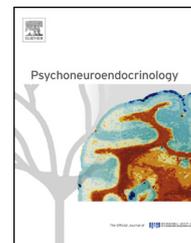




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Differentiating anticipatory from reactive cortisol responses to psychosocial stress

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Summary Most psychosocial stress studies assess the overall cortisol response without further identifying the temporal dynamics within hormone levels. It has been shown, however, that the amplitude of anticipatory cortisol stress levels has a unique predictive value for psychological health. So far, no “best practice” in how to investigate the anticipatory cortisol stress response has emerged. The goal of the current research was to develop a protocol that would allow for a sensitive and easy-to-implement laboratory-based investigation into anticipatory cortisol stress levels. We initially tested 26 healthy men in either an anticipation- or stress-only condition of the Trier Social Stress Test (TSST) to map the distinct timelines of anticipatory and reactive cortisol release profiles (study 1). Subsequently, we administered the TSST to 50 healthy men such that the cortisol responses to anticipatory and reactive stress components could be dissociated (study 2). In both studies we sampled saliva cortisol at high frequency (at baseline, during 10 min of anticipation and during and after 10 min of acute stress) and the current mood state pre- and post-stress. We found anticipatory responder rates of 20% and 40%, with peak anticipatory cortisol levels between 14 and 20 min after onset of anticipation. Visible changes in reactive cortisol levels occurred only after the termination of the acute stressor. We conclude that the best practice to detect a maximum number of anticipatory responders in the TSST would be to extend the anticipation phase to 15 min. In doing so, the anticipatory cortisol peak could be captured at a time-point of the actual stressor that is uninfluenced by reactive cortisol levels. Overall, we could reveal several features of anticipatory responders. Most importantly, there was a positive correlation between anticipatory and reactive stress responses. There was no association between anticipatory cortisol and alpha-amylase as well as subjective–psychological stress

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responses. Future studies will have to determine whether the anticipatory responders differ with respect to various stress-sensitive parameters like sex, personality, psychological wellbeing or chronic stress.

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1. Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is a major neuroendocrine stress system. Upon stimulation, levels of the HPA axis' final output product cortisol gradually increase until a peak is reached at 20–30 min after stressor onset. Cortisol levels fall back to baseline values within the following 90 min (Kirschbaum and Hellhammer, 2000). Psychosocial stress is a reliable trigger of the HPA axis in humans. A review of the literature shows that most psychosocial stress studies assess the overall cortisol stress response without further identifying the temporal dynamics within cortisol levels, i.e., anticipatory and reactive hormone surges are rarely distinguished (Dickerson and Kemeny, 2004; Foley and Kirschbaum, 2010).

However, previous studies have shown that the amplitude of an anticipatory cortisol stress response explains unique variance in psychological health. Specifically, early anticipatory rather than reactive cortisol stress responses were associated with early life adversity (Hardie et al., 2002), PTSD (Bremner et al., 2003), phobia (Alpers et al., 2003), resilience (Mikolajczak et al., 2008), alexithymia (de Timary et al., 2008) and depressive symptoms and aggression in children exposed to early peer victimization (Rudolph et al., 2010, 2011). Different studies pursued different strategies to investigate the anticipatory cortisol stress response, without a "best practice" approach emerging so far. Starcke et al. (2008) refrained from administering an acute stressor altogether. Studies interested in both anticipatory and reactive cortisol levels examined either naturalistic (Alpers et al., 2003) or laboratory-based (Hardie et al., 2002; Bremner et al., 2003; de Timary et al., 2008; Mikolajczak et al., 2008; Rudolph et al., 2010, 2011) stressors. In the naturalistic setting, participants are aware of the occurrence and nature of the impending stressor well in advance. There is hence ample time for the anticipatory stress response to fully develop. However, uncontrollable acute stressors may co-occur within the same time period and interfere with anticipatory stress. Since the anticipatory stress-sensitive participants already enter the experimental situation with elevated cortisol levels, the determination of a proper baseline for the reactive stress constitutes an additional problem. In the laboratory setting, only the response to short-term anticipation can be captured. However, by revealing the exact nature of the acute stressor just shortly prior to its onset, the occurrence of uncontrollable influences during the anticipation phase and differences in baseline hormone levels can be controlled for. Studies have not consequently exploited these advantages of laboratory-based stress induction. Often, baseline cortisol levels were utilized as a proxy of the unspecific anticipatory stress response to the testing situation per se, which implies that the source and timing of potential anticipatory stress remained unconsidered (Hardie et al., 2002; de Timary et al., 2008; Mikolajczak et al., 2008; Rudolph et al., 2010, 2011).

