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Long-term stability of the cortisol awakening response over adolescence

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Summary The cortisol awakening response (CAR) has been widely assessed as a measure of hypothalamic-pituitary-adrenal (HPA) axis activity. Short-term stability is high; however, little is known about the long-term stability of the CAR. Because there are indications that development in adolescence influences HPA axis activity, this study investigated the stability of the CAR over adolescence.

Participants were 229 boys and 181 girls from an adolescent general population sample who were assessed in three consecutive years, at mean ages of 15.0 (SD = 0.4), 16.0 (SD = 0.4) and 17.0 (SD = 0.4) years. Cortisol was analyzed in saliva sampled at awakening, and 30 and 60 min later. Stability was investigated both as rank-order and as mean-level stability. Effects of physical development during adolescence on stability were investigated as well.

Rank-order stability was moderate to low, with tracking coefficients (interpretable as stability coefficients over time) of .15 ($p < .001$) for cortisol at awakening and .24 ($p < .001$) for cortisol 30 and 60 min after awakening. Mean-levels of cortisol at awakening did not change, while the response to awakening increased over the years (linear slopes for cortisol 30 and 60 min after awakening all $p < .01$). The increase may reflect the physical development of the adolescents.

This is the first study, in a large population based sample, indicating that the rank-order of the CAR is stable over the course of several years. Interestingly, mean-levels of the cortisol response to awakening increased over the years, suggesting a maturation of HPA axis reactivity in relation to physical development over adolescence. Physical development should therefore be taken into account when investigating the CAR as a measure of HPA axis activity in adolescence.

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1. Introduction

The cortisol awakening response (CAR) has been widely assessed as a measure of hypothalamic-pituitary-adrenal (HPA) axis activity. The CAR is superimposed on the circadian rhythm of cortisol secretion, and in addition to basal activity as reflected by day-time cortisol, it also reflects the reactivity or flexibility of the HPA axis (Fries et al., 2009). It is often used as a biological marker for disorders related to HPA axis (dys)functioning, such as anxiety and depression which are associated with increased CARs (Pruessner et al., 2003a; Greaves-Lord et al., 2007), and disruptive behavior disorders, which are associated with fearlessness and decreased CARs (Popma et al., 2007; Alink et al., 2008). Short-term stability of the CAR (i.e. over several days) has been shown to be remarkably high in adult samples (Wust et al., 2000; Edwards et al., 2001). Because the CAR is increasingly being used in longitudinal studies predicting behavioral and emotional outcomes (e.g. Adam et al., 2010; Ruttle et al., 2011), it is of relevance to learn more about the long-term stability of this measure of HPA axis activity. At present, it is not clear whether the CAR is a stable biological marker. It is also unknown whether the CAR changes with age or normal development. This fundamental knowledge is essential for interpretation of associations with disorders related to HPA axis (dys)functioning. Particularly in adolescence, physical development is an issue, as it is likely to influence HPA axis activity (Kiess et al., 1995; Walker et al., 2001; Rosmalen et al., 2005). For these reasons, in this study the long-term stability of the CAR over adolescence was investigated, taking into account physical development. Stability is investigated both as rank-order and as mean-level stability. While rank-order stability reflects the stability of an individual's position within the group, mean-level stability reflects the stability or change in mean-levels for the whole group.

To date, long-term rank-order stability of the CAR has not been described. As for day-time cortisol levels, there are indications that HPA axis activity is stable over time, with correlations up to .60 over 1.5—2 years (Walker et al., 2001). This stability level was, however, calculated over only 21 adolescents. Therefore it remains unclear whether the CAR constitutes a stable biological characteristic. Further study of the rank-order stability of the CAR is warranted.

As for mean-level stability, there are indications that the typical cortisol awakening response is not yet mature in childhood and adolescence (Clow et al., 2004). When studied in adult samples (Clow et al., 2004), cortisol levels follow a specific response curve with a sharp increase after awakening, followed by a gradual decrease. Several studies in child and adolescent samples, however, have found smaller (Pruessner et al., 1997, commented on by Clow et al., 2004; Rosmalen et al., 2005) or no (Freitag et al., 2009) cortisol response to awakening. It has therefore been suggested that the typical CAR as seen in adults may be different for children. An age effect on the response curve of cortisol after awakening has not been found cross-sectionally in child or adult populations (Pruessner et al., 1997; Wust et al., 2000; Edwards et al., 2001), although an age affect has been reported in children on the level of the total CAR (Michels et al., 2012). However, in the developmental phase in which maturation of the CAR is most likely to occur (i.e. adolescence), an age effect has remained uninvestigated. Further, the cross-sectional designs compared different groups of individuals, making them unable to distinguish true differences with age, from differences between individuals of different ages. The only longitudinal study describing long-term stability of mean-level HPA axis activity in adolescents focused on day-time cortisol levels (Walker et al., 2001). They reported an increase over 1.5—2 years in cortisol levels measured four times during the day. However, because to date changes in the CAR over time have not been investigated in the same individuals, conducting such a study is a necessity, as this is the only way to elucidate the issue of stability or change in the CAR over adolescence.

