



## Salivary cortisone, as a biomarker for psychosocial stress, is associated with state anxiety and heart rate

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### ABSTRACT

**Background:** Stress activates the central nervous, the autonomic nervous, and the endocrine system. This study aimed to (1) test the usability of salivary cortisone in a standardized psychosocial stressor, (2) create a comprehensive profile of hormonal responses to determine laboratory parameters with high discriminatory power, and (3) analyze their association with psychometric and autonomic stress measures.

**Methods:** Healthy young men (18–35 years) completed either the Trier Social Stress Test (TSST) (n = 33) or a Placebo-TSST (n = 34). Blood and saliva were collected at 14 time points along with state-anxiety (STAI) and heart rate. Serum steroids (cortisol\*, cortisone\*, dehydroepiandrosterone-sulfate, androstenedione\*, progesterone\*, 17-hydroxyprogesterone\*, testosterone, estradiol\*, aldosterone\*), salivary cortisol\* and cortisone\*, copeptin\*, adrenocorticotropic hormone\*, corticosteroid-binding globulin, and salivary alpha-amylase\* were analyzed. We used mixed-design ANOVAs to test group differences, receiver operator characteristic (ROC) curve analyses to assess the discriminatory power of each measure, and Spearman correlation analyses to probe the association between measures.

**Results:** The largest area under the ROC curve was observed in salivary cortisone at 20 min after the end of the TSST (AUC = 0.909 ± 0.044, p < 0.0001). Significant time-by-group interactions were found in the parameters marked with \* above, indicating stress-induced increases. The peak response of salivary cortisone was significantly associated with those of STAI (rho = 0.477, p = 0.016) and heart rate (rho = 0.699, p < 0.0001) in the TSST group.

**Conclusion:** Our study found salivary cortisone to be a stress biomarker with high discriminatory power and significant correlations with subjective and autonomic stress measures. Our results can inform future stress studies of sampling time for different laboratory parameters.

### 1. Introduction

An adaptive response to a stressor (i.e., a demand or threat) comprises activation in the central nervous system, the autonomic nervous system (ANS), and the endocrine system (Chrousos and Gold, 1992).

The brain orchestrates the stress response and is central for the adaptation to current and future stressors (Ulrich-Lai and Herman, 2009). ANS stimulation is marked by a rapid increase of heart rate (Allen et al., 2014), the release of plasma catecholamines and salivary alpha-amylase (Granger et al., 2007; Rohleder and Nater, 2009). The response of the

**Abbreviations:** 11β-HSD2, 11 beta hydroxysteroid dehydrogenase 2; 17-OHP, 17-hydroxyprogesterone; AA, alpha-amylase; ACTH, adrenocorticotropic hormone; AND, androstenedione; ANS, autonomic nervous system; AUC, area under the curve; AVP, arginine vasopressin; BMI, body mass index; CBG, corticosteroid binding globulin; CRH, corticotropin-releasing hormone; CV, coefficient of variation; DHEAS, dehydroepiandrosterone sulfate; E, cortisone; F, cortisol; HPA, hypothalamus-pituitary-adrenal; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ROC, receiver operating characteristic; STAI, state-trait anxiety inventory; TSST, Trier Social Stress Test

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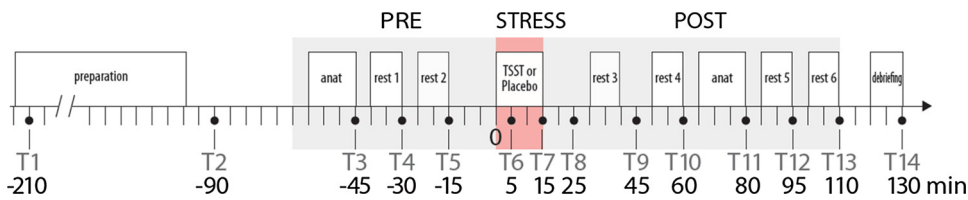
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**Fig. 1.** Study design with relative time stamps in minutes. Zero minute indicates the start of the stress intervention, the Trier Social Stress Test (TSST) or placebo TSST. Abbreviations: anat, Anatomical MRI scan (details not directly relevant for the current paper).

hypothalamus-pituitary-adrenal (HPA)-axis, key element of the endocrine stress response, consists of a cascade of hormone releases (Spanakis et al., 2016): corticotropin-releasing hormone (CRH) and copeptin from the hypothalamus (Katan et al., 2008; Urwyler et al., 2015), adrenocorticotropic hormone (ACTH) from the pituitary, and – the primary stress hormone – cortisol from the adrenal glands. More than 90% of the cortisol in circulation is bound to proteins (Lewis et al., 2005). The unbound fraction of cortisol is biologically active and can diffuse into the saliva (Hellhammer et al., 2009). Therefore, salivary cortisol has been used as a surrogate marker of serum free cortisol with its advantage of non-invasive sample collection. However, salivary cortisol is converted to cortisone rapidly and irreversibly, as the salivary glands exhibit high levels of 11 $\beta$ -hydroxysteroid dehydrogenase 2 (11 $\beta$ -HSD2) (Blair et al., 2017). In consequence, the concentration of cortisone in saliva is 2–6 times higher than that of cortisol in saliva (Bae et al., 2016). Recently, salivary cortisone was found to be a better surrogate marker of serum free cortisol than salivary cortisol, particularly when serum cortisol levels are low, or when hydrocortisone was administered as a therapeutic or experimental intervention (Blair et al., 2017; Debono et al., 2016; Perogamvros et al., 2010). Therefore, the first aim of our study was to investigate the usability of saliva cortisone as an endocrine stress marker in a standardized psychophysiological stress experiment, the Trier Social Stress Test (TSST) (Kirschbaum et al., 1993).

The second aim of our study was based on the observation that previous stress studies show a high degree of heterogeneity in terms of sampling time points and laboratory parameters. This makes their comparison difficult and can only insufficiently inform future stress studies. We, therefore, wanted to create a comprehensive response profile of laboratory parameters for a standardized psychosocial stressor, the TSST, in healthy young men (Kirschbaum et al., 1993) and a closely matching control task (Het et al., 2009). With a relatively high sampling frequency, we aimed to assess the time courses of measures from different stress axes before, during, and after the intervention and to determine the laboratory parameters which discriminate between the stress and the control group. In addition to the main stress axes (i.e., ANS and the HPA-axis), we investigated how stress affects the level of sex steroid hormones, considering that both chronic stress and the alteration of sex steroid hormones are related to a variety of medical conditions (Byun et al., 2013; Corona and Maggi, 2010; Toufexis et al., 2014).

