ARTICLE
Measuring Salivary Alpha-Amylase in the Undergraduate Neuroscience Laboratory

Maria S. Bañuelos, Aya Musleh, & Lisa E. Olson
Department of Biology, University of Redlands, Redlands, CA 92373.

Undergraduate courses in biopsychology, neuroscience, and physiology often include laboratory exercises that examine responses to stimulation of the sympathetic nervous system with measurements of heart rate, blood pressure, or galvanic skin levels (sweat response). A newer bioindicator of the sympathetic nervous system is salivary alpha-amylase (sAA) measured with a colorimetic enzyme assay. Undergraduate students successfully measured a rise in sAA due to the stress of giving a class presentation (n=13). Students were enthusiastic to measure a physiological response to a real-life anxiety-producing situation. We describe potential difficulties in the assay and our adaptations to the manufacturer's protocol to make it more feasible in the undergraduate setting.

Key words: salivary alpha-amylase (sAA); sympathetic nervous system (SNS); autonomic nervous system (ANS); stress

The physiological response to stressful stimuli is determined by the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic ("fight or flight") nervous system. The HPA response is a hormone cascade ultimately leading to the release of cortisol in the bloodstream. This is characterized by a time delay (~20 minutes) and longer-lasting effects than the sympathetic response. In contrast to cortisol release, the nerve-mediated sympathetic response is immediate and can be attenuated more quickly. Sympathetic responses such as increased heart rate and blood pressure, pupil dilation, and release of glucose from the liver are well-established (Bear et al., 2007). A more recently recognized indicator of sympathetic activity is the release of the enzyme alpha-amylase in saliva.

Salivary alpha-amylase (sAA), a digestive enzyme produced and excreted from norepinephrine-responsive salivary gland cells, is a marker of psychophysiological stress (Granger et al., 2007; Nater and Rohleder, 2009). Arousal of the sympathetic nervous system via the beta-adrenergic agonist isoprenaline causes an increase in the secretion of sAA (Speirs et al., 1974), and sympathetic inhibition with propanol decreases secretion (van Stegeren et al., 2006). The function of sAA is to digest carbohydrates by hydrolyzing glycosidic bonds between sugars (Granger et al., 2007). It is uncertain how or if this is adaptive to organisms under stress, although it is possible that it results in a short-term availability of energy; it is also possible that sAA's interaction with bacteria in the oral cavity may be important (Nater and Rohleder, 2009).

The incorporation of sAA experiments into undergraduate neuroscience courses can complement other non-invasive measures of the sympathetic nervous system such as heart rate and blood pressure (iWorx Systems, 2013), galvanic skin levels (sweat response; Colgan, 2012), or the minimally invasive measurement of blood glucose (Flint, 2004). sAA differs from these bioindicators in that it is not monitored in real time. Including sAA analysis in a course adds the opportunity to learn the basic methodology of colorimetric enzyme assays, a widely applicable method.

sAA and cortisol can be assayed from the same saliva sample, if comparing the sympathetic nervous system and HPA axis is of interest (Kalman and Grahn, 2004) and if serial samples are feasible, since sAA rises more quickly than cortisol (Takai et al., 2004). Measuring sAA may be more practical in an undergraduate laboratory setting than measuring cortisol, since sAA seems to be influenced less by sex, menstrual cycle, and oral contraceptives (Kudielka et al., 2009; Rohleder and Nater, 2009).

In this study, we present a laboratory exercise measuring the rise in sAA due to the stress of giving a class presentation. The learning objectives for this exercise included: 1) Students will be able to articulate differences between the sympathetic nervous system and hypothalamic-pituitary-system response to stress, 2) Students will be able to describe how a kinetic colorimetric assay can quantitate enzyme levels, and 3) Students will be able to statistically analyze data with a paired t-test.

