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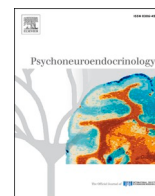
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Research paper

Conditioning cortisol in healthy young women – A randomized controlled trial☆

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ABSTRACT

Background: Learned placebo effects induced by pharmacological conditioning affect immune and endocrine outcomes and may offer new possibilities for clinical applications. Whether or not cortisol is subject to this type of associative learning processes, and whether conditioning may affect responses to stress, is currently unclear.
Method: A randomized placebo-controlled trial was conducted in 48 healthy young women. During acquisition, participants received a pill containing either 100 mg hydrocortisone (unconditioned stimulus) or placebo, paired with a gustatory conditioned stimulus on three consecutive days. During evocation, all participants received placebo paired with the conditioned stimulus, again on three consecutive days. During the third evocation trial, participants underwent a psychosocial stress task. The main outcome parameter salivary cortisol and secondary outcome parameters salivary alpha-amylase, self-reported positive affect and tension, heart rate, and skin conductance level were measured at several time points.
Results: Significant baseline group differences on cortisol were found at several time points, which complicate the interpretation of group differences. During the first evocation session, the conditioned group showed a moderately smaller cumulative decrease in salivary cortisol from baseline than the placebo control group. No significant differences were found between the groups on cortisol during the second and third evocation or in response to stress, nor on other outcome measures.
Conclusion: Although the results provide potential further indications for effects of conditioning on cortisol, baseline differences make it impossible to draw clear conclusions. No indications for possible effects of conditioning on the cortisol stress response or autonomous or affective responses to stress were found.

1. Introduction

Learned placebo effects can be induced by repeatedly pairing a pharmacologically salient unconditioned stimulus (UCS) with a previously neutral conditioned stimulus (CS) in a classical conditioning paradigm. After an association has been formed between UCS and CS, administration of the CS alone elicits physiological responses that are similar or counter regulatory to the responses elicited by the UCS (Pacheco-Lopez et al., 2007; Schedlowski and Pacheco-Lopez, 2010). Pharmacological conditioning has been effective for several immune

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and endocrine outcomes (Skvortsova et al., 2019a; Tekampe et al., 2017; Wendt et al., 2014), and may offer new possibilities for clinical applications (Luckemann et al., 2017; Tekampe et al., 2018).

As cortisol plays a key role in the regulation of the stress response, conditioned cortisol responses would be of great conceptual interest and possibly clinical relevance. Conceptually, conditioned responses within the hypothalamic–pituitary–adrenal axis (HPA axis) would provide more insight into the strong bi-directional communication mechanisms between the central nervous system, governing the HPA axis, and the adrenal glands that produce and secrete cortisol. Clinically, the ability to alter cortisol responses by pharmacological conditioning might in time offer new options for adjunct therapies in stress-related and possibly auto-immune disorders (Tekampe et al., 2018), in which dysfunction of the HPA axis plays a role (Fries et al., 2005; Geenen et al., 2006; Kemeny and Schedlowski, 2007; Yehuda and Seckl, 2011). Previous studies have shown that blunted cortisol responses to psychosocial stress are usually associated with more negative affect (Het et al., 2011; Putman and Roelofs, 2011), while pharmacologically altering cortisol levels may yield promising results regarding stress-related disorders (Aerni et al., 2004; van Peer et al., 2010; Het et al., 2011). Investigating the possible effects of conditioning on the stress response, which includes activation of the HPA axis and the autonomous nervous system (ANS), may thus provide an important starting point to provide first indications for the potential clinical relevance of conditioned HPA axis responses.

Despite its potentially high relevance for humans, HPA axis conditioning has predominantly been investigated in animals. A recent systematic review identified 15 studies measuring conditioned HPA axis responses (cortisol/corticosterone) in animals, of which 13 found significant results indicative of conditioning (Skvortsova et al., 2019a). In these studies various UCS were used, but only a single study (Kreutz et al., 1992) used a UCS directly involved in the regulation of the HPA axis (corticotropin-releasing hormone), resulting in a conditioned increase in corticosterone in rats. For the remaining studies it is unclear whether the HPA axis responses are a direct result of conditioning or more indirectly activated by other conditioned responses, e.g. conditioned stress or immune responses. Whether or not cortisol in humans is also subject to associative learning processes is currently unclear (Tekampe et al., 2017). The limited number of available studies that have investigated HPA axis conditioning in humans provide inconclusive results. Conditioning with sumatriptan, inhibiting the release of cortisol, led to conditioned decreases in plasma cortisol (Benedetti et al., 2003), while conditioning with dexamethasone, a synthetic glucocorticoid that also inhibits cortisol secretion, led to statistically inconclusive results, with – on a descriptive level - indications for a conditioned increase in cortisol (Sabbioni et al., 1997). In a recent study, conditioning with corticotropin-releasing hormone (CRH) led to an increased release of cortisol only in participants with above median cortisol levels at baseline (Petrakova et al., 2017). Taken together, there are indications that the activity of the HPA axis as measured by cortisol may be affected by conditioning. However, results are far from unequivocal.

Whether conditioning affects the cortisol secretion of the HPA axis not only under basal circumstances, but also in response to psychosocial stress, remains unclear. Previous studies indicate a key role of cortisol in the regulation of affective stress responses (Het et al., 2011; Putman and Roelofs, 2011) and an involvement of HPA axis dysfunctions in psychological disorders (Aerni et al., 2004; van Peer et al., 2010; Het et al., 2011). Therefore, investigating conditioned changes in the cortisol response to stress is especially relevant.

The goal of the current study was to provide more insight into whether or not cortisol secretion, as has been shown validly reflected by salivary cortisol concentrations (Hellhammer et al., 2009; Kudielka et al., 2009), can be conditioned with hydrocortisone and to explore whether this affects not only basal cortisol secretion but also cortisol secretion in response to stress. Therefore, the effect of pharmacological conditioning with hydrocortisone on the main outcome parameter salivary cortisol levels under basal conditions and in response to stress

was investigated in healthy female volunteers. Because activation of the stress response includes HPA axis activation as well as affective responses and activation of the ANS, we additionally explored possible effects of cortisol conditioning on secondary parameters reflecting affective and autonomic responses to stress. We hypothesized that conditioned decreases in cortisol may be a likely result of conditioning with hydrocortisone. Hydrocortisone is identical to cortisol and its administration affects negative feedback loops on different levels of the HPA axis. The conditioning paradigm applied in this study has been successfully used before, inducing conditioned responses in various immune and endocrine outcomes (e.g., Stockhorst et al., 1999; Goebel et al., 2002; Goebel et al., 2008; Stockhorst et al., 2011). To provide first indications for the potential clinical relevance of conditioned HPA axis responses, possible effects of conditioning on the cortisol response to stress and on secondary outcome parameters reflecting autonomous and affective responses to stress were explored by including the Trier Social Stress Test as a well-validated psychosocial stress task (Kirschbaum et al., 1993; Dickerson and Kemeny, 2004; Denson et al., 2009) in the evocation phase of this experiment. The feasibility of this addition of a real-world challenge to the conditioning paradigm has been tested in a pilot study (Tekampe et al., 2019). Investigating conditioning of cortisol in female volunteers adds to the relevance of this study, due to the higher prevalence of stress-related disorders in women compared to men (Gradus, 2017).

2. Method

The medical research ethics committee of the Leiden University Medical Center approved the study protocol (LUMC; P14–020, NL47105.058.14), which was published previously (Tekampe et al., 2019). The study was conducted according to the principles of the Declaration of Helsinki (21.10.2008) and the Dutch Medical Research Involving Human Subjects Act (WMO).

2.1. Design

This randomized, placebo-controlled study involved two groups and two experimental phases. After a screening to establish eligibility, participants were randomly assigned to either the hydrocortisone conditioning group or the placebo control group. During the first experimental phase, the acquisition phase, participants received a pill containing either 100 mg of hydrocortisone, which is identical to cortisol, used as an unconditioned stimulus (UCS), or placebo, paired with 150 ml of a distinctively tasting beverage used as the conditioned stimulus (CS) on three consecutive days. The beverage, which was similar to previous conditioning studies (e.g. Goebel et al., 2002, 2008), consisted of 150 ml of commercially available strawberry milk, to which one drop of lavender oil was added. It was colored green by adding the colorants Quinoline Yellow (E104) and Patent Blue (E131). During the second experimental phase, the evocation phase, all participants received placebo paired with the CS again on three consecutive days. On the third evocation day, all participants were additionally exposed to the Trier Social Stress Test (TSST; Kirschbaum et al., 1993).

2.2. Power analysis

The number of participants to be included in the study was predetermined by a power analysis run in G*Power (Faul et al., 2007, 2009). A pilot study (Tekampe et al., 2019) and comparable studies investigating conditioned effects on other endocrine outcomes (insulin, blood glucose) (Stockhorst et al., 1999; Stockhorst et al., 2004; Stockhorst et al., 2011) provided input for the expected effect size (d = 0.06) and a corrective design factor accounting for covariates (Borm et al., 2007). Based on the power analysis with an alpha level of 0.05 and a power of 0.80, it was decided to include a total of 48 participants.

2.3. Participants

Female participants between 18 and 30 years of age were recruited predominantly among student populations at various Dutch universities. Potential participants had to be fluent in Dutch, of good mental and physical health as established by an extensive screening procedure, free from medication including oral contraceptives, and not allergic or hypersensitive to any of the substances used in the study. Subjects were requested to refrain from using drugs, drinking alcoholic or caffeinated beverages, engaging in vigorous physical exercise, eating heavy meals, or smoking cigarettes before each study appointment as all of these behaviors could potentially affect the outcomes of this study. All participants provided written informed consent. Participants were blinded for the conditioning nature of the study by a cover story describing all study procedures except the administration of the CS and stating that the aim of the study was to further investigate the effects of hydrocortisone. Participants were thus informed that they could receive 100 mg hydrocortisone during each of the experimental sessions. After completing their participation all participants were fully debriefed.

