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Cortisol and salivary alpha-amylase trajectories following a group social-evaluative stressor with adolescents

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Abstract

Intraindividual variability in stress responsivity and the interrelationship of multiple neuroendocrine systems make a multisystem analytic approach to examining the human stress response challenging. The present study makes use of an efficient social-evaluative stress paradigm –the Group Public Speaking Task for Adolescents (GPST-A) – to examine the hypothalamic-pituitary-adrenocortical (HPA)-axis and Autonomic Nervous System (ANS) reactivity profiles of 54 adolescents with salivary cortisol and salivary alpha-amylase (sAA). First, we account for individuals' time latency of hormone concentrations between individuals. Second, we use a two-piece multilevel growth curve model with landmark registration to examine the reactivity and recovery periods of the stress response separately. This analytic approach increases the models' sensitivity to detecting trajectory differences in the reactivity and recovery phases of the stress response and allows for interindividual variation in the timing of participants' peak response following a social-evaluative stressor.

The GPST-A evoked typical cortisol and sAA responses in both males and females. Males' cortisol concentrations were significantly higher than females' during each phase of the response. We found no gender difference in the sAA response. However, the rate of increase in sAA as well as overall sAA secretion across the study were associated with steeper rates of cortisol reactivity and recovery.

This study demonstrates a way to model the response trajectories of salivary biomarkers of the HPA-axis and ANS when taking a multisystem approach to neuroendocrine research that enables researchers to make conclusions about the reactivity and recovery phases of the HPA-axis and ANS responses. As the study of the human stress response progresses toward a multisystem analytic approach, it is critical that individual variability in peak latency be taken into

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Conflict of Interest Documentation

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consideration and that accurate modeling techniques capture individual variability in the stress response so that accurate conclusions can be made about separate phases of the response.

Keywords

Cortisol; salivary Alpha-amylase; Adolescence; GPST-A; Stress Reactivity

1. Introduction

The bulk of research on the human neuroendocrine response to acute stress has focused on the hypothalamic-pituitary-adrenocortical (HPA)-axis, examining cortisol reactivity trajectories in isolation leading to only a partial understanding of the *overall* response to stress, which involves the responses of many stress-sensitive biological systems in the body (Hellhammer, Wüst, & Kudielka, 2009). Studies that adopt a multisystem approach to examine the stress response often utilize statistical techniques that do not account for interindividual variability among people, providing an incomplete picture of stress response system function. Given the health implications of dysregulation of regulatory systems (McEwen, 1998; Sapolsky, 1998), a multisystem approach is warranted to better understand the internal hormonal milieu and long-term health outcomes.

1.1 Connections between the ANS & HPA

The HPA axis and the sympathetic branch of the ANS are the main systems that generate the physiological response to stress. The ANS produces an immediate response, initiating changes in the cardiovascular and respiratory systems. The HPA system works more slowly through a cascade of hormones culminating in the release of cortisol (Chrousos & Gold, 1992). While the two systems are both contributing to the stress response, the HPA-axis response has been associated with negative affect as well as threats that are uncontrollable and/or social-evaluative whereas the ANS measures are less valenced and more broadly related to arousal (Cacioppo, Berntson, Larsen, Poehlmann, & Ito, 2000; Dickerson & Kemeny, 2004).

The HPA-axis and ANS are interrelated at the neural level, which allows the systems to potentially influence one another (van Stergeren, Wolf, Everaerd, Scheltens, Barkhof, & Rombouts, 2007) and many researchers are calling for studies that examine the coordination of these systems (Bauer, Quas, & Boyce, 2002; Laurent, Powers, & Granger, 2013). Salivary measures of these systems provide a potential way of examining these systems together in an ecologically valid way with saliva, which is easily collected and non-invasive to participants. While cortisol is the main marker of the HPA-axis, salivary alpha amylase (sAA) is used as a proxy marker of autonomic activity (Granger et al., 2007; Nater & Rohleder, 2009).

1.2 Multisystem Approach

Research suggests there is substantial interindividual variability in baseline and peak, concentrations of cortisol and sAA following acute stress (Kudielka, Hellhammer & Wüst, 2009; Lopez-Duran, Kovacs, & George, 2009). Typical response profiles of cortisol and sAA appear asymmetric, resulting from the rapid release and recovery of sAA and relatively

slower cortisol response post-stressor. Although the HPA-axis and ANS act in synchrony following a stressor, research suggests they exert bidirectional effects on one another (Boyce & Ellis, 2005; Sapolsky, 1998). Mounting evidence suggests examining the response of both the HPA-axis and ANS will contribute to a more complete understanding of stress system function and dysfunction across individuals and, specifically, in adolescents (Granger, Fortunato, Beltzer, Virag, Birght, & Out, 2012).

The magnitude of the cortisol response to an acute social-evaluative stressor increases over the course of development, peaking in mid-adolescence (14–15 years-old) for boys and girls (Gunnar, Wewerka, Frenn, Long, & Griggs, 2009; Sumter, Bokhorst, Miers, Van Pelt, & Westenberg, 2010). Predominantly, developmental studies focused on cortisol trajectories so less is known about interindividual variability in sAA. However, Stroud and colleagues (2009) found greater sAA reactivity in adolescents than children, highlighting adolescence as an important developmental period for studying the stress response.

