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An introductory guide to conducting the Trier Social Stress Test

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ABSTRACT

The Trier Social Stress Test (TSST) is a reliable biopsychological tool to examine the effects of acute stress on psychological and physiological functioning in humans. While the TSST reliably increases hypothalamic-pituitary-adrenal axis activation, amongst other biomarkers, through a combination of social evaluative threat and uncontrollability, the original protocol is limited in methodological detail that has impacted its reproducibility. Although many studies include a mock job interview and surprise arithmetic task, there are large variations in the timing of events, the number and method of biological (e.g., cortisol) sampling, the administration of a glucose drink, set-up of equipment and rooms, panel composition, and panel interaction with participants. We provide an overview of the potential impact of methodological variations on the stress (cortisol) response. Importantly, we also provide a step-by-step guide as a laboratory manual on how to conduct the TSST. This introductory guide may be a useful and time-saving resource that may also improve the scientific standard and reliability of the reported psychobiological stress effects in future studies.

1. Introduction

Emotional, physical and environmental stressors permeate several domains of daily living. For decades, stress has been researched in many experimental laboratories (Maes et al., 1998; Mason, 1975) and a wealth of knowledge about the impact of stress on human functions has been gained. Yet, stress is a complex function that affects various psychological and biological systems, particularly the hypothalamic-pituitary-adrenal (HPA) axis. Over time, chronic stress can have severe negative consequences on the population's health and well-being by contributing to the manifestation of many psychosomatic and psychiatric illnesses. Not surprisingly, stress has been reported by the World Health Organization as one of the most significant health concerns of the 21st century (Bebbington, 2001). Therefore, the importance of studying stress, especially the study of associations between stress and various physical and mental health problems, is abundantly clear.

The present paper aims to describe the main methodological details and provide a introductory guide for the administration of the Trier Social Stress Test (TSST) (Kirschbaum et al., 1993), a commonly used and very reliable laboratory tool for inducing a strong psychobiological stress response in humans. The novel contribution of this paper is the step-by-step introductory guide presented in the Supplementary material that provides much-needed information on how to conduct the TSST given that there are very limited details about the exact methodology of the TSST available from past studies. This paper and guide will be useful for applied researchers in psychology, neuroscience, psychiatry and allied health. Given the broad application of the TSST across fields, we further highlight that this is an introductory guide that may be most useful for those starting out with the TSST for the first time, and can be adapted based on specific research questions. We also highlight that our goal with this paper was not to provide a comprehensive review of the literature and its limitations, as this can be found elsewhere (e.g., Allen et al., 2014, 2017; Birkett, 2011; Goodman et al., 2017; Kudielka et al., 2007).

The first section of the paper introduces the psychological, physiological and neurohormonal mechanisms involved in the human stress response. This is important for a better appreciation of the complexity of the stress response and factors that could influence it. The second section of the paper provides a brief description of the TSST. This includes a discussion of the implications of variations to key methodological aspects of the administration of the TSST as evident from past studies.

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2. The neurobiology of stress

The body's response to stress serves a protective purpose, as it prepares the body to combat perceived or actual disruptions to homeostasis, or minimize the impact on the organism through activation of the fight, flight or freeze response (Ulrich-Lai and Herman, 2009). Several complex interconnected physiological systems are activated in response to stress, with these forming the basis for the adaptive survival mechanisms of the human body in the face of threat (Compas et al., 2006). Mechanistically, the two primary adaptive stress response systems consist of the autonomic nervous system, which involves the sympathetic and parasympathetic branches of the peripheral nervous system, and the HPA axis that integrates both the central nervous and endocrine systems (Compas et al., 2006).

As part of the autonomic nervous system via its *sympathetic* branch, the sympathetic adrenal medullary system provokes the most immediate "fast" response to stress through activation of the adrenal medulla and release of epinephrine and norepinephrine into the blood that causes rapid changes in physiological states (e.g. increased heart rate and blood flow due to excitation of the cardiovascular system; Ulrich-Lai and Herman, 2009). This response is also sometimes referred to as our "fight and flight" response. This autonomic nervous system excitation is brief due to the reflex activation of the *parasympathetic* branch, which acts to restore functions and relax the body by slowing and maintaining the body's basic needs (Boyle, 2013; Murison, 2016). By comparison, activation of the HPA axis is a relatively "slow" response, and involves the hypothalamus acting on the anterior pituitary gland to release andrenocorticotropic hormone into systemic circulation to reach its primary target organ, the adrenal cortex. Peak levels of plasma glucocorticoids (e.g., cortisol) are then released approximately ten minutes post initiation of a stressor and act to maintain homeostasis in the body (Droste et al., 2008).

However, there are various additional human systems involved in the stress response, including the immune system, the enteric (gastrointestinal) nervous system, other endocrine systems such as the thyroid and somatotropic axes, cognitive functions, such a reappraisal of the stressor, and even the brain's structure and function (De Kloet et al., 2005; Murison, 2016; Steptoe et al., 2007; Ziegler, 2012). Additionally, the hormones involved in the stress response are also more widespread than those mentioned here; for a review see Allen et al. (2014).

While these adaptive stress response systems are highly functional, long-term exposure to stress can lead to debilitating consequences. Chronic stress significantly increases an individual's vulnerability to detrimental medical outcomes, including autoimmune diseases, cardiovascular diseases, endocrine disorders, and obesity (Chrousos, 2009; Dallman et al., 2003; Khanam, 2017; Miller et al., 2007; Steptoe and Kivimaki, 2012; Stojanovich and Marisavljevich, 2008). In addition, stress is associated with increased vulnerability to a number of mental health conditions, with evidence showing it to be an important predictor of anxiety and depression (D'Angelo and Wierzbicki, 2003; Parrish et al., 2011). Furthermore, individuals faced with chronic stress have an increased likelihood of engaging in substance abuse and are more vulnerable to addiction relapse (Monroe and Hadjiyannakis, 2002; Sinha, 2008; Vgontzas et al., 1998), have sleep disturbance or symptoms of insomnia (Vgontzas et al., 1998), and have problems with cognitive functioning in both the short and long-term (Chen and Baram, 2016; Stawski et al., 2006). In the short-term, preoccupation with earlier stressors can lead to reduced ability to allocate attention and memory resources towards tasks at hand, and long-term chronic stress is associated with accelerated cognitive declines (Scott et al., 2015). Daily stress is further associated with declines in both an individual's health and mood (DeLongis et al., 1988). Such a broad range of negative and potentially long-term consequences of stress, and a fast-paced modern world, highlights the need to improve our understanding of how stress impacts our behavior, health, and biological systems.