2.4. Measures

2.4.1. Salivary cortisol and alpha-amylase

Salivary cortisol was measured as the main outcome parameter of this study and salivary alpha-amylase, reflecting the ANS response to stress, was a secondary outcome parameter. Salivary measures were chosen over blood sampling in this stress-related study, as it is non-invasive, inducing no or minimal amounts of stress (Hellhammer et al., 2009). Measuring cortisol in saliva is a frequently used and well-validated method to measure HPA axis activity in stress research (e. g. Hellhammer et al., 2009). Cortisol levels in saliva are thought to reflect the concentration of free or unbound cortisol that is bioactive. They correlate strongly, but not perfectly, with cortisol blood levels, as the latter reflect bound as well as unbound cortisol (Kudielka et al., 2009). All measurements took place in the afternoon or early evening. Saliva for subsequent cortisol and alpha-amylase measurement was collected using Salivettes (Sarstedt, Rommelsdorf, Germany; Nater et al., 2005; Rohleder et al., 2006; Hellhammer et al., 2009; Kudielka et al., 2012). Participants were asked to put the cotton swab of the Salivette in their mouth without touching it with their hands and move it around their mouth with their tongue for one minute. After collection, the Salivettes were centrifuged (1885 g, 5 min, room temperature) and stored at − 80 °C at the Leiden University Medical Center until analysis at the Radboud university medical center. Cortisol was analyzed in saliva by LCMSMS on a Xevo TQ-XS tandem quadrupole mass spectrometer coupled to an Acquity UPLC system (Waters, Milford, MA, USA) after solid phase extraction. A total CV is 4.7% at 3.2 nmol/L and 3.4% at 37.6 nmol/L (n = 11). Alpha-amylase in saliva was analyzed in a 1:60 dilution by an enzymatic colorimetric assay on a Cobas c702 analyzer (Roche, Mannheim, Germany).

2.4.2. Self-reported affect and stress

Positive and negative affect reflected in a Dutch version of the 20-item Positive and Negative Affect Schedule (PANAS (Watson et al., 1988)) were secondary outcome parameters in this study. The PANAS consists of 10 positive (e.g. 'attentive') and 10 negative emotion adjectives (e.g. 'hostile') that are to be rated on a 5-point Likert scale ranging from 'very slightly or not at all' (1) to 'extremely' (5). Internal consistency of the positive affect subscale was good to excellent (Cronbach's alphas between 0.86 and 0.92). For the negative subscale, internal consistency was compromised by floor effects, resulting in items with zero variance. Cronbach's alphas for this scale ranged from 0.38 to 0.86 but was overall low. Due to this lack of internal consistency for many of the measurement moments and the floor effects, the negative affect scale of the PANAS was excluded from further analyses.

In addition to the PANAS, 7 stress-related items (e.g. 'How relaxed do you feel at the moment?', 'How nervous do you feel at the moment?') were measured using visual analogue scales (Folstein and Luria, 1973; Childs et al., 2011; de Brouwer et al., 2011) anchored 'not at all' (0) to 'very much so' (100). Based on the good to excellent internal consistency of these items (Cronbach's alphas between 0.81 and 0.94), they were summarized in one single score reflecting tension by reversing the positive items and computing the mean of all seven items.

2.4.3. Heart rate, skin conductance, and heart rate variability

Heart rate and skin conductance were measured at baseline and during evocation as secondary outcome parameters of this study, reflecting activation of the ANS. Heart rate was measured with a Lead-II configuration involving one electrode to the chest of the participant and one to the lower left rib. A high pass filter of 0.5 Hertz was used. Skin conductance level was measured with two electrodes applied to the participants' non-dominant hand with a gain of 5 μƱ/V and a low pass filter of 10 Hertz. Both measurements were done simultaneously and non-invasively with the BIOPAC ECG100C Electrocardiogram Amplifier and the GSR100C module in combination with MP150 Data Acquisition System at a sampling rate of 1000 per second. For the analyses of HR and SC, mean levels of 5-minute measurement epochs were used, which were derived from standardized electronic markers that indicated the beginning and end of the interval in which participants were asked to sit still. For the TSST, mean levels of 5-minute epochs were derived from the preparation, speech, and mental arithmetic parts of the TSST separately, in order to reflect only the active parts of the TSST, excluding instructions by the experimenter or panel members. The subparts of the TSST were subsequently averaged for analysis. See procedure section and Section 1 of the Appendix for a more precise definition of the used measurement epochs. Analysis of the data was done using PhysioData Toolbox (Sjak-Shie, 2018).

Heart rate variability (HRV) was assessed as a measure of stress reflected in vagal withdrawal (Laborde et al., 2017). For the calculation of the Root Mean Square of the Successive Differences (RMSSD), two standardized 8-minute sequences recorded during a filler task in which participants watched a nature documentary and during the TSST was selected. The standardized 8-minute epoch during the TSST was based on markers set to indicate the sub-parts of the TSST (preparation, speech, arithmetic task). Both sequences were scanned for artifacts, ectopic beats and extra systoles and corrected if necessary.

2.4.4. Baseline characteristics of the participants

At baseline, demographic (e.g. age, education), health (e.g. BMI) and personality characteristics of the participants were assessed using questionnaires. Extraversion and neuroticism were assessed by the Eysenck Personality Questionnaire Revised Short Scale (EPQ-RSS-EN, (Sanderman et al., 1991)), optimism by the Revised Life Orientation Test (Lot-R, (Scheier et al., 1994)), and perceived stress by the Perceived Stress Scale (PSS, (Cohen, 1988; Cohen and Janiki-Deverts, 2012)). Internal consistency of these scales, reflected in Cronbach's alphas were 0.86 and 0.68 for the EPQ-RSS Extraversion and Neuroticism subscales respectively, 0.68 for the LOT-R total and 0.77 for the PSS.

2.4.5. Additional measures

After intake of the pill and CS, the participants were asked to rate the subjective taste quality of the CS on a nine-point Likert scale reaching from 'very unpleasant' (0) to 'very pleasant' (9) (Wirth et al., 2011; Grigoleit et al., 2012; Ober et al., 2012). Also participants completed a questionnaire checking for blinding at the end of the study. In this questionnaire, they were asked to indicate for each of the experimental sessions whether they thought to have received placebo or hydrocortisone.

2.4.6. Trier Social Stress Test

The TSST is a well-validated and frequently used laboratory stressor that takes about 20 min to complete and has been shown to reliably elicit

cortisol responses (Kirschbaum et al., 1993; Dickerson and Kemeny, 2004; Denson et al., 2009). It starts with a 5-minute preparation period in which participants prepare a presentation about their personal qualifications for their dream job. Subsequently they have to deliver this presentation as part of a 6-minute sham job interview in front of 2 panel members providing negative feedback. After the interview they also have to complete a 4-minute mental arithmetic task under time pressure.

2.5. Procedure

Before entering the study, all potential participants were screened for eligibility by completing the MINI Neuropsychiatric Interview for all DSM-IV conditions, and an assessment of their physical health, conducted by a physician. The screening appointment was scheduled at least one week prior to the first acquisition session and lasted about 1.5 h. During the screening, demographic, health-related and baseline characteristics (e.g. education, menstrual cycle phase, perceived stress, personality traits) of the participants were assessed using self-report questionnaires and a baseline measurement of self-reported affect and tension, saliva collection for subsequent cortisol and alpha-amylase measurement, and a 5-minute recording of heart rate and skin conductance commenced.

For an overview of the measurement points during the experiment see Fig. 1. The acquisition phase comprised three experimental sessions (session 1–3) taking place in the afternoons of three consecutive days, as cortisol levels are more stable during the second half of the day. Each of the sessions followed the same procedure and lasted approximately 10–15 min. After confirming participants still met the inclusion criteria by structured interview questions, saliva was collected and participants self-reported affect and tension were assessed. Then, a pill containing either hydrocortisone (UCS) or placebo was administered, together with the CS. After administration, participants were asked to rate the subjective taste quality of the CS. At the end of each session, participants were instructed to refrain from activities that could potentially influence the HPA axis and thus interfere with conditioning (e.g., drinking caffeinated or alcoholic beverages, consuming heavy meals, and exercising) for at least the following 4 h.

The evocation phase took place 4 days after the final acquisition session, again in the afternoons of three consecutive days (session 4–6). Session 4 and 5 lasted approximately 2.5 h and session 6 3 h. Before the start of the session it was confirmed again that the participant met the inclusion criteria. Then electrodes for the recording of heart rate and skin conductance were attached to the participant and a baseline measurement of self-reported affect and tension, saliva collection and a 5-minute recording of heart rate and skin conductance commenced. Afterwards, all participants received a pill containing placebo paired with the CS (T0) and were asked to rate the subjective taste quality of the CS. The sessions continued with filler tasks, interrupted by measurements of self-reported affect and tension, saliva collection and 5-minute recordings of heart rate and skin conductance every 30 min (see Fig. 1). Sessions 4 and 5 each contained 5 of such measurement points, which are indicated by their relative timing with regard to the administration of the CS (0): T-10 (baseline), T + 30, T + 60, T + 90 and T + 120. In the last evocation session, session 6, participants were exposed to the TSST after measurement 3 (T + 60) and a 6th measurements at T + 150, approximately 60 min after the end of the TSST was added. At the end of the 6th session, participants filled in a questionnaire to check for blinding. After that, participants were debriefed about the nature of the TSST as a stressor and not as an actual assessment of skills, and the conditioning protocol of the study. For their participation in the study participants received ascending amounts of monetary compensation after each experimental session, amounting to a total of €150,- upon completion of the study.
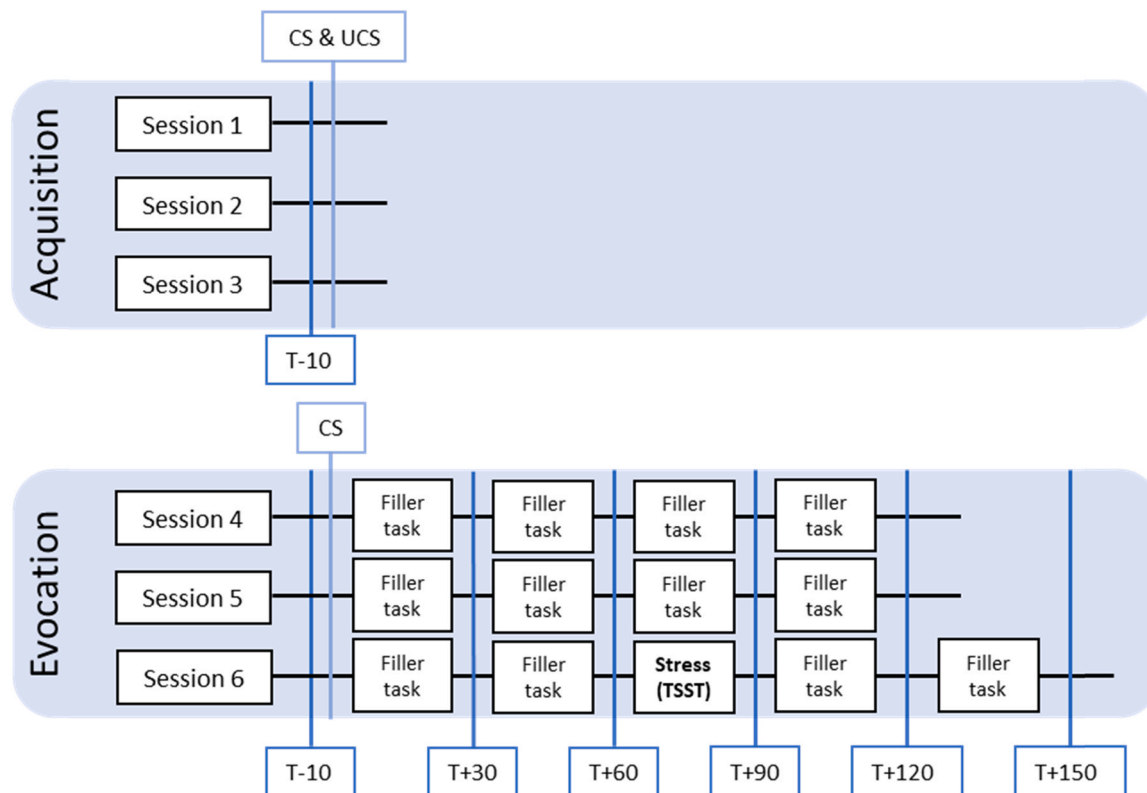

