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Psychometric properties of the diurnal cortisol profile in youth

Sivan Rotenberg

A Thesis

in

The Department

of

Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of Master of Arts (Clinical Psychology) at
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ABSTRACT

Psychometric properties of the diurnal cortisol profile in youth

Sivan Rotenberg

Cortisol is the end product of the hypothalamic pituitary adrenal axis (HPA). It is released in a diurnal profile with a noticeable morning rise (cortisol awakening response) and decline throughout the day (diurnal slope). Cortisol is of interest to many researchers due to its association with negative physical and mental health consequences. For the diurnal cortisol profile to be considered a stable individual difference, it must be reliable to measure. Current knowledge of the reliability of the diurnal cortisol profile is almost entirely based on adults. The reliability in youth may differ due developmental factors, such as puberty, among other possible covariates. The present study evaluated the reliability of calculated indices and individual measures of the diurnal cortisol profile in youth aged 9 to 18 years. Three groups of youth collected five to six saliva samples per day over two to three days. Cortisol assays and calculated indices were conducted using standardized methods. Results indicated maximum peak cortisol level, the total cortisol concentration over a day (AUC_{TG}), and the cortisol awakening response relative to ground (AUC_{AG}) can be moderately reliably assessed in children and adolescents when sampled over two to three days. At least seven days are needed to obtain reliable measures of the change in cortisol concentration (AUC_I, diurnal slope). Important covariates to consider include sleep duration, day of week, pubertal stage, time of awakening, and perceived stress. These findings suggest the diurnal cortisol profile in children and adolescents can be reliably assessed and reflects a stable individual difference. Methodological considerations and suggestions for future research are discussed.

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Homeostatic systems respond to changes in the environment to maintain equilibrium. The endocrine system is a homeostatic system comprised of networks, such as the hypothalamic pituitary adrenal (HPA) axis and hypothalamic pituitary thyroidal (HPT) axis, and organs, including the pancreas and parathyroid gland (Hiller-Sturmhofel & Bartke, 1998), which enable the body to respond and adapt to changes in the internal or external environment (Chrousos 2009). The HPA axis, and its end product cortisol, has been implicated in catabolic processes in nearly every system in the human body (e.g., arousal, mood, sleep, intermediary metabolism, maintenance of cardiovascular tone, immune and inflammatory responses; Chrousos & Kino, 2007). Cortisol promotes functions related to activation (e.g., arousal) and inhibits restorative processes (e.g., sleep). Cortisol is a measure that can be reliably assessed in adults (Edwards, Clow, Evans, & Hucklebridge, 2001; Smyth et al., 1997). The question remains whether cortisol is a stable individual difference that can be reliably measured in children and adolescents. The aim of the current study was to evaluate the reliability of cortisol measurement in children and adolescents. In the following sections, the HPA axis and pattern of cortisol release are first reviewed. Second, the developmental pattern and factors associated with the diurnal profile are considered. Third, methodological practices and knowledge regarding the reliability of cortisol measurement in adults are discussed, as well as unique issues relevant to cortisol measurement in youth. Fourth, the current state of knowledge regarding the reliability and stability of cortisol measurement in youth is reviewed. Finally, remaining questions and the justification for the psychometric evaluation of the diurnal cortisol profile in children and adolescents are presented.

HPA Axis Activation and Regulation

The HPA axis is a slow cascade of endocrine events initiated by the hypothalamus (McEwen, 1998). Activation of the HPA axis is controlled by the paraventricular nucleus of the hypothalamus, where corticotrophin-releasing hormone is secreted (Herman, Ostrander, Mueller, & Figeuiredo, 2005). The paraventricular nucleus is regulated by the suprachiasmatic nucleus, as well as segments of the limbic system, including the medial prefrontal cortex, hippocampus, and

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amygdala (Jacobson, 2005; Putnam, Pizzagalli, Gooding, Kalin, & Davidson, 2008; Weitzman et al., 1971; Windle et al., 1998). Corticotrophin-releasing hormone stimulates the anterior pituitary to release adrenocorticotropin hormone, which in turn stimulates the anterior adrenal cortex to release cortisol (Egliston, McMahon, & Austin, 2007).

Cortisol is released in a pulsatile fashion, in association with several biological processes (e.g., metabolism, inflammation, and cardiovascular tone), in a circadian rhythm, and in response to physical (noise) or psychological (perceived stress) stressors (Herman, et al., 2005; Hiller-Sturmhofel & Bartke, 1998). The duration and magnitude of the release of cortisol is regulated by a negative feedback loop, wherein cortisol inhibits the release of corticotrophin-releasing hormone (Windle, Wood, Shanks, Lightman & Ingram, 1998). There are at least two feedback loops: fast feedback and delayed feedback. The fast feedback loop appears to regulate the rate of cortisol release by acting at the level of receptors on the membrane of the paraventricular nucleus (Di, Malcher-Lopes, Halmos, & Tasker, 2003; Herman et al., 2005). The delayed feedback loop regulates the amount of cortisol released by interacting at the genomic level and modifying the activity of transcription factors (Tsigos & Chrousos, 2002). Cortisol is not stored; upon HPA axis activation, cortisol production is initiated and then released into circulation.

Diurnal Cortisol Profile and Awakening Response

The circadian rhythm of cortisol is characterized by a diurnal cortisol profile with increasing cortisol levels prior to awakening until 30 to 60 minutes after awakening, then gradually declining levels throughout the day that reach nadir around bedtime (Fries, Dettenborn, & Kirschbaum, 2009). Cortisol levels peak 30 to 60 min post-awakening, a phenomenon known as the cortisol awakening response or awakening challenge (Clow, Thorn, Evans, & Hucklebridge, 2004; Hanrahan et al, 2006). The awakening response typically yields a 50 to 75% increase in the cortisol volume (approximately 4-15 nmol/l; Wust, Wolf, et al., 2000), and appears to be a distinct phenomenon that is influenced by the awakening process (Wilhelm, Born, Kudielka, Schlotz, & Wust, 2007). In laboratory sleep conditions, Wilhelm and colleagues (2007)

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found that the awakening response was an added positive effect to the linear cortisol increase in the early morning. The average nighttime cortisol levels were inversely related to the peak level of the cortisol awakening response; suggesting that the cortisol awakening response is likely regulated by some of the same structures as the diurnal profile (Wilhelm et al., 2007).

Developmental Pattern of the Diurnal Cortisol Profile

The diurnal cortisol profile has a developmental pattern. It emerges at an early age and continues to develop throughout childhood and adolescence. Newborn infants do not show a diurnal pattern, but rather have two cortisol peaks that are 12 hours apart. The peaks appear to be unrelated to time of day or state of arousal (Egliston et al., 2007; Gunnar & Donzella, 2002). The diurnal cortisol profile emerges around 2 to 3 months of age. The trend of high levels in the morning and a decline throughout the day continues throughout childhood and adolescence (Kiess et al., 1995; O'Connor, Ben-Shlomo, Heron, Golding, Adams, & Glover, 2005; Oskis, Loveday, Hucklebridge, Thorn, & Clow, 2008; Wust, Federenko, Hellhammer, Kirschbaum, 2000); however, the amount of cortisol released changes across development. The decline in cortisol levels between the mid-morning and late afternoon is not stable until mid-childhood, when stable decreases begin to be observed (Gunnar & Donzella, 2002). This instability has been associated with developmental changes of the sleep/wake pattern in children (Gunnar & Donzella, 2002). Infants and young children tend to nap during the day, resulting in a suppression of the HPA axis and a decrease in circulating cortisol levels, which then rebound afterwards (Gunnar & Donzella, 2002).

During adolescence, the amount of cortisol released increases, but the exact timing and nature of the increase remains unclear (Gunnar, Wewerka, Frenn, Long & Griggs, 2009). Between the ages of 10 to 14 years, a longitudinal study reported a marked cortisol level increase (Walker, Walder, & Reynolds, 2001), while cross sectional studies suggest that cortisol levels increase gradually from childhood, through adolescence to adulthood (Lupien, King, Meaney, & McEwen, 2001; Tornhage, 2002). The increase in cortisol levels during adolescence has been

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associated with pubertal maturation (Kiess et al., 1995; Netherton, Goodyer, Tamplin, & Herbert, 2004). For example, Oskis and colleagues (2009) found post-menarche female adolescents had higher levels of cortisol throughout the day, compared to pre-menarche females. Adam (2006) found males and females at more advanced pubertal stages had steeper diurnal cortisol declines throughout the day and a reduced awakening response. Across adulthood, increasing age has been associated with a lower cortisol awakening response but higher cortisol levels upon awakening (Kudielka & Kirschbaum, 2003; but also see Edwards, Clow, Evans, & Hucklebridge, 2001); age does not seem to be related to the diurnal cortisol decline (Smyth et al., 1997). In older adults, cortisol levels increase with age, but the diurnal decline flattens (Ice, 2005). After 80 years of age, the diurnal profile begins to resemble newborn infants (two peaks of cortisol levels; Ice, 2005).

Factors Associated with the Diurnal Cortisol Profile

Several factors have been found to influence the diurnal cortisol profile, including waking time, sex, weight status, season of sampling, and day of the week (weekday vs. weekend), among others. Early risers tend to have greater cortisol awakening responses and typically secrete more cortisol throughout the day (Edwards, Evans et al., 2001; Kelly, Young, Sweeting, Fischer& West, 2008; Kudielka & Kirschbaum, 2003). While sex differences do not seem to influence the awakening response or evening cortisol level, the decline following the awakening response decreases more rapidly in males than females (Pruessner et al., 1997; Rosmalen et al., 2005; Wust, Wolf, et al., 2000).