3. Psychobiological stress in the laboratory: The Trier Social Stress Test

In the laboratory, the study of acute stress in humans, using a reliable and valid acute stressor, is essential for basic and translational research (Allen et al., 2014). The TSST (Kirschbaum et al., 1993) is one of the most widely used research tools for the induction of acute psychobiological stress in experimental research worldwide. In its original form (Kirschbaum et al., 1993), the TSST provides an ecologically valid stressor that elicits moderate acute stress through exposure to a psychosocial stressor. It consists of three main components: an anticipation period, a 5-minute mock job interview, and a 5-minute surprise mental arithmetic task; the interview and arithmetic task are performed in front of a panel.

Participants are unaware of the arithmetic task and therefore the TSST contains an element of deceit and uncontrollability. A review by Dickerson and Kemeny (2004) determined that ?? performance on tasks containing the central elements of both social evaluative threat (e.g., performance on the task is open to negative evaluation by others) and uncontrollability, were associated with the highest cortisol responses and lengthiest recovery periods. As such, the TSST is advantageous in having both of these elements embedded within the protocol, enabling researchers to examine the biological and psychological acute stress responses of individuals within the laboratory (Allen et al., 2017). Deceiving participants is commonly used in psychological research, although this is dependent on the discretion of individual ethics committees. Certain conditions are necessary for deceit to be acceptable in psychological research, including the condition that the study will make a valuable contribution to the existing scientific understanding, that the deception is not expected to cause significant, ongoing and/or severe harm or emotional distress to participants, and that participants are debriefed about the protocol as soon as the study protocol permits (Boynton et al., 2013); also see (Hertwig and Ortmann, 2008).

Most commonly in experimental research, acute psychological stressors are measured through assessment of physiological changes, such as increased HPA axis activity observed through cortisol release (Kirschbaum et al., 1993). Cortisol measurement, obtained from either saliva or blood sampling, has proven to be the most commonly used marker of stress (Goodman et al., 2017). However, a strong stress response following participation in the TSST can be observed via numerous other physiological and psychological markers of stress. This includes significant changes in norepinephrine, epinephrine, salivary amylase, cardiovascular functions, such as heart rate and blood pressure, and electrodermal activity, such as that generated by sweat glands (Miller and Kirschbaum, 2013), increased autonomic nervous system activity (e.g., heart rate variability; Xhyheri et al., 2012), impacts on the immune system (e.g., increased immune molecules/circulating inflammatory markers following exposure to stress; Steptoe et al., 2007), and changes in gastric function activity (e.g., exacerbating symptoms of irritable bowel syndrome; Kennedy et al., 2014).

Psychologically, the TSST has also been shown to influence cognitive functions. Specifically, the TSST has been shown to impair working memory for neutral stimuli in those who responded with high cortisol levels (e.g., using a digit span memory task; Elzinga and Roelofs, 2005) and to moderate more complex cognitive functions such as cognitive flexibility (Plessow et al., 2011) and creativity (Akinola and Mendes, 2008). Emotionally, the TSST can lead to increased self-reported stress, anxiety, and negative mood (Allen et al., 2014). Administration of the TSST also results in participants reporting increased wakefulness (Kirschbaum et al., 1999).

While the effectiveness of TSST to induce physiological and psychological changes is clear, research has revealed that there are considerable intra- and inter-individual variations in the psychobiological stress responses elicited. Demographical, environmental and physiological factors, such as age (Kudielka et al., 2007), sex (Kirschbaum et al., 1992), education (Fiocco et al., 2007), personality (Oswald et al.,

2006), nicotine, alcohol and caffeine consumption (Kudielka et al., 2007), culture (Laungani, 1993), genetics (Ising and Holsboer, 2006), consumption of substances and some medications (see Brody et al., 2002; Fries et al., 2006), and methodological aspects (Zänkert et al., 2018) are known to impact both the magnitude and the course of the biomarkers examined during stress research. The above factors likely contribute to both the intra- and inter-individual variations observed in stress response patterns during the TSST (Allen et al., 2014; Miller and Kirschbaum, 2013).

Methodologically, there is notable variability in how the TSST protocol is administered across different laboratories. This is largely due the relatively brief methodological description of the TSST protocol in the original paper using it (Kirschbaum et al., 1993), and because many studies to date have failed to report their specificmethodology in detail. Since the publication of the original TSST study in 1993, several attempts have been made to provide an update to the protocol and to review the empirical evidence on the TSST (e.g. Allen et al., 2014, 2017; Birkett, 2011; Goodman et al., 2017). In particular, the impact of variations of the TSST protocol was shown in Goodman et al.'s (2017) meta-analysis of 186 individual studies, which observed differences in effect sizes of the cortisol stress response when different methodological elements were employed across studies. Some of these variations included the time of day administered, sex of participant and other inter-individual differences, number of salivary cortisol measurements and method, panel composition, assessments completed before and during the TSST, speech anticipation times, subtraction number used in arithmetic task, and panel instructions and feedback. Therefore, despite clear evidence for the efficacy of the TSST in experimental research, the field has experienced various inconsistencies in its application and there is limited information available on the exact steps and setup of the TSST.

Developing a widely available and informative TSST guide is one way to reduce some of the intra- and inter-variability observed across studies, while also providing an accessible time-saving resource for those in the field of stress research. Such improvements in methodological aspects will significantly advance our understanding of how stress impacts health outcomes byproviding a better foundation from which to compare findings and in turn advance the research in this area.

4. Rationale for an introductory guide to the TSST

In light of the apparent lack of a consistent and detailed description of the TSST methodology, the aim of this paper is to provide an overview of the methodological inconsistencies in the administration of the TSST, and more importantly, to provide an introductory step-by-step guide in the form of a laboratory manual as a foundation for researchers to use when developing and administering the TSST. We focused only on the administration of the TSST to individual adult participants. This includes the specific components of a waiting period, task introduction, anticipatory phase, speech period, surprise arithmetic task, debrief, and recovery as per the original protocol (Kirschbaum et al., 1993). We acknowledge that there are various versions of the TSST, such as the group (TSST-G) version (von Dawans et al., 2011), the virtual reality (TSST-VR) version (Kotlyar et al., 2008), a version suited to children (TSST-C) (Buske-Kirschbaum et al., 1997), a control (placebo) version of the TSST (Het et al., 2009), a friendly TSST (f-TSST) (Wiemers et al., 2013), a modified version suited to both adults and children (TSST-M) (Yim et al., 2010) and an electronic version (e-TSST) (Hawn et al., 2015) published elsewhere.

