

Temporal Patterns, Heterogeneity, And Stability Of Diurnal
Cortisol Rhythms In Children With
Autism Spectrum Disorder

By

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CHAPTER I

INTRODUCTION

Individuals with autism spectrum disorder (ASD) demonstrate profound impairments in social interaction, communication, and stereotypic behaviors (APA, 2013) that are often manifest as difficulty responding to changes in daily routines. These difficulties may be related to atypical functioning of the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, it is well known that novelty, unpredictability, and change increase activation of the HPA axis and are associated with heightened levels of cortisol, a primary stress hormone and HPA end-product (e.g., Gunnar, Marvinney, Isensee, & Fisch, 1988; Levine, Coe, & Wiener, 1989). Because cortisol is crucial for homeostatic regulation and the ability to adapt to environmental challenges, it is important to assess whether children with ASD have a distinctive cortisol signature that differentiates them from typically developing (TD) peers.¹

Diurnal Rhythm of Cortisol

The focus of the present study was the diurnal rhythm of cortisol secretion, the functional form of which is well characterized across individuals. Cortisol is highest in the morning upon waking, and an estimated 77% of people experience a sharp rise in cortisol 30-minutes post waking, referred to as the cortisol awakening response (CAR) (Pruessner et al., 1997; Wust et al., 2000). The CAR has been conceptualized as a preparatory phenomenon as individuals anticipate daily events and challenges that may occur throughout the course of the day (Fries et al., 2009).

¹ The study in this thesis was published in August 2015. I am one of the authors of the article, and permissions have been obtained through Elsevier. The article can be found at the following citation:
Tomarken, A. J., Han, G. T., & Corbett, B. A. (2015). Temporal patterns, heterogeneity, and stability of diurnal cortisol rhythms in children with autism spectrum disorder. *Psychoneuroendocrinology*, 62, 217-226.

The CAR is followed by a steady decline of cortisol levels throughout the day until it reaches a nadir in the evening (Anders, 1982; Weitzman et al., 1971). Together, the CAR and subsequent decline are the two dominant featural components of the diurnal rhythm of cortisol secretion throughout the day.

One study of children and adolescents with and without ASD found that there were no significant differences in the overall amount of daily cortisol secretion between groups (Marinovic-Curin et al., 2008), thus suggesting the importance of studying more specific components of the diurnal cycle. Indeed, while afternoon levels of cortisol appear comparable between children with and without ASD, elevated evening cortisol levels in children with ASD have been reported (Corbett et al., 2008). This elevation in evening cortisol has been associated with measures of daily stress and sensory sensitivity (Corbett et al., 2009).

Featural Components of the Diurnal Cycle

Other investigations have focused on the featural components of the diurnal cycle, such as the aforementioned CAR and linear decline. To date, studies have yielded no differences between ASD and TD children in the CAR (Corbett and Schupp, 2014; Marinovic-Curin et al., 2008; Zinke et al., 2010) and mixed findings regarding whether or not there are differences in the slope of the peak-to-trough decline of cortisol throughout the day. Some studies have reported no between-group differences (Brosnan et al., 2009; Corbett et al., 2006; Kidd et al. 2012), while others have reported a dampened linear decline in the ASD group (Corbett et al., 2008; Corbett et al., 2009) that is likely concomitant with elevated evening cortisol levels.

The present study was designed to provide a more complete and nuanced picture of cortisol differences between children with ASD and TD peers and, in the process, elucidate

factors that might account for inconsistent results in previous studies. One source of variation in prior findings could be differences in the data-analytic procedures used to assess variations in the functional form of cortisol across the day. We used a more comprehensive approach than previous studies by including assessments of both changes in mean levels over time using omnibus repeated measures analyses and of specific featural components of the cortisol rhythm (i.e., the CAR and linear decline in cortisol from morning through evening). In addition, to enhance sensitivity of measurement, we used the precise times at which cortisol was sampled as a predictor in our featural models.

Heterogeneity within ASD

Heterogeneity within the ASD group is another factor that might account for inconsistencies across studies. It is well-known that ASD is highly heterogeneous across multiple domains (e.g., symptom severity, genetics, neurobiological abnormalities; for a review, see, e.g., Jeste & Geschwind, 2014; Lenroot & Yeung, 2013). Previous studies have generally reported that groups of children with ASD have more variability in diurnal cortisol values (Corbett et al., 2006; Corbett et al., 2008; Hoshino et al., 1987; Richdale and Prior, 1992; Yamazaki et al., 1975) and parents of ASD patients demonstrate heterogeneity in daily cortisol profiles (Dykens & Lambert, 2013). To our knowledge, however, no prior studies have explicitly assessed whether sub-groups of ASD children can be identified based on distinct patterns of the diurnal rhythm of cortisol. We used group-based trajectory modeling (GBTM; Nagin, 2005; Jones & Nagin, 2007) to assess whether subgroups could be identified that displayed distinct trajectories of cortisol across the day. To understand potential causes of differences in cortisol measures between ASD and TD children and any ASD subgroups identified, we assessed the relation between cortisol

and measures of daily stress, trait anxiety, and sensory sensitivity, as well as demographic and symptomatic features.

One limitation of previous studies on HPA functioning in ASD is the failure to assess psychometric properties of cortisol measures that also might account for differences across studies or in the specific pattern of effects observed in a given study. There are several reasons why an examination of reliability, stability, and variability is important. First, if basal cortisol levels truly reflect individual differences in HPA functioning that are linked to the differences between ASD and TD individuals or heterogeneity within the ASD group, it should demonstrate the temporal stability expected of an individual difference measure. Second, because increased measurement error tends to attenuate relations with external variables (e.g., Nunnally & Bernstein, 1978), patterns of significant and non-significant correlations and group differences could be linked to differences in the reliability or stability of the different measures of diurnal cortisol that can be extracted. Third, it is important to assess whether prior evidence for greater variability of cortisol within the ASD group is due to greater variability between individuals (perhaps due to subgroup heterogeneity) or greater fluctuations on a within-subjects basis. For all these reasons, we additionally compared the TSD and AD groups on the stability and variability of cortisol measures.

Current Study

In sum, the current study examined characteristics of diurnal cortisol variation in a sample of children with and without ASD by assessing mean differences at different times of day, featural components of the diurnal rhythm, subgroup heterogeneity in cortisol trajectories,

and psychometric properties (i.e., stability and variability) of cortisol. Considered as a whole, this approach is more comprehensive than that used in previous studies.

CHAPTER II

METHODS

Participants

The participants consisted of 63 unmedicated, healthy children between the ages of 7 and 16 years old, 36 with ASD (30 males, mean age=10.20, SD=1.96), and 27 TD controls (23 males, mean age=9.71, SD=1.54). Diagnoses were made in accordance with the Diagnostic and Statistical Manual (DSM-IV) criteria (APA, 2000) and were confirmed by a previous diagnosis by a psychologist, psychiatrist, or behavioral pediatrician with ASD expertise, clinical judgment at the time of participation (by a licensed psychologist experienced in the diagnosis of ASD), and the ADOS (Lord et al., 2000), which was administered by research-reliable personnel. Inclusion in the study required an estimated IQ of 70 or higher (see Table 1 for means, SDs, and ranges).