The relation between weight status and cortisol has been inconsistent. Researchers have reported that a larger weight status is associated with increased awakening response (Adam, 2006), a flattened decline (attributable to an elevated non-declining afternoon/evening sample or absence of a morning rise; Dekker et al. 2008), or no relation (Netherton et al., 2004; Rosmalen et al., 2005; Steptoe, Kunz-Ebrecht, Brydon, & Wardle, 2004). The influence of season of sampling on the diurnal profile has also been inconsistent, with some researchers reporting higher cortisol levels in the winter (short photoperiod; Walker, Best, Noon, Watt, & Webb, 1997), others

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reporting higher cortisol levels in the spring and summer (long photoperiod; Matchock, Dorn, & Susman, 2007; Rosmalen et al., 2005), and still others reporting no relation (Smyth et al., 1997). The association between day of the week and cortisol is likely influenced by the time of awakening; however, Scholtz and colleagues (2004) found that the cortisol awakening response was typically higher on the weekday, even after controlling for the effect of time of awakening. The influences of these factors, or covariates, on the diurnal profile are not reliably reported across researchers and may be attributable to the cortisol measure investigated, the sampling procedure used, or other factors not considered. The MacArthur Network (2000) suggests statistically controlling for the effects of possible covariates, such as between-person (e.g., sex), state (e.g., menstrual cycle stage), disease (e.g., liver disease), dynamic (e.g., sleep quantity, time of awakening), and psychological (e.g., affect) factors. The majority of this literature regarding the influence of covariates has been reported only for university-aged students and adults; much less is known about the effects of these possible covariates in children and adolescents.

Importantly, the activity of the HPA axis has implications for physical and mental health consequences; excessive (hyperactivity) or deficient (hypoactivity) responses can both result in health problems (Chrousos 2009). In adults, hyperactivity of the HPA axis, as characterized by a large awakening response (increase in cortisol greater than 2.5 nmol/L), a flattened decline, and/or high cortisol levels, has been associated with increased symptomotology for upper respiratory illness (Edwards, Hucklebridge, Clow. & Evans, 2003), greater central adiposity (Steptoe et al., 2004), and increased concentration of coronary calcifications (an indicator of atherosclerosis; Matthews, Schwartz, Cohen, & Seeman, 2006). Hypoactivity of the HPA axis is characterized by a blunted awakening response (increase in the cortisol less than 2.5 nmol/L) and/or reduced cortisol levels. In adults, these deficient responses have been associated with chronic fatigue syndrome, fibromyalgia (Crofford et al., 2004), and autoimmune problems, such as rheumatoid arthritis (Chrousos 2009). While data are limited in children and adolescent populations, variations in HPA axis activity appear related to health consequences including

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depression (Adam, Doane, Zinbarg, Mineka, Craske, & Griffith, 2010; Shirtcliff & Essex, 2008) and immune problems, such as asthma (Landstra, Postma, Boezen, & Van Aalderen, 2002). To further investigate the relationship between cortisol and health in youth, the reliability of the diurnal cortisol profile must be evaluated to establish it as a stable individual difference.

Measuring the Diurnal Profile of Cortisol

Cortisol can be measured in blood (serum), saliva, urine, and hair. The type of sample collected depends on the form of cortisol desired. Serum samples contain both bound and unbound cortisol and are often measured in laboratory-based studies (Kirschbaum, Strasburger, Jammers, & Hellhammer, 1989). Cortisol bound to carrier proteins, such as corticosteroid-binding globulin and albumin, is unable to leave the blood stream, and therefore, can only be measured in blood. Information regarding the concentration of carrier proteins in the blood provides additional knowledge regarding the levels of cortisol in the system. For example, high cortisol levels in obesity may be reflective of low carrier protein levels rather than overproduction of cortisol (Levine, Zagoory-Sharon, Feldman, Lewis, & Weller, 2007). Salivary cortisol levels contain only the unbound form of cortisol because protein-bound molecules are prevented from passing the lining of the saliva glands (Kirschbaum & Hellhammer, 1994). Salivary and serum samples are highly correlated across ages (Gunnar et al, 1989; Reid et al, 1992; Woodside et al, 1991) and they provide an index of the concentration of cortisol at the time of sampling; thus, these samples can be used to assess the cortisol awakening response and the diurnal profile. Urinary samples contain only 1% of unbound cortisol because more than 95% is metabolized in the liver. Adequate analysis requires at least 24-hour urine collection; which would reflect total cortisol production over the previous day (Hellhammer, Wust, & Kudielka, 2009). Recently, hair samples have emerged as another method of analyzing cortisol levels. Unbound cortisol is embedded on the hair shaft by circulating blood and a 2-3cm length sample reflects cortisol levels over the past two to three months (Raul, Cirimele, Ludes, & Kintz, 2004; Sauve, Koren, Walsh, Tokmakejian & Van Uum, 2007). Urine and hair samples are highly correlated and can be used as

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a global measure of unbound cortisol (Sauve et al, 2007). The standard international unit for measuring cortisol is nmol/l; however, some researchers also use μg/dl.

Salivary cortisol measurement is the preferred sampling method to assess the HPA axis in naturalistic settings. Saliva samples are commonly collected using the Salivette, a plastic dual vial tube with a cotton swab to absorb saliva. To assess the diurnal profile, saliva samples are typically taken once or twice in the morning, and at least once in the afternoon and evening over two to three consecutive days. The MacArthur Network (2000) recommends collecting five samples, regardless of age: immediately upon awakening, +45min post awakening, between 4-6pm, between 6-9pm, and between 9pm-bedtime. The cortisol awakening response is commonly measured using samples taken at awakening, +30, +45 and +60min post-awakening over two to three consecutive days. Multiple samples are collected over several days because single samples have been found to have low intra- and inter-individual reliability in adults (Coste, Strauch, Letrait, & Bertagna, 1994; Schulz, & Knabe, 1994; Wust, Wolf, Hellhammer, Federenko, Schommer, & Kirschbaum, 2000). In the adult literature, the methodological designs vary considerably regarding the number of samples obtained per day. Some researchers use two saliva samples while others use up to seven samples per day (cf. Edwards, Evans, Hucklebridge & Clow, 2001; Kelly et al., 2008; Nijm, Kristenson, Olsson & Jonasson, 2007; Smyth et al., 1997). With regards to the number of days of sampling, the MacArthur Network (2000) recommends 3 to 4 days of sampling to determine the amount of cortisol released, and at least 6 days to capture the diurnal decline.

In the child and adolescent literature, methodological practices are even more varied. Some researchers use only one or two samples (cf. El-Sheikh, et al., 2008; Kelly et al., 2008; Lupien et al., 2001), while others sample up to eight times per day (Adam et al., 2010; Kiess et al., 1995; Oskis et al., 2009). Researchers also collect saliva from one or two days (El-Sheikh et al., 2008; Oskis et al., 2009), up to seven days (Netherton, et al., 2004; Pruessner et al., 1997). Due to the discrepancy in the samples per day and number of days used to collect saliva,

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combined with the inherent pulsatile quality of cortisol release, and the developmental changes associated with the diurnal profile, there is high potential for considerable variability in the reliable measurement of cortisol. A psychometric evaluation of the reliability of the diurnal cortisol profile and the influence of covariates remains to be conducted. If individual differences in the diurnal profile are not consistent, then observed differences in the diurnal profile could be attributable to methodology (e.g., day or time of sampling), external factors (e.g., mood, amount of sleep, stress/hassles), or the instability of the diurnal profile itself.

To derive interpretable cortisol values, researchers calculate several different indices. There are generally four classes of indices including: 1) the awakening response, 2) diurnal slope, 3) total cortisol level over the day, and 4) individual time points (see Figure 1). The awakening response is most commonly calculated from at least three sequential time points upon awakening (Edwards, Clow, et al., 2001; Edwards, Evans, et al., 2001; Kudielka & Kirschbaum, 2003); although some researchers have calculated the awakening response based only on two time points (Backhaus, Junghanns & Hohagen, 2004; Rosmalen, Oldehinkel, Ormel, De Winter, Buitelaar, Verhulst, 2005). The area under the curve (AUC) relative to the cortisol level at the time of awakening describes the dynamic increase in the amount of cortisol that was secreted following awakening (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003), and is referred to as area under the curve relative to increase (**AUC_I**). In adults, the mean AUC_I typically ranges from 12.4 nmol/L (SD = 5.4; Stetler & Miller, 2005) to 13.4 nmol/L (SD = 1.06; Kudielka & Kirschbaum, 2003). In adolescents, AUC_I ranges from 9.16 nmol/L (SD = 7.42; Oskiset al., 2009) to 14.40 nmol/L (SD = 12.31; Dockray, et al., 2009). The dynamic increase of cortisol following awakening can also be represented by the mean increase (average of the post-awakening samples, +30, +45, +60min post-awakening samples, minus the awakening sample; Edwards, Hucklebridge, et al., 2003); which is be highly related to AUC_I ($r > .97$; Edwards, Hucklebridge, et al., 2003). The awakening response is also calculated relative to ground or zero (**AUC_{AG}**), which represents the total amount of cortisol that was released during the given period (Pruessner

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et al., 2003). Characterizing the awakening response with AUC_I is considered to be less reliable than AUC_{AG}, due to problems acquiring a dependable awakening sample (Clow et al., 2004).

