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Incidence, profiles and correlates of the Cortisol Awakening Response in high-functioning young males with ASD

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ABSTRACT

Background: The Cortisol Awakening Response (CAR) is sometimes dysregulated in young people with Autism Spectrum Disorder (ASD), but previous findings are mostly based upon group mean data and do not report individual responses. In addition, investigation of the correlates of CAR dysregulation has been limited.

Methods: To provide insight into the individual profiles and correlates of the CAR in young males with ASD, 32 high-functioning male participants with ASD aged between 9 yr and 18 yr completed several measures of anxiety and mood, and provided saliva samples at waking and 30 min later for calculation of the CAR.

Results: Although group mean data showed an expected CAR profile, over half of the participants had a dysregulated CAR. There was a significant interaction between cortisol concentrations at waking and 30 min later and CAR presence/absence, suggestive of the presence of hyper- and hypo-cortisolism. Unlike previous data regarding CAR and mood states in young females with ASD, there were no significant associations between anxiety or depression and CAR dysregulation in this sample of boys with ASD.

Conclusions: The use of the CAR in research and clinical settings must be accompanied by an awareness of the likelihood of individual variability.

1. Introduction

As well as being characterised by difficulties in understanding social interactions, and restricted interests and repetitive behaviour patterns, (APA, 2013) ASD in children is also often comorbid with elevated anxiety (Kim, Szatmari, Bryson, Streiner, & Wilson, 2000; White, Oswald, Ollendick, & Scahill, 2009), and that anxiety is sometimes reported to be many times more prevalent than in non-ASD peers (Hallett et al., 2013). For example, children with ASD have a clinically-diagnosed prevalence of Generalised Anxiety Disorder (GAD) of 20.9% and Social Phobia of 7.3%, respectively between twenty and seven times more common than in age- and IQ-comparable non-ASD peers (Bitsika & Sharpley, 2015); they are also vulnerable to a higher prevalence of depression (Strang et al., 2012).

Young people with ASD have also been reported to exhibit elevated concentrations of cortisol, a neurohormone that is released from the adrenal cortex following a cascade of events originating in the hypothalamus, and which is responsive to stress (Gaab, Rohleder, Nater, & Ehlert, 2005). There have been some data which report an association between elevated cortisol and rate of gastrointestinal disorders in people with ASD (Ferguson et al., 2016) (gastrointestinal disorders occur more commonly among

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children with ASD (McElhanon, McCracken, Karpen, & Sharp, 2014)), suggestive of wide-ranging effects of anxiety, stress, and elevated cortisol in this population.

In addition to its immediate ‘response to stress’ role, cortisol secretion follows an underlying pulsatile pattern that varies diurnally (Weitzman et al., 1971), but this diurnal variation may be disrupted by chronic stress (Smyth et al., 1997; Stone et al., 2001) and anxiety (O’Donovan et al., 2010). One aspect of the HPA-axis diurnal pattern that has received a great deal of attention is the ‘Cortisol Awakening Response’ (CAR), which is a measure of the increase in cortisol from the time of awakening to 30 to 45 min later. The CAR is a reliable indicator of overall HPA-axis activity (Wust et al., 2000) and is suggested to function to prepare the individual for the demands of the day (Fries, Dettenborn, & Kirschbaum, 2009). However, like the HPA-axis diurnal fluctuation, the CAR is also subject to dysregulation in some people who experience elevated stress and anxiety (Fries et al., 2009), particularly those who experience chronic stress (Bellingrath, Weigl, & Kudielka, 2008). As well as the link between elevated cortisol and stress, there is also some suggestion that *hypo*activiation of the HPA axis may be associated with chronic stress (Dykens & Lambert, 2013). These data support the suggestion that general dysregulation of the HPA-axis (including the CAR) is associated with stress and anxiety, and that it may be linked with downstream medical problems that have been found in high prevalence in the ASD population.

Although some data have reported that the CAR is present in children with ASD and is similar to that in non-ASD children (Corbett & Schupp, 2014; Zinke, Fries, Kliegel, Kirschbaum, & Dettenborn, 2010), other reports indicated that it was absent (Brosnan, Turner-Cobb, Munro-Naan, & Jessop, 2009) or present but characterised by significant between-subject variability (Corbett, Mendoza, Abdullah, Wegelin, & Levine, 2006). In their review of 16 studies of the CAR in children and adolescents with ASD, Taylor and Corbett (2014) reported little evidence of differences in the CAR between ASD and non-ASD participants, although the fact that one study reported an absence of the CAR in their adolescent ASD participants (Brosnan et al., 2009) is suggestive of the possibility of large-scale dysregulation of the CAR in this population. Other recent studies have also failed to find group-based differences in the CAR between ASD and non-ASD peers (Lydon et al., 2015; Muscatello & Corbett, 2018; Tomarken, Han, & Corbett, 2015).