The goal of the current research was to develop a protocol that would allow for a sensitive and easy-to-implement laboratory-based investigation into the anticipatory cortisol stress response. To this purpose, we administered the Trier Social Stress Test (TSST; Kirschbaum et al., 1993) in such a way that the cortisol responses to anticipatory and reactive stress components could be dissociated. Levine and Coe (1985) have reported that it takes 7 min until detectable increases in stress-induced circulating cortisol levels occur. To validate this timeline for the current context (i.e., to obtain an indication as to when measurable changes in salivary cortisol levels following anticipatory and reactive stress could be expected), we initially tested 26 participants in either an anticipation-only ($n = 14$) or a stress-only ($n = 12$) TSST condition. Saliva cortisol was sampled at high frequency: at baseline, in 2-min intervals throughout anticipation (10 min), acute stress (10 min) and the following 12 min and in 10-min intervals thereafter. In a subsequent study, 50 participants underwent the complete TSST procedure (including 10 min of both anticipatory and acute stress), again using a high-frequency sampling procedure. Based on the timeline results of study 1, we could thus capture the anticipatory stress response and determine its interaction with the onset of reactive stress. We hypothesized to find two groups with distinct cortisol release profiles: Anticipatory responders with a physiologically relevant increase in cortisol levels early into the task (before the acute stressor could have triggered HPA axis activity) and reactive responders with a physiologically relevant increase in cortisol levels only after the acute stressor could have had a measurable effect. A physiological relevant increase in cortisol levels was defined as an elevation of at least 2.5 nmol/l over the individual baseline level, as defined previously (Van Cauter and Refetoff, 1985). Aiming to more closely characterize the anticipatory stress response, we compared additional stress markers (salivary alpha-amylase and subjective–psychological stress responses) between anticipation- and stress-only (study 1) and responder (study 2) groups.

2. Materials and methods

2.1. Participants

Male participants between 18 and 30 years of age were recruited by posting ads on the electronic billboard of the McGill University website. Women were excluded to avoid the confounding effects of hormonal status on cortisol levels (Kajantie and Phillips, 2006). Given a potential influence on cortisol and alpha-amylase activity, information about recreational drug use, medical and psychological history was assessed in a telephone interview. Regular recreational drug users (cannabis within the past two months, any other recreational drug within the past year), habitual smokers (more than five cigarettes per week) and individuals

reporting chronic illnesses (including current psychological disorders) or taking medication that might influence HPA axis activity were excluded from participation. Altogether, 26 participants (mean age 22.21 years; SD 3.31) were included in study 1 and 50 participants (mean age 22.48 years; SD 3.65) were included in study 2. All participants gave written informed consent. Both studies were approved by the McGill University Research Ethics Board. The data from study 2 has previously been analyzed and published from the perspective of covariance of stress-induced salivary cortisol and alpha-amylase release (Engert et al., 2011).

2.2. General procedure

Since cortisol secretion is characterized by a strong circadian rhythm (Dallman et al., 2000; Fries et al., 2009), testing was performed between 1300 h and 1700 h. To establish a common baseline and control for the pre-test exposure to food, stress and physical exercise, participants had a little snack upon arrival at the laboratory after which they refrained from eating or drinking anything but water for the remainder of their stay. Given the high-frequency saliva sampling, participants were encouraged to drink water ad lib. except during the acute 10-min stress phase.

All participants underwent a 30-min adjustment and a 10-min baseline phase. Subsequently, as is the usual TSST procedure, participants of study 2 were brought to the TSST room, where they were informed about the details of the upcoming task. The 10-min anticipation phase, which took place inside the TSST room, was followed by the 10-min stress phase. After the stress phase, participants were brought back to the resting rooms where they remained seated for the remaining recovery phase (Fig. 1). In study 1, participants of the anticipation-only condition were informed that no stress test would take place after the 10-min anticipation phase. For reasons of comparability, they were asked to wait for the designated time of 10 min in a separate room and subsequently brought back to the resting rooms for recovery. Participants of the stress-only condition were told about the upcoming test only on their way to the TSST room (approx. 1 min prior to stressor onset). Otherwise, all procedures in studies 1 and 2 were identical.

2.3. Trier Social Stress Test

Participants were exposed to the TSST (Kirschbaum et al., 1993), the most frequently administered psychological paradigm to stimulate an endocrine stress response in the laboratory setting. The TSST is a social evaluative and mentally

challenging task, which was shown to provoke a robust HPA axis stress response when compared to several other laboratory stressors (Dickerson and Kemeny, 2004).

2.4. Assessment and analysis of cortisol

Cortisol was sampled using the salivette collection device (Sarstedt Inc., Quebec City, QC, Canada) and stored at -20°C until analysis. In study 1, saliva samples were taken at baseline (at -20 and -10 min), throughout the 10 min of anticipation (at -8 , -6 , -4 , -2 and 0 min), in 2-min intervals during acute stress and the following 12 min (at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 min) and in 10-min intervals thereafter (at 30, 40, 50 and 60 min). In study 2, we skipped the -8 , -6 , -4 , -2 and the 12 min measures, but added one sample at 70 min (Fig. 1). We followed the same labeling convention in both studies, i.e., although there was no acute stress phase in the anticipation-only group, the 0 min measurement time-point indicated the beginning of what would have been the acute stress phase. As recommended for the collection of salivary cortisol and alpha-amylase (Rohleder and Nater, 2009), participants were instructed to place the saliva collection swabs in their mouths and to refrain from chewing for exactly 2 min. Cortisol concentration (calculated and expressed in nmol/l) was determined using a time-resolved fluorescence immunoassay (Dressendorfer et al., 1992), with intra- and interassay variabilities of less than 10% and 12%, respectively.

