International Journal of Psychophysiology 69 (2008) 33–40

Contents lists available at ScienceDirect

International Journal of Psychophysiology

journal homepage: www.elsevier.com/locate/ijpsycho

Salivary alpha amylase and cortisol responses to different stress tasks: Impact of sex

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ARTICLE INFO

Article history:
Received 17 December 2007
Received in revised form 22 February 2008
Accepted 27 February 2008
Available online 5 March 2008

Keywords:
Amylase
Cortisol
Stress
Sex difference
Human

ABSTRACT

Neuro-endocrine markers such as salivary alpha amylase (sAA) and cortisol (CORT) play an important role in establishing human responses to stressful events. Whereas sAA levels reflect sympathetic system activity, salivary cortisol appears to be a valid measure for HPA axis activity. Although many studies looked at either sAA or CORT responses in reaction to stress, work still has to be done to look at the way these systems interact, especially when both systems are activated. Additionally, sex effects in CORT responses have been investigated relatively often, but possible sex differences in sAA levels and responses, or the way both systems interact has not been the focus of sufficient studies to yield a univocal conclusion.

In this study we presented a group of healthy participants (n=80) with two mildly stressful tasks, consisting of an aversive picture rating task and a cold pressor stress (CPS) task. The second task was compared with a control task. We expected a rise in sAA level in response to the first task and sAA as well as CORT responses on the second task and explored the interaction between the two responses.

Results indicate that sAA is indeed a sensitive marker in both psychologically and physically induced arousal paradigms, whereas a cortisol response was only observed in the CPS task. Men had higher sAA levels than women during the complete course of the study, but men and women were comparable in their responsivity to the tasks. No strong correlations between sAA and CORT responses were found.

1. Introduction

Neuro-endocrine markers play an important role in establishing the bodily reaction in studies on human responses to stressful events. Salivary cortisol (Cort) sampling has been used as a measure for HPA axis activity for quite some time (Kirschbaum and Hellhammer, 1994). The sympathetic adrenal medullar system (SAM) activation as part of the stress response is monitored by measurement of salivary alpha amylase (sAA) levels in several studies (Bosch et al., 1996; Granger et al., 2007; Nater et al., 2005; Rohleder et al., 2006). Studies show marked increases in sAA levels in response to stressful tasks or procedures, such as a parachute jump (Chatterton et al., 1997) or a stressful video game (Skosnik et al., 2000) as well as other types of psychological (e.g. pre-examination) stress-inductions (Bosch et al., 1996, 2003). The finding that psychosocial stress stimulates sAA were underlined in studies employing the Trier Social Stress Test (TSST) (Nater et al., 2006b, 2005; Rohleder et al., 2004) and a study in which subjects underwent a stressful fMRI procedure, involving negative emotional picture viewing (van Stegeren et al., 2006) or video stressors (Takai et al., 2004). Finally, pharmacological manipulation of the SAM system underscored the role of sAA amylase as an indicator of sympathetic activity. Stimulation of the SAM system by administration of yohimbine (an alpha-2 adrenergic receptor antagonist) was shown to significantly increase sAA levels (Ehlert et al., 2006), whereas application of the beta-adrenergic receptor blocker propranolol was successful in reducing stress-induced sAA increases (van Stegeren et al., 2006).

Activation of the stress response in reaction to a threatening, negative or unexpected experience evokes a chain of neuro-endocrine and nervous system reactions. The SAM system with catecholamines such as noradrenaline and adrenaline, in interaction with glucocorticoïds, plays a key role in both normal homeostasis and in sympathetically mediated responses to stress. The various roles of glucocorticoïds (GCs) in stress responses have been extensively reviewed (Sapolsky et al., 2000). GC actions permit, stimulate or suppress an ongoing stress response or can be preparative for a subsequent stressor. But the exact way these systems interact in the stress response is not univocal. Although many studies looked at either NA or CORT responses in reaction to stress, reality is that both systems are part of a coherent unity that needs to work in concert. Work has to be done to look at the way these systems interact, especially when both systems are activated.

This study was designed to investigate sAA and Cortisol responses to two different stressors. The first task was a mild (psychological) stress task in which subjects watched a large set of neutral and emotional pictures from the International Affective Picture Set (IAPS) (Lang et al., 1997). The task was intended to only activate the SAM system — but not the HPA axis and evoked increased amygdala

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doi:10.1016/j.ijpsycho.2008.02.008

activation measured with fMRI in a previous study due to its emotional arousal (van Stegeren et al., 2005). Additionally it evoked an increase in sAA levels in healthy subjects, but no cortisol increase (van Stegeren et al., 2006). Immediately after this task the group was split: half of the participants underwent a second stress task, the other half a control condition. This stress task consisted of a cold pressor stress (CPS) procedure that has been shown in earlier studies to evoke a CORT response in a substantial proportion of the participants (Andreano and Cahill, 2006; Cahill et al., 2003). Salivary sampling at several points in time during the experiment would serve as an indication of the sAA and CORT levels, the responsivity of both systems and its possible interactions.

Based on a scarce set of studies that show that consecutive exposure to stressors showed an accumulation of stress responses (Liu et al., 2007; Sabban and Serova, 2007), we hypothesized that the response on one stress task can affect the response on consecutive experiences.