The slope of the line from the peak morning cortisol value to the last measured point characterizes the **diurnal slope**. The diurnal slope can be calculated using standard linear regression (Giese-Davis, Di Micell, Sephton, & Spiegel, 2006). Normal declining slopes are characterized by negative values while flattened slopes are described by values closer to zero. In adults, the diurnal slope typically ranges from -.09 nmol/L (SD = .06; Stone et al., 2001) to -.82 nmol/L (SD = .5; Dekker et al., 2008). In adolescents, Adam and colleagues (2010) reported a diurnal slope of -.56 (SD = .38). The total cortisol concentration over a day is the area under the diurnal curve ($\mathbf{AUC_{TG}}$), and represents the overall secretory activity of the HPA axis. This measure is based on all measured time points following the cortisol awakening response until the last measured point. Alternatively, the diurnal mean, or the averaged value of all cortisol samples over a day (excluding samples of the awakening response), is often used as a proxy for the total concentration of cortisol (Edwards, Clow, et al., 2001; Smyth et al., 1997). Finally, individual time points along the diurnal profile are also reported, with the morning, maximum (peak), and random samples (samples not taken at a particular time) being the individual points most commonly used in analyses. In adults, morning cortisol samples typically range from 14.3 nmol/L (SD = 7.9; Dekker et al., 2008) to 20.4 nmol/L (SD = 11.4; Steptoe et al., 2004). In adolescents, morning cortisol values range from 10.5 nmol/L (SD = 8.1; Kelly et al., 2008) to 13.21 nmol/L (SD = 6.64; Tzortzi et al., 2009).

Each cortisol index is thought to provide unique information about HPA axis activity. Consider the relationship between these indices. The diurnal mean was positively related to the AUC_{AG} in adults (r_{avg} = .53; Edwards, Clow, et al., 2001), but not in adolescents (r = .05; Oskis et al., 2008). The diurnal mean was not related to AUC_I in adults (r_{avg} = .12; Edwards, Clow, et al., 2001) or adolescents (r = .15; Oskis et al., 2008). In turn, the AUC_I was negatively related to awakening samples (r = -.43; Rosmalen, et al., 2005), and positively related to diurnal slope (r =

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.40; Oskis, et al., 2008). Thus, individuals with greater values at awakening do not exhibit as elevated an increase in AUC_I compared to those with lower values upon awakening; and, having an elevated AUC_I leads to a greater decline in cortisol throughout the day. Finally, the individual awakening sample was positively related to AUC_{TG} (r = .71; Rosmalen, et al., 2005). The observed differences between these cortisol indices suggest that they are unique and measure distinct constructs or aspects of HPA activity. Alternatively, the differences may reflect psychometric characteristics of the time points and/or formulas used to calculate these indices or the unreliability of the diurnal cortisol profile.

Reliability of the Diurnal Profile

The current state of knowledge regarding the psychometrics, or reliability and stability, of the diurnal profile is predominately based on adults. Edwards, Clow, and colleagues (2001) found that patterns of cortisol activity, characterized by the cortisol awakening response and diurnal mean, had good intra-individual stability (AUC_{AG} r = .52; AUC_I r = .34; diurnal mean r = .65) across two consecutive days, and therefore represented a stable individual trait characteristic of the diurnal cortisol profile. Smyth et al. (1997) evaluated the test-retest reliability of the diurnal slope and mean level of cortisol over two days in adults. They found that 68% of the participants had either declining or flattened slopes across two days, while 31% had variable slopes. Despite an individual's slope, the diurnal mean was fairly stable over the two days (normal slope r = .61; flat slope r = .70; variable slope r = .57; Smyth et al., 1997). Coste and colleagues (1994) examined the efficacy of using a single morning cortisol sample as a measure of HPA axis activity and found that single samples over three non-consecutive days have an intraclass correlation (ICC) of 0.18. Furthermore, Coste found that to reach satisfactory reliability (ICC $\geq$ 0.8) 18 days of sampling using one sample per day would be needed. Schwartz (2000) suggests that reliable assessment of the AUC_{TG} necessitates five samples collected over three to four days, while reliable assessment of the diurnal slope requires more than five days of sampling (MacArthur Network, 2000). The inter-individual variation of the cortisol awakening response

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appears to have a genetic component (AUC_{AG}, $h^2 = .48$; mean increase, $h^2 = .40$), while the diurnal slope ($h^2 = .20$) does not (Wust, Federenko, et al., 2000). Overall, two day of measurement may be sufficient to reliably assess the diurnal profile of cortisol in adults; however, only one study evaluated the influence of covariates on the reliability of the diurnal cortisol profile (Edwards, Clow, et al., 2001) and found no effect for age, gender, or smoking status.

Fewer studies have examined the reliability of the diurnal cortisol profile in children and adolescents. Most of the studies that have examined the reliability of cortisol measurement in youth have had a small sample size and/or combined adult and adolescent participants. O'Conner and colleagues (2005) found moderate stability for individual samples taken across three consecutive days in a group of 74 children ($awake_0$ $\alpha = .49$; $awake_{30}$ $\alpha = .77$; afternoon $\alpha = .58$; bedtime $\alpha = .75$). Oskis et al (2008) also found moderate stability of the awakening reponse and diurnal profile in adolescents over two days (AUC_{AG} $r = .55$; AUC_I $r = .53$; diurnal mean $r = .56$; diurnal slope $r = .73$). The results of these two preliminary studies suggest the intra-individual reliability of individual measures and calculated indices in youth. No additional studies examining the reliability of cortisol measurement in youth exist (to the best of our knowledge). Further research is needed to address the influence of developmental factors, puberty, the sleep/wake cycle, and other covariates on the psychometric properties of the diurnal cortisol profile in children and adolescents.

The primary objective of this study was to evaluate the psychometric properties of the diurnal cortisol profile in children and adolescents. The intra-individual stability of the awakening response (AUC_{AG}, AUC_I), the diurnal slope, the total amount of cortisol over a day (AUC_{TG}), as well as the commonly assessed individual time points throughout the cycle were examined. We hypothesized that the calculated measures of the diurnal cortisol profile would be more reliable than individual time points. The reliability of these measures was also examined across time (2 vs. 3 days). We hypothesized that the reliability of the cortisol measures would be better when

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averaged over 3 days compared to 2 days. Finally, we hypothesized that controlling for the influence of covariates would improve the reliability of the cortisol measures. As a secondary objective, the amount of variance associated with each covariate (sex, age, perceived stress level, pubertal stage, season, time of awakening, and sleep duration) would be derived and the number of days of sampling need to reach optimal reliability (ICC $\geq$ .8) would be calculated.

Method

Participants

Youth aged 10-15 years were recruited to participate in the Healthy Heart Project using flyers, postcards, and bookmarks distributed throughout the Montreal community and to classrooms in schools approved by the Montreal English School Board. Healthy Heart I (March 2006 to September 2007) included 132 children (M = 12.51 years, SD = 1.81; 44% girls; Cohort 1 Cycle 1). Healthy Heart II (November 2007 to July 2009) included 168 new children (M = 12.21 years, SD = 1.92; 44.2% girls; Cohort 2) and 73 children from Cohort 1 who returned for a second cycle of sampling (M = 13.76 years, SD = 1.91; 51% girls; Cohort 1 Cycle 2). Children with serious psychopathology or medication use known to interfere with cardiovascular functioning were excluded from the study. Only aspects of the Healthy Heart Project relevant to the current project are described here.

Measures

Saliva sampling. Saliva samples were collected using the Salivette sampling device (Salimetric, Inc.). Children were instructed to place the cotton swab in their mouth and to hold it there for at least 30 seconds. When the cotton swab was saturated, children were instructed to place it in the Salivette tube and store it in their refrigerator at home until they returned to the laboratory for their second visit. Children were instructed not to eat or brush their teeth 10 minutes before taking a sample, and to complete a daily log, recording the time that each sample was taken. The child's parent initialed the recording, which was later used as an indicator of

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compliance. In the laboratory, the saliva samples were stored in a sub-zero freezer until they were packaged in dry ice and couriered to the University of Trier, Germany for cortisol assaying.

Healthy Heart I (Cohort 1 Cycle 1) collected saliva samples five times per day over three days. Samples were collected at awakening ($awake_0$), +30 minutes post-awakening ($awake_{30}$), +45 minutes post-awakening ($awake_{45}$), before lunch, and before dinner. Healthy Heart II (Cohort 2 and Cohort 1 Cycle 2) collected saliva samples six times per day over two days. A sixth sample before bedtime was added to the five sample times collected during Healthy Heart I.

Cortisol assaying. The saliva samples were stored at -20 °C until assaying. After thawing, saliva samples were centrifuged at 2000 g for 6 minutes, which resulted in a clear supernatant of low viscosity. Assays were completed in duplicate using 100ul of saliva (50µl per well). Cortisol levels were determined using a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA). Ninety-six well-Maxisorb microtiterplates (Nunc) were coated with swine-anti-rabbit immunoglobulin. After an incubation period of 48h at 4°C, plates were washed three times with a wash buffer (pH=7,4; contains sodium phosphate and the Tween-40). In the next step, the plates were coated with a rabbit anti-cortisol antibody and incubated for 48h at 4°C. Synthetic saliva mixed with cortisol in a range from 0-100nmol/l served as standards. Standards, controls (saliva pools), and samples were placed in duplicate wells. Fifty microliters of biotin-conjugated cortisol was added and after 30 minutes of incubation the non-binding cortisol / biotin-conjugated cortisol was removed by washing three times. Two hundred microliters of europium-streptavidin (Wallac, Turku, Finland) was added to each well; after 30 minutes and washing the wells six times, 200µl enhancement solution was added (Pharmacia, Freiburg, Germany). Within 15 minutes on a shaker, the enhancement solution induced the fluorescence, which was detected with a DELFIA-Fluorometer (Wallac, Turku, Finland). With a computer-controlled program, a standard curve was generated and the cortisol concentration of the sample was calculated. For the current samples, the intra-

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assay coefficients of variation were between 4.0 to 6.7%, and the corresponding inter-assay coefficients of variation were between 7.1 to 9.0%.

