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A fluid response: Alpha-amylase reactions to acute laboratory stress are related to sample timing and saliva flow rate

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ABSTRACT

Salivary alpha-amylase (sAA) is used as a sympathetic (SNS) stress marker, though its release is likely co-determined by SNS and parasympathetic (PNS) activation. The SNS and PNS show asynchronous changes during acute stressors, and sAA responses may thus vary with sample timing.

Thirty-four participants underwent an eight-minute memory task (MT) and cold pressor task (CPT). Cardiovascular SNS (pre-ejection period, blood pressure) and PNS (heart rate variability) activity were monitored continuously. Unstimulated saliva was collected repeatedly during and after each laboratory stressor, and sAA concentration (U/ml) and secretion (U/minute) determined.

Both stressors increased anxiety. The MT caused an immediate and continued cardiac SNS activation, but sAA concentration increased at task cessation only (+54%); i.e., when there was SNS–PNS co-activation. During the MT sAA secretion even decreased (−35%) in conjunction with flow rate and vagal tone. The CPT robustly increased blood pressure but not sAA.

In summary, sAA fluctuations did not parallel changes in cardiac SNS activity or anxiety. sAA responses seem contingent on sample timing and flow rate, likely involving both SNS and PNS influences. Verification using other stressors and contexts seems warranted.

1. Introduction

The discovery that the adrenal stress hormone cortisol can be measured reliably and non-invasively in saliva was a methodological breakthrough in stress research, and much effort has since been dedicated to determine if the assessment of other neuro-endocrine markers may benefit from the ease of saliva collection. As a promising candidate, salivary alpha-amylase (sAA) has gained rapid popularity as a noninvasive marker of sympathetic nervous system (SNS) activity (Granger, Kivlighan, El-Sheikh, Gordis, & Stroud, 2007; Nater & Rohleder, 2009; Rohleder & Nater, 2009). sAA is a digestive enzyme that breaks down starch into glucose and maltose, and enzymatic activity (in Units/ml) is used as a proxy for sAA concentration.[1] The use of sAA as a marker of SNS activity seems justified: sAA release from the salivary glands is under strong control of local sympathetic nerves (Proctor & Carpenter, 2007), its salivary concentration rapidly increases

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[1] sAA concentration is inferred from the amount of enzyme that catalyzes the conversion of 1 μmol of substrate (i.e., startch) per minute.

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during acute stress, and its use as a marker of sympathetic activation is also validated by pharmacological studies (Bosch et al., 1998; Bosch, de Geus, Veerman, Hoogstraten, & Nieuw Amerongen, 2003; Ehlert, Erni, Hebisch, & Nater, 2006; Takai et al., 2004; van Stegeren, Rohleder, Everaerd, & Wolf, 2006; van Stegeren, Wolf, & Kindt, 2008).

Whereas it is undisputed that sAA release is under sympathetic control, the inference that increases in sAA therefore signify sympathetic activation is nonetheless problematic. The inference is logically flawed (i.e., affirming the consequent), and there are also strong empirical arguments to question this inference (c.f. Bosch, Veerman, de Geus, & Proctor, 2011). Most of these arguments center around the fact that the parasympathetic nerves also play a significant role in sAA release. For example, several sAA-rich salivary glands, like the sublingual and minor glands, are almost exclusively under parasympathetic nervous system (PNS) control (Bosch et al., 2011). Further, experimental studies show that the sympathetic effects on sAA release are strongly moderated by concurrent PNS activity, a phenomenon denoted as 'augmented secretion' (see Proctor & Carpenter, 2007).

In order to better understand the differential contribution of the PNS and SNS to sAA responses during stress, we have previously compared sAA secretion in response to stressors that elicit distinct patterns of autonomic activity (Bosch et al., 2003). It was found that a stressor eliciting sympathetic-parasympathetic co-activation (i.e., viewing a surgical video) caused a marked sAA release (+65%), whereas a cognitive stressor causing a sympathetic activation in conjunction with parasympathetic inhibition (i.e., a memory search task) showed no significant change in sAA release (+10%). Importantly, the latter stressor caused a much stronger sympathetic activation (as measured by cardiac PEP, LVET, and blood pressure responses) than the stressful video (Bosch et al., 2003). These findings therefore are inconsistent with the idea that sAA reliably represents SNS activity, and consistent with a moderating effect of parasympathetic activity (Berntson, Cacioppo, & Quigley, 1991; Proctor & Carpenter, 2007).

On the basis that sAA release is orchestrated by joint activity of the two autonomic branches, we predicted that sample timing may be critical to the observed sAA responses during stress. This prediction builds on knowledge that activity in the autonomic branches is asynchronous over the course of an acute stressor, whereby the PNS tends to exhibit a faster off and onset than the SNS (Berntson et al., 1997; Berntson, Quigley, & Lozano, 2007; Somsen, Jennings, & Van der Molen, 2004). Studies have shown, for example, that the PNS withdrawal during acute stress rapidly restores immediately post-stress, at which time sympathetic activation still lingers (see Berntson et al., 2007). Some have even reported a parasympathetic rebound immediate post-stress, whereby PNS activity overshoots baseline levels, causing a transient sympathetic-parasympathetic co-activation (Mezzacappa, Kelsey, Katkin, & Sloan, 2001; Rottenberg, Wilhelm, Gross, & Gotlib, 2003). Hence, we predicted that the largest sAA increase will be observed immediately post stress, when the PNS will have little effect or possibly even an augmenting effect on sAA, and we further predicted that the smallest sAA changes will be observed during stress, when SNS effects on sAA may be attenuated by a PNS withdrawal. It is noteworthy that nearly all published studies have only sampled sAA at stressor termination, and the study by Bosch et al. (2003) – which found no effect of a cognitive stressor on sAA release – collected saliva during the stressor.