Some studies have examined ANS and HPA-axis synchrony using sAA and cortisol during social-evaluative stressors in adolescents. Gordis, Granger, Susman, and Trickett (2006) found asymmetry between sAA and cortisol release in adolescents using RMANOVAs — there was a rapid increase and recovery of sAA post-stressor whereas cortisol peaked later post-stressor, when sAA was almost recovered. Van den Bos and colleagues (2014) had similar results in a different sample of adolescents, finding increased cortisol and sAA concentrations post-stressor with sAA peaking and recovering much more quickly than cortisol. In both cases, cortisol and sAA were collected concurrently yet analyzed separately, limiting the ability to comment about cortisol and sAA reactivity profiles simultaneously.

Though research is mounting that suggests it is important to examine these two systems simultaneously, a variety of analytic approaches have been suggested. After substantial research in child behavior, health and development, Bauer and colleagues (2002) speculate that examining these systems separately may result in important relationships going undetected. In turn, they suggest additive or interactive analytic models. Vries-Bouw and colleagues (2012) used regression analyses to show cortisol and sAA reactivity had a combined impact on negative behavior in adolescents — low reactivity of both parameters was related to higher levels of disruptive behavior. Chen et al. (2015) argue that an interactive model is better than a simple additive model when examining the effects of cortisol and sAA secretion in behavioral outcomes. An interactive model assumes that two biomarkers work in coordination and can confirm differing relationships between one biomarker and a behavior depending on the pattern of release of the other biomarker. In their study utilizing this model, Chen et al. (2015) found that cortisol was negatively associated with externalizing problems only when sAA was at low levels. In turn, the effect of cortisol on behavioral outcomes was conditional on sAA concentrations, which would not have been obvious if cortisol and sAA had been analyzed separately. Together these studies support the hypothesis that the HPA-axis and ANS augment each other and predict biopsychosocial outcomes better when examined together than either system alone.

While research points to the interrelationship between the HPA-axis and ANS when cortisol and sAA reactivity profiles are examined, few studies have used more sophisticated analyses

that allow simultaneous modeling of sAA and cortisol trajectories or consider inter- and intraindividual differences in sAA or cortisol secretion. One exemplar study by Laurent, Powers and Granger (2013) assessed multiple types of stress response coordination with growth curve modeling (GCM). When they examined intraindividual coordination across the HPA-axis and ANS among young-adults, they found that participants' responses to an interpersonal stressor were aligned over time. The present study also utilizes a GCM model to focus in on the relationship between sAA and cortisol secretion post stressor.

1.3 Measuring the Stress Response

Studying the stress response from a multisystem perspective requires a stress paradigm that elicits neuroendocrine responses from both the HPA-axis and ANS. The Trier Social Stress Test (TSST; Buske-Kirschbaum et al., 1997) is a performance-oriented, social-evaluative stressor in which participants perform a speech and mental arithmetic task. The TSST is well-validated, consistently eliciting mild to moderate HPA-axis responses in children, adolescents, and adults (e.g., Gunnar et al., 2009). Typical physiological responses include peak concentration of sAA and cortisol approximately 10- and 30-minutes post-TSST onset, respectively, and returning to baseline levels within 60-minutes (Nater et al., 2005; Dickerson & Kemeny, 2004).

The Group Public Speaking Task for Adolescents (GPST-A) is a group version of the TSST developed to elicit a physiological response to stress in adolescents that is less time-consuming and resource-intensive compared to the single-subject version of the task (Hostinar, McQuillan, Mirous, Grant, & Adam, 2014). Participants are in the same room, separated by dividers and called on randomly to present a speech to confederate judges. Hostinar and colleagues (2014) found the task elicited a significant increase in cortisol in adolescents (approximately 60% above baseline), with no gender differences in reactivity. While Hostinar and colleagues (2014) showed the GPST-A is effective and efficient at examining adolescents' stress response, they did not examine ANS reactivity.

1.4 Present Study

To date, no studies have tested whether the GPST-A elicits a cortisol and sAA response via HPA-axis and ANS activation. The following study aims to test the feasibility of administering the GPST-A to elicit a stress response from both the HPA-axis and ANS. Furthermore, few studies have examined the coordination between the HPA-axis and ANS accounting for interindividual differences in time to peak allowing researchers to differentiate between the reactivity and recovery period of the response.

This study extends Lopez-Duran et al.'s (2014) two-piece multilevel GCM with landmark registration (LR) work on the HPA-axis to the ANS to take a multisystem approach to examining the response profiles of both sAA and cortisol. In turn, this study will determine if the HPA-axis and ANS show coordination following the GPST-A during adolescence, identifying features of this coordination. The present study is novel in that it uses a sophisticated analytic approach to examine the response profiles of these two systems to examine stress reactivity in a group of adolescents after the GPST-A.

2. Methods

2.1 Recruitment and Participants

Full-time public high school students from suburban towns surrounding a large research university who were currently enrolled in a regularly offered health class for students in grade 11 were recruited to participate in this study. Permission to recruit students and conduct the study in two schools was received from district and school administrators. Fifty-four adolescents ($n=40$ female) ages 14 to 18 years ($M=16.6$ years) participated in the study. All students spoke English fluently, had written consent from their parents, and provided assent to participate in the current study.

