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Abstract: This study examined the associations between emotional distress and HPA-axis dysregulations. Adolescents (29 boys and 29 girls, Mage = 15.06 years) completed standardized scales measuring depression, anxiety and emotional reactivity; a measure of internalizing problems was derived from the Child Behavior Checklist, completed by the parents and a teacher. For the measurement of HPA axis function, three saliva samples were collected over the course of a single week-end day (at awakening, at noon, in the evening).

Individuals with similar diurnal cortisol profiles were grouped using cluster analysis. We identified a subgroup of adolescents characterised by a flattened diurnal cortisol profile with elevated noon and evening cortisol;

these adolescents were more depressive, anxious and emotional reactive and scored higher on internalizing problems than all other adolescents. Moreover, repeated measurement analyses revealed that the diurnal cortisol evolution was related to all indices of self-reported emotional distress.

We can conclude that in a non-clinical sample of post-pubertal adolescents an endocrine abnormality - a high, flattened diurnal cortisol profile - is associated with symptoms of emotional distress across current nosological entities; this profile seems to present the severity of the affective symptomatology rather than its nature. These results may underscore the need to reconsider existing classification systems which are based on descriptive phenomenology.

Cover Letter

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KATHOLIEKE
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LEUVEN, January 31, 2007

Dear Editor,

Please find enclosed our manuscript entitled “The association between emotional distress and diurnal cortisol profile in post-pubertal adolescents”, which we would like to submit for publication in ‘Psychoneuroendocrinology”.

I hereby declare that all co-authors have seen and agree with the contents of the manuscript and that none have financial interest. This submission is not under review by any other journal or published previously. The local ethical committee approved the study and all participants and their parents gave their written informed consent.

We look forward to your comments and thank you in advance for your consideration regarding this manuscript.

Sincerely,

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* Manuscript

Running title: Adolescents' emotional distress and cortisol

The association between emotional distress and diurnal cortisol profile in post-pubertal adolescents

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Abstract

This study examined the associations between emotional distress and HPA-axis dysregulations. Adolescents (29 boys and 29 girls, M_{age} = 15.06 years) completed standardized scales measuring depression, anxiety and emotional reactivity; a measure of internalizing problems was derived from the Child Behavior Checklist, completed by the parents and a teacher. For the measurement of HPA axis function, three saliva samples were collected over the course of a single week-end day (at awakening, at noon, in the evening). Individuals with similar diurnal cortisol profiles were grouped using cluster analysis. We identified a subgroup of adolescents characterised by a flattened diurnal cortisol profile with elevated noon and evening cortisol; these adolescents were more depressive, anxious and emotional reactive and scored higher on internalizing problems than all other adolescents. Moreover, repeated measurement analyses revealed that the diurnal cortisol evolution was related to all indices of self-reported emotional distress.

We can conclude that in a non-clinical sample of post-pubertal adolescents an endocrine abnormality - a high, flattened diurnal cortisol profile – is associated with symptoms of emotional distress across current nosological entities; this profile seems to present the severity of the affective symptomatology rather than its nature. These results may underscore the need to reconsider existing classification systems which are based on descriptive phenomenology.

Key words: diurnal cortisol profile; post-puberty; internalizing problems, depressive symptoms, anxious symptoms, emotional reactivity

Introduction

Mood and anxiety disorders are associated with several HPA-axis dysregulations. In some studies, these dysregulations refer to changes in absolute levels of cortisol secretion at different times during the day (e.g., lowered or elevated morning cortisol, elevated evening cortisol) or to changes in overall cortisol secretion (e.g. overall hyper- or hyposecretion) (Chrousos, 1998; Sapolsky et al., 2000; Antonijevic, 2006; Halbreich, 2006). However, it is suggested that disturbances of temporal pattern of cortisol secretion may be even more relevant measures of HPA-axis dysregulation (Vedhara et al., 2003, 2006). These include a flattened diurnal profile due to elevated evening cortisol and associated with hypercortisolism (e.g., Deuschle et al., 1997; Chrousos and Gold, 1998; Rosmond et al., 1998; den Hartog et al., 2003; Abercrombie et al., 2004; Giese-Davis et al., 2006), flat diurnal profile due to low morning basal levels which remain constant throughout the day (Gunnar and Vazques, 2001), or a reduction in the length of the nocturnal quiescent period (Halbreich et al., 1985a,b).

It is proposed that individuals sharing a certain pattern of HPA-axis dysregulation are predisposed to develop some stress-related disorders while people sharing another pattern have a risk to develop other stress-related disorders (Chrousos et al., 2003). For instance, HPA-axis hyperactivation, including hypercortisolism, is associated with major depression and anxiety disorders while HPA-axis hypoactivation, including hypocortisolism may occur in patients with atypical depression and in patients with chronic fatigue syndrome (Chrousos, 1998; Chrousos et al., 2003; Raison and Miller, 2003; Heim et al., 2004; Van Praag et al., 2004; Van Den Eede et al., 2005; Antonijevic, 2006). However it is unclear whether these associations also hold for the subclinical range of mood and anxiety disorders observed in normal, non-clinical populations in which the severity of emotional distress symptoms is not assessed with a clinical interview but with self-report (cf. Pruessner et al., 2003; Vedhara et al., 2003, 2006) parent- and teacher-report questionnaires (Netherthon et al., 2004) and

whether they also hold for adolescents (Kaufman and Charney, 2001; Forbes et al., 2005; Tarullo and Gunnar, 2006). Furthermore, recent theories hold that many currently known endocrine abnormalities are not specific to current psychiatric diagnostic entities. Instead, endocrine abnormalities seem to be more specific to clusters of symptoms across current nosological entities. According to Halbreich (2006), neuroendocrine abnormalities may be due to internal or external stress; he proposes that at best they may present either the severity of the stress or the magnitude of the individual adaptation to stress or the severity of the affective symptomatology, but not its nature.

The aim of our current research is twofold. In a non-clinical sample of post-pubertal adolescents, we collected saliva cortisol samples at awakening, at noon and in the evening (at bedtime) and measured indices of emotional distress (self-reported depressive, anxiety and emotional reactivity symptoms and parent- and teacher-reported internalizing problems) to study: (1) whether one or more group(s) of individuals sharing a similar diurnal cortisol profile could be discerned in this sample and whether group(s) exhibiting an altered/abnormal diurnal cortisol profile also showed altered/abnormal indices of emotional distress; (2) the relationships between indices of emotional distress on the one hand and patterns of diurnal change in cortisol and absolute levels at awakening, at noon and in the evening (before going to sleep) on the other hand.