The present study had one further aim: to address the role of salivary flow rate as a factor relevant to sAA studies. The use of sAA as a SNS marker is based on the fact that sAA secretion (U/min) is under SNS control. However, most stress studies have instead measured sAA concentration (U/ml) (Bosch et al., 2011). The implicit assumption that these two parameters yield identical results has remained largely untested (Beltzer et al., 2010; Proctor & Carpenter, 2001; Rohleder & Nater, 2009). As shown in the formula below,[2] saliva flow rate (ml/min) is the sole determinant of the relationship between sAA secretion and concentration, and flow rate is almost exclusively under parasympathetic control (Garrett, 1987; Proctor & Carpenter, 2007). Accordingly, sAA concentration may provide an overestimation of sAA secretion when salivary flow rate decreases – reflecting reduced PNS activation of the salivary glands – but may provide an underestimation when saliva flow rate increases. This aspect of glandular physiology may also have clear implications for sample timing: during acute stress, when PNS activity shows a strong withdrawal, the largest effects on flow rate can be anticipated and hereby the largest discrepancy between sAA concentration and secretion (Bosch, Ring, de Geus, Veerman, & Nieuw Amerongen, 2002; Bosch et al., 2011).

In light of the preceding discussion, the present study examined the temporal dynamics of sAA during two acute laboratory stressors known to elicit distinct autonomic nervous system responses: i.e., a memory-search task (MT) and a cold pressor task (CPT) (Bosch et al., 2001, 2003; Willemsen et al., 1998; Willemsen, Carroll, Ring, & Drayson, 2002). The MT elicits a prototypical 'fight or flight' cardiac autonomic response pattern, characterized by a vagal withdrawal and enhanced sympathetic drive. In contrast, the CPT primarily elicits a localized vascular sympathetic activation characterized by a robust blood pressure response, but elicits little cardiac autonomic change (Allen et al., 1992; Willemsen et al., 1998, 2002; Winzer et al., 1999; Ring et al., 2000), and the data on sAA are mixed (see discussion). We anticipated the largest sAA increase at stressor off-set, when autonomic balance is shifted towards SNS–PNS co-activation, and we expected the smallest sAA changes during the stressor, when parasympathetic withdrawal may attenuate sympathetic effects on sAA secretion. We expected sAA during CPT to increase in parallel with pain, anxiety and pressor responses. Autonomic responses during CPT have rarely been determined beyond 3 min (Mourot, Bouhaddi, & Regnard, 2009) and this is the first study to investigate the temporal dynamics of sAA release during CPT. Correlation analyses were performed to explore associations between glandular responses and cardiovascular autonomic indices.

2. Method

2.1. Participants

Thirty-four university undergraduates (of which 18 were males) volunteered to take part in the study (Mean age = 22.1 yr, SD = 3.2; Mean BMI = 21.7 kg/m^2, range: 17.7–28.3). Participants received study credits for their participation. Inclusion criteria were: (a) no current medical treatment or prescribed medication, (b) no signs of colds or upper respiratory tract infection in the past two weeks. Participants signed informed consent, and the research protocol was approved by the local ethics committee of the Vrije Universiteit.

2.2. Procedure

In preparation, participants were instructed to refrain from using alcohol or nonprescription drugs 24 h before testing. Participants were asked not to deviate from their usual sleeping habits on the previous night, avoid vigorous exercise on the day of the experiment, and to abstain from smoking (five participants reported to be smokers), drinking caffeinated beverages, eating, and brushing teeth (to prevent gingival bleeding) one hour prior to the experiment. Women were scheduled within the seven days after their menses. Compliance with instructions was verified by a detailed health behaviour questionnaire. Experiments were set between 13:30 and 16:00 to minimize circadian effects (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007).

On arrival, the experimental procedure was explained to the participant and electrodes for electrocardiography (ECG) and impedance cardiography (ICG) were attached. After rinsing the mouth with tap water, participants were familiarized with the saliva-collection procedure and filled out questionnaires, followed by a

[2] sAA secretion (U/min) = sAA concentration (U/ml) × salivary flow rate (ml/min).

20-min resting baseline during which they engaged in leisurely reading. This was followed by one of the two experimental manipulations, counter-balanced across participants, each lasting for 8.5 min: i.e., (1) a computerized memory-search test (MT); (2) cold pressor task (CPT). Each task was followed by a 16 min recovery period, after which the other stressor was presented. With each stressor, six saliva samples were collected (collection procedure is detailed further below): a pre-task baseline saliva sample, followed by a sample at 2 and 5.5 min into the task, and a fourth sample was collected immediately upon termination of the task at 8.5 min (denoted task cessation or stressor offset). The latter corresponds with the usual stress-sample time point in sAA studies. During recovery two further saliva samples were taken at 4 min and 10 min post-task, when the participants were again engaged in quiet reading. Between the last recovery measure of the first task and the baseline measurement of the second task the participants rested for 20 min. Participants filled out a stress questionnaire in conjunction with the baseline, the stressor offset, and the 10-min recovery sample. Electrocardiogram (ECG) and an impedance cardiogram (ICG) were recorded continuously and blood pressure readings were taken at regular intervals that coincided with saliva sampling.

2.2.1. Memory-Search Task (MT)

For this task participants were instructed to memorize a set of four characters (letters and numbers) and to respond by pressing a lever (yes/no) within a set time (<2000 ms) if one of the memorized characters appeared on a screen among six random characters (Bosch et al., 2001, 2003; de Geus, van Doornen, & Orlebeke, 1993). The memory set was changed every minute. Participants received instantaneous feedback on correct or wrong responses, and a personal score as well as a group average was presented. False performance feedback was provided at random intervals. In order to sustain effort, available reaction time was automatically adjusted depending on performance.