2.2 Procedure

All data collection procedures were approved by the Institutional Review Board of a large research university. Data were collected in a classroom in the participant's school over a four-week period on a weekday after school hours (2:30–4pm) to control for the diurnal rhythm of cortisol and sAA. Participants attended one 90-minute appointment and were part of 3–5 person groups. Previous researchers concluded five participants is the ideal group size to produce a uniform HPA-axis response across individuals with the GPST-A (Hostinar et al., 2014). In this study, participants arrived to the test room after their school day ended (2:30–2:35pm). Participants sat alone at a desk and separated by opaque dividers. After giving assent, participants were instructed on how to provide saliva samples and gave a practice sample, Time (T) 0. Then participants completed computer-based questionnaires consisting of demographic information and psychosocial measures that took about 20 minutes. Overall, participants' acclimation period was 30 minutes long and occurred in the same room as the GPST-A before their baseline saliva sample (T1) was collected. After providing their baseline saliva sample (T1), participants then heard the task instructions for the GPST-A from a research assistant (Hostinar et al., 2014) and the judges entered the room to hear the speeches. Immediately following the GPST-A, the judges left the room and participants provided a second saliva sample (T2) and four more saliva samples at ten minute increments (T3–T6). After the final saliva sample (T6), the research assistant debriefed participants and answered questions about the study. At the end of their appointment, participants were compensated for their time with a \$20 gift certificate, offered pizza and were entered in a drawing for an iPad mini (for full protocol, see Katz & Peckins, under review).

2.3 Measures

2.3.1 Group Public Speaking Task for Adolescents (GPST-A)—The GPST-A (Hostinar et al., 2014) was utilized to elicit a stress response in this sample of youth. Participants were instructed to give a 1.5 minute speech in front of two judges and to imagine they were introducing themselves to new classmates. Participants were told they were being video recorded and their performance would be compared to the performance of their peers. Research assistants acting as judges were trained to have serious looks and to provide no feedback during the speech task. While performing the speech to the judges, participants could be seen by but not see their peers. The GPST-A lasted approximately 15 minutes.

2.3.2 Cortisol and Salivary Alpha-Amylase (sAA)—Saliva samples were collected at seven time points via passive drool before (T0, T1) and after (T2-T6) the GPST-A to capture peak reactivity and recovery of cortisol and sAA. Participants were allowed one minute to complete their saliva samples with a goal of 1mL of saliva. Studies utilizing a similar paradigm show clear effects of the task on hormone trajectories with sample sizes of N=20–60 (Dickerson & Kemeny, 2004). SAS Proc Power (version 9.4) indicates that, assuming a modest effect size ($r=0.35$; Cohen, 1988), to power this study at 80% requires a sample size of N=52. Cortisol and sAA assays were completed by the Biomarker Core Lab (University Park, PA) using Salimetrics assay kits (State College, PA). The first sample (T0) was not assayed because it reflects cortisol and sAA response to a novel experience and anticipatory stress (i.e., coming into the appointment for the experiment) and not cortisol or sAA at rest. Saliva samples were assayed for cortisol in duplicate, were averaged, then converted to nmol/L (cortisol). Inter-assay covariances were less than 10% and intra-assay covariances were less than 5%. Saliva samples were assayed for sAA in singlet and converted to U/mL. The inter-assay coefficient of variability was 5.05%. To correct for skew and kurtosis, a log transformation was applied to cortisol and sAA values, and those log-transformed values were used in analyses.

To test the relationship between the reactivity and recovery phases of the cortisol and sAA response, multiple indices of sAA activity were calculated. The sample at which each participant peaked in sAA was identified and used to calculate *sAA Reactivity Slope* and *sAA Recovery Slope*. *sAA Reactivity Slope* is the difference between each individual's baseline and peak sAA divided by the change over time in minutes between each individual's baseline and peak samples. *sAA Recovery Slope* is the difference between each individual's peak and recovery sAA divided by the change over time in minutes between each individual's peak and recovery samples. To test for effects of total sAA output across the six sampling times, Area Under the Curve with respect to Ground (AUCg) was calculated (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003).

2.4 Analytic Plan

Repeated measures ANOVAs were performed to test for significant change in cortisol and sAA over time and gender differences in change in cortisol and sAA. When the assumption of sphericity was violated, the Greenhouse-Geisser corrected degrees of freedom were used. A series of two-piece multilevel GCM-LR were fit using SAS ® software (Version 9.4) to examine associations between multiple measures of sAA activity (Level 2) and individuals' *reactivity* and *recovery* (Level 1) in cortisol in response to the GPST-A (eq. 1). LR captures the different phases of the stress response (i.e., reactivity, recovery) and accounts for *peak latency*—individual differences in post-stress peak hormone concentrations (Lopez-Duran et al., 2009; Lopez-Duran, Mayer, & Abelson, 2014). More traditional analytic approaches (e.g., linear and quadratic terms) account for the curvilinear nature of the cortisol response, but ignore individual and group differences in the time latency of post-stress peak concentrations (Lopez-Duran et al., 2009; Lopez-Duran et al., 2014). To carry out the two-piece multilevel GCM-LR, the sample at which participants peaked in cortisol was identified and used to create a Cortisol Inflection (CortInflect) variable. CortInflect was dummy coded as 0 at sampling times when the participant was in their reactivity phase and at their peak in

cortisol, and coded as 1 at sampling times when the participant was post-peak cortisol and in their recovery phase (Lopez-Duran et al., 2014; Singer & Willett, 2003). CortInflect was used to create the adjusted time variables *Cortisol Reactivity* and *Cortisol Recovery* for each participant. When participants were in their reactivity phase (CortInflect = 0), Cortisol Reactivity equals $(t_{\text{peak}} - t) \cdot (-1)$ where t_{peak} equals the time in minutes when participants reached their peak cortisol value. Cortisol Reactivity was set to 0 for all samples during the recovery phase (CortInflect = 1). When participants were in their recovery phase (CortInflect = 1), Cortisol Recovery equals $t - t_{\text{peak}}$. Cortisol Recovery was set to 0 for all samples during the reactivity phase (CortInflect = 0). Cortisol Reactivity and Cortisol Recovery are estimates of linear change in cortisol from baseline to peak and linear change post-peak through the final sample, respectively (see Table 1, adapted from Lopez-Duran et al., 2014; Singer & Willett, 2003). The same steps were followed to calculate *sAA Reactivity* and *sAA Recovery*.