2.5. Assessment and analysis of salivary alpha-amylase

The salivary enzyme alpha-amylase has received increasing attention as a stress marker of the sympathetic nervous system (SNS) within the past ten years (reviewed in Nater and Rohleder, 2009). We sampled alpha-amylase from the same salivette collection devices as cortisol. Therefore, measuring time-points were identical. Alpha-amylase activity (calculated and expressed in U/ml) was determined using an enzyme kinetic method (Winn-Deen et al., 1988; Lorentz et al., 1999).

2.6. Assessment of the subjective–psychological stress response

The Profile of Mood States (POMS; McNair et al., 1971) was administered to assess the subjective–psychological response to acute stress. Using a list of 65 adjectives, the POMS targets six transient, fluctuating mood states: tension–anxiety, anger–hostility, fatigue–inertia, depression–dejection,

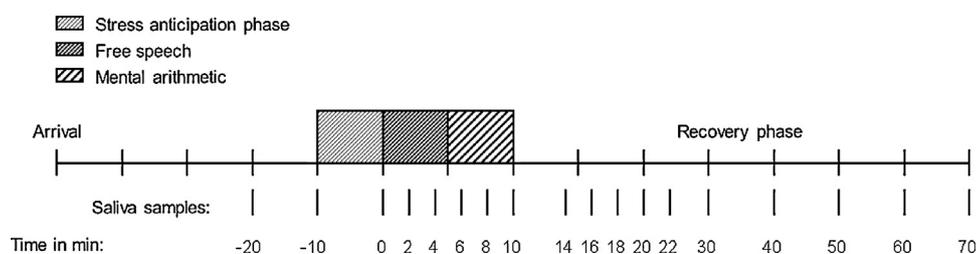


Figure 1 Timeline of the testing procedure in study 2.

vigor—activity and confusion—bewilderment. Participants reflected on their current state per adjective on a 5-item Likert scale ranging from “not at all” to “extremely”. In study 1, we attempted to assess the progression of mood changes at a high temporal resolution. Therefore, we created a mini version of the POMS which presented participants with only two adjectives per mood state (tense/anxious, depressed/unhappy, angry/hostile, confused/bewildered, vigorous/active, fatigued/worn-out) and took less than 30 s to complete. These items were administered at baseline (at –20 min), half-way through the anticipation phase (at –6 min), toward the end of anticipation/shortly before the onset of acute stress (at –2 min; immediately before taking participants to the TSST room), immediately after the termination of acute stress (10 min) and in 20 min intervals thereafter (at 30 and 50 min). In study 2, the complete POMS was administered at baseline (at –20 min) and shortly after the acute stressor (at 14 min).

2.7. Statistical analysis

All analyses were performed with the Predictive Analytics Software (PASW) version 17. In both studies, cortisol and alpha-amylase baseline levels were averaged from the –20 and –10 min samples. If applicable, violations of the assumption of sphericity were adjusted using the Greenhouse–Geisser correction. Significant effects were further investigated using Hochberg’s GT2 post hoc tests for unequal sample sizes and simple contrasts. Throughout all analyses, eta-squared (η^2) was used as an effect size estimate for one-way independent ANOVAs, partial eta-squared (η_p^2) for repeated-measures ANOVAs and Cohen’s *d* for pair wise post hoc comparisons. Log transformations were applied to correct for the positive skew in the cortisol and alpha-amylase data. All figures display original data.

To test for between group differences in age, body mass index (BMI) and the average baseline cortisol and alpha-amylase levels, one-way independent ANOVAs with the between subjects factor group were calculated for both studies.

2.7.1. Comparison of exclusively anticipatory and reactive stress profiles (study 1)

To investigate differences in the timelines of anticipatory and reactive cortisol release profiles, we calculated two-way mixed ANOVAs with the within subjects factor measurement time-point (average baseline, –8, –6, –4, –2, 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 30, 40, 50 and 60 min) and the between subjects factor group. Likewise, two-way mixed ANOVAs were performed to compare the alpha-amylase and subjective–psychological stress responses between groups.

2.7.2. Comparison of anticipatory, reactive and non-responder cortisol stress groups (study 2)

Based on the timeline results of study 1, participants were initially divided into two groups of either anticipatory or reactive cortisol stress responders (depending on the time window during which they displayed a stress-induced cortisol release of at least 2.5 nmol/l over their individual baseline level). Given a substantial number of participants who failed

to display this stress-induced cortisol release of at least 2.5 nmol/l over their individual baseline level altogether, a third group of non-responders was added. As in study 1, we examined group differences in cortisol, alpha-amylase and subjective–psychological response profiles by calculating two-way mixed ANOVAs with the within subjects factor measurement time-point (mean baseline to 70 min for cortisol and alpha-amylase; pre- and post stress of the respective POMS state for mood) and the between subjects factor group.