2.2.2. Cold Pressor Task (CPT)

Participants were instructed to immerse one arm until below the elbow in 8 °C water for 8 min (Willemsen et al., 1998). While CPT traditionally involves lower temperatures (2–4 °C) and shorter immersion (1–3 min), the 8 °C was chosen as it allows exposure that matched the timing of the MT while still eliciting distress and a robust pressor response (Willemsen et al., 1998). This longer CPT protocol has been validated using pharmacological blockade studies (Ring et al., 2000; Winzer et al., 1999), demonstrating that it induces a robust and sustained pressor response that is alpha-adrenergic dependent. CPT seemed relevant to include for two reasons: firstly, the highly localized (i.e., vascular) sympathetic activation that characterizes this task poses the question whether sAA will primarily follow a vascular or a cardiac activation pattern. Secondly, the evidence for an effect of CPT on sAA is mixed (with some studies showing increases and some observing no effect) and these studies lack independent verification of sympathetic activation other than blood pressure responses (Mourot et al., 2009; Willemsen et al., 2002).

2.3. Questionnaires

The state subscale of the Spielberger State-Trait Anxiety Inventory (STAI-Y) was used as an indicator of distress (Van der Ploeg, 1988). The state scale of STAI is among the most commonly used measures of anxiety and distress used in clinical and stress research, and also frequently used in laboratory stress studies (Campbell & Ehlert, 2012). The scale consists of 20 brief statements, rated on a four-point Likert scale (from "Very much" to "Not at all"), reflecting cognitions and feelings of nervousness, tension, and apprehension, or the lack thereof ("I am tense; I am worried" and "I feel calm; I feel secure."). The state scale of STAI boasts excellent psychometric qualities (Cronbach's alpha state subscale = .94 (Van der Ploeg, 1988).

Following the CPT, participants were asked to rate the pain caused by the CPT on a 6-grade Likert scale (1: not at all painful, 6: extremely painful). Participants also filled out a detailed self-report questionnaire on health (perceived health, use of medication, other medical treatment, use of contraceptives), habitual health behaviour (smoking; alcohol, tea and coffee consumption; physical exercise, sleep duration and quality), and health behaviours in the 24 h preceding the experiment. Height and weight were assessed using standard methods.

2.4. Saliva collection

Saliva was collected using the 'spitting method', described in detail by Navazesh (1993). This method exhibits a good retest reliability and is generally accepted as the preferred method in saliva research (Bosch et al., 2011; Bosch, 2014; Navazesh & Kumar, 2008; Rohleder & Nater, 2009). The saliva collecting method was practiced before the experiment to familiarize participants with the procedure. Participants were instructed to empty their mouth of saliva by swallowing and to let the saliva accumulate at the floor of the mouth without stimulating flow by oro-facial movements (i.e., 'unstimulated' saliva).

Saliva was expectorated into a pre-weighed, ice-chilled polypropylene test tube every 60 s, for a total of 2 min. Momentary salivary flow rate was determined gravimetrically, dividing the amount of saliva (grams corresponding to milliliters) by two, yielding ml/min. After collection, saliva was homogenized using a vortex mixer and clarified by centrifugation (at 10,000×*g*, 4 min) to eliminate buccal cells and oral microorganisms. The clear supernatant was divided into 500 μl aliquots and stored at −20 °C until analysis.

2.5. Amylase determination

Amylase activity was determined using the quantitative kinetic determination kit (from Roche; Mannheim, Germany), as described by Bosch et al. (1996, 1998). In short, saliva (10 μl, starting dilution 1:60 in PBS) was mixed with 190 μl of amylase reagent and incubated for 2 min at 37 °C. The increase in absorption (at 410 nm) over the subsequent 2 min was measured and compared with the activity of a multienzyme standard (Sigma Diagnostics). Amylase activity was expressed in units per milliliter (U/ml). All samples from each participant were assayed in quadruplicate in the same assay run. The intra-assay reliability (CV%) was 4%.

2.6. Cardiovascular assessment

Assessment of cardiovascular response focused on blood pressure and cardiac autonomic parameters. Blood pressure was measured with a Dinamap Vital Signs Monitor (Critikon model 845 XT). Blood pressure was assessed right after saliva collections except for the first stressor reading, which was obtained in the first minute of each task.

Indices of sympathetic and parasympathetic drive were obtained by analysis of Impedance Cardiography (ICG) and ECG signals (Berntson et al., 1997; de Geus & van Doornen, 1996), recorded continuously from six Ag/AgCl spot electrodes (AMI type 1650-005, Medtronic) using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) device (de Geus & van Doornen, 1996; Willemsen, DeGeus, Klaver, van Doornen, & Carroll, 1996). The ECG and ICG complexes were ensemble averaged with reference to the ECG R wave across 30-s periods. From these ensambles 2 min average levels were computed for heart rate (HR), root mean square of successive differences (RMSSD) and pre-ejection period (PEP), corresponding to each saliva collection (again with exception of the first task-reading, with corresponds to minutes 0–2). Changes in PEP were used to index changes in cardiac sympathetic drive (Sherwood et al., 1990), and RMSSD was used to index changes in cardiac parasympathetic/vagal tone (Berntson et al., 1997).