Initial models tested whether gender, race, speech order, group size (3, 4 or 5), and steroidal medication use were associated with cortisol reactivity and recovery. Only gender had a significant influence on cortisol secretion and was therefore the only covariate included in the final models (Table 2). When models were run without gender, findings remained the same. Time of day was not included as a covariate given all saliva samples were collected between 2:30–4pm. Final models were run for sAA Reactivity Slope, sAA Recovery Slope, and sAA AUCg separately to test how each phase of the sAA response is associated with each phase of the cortisol response to the GPST-A. Cortisol was used as the outcome in all models given sAA peaks and enters the recovery phase earlier than salivary cortisol. Additional analyses were performed with the intercept set to the baseline sample (i.e., Cortisol Reactivity = $t - t_{\text{baseline}}$ when CortInflect = 0, and t_{peak} when CortInflect = 1; Cortisol Recovery = $t - t_{\text{peak}}$ when CortInflect = 1, and 0 when CortInflect = 0). Findings from the baseline model are presented in the Appendix.

Supplemental analyses were performed using a joint modeling approach to test how cortisol and sAA intercept and growth (reactivity and recovery) parameters are linearly associated. Two-piece models with LR were run for cortisol and sAA separately, and the intercept, reactivity slope, and recovery slope parameters were extracted for both cortisol and sAA, resulting in six extracted terms for each participant. A Pearson Correlation analysis was performed on the extracted intercept and growth parameters in addition to participants' peak cortisol value, difference from baseline to peak cortisol, and time to peak in cortisol (in minutes) to test for linear associations between and within the cortisol and sAA response profiles.

$$\begin{aligned}
 \text{Level 1: } \text{LgCortisol}_{ij} &= \pi_{0j} + \pi_{1j}(\text{Reactivity}_{ij}) + \pi_{2j}(\text{Recovery}_{ij}) + e_{ij} \\
 \text{Level 2: } \pi_{0j} &= \beta_{00} + \beta_{01}(\text{Gender}) + \beta_{02}(\text{measure of sAA}) + r_{0j} \\
 \pi_{1j} &= \beta_{10} + \beta_{11}(\text{Gender}) + \beta_{12}(\text{measure of sAA}) + r_{1j} \\
 \pi_{2j} &= \beta_{20} + \beta_{21}(\text{Gender}) + \beta_{22}(\text{measure of sAA})
 \end{aligned}
 \tag{Equation 1}$$

3. Results

3.1 Group Cortisol and sAA Response

Repeated measures ANOVA revealed a significant change in cortisol concentration ($F_{1,62,85.78}=12.61$; $p<.001$) over time. Males had significantly higher levels of cortisol than females at every time point ($F_{1,52}=14.53$, $p<.001$) yet males and females did not differ in their change in cortisol over time, which is consistent with the adult cortisol literature (Nicolson, Storms, Ponds, & Sulon, 1997). Repeated measures ANOVA revealed a significant change in sAA concentrations over time ($F_{3,53,186.97}=15.41$, $p<.001$). There were no significant differences in sAA concentrations between males and females ($F_{1,52}=.74$, $p>.05$) which is consistent with other studies that used a social-evaluative stressor (Strahler, Mueller, Rosenloecher, Kirschbaum, & Rohleder, 2010).

There are no clear guidelines for determining a definite cortisol or sAA response, but 10% increase has been previously used as a meaningful cut-off (Gordis et al., 2006) and 78% ($n=42$) of the sample exceeded this threshold for cortisol and sAA. The average cortisol increase post-stressor was 50% over baseline levels, which is within the range typical of single-subject TSST studies with adolescents (Gunnar et al., 2009). The average sAA increase post-stressor was 115% over baseline levels. Consistent with previous work (see Dickerson & Kemeny, 2004 for a review of cortisol), in this sample, 65% of participants reached peak cortisol concentrations 21–30 minutes post-stressor (T3 or T4) and 80% of participants peaked in sAA concentrations 10–24 minutes post-stressor (T2 or T3). Twenty-four (44%) and 32 (59%) participants returned to their baseline levels of cortisol and sAA, respectively, by the final sample. Intraclass correlation coefficients (ICC) reveal the majority of variability in cortisol (ICC=74%) and sAA (ICC=65%) is accounted for by interindividual differences (see Figures 1 and 2).

3.2 Two-piece Multilevel Growth Curve Models (GCM) with Landmark Registration (LR)

Estimates for the two-piece multilevel GCM-LR are presented in Table 3. The findings are presented by measure of sAA activity, all while controlling for Gender. The Gender by sAA measure and Gender by Cortisol Reactivity interactions were not significant in any of the models; however, the Gender by Cortisol Recovery interaction was significant in each model ($\beta=.01$, $p<.01$) indicating on average, females had a less steep decline in cortisol during the post-peak, recovery period compared to males.