Here, we aimed to establish the shortest possible protocol and guide of the TSST for adult participants that considers participant burden and ease of application. Our approach has been to not only collate information from current research and reviews of the literature, but to also integrate this with details from our own experiences that are absent from the original protocol and that have appeared necessary to conduct the TSST (e.g., administer a glucose drink to participants to account for inter-individual variation in baseline blood glucose levels). Our personal recommendations have all been in consultation with experts in the field (see Acknowledgements) and based on existing evidence and theoretical knowledge. In some instances, we have highlighted where large effect sizes for some elements, such as using a three-person (vs. two-person) panel (Goodman et al., 2017), may prove practically difficult to implement in real experiments and may therefore not be the best choice. Moreover, these introductory guidelines may also be purposefully manipulated to test certain outcomes. For example, one may wish to explore the impact of acute stress on cognitive functioning by administering a cognitive battery during baseline, in anticipation of stress, immediately post-stress and after a period of rest during the TSST (e.g., Olver et al., 2015). We anticipate that this introductory guide will assist in establishing a scientific standard that can improve the rigor and reliability of the reported stress response in human research. However, such a guide provides only a foundation and by no means represents binding guidelines that all researchers must adhere to.

In the next section, we discuss the core elements of the TSST. We also provide suggestions, where empirically supported and suitable for the individual research question, to consider when employing this protocol and adapting it to the individual study.

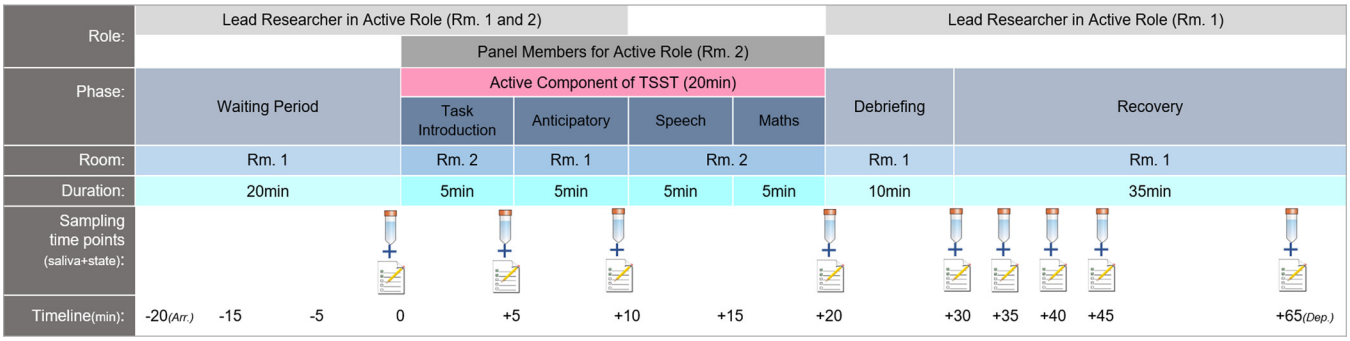

Fig. 1. An illustration of the various phases of a standardized TSST protocol. The top row (Researcher Roles) depicts the presence of each researcher for the separate Phases and Rooms of the TSST. The second row (Phase) depicts the separate phases involved in the TSST from beginning to end. Below the Phases, the Room row depicts where each of these phases takes place, i.e., Room 1 (Rm.1) being the waiting room, and Room 2 (Rm.2) being the TSST room. The Duration row includes the Duration, and depicts, in minutes, the length of each of the separate phases of the TSST. The red box in the duration row indicates the active components of the TSST and below this the TSST timeline is included. The TSST timeline is as per the original protocol (where time point 0 min indicates the onset of the active TSST, i.e., task instruction), with the participant arrival occurring at -20 min to TSST onset. As per the suggestions of this manuscript, the departure occurs at +65 min. Lastly, below the recommended TSST timeline, the figure includes the suggested timing of 9 sampling time points for cortisol and state psychological assessments, structured around the expected peak cortisol response.

5. A guide to conducting the TSST

In Fig. 1, we provide an overview of procedures for conducting the TSST. Briefly, a minimum of three investigators are required to run the protocol; one investigator to assume the role of the lead researcher who will oversee the entire protocol and remain with the participant from arrival to departure, along with two panel members who are present for the active component of the TSST. Two testing rooms are also required; see Supplementary material for illustrations.

The participant arrives to the TSST testing session 5 min prior to beginning the waiting (acclimation) period. The participant is introduced to the waiting room (Room 1) and its purpose throughout the laboratory session, and completes any required paperwork, after which the participant is directed to consume a glucose drink and rinse their mouth of any residue with a standardized allotment of water (100 milliliters). Immediately following this, the participant commences the waiting period (15 min until TSST onset; the 20-minute waiting period in Fig. 1 is inclusive of the 5 min for study particulars (e.g., arrival and consent), along with 15-minute acclimation). At time point 0, the TSST onset, the participant is led to the second room (Room 2) to begin the 5-minute task introduction period; during this time the participant enters Room 2 to face the awaiting panel (2 members). At this point, theyare introduced to the task they will complete via a standardized script read aloud by the lead researcher. The participant is then returned to Room 1 where they begin the 5-minute anticipatory stress period, during which they prepare their speech for the mock job interview as per the instructions provided in the task introduction (excess time remaining from the 5-minute task introduction period allows for transport to and from Room 1 and Room 2, and for completion of sampling during this period). The participant is then returned to Room 2 at +10 min where the lead researcher, while in view of the participant, begins the video recording. The researcher then proceeds to leave the room before the participant commences their 5-minute speech portion of the task (the active panel member will provide instructions to start their speech). This action of beginning the video recording, observed by the participant, is an important element of the TSST that largely contributes to the social evaluative component of the stress response. At the conclusion of the speech period (+15 min) the active panel member introduces the participant to the 5-minute surprise arithmetic task to be completed for the remaining 5 min of the active component of the TSST. At the conclusion of the arithmetic task (+20 min since TSST onset) the participant is returned to Room 1 for sampling, followed by a debriefing period, before then remaining in Room 1 for the recovery period.

