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

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Abstract

Repeated exposure to homotypic laboratory psychosocial stressors typically instigates rapid habituation in hypothalamic - pituitary - adrenal (HPA) axis-mediated stress responses in humans. However, emerging evidence suggests the combination of physical stress and social evaluative threat may be sufficient to attenuate this response habituation. Neuroendocrine, cardiovascular and subjective stress responses following repeated exposure to a combined physical and social evaluative stress protocol were assessed to examine the habituation response dynamic in this context.

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

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

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

1. Introduction

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

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

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

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

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

2. METHODS

2.1 Sample

Twenty-five medication-free, non-smoking males aged 19 – 32 years ($\bar{x}$ = 21.83, SD = 3.55) with a normal body mass index ($\bar{x}$ = 22.36, SD = 1.79 kg/m²) were recruited via email and poster advertisements around the University campus and local community. Exclusion criteria included endocrine, cardiovascular, or other chronic diseases (ascertained using a health screening questionnaire), smokers, BMI > 30 kg/m², current psychological affective/mood disorders (assessed by the Hospital Anxiety and Depression Scale [HADS]; Zigmond and Snaith, (1983); score on either scale > 8 excluded as potential 'caseness'; Bjelland, Dahl, Haug, and Neckelmann, 2002), and night shift work. Previous experience of a stress induction protocol was also an exclusion criterion.

2.2. Design

The study conformed to a repeated measures, crossover design comprising an initial counterbalanced control and stress visit in week one (separated by no more than three days), and a repeat stress visit after a six weeks delay. Stress visit 1 and the non-stress control day were counterbalanced to account for potential practise and order effects influencing performance on cognitive tasks. Participants completed three short, low demand cognitive tasks (2 back, Ospan, and an attention-switching task) post-stress in between measurement collection time points + 20 and + 40 (not reported here). Stress visit 2 was completed six weeks (± 2 days) after completion of stress visit 1. The study was approved by the University of Leeds' School of Psychology Research Ethics Committee and undertaken in accordance with the principles expressed in the Declaration of Helsinki (World Medical, 2013). An honorarium of £40 was paid upon completion of the study. All participants provided written informed consent prior to participation.

2.3 Procedure

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

<FIGURE 1>

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

2.4 Stress Protocol

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

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

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

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

2.5 Study Measures

2.5.1 Cortisol assessment

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

2.5.2 Cardiovascular data

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

2.5.3 Subjective stress measures

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

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

2.6 Nutritional status

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

2.7 Statistical Analysis

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

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

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

3. RESULTS

3.1 Sample characteristics

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

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

3.2 Salivary Cortisol Response

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

<FIGURE 2 >

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

<FIGURE 3>

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

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

3.3 Cardiovascular response

3.3.1 Blood pressure

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

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

<FIGURE 4>

3.3.2 Heart Rate

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

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

<FIGURE 5>

3.4 Subjective Response

3.4.1 Stress

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

3.4.2 Arousal

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

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 ± SEM) pre (- 10) and post
332 (+ 20) standardized meal intake on control and repeated stress protocol exposure visits

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Whilst a stress protocol characterised by comparatively stable stress responses over repeated exposures has utility in many contexts, it is acknowledged that a combined physical and mental stressor is not suitable for all study designs. In some contexts, it may be advantageous to be able to distinguish between the effects of an intervention on responses to mental and physical stress independently. For example, myocardial ischemic responses to mental stress induction offer superior prognostic capacity to predict future cardiac events compared to responses to physical stress (such as exercise; Jiang et al., 1996). Interventions have also been demonstrated to differentially attenuate myocardial ischemia to mental but not exercise induced stress (Jiang et al., 2013). Finally, without demonstration of response to the SECPT over a longer time period, it is not yet fully clear whether the SECPT alone is sufficient to reduce habituation or if the additional speech task is required. Further examination of the effects of combined physical and social evaluative stress protocols is needed to tease apart the relative effects of each component.

4.1 Conclusions

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

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Figure Captions:

Figure 1 Procedural timeline

Figure 2 Mean salivary cortisol response (nmol/L) to control and repeated stress exposures. ● Stress visit 2 > stress visit 1 and control ($p < .03$).■ Stress visit 1 and 2 > control ($p < .001$). ◆ Stress visit 1 > pre-stress levels ($p < .05$). ★Stress visit 2 > pre-stress levels ($p < .03$).

Figure 3 Mean aggregated salivary cortisol responses (nmol/L) to the control and repeated stress protocol exposures. AUCg and AUCi are plotted on the left axis, delta increase is plotted on the right axis. *****Significantly different from control

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

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