

## Stability and durability of salivary alpha-amylase across different storage conditions

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### ABSTRACT

Data collection in remote and field settings gains importance and popularity in stress research. Accordingly, existing stress induction paradigms have been successfully adapted to remote and field settings. However, guidelines for the comprehensive assessment of biomarkers such as salivary alpha-amylase (sAA) have yet to be sufficiently established for such contexts. In remote and field settings, swift freezing of saliva samples is not always possible, and samples must be returned to the laboratory for further processing. The current study investigated the robustness of sAA activity against external factors that may affect measurements obtained from saliva samples collected in field and remote settings. We compared sAA activity of samples that were stored in different vials (Salivettes® and Eppendorf® vials) and that were exposed to (1) up to three cycles of freezing and thawing, (2) different temperatures (4 °C, 20 °C, 30 °C, and 40 °C) for 3, 7, 14, or 28 days, or that were (3) sent via postal delivery. Results indicate sAA activity to be susceptible across different temperatures, different time intervals, and different vials. As a systematic pattern, sAA activity seems to decrease in treated samples with this effect being potentiated by more extreme conditions such as higher temperatures and longer time intervals. To conclude, sAA data collected in remote or field settings could be affected systematically by various external variables. Future studies collecting sAA should take factors influencing the durability and stability of sAA into account to ensure reliable and valid measurements of salivary data.

### 1. Introduction

Remote data collection is becoming increasingly common in stress research. This is also noticeable in adaptations of established stress induction protocols for use in remote settings (Pfeifer et al., 2021). It seems crucial to discuss how to collect, store, and ship saliva samples taken outside the laboratory for later analysis of salivary biomarkers such as cortisol and alpha-amylase (sAA) (Harvie et al., 2021; Nater and Rohleder, 2009; Pfeifer et al., 2021).

Different procedures for handling saliva samples in remote settings have been applied so far. Gunnar et al. (2021) developing an online version (TSST-OL) of the Trier Social Stress Test (TSST; Kirschbaum

et al., 1993), instructed participants to send saliva samples back to local research facilities within one day. Also using the TSST-OL, Meier et al. (2022) let participants store samples in their refrigerator until shipment. Other studies applying home-based stress induction did not include salivary measures (Eagle et al., 2021; Harvie et al., 2021).

Studies by Aardal and Holm (1995), Clements and Parker (1998), and Garde and Hansen (2005) univocally confirmed the robustness of cortisol against various external conditions. Cortisol as a more durable steroid hormone may be more robust when compared to sAA which is an enzyme. Indeed, a review by Nater and Rohleder (2009) concluded that the stability of sAA might be affected by: (1) room temperature/common fridges, (2) freezers of −20 °C or −80 °C, (3) multiple cycles of freezing

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and thawing, (4) collection method/vial of storage and (5) usage of preservatives.

Several studies have addressed these factors for sAA. The findings of Granger et al. (2007) suggested that sAA may be stable in refrigerators (no difference between samples stored at 4 °C and at -80 °C for storage of four days). Concerning repeated freezing and thawing, previous studies did not report any effects of up to three (Granger et al., 2007) or five (O'Donnell et al., 2009) thawing cycles. Concerning room temperature, studies reported no effect for exposure over 4 (Granger et al., 2007), 5 (O'Donnell et al., 2009), 7, 14, or 21 days (DeCaro, 2008). The extent to which higher temperatures could affect sAA activity has been investigated by DeCaro (2008) who did not reveal any effects for exposure to 37 °C for 7, 14, or 21 days.

Finally, several studies dealt with errors in sAA measurement induced by collection method or vial type. It was shown that cotton-based absorbent materials (i.e., pledgets that soak up saliva while being placed in the mouth) induce error variance for several salivary biomarkers but not salivary cortisol (Shirtcliff et al., 2001). Concerning sAA, the literature is inconsistent. While Granger et al. (2006) and Granger et al. (2007) did not find evidence for cotton, and hydro-cellulose (i.e., microspunge) to have an effect, DeCaro (2008) reported absorbent cotton rolls to impede sAA measurement. Factors causing these effects are under debate (DeCaro, 2008; Nater and Rohleder, 2009; Shirtcliff et al., 2001).