After identifying laboratory parameters with high discriminatory power, we also assessed their association with other stress measures; specifically, self-reported subjective negative affect (using the state-trait anxiety inventory, STAI (Spielberger et al., 1983)) and heart rate as autonomic stress marker.

## 2. Material and methods

### 2.1. Participants

Male participants (n = 67, age range: 18–35 years) were recruited at the Max Planck Institute for Human Cognitive and Brain Sciences in Leipzig, Germany. Exclusion criteria were: smoking, drug or excessive alcohol consumption, university degree in psychology or currently studying psychology, regular medication intake including steroid use, history of cardiovascular or neurological diseases, or a BMI higher than

27. Since magnetic resonance imaging (MRI) was used to acquire brain data for a different focus of the study (Reinelt et al., in preparation), standard MRI exclusion criteria additionally applied: participants with tattoos, irremovable metal objects (including retainers or piercing), tinnitus, or claustrophobia were excluded. Included participants were randomly assigned to either the stressor (n = 33) or the control group (n = 34). Due to unavailability of blood samples or heart rate data, 8 participants from the stress group and 9 participants from the control group were excluded from the statistical analysis. All appointments were scheduled for the same time of day (11:45) to control for diurnal fluctuations of hormones (e.g., cortisol (Katz and Shannon, 1964)). In addition, participants were asked to get at least 8 h of sleep before the day of the experiment, to wake up no later than 9:00 a.m. and to have their breakfast as usual. Participants were also requested not to exercise before their study appointment and refrain from drinking coffee or black tea. Written informed consent was obtained from all participants. The study was approved by the ethics committee of the medical faculty of the University of Leipzig. All procedures were performed in accordance with the current revision of the Helsinki Declaration. Participants received financial remuneration.

### 2.2. Procedure

in Fig. 1. Participants received a first salivary collection tube, Salivette Time points for sample collection are illustrated (Sarstedt AG & Co. KG) around 12:00 p.m. (T0). After T0, participants were equipped with a BioHarness3 chest strap (Zephyr Technology Corporation) recording an electrocardiogram (ECG). Participants then laid down on an examination couch and a physician placed an intravenous catheter in the left or right cubital vein. Immediately after placing the catheter, a first blood sample was acquired, and subjective experience and saliva were sampled a second time at T1, 210 min before the onset of the TSST or placebo-TSST. After this initial preparation, participants had a 15-minute lunch break, where they were given water and a snack. During the 10-minute walk to the study center, they were asked about their career aims and dream jobs. After 30 min of rest in the study center, a further sampling instance (T2, at 90 min before stress onset) of subjective rating, saliva, and blood samples was performed. Further samples (T3 at 45 min before stress onset, T4 at 30 min before stress onset, and T5 at 15 min before stress onset) were taken while participants were lying inside the MRI scanner (see 2.1). After T5, participants were brought to a different room, where they underwent either the TSST or the Placebo-TSST, which included the collection of subjective ratings, saliva, and blood (T6 at + 5 min after stress onset and T7 at + 15 min after stress onset) (see 2.3). Following the intervention, participants were brought back to the scanner room. In the MRI, six more samples of subjective rating, saliva, and blood were taken (T8 at + 25 min after stress onset, T9 at + 45 min after stress onset, T10 at + 60 min after stress onset, T11 at + 80 min after stress onset, T12 at + 95 min after stress onset, and T13 at + 110 min after stress onset). After exiting the scanner, participants were debriefed in a separate room. The experiment ended with a final sampling instance (T14 at + 130 min after stress onset).

### 2.3. Intervention

Participants completed either the Trier Social Stress Test (TSST)

(Kirschbaum et al., 1993) or a Placebo-TSST (Het et al., 2009).

### 2.3.1. TSST

Participants were accompanied from the MRI to a separate testing room, in which they encountered a committee of two professional actors introduced as “professional psychologists trained in the analysis of non-verbal communication”. Participants were told to imagine that they would apply for their dream job and asked to freely describe relevant personal qualifications. Additionally, participants were told (1) that, while talking, they would be observed by the committee and recorded by a video camera and a microphone, (2) that they would have to perform another task after their talk and (3) that they would have 5 min to prepare notes for their oral introduction. For this preparation, participants then sat down in front of the committee. After this preparation phase, a sampling time point followed (T6) before the participants stood in front of the microphone and began with their self-presentation without using their written notes. The committee members monitored the participant neutrally without any facial cues. Whenever the instructions were violated, committee members interrupted and repeated standardized instructions: for example, whenever a participant stopped talking, the committee waited for 20 s, silently looking at the participant before asking him to continue with the presentation. After 5 min, the participants were introduced to the next task, in which they had to count backward in steps of 17 from 2043 as fast and accurately as possible. Every time a participant made a mistake, he was asked to start from the beginning. After 5 min, participants sat down for another sampling instance (T7). Participants were then told that another task would follow in the MRI scanner, to which they were brought back to by the experimenter and the committee members. This additional information had the aim to maintain (subjective) stress levels until the participants were back in the MRI scanner. At T10, participants were then told that no more task would follow.

### 2.3.2. Placebo-TSST

The Placebo-TSST resembled the TSST but without the committee, the camera, and the microphone: participants were accompanied to the testing room and instructed to sit for 5 min and take notes about their career aims, which they would afterward talk about while standing alone in the room. Following this, they would perform a simple mental arithmetic task for 5 min, counting upwards from zero in increments of 15. Participants were then left without supervision in the testing room. After 5 min, the experimenter entered the room to collect psychometric, saliva, and blood samples (T6). After the experimenter had left, the participants had to stand up and read what they had prepared before. After 5 min, the experimenter re-entered the room and asked the participants to start with the arithmetic task. Following the 5 min of counting, the participants sat down for another sampling instance (T7), after which they were accompanied to the MRI.

## 2.4. Data acquisition

### 2.4.1. Endocrine data

While participants responded to the questionnaires with the free arm, the experimenter acquired blood samples (serum and plasma) using Sarstedt blood collection tubes (Sarstedt) from a catheter placed on the contra-lateral arm at the beginning of the procedure. Simultaneously, participants were chewing a Salivette® for Cortisol® (Sarstedt) for saliva sampling (duration: at least 2 min). Throughout the experiment, blood samples were collected at 14 time points (T1–T14) and saliva samples and subjective ratings at 15 time points (T0–T14). Saliva and blood samples were stored at  $-80^{\circ}\text{C}$  after centrifugation until the laboratory analysis.