2.7.3. Relationship between anticipatory and reactive cortisol stress responses (study 2)

The anticipatory cortisol stress response was operationalized by subtracting the individual peak anticipatory from the baseline cortisol levels. Likewise, the reactive stress response was operationalized by subtracting the individual peak reactive from the baseline cortisol levels. To investigate the relationship between anticipatory and reactive cortisol stress responses, we calculated a linear regression using the anticipatory cortisol stress response as predictor and the reactive cortisol stress response as dependent variable.

3. Results

In study 1, age ($F_{(1, 22)} < .01, p > .95$), BMI ($F_{(1, 23)} = .05, p > .80$), baseline cortisol ($F_{(1, 24)} = .19, p > .65$) and alpha-amylase ($F_{(1, 24)} = .56, p > .45$) levels did not differ between anticipatory- and stress-only groups. In study 2, age ($F_{(2, 47)} = .82, p > .40$), BMI ($F_{(2, 47)} = .86, p > .40$) and baseline alpha-amylase levels ($F_{(2, 48)} = .19, p > .80$) did not differ between cortisol stress responder groups. There was a significant between-group difference in baseline cortisol levels ($F_{(2, 47)} = 3.82, p = .029, \eta^2 = .14$). Post hoc tests showed that the anticipatory responders exhibited higher baseline cortisol than the non-responders ($p = .024, d = 1.15$). The difference between anticipatory and reactive responders showed the same direction but did not reach statistical significance ($p = .16, d = .74$).

3.1. Comparison of exclusively anticipatory and reactive stress profiles (study 1)

A two-way mixed ANOVA revealed a time main effect ($F_{(19, 456)} = 9.75, p < .001, \eta_p^2 = .29$) and a time by group interaction ($F_{(19, 456)} = 4.28, p = .007, \eta_p^2 = .15$) for cortisol release. There was no group main effect ($F_{(1, 24)} = .01, p > .95$) (Fig. 2). Simple contrasts showed that the groups differed from each other in their changes from baseline between 12 and 20 min after the onset of anticipation/2 and 10 min after the onset of acute stress (*p* values ranged between .042 and .001; η_p^2 values ranged between .16 and .35). It can be taken from Fig. 2 that anticipatory cortisol levels peaked at 20 min after the onset of anticipatory stress (with a 101% increase in cortisol levels from baseline). Reactive cortisol levels accordingly peaked 10 min later, i.e., at 20 min after the onset of acute stress (with an 87% increase in cortisol levels from baseline). The anticipation-only condition showed an obvious trend for a cortisol increase from baseline (by .35 nmol/l; 14%) at 0 min (10 min after the onset of anticipation and at the designated time-point of acute stressor onset). In the stress-only condition, no cortisol increase from baseline was

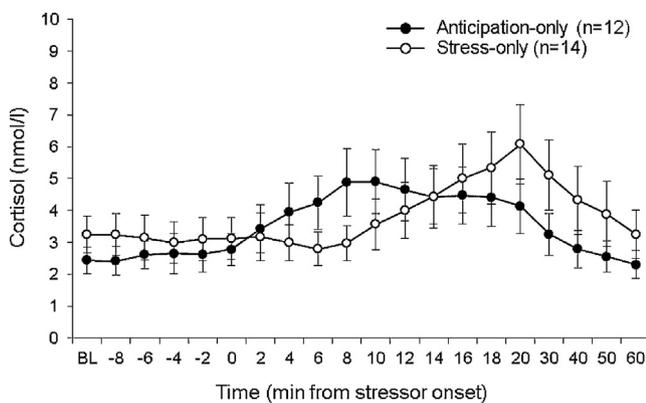


Figure 2 Means and standard errors of cortisol levels in anticipation- and stress-only groups (original data). There was a time main effect ($F_{(19, 456)} = 9.75, p < .001, \eta_p^2 = .29$), a time by group interaction ($F_{(19, 456)} = 4.28, p = .007, \eta_p^2 = .15$) but no group main effect ($F_{(1, 24)} = .01, p > .95$). Groups differed in their changes from baseline between 12 and 20 min after the onset of anticipation/2 and 10 min after the onset of acute stress (p values ranged between .042 and .001; η_p^2 values ranged between .16 and .35).

detected until including 8 min into the TSST. Only at 10 min after onset of acute stress (at its termination, actually), cortisol levels had increased from baseline by .33 nmol/l (10%). Considering both the current timeline results and Levine and Coe's (1985) finding of a 7-min threshold until detectable increases in stress-induced circulating cortisol levels occur, we determined the following criteria for the classification of anticipatory and reactive cortisol responder groups: Anticipatory responders display a stress-induced cortisol release of at least 2.5 nmol/l over their individual baseline level (Van Cauter and Refetoff, 1985; Schommer et al., 2003) between measurement time-points 0 (10 min after the onset of anticipation/immediately before the onset of acute stress) and 6 (16 min after the onset of anticipation/6 min after the onset of acute stress). Reactive responders display a stress-induced cortisol release of at least 2.5 nmol/l over their individual baseline level after measurement time-point 8 (18 min after the onset of anticipation/8 min after onset of acute stress). Applying these classification criteria, we detected 6 anticipatory responders in the anticipation-only group.