Physical development, e.g. pubertal development and changes in body mass index, is likely to influence the maturation and stability of the CAR during adolescence. Cross-sectional studies have shown cortisol levels to be higher in later compared to earlier stages of puberty (Kiess et al., 1995), while the CAR was found to differ by pubertal stage and gender (Matchock et al., 2007). Girls generally are about two years ahead of boys in pubertal development, and they have often been reported to show a higher CAR (Pruessner et al., 1997; Rosmalen et al., 2005). Furthermore, increasing body mass index (BMI) accompanying puberty is also likely to affect cortisol levels (Kiess et al., 1995; Rosmalen et al., 2005). Gender and physical development during adolescence should therefore be taken into account when investigating the stability of HPA axis activity.

Therefore, the aim of this study was to investigate the stability of the CAR as a measure of HPA axis activity over adolescence, in three annual assessments at ages 15, 16 and 17. Across these ages, (physical) maturation from adolescence to young adulthood may be captured (Root, 1973). Effects of gender and physical development during adolescence on stability of the CAR were investigated.

2. Methods

2.1. Participants

Participants in the final analyses were 410 adolescents (229 boys and 181 girls). They were part of the RADAR study (Research on Adolescent Development And Relationships), a Dutch population based cohort study on adolescent relationships with family members and friends and the development of personality and psychopathology. The RADAR study has been approved by the responsible Medical Ethics Committee, and all participants and their parents gave informed written consent. Participants were recruited from 230 elementary schools in urban and rural areas in the Netherlands. Of the 1569 randomly selected students, 364 (23%) did not meet inclusion criteria (both parents and sibling age $\geq$ 10 present, and sufficient understanding of the Dutch language) and 99 (6%) were not reachable. Of all eligible students, 636 (70%) agreed to participate in the study. However, 114 (7%) failed to provide written informed consent of all study members (adolescent, both parents, sibling and best friend) and could therefore not be included in the final study sample. The total RADAR cohort thus consists of 522 adolescents (294 boys and 228 girls), participating in annual assessment waves from age 13 on. Cortisol measurements were assessed from age 15 (wave 3) on.

Table 1 Main characteristics of participants taking part in the final analyses per year.

Age	Age 15	Age 16	Age 17
n[a]	375	325	295
Age	15.0 (0.4)	16.0 (0.4)	17.0 (0.4)
% Boys	56%	55%	56%
% Girls	44%	45%	44%
Sampling time Cort0	06:57 h (34 min)	07:01 h (34 min)	07:03 h (38 min)
Pubertal development[b]	3.3 (0.8)	3.6 (0.7)	–
BMI	20.1 (3.0)	20.7 (2.6)	22.3 (3.4)
Oral contraceptives[c]	–	39%	59%
Nicotine use[d]	16%	22%	25%
Alcohol use[d]	50%	74%	85%
Stressful experience[e]	18%	19%	16%

Note. Data represent means and SD or percentages.
[a] A total of 410 participants took part in the analyses at any time point.
[b] Mean and SD before transformation to Z-scores. Data present for ages 15 and 16.
[c] Percentages reflect OC use among female participants; data present for 67% of female participants at age 16 and 58% at age 17.
[d] Percentage of users per year after dichotomization.
[e] Percentage of participants reporting any stressful experience.

This study is based on data from the third (2008) to the fifth wave (2010) of RADAR, in this paper referred to as ages 15–17. Of the total RADAR cohort, 84% participated in the cortisol measurements at any year, consisting of 437 adolescents (243 boys and 194 girls, mean ages 15.0 (SD 0.4), 16.0 (SD 0.4), and 17.0 (SD 0.4). There was no difference in age, gender, pubertal development, BMI, or stressful experiences (all $p > .1$) between those who did and those who did not participate in the cortisol measurements. Participants were more often smokers than non-participants (16% vs. 5%, $\chi^2 = 7.704$, $p < .01$ at age 15; 25% vs. 12%, $\chi^2 = 6.820$, $p = .01$ at age 17). After exclusion on the basis of sampling errors, technical problems in the lab, or statistical outliers (see below), 410 participants took part in the final analyses. Main characteristics of these participants are described in Table 1.

