Pupil and Salivary Indicators of Autonomic Dysfunction in Autism Spectrum Disorder

Christa J. Anderson*, John Colombo, and Kathryn E. Unruh
The University of Kansas, Life Span Institute, 1000 Sunnyside Avenue, Room 1052, Lawrence
KS, 66045

Abstract

Dysregulated tonic pupil size has been reported in Autism Spectrum Disorder (ASD). Among the possible sources of this dysregulation are disruptions in the feedback loop between norepinephrine (NE) and hypothalamic systems. In the current study, we examined afternoon levels of salivary alpha-amylase (sAA, a putative correlate of NE) and cortisol (used to assess stress-based responses) in two independent samples of children with ASD. We found a larger pupil size and lower sAA levels in ASD, compared to typical and clinical age-matched controls. This was substantiated at the individual level, as sAA levels were strongly correlated with tonic pupil size. Relatively little diurnal variation in sAA taken in the home environment in the ASD group was also observed, while typical controls showed a significant linear increase throughout the day. Results are discussed in terms of potential early biomarkers and the elucidation of underlying neural dysfunction in ASD.

Keywords
Autism Spectrum Disorder; Autonomic Nervous System; Pupil Size; Norepinephrine; Alpha-amylase; Cortisol; Eye-Tracking

The past 15 to 20 years have seen an increase in the prevalence of Autism Spectrum Disorder (ASD) (Fombonne, 1999; Gernsbacher, Dawson, & Goldsmith, 2005). The Center for Disease Control and Prevention (CDC, 2012) has estimated the occurrence of ASD to be as high as 1 out of 88 births, making it one of the highest-incidence childhood neurodevelopmental disorders. As a result, ASD has been classified by the CDC as an urgent public health priority and have identified two interrelated goals to address this disorder: First to identify biomarkers that can serve to improve early detection of ASD, and second to investigate the physiological signatures of the disorder that might ultimately lead to greater insight into the neurodevelopmental processes that underlie ASD.

Characteristically, ASD has a late diagnosis, being diagnosed on average between 5 and 9 years of age (Hertz-Picciotto & Delwiche, 2009). The late diagnosis impedes the use of early behavioral and educational interventions when they appear to be most efficacious (i.e., before 24 months of age; Dawson, 2010; Harris & Handleman, 2000). Behavioral assessments for administration before 24 months of age have been developed and are commonly used to identify ASD, but due to the due to the subtle and heterogeneous nature
of ASD behavioral profiles at that early age (Pierce, Glatt, Liptak, & McIntyre, 2009; Zwaigenbaum et al., 2009) these measures have been criticized (Barbaro & Issanayake, 2009; Yirmiya & Charman, 2010) as lacking the sensitivity and specificity required for universal screening of the disorder. These problems highlight the desirability of biomarkers with high sensitivity and specificity to ASD into early detection and screening protocols. Because some of the neural impairments commonly found in ASD may be present during the prenatal period (Bauman & Kemper, 2005) it seems reasonable to posit the presence of a systemic physiological indicator of these impairments well before the common age of diagnosis. Such an indicator would be advantageous for early screening and detection, and could conceivably address the requisite need to obtain a better understanding of the early neurodevelopmental processes that occur in ASD (Dawson, 2010; Ratajczak, 2011; Zwaigenbaum, 2010).

**Rationale for the Current Study**

Previous studies have found dysregulated tonic pupil responses to consistently distinguish children with ASD from controls. Rubin found a smaller pupil size to a dark stimulus and a larger pupil size to a light stimulus in ASD compared to controls (Rubin, 1961). Anderson and Colombo (2009) subsequently found two- to five-year-old children with ASD to have a larger tonic pupil size to a light stimulus (5.1 lx) than age-matched controls. Fan et al. (2009) demonstrated alterations in pupil latency, amplitude and velocity to light stimuli in addition to dysregulated pupil responses to both light and dark stimulation in ASD. Finally, Martineau et al. (2011) found a smaller pupil response to a dark stimulus (0.0 lx) in ASD compared to controls. It is worth noting that Martineau et al. (2011) interpreted their results as being incongruent with previous findings, but given that the typical response to a light stimulus is a decrease in pupil size and increase to a dark stimulus their findings reported in these studies are quite consistent with the general finding of pupillary dysregulation in ASD. These findings are also congruent with other ANS investigations where altered resting and reflex autonomic responses such as cardiac and electrodermal activity (Bal et al., 2009; Ming, Julu, Brimacombe, Connor, & Daniels, 2005; Van Hecke et al., 2009), decreased sleep (Giannotti et al., 2008; Honomichl, Goodlin-Jones, Burnham, Gaylor, & Anders, 2002; Oyane & Bjorvatn, 2005), and blunted pain perception (Baranek & Berkson, 1994; Kientz & Dunn, 1997) in ASD. The current study sought to replicate and extend previous studies by examining the neural systems responsible for the control of pupil size in young children with ASD.

We view tonic pupil size as an exemplar of the type of measurement that would address the CDC priorities described above. Tonic pupil size is accessible before 24 months of age (Boev et al., 2005; Robinson & Fielder, 1990), and the potential sources of neurological dysfunction in pupil size are finite (Beatty & Lucero-Wagoner, 2000). This measure also has the advantage of being more sensitive to small-scale changes and small sample sizes than other autonomic measures such as heart-rate and electrodermal responses (Tursky, Shapiro, Crider, & Kahneman, 1969). The tonic size of the pupil is obtained by measuring reflexive pupillary responses to changes in luminance and accommodation efforts. Such measures must be independent of cognitive load, and change with alertness and diurnal variation.

**Neuroanatomy of the pupillary system**

Noted above, the neural pathways that regulate tonic pupil size are finite and limited; optimal pupillary function is determined by a balance of inhibitory and excitatory activity within the sympathetic and parasympathetic components of the ANS (Beatty & Lucero-Wagoner, 2000). The two most important components of the pupillary system are the norepinephrine (NE) [locus coeruleus (LC), ventrolateral medulla (A1), and ventrolateral pons (A5)] and hypothalamic systems (lateral and posterior hypothalamus); these systems...
provide both inhibitory and excitatory influences that are mediated by NE, and histamine and orexin, respectively (Guyenet, 1991; Hou, Langley, Szabadi, & Bradshaw, 2007; Stenberg, 2007; Szabadi & Bradshaw, 1996). As such, alterations in this balance can affect resting and reflex autonomic activity, sleep-wake cycle patterns, and the processing of incoming information, all of which have been found to be impaired in individuals with ASD as presented above. Therefore, dysregulated tonic pupil responses, along with other ANS mediated dysfunction in ASD, may be traced to perturbations in one or both of these systems—The role of the hypothalamic pituitary adrenal axis (HPA) is widely known and has been examined in depth (Corbett, Mendoza, Abdullah, Wegelin, & Levine, 2006; Corbett, Mendoza, Wegelin, Carman, & Levine, 2008; Spratt et al., 2012), but the case for the involvement of NE in ASD is less familiar; we have briefly reviewed the NE-system in the sections that follow.

