

The Cortisol Awakening Response In Children and Adolescents with a Parental History of
Anxiety

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Abstract

Objective: The current study used a high risk design to investigate whether the Cortisol Awakening Response (CAR) is a potential heritable trait marker of anxiety disorder risk.

Method: The sample consisted of 274 healthy offspring (7-18 years old) including 101 offspring with a parental history of panic disorder (PD), generalized anxiety disorder (GAD), or social anxiety disorder (SAD) and 173 offspring with no parental psychopathology. Salivary cortisol was collected at wake-up, 30, and 60 minutes later, as well as at 4pm and 8pm on two consecutive days. The CAR was calculated using area under the curve with respect to ground (AUCg) and increase (AUCi). Correlation analyses of covariates were conducted. **Results:** No differences between high and low risk groups were detected when the combined sample of high risk offspring was examined. However, when anxiety disorder subtypes were considered, offspring with parental GAD or SAD had a significantly lower CAR and diurnal cortisol response than those with no parental psychopathology. No differences in the CAR or diurnal cortisol were found in offspring with parental PD. Age and puberty status correlated negatively with AUCg and awakening values and anxiety sensitivity correlated positively with AUCg and awakening values. **Conclusions:** A blunted CAR and diurnal cortisol response may represent a possible heritable risk marker that is specific to GAD or SAD. Further research is needed to confirm these findings. Study results may have important implications in identifying children at risk for anxiety disorders and creating early interventions intended to change the trajectory of risk.

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Introduction

Background

Anxiety disorders are characterized by chronic, debilitating fear and anxiety, and are the most prevalent of all mental health disorders (Langlois et al., 2011). The term “Anxiety Disorders” is used as an umbrella term for the classification of several psychological conditions, primarily indicated by prolonged and intense feelings of distress that are out of proportion in relation to the actual threat, and that interfere with normal daily functioning (Health Canada, 2002).

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) currently recognizes multiple anxiety-related conditions associated with children and youth, including Panic Disorder (PD), Agoraphobia (AG), Panic Disorder with Agoraphobia (PD+AG), Generalized Anxiety Disorder (GAD), Social Anxiety Disorder (SAD), and specific phobias, (5th ed., American Psychological Association (APA), 2013a). Obsessive-Compulsive Disorder (OCD) and Post-Traumatic Stress Disorder (PTSD), which were previously classified as anxiety disorders in past editions of the DSM, are now separate from anxiety disorders and have their own diagnostic sections within the DSM-V (5th ed., APA, 2013b).

It is estimated that 12% of the Canadian population are affected by anxiety disorders, causing mild to severe impairment in social, occupational, and/or role functioning (Health Canada, 2002). Lifetime prevalence estimates for anxiety disorders are 28.8% (Kessler et al., 2005) and it is predicted that one in four people will have at least one anxiety disorder in their lifetime (Kessler et al., 1994). The one-year prevalence estimates of specific anxiety disorders for Canadians are: 0.7-1.5% for Agoraphobia (AG), 3% for Generalized Anxiety Disorder

(GAD), 6.7% for Social Anxiety Disorder (SAD) (Langlois et al., 2011), and 6.7% for specific phobias (Offord et al., 1996). For Panic Disorder (PD), the one-year prevalence estimates range from 1.6 to 3.7%, (Langlois et al., 2011), with reports of panic attacks ranging as high as 63% (Ollendick, Birmaher & Mattis, 2004).

In addition to high prevalence estimates of anxiety disorders, evidence suggests that they have early onsets in childhood and adolescence (Morris & March, 2004). In comparison with other mental health disorders, anxiety disorders have the earliest age of onset, with a median age of 11 years, and are associated with high rates of comorbidity, a presence of one or more additional disorders (Kessler et al., 2005). Among the disorders, specific phobias have the earliest age of onset at approximately age seven, while generalized anxiety disorder (GAD) typically occurs in middle childhood, between the ages of 9-10 (Morris & March, 2004). In contrast, the onset of social anxiety disorder and panic disorder typically occur in early adolescence, with the onset of panic disorder occurring around age 15 (Morris & March, 2004).

There also appears to be a considerable gender difference within the prevalence of anxiety disorders. Epidemiological research has demonstrated that across all ages, anxiety disorders are significantly more prevalent in females than in males (Beesdo, Knappe & Pine, 2009; Cartwright-Hatton, McNicol, & Doubleday, 2006; Ford, Goodman & Meltzer, 2003; Merikangas, Nakamura & Kessler, 2009). Although the rates of anxiety disorders increase throughout childhood and adolescence for both genders, rates of anxiety disorders in females significantly increase after age five, peaking in adolescence (Zahn-Waxler, Shirtcliff & Marceau, 2008). It is estimated that the lifetime and yearly prevalence of all anxiety disorders are 1.5 to 2 times more common among woman versus men (McLean, Asnaani, Litz & Hofmann, 2011), including panic disorder (PD; 5.0% vs. 2.0%), social anxiety disorder (SAD; 15.5% vs. 11.1%),

generalized anxiety disorder (GAD; 6.6% vs. 3.6%) and specific phobia (15.7% vs. 6.7%) (Kessler et al., 1994).

More than 80% of patients with anxiety disorders have at least one comorbid Axis I disorder (disorders listed at the top-level of the DSM system of diagnosis and representing acute symptoms that require treatment), particularly depression or another anxiety disorder (Brown, Campbell, Lehman, Grisham & Mancill, 2001). An epidemiologic survey by Grant et al. (2005) found that respondents with any anxiety disorder (including Panic Disorder with Agoraphobia (PD ± AG), Generalized Anxiety Disorder (GAD), Social Anxiety Disorder (SAD), or specific phobia) were significantly more likely to have at least one co-occurring substance-use disorder, particularly alcohol/drug dependence. The one-year prevalence rates of having any substance use disorder were: 17.30% for PD, 24.15% for PD + AG, 19.08% for GAD, 16.05% for SAD, and 13.83% for specific phobia. The presence of an anxiety disorder is also significantly associated with suicidal ideation and attempts, with odds ratios of 2.29 and 2.48, respectively (Sareen et al., 2005) and a ten-fold increase in overall suicide risk (Kahn et al., 2002).

The economic burden associated with anxiety disorders is also substantial. They are associated with high rates of lost productivity due to time off work and unemployment, as well as heavy use of emergency department services and primary care services (Health Canada, 2002). In the United States, costs associated with lost productivity for anxiety disorders were \$34 billion, 15 times greater than costs associated with mood disorders (Rice & Miller, 1998). Although less is known about the financial burden of anxiety disorders in Canada (Koerner et al., 2004), estimates on the economic burden associated with depression and distress (including anxiety symptoms) were calculated to be \$6.3 billion per year for healthcare usage and \$8.1 billion for lost productivity (Stephans & Joubert, 2001).

The preceding data highlights that anxiety disorders are serious mental health conditions with significant costs to the individual, their family, and society. The morbidity, early onset, functional disability, and economic burden associated with anxiety disorders suggest that the identification of premorbid trait markers that increase risk for the development of anxiety disorders is crucial to the development of prevention programs within the public-healthcare system. Accordingly, research regarding the etiology (causes or origin) of anxiety disorders is an essential step in the identification of such premorbid trait markers.

Etiology of Anxiety Disorders

At the present time, the etiology of anxiety disorders is still unknown (Merikangas, Asnanni, Litz & Hofmann, 2013). However, like other disorders, research has shown that the etiology of anxiety disorders is influenced by genetics, neurophysiological, personality and environmental factors (Gregory & Eley, 2007). It is evident that the pathways to pathological anxiety are very complex, involving a diverse multitude of interacting risk and resilience factors (Barlow, 2002). Due to this complexity, it is beyond the scope of this thesis to discuss the contribution of all of these etiological factors. Therefore, the present discussion will focus on two of these important factors; genetic loading and the hypothalamic-pituitary-adrenal (HPA) axis.

Genetic loading. Genetic predisposition appears to play an important role in the etiology of anxiety disorders. The familial aggregation of anxiety disorders has been strongly supported within the research literature and has been evidenced by both family studies and twin studies (Hettema, Neale & Kendler., 2001).

Family studies have shown that anxiety disorders run in families (Gregory & Eley, 2007) and that offspring of parents with anxiety disorders are at higher risk of developing an anxiety disorder themselves (Micco et al., 2009). For example, Hirshfeld-Becker, Micco, Simoes and

Henin (2008) found that rates of anxiety disorders ranged from 21% to 68% in offspring who had a parent with anxiety, compared to 0 to 26% in control offspring. Family studies have also calculated the summary odds ratio (OR) of anxiety disorders, which compares the relative odds of occurrence of anxiety disorders in offspring having a first-degree relative with the disorder (Szumilas, 2010). Summary odds ratios (OR) less than one are associated with lower odds, whereas an OR greater than one is associated with higher odds. A meta-analysis by Micco et al. (2009) found that offspring of parents with an anxiety disorder have an OR of 3.91 of developing an anxiety disorder than offspring with no parental psychopathology. Similarly, a meta-analytic review of the genetic epidemiology of anxiety disorders by Hettema et al. (2001) estimated a summary odds ratio ranging from 4 to 6, with specific odds ratios of 5.0 for panic disorder (PD), 6.1 for generalized anxiety disorder (GAD), and 4.1 for specific phobias and social phobia.

Although family studies are able to demonstrate the familiarity of anxiety disorders, twin studies go one step further in establishing whether anxiety disorders are indeed inherited (Gregory & Eley, 2007). By comparing the concordance rates of anxiety disorders in monozygotic (MZ) twins who share 100% of genetics with each other, and dizygotic (DZ) twins who share approximately 50% of their genetics, twin studies are able to distinguish genetic influences from environmental influences (Plomin, DeFries, McClearn & McGuffin, 2001). These studies are able to calculate heritability estimates, which estimate the proportion of variation in an anxiety disorder due to genetic contribution (Griffiths, Miller, Suzuki, Lewontin & Gelbart, 2000). In their meta-analytic study on the genetic epidemiology of anxiety disorders, Hettema and colleagues (2001) calculated estimated heritabilities ranging from 30-40% across all anxiety disorders, with specific estimates of 0.43 for PD, 0.32 for GAD, and .28 for specific phobias and social phobia.

Among all anxiety disorders, PD has the highest rates of heritability and the strongest degree of familial aggregation and appears to be specific to offspring of parents with PD (Hettema et al., 2001). Morbidity risk estimates for PD in first-degree relatives of individuals with PD, which calculates the likelihood of individuals developing PD, range from 7.9-17.3%, compared to 0.7-4.2% for first-degree relatives of individuals without PD (Shih, Belmonte & Zandi, 2004). Likewise, pair-wise concordance estimates for PD, which calculates the presence of PD in both individuals of a pair of twins, range from 24-73% for Monozygotic (MZ) twins, compared to 0-17% for dizygotic (DZ) twins (Shih et al., 2004). Morbidity risk estimates for GAD range from 2.1-4.1% for (Grant et al., 2005), and pair-wise concordance ratios range from 23-60% for MZ twins and 14-41% for DZ twins (Hettema et al., 2001). For phobias, including SAD, morbidity risk estimates are as high as 13%, and pair-wise concordance ratios range from 12-26% for MZ twins and 4-24% for DZ twins (Kessler, Petukhova, Sampson, Zaslavsky & Wittchen, 2012).

Molecular research has identified several specific candidate genes that may confer risk for anxiety disorders. Gene expression research by Le-Niculescu et al. (2011) has identified top gene candidates associated with anxiety disorders in humans, including gamma-aminobutyric acid B (GABA) receptor 1 (GABBR1), nuclear receptor subfamily 4 group A member 2 (NR4A2), dopamine receptor 1 (DRD1), adenosine A2a receptor (ADORA2A), Regulator of G-protein signaling 2 (RGS2), prostaglandin-H2 D-isomerase (PTGDS), dynein light chain LC8-type 2 (DYNLL2), cholecystokinin-B/gastrin receptor (CCKBR), D site of albumin promoter binding protein (DBP), as well as brain-blood biomarkers such as FBJ murine osteosarcoma viral oncogene homolog (FOS), quaking homolog KH domain RNA binding (QKI), and heat shock 70kDa protein 1B (HSPA1B).

In addition to multiple genes being associated with anxiety disorders in general, several gene candidates have also been linked to risk for specific anxiety disorders. Candidate genes that have been linked with PD risk include the serotonin 2 A receptor (5HT2AR), cholecystinin-B receptor (CCKBR), dopamine receptor DRD4, catechol-O-methyltransferase (COMT), transmembrane protein 32D, and neuropeptide S receptor (Sakolsky, McCracken & Nurmi, 2012). Additionally, the short allele of the promoter length polymorphism (5HTTLPR) and rs2710102 has been associated with social anxiety. Although evidence is preliminary and results have been infrequently replicated, the T941G single nucleotide polymorphism (SNP) of the monoamine oxidase A (MAOA) gene has been associated with GAD (Hettema et al., 2001; Marion, Hettema & Shlik, 2010; Smoller et al., 2009; Tadic et al., 2003).

Despite the identification of potential gene candidates, which may be responsible for the transmission and expression of anxiety disorders, anxiety disorders still remain relatively understudied from a genetic standpoint when compared to other major psychiatric disorders (Le-Niculescu, 2011). Furthermore, given the genetic complexity of anxiety disorders, the specific genes related to their risk have yet to be definitively identified, and most likely include an interaction between multiple genes, with small individual effects (Gregory & Eley, 2007; Smoller, Block & Young, 2009). For example, over 350 gene candidates have been investigated in the context of PD, however, results have remained inconsistent, negative, or unclearly replicated (Maron, et al., 2010).

Due to the difficulty in identifying specific gene candidates of anxiety disorders, genetic researchers have begun to examine and identify potential “endophenotypes” or markers of susceptibility for anxiety disorders. Endophenotypes are believed to have a clearer association with gene candidates than the disorder itself (Flint & Munafò, 2007). Endophenotypes are

measurable and can be neurophysiological, endocrinological, neuroanatomical, biochemical, or neuropsychological in nature (Gottesman & Gould, 2003). For example, recent research on children at familial risk for PD has identified important risk markers, such as impairments in saccadic eye movement (Zwanzger, Bradwejn, Diemer, Marshall & Koszycki, 2012) and facial affect recognition (Bilodeau, Bradwejn & Koszycki, 2014). Likewise, an increased startle-response in anticipation of an adverse stimulus has been identified as a potential vulnerability marker in children with a parental history of GAD (Grillon, Dierker & Merikangas, 1998), and high levels of behavioral inhibition have been found among children with a parental history of SAD (Biederman et al., 2001). Given the many potential risk factors for anxiety disorder that have been examined in the research literature, the current review will focus on one specific endophenotype; activity of the HPA axis.

The HPA axis. The HPA axis plays a pivotal role in regulating the body's reaction to stress, restoring deviations from homeostasis after encountering adverse stimuli (Campeau et al., 1998). As illustrated in Figure 1., when a threat is detected by the brain, the hypothalamus secretes corticotrophin releasing hormone (CRH), stimulating the anterior pituitary to produce adrenocorticotrophic hormone (ACTH), which triggers the release of cortisol, the body's primary stress hormone, from the adrenal glands (Saxbe, 2008). In turn, cortisol plays an inhibitory role (negative feedback) by signaling the system to stop the stress response.

At low levels, cortisol helps the body maintain homeostasis after exposure to stress. However, when the HPA axis is dysregulated or compromised by chronic stress, this cortisol secretion may become elevated or blunted, which can contribute to a number of adverse outcomes. It is hypothesized that dysregulation of the HPA axis may be associated with the development of mood and anxiety disorders (Abelson, Khan, Kiberzon & Young, 2007),

indicated by a higher basal and stress-induced HPA axis activity (Harris & Seckl, 2011). Despite inconsistencies, there is evidence to suggest that hypersecretion of cortisol is associated with major depressive disorder (MDD) and possibly anxiety disorders (Van Santen et al., 2011).

Among the various indicators of HPA axis activity, the most consistent association with affective disorders, particularly depression, has been the cortisol awakening response (CAR) (Cowen, 2010). Although most research on the CAR has focused on depression, these studies may have important implications for anxiety disorders. Depression and anxiety disorders have been shown to share similar pathophysiological mechanisms, including HPA axis dysregulation (Boyer, 2000), and it is possible that both disorders show similar CAR responses. Given these similarities, I will review the available literature on the CAR in both depression and anxiety.

The Cortisol Awakening Response (CAR)

Cortisol secretion demonstrates a distinct diurnal rhythm with highest production occurring in the second half of the night, a peak in the early morning hours, and a steady decline throughout the day (Fries, Dettenborn & Kirschbaum, 2009). In healthy individuals, morning awakening is associated with a 50% increase in cortisol secretion, and, as seen in Figure 2, generally peaks 30 to 60 minutes after awakening (Clow, Thron, Evans & Hucklebridge, 2010).

First recognized by Pruessner and colleagues (1997), the CAR has been extensively studied over the past several years and is considered a reliable measure of HPA axis function (Schmidt-Reinwald et al., 1999). In highly controlled studies on the CAR, an increase in awakening cortisol levels has been observed in approximately 75% of healthy research participants, and the CAR response shows moderate intra-individual stability over several days or weeks (Wust et al., 2000a) Additionally, reported changes in cortisol concentrations from

awakening to 30 minutes post-wakeup in healthy individuals has been shown to remain relatively consistent between studies (Clow et al., 2004).

The CAR has also been found to be largely determined by genetic influences, versus evening cortisol levels, which are largely influenced by the environment (Wust, Federenko, Hellhammer, & Kirschbaum, 2000b). Twin studies have demonstrated moderate-to-high heritability estimates for the CAR (Bartels et al., 2003), and a recent study has identified the mineralocorticoid receptor (MR) gene as a possible moderator of this response (Van Leeuwen et al., 2010). Furthermore, there is evidence to suggest that dysregulation of the CAR may be related to affective disorders, such as depression and anxiety disorders (Van Santen et al., 2011). As mentioned previously, most research on the CAR has focused on depression. However, anxiety disorders typically precede major depression, share similar clinical features and respond to similar treatments, suggesting that they may share a similar pathophysiology (Kessler & Wittchen, 2000). Considering this overlap, the following will briefly summarize the literature on the CAR in depression and unaffected offspring with a parental history of depression.