MATERIALS AND METHODS

Participants

All experiments were approved by the University of Redlands Institutional Review Board, and participants provided informed consent. Participants (n = 3 male, 10 female) were recruited from an undergraduate Introduction to Neuroscience course and received no incentive for voluntary participation. As part of the normal course requirements, students gave a 20-minute presentation on a research topic. On the day of the presentation, participants gave informed consent, rinsed out their mouths with water, took the state portion of the State-Trait Anxiety Inventory (Spielberger et al., 1970; Barnes et al., 2002), and provided a saliva sample via passive drool into a saliva collection aid (Salimetrics, State College, PA) prior to beginning their speeches. Additionally, participants took the same measure and provided a saliva sample on a

different day when they were only audience members for classmates' presentations (called a "nonpresentation day"). Data collection for both time points occurred at 9:30 AM. Participants were between the ages of 18 and 24 years, proficient in English, did not have any severe mental health issues that could compromise their ability to complete the assessment, were not pregnant, and did not use anxiolytic medication or nicotine. Participants were asked not to drink any alcohol in the 24 hours prior to the experiment, and not to eat, drink, exercise, or brush their teeth in the hour prior to testing. Participants were also instructed to drink caffeine according to their normal pattern of caffeine intake.

Due to the small class size and the potential for information associated with data collection (i.e., date of presentation) to compromise privacy coding, we chose to have students from a separate undergraduate course (Research Topics in Biology) carry out the enzyme assays. Given that some students may have anxiety disorders, we were particularly careful to protect anonymity. However, this would not be necessary with all research designs. This cross-course project was also a helpful collaboration because the Introduction to Neuroscience course was comprised of non-biology majors who had no experience using micropipetmen, and pipetting error was determined to be an important cause of variability (see Discussion). However, most biopsychology or neuroscience courses including molecular techniques would be appropriate settings for sAA assays and students could analyze their own (or coded) samples.

sAA analysis

All saliva samples were stored at -20°C immediately after collection and later analyzed using the sAA kinetic enzyme assay kit (Salimetrics, State College, PA). The kit provides an optically clear 96 well plate for use in a spectrophotometric plate reader, high and low amylase controls, a phosphate buffer diluent for saliva samples, and a chromagenic substrate (2-chloro-p-nitrophenol linked with maltotriose). sAA catalyzes the conversion of this substrate to yellow-colored 2-chloro-p-nitrophenol, which absorbs 405 nm light. sAA enzyme activity is proportional to the increase in absorbance over time.

On the day samples were diluted, we thawed the saliva at room temperature, briefly vortexed tubes, and then centrifuged at 1500 x g for 15 minutes. An overall 1:200 dilution was achieved in two serial steps: first, 10 µl saliva was added to 90 µl diluent (1:10 dilution), followed by 10 µl of this initial dilution added to 190 µl of diluent (1:20 dilution, making a 1:200 dilution overall). These diluted samples, as well as the remaining undiluted saliva, were stored at -20°C.

On the day of the assay, an introductory lecture on the topic of kinetic enzyme assays was provided while the substrate was warmed to 37°C in a waterbath. Topics included the shape expected for a graph of product of the reaction vs. reaction time, overlying curves expected for samples with more or less enzyme present, and how the slope of the curve could be used to estimate enzyme activity. We discussed which timepoint on the curve would be best for this estimate (i.e., prior to leveling off due to all the substrate being converted to product), and thus why timing is important in this assay.

To prepare the assay, 8 µl of each 1:200 diluted saliva sample was placed in one of eight wells in a strip. An eight channel pipetman was used to simultaneously add 320 µl of substrate into each well of the strip, and this marked the time of the start of the reaction.

The AgileReader™ ELISA Plate Reader (ACTgene, Inc., Piscataway, NJ) was used for the kinetic sAA assay. This plate reader has a heater and therefore could be warmed to remain at 37ºC internally during the assay, and also has a shaking setting to mix reagents thoroughly as recommended by the Salimetrics protocol. In our laboratory, we have also used a different plate reader without these features, placing the entire plate reader inside a large shaking incubator. Results were similar. Absorbance was measured at 405 nm at 1 min and then again at 3 min after substrate addition. The difference between these absorbances was calculated and multiplied by 328 to yield enzyme activity in U/mL, based on the formula provided by the manufacturer which accounts for the light path of the provided wells, millimolar absorptivity of 2-chloro-p-nitrophenol, and dilutions. The average coefficient of variation on inter-assay replicates of non-log-transformed values after the removal of outliers (see Discussion) was 12.4%.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistical software (International Business Machines Corporation, Armonk, NY) with an alpha of 0.05. sAA levels are often positively skewed (Rohleder and Nater, 2009), which led to a useful discussion of the assumptions of statistical tests, and the methods used when those assumptions are violated. Specifically, we applied a log10 transformation to the skewed data which then yielded a dataset with a normal distribution. This allowed us to proceed with a paired t-test on the transformed data. sAA levels and state anxiety on the presentation day versus non-presentation day were tested using separate paired t-tests. Correlations between sAA and state anxiety were tested using Pearson's product-moment correlation.

