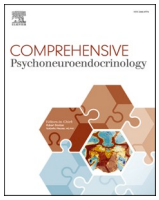
Comprehensive Psychoneuroendocrinology 18 (2024) 100232

Contents lists available at ScienceDirect

Comprehensive Psychoneuroendocrinology

journal homepage: www.sciencedirect.com/journal/comprehensive-psychoneuroendocrinology



The feigned annoyance and frustration test to activate the sympathoadrenal medullary system

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ARTICLE INFO

Keywords:
Acute stress
α-amylase
Stroop
Social evaluative threat
Sympathoadrenal medullary system

ABSTRACT

When perceived as threatening, social interactions have been shown to trigger the sympathoadrenal medullary system as well as the hypothalamic-pituitary-adrenal axis resulting in a physiologic stress response. The allostatic load placed on human health and physiology in the context of acute and chronic stress can have profound health consequences. The purpose of this study was to develop a protocol for a lab-based stress stimulus using social-evaluative threat. While several valid, stress-stimulating protocols exist, we sought to develop one that triggered a physiologic response, did not require significant lab resources, and could be completed in around 10 min. We included 53 participants (29 men and 24 women) and exposed them to a modified version of the Stroop Color-Word Interference Task during which the participants were made to feel they were performing the task poorly while the lead researcher feigned annoyance and frustration. After exposure to this Feigned Annoyance and Frustration (FAF) Test, both the men and women in this study demonstrated a statistically significant and clinically meaningful increase in subjective stress on the visual analog scale. Additionally, the men in this study demonstrated a statistically significant increase in heart rate and salivary α-amylase concentrations after exposure to the test. The women in this study did not demonstrate a statistically significant increase in the physiologic stress biomarkers. This protocol for the FAF Test shows promise to researchers with limited time and resources who are interested in experimentally activating the sympathoadrenal medullary system.

1. Introduction

It is well-established that stress is associated with increased risk of disease [1], increased severity of disease [1], increased rate of aging [1], higher likelihood of pain chronicity [2], and poorer disease prognosis [3]. The toll of psychosocial stress on the body has been repeatedly studied in the field of psychoneuroendocrinology and social-evaluative threat (SET) has been one of the key laboratory modalities used to trigger and study stress [4].

Acutely, stress triggers an increase in sympathetic nervous system activity through the sympathoadrenal medullary (SAM) system and the release of catecholamines. The intracellular norepinephrine triggers a cascade that results in the release of salivary α-amylase (sAA) [5]. This facilitates a second neuroendocrine cascade known as the hypothalamic-pituitary adrenal (HPA) axis. The HPA axis ultimately triggers the release of stress hormones – namely glucocorticoids – that facilitate the increased metabolism of fat and carbohydrates in order to mobilize glucose to accommodate for the increased physiologic demand of a "fight or flight" situation [6]. It is generally accepted that sAA is a valid representation of the SAM system [7–9].

Because of the negative impact stress has on overall health, it is critical to have a variety of valid laboratory stress tests to study the mechanism for the association between stress and disease. Many tests have been utilized with their appropriateness dependent on the focus of the research question. Studies have shown that the components of the most stressful triggers are (1) the uncontrollable nature of the stimulus, (2) the social-evaluative component of the stimulus, and (3) the threatening or challenging nature of the task [10–12].

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https://doi.org/10.1016/j.cpnec.2024.100232
Received 20 December 2023; Received in revised form 25 March 2024; Accepted 26 March 2024
Available online 3 April 2024

Our group was interested in a stress test that did not rely on cardiovascular stress, excluding exercise performance tasks such as the bicycle ergometer task. We also were in search of a task that was sufficiently robust, excluding stress tasks that are based solely on cognitive load, such as the Stroop Color-Word Interference Task [13] or the Serial Subtraction Task [14]. Additionally, we were interested in a task that did not involve physiologic pain, excluding an electric shock task [15] or the Cold Pressor Test [16]. Stress tasks that involve SET have been accepted as the most robust and effective at inducing a neuroendocrine response in research participants [4,10,12]. However, the protocol for the standard SET task, the Trier Social Stress Test [17,18] relies on additional lab resources that often are not available, namely space, time, and multiple researchers. For all these reasons, we attempted to develop our own protocol based on a previously published stress task, the modified Stroop Color-Word Interference Task [19]. In the 2004 study, participants were instructed in a cognitive load task based on the Stroop effect. To create additional stress, participants were misinformed that the task would be "on the second easiest setting" and that "they were expected to be excellent at the task." As participants performed the task, they were informed that they were not performing well. Additionally, the lead researcher attempted to passively communicate frustration with the participants' poor performance as well as annoyance with the other laboratory workers while participants' stress was measured.

Stress can be assessed in several ways [20]. Heart rate (HR) and sAA are physiologic biomarkers that have been correlated with laboratory induced stress [12,17,20,21]. It has been argued that while changes in HR are generally accepted to represent changes in autonomic activity, the influence of the parasympathetic nervous system holds greater sway over its function than sympathetic input [22]. Still, HR changes have been seen to directly correlate with acute stress [12,17–19,23].