The Vanderbilt University Institutional Review Board approved the study. Prior to participation in the study, parents provided informed written consent and participants provided verbal assent. Participants were recruited by IRB approved flyers and established recruitment systems (e.g., clinics, resource centers, support groups, school, recreational facilities).

Measures

The diagnostic and parent report measures and salivary cortisol collection training were administered during one visit to the University.

Autism diagnostic observation schedule (ADOS)

Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2000) is a semi-structured interview used to assess diagnostically characteristic behaviors of ASD. Test-retest reliability for

the domains include social (.78), communication (.73), social communication (.82), and restricted, repetitive behavior (.59). Internal consistency for all domains and modules ranges from .47 to .94. (Lord et al., 2000)

Wechsler abbreviated scale of intelligence (WASI)

Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler, 1999) is a measure of general intelligence used to estimate intellectual functioning.. Reported test-retest reliabilities range from .76 to .85 for each subtest, and are .95 for the full-scale estimated IQ (Wechsler, 1999).

Stress Survey Schedule (SSS)

Stress Survey Schedule (Grodin et al., 2001) is a parent-report measure of stress designed for individuals with autism and other developmental disabilities. The measure consists of 60 daily stress-related items rated on a five-point Likert scale and includes eight dimensions of stress. Internal consistency correlations range from 0.70 to 0.87. Based on evidence indicating linkages between cortisol and uncertainty and change (e.g., Gunnar et al., 1988), this study used the SSS total score and scores on the Anticipation/Uncertainty and Changes and Threats subscales.

Short Sensory Profile (SSP)

Short Sensory Profile (Dunn, 1999) is a parent questionnaire related to sensory sensitivity across several domains, including auditory, visual, tactile, oral, and multisensory processing. In this measure, lower scores reflect greater impairment.

Child Behavior Checklist (CBCL)

Child Behavior Checklist (Achenbach, 1992) is a parent-report measure that assesses behavioral and emotional problems in children. Eight lower-order and two higher-order

(internalizing, externalizing) behavioral domains are extracted. The CBCL also yields scores for DSM-IV diagnostic disorders. Across subscales, reported reliability coefficients range from .71 to .89. Given prior evidence linking cortisol in school-age children to internalizing problems (e.g., Granger, Weisz, Ikeda, McCracken, & Douglas, 1996), this study focused on the anxious/depressed, withdrawn/depressed, and overall internalizing scales and the DSM-IV anxiety diagnostic scale.

State-Trait Anxiety Inventory for Children (STAI-C)

State-Trait Anxiety Inventory for Children (Spielberger & Edwards, 1973) consists of two 20-item self-report scales designed to measure anxiety in children. One form measures current (state) anxiety, and the other measures persistent (trait) anxiety. The measure has been validated in typically developing individuals with a Cronbach's alpha of .91.

The CBCL and STAI-C were not administered to 9 children in the ASD group due to experimental constraints.

Cortisol Sampling Method

Following the diagnostic assessment, basal levels of salivary cortisol were collected from home over three (3) days at four (4) time points per day to obtain a representative aggregate of cortisol values to characterize the diurnal rhythm of each participant. For each of the three days, the sampling times were M1 (immediately upon waking), M2 (30-minutes post-waking), A (afternoon, approximately 3 PM), and E (evening, 30 min. before bedtime), thus resulting in 12 home samples in total. A well-established method (Corbett, Mendoza, Wegelin, Carmean, & Levine, 2008) was used, which included the provision of consistent collection materials and methods, controls for the intake and time of drinks, foods, and medications, and the use of

standardized procedures and protocols. For assessment of the CAR, participants were instructed to take the first sample immediately upon waking. Then they were allowed to get out of bed and go about their typical morning routines but were not allowed to eat or brush their teeth before the 30 minute post-waking sample. Samples were collected by passive drool.

Cortisol Assay

The salivary cortisol assay was completed using a Coat-A-Count® radioimmunoassay kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA) that was modified to accommodate lower levels of cortisol in human saliva compared to plasma. All saliva samples were stored at -20°C, and thawed and centrifuged at 2558 g for 15 minutes to separate the aqueous component from mucins and other suspended particles in the sample. The coated tube from the kit was substituted with a glass tube into which 100µl of saliva, 100µl of cortisol antibody (courtesy of Wendell Nicholson, Vanderbilt University, Nashville, TN), and 100µl of ¹²⁵I-cortisol were mixed. Following incubation at 4°C for 24 hours, 100µl of normal rat serum in 0.1% PO4//EDTA buffer (1:50) and precipitating reagent (PR81) were added. The mixture was centrifuged at 2558 g for 30 minutes, decanted, and counted. Serial dilution of samples indicated a linearity of 0.99. Interassay coefficient of variation was 10.4%. The cross-reactivity of cortisone with the cortisol antibody used is 2.6%. Given that normal human cortisone levels average 25 ng/ml and the fact that plasma cortisone is 16.2% free, this level of cross-reactivity implies that the contribution of cortisone to levels of cortisol can be estimated to be approximately .105 ng/ml. Most importantly, there is minimal variability in cortisone levels among healthy humans and levels do not appear to vary as cortisol secretion increases, even among patients with adrenocortical disorders (Morita, Isomura, Mune et al., 2004). Because cortisone levels are likely to be consistent between and within participants over time, they

represent a constant offset that would not affect the between- and within-subjects comparisons that are the primary focus of the study.

Data Analysis

Because cortisol is positively skewed, values were log transformed (base 10) prior to analyses. Analyses were conducted using SAS/STAT software, Version 9.4 of the SAS System for Windows™ (Copyright © 2002-2012 SAS Institute Inc. SAS and all other SAS Institute Inc. products or service names are registered trademarks of SAS Institute Inc., Cary, NC, USA) and STATA 12 (StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX: StataCorp LP.). Statistical procedures accommodating missing data were used, though the amount of missing data was small. 94.7 % and 98.1% of the possible cortisol samples were non-missing for the ASD and TD groups respectively. Zero-inflated negative binomial (ZINB) regression models indicated no significant group differences in numbers of missing observations per participant (likelihood ratio $\chi^2(1) = .66, p = .42$).

Effects of diagnosis on mean differences on cortisol levels

Main effects and interactive effects of Diagnosis (Dx), Day, and Time of Day (TOD) on mean cortisol levels were tested using the marginal linear model for correlated response data that is instantiated in SAS PROC MIXED (Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2006; Verbeke & Molenberghs, 2009). Restricted maximum likelihood (REML) was used for estimation and general F statistics were used for tests of fixed effects. A Kronecker Product (KP) structure (Galecki, 1994) for the effects of time of day and day was imposed on the residual covariance matrix to model non-independence. To control for sleep-waking variables, each participant's reported waking time and bedtime were used as covariates in the model. Because participants were instructed to take the evening sample 30 min. before bedtime, estimates of

bedtime were generated by adding 30 min. to the reported evening sample time. To replicate and extend prior findings indicating between-group differences in evening cortisol levels, Dx X Day simple effects analyses were conducted at each of the four times of day. A step-down Bonferroni approach (e.g., Westfall, Tobias, & Wolfinger, 2011) was used to control for multiplicity effects.