The NE-system and ASD—A relatively large literature exists on NE and ASD. Six studies have shown that measures of plasma NE are elevated in ASD (Cook, 1990; Israngkun, Newman, & Patel, 1986; Lake, Ziegler, & Murphy, 1977; Launay et al., 1987; Leboyer et al., 1994; Leventhal, Cook, Morford, Ravitz, & Freedman, 1990); two other studies yielded null results (Herault et al., 1994; Martineau et al., 1994). These findings of elevated NE were initially dismissed as a byproduct of heightened acute stress response to venipuncture in children with ASD (Cook, 1990; Minderaa, Anderson, Volkmar, Akkerhuis, & Cohen, 1994), and subsequent studies designed to address this issue used time-averaged measures of NE-release (which are not affected by acute stress). Interestingly, the majority of studies controlled in this manner yielded decreased time-averaged measures of NE release and its metabolites (Barthelemy et al., 1988; Lake, et al., 1977; Martineau, Barthelemy, Jouve, Muh, & Lelord, 1992; D. M. Young, Schenk, Yang, Jan, & Jan, 2010; J. G. Young, Cohen, Brown, & Caparulo, 1978; J. G. Young, Cohen, Caparulo, Brown, & Maas, 1979). Although the role of the NE-system in ASD had been de-emphasized by some (Cook, 1990; Minderaa, et al., 1994), recent research has renewed interest in pursuing the topic of NE-system functioning in ASD. This newer work includes findings of aberrant autonomic function in ASD (Anderson & Colombo, 2009; Bal, et al., 2009; Giannotti, et al., 2008; Ming, et al., 2005; Van Hecke, et al., 2009) and pharmacological studies showing positive improvements in higher-order ASD symptomology following the administration of β-adrenergic antagonists (e.g., propranolol; Beversdorf, Carpenter, Miller, Cios, & Hillier, 2008; Narayanan et al., 2010).

Salivary alpha-amylase (sAA)—In the current study we chose to examine a salivary correlate of NE-system activation, alpha-amylase (sAA). sAA is a digestive enzyme that is enriched with amylose, a salivary protein involved in the digestion of starches (Scannapieco, Torres, & Levine, 1993). The release of sAA is stimulated by parasympathetic and sympathetic innervations of α- and β-adrenergic receptors, leading to secretion of sAA from the parotid salivary gland (Turner & Sugiya, 2002). In most studies (Chatterton, Vogelsong, Lu, Eilman, & Hudgens, 1996; Rohleder, Nater, Wolf, Ehler, & Kirshbaum, 2004; Wetherell et al., 2000; but see Nater et al., 2006a) sAA has been found to be correlated with diurnal (daytime) variations and basal levels (from a single resting measure) of NE, along with phasic (task-specific) changes in plasma NE following the presentation of a physical or psychological stressor. Although sAA has never been examined in conjunction with pupil size, it has been found to vary with changes in other autonomic responses such as cardiac (Bosch, de Geus, Veerman, Hoogstraten, & Amerongen, 2003; Nater et al., 2006a) and electrodermal activity (El-Sheikh, Erath, Buckhalt, Granger, & Mize, 2008). Furthermore, like plasma NE, sAA decreases in response to β-adrenergic blockers (van Stregeren, Rohleder, Everaerd, & Wolf, 2006) and increases in response to α2-adrenergic antagonists.
(Nater et al., 2006b). These findings provide support for sAA as an indirect estimate of NE-system activation within the ANS (Nater & Rohleder, 2009).

Based on the difficulties associated with the venipuncture necessary for obtaining plasma levels of NE in children with ASD, the collection of sAA affords a number of important advantages. Correlates such as sAA have the potential to provide early and non-invasive biomarkers of ASD, and have the potential to substantially impact our knowledge of the underlying neural systems in ASD due to the link among sAA and ANS central NE-system pathways.

Finally, because previous findings of dysregulated NE in ASD had been attributed to a stress response arising from data collection procedures, we also included a measure of cortisol to provide an independent assessment of stress in response to the current study procedures. This enabled us to determine if sAA concentrations and tonic pupil size were affected by aversion to the testing environment and/or collection method, or if sAA and tonic pupil size are in fact accurate and viable indicators of altered ANS activation.

Current Studies

In this report we describe examinations of tonic pupil size, sAA, and cortisol in two independent samples. In Study 1 we examined single afternoon levels of sAA and cortisol taken at the laboratory and tonic pupil size in a small sample of children with ASD, along with clinical and typically-developing (TD) age-matched controls. In Study 2 we sought to replicate the findings of Study 1 with a larger sample of children with ASD compared to TD age-matched controls, and extended this examination to include diurnal variations of sAA taken at various time-points throughout the day in the child’s home environment to provide a more comprehensive investigation of sAA levels. Due to differences in the control subjects and measurement method we have reported on the samples separately, but we have also included an aggregated analysis of tonic pupil size and afternoon sAA from both studies comparing ASD and TD samples in an attempt to evaluate the discriminate ability of these measures with a larger sample size.

STUDY 1

Method

Participants—Children between the ages of 20 to 72 months of age were recruited for this study if they had a diagnosis of Autistic Disorder (AD), Pervasive-Developmental Disorder-Not Otherwise Specified (PDD-NOS), Down Syndrome (DS), or if they were typically-developing (TD). Children were recruited through mail from a variety of developmental disability organizations in metropolitan and suburban areas of Kansas City, KS and MO, and through a pre-established commercial list of families. Using this criterion, 37 children were recruited and seen for testing appointments and data from 32 participants were used in the final analysis for Study 1. Participants were given $50 per session ($100 total) to compensate for time and travel.

Exclusion/attrition criteria: Children were excluded from participation if they had impairments in vision and/or motor functioning that could significantly impede their ability to participate in the testing session (e.g., severe vision loss or inability to sit upright without assistance). With the exception of the ASD or DS diagnosis, none of the children had a history of chronic illness or medication use (i.e., none of the children were chronically taking any prescription or over-the-counter medications); the only exception to this was seasonal allergies and occasional anti-histamine use (n = 8). All children who participated in the current study were healthy (i.e., did not have any symptoms of acute illness such as cold.
flu, allergies, etc.) and were medication-free at least 48 hours prior to the testing sessions (i.e., those that were taking anti-histamines were asked to refrain from use for at least 48 hours and participated while they did not have allergy symptoms). Data from three participants was unusable due to inaccurate or insufficient calibration during both testing sessions, and two children were excluded because of the group-matching strategy.

**Group assignment:** The Autism Spectrum Disorder (ASD, \(n = 12\)) group consisted of children who were formally given a diagnosis of either AD (\(n = 8\)) or PDD-NOS (\(n = 4\)) by established diagnostic clinics in the Kansas City area that include teams of licensed professionals who have been formally trained and certified to provide an official diagnosis along the ASD spectrum. Diagnosis was verified by a trained member of our laboratory through Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord, Rutter, & DiLavore, 1997) administration of Module 1 or 2 (this was based on the child’s verbal ability). Both ADOS-G Modules utilize a semi-structured play observation designed to provide an opportunity to observe the presence or absence of the core deficits of AS and PDD-NOS.