Fig. 1. Overview of the study procedures. CS & UCS = administration of the conditioned and unconditioned stimulus; TSST = Trier Social Stress Test; T - 10 – T + 150 = measurement of salivary cortisol and alpha-amylase, self-reported affect and tension, 5-minute recording of heart rate and skin conductance level, numbers indicate timing from administration of the CS and UCS in minutes.

2.6. Statistical analysis

Statistical analyses were done in SPSS (version 25). As all outcome measurements were done in the evocation phase (sessions 4–6), only data of participants who completed at least one evocation session (session 4) were used in the analyses. Assumptions about the normality of sampling distribution, outliers and equal variances were checked for outcome variables. The sampling distribution was checked by inspecting histograms and calculating z-scores for skewness and kurtosis. The assumption of equal variances was checked by Levene's test. If many of the z-scores indicated significant skewness or kurtosis or if Levene's test indicated unequal variances, data was transformed using log 10 (salivary cortisol) or square root transformation (salivary alpha-amylase, VAS tension, heart rate, skin conductance), whichever yielded the most favorable results (again checked by histograms and z-scores). Outliers were identified by boxplots and confirmed by calculating z-scores. If boxplots showed outliers with $z > 2.58$ ($p < 0.01$), analyses were run with and without these outliers. For repeated measures ANOVAs the assumption of sphericity was checked by computing Mauchly's tests, Greenhouse-Geisser and Huynh-Feldt epsilon. In case the assumption of sphericity was not tenable, corrected test statistics were reported (Greenhouse-Geisser for epsilon < 0.75, indicated as $_{G-G}$ and Huynh-Feldt for epsilon > 0.75 indicated as $_{H-F}$). For all ANOVAs, partial eta squared (η_p^2) was calculated as an indicator of the effect size. Cut off values of η_p^2 of 0.01, 0.06, and 0.14 were used to indicate small, medium, and large effects, respectively.

Baseline differences between the two groups during screening were investigated by between-subject ANOVAs or in case of categorical variables (e.g. education) with Fisher's exact tests. To capture possible effects of hydrocortisone administration (session 2 and 3) on salivary cortisol and alpha-amylase, and self-reported affect and tension, between-subject ANOVAs were calculated per session and confirmed by sensitivity analyses excluding outliers if there were any, and with non-parametric Mann-Whitney *U* tests if assumptions were not met.

Possible effects of conditioning were investigated during the evocation phase. For the main outcome parameter salivary cortisol, untransformed data were aggregated into Area under the curves (AUC) using the formulas provided by Pruessner et al. (2003). To avoid inflating AUC values, time intervals were expressed in intervals of 10 min instead of 1 min. Two AUCs were computed for all measurements of session 4 and all measurements of session 5, respectively. For session 6, two AUCs were computed. The first one starting at baseline and ending with the measurement taken at 60 min after CS administration to reflect basal cortisol. A second AUC was calculated from 60 min after CS administration to the end of the session, to capture the response to the TSST. If significant baseline differences between the groups were found at the beginning of the respective session, the AUC increase (AUCi) with respect to baseline were computed, reflecting the cumulative increase or decrease in cortisol levels from baseline. Otherwise AUC with respect to ground (AUCg) was used. Possible covariates were added to the outcome analyses if they correlated significantly with the AUC of cortisol and significantly differed between groups. Thus Pearson correlations were computed between AUCs of cortisol and participant characteristics (PSS, EPQ, LOT) and baseline measurements of outcome variables (salivary cortisol, positive affect, tension, salivary alpha-amylase, heart rate and skin conductance level) measured during screening. Group differences in these possible covariates were analyzed by ANOVAs. Groups were compared by between-subject ANOVAs or ANCOVAs if covariates were identified. In case significant group differences in AUCs of cortisol were identified, these were investigated further by repeated measures ANOVAs including all measurements per evocation session as within-subjects factor Time, and Group as between-subjects factor. The response to the TSST in session 6 was analyzed by two repeated measures ANOVAs, one for each group. A significant increase in cortisol from the measurement taken just before the TSST to the measurement taken directly after the TSST was interpreted as indicative of the

effectiveness of the TSST. Additionally, the number of responders showing at least a 1.5 nmol/l increase in cortisol from the measurement taken just before the TSST to the measurement taken directly after he TSST was determined and proportions of responders were compared per group. Finally, as menstrual cycle phase has been shown to sometimes affect the cortisol stress response, two separate repeated measures ANOVAs were run per group to investigate whether there is an interaction between the change in cortisol levels over within-subject factor Time and between-subject factor menstrual cycle phase. In case a significant interaction was found, menstrual cycle phase would be added as a factor in subsequent analyses of the cortisol stress response in session 6. For secondary outcome parameters, repeated measures ANOVAs including all measurements per evocation session as within-subjects factor Time, and Group as between-subjects factor were conducted.

3. Results

3.1. Inclusion, drop-out and baseline characteristics of the participants

A total of 83 participants were screened for this study. Of these, 23 were excluded after screening. Seven participants were excluded for current or past mental complaints, 5 for current use of medication, 5 for being allergic or hypersensitive to one of the substances used in the study, 4 for current or past medical complaints and 2 for medical complaints in their family history. Eight participants dropped out before the evocation phase, leaving no outcome data to be analyzed. Reasons for dropout were missing an appointment or being more than half an hour late (3 participants), being unable to keep the appointments due to other obligations (2 participants), aversion to the CS (2 participants) and having the flu (1 participant, drop-out occurred before hydrocortisone administration). Another three participants dropped out during the evocation phase, two for reasons unknown (one after session 4, one after session 5) and one due to a stressful life event (after session 5). Additionally, data of session 6 of one participant was not usable as the participant refused to participate in the TSST. This left data of 52 participants for analyses on session 4, 51 participants for analyses on session 5, and 48 participants who completed the entire experiment (including the analyses on session 6).

The two experimental groups did not significantly differ on any of the baseline characteristics, see Table 1. Age of the participants ranged from 18 to 28 years. The educational level of the participants was high, with 79% of the participants being in, or having completed, tertiary education and the remainder secondary. The majority of participants were students, many of them working either paid or unpaid jobs next to their study. None of the participants had children and all indicated being unwed; half of the participants indicated being in a relationship. Most of the participants were Dutch nationals, one participant indicated having the Dutch and the Turkish nationality, and the remaining 4 participants had the British, German, Iraqi, or Italian nationality. The majority of participants in both groups had a normal BMI (between 18.00 and 24.90). Mean scores of the participants on perceived stress, neuroticism, extraversion and optimism did not differ between groups.

3.2. Check of assumptions and transformations of data

Untransformed cortisol levels showed a strong positive skewness, kurtosis and unequal variances for many of the measurements points. Logarithmic transformation (log10) resulted in close to normal distributions and equal variances for most, but not all, of the measurement points. Of the 20 measurements taken during the study, one showed an outlier. As a consequence, analyses were run on transformed cortisol levels and confirmed by sensitivity analysis without outliers where necessary, and non-parametric tests where possible. For the Area Under the Curve increase (AUCi) of cortisol, none of the transformations (log10, ln or square root) resulted in satisfactory

Table 1
Overview of baseline characteristics of participants.

	Hydrocortisone (N = 26)	Placebo (N = 26)	Total sample (N = 52)	Group differences
Demographic characteristics				
Age in years, mean (sd)	21.88 (2.30)	22.12 (2.29)	22.00 (2.28)	$F(1,50) = 0.31$, $p = 0.718$, $\eta_p^2 < 0.01$
Education, n (%)				$p = 0.173$ *Fisher's exact test*
Secondary	8 (31%)	3 (12%)	11 (21%)	
Tertiary	18 (69%)	23 (89%)[a]	41 (79%)	
Relationship status, n (%)				$p = 0.782$ *Fisher's exact test*
With partner	12 (46%)	14 (54%)	26 (50%)	
Without partner	14 (54%)	12 (46%)	26 (50%)	
Nationality, n (%)				$p = 0.350$ *Fisher's exact test*
Dutch	22 (85%)	25 (96%)	47 (90%)	
Other	3 (12%)	1 (4%)	4 (8%)	
Multiple	1 (4%)[a]	0 (0%)	1 (2%)	
Health-related characteristics				
BMI, mean (sd)	22.89 (3.35)	23.14 (2.92)	23.01 (3.11)	$F(1,50) = 0.08$, $p = 0.773$, $\eta_p^2 < 0.01$
Perceived Stress (PSS), mean (sd)	10.15 (4.13)	10.00 (4.14)	10.08 (4.10)	$F(1,50) = 0.02$, $p = 0.894$, $\eta_p^2 < 0.01$
Personality characteristics				
Neuroticism (EPQ-RSS), mean (sd)	1.65 (2.00)	2.46 (2.06)	2.06 (2.05)	$F(1,50) = 2.06$, $p = 0.158$, $\eta_p^2 = 0.04$
Extraversion (EPQ-RSS), mean (sd)	8.08 (3.50)	9.42 (2.53)	8.75 (3.10)	$F(1,50) = 2.53$, $p = 0.118$, $\eta_p^2 = 0.05$
Optimism (LOT-R total), mean (sd)	18.04 (2.99)	17.19 (2.51)	17.62 (2.77)	$F(1,50) = 1.22$, $p = 0.274$, $\eta_p^2 = 0.02$

Note. Data shown is for participants who completed at least session 4 and are included in the outcome analysis.