It is important to consider several variables when designing a study using a neuroendocrine biomarker such as sAA. Time of day must be considered since normal sAA levels rise and fall throughout the day promoting arousal and productivity [23,24]. SAA levels are known to reach their nadir about 30 min after awakening and gradually increase throughout the day [24]. Additionally, since sAA directly peaks in conjunction with sympathetic nervous activity, the peak salivary concentration is normally seen within 5 min of the application of the stressful stimulus [9,12].

Mixed findings have been reported regarding the effect of sex on sAA after exposure to stress. Some investigators report that men and women demonstrate similar changes in sAA in responses to stress [25–27]. However others have documented the influence sex or menstrual phase can have on sAA levels [28]. Because of these mixed reports, it is recommended to consider both male and female participants in the recruitment and analysis of stress research [29].

The purpose of this research study is to develop a protocol for the Feigned Annoyance and Frustration (FAF) Test designed to stimulate the SAM using SET and to test the validity of that protocol. A second purpose of this study is to determine if the FAF Test is effective in both men and women. We hypothesize that the exposure to the FAF Test will induce stress in the study population and that this change will be demonstrated by increases in HR, sAA, and subjective stress (VAS_{stress}). Additionally, we hypothesize that the FAF Test will be effective at inducing stress in male and female participants.

2. Methods

2.1. Participants

This study was approved by the Internal Review Board (IRB) at Loma Linda University (IRB # 5210188) and occurred as part of a study on the effect of stress on balance strategies for individuals with and without low back pain. Sixty participants were recruited from a convenience sample at Loma Linda University and the surrounding area. All participants in the study were consented before being enrolled. Participants were included if they were between the ages of 18 and 45 years of age, could balance on one leg, and did not have: a diagnosed anxiety disorder, history of low back surgery, current pregnancy (or pregnancy in the past 12 months), severe pain (current pain >6/10), or color blindness. All participants were compensated with a $25 gift card at the completion of the study. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

2.2. Quiescence period and subjective report outcome measures (SROMs)

All data collection was conducted between 2:30 p.m. and 7:00 p.m. to account for the diurnal variation in sAA levels. Upon arriving at the research facility, participants were seated to be consented. After being consented, participants were fitted with a polar H10 HR sensor which was then connected via Bluetooth to an iPad. Participants remained seated to allow their HR and stress levels to settle at baseline and to complete several self-report outcome measures: the Spielberger State-Trait Anxiety Inventory (STAI), the Perceived Stress Scale (PSS), and the Pittsburgh Sleep Quality Index (PSQI). The STAI is a 40-item questionnaire designed to quantify an individual's current anxiety level (state) and tendency toward anxiety (trait). It has been determined to be valid and reliable [30]. The PSS is a 10-item questionnaire designed to quantify an individual's perceived stress over the past 30 days as it relates to being overwhelming, unpredictable, and uncontrollable. The PSS has been determined to be valid and reliable [31]. The PSQI was developed as a representative quantification of patients' sleep experience over the past month. It is made up of seven component scores which are then combined to form the global score. It has been determined valid and reliable when used to distinguish good quality sleepers from poor quality sleepers [32].

2.2.1. Heart rate

HR was recorded using the mobile application, EliteHRV (Version 5.5.4, mobile app for IOS, EliteHRV.com, USA). HR data was exported from Elite HRV as raw inter-beat interval data. It was imported into Kubios HRV Scientific (v 4.0.1) where it was filtered for artifact and ectopic beats using the previously validated Kubios HRV algorithm [33,34]. HR data was visually inspected for missing data or erroneously marked beats. Average HR was calculated for 4 distinct time periods defined by 4 distinct saliva collection times: HR_1 (mean HR from the beginning of the study period until T_1), HR_2 (mean HR from T_1 to T_2), HR_3 (mean HR from T_2 to T_3), and HR_4 (mean HR from T_3 to T_4) (T_1 = time immediately following the completion of the SROM paperwork; T_2 = time immediately before the beginning of the stress stimulus; T_3 = time immediately after the completion of the stress stimulus; T_4 = 10 min after the completion of the stress stimulus; Fig. 1.

2.2.2. Visual analog scale

During the 25-min quiescence period, participants were asked to annotate their current stress level on a 10 cm line with one side of the line reading, "None", and the other side of the line reading, "As bad as it could be." At the end of the 90-min trial, participants were then again asked to rate their current stress on the same scale during the final 90-s saliva collection. The Visual Analog Scale (VAS) has been widely used in the literature and has been validated as a measure for subjective stress [35]. The minimal clinically important difference has not been determined for VAS_{stress}, however for pain it has been reported at 1.0 cm [36] and for anxiety it has been recommended between 1.2 and 1.3 cm [37]. We decided to use 1.2 cm as a cutoff for meaningful change in subjective stress appraisal.

