The psychosocial stress-induced increase in salivary alpha-amylase is independent of saliva flow rate

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Abstract
The stress response of salivary alpha-amylase (sAA) has been suggested as an index for sympathetic nervous system activation. However, concurrent inhibition of the parasympathetic nervous system is discussed as a confounder due to suppression of saliva flow rate. Here we set out to test the influence of stress-induced changes in flow rate on sAA secretion. Twenty-six subjects underwent the Trier Social Stress Test and a control condition. Saliva was sampled by passive drooling or salivettes. Saliva flow rate, sAA levels and output, salivary cortisol, and heart rate variability were measured. Flow rate increased only when sampled by passive drooling. Stress-induced increases in amylase levels were correlated with increases of amylase output but not with flow rate. Results indicate that flow rate is not a confounder of stress-induced sAA activation and suggest that valid measurements of sAA can be obtained by salivettes without the need for assessment of flow rate.

Descriptors: Psychological stress, Human, Saliva flow rate, Salivary alpha-amylase, Salivary amylase output

The salivary enzyme alpha-amylase has been proposed as a marker for stress-induced activity of the sympathetic nervous system. In the growing field of amylase research, recent studies have underscored the usefulness of salivary alpha-amylase in this regard. However, some methodological issues have to be resolved in order to integrate salivary alpha-amylase measurements as a standard tool into psychophysiological research. The present study set out to address one of these open questions, which is whether salivary amylase concentrations in response to stress are influenced by potential changes in saliva flow rate.

Alpha-amylase is one of the major protein components of saliva. The main function of salivary alpha-amylase is the enzymatic digestion of carbohydrates, but it is also important for mucosal immunity in the oral cavity, as it inhibits the adherence and growth of bacteria (Bosch, de Geus, Veerman, Hoogstraten, & Nieuw Amerongen, 2003; Scannapieco, Torres, & Levine, 1993). Among other proteins, alpha-amylase is synthesized and secreted by acinar cells, which make up more than 80% of the cells in the major salivary glands (Castle & Castle, 1998).

As early as 1979, Gilman, Thornton, Miller, and Biersner (1979) reported that intense physical exercise increased salivary alpha-amylase levels and attributed this to adrenoceptor activation of salivary glands. In 1996 Chatterton et al. reported a significant positive correlation between salivary amylase and plasma norepinephrine in response to a 20-min running exercise ($r = .64$). Correlations during psychological stress, that is, a written examination, were much lower ($r = .17$; Chatterton, Vogelsong, Lu, Ellman, & Hudgens, 1996). In the following, the same group reported marked increases of alpha-amylase in response to a parachute jump (Chatterton, Vogelsong, Lu, & Hudgens, 1997) and to a stressful video game (Skosnik, Chatterton, Swisher, & Park, 2000). Bosch et al. (1996) further reported higher salivary amylase levels on the day of an academic examination compared to two control days and, in a later study, stress-induced increases in alpha-amylase during a passive coping condition, that is, while watching a stressful video sequence (Bosch et al., 2003).

The findings that psychosocial stress stimulates increases of salivary amylase levels were corroborated in a series of studies employing the Trier Social Stress Test (TSST; Nater et al., 2005, 2006; Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004) or subjecting subjects to a stressful magnet resonance imaging procedure involving negative emotional pictures (van Stegeren, Rohleder, Everaerd, & Kirschbaum, 2004) or subjecting subjects to a stressful magnet resonance imaging procedure involving negative emotional pictures (van Stegeren, Rohleder, Everaerd, & Kirschbaum, 2004) or subjecting subjects to a stressful magnet resonance imaging procedure involving negative emotional pictures (van Stegeren, Rohleder, Everaerd, & Kirschbaum, 2004) or subjecting subjects to a stressful magnet resonance imaging procedure involving negative emotional pictures (van Stegeren, Rohleder, Everaerd, & Kirschbaum, 2004) or subjecting subjects to a stressful magnet resonance imaging procedure involving negative emotional pictures (van Stegeren, Rohleder, Everaerd, & Kirschbaum, 2004) of visual stressors (Takai et al., 2004). Similar findings have been obtained in children and adolescents using the TSST and other laboratory stressors (summarized in Granger et al., 2006; Granger, Kivlighan, El-Sheik, Gordis, & Stroud, in press).