2.2. Attrition analysis

At any year 410 participants took part in the final analysis, but the number of participants fluctuated per year, as participants could drop out or (re)participate at any year (see Table 1). At age 15, 383 boys and girls participated in the cortisol measurements, and 375 took part in the analysis after exclusion on the basis of sampling errors, technical problems in the lab, or statistical outliers (see below). Of the 383 participants at age 15, 263 (69%) completed all assessment years. At age 16, 66 (17%) dropped out; they did not differ from participants who completed assessments at ages 15 and 16, on age, gender, pubertal development, BMI, oral contraceptive (OC) use, nicotine or alcohol use, or stressful experiences (all $p > .1$). After exclusion, 325 took part in the analysis at age 16. At age 17, 54 (14%) dropped out; they did not differ from those who completed all years of cortisol measurements, on gender, pubertal development, BMI, OC use, nicotine or alcohol use, or stressful experiences (all $p > .1$), but were on average 0.13 years older ($t(315) = 2.00$, $p < .05$). After exclusion, 295 boys and girls took part in the analysis at age 17.

2.3. Cortisol assessment

Cortisol was measured in saliva. Saliva samples were collected by passive drooling, immediately after awakening (Cort0), and 30 min (Cort30) and 60 min (Cort60) later. These three samples constitute the cortisol awakening response (CAR; Pruessner et al., 1997). Cortisol sampling took place in February and March of each consecutive year. Participants were first given detailed verbal and written information regarding cortisol measurements. Subsequently, saliva sampling was planned for a suitable morning on a regular weekday. The first sample (at awakening) was planned before 08:00 h, taking into consideration the participant's normal schedule. Sampling times were set and written on a detailed instruction form.

Participants were instructed to rinse their mouths with water before sampling, and not to eat, drink milk or juice, smoke or brush their teeth before completing the third sample (Cort60). They were requested to report the exact sampling times on the instruction form on the day of sampling, and also to report if violations were made against any of the above. After collection, participants were asked to store the samples in the refrigerator and send them by mail to the research center the same day.

At the research center, all samples were checked for correctness of sampling. When necessary, e.g. when Cort0 was sampled after 08:00 h or sampling time of Cort30 or Cort60 was over 15 min late, or mistakes were made in any of the other instructions, participants were asked to collect new saliva samples, and a new sampling day was scheduled. A total of 3063 samples were collected. Per year, participants were excluded when they started sampling after 08:15 h ($n = 6$, 18 samples). Eight single samples were excluded because they were sampled over 15 min late on Cort30 or Cort60. When participants did not follow the sampling procedure correctly for one or more samples after repeated instruction, corresponding samples were excluded (14 samples). In total 40 samples were excluded for incorrect sampling, and 3023 samples remained.

Saliva was stored uncentrifuged at −20 °C until analysis. Salivary cortisol levels were analyzed using

electrochemiluminescence immunoassay ECLIA (E170 Roche, Switzerland). The lower detection limit was 0.5 nmol/l, and mean intra-assay and inter-assay coefficients of variation were, respectively, 3% and 12%. Due to technical problems in the lab (i.e. too little saliva present or contaminated samples), 151 samples could not be assayed, leaving 2872 samples available for further analysis.

2.4. Physical development

Physical development was assessed as pubertal development and body mass index (BMI). Pubertal development was assessed at ages 15 and 16. It was measured by a modification of the Pubertal Development Scale (PDS; Petersen et al., 1988), a self-report questionnaire consisting of seven questions regarding physical development (i.e. growth spurt, axillary hair, pubarche, menarche, thelarche, voice change and facial hair). In this modified version responses were coded on a five-point scale with respect to the time frame, from ''not yet noticed'' (1) to ''for more than two years'' (5). Also, a question concerning body hair was split into two questions (axillary hair and pubarche separately) and a general question concerning ''bodily change'' was added. The possible range of the mean scores was 0.9–4.4. Next, Z-scores were calculated separately for boys and girls to indicate the individual pubertal development compared to the whole sample. The body mass index was calculated as weight in kg/(length in m)^2 in all three assessments.

2.5. Control variables

Because oral contraceptives (OC), substance use and stressful experiences could affect the CAR (Clow et al., 2004; Gustafsson et al., 2010), these factors were controlled for. Whether or not girls used oral contraceptives at the moment of sampling was added to the assessment from age 16 on (yes/no). Substance use was evaluated in all three assessments as nicotine and alcohol use (Monshouwer et al., 2008). Nicotine use was assessed by a nine-option question ranging from ''I have never smoked'' to ''I smoke every day''. For presentation purposes in Table 1, nicotine use was dichotomized: ''I have never smoked'' to ''I smoke less than once a month'' was defined as not using nicotine, and ''I do not smoke weekly, but at least once a month'' to ''I smoke every day'' was defined as using nicotine. Alcohol use over the last four weeks was evaluated in all three assessments by means of a six-option question, ranging from ''none'' to ''daily''. In Table 1, alcohol use was also dichotomized: ''none'' was defined as not using alcohol, and ''1–3 days in the last four weeks'' to ''every day'' was defined as using alcohol. Stressful experiences in the past year, such as sexual assault, physical assault, and being threatened with violence, were assessed with a questionnaire based on the International Crime Victims Survey (ICVS; Nieuwbeerta, 2002), and specified by perpetrator (parent = 2, someone else = 1, not = 0). Being threatened with violence occurred most frequent, in 14% of the participants at age 15 and 13% at ages 16 and 17; in less than 1% was a parent the perpetrator. Physical assault was reported by 6% at age 15, 4% at age 16 and 3% at age 17. With the exception of one participant, someone other than a parent was the perpetrator. Sexual assault was reported by 3% at ages 15 and 17, and 4% at age 16; for 5 participants (1.2%) a parent was the perpetrator.