### 2.4.2. Psychometric data

Subjective stress was measured using the state version of the state-trait anxiety inventory (STAI) (Spielberger et al., 1983). This

questionnaire consists of 20 items such as “I am tense”; “I am worried” and “I feel calm”; “I feel secure”, which can be answered on a 4 point Likert-type scale ranging from 1 (“not at all”) to 4 (“very much so”).

### 2.4.3. Autonomic data

Outside the MRI scanner, heart rate data were obtained using a BioHarness3 (Zephyr) attached to the participants’ chest at the height of the xiphoid process to record an ECG. Inside the MRI scanner, a BrainAmp (Brain Products GmbH) was used for ECG acquisition. To reduce artifacts related to breathing (i.e., movement of the thorax), the three electrodes were placed on the participants’ backs (one adjacent to cervical spine c7, one above the coccyx, and one 15 cm below the left armpit). In the MRI scanner, pulse oximetry was recorded at a finger using an MP150 device (Biopac Systems, Inc.). Autonomic data were recorded continuously and heart rate was averaged over blocks of 3 min, which were centered at -40, -35, -25, -20, +3, +8, +13, +35, +40, +50, +55, +70, +75, +85, and +90 min with zero minute as the start of the stress or control intervention.

## 2.5. Laboratory analysis

Steroid hormones in serum, which include cortisol, cortisone, testosterone, androstenedione, 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEAS), progesterone, and estradiol, were simultaneously quantified using liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Gaudl et al., 2016). Cortisol and cortisone in saliva were also measured using LC–MS/MS (Bae et al., 2016). Inter-assay coefficient of variation for each steroid hormone was as follows: cortisol (0.8–3.1%), cortisone (2.0–9.6%), aldosterone (0.6–9.3%), testosterone (0.6–4.9%), androstenedione (0.4–5.6%), 17-OHP (0.7–5.9%), DHEAS (4.2–17.6%), progesterone (0.7–3.7%), and estradiol (0.4–5.6%) in serum and cortisol (4.5–5.1%) and cortisone (5.8–7.2%) in saliva. Other laboratory parameters were measured individually according to the product specification of the manufacturers. ACTH in plasma was measured using chemiluminescent immunoassay technology (DiaSorin) with inter-assay CVs of 5.5–8.9%. Copeptin in serum was measured by the BRAHMS copeptin ultrasensitive assay on the KRYPTOR Compact Plus system (Thermo Fisher Scientific) with inter-assay CVs less than 10% at concentrations above 4 pmol/L. Corticosteroid-binding globulin (CBG) in serum was measured using radioimmunoassay (Diasource Diagnostics) with inter-assay CVs of 4.8–10.8%. Alpha-amylase in saliva was measured using the enzymatic colorimetric test at Cobas 8000 c-module (Roche) after 1:300 dilution with 0.1% bovine serum albumin in phosphate buffer saline (Bae et al., 2016) with inter-assay CVs of 1.0–1.6%. Serum free cortisol was calculated using Coolen’s formula (Coolens et al., 1987).

## 2.6. Statistical analysis

Statistical analyses were performed using the software SPSS (Version 24) and MedCalc (Version 12.7.7). The group of participants who completed the TSST was defined as “stress group” and the one completing the Placebo-TSST was defined as “control group”. The demographic and anthropometric characteristics of age and BMI, respectively, were compared between the groups using independent t-tests. The reactivity of the investigated parameters was analyzed after baseline correction (i.e., dividing each value by the value at T5), using mixed-design analyses of variance (ANOVAs) with time (T0 or T1–T14) as within-subject factor and group (stress, control) as between-subject factor. Group means ( $\pm$  SEM) of each parameter are plotted in Fig. 2. Based on the non-normality of data tested with Kolmogorov-Smirnov test, Spearman correlation analyses were performed to test the associations between the peak responses (i.e., the maximal values after the TSST between T7 and T14 before the values start to decrease) of different parameters in the stress group.

To assess how efficiently each parameter could differentiate the

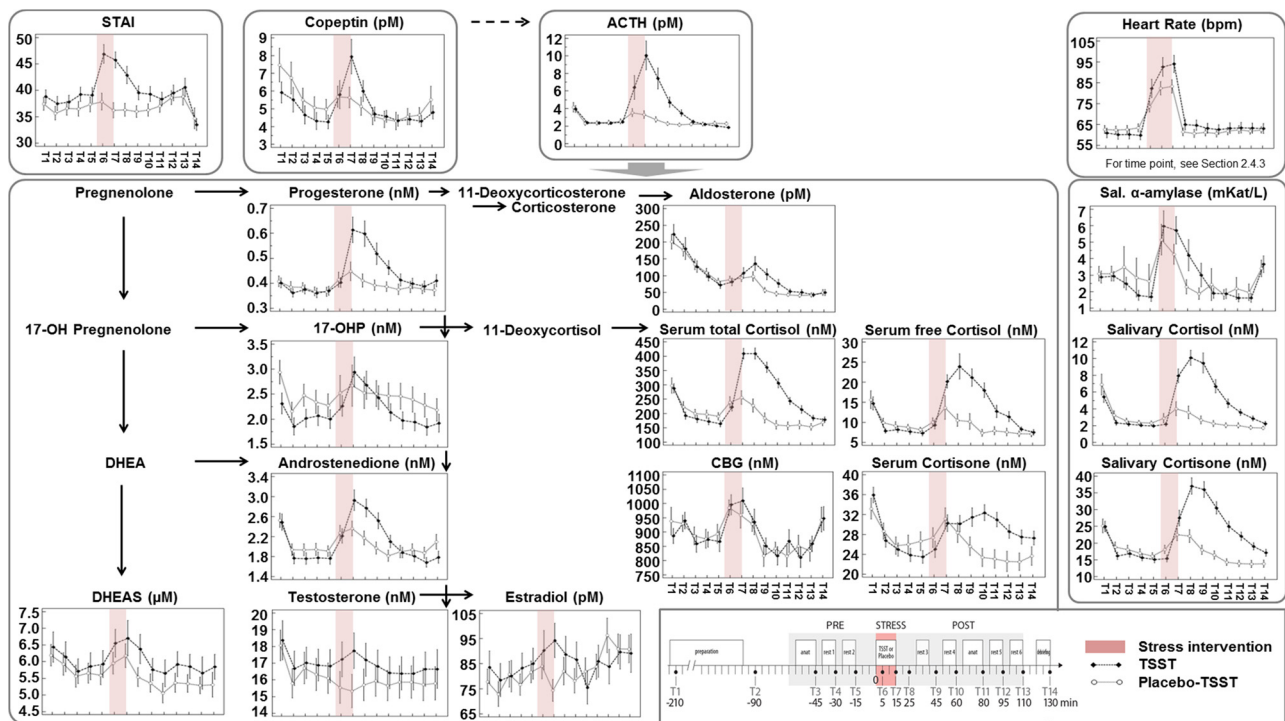


Fig. 2. Temporal trajectories of the laboratory parameters, state anxiety (state-trait anxiety inventory, STAI), and heart rate before, during, and after the Trier Social Stress Test (TSST) or the Placebo-TSST (Mean ± SEM). The x-axis represents the sampling time points, which are same for STAI and the laboratory parameters (For ECG, see Section 2.4.3). Sampling time points (x-axis) are specified at the lower right corner with the time frame of the stress intervention marked with a box.