The main effect for sAA Reactivity Slope was marginally significant ($\beta=50.26$, $p<.10$), indicating a steeper rate of change in sAA concentrations from baseline to peak was associated with higher peak levels of cortisol. Furthermore, sAA Reactivity Slope was associated with both Cortisol Reactivity ($\beta=1.38$, $p<.01$) and Cortisol Recovery ($\beta=-0.65$, $p<.01$) slopes. Adolescents with a steeper increase in sAA from baseline to peak presented with a steeper increase in cortisol from baseline to peak and steeper decline in cortisol from peak to recovery.

sAA Recovery Slope was not predictive of peak cortisol levels ($\beta=-29.87$, $p>.05$) yet the interaction with Cortisol Reactivity ($\beta=-0.71$, $p<.10$) approached statistical significance and Cortisol Recovery ($\beta=0.50$, $p<.01$) was statistically significant. These findings suggest a

steeper decline in sAA from peak to recovery is associated with both a steeper increase in cortisol from baseline to peak and steeper decline in cortisol from peak to recovery.

Total sAA concentration across the study (sAA AUCg) was marginally associated with peak cortisol and Cortisol Reactivity slope; however, was not associated with Cortisol Recovery slope. These findings suggest greater levels of sAA during the response to and recovery from a stressor are only associated with a steeper rise in cortisol and higher peak cortisol concentration and not the regulatory period following cortisol peak.

3.3 Correlation Analysis with Extracted Terms

Pearson product-moment correlations of the extracted sAA and cortisol intercept and growth parameters are presented in Table 4. Within the sAA profile, sAA intercept (indicating the estimated peak sAA level) was negatively correlated with sAA Recovery slope, indicating adolescents with higher peak levels of sAA had a steeper sAA Recovery slope. sAA Reactivity slope was negatively correlated with sAA Recovery slope, such that adolescents with a steeper Reactivity slope also had a steeper Recovery slope.

Within the cortisol profile, cortisol intercept (indicating the estimated peak cortisol level) was positively correlated with Cortisol Reactivity Slope and negatively correlated with Cortisol Recovery slope. This suggests adolescents with higher peak cortisol concentrations had steeper cortisol reactivity slopes and steeper cortisol recovery slopes. Cortisol Reactivity Slope was negatively correlated with Cortisol Recovery Slope, indicating adolescents with a steeper reactivity slope also had a steeper cortisol recovery slope. Peak cortisol concentration, the difference in cortisol from baseline to peak, and time to peak in cortisol (in minutes) were positively correlated with cortisol intercept and cortisol reactivity slope and with the exception of time to peak, negatively correlated with cortisol recovery slope.

sAA intercept was positively correlated with cortisol intercept and cortisol reactivity slope, indicating adolescents with higher peak levels of sAA had higher peak levels of cortisol and a steeper cortisol reactivity slope. sAA reactivity slope was positively correlated with cortisol reactivity slope and the difference in cortisol from baseline to peak, indicating individuals with a steeper sAA reactivity slope also had a steeper cortisol reactivity slope. sAA recovery slope was not associated with any of the cortisol parameters.

When correlational analyses were performed separately for each gender, differences emerge in the joint model. A steeper sAA reactivity slope was associated with a steeper cortisol reactivity slope and a steeper recovery slope *in females only*. In males, sAA reactivity slope was not correlated with any of the cortisol intercept or growth parameters. This gender difference suggests females may be driving the overall findings; however, we are unable to directly test this hypothesis due to the small number of males included in the study.

4. Discussion

4.1 Analytic Advances

The present study is novel in that it combined two analytic methods suggested by other scholars to appropriately model participants' response to a social-evaluative stressor. The

two-piece multilevel GCM suggested by Lopez-Duran et al. (2014) allowed us to examine the reactivity and recovery periods of the stress response trajectories separately and the use of LR accounts for peak latency, a phenomenon that is only recently being taken into consideration in neuroendocrine research. Past studies have used linear and quadratic terms at level 1 in a multilevel model and imposed a common peak time for everyone. These approaches do not account for interindividual differences in the timing of the response and can lead researchers to both Type 1 and Type 2 errors. Modeling the cortisol and sAA responses with two-piece multilevel GCM-LR allows the model to be more sensitive to detecting trajectory differences, particularly in the reactivity and recovery phases of the response. The present study replicates and expands upon the work by Lopez-Duran et al. (2014) that utilized two-piece multilevel GCM-LR to address the limitations of previously accepted analytic approaches, accounting for differences in the timing of the response and providing interpretable estimations of the reactivity and recovery slopes.

The present study also adopted the approach recommended by Laurent et al. (2014), to examine matched-phase coordination of the HPA-axis and ANS with a multilevel GCM. Previous studies have collected cortisol and sAA reactivity data simultaneously yet analyzed their response profiles separately, limiting the ability to test for coordination between these two systems. Results from the present study reveal how the HPA-axis and ANS work in synchrony, illustrating how each phase of an individual's sAA response maps onto each phase of their cortisol response. The present study also expands on the work of Hostinar et al. (2014) to show that a group social-evaluative stressor administered in a school setting can be used to elicit cortisol *and* sAA responses in adolescents. This is noteworthy given it is not always feasible to administer a single-subject stressor such as the TSST in studies of neuroendocrine function.

4.2 Significant Findings

While the aims of this paper were primarily methodological in nature, many of our findings are noteworthy and are discussed below.

4.2.1 Relationships between HPA-axis and ANS responses—The GPST-A elicited a cortisol and sAA response in the majority of adolescents and the rate of increase and decline in sAA as well as overall sAA secretion across the study were associated with steeper rates of cortisol reactivity and recovery. The finding that sAA reactivity and recovery are associated with the reactivity and recovery phases of the cortisol response to stress is consistent with previous literature reporting peak and AUC measures of sAA and cortisol in response to the TSST to be positively correlated among samples of youth (Gordis et al., 2006; Van den Bos et al., 2014). This finding is even more precise because of our statistical approach; we were able to account for individual differences in time to peak and duration of the recovery period.