It should be noted here that this paper and guide focus specifically on salivary cortisol sampling for measuring the biological stress response as it is a practical and cost-effective biomarker measurement, with minimal participant burden; for advantages of different biomarkers of the TSST response, see Allen et al. (2014). As per the original protocol (Kirschbaum et al., 1993), an alternative collection method to measure the physiological (cortisol) response to the TSST is blood sampling through venipuncture. However, in contrast to serum or plasma which allow only total cortisol measurement, salivary cortisol analysis represents the unbound biological active cortisol that is independent of flow rate and which lags behind serum levels, thereby presenting a better assessment of baseline cortisol that is not confounded by stress related to the environment, such as the venipuncture or meeting the researcher (Kirschbaum and Hellhammer, 1994). Given that the process of venipuncture can lead to increased psychological and physiological distress (i.e., from fear of the needle), a longer acclimation period, such as a 45-minute waiting period, may also be needed post venipuncture to allow for any heightened stress response to the venipuncture to return to baseline. That said, in their meta-analyses, Goodman et al. (2017) found that studies using intravenous catheters showed the highest cortisol stress response across all protocol variations. Thus, it is unclear if a longer waiting period is needed and whether the actual cortisol response is compromised. Regardless, the blood sampling time points for venipuncture can continue as per the recommendations outlined in Fig. 1.

In the following sections, we discuss suggestions for conducting the TSST and considerations for adapting and varying the components of the TSST.

5.1. Timing of administration

5.1.1. Time of day

The diurnal pattern of cortisol secretion in typically healthy adults has two components; a sharp increase in cortisol levels following awakening, known as the Cortisol Awakening Response (CAR) that is seen within the first hour post waking, and a subsequent decline in free cortisol concentration across the rest of the day (Edwards et al., 2001). Additionally, increases in cortisol are seen following the consumption of a meal (Kirschbaum et al., 1993), and a hypoglycemic state prior to the consumption of a meal has been associated with a decreased cortisol stress response (Davis et al., 1997). Given the fluctuations in normal (baseline) cortisol levels during the day, it is suggested that the TSST is conducted consistently at a set time that best reduces the systematic impact of these factors on the cortisol stress response to minimize inter-individual variation in baseline cortisol levels amongst participants.

With the expectation and understanding that baseline cortisol levels decrease as the day progresses, the afternoon has typically been regarded as the preferential TSST start time. Further, in consideration of post-meal cortisol increases and pre-meal hypoglycemia resulting in reduction in cortisol stress response, meal times are typically avoided (e.g., large meals should be avoided within one hour of the TSST). An opportune time window is after lunch and before dinner, which capitalizes on the natural low levels of cortisol concentration in the afternoon and avoids the influence of food consumption on cortisol levels and stress response. Recently, Goodman et al. (2017) determined in their meta-analysis that, for studies with TSST occurring in the A.M. period (morning: n = 17; Cohen's $d = 0.784$) or during the lunch-time period (12 pm – 2 pm: n = 30; $d = 0.811$), the cortisol stress response strength was slightly lower and exhibited more variability when compared to studies where the TSST took place between 2 pm −-5 pm (afternoon: n = 42; $d = 0.962$). In consideration of the above, the TSST should be conducted within a time period that factors in the diurnal pattern of cortisol secretion (e.g., avoiding the CAR period and periods of higher cortisol concentration at the beginning of the day/post-awakening) and that avoids pre-meal slumps in cortisol stress response and post-meal increases in cortisol levels. Other start times (e.g., late morning) may still be considered feasible alternatives, particularly for studies utilizing the TSST for purposes other than cortisol stimulation (e.g., sensor data such as galvanic skin response, blood pressure, heart rate, or psychological state data) but consideration should be paid to the pattern of cortisol secretion across the day and the impact of meals in proximity to testing. Ideally, timing of the TSST protocol should be kept consistent within a single experiment.

5.1.2. Waiting and speech anticipation time

External stressors unrelated to the individual's participation, or the participation in a research study itself, raise the possibility that participants may arrive at the laboratory in an already anxious state, such as from rushing to get to their appointment on time. To avoid elevated baseline cortisol levels prior to the TSST, study protocols typically include a waiting or acclimation period. Allowing time for a participant to acclimatize and recover from prior stressors, is necessary in facilitating a return to baseline (pre-study) cortisol levels (Dickerson and Kemeny, 2004).

The original protocol (Kirschbaum et al., 1993) outlined a participant acclimation period of 10 min (or 30 min for blood sample), with a speech anticipation period of 10 min. The Goodman et al. (2017) meta-analysis showed that studies with a 16–30 minute acclimation period had the highest effect size (n = 67; $d = 0.966$), but this decreased as

the waiting durations increased to 31–60 minutes (n = 65; d = 0.889) and beyond 60 min (n = 33; d = 0.799). As it stands, the ability of the TSST to produce cortisol stress responses has proven to be robust and ideally the overall protocol should aim to minimize participant burden and improve logistic operations and convenience. As such, it is suggested the participant should arrive 20 min prior to TSST onset (time point 0). This allocation allows 5 min for appropriate introduction and set-up, accompanied by a 15-minute acclimation period.

Regarding the speech anticipation period, the meta-analysis (Goodman et al., 2017) revealed similar mean effect sizes across studies with 3, 5, or 10-minute periods (*d's* = 0.891, .904, and .947, respectively). This is despite the original protocol allowing 10 min (Kirschbaum et al., 1993). Given the comparable effects, the speech anticipation period is one TSST element that can be kept relatively short to reduce the overall burden and improve logistics (Goodman et al., 2017). A 5-minute speech anticipation period may be most ideal to allow adequate time for any sampling to occur, while also providing sufficient preparation time for the participant.

5.1.3. Reduction in overall protocol time

The original TSST protocol operated across a range of 60–120 minutes, dependent upon the number of cortisol sampling collections and type of sampling, e.g., blood or saliva (Kirschbaum et al., 1993). In Fig. 1, we present a TSST protocol with a testing period of 85 min that we believe is the shortest and most practical length. This is inclusive of: arrival and acclimation (5-minute arrival; 15-minute acclimation), task introduction (5 min), speech anticipation (5 min), speech portion (5 min), arithmetic portion (5 min), debrief (10 min), and recovery (35 min). These time periods are consistent with the original protocol, except for the length of time for the task introduction period, which had no specified time. Here, we propose a standardized length of time of 5 min for the introduction period for consistency across studies. In addition to the task introduction itself, this 5-minute allocation allows timefor the participant to walk between the two testing rooms (i.e., participant goes to Room 2 to receive the task introduction after which they return to the initial waiting room), address any questions or concerns, and complete any required sampling before commencing their 5-minute speech anticipation period.

