

Alterations in HPA-axis and autonomic nervous system functioning in childhood anxiety disorders point to a chronic stress hypothesis

Dieleman, G.C., Huizink, A.C., Tulen, J.H., Utens, E.M., Creemers, H.E., Van der Ende, J., & Verhulst, F.C. (2015).

Psychoneuroendocrinology, 51, 135-150.

ABSTRACT

Background: It is of debate whether or not childhood anxiety disorders (AD) can be captured by one taxonomic construct. This study examined whether perceived arousal (PA), autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis measures can distinguish children with different primary diagnoses of clinical anxiety disorders (AD) from each other, and from a general population reference group (GP).

Methods: The study sample consisted of 152 AD children (comparing separation anxiety disorder, generalized anxiety disorder, social phobia and specific phobia), aged 8- to 12 years, and 225 same-aged reference children. HPA-axis functioning was measured by a diurnal cortisol profile. ANS functioning was measured by continuous measures of skin conductance level in rest and during a mental arithmetic task and high frequency heart rate variability in rest. PA was assessed by a questionnaire.

Results: The AD sample showed lower high frequency heart rate variability during rest, heightened anticipatory PA, higher basal and reactive skin conductance levels and lower basal HPA-axis functioning compared to the GP sample. The existence of 3 or more clinical disorders, i.e. a high clinical 'load', was associated with lower basal HPA-axis functioning, higher skin conductance level and lower posttest PA. Specific phobia could be discerned from social phobia and separation anxiety disorder on higher skin conductance level.

Conclusions: Our findings indicated that children with AD have specific psychophysiological characteristics, which resemble the psychophysiological characteristics of chronic stress. A high clinical 'load' is associated with an altered ANS and HPA-axis functioning. Overall, ANS and HPA-axis functioning relate to AD in general, except for specific phobia.

INTRODUCTION

Anxiety disorders (AD) are among the most prevalent psychiatric disorders in children and adolescents (e.g. Costello, Egger, Angold, 2005). They are associated with impaired functioning, such as school dropout, social isolation, alcoholism, and suicide attempts, and an increased risk for developing other psychiatric disorders (Bittner et al., 2007). Hence, research into causes and correlates of childhood anxiety disorders is imperative to improve treatment and prognosis for children affected by these disorders.

A complicating issue is whether distinctions between separate anxiety disorders in children can be made. High comorbidity rates between AD have been reported (e.g. Beesdo, Knappe, Pine, 2009). This underlines the hypothesis of one taxonomic construct capturing childhood anxiety disorders. However, substantial heterogeneity exists in the age of onset of specific anxiety disorders (Beesdo et al., 2010), providing, in terms of taxonomy, an indicator to separate different types of anxiety disorders (Beesdo, Knappe, Pine, 2009). Furthermore, age is also related to the clinical 'load'; i.e. the number of anxiety disorders increases with age. Anxiety load is significantly associated with poorer outcome (Woodward, Fergusson, 2001). One could argue that the causes and correlates of a high anxiety 'load' may differ from those of a low anxiety 'load'.

One way to test the taxonomic construct of AD is to study the physiological correlates of childhood anxiety disorders, i.e., the activity of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS), two major physiological stress systems. During non-stress conditions the HPA-axis shows a diurnal pattern of cortisol secretion, with peak levels approximately 30 minutes after waking up and a subsequent decline during the day (Wust et al., 2000). Glucocorticoids can act both to augment and suppress sympathetically mediated changes in e.g. cardiovascular function, metabolism, and immune function. Normally, activation of these stress systems leads to behavioral and physical adaptive changes that improve an organism's ability to survive. However, children with an anxiety disorder may perceive the world as full of stressors that demand endless vigilance and coping, with no possibility to relax and to regard things as safe (Sapolsky, 2002). It can be hypothesized that these children function under conditions of persistent stress, with an excessive and prolonged stress system activation. This would result in increased and prolonged production of hypothalamic corticotropin releasing factor (CRF), cortisol and catecholamines (Pervanidou, 2008), which, in turn, could result in hypoactivation of the HPA-axis, as a compensatory down-regulation (Charmandari, Tsigos, Chrousos, 2005).

Studies regarding the association between anxiety disorders and basal HPA-axis functioning in children are scarce and findings are inconclusive. Forbes et al. (2006) found higher peri-sleep-onset cortisol levels in children with an anxiety disorder compared to children with a depression or healthy control children, whereas Feder et al. (2004) found that anxious children exhibited significantly lower nighttime cortisol levels and a delayed rise in cortisol

during the nighttime when compared to depressed and healthy children. In other studies no association was found (e.g. Terleph et al., 2006; Greaves-Lord et al., 2007; Dieleman et al., 2010). However, Greaves-Lord et al. (2007) reported that young adolescents with persistent anxiety problems had higher morning cortisol levels and a higher awakening response.

Several problems may have contributed to the inconsistency of these findings. The groups under study were dissimilar in methods of sampling, age, developmental status, and diagnostic status. Some studies used general population samples (e.g. Greaves-Lord et al., 2007), whereas others focused on clinical groups (e.g. Forbes et al., 2006), which are likely to display major differences in symptom severity and comorbidity. Next to that, differences in functioning of the HPA-axis could depend on the stage of the disorder (Pervanidou, 2008).

Overall, research on ANS functioning in children with anxiety problems shows an increased activity of the sympathetic nervous system (e.g. Dietrich et al., 2007; Schmitz et al., 2011; Kossowsky et al., 2012) and a decrease of parasympathetic control (e.g. Schmitz et al., 2011). Furthermore, previous studies suggest that high anxious adults have an increased perception of physiological sensations (e.g. Hoehn-Saric, McLeod, 2000), i.e. increased perceived arousal, sometimes even in the absence of an actual difference in physiological measures (e.g. Edelman, Baker, 2002).

At present, it is still unclear as to what extent ANS or HPA-axis activity relate to anxiety in general, or whether they are specific correlates of the different types of anxiety disorders. Few studies have compared the endocrine and autonomic profiles between different pediatric anxiety disorders, and if so the focus was on one specific anxiety disorder with a disorder-specific stimulus (i.e. Kossowsky et al., 2012). To our knowledge this is the first study to test whether anxiety load is associated with altered HPA-axis and ANS functioning.

The present study's aim was to test whether HPA-axis (basal), ANS (basal and reactive) and perceived arousal (PA) measures can distinguish children (N=154) aged 8-12 years with different primary diagnoses (separation anxiety disorder (SAD), generalized anxiety disorder (GAD), social phobia (SoPh) and/or specific phobia (SpPh)) of clinical anxiety disorders (AD) from each other, and from a same-aged general population reference group (GP) (N=225). We hypothesized that children in the clinical anxiety sample show 1) elevated sympathetic and lowered parasympathetic activity compared to the general population sample. 2) Furthermore, we expected a pattern of hypoactivation of basal HPA-axis functioning, considering the severity and chronicity of perceived stress in a clinical AD sample, when compared to a general population reference group. 3) Within the AD sample we hypothesized that there would be no difference in activity of both ANS and HPA-axis between different types of anxiety disorders, because of the known high rates of comorbidity in childhood anxiety disorders. 4) Finally, we hypothesized that children with a high anxiety load in the clinical sample show hypoactivation of basal HPA-axis functioning, have higher sympathetic functioning and lower parasympathetic functioning, and higher perceived arousal in rest and stress conditions when compared to children with a low anxiety load.

METHODS

Participants

Eligible for participation in the clinical anxiety disorder group (AD) were N=152 children aged 8 to 12 years diagnosed with a primary diagnosis of separation anxiety disorder (SAD; N=57), generalized anxiety disorder (GAD; N=47), social phobia (SoPh; N=29) or specific phobia (SpPh; N=19), who had been referred to the outpatient clinic of the department of Child and Adolescent Psychiatry of either Erasmus MC in Rotterdam or Curium-LUMC in Leiden, The Netherlands. All children were diagnosed with the Anxiety Disorders Interview Schedule for DSM-IV (ADIS-C; Silverman, Albano, 1996).