CAR and depression. Most evidence for a link between CAR and affective disorders has come from research on adolescents and adults with current, subclinical, or remitted depression, as well as individuals at-risk for depression based on personality traits. In general, most studies have found an increased or elevated CAR associated with depression (van Santen et al., 2011), and to a less extent, a decreased or blunted CAR (Chida & Steptoe, 2008).

There is a substantial amount of evidence supporting an elevated CAR in MDD within community-based samples. A recent study by Dienes, Hazel and Hammen (2013) found that young women who met diagnostic criteria for MDD had a larger CAR compared to healthy

controls. A large community-based study by Vreeburg et al. (2009a) also found that persons with a current MDD had a significantly higher CAR compared to healthy controls. Similarly, in a community-sample of adults, Bhagwagar, Hafizi and Cowen (2005) found that those with a current MDD secreted approximately 25% more cortisol after awakening than healthy controls.

The hypersecretion of cortisol in the CAR is also present in individuals with subclinical depression -those displaying depressive symptomology, but not yet meeting diagnostic criteria for a depressive disorder. A study by Pruessner, Hellhammer, Pruessner, and Lupien (2003) found that higher levels of self-reported depressive symptoms in male university students were associated with a greater CAR. A more recent study by Heaney, Ginty, Carroll, and Phillips (2011) also found a higher CAR was related to higher self-reported depression scores among both male and female university students.

An elevated CAR has also been demonstrated in patients with remitted depression. A recent study found that patients in remission from a major depression episode displayed a 51% increase in CAR compared with controls (Aubrey et al., 2010). A similar increase in the CAR in patients with remitted depression was also found by Vreeburg et al. (2009a) and Bhagwagar et al. (2003). The finding that an increased CAR is present in the absence of depressive symptomology suggests that the CAR may represent a possible trait marker of depression, rather than a state marker (Vreeburg et al., 2009a).

Individuals with personality traits that are believed to confer risk for developing depression have also been reported to exhibit an elevated CAR. A recent study by Dienes et al., (2013) found that young women at risk for depression, based on trait-level negative and positive affect, had a larger CAR compared to control participants. Other studies have not only found an

elevated CAR among adolescents with high levels of neuroticism, but also found that the CAR predicted the subsequent onset of a clinical diagnosis of MDD (Adam et al., 2010; Vrshek-Schallhorn et al., 2013). Longitudinal prospective studies have also indicated that elevated waking cortisol levels, indexed by a single sample obtained after awakening in individuals at risk for depression, predicts future onset of depression and depressive symptoms. This has been demonstrated in at-risk samples of boys with internalizing/externalizing behaviour problems (Tyrka et al., 2012), adolescents with high life-adversity (Goodyer et al., 2000, 2009), adolescents exposed to post-natal maternal depression (Halligan, Herbert, Goodyer & Murray, 2007), and psychosocially vulnerable adult women (Harris et al., 2000).

Nevertheless, findings remain inconsistent, with some studies demonstrating a reduced (or blunted) CAR or waking cortisol associated with depression. A study by Huber, Issa, Schik and Wolf (2006), found a significantly lower CAR in patients suffering from depression compared to non-depressed controls. Likewise, a more recent study found that depressed outpatients had lower cortisol levels at time of awakening compared to healthy controls, and that severe levels of depression was associated with lower morning cortisol (Hsiao et al, 2010). A reduced CAR and morning cortisol levels have also been demonstrated in community-based samples of depressed women (Stetler & Miller, 2005; Strickland et al., 2002), depressed pregnant women (Shea et al, 2007), depressive symptomology among middle-aged men (Ahlberg et al., 2002), and university students with sub-clinical depression (Dedovic et al., 2010).

The inconsistencies between findings may be due to differences in the severity of depression being assessed (i.e. diagnosis of MDD versus depressive symptomology), failure to control for relevant factors known to influence the CAR, as well as differences in salivary

cortisol sampling methods across studies (Chida & Steptoe, 2008). Regarding sampling methods, it is important to note that some studies only collected salivary cortisol at one time-point after awakening (Goodyer et al., 2000, 2009; Halligan et al., 2007; Harris et al., 2000; Tyrka et al., 2012), which does not truly reflect the CAR response. Likewise, these studies only collected salivary cortisol samples on one day, which does not account for intra-individual variability in cortisol samples. These methodological issues are important to consider in future research on the CAR in depression, as well as anxiety.

Although the preceding evidence suggests that dysregulation of the CAR is associated with depression, it is not clear whether a dysregulated CAR constitutes a premorbid trait marker for depression. For this reason, the study of healthy, unaffected offspring of parents with depression is an important strategy to ascertain whether CAR dysregulation is a potential premorbid trait marker for depression risk.

The CAR and offspring with a parental history of depression. Studies investigating the CAR in offspring with a parental history of depression are limited and current findings remain inconsistent. Nevertheless, similar to patients with depression, there is evidence to suggest that offspring with a parental history of depression demonstrate an elevated CAR.

A large scale study by Vreeburg and colleagues (2010b) found that healthy, unaffected adults with a diagnosed parental history of depression showed a higher CAR compared to adults without a parental history of depression. Similarly, Mannie, Harmer and Cowen (2007) reported that young people, ages 17-21, with a parental history of depression exhibited an increase in waking salivary cortisol levels compared to controls. This also mirrors previous research by Halligan, Herbert, Goodyer and Murray (2004) who demonstrated higher, more variable morning

cortisol in adolescent offspring of mothers with postnatal depression. However, not all studies reveal an elevated CAR in at-risk offspring. A study by Young, Vazquez, Jiang and Pfeffer (2006) found morning cortisol levels did not differ in prepubescent children with and without a parental history of major depression.

As with studies on the CAR in individuals with depression, the inconsistencies between findings may be a result of methodological discrepancies. It is important to note that some studies had small sample sizes, did not adjust for important confounders, or used limited morning cortisol samples (Vreeburg et al., 2009). The use of limited morning cortisol samples also relates to the type of statistical analyses used in studies. Some studies only analyzed the mean of cortisol samples taken at single morning time-points across several sampling days (Young et al., 2006), while others analyzed the change in cortisol between multiple morning time-points, in addition to analyzing mean time-points across sampling days (Vreeburg et al., 2010). It is evident that the type of sampling and statistical methods used in studies may also explain inconsistencies in findings. As with limitations in research on patients with depression, these limitations must be addressed in future studies regarding the relationship between the CAR and affective disorders.

CAR in patients with anxiety. Although an extensive amount of research literature has demonstrated a link between depression and the CAR, less is known about anxiety disorders and the CAR (Vreeburg et al., 2010a). Across studies, findings have suggested that anxiety disorders may be associated with an elevated CAR (Adam et al., 2014), a normal CAR (Petrowski et al., 2010), or a blunted CAR (Dierckx et al., 2012).

In general, the majority of the research evidence has found that anxiety disorders are associated with a higher or elevated CAR. A study by Vreeburg et al., (2010a) found that

patients with anxiety disorders exhibited a higher CAR compared to controls, particularly for patients with PD with agoraphobia and anxious patients with comorbid depression. A similar elevation in morning cortisol was demonstrated in elderly patients with late-life GAD (Mantella et al., 2008). In children and adolescents, Kallen et al., (2008) found that low levels of anxiety in girls with an anxiety disorder were associated with a stronger CAR. Likewise, in a series of studies by Greaves-Lord and colleagues, adolescents with persistent anxiety problems (Greaves-Lord et al., 2007a) and increased self-reported anxiety levels (Greaves-Lord et al., 2009) displayed an elevated CAR.

A trend towards a higher CAR has also been demonstrated in patients with remitted anxiety disorders. A study by Vreeburg et al., (2010a) found that patients with a remitted anxiety disorder had a marginally higher CAR compared to controls. Similarly, Dierckx et al., (2012) found that over a one-year course of treatment, youth with a remitted anxiety disorder exhibited a higher CAR compared to those who remained symptomatic. Furthermore, a recent longitudinal prospective study by Adam and colleagues (2014) found that an elevated CAR in healthy adolescents significantly predicted first onset of anxiety disorders over a six-year follow-up, particularly for SAD.

As with depression, there is evidence to suggest that anxiety may not be associated with an elevated CAR or morning cortisol. Feder et al., (2004) found that children with anxiety disorders displayed peak levels of cortisol near time of awakening that were similar to depressed and healthy control children. Similarly, Petrowski et al., (2010) noted a similar CAR in patients with PD and healthy controls. In contrast to findings from their previous research, Greaves-Lord et al., (2009) found that young adolescents with persistently high self-reported anxiety did not show a higher CAR than those with low, increasing, or decreasing anxiety levels.

On the other hand, some studies have indicated that anxiety may be associated with a lower or blunted CAR and morning cortisol. Dierckx et al., (2012) found that non-remitters with persistent anxiety showed a blunted morning cortisol response compared to both early and late remitters. This is in line with findings from Kallen et al., (2008), who detected a lower CAR, 30 minutes after waking, in highly anxious girls. Likewise, one study found a lower CAR, 10-15 minutes after waking, in patients with PTSD compared to healthy controls (Wessa, Rohleder, Kirschbaum & Flor, 2006). Finally, in terms of prospective risk for anxiety, Tryka et al., (2012) found that morning cortisol concentrations did not predict future anxiety symptoms in boys with behavioural problems.

Similar to studies investigating the CAR in depression, inconsistent findings between these studies may reflect methodological differences. These studies did not use a control group of psychiatrically healthy individuals (Kallen et al., 2008; Dierckx et al., 2012), assess important confounding variables, such as pubertal status (Dierckx et al., 2012), had issues with compliance to sampling times (Kallen et al., 2008), or had a moderate sample size due to high attrition rates (Tryka et al., 2012). Additionally, discrepancies between findings could also relate to variations in morning sampling times, which ranged from one time-point (Tryka et al., 2012) to four time-points (Vreeburg et al., 2010a; Wessa et al., 2006), as well as the number of days samples were collected, which ranged from one day (Dierckx et al., 2012; Vreeburg et al., 2010a; Greaves-Lord et al., 2009; 2007a; Kallen et al., 2008; Wessa et al., 2006) to two consecutive days (Tykra et al., 2012; Petrowski et al., 2010; Mantella et al., 2008).

CAR and Parental History of Anxiety: A Premorbid Trait Marker?

In view of evidence supporting a higher CAR in individuals at familial risk of depression, there has been interest in examining whether dysregulation of the HPA axis also represents a heritable trait vulnerability marker (as opposed to only an illness marker) for anxiety disorders (Van Santen et al., 2011). To date, only four studies have examined the CAR in healthy, unaffected offspring of parents with anxiety disorders and/or anxious symptomatology.

One study, by O'Connor et al., (2005), examined awakening cortisol levels in a sample of 74 psychiatrically healthy, unaffected ten-year-olds with a history of maternal prenatal anxiety (i.e. pregnant mothers with anxiety). They found that maternal anxiety in late pregnancy was associated with a significant rise in cortisol from awakening to 30 minutes in offspring. Although this study suggests that prenatal anxiety influences awakening cortisol levels in offspring, the authors note that the study was limited by a small sample size, reliance on self-reported awakening and collection times that may be subject to bias, and failure to account for other factors that are known to influence cortisol levels, such as environmental factors. Additionally, salivary cortisol in offspring was only collected at two time-points during the morning (at awakening and 30 minutes after wakening), which may not be an adequate reflection of the CAR curve.

As part of a larger prospective study following 753 young Dutch adolescents, Greaves-Lord et al., (2007b) investigated morning cortisol levels in offspring of parents with high and low internalizing problems, including symptoms of anxiety. The group of children with high parental internalizing problems exhibited higher morning cortisol levels compared to offspring of parents with low internalizing problems. Further analysis revealed that the effects of high

morning cortisol in offspring of high parental internalizing problems were specific to parental symptoms of anxiety, not depression. However, the findings of this study are only specific to those with high and low parental internalizing problems (i.e. symptoms of anxiety), and therefore cannot be generalized to specific anxiety disorders or other populations. Similar to O'Connor et al's., (2005) study, salivary cortisol was only collected at awakening and 30 minutes later, and may not be an accurate reflection of the CAR.

A more recent study by Russ et al., (2012) examined awakening cortisol levels in response to starting school in children of mothers with social phobia. These children were also compared to children with a maternal history of generalized anxiety and those with no history of anxiety. Even though children of mothers with social phobia showed atypical elevations in evening cortisol in response to starting school, all children showed comparable elevated morning cortisol levels, which remained elevated from the first week of school until the end of the term. The researchers concluded that a persistent stress response to school in the morning (indicated by elevated cortisol levels) is typical for all children. Although these results suggest that elevated awakening cortisol levels may not be specific to children with a parental history of anxiety, this study examined awakening cortisol as a response to a major social stressor (starting school), and it is unclear whether elevations in awakening cortisol would have been seen in non-stressed conditions (i.e., out of school). Similarly, morning cortisol sampling was only performed within 30 minutes after awakening, which the researchers claim reflects both the diurnal cortisol profile and the CAR. However, in order to distinguish the components of the CAR separately from the diurnal profile, the researchers mention that it would have been more preferable to take a sample at awakening in addition to 30 minutes after awakening.

The strongest evidence supporting an elevated CAR as a premorbid trait marker for anxiety comes from The Netherlands Study of Depression and Anxiety by Vreeburg and colleagues (2010b). The researchers examined the CAR in a large sample of unaffected adults, 74 with a clinically-diagnosed parental history of depression or anxiety, 114 adults with a self-reported parental history, and 180 adults without a parental history. These three groups were additionally compared to 1262 people with depression or PD with agoraphobia. Salivary cortisol samples were obtained at awakening and 30, 45, and 60 minutes later. Compared to participants without a parental history, those with a diagnosed parental history of depression or anxiety showed a significantly elevated CAR (moderate effect-size, $d = 0.50$), similar to that of participants with depression or anxiety. Participants with a self-reported parental history of depression or anxiety did not differ from participants without a parental history. The researchers concluded that a higher CAR might be a putative trait marker for depression and anxiety disorders.

Nevertheless, as with the studies previously mentioned, there are several important limitations to the study by Vreeburg et al. (2010b). First, this study was cross-sectional in nature, and it is evident that longitudinal research is needed. Second, a majority of parents in the study had a primary diagnosis of depression, with or without comorbid anxiety, whereas less than 6% of participants had a primary diagnosis of anxiety only. The authors state that future research is needed in order to specify the relationship between the CAR and parental history of anxiety disorders.

The CAR and Personality Risk Markers

In addition to the CAR as a potential trait marker for anxiety disorders, there are also a number of individual risk markers that may be associated with the risk for developing anxiety disorders in children and adolescence. Like the CAR, the examination and identification of these individual risk markers in the context of offspring at risk for developing anxiety disorder is important for the early identification of vulnerable individuals and possible reduction in the development of anxiety disorders (Grillon et al., 2005). Research evidence has shown that there are certain personality traits that are strongly related to anxiety disorder risk (Clark, Watson & Mineka, 1994) and that the HPA axis may be influenced by variation in these traits (Walker et al., 2011). Among the personality traits that have been linked with anxiety disorder risk, behavioural inhibition, trait anxiety, and anxiety sensitivity, have been the most extensively discussed in the research literature.

Behavioural inhibition. Behavioural inhibition is a childhood temperament characterised by shyness and the tendency to withdraw when exposed to unfamiliar situations (Turner, Beidel & Wolff, 1996). It has been suggested that fearful, avoidant and withdrawn behaviours are characteristic of an inhibited temperament confer risk for developing pathological anxiety later in life (Rapee, 2002).

Overall, research evidence has shown that shy and inhibited children demonstrate an over-active HPA axis in response to social challenges (Hastings & Utendale, 2008). For example, in a series of studies by Kagan, Reznick and Snidman (1988), researchers found that toddlers who were classified as behaviourally inhibited, exhibited elevated cortisol levels in response to a behavioural stress task. Similarly, some researchers have found that behaviourally

inhibited children demonstrate high baseline cortisol levels (Kagan, Reznick & Snidman, 1987; Schmidt et al., 1997). However, a study by De Hann, Gunnar, Tout, Hart and Stansbury (1998) found that increased cortisol levels in toddlers, in response to starting preschool, was associated with more assertive and externalizing behaviour, rather than inhibited or anxious behaviour, suggesting that the association between cortisol and these traits is complex.

Although a relation between the CAR and behavioural inhibition has not been established, there appears to be a modest relationship between behavioural inhibition during the first 2 years of life and morning cortisol levels measured in early childhood. Early research by Kagen and colleagues (1987) found that children who were behaviourally inhibited at 21 months of age had higher morning cortisol levels at 5.5 years of age, compared to uninhibited children. Similarly, another study by Schmidt et al., (1996) found that infants who were behaviourally inhibited at 14 months of age displayed relatively high morning salivary cortisol at 4 years of age. Nevertheless, a more recent study by Russ et al., (2012) found that children who were classified as behaviourally inhibited at 14 months of age showed higher afternoon cortisol levels, but not elevated morning cortisol levels, when compared to children classified as uninhibited. From these results, it is evident that more research is needed in order to clarify the relationship between the temperament of behavioural inhibition and the CAR.

Trait anxiety. Trait anxiety is characterized by tendency to experience, report and attend to negative emotions such as fear, worry, and anxiety across different situations (Van den Bergh, Van Castler, Puissant, & Van Huffel, 2008). Research has shown that trait anxiety reflects a general risk marker for anxiety disorders (Grupe & Nitschke, 2013) and that high trait anxiety may be associated with attenuated HPA axis functioning (Jezova, Makatsori, Duncko, Moncek & Jakubek, 2004).

An early study by Zorrilla, DeRubeis, and Redei (1995) found that participants with high trait anxiety showed lower basal cortisol levels than those with low trait anxiety. This finding mirrors more recent research by Jezova and colleagues (2004), who found that participants with high trait anxiety showed lower levels of salivary cortisol in reaction to a psychosocial stressor. The researchers suggest that individuals with high trait anxiety may have an inability to respond to acute stressors with sufficient release of stress hormones.