Effects of diagnosis on featural components of the diurnal cycle

A piecewise linear mixed effects (LME) approach using REML estimation was used to examine the temporal pattern of the diurnal rhythm. The model included fixed effect terms denoting the CAR (i.e., the increase in cortisol from M1 to M2 after initial awakening) and the expected linear decline in cortisol from M2 through A to E. An additional goal was to maximize precision by using the exact sampling times reported by respondents. A random effects structure was implemented to model non-independence across days and time of day.

Heterogeneity within the ASD group

To assess heterogeneity of diurnal cortisol profiles within the ASD group, group-based trajectory modeling (GBTM) (Nagin, 1999; 2005) was used via the STATA *traj* plugin (Jones & Nagin, 2012). Results were also successfully replicated via SAS PROC TRAJ (Jones, 2004; Jones, Nagin, & Roeder, 2001). In this study, group-based trajectory analysis is designed to identify patterns of change in cortisol across time. These trajectories are estimated as polynomials of a given degree and allow us to identify the potential existence of latent subgroups within the same diagnostic (ASD) group. In our analysis, each individual's cortisol values were averaged across days for each time of day. For each iteration of model selection, I specified the number of latent classes (e.g., two, three, four, etc.) and their respective polynomial type (e.g., linear, quadratic, etc.), with a normal distribution of residuals. The *traj* plugin generated maximum likelihood (ML) estimates of regression coefficients for polynomial equations and of

probabilities of group membership.

Two types of models were specified. The first included all four times of day and the second separately assessed the CAR and the morning-to-evening decline. To examine different profiles in the overall diurnal patterning of cortisol, I followed the general procedure recommended by Nagin (2005) by first estimating quadratic polynomial models specifying one through four classes. After determining the number of classes associated with the best-fitting model, the possibility of a more parsimonious linear model was assessed.

To decide on the correct number of identifiable groups in the model, the researcher used the widely recommended Bayesian Information Criterion (BIC; Raftery, 1995, Schwarz, 1978). In the context of GBTM, application of the BIC involves estimating models with varying numbers of groups, followed by selecting the model with the largest BIC score. For the given model, the BIC is computed as: $BIC = \log(L) - 0.5k \log(N)$, where L denotes the value of the model's maximized likelihood, N denotes the sample size, and k denotes the number of parameters in the model. The number of parameters is determined by the order of the polynomial used to model each trajectory and the number of groups. For example, a two-group model in which the trajectories follow a quadratic form would involve $k=8$ parameters: for each of the two quadratic trajectories, there would be parameters for the intercept, first-order term, and second-order (quadratic) term; an additional parameter representing the random error of the residuals; and the number of groups minus 1, yielding $k=3*2+1+(2-1)=8$. Looking at the formula for the BIC, it becomes obvious that the BIC successfully penalizes for the number of parameters in the model to prevent overfitting and balances model complexity against goodness of fit to the data.

The researcher also calculated an estimate derived from the BIC values that indicates the relative probability that a model in a given set is the correct one (Kass & Wasserman, 1995).

This estimate is based on the Bayes factor, which is a construct from Bayesian statistics that provides a useful statistic for calibrating the substantive importance of a difference in the BIC scores of two models (Wasserman, 2000). The Bayes factor is computed as the ratio of the probability of a specified number of groups in the model compared to another specified number of groups (e.g., 2 groups vs. 3 groups). Kass and Wasserman (1995) provide an extension of the Bayes factor. In their metric for comparing more than two models, they show that the probability that a model in a give set is the correct one can be approximated by the following formula:

$$p = \frac{e^{BIC_j - BIC_{\max}}}{\sum_j e^{BIC_j - BIC_{\max}}}$$

where BIC_{\max} is the maximum BIC score of the J different models under consideration. In this study, the BIC and probability approximation by Kass and Wasserman (1995) were considered to determine the optimal model in the GBTM analyses.

Predictors of Cortisol

Correlational analyses assessed the relation between cortisol measures and scores on the SSS, SSP, ADOS, and CBCL measures of interest. Given that most of the measures of primary interest pertain specifically to ASD and that ASD and TD groups had substantial differences on most of the other measures, our primary focus was on correlations computed within the ASD group. Similarly, t tests were used to assess differences on these measures between ASD subgroups formed by the GBTM models.

Stability and variability of cortisol levels

To estimate the stability of cortisol across days for the ASD and TD groups, intraclass correlations (ICCs) (e.g., Shrout & Fleiss, 1979, Strube & Newman, 2007) were calculated at each of the four daily times of assessment (M1, M2, A, E). The ICC was defined according to the

following formula:

$$ICC = \frac{\sigma_u^2}{\sigma_u^2 + \sigma_\epsilon^2} \quad (0)$$

where σ_u^2 = variance of the random effects (denoting differences between subjects) and σ_ϵ^2 = variance of the residuals (denoting variability among the observations of a given subject). By applying the Spearman-Brown formula, ICCs were generated to estimate the test-retest stability of cortisol values aggregated across the three days with a hypothetical three-day average assessed under identical conditions (e.g., Shrout & Fleiss, 1979). Two types of ICCs are presented below: $\overset{\circ}{ICC}_1$ and $\overset{\circ}{ICC}_3$. In addition to the ICCs computed at each time of day, ICCs were also computed for overall daily mean, the CAR, and the linear decline in cortisol from M2 to E.

To estimate ICCs and compare groups, a Bayesian approach (Spiegelhalter, 2001; Turner, Omar, & Thompson, 2001) was implemented via SAS PROC MCMC. Vague prior distributions were specified for all parameters estimated (e.g., Spiegelhalter, 2001) and Markov Chain Monte Carlo (MCMC) simulation methods were used to generate a minimum of 10,000 samples from the posterior distribution of the parameters, after which medians and highest posterior density (HPD) confidence intervals (e.g., Christensen, Johnson, Branscum, & Hanson, 2011) were computed. To compare the ICC values of the TD and ASD groups, difference scores were computed for each posterior sample.

CHAPTER III

RESULTS

Preliminary Analyses

Two-sample *t* tests indicated the expected differences between the ASD and TD groups on IQ measures, although the ASD group scored in the average range on the WASI (see Table 1). The groups did not differ significantly in age (Table 1). The between-group differences on the SSS and SSP in Table 1 indicate that individuals with ASD have higher parent-reported ratings of daily stress and experience greater impairment due to sensory sensitivity. Group X Day mixed effects analyses indicated no significant effects on waking time (all *ps* > .15) or bedtime (all *ps* > .08) (see Table 1). Mean values of both measures were included as covariates in the analyses of cortisol because they predicted the latter and are potentially linked to circadian rhythms.