The age and gender composition of the ASD group dictated the recruitment and formation of the DS and TD control groups. A one-way univariate ANOVA indicated no between-group differences on chronological age (CA), \(F(2, 29) = .129, p = .879\), \(\eta^2 =.009\), indicating that the three groups were statistically matched on this variable. As expected, the groups varied significantly on mental age (MA), based on the Mullen Early Learning Composite Score (Mullen, 1995), \(F(2, 29) = 16.785, p < .001\), \(\eta^2 =.537\). A one-way MANOVA was also conducted to examine between-group differences in four subscale age-equivalents on the Mullen (Visual Reception, Fine Motor, Receptive Language, and Expressive Language); significant between-group differences were found among the four subscales of the Mullen, Wilk’s \(\Lambda = .287, F(8, 42) = 4.552, p < .001\), \(\eta^2 =.464\), with each of the subscales showing significant between-group differences (all \(p < .05\)). Follow-up comparisons of MA and Mullen subscales are presented with each control group’s description below.

The DS (\(n = 9\)) group consisted of children with a pre-established diagnosis that was independently verified by a genetics laboratory at birth, without a comorbid ASD diagnosis; this was confirmed by a trained member of our laboratory through ADOS-G Module 1 or 2 administration. The DS group was matched with the ASD group on CA (\(p = .791\)), MA (\(p = .714\)), subscale equivalents on the Mullen (all \(p > .10\)), and gender distribution. The TD group (\(n = 11\)) was comprised of children who had scores on all subscales of the Mullen not less than one standard deviation below the test mean, and who did not have a diagnosed developmental disability. The TD group was matched with the ASD group on CA (\(p = ns\)), and gender distribution, \(\hat{\eta}^2(2) = ns\), but not on MA (\(p < .001\)) or Mullen subscales (all \(p < .05\)). None of the children in the TD or DS groups had a biological parent or sibling with a diagnosis of ASD or other mental health issues such as schizophrenia, cognitive and language delays. See Table 1 for age, gender, and demographic information for each group.

**Testing Sessions**—Participants were seen at the laboratory for two testing sessions conducted at the same afternoon time (between 12:00 and 5:00 pm) on 2 different days (the difference in data collection times from Day 1 to Day 2 was quite small, \(M = 11.35\) minutes) to ensure that differences were not due to diurnal variations, as sAA and cortisol are less stable during morning hours (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007; Rohleder, et al., 2004). Study appointments were scheduled to be approximately one to two weeks apart (\(M = 14.04\) days between sessions). Participants did not consume any cafffeinated products within two hours, no food or milk (or other protein-enhanced products) within one hour, and no liquids within 10 minutes of their scheduled sessions to avoid interference with salivary collection (Granger, Kivlighan, El-Sheikh, Gordis, & Stroud, 2004).
2007; Hanrahan, McCarthy, Kleiber, Lutgendorf, & Tsalkian, 2006). Furthermore, appointments were scheduled on typical days that were free of stressful or atypical events (e.g., first day of school, birthday party, loss of a favorite toy, car wreck etc.) and parents were asked to reschedule their appointments if any unusual or stressful events occurred. Parents were also asked to keep their child inactive and ensure that their child did not engage in any substantial physical activity (e.g., running, biking, swimming, etc.) at least 1 h prior to the testing sessions; participants were reported to last engage in physical activity between 1.5 and 48 hours prior to the testing sessions (M = 12.63 hours). Parent confirmation of compliance with these restrictions was confirmed prior to the start of each session.

**Tonic Pupil Size and Laboratory Saliva Sample Collection:** During each testing session children were presented with a blank grey slide (3.0 lx) for three minutes to allow adequate time to collect tonic pupil size and a single afternoon measure of sAA and cortisol; thus the blank slide allowed us to obtain an un-stimulated or non-phasic measure of pupil size and saliva concentrations. Tonic pupil size was obtained during the first minute of the blank grey slide presentation, and saliva was collected during minutes 2 – 3 of the slide presentation. The luminance of the slide was measured using a hand-held photometer under the same distance and ambient conditions that were used during testing (see apparatus section). Luminance readings were taken repeatedly during study set-up to ensure accuracy and stability of measurement, and were checked regularly once data collection began to ensure that set luminance levels were maintained.

**Tonic pupil size:** To ensure an accurate “resting” pupil measure, experimenters and parents were asked to remain silent and not interact with the child during the first minute of slide presentation when tonic pupil size was being obtained. Parents were allowed to be in the room with the child but siblings were not. If the parent remained in the testing room, they were required to stay behind a partition where they could see their child on the television monitor but their child could not see or interact with them. The child was also unable to see the experimenter.

**Laboratory saliva sample collection:** While the parent was completing paperwork a “practice” saliva sample was taken from the child in the standardized testing room to familiarize them with the collection procedure; this practice procedure was done on both testing days. A single afternoon measure of salivary cortisol and sAA were taken one minute after the start of the blank grey slide by simultaneously placing three Sorbettes (provided by Salimetrics, LLC) under the child’s tongue for approximately two minutes. To minimize disturbance of the child during saliva collection, the experimenter discretely obtained samples by standing to the side and out of the child’s line of view and minimized their interactions with the child. Sorbettes were then placed into 2 mL cryovials and immediately frozen at -20° C. All samples were sent to Salimetrics to be assayed.

**Apparatus:** The stimulus was presented on a 40.6 cm computer monitor, which subtended a 21.6° visual angle at the viewing distance. Pupillary responses were recorded using an Applied Science Laboratory (ASL) E6 eye-tracking system, Model 504 (ASL, 2008) with the GazeTracker interface program (Eye-Gaze Response Interface Computer Aid, 2001) setup in a darkened interior room. The pan/tilt module, a component of the ASL system, uses infrared technology to illuminate the eye and telephoto an image of the eye onto an eye camera. The E6 control unit then extracts the pupil and reflection of the light source on the cornea and computes pupil diameter at a sampling rate of 60 Hz.

Each child was secured in a child-sized car seat using a five-point restraint to ensure the child’s safety and to minimize movement (i.e., the child was unable to move forward or

*Dev Psychobiol. Author manuscript; available in PMC 2014 July 01.*
backward to control for accommodation efforts). The car seat was secured onto a hydraulic chair, which enabled adjustment of the child’s eye height to be approximately centered with the mid-point of the stimulus monitor (124.5 cm). The car seat was slightly reclined to minimize head movement.

**Data Extraction and Reduction**

**Tonic pupil size:** Traces of pupil data that occurred within the screen coordinates (on the screen) for the first minute of the blank grey slide presentation was inspected and corrected for artifacts (blinks, loss of tracking, partial eyelid closures, head movements, and accommodation responses) using linear interpolation. Artifacts were identified as (a) a time difference between data points that were greater than 20 ms, (b) a discontinuity in pupil data, represented by a 0-value in the data file, or (c) a difference in pupil size that was greater than 0.20 mm. Each pupil trace included in the final analysis was at least 500 ms in length, with artifacts no longer than 500 ms and not exceeding 20% of the duration of the entire trace. An average pupil size (tonic pupil size) was computed from the corrected trace during minute-1 of the blank grey slide presentation (i.e., pupil data from the last two minutes of the baseline slide were not used) for both testing days. These artifact removal and average score computation methods are similar to those used in several previous studies (Granholm, Asarnow, Sarkin, & Dykes, 1996; Steinhauser, Siegle, Condray, & Pless, 2004; Verney, Granholm, & Marshall, 2004). Because luminance was held constant (3.0 lx) across both days for all groups, no data corrections were needed to adjust for changes in luminance as there were none.