2.2.3. Saliva collection and analysis

Participants were instructed to avoid rigorous physical activity within 24 h of the saliva collection. Additionally, they were instructed to abstain from alcohol 24 h before saliva collection. On the day of the scheduled session in the lab, participants were requested to avoid eating

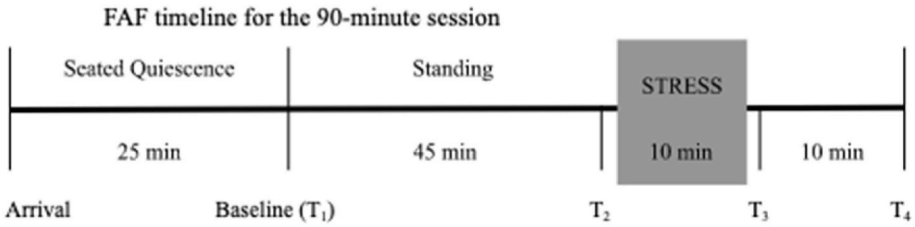

Fig. 1. FAF timeline for the 90-min session.

or drinking anything (including caffeine) for 1 h before coming in [5]. Plain water was permitted. Other than the aforementioned instructions, participants were advised to keep their regular routine regarding sleep, mealtimes, and daily activity. Saliva samples were collected, stored, shipped, and processed in accordance with the tier 1 BRISQ criteria [38]. Samples of saliva were collected using SalivaBio Oral Swab, 10 × 30mm (Item No. 5001.02). After being consented, each participant was instructed to rinse their mouth with a sip of plain, filtered water. After the quiescence period the participants were instructed to place the swab beneath the tongue directly from the packaging so as to not contaminate the swab with their hands. Next, participants were instructed to avoid swallowing, allowing saliva to pool while the swab was in place for 90 s. The swab was then removed, again without using the hands, by placing it directly from the mouth into a Swab Storage Tube, 17 × 100mm (Item No. 5001.05). Tubes were placed in a cooler during the trial and then stored in a freezer and kept at −80 °C until the time of processing (2 weeks–71 weeks). Saliva was collected at T_1, T_2, T_3, and T_4. Since this protocol took place as a part of another study on stress and balance, all participants performed two single leg balance tasks that took place in two identical 10-min trials: one just before T_2 and one between T_3 and T_4 (Fig. 1). None of the participants reported the balance task to induce significant fatigue.

Samples were shipped frozen and packaged with dry ice in accordance with the instructions provided by Salimetrics (Carlsbad, CA). Samples were assayed at the Salimetrics SalivaLab using the Salimetrics Salivary α-Amylase Assay Kit (Cat. No. 1–1902), without modifications to the manufacturer's protocol. Samples were thawed to room temperature, vortexed, and then centrifuged for 15 min at approximately 3000 RPM (1500×*g*) immediately before performing the assay. Samples were tested for sAA using a kinetic enzyme immunoassay (Cat. No. 1–1902). Sample test volume was 8 μl of 200X diluted saliva per determination. The assay had a lower limit of sensitivity of 0.4 U/mL, samples exceeding 400 U/mL needed further dilution, an average intra-assay coefficient of variation of 5.47%, and an average inter-assay coefficient of variation 4.7%, which met the manufacturer's criteria for accuracy and repeatability in salivary bioscience and exceeded the applicable NIH guidelines for enhancing reproducibility through rigor and transparency.

2.3. FAF test protocol

Fig. 1 contains the study timeline for each participant. Participants received standardized, verbal instruction in how to perform an application-based cognitive load task (Brain Test - Stroop Effect, Copyright Attila Hegedus) on an iPad. Standardized patient instructions are listed below:

Your task is to tap on the appropriate label at the bottom of the screen whose text denotes the ink color of the top label. Give as many correct answers as you can in 60 s. Correct answers are +1 and incorrect answers are −1. This task is the easiest setting, and most people don't have any trouble. The app measures your ability and quantifies your proficiency.

After participants began the first trial, the instructions were repeated when multiple incorrect attempts were made. No false feedback was given to the participants at any time. Examiner comments included:

Don't overthink it, it should be a lot easier than this. Just try to focus. Sorry, hang on a second. Would it help if I explain the instructions again?

If the participant answered Yes, the instructions were repeated. If the participant answered No:

Ok, let's start over and really try to focus this time.

At this time, the instructor made an effort to express frustration, disappointment, and annoyance at the participant's performance by heavily sighing, changing the tone of voice, and coarsely redirecting banter back to the task. This response was used for all participants regardless of the accuracy of their answers. If the participant was performing the task with a relatively high degree of accuracy, the researcher stated:

You're doing okay, but I need you to speed up a little. Actually, I need you to speed up a lot if we're going to be able to use any of this.

After completing the second attempt, the researcher expressed further disappointment, stating:

I'm not sure we're going to be able to use any of that. Let's try this instead: Starting from 999, subtract 7 out loud. For every incorrect answer you will hear a sound.

It was then clarified that a certain speed needed to be maintained in order to have an effective trial. A bell was rung for every incorrect answer and the researcher appeared to be marking a sheet of paper and checking the time throughout the trial. The researcher would express frustration with other lab personnel present during the study and make indirect comments about the success of the performance (i.e. "Do you know of any other participants who can come in tonight?") The same frustrated tone was maintained through the end of the research session. After the collection of the final saliva sample, all participants were informed of the ruse. The protocol was carried out by the same male investigator for all trials (TG).