2.6. Statistical analyses

Cortisol values over 3 SD above the mean were defined as outliers and excluded (30 samples), leaving 2842 of the 2872 samples assayed available for statistical analysis, for a total of 410 participants.

Analyses were initially performed on the raw data of cortisol at awakening (Cort0), and after 30 (Cort30) and 60 min (Cort60). Because the Area Under the Curve with respect to ground (AUCg) and Area Under the Curve with respect to increase (AUCi) are often used as measures of the CAR (Pruessner et al., 2003b), these were calculated as well. The AUCg reflects the total cortisol output during the first hour after awakening (AUCg = (((Cort30 + Cort0) * 30)/2) + (((Cort60 + Cort30) * 30)/2)). The AUCi reflects the cortisol output in response to awakening, and is computed as the AUC during the first hour after awakening with reference to Cort0 levels (AUCi = AUCg − (Cort0 * 60)).

Stability of the CAR measures over time was analyzed as rank-order and mean-level stability.[1] Rank-order stability was analyzed using generalized estimating equations (GEE; Zeger and Liang, 1986) for continuous outcome in SPSS 19.0. This method is suitable for analyzing longitudinal data and makes use of all available data. GEE accounts for dependence of repeated measures within one person by using a working correlation structure. An exchangeable correlation structure was used for all analyses. In order to assess rank-order stability, tracking of the CAR measures was analyzed by regressing the value at age 15 of each measure on the changes over ages 16 and 17 of the same measure (Twisk, 2003). The obtained standardized regression coefficient can be interpreted as a tracking coefficient, with a possible range between −1 and 1. All models were tested for interactions and confounding of time.

Mean-level stability or change trajectories of the CAR over time were examined by Latent Growth Modeling (LGM; e.g. Kline, 2005) within *Mplus* 6.0 (Muthén and Muthén, 2007) with maximum likelihood estimation (Satorra and Bentler, 1994). Mean-levels of the CAR at each year were used as indicators to estimate the latent intercept (i.e. mean-level of the CAR) and slope (i.e. mean change in the CAR over time) factors in LGM. In LGM, individual differences in change trajectories are captured by including variances for the latent growth parameters (i.e. the intercept and slope variance). Models with linear and quadratic growth functions were compared. The best fitting growth model for the CAR was based upon several goodness-of-fit indices (Kline, 2005): the comparative fit index (CFI), the root mean square error of approximation (RMSEA), and the chi-square difference test.

Gender differences and effects of physical development in the same year on rank-order and mean-level stability were examined. On rank-order stability, interaction terms for gender, pubertal development and BMI with the CAR measures were tested in the tracking analyses. In case of a significant

[1] The analyses were performed using all available data points. When reanalyzing the results including only those participants with >6 samples/data points available, the results did not change.

interaction effect, stratified analyses were performed. For BMI, groups were formed by a median split across all observations. Next, gender, pubertal development and BMI were added to the models to test for confounding effects. For mean-level stability of the CAR, gender differences were examined by multi-group LGM analyses. Using chi-square difference tests, an unconstrained model, allowing differences between boys and girls in growth parameters, was compared against constrained models with no differences between boys and girls in intercept mean or mean linear and quadratic growth parameters. To examine whether potential changes in the CAR would be due to physical development, in the final model the observed measures of the CAR per year were regressed on pubertal development and BMI assessed in the same year.

Next the influence of OC use, substance use and stressful experiences in the same year on rank-order and mean-level stability was controlled for. For rank-order stability, interaction terms of these control variables with the CAR measures were tested in the tracking analyses, and the control variables were added to the models to test for confounding effects. In case of a significant interaction effect, stratified analyses were performed. For alcohol use, two groups were formed based on whether alcohol use was reported for that year. For mean-level stability, to examine whether potential changes in the CAR would be due to OC use, stressful experiences, or substance use, in the final model the observed measures of the CAR per year were regressed on the control variables assessed in the same year.