2.7. Data reduction and analysis

Data were analyzed using full-within subject Task × Time ANCOVAs. Partial eta-squared (η_p^2) was used as a measure of effect size, which is comparable to adjusted R^2 obtained in regression analyses (Tabachnick & Fidell, 2001), whereby values of 0.02, 0.13 and 0.26 indicate small, medium, and large effect sizes, respectively (Cohen, 1992). Analyses were checked for possible confounding effects of task order and other potential confounders (sex, BMI, and smoking status). As inclusion of these covariates had little effect on the results, the detailed overview of these results is presented in the online supplement. Simple repeated-measures ANOVAs were conducted for follow-up analyses, and paired *t*-tests were used for post-hoc comparisons, using Bonferroni correction. Untransformed values are presented in figures, but for analyses data were transformed to restore normality (square root transformation (sqrt[x]): salivary flow rate, sAA concentration, and sAA secretion data; log transformation (ln[1 + x]) for RMSSD). Blood pressure readings were incomplete for five participants due to a technical fault. Outliers were defined as ±3.5SD and removed from analyses ($n = 1$ for PEP). When the sphericity assumption was violated (as determined by Mauchli's test), the p value was corrected using the Greenhouse-Geisser correction. In that case, respective epsilon (ε) values are presented along with corrected significance levels. For correlational analyses we used the percentage change values (Δ%, relative to baseline). Significance for these exploratory analyses was set at $p < .01$, to account for multiple comparisons. Data were analyzed using IBM SPSS Statistics 20.0 for Windows.

3. Results

3.1. Anxiety, pain and autonomic and cardiovascular responses

Verifying that both tasks were perceived as stressful, Fig. 1 shows that both tasks significantly increased state anxiety (time effect: $F(2, 54) = 52.50$, $\varepsilon = .59$, $p < .001$, $\eta_p^2 = .66$), whereby slightly higher increases were reported during the MT than during CPT ($t(30) = 3.35$, $p = .002$). There was no significant effect of task order or sex ($p > .20$).

Participants rated the subjective pain caused by the CPT to be 3.6 on a 6-grade scale, which was not significantly different ($t(46) = 0.21$, $p = .835$) from pain rating from published data derived from a more conventional brief CPT protocol (immersing the hand in 4 °C water for 90 s) (Allen, Shelley, & Boquet, 1992).

Next, analyses aimed at verifying if the tasks exhibited the expected patterns of cardiovascular and autonomic activity. As

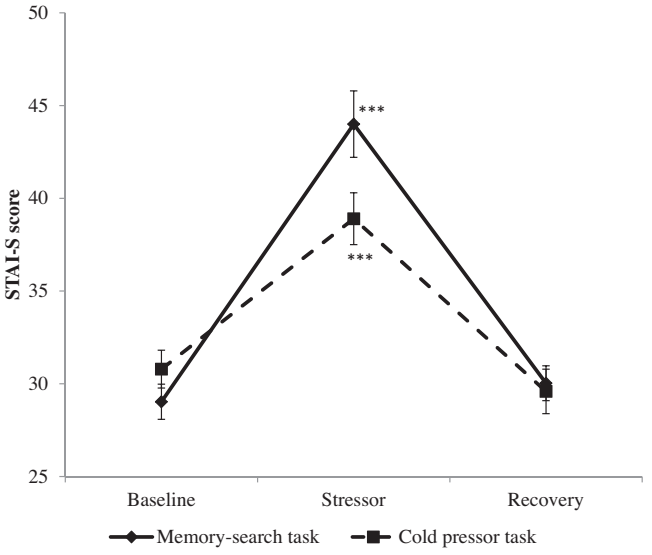

Fig. 1. Anxiety scores (Mean ± SE) during baseline, task, and recovery phases of Memory-search task and Cold pressor task. *** $p < .001$, representing difference from baseline.

shown in Table 1 and Fig. 2, the MT evoked the expected pattern of increased sympathetic activation (evidenced by reduced PEP and increased blood pressure) and a vagal withdrawal (reduced RMSSD). At MT offset, RMSSD was restored to baseline values whereas PEP was still significantly shorter, indicated ongoing sympathetic activation. In line with prior research, the CPT showed little effect on heart rate and cardiac autonomic measures (See Table 1), but exhibited a robust increase in blood pressure that was similar to the blood pressure increase during the MT. For both tasks, cardiovascular parameters returned to baseline levels within 5 min after stressor termination. Adjusting for task order, gender, BMI or smoking did not alter any of the reported results (see online supplement).

3.2. Salivary parameters

3.2.1. Salivary flow rate

Salivary flow rate responses are presented in Fig. 3. Repeated measures ANOVA yielded a significant Task × Time interaction ($F(5, 145) = 16.23$, $p < .001$, $\eta_p^2 = .35$) showing a different pattern of change for the two stressors. Separate analyses of each task showed that MT clearly altered flow rate ($F(5, 160) = 45.77$, $p < 001$, $\varepsilon = .76$, $\eta_p^2 = .59$). Flow rate decreased from baseline during the MT (−55% and −56% at 3.5 and 7 min respectively; $t(33) = 7.99$ and 6.99, respectively, $ps < .001$) which was followed by a modest rebound above baseline levels at stressor offset (+22%, $t(33) = -3.88$, $p < .001$). The CPT did not significantly affect salivary flow rate ($F(5, 160) = 1.00$, $p = 418$, $\eta_p^2 = .03$). Males had a higher average salivary flow rate than females ($F(1, 29) = 9.27$, $p = .005$, $\eta_p^2 = .24$), but adjusting for sex did not alter the results. Exploratory analyses did not identify other potential confounders (see online supplement).

3.2.2. Salivary α-amylase concentration

As can be seen on Fig. 4, the effect of the two tasks was different on sAA concentration over time, yielding a significant Task × Time interaction ($F(5, 150) = 5.90$, $\varepsilon = .67$, $p = .001$, $\eta_p^2 = .16$). Separate analyses of each task yielded a significant increase from baseline of sAA concentration in MT ($F(5, 155) = 10.60$, $p < 001$, $\varepsilon = .61$, $\eta_p^2 = .26$). Post-hoc analyses showed that during the MT sAA concentration did not change ($ps > .90$), and showed a significant increase at stressor offset (+54%, $t(33) = -6.28$ $p < .001$), and subsequently returned to baseline levels (ts < .81, $ps > .35$ for recovery). The CPT did not significantly affect sAA concentration ($F(5, 155) = 1.01$, $\varepsilon = .68$, $p = .396$, $\eta_p^2 = .03$). Exploratory analyses showed no response differences related to covariates (see online supplement).

