

CO₂ challenge-evoked hormonal changes predicting TSST changes in cortisol and subjective distress

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ABSTRACT

The importance of stressor response in relation to the development of psychopathology has been recognized for decades, yet the relationship is not fully understood. The Trier Social Stress Test (TSST) is an established conditioned stressor and frequently used to assess cortisol response to acute stress in different psychopathologies. The 35 % CO₂ Challenge is a biological stressor and has mostly been utilized to assess subjective responses in anxiety related disorders. In the current study (N=189), we assessed the hormonal effects (cortisol, testosterone) and subjective distress (stress, anxiety, and fear) of the 35 % CO₂ Challenge, and several days later, assessed the hormonal and subjective distress effects of the TSST in a mixed-sex, college-aged sample, to test for predictive effects of the 35 % CO₂ Challenge on TSST-evoked outcomes. No testosterone by cortisol interaction effects were found in females. In males, the 35 % CO₂ Challenge-evoked interaction of testosterone and cortisol predicted TSST-evoked subjective stress, anxiety, and fear, with higher concentrations of testosterone predicting subjective distress, but only at (relatively) low concentrations of cortisol (one standard deviation below mean concentrations). This result – in line with the dual-hormone hypothesis – suggests the 35 % CO₂ Challenge could be utilized in a wider array of laboratory stress response research.

1. Introduction

It is well established that how an individual responds to a stressor is linked to the development of psychopathology. Thus, laboratory stressors have been utilized for decades to assess the interaction of hormones and behavior in stress responses. Laboratory stressors vary in the putative neural mechanisms and methods used to elicit stress (Feinstein et al., 2013; Kirschbaum et al., 1993; Powers et al., 2009; Radley et al., 2015). Two widely used stressors believed to utilize different mechanisms to initially evoke stress are the Trier Social Stress Test (TSST; Kirschbaum et al., 1993) and the 35 % CO₂ Challenge (hereafter, CO₂ Challenge; Feinstein et al., 2013).

The TSST reliably elicits acute biological and subjective distress responses. The TSST upregulates hypothalamic-pituitary-adrenal (HPA) axis activation and causes a downstream increase in cortisol release (Taylor et al., 2008; Veer et al., 2011) by leveraging an individual's fear of social evaluation while giving a speech and performing arithmetic in a

public setting. Substantial evidence implicates HPA axis dysfunction in the etiology and maintenance of stress-linked mood and anxiety disorders (Burke et al., 2005; Zorn et al., 2017). Though the TSST is frequently used to assess for links between stress reactivity and psychopathologies, the resulting cortisol “risk profiles” are conflicting, even within the same mental health disorder (Bagley et al., 2011; Burke et al., 2005; Wichmann et al., 2017; Zorn et al., 2017). These contradictory results are suggestive of moderating factors.

Though cortisol is the predominant hormone in TSST literature, other hormones have been assessed, including testosterone. Testosterone research tends to focus on aggression/dominance (Mehta and Josephs, 2010), yet testosterone also has anxiolytic effects (Goldstat et al., 2003; Schmidt et al., 2004). Testosterone response to the TSST in “typical” individuals is inconsistent, suggesting the presence of moderator(s) (Bedgood et al., 2014; Lennartsson et al., 2012; Mehta and Josephs, 2010). Despite inconsistent hormone “risk profiles”, the TSST is considered a gold standard laboratory stressor.

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Another widely used laboratory stressor is the CO₂ Challenge. The CO₂ Challenge is less utilized in hormone research but is prominent in anxiety research (Feinstein et al., 2011; Schmidt and Zvolensky, 2007; Zvolensky and Eifert, 2001). The CO₂ Challenge can be thought of as a transdiagnostic laboratory stressor for anxiety propensity. The CO₂ Challenge induces stress through the inhalation of a medical grade gas mixture of 35 % CO₂ 65 % O₂. A variety of physiological sensations are reported during and directly after the CO₂ Challenge, including racing heart, lightheadedness, trembling, numbness in extremities, and breathlessness. These sensations are the result of respiratory acidosis, in which excess CO₂ causes the pH of bodily fluids to become hyperacidic (Ziemann et al., 2009). Subjective distress evoked by the CO₂ Challenge is strongly influenced by the appraisal of the sensations as threatening (i. e. their threat perception; Telch et al., 2011; Zvolensky and Eifert, 2001). Threat perception is known to play a role in reactions to stress and is a factor in the development and maintenance of mood and anxiety disorders (Beck, 1979; Zvolensky and Eifert, 2001). Though the CO₂ Challenge is a valid and powerful diagnostic tool to assess stress-evoked subjective responses, hormonal responses are less well studied. CO₂ Challenge-evoked cortisol response is inconsistent, as cortisol increase, decrease, and no change have been found (Argyropoulos, 2002; van Duinen et al., 2004, 2007; Josephs et al., 2017), suggesting the possibility of moderating factors.

The inconsistent hormonal findings associated with the two stressors are indicative of moderator(s), including possible coupling (Bedgood et al., 2014; Lennartsson et al., 2012; Mehta and Josephs, 2010; Shirtcliff et al., 2015). Coupling is a term derived by Shirtcliff et al., (2015) to describe the interplay of the HPA and HPG axes. In line with this idea, the dual-hormone hypothesis asserts increasing testosterone is “pathogenic” when cortisol is decreasing (Glenn et al., 2011; Grotzinger et al., 2018; Mehta and Josephs, 2010; Tackett et al., 2014; Zilioli et al., 2015). Additionally, one study by Josephs et al. (2017) found the coupling of testosterone by cortisol reactivity to the CO₂ Challenge was predictive of PTSD risk as a function of warzone stressors.

1.1. Underlying laboratory stressor differences

Evidence suggests the TSST and CO₂ Challenge evoke stress through different pathways. Psychosocial stressors (e.g., the TSST) are conditioned stressors, and require functioning amygdalae to assess if the situation is cause for alarm (Feinstein et al., 2011; Phillips and LeDoux, 1992). However, the CO₂ Challenge is a biological stressor, like caffeine and yohimbine. The CO₂ Challenge elicits initial responses through respiratory acidosis, an amygdala-independent physiological mechanism (Feinstein et al., 2013; Wemmie, 2011; Ziemann et al., 2009).

1.2. Importance of anxiety sensitivity & social evaluative threat

Two additional important factors to consider when assessing stress responses are anxiety sensitivity and social evaluative threat. Anxiety sensitivity refers to an individual's fear of arousal related behaviors and sensations e.g., appearing nervous, blushing, racing heart, and trembling (Taylor et al., 1992, 2007). Anxiety sensitivity is thought of as a transdiagnostic risk factor for anxiety propensity (Maller and Reiss, 1992; Taylor et al., 1992). Individuals with high anxiety sensitivity were found to report higher subjective distress during the TSST (Wearne et al., 2019) and the CO₂ Challenge (Richey et al., 2010). Social evaluative threat is a causal mechanism evoking social anxiety (Clark and Wells, 1995) and refers to how threatening or upsetting an individual views the potential negative evaluation of others. Social evaluative threat is the driving force of TSST-evoked distress. The appraisal of social concerns (ASC) is a measure that assesses an individual's level of social evaluative threat (Telch et al., 2004). We are not aware of studies that have included the ASC in the context of the TSST or that have assessed perceived social threat in the context of the CO₂ Challenge.