Similar to basal cortisol levels, trait anxiety also appears to be negatively associated with the CAR. A study by Walker et al., (2011) found that individuals high in trait anxiety secreted less cortisol following awakening compared to those with low trait anxiety. Therrien et al., (2008) also demonstrated a similar negative correlation between trait anxiety and the CAR in a sample of university women. In light of these findings, Walker et al., (2011) speculated that individuals high in trait anxiety react more negatively to stress, which in turn, may cause the HPA axis to become less responsive over time. Nevertheless, Van den Bergh et al., (2008) found that among post-pubertal adolescents, trait anxiety was associated with higher evening cortisol levels, but not awakening and noon cortisol levels. It is evident from these findings that the relationship between trait anxiety and the CAR is unclear, and that further research is needed to better elucidate this relationship.

Anxiety sensitivity. Anxiety sensitivity is a stable trait characterized by the tendency to appraise anxiety-related sensations as having negative psychological, physical, and social consequences (Reiss, Peterson & Gursky, 1988). Anxiety sensitivity is distinct from, yet related to, trait anxiety and may constitute a premorbid cognitive risk factor for anxiety disorders, particularly PD and panic attacks (Taylor, Koch & McNally, 1992). Several studies have found a negative association between the CAR and anxiety sensitivity (Therrien et al., 2008; Walker,

O'Connor, Schaefer, Talbot, & Hendrickx, 2011). However, another study, which examined the relationship between psychological traits and the CAR in a large sample of youth and adults, failed to find an association between AS and the CAR (Van Santen et al., 2011). It is evident that the relationship between anxiety sensitivity and the CAR remains unclear and that future research is needed.

The CAR and Environmental Variables

Although anxiety disorders appear to have a strong genetic component, with estimated heritabilities ranging from 30-40%, there still remains a large portion of variance that is most likely explained by environmental factors (Hettema, Neale & Kendler, 2001). Due to the large number of environmental factors that may be related to the CAR, it is beyond the scope of the current literature review to discuss all of them. Therefore, the current discussion will focus on three environmental variables that may impact the CAR; prenatal stress, child and adolescent life stressors, and gender of the affected parent.

Prenatal stress. Maternal stress during pregnancy has been shown to relate to dysregulation of the HPA axis in offspring (Gutteling, De Weerth & Buitelaar, 2005). More specifically, prenatal stress has been linked to fluctuations in morning cortisol levels in children (O'Connor et al., 2005). In a prospective longitudinal study that followed mothers and children from pregnancy, O'Donnell and colleagues (2013) found that prenatal stress was associated with a flattened diurnal slope and blunted CAR. Likewise, research suggests that prenatal exposure to stress may be associated with increased anxiety in offspring, as well as an increased risk for developing an anxiety disorder during adolescence (Davis & Sandman, 2012). Nevertheless, it is important to note that there is a general lack of consensus among researchers on the definition of

prenatal stress (Shea et al., 2007). Similarly, very few studies exploring prenatal stress have specifically distinguished the CAR from the diurnal cortisol profile. Indeed, further investigation of prenatal stress is warranted to better understand its effect on the CAR.

Child and adolescent life stressors. Similar to prenatal stressors, stressful life events during childhood and adolescence have been associated with changes in HPA axis regulation, as well as later development of anxiety disorders (Faravelli et al., 2012). In a sample of monozygotic and dizygotic twin pairs (mean age = 19.6 years), Wust et al. (2000a) found that chronic and social stressors were associated with an elevated CAR. Further evidence is provided from a meta-analytic study on adults by Chida and Steptoe (2008), who found that the overall volume of cortisol released during awakening (CARauc) and the cortisol increase following awakening (CARi) were positively related with general life stress. Nevertheless, some studies have failed to find association between life stressors and the CAR. In a sample of children (mean age = 10.7), Bevans, Cerbone and Overstreet (2008) found that life stressors within the past 12 months were associated with higher afternoon cortisol levels, but no association emerged for morning cortisol levels. Despite the inconsistent findings between studies, it is evident that research on life stressors and the CAR in children and adolescence is lacking. As with prenatal stressors, further research is needed in order to clarify the relationship between childhood and adolescent life stressors and the CAR.

Gender of affected parent. No studies to date have explored the relationship between the gender of the parent with an anxiety disorder and the CAR in offspring. Nevertheless, there is evidence to suggest that maternal anxiety may be specifically associated with vulnerability to anxiety disorders in children. A study by Kikkert, Middleburg and Hadders-Algra (2010) found that maternal but not paternal trait anxiety was associated with less optimal neurological

outcomes in infants. Similarly, it has been shown that maternal but not paternal anxiety disorders are predictive of anxiety disorders in children (McClure, Brennan, Hammen & Le Brocque, 2001). Further evidence is provided by Warren and colleagues (2003), who found that maternal PD was associated with higher salivary cortisol in infants. Nevertheless, the authors acknowledge that paternal psychopathology may also contribute to differences in infant neurophysiology. Additional research is needed to better understand the relationship between gender of the affected parent and the CAR offspring.

Covariates and Potential Confounds

Despite the CAR's potential as a premorbid trait marker for anxiety disorder risk, previous research has demonstrated that the CAR has both inter-individual and intra-individual variability (Pruessner et al., 1997; Wust et al., 2000a). Due to their influence on the observed variability within the CAR, identifying factors causing such variability is essential. As a result, an extensive amount of research over the last several decades has focused on identifying covariates and confounds related to the CAR. Among the many potential covariates and confounds that have been examined in the research literature, age, gender, and puberty status, are the most frequently researched.

Age. Studies on age-related differences in HPA axis activity have found that older age is associated with higher baseline cortisol levels in response to a stressor (Seeman, Singer, Wilkinson, & McEwen, 2001), as well as increased evening cortisol levels and CAR (Vreeburg et al., 2009b). Although Vreeburg et al (2009b) identified age as an important covariate in their study of the CAR in offspring with a parental history of anxiety disorders, a review of the CAR literature by Fries et al., (2009) found no clear relationship between age and the CAR. Most

studies have found that the CAR is negatively associated with age or not associated with age (Clow et al., 2004). The finding that age may not be related to the CAR has been documented in children, adolescents, and adults (Wust et al., 2000a; Oskis, Loveday, Hucklebridge, Thorn & Clow, 2009; Pruessner et al., 1997). Nevertheless, it should be noted that many of these studies are limited, in that they had assessed a very restricted age range. Additional research is therefore needed to determine the moderating effect of age on the CAR response.

Gender. Gender has received a considerable amount of attention as a potential factor influencing cortisol secretion, including the CAR. Studies on pre- and post- pubescent children have found that morning cortisol levels of mid-post pubescent females were 20-30% higher than males (Netherton, Goodyer, Tamplin, & Herber, 2004). Similarly, in a sample of 10 to 12 year old children, a higher CAR was found for females when measured by the area under the curve with respect to ground (AUCg) (Rosmalen et al., 2005). Interestingly, Vreeburg and colleagues (2009b) found that males showed a flattened CAR compared to females, further supporting gender as an important variable in the analysis of the CAR. In studies that have found a significant association between the CAR in offspring and parental history of anxiety disorders and/or anxious behaviour, gender has been used as a confounding factor (Greaves-Lord et al., 2007a) and covariate (O'Connor et al., 2005; Vreeburg et al., 2010) in the analysis of the CAR.

Despite this evidence demonstrating a link between gender and the CAR, it has been concluded that the influence of gender on the CAR is rather small (Fries et al., 2009), accounting for 1-3% of the total variability in the CAR (Wust et al., 2000a; Pruessner et al., 1997). These findings are in accordance with earlier research demonstrating no gender difference in morning cortisol levels in healthy children and adults (Kiess et al., 1995). As with age, the relationship

between gender and the CAR is in need of further research to better understand its function as a covariate, particularly in the context of children at risk for anxiety disorders.

Puberty status. Similar to age and gender, the biological maturation from childhood to adolescence may influence cortisol levels and the CAR. The gender difference reported by Netherton et al., (2004) was specific to mid-post pubescent girls, but not pre-early pubescent subjects, suggesting a change in the endocrine system at puberty. This notion is further supported by a study on 52 adolescents, ages 12-19, demonstrating that later puberty stages were associated with a smaller CAR (Adam, 2006). Nevertheless, in a large sample of girls and boys ages 10-12, Rosmalen and colleagues (2005) found no relationship between puberty stage and the CAR. Likewise, a lack of association between puberty status and the CAR was reported by Osksis et al., (2009) in their sample of 61 females, ages 9-18. In regards to studies on the CAR in children at risk for anxiety disorder, O'Connor et al. (2005) found that including puberty as a covariate did not alter the association between prenatal anxiety and the CAR in 10 year old offspring. Despite the obvious methodological limitations in age-range and sample size, there still remains a lack of clarity regarding the relationship between puberty status and the CAR. Further research is necessary to clarify this relationship.

CAR Responders versus Non-responders.

As mentioned previously, an increase in awakening cortisol levels occurs in approximately 75% of healthy individuals, with the remaining 25% of individuals demonstrating a blunted or decreased CAR (Wust et al., 2000a). The finding that approximately 25% of individuals show no increase in salivary cortisol levels after awakening suggests a distinct pattern of inter-individual variability in HPA-axis functioning (Wust et al., 2000a). However, it

is still up for debate whether CAR “non-responders” truly exist (Gribbin, Watamura, Carins, Harsh, & LeBourgeois, 2011), or are the result of participants’ non-adherence to sampling protocol (Clow, et al., 2004). Nevertheless, understanding factors that may influence a blunted or decreased CAR has important implications for CAR research (Gribbin et al., 2011), and it has been suggested by some researchers, that distinguishing between CAR “responders” and “non-responders” may be useful (Clow et al., 2004).

Diurnal Cortisol Response

Although the focus of the current study was to examine the CAR in offspring at familial risk for anxiety disorders, it is important to also examine diurnal cortisol response in these at-risk children. In contrast to the CAR, which reflects an immediate increase in cortisol levels 30–45 minutes after awakening in the morning (Edwards et al., 2001), diurnal cortisol secretion reflects a distinct circadian rhythm over 24-hours, with highest production occurring in the second half of the night, a peak in the early morning hours, and a steady decline throughout the day (Fries, et al., 2009). Similar to the CAR, elevated diurnal cortisol levels have been found to be associated with anxiety disorders such as PD (Bandelow et al., 2000). However, findings remain inconsistent, with several studies demonstrating no differences in diurnal cortisol levels in adolescents and adults with PD compared to healthy controls (Doane, et al., 2013). Furthermore, a study by O’Connor et al. (2005) found no association between prenatal anxiety and diurnal cortisol levels in offspring at age 10, suggesting that diurnal cortisol may not constitute a risk marker for anxiety disorders in children. Nevertheless, these studies were limited by a small sample size, reliance on self-reported collection times that may be subject to bias, or did not account for other factors that are known to influence cortisol levels. Further research on the relationship between the diurnal cortisol response and parental history of anxiety is warranted.

Similar to the CAR, a number of individual risk markers and covariates have been associated with the diurnal cortisol response. Evening cortisol levels have been found to be elevated in behaviourally inhibited children relative to uninhibited children (Russ et al., 2012), while trait anxiety has been found to be associated with a flatter diurnal curve and elevated evening cortisol levels (Van den Bergh, et al., 2008). Like the CAR, anxiety sensitivity has been shown to correlate negatively with cortisol responses to a social stressor (Sjörs, et al., 2010). Prenatal anxiety has been shown to be associated with a flatter diurnal slope (O'Donnell et al., 2013), a steeper diurnal response, and lower evening cortisol levels in children starting school (Bruce, Davis, & Gunner, 2002). Although findings are inconsistent, age has been shown to have a positive association with diurnal cortisol secretion (Clow et al., 2004), whereas gender and puberty status show varying associations with the diurnal cortisol response. As with research on the CAR, the effects of risk markers and covariates on the diurnal cortisol response warrant additional study.

Study Rationale

There is substantial evidence that anxiety disorders run in families and that children with parental anxiety are at increased risk for developing anxiety themselves. Recent research suggests that dysregulation of the CAR (e.g., elevated or blunted secretion) may represent a heritable pre-morbid vulnerability marker for anxiety risk (Van Santen et al., 2011). Although findings are still very preliminary, the CAR may be a promising mechanism for understanding the reported link between parental anxiety disorders and elevated risk for anxiety in offspring, and a potential target for preventive interventions that can shift a child's trajectory from risk to resilience. The present study investigated whether the CAR differed in unaffected children with and without parental anxiety disorders. This "high risk" design is a well-established strategy to

identify pre-existing heritable markers of anxiety disorder risk and elucidate possible mechanisms that explain the familial transmission of anxiety (Merikangas, Avenevoli, Dierker et al., 1999).

Objectives and Hypotheses of the Current Study

The primary aim of the current study was to examine whether awakening cortisol levels differed in children and adolescents with a parental history of PD, GAD or SAD (high risk children), compared to those with no parental psychopathology (low risk children). The study also examined if differences were specific to anxiety disorder subtype. Results of the direction of the CAR response (i.e. an elevated, decreased, or no response) have not been consistent. Therefore in the current study, I did not make a hypothesis regarding the direction of the CAR, but hypothesized that high risk children would exhibit a difference in their CAR compared to low risk controls. If such a difference exists, this would suggest that awakening cortisol might be a potential trait marker for anxiety disorder risk.

The secondary aim of the current study was to examine the relationship between the CAR and other variables of interest. The personality variables of behavioural inhibition, trait anxiety, and anxiety sensitivity have shown to be psychological risk-markers for anxiety disorders, and may be related to the CAR, although findings remain inconclusive regarding the directionality of this relationship. Based on the research evidence discussed previously, it was hypothesized that these traits would correlate with the CAR and potentially account for some variability in this marker.

In regards to other variables of interest, higher reports of prenatal stress and child stressors have been associated with risk for anxiety disorders, and these stressful events may be

related to the CAR. Although there is some evidence that maternal anxiety may be associated with vulnerability to anxiety disorders in offspring, a link between gender of the affected parent and the CAR in offspring has not been established. Based on the findings previously discussed, it was hypothesized that high risk children would be exposed to higher levels of prenatal stress and current/remote stressors compared to low risk control children. Furthermore, it is hypothesized that among high risk children, prenatal stress, current/remote stressors in offspring, and maternal anxiety disorder would be positively correlated with the CAR, and potentially account for some variability in this marker.

The potential covariates of age, gender, and puberty status have been shown to significantly influence the CAR in studies of offspring at risk for anxiety disorders. Based on the evidence reviewed previously, it was hypothesized that age and female gender would have a positive correlation with the CAR and later puberty stages would have a negative correlation with the CAR.

Although the primary focus of the current study was to examine the CAR as a potential pre-morbid trait marker in children with a parental history of anxiety, an additional set of analyses examined the relation of the diurnal cortisol response to children with a parental history of anxiety. Similar to the CAR, results of the direction of the diurnal cortisol response (i.e. an elevated, decreased, or no response) have been inconsistent. Although no specific hypothesis was made regarding the direction of the diurnal cortisol response, I predicted that children with a parental history of anxiety would exhibit a difference in diurnal cortisol secretion compared to controls.

It was also hypothesized that behavioural inhibition, trait anxiety, and anxiety sensitivity would correlate with diurnal cortisol response, and that these traits would account for some of the variability in this response. Additionally, it was hypothesized that prenatal stress, current/remote stressors in offspring, and maternal anxiety disorder would correlate with the diurnal cortisol response. It was further hypothesized that age, female gender, and later puberty stages would correlate with diurnal cortisol response.

The previously discussed hypotheses can be summarized as follows:

1. High risk children will exhibit a difference in their CAR compared to low risk children.
2. Behavioural inhibition, trait anxiety and anxiety sensitivity will correlate with the CAR and account for some of the variability in the CAR.
3. High risk children will report higher levels of prenatal stress and current/remote stressors.
4. Among high risk children, prenatal stress, current/remote stressors and maternal anxiety disorder will positively correlate with the CAR and account for some of its variability.
5. Age and female gender will positively correlate with the CAR and later puberty stages will have a negative correlation with the CAR.
6. High risk children will exhibit a difference in diurnal cortisol compared to low risk children.
7. Behavioural inhibition, trait anxiety and anxiety sensitivity will correlate with the diurnal cortisol response.
8. Age, female gender, and later puberty stages will correlate with the diurnal cortisol response.

Methods

Data was comprised from two cohorts from the Children at Risk for Panic Disorder (CAR) Study, an ongoing longitudinal and cross-sectional study designed to investigate biological and psychological risk markers for the development of panic disorder. This study was designed by Dr. Koszycki and her colleagues from the University of Ottawa's Faculty of Medicine and School of Psychology. This study was approved by the Research Ethics Board of the Royal Ottawa Mental Health Center (Cohort 1) and the University of Ottawa (Cohort 2), and was funded by the Canadian Institutes of Health Research.

Participants

Participants consisted of psychiatrically healthy offspring, between the ages 7-18 years of age at high and low genetic risk for PD with or without agoraphobia (PD±AG), GAD, and SAD. All families were recruited via advertisements placed in local newspapers and on the Internet, as well as flyers placed on public bulletin boards. In addition to these recruitment methods, some participants from Cohort 1 were recruited via their parents who had participated in a genetic study for PD.

Inclusion and Exclusion Criteria

High risk (HR) participants consisted of children who had a biological parent with a current or lifetime diagnosis of PD±AG, GAD, or SAD. Low risk (LR) participants consisted of children with no history of parental psychopathology. In order to control for possible effects of dual-parent psychopathology on risk markers, HR offspring with a second biological parent with a current or lifetime Axis I disorder other than PD±AG, SAD, or GAD were excluded. Offspring were excluded from the study based on the following criteria:

1. A documented history of threshold, subthreshold Axis-I disorders at the index visit.
2. An unstable and/or clinically significant medical condition (i.e. cardiovascular, respiratory, hematological, endocrine, or neurological disease).
3. Use of medications with effects on the peripheral and/or central nervous system.
4. Within the last three months prior to the study, participated in an experiment that involved medication intake.