Pubertal stage. Pubertal stage was only assessed during Healthy Heart II (Cohort 2 and Cohort 1 Cycle 2) using Tanner stage illustrations derived from the National Health and Nutrition Examination Survey, which depict five stages of pubertal development. There are ten pictures for the boys; five depict stages of genital development and five illustrate stages of pubic hair growth. There are also ten pictures for the girls; five depict the stages of breast development and five illustrate the stages of pubic hair growth. Pre- to early-puberty corresponds to Tanner stages I and II, while mid- to post-puberty corresponds to Tanner stage III, IV, and V. The children select gender-appropriate illustrations. These drawings have been used in several studies and demonstrate good reliability and validity (Dorn, Nottelmann, Inoff-Germain, Susman, & Chrousos, 1990; Netherton et al., 2004). Dorn et al. (1990) found a positive correlation between adolescent self-ratings and the ratings of a nurse practitioner for genital/ breast development (r_{boys} = .84; r_{girls}= .88) and pubic hair stages (r_{boys} = .77; r_{girls}= .91). Girls were also asked the age of their first menstrual period, if they had begun menstruation.

Seasonality. Season of sampling was based on the timing of the solstice and equinox occurrence (Matchock et al., 2007). Typically, winter is from December 21st to March 20th, spring is from March 21st to June 20th, summer is from June 21st to September 20th, and fall is from September 21st to December 20th; however, these dates vary slightly each year. Exact dates were determined using the United States National Oceanic and Atmospheric Administration calendar. Seasons were grouped based on photoperiod (fall/winter vs. spring/summer).

Sleep duration. The quantity of sleep for the night preceding saliva sampling was assessed using the child's daily logs. Sleep duration was calculated by subtracting the time of the bedtime sample from the time of the awake$_0$ sample. Sleep duration was only assessed during Healthy Heart II (Cohort 2 and Cohort 1 Cycle 2).

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Perceived stress. Stress was assessed using the Perceived Stress Scale, a self-report measure. The Perceived Stress Scale is a 14-item global measure of stress that assesses the degree to which individuals consider their life to be stressful (Cohen, Kamarck, & Mermelstein, 1983). Children answered questions about how often they have felt a certain way in the past month and responded on a five-point scale (0 = never; 4 = very often). For example, items include "In the last month, how often have you been upset because of something that happened unexpectedly?" and "In the last month, how often have you felt that you were unable to control the important things in your life?" The Perceived Stress Scale has been shown to be valid and reliable in a sample of young adults (α_{avg} = .85; Cohen et al., 1983).

Procedure

An initial telephone screening was conducted with interested parents to ensure their child was eligible to participate. Eligible children and their parents were then scheduled for two appointments. On the first visit, children and their parents completed questionnaires and anthropometric measures were taken (height, weight, body mass index, hip and waist circumference). The questionnaires included items assessing demographic information, pubertal stage, socioeconomic status, stress exposure, and sleeping patterns. One saliva sample was collected from the child using the Salivette device during the lab visit. At this time, the child was instructed how to use the Salivette device and given kits for saliva collection at home. At the second visit, participants returned the saliva samples and completed any remaining questionnaires. Families received monetary compensation for their time. The Concordia University Ethics Review Committee (UH2005-077-4) approved this study.

Data Preparation

Raw cortisol samples were square root adjusted to address non-normality and skewness. Five children from Cohort 1 Cycle 1 and Cohort 2, and two children from Cohort 1 Cycle 2 were excluded because no cortisol samples were provided (see Figure 2). Each calculated cortisol index was derived for every day of sampling. When calculating the cortisol awakening response

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(AUC_{AG}, AUC_I), the trapezoidal method described by Pruessner et al. (2003) was used, based on the $awake_0$, $awake_{30}$, and the $awake_{45}$ samples. The formula for calculating the AUC_{AG} and AUC_I are:

$$AUC_{AG} = (awake_{30} + awake_0) * t_1 / 2 + (awake_{45} + awake_{30}) * t_2 / 2$$

$$AUC_I = AUC_{AG} - awake_0 * (t_1 + t_2)$$

where t represents the time difference between when the samples were taken.

The diurnal slope was estimated using standard linear regression as seen in Giese-Davis et al. (2006). When calculating the diurnal slope, the highest cortisol sample from any morning sample ($awake_0$, $awake_{30}$ or $awake_{45}$) was used as the first value in the linear regression. The total concentration of cortisol over a day (AUC_{TG}) was calculated using all samples, except those associated with the awakening response ($awake_{30}$, $awake_{45}$). The formula for calculating the AUC_{TG} is (Pruessner et al., 2003):

$$AUC_{TG} = (lunch + awake_0) * t_1 / 2 + (dinner + lunch) * t_2 / 2 + (bedtime + dinner) * t_3 / 2$$

To select the individual morning and random measures, a random number generator was used. The morning measure was randomly selected from the $awake_0$, $awake_{30}$, or $awake_{45}$ samples, while the random measure was randomly selected from all collected samples on a given day. These random individual measures were chosen purposely to mimic methodological design choices observed in the literature (e.g., morning sample taken when child arrives at school; during classroom visit scheduled at convenient time). The maximum or peak measure was the highest cortisol value obtained for the day.

Missing data. Missing values resulted from children either missing specific saliva samples or having an insufficient number of samples to calculate a cortisol measure. The AUC_{AG} and AUC_I were set as missing in children who did not have an $awake_0$ sample or if the timing of the awakening response exceeded one hour. The AUC_{TG} was identified as missing in children without an $awake_0$ sample and/or at least two other samples. The diurnal slope was set as missing in children without any morning samples and/or at least two other samples (see Figure 2).

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Missing values were imputed using Amelia II (version 1.2-14, in order to retain as many data points as possible. Prior to imputation, Little's Missing Completely at Random (MCAR) test was conducted to determine if the missing data were correlated with an individual-level characteristic (Donders, van der Heijden, Stijnen, & Moons, 2006). When Little's MCAR test is not significant, the missing values are assumed to be missing completely at random (Donders et al., 2006). Alternatively, when Little's MCAR test is significant, data are not missing completely at random and may be either missing at random (MAR) or not missing at random (NMAR or non-ignorable). Data missing at random are assumed to be related to information collected about the individual (i.e., missingness depends on variables measured). Simple techniques (e.g., mean substitution) can be used to handle data that are missing completely at random, while complex imputation techniques (e.g., multiple imputation to derive unbiased estimates) are used to handle data missing at random. Multiple imputation uses various known individual characteristics to impute the missing value, resampling several times, akin to a bootstrapping technique (Donders et al., 2006). The aggregate of the resampled imputations is then used as the best estimate of the missing value. Data not missing at random (NMAR) is missing for a specific reason (e.g., question purposely skipped by participant) and must be replaced or deleted case-wise prior to analyses.

Statistical Analyses

Upon receipt of the assayed cortisol values from Germany, the four calculated cortisol indices (AUC_{AG}, AUC_{I}, AUC_{TG}, diurnal slope) were computed using equations previously reported (cf. Giese-Davis et al.,2006; Pruessner et al., 2003). For the calculated indices only, missing data procedures were performed to impute missing data. Preliminary data checks were conducted to ensure there were no differences across original data versus imputed data (see Table 1). Preliminary data checks also were conducted across the three groups of participants (Cohort 1 Cycle1; Cohort 2; Cohort 1 Cycle 2) for the means of the calculated and individual measures (see

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Table 3), as well as within each group across days to ensure that there were no systematic differences (see Table 4).

Hypothesis testing of the psychometric properties of the diurnal cortisol profile was guided by classical measurement theory. Specifically, multi-level modeling and intraclass correlational analyses were used to evaluate the reliability of seven cortisol measures: AUC_{AG}, AUC_I, diurnal slope, AUC_{TG}, individual morning cortisol sample, maximum (peak) cortisol sample, and a randomly selected cortisol sample (see Figure 1). The intra-individual reliability (within-person) was evaluated for each measure across time using intraclass correlation analyses. Covariates, including sex, age, perceived stress level, pubertal stage, season of sampling, time of awakening, day of sampling, and sleep duration, were then evaluated using multi-level modeling to identify their influence on the stability of cortisol measurement. Analyses were conducted using SAS 9.2 statistical software.

The intraclass correlation coefficient (ICC) is a classical index of reliability and represents the ratio of variance due to between-subject variability (σ_{BS}^2) over the sum of between-subject variability plus error variability (σ_{WS}^2; Coste et al., 1994). An intraclass correlation considers the relative position of the values as well as the quantitative difference between them; unlike a Pearson correlation, which only considers the relative order of the values. (Both ICCs and Pearson correlation coefficients were conducted for comparative purposes.) In the current study, the error variability represents the methodological error and within-subject variability. An ICC ranges from 0 to 1; values greater than 0.8 are deemed to have satisfactory reliability. To compare the reliability of the calculated indices to the individual time points, the average intraclass correlation (ICC_{AV}) was calculated. The ICC_{AV} represents the reliability of a measure averaged over multiple days of measurement (i.e., reliability over three days). To calculate the ICC_{AV}, the single day intraclass correlation (ICC_S) was first calculated (Shrout & Fleiss, 1979):

$$ICC_S = \sigma_{BS}^2 / (\sigma_{BS}^2 + \sigma_{WS}^2)$$

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Next, the ICC_{AV} was calculated using the Spearman-Brown Formula:

$$ICC_{AV} = N * ICC_S / 1+ (N-1)*ICC_S$$

where N is the number of days of measurement.