1.3. Overview

To our knowledge, the current exploratory study is the first to compare changes in hormonal and subjective distress in response to a CO₂ Challenge and the TSST. The primary objectives of this study are to address current gaps in research by: 1) evaluating subjective distress and hormone concentration alterations triggered by the CO₂ Challenge and the TSST, and 2) investigating whether the coupling of testosterone and cortisol in response to the CO₂ Challenge predicts subsequent subjective distress and cortisol response evoked by the TSST. We hypothesized the “pathogenic” dual-hormone hypothesis profile of increased testosterone and decreased cortisol response to the CO₂ Challenge would predict heightened subjective distress in response to the TSST (Mehta and Josephs, 2010). Based on evidence suggesting the CO₂ Challenge and the TSST initially utilize different brain pathways, we hypothesized the coupling of testosterone and cortisol in response to the CO₂ Challenge would not predict cortisol response to the TSST (Feinstein et al., 2011; Radley et al., 2015). Furthermore, recognizing the research gap and the significance of anxiety sensitivity and social evaluative threat, we controlled for the influence of anxiety sensitivity and perceived social evaluative threat.

2. Methods

2.1. Participants

Participants were recruited for participation in a two-day research study from an introductory psychology class at the University of Texas at Austin. Days 1 and 2 of the study were scheduled as close together as possible and ranged from 1 to 6 days apart (mean = 3.18, sd = 2.42). This research protocol was approved by the University of Texas at Austin Human Subjects Review Board, IRB Protocol # 2017– 11–0031.

Four hundred and eighty-seven participants completed the pre-screen measure for the study. Two hundred and six participants (63 females and 143 males) were included in the initial dataset. See [supplemental material](#) for a breakdown of excluded participants.

2.2. Inclusion & exclusion criteria

All participants who smoked cigarettes, used illegal substances, and/or took medication that influenced their hormone concentrations were excluded from the study. Female participants were required to have a regular menstrual cycle (length between 27 and 30 days) for the previous 6 months, could not have started or stopped hormonal birth control in the last 3 months, and could not be pregnant or breastfeeding.

2.3. Measures

2.3.1. Hormone sample collection

Approximately 3 ml of saliva was collected for each saliva sample in 4 ml sterile VWR vials (VWR International, United States). Experimenters wore gloves while handling cryotubes. Participants were instructed to refrain from drinking any alcohol or caffeine the evening before and/or morning of each scheduled lab session. Participant saliva samples were secured and stored in a –20 C laboratory freezer within 10-minutes of collection. All participant testing occurred between 1200 and 1800hrs to minimize the impact of diurnal variation on hormone concentrations (Dickerson and Kemeny, 2004; Hansen et al., 2008). Saliva samples were stored for 1–9 months before being shipped to Dresden LabService GmbH. Standard quality control procedures were used. The intra-assay variation coefficient was 4.3 % and the inter-assay variation coefficient was 5.1 %. Samples were analyzed for cortisol, testosterone, estradiol, progesterone, cortisone, corticosterone, and DHEAs using liquid chromatography mass spectrometry (LC-MS/MS). The present analyses focused on cortisol and testosterone.

2.3.2. Visual analogue scales (VAS)

VAS were used to measure subjective stress, anxiety, and fear, at multiple time points (Facco et al., 2011; Lesage and Berjot, 2011). Participants reported their levels of subjective distress by making a pencil mark at a point along a 100 mm line that represented their subjective experience (of stress, anxiety, and fear) at that moment. The end points of the VAS were labeled 0 for “not at all” and 100 for “extreme.” Three separate scales were administered (stress, anxiety, and fear) at each timepoint. All VAS were scored by the same person, author CAM, for consistency. Each VAS score was calculated by measuring the distance from 0 to the participant’s tic mark.

2.3.3. Anxiety sensitivity inventory (ASI-3)

The ASI-3 is a commonly used self-report measure to assess an individual’s fear of arousal-related sensations. The ASI-3 is an 18-item measure that assesses 3 dimensions of anxiety sensitivity (physical, cognitive, and social concerns; Taylor et al., 2007). Participants indicate, using a 5-point Likert scale ranging from 0 (very little) to 4 (very much), how concerned they are about experiencing the different anxiety symptoms listed on the measure. The maximum score on the ASI-3 is 72, with three subscale maximum scores of 24. Scores are calculated by summing the score of each relevant item. The ASI-3 is widely used in both clinical and non-clinical samples and has been shown to have good-to-excellent subscale reliability (Cronbach’s $\alpha = .79-.91$), internal consistency (Cronbach’s $\alpha = .88-.93$ and .91 for current study sample), and good construct validity (Taylor et al., 2007).

2.3.4. Appraisal of social concerns (ASC)

The ASC (Telch et al., 2004) is a 20-item self-report measure assessing respondents’ concern with potentially negative outcomes arising in social situations. Respondents select a number from the 0–100 scale which best describes the degree of concern for each particular social outcome when placed in a challenging social situation (e.g., “Talking to people at a party” and “Public speaking”). The ASC has shown excellent internal consistency (Cronbach $\alpha = .94$ and .96 for current study sample) and test-retest reliability with a non-clinical sample (Telch et al., 2004), good test-retest reliability ($r = .82$), factor validity, and good convergent and discriminant validity in a clinical sample (Schultz et al., 2006).

2.4. Procedure

2.4.1. Pre-lab screening & scheduling

Participants completed an initial online consent procedure, several pre-screening questionnaires (demographics and health) and several baseline measures focusing on trait and symptom psychopathology. After participants completed baseline measures, they were contacted by a project coordinator via an email script to assess their eligibility and to schedule their study timeslots (if eligible).

Male participants were scheduled based on their availability and study timeslot availability. Female participants were scheduled during the mid-luteal phase of their menstrual cycle (17–24 days after the start of their last menstruation), as female salivary cortisol stress response is most like males’ during females’ luteal phase (Wolfram et al., 2011). Eligible female participants were emailed with a one-week participation window for the study.

CO₂ Challenge procedure. For the CO₂ Challenge, a medical grade gas-mixture of 65 % O₂ and 35 % CO₂ was utilized (from Praxair). We followed standard practice for measuring hormonal reactivity to both stressors (Bagley et al., 2011; Josephs et al., 2017; Liu and Zhang, 2020; van Duinen et al., 2005, van Duinen et al., 2007). For the CO₂ Challenge, we took two saliva samples: the first sample was collected after a 30-minute rest period, and immediately before the CO₂ Challenge, and the second sample was collected 30-minutes after the completion of CO₂ Challenge. Subjective distress measures (VAS) were collected at three timepoints, during both saliva sample collections and an additional

measurement following the completion of the CO₂ Challenge (see Fig. 1 and Supplemental Materials for a detailed description of procedure).

A repeated breathing procedure for the CO₂ Challenge that streamlines the procedure and reduces avoidance was utilized. This repeated breathing procedure has been used with 5 %, 7 %, and 20 % CO₂ Challenges (Schmidt and Zvolensky, 2007), and recently the 35 % CO₂ Challenge (Zaizar et al., 2018). The repeated breathing procedure consists of the participant deeply inhaling and exhaling the medical grade gas-mixture for 10 seconds (Zaizar et al., 2018).