Regarding alpha-amylase, a time main effect ($F_{(19, 437)} = 4.93, p < .001, \eta_p^2 = .18$) and a time by group interaction ($F_{(19, 437)} = 5.50, p < .001, \eta_p^2 = .19$) but no group main effect ($F_{(1, 23)} = .34, p > .55$) were found (Fig. 3). Simple contrasts showed a different picture than for cortisol: Anticipation- and stress-only groups differed significantly in their changes from baseline at 6 min after the onset of anticipation (at measurement time-point -4 min) and between 16 and 22 min after the onset of anticipation/6 and 12 min after the onset of acute stress (p values ranged between .040 and .006, η_p^2 values ranged between .17 and .29). Fig. 3 shows that the anticipatory peak occurred at 12 min after the onset of anticipation (with a 25% increase in alpha-amylase levels from baseline). Reactive alpha-amylase levels peaked at 6 min after the onset of acute stress (with a 91% increase

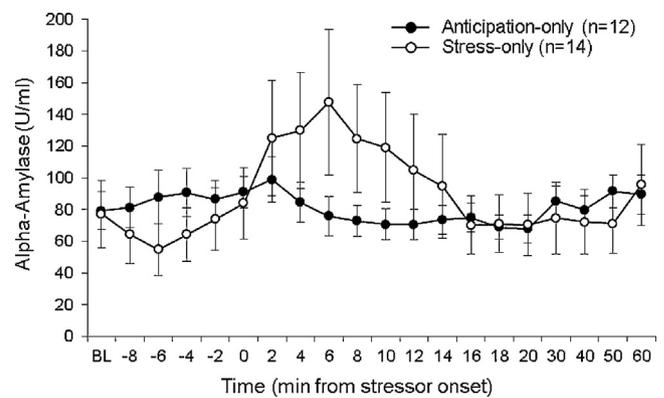


Figure 3 Means and standard errors of salivary alpha-amylase levels in anticipation- and stress-only groups (original data). There was a time main effect ($F_{(19, 437)} = 4.93, p < .001, \eta_p^2 = .18$), a time by group interaction ($F_{(19, 437)} = 5.50, p < .001, \eta_p^2 = .19$) but no group main effect ($F_{(1, 23)} = .34, p > .55, \eta_p^2 = .01$). Groups differed in their changes from baseline at 6 min after the onset of anticipation and between 16 and 22 min after the onset of anticipation/6 and 12 min after the onset of acute stress (p values ranged between .040 and .006, η_p^2 values ranged between .17 and .29).

in alpha-amylase levels from baseline). Consequently, groups did not differ at the time-point of peak anticipatory alpha-amylase release. It was mainly the stress-only group that drove the time by group interaction.

Regarding the subjective–psychological stress response, two-way mixed ANOVAs revealed overall and group-specific changes in mood over time. Simple contrasts showed that the anticipation-only group exhibited an increase in tension–anxiety at -2 min (shortly before acute stressor onset). The stress-only group exhibited increases in tension–anxiety, fatigue–inertia, depression–dejection and confusion–bewilderment at 10 min (immediately after the termination of acute stress) (see Table 1 for a summary of these results). To follow up on the relationship between cortisol and subjective–psychological stress responses in the anticipation-only group, the bivariate correlation between the maximum increases in cortisol (operationalized as the difference between peak and baseline cortisol levels) and tension–anxiety (operationalized as the difference between -2 min and baseline tension–anxiety levels) was calculated using the Pearson correlation coefficient. There was no significant association ($r = .039, p > .85$).

3.2. Group assignment (study 2)

Using our above determined classification criteria, we detected 10 anticipatory responders and 21 reactive responders. 19 participants who failed to display a stress-induced cortisol release of at least 2.5 nmol/l over their individual baseline level throughout the entire experiment were pooled in a group of non-responders. The 10 anticipatory responders were identified as early as 2 min into the acute stress phase (12 min after anticipation onset). Anticipatory cortisol levels in the anticipatory responder group peaked at 4 min (with a 144% increase in cortisol levels from baseline), reactive

Table 1 Results for stress-induced changes in the POMS mood states as determined in two-way mixed ANOVAs (study 1).