Several studies showed that men and women differ in their response to stressful events: in their personal emotional rating of emotional material (women almost always higher than men), or their memory performance of emotional information (Bradley et al., 2001; Cahill and van Stegeren, 2003; Canli et al., 2002; van Stegeren et al., 1998). Also, baseline differences between men and women were found on several cardiovascular measures such as heart rate and blood pressure (Saab et al., 1989; Suarez et al., 2004) as well as effects of sex and hormonal status on the physiological response to acute psychosocial stress (Kudielka et al., 1998; Kuhlmann and Wolf, 2005; Stark et al., 2006; Wolf et al., 2001). Several studies found a stronger salivary cortisol response in men than in women in reaction to stressors (Kirschbaum et al., 1999; Kudielka and Kirschbaum, 2005). However, sex differences do not substantially explain the variability in the CORT response to laboratory stressors in young subjects, as demonstrated in a recent large meta analysis (Dickerson and Kemeny, 2004). So, although several studies investigated sex differences in CORT response, only few studies focused on sex differences in sAA levels or responses and the studies led to ambiguous results (Kivlighan and Granger, 2006; Nater et al., 2006a; Takai et al., 2007).

The aim of the present study was twofold: the first research question is whether sAA and CORT responses are related during two consecutive stress tasks. We hypothesize that SAM system and HPA axis responses on the tasks are interconnected. More precisely, we hypothesize that subjects reacting with a sAA increase on the first task are more sensitive to a following stressful stimulus, setting up the system for a stronger response to the second task. So we hypothesized that a strong sAA response on the first task predicts a stronger sAA and CORT response on the second stress task. The second research question refers to the idea that men and women might differ in their stress response. We want to explore whether sex is affecting baseline sAA and CORT levels as well as the reactivity and the interaction of both hormonal systems.

2. Methods

2.1. Design

This design was part of a larger study in which the interaction effects of neuroendocrine responses on memory performance were investigated. The memory performance data will be reported elsewhere. Here the sAA and Cort responses and the possible correlation between these two measures in response to two consecutive stressors were examined and analyzed.

This is a mixed design in which Task 1 consists of watching Neutral (Neu) and Emotional (Emo) pictures as within subjects' variable. In Task 2 subjects were randomly assigned to one out of two conditions as between-subjects variable: a cold pressor stress (CPS) procedure versus control condition.

2.2. Participants

Eighty healthy subjects (21 male, 59 female, mean age = 20.7; SD = 3.2) participated in this study. Thirty-seven of the female participants were using oral contraceptives (OC), 22 were non-OC and of this last group 8 women were in the first half and 11 women in the second half of their cycle. Information on cycling day of 2 non-OC women was missing. Men and (OC and non-OC) women were equally divided over the CPS and control condition (Chi-square test: all n.s.). All participants were students of the Department of Clinical Psychology at the University of Amsterdam and received course credit for their participation. They were all free of use of medication or drugs and had no previous experience with experiments of this kind. The study-protocol was approved by the Ethics Committee of the Department of Psychology of the University of Amsterdam. Each subject was informed about experimental procedures and signed an informed consent form. One female subject withdrew after the first session.

2.3. Materials

2.3.1. Task 1: Picture watching and rating

Stimulus material consisted of 144 pictures derived from the International Affective Picture System (IAPS) (Lang et al., 1997). Pictures were divided in four categories, validated and used in earlier studies (van Stegeren and Everaerd, in preparation; van Stegeren et al., 2005) depicting neutral domestic items or tools (CAT1) to extremely negative emotional (CAT4) images, depicting mutilation or serious injuries. Categories ranging from 1 to 4 were related to IAPS norms ranging from neutral (5.0) to extremely negative pictures (2.0) and in arousal from low (3.2) to highly arousing (6.2). No positively valenced pictures were included. After each picture a rating form appeared on the computer screen with the question “how emotional did you feel the picture to be?”. Participants pressed one out of four buttons with their right hand to indicate emotionality on a four-point scale from 0 (“not emotional at all”) to 3 (“extreme emotionally intense”).

2.3.2. Task 2: Cold pressor stress (CPS) versus control condition

To evoke a physical stress response a cold pressor stress (CPS) task was used. In earlier studies this procedure has been shown to cause a cortisol response in healthy subjects (Buchanan et al., 2006; Cahill et al., 2003; McRae et al., 2006). Subjects had to place their arm in a tank containing water with ice cubes, with a temperature of below 3 °C. A container with 8 l of warm water with a temperature between 35 and 40 °C served as a control condition. Temperature was kept constant during the experiment.

2.3.3. Apparatus and software

A software program (WESP, version 1.9.8) timed the administration of the stimuli and marked change-over between stimuli and interstimulus intervals. The software program is developed at our research department (http://www.fmg.uva.nl/psy_research/ => Technical support unit). The data were stored on a PC for off-line analysis. All pictures were stored as bmp-files on the PC and were presented on a 17″-monitor at a distance of 80 cm from the subjects.