Whether maturation of the CAR occurs (i.e. the typical CAR was found in each year), was investigated by examining the changes in cortisol from awakening through 30 and 60 min after awakening using a multi-group multivariate LGM. The intercepts of cortisol, as well as the slopes of cortisol, were allowed to correlate for the three years.

3. Results

3.1. Rank-order stability: tracking

Rank-order stability of cortisol at awakening, and 30 and 60 min later over time, as well as the areas under the curve (AUCg and AUCi), are represented by standardized tracking coefficients. These are interpretable as coefficients of stability over time and have a possible range from −1 to 1. For Cort0 the tracking coefficient was low (β = .15, p = .001). For Cort30 and Cort60 the tracking coefficients were moderate (both β = .24, $p < .001$). For the AUCg the tracking coefficient was also moderate (β = .29, $p < .001$) and for the AUCi the tracking coefficient was low (β = .17, $p < .001$). The rank-order stability did not change across the three years.

No effect of gender was found on the rank-order stability of Cort0, Cort30, Cort60, or on the AUCg. On the rank-order stability of the AUCi, however, a gender interaction was found. Boys showed a tracking coefficient of .26 ($p < .001$), whereas girls did not show significant rank-order stability on the AUCi, with a tracking coefficient of .05 (p = .488).

For physical development, there was no effect of pubertal status on the rank-order stability of the CAR measures. For BMI an interaction effect was found with the rank-order stability of Cort30 and Cort60. Follow-up analyses after a median split across all observations on BMI, revealed higher tracking coefficients in the high BMI group (Cort30: β = .35, $p < .001$; Cort60: β = .31, $p < .001$) than in the low BMI group (Cort30: β = .12, $p < .05$; Cort60: β = .17, $p < .05$).

As for the control variables, only effects of substance use were found on rank-order stability, and only for Cort60. Concurrent nicotine use was a confounder of the rank-order stability of Cort60: controlling for nicotine use resulted in a decreased stability of Cort60 (β = .21, $p < .001$). Concurrent alcohol use interacted with the rank-order stability of Cort60. Follow-up analyses revealed higher tracking coefficients of Cort60 for alcohol use (β = .28, $p < .001$), compared to no alcohol use (β = .01, p = .944), although the latter may reflect a power problem, as this concerned only 84 (24%) observations. Oral contraceptives were not related to the CAR measures (all $p > .1$), and no effect of stressful experiences was found on the rank-order stability of either measure.

3.2. Mean-level stability: growth curve models

In Table 2 the observed mean levels and standard deviations of cortisol at awakening, and 30 and 60 min later, as well as

Table 2 Observed means and standard deviations for the CAR over adolescence.

Age	Age 15		Age 16		Age 17	
	M	σ	*M*	σ	*M*	σ
Boys						
Cort0	19.28	8.62	18.83	8.84	19.73	9.06
Cort30	16.06	7.44	16.53	7.61	19.61	9.47
Cort60	14.62	7.29	15.44	8.43	18.75	8.74
AUCg	997.83	347.75	1012.40	379.06	1138.40	438.35
AUCi	−164.06	403.06	−130.89	392.93	−21.06	341.48
Girls						
Cort0	18.81	7.37	18.37	8.28	20.46	9.70
Cort30	17.92	8.69	18.70	10.84	21.79	10.92
Cort60	16.87	9.10	17.87	10.54	22.02	11.31
AUCg	1078.01	396.47	1080.37	474.55	1283.38	511.19
AUCi	−45.40	394.12	−15.47	475.55	63.90	500.25

Note. AUCg = Area Under the Curve with respect to ground; AUCi = Area Under the Curve with respect to increase.

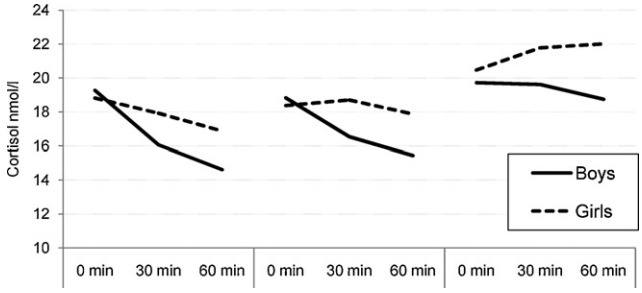

Figure 1 Cortisol awakening response over adolescence for boys and girls.

the AUCg and AUCi are presented. In Fig. 1 the course of the CAR across the three years is visually displayed. First, the stability or change in cortisol at awakening and 30 and 60 min later over the three years was estimated using a multivariate LGM. The observed scores of Cort0, Cort30 and Cort60 were allowed to correlate within each year. Results suggested linear growth for Cort0, Cort30 and Cort60 (see Table 3). For Cort0 the linear slope factor was not significantly different from zero, whereas Cort30 and Cort60 increased over time (see Table 4). The course of the AUCg and the AUCi can be seen in Figs. 2 and 3, respectively. Results of the LGMs on the AUCg and the AUCi suggested quadratic growth over the three years for the AUCg and linear growth for the AUCi (see Table 3).