groups, receiver operating characteristic (ROC) curves (Metz, 1978; Zweig and Campbell, 1993) were computed at each time point with the stress group as the positive (n = 25) and the control group as the negative group (n = 25) using MedCalc. The area under the ROC curve (AUC) was calculated using the empirical method (Lasko et al., 2005). AUCs higher than 0.80 indicate that two distributions (in this case: groups) can be separated well (Lasko et al., 2005). Confidence intervals, Youden index J, and its corresponding criterion value were calculated using the bias-corrected and accelerated interval bootstrap method (BCa) (Efron and Tibshirani, 1993). Estimated sensitivities at fixed specificities of 80%, 90%, and 95% were calculated along with BCa 95% confidence intervals. The AUCs from correlated ROC curves were compared nonparametrically with the DeLong method implemented in MedCalc (DeLong et al., 1988).

### 3. Results

There was no significant difference between the stress group (n = 25) and the control group (n = 25) in age or in BMI (Table 1).

#### 3.1. Characterization of the stress response

The temporal dynamics of the laboratory parameters, STAI scores, and heart rate in response to the TSST or Placebo-TSST are shown in Fig. 2. Significant time by group interactions were found for serum total cortisol, salivary cortisone, ACTH, androstenedione, salivary cortisol, serum cortisone, copeptin, progesterone, serum free cortisol, STAI scores, aldosterone, heart rate, estradiol, and 17-OHP, indicating stress-induced increases. No significant interactions were present for salivary alpha-amylase, testosterone, DHEAS, and CBG (see Table 2). Average responses peaked at different time points with a lag between the parameters: STAI scores peaked at T6 (+ 5 min after TSST onset), ACTH, progesterone, androstenedione, and estradiol at T7 (+ 15 min after TSST onset), serum total cortisol, serum free cortisol, salivary cortisol, and salivary cortisone at T8 (+ 25 min after TSST onset).

Table 1

Summary of participant characteristics in the stress and the control group.

	Stress group	Control group
<b>Included in the study</b>		
N (all males)	33	34
<b>Excluded from the analysis</b>		
N	8	9
Reason for the exclusion		
• Serum samples not available	3	5
• Heart rate data not available	5	5
• Serum samples and heart rate data not available	0	1
<b>Included in the analysis</b>		
N	25	25
Age (yr) Mean ± SD	25.20 ± 2.55	26.32 ± 2.78
BMI (kg/m <sup>2</sup> ) Mean ± SD	22.82 ± 2.05	23.96 ± 2.58

There was no significant difference in age or BMI between the groups.

#### 3.2. Discriminatory power of the laboratory parameters at each time point

AUCs higher than 0.80 were found at one or more time points between T7 (after the end of the stressor) and T12 in ACTH, serum total cortisol, serum free cortisol, salivary cortisol, salivary cortisone, and STAI scores (see Supplemental Table 1). The parameters with the highest AUC at each time point are summarized in Table 3. As shown in Fig. 3 and Supplemental Table 1, ACTH showed an AUC higher than 0.8 from T7 to T9, while salivary cortisone had an AUC higher than 0.80 from T8 to T12. The highest AUC among all parameters and time points was found for salivary cortisone at T9 (AUC = 0.909, standard error = 0.044, p < 0.0001). Comparison of AUCs between the ROC curves showed no significant difference between the parameters with an AUC larger than 0.80. Nevertheless, another important aspect of the ROC curves beside AUC is the sensitivity at a fixed specificity. Although their AUCs did not differ significantly, salivary cortisone showed a higher sensitivity with narrower confidence interval (CI) [Salivary cortisone sensitivity (CI): 60% (30.59–84.00) vs. salivary cortisol

**Table 2**

Summary of the time by group interaction effect of each parameter after baseline correction (i.e., dividing each value by the value at T5). Abbreviations:  $df^1$ , degree of freedom;  $df^2$ , degree of freedom for error; ACTH, adrenocorticotropic hormone; STAI, state trait anxiety inventory; DHEAS, dehydroepiandrosterone sulfate; CBG, corticosteroid binding globulin.

Parameter	F ( $df^1$ , $df^2$ )	p
Serum total cortisol	33.0 (2.2, 103.8)	< 0.001
Salivary cortisone	26.0 (2.5, 118.6)	< 0.001
ACTH	24.6 (1.6, 79.0)	< 0.001
Androstenedione	20.5 (3.3, 158.3)	< 0.001
Salivary cortisol	19.5 (2.0, 98.0)	< 0.001
Serum cortisone	14.0 (9.0, 142.0)	< 0.001
Copeptin	10.0 (1.9, 90.4)	< 0.001
Progesterone	9.9 (1.9, 90.9)	< 0.001
Serum free cortisol	7.4 (2.4, 114.5)	< 0.001
STAI score	7.3 (4.0, 192.5)	< 0.001
Aldosterone	5.3 (2.4, 113.9)	0.004
Heart rate	5.1 (2.8, 130.0)	0.003
Estradiol	4.8 (6.5, 312.5)	< 0.001
17-hydroxyprogesterone	3.1 (2.3, 111.6)	0.040
Salivary alpha-amylase	2.6 (2.4, 108.4)	0.071
Testosterone	2.4 (2.4, 113.5)	0.089
CBG	1.0 (6.2, 299.4)	0.454
DHEAS	0.8 (6.7, 322.9)	0.612

sensitivity (CI): 56.00% (8.00–92.00) at a specificity of 95% (Supplemental Table 1)].