TSST procedure. Standard practice in the assessment of TSST-evoked reactivity involves a minimum of three saliva collections (Bagley et al., 2011; Liu et al., 2017). The first, which follows a 30-minute rest period, establishes a baseline concentration; the second measures anticipatory or peak levels, and the third measures the start of recovery levels (see Fig. 2; Bagley et al., 2011; Liu et al., 2017). In the current study, we collected three saliva samples for the TSST - the first, immediately before participants were informed of the nature of the stressor (the speech and math challenges); the second, 15-minutes after completion of the speech/math challenge; and the third, 30-minutes after completion of the speech/math challenge. Subjective distress measures (VAS) were collected at five time points, during all three saliva sample collections, and additionally during the anticipatory period and following the completion of the TSST (see Fig. 1 and Supplemental Materials for a detailed description of procedure).

2.5. Data analysis plan

Statistical analyses were performed using R Studio 1.3.1093 (R Core Team, 2020). To confirm analyzing males and females separately was appropriate, due to concerns regarding heteroscedasticity, Levene’s tests were conducted on the main outcome variables.

The analyses were broken down into 2 main parts. The first part assessed subjective distress (stress, anxiety, and fear) and hormonal (testosterone and cortisol) responses to the CO₂ Challenge and the TSST. The second part utilized intra-individual analyses and assessed the ability of an individual’s CO₂ Challenge-evoked hormonal responses to predict the individual’s TSST-evoked subjective distress and hormonal responses.

For part 1, one-factor, repeated-measures ANOVAs, with elapsed time as the repeated-measures factor were used to test the effect of each of the two stressors on changes in cortisol concentrations, testosterone concentrations, and subjective distress (stress, anxiety, and fear). For significant ANOVA analyses, regressions were used to test the difference between time points. Using a medium effect size, the number of participants required for .80 power is 28 (Faul et al., 2009). Therefore, we had sufficient power for both females and males.

For the regression analyses in part 2, hormones and subjective distress were quantified using area under the curve increase (AUC_i). AUC_i is commonly used for hormones and other measures with multiple timepoints (Pruessner et al., 2003). AUC_i refers to change across timepoints of a variable when the y intercept is equal to time 1, or baseline level. AUC_i is an index of the increase of a variable over the variable level established at baseline. For part 2, regressions were utilized to assess on an intra-individual level whether 1) CO₂ Challenge-evoked (AUC_i) cortisol predicts TSST-evoked (AUC_i) cortisol, and 2) CO₂ Challenge-evoked interaction of (AUC_i) testosterone and cortisol predicts TSST-evoked (AUC_i) cortisol and subjective distress (stress, anxiety, and fear). Regression models were analyzed with covariate measures of anxiety sensitivity (ASI-3) and social evaluative threat (ASC). Simple main effects were utilized for significant regressions to further the understanding of the interactions. In general, assumption checks proved satisfactory, although in some cases the normality of the residuals was questionable. Therefore, though we report p-values from conventional analysis, all findings were confirmed via bootstrapping 10,000 times. Using a medium effect size, the number of participants required for .80 power is 55 (Faul et al., 2009). Thus, for females, we

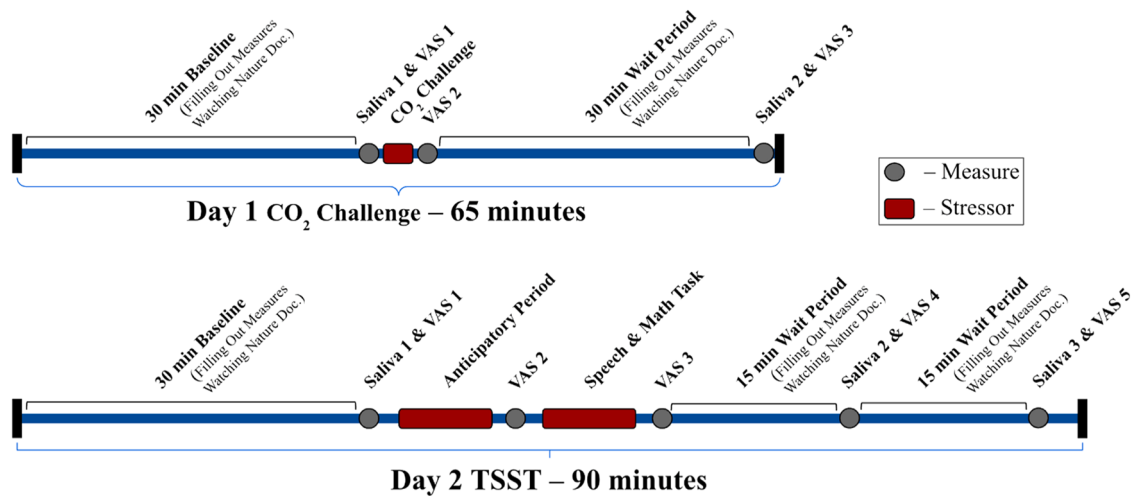


Fig. 1. CO₂ Challenge and TSST Protocol Timeline.

were slightly underpowered and for males we were sufficiently powered.

3. Results

3.1. Breakdown of missing data

Two hundred and six participants (63 females and 143 males) were included in the initial dataset. For all hormone analyses, 17 participants (4 females and 13 males) were removed due to cracked cryotubes or smeared writing on cryotubes. Five participants (1 female and 4 males) were removed for Day 1 hormone analyses and five participants (3 females and 2 males) were removed for Day 2 hormone analyses, as the samples have not been analyzed. For subjective distress analyses, 22 spring semester 2018 participants (11 females and 11 males) were removed because complete subjective distress timepoints were not implemented until the end of spring semester of 2018. The resulting datasets are: 132 for males and 54 for females for Day 1 subjective distress predicting Day 2 subjective distress analyses; 124 males and 56 females for Day 1 hormone predicting Day 2 hormone analyses; and 115 males and 49 females for mixed hormone and subjective distress analyses.

3.2. Empirical justification for separating analyses by biological sex

To assess whether the variances for the male and female samples were comparable, Levene's test was conducted for key outcome variables (AUC_i subjective stress $F(1184) = 2.42$, $p = 0.122$; AUC_i subjective anxiety $F(1184) = 3.988$, $p = 0.047$; AUC_i subjective fear $F(1184) = 5.02$, $p = 0.023$). A Fisher's combined p -value was subsequently calculated to assess whether, collectively, the variances for the male and female sample outcome variables are homogenous. The Fishers combined p value was 0.011, indicating that, collectively, the variances for the outcome variables are significantly different for males and females, i.e., the p -value indicates the presence of heteroscedasticity. Aggregating male and female samples into the same dataset would therefore violate the homogeneity of variance assumption underlying the assumptions of our regression models. Thus, males and females were analyzed separately.

3.3. Data transformation

As reported in previous studies, our hormone data had a right skew. Therefore for all regression analyses, hormone values were log transformed (Bedgood et al., 2014; Zilioli et al., 2015). Descriptive statistics

and ANOVAs were performed on untransformed hormone data.