The general population (GP) sample, consisting of 202 participants aged 8 to 12 years, was drawn from a larger general population sample study (Tick, Van der Ende, Verhulst, 2007). Study characteristics of the 2 groups are presented in Tables 1 and 2. All children were diagnosed with the Diagnostic Interview Schedule for Children-Parent version (DISC-P; Shaffer, Fisher, Lucas, 1998).

Exclusion criteria for both the AD and GP group were: IQ < 85, poor command of the Dutch language, serious physical disease, pervasive developmental disorder, selective mutism, schizophrenia or other psychotic disorders, pharmacotherapy that could interfere with HPA-axis or ANS functioning or pharmacotherapy aimed at treating anxiety or depressive symptoms. Methylphenidate treatment in children with ADHD was discontinued the day before and on the day of measurements (AD N=7, GP N=6) because methylphenidate treatment can increase heart rate and blood pressure (Ballard et al., 1976).

The Committees for Medical Ethics of Erasmus MC and LUMC approved the study.

Procedure

Parents and participants signed informed consent before participation. Prior to the physiological assessment, parents and children completed psychological questionnaires. To assess the diurnal cortisol profile, participants collected 4 saliva samples at home by filling tubes till a marker (at 500 µl): (1) a first sample immediately after awakening in the morning (Cort1), when the child was still in bed, (2) 30 minutes later (Cort2), (3) at 12h (Cort3), and (4) at 20h (Cort4). All samples were collected on a single regular school day, stored in the freezer at home, and taken to the assessment at the outpatient department one day later. Parents were asked to register general physical condition, activity levels, consumption pattern, and medication use of their child.

The physiological assessment took place in the hospital between 12h and 1830h. Participants were seated comfortably in a laboratory room where temperature and humidity levels were kept constant. Lunch was eaten before the assessment; the gap between lunch and baseline saliva samples was at least 1.5 h. After an acclimatization period of 45 minutes, the session began with a baseline period of 10 minutes in which the participant was asked to sit

still and relax. Subsequently, a mental arithmetic task (MAT) was administered. At the end of the baseline period and after the MAT, a questionnaire concerning physiological arousal (PAQ) was administered. A cortisol sample was collected after the baseline period (Cort5), to have a second basal cortisol measure next to the diurnal profile on a different day.

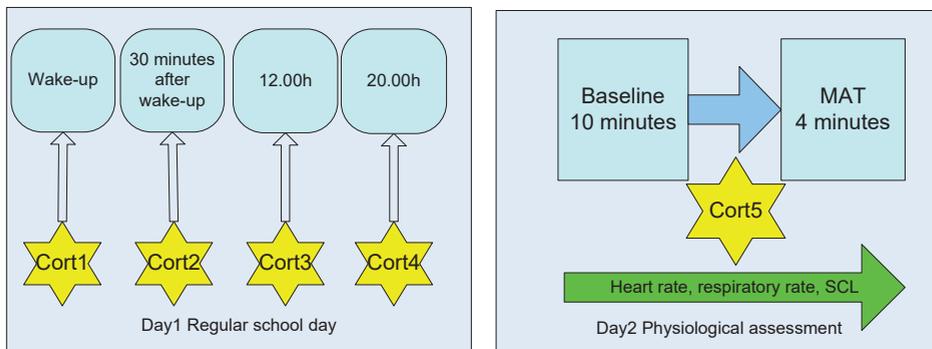


Figure 1 Procedure

Note. Cort1-Cort2-Cort3-Cort4-Cort5 = Cortisol sample 1-5, SCL= Skin conductance level, MAT=Mental arithmetic task.

Measures

Assessment of diagnostic status and symptom severity

Anxiety Disorders Interview Schedule for DSM-IV (AD-group)

ADIS-C (Silverman, Saavedra, Pina, 2001) is a semi-structured interview to assess DSM-IV AD in 7- to 17-year-olds. A trained psychologist conducted the interview with the child and parents separately. To obtain a diagnosis, both a symptom count of DSM-IV symptom criteria, as well as level of impairment according to the parent, child, and interviewer, are taken into account.

Diagnostic Interview Schedule for Children-Parent version (GP group)

The DISC-IV- P/C is a highly structured respondent-based psychiatric interview to assess DSM-IV disorders found in children. The DISC-IV-P showed good inter-rater and test-retest reliability (Shaffer, Fisher, Lucas, 1998; Shaffer et al., 2000). Last year's diagnoses were calculated according to the DISC-IV-P diagnostic algorithm.

Child Behavior Checklist

The CBCL is a parent questionnaire for assessing problems in 4- to 18-year-olds. It contains 120 items on behavioral or emotional problems during the past 6 months. The response format is 0 = not true, 1 = somewhat or sometimes true, and 2 = very true or often true. The good reliability and validity of the original version of the CBCL (Achenbach, Rescorla, 2001)

were confirmed for the Dutch translation (Verhulst, Van der Ende, 2013). Scores on two broad-band scales were computed: Internalizing and Externalizing.

Multidimensional Anxiety Scale for Children

The MASC is a 39-item self-report questionnaire that assesses anxiety symptoms concerning the last two weeks in children. Items were scored from 0 to 3 (0 = never true, 1 = rarely true, 2 = sometimes true, 3 = often true). The internal consistency (.93) and test-retest reliabilities (.81) are very good (March et al., 1997; Liber, 2008).

Children's Depression Inventory

The CDI is a 27-item self-report questionnaire that assesses depressive symptoms concerning the last two weeks in children. Items are scored from 0 to 2 (0 = never true, 1 = sometimes true, 2 = always true). The internal consistency (.82) (Liber, 2008) and 1-month test-retest reliabilities (.72) are adequate (Kovacs, 1981).

Stress task

The Mental Arithmetic Task was applied as a standardized laboratory stress task to induce measurable physiological changes (Kirschbaum, Pirke, Hellhammer, 1993). The MAT lasted 4 min, during which the child was asked to subtract numbers as quickly and accurately as possible. If the child made a mistake or did not respond, he or she was required to start all over again. Dependent on the child's age, the child was asked to subtract 7 from 100 (<12 years), or 23 from 1021 (≥ 12 years), and so on.

Physiological and hormonal measures

Cortisol assessment

The laboratory that analyzed our cortisol samples switched from solid-phase radioimmunoassay (RIA, Diagnostic Products Corporation, Los Angeles) to Enzyme-Linked Immuno Sorbent Assay (ELISA, DRG-kits, Marburg, Germany) during data collection. Thirty-one cortisol samples were analyzed by both RIA and ELISA. Correlation between both assays was high ($R=.994878$). The slope was not equal to 1 so the concentrations in the DRG standards were adjusted by the laboratory to guarantee equal results.

Cortisol samples of eighty-three children of the AD sample were analyzed by solid-phase radioimmunoassay (Kallen et al., 2008). An Enzyme-Linked Immuno Sorbent Assay (DRG Instruments GmbH, Marburg, Germany) was used to determine cortisol concentrations in 50 μ l duplicate samples for the rest of the samples. For technical specifics see (Dieleman et al., 2010). Cortisol values that were above 3 SD of the mean were excluded from the analysis to reduce the impact of outliers.

In addition to the separate cortisol variables, we used composite cortisol variables to analyze the diurnal profile. We calculated three composite measures according to Pruessner

et al. (2003): cortisol awakening rise with respect to ground (CAR_g), with respect to increase (CAR_i) and the area under the curve with respect to ground (AUC_g). The CAR_g and CAR_i were calculated using the following formulas:

$$CAR_g = ((Cort1+Cort2) * time_{cort2-cort1})/2, CAR_i = CAR_g - (Cort1 * time_{cort2-cort1}).$$

The formula to calculate AUC_g was:

$$AUC_g = AUC_{cort2-cort3} + AUC_{cort3-cort4} = ((Cort2+Cort3) * time_{cort3-cort2})/2 + ((Cort3+Cort4) * time_{cort4-cort3})/2.$$

If one of the cortisol samples was missing, an area under the curve could not be calculated. Therefore for the GP group, N=13 CAR_g (6.4%), N=13 CAR_i (6.4 %) and N=25 AUC_g (12.4%), for the AD group, N=17 CAR_g (11.0%), N=21 CAR_i (13.6%) and N=16 AUC_g (10.4%), were missing and excluded for those specific analyses.