Even though some studies dealt with the reliability of sAA measurement, no publication contained those variables at once that play a role in research in remote settings (i.e., freezing and thawing, exposure to different temperatures for variable time intervals, different vials). Moreover, the effects of postal delivery have not yet been examined for sAA even though mailing of saliva samples may be appropriate for different purposes. To further shed light onto these relations, we designed four experiments alongside the following hypotheses:

- (1) In an initial experiment we tested the effects of vial independently of other manipulations. We took measurements from a baseline saliva pool containing a homogenous level of sAA activity and compared them with other portions of the pool aliquoted onto different types of vials. For this first experiment, we hypothesized no effect of vial.
- (2) Since thawing of samples is recommended upon analysis and sAA should be inactive in a frozen state, we hypothesized that thawing and freezing should not affect sAA. To test this hypothesis, we exposed saliva samples to up to three cycles of freezing and thawing.
- (3) Previous literature suggested sAA to be resistant to storage at various temperatures. However, from a biological perspective, it is not plausible that sAA remains stable in non-frozen samples. Micro-organisms contributing to the degradation of sAA should find optimal conditions under higher temperatures and might cause measurement errors. Therefore, we assumed to find changes in sAA in non-frozen samples that would potentiate with increasing temperature and increasing exposure intervals. To test this hypothesis, we exposed saliva samples to 4 °C, 20 °C, 30 °C, or 40 °C for 3, 7, 14, or 28 days.
- (4) In a fourth experiment, we exposed saliva samples to postal delivery. No study so far examined the stability and durability of sAA in mailed samples. Assuming that samples would undergo different temperatures or further stimulation (e.g., screening/x-raying), we hypothesized to find changes in sAA activity after postal delivery.

## 2. Methods

### 2.1. Sample

This study was approved by the local ethic committee of the Ruhr

University Bochum. We collected fresh saliva from  $N = 19$  participants ( $N = 11$  females) (descriptive sample characteristics can be found in the Appendix A1.1). Participants were instructed to salivate in 50 ml tubes over the course of 24 h and to store full tubes in the refrigerator. Participants were asked to collect their saliva whenever possible during this 24-hour period. We instructed participants to adhere to predefined regulations that are provided in the Appendix A1.2 in order to ensure the purity of saliva.

Saliva out of 50 ml tubes was mixed to create a baseline saliva pool of 1255 ml in total (the amount of saliva contributed per individual varied between 10 ml-300 ml), which was subsequently preprocessed and exposed to the various experimental conditions in Salivettes® (Sarstedt, Nümbrecht, Germany) and Eppendorf 1.5 ml tubes (Eppendorf®, Hamburg, Germany). The number of vials used was determined by means of an a priori sample size calculation which can be found in the Appendix A1.3.

### 2.2. Generation and preprocessing of the saliva pool

To remove food residues and mucins from saliva, 50 ml collection tubes were first centrifuged at room temperature (20 °C) for 10 min at 10000 xg. This centrifugation can be assumed to have effectively removed debris of food and cells (also including mucins) from saliva (Zhang et al., 2016). In order to generate a homogenous baseline saliva pool, supernatant out of centrifuged 50 ml collection tubes was mixed in a measuring breaker. The resulting baseline pool comprised 1150 ml of saliva. Under constant stirring, 500 µl of saliva was aliquoted onto the pledgets of the Salivettes or into Eppendorf vials (10 µl would have been the minimal volume required by the assay used for sAA measurement). Vials were then exposed to different storage conditions.

### 2.3. Establishment of experimental conditions

Baseline condition:  $N = 60$  samples were drawn out of the original saliva pool, mixed, and immediately analyzed. That is, samples directly analyzed from the baseline saliva pool were pipetted onto 96-well plates for sAA measurement within a few minutes after finalizing the baseline pool. In addition,  $N = 60$  samples were pipetted onto Salivettes or in Eppendorf vials, respectively. Those vials were then frozen for 2 h, thawed, centrifuged at 10000 xg for 5 min, and the clear supernatant was immediately analyzed. It seems important to specifically highlight that samples analyzed directly out of the baseline pool were not frozen before sAA measurement (this concerns Salivettes and Eppendorf vials of the baseline condition). This different treatment of samples in the baseline condition may confound results regarding the additional factor of freezing and is debated in the "Discussion" in detail.

