

Title: Evidence of a unique and common genetic etiology between the CAR
and the remaining part of the diurnal cycle: a study of 14 year-old twins

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Abstract

Introduction: By and large, studies have reported moderate contributions of genetic factors to cortisol secreted in the early morning and even smaller estimates later in the day. In contrast, the cortisol awakening response (CAR) has shown much stronger heritability estimates, which prompted the hypothesis that the etiology of cortisol secretion may vary according to the time of day. A direct test of this possibility has, however, not yet been performed. Objective: To describe the specific and common etiology of the CAR, awakening level and cortisol change from morning to evening in an age-homogenous sample of twin adolescents. Methods: A total of 592 participants of the Québec Newborn Twin Study, a population-based 1995-1998 cohort of families with twins in Canada, have collected saliva at awakening, 30 minutes later, at the end of afternoon and in the evening over four collection days. Results: Multivariate Cholesky model showed both specific and common sources of variance between the CAR, awakening and cortisol diurnal change. The CAR had the strongest heritability estimates, which, for the most part, did not overlap with the other indicators. Conversely, similar magnitudes of genetic and environmental contributions were detected at awakening and for diurnal change, which partially overlapped. Conclusion: Our study unraveled differences between the latent etiologies of the CAR and the rest of the diurnal cycle, which may contribute to identify regulatory genes and environments and detangle how these indicators each relate to physical and mental health.

Keywords: HPA axis; Cortisol; Diurnal rhythm; Genes; Twin studies; Cortisol awakening response.

Word count: 238

1. Introduction

Cortisol, a glucocorticoid hormone secreted by the hypothalamus-pituitary-adrenal (HPA) axis, is involved in the regulation of many systems critical for well-being. Several cognitive and emotional functions also depend on it, such as attention, memory and regulating behavioral activation and inhibition. Cortisol typically follows a time-dependent pattern of secretion over the day, with higher levels normally present shortly after awakening followed by a rapid and then progressive decrease until a minimum is reached around midnight. This circadian rhythm emerges as a result of several ACTH driven pulses of cortisol, which are themselves under the control of several sources of influence coordinated by the central nervous system (Vis et al., 2012). Diurnal cortisol secretion receives a great deal of attention because of its proposed impact on a wide range of physical, psychological and behavioral difficulties (Fries et al., 2005; Susman, 2006). Central to this hypothesis is the great disparity in basal secretion between individuals of all ages, including in the pattern of *change* of cortisol during the day (Smyth et al., 1997).

Many factors have been proposed to affect the circadian rhythm. These factors could, ultimately, be grouped into those present at the individual level and those emerging from the environment. Diurnal cortisol secretion has been associated with personal traits, such as optimism (Jobin et al., 2014) and fearfulness (Gunnar et al., 2009). Diurnal secretion has also been linked to early adverse experiences, such as maltreatment and neglect (Cicchetti et al., 2010; Fries et al., 2008; Tarullo and Gunnar, 2006). Similar findings are also emerging in regards to peer victimization in adolescence (Ouellet-Morin et al., 2011a; Ouellet-Morin et al., 2011b; Vaillancourt et al., 2008), reflecting the sensitive nature of HPA axis activity to changing social environments. It remains unclear, however, to which extent these associations reflect acquired and/or inherited influences, as some experiences may partly arise as a function of the individuals' genetic makeup (Jaffee and Price, 2007). Delineating the etiology of cortisol circadian rhythm represents a key building block to ascertain its impact on health.

Most twin and parent-offspring studies conducted thus far have shown that cortisol secreted in the early hours of the morning is moderately inherited whereas lower estimates are detected later on (Bartels et al., 2003a; Franz et al., 2010; Kupper et al., 2005; Wüst, 2000). For example, Kupper & al. (2005) have

reported moderate heritability estimates (33 and 34%) at awakening and 30 minutes later in adulthood, whereas non-significant genetic contributions were detected subsequently (Kupper et al., 2005). A similar pattern was found at an earlier age (Bartels et al., 2003a; Ouellet-Morin et al., 2009; Schreiber et al., 2006). Gustafsson et al. (2011) have shown moderate-to-high heritability estimates at awakening (28%) and in the cortisol response to awakening (CAR; 60%), but a low genetic contribution in the evening (8%). Based on these findings, it is proposed that cortisol secreted at awakening and thereafter may have a distinct etiology. Such possibility may arise if distinct genetic and environmental factors are involved (i.e., qualitative differences) and/or because the magnitude of these contributions differs as the day goes by (i.e., quantitative differences) (Bartels et al., 2003a; Edwards et al., 2001; Gustafsson et al., 2011; Kupper et al., 2005; Schmidt-Reinwald et al., 1999; Wilhelm et al., 2007; Wüst, 2000). A direct test of this possibility requires performing multivariate genetic models estimating simultaneously the genetic and environmental contributions. To the best of our knowledge, no study has yet performed this test.

Our understanding of the etiology of diurnal cortisol secretion may also be limited by the fact that few studies have been conducted in youth. This is surprising on many accounts. First, basal cortisol secretion undergoes important changes during the first two decades of life, with decreasing levels noted from toddlerhood to mid-childhood followed by an opposite trend (Adam, 2006; Gunnar et al., 2009; Shirtcliff et al., 2011). These maturational changes may depend on changing social environments (e.g., daycare to formal schooling), neuroendocrine factors (e.g., sex hormones) and brain structures and functioning (e.g., prefrontal cortex; (Gunnar and Vazquez, 2006; Lupien et al., 2009). Second, the dearth of studies describing the genetic and environmental etiology of diurnal cortisol secretion in adolescence is at odds with the documented increase of mental health problems during this time. Third, the genetic and environmental estimates derived from the adult samples may not be generalized to younger samples, because older twin pairs may face greater disparity in their daily routines. The use of age-heterogeneous samples to describe the genetic and environmental etiology of cortisol secretion may help increase the precision of these estimates.

Obtaining precise etiology estimates of the cortisol circadian rhythm also depends on our capacity to summarize all the available information according to indicators that depart from single point analyses. Statistical approaches such as linear growth curve models (LGCM) are increasingly used, because they simultaneously estimate the morning cortisol level and the changes occurring thereafter. In addition to maximizing statistical power, they easily accommodate unequal observations across individuals (missing data) and control for time-varying covariates (e.g., time of collection). Combined with confirmatory factorial analyses, LGCM contribute to tease apart “trait-like” from “situation-specific” variation.