The reliability of the measures over two and three days of measurement (ICC_{AV}) were compared to examine the influence of an added day of measurement on the reliability of the calculated indices and individual measures. Finally to determine the influence of controlling for covariates on the reliability of a measure (adjusted ICC_{AV}), the between-subject and error variability were recalculated after controlling for all covariates (sex, age, perceived stress, pubertal stage, season of sampling, time of awakening, day of week, and duration of sleep). The adjusted ICC_{AV} was compared to the unadjusted ICC_{AV} (no covariates).

The secondary objectives of this study were addressed using multi-level modeling, regression analyses, and the Spearman-Brown Formula. The percent of variance associated with each covariate was found by comparing the total variability controlling for the covariate to the total variability without controlling for the covariate. The beta coefficients (β') of the regression analysis were used to determine which covariates were significantly related to each measure. As well, the number of days of measurement needed to obtain optimal reliability (ICC_{OR}), defined as greater than .8, was calculated by rearranging the Spearman-Brown formula:

$$N = ICC_{OR} (1 - ICC_S) / ICC_S (1 - ICC_{OR})$$

where ICC_{OR} represents optimal reliability.

Results

Missing Data

Across the three groups, 14% of the calculated cortisol indices and 1.3% of the individual measures were missing (see Table 1). The missing values were imputed 20 times with resampling techniques; sex, age, perceived stress score, pubertal stage, season of sampling, time of awakening, day of the week, and duration of sleep were used to inform the imputation. The final aggregates of the 20 imputations were not significantly different than the original values (see

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Table 1); thus, all statistical analyses were conducted using the imputed data. Missing individual measures were excluded using case-wise deletion (data imputation is not possible).

Preliminary Data Checks

Reliability analyses were conducted on Cohort 1 Cycle 1 (127 youth), Cohort 1 Cycle 2 (71 youth), and Cohort 2 (163 youth). While there were no sex differences between the groups, age, perceived stress score, pubertal stage, season of sampling, time of awakening, day of the week, and duration of sleep differed between the groups (see Table 2). Older youth in Cohort 1 (from Cycle 1 to Cycle 2) endorsed experiencing more stress. Compared to the other groups, Cohort 1 Cycle 1 sampled more on the weekend, woke up later, and collected more samples during the spring/summer season. Cohort 1 Cycle 2 was older, more advanced in pubertal stage, and had shorter sleep duration than Cohort 2.

After controlling for demographic variables (i.e., age, perceived stress, season of sampling, time of awakening, day of the week), no differences were found between groups for mean values of AUC_I, diurnal slope, morning measure, or random measure (see Table 3). Between group differences were observed for mean values of AUC_{AG}, AUC_{TG}, and maximum measure. However, when these differences were re-examined after controlling for sleep duration (only possible for Cohort 1 Cycle 2 and Cohort 2), the mean values were similar (AUC_{AG} $F(1, 200) = 3.89$, $p = .06$; AUC_{TG} $F(1, 200) = .02$, $p = .88$; maximum measure, $F(1, 222) = .49$, $p = .49$). Finally, the mean of the calculated indices and the individual measures were compared across days of sampling, within each group, using a one-way ANOVA (see Table 4). The AUC_I and the individual measures were higher in day one than day two for Cohort 2. No other differences were observed in Cohort 2; no differences were observed in Cohort 1 (Cycle 1 or 2).

Hypotheses Testing

Reliability of calculated indices versus individual measures. The unadjusted (no covariates) intra-class correlation coefficients (ICC) are presented in Table 5. (Pearson correlation coefficients are presented for comparative purposes in Table 6 and 7.) The first

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hypothesis that the calculated indices of the diurnal cortisol profile would be more reliable than individual measures was not supported. The reliability of the measures across cohorts ranged from ICC_{avg} .15 to .72. The rank order of reliability was: maximum measure, AUC_{TG}, AUC_{AG}, morning measure, AUC_I, diurnal slope, and random measure.

Contribution of covariates. To test the second hypothesis that adjusting for covariates would improve the reliability measures, the intraclass correlations were adjusted for all assessed covariates (see Table 5). (Partial correlations are presented for comparative purposes in Table 6 and 7.) Controlling for covariates did not yield consistent results across the three groups; sometimes the ICCs increased, sometimes they decreased, and other times they remained the same. The percent of variance accounted for by each covariate was examined using multi-level modeling for both the calculated indices (see Table 8) and the individual measures (see Table 9). On average, the covariates accounted for a small portion of the variance of the calculated cortisol indices and individual measures: sleep duration (3.75%), day of week (2.09%), pubertal status (2.05%), time of awakening (1.66%), age (1.45%), and perceived stress (1.14%). Standardized regression coefficients were calculated to examine the relative association across the covariates and the calculated cortisol indices and individual measures (see Tables 10 and 11). Day of week (β'_{avg} = -.12), sleep duration (β'_{avg} = -.11), and time of day (β'_{avg} = -.10) were inversely related to cortisol level, while perceived stress (β'_{avg} = .11), age (β'_{avg} = .09), and pubertal status (β'_{avg} = .08) were positively related to cortisol level.

Days of measurement. To test the third hypothesis, the reliability across three, two, and one day of measurement was compared (see Table 5). Healthy Heart I (Cohort 1 Cycle 1) provided three days of measurement; Healthy Heart II (Cohort 1 Cycle 2 and Cohort 2) provided two days of measurement. Reliability based on one day of measurement was statistically derived using intraclass correlation estimates for a single measure (ICC_S). The number of days of measurement needed to obtain optimal reliability (ICC_{OR} = .8) also was calculated (see Table 5).

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The hypothesis was partly supported: ICC values were greater from three days of measurement versus only two days; however, the difference was not meaningful. The number of days needed to obtain optimal reliability ranged from 3 days (maximum, peak) to 1 week (AUC_{AG}, AUC_{TG}, morning measure) to 1 month (AUC_I, diurnal slope, random measure) in children and adolescents.

Discussion

Cortisol is a common biomarker used in research with adults, adolescents, and children. Despite its frequent use, the psychometric properties of the diurnal cortisol profile have not been established in youth. The reliability of cortisol measures, and the contribution of important covariates and methodological sampling designs on reliability, remains to be evaluated in children and adolescents. The aim of the current project was to address this gap in the current state of knowledge by assessing psychometric properties of the diurnal cortisol profile in youth. There were three main objectives. First, the reliability of the calculated cortisol indices (AUC_{AG}, AUC_I, diurnal slope, AUC_{TG}) were compared to individual measures (morning measure, maximum measure, random measure). Second, the contribution of important covariates (sex, age, perceived stress, pubertal stage, season of sampling, time of awakening, day of week, sleep duration) on the reliability of the cortisol calculated indices and individual measures was evaluated. Third, the reliability of two versus three days of measurement and the number of days of sampling needed to reach optimal reliability (ICC $\geq$ .8) were calculated.

The first hypothesis that cortisol calculated indices would be more reliable than individual measures was not supported. The maximum (peak) individual measure was found to be most reliable, followed by AUC_{TG}, AUC_{AG}, morning measure, AUC_I, diurnal slope, and a random measure. The reliability of the calculated indices was largely consistent with that previously reported in the literature. AUC_{AG} and AUC_{TG} were found to have moderate reliability levels (ICC_{avg} = .58 and .61, respectively), which is consistent with adult (Edwards, Evans, et al. 2001; Edward, Clow et al., 2001) and child findings (Oskis et al., 2009). Previous researchers

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have concluded that the moderate reliability of these calculated indices suggest that the diurnal cortisol profile is a stable individual characteristic. AUC_I was found to have low reliability (ICC_{avg} = .36), which is consistent with adult findings (Edwards et al., 2001); but inconsistent with child findings in a single study that reported moderate two-day reliability (r = .53; Oskis et al., 2009). The observed difference in the AUC_I reliability may be due to day of week sampling (65% versus 90% consecutive weekdays) in the Oskis et al. study versus the current study. Finally, diurnal slope was found to have low reliability (ICC_{avg} = .21), which is inconsistent with both adult (r = .55; Edwards, Evans, et al. 2001) and adolescent studies (r = .73; Oskis et al., 2009). These inconsistencies may be attributable to the mathematical formula used to calculate diurnal slope (standard linear regression versus difference score). More rigorous studies have been found to use standard linear regression (cf. Cohen et al., 2006; Edwards et al., 2003; Sephton, Sapolsky, Kraemer, & Spiegel, 2000). Compared to a difference score, standard linear regression involves the inclusion of more data points, which results in the addition of error variation. These results raise questions about the reliability of frequently used cortisol measures and methodological distinctions (sampling day, calculation formula). Altogether, the maximum (peak), AUC_{AG}, AUC_{TG}, and morning measure appear to have moderate reliability in children and adolescents.

The second hypothesis that controlling for relevant covariates would enhance reliability of the cortisol measures was not supported. The covariates were selected as per recommendations from the MacArthur Network (2000) and included between-subject (sex, age, pubertal stage), dynamic (season of sampling, time of awakening, day of week, sleep duration), and psychological (perceived stress level) covariates. There was a considerable difference in the variance accounted for by the covariates across the seven cortisol measures examined, with none having consistent effects across all measures. On average, the covariates accounted for a small portion of the variance of the calculated cortisol indices and individual measures: sleep duration (3.75%), day of week (2.09%), pubertal status (2.05%), time of awakening (1.66%), age (1.45%),

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and perceived stress (1.14%). The reliability of the calculated indices and individual measures remained largely consistent after controlling for the covariates.