2.3.4. Salivary sampling

During the experiment salivary samples were obtained using Salivette sampling devices (Sarstedt, Nümbrecht, Germany) at five consecutive points in time (Fig. 1). The first sample was taken just before the picture presentation started (t1 = −30 min), a second sample was taken immediately after picture presentation but before the CPS task started (t2 = 0). The next three measurements took place at +10, +20 and +60 min after the start of the CPS (t3, t4, t5 respectively). Salivary Alpha Amylase (sAA) and cortisol levels were

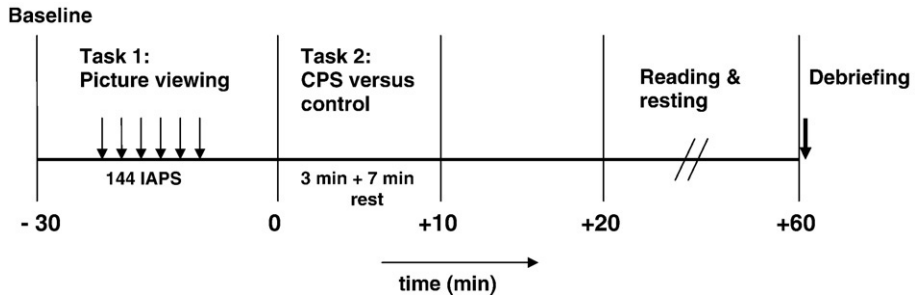

Fig. 1. Timeline of the study. Salivary sampling was carried out on five points in time during the study. The first task was carried out between ($t-30$) and (t0); the second task between (t0) and ($t+10$).

assessed out of the salivary samples. Free cortisol levels are measured using a commercially available immunoassay (IBL, Hamburg, Germany). Inter- and intra-assay variations are below 15%. Salivary Alpha Amylase (sAA) levels were assessed out of samples obtained using Salivette sampling devices (Sarstedt, Nümbrecht, Germany). Alpha amylase is measured by a quantitative enzyme kinetic method. Saliva is diluted 1:625 with double-distilled water. 20 µl of diluted saliva and standard are transferred into transparent 96-well microplates (Roth, Germany) in duplicates. Standard is prepared from "Calibrator f.a.s." solution (Roche Diagnostics, Mannheim, Germany) ranging from 5.01 to 326 U/l Amylase, and double-distilled water as zero standard. After that, 80 µl of substrate reagent (Alpha amylase EPS Sys; Roche Diagnostics) are added. The microplate is then warmed to 37 °C in a water bath for 90 s. After a first interference measurement at 405 nm using a standard ELISA-reader (Anthos HT2, Anthos, Krefeld, Germany), the plate is incubated for another 5 min at 37 °C, and the second measurement is done. Increases of absorbance of samples are transformed to sAA concentrations using a linear regression calculated for the standard curve on each microplate (GraphPad Prism 4.0b for MacOSX, GraphPad Software, San Diego, USA).

2.3.5. Procedure

Subjects were told they would participate in a study on "Emotional perception in the cold" and that the focus of the study was an interest in their physiological reactions on a mental task under cold versus control conditions.

Subjects were tested in the lab of the University of Amsterdam at weekdays. Bearing in mind that cortisol is subject to strong circadian fluctuations in morning hours (Kirschbaum and Hellhammer, 1994), subjects were only tested from 12 noon onwards till 1800 h. In the first session subjects were comfortably seated behind a table and measuring devices were applied. During an acclimatization period of 15 min they were presented with an on screen instruction that explained the procedure of the day and filled out the informed consent form hereafter.

The stimulus presentation of Task 1 started with three training pictures followed by an emotional rating a screen where they could indicate their personal evaluation of the emotional intensity of the pictures. Pictures were presented for 3 s, followed by the rating screen. If they pressed a button for their judgment, a black screen appeared for 1 s. If they did not respond within 8 s the next picture just appeared. Blocks of 24 pictures were randomly presented with a break of 15 s in between the blocks. The only restriction was that a maximum of 3 extreme emotional CAT4 pictures could be presented in a row. Total presentation time of the picture task lasted between 10–15 min.

Immediately after acclimatization and presentation of all pictures, lasting around 30 min altogether, a second salivary sample was obtained ($t=0$) and the CPS procedure began. Subjects were randomly assigned to either the CPS or the control condition. All subjects received the same instruction to place their left arm including their elbow in the water. Participants were asked to keep their arm in the water for 3 min, or as long as they could stand it. They were informed that the ice water was not dangerous and could not lead to freezing. The experimenter watched the participants keeping their arm under water and stepped back, but no conversation was allowed during the 3 min. After this time the experimenter indicated they could take their arm out of the water and supplied a towel to dry their arm.

Immediately after the CPS/control procedure subjects were asked to rate how stressful the procedure was for them ("Stress Rating"). They could score this with a mark between 0 (no stress) and 10 (most stressful experience ever). Hereafter they remained in the lab, reading and resting, and finally participants were informed on the full purpose of this study.

2.3.6. Statistical analysis

Basic analyses included evaluation of sex group differences in demographic characteristics with *t*-tests. Secondly, subject's personal rating of the experienced stress level in each condition was compared in an ANOVA with "Stress rating" as outcome and Stress Task and Sex as independent variables. Major outcomes of interest were the sAA and Cort levels during the experiment. All measurements were log-transformed due to non-normality (tested with Shapiro–Wilk).