3.4. Demographics & descriptive statistics

See Table 1 in Supplement that presents descriptive statistics for each timepoint, and AUC_i levels for CO₂ Challenge-evoked and TSST-evoked salivary cortisol and testosterone concentrations, and subjective distress (stress, anxiety, and fear).

3.5. Part 1 – Assessing stressor effects on subjective distress & hormones

3.5.1. Stressor effects on salivary cortisol & testosterone concentrations

The CO₂ Challenge did not produce a significant change in cortisol concentrations in females ($F(1, 114) = 0.33$, $p = 0.569$). However, in males, in support of a previous finding (van Duinen et al., 2004), the CO₂ Challenge produced a significant decrease in cortisol concentrations ($F(1, 250) = 9.97$, $p = 0.002$; see Fig. 2).

In line with previous TSST studies, ANOVA analysis indicated there was a significant change in at least one cortisol sample (for females $F(2, 168) = 3.78$, $p = 0.025$; for males $F(1, 254) = 13.61$, $p < 0.001$; Bedgood et al., 2014; Kirschbaum et al., 1993). Consistent with these findings, regression analyses revealed cortisol concentrations increased from time 1 (baseline) to time 2 (peak; for females $t(112) = 2.28$, $p = 0.024$; for males $t(254) = 5.03$, $p < 0.001$; see Fig. 2), followed by a decrease towards baseline concentrations from time 2 to time 3 (recovery) in both females and males (for females $t(112) = -2.05$, $p = 0.042$; for males $t(254) = -2.47$, $p < 0.014$; see Fig. 2).

Consistent with prior research, no significant (mean) testosterone level change was found for either biological sex in response to the CO₂ Challenge (for females $F(1, 114) = 1.79$, $p = 0.183$; for males $F(1, 250) = 0.89$, $p = 0.346$; Josephs et al., 2017; Table 1 in Supplement). Similarly, mean testosterone concentrations did not change in response to the TSST for either biological sex (for females $F(2, 168) = 2.32$, $p = 0.101$; for males $F(2, 381) = 0.46$, $p = 0.625$; Bedgood et al., 2014; Schoofs and Wolf, 2011; see Fig. 3).

3.5.2. Effects of CO₂ challenge & TSST on subjective distress

In line with prior research, there was a significant change in at least one of the Day 1 stress, anxiety, and fear measures for both females and males (for females stress $F(2, 159) = 24.91$, $p < 0.001$; anxiety $F(2, 159) = 27.74$, $p < 0.001$; and fear $F(2, 159) = 32.32$, $p < 0.001$; for males stress $F(2, 393) = 53.81$, $p < 0.001$; anxiety $F(2, 393) = 61.67$, $p < 0.001$; and fear $F(2, 393) = 78.18$, $p < 0.001$; see Supplement Fig. 1.). Increases in all three subjective distress measures were observed for both females and males, from time 1 (baseline) to time 2 (peak; for females stress $t(106) =$

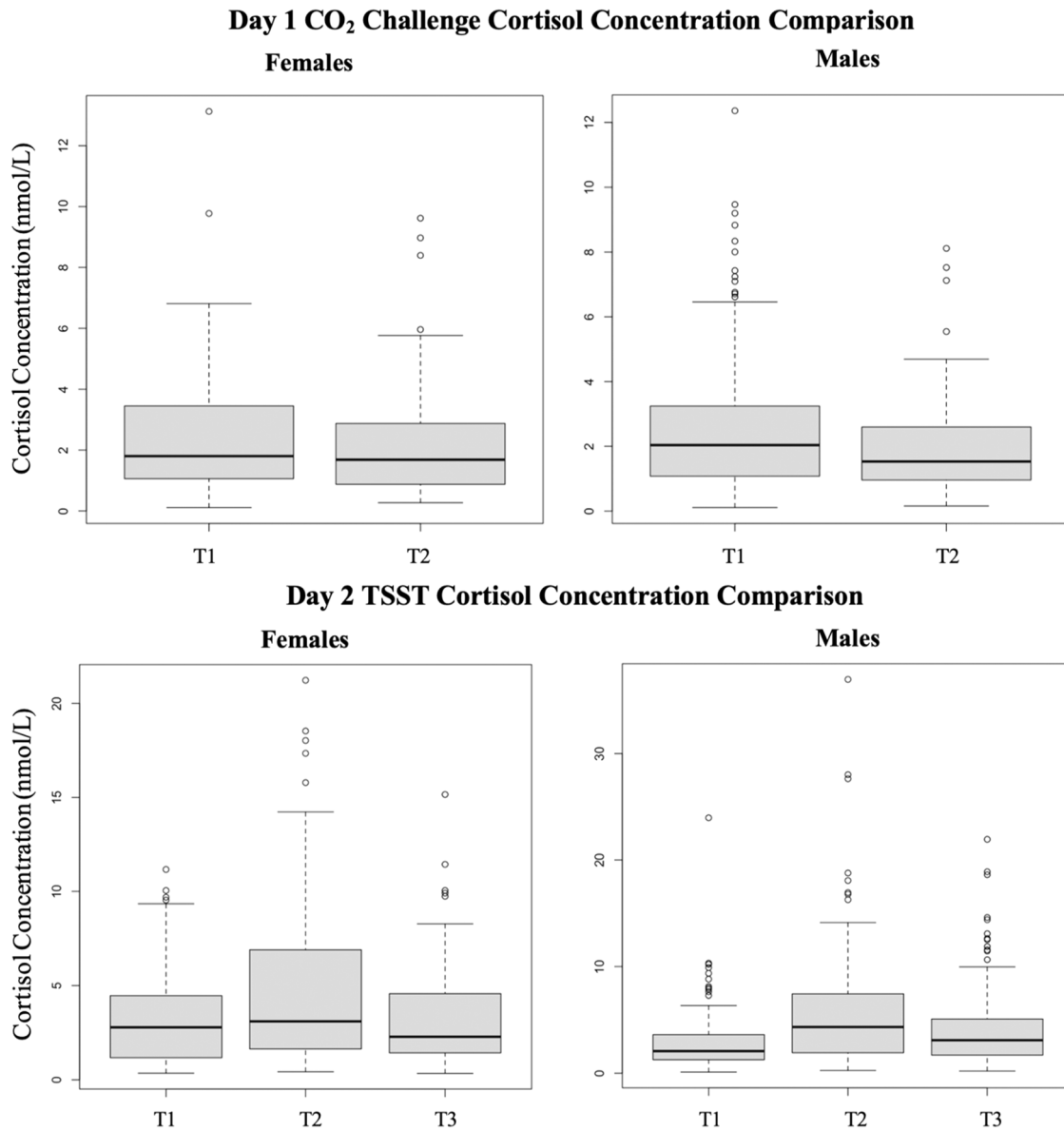


Fig. 2. The boxplots depict measures of cortisol (for each biological sex) for CO₂ Challenge timepoint 1 (baseline) and timepoint 2 (30-minutes post stressor), and TSST timepoint 1 (baseline), timepoint 2 (15-minutes post stressor), and timepoint 3 (30-minutes post stressor).