[a] percentages amount to 101 because of rounding. BMI = Body Mass Index, PSS = Perceived Stress Scale, EPQ-RSS = Eysenck Personality Questionnaire Revised Short scale, LOT-R = Life Orientation Test Revised.

distribution of scores. All of the AUCis showed outliers and removing these improved the distribution. Therefore, analyses were run on untransformed AUCis and confirmed by non-parametric sensitivity analyses. Positive affect scores did not violate assumptions and one of the 20 measurements taken during the study showed an outlier. Where necessary, analyses were therefore confirmed by sensitivity analyses without this outlier. VAS tension scores, salivary alpha-amylase levels, heart rate and skin conductance levels showed a moderate positive skewness and kurtosis. Analyses were therefore run on square root transformed variables, which had a normal or close to normal distribution. Of the 20 measurements taken during the study, outliers were identified on 4 VAS tension scores and 2 salivary alpha-amylase measurements. One outlier was identified for heart rate during the preparation period of the TSST. For skin conductance levels, 9 measurements showed an outlier (the same person). Where necessary, analyses of these outcomes were therefore confirmed by sensitivity analyses without outliers.

3.3. Baseline differences in outcome variables

On the baseline measurement taken during screening, the hydrocortisone conditioning group had moderately lower levels of salivary cortisol and heart rate than the placebo control group (see Table 2). However, for salivary cortisol, these differences were not confirmed in non-parametric sensitivity analyses (p = 0.09). The groups did not differ significantly on positive affect, VAS tension scores, salivary alpha-amylase levels and skin conductance level (see Table 2). All results were confirmed by non-parametric sensitivity analyses.

3.4. Identification of possible covariates

Of the participant characteristics (PSS, EPQ, LOT) and baseline outcome measures during screening that were investigated as possible covariates, only extraversion (EPQ-RSS-EN) correlated significantly with the AUCg of cortisol during session 6 (r = −0.30; p = 0.040), AUCg of cortisol during stress (r = −0.34; p = 0.018) and AUCi of cortisol during stress (r = −0.37; p = 0.010). As extraversion did not significantly differ between groups, it was not added to the subsequent outcome analyses as a possible covariate.

3.5. Group differences during acquisition

At the beginning of session 1, the hydrocortisone conditioning group again had moderately lower salivary cortisol levels than the placebo control group (see Table 3). At the beginning of session 2, 24 h after the first dose of hydrocortisone or placebo, the hydrocortisone conditioning group had lower cortisol levels compared to the placebo control group and a similar difference between the groups was found for session 3, 24 h after the second dose. Groups did not differ on positive affect scores, tension, and salivary alpha-amylase. All of these results were confirmed by non-parametric sensitivity analyses (see Table 3).

Table 2
Baseline measurements taken during screening.

	Hydrocortisone (N = 26)	Placebo (N = 26)	Total sample (N = 52)	Group differences
Salivary cortisol (nmol/l)	1.39 (0.74)	2.14 (1.51)	1.76 (1.23)	**$F(1,50) = 4.08$, $p = 0.049$, $\eta_p^2 = 0.08$[a,b]**
Positive affect	30.46 (5.77)	30.77 (7.64)	30.62 (6.70)	$F(1,50) = 0.03$, $p = 0.870$, $\eta_p^2 < 0.01$
VAS tension	17.11 (9.21)	19.15 (9.91)	18.13 (9.52)	$F(1,50) = 0.45$, $p = 0.504$, $\eta_p^2 < 0.01$[a]
Salivary alpha-amylase (u/ml)	138.71 (110.45)	140.96 (99.72)	139.84 (104.19)	$F(1,50) = 0.01$, $p = 0.912$, $\eta_p^2 < 0.01$[a]
Heart rate	65.82 (5.89)	70.96 (9.55)	68.39 (8.27)	**$F(1,50) = 5.12$, $p = 0.028$, $\eta_p^2 = 0.09$[a]**
Skin conductance level screening	3.17 (2.31)	3.81 (2.11)	3.49 (2.22)	$F(1,50) = 1.73$, $p = 0.194$, $\eta_p^2 = 0.03$[a]

Note. Data shown is for participants who completed at least session 4 and are included in the outcome analysis. Table displays means and (sd) for untransformed data including possible outliers. Test statistics of tests indicating significant group differences are printed in bold.

[a] tests were performed on transformed data;
[b] test outcomes were not confirmed by non-parametric sensitivity analyses.

Table 3
Measurements of outcome parameters during acquisition.

	Hydrocortisone (N = 26)	Placebo (N = 26)	Total sample (N = 52)	Group differences
Salivary cortisol session 1 (nmol/l)	2.20 (1.00)	3.33 (2.08)	2.76 (1.71)	**F(1,50) = 4.49, p = 0.039 η_p^2 = 0.08**[a]
Salivary cortisol session 2 (nmol/l)	1.09 (1.13)	2.80 (1.44)	1.94 (1.54)	**F(1,50) = 37.23, p < 0.001, η_p^2 = 0.43**[a]
Salivary cortisol session 3 (nmol/l)	0.73 (0.43)	2.75 (1.56)	1.76 (1.53)	**F(1,49) = 63.29, p < 0.001, η_p^2 = 0.56**[a]
Positive affect session 1	27.08 (5.95)	27.12 (7.43)	27.10 (6.66)	F(1,50) < 0.01, p = 0.984, η_p^2 < 0.01
Positive affect session 2	25.58 (6.70)	26.96 (7.16)	26.27 (6.90)	F(1,50) = 0.52, p = 0.475, η_p^2 = 0.01
Positive affect session 3	25.00 (6.37)	25.54 (7.26)	25.27 (6.77)	F(1,50) = 0.08, p = 0.777, η_p^2 < 0.01
VAS tension session 1	21.36 (13.15)	25.62 (12.33)	23.49 (12.80)	F(1,50) = 1.68, p = 0.201, η_p^2 = 0.03[a]
VAS tension session 2	23.77 (15.70)	24.43 (11.73)	24.10 (13.73)	F(1,50) = 0.25, p = 0.619, η_p^2 < 0.01[a]
VAS tension session 3	22.57 (13.76)	24.13 (14.10)	23.35 (13.81)	F(1,50) = 0.32, p = 0.573, η_p^2 < 0.01[a]
Salivary alpha-amylase session 1 (u/ml)	143.87 (102.89)	133.68 (91.39)	138.77 (96.49)	F(1,50) = 0.06, p = 0.804, η_p^2 < 0.01[a]
Salivary alpha-amylase session 2 (u/ml)	128.45 (104.02)	137.73 (78.59)	133.09 (91.40)	F(1,50) = 0.46, p = 0.500, η_p^2 < 0.01[a]
Salivary alpha-amylase session 3 (u/ml)	104.20 (70.96)	121.60 (78.02)	112.90 (74.36)	F(1,50) = 0.70, p = 0.406, η_p^2 = 0.01[a]

Note. Data shown is for participants who completed at least session 4 and are included in the outcome analysis. Table displays means and (sd) for untransformed data including possible outliers. Test statistics of tests indicating significant group differences are printed in bold.

[a] tests are performed on transformed data.

3.6. Effects of conditioning in session 4

3.6.1. Cortisol

At the beginning of session 4, the hydrocortisone conditioning group had moderately lower cortisol levels than the placebo control group (F (1,49) = 5.95, p = 0.018 η_p^2 = 0.11). For untransformed salivary cortisol levels see Table 4. This difference was statistically significant regardless of outliers (F(1,48) = 9.67, p = 0.003 η^2 = 0.17) and confirmed by non-parametric sensitivity analyses. To take these baseline difference between the groups into account, further analyses were done on the Area Under the Curve with respect to baseline (AUCi), which was computed from the untransformed cortisol levels and reflects the cumulative decrease in cortisol levels from baseline (see Fig. 2, panel A). Leaving out two outliers, the hydrocortisone conditioning group showed a moderately smaller cumulative decrease from baseline than the placebo control group (F(1,47) = 4,26; p = 0.045 η_p^2 = 0.08). This difference did not reach statistical significance when the two outliers were left in the

Table 4
Salivary cortisol levels in nmol/l for all measurements taken during the evocation phase.

	Hydrocortisone conditioning	Placebo	Total
Session 4	(N = 26)	(N = 26)	(N = 52)
T - 10	2.02 (1.39)	2.78 (1.54)	2.41 (1.51)
T + 30	1.40 (0.72)	2.05 (1.16)	1.73 (1.01)
T + 60	1.19 (0.54)	1.45 (0.64)	1.32 (0.60)
T + 90	1.01 (0.48)	1.22 (0.65)	1.12 (0.57)
T + 120	0.99 (0.57)	1.00 (0.52)	0.99 (0.54)
Session 5	(N = 26)	(N = 25)	(N = 51)
T - 10	2.47 (2.06)	3.01 (2.15)	2.74 (2.10)
T + 30	1.74 (1.01)	2.30 (1.58)	2.02 (1.34)
T + 60	1.43 (0.88)	1.56 (0.81)	1.49 (0.84)
T + 90	1.11 (0.62)	1.30 (0.69)	1.21 (0.65)
T + 120	1.07 (0.73)	1.07 (0.61)	1.07 (0.67)
Session 6	(N = 24)	(N = 24)	(N = 48)
T - 10	2.23 (1.28)	3.09 (1.70)	2.66 (1.55)
T + 30	1.75 (0.75)	2.28 (1.24)	2.01 (1.05)
T + 60	1.27 (0.52)	1.68 (0.88)	1.47 (0.75)
T + 90	2.54 (1.17)	2.92 (2.51)	2.73 (1.96)
T + 120	3.27 (2.55)	2.89 (2.49)	3.08 (2.50)
T + 150	2.09 (1.68)	1.88 (1.16)	1.98 (1.42)

Note. Table displays means and (sd) for untransformed data including possible outliers. Analyses reported in this article were done on transformed data.

analysis (F(1, 49) = 1.88; p = 0.177; η_p^2 = 0.04) or when tested non-parametrically (p = 0.052).