When examining the associations of the covariates with the cortisol measures, sleep duration and time of day were inversely related to cortisol such that children who slept longer or woke up later had lower levels of cortisol. Perceived stress, age, and pubertal status were positively related to cortisol, such that older children, at more advanced pubertal stages, who perceived more stress had higher cortisol levels. As well, cortisol levels measured on weekdays and in girls were higher. These associations are largely consistent with the previous findings in adults and children.

The third hypothesis that the measures derived from three versus two days of sampling would be more reliable was partly supported. The reliability of the cortisol measures was greater with three days of sampling than two days; however, the difference was not meaningful. While compared to one day of sampling, there was marked improvement in the reliability for two and three day. Follow-up analyses indicated that between three days to one month of sampling, depending on the cortisol measure of interest, are needed to reach optimal reliability levels. The maximum sample is adequately captured after three days of sampling. AUC_{AG}, AUC_{TG}, and the morning measure require seven days of sampling; AUC_I requires fourteen days; and diurnal slope and any given sample (no particular time) require thirty days. In a recent study with adults, Hellhammer and colleagues (2007) found that four days of sampling were sufficient to reach optimal reliability for AUC_{AG} (ICC_{AV} = .82) and moderate reliability for AUC_I (ICC_{AV} = .65). Schwartz (2000) suggests that reliable assessment of the AUC_{TG} requires over three to four days, while reliable assessment of the diurnal slope requires more than five days of sampling (MacArthur Network, 2000). No prior studies have examined the number of days needed for reliable cortisol measurement in children or adolescents.

There were several strengths to the present study. Importantly, the present research findings meaningfully contribute to the literature by addressing existing gaps in the current

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literature. While the psychometric properties of cortisol measurement have been previously examined in adults (cf., Edwards, Evans, et al., 2001; Smyth et al., 1997; Stone et al., 2001), the present study is the first psychometric evaluation of the diurnal cortisol profile in children and adolescents. Several measures emerged as moderately reliably measured in a youth population including maximum peak cortisol, AUC_{AG}, AUC_{TG}, and possibly a morning measure sampled across at least 3 days, or preferably more. The present study highlights covariates that affect the reliability of the diurnal cortisol profile. Namely, the findings suggest sleep duration, day of week, pubertal status, and time of awakening are particularly important given their association with the cortisol measures. This is also one of the first studies to demonstrate that children's perceived stress levels are an important covariate when measuring cortisol. Taken together, these findings have implications for methodological designs that should be considered and are further discussed below. A large sample size and a wide age range of children and adolescents (aged 9 to 18 years) permitted evaluation of a possible developmental pattern of the diurnal cortisol profile. The positive association between pubertal stage and total concentration of cortisol released over the day (AUC_{TG}), suggests a possible developmental increase in cortisol levels, which is consistent with previous research (Adam, 2006). Another strength of the current study is that when calculating the indices of the cortisol awakening response (AUC_{AG}, AUC_{I}) and the total concentration of cortisol over the day (AUC_{TG}), the child's actual reported time of sampling was used in the equations, rather than predetermined times not specific to each child. Finally, reliability measures were calculated using intraclass correlations, which are considered to be more psychometrically appropriate because intraclass correlations account for rank order and mean level differences, while Pearson correlations only account for rank order differences.

There were also limitations in the present study. Although three groups were included in the present study, there were differences between groups, including the sampling design protocols that made direct comparisons challenging. In Healthy Heart I, a final bedtime sample was not

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collected and measures of pubertal status and sleep duration were not collected. In the preliminary data checks, the groups appeared to differ on mean levels of AUC_{AG}, AUC_{TG}, and maximum measure after controlling for the covariates. However, upon further examination, sleep duration (which was only assessed during Healthy Heart II) likely accounted for this observed group difference. In fact, when the analyses were redone controlling for sleep duration, the mean differences were no longer observed. Another limitation was the methodological decision to allow participants to choose which day of the week they wanted to collect saliva samples. They always had to choose at least one weekday, but the other day or the other two days could be weekend days. There were observed mean differences between weekday and weekend cortisol levels, which contributed to the observations on the reliability of the cortisol measures. An additional limitation to this study is that the intraclass correlation for the diurnal slope of Cohort 2 could not be calculated because the error variance was the main source of variation for this measure. In the current study, the error variance represents both the methodological error as well as within subject variability. To address this, Pearson correlation coefficients were also calculated to provide proxy estimates. Finally, sampling over a longer period of time would be ideal for a psychometric evaluation (at least 1 week) because it may allow for a better approximation of the diurnal cortisol profile.

This study has implications for methodological designs and sampling protocols with children and adolescents. First involves the decision about the number of days of sampling. While three days will provide moderate reliability of the maximum peak cortisol level and the total amount of cortisol released (AUC_{AG}, AUC_{TG}), at least seven days are needed to obtain reliable measures of the change in cortisol concentration (AUC_I, diurnal slope). This measurement issue has practical implications because while more samples will increase reliability, participant burden, sampling compliance, and financial resources are important to consider. Researchers should carefully consider the cortisol measure of interest and then make methodological decisions to ensure it is reliably measured. In the current study five and six

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samples were collected each day; other researchers have used two (Kelly et al., 2008; Lupien et al., 2001), while still others sample up to eight times per day (Adam et al., 2010; Kiess et al., 1995; Oskis et al., 2009). The number of samples needed differs depending on the cortisol index or measure of interest.

Several covariates were evaluated in the present study and were largely selected based on the adult literature and recommendations from the MacArthur Network (2000). Sleep duration, time of awakening, day of the week, perceived stress, and pubertal stage were the covariates that predominantly contributed to the variance of the measures and were significantly associated with the calculated indices and individual measures. Thus, it is important to record the child's bedtime the night before sampling and time of awakening and to assess perceived stress level and pubertal stage. In adults, heightened and blunted cortisol profiles have been associated with negative health consequences (cf. Chrousos, 2009; Crofford et al., 2004; Matthews, et al., 2006); thus, understanding the role of stress on the diurnal profile as it develops is important. Other measures of stress, such as daily hassles, may be important to consider. Ellenbogen, Hodgins, Walker, Couture, and Adam (2006) found daily hassles were significantly associated with AUC_{AG} (r = .14) in a sample of youth. Finally, the influence of state affect may also be an important to consider. In adults, negative mood states are associated with cortisol levels throughout the day (van Eck, Berkhof, Nicolson, & Sulon, 1996); and may influence the reliability of cortisol measures. Using ecological momentary assessment, cortisol samples coinciding with higher reported stress throughout the day were higher compared to samples with lower reported stress (Smyth, et al., 1998). Finally, most children and adolescents who participated were compliant with collecting saliva samples (96.8%) and their parents initialed each collected sample time as an indicator of compliance. Similar to other studies, children and adolescents were able to comply with several saliva samples collected throughout the day (including school days), for several days.

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The results of the present study suggest that the diurnal cortisol profile can be moderately reliably assessed in children and adolescents. The role of covariates and number of days of sampling on the reliability of cortisol measures was underscored and has implications for methodological designs of future research. Maximum peak cortisol levels and the total amount of cortisol released can be reliably assessed using three days of measurement, but at least seven days are needed to obtain reliable measures of the change in cortisol concentration. Important covariates include day of week, sleep duration, pubertal status, and time of awakening; information regarding these should be recorded during data collection. When diurnal profiles are examined, there are several sources of variation to consider, between-subject, within-subject, and error. Controlling for sources of variation (e.g., between-subject covariates, dynamic covariates) maximizes reliability so that the diurnal cortisol profile reflects a stable individual difference.

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Figure Captions

Figure 1. The indices of the diurnal profile of cortisol; AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_I, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile

Figure 2. Participants included in the study.

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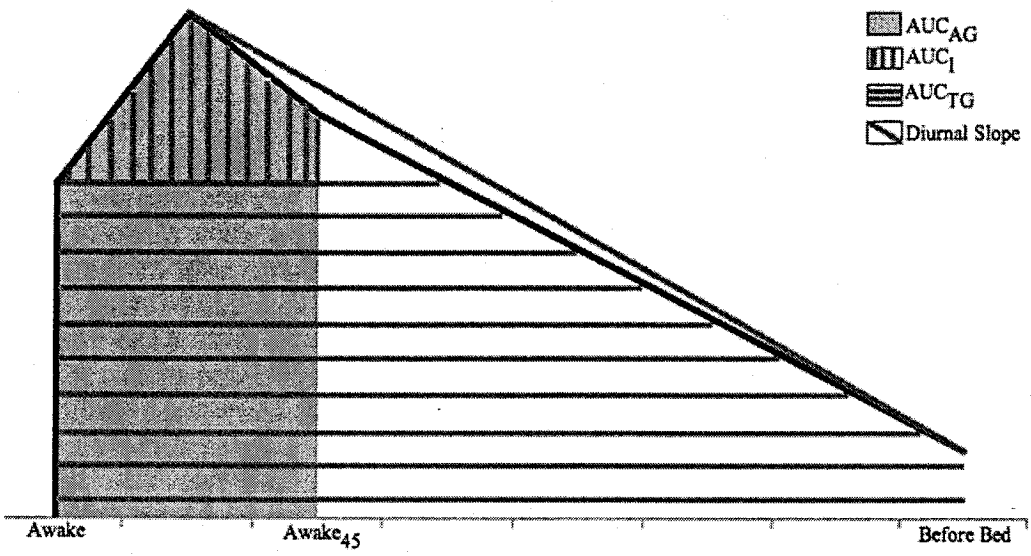