Thawing cycles: After initial freezing at -20 °C, samples were thawed by exposing them to room temperature (20 °C) for 4 h. Afterwards, samples were frozen at -20 °C again. This procedure was repeated for samples exposed to two, or three thawing cycles.

Exposure by time interval: Samples were stored (a) in a regular laboratory refrigerator (4 °C), (b) in a laboratory room (20 °C), or two incubators (drying and heating oven ED 115, BINDER, Germany), one set to (c) 30 °C and the other to (d) 40 °C. Exposure covered 3, 7, 14, or 28 days.

Postal delivery: For postal delivery, samples were mailed from Düsseldorf, Germany, to Bochum, Germany. As this study was conducted during April/May (drop into the mailing box: April 26th, 2022), postal delivery took place during spring, representing temperate climate in Germany. Salivettes and Eppendorf vials were sent in two parcels. Distribution was chosen randomly to ensure that both parcels equally contained 20 Salivettes and 20 Eppendorf vials. The two parcels were treated identically and dropped into the mailing box simultaneously. Both parcels arrived at the Ruhr University after six days (May 2nd, 2022) and were swiftly frozen at -20 °C.

## 2.4. Laboratory analysis

After exposure to different storage conditions, samples were frozen at  $-20^{\circ}\text{C}$ . Upon analysis, samples were thawed and centrifuged at 2000 xg for 5 min at room temperature. Again, this procedure can be considered an effective cleaning step (Worthman et al., 1990; Zhang et al., 2016). Importantly, samples of each condition (e.g., Salivettes exposed to one thawing cycle) were pooled to reduce variance produced at the level of single samples. However, the pool created per condition was then pipetted onto as many wells on 96-well plates as samples were exposed to the condition at hand (and given by our a priori power analysis). For instance, saliva out of the  $N = 40$  Salivettes that were exposed to the postal delivery, were pooled, and then pipetted onto 40 wells of 96-well plates. Laboratory analysis was performed at the local core facility joint laboratory of the Genetic Psychology and Cognitive Psychology at the Ruhr University Bochum. A colorimetric test using 2-Chloro-4-nitrophenyl- $\alpha$ -D-maltotriose (CNP-G3) as a substrate reagent was performed in duplicates on a Synergy2 plate reader (Biotek/Agilent, USA). Therefore, saliva samples were sequentially diluted in a ratio of 1:200 with assay diluent to measure sAA activity as described elsewhere (Lorentz et al. (1999)). Standard samples measured in 20 replicates on the same plate showed intra-assay coefficients of variability (CV) between 5.8 %–6.3 % and inter-assay CVs between 7.5 %–8 % as measured on different plates.

## 2.5. Statistical analysis

Data cleaning is described in the Appendix A1.4. After data cleaning, our final sample comprised  $N = 657$  samples. Of these, the baseline condition comprised  $N = 103$  samples. Conditions of thawing and freezing counted  $N = 221$  samples while  $N = 304$  samples were applicable for analysis out after exposure to varying temperatures over different time intervals. The postal delivery condition contributed with  $N = 74$  samples to our analysis. For an overview over the more fine-grained distribution of samples within the different experiments, see the Appendix (Table A2.1–A2.4).

Statistical analysis was performed in R (version 4.2.2; R Core Team, 2022). Raw data and analysis scripts can be found on OSF under the link [https://osf.io/z94yn/?view\\_only=9413be5f4d284ec0a8e0219642e61ce3](https://osf.io/z94yn/?view_only=9413be5f4d284ec0a8e0219642e61ce3). Since most of our data did not seem to fulfill required statistical assumptions for parametric ANOVA, we used the bestNormalize() function (Peterson, 2021) in R to approximate required distributions. Subsequently, we applied parametric univariate between-subject one-way analyses of variance (ANOVA) to test for the main and interaction effects of experimental factors in our four experiments separately. Since all four experiments included the same baseline measurements, the significance level was adapted to  $p = .0125$  ( $p = .05/4$ ). Due to the massive number of multiple comparisons and since we were more interested in the holistic trend of effects rather than differences between the single conditions, we did not conduct further post-hoc tests. Instead, for significant main effects of the four experiments, we further specified linear regressions modeling data as predicted by the factors rendering significant main effects in the previous ANOVA. This was done across the different types of vials (Salivettes and Eppendorf vials) and separately for the two types of vials. However, we did not specify regression models for the main effect of vial even though it turned out to be significant in an ANOVA.