Consent/Assent

For eligible offspring under 16 years of age, written informed consent was obtained from their legal guardian in addition to their assent, while eligible offspring 16 years or older provided their own consent.

Assessment of Parents

Parents underwent an initial telephone screening interview with a research assistant who explained the purpose and details of the study. Parents were then questioned about their own psychiatric history, as well as the psychiatric history of their spouse. Parents were also asked about their child's history of psychiatric symptoms, medical history and history of medication use. If the family was deemed eligible based on the inclusion/exclusion criteria, a second telephone interview was scheduled to evaluate the diagnostic status of the parent.

A structured clinical interview was administered by a licensed psychologist, with extensive experience using this instrument, to confirm the presence of current or lifetime history of primary panic PD \pm AG, GAD, or SAD. Parents who had comorbidity (other than dysthymia, unipolar major depression, or specific phobia) were excluded. The same structured interview was also used to confirm the absence of psychiatric illness in both parents of LR offspring and was

administered by a research assistant who was trained to high levels of inter-rater reliability and was supervised by the primary investigator throughout the study.

Assessment of Offspring

Offspring participated in a face-to-face structured clinical interview (conducted by doctoral level clinicians and trained research assistants who were blind to the parental diagnosis) to confirm the absence of current threshold or sub-threshold psychiatric problems, as well as a history of threshold and sub-threshold psychiatric problems. The childhood version of the SCID (KID-SCID; Matzner, Silvan, Chowdhury & Nastasi, 1996) was used for Cohort 1 and the Anxiety Disorders Interview Schedule for DSM-IV (ADIS)-Child Version (Silverman & Albano, 1996) was used for Cohort 2. Both interviews are reliable measures of Axis I disorders in childhood.

A research assistant interviewed parents independently in order to obtain information about the mother's pre- and postnatal history, as well as the child's developmental, social, and medical history, performance in school, and any remote or current psychosocial stressors that are listed in the DSM-IV (Axis IV) assessment. Eligible children were asked to complete a series of self-report questionnaires and were interviewed by a research assistant about stressful experiences. Younger participants were assisted by a research assistant in completing self-report measures. Participant's body mass index (BMI) was calculated and their physical activity level was assessed. After completion of the study visit, each child was paid \$25.00 as remuneration for the assessment. Participants were then provided with a salivary cortisol collection kit and asked to collect saliva samples at specified time points in their natural environment.

Clinical Interviews

Structured Clinical Interview for DSM-IV (SCID)-Adult Edition (First, Spitzer, & Gibbon, 1995) - The SCID is a semi-structured interview that assesses current and past DSM-IV Axis I diagnoses in adults ages 18 and older and is a widely used diagnostic instrument in psychiatry research. Reliability studies indicate high levels of diagnostic agreement for PD ($\kappa = .66$ to 1.0) with high diagnostic agreement for other Axis I disorders when the SCID is administered by experienced interviewers (APA, 2000). The SCID was used to confirm primary diagnoses of PD, GAD, and SAD in affected parents, and to confirm the absence of psychiatric illness in unaffected control parents.

Childhood Disorders Version of the SCID (KID-SCID) (Matzner, et al., 1996) – The KID-SCID is a semi-structured interview based on the adult edition of the SCID. It includes most adult disorders found in the adult version of the SCID, in addition to childhood disorders, such as Separation Anxiety Disorder. The KID-SCID has shown moderate to good inter-rater reliability and internal consistency (Roelofs, Muris, Braet, Arntz & Beelen, 2014).

Anxiety Disorders Interview Schedule for DSM-IV (ADIS)-Child Version (Silverman & Albano, 1996) – The ADIS-C is a semi-structured interview designed to assess DSM-IV anxiety disorders in children ages 6 to 17 years of age. In addition to anxiety disorders, the ADIS-C also includes modules for assessing mood disorders, externalizing disorders, tic disorders, schizophrenia and substance abuse/dependence, as well as screens for selective mutism, eating disorders, somatoform disorders, and learning disorders. The ADIS-C is considered to be a gold-standard diagnostic instrument for anxiety disorders in children (Stallings & March, 1995). The ADIS-C has excellent inter-rater reliability ($\kappa = .95$), high test-retest reliability ($>.90$) for child

interviews, as well as moderate to high kappa coefficients for other disorders (Silverman, Saavedra & Pina, 2001).

Self-report Measures

Baseline Screening Questionnaire – A baseline screening questionnaire was used to obtain information about the mother's pre- and postnatal history, including prenatal stressors, history of drug and alcohol use, psychiatric history, child medical and developmental history, and child stressors present in the last 12 months. Prenatal and child stressor categories included problems with primary support groups, problems related to social environment, education problems, occupational problems, housing problems, economic problems, problems with access to healthcare services, problems related to interaction with the legal system/crime and other psychosocial/environmental problems.

Tanner Scale (Tanner, 1962) – The Tanner scale is a self-report measure assessing physical development in children, adolescents, and adults. The scale is categorized into five consecutive puberty stages based on external sex characteristics, such as breast size for females, testicular volume in males, and pubic and auxiliary hair growth in both males and females. For the purpose of this study, the Tanner scale was used to control for the effect of pubertal status in the analysis of salivary cortisol. Participants were further grouped into two categories based on the Tanner stages of development; pre-early: Tanner stage <III, and mid-post: Tanner stage >II (Netherton, et al., 2004).

State-Trait Anxiety Inventory for Children (STAIC) (Spielberger, 1973) – The STAIC consists of two 20-item scales designed to assess trait (STAIT-T) anxiety in children and adolescents ages 6 to 18 years old. Items are rated on a 3-point likert scale, with higher scores

reflecting higher levels of trait anxiety. Internal consistency for the STAIT-T is satisfactory, with an alpha coefficient of 0.80 (Southam-Gerow, Flannery-Schroeder, & Kendall, 2003). Test-retest for the reliability for STAIT-T over an interval of 6-weeks ranges from 0.65 to 0.71, and construct validity correlates 0.75 with the Revised Children's Manifest Anxiety Scale (Southam-Gerow, et al., 2003).

Childhood Self-Report of Inhibition (CSRI)-Version 2 (Reznick, n.d.) – The CSRI is a 31-item scale designed to assess childhood behavioural inhibition (BI) and is based on the adult self-report Retrospective Childhood Inhibition Scale (RCIS) (Reznick, 1992). Items on the CSRI are rated on a 5-point likert scale, directly parallel those of the RCIS, and are written in a language that is appropriate for children as young as 7 years old. Respondents are questioned about a broad range of childhood behaviours associated with BI, including separation anxiety, social withdrawal, general and specific fears, and a wide range of complaints related to illness. There are currently no published psychometric data available for the CSRI (*Reznick, personal communication*), however, research on the RCIS using undergraduate students indicate high internal consistency of the scale (Cronbach's α from 0.79 to 0.91).

Childhood Anxiety Sensitivity Index (CASI) (Silverman et al., 1991) – The CASI is designed to assess children's beliefs about the negative consequences of anxiety. It includes 18 items that are rated on a 3-point likert scale. Psychometric evaluation of the CASI in children and adolescents ages 6-17 years reveal internal consistency estimates of 0.87 and test-retest reliability estimates of 0.76 and 0.79 for nonclinical and clinical samples respectively (Silverman, Ginsburg & Goedhart, 1999). The CASI correlates with childhood measures of fear and anxiety (Rabian, Embry & MacIntyre, 1999) and discriminates between children with and without anxiety disorders (Muris et al., 2001; Myers & Winters, 2002).

Biological Measures

Salivary Cortisol Collection and Assay – Participants collected salivary cortisol using salivettes at five time points within in their natural setting: Time 1 in the morning before getting out of bed, Time 2 at 30 minutes after awakening, Time 3 at 60 minutes after awakening, Time 4 at 4pm, and Time 5 at 8pm. Cortisol samples were collected over a period of two consecutive days in order to increase reliability of collection values. Mean values were obtained for each time point across the two-day collection period. Detailed instructions and an illustration of the collection process were provided to the participant and their parent in order to ensure proper collection of the samples. Participants were instructed about medications and foods to avoid during the days samples were collected and were asked to refrain from eating, drinking (except water), smoking, and brushing their teeth at least 60 minutes before collection. Participants were also instructed not to collect samples if they were ill or menstruating. A diary was provided to participants in order to record the exact time of sampling, as well as any deviation from the sampling protocol. They were also asked to document the presence of stressful events during sampling days. After collection, parents were instructed to store samples in their freezer and return the samples to the research unit at their child's next scheduled visit. Each child received \$30.00 for completing the two-day cortisol collection. Samples were stored in the lab at -20°C until assayed. For Cohort 1, salivary cortisol was measured via radioimmunoassay using ImmuChem Cortisol Coated Tube RIA Kits, provided by MP Biochemicals. For Cohort 2, salivary cortisol was measured in duplicate via radioimmunoassay using Cortisol Salivary Immunoassay Kits from Salimetrics.

Excluded Measures

Although the following measures were included in the original proposal for the current study, they were ultimately excluded from the study. These measures, body mass index (BMI) and fitness level, are described below in addition to the reasons why they were excluded.

Body Mass Index (BMI). Research staff obtained participant's height and weight at each visit in order to calculate BMI. BMI was obtained for 188 participants; the mean BMI score was 19.98. However, BMI was excluded as a covariate from the final analysis as it was not measured in the first 83 participants in Cohort 1, and it did not correlate with any measure of cortisol.

Fitness Level. Participants provided a log of the minutes/hours of physical activity they engaged in during a typical week. Activities include climbing stairs, riding a bike/scooter, taking a walk, physical education class, dancing, playing sports, swimming, raking leaves/shoveling snow, and other activities sufficient enough to "work up a sweat". Fitness level was excluded from the final analysis as a covariate as this measure was found to be an inadequate measure of physical activity due to inconsistencies in units of measure reported by participants.

Statistical Analyses

Sample Size and Flow of Participants

The sample for the current study was comprised of children who participated in two separate studies on children at risk for anxiety: study cohort 1 (N=125) and study cohort 2 (N=164). The initial combined sample consisted of N=289 participants (HR = 114, LR = 175). Consort diagrams, illustrating the flow of participants, are presented for cohort 1 (Figure 3) and cohort 2 (Figure 4) separately.

Cohort 1. As seen in Figure 3, cohort 1 initially consisted of 125 participants, who were further divided into HR (n=53) and LR (n=72) risk groups. Of the HR group, two participants withdrew from the study prior to sample collection. In the LR group, one participant did not collect cortisol samples and was excluded. Due to change in protocol, cortisol samples were only obtained at wakeup, 4pm and 8pm for one day, therefore, CAR and +30 and +60 awakening cortisol values were available for 74 children (13 high risk, 50 low risk) in this cohort.

Cohort 2. As seen in Figure 4, cohort 2 consisted of 164 participants (61 HR, 103 LR). Of the HR participants, four withdrew from the study prior to sample collection and two did not collect any cortisol samples. In the LR group, four withdrew from the study prior to sample collection and three did not collect any cortisol samples.

Final sample. The final sample size consisted of N=274 participants (HR = 101, LR = 173).

Data Cleaning and Missing Values

Extreme values. Cortisol values were converted to z-scores in order to examine potential extreme outliers (defined as 2.5 standard deviations above the mean as per Russ et al., 2012). Examination z-scores revealed that 3% of values in Cohort 1, and 3% of values in Cohort 2, were identified as extreme outliers and were subsequently deleted, as recommended by Adam and Kumari (2009) because of their potential to skew results. These deleted values were replaced by new values generated in the multiple imputation procedure (See section on Multiple Imputation of Missing Cortisol Values).

Skew & transformation. Since the distribution of cortisol values were positively skewed, the individual values were log transformed. For variables containing negative values

(AUCi), a constant of +2 was added before log transformation. Analyses of psychological variables (STAIC-T, CSRCI, and CASI) also revealed a positive skew, and were therefore log transformed as well. Non-transformed values are presented in the Results section.

Missing and insufficient data. An analysis of missing data revealed that 0.5% of cortisol values were missing for Cohort 1 and Cohort 2 together. For Cohort 1, 51 (8%) of cortisol values, and for Cohort 2, 179 (12%) of cortisol values were unable to be analyzed due to insufficient saliva volume to conduct analysis. BSQ data (i.e. presence of prenatal stressors and current/remote child stressors) were also missing for 28 participants in the total sample. As the percentage of missing and insufficient values in the data set was greater than 5%, Multiple Imputation (MI) was conducted as recommended by Graham (2009). Multiple Imputation is considered the gold standard for imputing missing data as it avoids issues that are characteristic of mean substitution and excluding cases with missing values (Walker et al., 2011).

Multiple imputation of missing cortisol values. Before MI, the data were divided into two separate datasets based on awakening cortisol values/AUC and diurnal/evening cortisol values. Missing cortisol values were first visually examined for patterns of missing data. The pattern of missing data values appeared random. Multiple imputations were run using Mersenne Twister random number generation and the set starting point for the Active Generator Initialization was set at a fixed value (2000000 by default). Imputation of missing values was performed using the automatic method, which automatically selects an imputation method based on a scan of the data for patterns of monotonicity. The number of imputations was set at 5, which is the default number of imputations for SPSS and is in accordance with Rubin (1984), who showed that only 3-10 imputations are needed.

Statistical Analyses

Cohort comparison. In order to investigate cohort as a potential covariate, mean cortisol values were compared between Cohort 1 and Cohort 2 using a one-way ANOVA.

Baseline characteristics. Differences between the risk groups on baseline demographic characteristics (gender, age, puberty status), personality measures (STAIC-T, CSRCI, CASI), prenatal stress and remote/current child stressors were analyzed using ANOVA for continuous data and chi-square (χ^2) statistic for categorical data.

Covariates. Pearson correlation analysis was used to examine the relationship between continuous variables (e.g., age, personality traits) and average cortisol values and AUC values and Point Biserial correlation was performed to examine the relationship between categorical variables (e.g. puberty status, gender, current/remote stressors) and average cortisol values and AUC values. As no significant association emerged for gender and indices of HPA axis function, gender was excluded as a covariate from the analyses. Pearson and Biserial correlations for the main covariates of age, puberty status, and gender are described in the Results section.

Awakening cortisol and CAR analyses.

Awakening Cortisol Averages. Averages of the 3 awakening sampling times across the 2 sampling days were obtained (Time 1 at awakening; Time 2 at 30 minutes after awakening, and Time 3 at 60 minutes after awakening). For the first 78 participants in cohort 1, samples were collected on one day only (as per protocol), and therefore, day 1 values were used in place of averages (means). A Generalized Linear Mixed-effects Model (GLMM) was used to analyze differences in awakening cortisol averages between HR and LR groups. Puberty status, age, and cohort were entered as covariates in the model. Risk group (LR and HR) was entered as a fixed

factor. As some families included more than one child, Family ID was entered as a random effect in the model.

CAR AUC analyses. An initial area under the curve analysis (AUC) was conducted using 3 morning salivary cortisol sample times to account for individual changes over time. AUC analysis accounts for the intensity and sensitivity of CAR using AUC_g (area under the curve with respect to ground), which is an estimate of total cortisol secretion over the first hour after waking, and AUC_i (area under the curve with respect to increase), which accounts for changes over time after awakening (Fekedulegn et al., 2007). AUC was calculated using Pruessner's (2003) linear trapezoidal method:

$$AUC_g = (((m_1 + m_2)/2) * 0.5) + (((m_2 + m_3)/2) * 0.5)$$

$$AUC_i = AUC_g - (m_1(0.5 + 0.5))$$

A GLMM was used to analyze differences in the cortisol awakening response (AUC_i and AUC_g) between HR and LR groups. Puberty status, age, and cohort were entered as covariates in the model. Risk group (LR and HR) was entered as a fixed factor and Family ID was entered as a random effect in the model.

Responders vs. non-responders. AUC_i values, which denotes changes in cortisol over the 60-minute after awakening, were used to determine if there was an overall increase or decrease in cortisol levels after awakening for each participant. Based on this increase or decrease in cortisol levels, participants were further divided into two sub-groups; Responders (participants

who showed an overall increase in cortisol between awakening and 60-minutes after awakening) and non-responders (participants who showed an overall decrease in cortisol between awakening and 60-minutes after awakening). Differences between responders and non-responders on baseline characteristics were assessed by ANOVA and chi-square (χ^2) statistics. Additionally, a two-way GLMM was conducted to test for interactions between responder status and risk group. If a significant interaction emerged, post hoc testing was performed to determine which groups differed. Puberty status, age, and cohort were entered as covariates in the model and Family ID was entered as a random effect.

Analysis of awakening cortisol averages. A GLMM analysis was used to examine awakening cortisol average values at each time point (Time 1 at awakening, Time 2 at 30 minutes after awakening, and Time 3 at 60 minutes after awakening between HR and LR groups). Puberty status, age, and cohort were entered as covariates in the model and Family ID was entered as a random effect.

Psychological variables. A Bivariate Pearson correlation analysis was run to investigate the relation between the CAR (AUC_i and AUC_g), individual awakening cortisol average values, and psychological variables of interest (STAIC, CASI, and CSRCI) for HR and LR participants separately. For significant correlations, a linear regression analysis was performed in order to examine the predictive relationship between psychological variables and the CAR and individual awakening cortisol average values.

Environmental variables. A Point Biserial correlation analysis was run to investigate the relation between the CAR (AUC_i and AUC_g), individual awakening cortisol averages and environmental variables of interest (gender of affected parent, current/remote participant

stressors, and prenatal stressors). For significant correlations, a linear regression analysis was performed in order to examine the predictive relationship between environmental variables and the CAR and individual awakening cortisol average values.

Diurnal and evening cortisol analysis.

Evening cortisol averages. Averages of the 2 evening sampling times across the 2 sampling days were obtained (Time 4 at 4pm and Time 5 at 8pm).