Autonomic measures

During the experiment, continuous measurements were made of heart rate (Stratakis, Chrousos, 1995), respiration rate (RESP) and skin conductance level (SCL). HR was recorded using a 3-lead ECG, sampled at 512 Hz. SCL was used as a measure of the sympathetic function of the ANS and was assessed using two adhesive disposable active Ag/AgCl electrodes attached to the volar surfaces of the medial phalanges of the index and ring fingers of the non-dominant hand. SCL was sampled at 8 Hz, and stored in μS . RESP was measured using an inductive plethysmography method (belts containing a magnetic coil, Respirace™, TEMEC Respiratory Inductive Plethysmography system; TEMEC Instruments B.V., Kerkrade, The Netherlands), sampled at 8 Hz.

Physiological Arousal Questionnaire

The PAQ is a 7-item self-report questionnaire developed at our department for assessment of the perceived state of physiological arousal. The child was asked to indicate on a 9-point scale (0-8) to what extent he or she felt aroused: 0 = not at all to 8 = very much. Cronbach's alpha was .64 (during baseline), .81 (during stress). For a description in detail see Dieleman et al. (2010).

Analysis of physiological data

HRV during rest

A customised software program calculated the interbeat intervals (IBI's) of the ECG using R-top detection, resulting in IBI time series during rest (3 min period of stationary signal; minutes 7-10 of resting period). These time series were inspected and artifacts were removed.

The IBI time series during the 3 min period of rest was further subjected to a discrete Fourier transform, based on non-equidistant sampling of the R-wave incidences (CARSPAN program, Groningen, The Netherlands (Mulder et al., 1988; Van steenis, Tulen, Mulder, 1994), to yield power spectra of the rhythmic oscillations over a frequency range of 0.02–0.50 Hz, with a resolution of 0.01 Hz. For each time segment, the power was calculated for the high-frequency band (HF: 0.14–0.50 Hz), in addition to mean HR and respiration rate. HF HR variability is a commonly used proxy for the vagal component of autonomic cardiac control. Because the HF of HR variability is strongly correlated with respiratory sinus arrhythmia (Kamath, Fallen, 1993), respiratory frequency was monitored separately to control for this effect. Time segments with more spectral power for respiration in the MF band than in the HF band were discarded from HF HR variability analyses. This resulted in the removal of the observations of 3 children from the GP group and 1 from the AD group. HF HR variability was not calculated during the MAT because of breathing irregularities as a consequence of speaking.

Data Analysis

All statistical analyses were performed with SPSS 20.0. For descriptive purposes, means and standard deviations were calculated based on untransformed variables. For further analyses, measures of SCL, Cort₁₋₅, HF HR variability, perceived arousal, CDI Total score and scores on the internalizing and externalizing scales of the CBCL were log-transformed to approach a normal distribution. Subsequently, stress responses in perceived arousal and SCL were calculated as the level during the MAT minus the pretest level for each of these measures. Age, gender and BMI might confound the cortisol-psychopathology relationship (e.g. see Rosmalen et al. (2005) for an overview). To control for possible effects of age, gender and BMI, these variables were entered as covariates. Effect sizes are reported as partial Eta squared, with .01 defined as a small effect size, .06 as a medium effect size and .14 as a large effect size (Cohen, 1988).

First, we investigated whether there was a difference between the AD and GP group in HPA-axis functioning using four two-way between groups ANCOVAs with sex and group as factors and age and BMI as covariates. The dependent variables were CAR_g, CAR_i, AUC_g and Cort₅ (after the baseline period). These analyses were repeated with the addition of the externalizing scale of the CBCL and the CDI Total score as covariates to control for the effects of comorbid behavior problems and depressive symptoms. Subsequently, a two-way MANCOVA with group and sex as factors, BMI and age as covariates, and Cort₁₋₄ as dependent variables was conducted.

Further, to analyze if there was a difference between the GP and AD group in autonomic functioning levels during rest, group differences in pretest levels (PAQ, HF HR variability and SCL) and in difference scores between pre- to post/during test levels (PAQ and SCL) corrected for pretest levels, were analyzed using ANCOVAs. All analyses were adjusted for age, gender, and BMI. These analyses were repeated with the addition of the externalizing scale

of the CBCL and the CDI Total score as covariates to control for the effects of comorbid behavior problems and depressive symptoms.

Third, to investigate if 'anxiety load' was associated with HPA-axis functioning and autonomic functioning, the AD group was subsequently divided into three groups: a group with one anxiety disorder, a group with two anxiety disorders and a group with three or more anxiety disorders. The analyses as described above for the comparison AD versus GP group were repeated for the comparison between the three groups with a different anxiety load. Posthoc comparisons tests were used to explore which of the three groups were significantly different from each other.

Fourth, to investigate if 'clinical load', which takes comorbidity (Depression, Dysthymic disorder, ADHD, ODD and CD) into account as well, was associated with HPA-axis functioning and autonomic functioning, the AD group was subsequently divided into three groups: a group with one clinical disorder, a group with two clinical disorders and a group with three or more clinical disorders. The analyses as described above for the comparison AD versus GP group were repeated for the comparison between the three groups with a different clinical load. Posthoc comparisons tests were used to explore which of the three groups were significantly different from each other.

Finally, in order to investigate whether different anxiety disorders have different endocrine or autonomic profiles the AD group was divided into four groups. Groups were based on the anxiety diagnosis with the highest severity score on the ADIS-C according to the clinician. This resulted in groups with, respectively, a main diagnosis of SAD, GAD, SoPh and SpPh. The analyses as described above for the comparison AD versus GP group were repeated for the comparison between the four groups with a different anxiety disorder. Posthoc comparisons tests were used to explore which of the four groups were significantly different from each other.

RESULTS

Table 1. Sample characteristics of AD and GP sample and comorbidity

Measures	AD (N=152) Mean (SD)	GP (N=200) Mean (SD)
Age	10.2 (1.5)	10.1 (1.5)
Gender (♀, ♂)	46.8%, 53.2%	50%, 50%
BMI	17.9 (4.9)	18.1 (3.2)
CBCL _{-intern} ***	20.6 (9.0)	7.3 (5.8)
CBCL _{-extern} ***	11.4 (7.9)	5.9 (5.9)
MASC Total score***	51.37 (17.72)	42.08 (14.09)
CDI Total score***	9.61 (7.04)	6.41 (5.49)
No medication in last two months [#]	60.4%	73.8%
Time of awakening	7.15 (0.5)	7.03 (0.85)
Percentage menarche	7.1% of girls	5.9% of girls

Table 1. Sample characteristics of AD and GP sample and comorbidity (*continued*)

Diagnoses	AD	GP
	Main diagnosis ADIS-C /Count anxiety diagnosis ADIS-C N (% of total sample)/N	Count anxiety diagnosis DISC-P N (% of total sample)
GAD	47 (30.5)/75	0 (0)
SoPh	29 (18.8)/49	2 (1.0)
SAD	57 (37.0)/46	2 (1.0)
SpPh	19 (12.3)/48	25 (12.4)
PD	-	-
OCD	-	-
PTSD	-	-
	Other diagnosis ADIS-C N (% of total sample)	Other diagnosis DISC-P N (%)
ADHD	15 (9.7)	17 (8.4)
ODD	10 (6.5)	15 (7.4)
CD	3 (1.9)	1 (0.5)
DD	2 (1.3)	-
DysD	8 (5.2)	-

Note. GP = general population sample, AD = clinical anxiety disorder sample, SD = standard deviation, BMI = Body Mass Index, CBCL_{intern} = Internalizing scale of Childhood Behavior Checklist, CBCL_{extern} = Externalizing scale of Childhood Behavior Checklist, MASC = Multidimensional Anxiety Scale for Children, CDI = Children's Depression Inventory, ADIS-C= Anxiety Disorders Interview Schedule for DSM-IV, DISC-P= Diagnostic Interview Schedule for Children-Parent version, GAD = generalized anxiety disorder, SoPh = social phobia, SAD = separation anxiety disorder, SpPh = specific phobia, PD = panic disorder, OCD = obsessive compulsive disorder, PTSD = posttraumatic stress disorder, ADHD = attention deficit hyperkinetic disorder, ODD = oppositional defiant disorder, CD = conduct disorder, DD = depressive disorder, DysD = dysthymic disorder. * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

For specification of medication use in the last two months see supplementary Figure 1.