Method

Participants

The current study involved a sample of 68 14- and 15-year olds, participating in a prospective follow-up study (see Van den Bergh et al., 2006). The 58 participants who had complete data for all cortisol measurements were included (29 boys and 29 girls, M_{age} = 15.06 years, *SD*=0.26 years). They had all reached Tanner stage 3 of pubertal development (M=4.13; SD=.526). According to Tanner (1962) there are 5 puberty stages with stage $\geq$ 3

indicating post-puberty. The local ethical committee approved the study and all participants and their parents gave their written informed consent.

Measures

Cortisol

Subjects provided samples of cortisol on a weekend day at home, spitting saliva in a salivette (Sarstedt, Inc., Newton, NC) at three times: immediately after awakening (*M*= 0851, *SD*= 0056), at noon (*M*= 1256, *SD*=0054) and in the evening (*M*= 2032 , *SD*=0115). Subjects were instructed not to brush their teeth or eat before spitting saliva. The tubes were kept refrigerated and brought along to a laboratory visit together with the questionnaires they had completed at home. All samples were stored at -60°C upon arrival. Cortisol in saliva was measured with a revised version of the protocol provided by the manufacturer of the Coat-a-Count Radio-Inmuno-Assay Kit (Euro DPC, Llanberis, Wales). Two hundred microliters of saliva and diluted standards is used, with incubation for 3 hours at room temperature, modified by an additional period overnight (15-18 hours) at +4°C (cf. O'Connor et al., 2005)

Emotional distress: self-report measures of depressive, anxious and emotional reactivity symptoms

Standardized Dutch versions of the scales were used; all Cronbach's alphas were between .78 and .95 in reference populations.

Depressive symptoms : The Child Depression Inventory (CDI, Kovacs) measures the severity of cognitive, affective and behavioural symptoms of depression over the last two weeks and contains 27 items, scored from 0 to 2 (Timbremont and Braet, 2002). According to the authors of the CDI, a score of 19 best distinguishes between non-depressed and clinically depressed persons rated with DSM-IV criteria (American Psychiatric Association, 1994), whereas levels between 13 and 18 are regarded as subclinical or minor depressive episode.

Anxiety symptoms: The State Trait Anxiety Inventory for Children (STAIC, Bakker et al., 1989) comprises two subscales of 20 items scored from 1 to 4. Trait anxiety refers to a disposition or proneness to react with anxiety, while State anxiety refers to a transient emotional state, characterized by subjectively experienced tension and increased activity state of the autonomous nervous system.

Emotional reactivity symptoms: We used the subscale Emotional reactivity (ER), containing 20 items with a yes/no format, of the Formal Characteristics of Behaviour-Temperament Inventory (FCB-TI; Strelau and Zawadzki, 1993). ER assesses the tendency to react intensively to emotion-generating stimuli. High reactive individuals need only low levels of stimulation to obtain an optimal level of arousal, while low reactive individuals need high levels of stimulation in order to reach an optimal level of activation. It is hypothesized that cortisol levels are higher and cortisol diurnal profiles more abnormal in individuals who are highly reactive, but research on this topic is rather sparse (Strelau, 1998).

Emotional distress: mother-report and teacher-report measures of internalizing problems. The mothers completed the Child Behavior Checklist (CBCL, Verhulst et al., 1996) and teachers completed the Teacher Report Form (TRF, Verhulst et al., 1997). These questionnaires measure behavioural or emotional problems during the past 6 months (CBCL) and 2 months (TRF). The response format is: 0=not true, 1=somewhat or sometimes true and 2=very true or often true. We focus on the broadband category of internalizing problems. To take all information from all the informants into account, a principal component analysis was conducted on mother and teacher Internalizing Problem Scales; it revealed one component that explained 70 % of the variance. We used this component as an index of internalizing problems.

Statistical analyses

To examine our first research aim, cluster analysis and ANOVA were performed. Cluster analysis is a commonly used technique in biology for grouping objects of similar kind into respective categories (clusters). This pattern recognition method has been used in the field of behavioural endocrinology to detect groups of individuals with homogeneous endocrine (e.g., cortisol) and biological (e.g., sleep EEG) patterns related to emotional distress indices (Gerra et al., 2003; Staner et al., 2003). To detect subgroups of subjects with a similar diurnal cortisol profile we used the FASTCLUS procedure in SAS v9.1. This uses a K-means algorithm based on Hartigan (1975) and MacQueen (1967). A solution with *k* clusters is found by minimizing the sum of squares of Euclidean distances between each observation and its cluster center. The cluster analysis is performed for several values of *k* (i.c., 2 to 8). Further, for a given value of *k*, we decided to execute the algorithm multiple times (i.c., 50 times), each time with another random selection of initial centers. This was done to anticipate on the effect of the initial cluster centers on the clustering result (e.g., Steinley, 2003). This leaves us with a set of solutions due to varying *k* and the initial centers. To decide on the optimal solution we relied on the interpretability and face validity of the solution as well as on numerical indices like the Calinski-Harabasz index (CH; Calinksi and Harabasz, 1974), the GAP statistic (Tibshirani et al., 2001), and the silhouette value (Rousseeuw, 1987). These indices can help in choosing the optimal number of clusters.

One-way ANOVAs were used to investigate whether the obtained clusters differed on the variables of interest. The ω^2 (omega squared) index was computed as a measure of effect size for each variable. This index tries to give a population-based estimate of the proportion of variance in the outcome that is accounted for by a predictor (Olejnik and Algina, 2000). Pairwise comparisons were used to investigate where the differences, if any, were located. No correction for multiple testing was used (e.g., using the Tukey-Kramer procedure) for several reasons: a) they are used to protect the overall alpha Type-I error at the expense of power

(Rothman, 1990), b) their results are influenced by the number of tests performed which is usually arbitrary, and c) we have a small sample such that power may already be limited. For pairwise comparisons, a contrast version of ω^2 is computed (Olejnik and Algina, 2000). The ω^2 index of effect size is more important than the *p*-value and is thus our primary measure (e.g., Olejnik and Algina, 2000). Effect size measures are less influenced by sample size.