A subsequent repeated-measures ANOVA for all 5 of the log-transformed salivary cortisol measurements (Time) in session 4, taking Group (Hydrocortisone or Placebo) into account as a between-subjects factor, showed a large main effect for Time ($F_{G-G}(2.03, 99.61)$ = 92.42; p < 0.001; η_p^2 = 0.65) and a moderate main effect for the interaction between Time and Group ($F_{G-G}(2.03, 99.61)$ = 3.26; p = 0.042 η_p^2 = 0.06). Tests of within-subjects contrasts of Time showed that each cortisol measurement was lower than the previous one (all p < 0.001, all η_p^2 ≥0.14) indicating a steady decrease across the session. Also, regarding the Time by Group interaction, there were moderately larger decreases in cortisol in the placebo control group compared to the hydrocortisone conditioning group from 30 to 60 (F(1,49) = 4.17; p = 0.047 η_p^2 = 0.08) and from 90 to 120 min after administration of the CS (F(1,49) = 5.38; p = 0.025 η_p^2 = 0.10). Comparable results were obtained when excluding one outlier on baseline cortisol, with the distinction that significant differences between the groups were found (F (1,48) = 4.72; p = 0.035 η_p^2 = 0.09). See Fig. 2 panel B for the mean untransformed salivary cortisol levels per measurement in both groups during session 4 (including the outlier on the first measurement); for actual values see Table 4.

3.6.2. Secondary outcome measures

Positive affect and VAS tension scores, heart rate and skin conductance levels fluctuated over time, but no significant effects of Group or interaction between Time and Group were found. For salivary alpha-amylase, levels did not significantly differ over time and again no
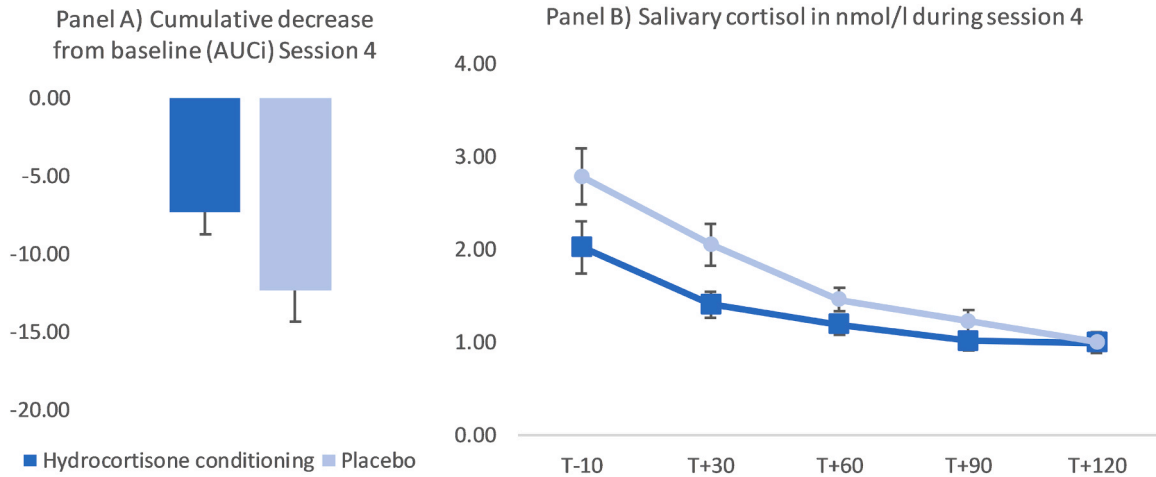
Fig. 2. Salivary cortisol during session 4 (untransformed). Panel A shows the mean cumulative decrease from baseline (expressed by AUCi) per group. Panel B shows the mean for each measurement per group. Error bars are SEM.

significant effects of Group or Time x Group interaction were found. For a detailed account of these analyses and the course these outcomes took across measurements, please view Section 2.1 of the Appendix.

3.7. Effects of conditioning in session 5

3.7.1. Cortisol

For cortisol levels in session 5, no significant baseline differences were found between the hydrocortisone and placebo control group parametrically ($F_{(1,49)} = 1.36$; $p = 0.249$; $\eta_p^2 = 0.03$) as well as non-parametrically ($p = 0.283$). However, for reasons of consistency, analyses were done on the AUCi of cortisol, thus taking baseline into account. Groups did also not differ on the AUCi of cortisol ($F_{(1,47)} = 0.26$; $p = 0.613$; $\eta_p^2 < 0.01$), regardless of outliers ($F_{(1,44)} = 1.45$; $p = 0.234$; $\eta_p^2 = 0.03$), see Fig. 3 panel A. This was again confirmed by non-parametric testing ($p = 0.617$). As the previous analyses did not indicate significant group differences, no repeated-measures analysis was carried out on the cortisol levels in session 5; for a descriptive view of the untransformed values see Fig. 3 panel B; for actual values see Table 4.

3.7.2. Secondary outcome measures

As for session 4, positive affect scores, heart rate and skin conductance levels fluctuated over time, but no significant effects of Group or interaction between Time and Group were found. For VAS tension scores

and salivary alpha-amylase, levels did not significantly differ over time and again no significant effects of Group or Time x Group interaction were found. For a detailed account of these analyses and the course these outcomes took across measurements, please view Section 2.2 of the Appendix.

3.8. Effects of conditioning on responses to stress in session 6

3.8.1. Cortisol

The hydrocortisone conditioning group had significantly lower cortisol levels than the placebo control group at baseline of session 6 when tested both parametrically ($F_{(1,46)} = 4.25$; $p = 0.045$ $\eta_p^2 = 0.09$) and non-parametrically ($p = 0.041$). Before commencement of the Trier Social Stress Test (TSST) the cumulative decrease in cortisol levels (AUCi from T1 to T3) did not differ between the two groups ($F_{(1,46)} = 1.57$; $p = 0.216$; $\eta_p^2 = 0.03$, see Fig. 4 panel A). This was regardless of outliers ($F_{(1,45)} = 3.64$; $p = 0.063$; $\eta_p^2 = 0.08$) and confirmed by non-parametric sensitivity analyses ($p = 0.398$).

At T3, just before the TSST, the log-transformed cortisol levels did not significantly differ between the two groups when tested parametrically ($F_{(1,46)} = 3.27$; $p = 0.077$; $\eta_p^2 = 0.07$) as well as non-parametrically ($p = 0.124$). Exposure to the TSST led to a large increase in cortisol levels in the hydrocortisone ($F_{(1,22)} = 24.39$; $p < 0.001$; $\eta_p^2 = 0.53$) as well as the placebo control group ($F_{(1,23)} =$
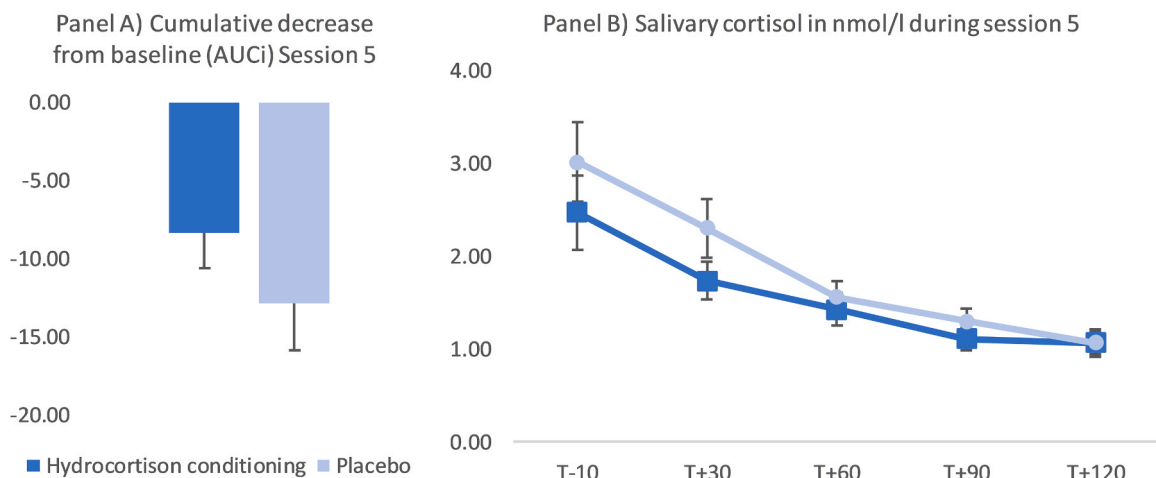

Fig. 3. Salivary cortisol during session 5 (untransformed). Panel A shows the mean cumulative decrease from baseline (expressed by AUCi) per group. Panel B shows the mean for each measurement per group. Error bars are SEM.
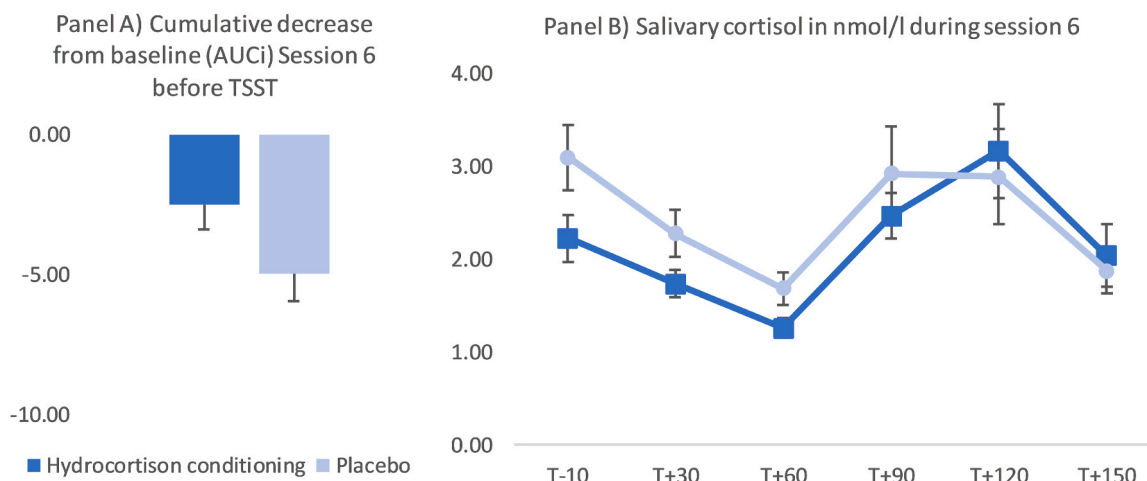
Panel A) Cumulative decrease from baseline (AUCi) Session 6 before TSST

Panel B) Salivary cortisol in nmol/l during session 6

Fig. 4. Salivary cortisol during session 6 (untransformed). Panel A shows the mean cumulative decrease from baseline (expressed by AUCi) per group from T1 to T3. Panel B shows the mean for each measurement per group. Error bars are SEM.