4.98, $p < 0.001$, $R^2 = 0.19$; anxiety $t(106) = 5.94$, $p < 0.001$, $R^2 = 0.25$; and fear $t(106) = 4.77$, $p < 0.001$, $R^2 = 0.24$; for males stress $t(262) = 8.08$, $p < 0.001$, $R^2 = 0.20$; anxiety $t(262) = 8.44$, $p < 0.001$, $R^2 = 0.21$; and fear $t(262) = 9.75$, $p < 0.001$, $R^2 = 0.27$), followed by a gradual decrease towards baseline concentrations from time 2 to time 3 (for females stress $t(106) = -6.59$, $p < 0.001$, $R^2 = 0.29$; anxiety $t(106) = -6.27$, $p < 0.001$, $R^2 = 0.27$; and fear $t(106) = -6.46$, $p < 0.001$, $R^2 = 0.28$; for males stress $t(262) = -8.70$, $p < 0.001$, $R^2 = 0.22$; anxiety $t(262) = -9.47$, $p < 0.001$, $R^2 = 0.26$; and fear $t(262) = -9.40$, $p < 0.001$, $R^2 = 0.25$).

Consistent with previous findings, we found a significant change in at least one of the Day 2 (TSST) stress, anxiety, and fear measures for both females and males (for females stress $F(4, 265) = 21.53$, $p < 0.001$; anxiety $F(4, 265) = 23.78$, $p < 0.001$; and fear $F(4, 265) = 21.71$, $p < 0.001$; for males stress $F(4, 655) = 41.69$, $p < 0.001$; anxiety $F(4, 655) = 47.92$, $p < 0.001$; and fear $F(4, 655) = 30.41$, $p < 0.001$; see [Supplement Fig. 2.](#); [Campbell and Ehler, 2012](#); Schlotz et al., 2008). Increases in all three subjective distress measures were observed for both females and males, from time 1 (baseline) to time 2 (peak; for females stress $t(106) =$

6.46, $p < 0.001$, $R^2 = 0.28$; anxiety $t(106) = 7.97$, $p < 0.001$, $R^2 = 0.37$; and fear $t(106) = 6.94$, $p < 0.001$, $R^2 = 0.31$; for males stress $t(262) = 9.26$, $p < 0.001$, $R^2 = 0.25$; anxiety $t(262) = 10.63$, $p < 0.001$, $R^2 = 0.30$; and fear $t(262) = 7.83$, $p < 0.001$, $R^2 = 0.20$), followed by a gradual decrease towards baseline concentrations from time 2 through time 3 (for females stress $t(106) = -7.50$, $p < 0.001$, $R^2 = 0.21$; anxiety $t(106) = -7.66$, $p < 0.001$, $R^2 = 0.22$; and fear $t(106) = -7.29$, $p < 0.001$, $R^2 = 0.19$; for males stress $t(262) = -11.26$, $p < 0.001$, $R^2 = 0.19$; anxiety $t(262) = -11.57$, $p < 0.001$, $R^2 = 0.20$; and fear $t(262) = -9.09$, $p < 0.001$, $R^2 = 0.14$).

3.5.3. Subjective distress peak comparison for TSST & CO₂ challenge

One-factor, repeated-measures ANOVAs, with “stressor type” as the repeated measures factor revealed comparable responding between the two stressors (CO₂ Challenge and TSST). Moreover, measures of subjective stress and anxiety were comparable across the two stressors for both males and females (for females: stress $F(1, 106) = 1.21$, $p = 0.275$; anxiety $F(1, 106) = 0.05$, $p = 0.818$; for males: stress $F(1, 262) = 2.42$, $p = 0.12$; anxiety $F(2, 262) = 1.78$, $p = 0.182$). In contrast, whereas peak

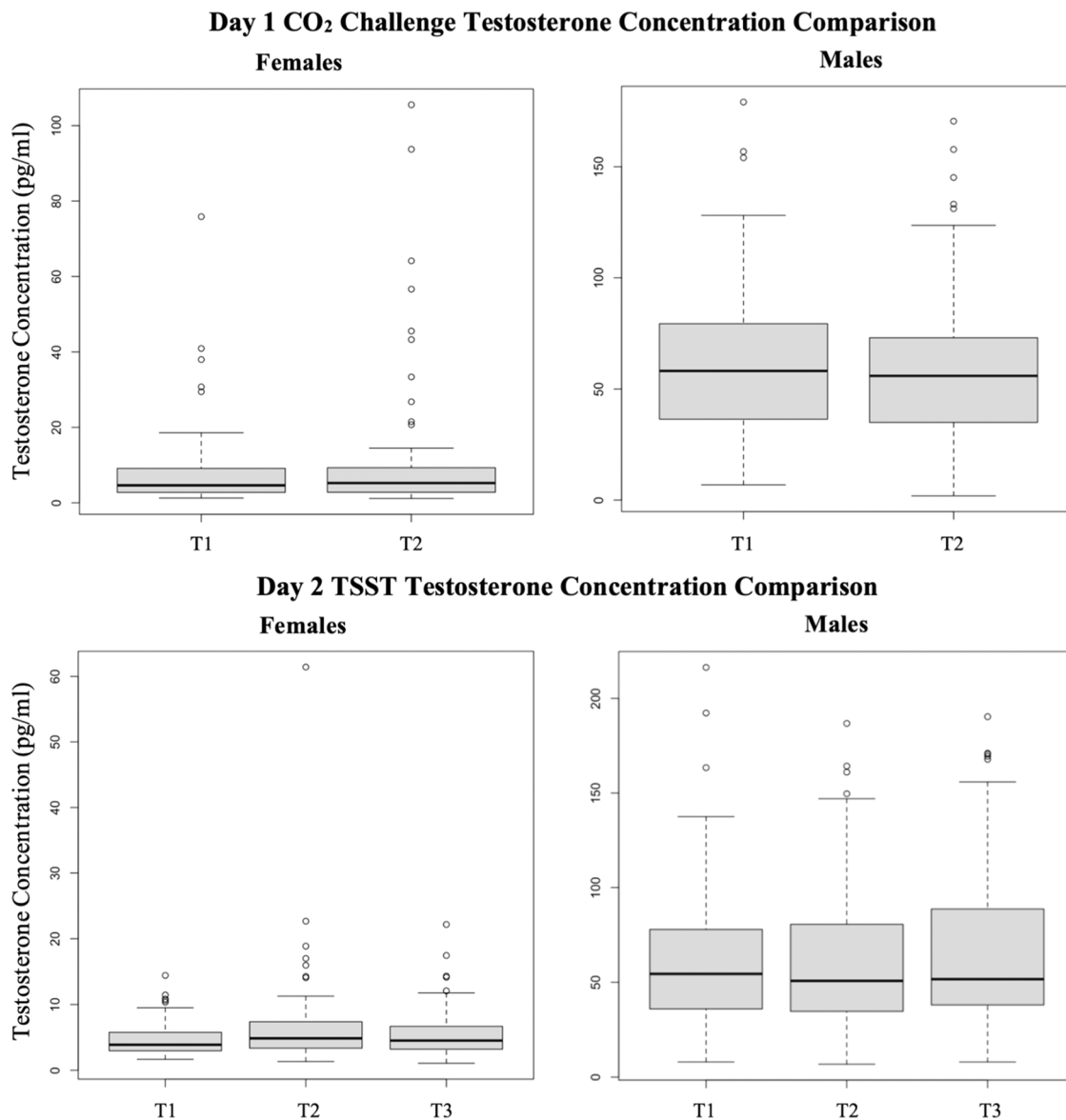


Fig. 3. The boxplots depict measures of testosterone (for each biological sex) for CO₂ Challenge timepoint 1 (baseline) and timepoint 2 (30-minutes post stressor), and TSST timepoint 1 (baseline), timepoint 2 (15-minutes post stressor), and timepoint 3 (30-minutes post stressor).

levels for subjective fear to the two stressors were similar for females ($F(1, 106) = 0.16, p = 0.686$), males reported significantly higher peak subjective fear to the CO₂ Challenge than to the TSST ($F(2, 262) = 6.01, p = 0.015$; see Fig. 4).