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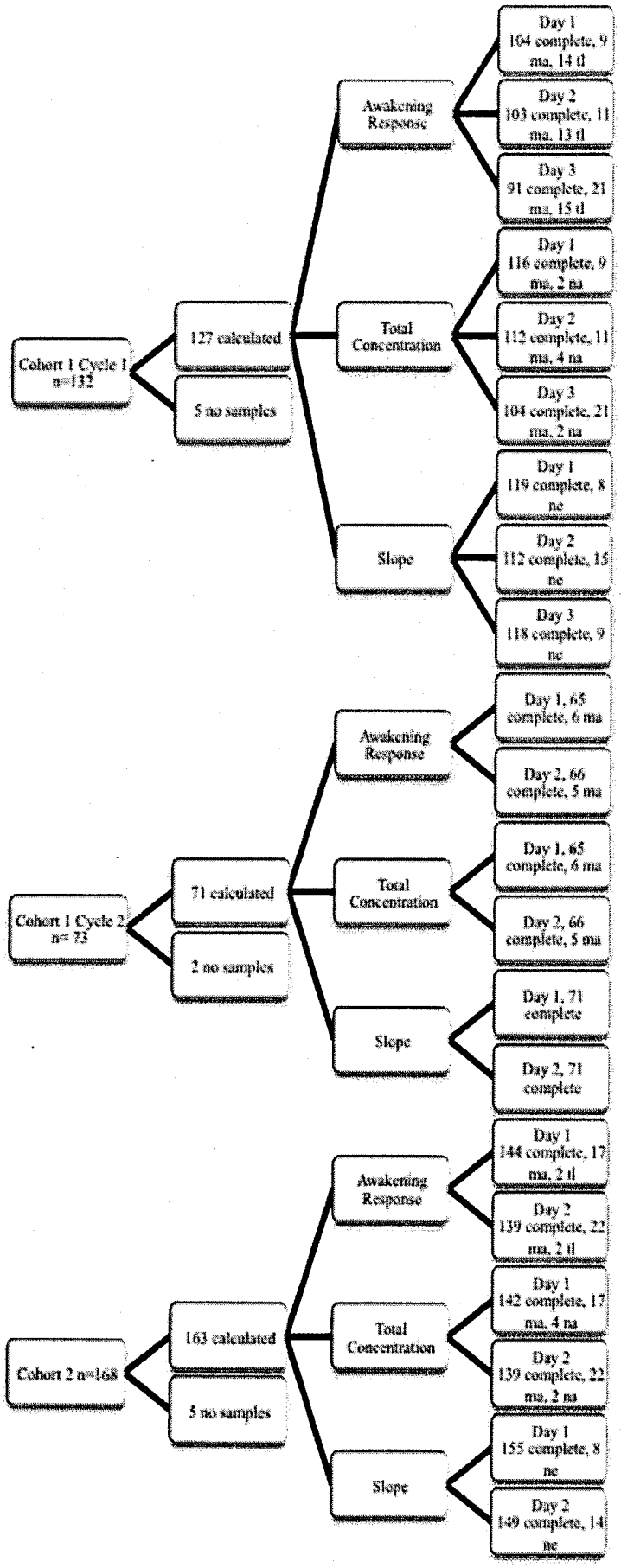
Healthy Heart I (3 days, 5 samples/ day)

Healthy Heart I (2 days, 6 samples/ day)



ma – missing awake$_0$ samples
tl - exceed time limit
na - no afternoon samples
ne- not enough samples

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Table 1

Descriptive Statistics of Imputed and Unimputed Calculated Indices

	Healthy Heart I						Healthy Heart II											
	Cohort 1 Cycle 1 (n = 127)						Cohort 1 Cycle 2 (n = 71)						Cohort 2 (n = 163)					
	Unimputed			Imputed			Unimputed			Imputed			Unimputed			Imputed		
Indices	*M*	*SD*	Missing	*M*	*SD*	*p*	*M*	*SD*	Missing	*M*	*SD*	*p*	*M*	*SD*	Missing	*M*	*SD*	*p*
AUC_{AG}	2.45	0.74	22%	2.44	0.67	0.93	2.86	1.06	14%	2.86	1.00	0.95	2.58	0.76	13%	2.60	0.74	0.83
AUC_{I}	0.18	0.56	22%	0.17	0.51	0.87	0.33	0.78	14%	0.31	0.74	0.82	0.28	0.68	13%	0.28	0.66	0.97
AUC_{TG}	21.41	7.16	23%	21.4	6.57	0.92	27.22	8.96	22%	27.3	8.97	0.54	27.31	8.22	9%	26.8	7.97	0.61
Slope	-.82	0.27	9%	-.82	0.26	0.91	-.85	0.20	7%	-.85	0.19	0.94	-.86	0.16	5%	-.86	0.16	0.99

Note. The mean values are presented in nmol/L. Group differences were examined with one-way ANOVA. AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_{I}, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile; Missing, percent of missing values.

Table 2

Between Group Differences on the Demographic Covariates

	Healthy Heart I		Healthy Heart II				
	Cohort 1 Cycle 1 (n = 127)		Cohort 1 Cycle 2 (n = 71)		Cohort 2 (n = 163)		
Covariates	*M*	*SD*	*M*	*SD*	*M*	*SD*	F
Sex, female (%)	(44)		(51)		(44)		0.35
Age	12.50	1.79	13.76	1.90	12.23	1.92	16.92
Perceived stress	13.94	6.62	15.74	6.98	14.47	6.75	3.64
Pubertal stage	n/a	n/a	3.37	1.44	2.83	1.52	
Season of sampling, fall/winter (%)	(39.37)		(36.60)		(50.30)		3.69
Time of awakening	7:56	1:32	7:31	1:26	7:36	1:28	5.76
Day of the week, weekday (%)	(65.90)		(97.30)		(89.60)		44.91
Sleep duration	n/a	n/a	8:35	1:18	9:10	1:29	

Note. Groups were compared using a one-way ANOVA.

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Table 3

Between Group Differences on the Calculated Indices and Individual Measures

	Healthy Heart I		Healthy Heart II				
	Cohort 1 Cycle 1 (n = 127)		Cohort 1 Cycle 2 (n = 71)		Cohort 2 (n = 163)		
Measures	*M*	*SD*	*M*	*SD*	*M*	*SD*	F
			Calculated indices				
AUC_{AG}	2.44	.53	2.60	.61	2.86	.83	8.51
AUC_I	.17	.33	.28	.51	.31	.59	1.98
Slope	- .82	.17	- .86	.11	- .85	.13	2.61
AUC_{TG}	21.45	5.13	26.80	6.49	27.39	7.71	21.04
			Individual measures				
Morning	3.10	.82	3.33	.97	3.40	.88	1.77
Maximum	3.58	0.92	3.95	1.05	4.10	1.11	4.01
Random	2.46	0.76	2.42	0.96	2.60	1.10	1.25

Note. Groups were compared with one-way ANOVA. AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_I, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile.

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Table 4

Within Group Differences on the Calculated Indices and Individual Measures

	Healthy Heart I							Healthy Heart II							
	Cohort 1 Cycle 1 (n = 127)							Cohort 1 Cycle 2 (n = 71)					Cohort 2 (n = 163)				
	Day One		Day Two		Day Three			Day One		Day Two			Day One		Day Two		
Measures	*M*	*SD*	*M*	*SD*	*M*	*SD*	*p*	*M*	*SD*	*M*	*SD*	*p*	*M*	*SD*	*M*	*SD*	*p*
Calculated Indices																	
AUC_{AG}	2.45	0.76	2.46	0.65	2.42	0.62	0.86	2.64	0.73	2.56	0.76	0.48	2.93	0.88	2.79	1.1	0.21
AUC_{I}	0.15	0.56	0.2	0.48	0.16	0.48	0.64	0.34	0.70	0.22	0.61	0.25	0.39	0.76	0.23	0.72	0.02
Slope	- .81	0.23	- .82	0.22	- .81	0.31	0.90	- .88	0.10	- .84	0.20	0.19	- .84	0.24	- .87	0.12	0.19
AUC_{TG}	21.16	6.15	22.18	6.41	22.02	7.13	0.49	27.67	8.12	25.94	7.78	0.20	27.89	9.24	26.82	8.66	0.23
Individual Measures																	
Morning	3.14	1.10	3.19	1.02	3.07	1.06	0.68	3.42	1.19	3.20	1.11	0.28	3.58	1.21	3.24	1.10	0.01
Maximum	3.57	1.15	3.71	0.98	3.50	1.08	0.27	4.07	1.20	3.79	1.14	0.16	4.30	1.18	3.90	1.33	0.005
Random	2.39	1.17	2.52	1.16	2.49	1.24	0.68	2.21	1.33	2.64	1.29	0.06	2.87	1.54	2.38	1.44	0.004

Note. Within group differences were examined with one-way ANOVA. AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_{I}, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile.

