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Developmental differences in infant salivary alpha-amylase and cortisol responses to stress

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Summary

This study examined developmental differences in infants' salivary alpha-amylase (sAA) and cortisol levels and responses to the well-baby exam/inoculation stress protocol at 2, 6, 12, and 24 months of age. Mother-infant pairs (N = 85; 45 girls) were assessed during well-baby visits and saliva was sampled before the well baby exam/inoculation procedure (pretest) and at 5, 10, and 20 minutes post inoculation stress. Older infants (24 months) had higher levels of sAA than younger infants (2, 6 and 12 months). Stress-related sAA increases were evident at 6 and 12 months, but not at 2 or 24 months of age. Stress-related cortisol increases were present at 2 and 6 months, but not at older ages. Mothers had higher sAA levels than their infants, but did not show sAA or cortisol increases to their infants' inoculation. Pre-test, maternal and infant sAA levels were positively correlated ($r_s.47$ to $.65$) at 6, 12, and 24 months of age, but not at 2 months. These findings suggest that the association between the sympathetic branch of the autonomic nervous system and the secretion of sAA develops between 2 and 6 months of age, when levels of sAA are responsive to exposure to a painful stressor.

Keywords

infant; cortisol; alpha-amylase; stress; maternal-infant synchrony

Perturbations early in life including exposure to stress during the prenatal and early postnatal period appear to have profound and lasting consequences for development that may be mediated through dysregulation of physiological stress systems (e.g., Cicchetti & Blender, 2004; Davis et al., 2007; Phillips, 2007; Gunnar & Quevedo, 2008). The biological stress response consists primarily of the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympathetic nervous system (SNS) (Mason, 1975; Chrousos & Gold, 1992). Evaluation of the

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Conflict of Interest

Dr. Davis has no conflict of interest regarding this manuscript.

Dr. Granger is the founder and CEO of Salimetrics LLC which performed the sAA assays for this manuscript.

Contributors

Authors Davis and Granger designed the study. Dr. Davis supervised the data collection, performed the statistical analyses and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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consequences of early life stress exposure requires assessment of the dynamic interplay between these two physiological stress systems during development. Studies evaluating biological stress systems in young children, however, are hampered by the need to use minimally invasive measures. Accessible measurement tools exist for the assay of cortisol, the end-product of the HPA axis, in saliva (e.g., Kirschbaum & Hellhammer, 1994). Thus, research evaluating the development of individual differences in stress regulation and the role that physiological stress systems play in shaping development have focused primarily on the correlates and concomitants of the activity of the HPA axis (e.g., Stansbury & Gunnar, 1994; Davis, Donzella, Krueger & Gunnar, 1999). Studies have yielded important information regarding both developmental changes and individual differences in stress regulation (see Gunnar & Davis, 2003 for review). Because of the difficulty of measuring SNS activity in young children, less is known about the role the SNS component of the stress response plays in these developmental processes. The availability of a salivary biomarker reflective of SNS activity (salivary alpha-amylase, sAA) has enabled simultaneous assessment of the two main components of the biological stress response in infants and young children.

The SNS and HPA axis

Contemporary theorists call for a multi-system approach, involving evaluation of multiple biological systems (Cicchetti & Blender, 2004), to extend our understanding of the development of individual differences in psychobiological stress regulation. This study assesses two salivary analytes representing the activity of the SNS and HPA axis. The sympathetic branch of the autonomic nervous system (ANS) is partially responsible for the “fight or flight” response (Cannon, 1915) which includes increased respiratory rate, cardiac tone and blood flow to skeletal muscles. Salivary alpha amylase is an enzyme that has been used as a surrogate marker for SNS activity. In contrast to many other salivary analytes, sAA is not actively transported, nor does it passively diffuse, into saliva from the general circulation. Salivary alpha amylase is produced locally in the oral mucosa by the salivary glands. The salivary glands are innervated by efferent sympathetic nerves and in adults levels of the enzyme alpha-amylase in oral fluids appear to reflect the activity of the SNS (adrenergic stimulation). At birth sAA is not present in the oral compartment (ODonnell & Miller, 1980) and sympathetic innervation of the salivary glands develops postnatally (Knox & Hoffman, 2008). In adults levels of sAA have been associated with the SNS component of the stress response. Increases in sAA are seen in response to physical (Walsh et al., 1999) as well as psychological stress (Chatterton, Vogelsong, Lu & Hudgens, 1997; Nater et al., 2005) peaking 5 to 10 minutes after the stressor. Further, stress related increases in sAA are inhibited by adrenergic blockers (e.g., Speirs et al., 1974; van Stegeren, Rohleder, Everaerd & Wolf, 2006) while adrenergic agonists stimulate sAA release (Ehlert et al., 2005). Salivary alpha amylase levels are additionally correlated with skin conductance levels providing further evidence that sAA is a surrogate marker of SNS activity (El-Sheikh et al., 2008).