Thirdly, baseline sAA and CORT levels by Stress task and Sex were analyzed with an ANOVA. All hormonal measurements during the experiment were then compared in a General Linear Model, with time as repeated measure, and Stress Task and Sex as between subject variables. Also, based on earlier studies (Nater et al., 2007), sAA level analyses were controlled for time of day, and men and women were evenly divided over the time-slots. Simple as well as repeated contrasts were calculated to compare neuro-endocrine levels from baseline to other time points and between consecutive time points where specific effects were expected. We expected a significant increase in sAA level between baseline ($t-30$) and immediately after the IAPS task (t0), but no difference in CORT level yet; we expected a significant increase in CORT level at +20 min after CPS task compared to previous time points (t0 and $t+10$).

Fourth, we computed difference scores (δ-scores) in sAA and CORT levels to test our hypotheses. Therefore we needed three δ-scores:

a) sAA response to the IAPS task calculated by: sAA level at $t=0$ minus $t-30$ = sAAresp_Task1;
b) sAA response to the CPS task (sAA level at $t+10$ minus t0 = sAAresp_Task2) and

Table 1
Demographic characteristics by gender

Cold	Pressor stress task (CPS) $n=39$			Control task $n=40$			
	Male	Female	Total	Male	Female	Total	*t*-test
Sample size	10	29	39	11	29	40	
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	p-value
Age	20.3 (0.9)	21.1 (2.3)	20.9 (2.1)	20.7(1.4)	20.4 (2.1)	20.5 (1.9)	n.s.
BMI	22.0 (2.6)	21.0 (1.8)	21.3 (2.1)	22.1 (1.3)	21.3 (2.2)	21.5 (2.0)	n.s.
Smoking	5	11	16	5	6	11	n.s.

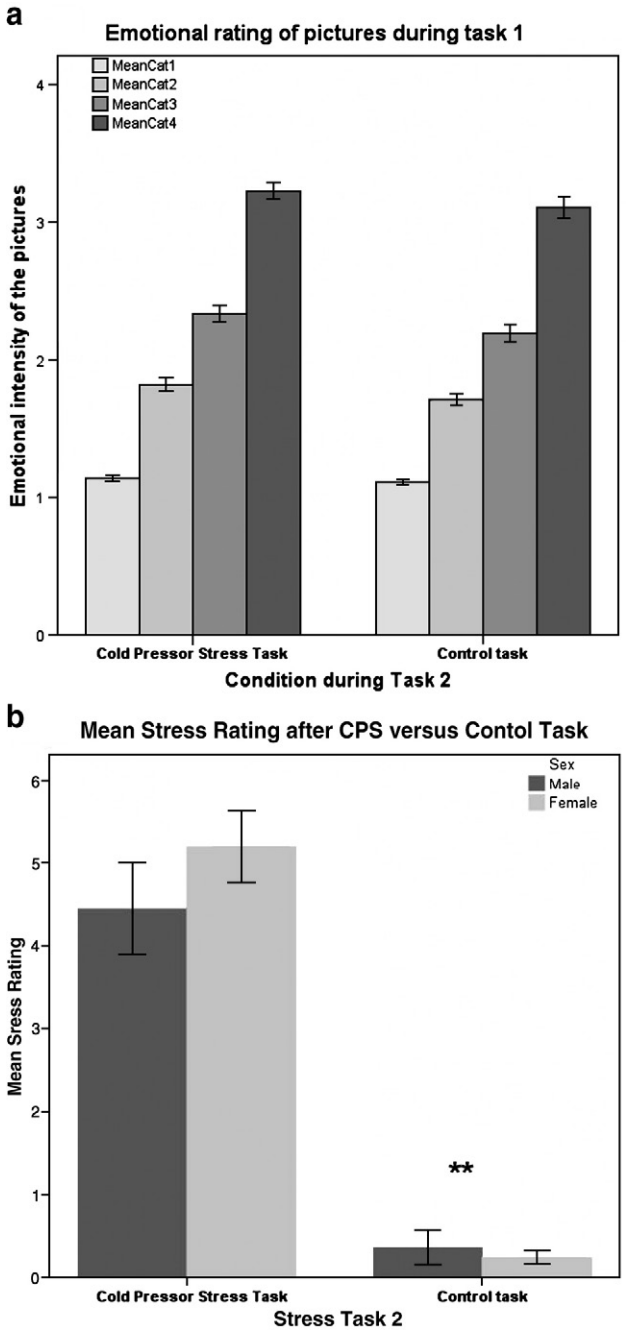

Fig. 2. (a) Emotional rating of pictures during Task 1. All subjects rated the pictures presented, by answering the question on screen after each picture: How emotionally intense did you find the picture to be? with 1 being 'not emotional at all' to 4 = extreme emotional intense. Ratings correlated highly with the original categorization based on the IAPS (Lang et al., 1997) validation ratings (r=.98). Ratings did not differ between groups that were formed after the first task, that then underwent the CPS versus the control task respectively. (b) Stress rating scores of Task 2 by men and women. Men and women rated the CPS task on a scale from 0 (not stressful at all) to 10 (most stressful experience ever). Stress ratings were significantly higher in the group that underwent the CPS task versus a control task (** = p<.001) with no effect of sex on the scores.

c) Cortisol response to the CPS task (Cortisol level at t+20 minus t0=CORTresp_Task2), since this was the time point we expected a possible cortisol response on the CPS task (Dickerson and Kemeny, 2004).