3.2.3. Salivary α-amylase secretion

As can be seen in Fig. 5, the two tasks elicited different sAA secretion patterns, yielding a significant Task × Time interaction ($F(5, 145) = 22.18$, $p < .001$, $\eta_p^2 = .43$). Separate analyses of each task response showed that MT elicited significant changes in sAA secretion ($F(5, 150) = 35.63$, $p < .001$, $\varepsilon = .69$, $\eta_p^2 = .54$), whereby sAA secretion was decreased from baseline during MT (−43% and −31%, $t(31) = 5.81$ and 5.95, respectively, $ps < .001$), followed by a rapid increase above baseline values at stressor offset (+60%, $t(33) = -5.92$, $p < .001$); during recovery sAA secretion returned to baseline values (ts = −1.36 and −0.63, $ps > .180$). The CPT did not significantly affect sAA secretion ($F(5, 155) = 0.51$, $p = .770$, $\eta_p^2 = .02$).

Men showed a higher sAA secretion than women ($F(1, 27) = 9.87$, $p = .004$, $\eta_p^2 = .27$), which is explained by higher salivary flow rates in men. However, adjusting for sex did not alter the results (see online supplement).

3.3. Correlational analyses

The increase in salivary flow rate at MT offset correlated positively with RMSSD change ($r(33) = .56$, $p = .001$), supporting the idea that enhanced parasympathetic tone was associated with increased flow rate. This finding was replicated for the association between RMSSD and sAA secretion ($r(33) = .43$, $p = .01$), but not for RMSSD and sAA concentration. No associations were observed between PEP changes and changes in salivary measures at any of the time points.

Relative changes in sAA concentration and sAA secretion were significantly correlated at all time points; correlation coefficients were .65, .76, .66, .71, and .56 during CPT, and .59, .61, .63, .57, and

Table 1
Statistical results for cardiovascular responses.

Measure	Task × Time interaction Statistic	η_p^2	Memory task time effect Statistic	η_p^2	Cold pressor task time effect Statistic	η_p^2
SBP	$F(6,156) = 2.46^{*}$	.09	$F(6,168) = 6.79^{***}$	.20	$F(6,180) = 7.88^{***}$	.21
DBP	$F(6,156) = 1.20$, $\varepsilon = .66$	.04	$F(6,168) = 11.60^{***}$, $\varepsilon = .67$	.29	$F(6,180) = 5.52^{***}$, $\varepsilon = .72$	.16
HR	$F(6,192) = 16.91^{***}$, $\varepsilon = .45$	.35	$F(6,192) = 5.32^{***}$, $\varepsilon = .33$	.36	$F(6,192) = 2.23$, $\varepsilon = .69$	.07
RMSSD	$F(6,192) = 17.31^{***}$, $\varepsilon = .63$	.35	$F(6,192) = 15.96^{***}$, $\varepsilon = .66$	.33	$F(6,192) = 1.82$, $\varepsilon = .63$	.05
PEP	$F(6,186) = 4.93^{**}$, $\varepsilon = .55$	.14	$F(6,186) = 8.44^{***}$, $\varepsilon = .64$	.21	$F(6,186) = 1.81$, $\varepsilon = .51$	.06

Note: MT: Memory-search Task, CPT: Cold Pressor Task, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HR: Heart rate, RMSSD: Root mean square of successive differences, PEP: Pre-ejection period. *F* values represent within-subject effects with adjustment for task order.

* $p < .05$.
** $p < .01$.
*** $p < .001$.