Gender differences were found in multi-group LGM analyses on Cort0, Cort30 and Cort60, and the AUCi. On Cort0, Cort30 and Cort60, differences were found between boys and girls in intercept means ($\Delta\chi^2(3) = 10.65$, $p < .05$), with girls having higher intercepts at Cort30 and Cort60 than boys (see Fig. 1). There were no gender differences in slope means ($\Delta\chi^2(3) = 1.16$, $p = .76$). On the AUCi there were differences between boys and girls in intercept mean ($\Delta\chi^2(1) = 9.08$,

Table 3 Fit Indices from hierarchical analyses of growth factors in latent growth modeling.

Growth factor	χ^2	df	CFI	RMSEA	90% CI RMSEA	$\Delta\chi^2$-test
Cort 0-30-60						
Linear growth	**35.050**	**21**	**.973**	**.040**	**[.013, .063]**	
Quadratic growth	28.392	18	.098	.037	[.000, .062]	.081
AUCg						
Linear growth	7.285	2	.879	.082	[.024, .150]	–
Quadratic growth	**0.273**	**1**	**1.000**	**.000**	**[.000, .108]**	**< .01****
AUCi						
Linear growth	**2.976**	**2**	**.915**	**.035**	**[.000, .113]**	**–**
Quadratic growth	1.788	1	.931	.045	[.000, .151]	.276

Note. The best-fitting growth functions are in boldface. CFI = comparative fit index; RMSEA = root mean square error of approximation; 90% CI RMSEA = 90% confidence interval of the root mean square error of approximation; $\Delta\chi^2$-test = chi-square difference test; AUCg = Area Under the Curve with respect to ground; AUCi = Area Under the Curve with respect to increase.
** $p < .01$.

Table 4 Mean-level stability of the CAR over adolescence, results of latent growth curve models.

	Boys		Girls	
	M (SE)	σ^2 (SE)	*M* (SE)	σ^2 (SE)
Cort 0-30-60				
IC 0	19.06 (0.58)**	18.89 (4.82)**	18.43 (0.58)**	5.55 (4.66)
SL 0	0.25 (0.42)	0.31 (2.93)	0.73 (0.51)	1.18 (4.17)
IC 30	15.76 (0.50)**	15.98 (3.69)**	17.68 (0.66)**	19.78 (6.85)**
SL 30	1.66 (0.39)**	0.00 (0.00)	1.86 (0.54)**	0.88 (5.64)
IC 60	14.29 (0.49)**	10.10 (3.68)**	16.41 (0.69)**	25.32 (7.41)**
SL 60	2.03 (0.39)**	0.00 (0.00)	2.61 (0.56)**	2.03 (5.88)
AUCg[a]				
IC	1021.28 (20.21)**	48551.90 (8106.18)**	1021.28 (20.21)**	48551.90 (8106.18)**
SL	−49.69 (50.36)	18.99 (1939.54)	−49.69 (50.36)	18.99 (1939.54)
QU	69.12 (26.00)**	0.00 (0.00)	69.12 (26.00)**	0.00 (0.00)
AUCi				
IC	−175.81 (27.22)**	34971.98 (9327.98)**	−50.17 (31.11)	6704.86 (12120.28)
SL	71.04 (17.96)**	1.88 (100.13)	54.21 (27.67)*	1.88 (100.13)

Note. IC = intercept, estimate of cortisol levels at age 15; SL = linear slope, estimate of change in cortisol levels over years; QU = quadratic slope, estimate of change in cortisol levels over years; AUCg = Area Under the Curve with respect to ground; AUCi = Area Under the Curve with respect to increase.
[a] As there were no gender differences on the AUCg, mean-level stability is estimated the same for boys and girls.
* $p < .05$.
** $p < .01$.

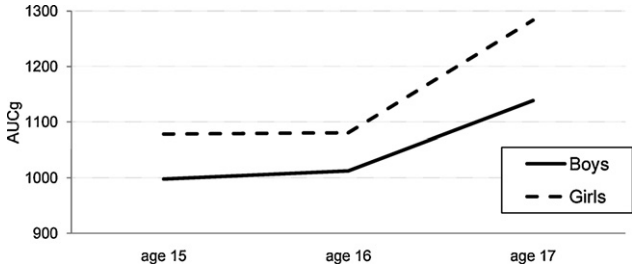

Figure 2 AUCg over adolescence for boys and girls.