To examine our second research aim, repeated measurements (RM) analyses were performed to investigate whether the different indices (depression, state and trait anxiety, emotional reactivity and internalizing problems) were associated with the pattern of changes in cortisol level during the day. Cortisol was measured at three time points: at awakening (0 hours), at noon (approximately 4 hours after awakening) and in the evening (just before bedtime; approximately 12 hours after awakening), so these moments were given value 0, 4, and 12 in the analysis. The associations among the cortisol measurements at different times of the day were best modelled using a heterogeneous fist-order autoregressive covariance structure. Each analysis included the main effect of the emotional distress variable (this investigates whether the awakening cortisol level depends on the emotional distress variable), a linear and quadratic time effect on cortisol (to investigate whether the cortisol level evolves in a linear and/or nonlinear way), and interactions between both time effects and the emotional distress variable (to investigate whether the level of emotional distress is associated with the linear and/or nonlinear diurnal cortisol evolution). Backward variable selection was performed by sequentially dropping non-relevant predictors, if any. We kept models hierarchical in the sense that the inclusion of an interaction effect implied the inclusion of all its higher order effects. Thus, together with the intercept we started the backward selection with a model containing 6 predictors which means that we have 9.7 subjects per predictor. These models do not directly assess the relationship between the emotional distress variables and absolute cortisol levels at noon and evening. Therefore, we additionally performed

univariate regression analyses to investigate the effect of this distress variable on the absolute cortisol levels. This allowed the computation of the ω^2 measure of effect size.

Some of the variables were positively skewed and were transformed. Cortisol levels were log-transformed because mainly the noon and evening levels were skewed. The CDI and internalizing problem scores benefited more from a square root-transformation to reduce skewness. Since internalizing problems had negative values, its transformation was sqrt (x – min (x)). Note that the results of the analyses for the untransformed variables (not reported) were similar to those for the transformed ones.

Results

Preliminary analysis: Descriptives of self-reported emotional distress and cortisol

Descriptives for all relevant variables are presented in Table 1. For the CDI only, cut-off scores are provided in the manual; using them we can tentatively conclude that 14 % (n=8; 4 female, 4 males) may suffer from a minor depressive episode (score range 13-18), while 9 % (n=5; 4 female, 1 male) may thus suffer from a major depressive period (score > 18). Probably more than the 25 % suffering from a major or minor depressive episode may suffer from 'depressed mood.' Although the available reference samples are on average two (CDI; Timbremont and Braet, 2002) and one year younger (STAIC, Bakker et al., 1989) than our sample, taking into account the range of scores obtained and the fact that the mean CDI-score is situated at deciles 5-7 and the mean STAIC-scores at deciles 6-8 of these samples, we can tentatively conclude that our sample covered the range of depressive and anxiety symptoms seen in non-clinical populations and that a substantial proportion scored in the higher range. For emotional reactivity there is no adequate reference sample available.

There was a clear downward trend in cortisol from awakening to evening (see Figure 1). These data are in accordance with other community samples including depressive outpatients and controls (Peeters et al., 2004; Young et al., 2006). The mean cortisol at

awakening is lower than, e.g., the mean in a sample of university students with a mean age of 24.3 (mean=17.64 nmol/liter (no SD given); Pruessner et al., 2003); this may be due to the younger age of our sample (Kudielka and Kirschbaum, 2003).

Research aim 1: Grouping of adolescents with homogeneous cortisol day-time profile and associations with indices of emotional distress.

The numerical indices in general favour a 3-cluster solution with two big clusters each containing 25 cases and one cluster containing 8 cases. This latter cluster contains three cases that differ from the other five because they have a clearly elevated evening cortisol level. Therefore, on interpretability and face validity grounds we prefer to choose a cluster solution that puts these three cases into a different, fourth cluster. This four cluster solution also scored very well on the numerical indices.

Of the four clusters, the cortisol daytime profile of cluster 3 (n=25) could be regarded as normal: cortisol was high at awakening and showed a steady trend downwards during the day. Compared to this group, the second cluster (n=25) had a relatively low, decreased awakening cortisol level but similar noon and evening levels as compared to the third group. The first cluster (n=3) showed rather normal awakening cortisol levels, elevated noon cortisol and clearly elevated evening cortisol. Finally, the fourth cluster (n=5) had a rather normal awakening level, clearly elevated noon level, and an evening cortisol that shows the expected decrease but was slightly elevated. Basically, the following deviances from the normal pattern of cortisol changes during the day were observed: the second cluster had decreased awakening cortisol, the fourth elevated noon cortisol and the first elevated noon levels and clearly elevated evening cortisol (see Figure 6). There was no evidence for an association between clusters and gender (Fisher's exact test $p = 0.75$).

One-way ANOVAs and pairwise comparisons between the 4 clusters (see Table 2 for a summary of the results) indicated that the mean of cluster 1 tended to differ from the other

clusters for several variables. The other clusters did not differ greatly from each other such that we only describe the results for cluster 1. The results suggested that cluster 1 contains adolescents which, compared to all other adolescents, describe themselves as experiencing more symptoms of depression and anxiety and being more emotionally reactive; according to their parents and teachers these adolescents show more internalizing problems. The effect sizes for cluster differences vary from .07 (trait anxiety) to .16 (emotional reactivity), such that 7 to 16 percent of the variability in emotional distress in the population could be explained by cluster membership only. These rather high effect sizes are mainly due to differences between cluster 1 and the other clusters, as the pairwise comparison effect sizes show.

Research aim 2: Associations between indices of emotional distress and absolute versus changes in cortisol levels during the day.

In the RM models of all emotional distress variables, similar linear (p's < 0.0001) and quadratic (p's between 0.03 and 0.04) time effects were found. This reflects the observation that cortisol levels decreased during the day and that the decrease slows down as the day progressed. Also, in all models there was no main effect of the distress variable suggesting that each of these variables was not clearly related to awakening cortisol. More importantly, significant interaction effects in the RM models of all self-report indices of emotional distress indicated that all these variables affected the cortisol day-time profile (see Table 3 for an overview of the results and see Figure 2 to 5).

For internalizing problems, which was the only measure based on questionnaires completed by parents and teachers, only the general linear and quadratic time effects were significant. This measure was not strongly related to the diurnal cortisol profile.