### 3.3. Association with anxiety levels and heart rate

Among the laboratory parameters with AUCs higher than 0.80 (see 3.2), the peak values of serum total and free cortisol, salivary cortisol, and salivary cortisone showed significant correlations with peak values of STAI scores and heart rate within the stress group ( $n = 25$ ). Pairwise correlations of the investigated parameters are summarized in the Supplemental Table 2. The peak response of salivary cortisone was significantly associated with those of STAI ( $\rho = 0.477$ ,  $p = 0.016$ ) and heart rate ( $\rho = 0.699$ ,  $p < 0.0001$ ) in the TSST group (Supplemental Fig. 1).

## 4. Discussion

We find that salivary cortisone can serve as a stress marker in experimental studies. It exhibits high discriminatory power and correlates significantly with subjective and autonomic stress markers such as state anxiety and heart rate. Furthermore, we have identified the time courses and the discriminatory power of a comprehensive set of laboratory parameters, which may inform future studies about when and which laboratory parameter to collect.

Salivary cortisone has recently emerged as a superior surrogate

**Table 3**

Summary of the laboratory parameters with the highest area under the curve in the receiver operating characteristic (ROC) analysis at each time point (after the stressor). Statistics from Mann-Whitney  $U$  tests. Abbreviations: ACTH, adrenocorticotropic hormone; AUC, area under the curve; J, Youden Index J; cut-off is the association criterion based on the Youden index J; STAI, state trait anxiety inventory. For the list of all parameters with  $AUC > 0.80$ , see Supplemental Table 1.

Parameter	Time point	Median (min-max)		p-value	ROC Analysis			
		Stress group	Control group		AUC	p-value	J	cut-off
STAI	6	45.00 (36.00 – 67.00)	37.00 (26.00 – 59.00)	< 0.0001	0.798	< 0.0001	0.48	> 37.00
ACTH (pmol/L)	7	7.38 (2.10 – 37.44)	2.65 (1.24 – 11.07)	< 0.0001	0.870	< 0.0001	0.64	> 3.83
Serum free cortisol (nmol/L)	8	20.20 (6.65 – 89.97)	8.47 (3.31 – 40.13)	< 0.0001	0.891	< 0.0001	0.68	> 11.62
Salivary cortisone (nmol/L)	9	36.34 (11.35 – 62.42)	17.53 (7.43 – 32.46)	< 0.0001	0.909	< 0.0001	0.76	> 23.91
Serum free cortisol (nmol/L)	10	14.89 (6.14 – 39.23)	6.96 (2.66 – 23.03)	< 0.0001	0.902	< 0.0001	0.76	> 9.56
Salivary cortisone (nmol/L)	11	24.47 (8.46 – 44.11)	14.15 (5.94 – 25.30)	< 0.0001	0.885	< 0.0001	0.72	> 19.78
Salivary cortisone (nmol/L)	12	22.05 (9.02 – 37.17)	13.68 (5.63 – 22.66)	< 0.0001	0.848	< 0.0001	0.64	> 16.20
Salivary cortisol (nmol/L)	13	2.51 (1.32 – 6.98)	1.44 (0.46 – 4.97)	0.001	0.778	< 0.0001	0.48	> 1.43
Salivary cortisone (nmol/L)	14	16.95 (9.27 – 31.35)	13.62 (6.02 – 33.57)	0.009	0.714	0.004	0.40	> 16.01

marker for serum free cortisol compared to salivary cortisol due to the irreversible conversion from cortisol to cortisone in saliva (Blair et al., 2014; Debono et al., 2016; Perogamvros et al., 2010; Raff, 2016). Perogamvros et al. (Perogamvros et al., 2010) found that salivary cortisone has a comparable fold-change to serum free cortisol after adrenal stimulation with the synthetic ACTH “Synacthen®”. After hydrocortisone administration, salivary cortisol measurement results showed falsely high values due to interference by hydrocortisone, whereas the response of salivary cortisone was very similar to that of serum free cortisol (Perogamvros et al., 2010). Salivary cortisone was also detectable at very low serum cortisol concentrations, which were induced by dexamethasone suppression of ACTH (Ceccato et al., 2012; Cornes et al., 2015; Debono et al., 2016; Mak et al., 2017). Salivary cortisol concentrations, on the other hand, often appeared to be below the limit of detection. This reflects the rapid conversion of cortisol to cortisone by  $11\beta$ -HSD2 in saliva, which was also observed in the present study (see Supplemental Fig. 2). In many research laboratories, salivary cortisol is measured using immunoassays. We previously showed that the physiological level of salivary cortisone can lead to falsely high values of salivary cortisol in immunoassays due to its cross-reactivity (Bae et al., 2016). The effect of this cross-reactivity in immunoassays is more pronounced for lower concentrations of cortisol, which may appear in afternoon samples due to circadian rhythm. This implies that stress research would benefit from using LC-MS/MS, which does not display such interference (Bae et al., 2016).

By assessing the temporal dynamics of a comprehensive panel of laboratory parameters, we were able to characterize their relative discriminatory power in terms of sampling time points. Among the laboratory parameters that showed significant interaction effects of time and group, ACTH, progesterone, and androstenedione showed a rapid increase, peaking immediately post stress test. In the ROC analysis, ACTH showed an AUC of 0.870 immediately following the TSST and could differentiate the two groups up to 45 min (T9) from the stress onset. Fast stress recovery of ACTH indicates a “healthy” stress hormone profile that is maintained by sensitive glucocorticoid-mediated negative feedback circuits (Sapolsky, 2000). Because of its short half-life (7–10 min) and low stability in vitro, ACTH has been regarded as a less preferable biomarker compared to cortisol in stress research (Livesey and Dolamore, 2010; MEAKIN et al., 1959). Elevation in progesterone and androstenedione concentration shows their synthesis and secretion from the adrenal glands through ACTH stimulation (Lennartsson et al., 2012; Schumacher et al., 2014). A fast peak response of these hormones with anti-glucocorticoid properties may imply protective mechanisms against exposure to an excess amount of cortisol during the stress response (Maninger et al., 2009; Morgan et al., 2004). The slow rise and recovery of cortisol and its metabolite cortisone may allow the body to prepare defense mechanisms during the early phase of stress and to adapt to the stressor (Bae et al., 2015; Qian et al., 2011).