**Laboratory saliva data:** Concentrations of sAA (U/mL) were subjected to square root transformations and cortisol concentrations (ug/dL) were subjected to a logarithm transformation to adjust for positive skew and variability, as in previous salivary studies (Fortunato, Dribin, Granger, & Buss, 2008; Gordis, Granger, Susman, & Trickett, 2006; Granger, et al., 2007).

**Data Analysis**

Tonic pupil size and afternoon measures of sAA and cortisol were first analyzed for effects of measurement order by evaluating within-group differences and interclass correlation coefficients (ICC) and Cronbach’s Alpha reliability statistics to assess response stability between Day 1 and Day 2. Because pupil and salivary measures from both testing days were necessary to evaluate within-group differences, the sample size for these analyses are smaller than that of the subsequent between-group analyses (six subjects had data for only one testing session). Prior to data analysis, univariate outliers were identified; using a criterion of scores greater than 4 SD from the group mean, three outliers for tonic pupil size (two for afternoon sAA and one for afternoon cortisol) were identified and removed. Because the age range of the current study had a fairly wide span (20 to 72 months of age) between-group differences in all pupil and afternoon saliva measures taken at the laboratory were evaluated with an ANCOVA, with CA entered as a covariate. To ensure that the ANCOVA met the homogeneity assumption, homogeneity of the variance-covariance matrices were evaluated at the $p < .05$ level using Levene’s estimate and were found to be non-significant for all analyses in Study 1. Follow-up analyses were evaluated using the LSD adjustment for multiple comparisons.

**Results**

**Tonic Pupil Size**—Within-group differences and ICCs among tonic pupil size, measured during appointment Day 1 and Day 2, were first examined. The results of the paired-samples $t$-test was non-significant, $t(22) = -.699$, with satisfactory ICCs, $r = .774$, and reliability,
alpha = .87; thus, tonic pupil size from both testing days was averaged to form one variable. A univariate Diagnosis (3) ANCOVA (controlling for CA) was then performed to evaluate between-group differences in tonic pupil size, and was significant, $F(2, 25) = 6.244, p = .006, \eta^2 = .333$. Follow-up analyses of the Diagnosis main effect indicated that the ASD group had a larger adjusted mean tonic pupil size ($M_{adj} = 5.593$) that varied significantly from the adjusted means of the DS ($M_{adj} = 4.199, p = .003$) and TD ($M_{adj} = 4.535, p = .018$) groups (whose tonic pupil size did not differ significantly, $p = .447$). We also found a strong correlation among tonic pupil size and total ADOS-G scores, $\rho(25) = .490, p = .013$. These results replicate the Anderson and Colombo (2009) study of an enlarged tonic pupil size in ASD, and demonstrate that the larger tonic pupil size in ASD is independent of CA. See Figure 1 for observed means of tonic pupil size.

**Afternoon Saliva Measures**—Within-group differences and ICCs for sAA and cortisol measurements, taken at one afternoon time point during Day 1 and Day 2 appointments, were also examined. The results of the paired-samples $t$-test was non-significant for both sAA, $t(24) = -.845, p = ns$, and cortisol, $t(23) = 1.650, p = ns$; the ICCs and reliability statistics were satisfactory for both sAA ($r = .808, alpha = .893$) and cortisol ($r = .434, alpha = .451$), therefore saliva measures from both testing days were averaged together for final analyses.

**Afternoon sAA**: The univariate Diagnosis (3) ANCOVA (with CA held constant) for between-group differences in afternoon levels of sAA revealed a significant main effect of Diagnosis, $F(2, 28) = 3.420, p = .047, \eta^2 = .196$. Follow-up analysis of the Diagnosis main effect indicated that the adjusted mean of the ASD group ($M_{adj} = 6.444$) was significantly lower than the adjusted mean of the DS ($M_{adj} = 8.783, p = .050$) and TD groups ($M_{adj} = 9.002, p = .025$); the afternoon sAA levels of these two latter groups did not differ significantly ($p = .852$). Furthermore, a significant negative correlation was found between afternoon sAA and tonic pupil size, $\rho(26) = -.462, p = .013$; this correlation became non-significant if diagnosis was held constant, $r_{ab,c}(25) = -.205, p = .296$, indicating that lower afternoon levels of sAA and the larger tonic pupil size of the ASD group is likely driven by a common underlying factor. Afternoon sAA levels were also significantly correlated with ADOS-G total scores, $\rho(27) = -.484, p = .011$.

**Afternoon salivary cortisol**: The univariate Diagnosis (3) ANCOVA (covarying CA) to examine between-group differences in afternoon cortisol revealed a significant main effect of Diagnosis $F(2, 28) = 4.382, p = .022, \eta^2 = .238$. However, this significant main effect was driven by a higher adjusted mean afternoon cortisol level for the DS group ($M_{adj} = -2.202$), which differed significantly from the adjusted mean of the ASD ($M_{adj} = -2.708, p = .038$) and TD groups ($M_{adj} = -2.888, p = .007$); the afternoon cortisol levels of the latter two groups did not differ ($p = .422$). As expected, the correlation between afternoon cortisol and tonic pupil size was non-significant, $\rho(24) = -.102, p = ns$, and remained non-significant if diagnosis was partialed $r_{ab,c}(23) = -.219, p = ns$. The observed and adjusted means for afternoon cortisol are negative values because they were subjected to a logarithm transformation to adjust for positive skew and variability for analysis, but the pre-transformed mean cortisol concentrations (ug/dL) are positive and presented in Figure 2.

**Gender**—Because the gender distribution of this sample was imbalanced, the above analyses were also examined with the female participants excluded to ensure that the overall results were not affected by the inclusion of females. When female participants were removed from the analyses, the results were unchanged; the univariate Diagnosis (3) ANCOVA retained significant between-group differences in tonic pupil size, $R(2, 21) =$. 

*Dev Psychobiol. Author manuscript; available in PMC 2014 July 01.*
4.499, p = .024, η² = .300, afternoon sAA, F(2, 23) = 7.899, p = .002, η² = .407, and afternoon cortisol levels, F(2, 21) = 7.648, p = .003, η² = .421.

**Classification of Group Membership**—The above group-based analyses indicate that both tonic pupil size and afternoon levels of sAA significantly differentiated the ASD group from both DS and TD controls. Afternoon measures of sAA were included in the current study as a means to investigate underlying neural functioning within the pupillary system, but we chose to include both tonic pupil size and afternoon sAA in a discriminant analysis to determine if the addition of afternoon levels of sAA would provide stronger discriminant ability than tonic pupil size alone. The overall Wilks’ lambda was significant, Λ = .487, χ²(4, N = 28) = 17.630, p = .001, with a non-significant residual, Wilks’ lambda, Λ = .951, χ²(1, N = 28) = 1.226, p = ns, thus only the first discriminant function will be interpreted. The discriminant analysis correctly classified 64.3% of the overall sample (53.6% cross-validated), producing a 20% false-positive and 30% false-negative rate to detect ASD. Specifically, we observed 80% correct classification (70% cross-validated) for the ASD group, 66.7% (55.6% cross-validated) for the DS group, and 44.4% (33.3% cross-validated) for the TD group. Table 2 presents the within-group correlations and standardized weights, and Figure 3 presents a scatterplot of tonic pupil size and afternoon sAA levels.