Diurnal cortisol curve. In order to examine the diurnal cortisol response, awakening cortisol values were averaged across the 3 morning time points (WU, WU+30, and WU+60) to derive an average morning value. Average morning cortisol values, 4pm average values, and 8pm average values for LR and HR participants were graphed using a scatter plot with markers and lines in order to visually inspect the diurnal cortisol response.

Diurnal AUC analysis. An AUC_g (area under the curve with respect to ground) analysis was conducted to estimate total diurnal cortisol secretion using average morning cortisol values, 4pm average values, and 8pm average values. In order to calculate diurnal AUC_g, an arbitrary collection time point of 8am was assigned to average morning cortisol values. AUC_g was calculated using Pruessner's (2003) linear trapezoidal method:

$$\text{AUC}_g = (((m_1 + m_2)/2) * 8) + (((m_2 + m_3)/2) * 4)$$

A GLMM was used to analyze differences in diurnal AUCg between HR and LR groups. Puberty status, age, and cohort were entered as covariates in the model. Risk group (LR and HR) was entered as fixed main effect and Family ID was entered as a random effect.

Analysis of evening cortisol averages. A GLMM analysis was used to examine evening cortisol average values at each time point (Time 4 at 4pm and Time 5 at 8pm) between HR and LR groups. Puberty status, age, and cohort were entered as covariates in the model. Risk group (LR and HR) were entered as fixed effects and family ID was entered as a random effect into the model.

Covariates. Pearson correlation analysis was performed to examine the relationship between continuous variables and diurnal AUCg, 4pm, and 8pm average cortisol values and . Point Biserial correlation was performed to examine the relationship between categorical variables and these indices of HPA axis function. Gender did not significantly correlate with diurnal AUCg, 4pm, and 8pm average cortisol values and was subsequently excluded as a covariate from analyses. Pearson and Biserial correlations for the main covariates of age, puberty status, and gender for diurnal AUCg, 4pm, and 8pm average cortisol values are presented in Table 7.

Psychological variables. A Bivariate Pearson correlation analysis was run to investigate the relation between diurnal AUCg, 4pm, and 8pm cortisol averages, and psychological variables of interest (STAIC, CASI, and CSRCI) for HR and LR participants separately. For significant correlations, a linear regression analysis was performed in order to examine the predictive relationship with diurnal AUCg and evening cortisol averages.

Environmental variables. A Point Biserial correlation analysis was run to investigate the relation between diurnal AUCg, 4pm and 8pm cortisol averages and environmental variables of interest (gender of affected parent, current/remote participant stressors, and prenatal stressors). For significant correlations, a linear regression analysis was performed in order to examine the predictive relationship between environmental variables and diurnal AUCg and evening cortisol averages.

Analyses of Anxiety Disorder Subgroups.

Analyses across 3 different anxiety risk groups (PD, GAD, and SAD) and cortisol values (morning AUC values, morning cortisol averages, diurnal AUCg, and evening cortisol averages) was conducted using a GLMM. Due to the small sample size of the SAD group (n=9), the SAD group was combined with the GAD group (n=20) and compared with the PD group (n=77). Puberty status, age, and cohort were entered as covariates in the model. Risk group (PD, SAD+GAD or LR) was entered as fixed effects and family ID was entered as a random effect into the model. For significant main effects, a Bonferroni post-hoc comparison test was performed using estimated marginal means in order to establish differences between groups.

Effect Sizes.

Effect sizes were calculated using Cohen's d for significant and non-significant findings. Cohen's (1988) classification of small ($d \geq .20$), medium ($d \geq .50$), and large ($d \geq .80$) was applied. Cohen's d was calculated using an online effect size calculator.

Results

Cohort Comparison

A one-way ANOVA between Cohort 1 and Cohort 2 revealed that Cohort 1 had significantly higher mean cortisol averages than Cohort 2; Average wake-up ($F(1, 264) = 140.034, p = .000$), Average wake-up + 30 ($F(1, 201) = 166.948, p = .000$), Average wake-up +60 ($F(1, 201) = 156.202, p = .000$), 4pm ($F(1, 263) = 90.213, p = .000$), and 8pm ($F(1, 249) = 48.677, p = .000$). Cohort was therefore included as a covariate in the model.

Baseline Characteristics.

As seen in Table 1, HR participants were about proportional in gender ratio, were approximately 11-years of age, and were comparative in puberty-status. PD was the prominent parental anxiety diagnosis and the affected parent was most commonly the mother. In contrast to the LR group, the HR group had a higher number of female participants, but was comparable in age, puberty status, and mean STAIC-T, CSRCI, and CASI scores. Compared to LR participants, HR participants were significantly more likely to report remote/current stressors ($\chi^2 = 9.298, df=1, p = .002$) and exposure to prenatal stressors ($\chi^2 = 11.091, df=1, p = .001$).

The CAR and Awakening Cortisol Values

AUC. Non-transformed AUC_g and AUC_i values and effect sizes are presented at the bottom of Table 3. There was no significant difference between risk-groups in AUC_g ($F(1,137.595) = 2.551, p = .126$) and AUC_i ($F(1,133.719) = 1.616, p = .259$).

Awakening cortisol. Mean awakening cortisol levels for LR and HR groups are presented in Figure 5. Both LR and HR groups showed a trend towards an increase in mean

awakening cortisol levels between wake-up and 30 minutes post-wake-up and a subsequent decrease between 30 minutes post wake-up and 60 minutes post wake-up. Overall, 64% of participants showed an overall increase in mean awakening cortisol levels between wake-up and 60 minutes (LR= 64.38% and HR=63.53%).

Responders vs. non-responders. Descriptive statistics (age, gender and puberty status) for responders and non-responders are presented in Table 2. ANOVA revealed a significant difference between responders and non-responders for age ($F(1, 218.693) = 22.765, p = .000$), with the responder group showing a higher mean age than non-responders ($M = 12.29, SD = 3.140$ versus $M = 11.41, SD = 3.026, d = 0.28$). Chi-square analyses revealed a significant difference between response groups in gender ($\chi^2 = 18.708, df = 1, p = .000$) and puberty status ($\chi^2 = 14.173, df = 1, p = .000$). Responders were significantly more likely to be male and classified as mid-post puberty status than non-responders.

Overall mean increase in awakening cortisol levels between LR and HR groups are presented in Figure 6 for responders and Figure 7 for non-responders. The interaction between risk-group and response status was not significant ($F(1, 203.404) = 1.175, p = .394$). However, post-hoc pairwise comparisons revealed that for the responder group, the difference between risk groups was marginally significant ($p = .078$), with the HR group exhibiting a smaller overall increase in awakening cortisol levels than the LR group ($M = 0.118, SD = 0.111$, versus $M = 0.161, SD = 0.138, d = 0.34$).

Average awakening cortisol values. Non-transformed average awakening cortisol values and effect sizes are presented in Table 3. There was no significant difference between LR and HR risk groups in average wake-up values ($F(1, 135.573) = 0.517, p = .480$ and average

wake-up +60 values ($F(1,140.281) = 2.060, p = .161$). However, there was a trend for wake-up +30 values to be lower in the HR group ($F(1,138.340) = 3.119, p = .089$; $M = 0.446, SD = 0.345$ versus $M = 0.523, SD = 0.396, d = 0.21$).

Association with demographic variables. Correlations for covariates of age, puberty status and gender and CAR values are presented in Table 4. An examination of the covariates revealed a significant negative correlation between age and average morning cortisol values and AUCg. A significant negative correlation also emerged between puberty status and average morning cortisol values and AUCg. The correlations between gender and CAR values were not significant.

Association with psychological and environmental variables. Correlations between CAR values and personality traits and environmental variables are presented in Table 5 for LR participants and in Table 6 for HR participants. For LR participants, there was a significant association between CASI scores and average WU cortisol values. However, regression analysis indicated that CASI scores did not significantly predict WU cortisol values ($\beta = .113$), only accounting for 1.3% of variance. All other correlations were not significant for LR participants. For HR participants, CASI scores correlated positively with average cortisol WU+30 values, WU+60 values, and AUCg. However, regression analysis indicated that CASI scores did not significantly predict WU+30 ($\beta = .138$) or AUCg ($\beta = .182$), only explaining 1.9% and 3.3% of variance, respectively. Although not significant, CASI scores marginally predicted WU+60 values ($\beta = .217$), accounting for almost 5% of the variance in WU+60 values. All other correlations were not significant.

Diurnal & Evening Cortisol

Diurnal cortisol. The diurnal cortisol response for LR and HR participants is depicted in Figure 8. Both LR and HR participants showed a steady decline in cortisol levels between the morning and 4pm, and a slower decline between 4pm and 8pm. LR participants also showed overall higher morning, 4pm, and 8pm values, compared to HR participants.

Diurnal AUCg analysis. Non-transformed diurnal AUCg and effect sizes are presented at the bottom of Table 7. There was no significant difference between risk-groups in diurnal AUCg ($F(1,171.736) = 0.743, p = .413$).

Average evening cortisol values. Non-transformed average evening cortisol values and effect sizes are presented in Table 7. There was no significant difference between LR and HR risk groups in average 4pm values ($F(1,167.56) = 1.383, p = .246$), but a trend difference emerged for 8pm values ($F(1,158.61) = 2.978, p = .090, d = 0.195$), with the HR group exhibiting lower values than the LR group.

Association with demographic variables. Correlations for covariates of age, gender, puberty status and 4pm and 8pm average cortisol values are presented in Table 8. An examination of the covariates revealed a significant negative correlation between age and diurnal AUCg, 4pm and 8pm average cortisol values. There was also a significant negative correlation between puberty status and diurnal AUCg, 4pm and 8pm average cortisol values. The correlations between gender and diurnal AUCg, 4pm and 8pm average cortisol values were not significant.

Association with psychological and environmental variables. Correlations for diurnal AUCg, 4pm and 8pm average cortisol values and psychological and environmental variables are

presented in Table 9 for LR participants and Table 10 for HR participants. Although there was a significant correlation between diurnal AUCg and CASI among HR participants, regression analysis indicated that the CASI did not significantly predict AUCg ($\beta = .154$), only accounting for 2.4% of variance. Among LR participants, none of the associations were statistically significant.

Additional Analyses

Additional analyses examined the relationship between specific risk groups (PD, GAD+SAD, LR) and cortisol values (WU, WU+30, WU+60, morning AUCg, AUCi, 4pm, 8pm, and diurnal AUCg). Exploratory analysis revealed a significant main effect of risk group for WU ($F(2, 135.597) = 3.428, p = .036$), WU+30 ($F(2, 138.364) = 3.386, p = .050$), WU+60 ($F(2, 140.362) = 3.339, p = .043$), morning AUCg ($F(2, 137.127) = 3.739, p = .035$), 4pm ($F(2, 166.552) = 4.092, p = .019$), 8pm ($F(2, 157.458) = 3.214, p = .046$), and diurnal AUCg ($F(2, 170.938) = 6.914, p = .002$). There was no significant effect of risk group for AUCi ($F(2, 135.206) = 0.896, p = .430$).

Post-hoc comparison between PD versus GAD+SAD groups are presented in Table 11. Analysis revealed that mean values were significantly higher for the PD group versus the GAD+SAD group for WU, 4pm and diurnal AUCg. There was a trend for morning AUCg values to be higher for the PD group versus the GAD+SAD group. There was no significant difference between PD and GAD+SAD groups for WU+30, WU+60 and 8pm. The difference between PD and GAD+SAD groups had large effect sizes for WU, 4pm, and diurnal AUCg and medium effect sizes for WU+30, WU+60, morning AUCg, and 8pm.

Post-hoc comparison between PD versus LR groups are presented in Table 12. Analysis revealed no significant difference between PD and LR groups for WU, WU+30, WU+60, morning AUCg, 4pm, 8pm and diurnal AUCg. The effect sizes for the difference between PD and LR groups were unremarkable.

Post-hoc comparison between the GAD+SAD group versus the LR group is presented in Table 13. Analysis revealed that mean scores were significantly lower for the GAD+SAD group versus the LR group for WU+30, WU+, morning AUCg, 4pm, 8pm, and diurnal AUCg. There was a trend for WU values to be lower for the GAD+SAD group. The difference between GAD+SAD and LR groups had medium effect sizes for WU, WU+30, WU+60, morning AUCg, 4pm, 8pm, and diurnal AUCg.

Discussion

Dysregulation of the HPA axis has been implicated in the pathogenesis of anxiety disorders. To assess whether this dysregulation may be a heritable trait marker of anxiety disorder risk, the principal objective of the current study was to examine the CAR in offspring at high and low genetic risk for anxiety disorders. The identification of the CAR as a possible trait marker of anxiety disorder risk may elucidate the mechanism by which anxiety disorders are transmitted from parent to child, provide researchers with a candidate phenotype for molecular genetics research, and provide a potential target for preventive interventions that can shift a child's trajectory from risk to resilience.

The CAR and Awakening Cortisol

The hypothesis that CAR and awakening cortisol levels would differ in high and low risk children was partially supported in this study. When the combined group of HR children was

compared to LR controls, no difference in total cortisol secretion over the first hour after awakening (AUC_g) and changes over time after wakening (AUC_i) were detected. Additionally, when individual morning time points were examined, no significant difference emerged for the average awakening cortisol levels at wake-up and 60 minutes post-wakeup, although 30-minute post-wakeup averages tended to be lower in HR children. This latter finding is interesting as other studies have detected a blunted 30-minute post wake-up response in children with a parental history of anxiety (Wessa et al., 2006; Dierckx et al., 2012) and anxious behaviour in girls (Kallen et al., 2008). Given that salivary cortisol levels typically peak around 30 minutes post wake-up (Edwards, et al., 2001), detectable differences between groups may be most evident at this time-point. In the current study, differences in the CAR may have emerged if samples were collected at more frequent time-points (i.e. 15 minutes post wake-up). Research has shown that delays of 15 minutes between wake-up and the commencement of sampling significantly reduce the magnitude of the CAR (Okun et al., 2010; Smyth, Clow, Thorn, Hucklebridge & Evans, 2013). However, sampling at more frequent intervals presents a challenge, as the collection of multiple samples in a restricted time-period increases the burden of sampling for parents (Smith & Dougherty, 2013) and raises issues of collecting saliva from children who may have difficulty generating saliva.

Although this study found no effect of risk group on the CAR for the combined sample of HR children, analysis of anxiety disorder subtype revealed several notable findings. Whereas children at risk for PD and LR controls had similar AUC_g, AUC_i and awakening cortisol values, those at risk for GAD or SAD exhibited significantly lower AUC_g as well as lower WU+30 and WU+60 values relative to control. There was also a trend for these children to demonstrate lower AUC_i values than controls, although the significance of this finding is unclear as the use of

AUC_i remains relatively limited in research compared to AUC_g (Fekedulegn et al., 2007) and is more strongly influenced by confounds such as time of awakening, quality, sleep duration and quality, light intensity at awakening, and subjective stressors the previous day (Hellhammer, Fries, Schweisthal, Schlotz, Stone, and Hagemann, 2007).

Overall, these findings suggest that a decreased or blunted CAR may be a possible heritable marker of risk that is specific to GAD or SAD but not PD. The finding that the CAR may be normal in children at risk for PD is consistent with previous research (Petrowski, 2010), although one study found a higher CAR in offspring with parental PD and agoraphobia relative to controls (Vreeburg et al., 2010a). However, this finding is difficult to interpret due to the preponderance of offspring with a parental history of depression, with less than 6% having a parental history of anxiety only.

The finding of a decreased or blunted CAR and awakening cortisol levels in unaffected children at risk for GAD or SAD is novel, and has yet to be reported in other published studies. In clinical samples, a blunted CAR has been observed in older adults diagnosed with GAD and SAD (Hek et al., 2013) and in adults with GAD (Fries, Ebert, Kling, Beesdo & Kirschbaum, 2008) and SAD (Shirotsuki et al., 2009; Furlan, DeMartinis, Schwiezer, Rickels, and Lucki, 2001; Beaton et al., 2006) following exposure to a psychosocial stressor. These findings suggest possible down-regulation of the HPA axis in these anxiety disorder subtypes. However, evidence of a blunted response in GAD and SAD has been inconsistent, with some studies reporting elevated (Condren, O'Neill, Ryan, Barrett & Thakore, 2002; Lenze et al., 2011; Mantella et al., 2008) and normal (Gerra et al., 2000; Martel et al., 1999) HPA axis function.

It has been proposed by some researchers that a blunted cortisol response may be a consequence of prolonged hyperactivity of the HPA axis resulting from exposure to chronic

stress (Fries, Hesse, Hellhammer & Hellhammer, 2005). The pattern of low cortisol levels seen in individuals with GAD and SAD may reflect changes in the HPA axis's response to stress, resulting from a life-long history coping with an anxiety disorder (Beaton et al., 2006). Indeed, in the current study, children with a parental history of GAD or SAD reported a significantly higher level of remote or current stressors compared to controls, suggesting an exposure to chronic stress. A blunted CAR and cortisol stress response has been well supported within the research literature on children exposed to traumatic events and life stressors (Bevans, Cerbone, & Overstreet, 2008; Ouellet-Morin et al, 2011; Ouellet-Morin et al, 2013), as well as adults with a history of adverse life events in childhood (Elzinga, Roelofs, Tollenaar, Bakvis, van Pelt, & Spinhoven, 2008; Carpenter et al., 2007). Nevertheless, exposure to stressors did not significantly correlate with the CAR within the GAD + SAD group, suggesting other factors likely account for the blunted CAR seen in these at-risk children.

It is interesting to note that mothers with PD but not those with GAD or SAD reported a significantly higher rate of prenatal stressors than control mothers. This is consistent with studies showing that mothers with PD report significantly more stressful life events during pregnancy than controls (Warren, Racu, Gregg & Simmens, 2006). Furthermore, it was found that the higher rate of prenatal stressors among mothers with PD was significantly associated with a blunted CAR in their offspring. Previous research that has found that prenatal stress is associated with an altered CAR in offspring (O'Connor et al., 2005; O'Donnell et al., 2013), as well as higher cortisol stress reactivity in infants (Tollenaar, Beijers, Jansen, Riksen-Walraven & De Weerth, 2011) and young adults (Entringer, Kumstra, Hellhammer, Wadha, & Wust, 2009). These findings have led to speculation that prenatal stress increases cortisol levels in utero, compromising fetal brain development, particularly the HPA axis (Beijers, Buitelaar &

DeWeerth, 2014). However, the mechanisms through which maternal stressors affect the developing fetus are not completely understood (Beijers et al., 2014), and further research is warranted to address this relationship.