POMS mood state	$F(df^a)$, p , η_p^2	$F(df)$, p , η_p^2 (for simple contrasts) ^b
Tension–anxiety		
Time:	7.53, <.001, .24	–2 min-BL: 7.88, .010, .25
Time \times group:	19.95, <.001, .45	10 min-BL: 31.91, <.001, .57
Group:	1.00, >.30	
Anger–hostility		
Time:	1.78, >.15	
Time \times group:	1.17, >.30	
Group:	0.51, >.45	
Fatigue–inertia		
Time:	4.92, .005, .17	10 min-BL: 3.34, .080, .12
Time \times group:	3.40, .026, .12	
Group:	0.16, >.65	
Depression–dejection		
Time:	2.79, .063, .10	10 min-BL: 4.09, .050, .15
Time \times group:	3.80, .024, .14	
Group:	0.11, >.70	
Vigor–activity		
Time:	3.12, .029, .12	
Time \times group:	0.49, >0.65	
Group:	0.38, >.50	
Confusion–bewilderment		
Time:	0.85, >.45	10 min-BL: 10.53, .003, .31
Time \times group:	3.90, .015, .14	
Group:	0.01, >.90	

POMS: Profile of Mood States.

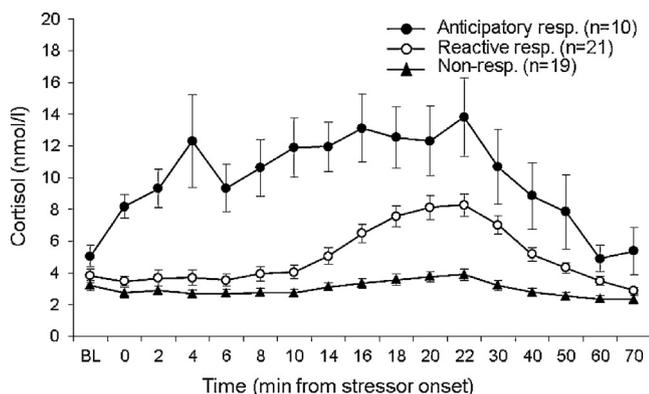
^a (5, 120) for the time and time by group effects, (1, 24) for the group effect.^b Simple contrasts are reported only for the time by group effect.

Figure 4 Means and standard errors of cortisol levels in the anticipatory, reactive and non-responder groups (original data). There was a time main effect ($F_{(16, 752)} = 32.89$, $p < .001$, $\eta_p^2 = .41$), a group main effect ($F_{(2, 47)} = 20.53$, $p < .001$, $\eta_p^2 = .47$) and a time by group interaction ($F_{(32, 752)} = 6.18$, $p < .001$, $\eta_p^2 = .21$). Groups differed significantly (p values ranged between <.001 and .005, d values ranged between 1.05 and 2.47) in their changes from baseline between 0 min (stressor onset) and 50 min after stressor onset (p values ranged between .026 and <.001; η_p^2 values ranged between .14 and .57).

cortisol levels in the reactive responder group peaked at 22 min (with a 118% increase in cortisol levels from baseline) after the onset of acute stress (14 and 32 min after the onset of anticipation, respectively) (Fig. 4). Importantly, as shown in Fig. 4, the anticipatory responders showed a double peak. This means that other than the name might initially suggest anticipatory responders were also reactive responders.

3.3. Comparison of anticipatory, reactive and non-responder cortisol stress groups (study 2)

A two-way mixed ANOVA revealed a time main effect ($F_{(16, 752)} = 32.89$, $p < .001$, $\eta_p^2 = .41$), a group main effect ($F_{(2, 47)} = 20.53$, $p < .001$, $\eta_p^2 = .47$) and a time by group interaction ($F_{(32, 752)} = 6.18$, $p < .001$, $\eta_p^2 = .21$) of stress-induced cortisol release (Fig. 4). Hochberg's post hoc tests and simple contrasts showed that the groups differed significantly (p values ranged between <.001 and .005, d values ranged between 1.05 and 2.47) in their change from baseline between 0 min (onset of the acute stressor) and 50 min after onset of the acute stressor (p values ranged between .026 and <.001; η_p^2 values ranged between .14 and .57).

Due to several successive missing values, one non-responder had to be excluded from all analyses of the alpha-amylase stress response and one reactive responder had to be excluded from all analyses of the subjective–psychological

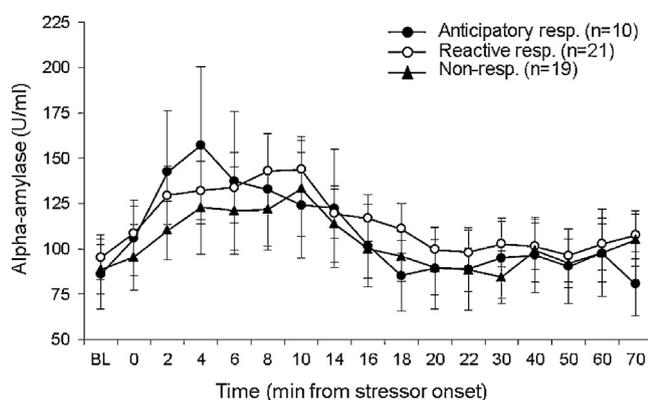


Figure 5 Means and standard errors of salivary alpha-amylase levels in the anticipatory, reactive and non-responder groups (original data). There was a time main effect ($F_{(16, 736)} = 8.03$, $p < .001$, $\eta_p^2 = .15$). No group main effect or group by time interaction were found (all $F < 1.00$, $p > .60$).