Table 1. Group Differences on Demographic and Background Variables

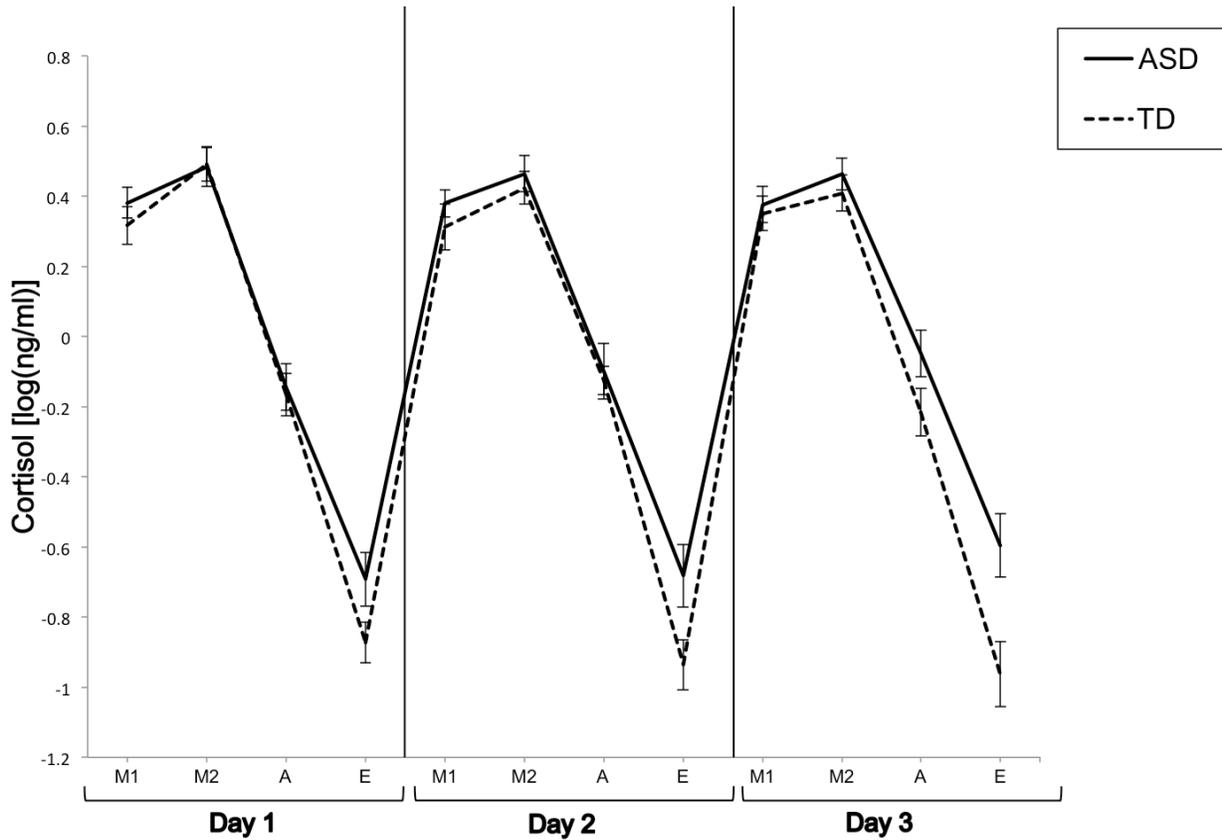
Measure	ASD			TD			<i>p</i> -value
	Mean	SD	Range	Mean	SD	Range	
Age	10.20	1.96	(7.66, 16.31)	9.72	1.54	(7.02, 12.85)	0.269
WASI IQ							
Full Scale	104.42	25.45	(70, 149)	120.45	13.97	(96, 151)	0.006
Performance	101.18	19.10	(68, 140)	113.10	14.34	(85, 143)	0.006
Verbal	101.18	24.07	(55, 150)	123.59	14.62	(96, 147)	<0.001
SSS	112.32	23.91	(58, 155)	64.66	12.50	(49, 93)	<0.001
SSP	121.46	17.69	(89, 155)	172.58	16.85	(117, 190)	<0.001
Waking Time	7:10 AM	1:15	(4:13 AM, 10:56 AM)	7:26 AM	1:04	(5:45 AM, 9:38 AM)	0.414
Bed Time	9:20 PM	0:49	(7:30 PM, 11:10 PM)	9:42 PM	0:50	(8:10 PM, 11:05 PM)	0.086

Note. WASI=Wechsler Abbreviated Scale of Intelligence; SSS=Stress Survey Schedule; SSP=Short Sensory Profile; Waking Time and Bed Time values shown in HH:MM clock time. Waking Time = average of reported times for M1 cortisol sampling. Bed Time = average of reported times for E cortisol sampling + 30 min.

Comparison of Mean Cortisol Levels

Figure 1 displays the predicted values on log cortisol yielded by the omnibus Dx (Diagnosis) X Day X TOD (Time of Day) ANCOVA, with waking time and bedtime serving as covariates (see Table 2 for both logged and unlogged ng/mL mean values). As shown, both the ASD and TD groups show a diurnal pattern of cortisol where cortisol is at a maximum in the morning and gradual declines throughout the afternoon until reaching a minimum in the evening. The omnibus ANOVA yielded a significant main effect of Dx ($F(1, 128)= 4.78, p =0.031$) that reflected higher levels of cortisol across days and times by the ASD relative to the TD group. Furthermore, the analysis also yielded a trend for the interaction between Dx X TOD ($F(3, 111)=2.30, p =0.082$). Planned step-down Bonferroni simple effects contrasts indicated that the ASD group had significantly higher evening cortisol levels ($F(1, 122)=7.86, \text{unadjusted } p = 0.006, \text{adjusted } p =0.024$) than the TD group. There were no significant differences between the two groups— at the M1 ($F(1,103) < 1, \text{unadjusted } p = .50$), M2 ($F(1,123)<1, \text{unadjusted } p =.76$), and A ($F(1,110)<1, \text{unadjusted } p =.40$) times of day.

Figure 1. Mean Cortisol Values Across Days and Times of Day



Note: Values are adjusted for waking time and bedtime.

Table 2. Mean Cortisol at Each Time of Day

Cortisol Units	Group	M1 (SD)	M2 (SD)	A (SD)	E (SD)	Total (SD)
ng/mL	ASD	2.86 (1.20)	3.58 (1.60)	1.40 (1.73)	0.47 (0.59)	2.09 (0.85)
	TD	2.54 (1.11)	3.23 (1.46)	0.83 (0.35)	0.18 (0.14)	1.69 (0.59)
Log ₁₀ (ng/mL)	ASD	0.38 (0.19)	0.47 (0.24)	-0.10 (0.34)	-0.68 (0.45)	0.03 (0.21)
	TD	0.33 (0.23)	0.44 (0.22)	-0.17 (0.22)	-0.92 (0.31)	-0.08 (0.17)

Note. ASD $n = 36$; TD $n = 27$; Cortisol values at each time of day (M1=Immediately upon waking; M2=30 minutes post-waking; A=Afternoon, approximately 3pm; and E=Evening, 30 minutes before bedtime), averaged across three days. Total cortisol was calculated as the average of the four times of day.