Sample characteristics (see Tables 1-3)

Scores on the internalizing and externalizing scale of the CBCL, the CDI Total score and MASC Total score were significantly higher in the AD group compared to the GP group. Correlations between psychiatric and physiological measures are presented in Tables 2 and 3. MASC Total score is significantly negatively correlated with CAR_i; high levels on the MASC Total score are associated with a low CAR_i in the AD group.

Table 2. Pearson correlations between physiologic and psychiatric measures in the anxiety disorder group

	MASC total Score	CDI total score	CBCL internalizing scale	CBCL externalizing scale	CAR _g CAR _i	AUC _g	HF HR	SCL baseline	SCL during test
MASC Total Score	1	.419**	.191**	.002	-.01	-.189*	-.066	-.095	-.036
CDI Total score		1	.297**	.235**	-.026	-.137	-.103	-.020	.034
CBCL internalizing scale			1	.498**	.086	.016	-.043	-.004	.041
CBCL externalizing scale				1	.048	-.052	-.060	.124	.172*
CAR _g					1	.164	.400**	-.012	.071
CAR _i						1	.149	-.032	.006
AUC _g							1	-.146	-.142
HF HR								1	-.127
SCL baseline									1
SCL during test									

Note. CBCL internalizing = Internalizing scale of Childhood Behavior Checklist, CBCL externalizing = Externalizing scale of Childhood Behavior Checklist, MASC = Multidimensional Anxiety Scale for Children, CDI = Children's Depression Inventory, CAR_g = cortisol awakening rise with respect to ground, CAR_i = cortisol awakening rise with respect to increase, AUC_g = area under the curve for Cortiz-Cort.4 with respect to ground, SCL = skin conductance level, HF HR= high frequency heart rate variability, * = p<.05, ** = p<.01.

Table 3. Pearson correlations between physiologic and psychiatric measures in the general population group

	MASC total Score	CDI total score	CBCL internalizing scale	CBCL externalizing scale	CAR _g	CAR _i	AUC _g	HF HR	SCL baseline	SCL during test
MASC Total Score	1	.468**	.412**	.223**	-.03	-.123	-.015	-.012	.024	-.052
CDI Total score		1	.469**	.429**	-.010	.109	.070	.055	-.012	-.022
CBCL internalizing scale			1	.545**	.053	.097	.118	-.023	-.113	-.096
CBCL externalizing scale				1	-.042	.126	-.038	-.038	.003	.011
CAR _g					1	.096	.574**	-.063	-.056	-.103
CAR _i						1	.207**	.002	.065	-.009
AUC _g							1	.033	-.095	-.031
HF HR								1	-.088	-.058
SCL baseline									1	.836**
SCL during test										1

Note. CBCL internalizing = Internalizing scale of Childhood Behavior Checklist, CBCL externalizing = Externalizing scale of Childhood Behavior Checklist, MASC = Multidimensional Anxiety Scale for Children, CDI = Children's Depression Inventory, CAR_g = cortisol awakening rise with respect to ground, CAR_i = cortisol awakening rise with respect to increase, AUC_g = area under the curve for Cortiz-Cort4 with respect to ground, SCL = skin conductance level, HF HR= high frequency heart rate variability, *= $p < .05$, **= $p < .01$

Daytime cortisol measures (see Table 4)

Both the AD and GP group included several subjects with negative values for CAR_i (GP: 24.8%, AD 21.4%). There were no differences in cortisol awakening measures between both groups. CAR_g was lower for boys (mean=7.66, SD=2.12) compared to girls (mean=8.46, SD=2.21) ($F=7.31(1, 288)$, $p<.01$, partial Eta squared=.025 (small)).

For AUC_g , there was a highly significant between groups difference (see Figure 2). The difference in AUC_g between the AD group and the GP group was determined by lower values of Cort3 and Cort4 (see Table 4) for the AD group. The same pattern was seen for baseline cortisol levels preceding the stress task. These results remained significant after correcting for externalizing behavior and depressive symptoms. Effect sizes varied between medium to large effects (partial Eta squared .11-.25).

To check that our significant results were not due to medication effects, we did a secondary analysis only for children that did not use any medication. Results indicated that our findings were not influenced by the use of medication.

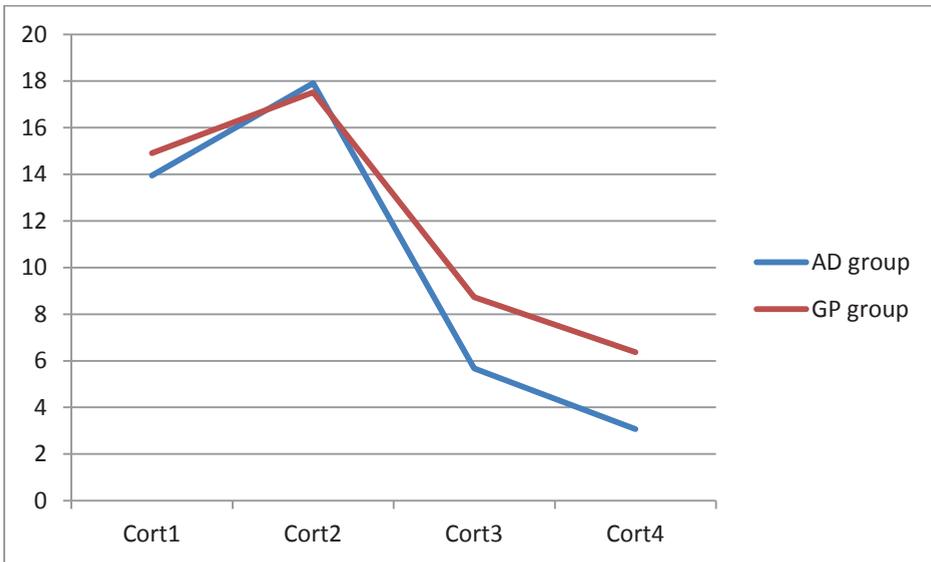


Figure 2. Cortisol diurnal profile

Note. GP = general population group, AD = anxiety disorder group, Cort1 = cortisol directly after awakening, Cort2 = cortisol half an hour after awakening, Cort3 = cortisol at 12.00 p.m., Cort4 = cortisol at 8.00 p.m.

Baseline and stress response measures of perceived arousal and autonomic functioning

The MAT elicited a significant increase in PAQ Total score in the GP group ($T= 5.66$, $p<.001$), thus reflecting a perceived physiological stress response. At pretest, the AD group had significantly higher levels of perceived arousal and SCL, and lower levels of HF HR variability when compared to controls (see Table 4). During the stress task, AD children had a higher

Table 4. Means, as well as group differences in baseline HPA measures and in baseline and reactive autonomic and perceived arousal measures