4.2.2 Gender differences—Although the GPST-A evoked typical cortisol and sAA responses in both males and females, males' cortisol concentrations were significantly higher than females' during each phase of the response. Previous studies show mixed results with respect to gender differences in adolescent cortisol reactivity. Some studies found no

differences in the cortisol reactivity among adolescent boys and girls (Gunnar et al., 2009; Stroud et al., 2009; Sumter et al., 2010; Hostinar et al., 2014). Others found higher cortisol concentrations at baseline and peak phases of the response in males compared to similar-aged females (Strahler et al., 2010; Zijlmans, Beijers, Mack, Pruessner, & de Weerth, 2013; Zoccola, Quas, & Yim, 2010). While findings are mixed, the analytic approaches also vary widely. The approach of previous studies may lack sensitivity to detect subtle differences because they focus only on mean differences with repeated measures ANOVAs (Gunnar et al., 2009; Stroud et al., 2009; Sumter et al., 2010; Zijlmans et al., 2013; Zoccola, Quas, & Yim, 2010) or multilevel models with linear and quadratic terms (*Time* & *Time*²) that do not take into consideration individual differences in peak latency (Hostinar et al., 2014). As Lopez-Duran and colleagues (2014) points, the use of two-piece multilevel GCM-LR allows researchers to examine different phases of the cortisol response more accurately, reducing the risk of false negatives. In this study, this technique allowed us to detect gender differences in cortisol release among the participants even with a small number of males included in the study.

The lack of gender differences in the sAA response to a social-evaluative stressor in the present study is consistent with previous research (Strahler et al., 2010; Stroud et al., 2009). One study showed increased sAA reactivity in males compared to females (van Stegeren, Wolf, & Kindt, 2008). However, van Stegeren and colleagues (2008) used the cold-pressor task to elicit a stress response, which is physical in nature rather than social-evaluative.

4.3 Limitations

This study has several limitations worth noting. The small sample size and ethnically homogenous sample limits the ability to generalize the findings to all adolescents. As such, the findings from this study are representative of adolescents living in suburban US. However, the present study is the first of its kind to use the GPST-A in a school and utilize two-piece multilevel GCM-LR to examine cortisol and sAA trajectories simultaneously, and findings from this study can be used as a launching point for future studies with larger and more ethnically diverse samples. Furthermore, the findings of gender differences in cortisol trajectories indicate separate models for males and females are necessary yet due to the imbalance between genders (females=40, males=14) and overall small sample size, gender stratified two-piece multilevel GCM-LR were not run. In the future, studies should collect a larger, more gender-balanced sample in order to appropriately examine each phase of the stress response in males and females.

While the HPA-axis response to stress is most commonly measured with cortisol, the ANS is measured in a variety of ways. sAA is only a surrogate marker of the ANS and is less precise than other measures (e.g., vagal tone) that capture the rapid response through effects on the cardiovascular and respiratory systems. In turn, using sAA as a marker of ANS activity limits conclusions that can be made about the immediate response of this system following a stressor. It should be noted that the social context of the GPST-A remains unexplored in this study and therefore, we are unable to discern which aspect of the GPST-A influenced participants' responses. For example, while the two judges were different ages and races (Caucasian 25 years old and African American 56 years old), both judges were female

throughout the study. This may have influenced the participants' reaction to the task. Ideally, judges should include one male and one female to control for gender biases participants may have (Kirschbaum, Pirke, & Hellhammer, 1993).

Additionally, participants' response to the stressor may be influenced by their relationship with the other participants in the group. Previous literature suggests that both social support and negative relationships influence participants' responses to the TSST (Kirschbaum, Klauer, Filipp, & Hellhammer, 1995; Knack, Jensen-Cambell, & Baum 2011). Because participants in this study were classmates with one another, their previous social interactions or social status within the school may contribute to the social-evaluative nature of the stressor and influence their HPA-axis and ANS response.

4.4 Future Directions

An unexplored aspect of the GPST-A is how the relationships between the group members may have affected their reaction to, and ability to cope after, the stressor. For some adolescents, having classmates and/or friends in the group may have made the experience less stressful, and for others it may have made it more so. Recent literature suggests that social connections can affect people's physiological response to stress (Hostinar & Gunnar, 2013). Given the increased social sensitivity during adolescence (Somerville, 2013), it is logical to hypothesize that adolescents' physiological response and coping strategies may be affected by positive or negative relationships in their GPST-A group. Future experiments utilizing the GPST-A should explore the relationship between group members with social-network analysis, especially when settings such as this experiment take place when students are all in the same school and likely know one another. Additionally, future research should examine how psychological constructs (e.g., emotion regulation strategies) might be influencing individuals' neuroendocrine response trajectories and could therefore be utilized as predictors in these models.

4.5 Conclusion

This study highlights the importance of accurately modeling the response trajectories of salivary biomarkers of the HPA-axis and ANS when taking a multisystem approach to neuroendocrine research and making conclusions about the reactivity and recovery period of the HPA-axis and ANS. As studies incorporate a multisystem analytic approach, it is critical that individual variability in peak latency be taken into consideration and that accurate modeling techniques capture individual variability in the stress response. Moving forward, we suggest that future studies incorporate analytic approaches outlined in the present study when examining multiple systems of the stress response.