2.4. Data analysis

Mean and standard deviation were computed for quantitative variables and frequency (percentage) for categorical variables at baseline. Normality of quantitative variables were assessed using the Shapiro-Wilk test and box plots. Log transformation was applied to raw sAA concentrations to address non-normality and skewness. The independent *t*-test was used for quantitative variables at baseline and the Mann-Whitney *U* test was used for non-normal and ordinal data. The independent chi square test was used for categorical variables at baseline. Linear mixed effects models (repeated measures) were used to examine the effect of the between-group factor (sex) and within-group factor (time) on the dependent variables (HR, sAA, and VAS_{stress}) [39]. A Bonferroni correction was used to adjust for multiple post-hoc comparisons. A power calculation was performed using G*Power (Version 3.1.9.2; Heinrich-Heine Universität, Düsseldorf, Germany) with a similar method as has been previously reported [10,26]. A minimum sample size of n = 52 was required to provide 80% power at the 5% level of significance to capture a small effect size of 0.20 or higher. The data was analyzed using SPSS Statistics Software version 29.0 (SPSS Inc, Chicago, IL, USA). All analyses were performed at an alpha level of 0.05.

3. Results

Of the 60 participants who were recruited, consented, and completed the FAF Test protocol, 53 participants (29 men and 24 women) were included in the final analysis due to missing data. No participants opted to terminate the trial before completion. The average age of the men was significantly higher than the average age of the women (mean ± SD: men: 30.2 ± 4.5 years, women: 27.9 ± 5.2 years, $p = 0.033$). Mean BMI for the men was also significantly higher than the mean BMI for the women (men: 25.5 ± 3.3 kg/m^2, women: 23.6 ± 4.0 kg/m^2, $p = 0.037$). Resting HR for the men was lower than the resting HR for the women (men: 71.4 ± 8.6 bpm, women: 83.1 ± 10.6 bpm, $p < 0.001$). All other demographic data was not significantly different between groups at baseline ($p > 0.05$) (Table 1).

There was a significant increase in HR for both groups over time ($p < 0.001$) (Table 2 and Fig. 2a). There was no interaction between time and sex, however the between groups analysis revealed a significant difference between men and women ($p = 0.003$) (Table 2). For men there was a statistically significant increase in HR after stress (HR_4 compared to HR_2, $p = 0.040$). For women, HR was not significantly different after stress (HR_4 compared to HR_2, $p = 0.059$). However, HR_4 (after stress) increased significantly compared to HR_3 (during the stress task) ($p = 0.001$) (Table 2 and Fig. 2a). Both groups exhibited a significant increase in sAA over time ($p < 0.001$) (Table 2 and Fig. 2b). Men demonstrated a

Table 1
Demographics and baseline data.

Characteristics	Total (n = 53)	Men ($n_1 = 29$)	Women ($n_2 = 24$)	P – value
Age (years)	29.2 ± 4.9	30.2 ± 4.5	27.9 ± 5.2	**0.033**
BMI (kg/m^2)	24.6 ± 3.7	25.5 ± 3.3	23.6 ± 4.0	**0.037**
NPRS[a]	0 (0, 5)	0 (0, 4)	0 (0, 5)	0.581
Occupation[b]				0.063
Medical	9 (17)	6 (21)	3 (12)	
Student	36 (68)	16 (55)	20 (83)	
Other	8 (15)	7 (24)	1 (4)	
STAI-State Subscale	29.6 ± 7.9	29.1 ± 7.0	30.2 ± 9.1	0.986
STAI-Trait Subscale	37.1 ± 10.2	34.6 ± 8.6	40.2 ± 11.3	0.093
PSQI	5.3 ± 2.4	5.2 ± 2.6	5.4 ± 2.2	0.639
PSS	14.8 ± 5.9	13.6 ± 5.0	16.2 ± 6.7	0.109
VAS_{stress} (cm)[a]	1.1 (0, 7)	1.0 (0, 5)	1.6 (0, 7)	0.180
Resting HR (bpm)	76.6 ± 11.1	71.4 ± 8.6	83.1 ± 10.6	**<0.001**
sAA (U/mL)	111.9 ± 101.5	113.5 ± 87.7	110 ± 118.1	0.437[c]

Abbreviations: BMI: Body Mass Index, NPRS: Numerical Pain Rating Scale, STAI: State-Trait Anxiety Inventory; TSK: Tampa Scale of Kinesiophobia, PSQI: Pittsburgh Sleep Quality Index; PSS: Perceived Stress Scale; VAS_{stress}: Visual Analog Scale for Stress; ODI: Oswestry Disability Index; HR: Heart Rate; sAA: Salivary α-Amylase. Values are presented as mean ± SD unless otherwise indicated.
[a] Median (min, max).
[b] Frequency (percentage).
[c] p-values for log-transformed data.

Table 2
Stress reactivity to the FAF overall with within (time) and between (sex) groups comparison.