An alternative explanation for the blunted CAR seen in children with a parental history of GAD or SAD is that hypocortisolism may lead to elevated anxiety awareness and elicitation of worry, which in turn, increases risk for developing anxiety disorders (Steudte et al., 2010). Evidence for this theory is supported by studies demonstrating an association between higher cortisol levels and lower fear and anxiety ratings in response to a social stressor. For example, one study found that administration of cortisol subsequently reduced fear ratings of a social stressor in patients with SAD (Soravia et al., 2006). Likewise, another study found that cortisol responses to a laboratory social stressor were negatively associated with subsequent ratings of anxiety in healthy research participants (Schlotz, Kumstra, Layes, Entringer, Jones & Wust, 2008). This pattern of hypocortisolism, seen in this study and others, provides further evidence for a blunted CAR as a potential trait marker for the development of GAD or SAD.

The question remains why a dysregulated CAR and awakening cortisol levels are evident in children with a parental history of GAD or SAD, but not PD. A possible explanation for this finding may relate to the age-range of the current sample and the age-of-onset of different anxiety disorders. Both GAD and SAD have early ages of onset, typically beginning in childhood or early adolescence, whereas PD usually begins in late adolescence or early adulthood (Health Canada, 2002). The sample in the current study consisted of children and adolescents (mean age of 11-years old). If a dysregulated CAR and awakening cortisol levels are more evident closer to the age of disorder onset, this could help explain why the current study

failed to find an effect for children at risk for PD. To explore this possibility, future high risk research should include a group of older at-risk offspring.

The finding that alterations in the CAR may be diagnostic-specific rather than a marker of risk across anxiety disorders is intriguing and has important implications for future investigation of the HPA axis in unaffected at-risk offspring. It is plausible that research on the CAR as a vulnerability marker of anxiety disorder risk has yielded mixed findings, in part, because researchers have investigated offspring of parents with heterogeneous anxiety disorders, obscuring important differences between groups. It is important to highlight that the number of offspring at risk for GAD or SAD in the current study was relatively small and findings should therefore be viewed with caution. Nevertheless, it will be worthwhile for future research to ascertain whether HPA axis dysregulation is specific to any of the anxiety disorders or a general feature of all anxiety disorders.

Influence of Child Characteristics on the CAR

The current study found that the CAR decreased with increasing age. This is in line with findings from Kudielka and Kirshchbaum (2003) who found that age had a small negative correlation with the CAR in a community sample of adults, youth, and children. Nevertheless, findings between studies remain inconsistent; with some studies suggesting that age may be unrelated to the CAR (Wust et al., 2000b). It has been reported that the CAR is unrelated to age in adolescents (Ellenbogen, Hodgins & Walker, 2004) and children under age ten (Pruessner et al., 1997). It is important to note that most studies have investigated the relationship between age and the CAR in adults and further research is needed to clarify whether age is an important moderator of the CAR in children and adolescents.

As the current study found that age and puberty status were highly correlated, it was not surprising that puberty status was found to have a small but significant negative correlation with the CAR, as measured by AUCg and morning cortisol means. This finding suggests that the CAR decreases as children progress from pre-early to mid-post puberty. However, there is lack of clarity between studies regarding the relationship between the CAR and pubertal maturation (Oskis, Loveday, Hucklebridge, Thorn & Clow, 2009). A study by Adam (2006) found that among adolescents, ages 13-19, higher stages of puberty were associated with a smaller CAR. Nevertheless, Rosmalen et al (2005) found that the CAR was not influenced by puberty status in 10-12 year old children; however, as this study used a restricted age range the researchers were unable to fully examine different puberty stages. It is possible that an association between puberty status and the CAR is only present in studies that employ a wide age-range of children and youth, such as in the current study.

Unlike age and puberty status, gender was not associated with any measure of the CAR. This result is in line with early findings from Kiess et al. (1995), who also failed to demonstrate a gender difference in awakening cortisol levels in children and adults. On the other hand, this result contradicts previous studies that have demonstrated a marked gender difference, finding a higher CAR and morning cortisol levels in girls compared to boys (Netherton et al., 2004; Rosmalen et al., 2005), as well as in women compared to men (Vreeburg et al., 2009b). It is important to note that studies that have reported gender differences in the CAR have found that gender has a minimal influence on the CAR (Fries et al., 2009), accounting for only 1-3% of its total variance (Wust et al., 2000a).

Influence of Anxiety-Related Traits on the CAR

The hypothesis that anxiety-related personality traits would influence the CAR was not supported in this study. Although a significant, albeit small association emerged between anxiety sensitivity and parameters of the CAR in high and low risk children, anxiety sensitivity was not a significant predictor of the CAR. Similar findings have been reported by van Santen et al. (2011) who found that anxiety sensitivity did not significantly influence the CAR in adults with a parental history of depression and anxiety disorders.

Similarly, behavioural inhibition did not influence the CAR in high or low risk children in the current study. Previous research has suggested that behavioural inhibition in early childhood is associated with an over-active HPA axis (Hastings & Utendale, 2008). A series of earlier studies by Kagan et al. (1987; 1988) found behavioural inhibition in toddlers to be associated with higher baseline and reactive cortisol. These findings were further supported by Schmidt et al., (1996), who found that infants who were behaviourally inhibited at 14 months of age displayed relatively high morning salivary cortisol at 4-years of age. Although these early studies suggested a positive relationship between behavioural inhibition and overall cortisol activity, they did not specifically measure the CAR in the context of children with a parental history of anxiety disorders. Moreover, previous studies have assessed behavioural inhibition in samples of young children, unlike the current study that assessed behavioural inhibition in children and adolescents ages 7-18 years of age.

The lack of relationship between trait anxiety and the CAR is consistent with findings from van den Bergh et al. (2008), who failed to detect an association between trait anxiety and morning cortisol levels in pre-pubescent adolescents. However, these findings contrast with other research demonstrating a negative relationship between trait anxiety and the CAR (Therrien et

al., 2008; Walker et al., 2011). In the context of HPA axis activity, higher levels of trait anxiety have also been found to have a negative association with basal cortisol levels (Zorrilla et al., 1995) as well as cortisol reactivity (Jezova et al., 2004).

In sum, the current study suggests that anxiety-related personality traits may not be important predictors of the CAR in high and low risk children. Nevertheless, given the paucity of research examining the association between these traits and HPA axis function in vulnerable children, more research is necessary to further clarify this relationship.

Responder Status

Previous research has shown that an increased CAR occurs in approximately 75% of healthy individuals, with the remaining 25% showing a decreased or blunted CAR, suggesting a distinct pattern of inter-individual variability in the CAR (Wust et al., 2000a). As a result, it has been suggested by some researchers that distinguishing between CAR “responders” (i.e. those who show an increased CAR) and “non-responders” (i.e. those who show a decreased or blunted CAR) may be useful (Clow et al., 2004). The current study was able to demonstrate a typical CAR curve, with values peaking at 30-minutes post-wakeup, and a subsequent decline thereafter. Overall, 64% of participants showed an increase in salivary cortisol levels within 1-hour after awakening. This response rate is similar to that reported by Vreeburg et al. (2010b), who found an increase in morning salivary cortisol levels in 68.5% of adult participants with a parental history of anxiety.

Comparison of responders and non-responders revealed that responders were more likely to be older, female, and classified as mid-post puberty. Although the age difference found between responders and non-responders was relatively small (less than 1 year), it is in line with

research suggesting that older children are more likely to be responders than younger ones (DeCaro & Worthman, 2008). The observed gender difference in the CAR response status is in contrast to previous research demonstrating no difference between males and females in response rate (Wust et al., 2000b). However, it is possible that the moderating effect of gender on response status in the current study was mediated by pubertal stage status, as it has been shown that females tend to enter and complete puberty at an early age compared to males (Kail, 2010).

The interaction between risk-groups and response-status was not significant, suggesting that children with a parental history of anxiety are similar to control children in their response-status. This finding is novel, in that to date, no other studies have examined CAR response-status in children at risk for anxiety. Although no interaction between risk-group and response status was detected, there was a trend for HR responders to exhibit a smaller overall increase in awakening cortisol levels than LR responders; however, the effect size for the difference between risk groups was small. For the non-responder group, who showed no awakening response or a decreased response, it is interesting to note that HR participants showed less of a decrease compared to LR participants, although the difference was not statistically significant.

These findings further support a potential trend towards a blunted CAR in children with a parental history of anxiety; however, it may only be present in individuals identified as cortisol responders. It is important to note that, in the current study, responder status was derived from AUC_i values which are strongly influenced by subjective factors (Hellhammer et al., 2007). Furthermore, there remains a lack of agreement within the research literature about threshold criteria to distinguish responders from non-responders, with the most common criterion of 2.5-nmol/l above baseline being considered too conservative (Miller, Plessow, Kirschbaum & Stalder, 2013). Given that the application of response status to the CAR has been limited (Wust

et al., 2000b; Federenko et al., 2004), future research is necessary to ascertain how best to define response status to the CAR.

Diurnal Cortisol and Evening Values

As expected, both risk groups demonstrated a characteristic decline in cortisol levels between the morning and evening, which is in line with previous research on the diurnal cortisol cycle (Fries et al., 2009). However, contrary to prediction, there was no significant difference between the combined HR groups and LR controls in diurnal cortisol secretion, although 8 pm cortisol levels tended to be lower among high risk offspring. In contrast, analysis of anxiety disorder subtypes revealed some notable differences. As with the CAR and awakening cortisol levels, diurnal response and 4pm and 8pm values were comparable for PD and LR risk groups. However, the diurnal response, and 4pm and 8pm cortisol values were significantly lower for the GAD+SAD risk group versus LR controls, with moderate effects. As well, diurnal cortisol response and 4 pm values were lower for the GAD + SAD versus the PD group, with moderate to large effects. Similar to the CAR results, these analyses indicate that a blunted diurnal cortisol response may represent a heritable trait marker that is specific to GAD or SAD.

It should be noted that findings have been inconsistent regarding the direction and specificity of the diurnal cortisol response. For example, Russ and colleagues (2012) found elevated bedtime cortisol levels in children of mothers with SAD, but not in children of mother with GAD or controls. Although, a blunted diurnal response has been found in clinically depressed youth with comorbid anxiety disorders (Doane et al., 2013), a normal diurnal response has been found in patients with GAD (Stedte et al., 2010).

There was no evidence that current and remote stressors moderated diurnal cortisol response in the GAD/SAD group. However, unlike the CAR, the diurnal cortisol response did not correlate with prenatal stressors in the PD group, suggesting that early morning cortisol secretion may be more sensitive to the effects of prenatal stressors than diurnal secretion in at-risk PD offspring. As mentioned earlier, further research is needed to elucidate the exact mechanisms through which maternal stressors impact fetal development of the HPA axis (Beijers et al., 2014).

Influence of Child Characteristics on the Diurnal Response

As with findings reported on the CAR, diurnal cortisol was found to be negatively associated with age and puberty status. This is consistent with early findings from Deuschle et al (1997) that diurnal cortisol shows an age-related decline in amplitude, as well as more recent findings from Adam (2006), showing a steeper decline in diurnal cortisol in adolescents at higher stages of pubertal development. However, there remains little consensus within the literature, with some studies reporting no association between age and diurnal cortisol secretion (Edwards, Clow, Evans & Hucklebridge, 2001; Wolf, Convit, Thorn & de Leon, 2002), as well as pubertal stage and diurnal cortisol (Rosmalen et al., 2005). Nevertheless, it is important to note that some of these studies used very broad age ranges, rather than distinct age groups as in the current study (Heaney, Phillips & Carroll, 2012).

Similar to the CAR, gender was not associated with the diurnal cortisol response. This is consistent with previous research demonstrating a lack of association between gender and diurnal cortisol levels in children (Knutsson et al., 1997) and adults (Edwards et al., 2001), although some studies have reported consistently higher diurnal cortisol levels among women (Larsson,

Gulberg, Rastam & Lindbald, 2009). However, studies that have reported a gender difference in diurnal cortisol have typically been based on adults and elderly subjects, unlike the current study. It is possible that gender differences in diurnal cortisol may be more evident in adults.

Influence of Anxiety-Related Traits on the Diurnal Response

The current study found that anxiety-related personality traits did not significantly influence the diurnal cortisol response. Despite a small yet significant association between anxiety sensitivity and diurnal cortisol emerging for high risk children, anxiety sensitivity was not a significant predictor of the diurnal cortisol response. However, as with research on anxiety-related traits and the CAR, findings regarding the diurnal response remain inconsistent. A study by Sjors et al. (2010) found that anxiety sensitivity was negatively associated with diurnal cortisol in women suffering with chronic pain. As previously discussed, behavioural inhibition has been found to be associated with higher baseline and reactive cortisol in toddlers (Kagan et al., 1987; 1988) and trait anxiety has been found to have a negative association with basal (Zorrilla et al., 1995) and reactive cortisol (Jezova et al., 2004). It is evident that further research is warranted in order to clarify the relationship between these personality traits and diurnal cortisol.

Strengths and Limitations of the Study

This is the first study, to date, to demonstrate a blunted CAR and diurnal response in children with a parental history of GAD or SAD. The current study expands upon previous studies in several important ways. First, this study was able to address methodological issues of the CAR by collecting samples at awakening and 30 and 60 minutes post-wakeup. Previous studies that have analyzed the CAR have only collected one sample at awakening (Russ et al.,

2012) or samples at awakening and 30 minutes post-wakeup (Greaves-Lord et al., 2007b; O'Connor et al., 2005). As the CAR has been thought to constitute 2-4 secretory bursts (Chida & Steptoe, 2008), peaking 30 to 60 minutes after awakening (Clow et al., 2010), sampling at awakening and 30 and 60 minutes post-wakeup may be a more accurate reflection of the CAR.

A second strength of the current study is that it was able to assess the CAR in a sample of children of parents with a primary diagnosis of anxiety disorder. Given the paucity of research on anxiety disorders and the CAR, previous studies have focused on samples of parents with primary depression, with limited comorbidity with anxiety, therefore restricting these findings to depression with or without comorbid anxiety (Vreeburg et al., 2010b). The current study is able to extend these findings to include anxiety disorders.

A final, albeit important, strength of the current study is the inclusion of psychiatrically healthy children with and without parental anxiety. Previous studies are potentially confounded because they included individuals with pre-existing anxiety and/or depressive disorders. By studying unaffected offspring, the current study was able to exclude the effects of these psychopathologies on HPA axis function, and obtain a clearer picture of whether HPA axis dysregulation is possible premorbid vulnerability markers of anxiety disorder risk.

Although the current study has reported some noteworthy and statistically significant findings, there are several limitations that should be mentioned. First, this study was not able to control for important confounding factors such as time of awakening, sleep duration, season, and physical activity, all of which have been reported to have an effect on salivary cortisol levels (Vreeburg et al., 2009b), and strongly influence sensitive parameters like AUC_i (Hellhammer, et al., 2007). Even though the current study obtained participants' time of awakening and physical

activity level, participants' reports of these factors were found to be inconsistent or largely unreported. Although the inclusion of all these potential covariates would be ideal, it was deemed unfeasible in the current study. Future research on the CAR and diurnal response should consider accounting for these covariates if it is feasible to the study.

Second, the number of offspring with a parental history of GAD or SAD was relatively small. Most HR participants had a parental history of PD, with only 27% having a parental history of GAD or SAD. Although the current study found a large and significant effect of the CAR and diurnal cortisol within this group, it is possible that a larger sample size may have yielded an even larger effect. Given the small number of participants with a parental history of GAD or SAD, these two groups were combined in order to provide a larger sub-sample. Therefore, it is impossible to know if the results of this study are attributable primarily to GAD, SAD, or both. If possible, future research should explore the CAR and diurnal response in these groups separately, as well as consider including offspring at risk for other anxiety disorders, such as specific phobias.

Third, the current study only investigated one indicator of stress; cortisol. Other hormonal indicators of stress reactivity such as the adrenal steroid dehydroepiandrosterone (DHEA) (Bourdarene, Legros & Timsit-Berthier, 2002) and protein enzyme Alpha amylase (Nater, et al., 2005), have also been proposed to reflect autonomic nervous system (ANS) activity and stress reactivity. Furthermore, a significantly lower DHEA to cortisol ratio has been found in socially anxious males (Shirotzuki et al., 2009), and elevated alpha-amylase has been associated with GAD (van Veen et al., 2008), suggesting that these indicators may also be prime risk markers for the development of anxiety disorders. In addition to cortisol, future research should consider exploring DHEA and alpha-amylase as potential trait markers of anxiety disorder risk.

Implications

Despite the limitations discussed, the results of the current study have several important implications. The identification of a blunted CAR and diurnal response as a risk marker in children with a parental history of GAD or SAD may allow for early identification of children at risk for these and possibly other disorders. The early identification of at-risk children allows for the creation of specific counselling interventions through public health care system, with the purpose of shifting children's trajectory from risk to resilience.

As dysregulation of the HPA-axis is hypothesized to contribute to the development of anxiety disorders (Abelson, et al., 2007), interventions aimed at altering the stress system in at-risk children may be beneficial. Interventions that have been well supported for having a positive influence on the HPA-axis by reducing symptoms of stress are Mindfulness Based Stress Reduction (MBSR) and Mindfulness Based Cognitive Therapy (MBCT) (Fjorback, Arendt, Ornbol, Fink & Walach, 2011). Mindfulness-based therapies improve anxiety and mood symptoms in adults and have recently been shown to promote psychological well-being in children and adolescents (Burke, 2010).