The most time-dependent element is the number of biomarker sampling points throughout the protocol. Peak salivary cortisol levels are reported to occur 10 min post cessation of the active stress, i.e., 10 min post conclusion of the surprise arithmetic task (Kirschbaum et al., 1993). Results from the Goodman et al (2017) analyses determined that the highest cortisol levels typically occurred between 35–45 minutes post TSST onset. In light of the peak salivary cortisol levels observed in the original study (Kirschbaum et al., 1993) and current evidence from the meta-analysis (Goodman et al., 2017), frequent saliva sampling from time point 30–45 minutes after TSST onset (i.e., minimum of 4 collections; see Fig. 1) is suggested to capture the full extent of the salivary cortisol response post-acute stressor.

Following the gradual peak in cortisol post-TSST, a gradual decline in cortisol levels is expected. The recovery period of 35 min, which includes samples from 30 to 65 minutes after the onset of the task (Fig. 1), is enough time for the peak cortisol stress response to be captured and for the cortisol levels to begin returning to baseline levels. However, if a complete return to baseline (pre-stimulation basal diurnal levels) values of cortisol is of particular interest, then the recovery period should be extended to at least 60 min (i.e., 90 min post onset of the TSST), resulting in an overall testing period of 110 min (Kirschbaum et al., 1993).

- ***Summary:*** An afternoon (e.g., between 2 pm −5 pm) scheduling of the TSST may minimize inter-subject fluctuations in baseline cortisol levels. Other times may be viable, so long as researchers factor meal consumption and patterns of cortisol secretion into the chosen timing of administration of the TSST. Moreover, a 20-minute pre-TSST onset arrival is suggested, allowing for 5 min of introduction and set up, followed by an acclimation period of 15 min. A 5-minute speech anticipation period is suggested. For studies using saliva sampling, the protocol length may be reduced to 85 min total duration to reduce participant burden while capturing the peak cortisol stress response levels and some recovery. Should capture of return to basal diurnal levels of cortisol be desired, the duration of the recovery phase should be increased from 35 to 60 min resulting in 110 min total duration. Timing of the protocol should be kept consistent within a single experiment.

5.2. Participants

5.2.1. Physiological and environmental factors

As previously stated, considerable intra- and inter-individual variations in the psychobiological stress responses to the TSST have been observed due, in part, to a range of environmental and physiological factors. These factors should be considered when screening participants for the TSST and on the day of the TSST. Age and gender are known factors influencing the stress response, with the impact of genetic and cultural factors also requiring consideration (Allen et al., 2014). Sleep cycles may also impact on salivary cortisol results, therefore deviations from the expected sleep routine, such as night shift or insomnia, should be screened for. Some medications (e.g., oral steroids) also result in blunted or false salivary cortisol results and should be excluded from sampling.

Also as previously noted, in addition to the normal diurnal variation, cortisol levels in the body are known to rise after each meal (Follenius et al., 1982; Quigley and Yen, 1979). Therefore, participants should avoid consuming substantial meals/food or beverages other than water in the hour leading up to the TSST. Additionally, no vigorous exercise should occur within the hour prior to the TSST, as there is evidence showing that moderate to high intensity exercise can provoke an increase in circulating cortisol levels (Hill et al., 2008). A record of these details should be kept for each participant so that potential variations may be accounted for where relevant.

5.2.2. Menstrual cycle and sex hormones

Research has shown large gender variability in the salivary cortisol response to acute stressors, including in response to the TSST, when no appreciable differences in cortisol levels were noted in the pre-stress levels between genders (Kudielka et al., 2009). This is because sex hormones differentially affect salivary cortisol response (Lennartsson et al., 2012; Stephens et al., 2016).Specifically, for healthy adults the salivary cortisol response to acute stress in the laboratory is significantly larger for men than women (Earle et al., 1999; Lovallo et al., 2006; Nicolson et al., 1997; Seeman et al., 2001; Steptoe et al., 1996).

Moreover, there is also evidence of female menstrual cycle phase significantly influencing cortisol levels to psychosocial stressors. As evidenced by Kirschbaum et al. (1999), women completing the TSST during the luteal phase of their menstrual cycle (between 14 to 28 days since the first day of their last menstruation of a regular 28-day cycle), produced salivary cortisol responses comparable to males, while women in the follicular phase or women using hormonal contraceptives exhibited significantly lower salivary cortisol responses (Kirschbaum et al., 1999). The same study also found that women on oral contraceptives produced lower cortisol response to the TSST (Kirschbaum et al., 1999). Others found that, compared to women in their follicular phase of their cycle, those in the luteal phase showed significantly higher cortisol levels following the TSST-VR (Montero-Lopez et al., 2018), although this finding was in contrast to that of another study (Maki et al., 2015).

It would therefore be ideal to control for contraceptive use and menstrual cycle phase in experiments involving women. Future studies need to determine which phase of the menstrual cycle make women more comparable to men, although some evidence is suggesting that

this is the luteal phase (Kirschbaum et al., 1999). For an in-depth review of the potential influences of gender, endogenous sex steroids, menstrual cycle phase, oral contraceptives and corticosteroid binding globulin on the salivary cortisol response to stress, see Kudielka and Kirschbaum (2005) and Villada et al. (2017).

- ***Summary:*** It is recommended that females are free from contraceptives and tested during the same menstrual cycle phase, e.g., luteal phase. However, this may be manipulated depending upon the research question. At a minimum, a record of menstrual cycle phase and of any hormonal contraceptives taken at the time of the TSST should be kept. Moreover, determining the timing of phases in female participants can be difficult and thus researcher may want to consider using a urine luteinizing hormone kit as a more objective measure.

5.3. Salivary cortisol sampling

5.3.1. Collection time points

Across TSST studies, there are large variations in both the number of saliva samples collected and the time points at which these samples are collected. The profile of the cortisol response from the stressor indicates a gradual rise, rather than a peak, that is followed by a gradual fall in cortisol values (Dickerson and Kemeny, 2004). The original TSST paper reported peak salivary cortisol levels at time-point +30 min post the onset of the TSST (i.e., time-point 0 being the task introduction), therefore 10 min after the cessation of the arithmetic task (Kirschbaum et al., 1993). Results from the Goodman et al. (2017) analyses determined that the highest cortisol levels typically occurred between 35–45 minutes post the onset of the TSST, therefore 15–25 minutes after the cessation of the arithmetic task. Considering this, frequent sampling between 30 and 45 min after TSST onset is suggested (e.g., +30; +35: +40; +45; see Fig. 1) to ensure the full extent of the salivary cortisol response post-acute stressor is captured.