In both the *depressive symptoms (*square root-transformed CDI-scores) and *trait anxiety* RM models, the interaction between the distress variable and the linear time effect

suggested that the degree of linear cortisol decrease during the day depended on the level of distress. It appears that depressive symptomatology (see Figure 3) and trait anxiety symptomatology (see Figure 4) are both related to a flattened cortisol day profile characterized by decreased awakening cortisol and elevated evening cortisol. In both the *state anxiety* and *emotional reactivity* RM models the interaction between the distress variable and the quadratic time effect indicated that the level of non-linear decrease depended on the level of distress. If distress was high, cortisol had a more non-linear decrease. For highly state anxious adolescents cortisol levels appear to decrease faster before noon but slower after noon (see Figure 5); highly state anxious adolescents appear to have higher morning and evening cortisol levels even though the main effect of state anxiety is small and not significant. Further, highly emotionally reactive adolescents had a more non-linear cortisol profile because relative to other adolescents their cortisol levels between noon and evening decreased slower; evening cortisol is elevated (see Figure 6).

Univariate regression analyses revealed that emotional distress was mainly related to (log of the) evening cortisol level (CDI: $\omega^2 = 0.02$, $p = 0.1333$; trait anxiety: $\omega^2 = 0.07$, $p = 0.0281$; state anxiety: $\omega^2 = 0.07$, $p = 0.0224$; ER: $\omega^2 = 0.08$, $p = 0.0154$; internalizing problems: $\omega^2 = 0.01$, $p = 0.2061$). The effect sizes (ω^2) for evening cortisol were decent, while for (log of the) awakening cortisol the effect sizes varied from 0.00 to 0.02 (with p-values > 0.15) and for (log of the) noon cortisol the effect sizes were all 0.00 (with p-values > 0.53). Note that, consistent with the RM models, no strong relation between emotional distress and awakening cortisol was found.

Discussion

We studied the relationship between cortisol and emotional distress in a sample of post-pubertal adolescents and have obtained several interesting findings.

A specific pattern of diurnal cortisol secretion is associated with high emotional distress.

Our first aim was to group adolescents with homogenous patterns of changes in cortisol levels during the day, if possible, and to examine eventual associations with emotional distress variables.

First, cluster analysis identified four groups; one group with a normal diurnal cortisol profile and three groups with deviations from the normal profile. We can conclude that in a non-clinical sample of adolescents differences in diurnal cortisol profile exists which can be discerned with cluster analysis.

Second, the most remarkable result was that a small subgroup (cluster 1) showing elevated noon and strongly elevated evening cortisol secretion, consistently differed from one or more of the other subgroups on all indices of emotional distress. These adolescents rated themselves as more depressive, more anxious and more emotionally reactive and were by their mother and teacher scored as having more internal problems compared to all other (subgroups of) adolescents in this sample. These adolescents seem to share a particular dysregulation of their HPA-axis observed as blunted cortisol decrease in the afternoon and evening; this pattern may be interpreted as reflecting HPA-axis hyperactivity. We were however not able to prove that their HPA-axis was indeed hyperactive; we can only infer this link by combining information from other studies (see also Miller et al., 2007). A comparable diurnal profile with elevated afternoon and evening cortisol, also based on salivary cortisol measures, has been linked with inadequate suppression of morning cortisol by overnight dexamethasone, in chronically distressed (Rosmond et al., 1998) as well as in depressed persons (Young et al., 2006). According to Chrousos and Gold (1998), the findings of Rosmond et al. (1998) suggest chronic hypersecretion of CRH and a reset of their HPA axis; earlier results of Deuschle et al. (1997; 24-h blood sampling) lend support to this interpretation of a flattened profile.

Our 12 hours (diurnal) profile seem to suggest that secretion of cortisol will also be elevated during the night and until the morning, however only the use of a circadian profile would have enabled us to verify the existence of hypercortisolism over 24 hours. This blunting of the diurnal/circadian rhythm can be detrimental because the organism may for several hours (e.g. starting in late afternoon, during the evening dip and until awakening) be exposed to higher than optimal levels of cortisol (Chrousos and Gold, 1998; McEwen, 2002).

Third, our data lend support to the observation that the presumed HPA-axis dysregulation - i.e. HPA-axis hyperactivity reflected in a flattened profile – is not specific to current psychiatric diagnostic entities (Halbreich, 2006). In stead, in our sample the endocrine abnormality seem to be more specific to clusters of symptoms across the current nosological entities of at least depressive symptomatology and anxiety symptomatology. Identifying neurobiological distinct subtypes of disorders irrespective of existing diagnostic borders may lead to more optimized strategies for prevention and treatment and is therefore preferred above current classification systems of depression that are based on a descriptive, phenomenological approach (Gottesman and Gould, 2003; Halbreich, 2006).

Fourth, for cluster 2 and 4, no significant alterations in emotional distress characteristics arose from the ANOVA's. However, this does not exclude the possibility that e.g. for cluster 2 other measures than the CDI, STAIC, FCB-TI and CLCL/TRF which we used, might have revealed significant associations. A flattened diurnal profile with lowered morning cortisol, as observed in cluster 2, has e.g., been seen in children reared in orphanages and foster care and is suggestive of underlying psychopathology (see Gunnar and Vazques, 2001; Tarullo and Gunnar, 2006).

A flattened cortisol decrease after noon, related to higher predicted cortisol levels in the evening, presents the severity of the affective symptomatology.

Our second aim was to examine associations between several indices of emotional distress on the one hand and cortisol daytime profile and absolute levels of cortisol secretion at awakening, at noon and in the evening (at bedtime) on the other hand.

First, the RM analyses can be summarized by stating that all four self-report indices of emotional distress are associated with alterations in the normal cortisol day time profile: adolescents with high emotional distress showed a flattened cortisol decrease after noon related to higher predicted cortisol levels in the evening. Because of the small sample size, we feel that not too much importance should be attached to the fact that some distress variables interact with time and others with time^2. More research on large, representative samples is needed to examine the eventual meaning of these specific associations between time, time^2 and different indices of emotional distress. Although are results corroborate findings of Sachar et al. (1970), Deuschle et al. (1997), den Hartog (2003), and Young (2006) on depression, and diurnal/circadian cortisol rhythm, our results are also consistent with the hypothesis of Halbreich (2006) that HPA-axis dysregulation may represent the severity of the affective symptomatology, but not necessary its nature.

Second, the main effect for the emotional distress variable was never significant in the RM models, suggesting that emotional distress is not clearly related to awakening cortisol. In line with this finding, subsequent univariate regressions show very small effect sizes for the relationship between the emotional distress measures and morning cortisol. However, several figures give the impression that high distress is related to changes in awaking cortisol. The lack of a main effect may reflect the fact that saliva cortisol was collected immediately after awakening and not after 30 minutes (i.e., when the post- awakening response occur; e.g., Wüst et al., 2000; Pruesner et al., 2003); alternatively it could be caused by a combination of small sample size and high variability in awakening cortisol.