To test if the neuroendocrine response on Task 2 could be predicted by the response on Task 1 the delta scores of sAA and CORT on both tasks were correlated. Since we did only expect a neuroendocrine response in the CPS condition, data were analyzed split by group (CPS versus control). A regression analysis was carried out with the CORT response on the CPS task (CORTresp_Task2) as dependent variable and the sAA responses on task1 and 2 as predictors. We also investigated whether the sAA response on the IAPS task was correlated with the sAA response on Task 2 with a non-parametric correlation measure (Kendall's tau-b).

Finally, we used the untransformed means of sAA and CORT concentrations during the experiment for the graphic presentation in the figures. For all statistical analyses, SPSS 15.0 was used with a significance level of p=.05 (two-tailed).

3. Results

3.1. Demographics

Demographic characteristics for the subjects undergoing the CPS and control task are summarized in Table 1. CPS versus Control task groups did not differ on demographic variables in terms of age, weight, length, BMI or smoking behavior (all p>.20).

3.2. Emotionality rating of Task 1

Mean ratings for each picture category were calculated for all subjects and correlated highly with the original categorization from 1 to 4 (r=.98). There was no difference in emotional rating of Task 1 for subjects that belonged to the CPS or control condition in Task 2 (Fig. 2a). Mean stress rating of Task 1 did not differ between men and women and were neither correlated to sAA or CORT baseline levels nor to the responses to the first task.

3.3. Stress rating scores on Task 2

Participants rated the CPS procedure (5.0±2.19) as significantly more stressful than the control task (2.6±2.85) (F(1,75)=125.11, p<.001) but there was no main effect of sex or interaction effect of sex by stress task (Fig. 2b).

3.4. Stress hormone measurements

3.4.1. Baseline levels by stress task and sex

All subjects underwent the IAPS picture viewing and rating during Task 1. There were no baseline (t=−30) differences in sAA or cortisol

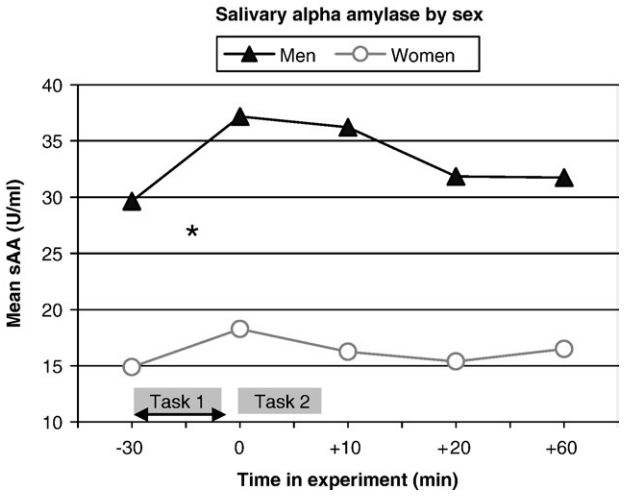

Fig. 3. sAA response by sex during the experiment. (not split for second stress task groups). a) Baseline sAA levels differed between men and women, with men having significantly higher sAA levels than women (p<.05 *). b) This difference remained significant throughout the experiment with no interaction of sex by stress task or by time.

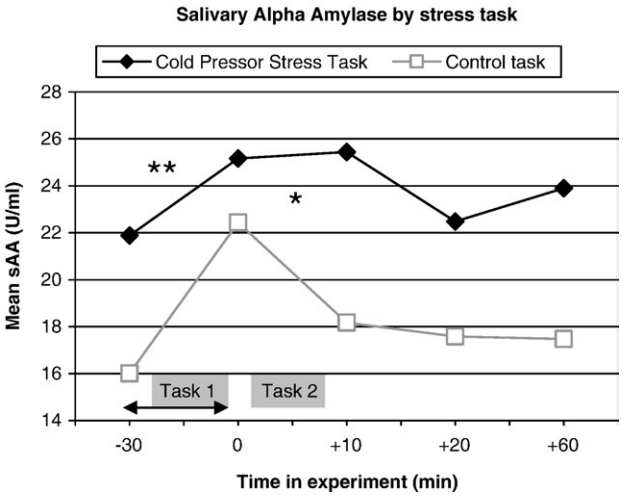

Fig. 4. sAA responses on both tasks. The CPS versus control task grouping took place after Task 1. ** = $p<.01$ is related to a significant increase in sAA level from before (−30) to after the aversive picture rating task (0). * = $p<.05$ refers to a significant interaction effect between the sAA levels of CPS versus control task groups over time. The sAA level in the CPS group further increased in response to the stress task, whereas sAA levels in the control group returned to baseline.

levels by groups that were later assigned to one of the stress conditions in Task 2 (CPS versus control task) ($F(1,62)=.329$, $p=.57$ and $F(1,72)=1.651$, $p=.20$ respectively). There was however a significant difference when comparing baseline sAA level by sex ($F(1,62)=5.141$, $p<.05$; Cohen's $d=0.66$) where men had higher baseline sAA levels than women at −30 min (Fig. 3). The power (calculated that with Gpower 3.0) was .73 (two-tailed t-test for independent means). Although the power to detect a similar sex differences in the baseline CORT levels was .73, no difference in baseline CORT level by sex ($p>.10$) was found.