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Appendix A

Table A1

Example of Data in Long Format with Adjusted Time Variables when Intercept Set at Baseline

Participant	Sample	Cortisol	Time	CortInflect	Reactivity	Recovery
2	2.1	1.94	0	0	0	0
2	2.2	2.15	13	0	13	0
2	2.3	2.02	22	1	13	9
2	2.4	1.88	32	1	13	19
2	2.5	2.04	41	1	13	28
2	2.6	1.84	51	1	13	38
3	3.1	1.38	0	0	0	0
3	3.2	1.52	13	0	13	0
3	3.3	1.74	22	0	22	0
3	3.4	1.64	32	1	22	10
3	3.5	1.56	41	1	22	19
3	3.6	1.5	51	1	22	29

Note. Time=minutes since baseline sample (S1); Cortisol = log transformed cortisol value in nmol/L units; **Bolded** value = Peak cortisol; CortInflect = the inflection point of the participant's cortisol response, 0 = samples up until their peak cortisol and including the peak, 1 = recovery period; Reactivity = the minutes to the peak, the reactivity period of the response for participant X; Recovery = the minutes since peak, the recovery period of the response for participant X. Adapted from Lopez-Duran et al., 2014 and Singer & Willet, 2003.

Table A2

Model Building when Intercept Set at Baseline

	Model 1		Model 2	
	Estimate	SE	Estimate	SE
Fixed Effects				
Intercept, γ_{000}	1.44 **	0.06	1.79 **	0.11
Reactivity Slope, γ_{100}	0.01 **	0.002	0.02 **	0.004
Recovery Slope, γ_{200}	-0.01	0.001	-0.02 **	0.002
Gender, γ_{010}	---	---	-0.47 **	0.13
Reactivity Slope by Gender, γ_{110}	---	---	-0.00	0.01
Recovery Slope by Gender, γ_{210}	---	---	0.01 **	0.003
Random Effects				
Variance Intercept, σ^2_{r0}	0.20 **	0.04	0.16 **	0.03
Variance Reactivity Slope, σ^2_{r1}	2.0×10^{-4} **	5.1×10^{-5}	2.2×10^{-4} **	5.2×10^{-5}
Variance Recovery Slope, σ^2_{r2}	5.5×10^{-5} **	1.9×10^{-5}	3.2×10^{-5} *	1.5×10^{-5}
Covariance, Intercept, Reactivity Slope, $\sigma^2_{r0,r1}$	-0.002	0.001	-0.002	0.001
Covariance, Intercept, Recovery Slope, $\sigma^2_{r0,r2}$	-0.001 *	5.9×10^{-4}	-2.2×10^{-4}	4.5×10^{-4}
Covariance, Reactivity Slope, Recovery Slope, $\sigma^2_{r1,r2}$	-7.0×10^{-5} *	2.6×10^{-5}	-6.0×10^{-5} *	2.3×10^{-5}
Residual Variance, σ^2_e	0.01 **	0.002	0.01 **	0.002
Indices of Model Fit				

	Model 1		Model 2	
	Estimate	SE	Estimate	SE
AIC	-37.4		-36.8	
BIC	-23.5		-22.9	

Note. Models based on up to 6 occasions nested within 54 participants for a total of 324 observations. Dependent variable is log transformed cortisol concentration, nmol/L. Model 1 includes intercept (baseline cortisol), minutes from baseline through peak cortisol (Reactivity), minutes from Peak to appointment end (Recovery) at Level 1. Model 2 includes intercept (baseline cortisol), Reactivity and

Recovery at Level 1 and gender as an independent variable at Level 2.

**

$p < .01$,

*

$p < .05$,

$p < .10$.

AIC = Akaike Information Criterion. BIC = Bayesian Information Criterion.

Table A3

Estimates from Two-piece Multilevel Growth Curve Models with Landmark Registration when Intercept Set at Baseline

	β	SE
Estimates for cortisol concentration		
Intercept, γ_{000}	1.76**	(0.14)
Gender, γ_{010}	-0.49**	(0.16)
sAA Reactivity Slope	-2.85	(27.22)
sAA Recovery Slope	-8.85	(18.56)
sAA AUCg	0.12	(0.23)
Reactivity π_1 (Estimates for slope from baseline to peak)		
Intercept, γ_{100}	0.01**	(0.002)
Gender, γ_{110}	-0.003	(0.005)
sAA Reactivity Slope	1.43**	(0.38)
sAA Recovery Slope	-0.69	(0.39)
sAA AUCg	0.01*	(0.01)
Recovery, π_2 (Estimates for slope from Peak to End)		
Intercept, γ_{200}	-0.02**	(0.002)
Gender, γ_{210}	0.01**	(0.003)
sAA Reactivity Slope	-0.59**	(0.20)
sAA Recovery Slope	0.36	(0.19)
sAA AUCg	-3.37×10^{-3}	(3.31×10^{-3})

Note.

**

$p < .01$,

*

$p < .05$,

$p < .10$.

Highlights

- Group stressor (GPST-A) used in school setting among adolescent peers
- Interindividual differences in time to peak hormone concentration examined
- Differentiates between reactivity and recovery phases of response of individuals
- Multisystem analytic approach with two-piece GCM with landmark registration
- Stressor protocol effectively elicited response from HPA and ANS

Figure 1.
Cortisol Trajectories in Response to the Group Public Speaking Task for Adolescents
Note. Cortisol values are log transformed in nmol/L units. Gray lines represent cortisol trajectories for each participant. Black line represents the mean cortisol trajectory across participants.

Figure 2.
sAA Trajectories in Response to the Group Public Speaking Task for Adolescents
Note. sAA values are log transformed in U/mL units. Gray lines represent sAA trajectories for each participant. Black line represents the mean sAA trajectory across participants.