In the context of existing interventions within the public education system, there are a number of universal programs aimed at preventing anxiety through psychoeducation. These universal school-based interventions have been shown to be successful in reducing anxiety, are cost-effective, and avoid potential stigmatization which may occur in targeted or individual interventions (Barrett & Turner, 2001; Briesch, Hargmoser, Santetti & Briesch, 2010). One such program, the FRIENDS for Life program (FFL), is a CBT-based program aimed at preventing anxiety disorders in children and adolescents by using a social-emotional learning

approach (Barrett & Pahl, 2006). The FFL program targets thoughts and behaviours associated with anxiety and teaches children to identify and understand their own body's anxiety response and how to manage these responses (Barrett & Pahl, 2006). Although it is suggested that the FFL program is most beneficial for children diagnosed with anxiety disorders, FFL benefits all children and has shown an overall positive effect on their outcomes (Briesch et al., 2010).

An additional finding of the current study was that higher levels of prenatal stressors reported by mothers with PD were associated with a blunted CAR in their offspring. Considering growing evidence that fetal exposure to elevated levels of cortisol may alter programming of the stress system and increase future risk for psychopathology, stress-reduction interventions during pregnancy may help mitigate risk for the development of anxiety disorders in offspring. Studies have shown that various types of yoga practices during pregnancy greatly reduces trait anxiety and perceived stress in women (Beddoe, Yang, Kennedy, Weiss & Lee, 2009; Field, Diego, Delgado & Medina, 2013) and lowers cortisol levels (Field et al., 2013). Other therapies such as mindfulness-based interventions (Vieten & Astin, 2008) and relaxation training (Urech et al., 2010) have also been found to significantly reduce prenatal anxiety.

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Tables & Figures

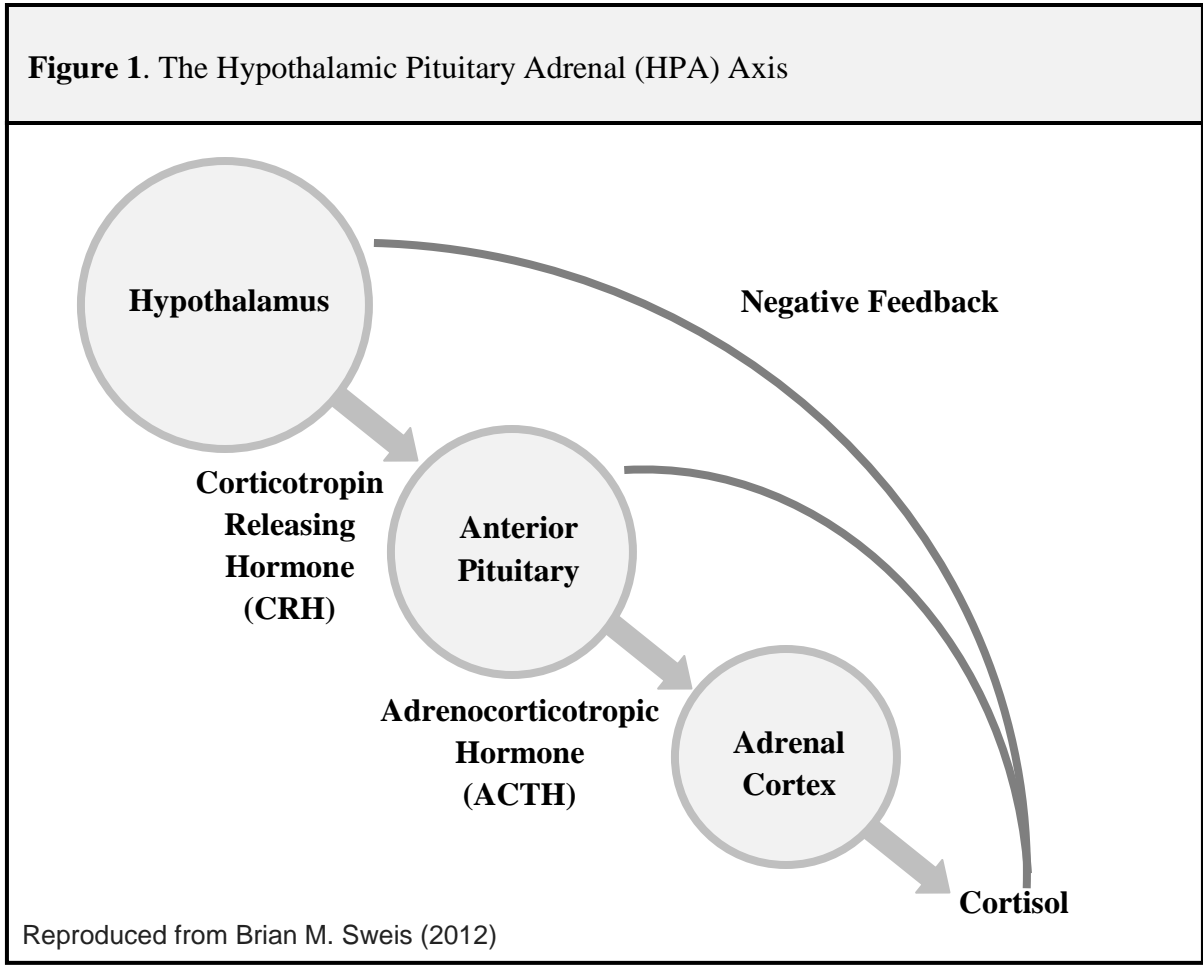


Figure 2. Graph depicting a typical cortisol awakening response (CAR), with cortisol concentration levels peaking at 30 minutes after awakening. (Salimetrics, 2010)

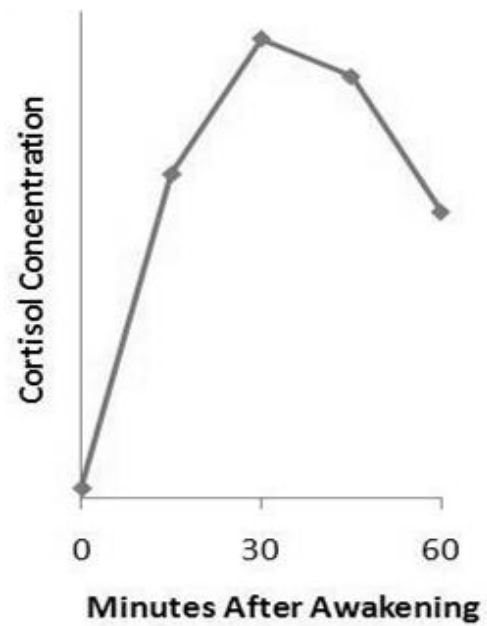


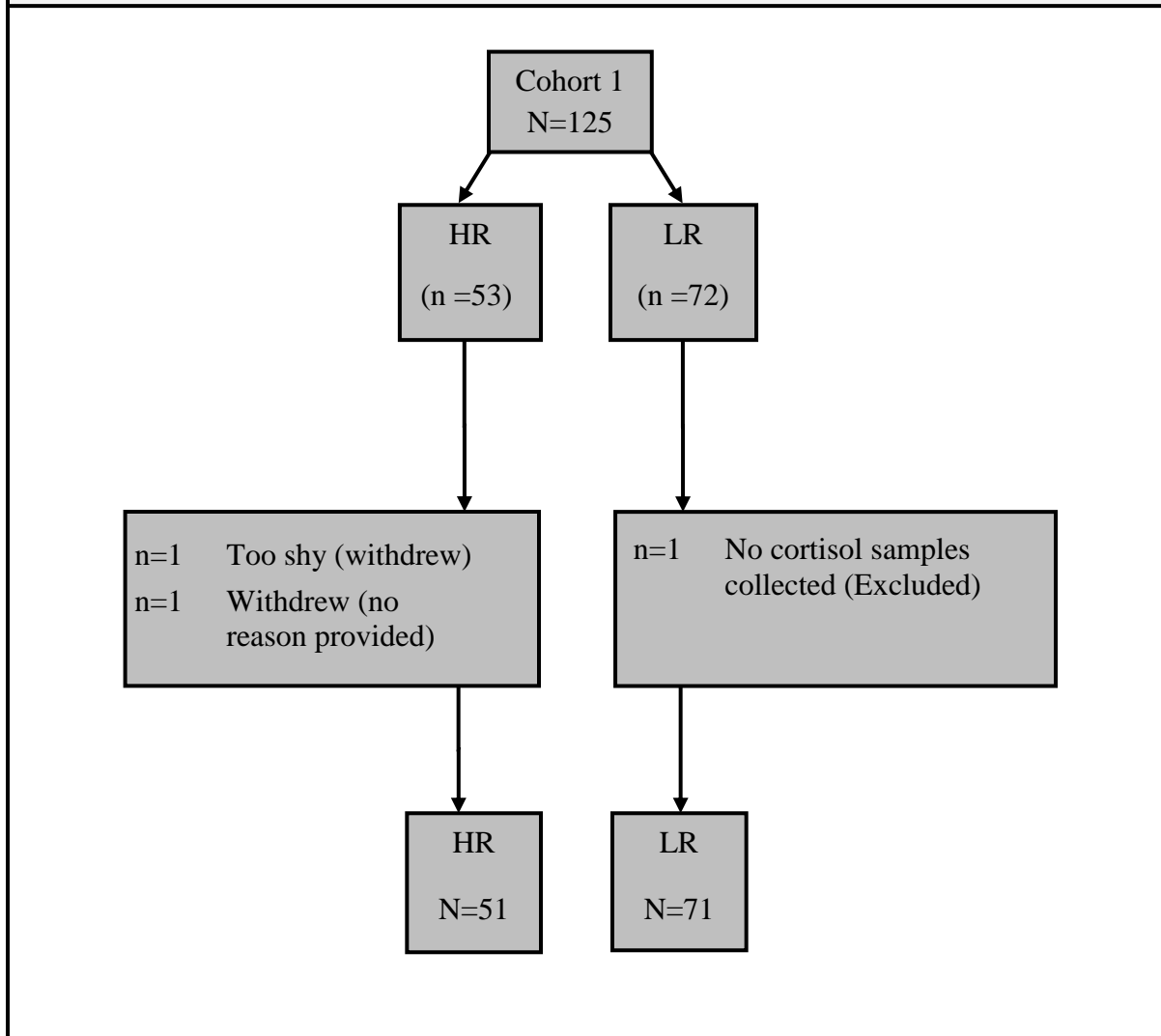
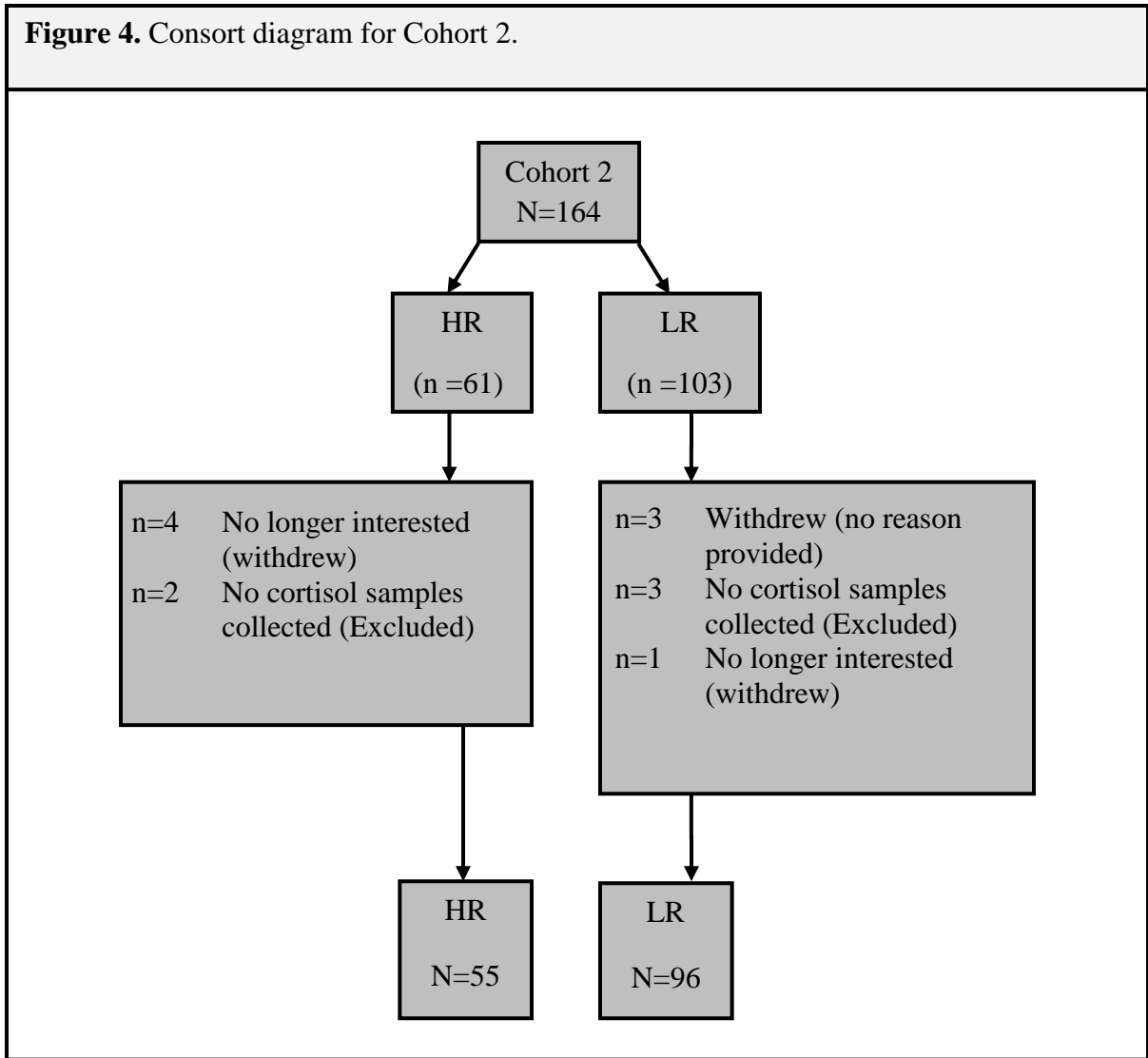
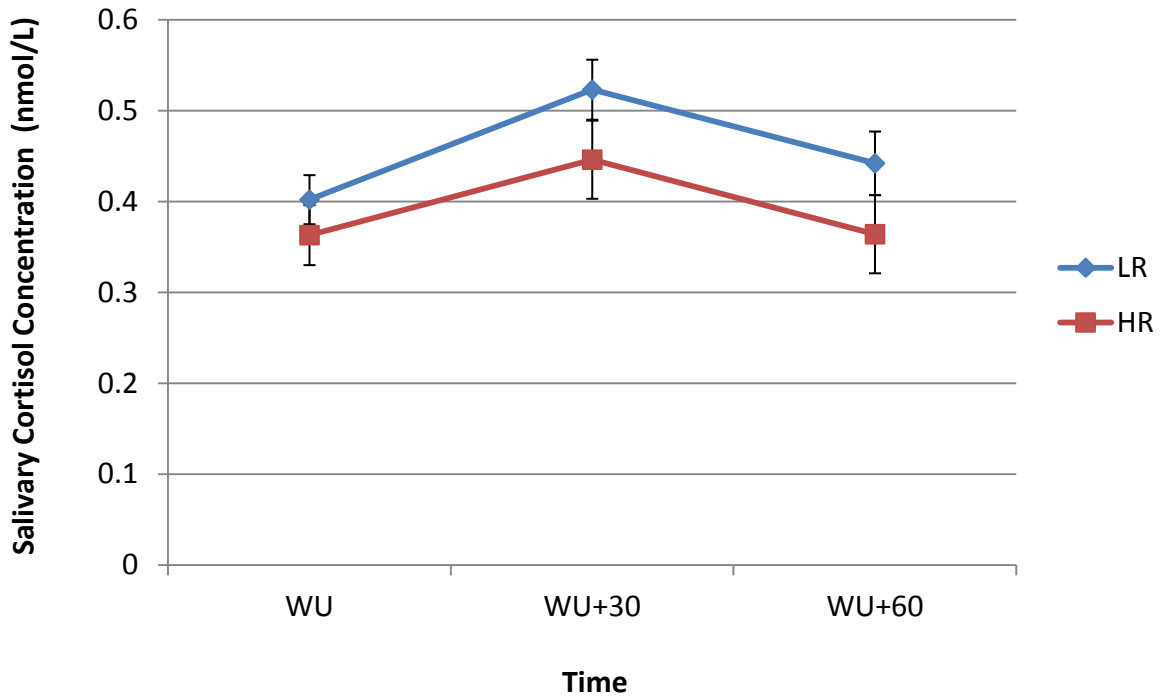
Figure 3. Consort diagram for Cohort 1.

Figure 4. Consort diagram for Cohort 2.



| Table 1. Sample Characteristics | | |
|--|------------------------|------------------------|
| | LR (n = 173) | HR (n = 101) |
| Participant gender, Female, % | 43.40 | 48.10 |
| Age, years: mean (sd) | 11.54 (3.062) | 11.97 (3.311) |
| Pre-early puberty, % | 50.00 | 44.30 |
| STAIC-T total, mean (sd) | 30.072 (6.373) | 31.283 (6.783) |
| CSRCI total, mean (sd) | 1.827 (0.363) | 1.831 (0.324) |
| CASI total, mean (sd) | 25.500 (5.151) | 25.680 (5.551) |
| Child stressors (remote/12 months), % | 38.10 | 57.40* |
| Prenatal stressors, % | 17.90 | 37.10* |
| Affected parent gender, Female, % | N/A | 63.60 |
| HR parental anxiety: | | |
| PD, % | N/A | 72.60 |
| GAD, % | N/A | 18.90 |
| SAD, % | N/A | 8.50 |
| *P<0.01 | | |

Figure 5. Mean Awakening Cortisol Levels for LR and HR Groups



| Table 2. Descriptive Statistics for Responders and Non-Responders | | |
|--|-------------------------------|----------------------------------|
| | Responders (n=137) | Non-Responders (n=77) |
| Participant gender, Female, % | 49.80* | 36.90 |
| Age, years: mean (sd) | 12.29 (3.140)* | 11.41 (3.026) |
| Pre-early puberty, % | 40.40* | 51.60 |
| *P<0.001 | | |

Figure 6. Overall Mean Increase in Awakening Cortisol Levels Between LR and HR Groups for Responders.