Third, univariate regressions revealed that emotional distress was mainly related to evening cortisol level and not to awakening and noon levels. This finding is in line with findings of elevated evening salivary cortisol in depression in 8- to 16-year olds (Goodyer et al., 1996) and in 13- to 17-year olds (Goodyer et al., 2001), and in adolescents having reached Tanner stage III (Dahl et al., 1991; Rao et al., 1996; Forbes et al., 2006). However, this result does not corroborate the findings of Vedhara and colleagues (2003), who did not find associations between indices of emotional distress and absolute levels of cortisol on any time of the day, and the findings of Adam (2006) who, in a study of normal adolescents, did not found associations between any of the diurnal cortisol parameters levels and trait anxiety. Moreover, Goodyer et al. (2003) found raised morning cortisol in depressive adolescents, while in the study of Forbes et al. (2006) anxious symptoms were not associated with peri-sleep-onset cortisol. The inconsistency in these results may be related to the use of different measures to obtain and analyze cortisol and to the fact that the puberty stages is not always taken into account in studies with adolescents. The latter is however necessary because the HPA-axis reaches its full maturation, including full diurnal rhythms, only after the pubertal transition, i.e. with the attainment of Tanner phase III.

Fourth, internalizing problems, the only other-report variable, was the only variable that was not associated with the diurnal cortisol profile. This may be seen as a confirmation that adolescents may be the best informants about their own affect, but does not withhold the fact that parents or teachers may be better at describing observable depressive symptoms such as changes in eating and reduced energy level (Hankin and Abramson, 2001).

Our study has a few limitations that necessitate some caution in interpreting the results and their implications. *First*, the sample was rather small which hampers a very reliable assessment of the effects. An argument in favour of our results, however, is that both in the cluster analyses and in the repeated measurement analyses consistent findings were obtained

across different measures of emotional distress. *Second,* the HPA-axis is connected with other biological systems (e.g., monoamine neurotransmitters, immune system) which we did not measure. The simultaneous study of several systems may have given a more complete picture of pathophysiological pathways in emotional distress. *Third,* as it is known that saliva cortisol shows day-to-day variability, assessment of salivary cortisol on more than one day could have increased the data reliability (Pruessner et al., 1997; Wüst et al., 2000). Moreover, we planned the saliva collection on a week-end day for reasons of feasibility, not a week day when the schedules of adolescents are more stable.

In conclusion, our data reveal that in a non-clinical sample of post-pubertal adolescents, individuals sharing a pattern of HPA-axis dysregulation that is reflected in a flattened day time profile due to elevated noon and evening cortisol levels, share also the expression of symptoms of depression, anxiety, emotional reactivity and internalizing problems as respectively measured with standardized self- and standardized other-report scales. This neuroendocrine abnormality is specific to clusters of symptoms across current nosological entities and furthermore; it presents the severity of the affective symptomatology rather than its nature and may be important in guiding the choice for efficient therapeutic interventions. These results underscore the need to reconsider existing classification systems which are based on descriptive phenomenology, expressed by several authors (Gottesman and Gould, 2003; Halbreich, 2006).

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Figure captions

Figure 1

Median cortisol secretion (with interquartile range) at awakening, noon and evening.

Figure 2

Cortisol profile for each cluster

Figure 3

Child Depression Inventory-scores and cortisol secretion across the day in adolescence (the cortisol profile for adolescents with very low (mean CDI – 1.96SD), average (mean CDI) and very high CDI-score (mean CDI + 1.96SD) is shown).

Figure 4

Trait Anxiety score and cortisol secretion across the day in adolescence (the cortisol profile for adolescents with very low (mean CDI – 1.96SD), average (mean CDI) and very high CDI-score (mean CDI + 1.96SD) is shown).

Figure 5

State Anxiety score and cortisol secretion across the day in adolescence (the cortisol profile for adolescents with very low (mean CDI – 1.96SD), average (mean CDI) and very high CDI-score (mean CDI + 1.96SD) is shown).

Figure 6

Emotional reactivity subscale –score across the day in adolescence (the cortisol profile for adolescents with very low (mean CDI – 1.96SD), average (mean CDI) and very high CDI-score (mean CDI + 1.96SD) is shown).

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Role of funding source

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*** Conflict of Interest**

Conflict of interest

All authors declare that they have no conflicts of interest.

Contributors

Author Van den Bergh designed the study and wrote the introduction and discussion sections of the manuscript. Authors Van Calster and Van Huffel undertook the statistical analyses; author Van Calster wrote the statistical analysis and results sections. Author Pinna Puissant managed the literature searches and wrote the participants and measures sections. All authors contributed to and have approved the final manuscript.

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Figure 1

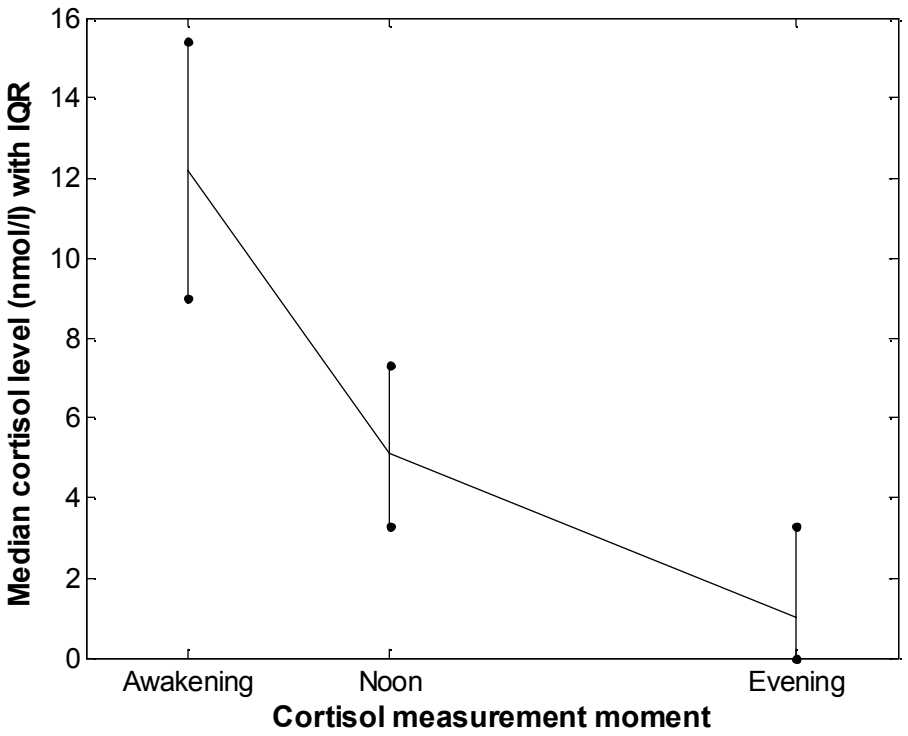


Figure 1 Median cortisol secretion (with interquartile range) at awakening, noon and evening