The present study aims to describe the genetic and environmental etiology of diurnal cortisol secretion in mid-adolescence. More specifically, we tested whether it is possible to derive stable patterns of diurnal cortisol secretion from samples collected across multiple days. We then estimated the genetic and environmental contributions of individual differences in the CAR and cortisol change from morning to evening and examined whether these factors are shared or are rather specific to each indicator.

2. Methods

2.1 Sample

Participants were part of the Québec Newborn Twin Study, a sample of twins recruited between 1995 and 1998 in the greater Montréal area. A total of 989 families with twins were contacted after the twins' birth, of which 672 agreed to participate (68.0%). Twins were first seen when they were 5 months of age and then prospectively assessed for a variety of child and family characteristics. The present study focuses on data collected when the twins were 14 years-old [mean (standard deviation or SD), 14.00 (.28)]. Valid data was available for 592 twins [280 monozygotic (MZ), 204 same-sex dizygotic (DZ) and 108 mixed-sex DZ twins] from whom most (74%) had collected saliva at each of four collection days. The families were comparable to another sample of single births in the province of Québec. At the time of their children's birth, 95% of parents lived together, 44% of the twins were the firstborn children, 66% of mothers and 60% of fathers were between 25 and 34 years old, 17% of mothers and 14% of fathers had not finished high school, 28% of mothers and 27% of fathers held a university degree, 83% of the parents were employed, 10% of the families received social welfare or unemployment insurance, and 30% of

families had an annual income of < \$30,000. Most families were of European descent (87%), 3% were of African descent, 3% were of Asian descent, and 1% were Native North Americans. Zygosity was assessed by using 8-10 highly polymorphous genetic markers. Twins were diagnosed as MZ when concordant for every genetic marker. When genetic material was insufficient or unavailable due to parental refusal (43% of cases), zygosity was determined based on physical resemblance questionnaires at 18 months and again at age 9 (Spitz et al., 1996). The comparison of both methods in a subsample of 237 same-sex pairs revealed a 94% correspondence rate.

2.2 Procedure

Letters explaining the objectives of the study were sent to the families, followed by a home visit. After informed consent from the parents and assent from the participants were obtained, the research assistants explained the collection protocol, which consisted in sampling saliva at four time points during the day (at awakening, 30 minutes later, late in the afternoon and bedtime) on four collection days (Tuesdays and Thursdays on two consecutive weeks). The research assistants made sure that the participants (and their parents) were familiar with the material. The families were visited a second time to gather the saliva tubes. All instruments and study procedures were approved by the Ethics Committee of the Ste-Justine Hospital Research Center.

2.3 Measures

2.3.1 *Saliva collection and cortisol analysis*

Participants were provided with saliva tubes (Sarstedt©), diaries to report collection times by the twins (supervised by their parents) and instructions for collection. Saliva samples were first placed in the participants' refrigerator during data collection days and then stored in freezers at -20°C once returned to the laboratory until cortisol determination using a high sensitivity enzyme immune assay kit (Salimetrics® State College, PA, Catalogue No. 1-3102). Frozen samples were brought to room temperature to be centrifuged at 15000xg (3000rpm) for 15 minutes and all analyzed in one batch. The range of detection for this assay is between 0.012-3ug/dL (.33-82.76 nmol/L). Of the possible 9472 saliva samples from 592 participants, 2037 (21.5%) were missing due to participants lapses, insufficient saliva collection or technical

problems (on average, 25.2% were missing at awakening, 17.7% at +30min, 8.7% at the end of afternoon and 25.9% in the evening). We identified 75 cortisol samples (1.0%) with a value greater than 3 times the SD above the mean of their respective sampling time and replaced them by the last value within the 3SD. Participants were considered “compliant” if their awakening and +30min samples were separated from at least 20 min and less than 40 min, the awakening collection was completed within the first 15 min following awakening and not distinct between the twins (\leq eight min). A total 8.61% of the samples were discarded due to noncompliance. Cortisol values were converted in nmol/L (to convert ug/dL to nmol/L, multiply by 27.588) and naturally log transformed prior to data analyses.

2.4 Statistical analyses

2.4.1 *Preliminary analyses*

Our preliminary analyses were conducted in four steps. First, we derived an indicator of CAR for each day of saliva collection by subtracting the awakening level from the one collected 30 minutes later. Second, we performed growth curve analyses using mixed modeling for longitudinal data to capture the cortisol diurnal rhythm at each collection day by estimating the mean level of cortisol at awakening (Intercept) and the change that took place thereafter (Slope). To this end, we chose an unspecified curve model to allow for slightly varying assessment times between individuals and to obtain an optimal estimate of change without imposing any particular shape of change across individuals (Duncan et al., 1997). The model contained both fixed and random estimates, corresponding to the parameters’ mean and variance between individuals. Models were fitted in Mplus Version 6.11 using maximum likelihood estimation and the COMPLEX option adjusting standard error estimates to correct for the non-independence of observations. Third, we tested whether the estimates of Intercepts, Slopes and CAR were affected by a wide range of potential confounders (e.g., sexual maturity, medications and health-related characteristics such as cold, fever, allergies). Only a few (i.e., sex, awakening time, hours of sleep, sleeping problems, exercises and alcohol or drug consumption) were uniquely associated with at least one indicator and were statistically controlled for in the subsequent analyses. Forth, the four intercept estimates (one for each collection day) were included in a confirmatory factorial analysis (CFA) to

examine whether a more stable indicator could be derived and thus be free from situational-specific variation. Similar CFAs were conducted for the Slopes and CAR estimates. Supplementary Figure 1 presents an overall representation of these analyses.

2.4.2 Genetic modeling

The twin design makes it possible to assess the relative role of latent genetic and environmental factors associated with a measured phenotype (e.g., cortisol; Neale and Cardon, 1992). Specifically, by comparing within-pair correlations for MZ twins, who share 100% of their genes, to same-sex DZ twins, who share ~50% of their genes, sources of variability of a phenotype can be estimated in terms of additive (A) and non-additive (D) genetic factors as well as shared (C) and non-shared environmental factors (E). Additive genetic effects are suggested when the MZ intra-pair (i.e., intra-class) correlation is up to twice the same-sex DZ correlation. Larger differences may indicate a dominance genetic effect, which emerges, for instance, when a dominant gene inherited from one parent has a stronger impact on the phenotype than a recessive gene inherited from the other parent. Dominant genetic mechanisms may also arise from gene-gene interactions (i.e., epistasis). The relative effect of shared environmental factors can be approximated by subtracting the MZ intra-pair correlation from twice the DZ intra-pair correlation and refers to environments affecting twins within a pair in a similar way. Non-shared environmental effects are approximated by the extent to which the MZ correlation is lower than 1 and comprises environments that differently impact the twins of a same pair. Any measurement error is also captured in the E variance component.