However, those studies were based upon total sample data and did not investigate each individual’s HPA axis responses to better describe the range of CAR activity across participants. Further, there were differences in the sample characteristics used across several studies as well as some limitations in sample sizes, and these were sometimes associated with results. For example, Brosnan et al. (2009) observed no CAR in 20 male adolescents with ASD aged 11 yr to 16 yr who were institutionalised. By contrast, Corbett and Schupp (2014) observed the presence of the CAR but found no differences in CAR between ASD and non-ASD participants in a large sample consisting of 94 pre-pubescent males. Zinke et al. (2010) recruited their 15 participants aged 6 yr to 12 yr from a clinic setting and found no CAR differences across ASD and non-ASD participants. Corbett et al. (2006) included only 12 ASD participants aged 8 yr to 12 yr, finding no differences in CAR across ASD and non-ASD participants. In contrast to these group-based investigations, a few recent studies have focussed upon individual participant data rather than simply reporting group means, and have found that the CAR was present in their ASD samples but that it was not universal across all participants. In particular, some degree of dysregulation in the diurnal fluctuation (DF) from morning to evening of the HPA axis in young people with ASD was found in about 14% of samples of 150 boys aged 6 yr to 18 yr (Bitsika, Sharpley, Andronicos, & Agnew, 2015) and 39 girls of the same age range (Sharpley, Bitsika, Agnew, & Andronicos, 2016), and dysregulation of the CAR was also found in about 50% of those girls (Sharpley, Bitsika, Agnew et al., 2016; Sharpley, Bitsika, Andronicos, & Agnew, 2016).

To date, no study has reported on the individual participant CAR profiles from boys and male adolescents with ASD. Similarly, although the effects of age and ASD symptoms were found to be associated with a blunted DF in a predominantly male sample of children and adolescents with ASD (Muscatello & Corbett, 2018), the associations between CAR and age, ASD symptoms and other demographic factors such as IQ, anxiety and depression have not been reported for the individuals comprising the samples of young males with ASD used in previous research. Because of the repeatedly-reported lack of differences in mean CAR values across ASD and non-ASD children cited above, the continued comparison of these two groups is unlikely to provide data of value in understanding how the CAR functions in ASD populations. Instead, the most relevant issue for current research is the individual variability of the CAR in ASD samples, and the investigation of correlates of that variability.

Several research issues emerge from this current literature. First, the need to examine the CAR at both group and individual levels in a sample of high-functioning boys with ASD from a relatively homogeneous age range. Second, because it has been suggested that dysregulation of the CAR in the ASD population may be due to ASD-related factors including severity of ASD or the age of the children studied (Corbett & Schupp, 2014), these variables should be investigated as correlates of the presence of the CAR. Third, because previous studies have suggested that stress- or anxiety-related factors influence the DF and CAR in these children (Bitsika et al., 2015), anxiety and depression need to be investigated as predictors of the CAR. Finally, there is some evidence of an interaction between the actual concentrations of cortisol at waking and the presence of the DF in boys with ASD (Bitsika et al., 2015), but no significant association between waking cortisol concentration and the CAR in girls with ASD (Sharpley, Bitsika, Agnew et al., 2016). Those studies did not investigate the association between waking cortisol and CAR in boys with ASD, and so the effects of waking cortisol concentration upon the presence of the CAR are also salient to further understanding of this issue. Identification of the possible correlates of dysregulation of the CAR in ASD samples might lead to further research regarding pathways from those factors to the CAR, and could inform clinical practice by helping to identify those young people with ASD who are most at-risk of the physiological effects of chronic stress (i.e., HPA-axis dysregulation).

Therefore, the current study aimed to (i) investigate the presence of any dysregulation to the CAR in a sample of young males with ASD at a group and at an individual level, (ii) evaluate the association between waking cortisol and CAR, and (iii) compare demographic (age), cognitive (IQ), ASD-diagnostic factors (scores on a measure of ASD), and anxiety (Generalised Anxiety Disorder, Social Phobia) and depression (Major Depressive Disorder) factors as correlates of CAR dysregulation. Because of the average 4:1 male:female ratio for prevalence of ASD, plus the lack of previous reports of the association between these factors and dysregulation of the CAR in a sample of young males with ASD, a male-only sample was chosen to enhance generalisability to the larger fraction of the ASD population and to fill this gap in the literature. It was hoped that the results of this study might inform individualised treatment models for young males with ASD, particularly in the light of the associated effects of elevated cortisol upon gastro-intestinal problems (Ferguson et al., 2016).