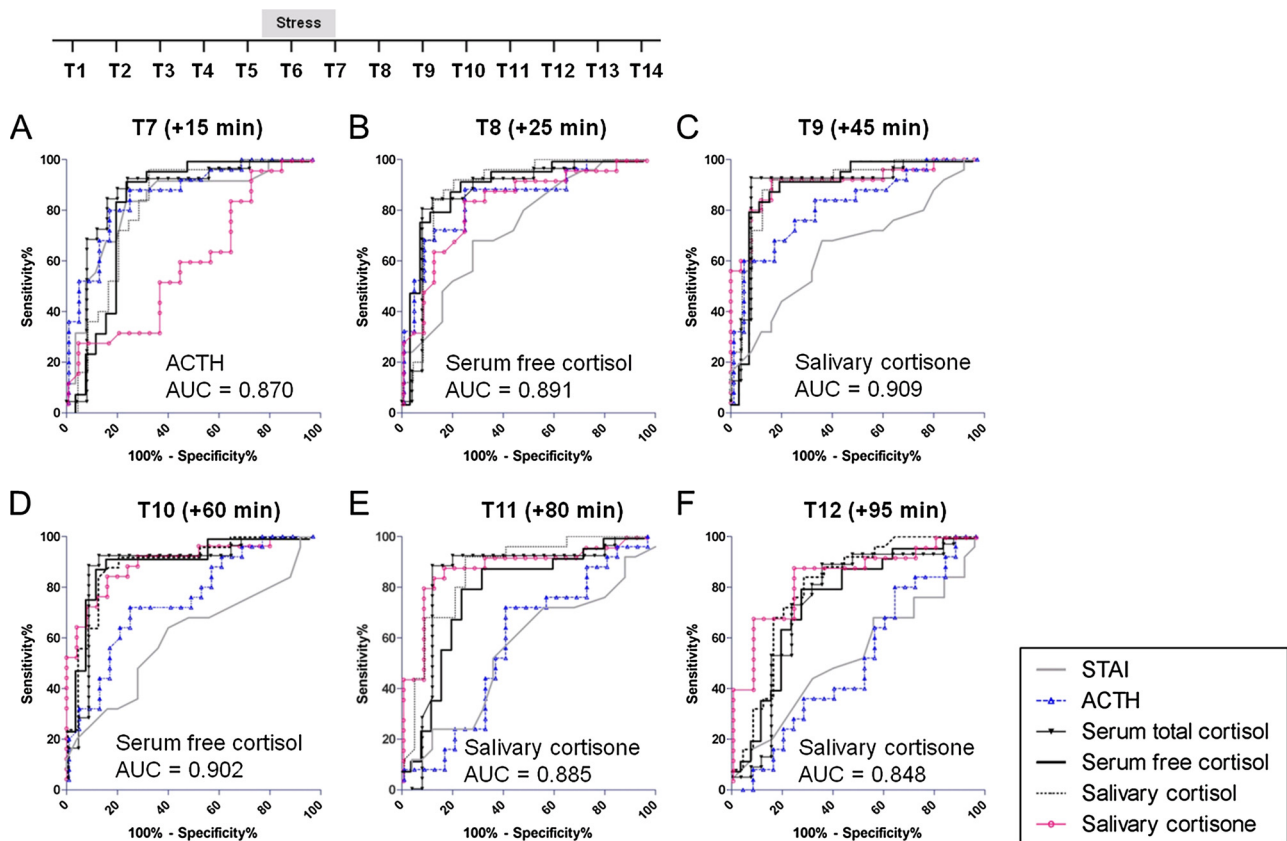


Fig. 3. Receiver operating characteristic (ROC) curves of laboratory parameters which had an area under the ROC curve (AUC) greater than 0.80 are plotted from time point 7 to time point 12 (from A to F). The highest AUC for each time point is specified with the respective parameter.

While both serum and salivary cortisol showed high discriminatory power between the two study groups, cortisol in serum necessitates invasive venipunctures, which may induce stress and evoke a cortisol response in itself (Weckesser et al., 2014). According to the “free hormone hypothesis” (Mendel, 1989), which states that the biological activity of a hormone is determined by its free concentration, free cortisol in the serum would be the most accurate stress marker. However, direct measurements of free cortisol require very labor- and time-intensive procedures, such as equilibrium dialysis or ultrafiltration (Perogamvros et al., 2009). Indirectly, serum free cortisol can be calculated using Coolen’s formula, which requires the additional measurement of CBG, as we performed in this study (Bae and Kratzsch, 2015). It should be noted that calculated serum free cortisol was previously found to underestimate measured serum free cortisol in the context of ACTH stimulation (Perogamvros et al., 2010). Salivary cortisol has been used extensively as a surrogate marker for serum free cortisol. However, salivary cortisol is rapidly converted to salivary cortisone by  $11\beta$ -HSD2 in salivary glands (Blair et al., 2017; Perogamvros et al., 2009). In agreement with these findings, salivary cortisol was found to be lower than serum free cortisol in our current study (Fig. 2) and even below the limit of detection when serum cortisol was very low in a recent study (Dennedy, 2018). Salivary cortisone showed high discriminatory power approximately 10 min after the peak of salivary cortisol. This time lag can be assumed to be due to the time required for the conversion from cortisol to cortisone by  $11\beta$ -HSD2 (Bae et al., 2015). Our findings emphasize that consideration of the timing of sample collection is essential to obtain meaningful and reproducible results in stress research. Our comprehensive temporal profiling of the stress response allows for the optimal selection of laboratory biomarkers in future stress research, in line with ethical considerations and/or financial constraints. For studies that want to capture both stress reactivity and stress recovery, we suggest measuring salivary cortisone at least at following time points:

T1) 20–30 min before the beginning of the stress intervention, T2) immediately after the end of the stress intervention, T3) 10 min after T2, T4) 20 min after T2, and T5) one hour after T2.

A limitation of our study was that we investigated the stress response only in healthy young males. We excluded females due to the influence of sex hormones which fluctuate with the menstrual cycle and the influence of oral contraceptives on cortisol levels. The generalizability of our results to other participant samples, such as females, thus remains to be tested in further research. It should also be noted that measurement conditions before and after the TSST could deviate from sample collection in a TSST study design without MRI (which was acquired for a different focus of the study; cf. Reinelt et al., in preparation). However, factors such as supine position in the MRI, the MRI noise, or the movement in and out of the scanner itself were closely matched between stress and control group in our experimental setup.

In summary, our study with a laboratory stressor identified salivary cortisone as a promising stress marker that showed a high discriminatory power and significant associations with other (subjective and autonomic) stress measures. This finding suggests the usability of salivary cortisone in psychophysiological stress research in addition to its potential diagnostic usage in clinical settings. Furthermore, our finding emphasizes the importance of optimal timing of sample collection for specific laboratory parameters, thus aiding study design in future stress research.

#### Conflicts of interest

None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.psyneuen.2018.10.015>.