## 3. Results

Descriptive statistics of raw (untransformed) sAA activity can be found in the Appendix for all four experiments (Table A2.1: baseline condition, Table A2.2: thawing cycles, Table A2.3: exposure by time interval, Table A2.4: postal delivery). For the data transformation, the bestNormalize() function applied an orderNorm data transformation for data of the baseline saliva pool and the exposure by time interval

condition. Data of the thawing cycles were transformed using a BoxCox transformation while a Yeo-Johnson data transformation was used for the postal delivery condition.

In the following, we report results for ANOVA and regressions for the different experiments as modeled across the two types of vials. Results for regressions modeled separately for the different types of vials can be found in the Appendix (Table A3.1: baseline condition, Table A3.2: thawing cycles, Table A3.3: exposure by time interval – temperature, Table A3.4: exposure by time interval – time, Table A3.5: postal delivery). Fig. 1 shows an overview of plotted regression models for the different experiments.

### 3.1. Baseline saliva pool

A one-way ANOVA revealed a statistically significant difference in sAA activity between different vials in the baseline condition,  $F_{(2,173)} = 36.28$ ,  $p < .001$ ,  $\eta_p^2 = .30$ . A subsequent linear regression confirmed the factor of vial to be a significant predictor of sAA activity, which explained 30 % of the variance, adjusted  $R^2 = .29$ ,  $F_{(2,173)} = 36.28$ ,  $p < .001$ , Figure A1). Compared to the samples taken out of the baseline pool directly, aliquots onto Salivettes,  $b = -0.58$ ,  $t_{(173)} = -3.70$ ,  $p < .001$ , as well as aliquots onto Eppendorf vials,  $b = -1.32$ ,  $t_{(173)} = -8.49$ ,  $p < .001$  showed smaller sAA activity.