Table 5

Adjusted and Unadjusted Intraclass Correlations for Calculated Indices and Individual Measures

	Healthy Heart I			Healthy Heart II					
	Cohort 1 Cycle 1 (n = 127)			Cohort 1 Cycle 2 (n = 71)			Cohort 2 (n = 163)		
Measures	ICC_S	ICC_{AV}	*m*	ICC_S	ICC_{AV}	*m*	ICC_S	ICC_{AV}	*m*
Unadjusted Model									
Calculated									
AUC_{AG}	.41	.67	6	.34	.51	8	.38	.55	6
AUC_I	.14	.34	24	.20	.33	16	.26	.41	12
Slope	.15	.34	23	.04	.08	86	.0[a]	n/a	n/a
AUC_{TG}	.41	.68	6	.34	.51	8	.47	.64	4
Individual									
Morning	.32	.59	8	.34	.51	8	.10	.18	37
Maximum	.54	.78	3	.54	.70	3	.53	.70	3
Random	.11	.26	34	.06	.11	65	.05	.09	82
Adjusted Model									
Calculated									
AUC_{AG}	.43	.70	5	.25	.40	12	.40	.57	6
AUC_I	.16	.37	20	.21	.35	15	.21	.34	15
Slope	.14	.32	26	.07	.13	53	.0[a]	n/a	n/a
AUC_{TG}	.49	.75	4	.35	.51	8	.44	.61	5
Individual									
Morning	.31	.57	9	.27	.42	11	.11	.20	32

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Maximum	.53	.77	4	.51	.68	4	.56	.72	3
Random	.07	.17	57	.02	.04	191	.03	.06	118

Note. Model adjusted for all covariates included in the study (sex, age, perceived stress, pubertal stage, season, time of awakening, day of week, and duration of sleep). Dashes indicate that the measure could not be calculated. AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_{I}, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile; ICC_S, single day reliability; ICC_{AV}, average reliability; *m,* number of days sampling need to reach an ICC = .8.

[a]ICC could not be calculated because the error coefficient was the major source of variation.

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Table 6

Pearson Correlations and Partial Correlations Across Days for Calculated Cortisol Indices

	Healthy Heart I								Healthy Heart II							
	Cohort 1 Cycle 1 (n = 127)								Cohort 1 Cycle 2 (n = 71)				Cohort 2 (n = 163)			
	AUC_{AG}		AUC_I		Slope		AUC_{TG}		AUC_{AG}	AUC_I	Slope	AUC_{TG}	AUC_{AG}	AUC_I	Slope	AUC_{TG}
	Day 2	Day 3	Day 2	Day 3	Day 2	Day 3	Day 2	Day 3	Day 2	Day 2	Day 2	Day 2	Day 2	Day 2	Day 2	Day 2
	Pearson Correlations															
Day 1	.44^	.35^	.17	.07	.03	.35^	.46^	.53^	.32″	.21	.05	33″	.40^	.22″	-.01	.48^
Day 2	-	.44^	-	.21*	-	.03	-	.27″	-	-	-	-	-	-	-	-
	Partial Correlations															
Day 1	.44^	.34^	.17	.07	.02	.31″	.47^	.54^	.26*	.15	.04	.24	.40^	.26″	-.02	.50^
Day 2	-	.44^	-	.25″	-	.02	-	.33^	-	-	-	-	-	-	-	-

Note. Partial correlations included all covariates (sex, age, perceived stress, pubertal stage, season, time of awakening, day of week, and duration of sleep). AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_I, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile

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Table 7

Pearson Correlations and Partial Correlations Across Days for Individual Cortisol Measures

	Healthy Heart I						Healthy Heart II					
	Cohort 1 Cycle 1 (n = 127)						Cohort 1 Cycle 2 (n = 71)			Cohort 2 (n = 163)		
	Morning		Maximum		Random		Morning	Maximum	Random	Morning	Maximum	Random
	Day 2	Day 3	Day 2	Day 3	Day 2	Day 3	Day 2	Day 2	Day 2	Day 2	Day 2	Day 2
	Pearson Correlations											
Day 1	.23*	.32″	.56^	.51^	.15	.03	.35″	.56^	.07	.11	.57^	.08
Day 2	-	.32″	-	.47^	-	.08	-	-	-	-	-	-
	Partial Correlations											
Day 1	.26″	.30″	.53^	.53^	.13	.03	.22	.53^	.07	.10	.59^	.07
Day2	-	.30″	-	.47^	-	.02	-	-	-	-	-	-

Note. Partial correlation included all covariates in the study (sex, age, perceived stress, pubertal stage, season, time of awakening, day of week, and duration of sleep).

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Table 8

Percent of Variance of Calculated Indices Accounted by Covariates

	Healthy Heart I				Healthy Heart II							
	Cohort 1 Cycle 1 (n = 127)				Cohort 1 Cycle 2 (n = 71)				Cohort 2 (n = 163)			
Covariates	AUC_{AG}	AUC_I	Slope	AUC_{TG}	AUC_{AG}	AUC_I	Slope	AUC_{TG}	AUC_{AG}	AUC_I	Slope	AUC_{TG}
Between Subjects Covariates												
Sex	0.02	0.00	0.00	0.82	0.00	1.48	0.00	0.00	0.00	0.05	0.00	0.00
Age	0.54	0.43	0.01	0.00	5.45	1.87	0.00	5.73	0.00	3.40	0.00	1.00
Perceived Stress	0.48	0.00	0.00	2.78	9.14	0.00	0.22	3.93	0.00	0.67	0.00	4.35
Puberty	n/a	n/a	n/a	n/a	0.87	1.22	0.00	10.24	0.00	4.09	0.00	0.00
Within Subject Covariates												
Season	0.00	0.00	0.01	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00
Time Awake	0.59	1.50	2.49	3.06	0.13	0.50	0.00	13.7	1.39	0.00	0.27	1.89
Day of Week	4.00	4.73	0.25	2.24	0.00	4.03	0.00	1.86	0.65	0.02	0.00	0.71
Sleep Duration	n/a	n/a	n/a	n/a	6.84	1.57	0.00	14.26	0.83	9.62	0.00	10.8

Note. AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_I, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile.

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Table 9

Percent of Variance of Individual Measures Accounted by Covariates

	Healthy Heart I			Healthy Heart II					
	Cohort 1 Cycle 1 (n = 127)			Cohort 1 Cycle 2 (n = 71)			Cohort 2 (n = 163)		
Covariates	Morning	Maximum	Random	Morning	Maximum	Random	Morning	Maximum	Random
Between Subjects Covariates									
Sex	2.49	2.65	2.77	0.00	1.31	0.00	0.00	0.00	0.00
Age	0.35	0.10	0.00	8.87	3.02	0.00	0.00	0.03	0.00
Perceived Stress	0.00	0.00	0.00	0.13	1.83	0.00	0.00	0.00	2.34
Puberty	n/a	n/a	n/a	0.00	0.00	0.00	0.00	0.00	0.00
Within Subjects Covariates									
Season	0.00	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00
Time Awake	0.81	0.13	0.00	2.64	2.64	0.69	0.00	1.21	2.58
Day of Week	5.08	5.40	4.00	1.87	1.93	1.28	0.47	1.54	2.08
Sleep Duration	n/a	n/a	n/a	3.71	4.24	0.00	0.37	3.81	0.01

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Table 10

Standardized Regression Coefficients (β') for Calculated Cortisol Indices

	Healthy Heart I				Healthy Heart II							
	Cohort 1 Cycle 1 (n = 127)				Cohort 1 Cycle 2 (n = 71)				Cohort 2 (n = 163)			
Covariates	AUC_{AG}	AUC_I	Slope	AUC_{TG}	AUC_{AG}	AUC_I	Slope	AUC_{TG}	AUC_{AG}	AUC_I	Slope	AUC_{TG}
Between Subjects Covariates												
Sex	.07	.03	.03	.11	.09	.15	-.02	.003	.04	.07	.00	.07
Age	.10	.09	-.06	.04	.25″	.16	.05	.26″	.02	.19^	-.03	.12
Perceived Stress	.14″	-.03	.12*	.21^	.30^	.02	.14	.23″	.12*	.08	.08	.15*
Puberty	n/a	n/a	n/a	n/a	.21*	-.001	.05	.31^	.04	.13*	.02	.13*
Within Subject Covariates												
Season	-.02	-.03	.06	.05	-.03	-.10	.01	-.06	-.03	-.02	-.04	.01
Time Awake	-.12″	-.13″	.17″	-.22^	-.10	-.11	.03	-.38^	-.13*	-.01	-.06	-.15″
Day of Week	-.20^	-.22^	.07	-.16″	-.06	-.22″	.05	-.16	-.10	-.06	-.03	-.10*
Sleep Duration	n/a	n/a	n/a	n/a	-.29″	-.19	.03	-.40^	-.11	-.32^	.82	-.34^

Note. AUC_{AG}, area under the curve relative to ground for the awakening response; AUC_I, area under the curve relative to increase ($awake_0$); AUC_{TG}, area under the curve relative to ground for the diurnal profile; * $p < .05$; " $p < .01$; ^ $p < .001$.

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Table 11

Standardized Regression Coefficients (β') for Individual Cortisol Measures

	Healthy Heart I			Healthy Heart II					
	Cohort 1 Cycle 1 (n = 127)			Cohort 1 Cycle 2 (n = 71)			Cohort 2 (n = 163)		
Covariates	Morning	Maximum	Random	Morning	Maximum	Random	Morning	Maximum	Random
				Between Subjects Covariates					
Sex	.17″	.17″	.18″	.09	.15	-.01	.03	.04	.06
Age	.09	.08	-.04	.31^	.20*	.05	-.03	.07	.02
Perceived Stress	.09	.13	.09	.12	.14	.005	.07	.12	.02
Puberty	n/a	n/a	n/a	.13	.07	.04	-.09	.09	.03
				Within Subjects Covariates					
Season	.01	-.01	-.003	-.09	-.09	.12	.01	-.04	.03
Time Awake	-.12*	-.10*	-.02	-.18*	-.18*	-.12	-.04	-.13*	-.17″
Day of Week	-.17″	-.19^	-.12*	-.16	-.16	-.14	-.09	-.14*	-.16″
Sleep Duration	n/a	n/a	n/a	-.23″	-.24″	-.09	-.08	-.21^	.07

Note. * $p < .05$; ″ $p < .01$; ^ $p < .001$.