Cortisol is the primary end-product of the HPA axis. Individual differences in cortisol levels are associated with psychological stress, arousal, and negative emotionality (e.g., Kirschbaum & Hellhammer, 1994). The HPA axis response involves a cascade of events resulting in the biosynthesis and release of cortisol from the adrenal cortex. Through passive diffusion, the unbound or biologically active component of cortisol is transferred to saliva and salivary and plasma levels of cortisol are highly correlated as early as infancy (Calixto et al., 2002).

Multi-System Measurement of the Psychobiology of the Stress Response

Although the benefits of multi-system measurements of biological processes in behavioral research are clearly recognized, because of issues related to feasibility, few studies of stress regulation in young children have included assessment of both HPA and SNS activity. Studies

that have measured salivary cortisol and sympathetic control of heart rate (pre-ejection period) have identified a distinctive profile related to fearful temperament (Buss, Davidson, Kalin & Goldsmith, 2004; Buss, Goldsmith & Davidson, 2005). The assessment of biological analytes in saliva has enabled researchers to measure both HPA and SNS activity non-invasively in ecologically valid social contexts and in special populations (i.e., infants) and has yielded new information about the development of individual differences in HPA and SNS regulation. Individual differences in the relation between cortisol and sAA have been shown to be related to the maternal/infant attachment relationship (Hill-Soderlund et al., 2008), maltreatment (Gordis, Granger, Susman & Trickett, 2008), problem behavior (Gordis, Granger, Susman & Trickett, 2006), and affective behavior (Fortunato, Dribin, Granger & Buss, 2008). These findings highlight the importance of assessing the coordination between HPA and SNS activity in biosocial models of development.

Although the multi-system approach appears to have significant potential to extend our understanding of the role that early experiences play in shaping individual differences in stress-related psychobiology, only a handful of studies (Buss et al., 2004; Hill-Soderlund et al., 2008; Fortunato et al., 2008) have applied this approach in studies with infants and young children. Virtually nothing is known about developmental changes in sAA in response to stress across infancy or the appropriate time course for evaluation of infant sAA responses. In the present study, we document developmental differences in sAA and cortisol in the context of the nociceptive stress of standard inoculations administered at infant well-baby visits.

The well-baby exam/inoculation stressor protocol has been applied as a standardized manipulation for measuring stress responses (e.g., Lewis & Ramsay, 1995; Gunnar, Brodersen, Krueger & Rigatuso, 1996) and represents an excellent paradigm for investigating sAA responses to stress. Cortisol and behavioral responses to this stressor have been clearly characterized. Prior studies have documented that this stressor reliably elicits an increase in salivary cortisol levels that peak between 15 and 25 minutes after the stressor at two, four and 6 months of age (Lewis & Ramsay, 1995; Gunnar et al., 1996; Ramsay & Lewis, 2003). During the second postnatal year infants are generally less reactive to stress and cortisol increases to the well-baby/inoculation procedure are not consistently observed (Lewis & Ramsay, 1993; Gunnar et al., 1996). Using this protocol we can begin to understand at what age sAA can be used to evaluate infant stress responses and the appropriate time course for evaluation of these responses.

Maternal regulation of infant stress responses

In the context of early development several specific models emphasize the importance of the caregiver in the direct and indirect regulation of the child's behavioral and biological systems (Calkins & Fox, 2002; Hofer, 2006; Gunnar & Quevedo, 2008). This "co-regulation" is considered the process by which behavioral or physiological systems, in particular those associated with stress responding, of the mother and child are coordinated to support the development of the infant's own regulation systems (Field, 1992; Hofer, 2006). In several studies, it has been observed that salivary cortisol and/or sAA are associated or synchronized between individuals. These associations are apparent between mothers and infants, preschoolers, elementary school-aged children, and adolescent offspring (Granger et al., 1998; Sethre-Hofstad, Stansbury & Rice, 2002; Kivlighan, Granger, Blair & Investigators, 2005; Thompson & Trevathan, 2008; van Bakel & Riksen-Walraven, 2008) as well as between individuals who are not genetically related, including young adults who are dating and spouses in established marriages (Brandtstadter, Baltes-Götz, Kirschbaum & Hellhammer, 1991; Powers, Pietromonaco, Gunlicks & Sayer, 2006). In this study, we explore developmental differences in the degree of synchrony between mothers and their infants' physiological activity. Moreover, we examined the dyads "shared experience" by, in the presence of the

mother, subjecting her infant to an inoculation stressor, and testing maternal-infant synchrony in physiological responses to infant distress.