**Discussion**

In Study 1 we observed enlarged tonic pupil size and lower afternoon levels of sAA to significantly distinguish the ASD group from DS and TD controls. We then chose to replicate and extend these findings in an independent sample of children with ASD to address some of the limitations of Study 1. In Study 2, we measured tonic pupil size and afternoon sAA and cortisol measures in the laboratory, in addition to diurnal variations of sAA taken in the home environment at four time points across two days to ensure response stability, in a larger sample of only males with ASD along with age-matched TD controls. Because ICCs indicated response stability in tonic pupil size and afternoon sAA and cortisol measures in Study 1 we chose to take only one laboratory measurement of each in Study 2. Consequently, the methods employed in Study 2 were similar to those used in Study 1; for brevity the reader will be referred to information in the Study 1 Method section where appropriate and methodological alterations in Study 2 are highlighted and discussed when applicable.

**STUDY 2**

**Method**

**Participants**—Male children between the ages of 33 to 79 months of age were recruited for Study 2 if they had a diagnosis of AD, PDD-NOS, or if they were TD. Thirty-seven children were recruited using methods described above for Study 1 and were seen for one testing appointment. Participants were given $50 for completing home saliva samples and $40 for attending one laboratory session ($90 total) to compensate for time and travel.

**Exclusion/attrition criteria:** The exclusion criteria described in Study 1 was applied to the recruitment of children in Study 2; none of the children were taking any chronic or acute medications and all were free of any comorbid impairments.

**Group assignment:** The ASD group (n = 18) group consisted of children who were formally given a diagnosis of either AD (n = 11) or PDD-NOS (n = 7) by established ASD diagnostic clinics, which was confirmed through ADOS-G administration of Module 1 or 2 (based on the child’s verbal ability). The age of the ASD group dictated the recruitment and formation of the TD group (n = 19). A one-way univariate ANOVA indicated no between-
group differences on CA, \( F(1, 35) = 2.052, p = .161, \eta^2 =.055 \), or MA, \( F(1, 35) = 2.053, p = .161, \eta^2 =.055 \), indicating that the two groups were statistically matched on both variables. A one-way MANOVA was conducted to examine between-group differences in the four subscale age-equivalents on the Mullen; a significant main effect of Diagnosis was found, Wilk’s \( \Lambda =.638, F(4, 32) = 4.538, p < .01, \eta^2 =.362 \); follow-up tests revealed significant between-group differences on the Expressive Language subscale \( (p = .016) \) with the ASD group having a lower mean MA \( (M = 45 \text{ m}) \) than the TD group \( (M = 59 \text{ m}) \), none of the other Mullen subscales varied significantly between the groups \( (p_s > .05) \). None of the children in the TD group had a biological parent or sibling with a diagnosis of ASD or other mental health issues such as schizophrenia and/or cognitive and language delays. See Table 3 for age and demographic information for both groups in Study 2.

**Testing Sessions**—Upon enrollment in Study 2, parents were sent a kit to obtain diurnal variations in sAA from their child in the home environment at four pre-specified time points; this was completed on two separate days to ensure response stability (see section below on Diurnal Variations for sAA). Once both home samples were completed, one afternoon laboratory session was scheduled (between 12:00 and 5:00 pm) to obtain tonic pupil size and an afternoon sample of sAA and cortisol. Parents were asked to ensure that their child adhered to the food, liquid, caffeine, and physical activity restrictions outlined in the Testing Sessions section presented in Study 1; they were asked to adhere to these restrictions for both diurnal sAA collection days and the laboratory session. Parents were also instructed to collect the diurnal sAA samples and attend the laboratory session on “typical” days that were free of stressful or atypical events, and were asked to reschedule their home collection days and/or laboratory appointment if any unusual or stressful events occurred. Parent confirmation of compliance with these restrictions was confirmed via questionnaire for all three testing days (two home days and one laboratory session).

**Diurnal sAA Variations:** Parents were sent a salivary collection kit that contained (a) an instruction sheet with detailed instructions and diagrams on how to properly collect and store saliva samples in addition to our lab contact information, (b) collection supplies that included eight Salimetrics Collection Swabs (SCS), eight cryovials, two storage bags, and (c) a questionnaire to document the child’s current health, food intake, and stress levels for each of the two collection days, along with collection times. Parents were instructed to collect saliva samples at the following pre-determined times: 1) upon waking, 2) 30 minutes after waking, 3) at 11:50 am, and 4) at 4:50 pm. Once collected parents were instructed to record the exact time the sample collection began on the questionnaire and indicate the corresponding cryovial number. Parents were instructed to immediately place the collected saliva sample in their home freezer. Once both collection days were completed \( (M = 10 \text{ days between collection days}) \), one afternoon laboratory session was scheduled \( (M = 24 \text{ days between last home collection date and laboratory session date}) \). Parents were asked to bring the frozen saliva samples with them in a cooler to their scheduled appointment. Once they arrived, the samples were immediately placed in our laboratory freezer and frozen at -20° C, until they were sent to Salimetrics, LLC.

**Laboratory Measurement of Tonic Pupil Size and Afternoon sAA and Cortisol:** The collection of tonic pupil size and afternoon measurement of sAA and salivary cortisol was identical to the methods from Study 1; the only exception to this was that both measures were only recorded on one testing day instead of two (see the Tonic Pupil Size and Laboratory Saliva Sample Collection section from Study 1 for methodology). In the following section, methods were identical to Study 1 unless otherwise specified.
Afternoon saliva collection: The session began with a “practice” saliva sample even though the children were familiar with the saliva collection method from the home samples that were previously obtained by their parents; thus, Study 2 subjects did have more exposure to the collection method than those in Study 1. The saliva sample was collected by placing one SCS under the child’s tongue for approximately two minutes (the use of Sorbettes was discontinued by Salimetrics between the conduct of the two studies, but the two collection devices provide identical assay results).

Apparatus: The stimulus was presented and tonic pupil size was recorded using the same apparatus, visual angle, interface program, sampling rate, and subject seating arrangement as described in Study 1. The only exception to this was the incorporation of a video head tracker (VHT) in Study 2 that is integrated into the ASL pan/tilt module and allows for auto-detection of the child’s facial features to automatically find the child’s eye. The VHT therefore decreases calibration time and allows for more precise tracking of the child’s eye and head movements.

Data Extraction and Reduction

Tonic pupil data: Traces of pupil data that occurred within the screen coordinates for the first minute of the blank grey slide were inspected and corrected for artifacts using the same criteria outlined in Study 1, and an average tonic pupil size was computed from adequate pupil traces.

Salivary data: Concentrations of sAA (U/mL) were subjected to square root transformations, and cortisol concentrations (ug/dL) were subjected to a logarithm transformation to adjust for positive skew and variability.