RESULTS

We tested participants on a day they were scheduled to give a class presentation and a day they were simply a member of the audience. sAA levels were significantly higher on the presentation day, $F(1, 11) = 6.051$, $p < 0.05$, as was state anxiety, $F(1, 10) = 59.084$, $p < 0.001$ (Figure 1). The levels of sAA were not correlated to state anxiety scores on either day, nor was the change in sAA correlated to the change in state anxiety (p's > 0.05), but the power to detect a correlation was limited in the small sample size ($n = 13$).

DISCUSSION

We were able to successfully measure sAA and validate its rise due to the stress of an academic presentation even

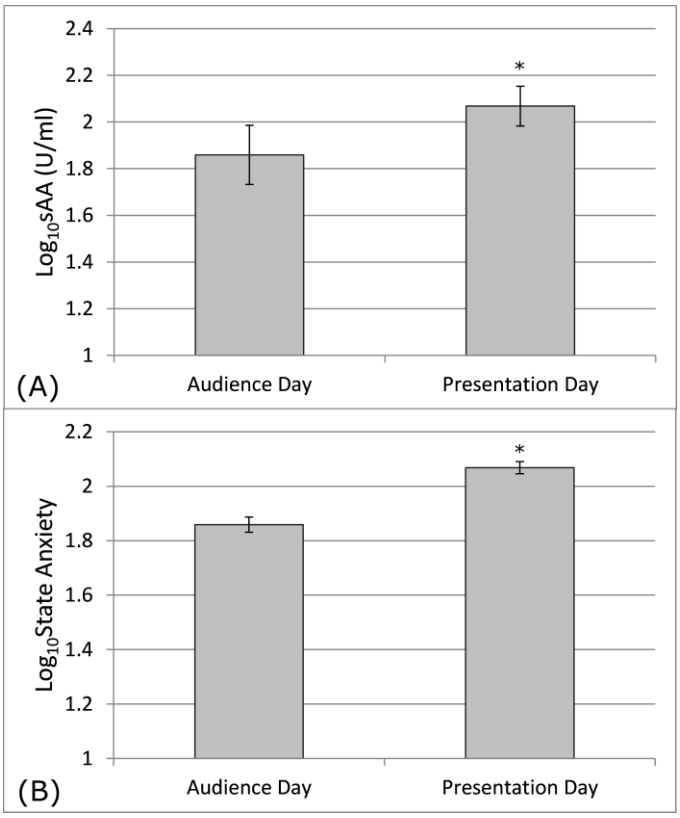

Figure 1. sAA level as a marker of stress due to giving a class presentation. sAA level *(A)* and state anxiety *(B)* increased on the day students gave presentations compared to a day in the audience. Data were log-transformed due to violations of normality. *$p < 0.05$, $n = 13$

with a small sample size (n = 13). sAA could be used as a dependent variable for a variety of student-designed experiments. For example, sAA has been shown to rise during acute laboratory stressors, such as the Trier Social Stress Test (Rohleder et al., 2004), a math task (Noto et al., 2005), watching a stressful video (Takai et al., 2004), experiencing physical stressors such as exercise and temperature extremes (Chatterton et al., 1996), and real-world settings such taking an academic examination (Bosch et al., 1996; Chatterton et al., 1996; Kang, 2010). Serial timed samples could also be used to demonstrate the diurnal variation of sAA (Nater et al., 2007; O'Donnell et al., 2009).

Measurements of sAA could also complement data collection for other indicators of the stress response, with a discussion of how well or if they correlate. The detection of a correlation between sAA levels and plasma catecholamines has varied depending on study design (reviewed in Nater and Rohleder, 2009), with possibly a stronger correlation to norepinephrine than to epinephrine (Thoma et al., 2012). Although blood pressure and sAA both rise due to a variety of stimuli, individual's responses are not necessarily correlated (Nagy et al., 2015), nor are sAA and heart rate variability measures (Kobayashi et al., 2012). How each of these different aspects of autonomic activity relate to each other is an area of active investigation. The symmetry or asymmetry of sAA and cortisol responses has been investigated as its own indicator of psychological health (Gordis et al., 2008; Ali and Pruessner, 2012).