## References

- Allen, A.P., Kennedy, P.J., Cryan, J.F., Dinan, T.G., Clarke, G., 2014. Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. *Neurosci. Biobehav. Rev.* 38, 94–124. <https://doi.org/10.1016/j.neubiorev.2013.11.005>.
- Bae, Y.J., Kratzsch, J., 2015. Corticosteroid-binding globulin: modulating mechanisms of bioavailability of cortisol and its clinical implications. *Best Pract. Res. Clin. Endocrinol. Metab.* 29, 761–772. <https://doi.org/10.1016/j.beem.2015.09.001>.
- Bae, Y.J., Stadelmann, S., Klein, A.M., Jaeger, S., Hiemisch, A., Kiess, W., Ceglarek, U., Gaudl, A., Schaab, M., Klitzing, Kvon, Thiery, J., Kratzsch, J., Döhnert, M., 2015. The hyporeactivity of salivary cortisol at stress test (TSST-C) in children with internalizing or externalizing disorders is contrastively associated with  $\alpha$ -amylase. *J. Psychiatr. Res.* 71, 78–88. <https://doi.org/10.1016/j.jpsychires.2015.09.013>.
- Bae, Y.J., Gaudl, A., Jaeger, S., Stadelmann, S., Hiemisch, A., Kiess, W., Willenberg, A., Schaab, M., Klitzing, Kvon, Thiery, J., Ceglarek, U., Döhnert, M., Kratzsch, J., 2016. Immunoassay or LC–MS/MS for the measurement of salivary cortisol in children? *Clin. Chem. Lab. Med.* 54, 811–822. <https://doi.org/10.1515/cclm-2015-0412>.
- Blair, J., Adaway, J., Keevil, B., Ross, R., 2017. Salivary cortisol and cortisone in the clinical setting. *Curr. Opin. Endocrinol. Diabetes Obes.* 24, 161–168. <https://doi.org/10.1097/MED.0000000000000328>.
- Blair, J., Lancaster, G., Titman, A., Peak, M., Newlands, P., Collingwood, C., Chesters, C., Moorcroft, T., Wallin, N., Hawcutt, D., Gardner, C., Didi, M., Lacy, D., Couriel, J., 2014. Early morning salivary cortisol and cortisone, and adrenal responses to a simplified low-dose short Synacthen test in children with asthma. *Clin. Endocrinol.* 80, 376–383. <https://doi.org/10.1111/cen.12302>.
- Byun, J.S., Lyu, S.W., Seok, H.H., Kim, W.J., Shim, S.H., Bak, C.W., 2013. Sexual dysfunctions induced by stress of timed intercourse and medical treatment. *BJU Int.* 111, E227–34. <https://doi.org/10.1111/j.1464-410X.2012.11577.x>.
- Ceccato, F., Albiger, N., Reimondo, G., Frigo, A.C., Ferasin, S., Occhi, G., Mantero, F., Terzolo, M., Scaroni, C., 2012. Assessment of glucocorticoid therapy with salivary cortisol in secondary adrenal insufficiency. *Eur. J. Endocrinol.* 167, 769–776. <https://doi.org/10.1530/EJE-12-0534>.
- Chrousos, G.P., Gold, P.W., 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *JAMA* 267, 1244–1252.
- Coolens, J.L., van Baelen, H., Heyns, W., 1987. Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. *J. Steroid Biochem.* 26, 197–202.
- Cornes, M.P., Ashby, H.L., Khalid, Y., Buch, H.N., Ford, C., Gama, R., 2015. Salivary cortisol and cortisone responses to tetracosactin (synacthen). *Ann. Clin. Biochem.* 52, 606–610. <https://doi.org/10.1177/0004563215577838>.
- Corona, G., Maggi, M., 2010. The role of testosterone in erectile dysfunction. *Nat. Rev. Urol.* 7, 46–56. <https://doi.org/10.1038/nrurol.2009.235>.
- Debono, M., Harrison, R.F., Whitaker, M.J., Eckland, D., Arlt, W., Keevil, B.G., Ross, R.J., 2016. Salivary cortisone reflects cortisol exposure under physiological conditions and after hydrocortisone. *J. Clin. Endocrinol. Metab.* 101, 1469–1477. <https://doi.org/10.1210/jc.2015-3694>.
- DeLong, E.R., DeLong, D.M., Clarke-Pearson, D.L., 1988. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 44, 837–845.
- Dennedy, M.C., 2018. Salivary cortisone and cortisol following synacthen, a future replacement for serum cortisol? Commentary to: use of salivary cortisol and cortisone in the high and low dose synacthen test. *Clin. Endocrinol.* 88, 770–771. <https://doi.org/10.1111/cen.13585>.
- Efron, B., Tibshirani, R.J., 1993. *An Introduction to the Bootstrap*. Chapman & Hall, New York, London.
- Gaudl, A., Kratzsch, J., Bae, Y.J., Kiess, W., Thiery, J., Ceglarek, U., 2016. Liquid chromatography quadrupole linear ion trap mass spectrometry for quantitative steroid hormone analysis in plasma, urine, saliva and hair. *J. Chromatogr. A* 1464, 64–71. <https://doi.org/10.1016/j.chroma.2016.07.087>.
- Granger, D.A., Kivlighan, K.T., el-Sheikh, M., Gordis, E.B., Stroud, L.R., 2007. Salivary alpha-amylase in biobehavioral research: recent developments and applications. *Ann. N. Y. Acad. Sci.* 1098, 122–144. <https://doi.org/10.1196/annals.1384.008>.
- Hellhammer, D.H., Wüst, S., Kudielka, B.M., 2009. Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology* 34, 163–171. <https://doi.org/10.1016/j.psyneuen.2008.10.026>.
- Het, S., Rohleder, N., Schoofs, D., Kirschbaum, C., Wolf, O.T., 2009. Neuroendocrine and psychometric evaluation of a placebo version of the 'Trier Social Stress Test'. *Psychoneuroendocrinology* 34, 1075–1086. <https://doi.org/10.1016/j.psyneuen.2009.02.008>.
- Katan, M., Müller, B., Christ-Crain, M., 2008. Copeptin: a new and promising diagnostic and prognostic marker. *Crit. Care (Lond., Engl.)* 12, 117. <https://doi.org/10.1186/cc6799>.
- Katz, F.H., Shannon, I.L., 1964. Identification and significance of parotid fluid corticosteroids. *Acta Endocrinol.* 46, 393–404.
- Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The 'Trier Social Stress Test'—a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology* 28, 76–81.