Measuring Biological Stress Responses during Infancy

Few published studies have applied this multi-system measurement strategy in infancy or early childhood to evaluate whether links between individual differences in HPA responses are moderated by SNS activation (Buss et al., 2004; Fortunato et al., 2008). Before progress can be made, however, several basic questions must be answered related to developmental changes in sAA and the ability to use sAA as an indication of stress reactivity during early childhood. For instance, the oral biology literature reveals that at birth, sAA is not present in the oral compartment and that sAA activity shows a sharp rise in the 0.9–1.9 year period reaching maximum levels by 5–6 years of age (O'Donnell & Miller, 1980). The age of onset in the rise of sAA levels is thought to be partially related to the timing of the introduction of solid foods in the diet and the emergence of dentition needed to chew those solids. Key unanswered questions include: (i) what is the time course of the sAA response to stress (i.e., at what time from 5 to 20 minutes post stress do levels peak and recover) during the first 2 postnatal years? and (ii) are there developmental differences in sAA responses to stress during the first 2 postnatal years? In this study we address these specific knowledge gaps.

Purpose of the Study

Using a cross-sectional design, we examine developmental differences across the first two postnatal years in sAA and cortisol pre-test and in response to inoculation stress administered as part of routine well-baby visits. The primary study goal was to document the time course of the sAA response in infants to the nociceptive stimuli and to evaluate developmental changes in this response across the first two postnatal years. Secondary aims were to assess synchrony between maternal and infant sAA/cortisol levels and the coordination between the sAA and cortisol.

Methods

Participants

Study participants included 85 mother-infant pairs (45 girls) recruited from an ongoing study of prenatal stress and development. Using a cross-sectional design mother infant pairs were assessed when their infants were 2 (n = 22, 11 girls), 6 (n = 19, 10 girls), 12 (n = 22, 11 girls) or 24 (n = 22, 13 girls) months of age. Mothers gave informed consent for all aspects of the protocol, which was approved by the Institutional Review Board for protection of human subjects. Selection criteria included: full term at birth, appropriate weight for gestational age at birth, Apgar scores greater than 7, and no evidence of alcohol or drug use during pregnancy based on data abstracted from prenatal and delivery medical records. Descriptive information for the study sample is shown in Table 1. Demographic factors including maternal age, marital status, household income, education, race/ethnicity and birth order of the child were obtained using a maternal interview and did not differ between the four age groups (all p 's > .2). On the study day all of the infants were in good health and none had a fever.

Procedure

Mother-infant pairs were assessed at the infant's routine well-baby examination at 2, 6, 12 or 24 months. Participants were met by a research assistant in the waiting room of the doctor's office. Data collection occurred at the time of infants' well-baby visits. Thus, sample collection time varied between 8:15 AM and 6:00 PM. Time of sample collection did not differ between the four study groups ($p = .48$). Mothers were asked to report on the infant's state in the car

ride, breastfeeding status and the time of last feeding (See Table 2). Infants were significantly more likely to sleep in the car ride to the well-baby visit at 2 and 6 months, as compared to 12 and 24 months (p 's $<.05$). The duration of time since the infant last ate did not significantly differ by age group ($p = .68$). Infants were significantly more likely to be fed breast milk at 2 months as compared to all other ages and least likely to be fed breast milk at 24 months (p 's $<.05$). Mothers had been asked to schedule their infant's feedings so that they did not occur during the visit and were reminded by the research assistant not to feed their infant, or themselves, during the study period.

Upon arrival to the doctor's office pre-test saliva samples were collected (for cortisol and alpha amylase analysis) from both the mother and her infant and the time of sampling was recorded. The research assistant then accompanied the mother-infant pair into the examination room. Infants were undressed by their mother, weighed and measured by a nurse and then examined by the infant's pediatrician. At the end of the visit the nurse gave the infant the standard set of inoculations administered via intramuscular injections while infants were on their back on the exam table restrained by their mother and nurse. Time of inoculations and number of injections were recorded. The duration of time between the pretest samples and the inoculation did not differ across the study groups (all p 's $>.3$). The number of injections received at 2, 6 and 12 months did not significantly differ (p 's $>.10$). Infants received significantly fewer injections at 24 months ($p <.05$). Following the inoculation, infants were soothed and dressed by their mother. Saliva samples were collected simultaneously from the mother and her infant at 5, 10 and 20 minutes after the first inoculation for sAA assessment and at 20 minutes post inoculation for cortisol analysis. The eight infants who were fed during the visit and their mothers (2 months $n = 3$, 6 months $n = 2$, 12 months $n = 3$) were excluded from all analyses.