Figure 2

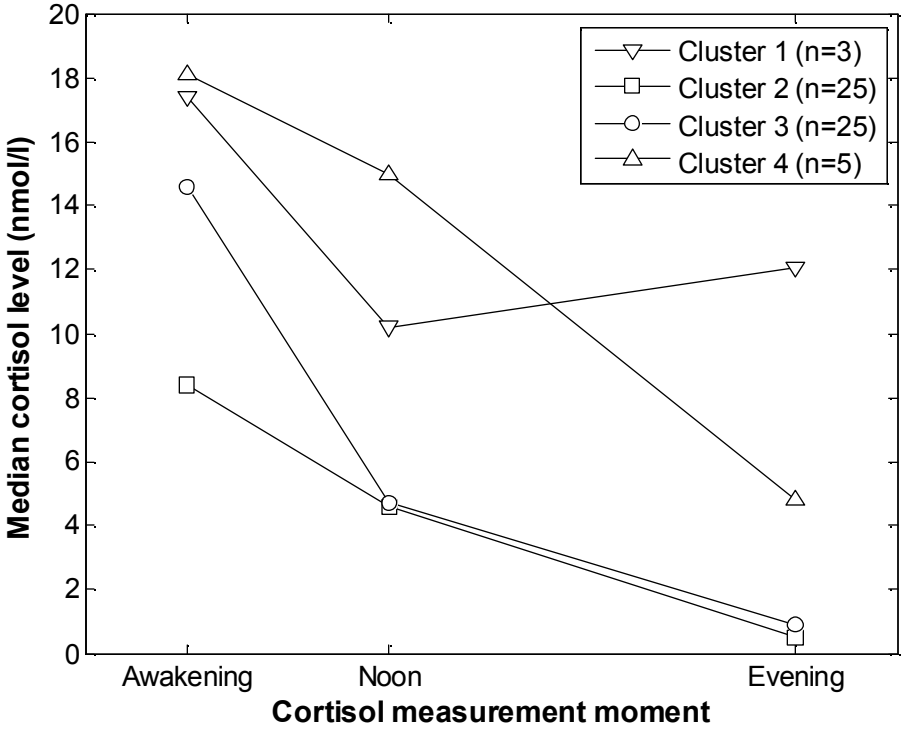

Median cortisol level (nmol/l)
20
18
16
14
12
10
8
6
4
2
0
Awakening
Noon
Evening
Cortisol measurement moment
Cluster 1 (n=3)
Cluster 2 (n=25)
Cluster 3 (n=25)
Cluster 4 (n=5)

Figure 3

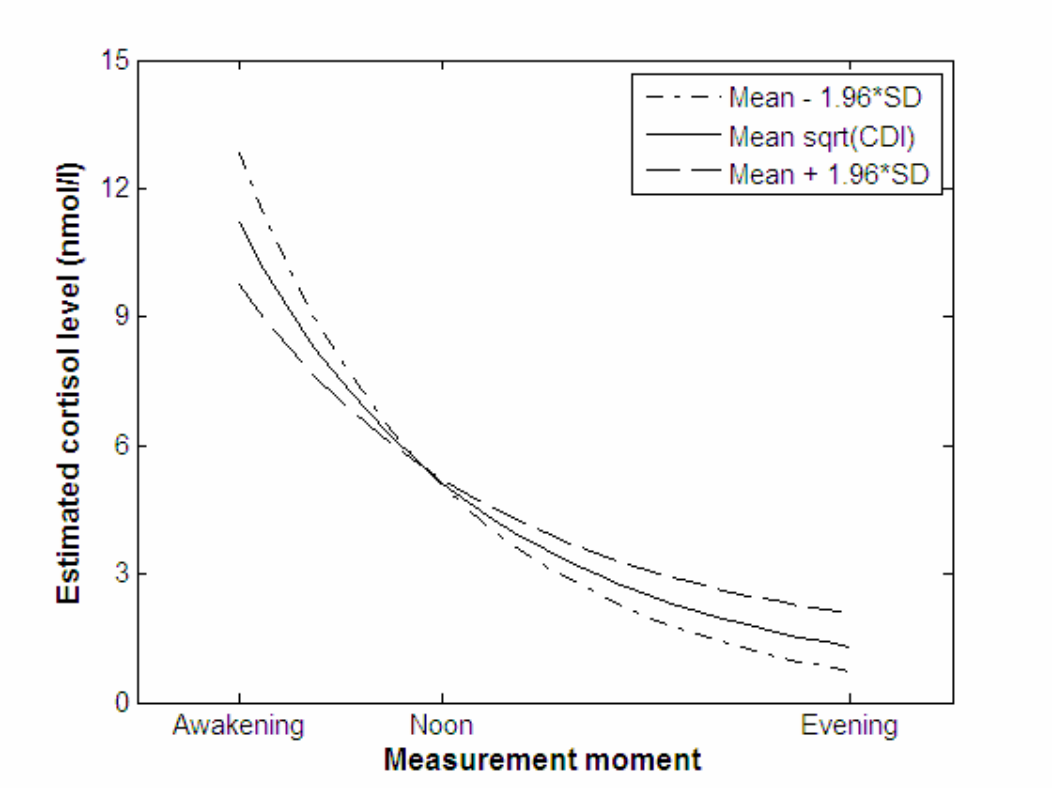

15
12
9
6
3
0
Estimated cortisol level (nmol/l)
Mean - 1.96*SD
Mean sqrt(CDI)
Mean + 1.96*SD
Awakening
Noon
Evening
Measurement moment