Where measured, associations with plasma norepinephrine levels were weaker in these psychological stress paradigms compared with the exercise data reported by Chatterton et al. (1996).
et al., 2003) and after a computerized color word task (Bakke videos in contrast to reduced flow rate in a memory task (Bosch increased flow rate was reported in response to watching stressful to physical exercise by Li and Gleeson (2004). On the other hand, increased flow rate was stimulated by chewing on a cotton roll.

Given the considerable variance in the data on flow rate responses to stress and its impact on salivary alpha-amylase measurements, we set out in the present study to investigate the following questions: (1) Does saliva flow rate change in response to typical laboratory stress (i.e., TSST)? (2) Do flow rate changes depend on sampling method; that is, does the use of salivettes versus passive drooling influence stress responses? (3) Do potential changes in flow rate and/or the sampling method influence the salivary alpha-amylase response to stress? Answers to these questions will allow us to determine whether saliva flow rate is a confounder and has to be assessed in future studies and whether the use of salivettes is advisable when measuring salivary alpha-amylase. To be able to evaluate salivary alpha-amylase responses in the context of the typically used markers of psychological stress, we additionally measured heart rate variability and salivary cortisol.

Methods
Participants
Twenty-six healthy participants were recruited at the Dresden University of Technology. All participants were Caucasian men. Women were excluded because it is not known at this time whether varying gonadal steroid levels influence amylase stress response, as they do, for example, with cortisol (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). All participants underwent a comprehensive medical examination for past or current health problems. Exclusion criteria were any psychiatric, endocrine, cardiovascular, or other chronic disease, medication with psychoactive drugs, beta-blockers, or glucocorticoids, and a body mass index (BMI) <20 or >28 kg/m², and age <20 or >35 years, respectively. Participants were randomly assigned to one of the two sampling conditions (i.e., salivette or passive drooling). Participants in the salivette condition had a mean age of 23.2 years ± 2.5 SD (range: 21–30 years) and a mean BMI of 22.3 kg/m² ± 1.7 SD (range: 20.1–24.9 kg/m²); participants in the passive drooling condition had a mean age of 24.8 years ± 4.1 SD (range: 21–35 years) and a mean BMI of 22.6 kg/m² ± 1.8 SD (range: 20.6–27.2 kg/m²). To assess participants’ psychological health, a German version of the 12-item General Health Questionnaire (GHQ-12) was administered (Goldberg, 1992; Schmitz, Kruse, & Tress, 1999). Mean GHQ scores as well as age and BMI did not differ between participants assigned to the respective sample conditions (GHQ: salivette vs. passive drooling: 3.21 ± 0.48; p = .25). No changes in flow rate were found after watching stressful (as well as soothing) videos (Takai et al., 2004) or in one of the TSST studies by Nater et al. (2006). The latter study was the only one in which salivettes were used, that is, in which flow rate was stimulated by chewing on a cotton roll.

Experimental Protocol
After a telephone interview regarding basic inclusion criteria (e.g., age, BMI, or medication), participants were scheduled for two laboratory sessions on consecutive weekdays, always beginning between 14.00 and 17.00 h. At the first laboratory session, a written explanation of the study goals and protocol was handed to the participants. Upon agreement to the study protocol, all participants signed a written informed consent letter, followed by
a brief medical examination and testing of overall psychological health by administration of the GHQ-12 (see above). This first laboratory session served as a control condition, whereas the second laboratory session served as a stress condition. This fixed sequence was chosen to avoid carryover effects; that is, to avoid a situation in which participants who have completed the TSST suspect or anticipate social evaluation during the control condition, the TSST always followed the control condition. The study protocol was approved by the ethics committee of the German Psychological Association (DGPs). Participants were paid EUR 15 for their participation.