Measures

Determination of Salivary Biomarkers—Saliva was obtained (without any stimulant) by placing a swab in the mother or infant's mouth. The swab was then placed in a saliva extraction tube (Roche Diagnostics). Samples were placed into a centrifuge (for ten minutes at 3000 rpm) to extract saliva and then stored in a freezer at -70°C until assayed.

Alpha-Amylase: All samples were transported on dry ice to Pennsylvania State University and stored frozen at -80°C until assayed for sAA. On the day of testing, all samples were centrifuged to remove mucins. Following Granger et al (2007), samples were assayed for sAA using a commercially available kinetic reaction assay (Salimetrics, State College PA). The assay employs a chromagenic substrate, 2-chloro-p-nitrophenol, linked to maltotriose. The enzymatic action of sAA on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm using a standard laboratory plate reader. The amount of α -amylase activity present in the sample is directly proportional to the increase (over a 2 minute period) in absorbance at 405 nm. Results are computed in U/mL of α -amylase using the formula: [Absorbance difference per minute x total assay volume (328 ml) x dilution factor (200)]/[millimolar absorptivity of 2-chloro-p-nitrophenol (12.9) x sample volume (.008 ml) x light path (.97)]. On average, the intra- and inter-assay coefficients of variance for sAA were less than 10% and 15%, respectively.

Cortisol—Saliva samples were assayed for cortisol determination employing a competitive luminescence immunoassay (LIA; IBL-America, Minneapolis, MN) with reported detection limits of 0.015 $\mu\text{g/dl}$. The cross reactivity of the assay was $<2.5\%$ with cortisone, prednisone and corticosterone and $<0.1\%$ with other naturally occurring steroids. Thawed samples were centrifuged at 3000 rpm for 15 minutes before assay. All of the samples from an infant were included in the same assay batch to eliminate within subject inter-assay variance. Each batch contained subjects from the four age groups. Data reduction for the LIA assay was done by an

automated four-parameter logistics computer program (software Mikro Win 2000; Berthold Microplate Luminometer). All samples were assayed in duplicate and averaged. The intra- and inter-assay coefficients of variance are 5.5% and 7.6%, respectively.

Data Transformation and Analysis Plan—Two of the response samples collected at the 2-month visit did not contain sufficient saliva for cortisol analysis. We screened mothers and infants separately for outliers greater than 3 standard deviations above the mean at each sampling interval. Using this criterion, two sAA samples from one mother were removed at the 12-month visit and one infant response sample was removed at the 6-month visit. These subjects were only excluded from analyses involving these samples. A square root transformation was then implemented to reduce skewedness. All values reported in the text are raw U/mL (sAA) or $\mu\text{g/dL}$ (cortisol) levels to facilitate interpretation.

Preliminary analyses were performed using correlations and t-tests to identify factors (e.g., time of sample collection, time since last feeding, state in car ride, breastfeeding status, race, birth order) that might influence sAA or cortisol levels. Factors significantly associated with salivary biomarkers (e.g., time of day) were included as covariates in subsequent analyses. To evaluate age-differences in infant sAA/cortisol levels, separate ANOVAs were computed with pre-test sAA/cortisol levels as the dependent variable and infant age group and sex as between subject factors. Planned t-tests were implemented to determine at what age differences in pre-test sAA/cortisol levels emerged. To identify differences between maternal and infant sAA/cortisol paired t-tests were performed with pre-test sAA/cortisol levels at each infant age group. To determine the sAA/cortisol response to inoculation for the mother and her infant separate 4 (sample collection time) by 4 (infant age group) by 2 (infant sex) repeated measures analyses of variance were implemented with age and sex as between group factors. Time of day entered as a covariate for maternal analyses. Where the overall model was significant, Bonferroni corrected t-tests were implemented to identify the sample collection times at which sAA/cortisol, differed from pretest levels. The relation between maternal and infant biomarkers and between sAA and cortisol were evaluated using Pearson correlations with time of day covaried in analyses including maternal samples. For these analyses, in addition to associations between pretest cortisol and sAA levels, the magnitude of the response to inoculation was calculated using delta scores.