- Lasko, T.A., Bhagwat, J.G., Zou, K.H., Ohno-Machado, L., 2005. The use of receiver operating characteristic curves in biomedical informatics. *J. Biomed. Inform.* 38, 404–415. <https://doi.org/10.1016/j.jbi.2005.02.008>.
- Lennartsson, A.-K., Kushnir, M.M., Bergquist, J., Billig, H., Jonsdottir, I.H., 2012. Sex steroid levels temporarily increase in response to acute psychosocial stress in healthy men and women. *Int. J. Psychophysiol.* 84, 246–253. <https://doi.org/10.1016/j.ijpsycho.2012.03.001>.
- Lewis, J.G., Bagley, C.J., Elder, P.A., Bachmann, A.W., Torpy, D.J., 2005. Plasma free cortisol fraction reflects levels of functioning corticosteroid-binding globulin. *Clin. Chim. Acta* 359, 189–194. <https://doi.org/10.1016/j.cccn.2005.03.044>.
- Livesey, J.H., Dolamore, B., 2010. Stability of plasma adrenocorticotrophic hormone (ACTH): influence of hemolysis, rapid chilling, time, and the addition of a maleimide. *Clin. Biochem.* 43, 1478–1480. <https://doi.org/10.1016/j.clinbiochem.2010.09.020>.
- Mak, I.Y.F., Au Yeung, B.Y.T., Ng, Y.W., Choi, C.H., Iu, H.Y.P., Shek, C.C., Tiu, S.C., 2017. Salivary cortisol and cortisone after low-dose corticotropin stimulation in the diagnosis of adrenal insufficiency. *J. Endocr. Soc.* 1, 96–108. <https://doi.org/10.1210/je.2016-1056>.
- Maninger, N., Wolkowitz, O.M., Reus, V.I., Epel, E.S., Mellon, S.H., 2009. Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). *Front. Neuroendocrinol.* 30, 65–91. <https://doi.org/10.1016/j.yfrne.2008.11.002>.
- Meakin, J.W., Bethune, J.E., Despointes, R.H., Nelson, D.H.O.N., 1959. The rate of disappearance of ACTH activity from the blood of humans. *J. Clin. Endocrinol. Metab.* 19, 1491–1495. <https://doi.org/10.1210/jcem-19-11-1491>.
- Mendel, C.M., 1989. The free hormone hypothesis: a physiologically based mathematical model. *Endocr. Rev.* 10, 232–274. <https://doi.org/10.1210/edrv-10-3-232>.
- Metz, C.E., 1978. Basic principles of ROC analysis. *Semin. Nucl. Med.* 8, 283–298. [https://doi.org/10.1016/S0001-2998\(78\)80014-2](https://doi.org/10.1016/S0001-2998(78)80014-2).
- Morgan, C.A., Southwick, S., Hazlett, G., Rasmussen, A., Hoyt, G., Zimolo, Z., Charney, D., 2004. Relationships among plasma dehydroepiandrosterone sulfate and cortisol levels, symptoms of dissociation, and objective performance in humans exposed to acute stress. *Arch. Gen. Psychiatry* 61, 819–825. <https://doi.org/10.1001/archpsyc.61.8.819>.
- Perogamvros, I., Keevil, B.G., Ray, D.W., Trainer, P.J., 2010. Salivary cortisone is a potential biomarker for serum free cortisol. *J. Clin. Endocrinol. Metab.* 95, 4951–4958. <https://doi.org/10.1210/jc.2010-1215>.
- Perogamvros, I., Owen, L.J., Newell-Price, J., Ray, D.W., Trainer, P.J., Keevil, B.G., 2009. Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 877, 3771–3775. <https://doi.org/10.1016/j.jchromb.2009.09.014>.
- Qian, X., Droste, S.K., Gutiérrez-Mecinas, M., Collins, A., Kersanté, F., Reul, J.M.H.M., Linthorst, A.C.E., 2011. A rapid release of corticosteroid-binding globulin from the liver restrains the glucocorticoid hormone response to acute stress. *Endocrinology* 152, 3738–3748. <https://doi.org/10.1210/en.2011-1008>.
- Raff, H., 2016. Measurement of salivary cortisone to assess the adequacy of hydrocortisone replacement. *J. Clin. Endocrinol. Metab.* 101, 1350–1352. <https://doi.org/10.1210/jc.2016-1228>.
- Rohleder, N., Nater, U.M., 2009. Determinants of salivary alpha-amylase in humans and methodological considerations. *Psychoneuroendocrinology* 34, 469–485. <https://doi.org/10.1016/j.psyneuen.2008.12.004>.
- Sapolsky, R.M., 2000. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch. Gen. Psychiatry* 57, 925–935.
- Schumacher, M., Mattern, C., Ghoumari, A., Oudinet, J.P., Liere, P., Labombarda, F., Sitruk-Ware, R., Nicola, A.Fde, Guennoun, R., 2014. Revisiting the roles of progesterone and allopregnanolone in the nervous system: Resurgence of the progesterone receptors. *Prog. Neurobiol.* 113, 6–39. <https://doi.org/10.1016/j.pneurobio.2013.09.004>.
- Spanakis, E.K., Wand, G.S., Ji, N., Golden, S.H., 2016. Association of HPA axis hormones with copeptin after psychological stress differs by sex. *Psychoneuroendocrinology* 63, 254–261. <https://doi.org/10.1016/j.psyneuen.2015.10.009>.
- Spielberger, C., Gorsuch, R., Lushene, R., Vagg, P., Jacobs, G., 1983. *Manual for the State-Trait Anxiety Inventory. Consulting Psychologists' Press, Palo Alto, California*.
- Toufexis, D., Rivarola, M.A., Lara, H., Viau, V., 2014. Stress and the reproductive axis. *J. Neuroendocrinol.* 26, 573–586. <https://doi.org/10.1111/jne.12179>.
- Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses. *Nat. Rev. Neurosci.* 10, 397–409. <https://doi.org/10.1038/nrn2647>.
- Urwyler, S.A., Schuetz, P., Sailer, C., Christ-Crain, M., 2015. Copeptin as a stress marker prior and after a written examination—the CoEXAM study. *Stress (Amsterdam, Netherlands)* 18, 134–137. <https://doi.org/10.3109/10253890.2014.993966>.
- Weckesser, L.J., Plessow, F., Pilhatsch, M., Muehlhan, M., Kirschbaum, C., Miller, R., 2014. Do venepuncture procedures induce cortisol responses? A review, study, and synthesis for stress research. *Psychoneuroendocrinology* 46, 88–99. <https://doi.org/10.1016/j.psyneuen.2014.04.012>.
- Zweig, M.H., Campbell, G., 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin. Chem.* 39, 561–577.