stress response. A two-way mixed ANOVA revealed a time effect of stress-induced alpha-amylase release ($F_{(16, 736)} = 8.03$, $p < .001$, $\eta_p^2 = .15$). No group main effect ($F_{(2, 46)} < 1.00$, $p > .70$) or group by time interaction ($F_{(32, 736)} < 1.0$, $p > .60$) were found (Fig. 5). Regarding the five POMS mood states, two-way mixed ANOVAs revealed a marginal decrease in depression–dejection, marginal increases in tension–anxiety and confusion–bewilderment and significant increases in anger–hostility and vigor–activity over time (see Table 2 for a summary of these results). No group main effects or time by group interactions were found (all $F_{(2, 46)} < 2.00$, $p > .10$).

3.4. Relationship between anticipatory and reactive cortisol stress responses (study 2)

There was a positive association between the anticipatory and reactive cortisol stress responses ($R = .82$). The anticipatory stress response accounted for 67% of the variation in reactive cortisol levels (see Table 3 for a summary of the regression results).

4. Discussion

The goal of our current research was to establish a protocol that would allow for a sensitive, easy-to-implement

Table 2 Time effects (pre–post stress comparisons) for the POMS mood states as determined in two-way mixed ANOVAs (study 2).

POMS mood state	$F_{(1, 46)}$	p	η_p^2
Tension–anxiety	3.66	.062	.07
Anger–hostility	4.32	.043	.09
Fatigue–inertia	.02	>.85	
Depression–dejection	3.34	.074	.07
Vigor–activity	9.61	.003	.17
Confusion–bewilderment	3.07	.086	.06

POMS: Profile of Mood States.

Table 3 Output from the simple regression analysis of the relationship between anticipatory and reactive cortisol stress responses (study 2).

	B	$SE\ B$	β
Constant	3.86	.72	
Anticipatory cortisol stress response	.87	.09	.82*

$R^2 = .67$ ($p < .001$).

* $p < .001$.

laboratory-based investigation into the anticipatory cortisol stress response. In an initial study, we explored the time dynamics of anticipatory and reactive cortisol release profiles. After purely anticipatory stress, a trend for a cortisol increase from baseline (by .35 nmol/l; 14%) was first detectable at 0 min (10 min after the onset of anticipation and at the designated time-point of acute stressor onset). After purely reactive stress, a respective trend (an increase by .33 nmol/l; 10%) was detected at 10 min after onset of the acute stressor (corresponding to the time-point of stressor termination). We conclude from these results that a reasonable and conservative time window for the detectability of an anticipatory cortisol stress response lies between 10 and 16 min after the onset of anticipatory stress.

In a subsequent study, based on the timeline results of study 1, we captured the anticipatory stress response and determined its interaction with the onset of reactive stress in an independent participant sample. As hypothesized, we identified two groups with distinct cortisol release profiles: anticipatory responders with a physiologically relevant increase in cortisol levels between 10 and 16 min after the onset of anticipation (immediately before to 6 min after the onset of acute stress) and reactive responders with a physiologically relevant increase in cortisol levels 8 min or later after the onset of acute stress (18 min after the onset of anticipation). In addition, we observed a numerically strong third group of participants without any physiologically relevant cortisol increase. Cortisol levels in the anticipatory responders increased by 62% within 10 min of anticipatory stress and by 144% within 4 min of acute stress (corresponding to 14 min of anticipatory stress). 10 out of 10 anticipatory responders were identified as early as 2 min into the acute stress phase (corresponding to 12 min of anticipatory stress). Based on the cortisol sample collected immediately after anticipation/prior to acute stress, only 4 out of 10 anticipatory responders would have been detected.

Other than in study 2 (at 14 min after the onset of anticipation/4 min after the onset of acute stress), the anticipatory peak in the anticipation-only condition manifested at what would be 20 min after anticipation onset/10 min after acute stressor onset in a conventional TSST – i.e., at a time-point when reactive cortisol levels already show visible increases. We conclude that the best practice to detect a maximum number of anticipatory responders in the TSST while only minimally changing the testing procedure would be to extend the anticipation phase to 15 min. In doing so, the peak of the anticipatory cortisol response (between 14 and 20 min after anticipation onset) could be captured at a

time-point of the actual stressor (5 min after onset) that is definitely uninfluenced by reactive cortisol levels.

Overall, study 2 revealed several features of anticipatory cortisol stress responders. First, next to exhibiting significant cortisol release in anticipation of a stressful event, the anticipatory responders exhibited a two-fold higher reactive stress-induced cortisol release than the reactive responders. The highly significant association between anticipatory and reactive cortisol stress responses was consequently positive. Alternatively, it would have been conceivable that the presence of an anticipatory cortisol stress response blunts the response to the acute stressor itself, as has been shown for elevated baseline cortisol levels in students during midterm exam week (Young and Nolen-Hoeksema, 2001).