Results

Preliminary Analyses

Neither infant sAA nor cortisol levels were associated with sample collection time (all p 's > .29). Furthermore, the duration of time since the infant last ate was not associated with sAA or cortisol (all p 's > .26). After controlling for infant age, whether or not infants slept in the car ride, the number of injections, and breastfeeding status were not significantly related to cortisol or sAA (all p 's > .2). As expected based on circadian variation of these hormones, maternal sAA levels were positively associated with sample collection time of day, $r(77) = .23$, $p < .05$, and maternal cortisol was negatively associated with time of day, $r(77) = -0.32$, $p < .01$. Time of day was entered as a covariate in all analyses including maternal samples. Maternal factors that have been shown to affect cortisol (Adam & Gunnar, 2001; Heinrichs et al., 2001) including age, marital status, whether this was her first child, breastfeeding status and race were not associated with maternal sAA or cortisol levels (p 's > .10).

Pre-test sAA Levels

Pre-test infant sAA levels differed by age group, $F(3,73) = 8.2$, $p < .001$. Twenty-four-month-old infants produced higher levels of pre-test sAA as compared to infants at 2, 6, and 12 months (all p 's < .01). Additionally, there was a trend for 12-month-old infants to produce higher pre-

test sAA levels as compared to infants younger than 12 months ($p's < .08$). Neither the main effect of sex ($p = .18$) nor the sex by age interaction was significant for infant pre-test sAA ($p = .97$). Infant sAA levels were significantly lower than maternal levels at 2, 6, and 12 months (all $p's < .05$), but not 24 months of age ($p = .89$). Maternal sAA levels were not affected by the age or sex of her child ($p's > .15$). See Figure 1 for maternal and infant sAA levels.

Maternal and infant pretest sAA levels were significantly associated, $partial\ r(74) = .31, p < .01$. Analysis of the association by age-group revealed a significant association at 6 months, $partial\ r(14) = .65, p < .01$, 12 months, $partial\ r(16) = .58, p < .01$, and 24 months, $partial\ r(19) = .47, p < .05$, but not 2 months, $partial\ r(16) = -.06, ns$.

sAA Stress Responses

For infants, there was a main effect of sampling time, $F(3,204) = 3.4, p < .05$, revealing that, on average, infants' sAA levels were higher at 5 minutes post inoculation as compared to all other time points (all $p's < .05$). Additionally, as shown in Figure 1, there was an infant age by sampling time interaction, $F(9,204) = 2.8, p < .01$. Planned post hoc tests revealed that at 2 months of age sAA levels did not significantly change in response to the inoculation ($p's > .38$). At 6 and 12 months infants' 5 minute post inoculation sAA levels were significantly higher than pre-test levels (all $p's < .05$) while 10 and 20 minute post inoculation sAA levels did not significantly differ from pre-test levels (all $p's > .15$). By contrast, at 24 months, sAA did not significantly differ between pre-test and 5 minutes post inoculation levels ($p = .45$) and showed a significant decline from pre-test to 10 and 20 minutes post-inoculation (all $p's < .05$), suggesting the possibility that sAA levels were elevated in anticipation of the well-baby visit and declined over the session. There was no significant main effect of infant sex nor were the interactions between sample collection time and infant sex significant (all $p's > .19$).

As shown in Figure 1, maternal sAA levels did not increase in response to the infant's reaction to the inoculation stressor, $F(3,201) = 0.06, p = .95$, nor did their pattern of sAA differ based on infant age ($p = .83$) or sex ($p = .10$). Because mothers did not display an sAA increase to the inoculation procedure, we did not have a measure of maternal sAA reactivity and thus we could not look at synchrony in maternal and infant sAA responses to stress.

Pre-test Cortisol Levels

Age and sex did not affect pre-test cortisol levels (main effect and interaction $p's > .20$). Further, maternal and infant pretest cortisol levels did not significantly differ ($p = .19$; see Figure 2). To examine synchrony of maternal and infant cortisol levels, partial correlations were computed using pre-test cortisol levels. The association between maternal and infant cortisol was not significant ($p = .45$).