2. Methods

2.1. Participants

A total of 32 young males 9 to 18 years of age (M = 14.3 yr, SD = 2.7 yr) with ASD, plus one of their parents (30 mothers, 2 fathers) were recruited from parent support groups and other service organisations in Queensland Australia, for a study about “How it feels to have ASD”. All these young males with ASD had received a diagnosis of ASD via administration of the second edition of the Autism Diagnostic Observation Schedule (ADOS^{-2}) (Lord et al., 2012) by a research-reliable ADOS-2 trained assistant during recruitment; they all also had a Full Scale IQ above 70 on the Wechsler Abbreviated Scale of Intelligence (2nd ed.) (WASI-II) (PsychCorp, 1999) measured as part of the recruitment process. Because they were able to sufficiently self-care and attended mainstream schools, they were classified as ‘high-functioning’ by their parents. All participants were Anglo-Saxon in ethnicity and all had been born in Australia. The parents gave written informed consent for their sons to participate and their sons gave verbal or written assent to participate, depending upon their age. The parents reported that none of their sons had any concurrent genetic or neurological conditions or previous DSM-5 (APA, 2013) classification of comorbid psychiatric disorder.

2.2. Materials

The ADOS-2 (Lord et al., 2012) is considered to be the ‘gold standard’ in identifying the presence of ASD and has been recommended in several Best Practice Guidelines as an appropriate standardized diagnostic observation tool (Filipek et al., 1999; National Research Council, 2001). From a series of standardized interactive activities that focus upon social interactions, communication and repetitive behaviours, a diagnosis of ASD may be obtained based upon the Overall Total Score, which is derived from scores for Social-Affective Interaction and Restrictive and Repetitive Behaviours. The Overall Total Score may range from 0 to 28, with the ADOS-2 cutoff for Autism Spectrum Disorder as it is defined under the DSM-5 (APA, 2013) being an Overall Total Score of at least 7.

WASI-II. The WASI-II is a short form intelligence test designed to screen individuals to determine their level of cognitive ability, and correlates strongly with the Wechsler Intelligence Scale for Children (PsychCorp, 1999). Minshew, Turner, and Goldstein, (2005) found good evidence of the scale’s predictive validity in individuals with ASD for research applications, supporting its use in the present study. A Full Scale IQ score is produced from two composite scales on the WASI-II: Verbal Comprehension and Perceptual Reasoning.

Child and Adolescent Symptom Inventory (4th ed.) (CASI-4). The CASI-4 consists of 148 items drawn from *DSM-IV* diagnostic criteria for a range of psychiatric disorders (Gadow & Sprafkin, 2010). The subscales relating to General Anxiety Disorder (GAD) and Social Phobia (SP) are congruent with the current diagnostic criteria for these disorders as set out in the DSM-5 (APA, 2013) and were used in the present study as indicators of overall anxiety and anxiety specifically related to social situations (which young people with ASD find stressful). Responses indicate the frequency of symptoms for these two forms of anxiety, rated on a four-point Likert scale ranging from 0 (never) to 3 (very often). Gadow and Sprafkin (2010) reported satisfactory internal consistency for the overall scale (r = .74) and for these two subscales. Prior research has supported the use of the CASI-4 with ASD children (Gadow, Devincent, Pomeroy, & Azizian, 2005) and the CASI-4 test manual provides normative data for ASD children (Gadow & Sprafkin, 2010). Some previous research has indicated that the self-reports of anxiety in young people with ASD had stronger agreement with cortisol as a physiological index of anxiety and stress than reports from adults who knew those children (Bitsika, Sharpley, Sweeney, & McFarlane, 2014) and so self-reported data from the young males with ASD on the CASI-4 GAD and SP subscales were utilised in this study as an index of their general and social anxiety status, and referred to as CASI-GAD and CASI-SP. Another of the CASI-4 subscales is focused upon symptoms of Major Depressive Disorder (MDD) in children as they are defined by the DSM-5 (APA, 2013). This subscale consists of 10 items, 6 of which measure the symptoms of irritability, feeling depressed, anhedonia, suicidal ideation, feelings of worthlessness or guilt, and fatigue on the four-point rating scale described above. Four other items measure the degree of change in appetite or weight, sleeping habits, activity level, and ability to concentrate or make decisions, for which responses are “Yes” or “No”, scored as 1 or 0 respectively. These scores were referred to as CASI-MDD.

Cortisol. Cortisol was extracted from saliva samples collected at waking (Waking Cortisol) and 30 min later (Morning Cortisol) using a Salivette (Sarstedt Australia, Mawson Lakes, Australia). The CAR was derived from these data by subtracting the Waking Cortisol from Morning Cortisol. All participants reported that they had gone to bed at their usual time the night before collection, and woken at their usual time, although this varied between participants. None of the participants engaged in any major activity between waking and the second cortisol collection, and none ate or drank any food or liquid during that period.