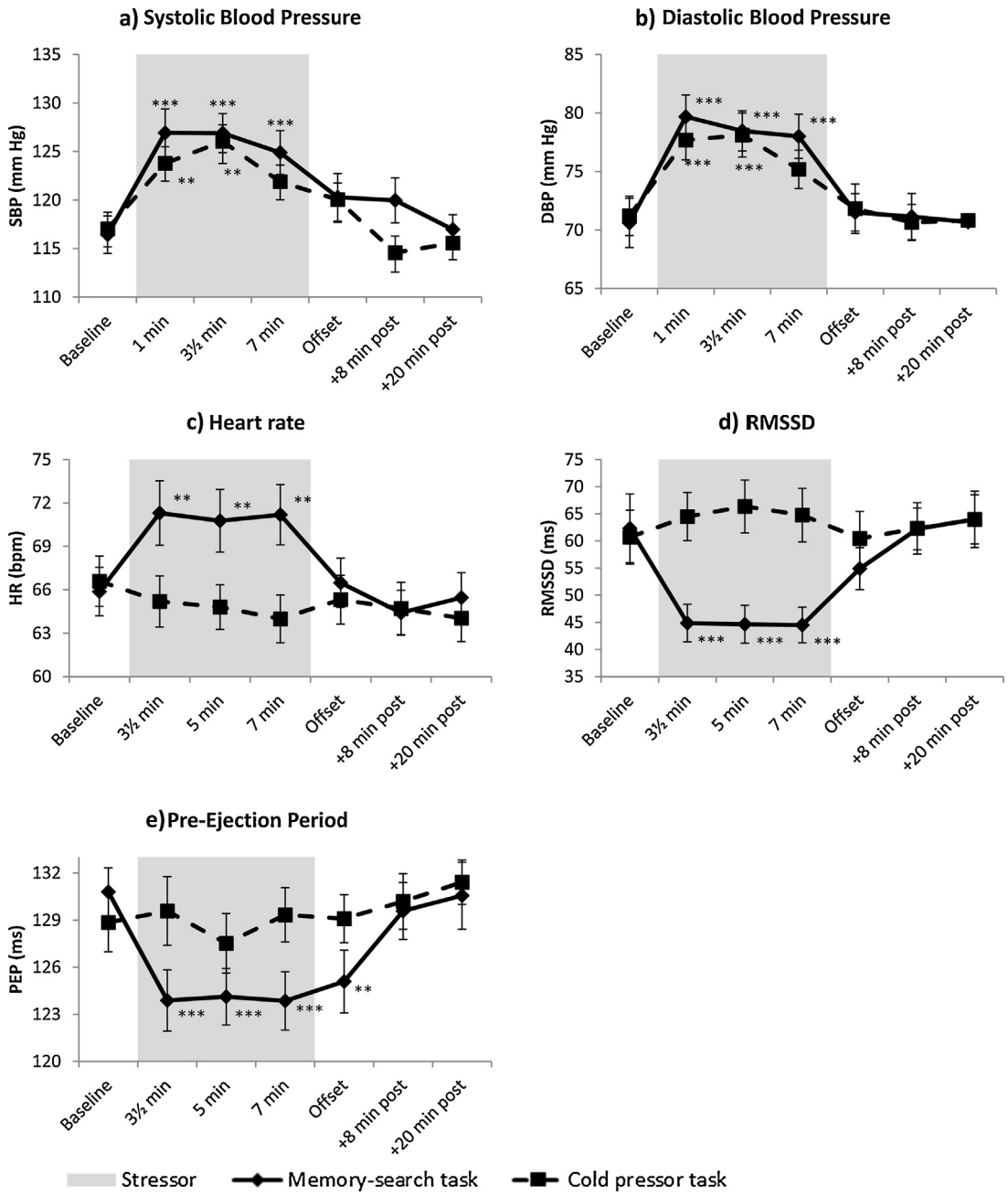

Fig. 2. Cardiovascular parameters (Mean ± SE) during Memory-search task and Cold pressor task. Asterisks indicate significant pair-wise differences from baseline values respectively, using Sidak correction; the timings on the x-axis indicate the end of each 2-min ECG/ICG epoch. $^{**}p < .01$, $^{***}p < .001$.

.55 during MT (all $ps \leq .001$ for 3.5, 7, 8.5 (offset), +8, and +20 min, respectively).

4. Discussion

For the present study it was predicted that sample timing may be an important determinant of sAA responses to stress, and this idea was tested by comparing sAA responses during two stressors known to elicit distinct patterns of cardiac autonomic activity. The results appeared consistent with expectations: over the course of a cognitive stressor (MT) an increase in sAA was evident at stressor offset only. Moreover, during this stressor sAA concentration was virtually unaffected and sAA secretion even decreased, despite evidence of consistent and robust sympathetic activation (i.e., decreased PEP, increased BP). These findings indicate that sAA increases upon stressor termination – the measurement time point used in most studies – may not be representative of what is happening during stress. Also notable is that the CPT had little effect on sAA levels, although participants reported pain and showed a marked increase in anxiety as well as vascular sympathetic activation (i.e., elevated blood pressure). Further, the change of sAA and PEP did not correlate at any time point. Overall, these results suggest that the interpretation of sAA as a marker of stress/arousal or sympathetic activity is less straightforward than perhaps assumed, and the role of concurrent parasympathetic activity (related to aspects like measurement timing and saliva flow rate) warrant more attention in future studies.

The present study also aimed to address the ongoing debate on the possible confounding role of salivary flow rate in determining sAA concentration. The empirical literature has been

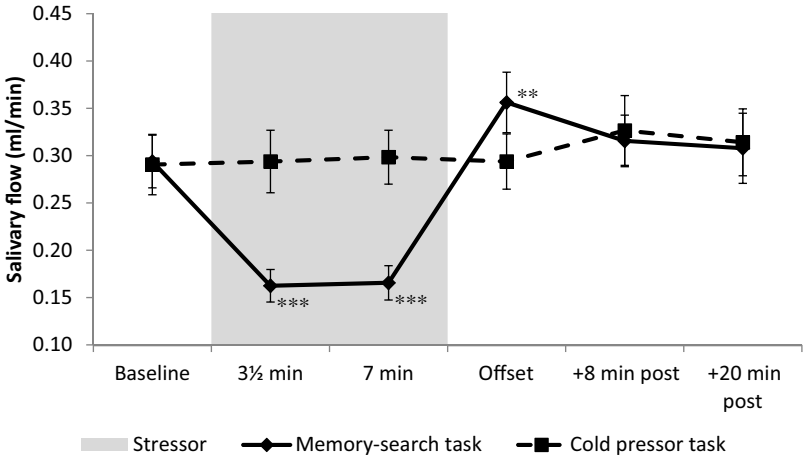

Fig. 3. Salivary flow rate (Mean ± SE) during Memory-search task and Cold pressor task. $^{**}p < .01$, $^{***}p < .001$, representing Sidak corrected difference from baseline values. The timings on the x-axis indicate when each 2-min saliva collection finished.

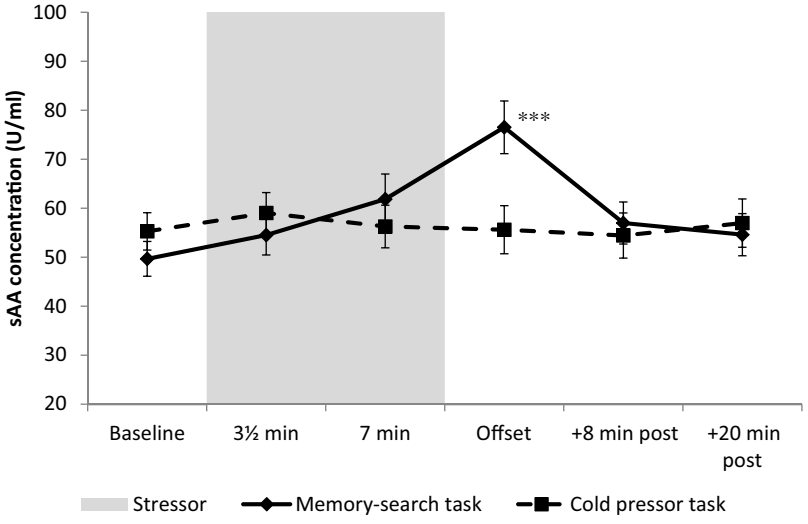

Fig. 4. Salivary alpha-amylase concentration (Mean ± SE) during Memory-search task and Cold pressor task. $^{***}p < .001$, representing Sidak corrected difference from baseline values for each task. The timings on the x-axis indicate when each 2-min saliva collection finished.