Cortisol Stress Responses

For infants, there was a main effect of sampling time [$F(1,68) = 34.9, p < .01$] revealing that infant cortisol levels were higher 20 minutes following the inoculation as compared to pre-test levels. The infant age by sampling time interaction [$F(3,68) = 10.6, p < .01$], illustrated in Figure 2, indicates that infants displayed a significant increase at 2 and 6 months ($p's < .01$), but not at 12 and 24 months ($p's > .16$). The significant infant sex by sampling time interaction [$F(1,68) = 4.6, p < .05$] displayed in Figure 3 reveals that although both boys and girls displayed a significant increase in cortisol ($p's < .05$) boys displayed a larger cortisol response to the inoculation as compared to girls ($p < .05$).

As shown in Figure 2, even after controlling for time of day, maternal cortisol levels significantly decreased from pre-test to 20 minutes post infant inoculation, $F(1,68) = 10.7, p < .01$ possibly reflecting anticipatory concern or stress related to bringing the infant to the

pediatricians office. The pattern of maternal cortisol did not differ based on infant age or sex (p 's > .3). Mothers did not display a cortisol increase compared to pre-test levels in response to the inoculation procedure, thus we could not look at synchrony in cortisol responses to inoculation.

Relations between sAA and cortisol

Neither overall nor within the four age groups was pre-test infant sAA correlated with cortisol (p 's > .18). For infants overall the change in sAA from pre-test to peak response (5 minutes post) was not significantly correlated with the change in cortisol from pre-test to response (20 minutes post) [$r(75) = .20$ $p = .10$]. However, when the pattern of association was examined within age group, 6-month old infants who displayed a greater sAA response also displayed a greater cortisol response [$r(16) = .55$ $p < .05$]. No significant associations were noted at other age groups (p 's > .17). We did not obtain a measure of cortisol or sAA reactivity for mothers. Maternal pre-test sAA and cortisol, however, were not correlated [partial $r(74) = .05$, $p = .67$].

Discussion

This study applied a minimally-invasive and multi-system measurement approach to assess developmental differences in infants' biological stress responses to a standardized stressor. To our knowledge, this is the first study to evaluate age-related changes during the first two postnatal years in both sAA and cortisol responses to a standardized stressor (the well baby exam/inoculation procedure). Our findings are consistent with prior work evaluating developmental differences in salivary cortisol responses, but add the novel component of assessment of a proposed marker of SNS activity, sAA. Unique to this study, we identified age-related differences in both pre-test sAA and sAA responses to stress as well as in synchrony of sAA between the mother and her infant. Specifically, the findings suggest that by 6 months of age, but not at 2 months, levels of sAA are responsive to environmental stimuli. This study specifically addresses previously unanswered questions related to the time course of the sAA response to stress in infancy and developmental differences in sAA stress responses.

What is the time course of sAA response to stress during the first two postnatal years?

This study evaluated sAA levels prior to the well-baby exam and inoculation procedure (pre-test) and at 5, 10 and 20 minutes post injection. Our data document that, on average, the peak sAA response to this stressor occurs at 5 minutes after the inoculation and returns to pre-test levels by 10 minutes post inoculation. These data are consistent with findings from studies with older research participants (Gordis et al., 2006; Stroud et al., in press) and have important implications for researchers interested in capturing the sAA stress response. Several prior studies with infants and young children have used samples collected for the purposes of cortisol (i.e., at 20 minutes post stressor) analysis to evaluate sAA responses to an event (Granger et al., 2007; Hill-Soderlund et al., 2008; Fortunato et al., 2008). It is likely that these studies missed the sAA response to stress which occurs at 5 minutes post stressor and recovers by 10 minutes post stressor. The current study underscores the importance of considering the timing of sampling when interpreting patterns and themes in sAA findings across studies.

Are there developmental differences in sAA responses to stress and how do these differences compare to developmental changes in salivary cortisol responses?

As anticipated, levels of pre-test sAA increased across the first two postnatal years. Levels were lowest at 2 and 6 months and highest at 24 months. Maternal sAA levels were higher than infant levels at 2, 6, and 12 months, but not at 24 months of age. This developmental difference in sAA levels is consistent with the oral biology literature which suggest that alpha amylase levels in oral fluids is partially related to the emergence of dentition and the corresponding dietary shift towards regular consumption of solid foods (O'Donnell & Miller, 1980).