Saliva assays for cortisol. Saliva was collected using Salivettes (Sarstedt Australia, Mawson Lakes, Australia). Cortisol in saliva was measured using a specific salivary cortisol ELISA kit from Abnova Corporation (KA1885, Taipei, Taiwan). This is a solid-phase ELISA using a polyclonal rabbit antibody directed against cortisol. The assay is based on the principle of competitive binding, and endogenous cortisol in the sample competes with a cortisol-horseradish peroxidase conjugate for binding to the antibody. This ELISA has an intra-assay variability of 8.27% and inter-assay variability of 8.33%, with a spiking recovery of 100% and calibration range of

0.1 to 30 ng/ml. Salivary cortisol was assayed according to the manufacturer's instructions. Briefly, 50 µl of neat saliva from participants or cortisol standards (0.1–30 ng/ml) were transferred to the appropriate wells of the 96-well microtitre plate. Fifty µl enzyme-conjugate was dispensed into each well with thorough mixing. The plate was incubated at room temperature with gentle rocking for 60 min. The contents of each well were aspirated followed by four rinses with 300 µl wash solution. Substrate (200 µl) was added to each well and the plate was incubated at room temperature for 30 min. The reaction was stopped with the addition of 50 µl stop solution and absorbance was read at 450 nm immediately. All standards, controls and samples were assayed in duplicate and results were calculated using a 4-parameter logistics curve fit.

2.3. Procedure

Participants were given instructions for collection of the saliva samples, and compliance with these instructions was enhanced via written directions, plus a visit to each participant's home by a researcher before the day of saliva collection and again afterwards to model the process. The saliva collection procedure was rehearsed by parents and their sons in the presence of the researcher prior to the collection day. The follow-up visit collected data on the reported time of saliva collection, confirming adherence to the protocol. After collection from families, the Salivette was placed in a freezer and stored at −20 °C until processed. The saliva was extracted from the Salivettes by centrifugation according to the manufacturer's instructions, aliquotted and archived at −80 °C until cortisol assays were performed. Although some studies have collected two or three days' repeated samples of cortisol, data from those studies have consistently reported very high levels of agreement between samples collected at the same time of day over several days (e.g., Tomarken et al., 2015). Further, recent studies reported significant test-retest reliability of cortisol concentrations collected over eight-month and 2.4-year periods (Sharpley, Bitsika, Agnew, & Andronicos, 2015; Sharpley, Bitsika, Sarmukadam, McMillan, & Agnew, in press), and a review of 14 other studies that used repeated measurements of salivary cortisol has argued that a single sample is of sufficient reliability (Sharpley, Bitsika, Agnew et al., 2016). These data reduce the need for repeated sampling of cortisol over several days, particularly since this could unnecessarily increase the research burden upon children with ASD. The ADOS-2 and WASI-II were administered as part of the recruitment process for this study. The CASI-4 GAD, SP and MDD subscales were completed by the boys on the same day as the saliva collection.

2.4. Statistical analysis

Data were analysed via IBM SPSS 20. Descriptives was used to obtain mean, SD, 5% trimmed mean, ranges, skewness and kurtosis values, and normality was assessed by reference to the distribution and the Q-Q plots as well as Kolmogorov-Smirnov statistics. A paired samples *t*-test was used to detect a significant CAR-related change in cortisol concentrations from waking to 30 min later. Two-way ANOVA was used to test for the presence of main and interaction effects between time of sampling cortisol, whether the participants fell into the expected CAR pattern of responses, and cortisol concentrations. Logistic regression was used to test for the associations between CAR presence/absence and the three sets of 'predictor' variables (Age, WASI-II Full Scale IQ, ADOS-2 score; CASI-GAD, -SP, -MDD; Waking cortisol, Morning cortisol). *a priori* power analysis indicated that a sample of 32 was sufficient to detect a medium-strength correlation (.5) with 95% power and $p < .05$ (Cohen, 1988).

3. Results

3.1. Overall data

Table 1 shows the mean, 5% mean, SD, range, normality, skewness and kurtosis values for ADOS-2, WASI-II, CASI-GAD, -SP and -MDD scores respectively, plus the two cortisol measures and the CAR. All 5% trimmed means closely approximated the actual means, and only one of the eight tests for normality was significant at the Bonferroni-corrected $p <$ (.05/8) .006 level (for the ADOS-2). This was caused by a single outlier raw score of 17, which also accounted for the skewness and kurtosis values for those data. However, the score was accepted as genuine and within the range used in the ADOS-2 for indicating the presence of ASD and so was included in its raw form. Although some of the other skewness and kurtosis values were > 1.0, examination of the histograms and normal Q-Q plots

Table 1
Descriptive data for all predictor and cortisol measures.