On both experimental days, a Polar S810 heart rate monitor (Polar Electro Oy, Kempele, Finland) was attached to the participants immediately after arrival and continuous beat-to-beat measurement started. After a resting period of 30 min to reduce the impact of the possibly stressful process of reporting to an unknown laboratory, a first saliva sample was taken (as outlined below), followed by a control condition at laboratory session one or by a stress condition at laboratory session two. Psychosocial stress was induced on the second day by exposing all participants to the TSST, which consists of a 5-min anticipation period, followed by a 5-min free speech in a hypothetical job interview, and another 5 min of mental arithmetics, all in front of an audience and a video camera (Kirschbaum, Pirke, & Hellhammer, 1993). During the control condition, participants were taken to the same room in which the TSST would be held on the second day, but without an audience or video camera. After quietly reading a newspaper article while sitting at a table 5 min, participants were asked to assume a standing position and read aloud from the newspaper for 10 min. After the TSST and the control condition, participants were taken back to the waiting area and three additional saliva samples were taken 1, 10, and 20 min relative to cessation of the stress or control paradigm. These sampling intervals were chosen based on previous studies showing that peak levels of alpha-amylase occur immediately after stress and return to baseline within the following 10–20 min (Nater et al., 2005).

Saliva was collected using salivettes (Sarstedt, Nümbrecht, Germany) or the passive drooling method. Participants using salivettes were instructed to place the cotton swab into their mouths for exactly 2 min and chew slightly. The passive drooling method was modified from the original procedure suggested by Navazesh (1993). In accordance with Navazesh, participants were instructed to first void their mouths of saliva by swallowing. In contrast to the original method, saliva was then allowed to accumulate for exactly 2 min (instead of 5, to allow more frequent sampling points). Participants then spit all saliva into 2-ml tubes through a straw. This procedure was practiced during the first 30 min of each experimental day. All samples were obtained with one of the investigators attending the sampling procedure.

Heart Rate Variability
Heart rate variability parameters were measured using the software tool “Heart Rate Variability Analysis” (Niskanen, Tarvainen, Ranta-Aho, & Karjalainen, 2004) based on interbeat (R-R) intervals obtained using a Polar S810i heart rate monitor (Polar Electro Oy, Kempele, Finland). The software extracts heart rate variability in various frequency bands using fast Fourier transformations (FFT). Here, we used the variance of the high frequency band (HF; 0.15 to 0.4 Hz) as an index for vagal tone (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). HF variance was expressed as ms² and log-transformed to achieve normal distribution. HF variance and heart rate (HR) were calculated for six 2-min intervals relative to the stress and control conditions (parallel to saliva sampling and one additional time point during the stress or control procedure and at baseline, starting 5 min before the first saliva sample).

Laboratory Analyses
Saliva samples were frozen immediately after each laboratory session and stored at −20°C until analysis. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Sampling tubes used for passive drooling were centrifuged at 20,000 rpm for 5 min, resulting in mucous compounds being restricted to the lower part of the tube.

Concentration of alpha-amylase in saliva was measured by an enzyme kinetic method: Saliva was processed on a Genesis RSP8/150 liquid handling system (Tecan, Crailsheim, Germany). First, saliva was diluted 1:625 with double-distilled water by the liquid handling system. Twenty microliters of diluted saliva and standard were then transferred into standard transparent 96-well microplates (Roth, Karlsruhe, Germany). Standard was prepared from “Calibrator f.a.s.” solution (Roche Diagnostics, Mannheim, Germany) with concentrations of 326, 163, 81.5, 40.75, 20.38, 10.19, and 5.01 U/l alpha-amylase, respectively, and bidest water as zero standard. After that, 80 μl of substrate reagent (α-amylase EPS Sys; Roche Diagnostics, Mannheim, Germany) were pipetted into each well using a multichannel pipette. The microplate containing sample and substrate was then warmed to 37°C by incubation in a waterbath for 90 s. Immediately afterward, a first interference measurement was obtained at a wavelength of 405 nm using a standard ELISA reader (Anthos Labtech HT2, Anthos, Krefeld, Germany). The plate was then incubated for another 5 min at 37°C in the waterbath, before a second measurement at 405 nm was taken. Increases in absorbance were calculated for unknowns and standards. Increases of absorbance of diluted samples were transformed to alpha-amylase concentrations using a linear regression calculated for each microplate (Graphpad Prism 4.0c for MacOSX, Graphpad Software, San Diego, CA). Inter- and intra-assay variation was below 10%. Salivary free cortisol concentrations were measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/ml (IBL, Hamburg, Germany).

Saliva flow rate was calculated by the gravimetric method assuming the density of saliva being 1.0 g/ml (Chicharro, Lucia, Perez, Vaquero, & Urena, 1998). Saliva obtained after 2 min of passive drooling or using the salivette was weighed and flow rate was expressed in ml/min (g/min). Amylase output was then computed as U/min by multiplication of flow rate (ml/min) with amylase concentrations (U/ml) of the respective sample.