Table 1

Example of data in long format with adjusted time variables

Participant	Sample	Cortisol	Time	CortInflect	Reactivity	Recovery
2	2.1	1.94	0	0	-13	0
2	2.2	2.15	13	0	0	0
2	2.3	2.02	22	1	0	9
2	2.4	1.88	32	1	0	19
2	2.5	2.04	41	1	0	28
2	2.6	1.84	51	1	0	38
3	3.1	1.38	0	0	-22	0
3	3.2	1.52	13	0	-9	0
3	3.3	1.74	22	0	0	0
3	3.4	1.64	32	1	0	10
3	3.5	1.56	41	1	0	19
3	3.6	1.5	51	1	0	29

Note. Time=minutes since baseline sample (S1); Cortisol = log transformed cortisol value in nmol/L units; **Bolded** value = Peak cortisol; CortInflect = the inflection point of the participant's cortisol response, 0 = samples up until their peak cortisol and including the peak, 1 = recovery period; Reactivity = the minutes to the peak, the reactivity period of the response for participant X; Recovery = the minutes since peak, the recovery period of the response for participant X. Adapted from Lopez-Duran et al., 2014 and Singer & Willet, 2003.

Table 2**Model Building**

	Model 1		Model 2	
	Estimate	SE	Estimate	SE
Fixed Effects				
Intercept, γ_{000}	1.86 **	.08	2.30 **	.15
Reactivity Slope, γ_{100}	0.01 **	0.002	0.02 **	0.004
Recovery Slope, γ_{200}	-0.01 **	0.001	-0.02 **	0.002
Gender, γ_{010}	---	---	-.61 **	.17
Reactivity Slope by Gender, γ_{110}	---	---	-0.004	.005
Recovery Slope by Gender, γ_{210}	---	---	0.01 **	0.003
Random Effects				
Variance Intercept, σ^2_{r0}	.36 **	.07	.29 **	.06
Variance Reactivity Slope, σ^2_{r1}	2.18×10^{-4} **	4.9×10^{-5}	2.17×10^{-3} **	4.9×10^{-5}
Variance Recovery Slope, σ^2_{r2}	5.5×10^{-5} **	1.9×10^{-5}	3.3×10^{-5} *	1.6×10^{-5}
Covariance, Intercept, Reactivity Slope, $\sigma^2_{r0,r1}$	.01	.002	.005 **	.001
Covariance, Intercept, Recovery Slope, $\sigma^2_{r0,r2}$	-0.004 **	.001	-.002 **	.001
Covariance, Reactivity Slope, Recovery Slope, $\sigma^2_{r1,r2}$	-7.0×10^{-5} *	2.6×10^{-5}	-6.0×10^{-5} *	2.3×10^{-5}
Residual Variance, σ^2_e	0.01 **	0.002	0.02 **	0.002
Indices of Model Fit				
AIC	-34.1		-31.4	
BIC	-20.2		-17.4	

Note. Models based on up to 6 occasions nested within 54 participants for a total of 324 observations. Dependent variable is log transformed cortisol concentration, nmol/L. Model 1 includes intercept, minutes from baseline through peak cortisol (Reactivity), minutes from Peak to appointment end (Recovery) at Level 1. Model 2 includes intercept, Reactivity and Recovery at Level 1 and gender as an independent variable at Level 2.

**
p<.01,

*
p<.05.

AIC = Akaike Information Criterion. BIC = Bayesian Information Criterion.

Table 3

Estimates from Two-piece Multilevel Growth Curve Models with Landmark Registration

	β	SE
Estimates for cortisol concentration		
Intercept, γ_{000}	2.07**	(0.19)
Gender, γ_{010}	-0.57**	(0.21)
sAA Reactivity Slope	50.26	(28.70)
sAA Recovery Slope	-29.87	(20.54)
sAA AUCg	0.53	(0.27)
Reactivity π_1 (Estimates for slope from baseline to peak)		
Intercept, γ_{100}	0.01**	(0.004)
Gender, γ_{110}	-0.004	(0.004)
sAA Reactivity Slope	1.38**	(0.37)
sAA Recovery Slope	-0.71	(0.38)
sAA AUCg	0.01	(0.01)
Recovery, π_2 (Estimates for slope from Peak to End)		
Intercept, γ_{200}	-0.02**	(0.002)
Gender, γ_{210}	0.01**	(0.002)
sAA Reactivity Slope	-0.65**	(0.20)
sAA Recovery Slope	0.50**	(0.19)
sAA AUCg	-0.004	(0.003)

Note.

**
p<.01,*
p<.05,

p<.10.

Table 4

Bivariate Correlations of sAA and Cortisol Extracted Intercept and Growth Parameters

	1.	2.	3.	4.	5.	6.	7.	8.
1. sAA Intercept	---							
2. sAA Reactivity Slope	.17	---						
3. sAA Recovery Slope	-.31*	-.39**	---					
4. Cortisol Intercept	.28*	.13	-.10	---				
5. Cortisol Reactivity Slope	.33*	.36*	-.05	.57**	---			
6. Cortisol Recovery Slope	-.17	-.18	.15	-.69**	-.53**	---		
7. Peak Cortisol Value	.21	.16	-.03	.93**	.45**	-.66**	---	
8. Peak Cortisol – Baseline Cortisol	.22	.30*	.06	.75**	.78**	-.55**	.77**	---
9. Minutes to Peak Cortisol	.04	.16	.09	.35*	.46**	-.18	.19	.42**

Note.

**
 $p < .01$,*
 $p < .05$.