Data Analysis

Prior to data analysis univariate and multivariate outliers were identified using the criterion of scores greater than 4 SD from the group mean and Mahalanobis distance at \( p < .001 \), respectively; no outliers were found for tonic pupil size or afternoon cortisol measures taken at the laboratory, but one outlier in the ASD group and two in the TD group for afternoon sAA obtained at the laboratory, and two in the ASD group and one in the TD group for diurnal sAA were identified and removed. As in Study 1, between-group differences in tonic pupil size and measures of sAA and salivary cortisol from the laboratory session, and diurnal sAA measures were evaluated using an ANCOVA, with CA entered as a covariate. In addition, all analyses were evaluated for violations of homogeneity assumptions using the Levene’s estimate at the \( p < .05 \) level; this assumption was not violated unless specified.

Results

Tonic Pupil Size—A univariate Diagnosis (2) ANCOVA again demonstrated a significant between-group difference in tonic pupil size, \( F(1, 34) = 31.112, p > .001, \eta^2 = .478 \), with the ASD group having a significantly larger adjusted mean tonic pupil size (\( M_{adj} = 5.573 \)) than the TD group (\( M_{adj} = 4.304 \)). We also observed a significant correlation among tonic pupil size and ADOS-G total scores, \( r(37) = .526, p = .001 \), replicating the results obtained in Study 1 and the Anderson and Colombo (2009) study. Taken together, these results demonstrate a larger tonic pupil size in three independent samples of children with ASD. See Figure 4 for observed means of tonic pupil size.

Laboratory Afternoon Saliva Measures

sAA: The homogeneity of variance was violated (\( p < .05 \)) for this variable therefore, the Welch’s variance-weighted ANOVA was used. This analysis revealed a significant main
effect of Diagnosis, [Welch’s $F(1, 19.258) = 17.843, p < .001, \eta^2 = .332$], with the adjusted mean of the ASD group ($M_{adj} = 8.070$) being significantly lower than the adjusted mean of the TD group ($M_{adj} = 12.985$). Correlations among afternoon sAA levels and ADOS-G total scores were significant, $r(34) = -.476, p = .004$. As in Study 1, a significant and sizeable negative correlation was also found between afternoon sAA and tonic pupil size, $r(32) = -.404, p = .018$, which became non-significant once diagnosis was held constant, $r_{abc}(31) = .034, p = .853$. These findings provide replication of those obtained in Study 1, demonstrating lower afternoon levels of sAA taken in the lab environment in two independent samples of children with ASD that is negatively correlated with tonic pupil size.

**Cortisol:** The univariate Diagnosis (2) ANCOVA revealed no significant between-group differences in afternoon cortisol measures, $F(1, 34) = .513, p = .479$, $\eta^2 = .015$, (ASD, $M_{adj} = -2.353$; TD, $M_{adj} = -2.472$), replicating the results obtained in Study 1. See Figure 5 for pre-transformed mean concentrations of both laboratory measures of afternoon sAA (U/mL) and salivary cortisol (ug/dL).

**Diurnal sAA Variations**—Because parents were allowed to choose the two collection days, the saliva samples were collected on both weekdays and weekends. Therefore, correlations among weekend versus weekday variations in home sAA levels were examined and revealed non-significant correlations for each of the four pre-specified collection times during Day 1 and Day 2 ($ps > .05$). Next, within-group differences and ICCs during collection Day 1 and Day 2 were examined; the results of the paired-samples $t$-tests were non-significant for all four sampling times ($ps > .05$), with satisfactory ICCs, $r = .913$ and reliability, alpha = .93. As a result, diurnal sAA levels from both home testing days were averaged together for each of the four time points.

The repeated measure ANCOVA for between-group differences in diurnal sAA variations in the home environment revealed a significant main effect of Diagnosis, $F(3, 30) = 3.126, p = .040, \eta^2 = .238$. Follow-up polynomial contrasts indicated a significant linear effect $F(1, 32) = 7.138, p = .012$, partial $\eta^2 = .182$. As can be seen in Figure 6, the diurnal sAA levels of the TD group significantly increased over time ($ps < .05$), while those of the ASD group remained unchanged ($ps > .05$) in the home environment.

**Classification of Group Membership**—As in Study 1, group-based analyses indicated that tonic pupil size and afternoon sAA levels obtained during the laboratory session significantly differentiated the ASD group from the TD group; in addition, Study 2 yielded significant between-group differences in diurnal sAA variations from the home environment. The next step was to conduct a discriminant analysis to determine whether we could correctly classify ASD using tonic pupil size and afternoon sAA as in Study 1, and to determine if the addition of diurnal sAA levels would increase the overall discriminate ability of the model. The Wilks’ lambda for the analysis was significant, $\Lambda = .279$, $\chi^2(6, N = 37) = 21.707, p = .001$, diagnostic group classification was successfully predicted for 91.9% of the overall sample (78.4% cross-validated), yielding a 16% false-positive rate and a 0% false-negative rate to detect ASD. Specifically, we observed 83.3% correct classification (66.7% cross-validated) for the ASD group and 100% (89.5% cross-validated) for the TD group. These classification results replicate and extend our findings from Study 1 and demonstrate that the addition of diurnal sAA rhythms taken throughout the day in the home environment provide an added benefit to the overall discriminant model, decreasing the number of false negatives from 30% to 0%. Table 4 presents the within-group correlations and standardized weights.
Combined Results from Studies 1 and 2

Although the data for Studies 1 and 2 were collected under differing circumstances the methods used to obtain tonic pupil size and afternoon sAA levels in the laboratory environment were essentially the same. Therefore, we conducted an aggregate analysis using the ASD and TD participants from both studies to provide overall analyses and determine the degree of improved discrimination with the entire sample on hand.

Tonic Pupil Size and Afternoon sAA—As expected, a univariate Diagnosis (2) ANCOVA (with CA entered as a covariate) yielded a significant between-group difference in tonic pupil size, $F(1, 54) = 38.776, p > .0001, \eta^2 = .418$. The ASD group ($n = 29$) had a significantly larger adjusted mean tonic pupil size ($M_{adj} = 5.612$) than the TD group ($n = 28; M_{adj} = 4.347$).

Similarly, a univariate Diagnosis (2) ANCOVA (with CA again entered as a covariate) yielded a significant between-group difference in afternoon levels of sAA obtained during the laboratory session, $F(1, 49) = 6.041, p = .018, \eta^2 = .110$. The adjusted mean of the ASD group ($n = 27; M_{adj} = 8.958$) was significantly lower than the adjusted mean of the TD group ($n = 25; M_{adj} = 11.963$).

Classification of Group Membership—The discriminant analysis using the aggregated samples from both studies was conducted using tonic pupil size and afternoon laboratory obtained measures of sAA to classify ASD. The Wilks’ lambda was significant, $\Lambda = .529, \chi^2(2, N = 49) = 29.269, p < .001$, and successfully predicted diagnosis for 85.7% of the overall sample (83.7% cross-validated), producing a 19.2% false-positive rate and an 8.7% false-negative rate to detect ASD. This yielded 80.8% correct classification (80.8% cross-validated) for the ASD group and 91.3% (87% cross-validated) for the TD group.