Statistical Analyses
All laboratory data were tested for normality using the Kolmogorov–Smirnov test prior to analyses. No transformations were necessary for any of the variables except for HF variance, which was transformed by applying the formula ln x = ln(x + 1). ANOVAs for repeated measures with the between-subjects factor Sampling Condition (salivette vs. passive drooling), and the within-subject factors Stress (control vs. stress), and Time (−1 min, +1 min, +10 min, +20 min) were calculated to test for stress-induced changes in sAA concentrations, sAA output, and salivary cortisol and to test for the impact of sampling method.
Greenhouse-Geisser corrections for repeated measures were calculated where appropriate (indicated by decimal degrees of freedom values). Similar analyses were performed for heart rate variability parameters, except that the factor Time had six levels. For all parameters, we also calculated the relative increase with respect to the baseline (i.e., the baseline level of each parameter was subtracted from the posttreatment level), as additional indices of stress-induced changes. Pearson correlations were calculated to test for associations between relative increases of salivary and heart rate parameters. All analyses were performed using SPSS 11.0.1 for Mac OSX (SPSS Sciences, Munich, Germany). Data are expressed as means ± SEM, and \( p < .05 \) was set as the criterion for significance.

**Results**

The first section presents data that answer the first and the second research questions, that is, whether saliva flow rate changes in response to stress and whether flow rate changes differ between saliva obtained using salivettes versus saliva obtained using the passive drooling method. In the following sections we first present stress responses of salivary amylase concentrations (U/ml) and amylase concentrations corrected for saliva volume (i.e., amylase output in U/min). This together with the correlation of relative increases of uncorrected and corrected amylase data will answer the third research question, that is, whether stress-induced increases of salivary amylase are independent of flow rate and sampling method. To be able to evaluate salivary alpha-amylase responses in the context of the typically used markers of psychological stress, responses of heart rate variability and salivary cortisol are presented in the last section.

**Impact of Stress and Sampling Method on Saliva Flow Rate**

Saliva flow rate was calculated by the gravimetric method assuming the density of saliva being 1.0 g/ml as described in the Methods section. Figure 1 shows saliva flow rate over the course of the experiment. Repeated measures ANOVA revealed a significant Time \( \times \) Condition interaction, \( F(3,72) = 3.94; p = .012 \), whereas the three-way interaction was not significant (Stress \( \times \) Time \( \times \) Condition: \( F[3,72] = 0.85; p = .47 \)). This indicates that changes in flow rate are dependent on the sampling method. To further analyze the impact of sampling condition, separate repeated measures ANOVAs for the salivette and the passive drooling condition were calculated. Although flow rate did not respond to stress in the salivette condition, all \( F < 1 \), stress and time effects in the passive drooling condition indicate that saliva flow increases in response to the TSST but not to a control condition when saliva is obtained by passive drooling (Stress effect: \( F[1,11] = 5.11, p = .045 \); Time effect: \( F[3,33] = 3.50, p = .026 \); Stress \( \times \) Time interaction: n.s.). This is further underscored by the fact that relative increases of flow rate are significantly higher in saliva obtained by passive drooling than in salivettes (main effect Condition: \( F[1,24] = 7.63, p = .01; \) data not shown).

**Impact of Stress and Sampling Method on Salivary Amylase Levels**

As shown in Figure 2, exposure to the TSST induced significant increases in salivary alpha-amylase levels (Stress effect: \( F[1,24] = 20.54, p < .001 \); Stress \( \times \) Time interaction: \( F[1,51,33,23] = 19.47, p < .001 \)). Amylase concentrations in saliva were significantly lower in samples obtained by the passive drooling method compared to the salivette (main effect Condition: \( F[1,24] = 10.44, p = .004 \)). However, this overall difference had no influence on stress reactivity as indicated by nonsignificant interactions of Condition with the factors Time and Stress (Stress \( \times \) Condition: \( F[1,24] = 0.24, p = .63 \); Time \( \times \) Condition: \( F[1,94,42,59] = 1.41, p = .26 \); Stress \( \times \) Time \( \times \) Condition: \( F[1,51,33,23] = 0.51, p = .55 \)).