Variable		Total (n = 53)	Men ($n_1 = 29$)	Women ($n_2 = 24$)	p°°
		Mean ± STD	Mean ± STD	Mean ± STD	
HR (BPM)	HR_1	76.6 ± 11.1	71.4 ± 8.6	83.1 ± 10.6	**0.003**
	HR_2	82.6 ± 11.4*	77.8 ± 9.5*	88.4 ± 11.0**	
	HR_3	83.3 ± 13.8*	79.6 ± 14.0**	87.8 ± 12.5*	
	HR_4	86.9 ± 14.8***	83.0 ± 16.0†	91.6 ± 11.9††	
p°		**<0.001**	**<0.001**	**<0.001**	
sAA[a]	T_1	4.4 ± 0.9	4.5 ± 0.8	4.2 ± 1.0	0.294
	T_2	4.5 ± 1.0†††	4.5 ± 0.9†††	4.4 ± 1.2	
	T_3	4.6 ± 0.9*	4.9 ± 0.7†††	4.4 ± 1.0	
	T_4	4.9 ± 0.8***	5.0 ± 0.6***	4.7 ± 1.0**	
p°		**<0.001**	**<0.001**	**<0.001**	
VAS_{stress} (cm)	T_1	1.8 ± 2.0	1.3 ± 1.3	2.4 ± 2.5	0.233
	T_4	3.6 ± 2.2**	3.5 ± 2.0**	3.7 ± 2.4**	
p°		**<0.001**	**<0.001**	**<0.001**	

Abbreviations: VAS_{stress}: Visual Analog Scale for stress; HR: Heart Rate, BPM: Beats Per Minute; sAA: Salivary α-Amylase; HR_1: mean Heart Rate from beginning of the collection until T_1; HR_2: mean Heart Rate from T_1 to T_2; HR_3: mean Heart Rate from T_2 to T_3; HR_4: mean Heart Rate from T_3 to T_4; T_1: after 25-min quiescence period, T_2: immediately pre-stress, T_3: immediately post-stress, T_4: 10-min post-stress.
*p-value<0.05 for within groups compared to T_1 and T_4.
**p-value <0.003 for within groups compared to T_1.
***p-value<0.05 for within groups compared to T_1, T_2, and T_3.
†p-value<0.05 for within groups compared to T_1 and T_2.
††p-value<0.05 for within groups compared to T_1 and T_3.
†††p-value<0.05 for within groups compared to T_4.
° p-value for the null hypothesis that there is no significant difference within groups (variable x time).
°° p-value for the null hypothesis that there is no significant difference between men and women.
[a] Log transformations of raw α-Amylase concentrations in U/mL.