Overall Discussion

Major Findings—The goal of the current study was to examine the potential for biomarkers of ASD that might be obtained during infancy and may identify potential sources of neurological dysfunction to address the priorities outlined by the CDC. Previous studies have found dysregulated tonic pupil size in ASD (Anderson & Colombo, 2009; Anderson, Colombo, & Shaddy, 2006; Fan, et al., 2009; Martineau, et al., 2011; Rubin, 1961). We examined and replicated the dysregulated tonic pupil size in two independent samples of children with ASD in the current study and extended this finding to include the measurement of afternoon laboratory levels and home diurnal variations of sAA, which provides a potential non-invasive measure of NE-mediated regulation of pupil size. While sAA may not be a perfect correlate of NE, it has been found in most studies to be correlated with NE-system activity and the current findings showed it as a non-invasive stress-free alternative to venipuncture in assessing NE activity. Owing to the fact that our understanding of the sAA-NE connection is still not definitive, the results of the current study should be interpreted with appropriate caution.

In both Study 1 and Study 2, the ASD group was significantly distinguished from controls through a larger tonic pupil size and lower afternoon laboratory levels of sAA, and found to be significantly related to tonic pupil size and ADOS-G scores. These results provide replication of the larger tonic pupil size to a light stimulus (Anderson & Colombo, 2009) in two independent samples of children with ASD, and provide further support for the prevalence of dysregulated pupillary responses in ASD (Fan, et al., 2009; Martineau, et al., 2011). The sAA results extend the pupillary findings and potentially implicate the involvement of the NE component of the pupillary system in producing this response. The fact that, in both
studies, salivary cortisol did not vary between ASD and TD groups suggests that afternoon laboratory sAA results were not mediated by stress from data collection.

In addition, Study 2 revealed differences in diurnal patterns of sAA between ASD and TD participants; sAA concentrations did not vary in the ASD group but the TD group showed significant increases in sAA levels throughout the day.

**Age Range of the Study**—The 20 to 79 month age range was chosen for the current study because this is currently the youngest age group that most researchers agree ASD can be diagnosed using “gold standard” measures such as the ADOS-G (Chawarska, Klin, Paul, & Volkmar, 2007; Cox et al., 1999; Lord et al., 2006), and was therefore the target age group for the initial evaluation of potential biomarkers of ASD. Although this was a broad age range, we controlled for age by matching the groups on CA and MA, and by entering age as a covariate in the analyses.

**Cortisol Findings in Down Syndrome Controls**—Finally, in Study 1, we unexpectedly found the DS group to be distinguished from both the TD and ASD groups by heightened tonic levels of salivary cortisol. It may be that the heightened salivary cortisol response in the DS group could represent an increased risk for the co-occurrence of a variety of chronic conditions such as suppressed growth or immune and inflammatory responses as these conditions are commonly associated with increased cortisol levels (Charmandari, Kino, Souvatzoglou, & Chrousos, 2003) and commonly occur in DS (Cronk et al., 1988; Kusters, Verstegen, Gemen, & de Vries, 2009). While none of the DS participants in the current study were reported to have any chronic conditions, these differential heightened cortisol levels warrant further investigation.

**Implications**—The results of the current study have two primary implications. First replication of a dysregulated tonic pupil size and altered sAA in ASD implicates their potential as biomarkers for early identification. Second, both measures have the potential to provide information about neurological development in ASD and could provide potential targets for pharmacological intervention.

**Potential influence of the NE-system in ASD:** The NE-system is involved in a number of processes found to be commonly impaired in ASD, such as resting and reflex ANS activity (Anderson & Colombo, 2009; Bal et al., 2009; Fan et al., 2009; Ming et al., 2005; Rubin, 1961; Van Hecke et al., 2009), decreased sleep-wake cycle activity (Honomichl et al., 2002; Oyane & Bjorvatn, 2005; Williams, Sears, & Allard, 2004). The early development of the NE-system and vast NE innervations allow for fundamental influences in central nervous system (CNS) development (Aston-Jones, Foote, & Bloom, 1984; Coyle, 1977; Foote, Bloom, & Aston-Jones, 1983; Schlumpf, Shoemaker, & Bloom, 1980; Sievers, Lolova, Jenner, Klemm, & Sievers, 1981), including cortical structures (Schlumpf et al., 1980). Therefore, it is not surprising that improvements in aggression, irritability, anxiety, self-injurious behaviors, increased sleep time, task performance and working memory have all been found following the administration of β-adrenergic antagonists such as propranolol in ASD (Beversdorf et al., 2008; Narayanan et al., 2010), which reduces NE-modulated connections within cortical regions responsible for higher-order processing of communication and social responsiveness (Hasselmo, Linster, Patil, Ma, & Cekic, 1997).

**Conclusion:** If tonic pupil size and sAA do provide an indication of altered tonic ANS functioning in ASD, then assessment of this system early in life could lead to a greater understanding of how the pathology and functional impairment within this system may be involved in the neural pathology of ASD and the resulting cognitive and behavioral...
symptomology. In addition, this knowledge may provide a target for pharmacological remediation. Given the potential impact of the identification of early biomarkers of ASD, the results of the current study emphasize the need to further evaluate tonic pupil size and sAA as indicators of the disorder through replication with larger samples, and extension of these findings to infants and young children, pre-diagnosis, to further evaluate the early identification potential of tonic pupil size and sAA.

Acknowledgments

We are obliged to the parents and children in Kansas and Missouri who participated in this study, and the Autism and Down Syndrome organizations that helped us to promote this study to potential participants. We are also grateful for the expertise and assistance provided by Dr. Douglas Granger at Salimetrics, LLC and to the undergraduate independent study students who assisted in the coding and input of data.

This research was supported by NIMH grant R03 MH86061 and the University of Kansas General Research Fund. The research also received core support from the Kansas IDDRC, P30 NICHD HD 002528.

References


Dev Psychobiol. Author manuscript; available in PMC 2014 July 01.


Figure 1.
Study 1, average observed mean tonic pupil size from both testing days for the Autism Spectrum Disorder (ASD), Down syndrome (DS), and Typically-developing (TD) groups, presented in the bar graph. Observed mean pupil size and standard error for each individual subject, organized by group, presented in the scatterplot; the ASD group is separated by Autistic Disorder (AD) and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS).

**p < .025
Figure 2.
Study 1 mean afternoon laboratory concentrations of salivary alpha-amylase (sAA) and cortisol for the Autism Spectrum Disorder (ASD), Down syndrome (DS), and Typically-developing (TD) groups, presented in the bar graph. Mean concentrations and standard errors for each individual subject, organized by group, are presented in the scatterplot; the ASD group is separated by Autistic Disorder (AD) and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS). The means are presented as the pre-transformed concentrations of afternoon laboratory sAA and cortisol, but it was the square root and logarithm transformations that were used in the respective analyses to adjust for positive skew and variability.

** p < .025; * p < .05.
Figure 3.
Study 1, scatterplot of tonic pupil size to afternoon laboratory salivary concentrations of alpha-amylase (sAA) for the Autism Spectrum Disorder (ASD), Down Syndrome (DS), and Typically-developing (TD) groups.
Figure 4.
Bar graph for Study 2 displaying the average observed mean tonic pupil size for the Autism Spectrum Disorder (ASD) and Typically-developing (TD) groups; observed mean tonic pupil size and standard error for each individual subject is organized by group and presented in the scatterplot. The ASD group is separated by Autistic Disorder (AD) and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS).