This is further underscored by the finding that relative increases of sAA in saliva obtained by salivettes versus passive drooling did not differ (main effect Condition: \( F[1,24] < 0.001 \); Condition \( \times \) Stress interaction: \( F[1,24] < 0.001 \); data not shown).

**Impact of Stress and Sampling Method on Salivary Amylase Output**

Salivary alpha-amylase output (mg/min) is computed by multiplication of flow rate (ml/min) and amylase concentration (U/ml) as described in the Methods section. Figure 3 shows salivary amylase output separately for the stress and the control condition. Psychosocial stress induced significant increases of sAA output in both sampling conditions as indicated by a significant Stress effect, \( F(1,24) = 26.02, p < .001 \), Time effect, \( F(1,54,36,83) = 12.25, p < .001 \), and a significant Stress \( \times \) Time interaction, \( F(1,79,42,86) = 9.85, p < .001 \). Sampling condition influenced overall level of amylase output (main effect Condition: \( F[1,24] = 7.07, p = .014 \)), but had no impact on stress reactivity as indicated by nonsignificant interactions (Stress \( \times \) Condition: \( F[1,24] = 0.72, p = .40 \); Time

![Figure 1](image-url). Saliva flow rate in response to the TSST and control condition. Saliva was obtained by (A) salivettes vs. (B) passive drooling (graphs show means ± SEM).
x Condition: $F[1.54,36.83] = 1.94$, $p = .17$; Stress $\times$ Time $\times$ Condition: $F[1.79,42.86] = 0.73$, $p = .48$).

A further ANOVA revealed that relative increases of amylase output were significantly higher in response to stress (Stress effect: $F[1,24] = 14.70$, $p < .01$). There was a trend toward higher amylase output when saliva was obtained by passive drooling compared to salivettes (main effect Condition: $F[1,24] = 3.06$, $p = .09$). There was no interaction between Sampling Condition and Stress, $F(1,24) = 0.99$, $p = .33$ (data not shown).

**Association of Amylase Concentration and Output**

As shown in Figure 4, there was a positive association between the relative increase of amylase concentration and amylase output in the TSST condition ($r = .62$, $p = .002$). Separate analyses for the two sampling conditions revealed a higher correlation when salivettes were used ($r = .73$, $p = .01$) compared to passive drooling ($r = .56$, $p = .06$). No correlation was found between the relative increase of amylase concentration in the TSST condition with the change in salivary flow (total group: $r = -.06$, $p = .77$; salivette: $r = -.16$, $p = .59$; passive drooling: $r = -.002$, $p = .996$). In contrast, the relative increase of flow rate was positively associated with increase of amylase output (total group: $r = .65$, $p < .001$; salivette: $r = .75$, $p = .002$; passive drooling: $r = .57$, $p = .05$).

**Stress Response of Heart Rate Variability and Salivary Cortisol**

Free cortisol in saliva and heart rate variability was measured to verify the effectiveness of the TSST paradigm. Heart rate increased in response to both the stress and control conditions (Time effect: $F[3.58,78.81] = 76.97$, $p < .001$). HR increases tended to be higher during the TSST compared to the control condition (Stress $\times$ Time interaction: $F[3.01,66.25] = 2.16$, $p = .10$). The effect of Sampling Condition failed to reach significance (main effect Condition: $F[1,22] = 2.77$, $p = .11$; Stress $\times$ Condition interaction: $F[1,22] = 1.95$, $p = .18$). All other interactions were not significant (Time $\times$ Condition: $F[3.58,78.81] = 1.92$, $p = .12$; Stress $\times$ Time $\times$ Condition: $F[3.01,66.25] = 1.57$, $p = .21$; see Figure 5A).

As an index for vagal tone, the variance of the high frequency (0.15 to 0.4 Hz) component of beat-to-beat variability was calculated. HF variance responded to the stress and the control conditions with a decrease during experimental treatment and an increase thereafter (Time effect: $F[3.94,90.63] = 6.38$, $p < .001$). There was no difference between stress and control condition (Stress effect: $F[1,23] = 0.77$, $p = .39$; Stress $\times$ Time interaction: $F[3.73,85.77] = 0.41$, $p = .79$) and no impact of sampling condition (Condition effect: $F[1,23] = 0.52$, $p = .48$; Stress $\times$ Condition interaction: $F[1,23] = 0.14$, $p = .72$; Time $\times$ Condition: $F[3.94,90.63] = 1.42$, $p = .23$; Stress $\times$ Time $\times$ Condition: $F[3.73,85.77] = 1.15$, $p = .34$; data not shown).