3.4.2. Salivary alpha amylase

A GLM showed a main effect of time on sAA level ($p<.01$), with repeated contrasts showing that sAA level was significantly rising between −30 min and 0 ($F(1,51)=9.16$; $p<.01$), so just after the IAPS viewing and rating task (Fig. 4). Analyzing the sAA response in reaction to the CPS versus control task revealed a just significant interaction effect of sAA x task ($F(1,51)=3.93$, $p=.05$), in which the CPS group exhibited a further rise in sAA level compared to the control group, that showed a drop in sAA level. There also was a main effect of Sex on sAA levels during the experiment: men had higher sAA levels than women throughout the experiment ($F(1,49)=5.191$, $p<.05$), with no interaction of sex by stress task or by time (Fig. 3).

3.4.3. Cortisol

Analogously cortisol levels were tested in a multivariate GLM, that showed an overall effect of time on CORT level ($F(4,59)=11.283$, $p<.001$), and an overall interaction effect of Time×Stress Task ($F(4,59)=2.614$, $p<.05$). Repeated contrasts showed that this interaction took place between +10 min and +20 min after the second task where CORT levels were significantly rising in the CPS compared to the control group ($F(1,62)=7.999$, $p<.01$) for all subjects. This remained significant when analyzed split by sex: men and women both showed a significant increase in response to the CPS task compared to the control task at +20 min after the start of the cold pressor stress ($p<.05$) (Fig. 5). No main or interaction effect of sex on CORT level during the experiment was found.

3.5. Regression and correlation analysis

Finally, answering the question whether the response on the first task was predictive for the response on the second task, difference scores on both tasks were correlated. The correlation was analyzed for the CPS and control group separately, since no CORT response was

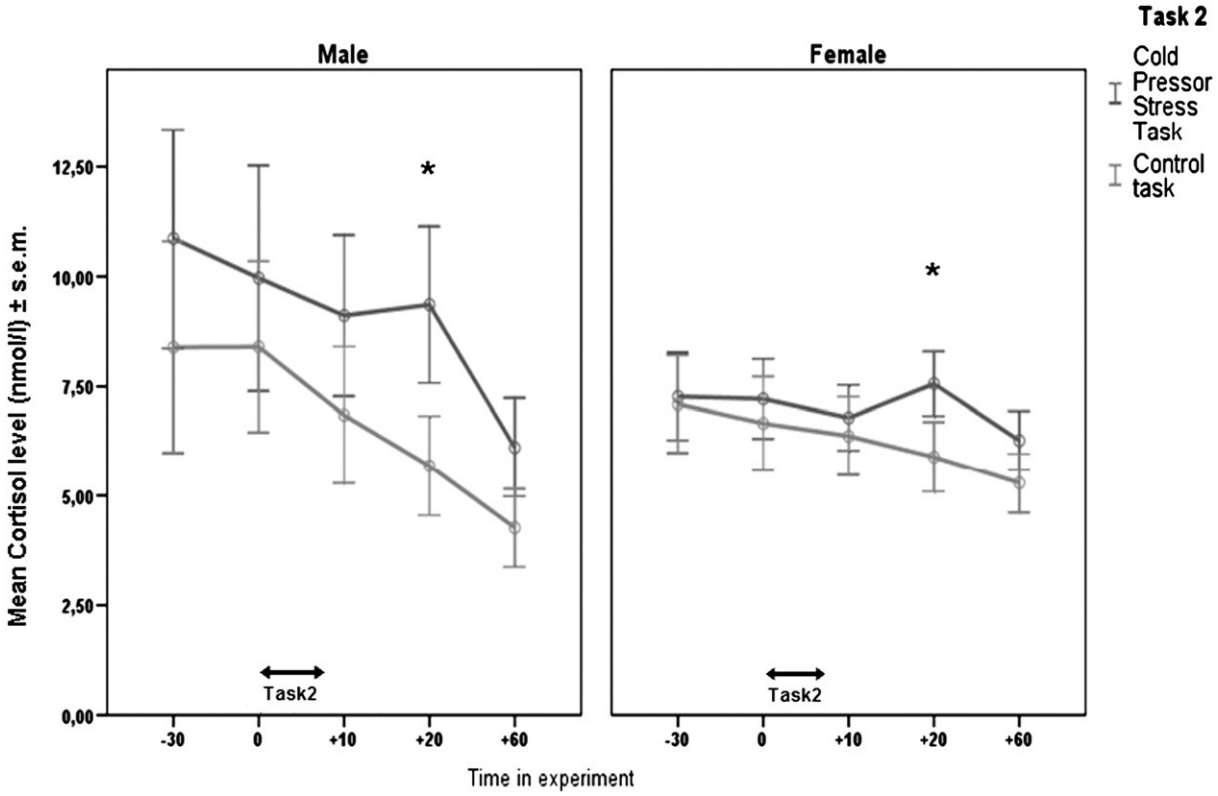

Fig. 5. Mean Cortisol level during the experiment for CPS versus Control condition by sex. Cortisol levels were significantly rising in the CPS compared to the control group, between +10 and +20 min after the start of the second task in both men (* = $p<.05$) and women (* = $p<.01$).

expected (nor found) in the latter group. No significant correlations were found in the analyses of the control task.