Sample	Mean	5% mean	SD	Range	Kolmogorov-Smirnov	Skewness	Kurtosis
ADOS-2	8.41	8.11	2.36	7-17	.296*	2.17	5.71
WASI-II	94.34	93.93	10.54	74-124	.119	.69	1.13
CASI-GAD	7.59	7.48	4.19	1-17	.118	.35	−.42
CASI-SP	4.43	4.31	3.11	0-12	.147	.43	−.39
CASI-MDD	5.12	4.99	2.95	1-12	.179	.68	−.33
Waking cortisol (nmol/L)	12.27	11.64	6.93	3.31 to 32.71	.173	1.57	2.33
Morning cortisol (nmol/L)	17.46	16.27	11.74	4.60 to 56.17	.175	1.72	3.16
CAR (nmol/L)	5.18	4.53	14.23	−21.42 to 47.95	.133	1.05	2.06

* $p < .006$.

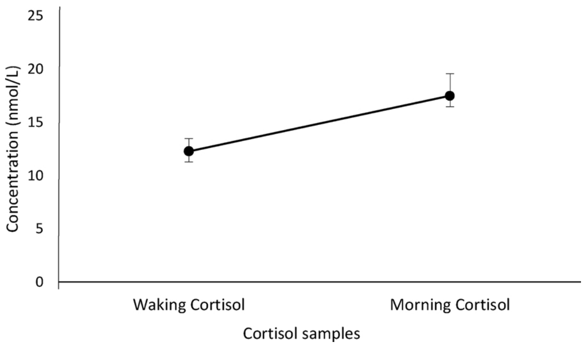

Fig. 1. Means and standard errors for a sample of 32 boys' cortisol concentrations at Waking and Morning (30 min later) cortisol samples.

revealed almost straight lines, signifying that these data were of sufficient normality so as to not need transformation. The scores for the three CASI-4 subscales are all much higher than those for comparable age- and IQ-matched non-ASD samples of boys (Gadow & Sprafkin, 2010) and indicate the presence of elevated GAD, SP and MDD in the current sample. The change in cortisol concentrations from waking to 30 min later is not dissimilar to that reported by Rosmalen et al. (2005) in their sample of 874 healthy boys (11.20 nmol/L, 14.72 nmol/L).

Fig. 1 shows the group means and standard errors for Waking and Morning Cortisol, indicative of the presence of the expected CAR increase within that time period at a sample level, which was statistically significant ($t(31) = 2.060$, $p < .05$). Individual cortisol responses were then examined, and Fig. 2 presents the CAR data for each of the 32 participants, indicating that 14 (43.75%) of the sample showed a CAR that was in the opposite direction to that hypothesized (i.e., their cortisol concentrations decreased from waking to 30 min later) and a further four participants showed increases of less than 1.0 nmol/L, much lower than the increase of 2.49 nmol/L recommended by Wust et al. (2000) as indicative of the CAR (although those data were based upon adults aged 18 yr to 71 yr with no diagnosis of ASD), and even lower than the average CAR of 3.2 nmol/L reported by Rosmalen et al. (2005) for their sample of healthy boys. Thus, the current findings of over 52% of the sample failing to show the CAR as it has been previously defined are suggestive of a large-scale departure from the 'expected' CAR increase in cortisol concentrations. Figs. 3(a) and (b) show the individual changes in cortisol concentrations from waking to 30 min later for each participant, broken down into those who demonstrated the expected CAR increase in concentrations (Fig. 3 (a)) and those who demonstrated a decrease in cortisol concentrations during that period (Fig. 3 (b)). The inter-participant variability is clear from these data, with some participants showing very steep CAR slopes, some showing much flatter increases, and some who showed a decrease in cortisol concentration from waking to 30 min later (referred to here as 'the Reverse CAR').

3.2. Waking cortisol and CAR

Fig. 4 shows an interaction between Time of sampling and Expectedness of CAR, which was verified by two-way (time x CAR presence/absence) ANOVA on cortisol concentrations for Time of sampling ($F(1,63) = 4.175$, p = .045, $\mu^2 = .065$), Presence of CAR ($F = 5.716$, $p = .020$, $\mu^2 = .087$) and the interaction of Time of sampling and Presence of CAR ($F = 13.953$, $p < .000$, $\mu^2 = .189$). That is, the cortisol slope differences found between the expected CAR versus the Reverse CAR subgroups were a function of differences in both their Waking and Morning cortisol concentrations, wherein the Reverse CAR subgroup had a higher cortisol concentration at waking and a lower concentration 30 min later. These results suggest that the presence/absence of the expected CAR