Pearson correlations revealed that there were no associations of relative increases in saliva flow rate, amylase concentration, and amylase output with changes of any of the heart rate parameters (all $R_s < .26$, all $p > .05$; data not shown).

Free cortisol significantly increased in response to psychosocial stress (Stress effect: $F[1,24] = 31.12$, $p < .001$; Time
Discussion

In the present study, saliva flow rate, alpha-amylase concentrations in saliva, and output were measured in response to the TSST (Kirschbaum et al., 1993) and to a control condition involving reading a newspaper article in a standing position but without other individuals present. The latter condition was chosen to mimic the physiological properties of the TSST, for example, the strain of orthostasis, without the psychologically stressful social evaluation, which appears to be the major component of these laboratory stress paradigms (Dickerson & Kemeny, 2004).

Results show that stress induces slight increases in saliva flow rate that were only detectable when saliva was obtained by passive drooling. No stress-induced flow-rate changes were measurable when salivettes were used. Stress induced significant increases in alpha-amylase concentrations in saliva in both sampling conditions, with generally higher concentrations in the salivette condition. Salivary amylase output, as calculated by multiplication of amylase concentrations with saliva flow rate, consequently increased significantly in response to stress. Amylase output was therefore slightly but nonsignificantly higher in samples obtained by passive drooling, whereas overall amylase output was higher with salivettes. There was no association of increases in flow rate and amylase levels, but a strong correlation of increases in amylase levels and amylase output in both sampling conditions. Amylase and flow-rate changes were not associated with changes in cortisol, heart rate, or heart rate variability.

The increase of amylase concentrations is consistent with earlier studies on amylase stress responses (Chatterton et al., 1996; Morse et al., 1981) as well as more recent studies by our group and others (Bosch et al., 2003; Nater et al., 2005, 2006; Rohleder et al. 2004). The finding of increasing saliva flow rate in response to stress when saliva was collected by passive drooling is in contrast to some of the earlier studies by Morse et al. (1981), to an academic stress study by Queiroz et al. (2002), who found lower flow rate on a day of a stressful academic exam, and to the flow rate declines reported by Li and Gleeson (2004) in response to exercise stress. Our data are in accordance with the increases in flow rate after watching stressful videos reported by Bosch et al. (2003) and the increases in response to the computerized color word test in young and elderly women found by Bakke et al. (2004). It appears that acute psychological stressors, as used in the latter studies by Bosch et al. (2003) and Bakke et al. (2004), evoke increases in flow rate, as found in the present study, whereas tasks with a physiological component, such as endodontal therapy or exercise, appear to decrease salivary flow. It is not surprising that saliva flow rate does not show detectable changes when salivettes are used, as also reported by Nater et al. (2006). It can be assumed that the use of salivettes leads to a rather uniform stimulation of salivary flow due to tactile stimulation by the presence of the cotton roll in the oral cavity, even if the instruction is not to chew on the cotton roll (Guinard et al., 1997).

Amylase output expressed as U/min increased in response to stress in both sampling conditions. No significant differences were found between sampling conditions, although variance and relative increase were higher, whereas absolute levels were lower in the passive drooling conditions. Similar results with respect to

Figure 4. Association of relative increases of amylase output (U/min) and amylase concentrations not corrected for saliva flow rate (U/ml). Correlation coefficients were higher in the salivette than in the passive drooling condition (r = .73, p = .011, and r = .56, p = .06, respectively).

Figure 5. Heart rate (A) and free cortisol response (B) to the psychosocial stress test TSST and control condition. (Graph shows means ± SEM. Cortisol and HR values did not differ significantly between passive drooling and salivettes and are thus presented collapsed over the two sampling conditions.)
stress responsiveness have also been reported in the study by Bosch et al. (2003), whereas in the Nater et al. (2006) study, increases failed to reach significance. No other studies in healthy adult subjects explicitly report stress-induced changes in amylase output expressed as units per minute. The generally higher concentrations of salivary alpha-amylase in the salivette condition may result from stimulation of the salivary glands by chewing on the cotton roll. Mastication does not only increase flow rate, but has also been shown to increase protein output by acinar cells (Froehlich, Pangborn, & Whitaker, 1987).