Comparison of Featural Components

The piecewise mixed effects analysis with sleep-wake variables as covariates indicated significant main effects of CAR ($F(1, 60.5)=14.92, p < 0.001$) and of the linear decline from M2 to E ($F(1, 76.7)=633.28, p < 0.0001$). This result serves to confirm that the two pieces are distinguishable and are representative of major featural components of the diurnal cycle.

Consistent with the visual representation in Figure 1, the analysis also revealed a marginally significant Group X linear decline interaction ($F(1, 76.5)=3.84, p = 0.054$) due to the fact that the slope of the linear decline in the ASD group was attenuated relative to the TD group ($B_{ASD} = -2.05, B_{TSD} = -2.40$). There was no significant Dx X CAR interaction ($F(1, 60.4) < 1$).

Group-based Trajectory Models within the ASD Group

In the first set of group-based trajectory models (GBTMs), the researcher assessed diurnal variation of cortisol across all four times of day (M1, M2, A, and E). Following Nagin's (2005) recommendations for model selection, I specified a quadratic polynomial model and assessed fit for numbers of groups from one (signifying no distinct grouping) to four. The BIC values and model probability indices showed that the best-fitting model was a two-group model. In this model, 27% of the participants with ASD were designated into one group, and 73% were designated into the other group. Subsequent analyses showed that this model fit better than linear alternatives.

Examination of the predicted quadratic polynomial curves across the 4 times of day clearly indicated that the most pronounced differences between the two groups were in the decline in cortisol from M2 to A and E. The results of the featural GBTM analyses were consistent with this observation. An analysis of the CAR (linear due to the presence of only two time points) clearly indicated that the best-fitting model specified only one group. In contrast, the

best-fitting quadratic and linear models for the decline across M2, A, and E both specified two groups (see Table 3). A comparison of the 2-group linear and quadratic models indicated superior fit for the linear model based on BIC and probability-correct values (Table 3). Several additional criteria (Nagin, 2005; Nagin & Odgers, 2010) indicated good fit of the two-group linear model, including high posterior probabilities of group membership for individual participants (group 1 mean $p = .92$, group 2 mean $p = .97$), a close correspondence between the estimated population probabilities of group membership (.252 and .748 respectively), and the proportion of participants assigned to groups (.25 and .75 respectively). The two groups did not significantly differ in waking time ($p > .45$) or bedtime ($p > .58$).

Table 3. BIC Values and Model Comparisons for Group-based Trajectory Models (Morning to Evening Decline)

Polynomial	# Groups	BIC (n=36)	BIC (n=104)	Probability Correct (within Polynomial)	Probability Correct (across Polynomials)
Linear	1	-41.66	-43.25	.00	.00
	2	-34.14	-37.33	.99	.87
	3	-36.60	-41.37	.01	.00
	4	-40.27	-46.64	.00	.00
Quadratic	1	-41.81	-43.93	.00	.00
	2	-34.71	-38.96	.99	.13
	3	-37.80	-44.17	.00	.00
	4	-42.65	-51.14	.00	.00

BIC=Bayesian Information Criterion. Higher (more positive) values of the BIC indicate better fit. N=36 = number of participants, N=104 = total number of observations. The two BIC scores bracket the theoretically correct value (Nagin, 2005). Within polynomial probabilities are comparisons among the J=4 models within a given type (linear or quadratic). Across polynomial probabilities are comparisons among all J=8 models. Probabilities were computed

using the formula
$$p = \frac{e^{BIC_j - BIC_{max}}}{\sum_j e^{BIC_j - BIC_{max}}}$$
 (Kass & Wasserman, 1995), where BIC_{max} = the best-fitting model in the

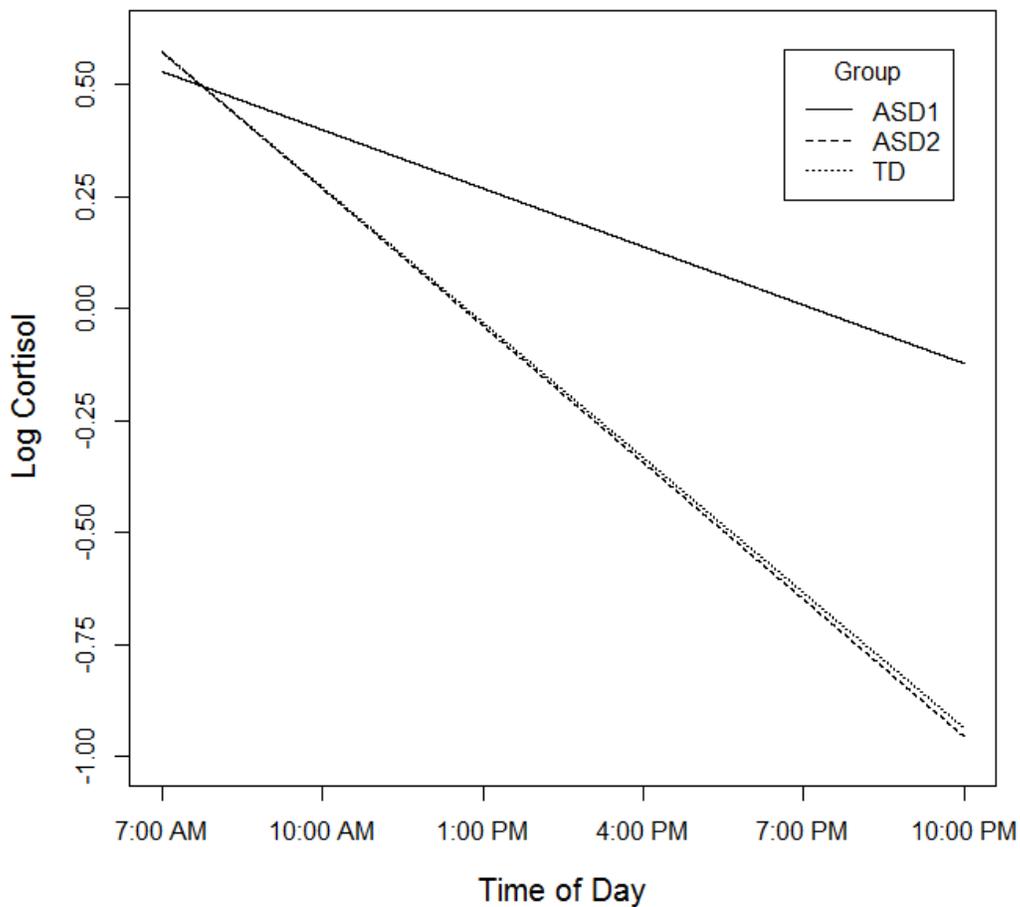
set and J = the number of models in the set.