In their meta-analysis (Goodman et al., 2017), the majority of TSST studies collected an average of 4.1 samples in the 30 min after the cessation of the arithmetic task. The total number of samples collected depends on the research question and needs to consider cost and participant burden. In Fig. 1, a maximum of 9 samples is depicted to illustrate potential sampling time points to capture baseline cortisol level, gradual cortisol stress response, and a return to baseline. Alternatively, as little as 2 samples may suffice for some research questions (e.g., a manipulation check in a cognitive study). Where the profile of cortisol response is of interest, we suggest 5–6 samples at a minimum, including one collection at pre-stress post acclimation baseline (time point -5), 3–4 collections occurring after the TSST onset to best capture the gradual rise and fall of the peak cortisol response (+30, +35, +40, +45), and a final recovery collection (~ +90 min post TSST onset) to capture a return to baseline cortisol levels.

5.3.2. Salivary collection method

There are various commercially available methods for collecting saliva that include passively drooling saliva into a tube, or absorbing saliva onto a device such as an oral swab (e.g., SalivaBio swabs from Salimetrics) (Rohleder and Nater, 2009) or a salivette (Sarstedt). These collection methods are minimally invasive, do not require specialized skills, and are convenient for repeated collections. For the TSST, the absorption method (e.g., using the commercially available synthetic oral swabs) is recommended as both a hygienic and a practical tool that comes with greater ease of use for multiple collections as long as only a small volume of saliva is of interest, i.e., to analyze cortisol levels only. Participants are required to place the oral swab in the mouth positioned under the front of the tongue, which is then left there for 1–2 min to absorb the saliva. Saliva volumes are then extracted by centrifuge.

However, where additional analytes are of interest, the passive drool method (e.g., SalivaBio, Salimetrics) will be more appropriate as it collects a larger volume of saliva. This method is approved for use with nearly all analytes and is considered the gold-standard for collecting whole saliva. It requires participants to passively pool saliva at the bottom of the mouth and expel this into a collection device, such as a plain tube with or without a straw.

For the measurement of cortisol specifically, both the absorption and the passive drool methods have been widely used. However, there is evidence that the collection method significantly influences the accuracy of cortisol measurement in saliva (Gallagher et al., 2006). The use of the absorption method with salivettes has been found to be a more reliable predictor of total and free serum cortisol than passive drool (Poll et al., 2007). In the latter study, both participants and technical staff also preferred the absorption method for saliva collection. Of note, salivettes produced lower cortisol concentration levels compared to the passive drool in the latter study (Poll et al., 2007), which is in line with evidence that cotton can reduce cortisol levels and create random errors (Shirtcliff et al., 2001; Strazdins et al., 2005). There are also variations in analyte levels depending on the positioning of the absorbent device that may result in collection of localized saliva rather than whole saliva (Granger et al., 2007).

Our recommendation of using oral swabs (e.g., SalivaBio; Salimetrics) for the multiple collection time points in the TSST relates to this method providing potentially quicker collection (as only a small volume is required) and because oral swabs are synthetically made from a non-toxic inert polymer, to eliminate the variable and inconsistent results demonstrated through the use of a biologic material, such as cotton (Shirtcliff et al., 2001). Researchers may also opt to conduct pre- and a post-TSST saliva collections using the passive drool method to provide a larger volume suitable for additional analytes, such as measurement of other hormones or metabolites, over only two sampling time points. Ultimately, care should be taken when comparing studies that used different salivary cortisol collection methods. It is vital that all studies carefully report the collection information, such as the method of collection including the type of swabs, location of these in the mouth, and duration of collection.

5.3.3. Glucose drink and baseline saliva

Details about the inclusion of a glucose drink in the TSST protocol were omitted from earlier descriptions of the protocol including the original study (Kirschbaum et al., 1993). This methodological detail has not been explicitly outlined in any of the previously published TSST protocols (Birkett, 2011; Kirschbaum et al., 1993). The inclusion of the glucose drink has only been included in studies where the researchers were intuitively aware of this addition and its purpose (e.g., through word of mouth). Glucose loading prior to the TSST is thought to account for inter-individual variation in baseline blood glucose levels prior to testing, which aids in the production of a reliable cortisol response curve when compared to water (Kirschbaum et al., 1997).

Evidence suggests that the response of the HPA axis is closely related to the physiological systems that are responsible for caloric movement and energy availability within the body (Dallman et al., 1993). To explore the impact of glucose levels and subsequent caloric loading on the free cortisol response to acute stress, Kirschbaum et al. (1997) manipulated the blood glucose levels of participants prior to inducing acute psychosocial stress. For healthy individuals with low glucose levels, an inhibited adrenocortical response was observed to the TSST, while those with high blood glucose levels displayed the expected doubling of the amount of free cortisol in the system following the stressor (Kirschbaum et al., 1997). Thus, readily accessible energy, achievable through the recommended glucose loading prior to stimulation, is recommended as a pre-requisite for a strong HPA stress response (Kirschbaum et al., 1997), in line with animal studies (Akana et al., 1994; Hanson et al., 1994). Depending on the research question however, some researchers may opt to omit the glucose drink, for example, if there is a specific research question about normal/baseline variations between certain groups of interest. Following consumption of

the glucose drink (to be administered at the beginning of the waiting period), it is imperative that participants use a standardized allocation of water to rinse their mouths and remove any glucose drink residue to avoid interfering with saliva sampling; see Supplementary materials.

- ***Summary:*** The number of samples to be collected is dependent upon the specific research question and the affordability of sampling. The proposed 9 collections represents an optimal number of samples to capture the full cortisol response profile. Oral swabs are practical and relatively easier than other methods for multiple cortisol collections, but are limited in saliva volume, and may need to be supplemented with passive drool sampling (e.g., at two sample time points, pre- and post-TSST). Careful adherence to procedures to avoid contamination of samples must occur, along with reporting in future studies of detailed descriptions of the collection methods used.. The inclusion of glucose loading via a glucose drink needs to be considered in light of the research question.

5.4. Panel arrangements

5.4.1. Age, sex and number of panel members

Since the protocol's conception in 1993, there have been a number of variations to the social interaction component of the TSST. Specifically, there have been variations in the number,age and gender composition of the panel members, the age and sex matching of the active panel member to the participant, and the level of interaction between the panel members and the participant.