Second, compared to reactive and non-responders, and again in contradiction to Young and Nolen-Hoeksema's findings, the anticipatory responders had an elevated cortisol baseline, even after 30 min of rest preceding saliva sampling. Future studies should investigate whether an elevated cortisol baseline is a consistent finding in anticipatory responders and whether it coincides with the experience of chronic stress – as would be suggested by the reliable finding of basal HPA axis hyperactivity in diverse situations of long-term stress (for a review see Anagnostis et al., 2009).

It could be argued that the occurrence of an anticipatory cortisol stress response is a manifestation of increased subjective stress sensitivity. We can take from study 1 that anticipation alone is, indeed, perceived as a psychological challenge. Based on the assumption of psychoendocrine covariance (Schlotz et al., 2008), the anticipatory stress responders should exhibit the highest levels of subjective–psychological stress. However, and third, we failed to find an association between anticipatory cortisol and subjective–psychological responses to both anticipatory (study 1) and acute (study 2) stress. Likewise, levels of the salivary enzyme alpha-amylase appeared to be insensitive to anticipatory stress. In study 1, a significant time by group interaction was driven by the course of alpha-amylase levels in the stress-only group. At the time-point of peak anticipatory alpha-amylase release (12 min after the onset of anticipation), anticipation- and stress-only groups did not differ from each other. In study 2, alpha-amylase release did not differ between the three stress responder groups. It has proven difficult in previous TSST studies to establish stable associations between endocrine, autonomic and subjective–psychological stress measures (Buchanan et al., 1999; Cohen et al., 2000; Schommer et al., 2003; Nater et al., 2005, 2006). Taking into consideration the distinct temporal dynamics of the respective stress measures might be critical to finding high associations between them (Schlotz et al., 2008; Engert et al., 2011). The lack of group differences in subjective–psychological and alpha-amylase measures might thus simply be due to a lack of power of the utilized statistical tests. Alternatively, it might indicate a dissociation of HPA axis and SNS as well as psychological stress responses in the anticipatory responders. This hypothesis could be tested by investigating correlational patterns (i.e., using cross-correlation analysis) between levels of anticipatory cortisol and other stress markers in a larger sample of anticipatory stress responders.

There are several limitations to the current study. First, no psychological trait measures were collected to further characterize the responder groups. It seems likely that the anticipatory responders display e.g., lower self-esteem and higher social anxiety than the reactive and non-responders. A second limitation is that by sampling saliva in 2-min intervals for 30 and 22 min in studies 1 and 2, respectively, we introduced a change to the conventional TSST procedure. The fact that participants sampled saliva while performing the stress test may have influenced the results by interfering with the speech portion of the TSST. However, participants reported no difficulties with having to talk while keeping a saliva collection swab in the mouth. Since the distribution and collection of salivettes were performed by a research assistant, if anything, the additional sampling and observer presence in the room might have increased the stressfulness of the situation. Third, the relatively high number of non-responders found in the current study has to be addressed. This finding confirms an observation that we have repeatedly made in the past years. At this point, we can only speculate on why the TSST seems to elicit lower cortisol responses. One possibility could be that today, students are better prepared and more accustomed to giving oral presentations than several years ago. An alternative explanation may be that after a considerable time of usage, a paradigm like the TSST reaches a certain degree of familiarity among students, thus interfering with the test's novelty aspect. Potential causes of this development should be considered in future studies. A final remark concerns doubts regarding the validity of alpha-amylase as a measure of SNS activity: Although the stimulation of salivary proteins is clearly ascribed to sympathetic activity, the stimulation of saliva flow rate is mainly mediated by parasympathetic nerves (Anderson et al., 1984; Garrett, 1987). In this regard, Rohleder et al. (2006) could demonstrate that stress-induced increases in salivary alpha-amylase levels were correlated with increases in amylase output but not with increases of flow rate. These results indicate that saliva flow rate does not seem to be a confounder of stress-induced alpha-amylase activation and that valid alpha-amylase measurements can be obtained by the use of salivettes.

In summary, we present a simple and easy-to-implement technique to identify anticipatory cortisol stress responders in the laboratory setting. The anticipatory cortisol stress response accounted for a substantial amount of variation in the reactive cortisol levels. Although previous studies have shown relatively increased anticipatory cortisol stress responses in several patients and vulnerability groups (Hardie et al., 2002; Alpers et al., 2003; Bremner et al., 2003; de Timary et al., 2008; Mikolajczak et al., 2008; Rudolph et al., 2010, 2011), here, anticipatory responder rates of 40% and 20% were revealed in two samples of healthy participants. Associations of anticipatory cortisol stress responsivity with various stress-sensitive parameters like sex, personality, psychological wellbeing or chronic stress remain to be addressed in future research. As a best practice approach, we recommend extending the anticipation phase to 15 min and introducing one additional cortisol measurement time-point at 5 min into the acute stress phase as a standard procedure in TSST studies. By this means, valuable information about interindividual differences in endocrine stress responsivity can be gained at a very low cost.

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Conflict of interest statement

All of the authors declare that they have no conflicts of interest.

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