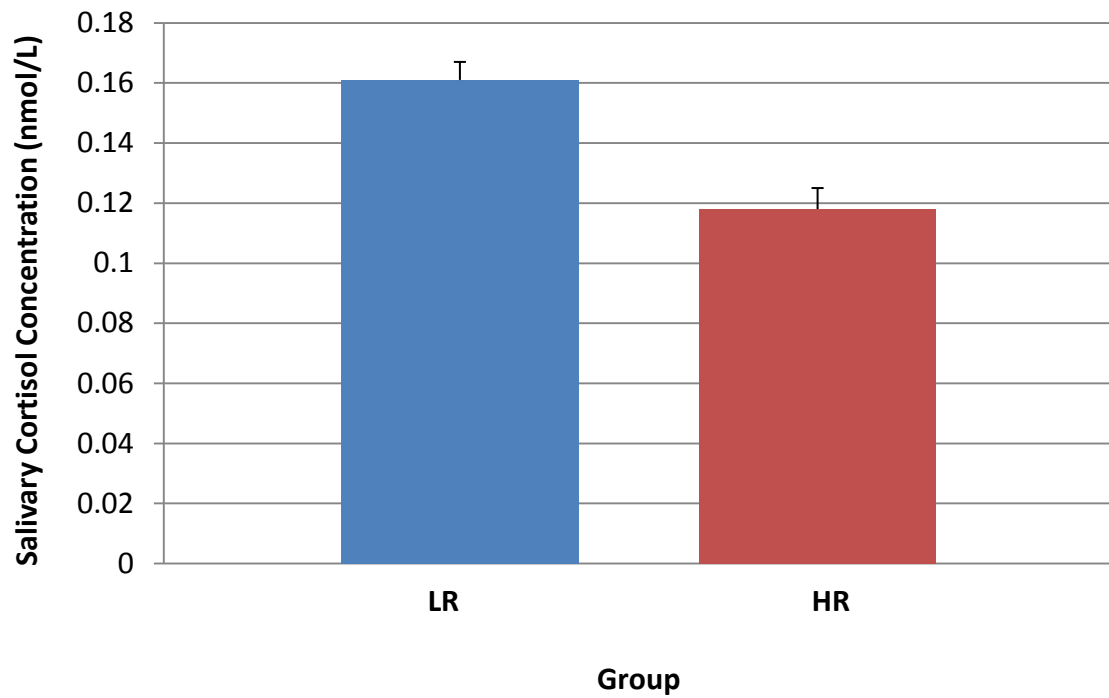
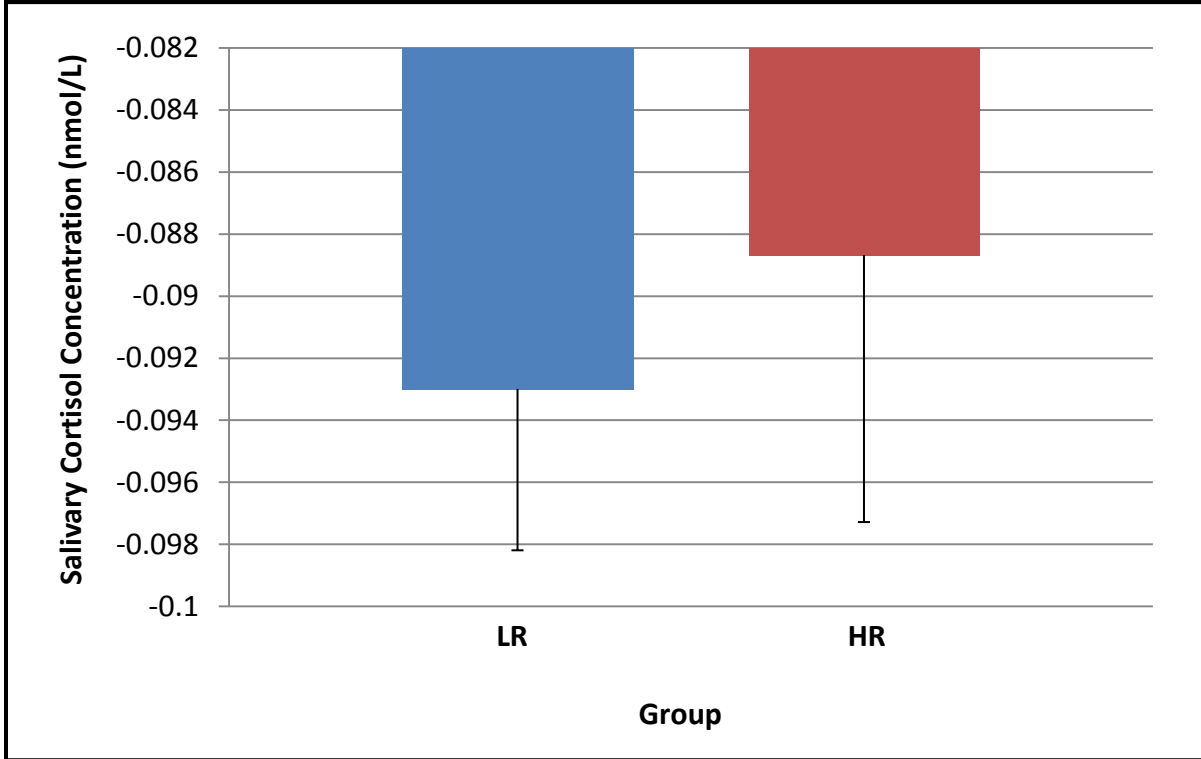


Figure 7. Overall Mean Decrease in Awakening Cortisol Levels Between LR and HR Groups for Non-Responders.

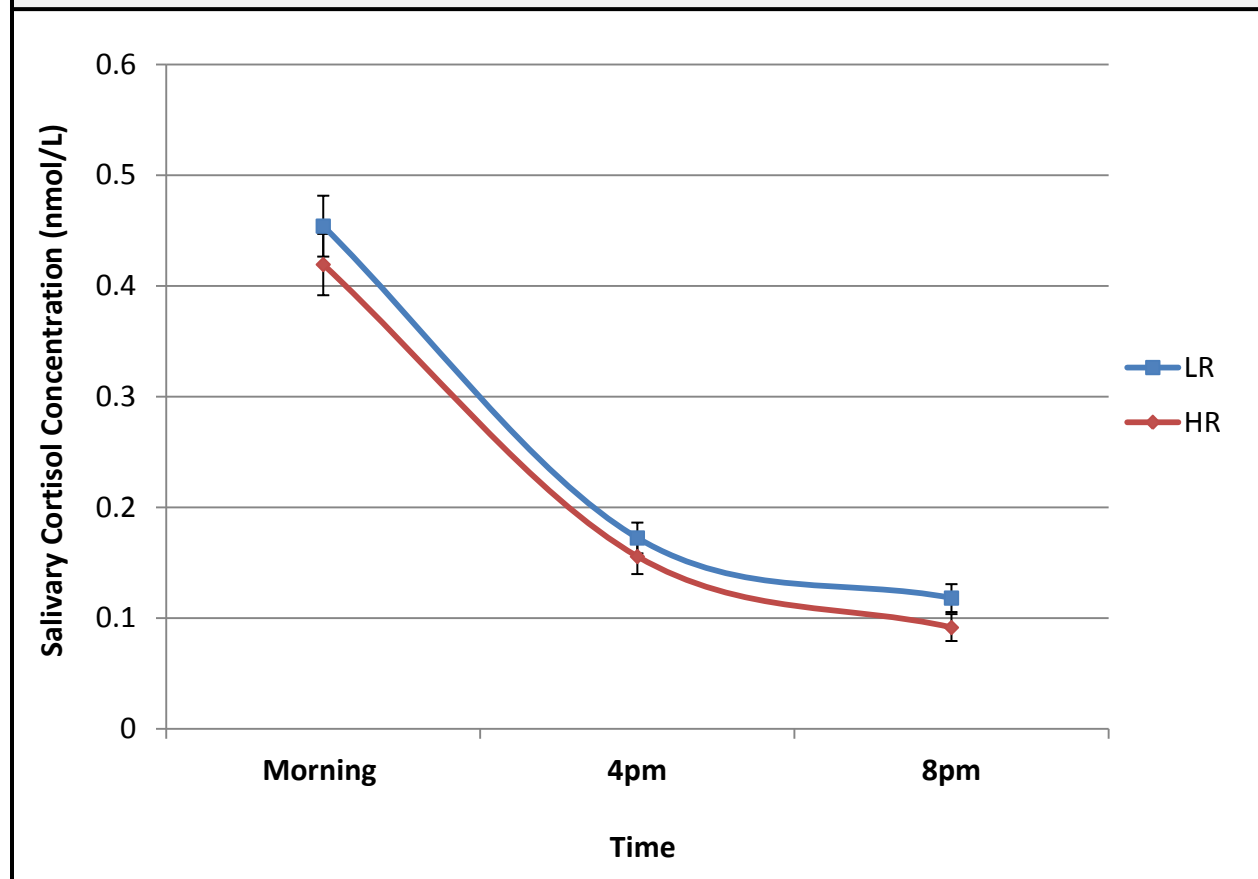


| Table 3. Non-transformed average awakening cortisol values and AUC values | | | |
|--|------------------------|------------------------|------------------------------------|
| | LR Mean (SD) | HR Mean (SD) | Effect Size (<i>d</i>) |
| Ave. Cort. WU | 0.402 (0.330) | 0.363 (0.265) | 0.130 |
| Ave. Cort. WU +30 | 0.523 (0.396) | 0.446 (0.345) | 0.207 |
| Ave. Cort. WU +60 | 0.442 (0.423) | 0.364 (0.348) | 0.201 |
| AUC _i | 0.070 (0.173) | 0.042 (0.147) | 0.174 |
| AUC _g | 0.472 (0.362) | 0.405 (0.303) | 0.201 |
| | | | |

| Table 4. <i>Correlations (r) for covariates of age, gender, and puberty status and CAR values.</i> | | | | | |
|---|--------------------------|-----------------------------|-----------------------------|-------------|-------------|
| | Ave. Cort. WU | Ave. Cort. WU+30 | Ave. Cort. WU+60 | AUCi | AUCg |
| Age | -0.294** | -0.180* | -0.203** | 0.082 | -0.225** |
| Gender | 0.017 | -0.014 | 0.029 | -0.022 | 0.035 |
| Puberty | -0.283** | -0.158* | -0.206** | 0.091 | -0.211** |
| *P<0.05, **P<0.01 | | | | | |

| Table 5. <i>Correlations (r) between CAR values and STAIC-T, CASI, CSRCI, child stressors, and prenatal stressors for LR participants.</i> | | | | | |
|---|--------------------------|-----------------------------|-----------------------------|-------------|-------------|
| | Ave. Cort. WU | Ave. Cort. WU+30 | Ave. Cort. WU+60 | AUCi | AUCg |
| STAIC-T | 0.018 | 0.022 | -0.020 | -0.013 | 0.010 |
| CASI | 0.171* | 0.080 | 0.153 | -0.059 | 0.127 |
| CSRCI | -0.107 | -0.141 | -0.133 | -0.090 | -0.140 |
| Child Stressors | 0.162 | 0.043 | 0.103 | -0.119 | 0.090 |
| Prenatal Stressors | 0.041 | 0.121 | 0.088 | 0.135 | 0.101 |
| *P<0.05 | | | | | |

| Table 6. <i>Correlations (r) between CAR values and STAIC-T, CASI, CSRCI, child stressors, prenatal stressors, and gender of affected parent for HR Participants.</i> | | | | | |
|--|----------------------|-------------------------|-------------------------|-------------|-------------|
| | Ave. Cort. WU | Ave. Cort. WU+30 | Ave. Cort. WU+60 | AUCi | AUCg |
| STAIC-T | 0.029 | -0.094 | -0.084 | -0.198 | -0.071 |
| CASI | 0.221 | 0.258* | 0.252* | 0.153 | 0.268* |
| CSRCI | 0.146 | -0.031 | 0.170 | -0.131 | 0.063 |
| Child Stressors | 0.083 | -0.015 | -0.087 | -0.182 | -0.016 |
| Prenatal Stressors | -0.212 | -0.207 | -0.180 | -0.062 | -0.216 |
| Affected Parent Gender | -0.135 | -0.133 | -0.173 | -0.075 | -0.155 |
| *P<0.05 | | | | | |

Figure 8. *The diurnal cortisol response for LR and HR participants.*

| Table 7. Non-transformed average evening cortisol values. | | | |
|--|-------------------------|-------------------------|-----------------------------------|
| | LR Mean (SD) | HR Mean (SD) | Effect Size (<i>d</i>) |
| Ave. Cort 4pm | 0.172 (0.176) | 0.155 (0.162) | 0.101 |
| Ave. Cort. 8pm | 0.118 (0.157) | 0.091 (0.117) | 0.195 |
| Diurnal AUCg | 3.091 (2.576) | 2.793 (2.137) | 0.126 |

Table 8. *Correlations (r) for covariates of age, gender, puberty status and 4pm and 8pm average cortisol values.*

| | Ave. Cort. 4pm | Ave. Cort. 8pm | Diurnal AUCg |
|-------------------|-----------------------|-----------------------|---------------------|
| Age | -0.150* | -0.143* | -.185** |
| Gender | 0.061 | 0.080 | .047 |
| Puberty | -0.192** | -0.187** | -.214** |
| *P<0.05, **P<0.01 | | | |

| Table 9. <i>Correlations (r) for 4pm, 8pm average cortisol values, and diurnal AUCg and STAIC-T, CASI, CSRCI, child stressors and prenatal stressors for LR Participants.</i> | | | |
|--|-----------------------|-----------------------|---------------------|
| | Ave. Cort. 4pm | Ave. Cort. 8pm | Diurnal AUCg |
| STAIC-T | 0.016 | 0.021 | .000 |
| CASI | 0.136 | 0.105 | .132 |
| CSRCI | -0.093 | -0.049 | -.107 |
| Child Stressors | 0.122 | 0.143 | .144 |
| Prenatal Stressors | 0.158 | 0.145 | .139 |
| | | | |

Table 10. *Correlations (r) for 4pm, 8pm average cortisol values and diurnal AUCg and STAC-T, CASI, CSRCI, child stressors, prenatal stressors and gender of affected parent for HR Participants.*

| | Ave. Cort. 4pm | Ave. Cort. 8pm | Diurnal AUCg |
|---------------------------|----------------|----------------|--------------|
| STAIC-T | -0.123 | -0.137 | -.085 |
| CASI | 0.145 | 0.171 | .220* |
| CSRCI | -0.044 | 0.011 | .002 |
| Child Stressors | 0.067 | 0.066 | .058 |
| Prenatal Stressors | -0.167 | -0.168 | -.163 |
| Gender of Affected Parent | -0.115 | -0.179 | -.148 |
| *P<0.05 | | | |

| Table 11. Post-hoc comparison between PD and GAD+SAD groups. | | | |
|---|------------------------|-----------------------------|------------------------------------|
| | PD Mean (SD) | GAD+SAD Mean (SD) | Effect Size (<i>d</i>) |
| Ave. Cort. WU | 0.452 (0.308)* | 0.243 (0.113) | 0.901 |
| Ave. Cort. WU +30 | 0.530 (0.405) | 0.334 (0.195) | 0.617 |
| Ave. Cort. WU +60 | 0.452 (0.407) | 0.246 (0.186) | 0.651 |
| AUCi | 0.039 (0.170) | 0.046 (0.111) | 0.049 |
| AUCg | 0.491 (0.367) | 0.289 (0.142) | 0.726 |
| Ave. Cort 4pm | 0.184 (0.117)* | 0.079 (0.064) | 1.113 |
| Ave. Cort. 8pm | 0.109 (0.131) | 0.045 (0.042) | 0.658 |
| Diurnal AUCg | 3.337 (2.309)** | 1.641 (0.873) | 0.924 |
| *P<0.05, **P<0.01 | | | |

| Table 12. Post-hoc comparison between PD and LR groups. | | | |
|--|------------------------|------------------------|------------------------------------|
| | PD Mean (SD) | LR Mean (SD) | Effect Size (<i>d</i>) |
| Ave. Cort. WU | 0.452 (0.308) | 0.402 (0.330) | 0.157 |
| Ave. Cort. WU +30 | 0.530 (0.405) | 0.523 (0.396) | 0.017 |
| Ave. Cort. WU +60 | 0.452 (0.407) | 0.442 (0.423) | 0.024 |
| AUCi | 0.039 (0.170) | 0.070 (0.173) | 0.181 |
| AUCg | 0.491 (0.367) | 0.472 (0.362) | 0.052 |
| Ave. Cort 4pm | 0.184 (0.117) | 0.172 (0.176) | 0.080 |
| Ave. Cort. 8pm | 0.109 (0.131) | 0.118 (0.157) | 0.062 |
| Diurnal AUCg | 3.337 (2.309) | 3.091 (2.576) | 0.076 |

| Table 13. <i>Post-hoc comparison between GAD+SAD and LR groups.</i> | | | |
|--|-----------------------------|------------------------|------------------------------------|
| | GAD+SAD Mean (SD) | LR Mean (SD) | Effect Size (<i>d</i>) |
| Ave. Cort. WU | 0.243 (0.113) | 0.402 (0.330) | 0.645 |
| Ave. Cort. WU +30 | 0.334 (0.195)* | 0.523 (0.396) | 0.606 |
| Ave. Cort. WU +60 | 0.246 (0.186)* | 0.442 (0.423) | 0.600 |
| AUCi | 0.046 (0.111) | 0.070 (0.173) | 0.165 |
| AUCg | 0.289 (0.142)* | 0.472 (0.362) | 0.666 |
| Ave. Cort 4pm | 0.079 (0.064)* | 0.172 (0.176) | 0.702 |
| Ave. Cort. 8pm | 0.045 (0.042)* | 0.118 (0.157) | 0.635 |
| Diurnal AUCg | 1.641 (0.873)** | 3.091 (2.576) | 0.754 |
| *P<0.05, **P<0.01 | | | |