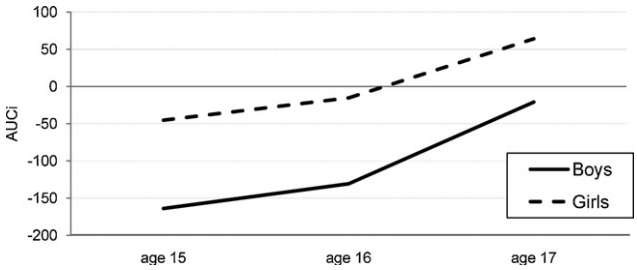

Figure 3 AUCi over adolescence for boys and girls.

$p < .01$), with girls scoring higher than boys. There were no gender differences in slope mean ($\Delta\chi^2(1) = .28$, $p = .60$). On the AUCg there were no differences between boys and girls in intercept mean ($\Delta\chi^2(1) = 3.06$, $p = .08$) or slope mean ($\Delta\chi^2(2) = 1.44$, $p = .49$) (see Table 4 and Fig. 3).

After controlling for physical development there were no significant slopes of Cort30 and Cort60 for either boys or girls. Controlling for physical development also resulted in a non-significant quadratic slope for both the AUCg and AUCi.

Controlling for substance use resulted in a non-significant slope of Cort30 and Cort60, and a non-significant quadratic slope of the AUCg, for both boys and girls. For girls only, controlling for substance use resulted in a non-significant slope of the AUCi. Oral contraceptives were not related to the CAR measures, and no significant effect of stressful experiences was found on the mean-level stability of either CAR measure.

Finally, whether the typical cortisol response to awakening was found in each year for boys and girls was examined. For boys the linear slope factor was significant and negative at age 15 ($SL = -2.16$, $p < .001$) and at age 16 ($SL = -1.70$, $p < .001$) and not significantly different from zero for cortisol at age 17 ($SL = -0.56$, $p = .144$). For girls the linear slope factor was significant and negative at age 15 ($SL = -1.06$, $p < .01$), not significantly different from zero at age 16 ($SL = -0.16$, $p = .742$), and positive and marginally significant at age 17 ($SL = .96$, $p = .074$). Correspondingly, the percentage of participants showing an increase in cortisol from awakening to 30 min later (i.e. responders) increased over time. An increase was observed in 43% of the participants at age 15, 47%, at age 16 and 54% at age 17. The percentage of responders was larger among girls than among boys at age 15 (51% vs. 38%, $\chi^2 = 6.197$, $p < .05$), but not at age 16 or 17.

4. Discussion

This study investigated the rank-order and mean-level stability of HPA axis activity as measured by the cortisol awakening response in adolescence, from ages 15 to 17. Our findings showed moderate to low rank-order stability for the CAR. Mean-levels of the CAR and specifically the response to awakening increased over adolescence. Changes in stability could largely be explained by physical development during adolescence.

The rank-order stability found in this study is in line with the variance in cortisol levels previously reported to remain constant over one year (Kirschbaum et al., 1990; Shirtcliff et al., 2005). The current study adds that even across three years there is constant rank-order stability. The moderate to low level in rank-order stability is not surprising, considering the substantial influence of contextual factors on HPA axis activity (Kirschbaum et al., 1990; Shirtcliff et al., 2005). Together this suggests that HPA axis activity is partly due to individual and stable trait factors, on top of which variation due to state factors occurs. As previously reported, this variation is in part due to day-to-day changes and analysis error (Kirschbaum et al., 1990; Shirtcliff et al., 2005).

Adolescence is pre-eminently a period characterized by change and development. This could also account for changes in rank-order of HPA axis activity over time when, for instance, one individual matures faster than another, or someone starts smoking and drinking alcohol. We therefore incorporated the most important factors that have previously been found to influence HPA axis activity in cross-sectional studies (Clow et al., 2004; Gustafsson et al., 2010). Physical development is prominent in adolescence, and as one might expect, rank-order stability was higher for individuals who were physically more mature. The influence of substance use was small and limited to cortisol 60 min after awakening. OC use and stressful experiences did not influence rank-order stability.

Other changes could also have accounted for rank-order changes in HPA axis activity, such as life events and (psycho)social factors. Many emotional and behavioral problems, such as substance use, disruptive behavior disorders, but also depression and anxiety, are known to significantly increase in prevalence during the period of adolescence (McGee et al., 1992). As these problems or disorders are related to HPA axis (dys)functioning (Pruessner et al., 2003a; Greaves-Lord et al., 2007; Popma et al., 2007; Alink et al., 2008), the onset of such disorders will most likely affect an individual's rank-order in HPA axis activity as well. These factors fell outside the scope of this study, as we aimed to investigate the stability of the CAR over adolescence, taking into account normal adolescent development. This fundamental knowledge of HPA axis activity is in turn essential for future longitudinal studies to better understand associations with, for instance, life events, (psycho)social factors and disorders related to HPA axis (dys)functioning. It would also be of importance for future studies to investigate whether (psycho)social factors, life events, or substance use may interact with HPA axis activity, and thereby lead to changes in disorders related to HPA axis (dys)functioning.