Regarding the gender composition of the TSST panel, psychological and neuroendocrine studies suggest that interactions with the opposite gender result in increases in anxiety and discomfort for both men and women (Chorney and Morris, 2008; McCubbin et al., 1991). In relation to the TSST, both men and women (in the follicular phase) present with greater cortisol increases from the acute stressor when exposed to panel compositions that include the opposite sex (Duchesne et al., 2012). Meta-analyses revealed that an all-female panel resulted in one of the lowest effect sizes for cortisol stress response strengths (n = 11; d = 0.547), with a mixed-gender panel eliciting cortisol stress responses of a very large effect (n = 146; d = 0.975) (Goodman et al., 2017). Although there was no evidence on an all-male panel, a mixed-gender panel may be the best option given the known evidence of heightened reactivity in men and women to the opposite gender.

What it is as yet unclear is whether the active panel member needs to be gender-matched, cross-gender matched, or randomly allocated. The majority of TSST studies have not reported this information (Goodman et al., 2017). Given the evidence that men and women have heightened reactivity, such as cortisol increase, when presented with the opposite gender, it may be feasible to suggest that the active panel member is cross-gender matched (e.g., male participant and female active panel member). However, strong recommendations regarding the gender orientation of the active panel member cannot be made at this point as more research is required. It is recommended that future research studies explicitly report such details. This is particularly important as there are significant differences in the reactivity of men and women's stress responses during social interactions (Verma et al., 2011) that requires further examination.

Although there is evidence of ageing effects on cortisol reactivity to an acute stress, such that older adults respond with lower cortisol responses to the TSST (Hidalgo et al., 2015), few studies comment on the age of panel members. To date, there is no evidence on the potential impact of the age of panel members on participants' cortisol reactivity during the TSST. It is recommended future research records the ages of panel members to determine whether thismay impact the observed cortisol stress response. Given evidence of own-age bias in face processing (Ebner and Johnson, 2010), it is possible that age concordance between participant and panel members (e.g., young participant and young panel members) may result in relatively lower cortisol reactivity compared to an age discordance (e.g., young participant and old panel members). However, this remains to be empirically tested.

Regarding the number of panel members, the original TSST protocol recommended three members (Kirschbaum et al., 1993). However, of the 186 studies analyzed by Goodman et al. (2017), large effects were obtained in studies that utilized two-member panels (n = 153; d = 0.891). Althoughlower, these effect sizes were relatively similar to those reported for three-member panels (n = 32; d = 1.064). Whilea three-member panel may be induce a somewhat stronger cortisol stress response, it is more labor-intensive as it requires significant coordination in obtaining volunteers for a three-member panel for each participant that also adheres to the mixed-gender panel composition. Thus, a less burdensome two-member mixed-gender panel may be better suited to most research environments.

5.4.2. Demeanor of the panel member

In the active part of the TSST, the experiences of uncontrollability and social-evaluation are elicited through a mock job interview that is followed by a surprise arithmetic task in front of a panel. The behavior of the panel towards the participant is a key element for contributing to a participant's feelings of uncontrollability and threat to self. A Dickerson and Kemeny (2004) meta-analytic review of over one hundred stress studies, determined that methods that incorporate elements of uncontrollability and threat to the social self and self-esteem (i.e., social-evaluative threat) exhibit the greatest efficacy for inducing significant increases in cortisol stress responses, with these elements incorporated into a number of psychosocial stress paradigms, including the TSST. The significance of these factors was subsequently tested when a control (placebo) version of the TSST was introduced by Het et al. (2009). The placebo protocol was similar to the original TSST protocol but with the removal of uncontrollability and social-evaluative threat (i.e., no panel members and no video recording), resulting in a significantly reduced salivary cortisol response (Het et al., 2009).

As per the original protocol (Kirschbaum et al., 1993), the recommendation remains that all panel members present with a strictly neutral demeanor towards the participant during the active component of the TSST (speech and arithmetic tasks) and take on the role of evaluating the participant's performance without the provision of any signs or indications of social support or cues. This neutral demeanor of the panel members contributes to the participant's experience of *uncontrollability* and *threat to the social self and self-esteem*, and to the successful activation of the HPA and sympathetic nervous system (e.g., Kirschbaum et al., 1999).

Meta-analyses of 131 studies determined that cortisol stress responses were significantly lower for studies in which the panel explicitly provided negative feedback to participants (n = 24, d = 0.713) as opposed to neutral feedback (n = 107, d = 0.869) (Goodman et al., 2017). It is possible that the ambiguity of a neutral expression contributes to a higher cortisol stress response. We commonly deal with positive feedback and interactions in daily life, and have become accustomed to handling negative feedback or criticism (Swann et al., 1992). It is additionally important that panel members ensure they do not unknowingly display negative mannerisms to the participant. Thus, panel members should be engaged with the participant, such as by showing interest in the participant's speech, but should notpresent a stone-cold manner that could be interpreted as negative.

5.4.3. Appearance

For consistency and to emulate a professional interview environment, it is suggested the panel members and lead researcher wear white lab coats, or work wear that matches the environment, throughout the entire protocol (except during the debriefing period when the lab-coats are to be removed to encourage a greater reduction in stress levels in the participant). Panel members and the lead researcher should also wear neat professional dress, with appropriate footwear, especially where feet are visible from underneath the panel's table in order to

maintain the professional environment.

5.4.4. Panel familiarity

To maintain a convincing interview environment and uphold the integral theme of uncontrollability and social evaluation, participants should be unfamiliar to the panel members. This is particularly relevant in teaching environments where participating students may know the research staff on the panel (e.g., if staff on the panel are also teaching or administrative staff).

- ***Summary:*** A two-member panel that is gender-balanced is ideal. Details of the age of the panel members and whether the gender of the active panel member was matched to the participant should be reported for future studies to consider. All panel members are to remain neutral (but not negative) towards the participant across the active component of the TSST. The panel members are also to wear professional clothing and should be unknown to the participant.

5.5. Assessments during the TSST

Participants are often required to complete several assessments (task-based and questionnaires) at baseline, prior to the commencement of the TSST. Meta-analyses revealed that the completion of assessments, such as questionnaires, during the pre-TSST acclimation period produced a slightly lower stress response effect size (n = 109; $d = 0.823$) when compared to a waiting period with no such activity (n = 49; $d = 1.031$) (Goodman et al., 2017). The number and type of assessments administered pre-TSST, and the potential for these assessments to induce stress in the participant and impact baseline cortisol levels, should be considered as these factors could reduce the overall stress response.