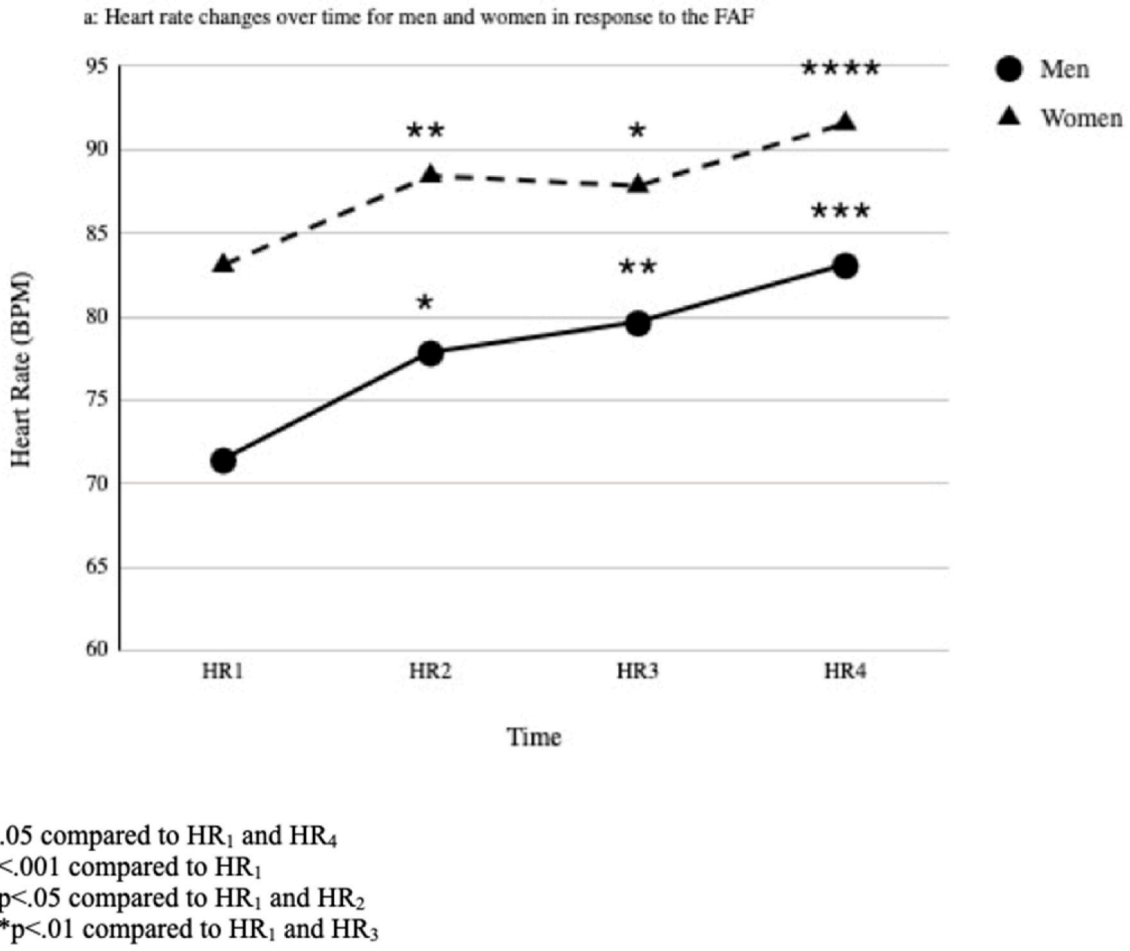

Fig. 2a. Heart rate changes over time for men and women in response to the FAF.

significant increase sAA concentrations after stress (T_4 compared to T_2, $p < 0.001$). While women demonstrated a significant increase in sAA concentrations compared to baseline (T_4 compared to T_1, $p = 0.003$), there was no statistically significant increase in sAA concentration comparing T_4 and T_2 ($p = 0.086$). There was no significant difference between groups ($p = 0.294$) (Table 2 and Fig. 2b). There was a significant increase in VAS_{stress} for both the men and women over time ($p < 0.001$) (Table 2 and Fig. 2c). There was no significant difference between groups ($p = 0.233$) (Table 2).

4. Discussion

In this study, we sought to develop a valid protocol to induce acute stress in men and women. We introduced the FAF Test as a feasible stress stimulus that offers several advantages. Unlike other methods reliant on SET, the FAF Test can be efficiently administered in 10 min and requires minimal resources, making it highly suitable for most laboratory settings. While a similarly modified version of the Stroop Color-Word Interference Task has been previously described in brief [19], we felt it would offer greater utility if we sought to validate a scripted protocol to maximize reproducibility for future investigation. Our proposed protocol for the FAF Test appeared to be effective at stimulating the SAM in men, as evidenced by the significant increase in sAA concentrations and HR after exposure to the FAF Test. The protocol was not as robust in activating the SAM in women as the changes in HR (HR_4 compared to HR_2) and sAA (T_4 compared to T_2) after the FAF Test failed to reach significance ($p = 0.086$ and $p = 0.059$, respectively). Despite the lack of physiologic change for women, both men and women reported a significant increase in subjective stress on the VAS_{stress}. It has been recommended and is of critical importance to include the subjective report of stress alongside biomarkers when assessing the negative impact of stress [40].

Our findings suggest that men and women were both influenced by the FAF Test. Other studies on stress reactivity in women often control for menstrual phase or for the use of oral contraceptives. We intentionally did not control for these variables to broaden the applicability of our study findings. In retrospect, we may have encountered a more robust physiologic change for the female group had we provided this control since women have been shown to demonstrate blunted changes in sAA concentrations during the follicular phase of the menstrual cycle [28]. It has also been suggested that menstrual phase may influence subjective stress appraisal [41], however our findings do not suggest that this was the case in our study. Our findings for women may have been more robust had we only considered individuals on oral contraceptives or in the luteal phase of the menstrual cycle.

We did find that the female participants exhibited a higher HR than the men at all 4 time points during the study period. From this, we propose that the difference in HR between groups may be due to sex and not differences in reactivity to SET. When examining generalized HR differences in men and women, women have been shown to have a higher HR than men [42] perhaps due to the anatomical size difference and other autonomic discrepancies between male and female physiology [43]. It is important to note that the investigator administering the FAF Test protocol in this study was male. It has been suggested that stress reactivity to SET is more robust when the examiner is of the opposite sex than the participant [44], however we did not appear to demonstrate