A novel component of the current investigation involved the identification of developmental differences in the sAA response to stress. We found that at 2 months, not only were pre-test sAA levels low, but infants did not display significant increases sAA in response to the inoculation stressor. The current findings are consistent with a recent study demonstrating a cardiac, but not a sAA response to a heelstick stressor in newborns (Schaffer et al., 2008). Sympathetic innervation of the salivary glands develops postnatally (Knox & Hoffman, 2008). Given that an autonomic response to stress is present even among neonates (Pineless et al., 2007; Schaffer et al., 2008), these data suggests that at 2 months the association between SNS activity and sAA may not be mature and thus, at this age (and younger) sAA can not be used as a surrogate marker of individual differences in SNS stress responses. At 6 and 12 months of age infants displayed a significant increase in sAA that peaked at 5 minutes and returned to pre-test levels by 10 minutes post stressor indicating sAA responses to stress can be measured as young as 6 months of age. The pattern displayed by the 24-month-old infants was distinctive with the highest sAA levels occurring at the pre-test assessment and declining across the assessment period. The explanation for this finding is unclear. At 24 months of age infants also did not display a significant cortisol increase to this stressor; possibly the inoculation procedure is not stressful at this age. It is important to note that infants received significantly fewer injections at 24 months perhaps making the procedure less aversive. The decrease in sAA across the assessment period may reflect an *anticipatory* response suggesting these older infants may already be aroused at the pre-test sample, a response that diminishes after the stressor has passed. Future studies will need to collect time matched samples at home and on days other than the day of the well-baby visit to address this issue.

By contrast, cortisol levels increased in response to the well-baby exam/inoculation stressor at 2 and 6 months of age, but not at 12 and 24 months. The robust increase in cortisol at 2 and 6 months in response to this stressor is consistent with previous studies evaluating developmental changes in cortisol stress reactivity (Lewis & Ramsay, 1995; Gunnar et al., 1996). At 12 months the increase in cortisol in response to the well-baby exam/inoculation did not achieve statistical significance, possibly due to our small sample size and the greater variability in cortisol responses at this age. As expected at 24 months infants did not display an increase in cortisol to the inoculation stressor. Numerous studies have shown that during the second postnatal year it becomes increasingly difficult to elicit a cortisol response to a variety of mild stressors (see Gunnar & Donzella, 2002 for review) and there is variability among studies as to whether the well-baby exam/inoculation elicits a cortisol response between 12 and 24 months (Lewis & Ramsay, 1993; Gunnar et al., 1996). Analyses additionally revealed sex differences in cortisol stress responses to this event with boys displaying a significantly greater cortisol stress response. This finding is inconsistent with prior studies of infant inoculation demonstrating either no sex difference (Lewis & Ramsay, 1995) or larger cortisol responses among girls (Gunnar et al., 1996), but consistent with adult work demonstrating larger stress responses in males (Kirschbaum & Hellhammer, 1994). These data evaluating sex differences should be considered preliminary due to the relatively small sample size within each age group and interpreted with caution.

Is there synchrony in sAA and cortisol between mothers and their infants?

A secondary aim of this investigation was to evaluate whether maternal and infant sAA and cortisol levels were correlated during the well baby exam/inoculation procedure. Synchrony was only evaluated among pre-test levels as mothers did not display significant sAA or cortisol increases in response to the well baby exam/inoculation procedure. Maternal and infant pretest sAA levels were correlated at 6 through 24 months, providing evidence for synchrony in maternal and infant SNS regulation. Levels were not associated at 2 months; consistent with evidence that sympathetic regulation of sAA is immature in 2-month-old infants. In contrast, no such association was found between maternal and infant cortisol levels. Although prior

work has noted synchrony between maternal and infant cortisol, (Spangler, 1991; Sethre-Hofstad, Stansbury & Rice, 2002; Stenius et al., 2008; Thompson & Trevathan, 2008) there is evidence that synchrony is only observed if mothers were high in maternal sensitivity (Sethre-Hofstad et al., 2002; van Bakel & Riksen-Walraven, 2008). Since quality of maternal care was not evaluated in the current investigation we were not able to assess the moderating role of maternal sensitivity.

Is there coordination between the HPA and SNS as measured by sAA and cortisol?

No association was detected between pre-test cortisol and sAA for mothers or infants. These data are consistent with the majority of published studies in the area (Chatterton et al., 1996; Nater et al., 2006; van Stegeren, Rohleder, Everaerd & Wolf, 2006; see Kivlighan and Granger 2006 for an exception). The pattern of association for stress reactivity in our study was less consistent. We did not have an index of maternal reactivity. Evidence for coordination between sAA and cortisol reactivity in infants emerged at 6 months, but not other ages. It is possible that the effect was limited to the 6-month time point because it was the only age at which *both* sAA and cortisol reactivity were observed. The HPA and SNS clearly work together to generate physiologic changes associated with stress responses; the nature of this association, however, remains unclear. These results should be interpreted with caution and replication with a larger sample size and additional cortisol samples is needed.