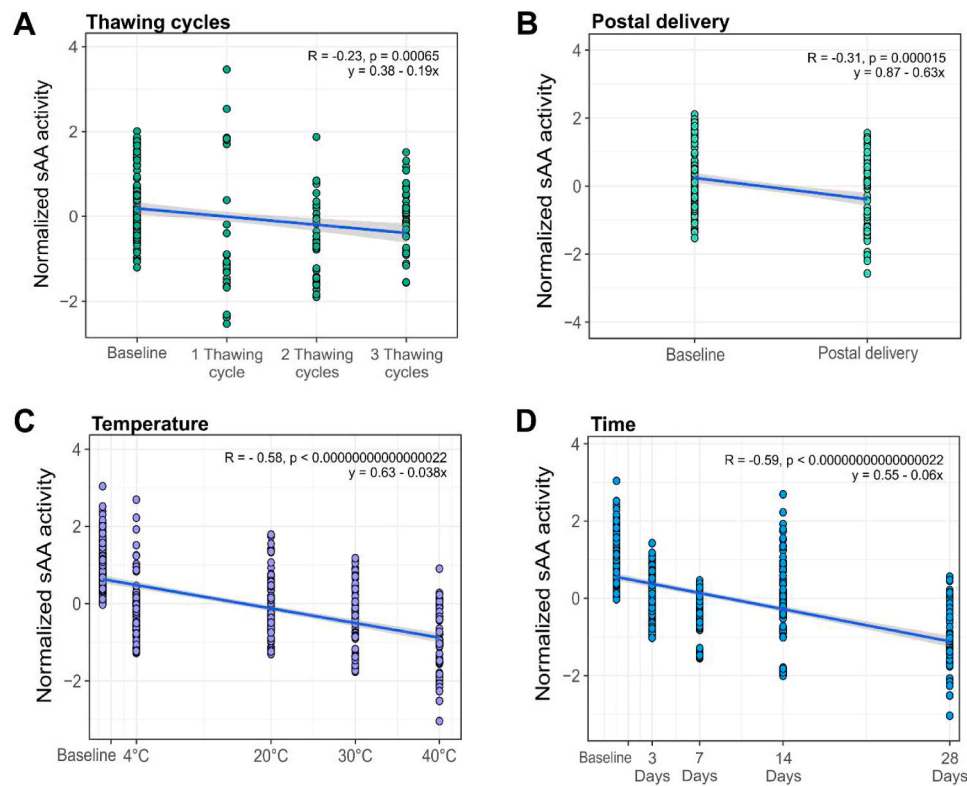
### 3.2. Thawing cycles

A two-way ANOVA revealed a significant main effect of vial (Salivettes vs. Eppendorf vials),  $F_{(1,213)} = 108.90$ ,  $p < .001$ ,  $\eta_p^2 = .32$ , as well as a significant main effect of thawing cycle (i.e., number of thawing cycles),  $F_{(3,213)} = 14.39$ ,  $p < .001$ ,  $\eta_p^2 = .17$ . The interaction of the factors vial and thawing cycle was also significant,  $F_{(3,213)} = 17.01$ ,  $p < .001$ ,  $\eta_p^2 = .19$ . A subsequent linear regression revealed the number of thawing cycles to explain 12 % of the variance, adjusted  $R^2 = .11$ ,  $F_{(3,217)} = 10.30$ ,  $p < .001$ . In comparison to the baseline condition, sAA activity was significantly lower after one thawing cycle,  $b = -0.78$ ,  $t_{(217)} = -3.87$ ,  $p < .001$ , and two thawing cycles,  $b = -0.82$ ,  $t_{(217)} = -4.72$ ,  $p < .001$ , but missed statistical significance for three thawing cycles,  $b = -0.34$ ,  $t_{(217)} = -1.94$ ,  $p = .054$ .

### 3.3. Exposure by time interval

A three-way ANOVA revealed a significant main effect of vial (Salivettes vs. Eppendorf vials),  $F_{(1,388)} = 36.88$ ,  $p < .001$ ,  $\eta_p^2 = .07$ , a significant main effect of temperature,  $F_{(4,388)} = 315.03$ ,  $p < .001$ ,  $\eta_p^2 = .39$ , as well as a significant main effect of time,  $F_{(3,388)} = 138.59$ ,  $p < .001$ ,  $\eta_p^2 = .51$ . The interaction effect of the factors vial and temperature was also significant,  $F_{(4,388)} = 109.51$ ,  $p < .001$ ,  $\eta_p^2 = .52$ . Likewise, the interaction effect of the factors vial and time,  $F_{(3,388)} = 5.26$ ,  $p < .001$ ,  $\eta_p^2 = .03$ , and the interaction effect of the factors temperature and time,  $F_{(9,388)} = 32.40$ ,  $p < .001$ ,  $\eta_p^2 = .43$  was significant. In addition, the three-way interaction between the factors of vial, temperature and time was significant,  $F_{(9,388)} = 3.22$ ,  $p < .001$ ,  $\eta_p^2 = .07$ . Consecutively, we performed separate linear regressions for the factors of temperature and time. Temperature explained 44 % of the variance, adjusted  $R^2 = .43$ ,  $F_{(4,417)} = 81.8$ ,  $p < .001$ . Compared to the baseline condition, sAA activity seemed to decrease with increasing temperature. This decay in sAA was significant for temperatures of  $4^{\circ}\text{C}$ ,  $b = -1.08$ ,  $t_{(417)} = -9.81$ ,  $p < .001$ ,  $20^{\circ}\text{C}$ ,  $b = -1.01$ ,  $t_{(417)} = -9.14$ ,  $p < .001$ ,  $30^{\circ}\text{C}$ ,  $b = -1.33$ ,  $t_{(417)} = -12.09$ ,  $p < .001$ , and  $40^{\circ}\text{C}$ ,  $b = -1.91$ ,  $t_{(417)} = -16.96$ ,  $p < .001$ , respectively.