Figure 4

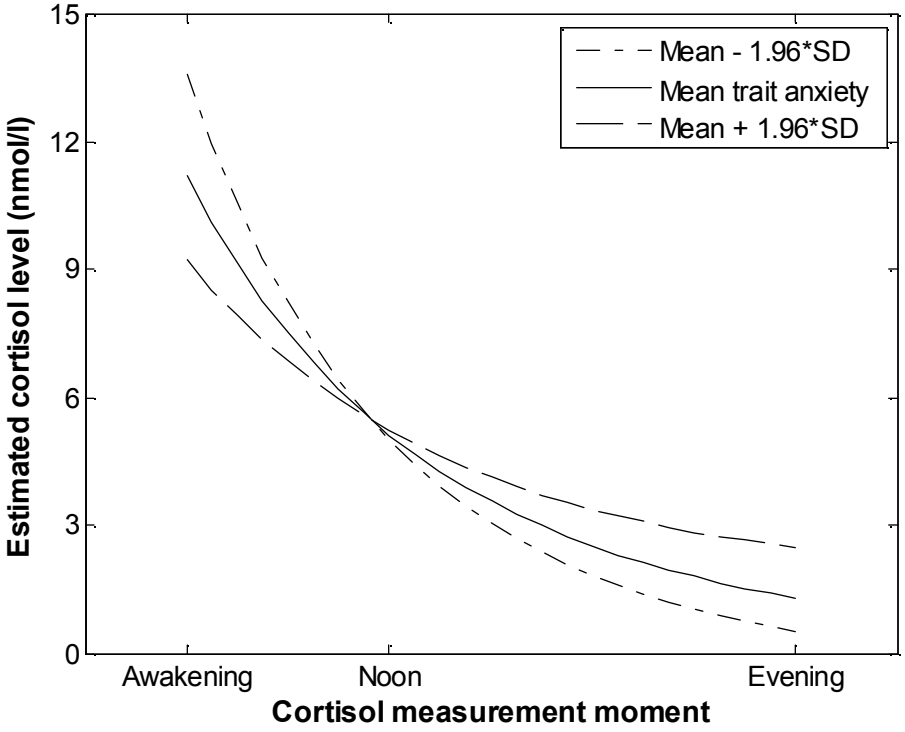

15
12
9
6
3
0
Estimated cortisol level (nmol/l)
Mean - 1.96*SD
Mean trait anxiety
Mean + 1.96*SD
Awakening
Noon
Evening
Cortisol measurement moment

Figure 5

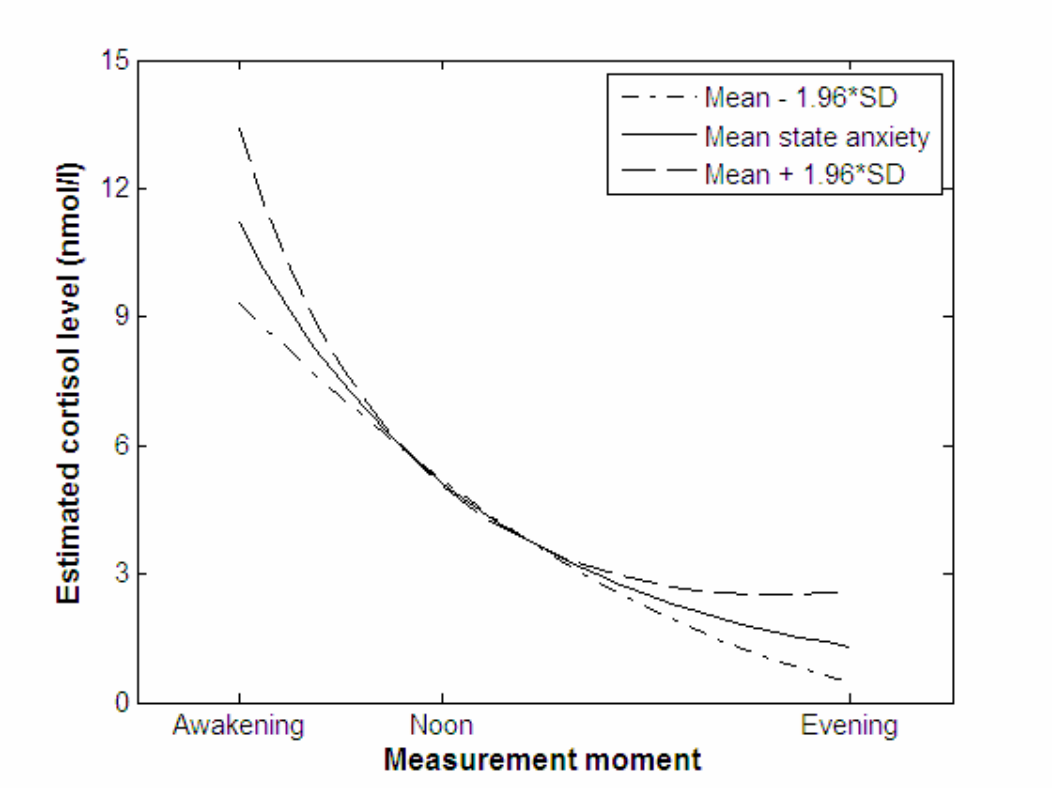

Mean - 1.96*SD
Mean state anxiety
Mean + 1.96*SD
Estimated cortisol level (nmol/l)
15
12
9
6
3
0
Awakening
Noon
Evening
Measurement moment