Structural equation modeling (SEM) using a maximum likelihood fit function enables a more precise estimation of the genetic and environmental parameters that also includes the confidence intervals (CIs) and the statistical significance of the estimated parameters (Neale & Cardon, 1992). To this end, a two-group model is fitted to the data where (1) the latent genetic correlations between the two twins of a same pair are fixed to 1.0 for MZ twins and to 0.5 (to estimate latent additive genetic effects) or to 0.25 (to estimate latent dominance genetic effects) for DZ twins; (2) the latent shared environmental correlations

between the two twins of a same pairs are fixed to 1.0 for both MZ twins and DZ twins and; (3) the non-shared environmental correlations between the two twins of a same pair are fixed to zero for MZ twins and DZ twins. The estimated coefficients a , d , c , and e provide information about the relative contribution of the latent factors A , D , C , and E to the total variance of each phenotype P , with the variance of $P = a^2 + d^2 + c^2 + e^2$. Notably, it is not possible to estimate c and d in the same model with data from twin pairs reared together because the estimation of c and d both rely on the same information (i.e., difference between the MZ and DZ intra-pair twin correlations). As such, the observed variances and covariances provide sufficient information to model either an ACE model or an ADE model, but not both (Neale & Cardon, 1992). We therefore tested separate ACE and ADE models for each of the three phenotypes in preliminary univariate analyses. Mixed-sex twin pairs, who are not essential to genetic modeling, were excluded from the genetic analyses, as their pattern of intra-pair correlations significantly differed from that found for same-sex twin pairs. Model fit was assessed based on the χ^2 -statistic, the Akaike information criterion (AIC), the Bayesian information criterion (BIC), the comparative fit index (CFI), and the root mean square error of approximation (RMSEA). Non-significant χ^2 , lower AIC and BIC, CFI of $\geq .9$, and RMSEA $< .08$ indicate good model fit and parsimony. Using nested χ^2 -difference tests, the full ACE (or ADE) model was compared to nested models, which made it possible to determine the best fitting and more parsimonious models based on the fit criteria, the significance and estimated values of the a , d , c , and e parameters and the significance of the nested χ^2 -difference tests.

2.4.3 *Multivariate Genetic Models*

Using the Mplus software package, univariate models were fitted separately for each of the cortisol indicator in preliminary analyses (available upon request). The results were used to guide the selection of the models considered in the multivariate analyses used to examine the sources of covariation between the CAR, intercept and slope. To this end, a multivariate Cholesky model (see Figure 1) estimated the covariance between the cortisol indicators partitioned into: (1) a “common” additive genetic factor A_{CAR} and a “common” non-shared environmental factor E_{CAR} that not only influence the CAR (denoted by the subscript CAR) but also the awakening level (intercept, denoted by the subscript I) and subsequent

diurnal change (slope, denoted by the subscript s); (2) a “common” genetic factor A_I , a “common” shared-environmental factor C_I and a “common” non-shared environmental factor E_I that influence both awakening level and diurnal change; (3) a “unique” genetic factor A_S , a “unique” shared environmental factor C_S and a “unique” non-shared environmental factor E_S that are specific to cortisol diurnal change and; (4) a “unique” genetic dominance factor D_{CAR} for the CAR as it was the only cortisol indicator for which the ADE model showed a better a fit than the ACE model in univariate models.

Coefficients a_{CAR} , d_{CAR} and e_{CAR} indicate the effect of additive, dominance genetic and non-shared environmental factors on CAR. Coefficients a_{CAR-I} and e_{CAR-I} as well as a_{CAR-S} and e_{CAR-S} indicate to what extent genetic or non-shared environmental factors that influence CAR also explain cortisol secreted at awakening and in the diurnal change (slope), respectively. Coefficients a_I , c_I and e_I indicate to what extent awakening cortisol is affected by genetic and shared or non-shared environmental factors that are not associated with CAR. Coefficients a_{I-S} , c_{I-S} and e_{I-S} indicate to what extent genetic, shared or non-shared environments associated with awakening cortisol are also related to diurnal change. Finally, coefficients a_S , c_S and e_S indicate to what extent diurnal change is affected by genetic and environmental factors that are not associated with CAR or awakening levels.

3. Results

3.1 *Deriving stable indicators of daytime cortisol secretion*

Consistent with the expected diurnal variation in cortisol from awakening to the evening, Table 1 shows higher cortisol levels 30 minutes following the awakening and decreasing levels from that point on. This suggests that grouping these data may be indicated, as few differences are noted across the collection days. Table 1 also reports different number of observations at each time point. This can be partly explained by the fact that some participants did not collect saliva each day, although 74% of the participants did it every day. A varying number of observations may also be the result of incomplete saliva samples. We observed 3.76%, 4.12%, 3.44% and 4.53% missing data points within each collection day from the first day to the last, respectively. The growth curve models easily accommodate unequal observations across individuals.

As illustrated in Supplementary Figure 1, we summarized the information available for each collection day by estimating the patterns of diurnal cortisol change using growth curve analyses. Table 2 presents the fixed and random effect estimates of the mean awakening levels (intercept) and diurnal change (slope). We excluded the second sample (30 minutes following awakening) from these analyses, as estimation of spline models would require more than the available four time points. Moreover, the rapid rise in cortisol is specifically captured by the CAR, which was also considered in the genetic analyses. The estimates showed that a significant decrease in cortisol levels took place from awakening to evening each day. Moreover, the random parameters suggested that the intercepts and slopes each had sufficient heterogeneity (i.e., variance), allowing further analyses. Finally, the results indicate that participants with higher awakening levels showed a steeper cortisol decrease thereafter. CAR values were also highly similar from one day to the other (3.66, 3.45, 3.42 and 2.92). The CFA confirmed that the estimates derived at each collection day could be grouped into single factors: the CAR, intercept and slope.