Variable	AD group Mean (SD-N)	GP group Mean (SD-N)	Group differences [#] ((M)ANCOVA S//partial Eta squared)	Group differences [#] ((M)ANCOVA S//partial Eta squared)	Group differences ^{###} ((M)ANCOVA S//partial Eta squared)
CAR _g	.84 (1.90-117)	.69 (1.44-184)	ns	ns	ns
CAR _s	7.86 (2.44-116)	8.11 (2.04-184)	ns	ns	ns
AUC _g	87.7 (28.9-111)	119.1 (31.7-172)	F(1,277) = 67.95 ^{***} // .20	F(1,271) = 48.34 ^{***} // .15	F(1,268) = 57.49 ^{***}
Cort-4			F(1,274) = 22.88 ^{***} // .25	F(1,268) = 15.73 ^{***} // .19	F(1,265) = 19.06 ^{***} // .22
Cort1(nmol/l)	13.95 (5.14)	14.91 (4.84)	ns	ns	ns
Cort2(nmol/l)	17.91 (6.58)	17.52 (5.26)	ns	ns	ns
Cort3(nmol/l)	5.67 (2.75)	8.73 (2.95)	F(1,274) = 56.62 ^{***} // .17	F(1,271) = 36.15 ^{***} // .12	F(1,268) = 44.31 ^{***} // .14
Cort4(nmol/l)	3.07 (3.00)	6.37 (2.82)	F(1,274) = 85.95 ^{***} // .24	F(1,271) = 59.20 ^{***} // .18	F(1,268) = 72.28 ^{***} // .21
Cort5(nmol/l)	5.74 (4.24-120)	7.76 (2.56-191)	F(1,305) = 46.02 ^{***} // .13	F(1,298) = 36.86 ^{***} // .11	F(1,295) = 37.33 ^{***} // .11
PAQ pretest	.88 (3.4-146)	.76 (.35-198)	F(1,339) = 9.01 [*] // .03	F(1,332) = 6.82 [*] // .02	ns
PAQ posttest	.92 (33-147)	.87 (.36-199)	ns	ns	ns
PAQ diff	.04 (23-145)	.11 (.28-198)	ns	ns	ns
SCL (μS) baseline	.54 (26-121)	.39 (.16-193)	F(1,308) = 37.26 ^{***} // .11	F(1,301) = 31.40 ^{***} // .09	F(1,301) = 31.40 ^{***} // .09
SCL (μS) during test	.67 (27-120)	.48 (.15-189)	F(1,303) = 55.0 ^{***} // .15	F(1,296) = 43.68 ^{***} // .13	F(1,294) = 48.06 ^{***} // .14
SCL (μS) diff	.13 (1.0-119)	.09 (.09-189)	F(1,301) = 17.46 ^{***} // .06	F(1,294) = 12.62 ^{***} // .04	F(1,292) = 14.15 ^{***} // .05
HF HR	3.40 (0.42-119)	3.57 (0.42-192)	F(1,305) = 10.78 ^{**} // .03	F(1,298) = 7.11 ^{**} // .02	F(1,298) = 10.24 ^{**} // .03

Note. SD = standard deviation, GP = general population group, AD = anxiety disorder group, Cort1 = cortisol directly after awakening, Cort2 = cortisol half an hour after awakening, Cort3 = cortisol at 12.00 p.m., Cort4 = cortisol at 8.00 p.m., Cort5 = cortisol during baseline, CAR_g = cortisol awakening rise with respect to ground, CAR_s = cortisol awakening rise with respect to increase, AUC_g = area under the curve for Cort2-Cort4 with respect to ground. SCL = skin conductance level, HF HR = high frequency heart rate variability, PAQ = Perceived Arousal Questionnaire. [#] covariates: age, sex, bmi. ^{**} covariates: age, sex, bmi. ^{###} covariates: age, sex, bmi, CDI Total score. * = p < .05, ** = p < .01, *** = p < .001.

difference in SCL when compared to controls. SCL during stress was significantly higher in the AD group (see Figure 3). These results remained significant after correcting for externalizing and depressive symptoms. Effect sizes varied between .01-.16, with medium to large effects for SCL and small effects for perceived arousal and HF HR variability.

To check that our significant results were not due to medication effects, we did a secondary analysis only for children that did not use any medication. Results indicated that our findings were not influenced by the use of medication.

Comorbidity

As shown in Table 5 comorbidity rates are high; 55.3% had a single clinical anxiety disorder, 30.9% had two clinical anxiety disorders, 11.8% had three clinical anxiety disorders and 2.0% had four clinical anxiety disorders. In the group with a main diagnosis of SAD the proportion of children with three or more clinical anxiety disorders was relatively high. SpPh is more often a comorbid than a main diagnosis, whereas SAD is more often a main than a comorbid diagnosis. One third of children with a main diagnosis of SoPh have a comorbid diagnosis of GAD.

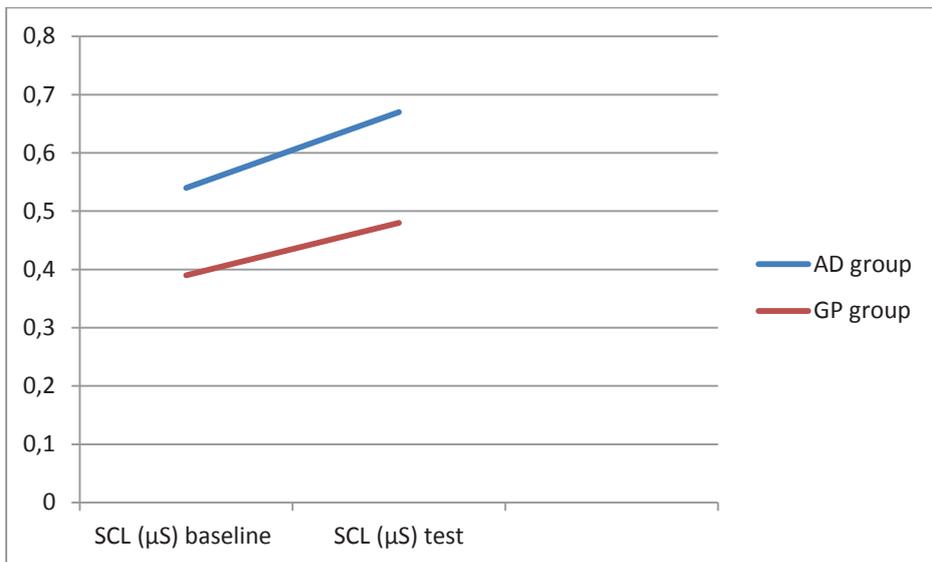


Figure 3. Skin conductance levels, baseline and during test

Note. GP = general population group, AD = anxiety disorder group, Cort1 = cortisol directly after awakening, SCL = skin conductance level.

Anxiety 'load' versus daytime cortisol, autonomic measures and perceived arousal

For AUC_g , there was a non-significant trend between groups. Means show that this trend is a consequence of the difference between the groups with one and two clinical anxiety disorders as compared to the group with three or more clinical anxiety disorders. Further analyses showed that there was no difference in Cort3 and a trend in Cort4. Mean scores in Cort3 and

Table 5. Cross tabulation of main diagnosis and comorbid disorders

		Comorbid diagnoses N(% per main anxiety diagnosis)									Total comorbid diagnoses per main anxiety diagnosis
		SAD	SoPh	SpPh	GAD	ADHD	ODD	CD	DYS	DD	
Main diagnosis	SAD=57	-	13(22.8)	15(26.3)	14(24.6)	8(14.0)	5(8.8)	1(1.8)	1(1.8)	2(3.5)	59
	SoPh=29	3 (10.3)	-	2(6.9)	9(31.0)	1(3.4)	1(3.4)	0(0)	3(10.3)	0(0)	19
	SpPh=19	5(26.3)	2(10.5)	-	5(26.3)	2(10.5)	2(10.5)	1(5.3)	0(0)	0(0)	17
	GAD=47	5(10.6)	6(12.8)	12(25.5)	-	4(8.5)	2(4.3)	1(2.1)	4(8.5)	0(0)	34
Count of comorbid diagnosis		13	21	29	28	15	10	3	8	2	129

Note. GAD = generalized anxiety disorder, SoPh = social phobia, SAD = separation anxiety disorder, SpPh = specific phobia

Cort₄ showed the same pattern as AUC_g between groups. Results are presented in table 6. There were no significant between groups differences for CAR_g, CAR_i, Cort₁, Cort₂ and Cort₅.

Regarding ANS functioning significant group differences were found in pretest and test levels of SCL. Posthoc analyses revealed that the group with the most clinical anxiety disorders had significantly higher pre- and posttest SCLs than the group with one or two anxiety disorders. These results remained significant after correcting for externalizing and depressive symptoms. There were no significant between group differences for the SCL stress response and HF HR variability.

Pre- and posttest perceived arousal was significantly different between groups. The group with 3 or more clinical anxiety disorders had lower pre- and posttest PAQ-scores in comparison with the groups with one and two anxiety disorders. These results remained significant after correcting for externalizing behavior. When the results were corrected for depressive symptoms, only posttest PAQ remained significantly different between groups. There was no significant between group difference for the PAQ stress response.

For all significant results described above overall effect sizes were medium and varied between .05-.10.

Clinical ‘load’ versus daytime cortisol, autonomic measures and perceived arousal

For AUC_g, there was a significant between groups difference. Post hoc analyses showed that the difference in AUC_g between the groups was determined by the difference between the groups with one and two clinical disorders as compared to the group with three or more clinical disorders. Thus, the group with the most clinical disorders had a significantly lower AUC_g. Further analyses showed that the difference was determined by lower values of Cort₃, Cort₄ showed a trend. The results remained significant for AUC_g and Cort₃ after correcting for externalizing behavior. Only the results for AUC_g remained significant after correction for depressive symptoms. Significant results are presented in table 7. There were no significant between groups differences for CAR_g, CAR_i, Cort₁, Cort₂ and Cort₅.