Although we did not collect quantitative data on the students' attitudes toward this project, student comments were indicative of engagement and learning. Some of these include: "I was actually really excited to learn how to do the amylase assays," "Measuring the stress response in saliva was a great way to learn about physiological responses," and more generally "I liked the hands-on aspects of this class," and "Labs were most important in helping me learn because we had to apply what we learned in class."

Methodological Recommendations for Undergraduate Laboratories

Particular care should be taken to protect the privacy of subjects when handling psychological questionnaire data. One student expressed concern that she would be embarrassed by scoring highly on the state anxiety measure, and our coding system included sample collection dates on tubes which could be used to disclose the participant's identity since students made presentations on different dates. Our cross-course collaboration resolved this issue, but we recommend that faculty consider the appropriate anonymizing scheme for their courses. Students may not even want to know their own personal data, as discovering that they score higher or lower than their peers may be uncomfortable. This can be addressed by having students initially label tubes, a faculty member conduct recoding and cover tubes with new labels, and then randomly distribute tubes to the class for assays. Alternatively, data could be pooled and only presented to students as means and standard deviations.

The data here were derived from saliva samples via passive drool into a saliva collection aid attached to a 2 ml cryovial (Salimetrics, State College, PA). Our participants commented that repeatedly spitting into the collection aid over several minutes was unpleasant and messy. Additionally, we found the viscous mucins present in the samples to be a hindrance to later pipetting. In subsequent pilot experiments, we used the oral swabs from Salimetrics instead. These swabs were placed under the tongue and held for 1-2 minutes until saturated, then placed into a double-walled tube designed to separate the liquid saliva components. The oral swabs caused no mess for the participants and the mucins remained in the swab after centrifugation, making pipetting much easier. Thus, we recommend the oral swabs (~$1.75 per sample) over the saliva collection aids (~$2.10 per sample). It should be noted that students who handle saliva samples should wear personal protective equipment (gloves at minimum) and dispose of swabs as biohazardous waste after centrifugation.

All of our samples were frozen at -20°C (as recommended by Salimetrics) prior to the enzyme assays.

For other research designs, participants could collect and freeze their own samples at home and transport them to the lab on ice. Such care in storage may be unnecessary, however, as some investigators have found sAA to be stable at room temperature up to five days (O'Donnell et al., 2009). We have repeated testing for undiluted samples that have been frozen for 1-2 years without a significant loss of enzyme activity. Additionally, we have frozen the diluted samples (see below) for up to six months and the reassayed samples have similar values. This is a deviation from the Salimetrics protocol, which states that dilutions should be conducted on the same day as analysis. We asked Salimetrics representatives if the dilutions could be frozen, and were told no, but the staff could not provide an explanation for why samples would not be stable. Thus, our adaptation of the protocol allows for more flexibility in terms of timing the various steps of the assay.

In our students' hands, we found that the initial centrifugation and dilution steps were the most time-consuming, and we needed to perform these steps on a different day than the enzyme assay due to time constraints of the 2-hour 50-minute class period. For samples collected with the saliva collection aid attached to a 2 ml cryovials (as suggested by the manufacturer), we belatedly realized that the 2 ml cryovials did not fit into a standard microcentrifuge, and samples had to be transferred to 1.7 ml microcentrifuge tubes prior to the spin. Alternatively, if oral swabs are used, the spin must be conducted in a variable speed clinical centrifuge which can hold 7 ml conical tubes (we used rotors designed for 15 ml conical tubes with success). If rotor positions in clinical centrifuges are limited, these spins may need to be conducted serially in 15-minute batches, extending the time required.

The dilution step was unexpectedly the source of a great amount of variability in our students' hands. The Salimetrics protocol suggests a 1:200 dilution in two serial steps: first, 10 µl saliva was added to 90 µl diluent (1:10 dilution), followed by 10 µl of this initial dilution added to 190 µl of diluent (1:20 dilution, making a 1:200 dilution overall). Despite instruction on pipetting technique and the importance of accuracy, it was observed that students had bubbles within or droplets on the outside of pipet tips too frequently. It may also be that our teaching lab pipetmen are not calibrated as accurately as those in a research-focused laboratory. Thus, we had students perform two completely separate dilutions from each original sample to demonstrate the importance of reproducibility. This meant each original sample required four tubes due to the serial nature of the dilution. For students unfamiliar with the concept of serial dilution, it took a nontrivial amount of time to ensure tubes were being labeled appropriately and the correct dilutions occurred at each step. Thus, we chose to freeze the samples at that point and conduct the actual enzyme assay a different day (another 2-hour 50-minute class period).