The changes in heart rate and heart rate variability show that cardiovascular reactivity did not discriminate between the two testing conditions. Nater et al. (2006) reported comparable changes of HRV parameters in response to the TSST, but did not report how HRV parameters responded to a control condition that also involved body posture changes. It remains unresolved why HR and HRV responses are not correlated with amylase responses, even though both are mediated by the sympathetic nerve. As the HRV response pattern during our control condition is a typical orthostatic response (Houtveen, Groot, & Geus, 2005; Nonell et al., 2005) and amylase apparently does not respond to orthostatic challenge, it may be hypothesized that differential activation patterns of the sympathetic nerve or further unknown factors are involved. It appears, however, that the response of heart rate parameters is more influenced by physiological processes involved in orthostasis, whereas salivary alpha-amylase responses are more influenced by psychological stress.

What do the results of the present study tell us about the role of saliva flow? First, the results show that flow rate does respond to psychosocial stress with a small but significant increase. This small increase appears to be only detectable when saliva is obtained by passive drooling. Although this difference is explainable by the fact that flow rate might be constantly stimulated by the sampling process, the finding that saliva flow does not markably decrease in response to stress is of utmost interest. This finding clearly contradicts the central criticism, which is that a hypothetical stress-induced decrease in salivary flow might artificially increase salivary amylase concentrations, as more amylase molecules would be present in a lower volume of saliva, without the need for higher protein secretion by the acinar cells of the salivary glands. In other words, the increase of alpha-amylase concentrations in saliva is necessarily a result of increased secretion of this protein, most likely mediated by sympathetic fibers, and not an artifact of unchanged protein amounts in a decreasing volume of saliva.

Our second goal was to determine the relative impact of flow-rate changes on salivary alpha-amylase secretion. The comparison of stress amylase concentrations with amylase output, that is, concentration corrected for flow rate, revealed that flow rate indeed has a small but nonsignificant impact on amylase stress responses. However, the substantial correlation between amylase concentrations and output shows that the impact of flow rate changes is negligible. Because previous studies have shown that relative increases and not absolute concentrations of amylase might be related to sympathetic nervous system activation (Nater et al., 2005; Rohleder et al., 2004; van Stegeren et al., 2006), it is of lesser importance that amylase concentrations appear to be lower when saliva is obtained by passive drooling.

These findings have several implications for the further use of salivary alpha-amylase in future studies in the field of psychophysiology. The relative independence of salivary alpha-amylase secretion from changes in saliva flow rate, the fact that the flow-rate changes are small, and that they are in the opposite direction of the central criticism cited above imply that alpha-amylase can be safely measured using salivettes. To obtain valid results, it is not necessary to employ the more complicated method of passive drooling and it is not necessary to determine the volume of saliva samples and to calculate flow rate. The use of salivettes furthermore appears to reduce variability of amylase output, possibly by reducing variance in flow rate.

The results of the present study have to be interpreted in the light of several limitations. First, only healthy young Caucasian men were investigated in the present study. Future studies will have to include women, as well as different age groups and ethnicities, to further generalize the findings. Also, the relatively small number of subjects may have led to an underestimation of the differences in amylase output between sampling conditions. On the other hand, the correlation between stress responses of amylase concentration and output was significant despite the low number of participants. A final methodological limitation might be the fixed order of control and stress conditions. A randomization of sequence would have allowed the determination of the impact of anticipatory stress in the control condition. However, this would have increased the number of participants needed, and the order of conditions used here, that is, TSST following control condition, has proven effective in reducing anticipatory stress in the control condition in previous studies (Rohleder et al., 2006).

Conclusion

The present results lead us to conclude that saliva flow rate is not a confounder of stress-induced activation of amylase secretion and that valid measurements of salivary alpha-amylase can be obtained by using salivettes and without assessing flow rate. Saliva flow rate increases in response to a psychosocial stress paradigm, but changes are only detectable when saliva is obtained by passive drooling, and not by salivettes. This, however, is not a problem for the use of salivettes, as saliva flow rate and sampling method have a negligible impact on salivary amylase output, as shown by the substantial correlation of amylase concentration with output and the absent association with flow rate. Presuming that further studies succeed in substantiating associations of amylase increases with sympathetic activation, the measurement of amylase will be an important tool for stress research in psychophysiology.

**REFERENCES**