Table 6. Daytime cortisol, ANS measures and perceived arousal versus 'anxiety load'

Variable	Single clinical anxiety disorder Mean (SD-n)	Two clinical anxiety disorders Mean (SD-n)	Three or more clinical anxiety disorders Mean (SD-n)	Group differences [#] (ANCOVA'S //partial Eta squared)	Group differences ^{##} (ANCOVA'S //partial Eta squared)	Group differences ^{###} (ANCOVA'S //partial Eta squared)
AUC _g	.89.52 (31.63-62)	92.17 (24.54-33)	71.45 (21.09-16)	ns, trend		
Cort3	.83 (.20-68)	.84 (.15-37)	.72 (.18-17)	ns		
Cort4	.56 (.32-65)	.54 (.31-37)	.38 (.24-17)	ns, trend		
SCL (µS) baseline	.48(.22-62)	.55 (.27-36)	.67(.26-23)	F(2,113)= 4.53 [*] // .07	F(2,108) = 3.27 [*] // .06	F(2,106) = 4.76 ^{***} // .08
SCL (µS) during test	.61 (.24-61)	.66 (.27-37)	.83 (.29-22)	F(2,112)= 5.07 ^{***} // .08	F(2,107)= 3.49 [*] // .06	F(2,105)= 4.74 [*] // .08
PAQ pretest	.89 (31-70)	.93 (30-36)	.72 (.49-17)	F(2,115)= 4.05 [*] // .07	F(2,110)= 4.90 ^{***} // .08	ns, trend
PAQ posttest	.96 (.29-72)	.94 (.32-35)	.70 (.43-17)	F(2,116)= 5.73 ^{***} // .07	F(2,111)= 5.80 ^{***} // .10	F(2,108)= 5.52 ^{***} // .09

Note. SD = standard deviation, GP = general population group, AUC_g = area under the curve for Cortz-Cort4 with respect to ground, Cort3 = cortisol at 12.00 p.m., Cort4 = cortisol at 8.00 p.m., SCL = skin conductance level, PAQ = Perceived Arousal Questionnaire. All variables, except AUC_g, were log-transformed. [#] covariates: age, sex, bmi. ^{##} covariates: age, sex, bmi, CBCL externalizing behavior, ^{###} covariates: age, sex, bmi, CDI Total score. * = p<.05, ***=p<.01.

Table 7. Daytime cortisol, ANS measures and perceived arousal versus 'clinical load'

Variable	Single clinical disorder Mean (SD-n)	Two clinical disorders Mean (SD-n)	Three or more clinical disorders Mean (SD-n)	Group differences* (ANCOVA'S //partial Eta squared)	Group differences** (ANCOVA'S //partial Eta squared)	Group differences*** (ANCOVA'S //partial Eta squared)
AUC _g	.87.82(28.01-54)	.9772 (30.33-35)	71.49(21.70-22)	F (2, 103) = 5.42 **//.10	F (2,99) = 5.05 **//.09	F (2,99) = 4.37 **//.08
Cort3	.82 (19-59)	.86 (16-39)	.73 (18-24)	F (2, 115) = 3.93 *//.07	F (2, 110) = 3.53 *//.06	ns, trend
Cort4	.55 (.33-57)	.57 (.30-38)	.40 (.24-24)	ns, trend		
SCL (µS) baseline	.48(.22-62)	.55 (.27-36)	.67(.26-23)	F(2,113) = 4.53 *//.07	F(2,108) = 3.27 *//.06	F(2,106) = 4.76 **//.08
SCL (µS) during test	.61 (.24-61)	.66 (.27-37)	.83 (.29-22)	F (2,112) = 5.07 **//.08	F (2,107) = 3.49 *//.06	F (2,105) = 4.74 *//.08
PAQ pretest	.88 (.32-62)	.95 (.30-38)	.76 (.43-23)	ns		
PAQ posttest	.95 (.31-63)	.95 (.32-38)	.76 (.39-23)	F (2,116) = 3.51 *//.06	F (2,111) = 3.49 *//.06	F (2,108) = 3.32 *//.06

Note. SD = standard deviation, GP = general population group, AUC_g = area under the curve for Cortz-Cort4 with respect to ground, Cortz = cortisol at 12.00 p.m., Cort4 = cortisol at 8.00 p.m., SCL = skin conductance level, PAQ = Perceived Arousal Questionnaire. All variables, except AUC_g, were log-transformed. # covariates: age, sex, bmi. ## covariates: age, sex, bmi, CBCL externalizing behavior, ### covariates: age, sex, bmi, CDI Total score. * = p<.05, ** = p<.01.

Regarding ANS functioning significant group differences were found in pretest and test levels of SCL. Posthoc analyses revealed that the group with the most clinical anxiety disorders had significantly higher pre- and posttest SCLs than the group with one or two anxiety disorders. These results remained significant after correcting for externalizing and depressive symptoms. There were no significant between group differences for the SCL stress response and HF HR variability.

Posttest perceived arousal was significantly different between groups. The group with 3 or more clinical anxiety disorders had a lower posttest PAQ-score in comparison with the group with only one anxiety disorder. Although children with two clinical anxiety disorders had rather similar scores on posttest PAQ as the group with one anxiety disorder, there was no significant difference with the group with 3 or more disorders, which may be due to a lack of power. These results remained significant after correcting for externalizing behavior. There was no significant between group difference for pretest PAQ or the PAQ stress response.

For all significant results described above overall effect sizes were medium and varied between .05-.10.

Type of main anxiety diagnosis versus daytime cortisol, autonomic measures and perceived arousal

As presented in Table 8, significant group differences were found in pretest and test levels of SCL. Posthoc analyses revealed that children with a main diagnosis of SpPh showed higher SCL's compared to the SAD and the SoPh-groups. Effect sizes were medium and varied between .11-.13. These results remained significant after correcting for externalizing and depressive symptoms. GAD was not significantly different from SpPh. There was no significant between-groups difference for any of the cortisol measures, perceived arousal or HF HR variability.

Children in the AD group with a main diagnosis of SpPh differed from children in the GP group with a diagnosis of SpPh (DISC-IV- P/C (N=25)) on several measures. Children with SpPh from the AD group have higher CBCL_{extern} scores (AD mean = 1.06 (sd =.27), GP mean = .78 (sd =.35) $T = 3.32$, $df 54$, $p < .01$), lower AUC_g (AD mean = 82.24 (sd = 28.17), GP mean = 106.17 (sd = 31.8) $T = -2.90$, $df 51$, $p < .01$), higher baseline SCL (AD mean = .69 (sd = .25), GP mean = .42 (sd = .16) $T = 4.77$, $df 52$, $p < .001$) and higher during test SCL (AD mean = .83 (sd = .25), GP mean = .51 (sd = .17) $T = 5.45$, $df 52$, $p < .001$). These results are in line with the general differences between the GP and the AD group. Thus the elevated levels of SCL's of SpPh are specific for clinical SpPh.

Table 8. ANS measures versus type of anxiety disorder

Variable	Separation anxiety disorder		Social anxiety disorder		Generalized anxiety disorder		Specific phobia		Group differences [#]		Group differences ^{##}		Group differences ^{###}	
	Mean (SD-n)		Mean (SD-n)		Mean (SD-n)		Mean (SD-n)		(ANCOVA'S//partial eta squared)		(ANCOVA'S//partial eta squared)		(ANCOVA'S//partial eta squared)	
SCL (μ S) baseline	.50 (.20-46)	.48 (.21-22)	.53 (.29-36)	.75 (.26-17)					F(3,111) = 4.36 **//.11	F(3,105) = 3.96 **//.11	F(3,104) = 4.49 **//.12			
SCL (μ S) during test	.62 (.19-46)	.58 (.24-22)	.68(.31-35)	.89 (.29-17)					F(3,110)= 5.31 **//.13	F(3,104)= 5.23 **//.13	F(3,103) = 5.91 **//.15			

Note. SD = standard deviation, SCL = skin conductance level, All variables were log-transformed. [#]covariates: age, sex, bmi. ^{##} covariates: age, sex, bmi, CBL externalizing behavior, ^{###} covariates: age, sex, bmi, CDI Total score. * = p < .05, ** = p < .01.