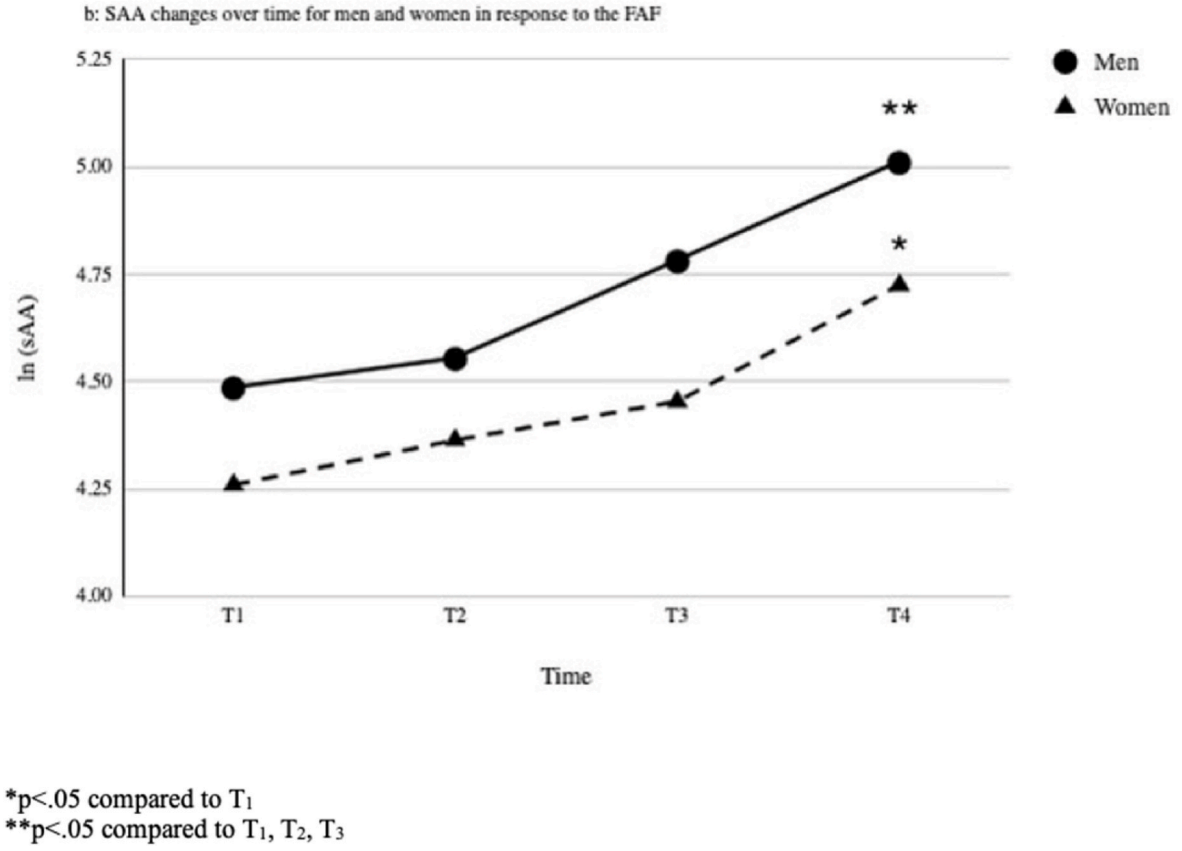

Fig. 2b. SAA changes over time for men and women in response to the FAF.

this phenomenon in our results.

The HR changes demonstrating stress reactivity for our participants was small. Other studies that use HR as a biomarker for stress compare baseline to peak HR [12] which may introduce a bias exaggerating HR reactivity. We chose to quantify HR by calculating the mean for a given time epoch which we feel was a more accurate representation of the participants' physiologic state for a given time period. Additionally, 4 mean HRs fits well with our repeated-measures design for the saliva analysis. Other studies have quantified HR by calculating minute-to-minute averages [17,19] which may demonstrate a higher peak HR than the means we reported. Future studies may consider the role of HR variability in this analysis which may provide greater insight into autonomic reactivity than mean HR.

A common biomarker used in other studies on SET is salivary cortisol. We chose to not include this in our analysis due to the additional time requirements it would add to our protocol. We were interested in a relatively short stress stimulus (10 min) with a short post-stress reassessment period (10 min). Studies that have documented the cortisol response to SET suggest post-stress peaks in cortisol between 15 and 35 min [4,12,17,45]. Other researchers even recommend the use of sAA over the use of cortisol to capture overall stress reactivity [9].

Several limitations should be considered when interpreting the findings of this study. First, this validation study was based on a subset of a data from another study on the effects of stress and balance. As such, there are some components of the study design that may have been different had the primary goal during data collection been to validate this protocol. For instance, the validity of this protocol would be greater if a control group was included in the study design.

To increase generalizability of our findings, both men and women were included in this study. However, we acknowledge that the baseline heterogeneity of the two groups exceeded what would be considered ideal. The men were a slightly older cohort than the women, perhaps having to do with the higher number of students in the female cohort. The lack of change in stress biomarkers for the women in this study limit the implications that can be made for females regarding the FAF Test. It may strengthen the findings of this study to further investigate inter-rater reliability of the protocol, perhaps carried out by an investigator who is not male.

For researchers interested in utilizing this protocol for future projects, we have a few small recommendations that may further enhance the sympathetic response to the FAF Test. First, the serial subtraction task that took place at the end of the FAF Test may induce a more robust stress response if participants were instructed to start the task over after every incorrect answer, as is the protocol during the Trier Social Stress Test [18].

A second recommendation is that baseline posture should be considered. HR_1 was calculated while participants were sitting to complete the paperwork. Since the FAF Test was conducted in standing, the baseline physiologic measures may be better contextualized if the paperwork was completed while the participants were standing. This would allow any changes in the SAM to be free from postural influence and may better demonstrate isolated changes due to the SET of the FAF Test. Since it has been recommended that both psychological and physiological responses to stress should be considered in the validation protocol [44], future researchers may do well to incorporate a repeat administration of the STAI-S at T_4 which would corroborate the subjective response already captured with the VAS_{stress}.