5.18; $p = 0.032$; $\eta_p^2 = 0.18$). For an overview of the mean untransformed salivary cortisol levels per measurement in both groups during session 6 see Fig. 4 panel B. There was no significant interaction between the time of measurement (before or directly after the TSST) and menstrual cycle phase on cortisol in both groups (hydrocortisone: $F(1,18) = 0.06$; $p = 0.805$; $\eta_p^2 < 0.01$, placebo: $F(1,15) = 0.05$; $p = 0.832$; $\eta_p^2 < 0.01$). A total of 19 participants (40%), eleven (48%) in the hydrocortisone conditioning group and 8 (33%) in the placebo control group, responded to the TSST with a cortisol increase of at least 1.5 nmol/l from T3 to T4, which has been defined as a cut-off for being a responder to the TSST (Miller et al., 2013). These proportions did not significantly differ between the two groups ($p = 0.380$ *Fisher's exact test*). The two groups did also not differ in their cortisol response to the TSST expressed in the AUCi from T3 to T6 ($F(1,45) = 0.62$; $p = 0.434$; $\eta_p^2 = 0.01$). This again was regardless of outliers ($F(1,44) = 0.19$; $p = 0.668$; $\eta_p^2 < 0.01$) and confirmed by non-parametric sensitivity analyses ($p = 0.202$). (Fig. 5).

3.8.2. Secondary outcome measures

Exposure to the TSST induced large increases in VAS tension scores ($F(1,46) = 134.27$; $p < 0.001$; $\eta_p^2 = 0.76$), salivary alpha-amylase ($F(1,43) = 48.06$; $p < 0.001$; $\eta_p^2 = 0.53$), and heart rate ($F(1,39) =$

AUCi Session 6 response to TSST

Fig. 5. Cortisol response to Trier Social Stress Test expressed in mean AUCi (T3 to T6 of session 6) per group and SEM.

136.75; $p < 0.001$; $\eta_p^2 = 0.78$). Heart rate variability decreased in response to the TSST ($F(1,40) = 40.72$; $p < 0.001$; $\eta_p^2 = 0.50$). Positive affect scores fluctuated over time, but did not significantly decrease in response to the TSST. For all of these outcomes, no effects of Group or Time x Group interaction were found. For a detailed account of these analyses and the course these outcomes took across measurements, please view Section 2.3 of the Appendix. Skin conductance level fluctuated over time ($F_{G-G}(1.85,73.78) = 25.08$; $p < 0.001$; $\eta_p^2 = 0.39$), with no significant effect of Group ($F(1,40) = 0.92$; $p = 0.344$; $\eta_p^2 = 0.02$), but a moderate interaction effect between Time and Group ($F_{G-G}(1.85,73.78) = 3.89$; $p = 0.028$; $\eta_p^2 = 0.09$). Within-subject contrasts showed a large increase in skin conductance from the measurement taken at the beginning of the session, about 10 min before administration of the CS, to 30 min after administration of the CS ($F(1,40) = 21.75$; $p < 0.001$; $\eta_p^2 = 0.35$) and again during the TSST ($F(1,40) = 93.44$; $p < 0.001$; $\eta_p^2 = 0.70$). After the TSST, from the measurement taken at 90–120 min after administration of the CS, skin conductance decreased ($F(1,40) = 136.16$; $p < 0.001$; $\eta_p^2 = 0.77$). This decrease was significantly larger in the placebo control group compared to the hydrocortisone conditioning group ($F(1,40) = 5.00$; $p = 0.031$; $\eta_p^2 = 0.11$). Analyses without outliers yielded similar results.
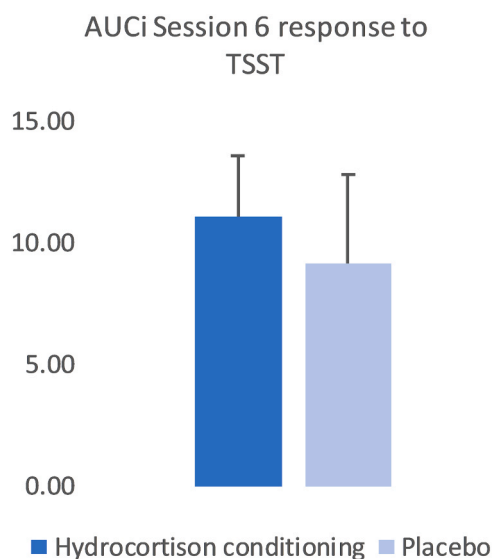
3.9. Blinding of the participants

At the end of the experiment, participants indicated for each session whether they thought to have received hydrocortisone or placebo. In total, participants indications were true 59% of the time. Participants in the hydrocortisone conditioning group indicated the true treatment per session 46% of the times. As in general participants much more frequently believed to have received placebo than hydrocortisone, the placebo control group indicated the true treatment more frequently, namely 73% of the time.

4. Discussion

The current study set out to investigate whether cortisol can be conditioned in healthy young women by repeatedly pairing the administration of hydrocortisone with a gustatory conditioned stimulus (CS). As cortisol plays a key role in the regulation of the stress response, conditioned responses were investigated both under basal conditions and in response to psychosocial stress. Investigating conditioned cortisol responses is of conceptual and clinical relevance, as it provides more insight in the mechanisms that govern the regulation of the HPA axis and may in time offer possibilities for the treatment of stress-related and auto-immune disorders in which the dysregulation of the HPA axis plays

a role.

Due to unanticipated baseline differences in cortisol between the groups, the current study has not been able to provide a definitive answer to whether or not salivary cortisol levels, as a reflection of cortisol secretion, are subject to conditioning, despite attempts to strengthen our knowledge base by increasing power and using hydrocortisone as UCS in a well-tested pharmacological conditioning design (Tekampe et al., 2019). Because of differences in the designs of all of the studies conducted on cortisol conditioning so far, for example with regard to the UCS used that affect cortisol release in different directions and potentially through different regulatory mechanisms, it is difficult to compare the studies directly. Despite some evidence for cortisol conditioning in animal studies (Hadamitzky et al., 2020), the majority of the previous studies on conditioning cortisol in humans yielded inconclusive indications for conditioned cortisol responses (Sabbioni et al., 1997; Petrakova et al., 2017). The one study that did show conditioned cortisol decreases in humans (Benedetti et al., 2003) used Sumatriptan as a UCS instead of an agent directly involved in the HPA axis (CRF, dexamethasone or hydrocortisone). As discussed more elaborately in a paper describing the design of the current study (Tekampe et al., 2019), hydrocortisone was chosen as UCS, because it is the pharmacological equivalent of cortisol, binding to both mineralocorticoid and glucocorticoid receptors. Also, the current study adds to the previous ones by including a psychosocial challenge of the stress system in which cortisol plays such an important role.

Results of this study may provide indications for a conditioned suppression of the HPA axis during the first evocation session (session 4). This would be consistent with the suppression of the HPA axis, as reflected in lower cortisol levels in response to the hydrocortisone administration in the conditioned group, during the acquisition phase. Despite efforts to statistically control for it, relatively consistent lower baseline salivary cortisol in the hydrocortisone conditioning group than in the placebo control group may offer an alternative explanation for the results of this study. The cause of these baseline differences is unknown and they may be due to chance. As the block-randomization scheme and delivery of the containers with study medication was executed by the pharmacy, blinding of the experimenters has not been compromised. Therefore, these baseline differences as well as any other effects observed in this study are not due to a lack of blinding. Another alternative explanation for the lower salivary cortisol levels in the hydrocortisone conditioned group would be the possible presence of a residual negative feedback effect of the repeated hydrocortisone administrations during the acquisition phase. This would call for a conditioning paradigm in which the acquisition and evocation trials are spaced further apart or for a design in which these possible residual effects are measured in a conditioned but not evoked control group.

Baseline levels of cortisol at the beginning of the experimental sessions were relatively high, comparable to levels in response to the TSST, and consistently decreased across repeated measurements during the evocation sessions, both in the hydrocortisone conditioning and the placebo control group. Together with the consistent decrease found in heart rate from the beginning of each evocation session, this might indicate the presence of anticipation stress in the participants. However, these effects were not present in other outcome parameters (skin conductance, salivary alpha-amylase) and self-report-data, and the study procedures were highly repetitive and therefore predictable, designed to prevent stress responses other than to the TSST. Therefore, the decrease in cortisol might at least partially be explained by circadian effects, as all sessions took place in the afternoon or early evening, where cortisol levels gradually decrease. The decrease in heart rate may also be due to the relative physical inactivity of the participants during the experimental sessions.