DISCUSSION

Our results show that children with a clinical anxiety disorder have a pattern of hypoactivation of basal HPA-axis functioning, elevated sympathetic, and lowered parasympathetic activity compared to a general population sample. Anxiety 'load', as a marker of severity, is associated with differences in functioning ANS. A high anxiety 'load' in the clinical sample is associated with higher sympathetic functioning in comparison to children with a low anxiety 'load'. There is no relationship between anxiety 'load' and parasympathetic functioning. Results for children with a high anxiety 'load' show a trend towards hypoactivation of basal HPA-axis functioning. In contrast with our expectations, children with a high anxiety 'load' have lower posttest perceived arousal in comparison with children with a low anxiety 'load'. When adding comorbid disorders to the 'load', the same pattern is seen. Moreover, a high clinical 'load' is associated with hypoactivation of basal HPA-axis functioning in comparison to children with a low clinical 'load' even after correcting for depressive and externalizing symptoms. Although we did not expect a difference in functioning of ANS or HPA-axis between the different types of anxiety disorders, our results show that children with a main diagnosis of specific phobia can be discerned from children with a social phobia and separation anxiety disorder on higher sympathetic functioning during basal and stress conditions, even in the presence of high comorbidity rates, although comorbidity rates were comparable to or even lower than other studies (e.g. Kendall et al., 2010).

Anxiety 'load'

Our results are in support of the hypothesis that a child with a clinical disorder functions under chronic stressful conditions, with concomitant changes in the activity of both the stress systems. This notion is further underlined by the finding that a high clinical 'load' in the clinical sample was associated with lower basal HPA-axis functioning and higher sympathetic functioning in comparison to children with only one or two clinical anxiety disorders, after correcting for externalizing and depressive symptoms. The analyses for anxiety 'load' show the same pattern, but show only a trend in the results for HPA-axis functioning which may be due to a lack of power. Our findings might indicate that there are already biological underpinnings of the 'load' of anxiety disorders in childhood, which might be predictive of adverse outcomes later in life. The 'load' of anxiety disorders during adolescence has been associated with later risks of anxiety disorders, major depression, substance dependence, suicidal behavior and other adverse outcomes, such as educational underachievement and early parenthood (Woodward, Fergusson, 2001).

Specificity of psychophysiological correlates

Our study underlines the hypothesis that basal ANS and HPA-axis functioning relate to anxiety disorders in general, and only heightened sympathetic (re)activity is a specific cor-

relate for specific phobia. Other lines of research also support the idea of specific phobia as a specific taxonomic entity. In a population-based twin registry, lifetime diagnoses for six anxiety disorders (generalized anxiety disorder, panic disorder, agoraphobia, social phobia, animal phobia, and situational phobia) were obtained during personal interviews (Hettema et al., 2005). The authors concluded that genetic factors predispose to two broad groups of disorders dichotomized as panic-generalized-agoraphobic anxiety versus the specific phobias. Social phobia was regarded as an intermediate as it was influenced by both genetic factors. Further evidence for specific phobia as a specific taxonomic entity comes from longitudinal studies: specific phobia exclusively predicts specific phobia from childhood or adolescence to adulthood (e.g. Pine et al., 1998). In other words: there is strong evidence for longitudinal specificity of specific phobia. The psychophysiological correlates of clinical specific phobia in our study, confirm the existing genetic and longitudinal evidence for specific phobia as a separate taxonomic construct. The lack of specificity of psychophysiological correlates for the other anxiety disorders is in line with the findings of Hettema et al. (2005).

Daytime cortisol

One of the most robust and striking results of our study is the low diurnal cortisol profile at noon and in the evening in children with AD. The same difference is seen in pretest cortisol levels, with lower levels of cortisol preceding the MAT. Within the AD group the severity of the disorder, or the clinical 'load', resulted in an even lower diurnal cortisol profile at noon and a trend for a lower diurnal cortisol profile in the evening. At least two causal pathways leading to hypocortisolism in clinical anxiety disorders are conceivable.

We propose that chronic and pathological anxiety problems, interfering with daily life and leading to a clinical anxiety disorder, have a different effect on physiological functioning than temporarily heightened, subclinical anxiety symptoms. In subclinical anxiety, the first symptoms of exacerbated worrying, fear and anxiety might lead to an initial increase in adrenocortical activity, i.e. hypercortisolism. When anxiety symptoms become clinical and persist, compensatory mechanisms become activated and gradually result in an attenuation of cortisol secretion, i.e. hypocortisolism. A recent study by Steudte et al. (2011) found evidence in support of this theory. The authors found lower concentrations of cortisol in hair in adults with GAD. Cortisol in hair reflects cortisol secretion over a prolonged period of time. This suggests that under naturalistic conditions GAD in adults is associated with hypocortisolism. Other evidence in support of this theory comes from animal studies. Herman et al. (2005) state that chronic stress leads to two mechanisms in animals. First, facilitation: a new stressor leads to a stronger increase in cortisol in chronically stressed animals compared to non-stressed animals, and second, habituation: a chronic stressor in chronically stressed animals leads to a progressive decrease in cortisol. Our results are in line with the habituation hypothesis of chronic stress in animal research.

Alternatively, altered physiological functioning, as a vulnerability factor, could influence the expression of clinical anxiety disorders, but is not related to normal variation in levels of anxiety. Following this line of reasoning, moderate changes in HPA-axis functioning might be adaptive, whereas large alterations in physiological functioning may mark dysregulated emotions. Studies in support of this theory showed that cortisol administration reduced fear reports in patients with social phobia (Soravia et al., 2006) and that cortisol responses to psychosocial stress were inversely related to subsequent anxiety ratings (Schlotz et al., 2008).

Autonomic measures

Although effect sizes varied, our study confirms previous findings in studies with children with anxiety symptoms regarding autonomic functioning: AD children show heightened sympathetic functioning in rest and stress (SCL as a proxy for sympathetic ANS) and lower parasympathetic functioning in rest (HF HR variability as a proxy for parasympathetic ANS) compared to controls. With respect to sympathetic ANS functioning there seems to be a dose-response effect; children with a high anxiety 'load' exhibit even higher levels of sympathetic activity in comparison with children with a low anxiety 'load'. Interestingly, in studies finding evidence of low cortisol in association with PTSD or PTSD risk, there was also evidence of greater sympathetic arousal as reflected by catecholamine levels (Radley et al., 2011). Possibly, hypocortisolism in non-stressful conditions due to 'habituation' in children with a clinical anxiety disorder (i.e. a chronic stressor leads to a progressive decrease in cortisol) alters the ability of the HPA-axis to restore homeostasis following exposure to stress, resulting in increased sympathetic activation.

Perceived arousal

Previous studies suggest that high anxious subjects tend to perceive physiological sensations as more severe than non-anxious subjects (Hoehn-Saric, McLeod, 2000), sometimes even in the absence of an actual difference in physiological measures (Edelmann, Baker, 2002). In our study, the perceived arousal during rest in children with an AD compared to controls was higher. Apparently, anticipatory anxiety in children with an AD is high and has reached a plateau even before the stress task begins. This finding is supported by a recent study in healthy adults, relating neuroticism to exaggerated anticipatory anxiety experience (Drabant et al., 2011).

In contrast with these findings is the result that within the AD group children with a high anxiety 'load' experienced less perceived arousal in comparison with children with a low anxiety 'load'. There are several possible explanations: 1) children with a high anxiety 'load' are alexithymic and can not register their own perceived arousal, 2) children actively direct their attention away from threatening bodily sensations and nervous feelings, or 3) children with a high anxiety 'load' already have a continuous plateau level of arousal which they rate

as not deviant from normal. This last hypothesis could be in line with the general hypothesis that children with a high anxiety 'load' function under chronic stress conditions.