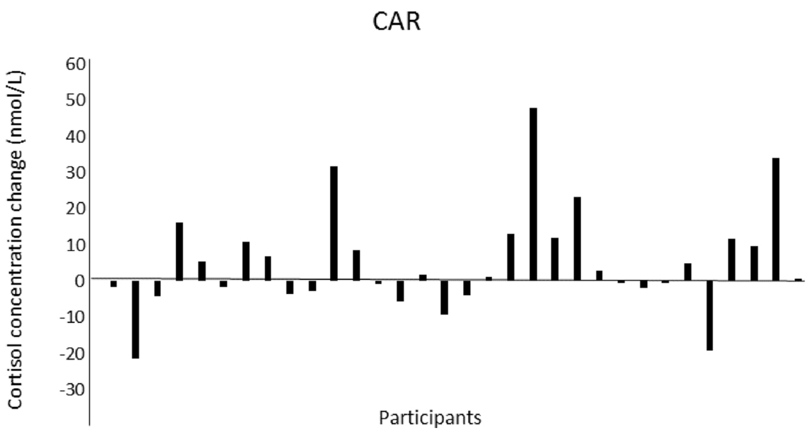

Fig. 2. Changes in cortisol concentration (nmol/L) from waking to 30 min later (i.e., CAR) for individual participants.

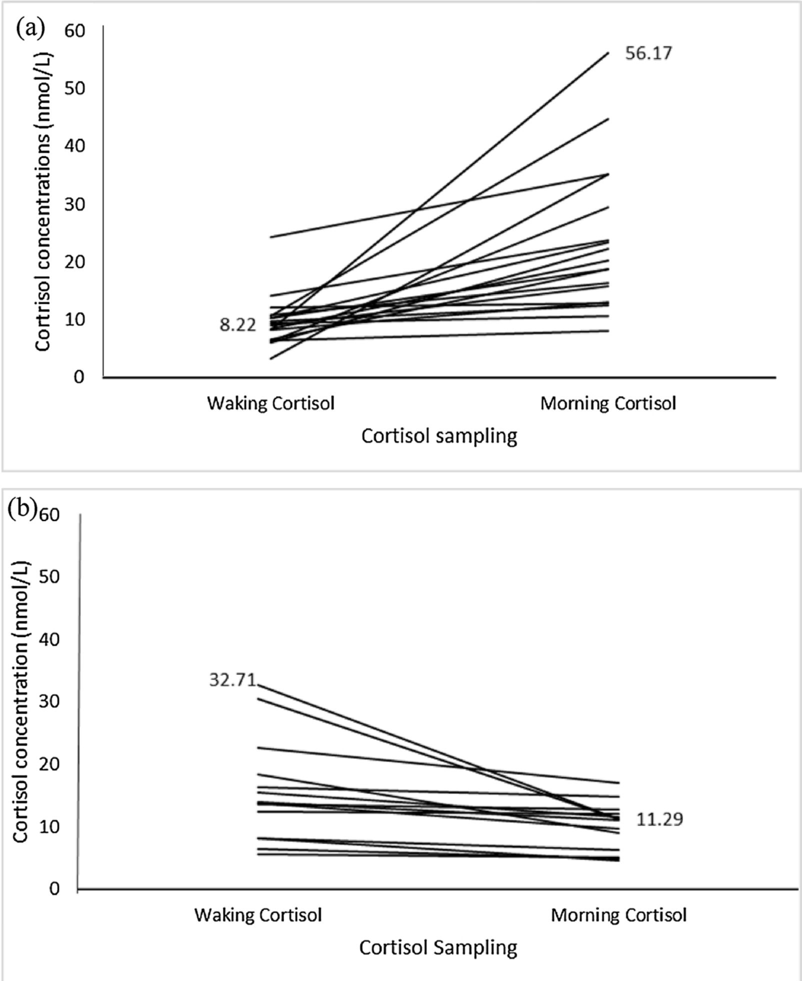

Fig. 3. (a) Individual CAR profiles for participants who showed 'expected' increases in cortisol concentrations. (b)Individual CAR profiles for participants who showed 'unexpected' decreases in cortisol concentrations.

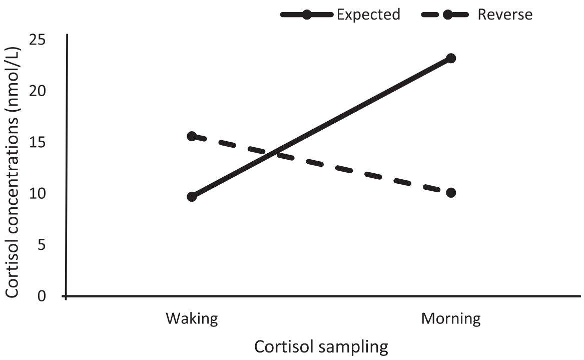

Fig. 4. Waking and Morning cortisol concentrations of 'Expected' *vs* 'Reverse CAR' subgroups.