5. Conclusion

This protocol for the FAF Test appears to be a valid stimulus to trigger an increase in the SAM in men. While the same physiologic increase in women was not demonstrated, both sexes subjectively reported a statistically significant and clinically meaningful increase in stress

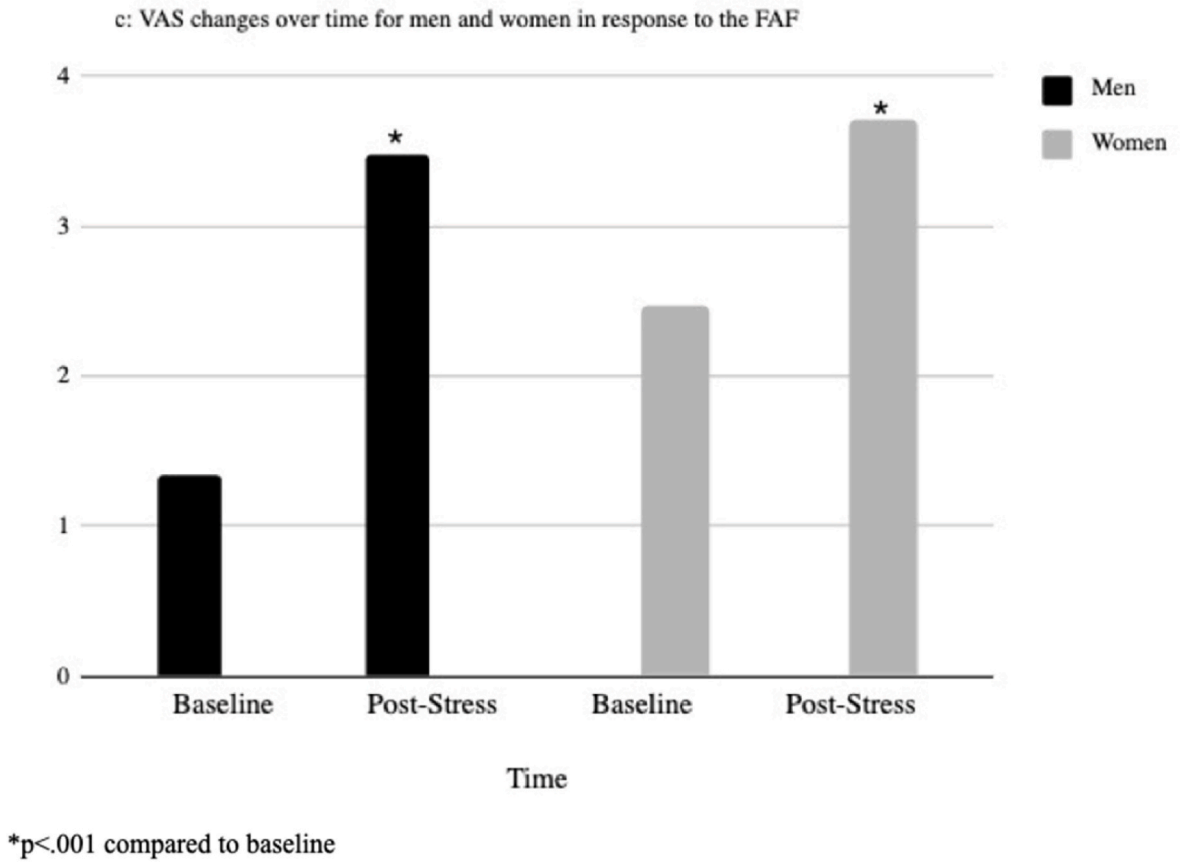

Fig. 2c. VAS changes over time for men and women in response to the FAF.

after the protocol. If women are to be included in future studies utilizing the FAF Test, investigators may do well to be more selective in their recruitment of participants regarding menstruation and the use of oral contraceptives. The FAF Test may be helpful for researchers interested in triggering the SAM system without using pain or exercise. Additionally, this protocol can be completed in 10-min, requires little space, and relies on few personnel.

Comprehensive Psychoneuroendocrinology submission checklist

By submitting the enclosed manuscript to Comprehensive Psychoneuroendocrinology the authors attest the following.

- The authors have read through the guidelines described in the Guide for Authors for the preparation of the manuscript and the report of their results
- The standard reporting guidelines appropriate to the studies reported to the manuscript have been followed
- All precautions have been taken to ensure that the studies described in the manuscript are not underpowered and appropriate power analyses have been conducted
- Guidelines for specific types of studies have been followed
- Care has been taken to ensure the manuscript is written in clear scientific English

CRediT authorship contribution statement

Ted W. Gehrig: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lee S. Berk:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Robert I. Dudley:** Writing – review & editing, Supervision. **Jo A. Smith:** Writing – review & editing, Resources. **Lida Gharibvand:** Writing – review & editing, Writing – original draft, Resources, Formal analysis, Data curation. **Everett B. Lohman:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest affecting the findings of this paper.

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