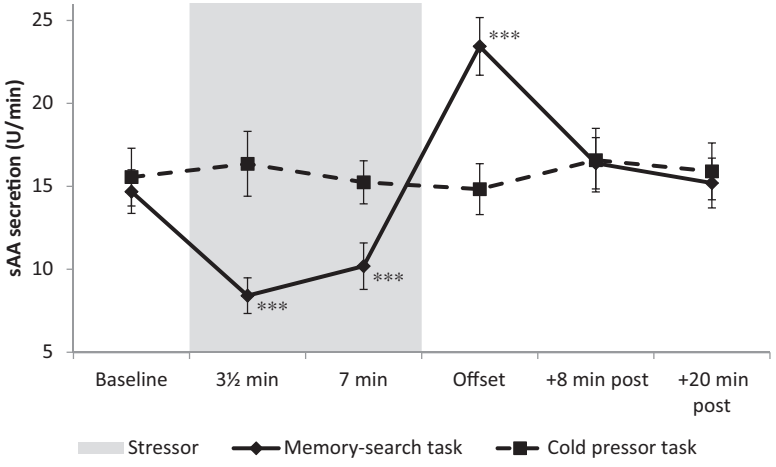

Fig. 5. Salivary alpha-amylase secretion (Mean ± $^{***}p < .001$, representing Sidak corrected difference from baseline values. The timings on the x-axis indicate when each 2-min saliva collection finished.

somewhat inconclusive on this issue, with some reports indicating that the influence of flow rate is fairly modest (Bosch et al., 1996; Rohleder, Wolf, Maldonado, & Kirschbaum, 2006; Sánchez-Navarro, Maldonado, Martínez-Selva, Enguix, & Ortiz, 2012). However these cited studies found very little effect on flow rate to begin with, which makes substantial confounding unlikely. The relation between sAA concentration and sAA secretion is mathematically determined by flow rate, and a different conclusion should, and indeed does, emerge when looking at studies in which flow rate is more strongly affected: in those instances sAA concentration and sAA secretion clearly diverge (Arhakis, Karagiannis, & Kalfas, 2013; Beltzer et al., 2010; Bosch et al., 2003; Proctor & Carpenter, 2001). This observation is reinforced by the present study, in which sAA concentration and (flow-rate adjusted) sAA secretion showed a strong discrepancy, i.e., sAA secretion showed a decrease during the MT while concentration did not change. Moreover, changes in sAA concentration and secretion showed only modest shared variance. We conclude therefore that sAA concentration cannot be confidently used as a proxy for sAA secretion and requires adjustment for salivary flow rate under some conditions (Beltzer et al., 2010).

Replicating prior research, salivary flow rate showed a modest rebound immediately post-MT, overshooting baseline levels, which suggests enhanced glandular parasympathetic activation (Bakke et al., 2004; Rohleder et al., 2006). This observation is somewhat reminiscent of a phenomenon known as vagal rebound (Blascovich & Mendes, 2010; Mezzacappa et al., 2001; Rottenberg et al., 2003), and it is therefore interesting that the increase in salivary flow rate positively correlated with concurrent vagal activity (RMSSD) at stressor offset. As outlined in the introduction, a PNS rebound in combination with a lingering sympathetic activation was predicted to augment sAA secretion as a result of autonomic co-activation of salivary glands (Proctor & Carpenter, 2007). On a speculative note, this observation made us wonder if the typical sAA increase post-stress might reflect a relief response, rather than a stress response. Admittedly a similar relief could be anticipated (but was not seen) with the CPT. Perhaps somewhat reassuring is that whereas the samples collected during stress showed a strong discrepancy between sAA concentration and secretion, at MT offset this discrepancy seemed more modest.

To ascertain that MT and CPT were comparable on a temporal level, we used an extended 8-minute CPT protocol which has been validated elsewhere (Ring et al., 2000; Winzer et al., 1999). Reassuringly our data, and that of others, showed that this CPT protocol faithfully replicated what has been shown for more conventional short CPT protocols: i.e., it induced a robust elevation in blood pressure that was sustained for the full duration of the immersion, it increased anxious distress (as measured by the Spielberger state anxiety questionnaire), and the average pain ratings were comparable to that of a brief CPT protocol (Allen et al., 1992). Importantly, likewise replicating prior reports was the observation that the CPT had no effect on sAA levels (Felmingham, Tran, Fong, & Bryant, 2012; Giles, Mahoney, Brunyé, Taylor, & Kanarek, 2014; O'Donnell, Kammerer, O'Reilly, Taylor, & Glover, 2009; Skoluda et al., 2015), although increases have been reported as well (Lord, Hall, Soares, & Steiner, 2011; Van Stegeren et al., 2008). It has been speculated that these inconsistencies might, in part, be explained by confounding effects of inadvertent stimulation of flow rate, e.g., clenching or chewing during this painful stimulus (Arhakis et al., 2013; Bosch et al., 2011). Further studies using standardized collection of unstimulated saliva may be able to refute or confirm this possibility.