3.2 Is the CAR's etiology distinct from the remaining part of the diurnal circadian rhythm?

Intra-pair correlations performed separately for MZ and same-sex DZ twin pairs across the cortisol indicators are presented in Table 3. The pattern of correlations was consistent with an ADE model for the CAR, as suggested by the fact that the MZ intra-pair correlation was larger than twice the DZ intra-pair correlation (.48 versus .13). Conversely, ACE models appeared indicated for the awakening and diurnal slope, because the MZ intra-pair correlations were approximately twice the DZ intra-pair correlation (or less) and both MZ and DZ twins showed some degree of intra-pair similarity. Preliminary univariate genetic analyses thus indicated that the multivariate Cholesky model should be as follows: ADE (CAR) – ACE (intercept) – ACE (slope). This multivariate model was compared with the saturated model (-2LL = -1424.84, d.f. = 30), which indicated that the former fit the data well ($\chi^2 = 88.05$, d.f. = 11, $p = 0.71$, AIC = 2895.73, BIC = 2961.62, RMSEA = .07, CFI = .94). Additionally, we also estimated a full ACE (CAR) – ACE (intercept) – ACE (slope) to evaluate whether the sole inclusion of an A parameter to depict the genetic factors for the CAR would be preferable. Based on the fit and parsimony indices, it was deemed not to be the case ($\chi^2 = 9.40$, d.f. = 9, $p = 0.40$, AIC = 2901.07, BIC = 2973.90, RMSEA = .08, CFI =

.93), suggesting that an ADE model was preferable to depict the CAR's etiology in the multivariate Cholesky analyses.

The unstandardized parameters and their standard errors are summarized in Figure 2. Both dominance and additive genetic factors explained individual differences in the CAR. The dominance genetic factor was specific to the CAR, as that parameter was not estimated for the awakening and diurnal change ($d_{CAR} = .95$; C.I. = .35-1.55). In contrast, the additive genetic factor influencing the CAR ($a_{CAR} = .48$; C.I. = .07-.89) also influenced the awakening level ($a_{CAR-I} = .30$; C.I. = .06-.54) and the diurnal change ($a_{CAR-S} = .11$; C.I. = .00-.22), suggesting a significant shared genetic etiology between the CAR and the other cortisol indicators. Thus, the additive genetic factors influencing the CAR, albeit small, overlapped to a large extent with the additive genetic factors influencing the awakening and diurnal change ($r = .59, p < .001$ and $r = .49, p < .001$, respectively). A similarly strong correlation was noted between the genetic factors influencing the awakening cortisol and the diurnal change ($r = .41, p < .001$). The awakening cortisol was also under the influence of additive genetic factors unshared with the CAR ($a_I = .41$; C.I. = .00-.81), but shared with the diurnal change ($a_{I-S} = -.20$; C.I. = -.35- -.05). Once all of these genetic contributions were taken into account, the slope did not have a specific genetic etiology. There was also an overlap between the shared-environmental factors estimated for the awakening levels ($c_I = .37$; C.I. = .03-.71) and the diurnal change ($c_{I-S} = -.18$; C.I. = -.33- -.04). In addition, all three cortisol indicators had specific and common non-shared environmental factors (see Figure 2).

In addition to delineating the pattern of specific and common etiology between the indicators of daytime cortisol secretion, the multivariate analysis allowed us to describe the relative portion of variance explained by each source of influence (see Figure 3). Genetic factors accounted for half the variance of the CAR (49.5%), while the other half (50.5%) was explained by non-shared environmental factors. The genetic etiology was mostly due to a non-additive genetic effect (39.5% versus 10.0% for the additive effect). Smaller genetic contributions were noted for the awakening level and diurnal cortisol change (27.8% and 31.4%, respectively). Also indicative that a different etiology may be present for the CAR is that a significant contribution of shared-environment was only observed for the last two indicators.

4. Discussion

The present study offers additional support, in a large age-homogeneous sample of adolescent twins, to existing evidence suggesting a genetic etiology of daytime cortisol secretion. Similarly to others studies, cortisol samples collected in the morning tended to have moderate heritability (Bartels et al., 2003b). Our own estimate of heritability at awakening (28%) is indeed comparable to findings noted elsewhere [39% (Wüst, 2000) and 33% (Kupper et al., 2005)], although a higher estimate has been reported in a laboratory setting in adults [56% (Franz et al., 2010)]. Studies conducted in childhood also fall in line with this overall pattern of findings (Bartels et al., 2003a; Gustafsson et al., 2011; Ouellet-Morin et al., 2009; Van Hulle et al., 2012). For example, about one-third of the variance of cortisol excretion was under genetic influences at awakening in infancy [32% (Ouellet-Morin et al., 2009)] and in the morning in childhood [31% (Van Hulle et al., 2012)].

Far fewer studies have specifically examined the genetic and environmental etiology of the CAR, from which more inconsistent findings have emerged. While some studies have reported moderate-to-strong genetic contributions [48% (Wüst, 2000) and 50% (Gustafsson et al., 2011)], others did not (Franz et al., 2010). In a distinct but complementary analysis, no added (unique) genetic contribution was detected for the CAR once the genetic influences of the awakening and +30 minutes levels were controlled for (Kupper et al., 2005). Also rarely investigated is the genetic and environmental etiology of the *diurnal pattern* of cortisol secretion taking place from morning to evening. The few studies conducted thus far have reported no genetic influences (Franz et al., 2010; Wüst, 2000). To the best of our knowledge, only one study has investigated this question using *stable* indicators of diurnal change, from which moment- and day-specific variations were isolated from stable inter- and intra- individual patterns of secretion (Van Hulle et al., 2012). In that study, the cortisol diurnal change from morning-to-afternoon was under partial genetic influences (32%), which is highly similar to what we found (31%). Moreover, and again in contrast to studies averaging cortisol levels collected over several days (or using single assessments), a shared-environmental contribution was detected [30% (Van Hulle et al., 2012) and 20% in our own]. One could thus hypothesize that deriving stable diurnal cortisol indicators increases the statistical power to

distinguish the shared-environmental from the additive genetic contributions. Additionally, the estimation of the etiology of diurnal cortisol secretion may vary according to whether moment-specific variation has been removed from stable individual differences. Future research should clarify this key methodological point.

Altogether, our findings offer evidence of distinct patterns of genetic and environmental etiology across the indicators of daytime cortisol secretion. Combined with the weak associations frequently observed between the CAR and the remaining two indicators, our findings support the idea that the CAR is regulated by distinct mechanisms from cortisol secreted later on. This hypothesis is not new (Clow et al., 2010; Fries et al., 2009), but has so far remained untested in a twin study design. The CAR may indeed reflect the action of two concurrent phenomena - the actual “response” to awakening and a “deeper current” related to the 24h-long variation in cortisol secretion (Wilhelm et al., 2007). Our study extends previous reports by formally testing whether the CAR has a specific (unique) latent etiology and/or whether genetic and environmental sources of variance overlap with the rest of the diurnal cycle.