Focusing on two-group linear model for the diurnal decline, the smaller group (25% of participants) is denoted as ASD1 and the larger group (75%) as ASD2. Figure 2 shows the average predicted trajectories across the M2, A, and E time points for each of these two groups and also displays the predicted decline for the TD group as a whole based on a linear fit. As clearly seen in Figure 2, the ASD1 group shows an attenuated linear decline across time relative to the other two groups, whereas the ASD2 and TD groups are nearly indistinguishable.

Consistent with these observations, ANOVAs and subsequent comparisons indicated that cortisol values for the smaller ASD subgroup at both the A and E time points were lower than those of both other groups (all ANOVA and contrast $ps < .0001$). There were no differences or even

trends when the larger ASD and TD groups were compared (both $ps > .38$). Similarly, a direct comparison of the linear decline indicated that the slope of the ASD1 group was attenuated relative to the slope of the other two groups (Group X Linear trend $F(2,61) = 25.18, p < .0001$, ASD1 vs ASD2 contrast $t(61) = 6.70, p < .0001$, ASD1 vs TD contrast $t(61) = 6.58, p < .0001$). The slope coefficients for the ASD2 and TD groups were almost identical (ASD $B = .112$, TD $B = .109, t(61) < 1, p = .82$). Results were unchanged when the analyses controlled for waking and bed time by including them as covariates in the analyses.

Figure 2. Predicted linear decline across ASD1, ASD2, and TD groups.



As seen, the ASD2 and TD groups look strikingly similar, while the ASD1 group, comprising 25% of the ASD group, shows a noticeably attenuated linear decline.

Correlates of Cortisol Measures

Within the ASD group correlations were computed between age, the ADOS, the SSS, SSP, CBCL, and STAIC and measures of cortisol at each of the four times of day, the CAR, and the linear decline from A2 to E. Because scatterplots indicated a tendency toward outliers, robust percentage bend correlations (Wilcox, 1994) were used. The only statistically significant correlations indicated that higher scores on the CBCL anxious-depressed ($r = -0.43, p = .03$) and anxiety diagnostic scales ($r = -0.39, p = .048$) were associated with lower cortisol levels upon awakening. *T* tests assessing differences between the two ASD groups with distinct patterns of linear decline failed to indicate significant differences on any measures (all $ps > .15$), as did multiple logistic regression analyses predicting group assignments from sets of inter-related variables (e.g., SSS sub-scales). Tests of differences between those ASD participants who demonstrated a CAR greater than 0 and those who did not were not significant. Although there was a trend toward higher ADOS total scores among those who did not demonstrate a CAR ($t(33)=1.77, p = .09$), no other effects approached significance (all $ps > .05$).

Stability of Cortisol

Table 4 shows ICC estimates and group differences for each time of day, the daily average across times of day, the CAR and the linear decline. Several notable patterns are evident. First, given that the ICCs indicate the proportion of between-subject variability that is consistent across days, all measures indicated a statistically significant individual differences component. Even the values indicating the stability of cortisol assessed on a single day (ICC_1) generally indicated a high proportion of variance due to individual participants (i.e., ICC values range from .31 to .63) that encompasses not just the specific times of day but the two featural components. Second, consistent with well-known results concerning the benefits of aggregation, the ICC_3

values indicating the predicted consistency of cortisol averaged across three days typically represented a substantial improvement over the ICC₁ values and, with a few exceptions approach the expected range (e.g., $\geq .70$) for measures of individual differences in the area of personality and temperament. Although there were no significant between-group differences, there was a tendency for the TD group to demonstrate greater stability than the ASD group during the morning, with the reverse pattern in the afternoon and evening. The pattern of results remained unchanged when we adjusted morning ICCs for waking time and evening ICCs for bedtime.

Table 4. Intraclass Correlations Assessing Stability of Cortisol Across Three Days

Measure	TD (n=36)		ASD (n=27)		ICC Difference (ASD-TD)	
	ICC ₁ (95% CI)	ICC ₃ (95% CI)	ICC ₁ (95% CI)	ICC ₃ (95% CI)	ICC ₁ (95% CI)	ICC ₃ (95% CI)
Morning 1	.45 (.22, .66)	.71 (.49, .87)	.31 (.10, .52)	.57 (.29, .79)	-.14 (-.44, .17)	-.13 (-.49, .17)
Morning 2	.63 (.43, .79)	.84 (.72, .93)	.41 (.20, .61)	.67 (.47, .85)	-.22 (-.49, .06)	-.16 (-.39, .07)
Afternoon	.32 (.07, .56)	.58 (.24, .83)	.41 (.20, .61)	.67 (.46, .84)	.09 (-.22, .41)	.09 (-.24, .51)
Evening	.44 (.21, .66)	.70 (.47, .87)	.56 (.35, .73)	.79 (.64, .91)	.11 (-.19, .42)	.09 (-.16, .36)
Daily Mean	.62 (.40, .79)	.83 (.68, .93)	.56 (.36, .75)	.80 (.64, .91)	-.05 (-.34, .22)	-.03 (-.24, .16)
CAR (M1-M2)	.38 (.14, .61)	.64 (.38, .84)	.48 (.28, .68)	.73 (.55, .87)	.10 (-.22, .40)	.09 (-.20, .41)
Linear Decline (M2-E)	.33 (.10, .60)	.59 (.26, .83)	.57 (.35, .75)	.80 (.64, .91)	.24 (-.10, .54)	.20 (-.11, .55)

Cortisol values are log-transformed. ICC₁=Estimated intraclass correlation of cortisol assessed on a single day. ICC₃ = Estimated intraclass correlation of cortisol aggregated across three days. ICC estimates are the medians of the Bayesian posterior distribution. CI = 95% Bayesian Highest Posterior Density (HPD) interval of the posterior distribution.

Between-group comparisons on the random and residual variance parameters for each measure indicated only isolated effects of small magnitude. The most notable difference was the significantly greater variability in the random variance for the ASD (estimated variance = .57) relative to the TD (estimated variance = .16) group on the measure of linear decline from M2 to

E (median difference = .40, HPD = .02 to .96). This effect is consistent with the results of the GBTM analyses indicating heterogeneity within the ASD group in patterns of linear decline. Computation of the ICCs for the linear decline within the subgroups defined by the GBTM analyses yielded lower values of the ICCs than for the ASD group as a whole (e.g., ICC3 = .36 for ASD1 and .64 for ASD2) that were largely due to the sharply reduced between-subjects random variance estimates within each ASD subgroup (variances = .10 for ASD1 and .20 for AD2) relative to the value observed when both groups were combined (variance =.57). These effects are not problematic and, indeed, are expected given that the goal of the GBTM analyses is to identify maximally homogeneous sub-groups.