On the day of the enzyme assay, the 96 well plate from the Salimetrics sAA kit was divided into strips of eight wells. It can be divided into groups of even fewer wells if desired, but with fewer wells, it is easy to lose orientation as there is not a way to mark individual wells. A strip of eight retains an asymmetric tab which can be used to orient the wells. We used a written, blank grid in the pattern of a 96 well plate for students to record which sample went in each well/strip. Analyzing one strip at a time in the plate reader was most feasible, since the assay is time-dependent and the coordination of multiple strips is difficult. A multichannel pipetman and reagent reservoir were necessary to add the substrate to the eight wells in a strip simultaneously. It could be possible to conduct the assay without a multichannel pipetman, but more replicates should be tested to determine variability due to timing. The Salimetrics protocol suggests reverse pipetting to avoid the introduction of bubbles, but our digital multichannel pipetman did not have this feature. Our solution was to pop the bubbles in each well with a needle prior to placing the strip in the plate reader. We used the same needle throughout the strip and wiped it on a laboratory tissue in between samples. We assumed that the cross-contamination of enzyme activity between samples would be negligible given the volume potentially present on the needle after wiping, and that the speed gained by not changing needles was advantageous due to the time-sensitive assay. Students should be instructed to place a laboratory tissue on the bench for the wiping, rather than hold the tissue in their hands. This prevents accidental needle sticks during the wiping process.

Salimetrics provides "high" and "low" amylase standards to include in each analysis to confirm that values fall in the recommended range. These were stored at 4°C as instructed. These controls usually have the earliest expiration date of all the components of the kit, and we found that the controls yielded lower than expected values when they had been stored for more than a few months. Since undergraduate laboratories often need to minimize cost and maximize the lifetime of reagents, it may be advantageous to freeze some aliquots of the standards given our positive experience with freezing diluted samples.

We found that duplicate assays from the same dilution of a sample had high reproducibility, but separate dilutions often diverged in values (coefficient of variation up to 50%). These are all technical replicates of the same biological sample, but they test reproducibility of different steps of the protocol. Given that Salimetrics does not include a recommendation for duplicate *dilutions* (just duplicate assays of the same dilution), we think this is something faculty should keep in mind when incorporating sAA assays into undergraduate laboratories. It was an excellent lesson for our students in the importance of pipetting accuracy. We decided that any two dilutions of a sample with a coefficient of variation greater than 30% meant that the sample needed to be rediluted and assayed again, and outliers removed. However, this did increase the total cost of the experiment. The current cost for the assay is approximately $2-3/well when purchased as a kit

from Salimetrics, although it is possible that reagents could be purchased separately from a different vendor at lower cost.

In summary, sAA can be used for a variety of laboratory exercises regarding the stress response in biopsychology, neuroscience, or physiology courses. We hope that our observations of potential difficulties and adaptations to the manufacturer's protocol aid in the adoption of this technique in undergraduate settings.

REFERENCES

Ali N, Pruessner JC (2012) The salivary alpha amylase over cortisol ratio as a marker to assess dysregulations of the stress systems. Physiol Behav 106:65-72

Barnes LLB, Harp D, Jung WS (2002) Reliability generalization of scores on the Spielberger State-Trait Anxiety Inventory. Educ Psychol Meas 62:603-618.

Bear MF, Connors BW, Paradiso MA (2007) Neuroscience: exploring the brain. Baltimore, MD: Lippincott Williams & Wilkins.

Bosch JA, Brand HS, Ligtenberg TJ, Bermond B, Hoogstraten J, Nieuw Amerongen AV (1996) Psychological stress as a determinant of protein levels and salivary-induced aggregation of Streptococcus gordonii in human whole saliva. Psychosom Med 58:374-382.

Chatterton RT Jr, Vogelsong KM, Lu YC, Ellman AB, Hudgens GA (1996) Salivary alpha-amylase as a measure of endogenous adrenergic activity. Clin Physiol 16:433-448.

Colgan W (2012) Classic clinical technique adapted to demonstrate autonomic nervous system physiology in an undergraduate laboratory course. J Undergrad Neurosci Educ 11:A158-A160.