Four findings from the present analyses stand out. First, our study revealed that the CAR has, for the most part, a different genetic etiology from awakening levels and diurnal change. The dominance genetic factor, unique to the CAR, explained the greater portion (80%) of its estimated heritability. Similar patterns of intra-pair correlations in the early morning, suggestive of a dominance genetic effect, have been reported before (Kupper et al., 2005). However, in that previous study, the dominance effect could not be distinguished from an additive genetic effect. The measurement of cortisol in a single day, which increases situation-specific influences, may have indeed limited statistical power despite their relatively large sample. More generally, the CAR’s largely distinct genetic etiology is consistent with previous suggestions that the CAR is regulated, at least partially, by distinct neurobiological mechanisms (Edwards et al., 2001; Wilhelm et al., 2007). For instance, the cortisol increase following awakening is thought to depend on a functional switch triggered by the sleep-wake transition in response to neuronal signals from the hippocampus and the light-sensitive suprachiasmatic nucleus (Clow et al., 2010; Pruessner et al., 2007). Additionally, the CAR may be the result of a change in adrenal sensitivity to ACTH (Wilhelm et

al., 2007), which is in itself linked with the time of day and the SNC (Bornstein et al., 2008; Clow et al., 2010).

We speculate that the dominance effect detected for the CAR could reflect the joint action of several regulatory systems and/or the interaction occurring between multiple alleles belonging to genes involved in its regulation. On the one hand, the detection of a dominance effect for the CAR may emerge as a result of the interaction taking place between the MR and the serotonin system, as suggested by the impact of 5-HT and 5-HT_{1A} receptors to MR and GR expression *in vivo* and *in vitro* (Robertson et al., 2005). On the other hand, a dominance effect may indicate an inflated MZ versus DZ twin intra-pair correlation because of the interplay between genetic and shared-environmental factors. Consistent with that possibility are the findings of the MR genetic polymorphisms (MRI180V and MR-2 G/C) being associated with the CAR in a context-dependent manner, such as following the intake of SSRI (Klok et al., 2011). The sex-specific association found between the *5-HTT* gene and the CAR (Wust et al., 2009) also suggests that a hidden interaction may take place between genes, such as sex-linked genes like the *MAOA*, and the environment. Sexual dimorphic associations can also originate from genetically-mediated differences in the cellular environment of men and women (e.g., secretion of androgens) which, in turn, modulate the expression of the genes involved in the CAR. The interplay between sex hormones and genes involved in cortisol secretion, such as the *GR* gene (Kumsta et al., 2007), further documents this possibility. More research is needed to explore this possibility.

Second, and again consistent with the idea that the CAR is regulated by distinct mechanisms, is the absence of a common shared-environmental source of variance between the CAR and the other indicators. This finding points to the possibility that the CAR may not be as responsive as the awakening levels and the diurnal profile to environmental influences that affect twins within a pair in a similar way. Conversely, non-shared environmental factors are shown to be both common and unique to the CAR, awakening levels and diurnal change. Theoretically, these findings may arise either from the exposure to distinct environmental experiences or from idiosyncratic perceptions of common environmental experiences. Future studies including measured environments in the multivariate genetic models could

expand on the present findings and test whether they explain part of the latent common or unique non-shared environment estimated for (and between) each cortisol indicator. In sum, researchers interested in investigating the neurophysiological processes affecting individual sensitivity to the environments - whether genetic (Belsky and Pluess, 2009) or biological (Boyce and Ellis, 2005) - may expect distinct mechanisms to take place according to the selected indicators (e.g., CAR versus diurnal change).

More generally, the distinct genetic and environmental etiology of the CAR relative to awakening cortisol and diurnal change may mirror the distinct functions the different indicators carry for the organism. The CAR is thought to have a unique role of insuring that individuals are ready for the demands expected later that day. This possibility is compatible with disrupted CAR noted in individuals under high levels of stress or in burn-out (Fries et al., 2009). Based on the present findings, we speculate that this physiological “jump start” may be influenced by the individuals’ inherited characteristics affecting both environmental exposure and cognitive and emotional processing. Interestingly, the CAR is thought to depend on the activation of memory representations about the self and orientation in time and space (Fries et al., 2009). The connection between the CAR and the hippocampus, a structure involved in memory processing, is consistent with this hypothesis (Clow et al., 2010; Fries et al., 2009; Gostisha et al., 2014; Pruessner et al., 2007).

Third, we found evidence for the influence of common genetic factors between the CAR, awakening level and the diurnal cycle, despite contributing only weakly to the CAR. To start with - and notwithstanding the time of day when cortisol is being secreted - glucocorticoids exert their influence by binding onto two types of receptors: the MR and the GR. MR show a ten-fold increased affinity to cortisol in comparison to the GR and bind to cortisol at lower levels such as during basal activity. Conversely, and because of its lower affinity to cortisol, GR bind to cortisol mainly under stressful circumstances and during the CAR (De Kloet et al., 1998). Several genetic polymorphisms within these receptors have been found to correlate with corticosteroids in rodents and with cortisol in humans, either during basal activity (Kuningas et al., 2007; Rosmond et al., 2000) or during response to stress (Derijk and de Kloet, 2008; Derijk et al., 2008). For example, *GR* genetic variants were associated with cortisol

responses to the Trier Social Stress Test, with higher levels noted for the *363S* allele carriers in comparison to the *BclI* CC and *N363S* AA allele carriers. The lowest responses were detected for *BclI* genotype GG (Wust et al., 2004). Consistent with the partly shared additive genetic etiology between the cortisol indicators is the evidence of an association between the oxytocin receptor gene and the cortisol awakening levels and the diurnal change (Norman et al., 2012). Alternatively, the common additive genetic influence on the CAR and the other indicators may arise because of spill-over effects over the day, as the HPA axis does not exist in a closed system. For example, cortisol secreted in response to awakening is still circulating after the CAR, possibly affecting individual differences later on and thus, the estimation of its etiology. It may thus be premature, solely on the basis of this common genetic etiology, to initiate molecular studies aiming to identify the specific genes involved in all three indicators. More twin studies for which similar data have been collected should first attempt to replicate this finding.