CHAPTER IV

DISCUSSION

The current study used a multifaceted approach to examine between- and within-group differences in diurnal cortisol in children with and without ASD. This study compared groups on mean cortisol sampled at specific times throughout the day and on featural components of the variation in cortisol across times of day. In addition, two important measurement issues were assessed: heterogeneity within the ASD group and the stability of individual differences in cortisol parameters.

Between- and Within-Group Differences in Diurnal Cortisol

The children with ASD demonstrated significantly higher cortisol levels overall than TD children. Although the absence of a significant Group X Time of Day interaction necessitates some caution, examination of group differences at specific times of day indicated significantly higher evening values for the ASD group and no significant differences or even trends at the other three times. The results of the featural analyses indicated no group differences on the CAR but a marginally significant effect ($p = .054$) indicating that, relative to the TD group, children with ASD demonstrated an attenuated decline in cortisol from M2 to E than TD children. Importantly, such late-day effects were not confounded by sleep- and waking-time. The overall pattern of results is consistent with previous findings that ASD and TD samples differ in evening cortisol (Brosnan et al. 2009; Corbett & Schupp, 2014; Corbett et al., 2008; Nir et al., 1995) but fail to differ in the CAR and morning (Brosnan et al., 2009; Corbett & Schupp, 2014; Marinovic-Curin et al., 2008) and afternoon (e.g., Corbett et al., 2008; Nir et al., 1995) levels. One caveat of

this study, however, is that the sample was comprised of high-functioning children and therefore it is unclear the extent to which our findings and conclusions generalize to lower functioning children

The group-based trajectory modeling analyses significantly extended previous results. For the M2 to E decline, eight alternative models were evaluated and a linear model specifying two groups was clearly the best fitting according to multiple criteria. The best-fitting model indicated that only a subgroup of ASD participants (an estimated 25%) demonstrate a flatter diurnal profile than the TD group. Looking more closely at the ASD subgroups and the TD group, there were notable differences between the smaller ASD subgroup and the other two groups, but the diurnal pattern in the larger ASD subgroup was remarkably similar to that of the TD group. An identical pattern was observed on analyses of cortisol levels at the afternoon and evening assessments. The marked differences between the ASD1 and ASD2 groups on these follow-up analyses are at least somewhat predictable given that the GBTM analysis was designed to form groups characterized by maximal between-group heterogeneity and within-group homogeneity. The TD group was not, however, included in the GBTM analysis. The marked similarity between the TD and ASD2 groups is especially notable and, indeed, was unanticipated. In a broader context, our findings are consistent with evidence that ASD is a highly heterogeneous disorder (e.g., Jeste & Geschwind, 2014; Lenroot & Yeung, 2013) and suggest the need for future studies that replicate and extend our findings by linking them to other markers of heterogeneity within ASD.

While our GBTM analyses have potentially important implications, a few caveats are warranted. The sample size within the ASD group (n=36) was smaller than typical for an analysis designed to identify subgroups. In addition, although the two-group model clearly had

the best fit and the probabilities of group assignment were quite high, no measures of ASD symptomatology, stress, and temperament discriminated the two groups. One likely reason for these null effects is likely the small sample size within the ASD1 group, which consisted of only 9 individuals. Furthermore, two of the measures were not administered to all ASD participants. This factor further lowered n's for some comparisons. Thus, this set of results would benefit from replication and extension in a larger sample.

It is also important to note that the groupings formed by GBTM and related statistical approaches (e.g., mixture modeling) do not necessarily correspond to taxonomically distinct entities (e.g., Bauer & Curran, 2003). However, as stated by Nagin and Odgers (2010), even if the GBTM analyses do not imply the identification of non-overlapping subtypes with unique etiologies, the results of the GBTM analyses allow researchers to identify salient profiles in the ASD population that can inform future studies. In other words, the identified differences between the ASD1 group and both the ASD2 and TD groups provides the motivation for studying ASD individuals with a flattened diurnal profile in future studies.

Diurnal Rhythm of Cortisol in ASD

It is presently unclear why some ASD children demonstrate a flatter diurnal rhythm of cortisol when compared to TD children or other ASD children. One obvious hypothesis is that this pattern reflects the cumulative effects of stress. Indeed, in a meta-analysis of a large number of studies Miller, Chen, and Zhou (2007), found that exposure to chronic stress is associated with significantly higher concentrations of afternoon and evening cortisol, a flatter diurnal rhythm, and a higher daily volume of output. In the present study, these outcomes were all characteristics of the ASD1 group (see, e.g., Figure 2) and of the ASD group as a whole when compared to the TD group. Corbett et al. (2009) found an association between evening cortisol and parent-

reported measures of daily stress and sensory sensitivity among children with ASD. In contrast, in the present study, these measures were not significantly correlated with any cortisol measures among the ASD group as a whole, and were not linked to differences between the two ASD subgroups. Subject characteristics (autistic disorder vs. ASD participants) and differences in experimental design (e.g., 6 vs. 3 days of sampling) may have contributed to this discrepancy (Taylor & Corbett, 2014). The measure of stress was also administered on only one occasion in the present study. Therefore, an important goal for future studies is the implementation of measures and methods (e.g., experience sampling) that validly assess daily stress and affect to allow for an understanding of the relationship between daily stress and cortisol variation in children with ASD.

Dimensions of Stress Linked to Elevated Cortisol

An additional goal is to clarify what precise dimensions, correlates, or consequences of stress might be linked to elevated cortisol and heterogeneity in diurnal cortisol profiles among ASD children. As they age, children with ASD become especially susceptible to social-evaluative and interpersonal anxiety (Bellini, 2006) and this pattern is linked to heightened cortisol responses during social interactions with TD children (Schupp, Simon, & Corbett, 2013). It is important to assess whether children in the ASD1 group identified by the GBTM analyses are more socially anxious and physiologically responsive to social interactions. One particularly promising avenue for future research may be the study of perceived stigmatization and resilience (compare, e.g., Hebron & Humphrey, 2014, to Chi et al. 2015). Studies have also shown that increased relational victimization (e.g., bullying, gossip, aggression, and exclusion) and poor friendship quality (e.g., low friendship responsiveness and neglect) contribute significantly to the social experience of individuals with autism (for a review, see Sreckovic, Brunsting, & Able,

2014). In previous studies, individuals characterized by high relational victimization and negative friendship quality exhibit greater secretion of cortisol throughout the day, as well as a prolonged cortisol response to acute stressors (Calhoun et al., 2014). These findings are consistent with the results from the current study, as the elevations in cortisol demonstrated by some ASD children are late in the day, reflecting an inability to disengage from, or adapt to, the various demands or stressors that accumulate throughout the day (e.g., Corbett et al, 2009; Sapolsky et al., 1986). Thus, it would be valuable to understand specific aspects of friendship quality in individuals with ASD to investigate whether differential levels of friendship quality and relational victimization help to distinguish the ASD1 and ASD2 groups in a larger follow-up study.