3.6. Part 2 – ability of CO₂ challenge-evoked responses to predict TSST-evoked responses

3.6.1. CO₂ Challenge-evoked change in cortisol does not predict TSST-evoked change in cortisol

CO₂ Challenge-evoked change in (AUC_i) cortisol did not predict TSST-evoked change in (AUC_i) cortisol in either females or males (for females: $t(49) = -0.02, p = 0.987$; for males: $t(118) = -0.69, p = 0.493$). Simple slopes analyses indicated no statistically significant slopes.

3.6.2. CO₂ challenge-evoked interaction of cortisol and testosterone changes predicting TSST-evoked cortisol & subjective distress changes

As hypothesized, CO₂ Challenge-evoked (AUC_i) testosterone by cortisol did not interact to significantly predict TSST-evoked change in

(AUC_i) cortisol for either males or females (for females: $t(49) = -0.02, p = 0.987$; for males: $t(118) = -0.69, p = 0.493$).

Contrary to our hypothesis, CO₂ Challenge-evoked testosterone by cortisol (AUC_i) did not interact to significantly predict TSST-evoked change in (AUC_i) subjective distress for females (stress $t(42) = 0.65, p = 0.521$; anxiety $t(42) = 0.60, p = 0.555$; or fear $t(42) = -0.91, p = 0.367$). However in line with our hypothesis, for males, CO₂ Challenge-evoked (AUC_i) testosterone by cortisol interacted to predict TSST-evoked change in subjective (AUC_i) distress (stress $t(109) = -2.50, p = 0.014, R^2 = 0.09$; anxiety $t(109) = -1.74, p = 0.086, R^2 = 0.09$; fear $t(109) = -1.79, p = 0.076, R^2 = 0.07$; see Fig. 5). The two models predicting TSST-evoked anxiety and fear were an exception to the confirmatory bootstrapping of analyses. In this case, the bootstrapped anxiety and fear models indicated the p-value was less than 0.05.

Simple slopes analyses indicated CO₂ Challenge-evoked (AUC_i) testosterone positively predicted TSST-evoked subjective stress when (AUC_i) cortisol concentrations were one standard deviation below the mean (stress $\beta = 15.12, p = 0.02$), a pattern consistent with the dual-hormone hypothesis (Mehta and Josephs, 2010; Zilioli et al., 2015). The same pattern was observed for models predicting subjective anxiety

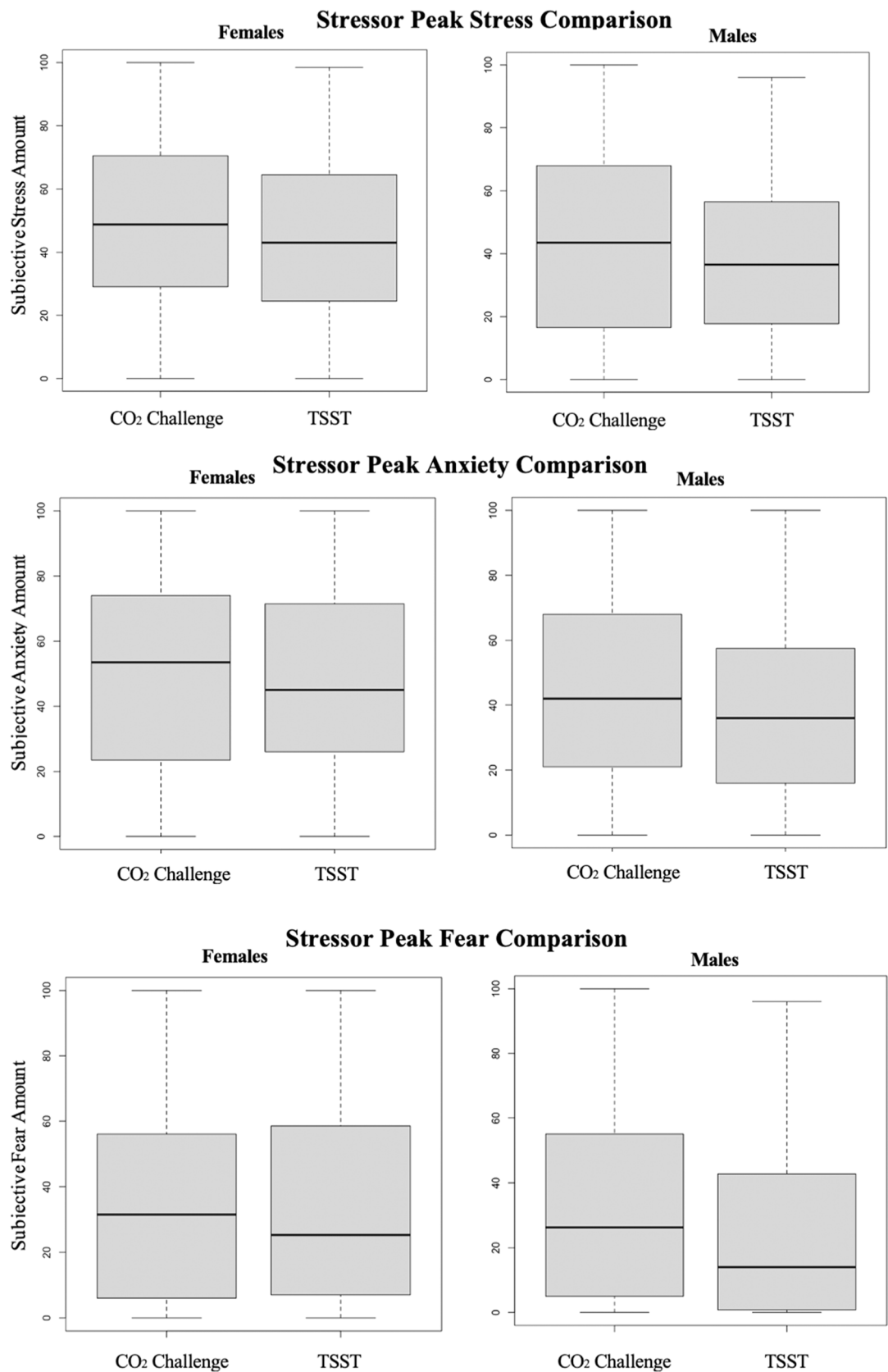


Fig. 4. The boxplots depict the visual comparison of Day 1 (CO₂ Challenge) and Day 2 (TSST) peak subjective stress, anxiety, and fear for each biological sex.