Limitations

This study was conducted in the clinical setting of the infant well-baby exam. Because visits were scheduled at the mother's convenience, we were not able to control time of day. This may have created noise in the data that may have masked associations between maternal and infant cortisol. However, these data clearly document the expected stress-related increases in cortisol in response to the inoculation procedure and, for the first time, document developmental changes in the sAA response to this stressor. Second, the small sample size at each age limits the ability to address secondary questions related to synchrony between mother-infant pairs and coordination between sAA and cortisol. Further, because mothers did not respond to the infant inoculation with an increase in sAA or cortisol, we were not able to evaluate synchrony in maternal and infant biological stress reactivity. Third, the current data suggest the possibility that mothers and older infants may be displaying physiological arousal in anticipation of the well-baby visit/inoculation. Future studies would benefit from the collection of samples on a typical day without a major stressor in order to address this issue. Fourth, sAA regulation is influenced by numerous factors including the development of sympathetic innervation of the salivary glands, dietary changes, and emerging dentition. Further research is necessary to evaluate the sources of individual variation in sAA levels in early life. Clearly, we need more information about the basic oral biology from a developmental perspective.

Conclusions

This study is the first to characterize developmental changes in both sAA and cortisol responses to stress over the first two postnatal years. The primary goal of this investigation was to address critical unanswered questions necessary for the evaluation of sAA during infancy and early childhood. We have shown that the sAA response to stress peaks by 5 minutes after the stressor and returns to pretest levels by 10 minutes post stress. Thus, inclusion of this early assessment time point is necessary to evaluate sAA changes in response to stress. This finding raises serious concern about the practice of assaying samples timed for cortisol collection (20 min post stressor) for sAA analysis. Second, we document that it is possible to evaluate sAA responses to a stressor in infants as young as 6 months of age. Further, by 6 months, as in older infants, sAA levels are correlated in maternal infant pairs. This evidence that sAA is influenced by

social and nociceptive stimuli, suggests that sAA can be used as a marker of adrenergic responses to stress in infants as young as 6 months.

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Figure 1.
Infant and maternal sAA responses to infant inoculation

Figure 2.
Infant and maternal cortisol responses to infant inoculation

Figure 3.
Sex differences in infant cortisol response to inoculation

Table 1

Demographic information for the study sample

	2-Months	6-Months	12-Months	24-Months
Gestational age at birth (wks.)	39.0 (1.2)	39.2 (.10)	39.1 (1.1)	39.2 (1.2)
Birth weight (g.)	3398 (428)	3601 (458)	3459 (414)	3334 (381)
Apgar score (median & range)	9 (8–10)	9 (8–10)	9 (8–10)	9 (8–10)
Maternal age (yrs.)	29.3 (5.1)	28.4 (5.5)	28.8 (3.6)	29.6 (5.5)
Married	81.8%	63.2%	77.3%	86.4%
First born	50%	53%	46%	28%
Education				
High school or equivalent	90.5%	100%	100%	100%
College graduate	61.9%	53.0%	59.0%	45.4%
Annual household income				
\$0 to \$30,000	9.6%	27.7%	9.0%	19.1%
\$30,001 and \$60,000	14.3%	33.4%	13.7%	33.3
\$60,001 and \$100,000	52.3%	16.7%	50.0%	23.8%
Over \$100,000	23.8%	22.2%	27.3%	23.8%
Race/Ethnicity				
Non-Hispanic white	40.9%	52.6%	36.4%	45.5%
Hispanic	28.6%	29.4%	36.4%	36.4%
Asian	18.2%	5.3%	22.7%	4.5%
Other	12.3%	12.7%	4.5%	13.6%

Table 2

Infant factors that potentially influence sAA or cortisol (Mean and SD or percent)

	2-Months	6-Months	12-Months	24-Months
Time of inoculation	12:24 (2:33)	12:22 (2:00)	11:21 (2:39)	12:55 (2:40)
Percent asleep in car ride	47.4%	47.1%	5.3%	13.6%
Time between inoculation and pre-test sample (min.)	63 (27)	58 (24)	56 (18)	47 (19)
Time between inoculation and last infant feeding (min.)	94.8 (63)	105.9 (60.9)	86.0 (46.2)	99.1 (66.7)
Percent breastfed	86.4%	36.8%	27.3%	4.5%
Number of injections	3.6 (0.7)	3.2 (0.7)	3.1 (1.0)	1.0 (0.2)