A regression analysis with CORTresp_Task2 as dependent and the sAA responses as predictors revealed no significant correlation. So, the sAA response on Task 1 or Task 2 was not predictive for the Cortisol response on the CPS task.

Finally, the sAA response on Task 1 was mildly, yet negative correlated with the sAA response on the CPS task (Kendall's tau-b= −0.29, p<.05; Spearman's rho=−0.38, p<.05). This means that subjects with a small or no sAA response on the first task had a stronger sAA response on the second task or that a strong sAA response on the first task was related to a decrease in sAA after the second task.

Further splitting the CPS group by sex to explore possible sex differences was leading to too small numbers per group (e.g. only 9 male subjects) to allow any meaningful conclusions regarding sex differences.

4. Discussion

The present study shows significant increases in sAA in response to the presentation of a psychological stress task (IAPS pictures) as well as in response to a physiological stressor (CPS). For cortisol, only the second stress task leads to a significant response. For sAA, but not for cortisol a sex difference was observed with men having higher levels than women at baseline and throughout the entire study. Finally, the sAA response on the first task was mildly, yet inversely correlated with the sAA response on the second task, but was not predictive for the CORT response. These three major findings will be discussed in turn.

4.1. Effects of the stress task on sAA and CORT

This study shows that sAA is sensitive to emotional arousal induced by affective pictures as well as to a physiological stressor, the CPS task. Earlier studies show that salivary alpha amylase is responsive to various types of challenging situations including heat stress, socially and cognitively oriented laboratory tasks and physical exercise (Chatterton et al., 1996; Gordis et al., 2006; Nater et al., 2006b, 2005). Recently these findings were extended by a study that revealed that alpha amylase changes in response to but not in anticipation of an athletic competition (Kivlighan and Granger, 2006). We were successful in inducing a second stress response by means of the CPS. Subjects rated this procedure as much more stressful than subjects who were in the control condition. As has been shown in previous studies (Andreano and Cahill, 2006; Cahill et al., 2003), cortisol levels rise in response to a cold pressor stress procedure. However, cortisol did not rise in reaction to the (first) emotional rating task. It could be argued that this lack of CORT response to Task 1 might be due to the collection time, because Task 2 (CPS or control) immediately followed Task 1. This procedure does not allow for the assessment of any potential cortisol increase after the sAA increase at that point in time. However, based on earlier studies of our lab in which we used the same set of pictures we showed that the task did evoke a significant noradrenergic response, measured as an increase in sAA levels (van Stegeren et al., 2006), yet not an increase in CORT levels (van Stegeren et al., 2008, 2007) . Our first conclusion is that sAA is a sensitive marker in both psychologically and physically induced arousal paradigms, whereas a cortisol response can be expected in more challenging stress tasks.

4.2. Sex difference in sAA levels

Sex differences were found in basal sAA level as well as in the sAA levels throughout the experiment: men had higher sAA levels than women. Only a few previous studies have addressed the issue of sex differences in sAA levels and results have been conflicting. In one study sex differences were studied (Kivlighan and Granger, 2006) and although sAA levels of men were higher than women's sAA levels, the difference was not significant. In a previous study of our lab (van Stegeren et al., 2006) sAA levels were determined during an fMRI experiment in which subjects saw the same set of emotional and neutral pictures during scanning. Salivary alpha amylase levels rose in anticipation of and during the scanning procedure and the emotional picture viewing. This rise in sAA level was blocked when subjects received a beta-adrenergic antagonist. Results in a previous publication (van Stegeren et al., 2006) were presented for the whole group, that consisted of 14 men and 14 women. Re-analyzing this data set with sex as between-subjects factor reveals that men under both conditions (BB or PL) have higher sAA levels than women, although statistically the baseline difference shows only a trend (p=.069) (additional data, analysis and graphs are available on request). The difference, seen at baseline, remains during the experiment (main effect of sex during the 3 time points) but is statistically also only a trend (p<.10). Variance in sAA values is relatively high in both men and women in that experiment.

In this study men show a significantly higher baseline sAA level than women. Based on these studies there appears to be a difference in baseline sAA levels between men and women. This is in contrast with a study on the diurnal profile of sAA in a group of men and women that were monitored during a day (Nater et al., 2007). In that study salivary samples were collected immediately after waking-up, 30 and 60 min later, and each full hour between 0900 and 2000 h in 76 healthy volunteers. Two differences might contribute to a contrast in their and our finding. In their study participants were monitored in real life during the day whereas we tested our subjects in the lab in the afternoon. Secondly, our participants came to the lab for a psychological experiment, and knew that they were going to be emotionally challenged. Perhaps this aspect affects the SNS and sAA levels of men more than that of women. Since men and women were equally divided over the time-slots in the afternoon, it cannot be attributed to an effect of time of day. In an earlier study (Yamaguchi et al., 2006) sAA activity was measured before and after walking in both urban and forest environments using a hand-held monitor. The researchers concluded that the *stressor* induced variations were much bigger than the circadian rhythm fluctuations in sAA activity in men. It remains to be investigated, in which context or under which circumstances sex differences in sAA levels occur. The dynamic pattern, hence the responsivity of men and women in reaction to the two tasks is completely comparable. Based on these findings our second conclusion is that baseline sAA values should be taken into account when using sAA responses on stress tasks in a mixed (M and F) population. As far as we can conclude now, the (dynamic) sAA response pattern appears to be comparable between men and women, but should be investigated in greater detail in following studies.