may well have been an outcome of the level of cortisol concentrations present within participants when they awoke as well as 30 min later. In turn, this may be indicative of a higher resting level of HPA-axis activity in the Reverse CAR participants in addition to a blunting of the CAR response when measured at 30 min after waking. These data are similar to those reported for the DF (morning *vs* afternoon cortisol) by Bitsika et al. (2015) for their sample of 150 boys with ASD (which showed a similar interaction effect for time of sampling x expectedness of DF change) but different to those reported for the CAR by the same authors in their sample of 39 girls with ASD (Sharpley, Bitsika, Agnew et al., 2016), which failed to find any significant difference in waking cortisol concentrations between the CAR versus Reverse CAR subgroups. These differences are discussed below.

3.3. Correlates of CAR dysregulation

A series of logistic regressions was performed to test for the contributions made by the three sets of variables to the CAR. The dependent variable was the presence/absence of the CAR as described above, and the first regression equation was for age, WASI-II Full Scale IQ, and ADOS-2 Overall Total Score, producing no significant effect for either the entire model or any of the three variables separately (χ^2 (3) = 2.637, p = .451). The second regression equation was for CASI-GAD, -SP, and –MDD, and also failed to reach significance (χ^2 (3) = 0.975, p = .807); and the third regression equation using Waking cortisol and Morning cortisol also failed to reach significance (χ^2 (2) = 4.397, p = .111).

4. Discussion

4.1. Overall findings on the CAR

As in several previous studies, the group-based data indicated the presence of a significant CAR in this sample of young males with ASD. However, when examined at the individual participant level, over half of the sample did not show the expected slope or magnitude of cortisol change from waking to 30 min later, with either relatively flat profiles or inverse profiles. These results highlight the benefit of examining CAR data at an individual as well as group mean level, and suggest that the CAR is by no means a universal phenomenon among young males with ASD, complementing previous data reported for a sample of girls with ASD (Sharpley, Bitsika, Agnew et al., 2016). These findings are also congruent with those from non-ASD populations reported by Smyth et al. (1997) and Stone et al. (2001), and therefore suggest that the failure to find a consistent CAR across all the young males in this study does not constitute an indication of an effect that is restricted to persons with ASD-alone, but rather an extension of the suggestion made by Stone et al. (2001) and Smyth et al. (1997) that chronic stress and anxiety can influence the CAR among people who do not meet the diagnostic criteria for ASD as well as those who do fit that diagnosis. These findings may help to explain some of the inconsistencies in the previous research into the CAR in young people with ASD, and suggest that some aspect of chronic stress might be a more fruitful factor to explore than ASD *per se* when seeking to understand the HPA-axis profiles of people with ASD. As mentioned in the Introduction, the prevalence of gastrointestinal disorders among people with ASD is higher than the non-ASD population, and there is some evidence that these disorders may be associated with cortisol elevation. The future investigation of this association holds promise for development of treatment protocols, particularly when those protocols emerge from an individualised model, such as that which underlies precision medicine (Insel, 2013).

4.2. Correlates of the CAR

That suggestion is challenged in a minor way by the fact that, although CASI-4 scores for GAD, SP and MDD in this study were similar to those from previous studies and clearly elevated above those for comparable non-ASD peer samples (Gadow & Sprafkin, 2010), those psychological variables were not significantly associated with CAR status in the present study. Unfortunately, this study did not measure the diagnostic criteria for Post-Traumatic Stress Disorder (PTSD), which focus upon longer-term stressors and stress reactions. Because of the well-established difficulty that young people with ASD have in understanding and responding to social interactions, their stress responses may have been present for several years rather than a few weeks, and may have been more effectively measured by the diagnostic criteria for PTSD. That is a subject for future investigation.

It is noteworthy that these data for the psychological correlates of dysregulated CAR in boys are different to those previously reported for girls (Sharpley, Bitsika, Agnew et al., 2016), where there was a significant correlation between MDD and dysregulated CAR, which is also found in non-ASD adults (Huber, Issa, Schik, & Wolf, 2006). Chida and Steptoe's (2009) review of nearly 150 studies of the effect that psychosocial stress has upon the CAR pointed out that, while job stress and general life stress were associated with elevated CAR, fatigue, burnout and exhaustion were linked with reduced CAR and the reversal of CAR that was identified as 'Reverse CAR' in this study. However, fatigue, burnout and exhaustion were not measured in this study, and the association between MDD and CAR found for girls previously was not significant for boys in this study. This may be a gender effect within young people with ASD, and requires direct comparison of a male-female sample for the association between CAR and MDD to provide confirmation of that suggestion.