Fourth, the mix of positive and negative genetic and environmental correlations between the CAR, awakening levels and diurnal change likely mirror the phenotypic correlations observed between them. Specifically, the presence of a positive genetic correlation, but a negative non-shared environment correlation between the CAR and the awakening levels (or diurnal change) may explain why these phenotypes are not significantly associated. Alternatively, the fact that higher cortisol levels at awakening are generally followed by a stronger cortisol diurnal decline may be due to common regulating genes, shared and non-shared environments working in synergy. Although replication is needed, these findings suggest that the apparent lack of overlap between the CAR and the cortisol awakening levels (and diurnal change) may be misleading, as both common genetic and environmental factors are at play, although these factors seem to exert opposite influences that cancel each other out.

Despite providing a direct test of the distinct etiology between the CAR and the awakening and diurnal change, the present study also has a number of limitations. First, we could only estimate the relative magnitude of latent genetic and environmental contributions to each indicator. Although informative, this exercise does not identify the specific genes and environments involved. The twin design may, however, represent a useful tool in targeting more effectively where these investigations should start. Second,

caution is needed while interpreting the relative importance of shared-environmental factors between studies that include participants of different ages. Unlike most adult twins, the adolescent twins in our sample still lived together and went to the same schools, thus potentially increasing the relative importance of the shared- versus the unique-environments. Third, we did not evaluate the impact of non-compliance to the collection protocol through the use of electronic devices but instead relied on written records provided by the participants. However, in addition to statistically controlling for the awakening time, we also found that MZ and DZ twins did not differ in regard to their reported compliance to the protocol. Random patterns of non-compliance may have, however, enhanced measurement errors, which could have constrained the statistical power available for the genetic analyses.

In conclusion, our study offers additional empirical evidence that the CAR may distinguish itself from the remaining diurnal cycle. Our findings may help identify the genes (or environments) that should be targeted in priority. Our results also emphasize that cortisol measured over multiple collection days and combined through the use of analytical techniques that minimize moment-specific variation may be desirable to improve statistical power.

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Table 1. Descriptive statistics of cortisol sampled across the four collection days

	N	Raw mean cortisol levels in nmol/L (SD)	Mean time of sampling (SD)
<u>Day 1</u>			
Awakening	520	8.71 (5.06)	6h49 (0h53)
Awakening + 30 minutes	520	12.16 (5.93)	7h20 (0h48)
End of afternoon	514	2.83 (2.44)	16h33 (0h47)
Evening	402	1.56 (2.37)	21h39 (0h43)
<u>Day 2</u>			
Awakening	518	8.71 (4.53)	6h49 (0h48)
Awakening + 30 minutes	519	11.37 (5.80)	7h21 (0h48)
End of afternoon	501	2.65 (2.30)	16h35 (0h50)
Evening	385	1.57 (2.27)	21h51 (1h01)
<u>Day 3</u>			
Awakening	525	8.62 (5.21)	6h51 (0h57)
Awakening + 30 minutes	526	11.72 (6.10)	7h23 (0h55)
End of afternoon	509	2.69 (2.43)	16h37 (0h48)
Evening	410	1.47 (1.33)	21h45 (0h46)
<u>Day 4</u>			
Awakening	493	8.48 (5.47)	6h53 (0h57)
Awakening + 30 minutes	494	11.45 (7.06)	7h25 (0h57)
End of afternoon	474	2.85 (2.97)	16h37 (0h50)
Evening	354	1.49 (1.64)	22h01 (0h55)

Table 2. Fixed, random and covariance naturally log 10 transformed nmol/L estimates of cortisol levels at awakening and in the remaining part of the day according to each collection day

	Day 1			Day 2			Day 3			B
	B	SE	p	B	SE	p	B	SE	p	
<i>Fixed</i> (means)										
Intercept (y_0)	21.09	.29	< .001	21.08	.27	< .001	20.81	.30	< .001	20.85
Slope (y_s)	-.92	.03	< .001	-.93	.02	< .001	-.89	.03	< .001	-.89
<i>Random</i> (variances)										
Intercept (σ_0)	13.20	5.27	.012	11.60	3.55	.001	14.37	4.29	.001	17.85
Slope (σ_s)	.08	.05	.111	.05	.03	.067	.07	.03	.020	.08
<i>Covariances</i>										
Intercept –Slope (y_0, y_s)	-.70	.48	.145	-.47	.031	.133	-.73	.32	.025	-.81

Note. The fixed estimate of the intercept represents the mean cortisol level at awakening while the fixed estimate of the slope reflects the change of cortisol occurring from that point on. These estimations take into account the exact time of saliva collection and the previously identified confounders. B = Unstandardized beta estimate; S.E. = Standard error. The critical ratio refers to the ratio of the unstandardized beta estimate over the standard error (B/S.E.). An unspecified curve model was selected to optimally capture estimates of cortisol diurnal rhythm without imposing any particular shape of change across individuals (Duncan et al., 1997).

* = $p < .05$; ** = $p < .01$; *** = $p < .001$.

Table 3. Cross-twin cross-trait correlations according to zygosity groups for the CAR, awakening and diurnal cortisol change.

Correlations		Twin 1			Twin 2		
		CAR	Intercept	Slope	CAR	Intercept	Slope
Twin 1	CAR	-	-.04	.20	.48	.14	.03
	Intercept	-.14	-	-.58	.14	.44	-.29
	Slope	.33	-.59	-	.03	-.29	.49
Twin 2	CAR	.13	-.004	.12	-	-.04	.20
	Intercept	-.004	.26	-.24	-.14	-	-.58
	Slope	.12	-.24	.38	.33	-.59	-

Note. The values above the diagonal are for the MZ twins ($n = 280$) and those under the diagonal are for the same-sex DZ twins ($n = 204$). Mixed-sex twin pairs, who are not essential to genetic modeling, were excluded from the genetic analyses as their pattern of intra-pair correlations significantly differed from that found for same-sex twin pairs. The values in bold form refer to the intraclass coefficients calculated within each cortisol indicator. All the estimates have been derived while controlling for the confounders (i.e., sex, awakening time, hours of sleep, sleeping problems, exercises and alcohol or drug consumption).

