alone is sufficient to reduce habituation or if the additional speech task is required. Further  
527 examination of the effects of combined physical and social evaluative stress protocols is  
528 needed to tease apart the relative effects of each component.

#### 529 **4.1 Conclusions**

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530 A combined physical and social evaluative stressor elicited significant elevations in  
531 neuroendocrine, cardiovascular and subjective stress responses over repeated exposures in  
532 young healthy males. The stressor was sufficient to attenuate the commonly reported rapid  
533 post-stress exposure habituation in HPA axis-mediated stress responses to laboratory  
534 protocols. The findings suggest a combined physical and social evaluative stressor is a  
535 potentially useful tool for study designs that require repeated presentation of a homotypic  
536 stressor.

537

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695

## 696 **Figure Captions:**

697 Figure 1 Procedural timeline

698 Figure 2 Mean salivary cortisol response (nmol/L) to control and repeated stress exposures.

699 ● Stress visit 2 > stress visit 1 and control ( $p < .03$ ). ■ Stress visit 1 and 2 > control ( $p <$   
700  $.001$ ). ◆ Stress visit 1 > pre-stress levels ( $p < .05$ ). ★ Stress visit 2 > pre-stress levels ( $p <$   
701  $.03$ ).

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702 Figure 3 Mean aggregated salivary cortisol responses (nmol/L) to the control and repeated  
703 stress protocol exposures. AUC<sub>g</sub> and AUC<sub>i</sub> are plotted on the left axis, delta increase is  
704 plotted on the right axis. \*Significantly different from control

705 Figure 4 Mean systolic (a) and diastolic (b) blood pressure (mm/Hg) and heart rate (c; bpm)  
706 response to control and repeated stress exposures. ■ Stress visits 1 and 2 > control ( $p <$   
707 .03). ◆ Stress visits 1 and 2 > pre-stress levels ( $p <$  .03). ⊕ Stress visit 2 + 5 > + 15 and +  
708 20 min ( $p <$  .04). ● Stress visit 2 > control ( $p <$  .04)

709 Figure 5 Mean subjective stress and arousal response (SACL) to control and repeated  
710 stress exposures. ■ Stress visits 1 and 2 > control ( $p <$  .03). ◆ Stress visit 1+ 20 > pre-  
711 stress levels ( $p <$  .01). ● Stress visit 2 > stress visit 1 and control ( $p <$  .001). ⊗ Stress visit 1  
712 > control ( $p <$  .001)

713