4.3. Hypocortisolism

The significant interaction between time of sampling and presence of the CAR raises the possibility that the Reverse CAR was a function of elevated cortisol concentrations prior to awakening as well as deflated concentrations 30 min later. Fig. 4 indicates the differences at waking and 30 min later for the CAR and Reverse CAR subgroups, but data were not collected prior to waking. While the CAR subgroup followed the expected trajectory of cortisol concentration change after waking, the Reverse CAR subgroup may have exhibited hypercortisolism at the waking measure and hypocortisolism at the 30 min measure. Hypocortisolism is thought to emerge from prolonged periods of hypercortisolism (Fries, Hesse, Hellhammer, & Hellhammer, 2005), when the HPA-axis becomes fatigued by repeated and/or prolonged very high levels of circulating cortisol which, when fed back to the hypothalamus, result in lowered production of the prohormone instigators of cortisol and thence cortisol itself (Hellhammer & Wade, 1993). This can lead to some people who experience chronic stress exhibiting unusually deflated cortisol concentrations, or impairment of the usual CAR as a preparation for the day's challenges (Fries et al., 2009), which may have been the case in the present sample for those participants who demonstrated the Reverse CAR. The fact that their waking cortisol concentrations were elevated compared to those participants who showed the expected CAR may have been due to an exacerbated pre-waking arousal, perhaps arising from chronic stress and hypervigilance which failed to allow a decrease in cortisol concentration during the hours of sleep in the late evening and early morning. The subsequent lack of a formal CAR increase may have been an outcome of hypocortisolism arising from the chronic stress and hypervigilance and its effect upon pre-waking cortisol. These hypotheses are tentative at this time and require further investigation before they can be accepted as more than suggested explanations of the findings reported here.

Hypocortisolism has been previously reported in children who have suffered chronic and severe social and interactional stress (Cicchetti & Rogosch, 2001), and these young people have been described as similar to people suffering from PTSD (King, Mandansky, King, Fletcher, & Brewer, 2001). Young people with ASD also often suffer prolonged and severe social rejection and consequent stress, anxiety and depression due to their difficulties in understanding and interaction socially (APA, 2013). As well as being a potential indicator of chronic severe stress, hypocortisolism may also have negative effects upon overall health by over-activating the immune system by way of reducing the anti-inflammatory effects of cortisol (Fries et al., 2005; Raison & Miller, 2003). Chrousos (2009) described the dysregulation of cortisol synthesis, arguing that "Malfunction of the stress response might impair growth, development, behavior and metabolism, which might potentially lead to various acute and chronic disorders" (p. 380). The suggestion that about half of the current sample may have demonstrated hypocortisolism holds implications for their current stress states as well as for their longer-term health. **However, as mentioned above, this explanation is hypothetical at this stage and would require collection of cortisol data over a longer period of time to be confirmed.**

4.4. Limitations

These findings are subject to several methodological limitations. First, and as explained in the Introduction, the sample was restricted to males of a reasonably homogeneous age range, who were high-functioning. The restrictions on generalisability of these results to females has been discussed above, but it should also be mentioned that young males of different ages, IQ or functioning levels may show different results to those reported here. The sample was also restricted in terms of its ethnicity, and it may be that other social settings could influence the outcomes for different samples. Although of sufficient size to provide satisfactory statistical power, the current sample represents only a very small fraction of the population of young males with ASD. Also in terms of generalisability, these data were collected once, and it may be that greater reliability could emerge from repetitions of the protocol, although the issue of participant burden always needs to be considered and there are data which argue against the need for such repeated measurements of cortisol in this population. The data on GAD, SP and MDD were self-reports, based upon the greater validity of these with cortisol concentrations demonstrated in a previous study (Bitsika et al., 2014), but triangulation of those psychological data with parent and clinician evaluations of the ASD participants would boost the validity of those data.

5. Conclusions

Taking these limitations into account, the findings from this study contribute to an understanding of the nature of the CAR in young males with ASD, and suggest that there may be some gender-based differences between the ways that the HPA-axes of young males and young females with ASD respond to chronic stress and are related to standardised indices of mental health. By examining the group data and also the individual data for each participant, it was possible to demonstrate that group effects might hide individual variability in the CAR. In addition, it appears from these data that assumptions regarding the 'average' CAR in young males with ASD (i.e., group effects) might be restricted when seeking to identify CAR dysfunction among individual boys and male adolescents with ASD. From this, the implications for clinical and research protocols flow as urging caution in drawing any firm conclusions regarding the effect that the core ASD diagnostic features (such as difficulties in social understanding and communication) or mood states might have upon the physiological stress responses of young people with ASD. Instead, adopting an 'individualised' or 'precision medicine' approach (Insel, 2013) to the assessment and treatment planning for these young people with ASD appears to be supported by these findings.

Conflict of interest

None of the authors has any conflict of interest to report.

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