Figure 1. Overview of the multivariate Cholesky model testing the common and specific genetic and environmental etiologies of the CAR, the awakening cortisol levels and diurnal cortisol change

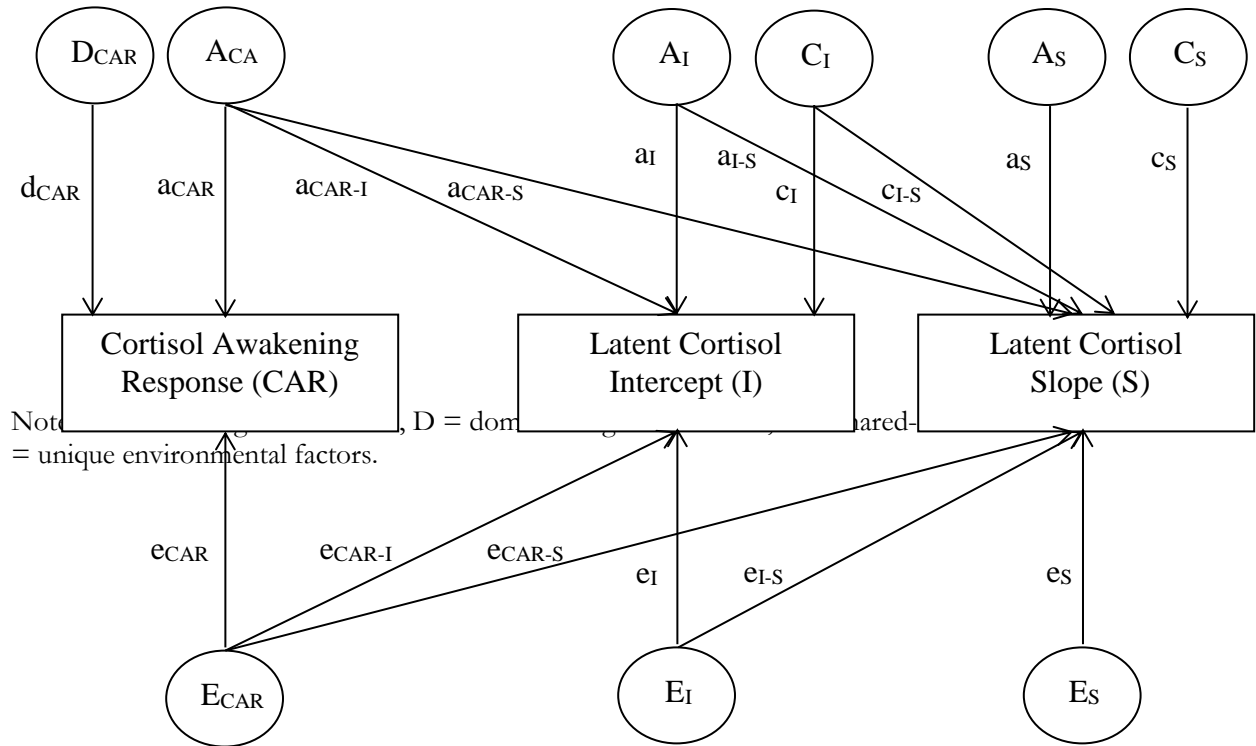


Figure 2. The unstandardized path coefficients (and standard deviation) derived from the Cholesky model including simultaneously the CAR, awakening levels and diurnal cortisol change.

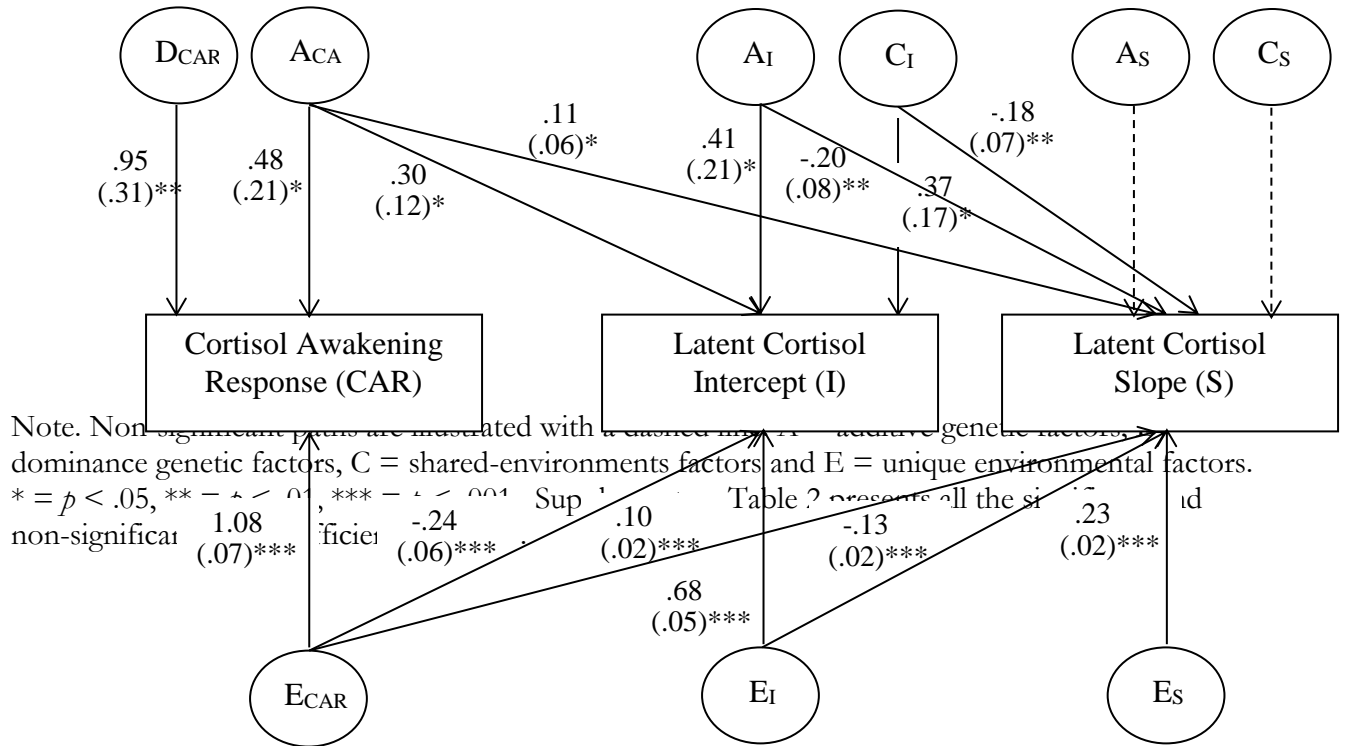
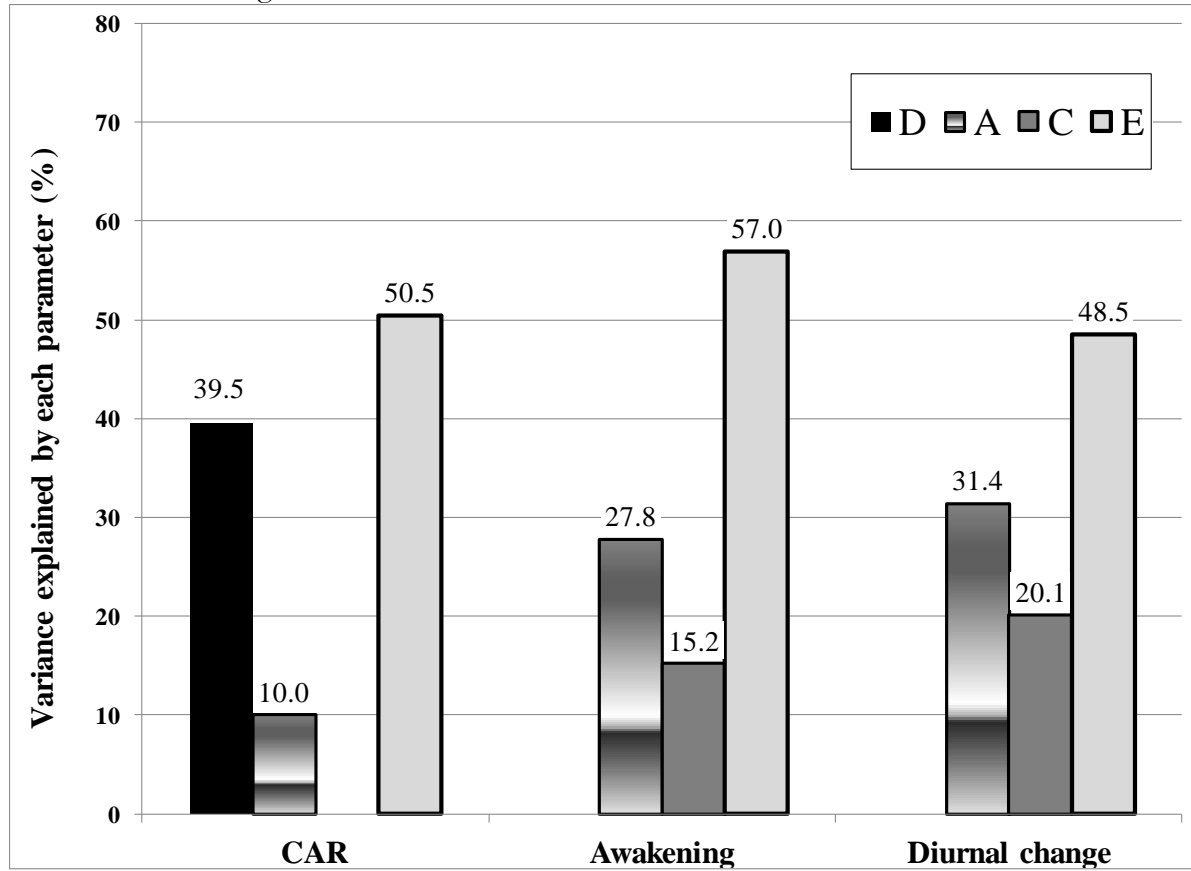