Figure 6

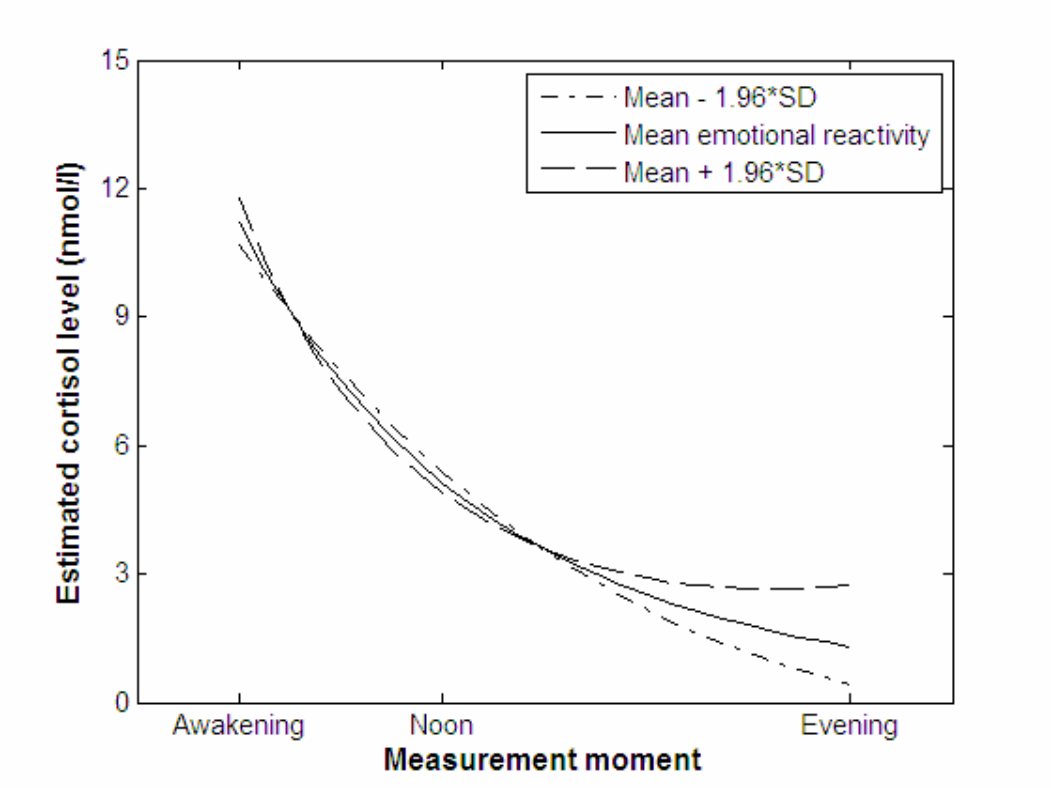

Mean - 1.96*SD
Mean emotional reactivity
Mean + 1.96*SD
Estimated cortisol level (nmol/l)
15
12
9
6
3
0
Awakening
Noon
Evening
Measurement moment

Table 1

Table 1 Descriptive statistics for key variables.

Variable	Mean (SD)	Median	Q1-Q3	Min-Max
Emotional distress				
Child Depression Inventory (CDI)	8.9 (5.44)	7.5	5 – 12	1 – 25
Trait anxiety (STAIC)	33.3 (6.68)	32.0	29 – 38	22 – 49
State anxiety (STAIC)	31.4 (2.85)	31.0	30 – 32	27 – 41
Emotional reactivity)(FCB-TI)	0.50 (0.208)	0.49	0.35 – 0.65	0.12 – 1
Internalizing problems:				
Mother/teacher scale (CBCL/TRF) [a]	0.029 (1.063)	-0.22	-0.76 – 0.48	-1.23 – 3.33
Cortisol				
Awakening cortisol level	12.2 (4.99)	11.8	9 – 15.4	1.5 – 25.4
Noon cortisol level	6.1 (4.24)	5.1	3.3 – 7.3	0.4 – 22.2
Evening cortisol level	2.3 (3.26)	1.3	0 – 3.3	0.0 – 17.8

Table 2

Table 2 Summary of results : ANOVA's and Tukey-Kramer pairwise comparisons between the 4 clusters

Emotional distress variable	ω^2	*P*	Cluster means 1 n=3	2 N=25	3 n=25	4 n=5	Pairwise comparisons: ω^2 (p-value)[b] 1 vs 2	1 vs 3	1 vs 4
Child depression inventory (CDI) [a]	.08	.06	4.08	2.94	2.66	2.64	.06 (.04)	.10 (<.01)	.07 (.03)
State anxiety (STAIC)	.11	.03	36.0	30.8	31.5	31.2	.14 (<.01)	.10 (<.01)	.08 (.02)
Trait anxiety (STAIC)	.07	.08	41.0	33.9	31.3	34.8	.04 (.08)	.08 (.02)	.01 (.19)
Emotional reactivity (FCB-TI)	.16	<.01	0.84	0.52	0.43	0.56	.10 (<.01)	.17 (<.01)	.04 (.05)
Internalizing problems: Mother/teacher scale (CBCL/TRF)	.11	.03	1.80	1.00	0.94	1.02	.12 (<.01)	.14 (<.01)	.07 (.02)

ω^2 : omega squared

[a] sqrt transformed

[b] pairwise comparisons between clusters 2, 3, and 4 are not shown (all $\omega^2 < .03$, all *p*-values > 0.11)

Table 3

Table 3 Overview of results of the final repeated measurements models

Emotional distress variable	Predictor	Parameter estimate (SD)	F	Df	P
Depression	Intercept	2.50 (0.061)	1702	1, 56	<.0001
(CDI)	Sqrt(CDI)	-0.064 (0.059)	1.17	1, 56	.2834
	Time	-0.19 (0.025)	55.9	1, 113	<.0001
	$Time^2$	0.0042 (0.0020)	4.51	1, 113	.0358
	Time*sqrt(CDI)	0.018 (0.0088)	4.07	1, 113	.0459
Trait anxiety	Intercept	2.50 (0.061)	1709	1, 56	<.0001
(STAIC)	Trait anxiety	-0.091 (0.059)	2.37	1, 56	.1294
	Time	-0.19 (0.025)	56.8	1, 113	<.0001
	$Time^2$	0.0042 (0.0019)	4.62	1, 113	.0338
	Time* trait anxiety	0.025 (0.0085)	8.75	1, 113	.0038
State anxiety	Intercept	2.50 (0.060)	1727	1, 56	<.0001
(STAIC)	State anxiety	0.084 (0.060)	1.94	1, 56	.1695
	Time	-0.19 (0.025)	56.5	1, 112	<.0001
	$Time^2$	0.0042 (0.0020)	4.58	1, 112	.0344
	Time*state anxiety	-0.040 (0.025)	2.48	1, 112	.1181
	$Time^2$*state anxiety	0.0043 (0.0020)	4.87	1, 112	.0294
Emotional	Intercept	2.50 (0.061)	1662	1, 56	<.0001
Reactivity	Emotional reactivity	0.023 (0.062)	0.14	1, 56	.7126
(FTB-CI)	Time	-0.19 (0.025)	56.4	1, 112	<0.0001
	$Time^2$	0.0042 (0.0019)	4.61	1, 112	.0339
	Time*ER	-0.026 (0.025)	1.02	1, 112	.3148
	$Time^2$*ER	0.0037 (0.0020)	3.50	1, 112	.0638
Internalizing	Intercept	2.50 (0.061)	1709	1, 57	<.0001
Problems	Time	-0.19 (0.025)	55.2	1, 114	<.0001
(CBCL/TRF)	$Time^2$	0.0042 (0.0020)	4.43	1, 114	.0374

$p<0.10$: borderline significant; $p<0.05$: significant