Time explained 50 % of variance, adjusted  $R^2 = .49$ ,  $F_{(4,417)} = 103.00$ ,  $p < .001$ . Compared to the baseline condition, sAA activity seemed to decrease with an increase in exposure time. This decay in sAA activity was significant for exposure times of 3 days,  $b = -0.98$ ,  $t_{(417)} = -9.47$ ,  $p < .001$ , 7 days,  $b = -1.35$ ,  $t_{(417)} = -12.92$ ,  $p < .001$ , 14



**Fig. 1.** Plot of linear regression models for different experimental conditions. Note. The figure shows plotted linear regression models for the different experimental conditions. Panel A illustrates the linear regression model for the thawing cycles. Panel B shows the linear regression model for the postal delivery condition. Panel C and D illustrate linear regression models for the exposure by time condition. Specifically, panel C shows the linear regression for exposure by time condition with “temperature” as a predictor. For panel D, we modeled exposure by time condition with “time” as predictor. Importantly, all regressions are modeled across the factor of vial so that Salivettes and Eppendorf vials are combined. Data on sAA activity are transformed. The confidence band refers to a 91.7 % interval, as given by the upper border of our inter-assay variability.

days,  $b = -0.91$ ,  $t_{(417)} = -8.68$ ,  $p < .001$ , and 28 days,  $b = -2.04$ ,  $t_{(417)} = -19.44$ ,  $p < .001$ , respectively.

### 3.4. Postal delivery

A one-way ANOVA revealed a significant main effect of vial (Salivettes vs. Eppendorf vials),  $F_{(1,188)} = 138.86$ ,  $p < .001$ ,  $\eta_p^2 = .43$ , as well as a significant main effect of condition (baseline vs. postal delivery),  $F_{(1,188)} = 40.60$ ,  $p < .001$ ,  $\eta_p^2 = .18$ . The interaction effect of the factors vial and condition was also significant,  $F_{(1,188)} = 23.64$ ,  $p < .001$ ,  $\eta_p^2 = .11$ . A subsequent linear regression confirmed that sending samples via postal delivery explained 9 % of the variance,  $R^2 = .09$ ,  $F_{(1,190)} = 19.69$ ,  $p < .001$ . Indeed, samples having undergone postal delivery contained less sAA activity than baseline samples,  $b = -0.63$ ,  $t_{(190)} = -4.44$ ,  $p < .001$ . Notably, we ran separate linear regressions for the different types of vials separately in the thawing cycle condition, the exposure by time interval condition, and the postal delivery condition. Results of these linear regression models for each vial separately can be found in the Appendix A3.5. However, we omit a detailed discussion of the results at this point.

## 4. Discussion

This study investigated the reliability of sAA measurement in samples exposed to external conditions that are of relevance when collecting, storing, or transporting saliva outside the laboratory. In particular, we exposed samples obtained from an initial baseline saliva pool of homogenous sAA activity to up to three thawing cycles, different temperatures for varying time intervals, and to a real postal delivery in different types of vials (Salivettes and Eppendorf vials).

To account for the factor of vial, we compared measurements directly taken from the baseline saliva pool to measurements taken from aliquots of the same pool stored in Salivettes or Eppendorf vials. Contrary to our hypothesis, measurements of sAA activity were lower when taken out of Salivettes and Eppendorf vials compared to portions that were analyzed directly out of the baseline pool. Several factors are under debate to explain this effect. Absorbent materials are discussed to either include substances that may interfere with laboratory assays, or to help filtering out substances that would otherwise cause such interference (Shirtcliff et al., 2001). However, and importantly, this may only concern cotton-based materials. Nowadays, Salivettes, that were also used in the current study, are made of synthetic fiber. Alternatively, absorbent materials may provide an increased surface that contributes to increased evaporation of saliva thereby affecting salivary agents. Similarly, molding of and bacterial growth on the absorbent material could affect salivary biomarkers (DeCaro, 2008; Nater and Rohleder, 2009). This may still apply to Salivettes nowadays. However, the latter factors may only come into play considering storage at higher temperatures or longer time intervals. This is why we systematically exposed Salivettes and Eppendorf vials to different experimental conditions. Still, this is also why we hypothesized no effect of vial in the baseline condition where sAA activity was analyzed very soon after aliquoting saliva onto Salivettes and Eppendorf vials. However, it seems worth mentioning that Salivettes and Eppendorf vials were frozen (and thawed) before analysis. Freezing of samples represents an established routine prior to laboratory analysis and has been suggested to destroy mucopolysaccharides that may disturb pipetting processes (Worthman et al., 1990), for instance. Therefore, it is recommended or even required by analysis manuals. Of special note, the manual covering the assay used for sAA determination in the current study (TECAN, IBL International)