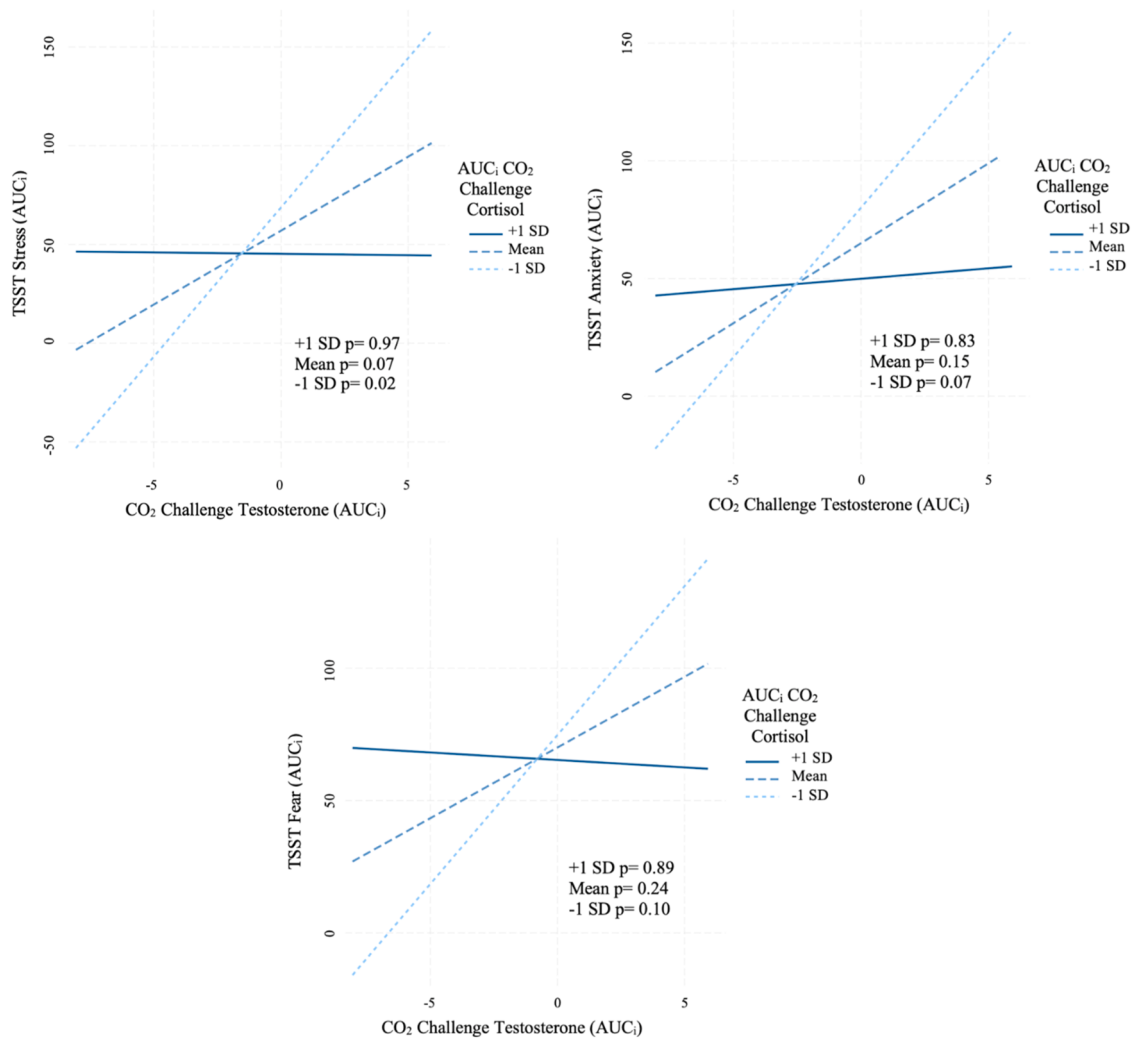


Fig. 5. CO₂ Challenge Testosterone by Cortisol predicting TSST Subjective Stress, Anxiety, & Fear. These graphs illustrate the predictive utility of the CO₂ Challenge-evoked testosterone by cortisol interaction for TSST subjective stress. Males who had increased/high testosterone and -1 SD cortisol to the CO₂ Challenge experienced increased/higher stress to the TSST.

and fear. Simple slopes analyses indicated CO₂ Challenge-evoked (AUC_i) testosterone was associated (trend) with TSST-evoked subjective anxiety/fear when cortisol concentrations were one standard deviation below the mean (anxiety $\beta = 12.71$, $p = 0.07$; fear $\beta = 11.25$, $p = 0.10$; see Fig. 5).

4. Discussion

The main aim of the present study was to assess hormonal reactivity (cortisol and testosterone) and subjective distress (stress, anxiety, and fear) evoked by the CO₂ Challenge and the TSST. The TSST is a conditioned stressor and a well-established diagnostic tool to assess hormonal and subjective stress response in mood, anxiety, and trauma-related disorders (Burke et al., 2005; Wichmann et al., 2017; Zorn et al., 2017). The CO₂ Challenge is a biological stressor and is considered a transdiagnostic measure for anxiety propensity. Due to biological sex-imposed heteroscedasticity, our analyses were segregated by biological sex. Although the analyses indicated these laboratory stressors have similarities in some areas, potential differences also emerged.

Our first aim was to evaluate the hormonal and subjective distress reactions to the two laboratory stressors. We accomplished this by assessing levels of hormonal (testosterone and cortisol) reactivity and subjective distress (stress, anxiety, and fear) evoked by the two stressors. Our findings regarding cortisol response to the TSST were in line with

previous studies and showed a characteristic increase in cortisol in anticipation of, and during the speech/arithmetic tasks, followed by a return to baseline concentrations (Bedgood et al., 2014; Kirschbaum et al., 1993). With respect to the CO₂ Challenge, our finding of a decrease in cortisol in males was consistent with a study by van Duinen et al. (2004). However, we found no significant change in cortisol in females (Josephs et al., 2017). This lack of significance is potentially due to power, as the cortisol response looks relatively equivalent in males and females (see Fig. 2). Both the CO₂ Challenge and the TSST showed the expected increase in subjective distress from baseline in response to the stressor, followed by a recovery to baseline levels (Campbell and Ehler, 2012). The CO₂ Challenge and the TSST produced similar peak levels of subjective distress, in males and females. The only exception was in males the CO₂ Challenge produced significantly *higher* subjective fear than the TSST.