Interestingly, when the state version of the State Trait Anxiety Inventory (STAI; Spielberger et al., 1983) was administered during the speech anticipation period (n = 42; $d = 0.846$), effect sizes for cortical stress responses were comparable to when no questionnaires were given during this period (n = 62; $d = .849$) (Goodman et al., 2017). Therefore, completing assessments at a later stage, such as during the speech anticipation period rather than the waiting period, may have the least amount of influence on the cortisol stress response. We suggest that an ideal time to administer a brief psychological assessment (e.g., the STAI) would be at the end of the acclimation period or immediately following the task introduction, but before the speech anticipation period.

These state assessments could also be repeated during the post-test recovery period to provide a measure of changes in state anxiety across the duration of the TSST. Additionally, included alongside each cortisol sampling time-point, a brief state assessment may be repeatedly administered in order to provide a subjective assessment of the participant's experience during the TSST. For example, participants can be asked to rate the way they feel at the time using a short visual analogue scale assessing dimensions of happiness, sadness, tiredness, anxiety and withdrawal; see Supplementary material for an example of a visual analogue scale.

The type and number of assessments to be administered during the TSST is at the discretion of the researcher and relates to the specific research question, but these should be considered in light of the potential burden of any assessments on the participants. Assessments with minimal demands and impact on mood, such as brief visual analogue scales (Aitken, 1969), may be preferred over those in which sensitive information, such as traumatic life events, is assessed.

- ***Summary:*** If assessments are utilized pre-TSST, these should be administered towards the end of the acclimation period or alternatively immediately following the task introduction prior to the speech anticipation phase. Selected assessments should involve minimal demands and have little impact on the mood of the participant but may vary depending on the research question. A subjective assessment of the participant's experience during the TSST should be collected.

5.6. Other psychobiological markers: Heart rate

In addition to cortisol measures, some researchers may be interested in obtaining other psychobiological markers of stress, such as heart rate and heart rate variability. Heart rate was measured in one of the studies included within the original TSST protocol (Kirschbaum et al., 1993); this was obtained while the participant was standing in front of the panel (this detail was unspecified in the original protocol). There is evidence to suggest that body position, such as prone, supine and sitting, significantly affects blood pressure and heart rate (Watanabe et al., 2007). For example, blood pressure was higher and heart rate was lower in the prone (vs. sitting) position. It is recommended that researchers interested in the recording of heart rate note the position of the participant during the active component of the TSST and collect a baseline measure of heart rate accordingly. For example, if participants are standing during the active TSST (speech and arithmetic task), it is ideal to also obtain an equivalent standing baseline heart rate measurement (e.g., for 10 min) that is collected during the acclimation period (pre-TSST). For a detailed description of psychobiological markers of stress other than salivary cortisol and heart rate discussed here, see Allen et al (2014).

- ***Summary:*** Other physiological markers of stress, such as heart rate, may be added to the TSST protocol.

5.7. Debriefing

At the conclusion of the speech and arithmetic task, the participant is returned to the waiting room where they are debriefed. During debriefing, the lead researcher informs the participant that the TSST is specifically designed to elicit psychobiological stress inside a laboratory environment, and that the speech and arithmetic task were intentionally difficult and do not in any way reflect their aptitude or ability. The prop elements such as the use of the camera should also be explained, along with an explanation that the panel was instructed to provide no feedback or encouragements during the task. Participants are also toldthat they are not actually evaluated or compared to others' performances on the task, with the actual measurement of interest being their physiological (e.g., cortisol) response. Participants are also given time to ask questions. Following the debriefing and questions, the panel members should be welcomed into the waiting room, with their lab coats removed, so that they may greet the participant. The latter allows for improved debriefing and recovery periods and a return to baseline in cortisol levels (see Supplementary material for additional details, such as a debriefing script).

- ***Summary:*** Debriefing is a crucial part of the TSST and should contain a detailed exchangebetween the participant and lead researcher, aided by the panel members, to ensure a thorough recovery process. This is also a vital component for managing the element of deception used in the TSST protocol.

6. Limitations

There are limitations to consider in this critical review and introductory guide. Firstly, we did not provide a comprehensive review of the TSST as such detailed descriptions of the key elements and related variabilities has been done elsewhere (e.g. Allen et al., 2014, 2017; Birkett, 2011; Goodman et al., 2017; Kudielka et al., 2007). Instead, we focused on developing and providing the most detailed step-by-step guide and laboratory manual for researchers to use to improve the administration and rigor of TSST research and to provide those unfamiliar with the protocol with significant details. As more research is

being conducted, it is likely that some of recommendations will need to be updated. For example, is it unclear whether the age of the panel members and other cultural factors may influence the cortisol stress response. Secondly, we only focused on the individual adult version of the TSST as it is the most commonly used version. Ultimately, detailed guides for other versions of the TSST will also be needed, and our current guide and recommendations provide the foundation for additional detailed protocols to be developed. Thirdly, we focused our attention on only salivary cortisol, with some mention of considerations for heart rate and venipuncture. We focused on salivary cortisol as it is the most reliable and practical biological marker for stress response to date. However, we acknowledge that there are additional biological markers (e.g., ACTH, vasopressin) and bodily systems (e.g., immune system, cardiovascular system, sympathetic adrenal-medullary system) that may be of interest, but inclusion of these was beyond the scope of this review and online guide. Relatedly, we did not cover in detail the use and role of subjective measures of stress, however such a measure is included in our guide in the Supplementary material. Finally, we acknowledge that the use of a consistent protocol across TSST studies is not enough to create more homogenous outcomes as various other factors will still create variability. One such factor is the various assaying techniques across laboratories, whether internally developed or commercial. Other variability may also come from sample characteristics, infrastructure (e.g., rooms), and questionnaires (e.g., in the waiting period). Yet, for researchers interested in conducting the TSST, this introductory guide will provide a useful and time-saving resource that may help improve consistency in the literature.

7. Conclusion

With this paper, we provide an overview of the TSST for adult participants and the use of salivary cortisol measures. Most importantly, we provide a detailed step-by-step introductory guide that presents the shortest possible protocol for TSST administration, taking into account participant burden and practicality (in the Supplementary material). We also provide guidance regarding adapting the protocol to the individual research question. This paper and introductory guide make an important contribution to stress research as the TSST remains one of the most widely used protocols for inducing acute psychobiological stress in humans. We hope to improve the research standards in the field and to ultimately advance understanding of how stress impacts everyday functions that have important implications for future health.

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Contributors

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Declaration of Competing Interest

All authors report no conflict of interest.

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Appendix A. Supplementary data

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