No significant differences between the hydrocortisone conditioning and the placebo control group were found during the second and third evocations or in response to stress (session 5 and 6), which might be due to extinction of the already moderate conditioned response after repeated unreinforced evocations. A recent study on the hormone oxytocin indicated that conditioned hormonal responses may be subject to fast extinction processes and the same may be true for cortisol (Skvortsova et al., 2019b). Studies on conditioned immunological responses showed that extinction of conditioned responses can be prevented by booster sessions (Doering and Rief, 2012) in which the association of CS and UCS is renewed or by administration of subclinical doses of the UCS during the evocation (Albring et al., 2014). This prevention of extinction processes widens the possibilities for using conditioned responses in clinical settings (Tekampe et al., 2018) and may form an interesting target for future studies.

Administration of the Trier Social Stress Test during the final evocation session led to marked increases in cortisol, self-reported stress, salivary alpha-amylase as well as heart rate and skin conductance, as well as a decrease in HRV in both the hydrocortisone conditioning and the placebo control group. The participants in this experiment were all young female volunteers, who as part of their education had ample recent experience in presenting, receiving critical feedback, and job interviews. Resultantly, the TSST might not have been as stressful for this population as it could have been for others. Despite this, 40% of the participants could be identified as cortisol responder to the TSST, indicating a strong stress response (Miller et al., 2013). The cortisol response to the TSST indicates that, at least during the third evocation trial, there was no adrenocortical insufficiency at this time after repeated hydrocortisone administration during the acquisition phase. In their immediate response to the TSST as well as in their recovery, both groups did not significantly differ from each other. Whether this absence of a conditioned effect is due to extinction of the conditioned response after repeated unreinforced evocations or because conditioning of cortisol does not affect the response to stress remains unclear at this point. Including a stressor in the first evocation session, where extinction has not yet taken place, could enable future studies to provide more clarity. In this study it was chosen only to include the psychosocial stress task at the very end of the experiment in order to prevent anticipation stress in the participants facing subsequent evocation trials, which could potentially disturb the evocation of a conditioned response.

Regarding the secondary outcome parameters self-reported affect and tension, salivary alpha-amylase, heart rate, skin conductance and, in response to stress, HRV, no indications for changes due to conditioning were found. Hydrocortisone administration with the dose and frequency as performed in this study is not known to affect these outcome parameters directly, which may explain the absence of conditioned responses under basal conditions. However, in response to stress, changes in cortisol levels have an effect on affective responses to stress (Het et al., 2011; Putman and Roelofs, 2011) and could in turn affect responses of the autonomic system as well. Whether the apparent absence of these effects in this study is due to ineffectiveness of cortisol conditioning in altering responses to stress or due to extinction processes, as described earlier, is currently unclear.

Regarding blinding, in general, participants were not able to guess reliably whether or not they had received hydrocortisone during the experiment. Due to the lack of symptoms or side effects, the majority of the participants believed to have received placebo. Therefore, the number of participants indicating the actual treatment they received at the end of the study was relatively larger in the placebo group.

Limitations of this study include the relatively small sample size, although it was determined based on a power calculation (Tekampe et al., 2019) and is larger than many of the previous studies addressing immune and endocrine conditioning (Tekampe et al., 2017). Also the sample under investigation in the current study was highly selective including only young healthy female volunteers who were not using oral contraceptives. Investigating a female sample bears the potential risk of possible effects of menstrual cycle phase affecting the study outcomes. While basal cortisol remains unaffected by menstrual cycle phase, the cortisol response to stress may be blunted in the follicular phase

(Kudielka et al., 2009). However, no difference in the response of participants in the follicular compared to the luteal phase could be observed in this study. Furthermore, women may be a more relevant target group when investigating HPA axis conditioning, as they are more prone to developing stress-related diseases (Gradus, 2017) and are generally underrepresented in experimental research. The oral administration of hydrocortisone as a UCS in this study makes it challenging to pinpoint the exact timing and strength of the UCS and therefore also the unconditioned response due to individual differences in intestinal uptake, and thereby biological availability of hydrocortisone. In contrast, administering hydrocortisone intravenously would provide better control over the timing and actual plasma levels. However, the invasive nature of intravenous administration may itself trigger a stress response in the HPA axis, which may interfere with conditioning (Kirschbaum and Hellhammer, 1994). Next, due to the administration of hydrocortisone during the three acquisition trials, it is not possible to examine whether a conditioned response might have been established already after only one acquisition session. Because the measurements in session 2 and 3 are taken after one or two days of hydrocortisone administration in the conditioned group, it is impossible to interpret any differences between the groups as a conditioned response rather than an effect of HPA axis suppression due to hydrocortisone treatment. Finally, the four days in between the acquisition and evocation phase in the current study, which were needed to certify sufficient wash-out of the repeated hydrocortisone administration in the conditioned group, might have led to passive forgetting, leading or contributing to the inconclusive results of the study. However, this seems unlikely as conditioned effects have been shown for varying intervals between the acquisition and evocation phase in other pharmacological conditioning studies (Tekampe et al., 2019, Hadamitzky et al., 2020), with conditioned responses even having been found until 30 days after conditioning (Hörbelt et al., 2019).

The use of a study design that has previously been effective in conditioning immune responses (Tekampe et al., 2017) can be considered a clear strength of this study. Also the addition of a psychosocial stressor at the end of the study, to investigate possible effects of conditioning on psychophysiological stress responses, can be considered a valuable innovation. Due to the systematic baseline differences found in this study, a replication of the results is needed to draw firm conclusions about the effectiveness of HPA axis conditioning. The possible mechanisms behind conditioning of the HPA axis may be another target for future investigations. In this regard, measuring other regulatory hormones of the HPA axis such as ACTH and CRH in animals may provide valuable insight.

For possible future replications, a few points are worth closer consideration. To control for a suppression of the HPA axis due to the administration of hydrocortisone, the length of the wash-out period between acquisition and evocation phase could be increased. Also adding a dose control group that receives hydrocortisone throughout both acquisition and evocation phase might be useful in distinguishing possible effects of HPA axis suppression from conditioned responses. Furthermore, if possible effects of conditioning in response to stress are of interest, introducing the stressor earlier in the evocation phase could be an option.

This study investigated whether cortisol can be conditioned in healthy young women by repeatedly pairing the administration of hydrocortisone with a gustatory conditioned stimulus (CS) and whether conditioning would affect cortisol responses to stress. Additionally possible effects of conditioning on autonomous and affective parameters were explored. Even though the current study provides further cautious indications for the potential effects of conditioning on cortisol, due to unanticipated baseline differences in cortisol between the groups, no definitive answer could be provided, despite attempts to strengthen our knowledge base by increasing power and using hydrocortisone as UCS in a well-tested pharmacological conditioning design. No indications were found for possible effects of conditioning on the cortisol stress response or on autonomous or affective outcomes under basal circumstances or in responses to stress.

Appendix

A.1) Additional information on the HRV measurement epochs

To investigate possible differences in HRV expressed in RMSSD between the hydrocortisone conditioned and placebo control group, 2 measurement epochs were selected. For the first epoch, serving as a baseline, a standardized part of HR recorded during a filler task in which participants watched a nature documentary was selected. This baseline recording started 2 min after the start of the task and ended ten min after the start of the task, resulting in an 8 min epoch. The second epoch was taken from HR data recorded during the TSST. To ensure that equal parts of the speech and mental arithmetic tasks of the TSST were included for each participant, a marker was inserted in between these two tasks. The position of this marker minus 4 min was used as the starting point of this epoch, while the marker plus 4 min was used as the endpoint of the epoch. This again resulted in a measurement epoch of exactly 8 min.

A.2.1) Course of additional outcome measures during session 4

Positive affect scores fluctuated over Time ($F_{H-F}(4,200) = 18.53$; $p < 0.001$; $\eta_p^2 = 0.27$). However there was no significant main effect for Group ($F(1,50) = 0.38$; $p = 0.542$, $\eta_p^2 < 0.01$) or interaction between Group and Time ($F_{H-F}(4,200) = 1.70$; $p = 0.151$; $\eta_p^2 = 0.03$). Tests of within-subjects contrasts showed that positive affect scores increased from 30 to 60 min after administration of the CS ($F(1,50) = 37.58$; $p < 0.001$; $\eta_p^2 = 0.43$) and from 90 to 120 min after administration of the CS ($F(1,50) = 25.16$; $p < 0.001$; $\eta_p^2 = 0.34$). When one outlier on the first measurement was left out of the analysis, the within-subject contrast also showed a significant moderate increase in positive affect from the first measurement to 30 min after administration of the CS ($F(1,49) = 4.87$; $p = 0.032$ $\eta_p^2 = 0.09$).

Tension scores fluctuated slightly over Time ($F_{H-F}(3.55,177.43) = 3.05$; $p = 0.023$; $\eta^2 = 0.06$). There were no significant main effects for Group ($F(1,50) = 0.51$; $p = 0.480$; $\eta_p^2 = 0.01$) or interaction between Group and Time ($F_{H-F}(3.55,177.43) = 0.24$; $p = 0.895$). Test of within-subject contrasts did not reach statistical significance.

For salivary alpha-amylase levels, there were no significant changes over Time ($F_{H-F}(3.08,154.03) = 0.32$; $p = 0.817$; $\eta_p^2 < 0.01$) and no effect for Group ($F(1,50) = 0.30$; $p = 0.590$; $\eta_p^2 < 0.01$) or interaction

effect between Group and Time ($F_{H\text{-}F}(3.08,154.03) = 0.96$; $p = 0.413$; $\eta_p^2 = 0.02$). Sensitivity analyses in which one outlier on the measurement taken 90 min after administration of the CS was excluded yielded similar results.

Heart rate decreased over Time ($F_{G\text{-}G}(2.36,108.52) = 62.32$; $p < 0.001$; $\eta_p^2 = 0.58$), but no significant main effect for Group ($F(1,46) = 0.84$; $p = 0.364$; $\eta_p^2 = 0.02$) or interaction between Time and Group ($F_{G\text{-}G}(2.36,108.52) = 0.68$; $p = 0.532$; $\eta_p^2 = 0.02$) was found. Test of within-subject contrasts showed that heart rate decreased from the beginning of the session to 30 min after administration of the CS ($F(1,46) = 36.72$; $p < 0.001$; $\eta_p^2 = 0.44$) and from 60 to 90 min after administration of the CS ($F(1,46) = 124.64$; $p < 0.001$; $\eta_p^2 = 0.73$). A moderate decrease was found from 90 to 120 min after administration of the CS ($F(1,46) = 4.15$; $p = 0.047$; $\eta_p^2 = 0.08$).