The lack of an sAA response during CPT also illustrates a more general point about the use of SNS markers in psychophysiology; i.e., SNS responses tend to show a high level of anatomical specificity (c.f. Folkow, 2000). Just as the CPT induced a strong vascular activation without a comparably strong sympathetic activation on a cardiac level (Allen et al., 1992; Willemsen et al., 1998, 2002), the CPT also did not induce significant SNS activation at the level of the salivary glands. Such anatomical specificity has been observed even within organs, including the salivary glands. For example, the secreto-motor sympathetic nerve fibers – responsible for the glandular secretion of sAA – are activated independently of the vasoactive sympathetic nerve fibers that regulate vasoconstriction in glandular tissue (Proctor & Carpenter, 2007). By implication, such specificity would suggest that it is better to denote sAA as a marker of glandular autonomic activity, which would be consistent with how other SNS markers are typically described (e.g., PEP as a marker of cardiac sympathetic activity).

Several limitations of this study should be noted. It is possible that the non-response of sAA to stress might reflect that, different from cardiac and pressure responses, sAA needs several minutes to develop. However, this possibility appears to be contradicted by available evidence. For example, electrical stimulation of the glandular nerves as well as reflex activation of these nerves (e.g., by chewing) increases sAA secretion within seconds (Garrett, 1987; Proctor & Carpenter, 2001). Further, sAA has been shown to increase within 3 min or less in response to psychological stressors, such as a stressful video (Engert et al., 2011; Takai et al., 2004) or a brief cold pressor test (Lord et al., 2011; Van Stegeren et al., 2008). However, to date only a few studies have investigated temporal dynamics of sAA release and even fewer investigated such dynamics during psychological stimuli, and clearly more research is needed to determine optimal sampling strategies.

Another limitation is that the correlational analyses – linking cardiovascular autonomic measures with salivary responses – were exploratory and of modest statistical power, and replication in larger samples is needed to provide more conclusive data. The results of the present exploratory analyses seemed consistent with other studies correlating measures of sympathetic activity with sAA, whereby associations were relatively small (Bosch et al., 2003; El-Sheikh, Erath, Buckhalt, Granger, & Mize, 2008; Granger, 2006; Nater et al., 2006; Thoma, Kirschbaum, Wolf, & Rohleder, 2012). This low correspondence with other measures of sympathetic activity also appears consistent the phenomenon of anatomical specificity discussed earlier, and low correspondence is the general observation in studies investigating correlations among various markers of SNS activity (e.g., skin conductance, catecholamines, PEP, pupil dilation) (c.f., Bosch et al., 2011). Future studies may therefore utilize pharmacological manipulations or direct nerve stimulation instead of correlations for validation of sAA (Kuebler et al., 2014; Mills, Goebel, Rehman, Irwin, & Maisel, 2000; Ring et al., 2000; Van Stegeren et al., 2006; Winzer et al., 1999). However, a recent small-scale study observed a strong association between catecholamines and sAA using a sophisticated statistical approach (Ditzen, Ehlert, & Nater, 2014). Another possible limitation is the lack of a resting control condition. Reassuringly, prior studies have consistently failed to demonstrate salivary changes during no-manipulation control conditions (Bosch et al., 2001, 2003; Willemsen et al., 2002), suggesting that repeated measurement by itself is unlikely to account for the observed sAA changes. The two stress task were separated by 20 min, and therefore carryover effects could be a concern. This possibility seems, however, less likely in light of the facts that: (1) When the 2nd task started, autonomic/cardiovascular variables had returned to baselines values for at least 15 min; (2) The baseline levels did not significantly differ between tasks; (3) The order of the tasks was counterbalanced across participants. Importantly, the inclusion of task order as a covariate did not change any of the results (see online supplement).

Finally, it would be relevant to determine how the present findings generalize to other types of stressors, such as those involving social evaluation. For example, a recent study which compared

psychological and physiological responses to several commonly used laboratory stressors (Skoluda et al., 2015), observed that the TSST elicits a more potent sAA response than CPT or a cognitive stressor. As most studies, this study collected saliva immediately upon completion of the stressor, but not during the stressor, which are the time points where we observed a zero or negative sAA response.

A notable strength of the present study is the collection of unstimulated saliva using the spitting method. This method is generally accepted as a 'gold standard', and prevents the noise associated with absorbent materials, such as incomplete recovery of sAA, inducing flow rate by accidental chewing, and poor quantification of saliva production (Beltzer et al., 2010; Bosch et al., 2011; Bosch, 2014; Proctor & Carpenter, 2001; Rohleder et al., 2006). There is also evidence that the use of absorbent materials may attenuate stress effects. For example, Rohleder et al. (2006) found that the stress-induced sAA increases measured with salivettes were much smaller than those of whole saliva (respectively, +70% versus +130% for sAA concentration, and +80% versus +320% for secretion). It might be speculated that the current nonresponse of sAA concentration during the tasks was caused by the saliva collection causing some sort of interruption to the task, e.g., by being distracting. This interference appears less likely as we did not observe significant differences in cardiovascular and autonomic responses between the time points with and without saliva collection. This speculation also seems inconsistent with the fact that sAA increased only when the cognitive task had actually stopped. Future research may therefore further compare different saliva sampling techniques.

In conclusion, the present study demonstrated timing-dependent and stressor-specific changes in sAA in response to acute laboratory stressors. Although the MT caused a clear and continuous elevation of cardiac sympathetic activity, sAA increases were only observed immediately post-stress. Moreover, although the CPT was perceived as stressful, painful, and robustly elevated blood pressure, no changes in sAA were seen. Together these observations lead us to conclude that the interpretation of sAA as a measure of SNS activity, or as a physiological marker of stress, is less solid than often assumed. Lastly, our results indicate that, depending on changes in saliva flow rate, sAA concentration and secretion may diverge substantially and that adjustment of sAA for salivary flow rate is warranted in future studies.

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Conflict of interest

The authors declare no conflict of interest and declare no financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopsycho.2015.04.012

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