explicitly required freezing of samples prior to analysis. Indeed, this freezing step was omitted for the baseline pool. Hence, we cannot rule out that results concerning Salivettes and Eppendorf vials in the baseline condition as well as all other samples of further conditions are confounded by the factor of freezing and thawing. Likewise, in the current experimental design, we may not have captured the entire debate surrounding the factor of vial. That is, because we approached the matter solely concerning storage of saliva as we pipetted saliva onto the different types of vials. In applied research, however, different types of vials also imply differences in the method of actual saliva collection (i. e., absorption or drooling). This is of relevance since fixed saturation thresholds of absorbent materials have been suspected to induce measurement errors (Beltzer et al., 2010). Last but not least, Salivettes and passive drooling techniques differ in their applicability. Salivettes provide easier handling, also for populations such as children and elderly (Shirtcliff et al., 2001). To conclude, it seems crucial for researchers to make an informed choice on the vial used for collection and storage of saliva.

In a second experiment, we exposed samples in both types of vials to up to three cycles of freezing and thawing. Contrary to our hypothesis, results indicate that sAA activity seems to decrease with an increasing number of thawing cycles in both types of vials. This finding contradicts previous research, which suggests sAA activity to be robust against multiple cycles of freezing and thawing (Granger et al., 2007; O'Donnell et al., 2009). Of note, our study used a larger sample size which was based on an a priori power analysis. However, it seems crucial to note that, especially for the Salivettes exposed to one thawing cycle, we were forced to exclude  $N = 12$  out of 20 samples due to technical difficulties in the laboratory analysis. Therefore, data of this condition might be considered with some caution.

In a third experiment, we exposed Salivettes and Eppendorf vials to 4 °C, 20 °C, 30 °C, and 40 °C for time intervals of 3, 7, 14, and 28 days. For this experimental condition, we could confirm our hypothesis that sAA activity may not be as stable as previously reported (DeCaro, 2008), but decreases with increasing temperature and exposure time. These changes in sAA activity may be either due to a decay or a denaturation of sAA, or rather due to measurement inaccuracy caused by other micro-organisms such as bacteria or fungi reacting under higher temperatures. Concerning stress research in remote settings, these results might be taken seriously as they portray the risk of invalid data originating from non-frozen saliva samples. While cortisol seems to withstand thermic conditions at least to some extent (Aardal and Holm, 1995; Garde and Hansen, 2005), researchers should not assume that a similar stability also exists for sAA.

Lastly, in a fourth experiment, we subjected Salivettes and Eppendorf vials to a real postal delivery. This experimental condition can be considered relevant as mailing of samples has been applied in a number of existing studies. In line with our hypothesis for this experiment, we found a change in sAA activity in samples sent via postal delivery: sAA activity was lower in mailed samples when compared to the baseline condition. However, due to our limited experimental control, we can only speculate about the factors that might have caused changes in sAA activity. To some extent, the postal delivery condition replicates the results reported for exposure to higher temperatures across different time intervals. Indeed, during mailing, samples probably underwent higher temperatures. Beyond the factor of temperature, we cannot exclude that mailed samples were exposed to other strains such as screening, x-raying of parcels, or vibration/shocks.

It seems important to also discuss findings presented above from the perspective of applied stress research. That is, changes in sAA should be evaluated concerning their physiological relevance by looking at proportional changes in the different conditions relative to the baseline samples. For the thawing cycles, sAA activity changed most for Salivettes after one thawing cycle (increase by 9.50 %), while the lowest percentage of change was found in Eppendorf vials after three thawing cycles (decrease by 2.70 %). Concerning exposure by time interval, sAA