For skin conductance level, a large significant main effect for Time ($F_{G\text{-}G}(1.63,78.26) = 24.90$; $p < 0.001$; $\eta_p^2 = 0.34$), but not for Group ($F(1,48) = 0.03$; $p = 0.862$; $\eta_p^2 < 0.01$) or interaction between Time and Group ($F_{G\text{-}G}(1.63,78.26) = 1.68$; $p = 0.197$; $\eta_p^2 = 0.03$) was found. Test of within-subject contrasts indicated a large increase in skin conductance level from the beginning of the session to 30 min after administration of the CS ($F(1,48) = 49.67$; $p < 0.001$; $\eta_p^2 = 0.51$). Sensitivity analyses in which outliers were excluded yielded similar results.

A.2.2) Course of additional outcome measures during session 5

For positive affect, results were similar to session 4. Large fluctuations over Time were found ($F_{H\text{-}F}(3.52,169.01) = 12.87$; $p < 0.001$; $\eta_p^2 = 0.21$), but no significant main effect for Group ($F(1,48) = 0.69$; $p = 0.411$; $\eta_p^2 = 0.01$) or interaction between Group and Time ($F_{H\text{-}F}(3.52,169.01) = 0.32$; $p = 0.840$; $\eta_p^2 < 0.01$). Tests of within-subjects contrasts showed that positive affect scores significantly increased from 30 to 60 min after administration of the CS ($F(1,48) = 20.43$; $p < 0.001$; $\eta_p^2 = 0.30$) and from 90 to 120 min after administration of the CS ($F(1,48) = 25.60$; $p < 0.001$; $\eta_p^2 = 0.35$).

Tension scores did not significantly differ between the measurement points ($F_{H\text{-}F}(3.95,189.78) = 2.33$; $p = 0.058$; $\eta_p^2 = 0.05$) or between the groups ($F(1,48) = 0.82$; $p = 0.370$; $\eta_p^2 = 0.02$). Also no significant interaction between Group and Time was found ($F_{H\text{-}F}(3.95,189.78) = 0.70$; $p = 0.594$; $\eta_p^2 = 0.01$).

For salivary alpha-amylase levels, there were no significant changes over Time ($F_{H\text{-}F}(3.48,170.63) = 0.91$; $p = 0.449$; $\eta_p^2 = 0.02$) and no effect for Group ($F(1,49) = 0.07$; $p = 0.800$; $\eta_p^2 < 0.01$) or interaction effect between Group and Time ($F_{H\text{-}F}(3.48,170.63) = 0.60$; $p = 0.641$; $\eta_p^2 = 0.01$).

Heart rate decreased over Time ($F_{G\text{-}G}(2.39,109.71) = 59.02$; $p < 0.001$; $\eta_p^2 = 0.56$) with no significant main effect for Group ($F(1,46) = 0.04$; $p = 0.839$; $\eta_p^2 < 0.01$) or interaction between Time and Group ($F_{G\text{-}G}(2.39,109.71) = 2.35$; $p = 0.090$; $\eta_p^2 = 0.05$). As for session 4, tests of within-subject contrasts showed that heart rate decreased from the beginning of the session to 30 min after administration of the CS ($F(1,46) = 32.66$; $p < 0.001$; $\eta_p^2 = 0.42$), from 60 to 90 min after administration of the CS ($F(1,46) = 70.51$; $p < 0.001$; $\eta_p^2 = 0.61$) and from 90 to 120 min after administration of the CS ($F(1,46) = 5.57$; $p = 0.023$; $\eta_p^2 = 0.11$).

Skin conductance level increased over Time ($F_{G\text{-}G}(1.56,73.10) = 20.51$; $p < 0.001$; $\eta_p^2 = 0.30$) with no significant effects of Group ($F(1,47) = 0.05$; $p = 0.823$; $\eta_p^2 < 0.01$) or interaction between Time and Group ($F_{G\text{-}G}(1.56,73.10) = 2.07$; $p = 0.143$; $\eta_p^2 = 0.04$). Test of within-subject contrasts indicated a large increase in skin conductance level from the beginning of the session to 30 min after administration of the CS ($F(1,47) = 47.33$; $p < 0.001$; $\eta_p^2 = 0.50$).

A.2.3) Course of additional outcome measures during session 6

Positive affect significantly fluctuated over Time ($F_{G\text{-}G}(3.10, 142.80) = 11.43$; $p < 0.001$; $\eta_p^2 = 0.20$), and there was no significant main effect for Group ($F(1.46) = 0.05$; $p = 0.819$; $\eta_p^2 < 0.01$), or interaction between time and group ($F_{G\text{-}G}(3.10, 142.80) = 1.49$; $p = 0.220$; $\eta_p^2 = 0.03$).

Within-subjects contrasts showed a large decrease in positive affect before the TSST, from 30 to 60 min after administration of the CS ($F(1,46) = 65.56$; $p < 0.001$; $\eta_p^2 = 0.59$), a large increase after the TSST, from 90 to 120 min after administration of the CS ($F(1,46) = 17.16$; $p < 0.001$; $\eta_p^2 = 0.27$) and a large decrease from 120 to 150 min after administration of the CS ($F(1,46) = 12.53$; $p = 0.001$; $\eta_p^2 = 0.21$).

Tension scores also fluctuated over Time ($F_{G\text{-}G}(2.36,108.52) = 72.57$; $p < 0.001$; $\eta_p^2 = 0.61$), but there was no significant main effect for Group ($F(1,46) = 1.00$; $p = 0.322$; $\eta_p^2 = 0.02$). Also, no significant interaction between Time and Group was found ($F_{G\text{-}G}(2.36,108.52) = 0.996$; $p = 0.383$ $\eta_p^2 = 0.02$). Within-subject contrasts showed a large decrease in tension scores before the TSST, from 30 to 60 min after administration of the CS ($F(1,46) = 7,80$; $p = 0.008$; $\eta_p^2 = 0.15$). In response to the TSST, from 60 to 90 min after administration of the CS, tension scores increased ($F(1,46) = 134.27$; $p < 0.001$; $\eta_p^2 = 0.76$). After the TSST, from 90 to 120 ($F(1,46) = 97.00$; $p < 0.001$; $\eta_p^2 = 0.68$) and 120 to 150 min $F(1,46) = 13.77$; $p = 0.001$; $\eta_p^2 = 0.23$) after administration of the CS, tensions scores decreased. Analyses without outliers yielded similar results.

Salivary alpha-amylase fluctuated over Time ($F_{G\text{-}G}(2.82, 121.45) = 29.62$; $p < 0.001$; $\eta_p^2 = 0.41$), and no significant effect of Group ($F(1,43) = 0.04$; $p = 0.838$; $\eta_p^2 < 0.01$) or interaction between Time and Group ($F_{G\text{-}G}(2.82, 121.45) = 1.01$; $p = 0.388$; $\eta_p^2 = 0.02$) were found. Within-subject contrasts showed that salivary alpha-amylase increased slightly before the TSST from 30 to 60 min after administration of the CS ($F(1,43) = 4.64$; $p = 0.037$; $\eta_p^2 = 0.10$) and to a large extent in response to the TSST from 60 to 90 min after administration of the CS ($F(1,43) = 48.06$; $p < 0.001$; $\eta_p^2 = 0.53$). After the TSST from 90 to 120 min after administration of the CS, salivary alpha-amylase decreased ($F(1,43) = 26.65$; $p < 0.001$; $\eta_p^2 = 0.38$). Analyses without outliers yielded similar results.

Heart rate fluctuated over Time ($F_{G\text{-}G}(2.07,80.57) = 119.59$; $p < 0.001$; $\eta_p^2 = 0.75$), with no significant main effect of Group ($F(1,39) = 1.49$; $p = 0.229$; $\eta_p^2 = 0.04$) or interaction between Time and Group ($F_{G\text{-}G}(2.07,80.57) = 0.83$; $p = 0.444$; $\eta_p^2 = 0.02$). Within-subject contrasts showed a large decrease in heart rate from the measurement taken at the beginning of the session to 30 min after administration of the CS ($F(1,39) = 49.22$; $p < 0.001$; $\eta_p^2 = 0.56$) and from 30 to 60 min after administration of the CS ($F(1,39) = 7.79$; $p = 0.008$.; $\eta_p^2 = 0.17$). Heart rate increased during the TSST ($F(1,39) = 136.75$; $p < 0.001$; $\eta_p^2 = 0.78$) and then decreased after the TSST ($F(1,39) = 294.62$; $p < 0.001$; $\eta_p^2 = 0.88$) and from 120 to 150 min after administration of the CS ($F(1,39) = 10.80$; $p = 0.002$; $\eta_p^2 = 0.22$).

Skin conductance level fluctuated over Time ($F_{G\text{-}G}(1.85,73.78) = 25.08$; $p < 0.001$; $\eta_p^2 = 0.39$) with no significant main effect for Group ($F(1,40) = 0.92$; $p = 0.344$; $\eta_p^2 = 0.02$). A significant interaction effect was found between Time and Group ($F_{G\text{-}G}(1.85,73.78) = 3.89$; $p = 0.028$; $\eta_p^2 = 0.09$). Within subject contrasts showed a large increase in skin conductance level from the measurement taken at the beginning of the session to 30 min after administration of the CS ($F(1,40) = 21.75$; $p < 001$; $\eta_p^2 = 0.35$), a large increase during the TSST ($F(1,40) = 93.44$; $p < 001$; $\eta_p^2 = 0.70$) and a large decrease after the TSST ($F(1,40) = 136.16$; $p < 001$; $\eta_p^2 = 0.77$). A moderate interaction effect between Group and Time was found for the recovery after the TSST ($F(1,40) = 5.00$; $p = 0.031$; $\eta_p^2 = 0.11$), where the decrease in skin conductance level was larger in the placebo control group than in the hydrocortisone conditioning group.

Heart rate variability decreased during the TSST ($F(1,40) = 40.72$; $p < 0.001$; $\eta_p^2 = 0.50$), with no significant effects of Group ($F(1,40) = 2.67$; $p = 0.110$; $\eta_p^2 = 0.06$), or interaction between time and group ($F(1,40) = 0.07$; $p = 0.800$; $\eta_p^2 < 0.01$).

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