4.3. Interaction between consecutive stress responses

The few studies that focused on a joined response between CORT and sAA levels in a stress paradigm did not find any significant correlation (Nater et al., 2005). In line with this study we were also not able to find a correlation between the sAA and CORT response in the two consecutive tasks. The difference in designs in our study compared to previous studies is that in our study two tasks were presented in a row and that both tasks lead to relatively mild stress responses. Many studies on stress paradigms use for example the Trier Social Stress Test (TSST) that leads to much more intense neuroendocrine responses than the CPS procedure (McRae et al., 2006).

Central control of glucocorticoid secretion is regulated principally by a select population of neurosecretory neurons in the hypothalamic paraventricular nucleus (PVN). Stressors involving an immediate physiologic threat ('systemic' stressors) are relayed directly to the PVN, probably via brainstem catecholaminergic projections. By contrast, stressors requiring interpretation by higher brain structures

('processive' stressors) appear to be channeled through limbic forebrain circuits (Herman and Cullinan, 1997). The fact that we did not find strong relationships between sAA responses of the first task and CORT responses to the second task might be due to the fact that both tasks tap into different parts of this system. The first task is psychological in nature and by cognitive interpretation of the pictures leading to an activation of the SAM system via top-down processes. The stress response induced by the second task likely results more from bottom-up signals. These are relayed through the more direct pathway to the PVN owing to its physiological nature (Herman and Cullinan, 1997).

This can explain the absence of a correlation between sAA responses in Task 1 and CORT responses in Task 2. Using two consecutive psychological stressors (e.g. CPS combined with TSST) could be an interesting idea for a future study to observe a stress task and sex interaction. Since men are known to react more to psychological stressors than women (Wolf et al., 2001) it could be interesting to investigate if men primed by a mild psychological stressor are more reactive to a subsequent stressor.

So, although we did not find an indication for a correlated response, it could also be true that there is a dose-dependency in this relationship between sAA and CORT levels and that the correlation only appears when specific levels of sAA and/or CORT are provoked. But it can be concluded that the reaction of the SAM and HPA axis systems are not always correlated and apparently serve their own specific purposes in the stress response.

Additionally, although several studies looked at the effect of sex on HPA axis activity in reaction to stress tasks (Kirschbaum et al., 1999; Kudielka et al., 1998; Kudielka and Kirschbaum, 2005), not many studies looked at correlations between sAA and CORT in response to stress tasks and also took participants sex into account. Future studies should shed light on this issue by comparing the neuro-endocrine responses on different stress tasks within men and women separately.

Based on a scarce set of studies that show that consecutive exposure to stressors showed an accumulation of stress responses (Liu et al., 2007; Sabban and Serova, 2007), we hypothesized that the response on one stress task can affect the response on consecutive experiences. We find partial support for this idea in this study. Subjects with a strong sAA response on the first task showed a less outspoken sAA response on the second task and vice versa. So, in this study a strong SNS response to the affective pictures was associated with a lower response to the subsequent physiological stressor. This points more towards a complementary response model when confronted with two subsequent arousing or stressful tasks.

However, the negative correlation between the sAA responses on both tasks might be the result of a methodological issue. The salivary sampling after the CPS task was carried out 10 min after the start of this second task. Since the CPS procedure itself took only 3 min there were an additional seven min for the sAA level to respond, casu quo possibly return to baseline in this case. In earlier studies the peak in sAA response to a stressor was measured at a 5 min interval after the stress task or during the stress task, and returned to baseline between 5 and 10 min after the task (Nater et al., 2006b, 2005; Rohleder et al., 2004). So, although we found an increase in mean sAA response on the CPS task compared to the control task at a +10 min interval, it could be true that:

a) the response could have been even higher when measured immediately after the CPS task (so within 5 min) and b) sAA levels were perhaps already returning to baseline at this 10 min interval. So it might be the case that the subjects who had a strong sAA response on the first (IAPS) task, were measured after their peak response on the second task and were already even below the sAA level of t0 (start of the second task). Our conclusion of correlated sAA responses on two consecutive stress tasks has to be very modest for this reason.

Taken together the present study indicates that sAA levels respond to emotionally arousing pictures as well as to a physiological stressor, whereas CORT reacted only to the physical stressor. Baseline sex differences in sAA were observed, but importantly men and women do appear to show a comparable reactivity in sAA levels on arousing tasks.

Future studies should be designed to establish more knowledge on the way the SAM system and HPA axis responses are related in men and women. Although our findings on this subject are presented with caution, it remains interesting to test whether sex differences in sAA levels exist and under what conditions they might occur. This can lead to a refined view on the individual response to stressful tasks and conditions in our field.

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