Neurobiological Mechanisms for Blunted Cortisol Profile

It is also important to assess the underlying neurobiological mechanisms for elevated cortisol levels and a blunted cortisol profile throughout the day. Although most theories of HPA activation linked to individual differences explicitly or implicitly posit heightened CNS drive as the critical mechanism (e.g., Sapolsky et al., 1986), more peripheral mechanisms involving metabolism and clearance cannot be ruled out. Identification of those components of the HPA axis that account for these effects requires challenge studies (e.g., Heuser, Yassouridis, & Holsboer, 1994) and other procedures that specifically assess cortisol metabolism (e.g., Boonen, Vervenne, Meersseman, et al., 2013). Furthermore, the fact that some biological mothers of children with ASD also demonstrate a blunted trajectory of diurnal cortisol (e.g., Dykens & Lambert, 2013; Seltzer, Floyd, Song, Greenberg, & Hong, 2011) suggests the possibility of an intergenerational pattern of transmission. One note of caution, however, is that in the present study, the ASD participants with a flatter slope had higher levels of cortisol during the afternoon

and evening than other groups, while mothers of ASD children with a blunted trajectory have exhibited overall lower levels of cortisol in prior studies. The latter profile indicative of generalized HPA hypo-activity has been found in other studies of parents experiencing chronic stress and in specific disorders (e.g., PTSD) (e.g., Yehuda, Kahana, Binder-Brynes, Southwick, Mason, & Giller, 1995).

It is also crucial to examine the functional significance and long-term consequences of the flatter slope of diurnal cortisol seen in some children with ASD. Just as the CAR plays a facilitating role in preparation for the day, reduced cortisol in the evening may contribute to rest and sleep. Sleep problems are common in ASD (e.g., Reynolds and Malow, 2011) and pre-sleep arousal is associated with greater sleep disturbance and psychopathology in youth with ASD (Richdale, Baker, Short & Gradisar, 2014). Although the only association between cortisol and indicators of psychopathology was found between early morning cortisol and anxiety, it is conceivable that children with a flatter diurnal profile are at heightened risk for subsequent affective and anxiety disorders, and psychopathology involving emotion dysregulation. In addition, the emerging evidence that a flatter slope of cortisol predicts negative health outcomes among adult populations (e.g., Kumari, Shipley, Stafford, & Kivimaki, 2011; Sephton et al., 2000; 2013) suggests the importance of future longitudinal studies assessing the long-term consequences of this diurnal profile. Interestingly, this evidence indicates that cortisol levels like those observed in the present study that do not meet clinical criteria for hyper-cortisolemia can nevertheless have important functional consequences. This is not surprising given the extensive evidence that the HPA axis is centrally involved in the orchestration and regulation of many essential bodily processes (Herman & Cullinan, 1997).

Finally, a better understanding of the unique diurnal cortisol profiles seen in children with

autism may be achieved through concomitant investigations of other biological measures implicated in the stress response. For example, future studies may examine baseline inflammatory reactivity to stress by measuring levels of key pro-inflammatory cytokines that have been shown to physiologically differentiate ASD individuals from TD individuals when confronted with psychosocial stressors (Tsiloni, Taliou, & Theoharides, 2015). Cortisol and inflammatory proteins are both released into the bloodstream in response to stressors. Studies have suggested that levels of certain pro-inflammatory cytokines (e.g., interleukin-6 and tumor necrosis factor-alpha) have a direct relationship with symptom severity in children with ASD (Yang et al., 2015). Chronically insufficient amount of sleep has also been associated with elevated cortisol levels, while chronic circadian misalignment has been associated with elevated pro-inflammatory cytokines (Wright et al., 2015). Thus, in conjunction with findings regarding HPA-axis functioning, an understanding of inflammatory reactivity to stress provides a useful avenue to better understand the unique biological profiles of individuals with autism. Investigation of inflammatory cytokines may also integrate evidence regarding immune dysfunction, unique reactivity and regulation of stress, sleep difficulties, and ability (or inability) to recruit adaptive social cognitive processing when confronted with psychosocial stressors (Moieni et al., 2015; Rossignol and Frye, 2014) in children with ASD.

Stability and Variability of Cortisol

The present investigation also examined the stability and variability of cortisol in ASD and TD children. For both the ASD and TD groups, all calculated ICC measures indicated a significant proportion of variance attributable to differences among participants that were stable over the time interval assessed. Indeed, the ICC₃ values obtained in this study indicate that aggregation of measures across a three-day interval yields measures with stability estimates that

are acceptable from a psychometric perspective. Although there was a trend indicating higher stability on morning assessments for the TD and on evening assessments for the ASD group, there were no significant between-group differences on any of the ICC measures.

For both the TD and ASD groups, evening cortisol measures were the only ones that attained ICC₃ values greater than or equal to .70 among both children with ASD and TD children. This suggests that the between-group mean differences on evening cortisol observed in previous studies may be due to stable dispositional, biological, and/or environmental factors. It is also important to note that the ICC₃ values for the earlier times of day are sufficiently high to render it unlikely that differences in the precision of measurement are a major factor accounting for the absence of significant differences between groups on these measures. In a similar vein, while groups marginally differed in the linear decline but not in the CAR, the ICC values for these two featural components differ only slightly, with no systematic trends evident.

Our stability analyses merit two cautions. First, while the ICC₁ results indicate that a notable proportion of the variance in cortisol assessed on a given day is due to within-subjects homogeneity, a substantial proportion of variance – in some cases the major proportion – appears due to the combination of day-specific factors and/or random error. Second, cortisol was assessed only during three consecutive days. Conclusions yielded by this narrow window of assessment may not adequately generalize to wider time intervals (e.g., 6-24 months). Indeed, the results of prior studies using non-ASD normative samples have indicated a notable decline in the strength of more stable, trait-like components of variance as time intervals increase (e.g., Ross, Murphy, Adam, Chen, & Miller, 2014; Shirtcliff, Allison, Armstrong, Slattery, Kalin, & Essex, 2012).

Finally, there was a notable reduction in the within-group variability of the linear decline

when random variance parameters were estimated within the ASD trajectory sub-groups. This result is consistent with the objective of the GBTM analyses to detect groups that are maximally homogeneous. It also suggests that the presence of identifiable subgroups may account for the greater variability among ASD children found in previous studies (e.g., Corbett et al., 2006, 2008).

Summary

This study aimed to provide a comprehensive assessment of the between- and within-group differences in the temporal patterning of the diurnal cortisol rhythm in children with and without ASD. Our findings indicate that children with ASD have elevated evening cortisol levels compared to their TD peers, and that children with ASD also exhibit a dampened linear decline of cortisol throughout the day. Subsequent analyses indicated that this latter pattern is characteristic of a subgroup of ASD children. Cortisol aggregated across the three-day sampling interval had acceptable psychometric properties in both groups. Follow-up studies are warranted to investigate heterogeneity and functional significance of the diurnal cortisol profiles in children with ASD and the long-term stability of cortisol.

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