Figure 3. Proportion (%) of variance due to the dominance genetic, additive genetic, shared-environmental and non-shared environmental factors for the CAR, awakening cortisol levels and cortisol diurnal change.



Notes. D = Dominance genetic factors; A = Additive genetic factors; C = Shared-environment factors; and E = Unshared-environment factors.

Supplementary Table 1. A summary of the confirmatory factorial analyses conducted for the CAR, intercepts and slopes derived at each collection day

Parameters		Estimate (C.I.)	S.E.	p	χ^2 (df)	p	CFI	TLI	RM
<u>CARs</u>									
Loading	intercept	1.00	--	--	1.946 (2)	.378	1.00	1.00	
	intercept	1.27 (.48-2.06)	.40	.002					
	intercept	1.27 (.25-2.29)	.52	.015					
	intercept	1.02 (.22-1.81)	.41	.012					
Common factor Variance		5.25 (-.86-11.37))	3.12	.092					
<u>Intercepts</u>									
Loading	intercept	1.00	--	--	.002 (1)	.963	1.00	1.00	
	intercept	1.11 (.81-1.41)	.15	< .001					
	intercept	1.36 (.95-1.76)	.21	< .001					
	intercept	1.54 (1.12-1.95)	.21	< .001					
Common factor Variance		1.70 (.88-2.52)	.42	< .001					
<u>Slopes</u>									
Loading	Slope 1	1.00	--	--	.007 (1)	.934	1.00	1.05	
	Slope 2	1.03	.23	< .001					
	Slope 3	1.50	.37	< .001					
	Slope 4	1.44	.36	< .001					
Common factor Variance		.33	.13	.010					

Note. Unstandardized beta estimate; S.E. = Standard error. The critical ratio refers to the ratio of the unstandardized beta estimate over the standard error (B/S.E.).

Supplementary Table 2. All significant and non-significant path coefficients estimated for the CAR, the awakening cortisol levels and the diurnal cortisol change.

Parameters	Estimate	S.E.	Critical ratio	Confidence Intervals
d_{CAR}	.95	.31	3.12**	(.35, 1.55)
a_{CAR}	.48	.21	2.30*	(.07, .89)
a_{CAR-I}	.30	.12	2.42*	(.06, .54)
a_{CAR-S}	.11	.06	2.01*	(.00, .22)
a_I	.41	.21	1.98*	(.00, .81)
a_{I-S}	-.20	.08	-2.54**	(-.35, -.05)
a_S	.00	.06	-.003	(-.12, .12)
c_I	.37	.17	2.14*	(.03, .71)
c_{I-S}	-.18	.07	-2.47**	(-.33, -.04)
c_S	.00	.05	.002	(-.10, .10)
e_{CAR}	1.08	.07	15.60***	(.94, 1.21)
e_{CAR-I}	-.24	.06	-4.27***	(-.35, -.13)
e_{CAR-S}	.10	.02	4.13***	(.05, .14)
e_I	.68	.05	14.07***	(.59, .78)
e_{I-S}	-.13	.02	-.622***	(-.17, -.09)
e_S	.23	.02	15.39***	(.20, .26)

Note. a = additive genetic factors, d = dominance genetic factors, c = shared-environments factors, e = unique environmental factors, CAR = cortisol awakening response, I = intercept (i.e., cortisol level at awakening) and S = Slope (i.e., cortisol diurnal change). * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

Supplementary Figure 1. Overall analytical strategy to derive continuously distributed, stable indicators of daytime cortisol change over the four collection days