activity changed most when stored in Salivettes at 40 °C for 28 days (decrease by 99.10 %), and least when exposed to 30 °C for 3 days in Eppendorf vials (increase by 0.38 %). Lastly, sAA activity after postal delivery decreased less when stored in Salivettes (decrease by 1.67 %) compared to Eppendorf vials (decrease by 7.17 %). To set these numbers into context, studies including experimental stress exposure showed sAA activity to vary somewhere in a window between 100 u/ml (baseline or control condition) and 500 u/ml (peak after stress exposure; e.g., Nater et al., 2006; Nater et al., 2005; Petrakova et al., 2015; Thoma et al., 2012). As a result, changes found in our study may have a physiological relevance, or not. Likewise, the decay in sAA activity as caused by investigated external variables may statistically affect the investigation of real experimental manipulation and its impact on sAA activity, or not. Eventually, we cannot provide a clear and universal interpretation concerning the relevance of our findings. In contrast, decisions on whether to tolerate reported changes in sAA activity for the sake of ecologically valid data, or whether to prefer the one option that was shown to cause more or less alterations in sAA activity, do ultimately depend on individual research questions.

This study comes with several methodological limitations. Variance homogeneity was not consistently given due to uneven sample sizes in our different experimental conditions. Still, we attempted to overcome this issue by means of data transformation. Additionally, despite our efforts to establish a baseline saliva pool of homogenous sAA activity at the onset, it is possible that the samples drawn from this pool did not contain equal amounts of sAA activity prior to exposure to the various conditions. Likewise, the absolute sAA activity of the initial baseline saliva pool might play a role with respect to observable effects. For instance, certain effects may only become apparent with a certain higher or lower absolute dosage of sAA activity as sAA may respond nonlinearly to different external conditions. A potential shortcoming further addressed the fact that we refraining from sharp exclusion criteria with respect to our participants. However, with that we aimed to further increase ecological validity of our study. Indeed, future research may examine in how far medication, hormonal contraception, or smoking may cause alterations in saliva composition, thereby affecting the stability and durability of sAA (Nater et al., 2007). Last but not least, an aspect that partly limits ecological validity of our findings concerns the fact that we cleaned saliva from food and cell particles via centrifugation prior to exposure to the different conditions. Indeed, centrifugation or other cleaning steps are not applicable collecting and storing saliva in real field settings. However, with our procedure, we wanted to ensure that our laboratory analysis yields valid results. Moreover, our study was rather thought to shed light onto how micro-organisms such as bacteria and fungi (which were not eliminated by the applied procedure) may affect sAA under the different conditions.

## 5. Conclusion

In conclusion, our study indicates that in order to obtain reliable and valid data for sAA stored or transported outside the laboratory, several external variables need to be considered. An increasing number of thawing cycles, higher temperatures, and longer exposure intervals generally lead to a decrease in sAA activity. In addition, sending samples via postal delivery may lead to a decrease in sAA activity depending on the specific external conditions that accompany the delivery process. Lastly, the storage vial needs to be cautiously selected when designing future studies, as the type of vial might interact with specific storage conditions. sAA as analyzed out of samples that were not stored according to common recommendations should be interpreted with some caution. Likewise, looking at relative changes in sAA, the different experimental conditions rendered more or less deviation in comparison to baseline samples. In total, changes in sAA activity ranged from a minimum of 0.38 % to a maximum of 99.1 %. Therefore, future studies might further explore the exact limits to which we might tolerate biases in salivary data at the benefit of ecologically valid research. That is,

stress research in remote and field settings is crucial for progressing our knowledge and investigating relations of interest in naturalistic contexts. Moreover, not assessing salivary data in remote and field studies cannot be regarded an optimal solution. Whenever it is not possible to handle saliva samples according to the common recommendations, we rather suggest researchers to opt for such procedures that affect sAA measurement the least. Ideally, researchers should use several different stress outcome parameters to cross-validate stress effects and should be transparent in describing their handling of the challenges covered by the current study.

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## CRedit authorship contribution statement

**Katrin Heyers:** Conceptualization, Formal analysis, Writing – original draft, Project administration. **Lena Sophie Pfeifer:** Conceptualization, Formal analysis, Writing – original draft, Project administration. **Kim Walusiacki:** Formal analysis. **Petunia Reinke:** Conceptualization, Writing – review and editing. **Dirk Moser:** Conceptualization, Formal analysis, Writing – review and editing. **Sebastian Ocklenburg:** Conceptualization, Writing – review and editing, Supervision. **Oliver T. Wolf:** Conceptualization, Resources, Writing – review and editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

None.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.psyneuen.2023.106929](https://doi.org/10.1016/j.psyneuen.2023.106929).

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