Flint RW (2004) Emotional arousal, blood glucose levels, and memory modulation: three laboratory exercises in cognitive neuroscience. J Undergrad Neurosci Educ 3:A16-A24.

Gordis EB, Granger DA, Susman EJ, Trickett PK (2008) Salivary alpha amylase–cortisol asymmetry in maltreated youth. Horm Behav 53:96-103.

Granger DA, Kivlighan KT, el-Sheik M, Gordis EB, Stroud LR (2007) Salivary alpha-amylase in biobehavioral research: recent developments and applications. Ann N Y Acad Sci 1098:122-144.

iWorx Systems (2013) Human circulation experiment 02: Blood pressure, peripheral circulation, and imposed conditions. Dover, NH: iWorx Systems, Inc. http://www.iworx.com/documents/LabExercises/BloodPressure-ImposedConditions.pdf

Kalman BA, Grahn RE (2004) Measuring salivary cortisol in the behavioral neuroscience laboratory. J Undergrad Neurosci Educ 2:A41-A49.

Kang Y (2010) Psychological stress-induced changes in salivary alpha-amylase and adrenergic activity. Nurs Health Sci 12:477-484.

Kobayashi H, Park B-J, Miyazaki Y (2012) Normative references of heart rate variability and salivary alpha-amylase in a healthy young male population. J Physiol Anthropol 31:9.

Kudielka BM, Hellhammer DH, Wust S (2009) Why do we respond so differently? Reviewing determinants of human salivary cortisol responses to challenge. Psychoneuroendocrinology 34:2-18.

Nagy T, van Lién R, Willemsen G, Proctor G, Efting M, Fülöp M, Bárdos G, Veerman EC, Bosch JA (2015) A fluid response: alpha-amylase reactions to acute laboratory stress are related to sample timing and saliva flow rate. Biol Psychol 109:111-119.

Nater UM, Rohleder N (2009) Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. Psychoneuroendocrinology 34:486-496.

Nater UM, Rohleder N, Schlotz W, Ehlert U, Kirschbaum C (2007) Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology 32:392-401.

Noto Y, Sato T, Kudo M, Kurata K, Hirota K (2005) The relationship between salivary biomarkers and State-Trait Anxiety Inventory score under mental arithmetic stress: a pilot study. Anesth Analg 101:1873-1876.

O'Donnell K, Kammerer M, O'Reilly R, Taylor A, Glover V (2009) Salivary alpha-amylase stability, diurnal profile and lack of response to the cold hand test in young women. Stress 12:549-554.

Rohleder N, Nater UM (2009) Determinants of salivary alpha-amylase in humans and methodological considerations. Psychoneuroendocrinology 34:469-485.

Rohleder N, Nater UM, Wolf JM, Ehlert U, Kirschbaum C (2004) Psychosocial stress-induced activation of salivary alpha-amylase: an indicator of sympathetic activity? Ann N Y Acad Sci 1032:258-263.

Speirs R, Herring J, Cooper W, Hardy C, Hind C (1974) The influence of sympathetic activity and isoprenaline on the secretion of amylase from the human parotid gland. Arch Oral Biol 19:747-752.

Spielberger C, Gorsuch R, Lushene R (1970) Manual for the State-Trait Anxiety Inventory. Palo Alto, CA: Consulting Psychologists Press.

Takai N, Yamaguchi M, Aragaki T, Eto K, Uchihashi K, Nishikawa Y (2004) Effect of psychological stress on the salivary cortisol and amylase levels in healthy young adults. Arch Oral Biol 49:963-968.

Thoma MV, Kirschbaum C, Wolf JM, Rohleder N (2012) Acute stress responses in salivary alpha-amylase predict increases of plasma norepinephrine. Biol Psychol 91:342-348.

van Stegeren A, Rohleder N, Everaerd W, Wolf OT (2006) Salivary alpha amylase as marker for adrenergic activity during stress: effect of betablockade. Psychoneuroendocrinology 31:137-141.

Received June 08, 2017; revised July 18, 2017; accepted July 19, 2017.

Address correspondence to: Dr. Lisa E. Olson, Biology Department, 1200 E. Colton Ave., Redlands, CA 92373. Email: lisa_olson@redlands.edu

Maria Bañuelos and Aya Musleh are undergraduate coauthors of this manuscript.

Copyright © 2017 Faculty for Undergraduate Neuroscience
www.funjournal.org