Mean-levels of HPA axis activity, and specifically the response to awakening, increased over adolescence. As this is the first longitudinal study repeatedly measuring the CAR within individuals, our results indicate that the response to awakening matures over adolescence. This is in concordance with previous cross-sectional studies that have suggested

that the CAR is not yet mature in childhood and adolescence (Clow et al., 2004; Rosmalen et al., 2005).

Our results further show that the increase in the response can be explained by physical development, and that the maturation of the CAR is stronger for girls, synchronous with the gender difference in pubertal timing. This suggests that the CAR maturation may be part of normal physical development in adolescence. This hypothesis is in line with the current perspective on the maturation of basal HPA axis activity in adolescence (see review by Gunnar and Vazquez, 2006). Possible mechanisms may lie in interactions with the hypothalamic-pituitary-gonadal (HPG) axis. Reciprocal interactions between the HPA and HPG axes have been described (Viau, 2002), and in adolescence the HPG axis is activated, resulting in a substantial increase in gonadal hormones (Sisk and Zehr, 2005). Although there are indications from animal research that both HPG and HPA axis activity changes substantially with adolescent maturation, the exact mechanisms are still poorly understood (for review see Romeo, 2010), and further investigation is warranted. In order to fully assess the maturation of the CAR, this interesting finding and its possible mechanisms should be further investigated in longitudinal studies covering the development from child to adult.

On mean-level stability, the same factors were of influence as on rank-order stability. In addition to physical development, substance use influenced the mean-level stability of HPA axis activity, but oral contraceptive use and stressful experiences did not. Since many individuals use substances like nicotine and alcohol, future studies assessing HPA axis activity should take substance use into account. As substance use generally commences in adolescence, our results indicate that longitudinal studies especially should monitor if and when participants start using substances.

The results of this study should be interpreted in light of some methodological limitations. First, the CAR was assessed in February and March of each year; sampling in these dark months could explain the small CARs in this study, through lack of light exposure at awakening (Scheer and Buijs, 1999; Thorn et al., 2004; Rosmalen et al., 2005). A strength, however, is that all participants sampled in these months on all annual assessments, so the influence of sampling month on rank-order and mean-level stability could only be minimal in this study.

Second, saliva was sampled on one day only; correcting for day-to-day variation was therefore not possible. Previous studies have, however, already shown that day-to-day variation of the CAR is low (Wust et al., 2000; Edwards et al., 2001), and despite possible day-to-day variation, there was still significant stability over the years.

Third, saliva sampling was done at home. Although we took all possible precautions in the sampling procedure, including self-report of exact sampling times, directly monitoring participants' compliance was not possible. This may have been a factor in the absence of a typical response to awakening as observed in the first annual assessment. However, the typical response did develop over subsequent assessments, and there is no reason to assume that participants' compliance would increase over time. Also, sampling of the CAR at home was previously found not to differ from sampling in a controlled laboratory environment (Wilhelm et al., 2007). Self-reported times have even been found to be preferable to automatic time recording (Kraemer et al., 2006). More importantly, stability of the CAR using home sampling is highly relevant, as it is common procedure in many (ongoing) longitudinal studies in the field of HPA axis research, such as the Whitehall II study (Steptoe et al., 2003; Kunz-Ebrecht et al., 2004), TRAILS (Rosmalen et al., 2005) and NESDA (Penninx et al., 2008). The current study shows that with a home sampling design, significant stability of the CAR can be found.

Fourth, pubertal development was only assessed at ages 15 and 16. As BMI is highly correlated with pubertal development, we included BMI and combined both measures to assess physical development. When analyzing BMI and pubertal development separately, BMI showed more variation and development over time, and was also more strongly related to the CAR. Pubertal development should be assessed more accurately and completely in future studies, as the effect of physical development on the stability of the CAR may be even stronger. OC use in girls was only assessed at ages 16 and 17, with a response rate of 58—67% of the girls. OC use was not associated with any of the CAR measures in this subsample, yet it would be important to study the influence of OC use in more detail in future studies.

In conclusion, over adolescence, a period characterized by change and development, the rank-order between individuals in HPA axis activity remains moderately stable. This indicates that the CAR is a stable biological marker for use in longitudinal studies on the relation between HPA axis activity and disorders related to HPA axis (dys)functioning such as anxiety, depression, and disruptive behavior disorders in adolescence. With physical development and commencing substance use typical for adolescence, however, mean-levels of HPA axis activity increased and the typical response to awakening matured. This underlines the importance of taking physical development and substance use into account when investigating HPA axis activity in adolescence.

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Conflict of interest

All authors declare that they have no conflict of interest.

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