***p < .001
Figure 5.
Study 2 mean afternoon laboratory concentrations of salivary alpha-amylase (sAA) and cortisol for the Autism Spectrum Disorder (ASD) and Typically-developing (TD) groups, presented in the bar graph. Mean afternoon salivary concentrations and standard error for each individual subject is organized by group and presented in the scatterplot. The ASD group is separated by Autistic Disorder (AD) and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS). The means are presented as the pre-transformed concentrations of afternoon sAA and cortisol, but it was the square root and logarithm transformations that were used in the respective analyses to adjust for positive skew and variability.

*** $p < .001$. 

Dev Psychobiol. Author manuscript; available in PMC 2014 July 01.
Figure 6.
Study 2 observed means of diurnal variations in salivary concentrations of alpha-amylase (sAA) for the Autism Spectrum Disorder (ASD) and Typically-developing (TD) groups. The means are presented as the pre-transformed concentrations of sAA, but it was the square root transformations that were used in the respective analyses to adjust for positive skew and variability.
* *p < .05.
Table 1

Study 1: Age, Gender, and Demographic Information for All Groups

<table>
<thead>
<tr>
<th>Gender distribution</th>
<th>ASD</th>
<th>DS</th>
<th>TD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11 male, 1 female n = 12</td>
<td>7 male, 2 female n = 9</td>
<td>10 male, 1 female n = 11</td>
</tr>
<tr>
<td>CA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>M</td>
<td>Range</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>50.25</td>
<td>30 – 69</td>
<td>48.67</td>
</tr>
<tr>
<td>Mullen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visual Reception&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36.17</td>
<td>16 – 54</td>
<td>32.22</td>
</tr>
<tr>
<td>Fine Motor&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34.42</td>
<td>16 – 53</td>
<td>29.67</td>
</tr>
<tr>
<td>Receptive Language&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>31.70</td>
<td>14 – 49</td>
<td>34.63</td>
</tr>
<tr>
<td>Expressive Language&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>31.90</td>
<td>10 – 46</td>
<td>31.50</td>
</tr>
<tr>
<td>Composite (MA)&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>33.33</td>
<td>14 – 47</td>
<td>31.44</td>
</tr>
<tr>
<td>ADOS-G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social</td>
<td>10</td>
<td>6 – 15</td>
<td>0.75</td>
</tr>
<tr>
<td>Communication</td>
<td>10.10</td>
<td>3 – 18</td>
<td>3.63</td>
</tr>
<tr>
<td>Behavior</td>
<td>2.5</td>
<td>0 – 5</td>
<td>0.13</td>
</tr>
<tr>
<td>Parent education level&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.45</td>
<td>8 – 18</td>
<td>14.44</td>
</tr>
</tbody>
</table>

Note: ASD = Autism Spectrum Disorder; DS = Down syndrome; TD = typically developing; CA = Chronological age; Mullen = Mullen Scales of Early Learning (Mullen, 1995); Composite = Early Learning Composite; MA = Mental age; ADOS-G = Autism Diagnostic Observation Schedule Generic (Lord et al., 1997); Social = Qualitative impairments in reciprocal social interaction; Behavior = Stereotyped behaviors and restricted interests.

*Presented in months.

<sup>a</sup>Age equivalent score.

<sup>c</sup>Years beyond high school for both parents.
### Table 2

Study 1: Discriminant Analysis Coefficients for Prediction of Diagnosis

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Correlation coefficient</th>
<th>Standardized coefficients&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afternoon sAA</td>
<td>.699</td>
<td>.745</td>
</tr>
<tr>
<td>Tonic pupil size</td>
<td>.221</td>
<td>-.536</td>
</tr>
</tbody>
</table>

Note. Afternoon sAA = baseline measures of salivary alpha-amylase (AA) taken across two testing days in the laboratory environment; Tonic pupil size = baseline measures of pupil size, recorded across two testing days in the laboratory.

<sup>a</sup>Standardized canonical discriminant function coefficients.
## Table 3

### Study 2: Age and Demographic Information for Both Groups

<table>
<thead>
<tr>
<th></th>
<th>ASD</th>
<th>TD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 18</td>
<td>n = 19</td>
</tr>
<tr>
<td><strong>CA</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>M</td>
<td>Range</td>
</tr>
<tr>
<td><strong>MULLEN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visual Reception&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>48.89</td>
<td>8 - 69</td>
</tr>
<tr>
<td>Fine Motor&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>49.61</td>
<td>5 - 68</td>
</tr>
<tr>
<td>Receptive Language&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>48.00</td>
<td>4 - 69</td>
</tr>
<tr>
<td>Expressive Language&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>44.72</td>
<td>1 - 70</td>
</tr>
<tr>
<td>Composite (MA)&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>47.81</td>
<td>4 - 66</td>
</tr>
<tr>
<td><strong>ADOS-G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social</td>
<td>9.56</td>
<td>4 - 16</td>
</tr>
<tr>
<td>Communication</td>
<td>8.69</td>
<td>2 - 24</td>
</tr>
<tr>
<td>Behavior</td>
<td>4.22</td>
<td>1 - 7</td>
</tr>
<tr>
<td><strong>Parent education level</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.33</td>
<td>4 - 17</td>
</tr>
</tbody>
</table>

**Note.** ASD = Autism Spectrum Disorder; TD = typically developing; CA = Chronological age. Mullen = Mullen Scales of Early Learning (Mullen, 1995); Composite = Early Learning Composite; MA = Mental age; ADOS-G = Autism Diagnostic Observation Schedule Generic (Lord et al., 1997); Social = Qualitative impairments in reciprocal social interaction; Behavior = Stereotyped behaviors and restricted interests.

<sup>a</sup>Presented in months.

<sup>b</sup>Age equivalent score.

<sup>c</sup>Years beyond high school for both parents.
Table 4
Study 2: Discriminant Analysis Coefficients for Prediction of Diagnosis

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Correlation coefficient</th>
<th>Standardized coefficients&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Home Diurnal sAA Times</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waking</td>
<td>.321</td>
<td>.837</td>
</tr>
<tr>
<td>30 min + Waking</td>
<td>.043</td>
<td>-.351</td>
</tr>
<tr>
<td>Afternoon</td>
<td>-.115</td>
<td>.346</td>
</tr>
<tr>
<td>Evening</td>
<td>-.101</td>
<td>-.246</td>
</tr>
<tr>
<td><strong>Laboratory Measures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afternoon sAA</td>
<td>-.183</td>
<td>-.789</td>
</tr>
<tr>
<td>Tonic pupil size</td>
<td>.678</td>
<td>.910</td>
</tr>
</tbody>
</table>

Note. sAA = salivary alpha-amylase; Waking sAA = sAA taken across two testing days upon waking; 30 min + Waking sAA = sAA taken across two testing days 30 minutes after waking time; Afternoon sAA = sAA taken across two testing days at 11:50 am; Evening sAA = sAA taken across two testing days at 4:50 pm; Laboratory sAA = sAA taken during one afternoon laboratory session; Tonic pupil size = baseline measure of pupil size taken during one afternoon laboratory session.

<sup>a</sup>Standardized canonical discriminant function coefficients.