Our second aim furthers prior research that focused on examining differences in cortisol and subjective distress response to stressors (Buske-Kirschbaum et al., 2003; Dickerson and Kemeny et al., 2004; Kirschbaum et al., 1999). This second aim involved assessing on an intra-individual level whether hormonal responses to the CO₂ Challenge predicted cortisol and subjective distress responses to the TSST. As hypothesized, intra-individual CO₂ Challenge-evoked testosterone by cortisol response did not predict TSST-evoked cortisol for either biological sex. This is supported by evidence suggesting the neural fear

pathways for the CO₂ Challenge and the TSST differ (Feinstein et al., 2011; LeDoux, 2000; Phillips and LeDoux, 1992; Taugher et al., 2020). For conditioned fears (e.g., the TSST, spiders, heights, and darkness) the standard neural pathway appears to be centered around the amygdala and requires the individual to have *learned* directly/indirectly that the situation should be feared (Feinstein, 2013). The amygdala receives information from sensory areas of the cortex that help increase/decrease the amygdala's response and has pathways to brainstem regions that control heart and respiratory rate etc. (LeDoux, 2000; Veer et al., 2011). However, evidence that the neural circuitry initially involved in the CO₂ Challenge (a biological stressor) response is independent of amygdala activation is supported by reports of fear evoked by the CO₂ Challenge in the absence of amygdala involvement. Feinstein et al. (2013) reported individuals with focal bilateral amygdala lesions (who otherwise reported never experiencing fear) had strong physiological and emotional reactions to each trial of the CO₂ Challenge. This differed from control participants, with intact amygdalae, who showed habituation to repeated CO₂ Challenge trials (Feinstein et al., 2013; Taugher et al., 2020). Altogether, this suggests though the CO₂ Challenge appears to initially elicit a response through a different mechanism, the amygdala-centered mechanisms are also likely at play. Additionally, in line with the idea of differing initial neural pathways, though the TSST reliably elicits a cortisol increase in the majority of participants (Taylor et al., 2008; Veer et al., 2011), the TSST-evoked cortisol response has a difficult time differentiating between types of psychopathology (Burke et al., 2005; Wichmann et al., 2017; Zorn et al., 2017). This may help further explain why the CO₂ Challenge-evoked coupling of testosterone and cortisol response failed to predict TSST-evoked cortisol response. Therefore, unlike the peak subjective distress result, the lack of overlap in stress-evoked hormone responses to the two stressors is consistent with the notion that these stressors differ in fundamental ways.

In male participants, our results were in line with our hypotheses and replicated the dual-hormone hypothesis (i.e., increasing testosterone by decreasing cortisol is “pathogenic”; Mehta and Josephs, 2010; Zilioli et al., 2015), i.e., for a male participant for whom cortisol decreased in response to the CO₂ Challenge, a positive relationship was found between CO₂ Challenge-evoked changes in testosterone, and TSST-evoked changes in subjective stress, anxiety, and fear reactivity. For female participants, contrary to expectation, no dual-hormone hypothesis pattern or other significant interaction pattern of CO₂ Challenge-evoked testosterone by cortisol predicting TSST-evoked subjective distress emerged. Notably, the *only* significant result for females was overall, female subjective distress peak was comparable between the CO₂ Challenge and the TSST. Unexpectedly, there were no significant CO₂ Challenge-evoked testosterone by cortisol interactions predicting TSST-evoked subjective distress for females. One possible explanation may be a lack of power due to the smaller sample size of female participants. Another factor for null findings in women may be related to menstrual cycle phase. Female participants only participated in the present study during their mid-luteal phase. As previously mentioned, the mid-luteal phase was chosen due to evidence that females have cortisol responses most similar to males during the luteal phase (Kirschbaum et al., 1992). However, since females have higher reported rates of stress, anxiety disorders, and mood disorders than males, choosing the phase when male and female responses are most similar may not be the best time to assess “pathogenic” hormone profiles. Perhaps the follicular phase, when females typically have hormonal responses that are less similar to males would increase the likelihood of detecting “pathogenicity” in female participants (Kirschbaum et al., 1992).

As previously discussed, anxiety sensitivity and social evaluative threat are two important dispositional variables that should be included in models assessing psychological distress. Thus, it is important to note the significant CO₂ Challenge-evoked coupling of testosterone by cortisol predicting TSST-evoked subjective distress models *included* anxiety sensitivity and social evaluative threat. This finding is

particularly noteworthy as it suggests the coupling of testosterone and cortisol response to the CO₂ Challenge is a meaningful predictor of emotional stress response above and beyond the variance predicted by these two well established dispositional predictors.

4.1. Limitations

The present study is subject to several limitations. Firstly, our study sample was drawn exclusively from a large research university, resulting in a cohort consisting solely of undergraduate students. Consequently, while our participants displayed diversity in terms of race and ethnicity, they lacked variation in age range, years of schooling, and socioeconomic status compared to a community-based sample. Secondly, despite our efforts to collect saliva samples at standardized intervals throughout the protocol, some participants experienced delays in producing the required amount of saliva, potentially introducing variability in salivary hormone concentrations. Thirdly, due to the need to maintain an acceptable duration for participation among our student cohort, we restricted the number of recovery saliva samples, precluding the assessment of hormone recovery period. To overcome this limitation, future studies should incorporate a minimum of three recovery samples over a 60-minute recovery period, as previous research suggests that examining hormone recovery patterns following a stressor can offer insights into psychopathology (Burke et al., 2005). Fourthly, because the saliva samples were stored at −20°C for 1–9 months before analysis instead of −80°C it is possible cortisol and testosterone were susceptible to degradation which could have impacted the reliability of hormone measurements (Toone et al., 2013; Rosenbaum et al., 2018). Fifthly, female menstrual cycle phase was determined via self-report rather than more rigorous methods such as sonography or biochemical measurements, leading to increased variability in cycle phase and reduced result reliability. Sixthly, the sequence in which the two stressors were administered was not randomized; the CO₂ Challenge preceded the TSST. This deliberate choice aimed to investigate whether changes in endocrine activity induced by the CO₂ Challenge, a rapid and low-resource stressor relative to the TSST, could predict TSST evoked outcomes. These limitations warrant attention in future research endeavors.

4.2. Conclusion

This study was the first assessment of hormonal and subjective distress responses to two very different, but widely used laboratory stressors. The dual-hormone hypothesis, as applied to clinical outcomes (i.e., high/increasing testosterone by low/decreasing cortisol is “pathogenic”; Mehta and Josephs, 2010; Zilioli et al., 2015) was confirmed in CO₂ Challenge-evoked testosterone by cortisol models predicting TSST-evoked subjective stress, anxiety, and fear in males. Unexpectedly, no significant coupling of testosterone by cortisol was found in females. Yet in both males and females, participants' peak subjective distress responses to the CO₂ Challenge and the TSST were comparable. This research contributes to the understanding of the coupling of testosterone and cortisol response to two distinct laboratory stressors. The current study also potentially suggests a wider role for the CO₂ Challenge – a role that could include being utilized to assess psychopathologies linked to dysfunction in the acute stress response (Rauschenberg et al., 2017). In conclusion, we propose it may be beneficial for future research to consider including both powerful laboratory stressors in their protocols, as the role of dysfunction in the acute stress response in the development of various psychopathologies has yet to be fully elucidated.

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CRediT authorship contribution statement

Michael J. Telch: Writing – review & editing, Resources, Methodology, Conceptualization. **Annabelle DiVita:** Writing – review & editing, Project administration, Investigation. **Frances A. Champagne:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Robert A. Josephs:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Ciara McAfee:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ciara A. McAfee Ph.D. reports financial support was provided by National Science Foundation Graduate Research Fellowship. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.psyneuen.2024.107187](https://doi.org/10.1016/j.psyneuen.2024.107187).

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