Limitations

Several limitations of our study need to be taken into consideration. First, the availability of only one cortisol diurnal profile can be seen as a limitation. Rotenberg et al. (2012) show that the cortisol awakening response requires at least 3 weekdays of sampling to yield a stable estimate. However, a review by Golden et al. (2011) states that the use of sampling multiple salivary cortisol measures across the diurnal curve (including awakening cortisol), likely reflects chronic cortisol burden and has the highest reliability of all cortisol measures ($r = 0.63-0.84$).

Second, sampling compliance was based on child-reported time of saliva collection combined with parent-initiated times on a daily log sheet. Use of electronically monitored timing would improve the precision and accuracy of sample timing, as well as the ability to screen for potentially invalid samples. Furthermore, our participants had lunch on the day of the assessment. Although lunch was eaten before the assessment and the gap between lunch and baseline saliva samples was at least 1.5 h, it might still have had an effect on pretest cortisol levels (Rotenberg et al., 2012). Other variables such as socioeconomic status and Tanner stage might have influenced cortisol values, although in a sample of 8 to 12 year olds the influence of Tanner stage will be limited. These data are not available for our subjects. This is a limitation of the study.

Another notable weakness of the current study is the lack of heart rate variability measures and cortisol measures during stress. The MAT is a cognitive task that compared to a public speaking/cognitive task combination is less capable of eliciting a substantial cortisol stress response (Kirschbaum, Pirke, Hellhammer, 1993; Dickerson, Kemeny, 2004). Furthermore, it is a task in which speech limits the possibility to control HF HR variability for respiratory frequency, therefore, HF HR variability was not analyzed during the MAT.

Comorbidity rates and the distribution of main anxiety diagnoses in this sample will exert its effects on the functioning of both stress systems. This is also applicable for the method of sampling and age, although we tried to minimize variation in developmental status through the inclusion of only children below 13 years. Considering these limitations, results regarding our sample cannot be directly extrapolated to other clinical samples. Nonetheless, selecting samples in which no comorbidity exists represent an artificial reality not found in most clinical settings.

CONCLUSION

Our findings indicate that children with an anxiety disorder can be distinguished on several psychophysiological characteristics from healthy children. The results underline the hypothesis that ANS and HPA-axis functioning relate to anxiety disorders in general, and only heightened sympathetic (re)activity is a specific correlate for specific phobia, which confirms the existing genetic and longitudinal evidence for specific phobia as separate taxonomic construct. Furthermore, our study found some evidence in support of the hypothesis that a child with a clinical anxiety disorder functions under chronic stressful conditions, with concomitant changes in the activity of both the stress systems. This notion is underlined by the finding that a high clinical 'load' in the clinical sample was associated with an even further deviation of basal HPA-axis functioning and sympathetic functioning. This might indicate that there are already biological underpinnings of the 'load' of anxiety disorders in childhood, which might be predictive of adverse outcomes later in life.

Future research is needed to replicate our findings in other clinical samples. Furthermore, the cross-sectional character of this study makes it difficult to draw conclusions regarding the causality of deviations in HPA-axis and ANS functioning in children with an AD, therefore longitudinal studies in children in which the course and severity of anxiety symptoms and disorders are related to basal and reactive HPA and ANS functioning are needed.

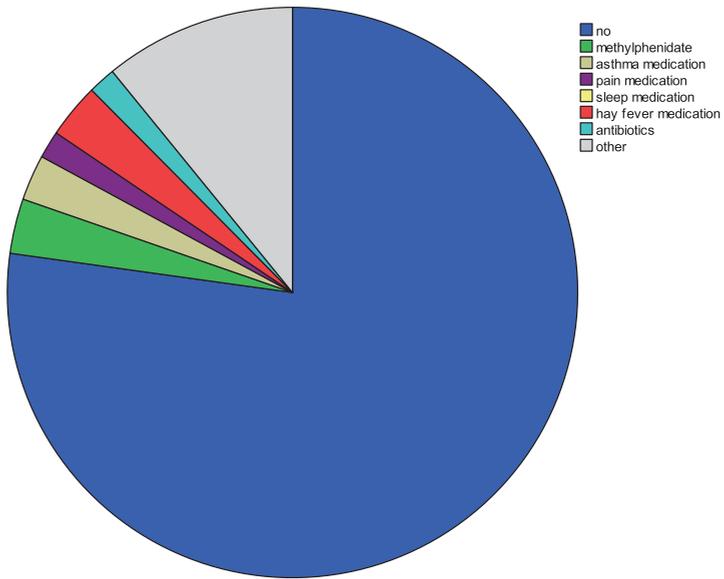
REFERENCES

- Achenbach, T.M., & Rescorla, L.A. (2001). *Manual for the ASEBA school-age forms & profiles*. Burlington, VT: University of Vermont, Research Center for Children, Youth, & Families.
- Ballard, J.E., Boileau, R.A., Sleator, E.K., Massey, B.H., & Sprague, R.L. (1976). Cardiovascular responses of hyperactive children to methylphenidate. *Journal of the American Medical Association*, *236*, 2870-2874.
- Beesdo, K., Knappe, S., & Pine, D.S. (2009). Anxiety and anxiety disorders in children and adolescents: developmental issues and implications for DSM-V. *Psychiatric Clinics of North America*, *32*, 483-524.
- Beesdo, K., Pine, D.S., Lieb, R., & Wittchen, H.U. (2010). Incidence and risk patterns of anxiety and depressive disorders and categorization of generalized anxiety disorder. *Archives of General Psychiatry*, *67*, 47-57.
- Bittner, A., Egger, H.L., Erkanli, A., Costello, E.J., Foley, D.L., & Angold, A. (2007). What do childhood anxiety disorders predict? *Journal of Child Psychology and Psychiatry*, *48*, 1174-1183.
- Charmandari, E., Tsigos, C., & Chrousos, G. (2005). Endocrinology of the stress response. *Annual Review of Physiology*, *67*, 259-284.
- Cohen, J. (1988). *Statistical power analysis for the behavioral sciences (2nd ed.)*. Hillsdale, NJ: Erlbaum.
- Costello, E.J., Egger, H.L., & Angold, A. (2005). The developmental epidemiology of anxiety disorders: phenomenology, prevalence, and comorbidity. *Child and Adolescent Psychiatric Clinics of North America*, *14*, 631-648.
- Dickerson, S.S., & Kemeny, M.E. (2004). Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychological Bulletin*, *130*, 355-391.
- Dieleman, G.C., Van der Ende, J., Verhulst, F.C., & Huizink, A.C. (2010). Perceived and physiological arousal during a stress task: can they differentiate between anxiety and depression? *Psychoneuroendocrinology*, *35*, 1223-1234.
- Dietrich, A., Riese, H., Sondejker, F.E., Greaves-Lord, K., Van Roon, A.M., Ormel, J., Neeleman, J., & Rosmalen, J.G. (2007). Externalizing and internalizing problems in relation to autonomic function: a population-based study in preadolescents. *Journal of the American Academy of Child and Adolescent Psychiatry*, *46*, 378-386.
- Drabant, E.M., Kuo, J.R., Ramel, W., Blechert, J., Edge, M.D., Cooper, J.R., Goldin, P.R., Hariri, A.R., & Gross, J.J. (2011). Experiential, autonomic, and neural responses during threat anticipation vary as a function of threat intensity and neuroticism. *Neuroimage*, *55*, 401-410.
- Edelmann, R.J., & Baker, S.R. (2002). Self-reported and actual physiological responses in social phobia. *British Journal of Clinical Psychology*, *41*, 1-14.
- Feder, A., Coplan, J.D., Goetz, R.R., Mathew, S.J., Pine, D.S., Dahl, R.E., Ryan, N.D., Greenwald, S., & Weissman, M.M. (2004). Twenty-four-hour cortisol secretion patterns in prepubertal children with anxiety or depressive disorders. *Biological Psychiatry*, *56*, 198-204.
- Forbes, E.E., Williamson, D.E., Ryan, N.D., Birmaher, B., Axelson, D.A., & Dahl, R.E. (2006). Peri-sleep-onset cortisol levels in children and adolescents with affective disorders. *Biological Psychiatry*, *59*, 24-30.
- Golden, S.H., Wand, G.S., Malhotra, S., Kamel, I., & Horton, K. (2011). Reliability of hypothalamic-pituitary-adrenal axis assessment methods for use in population-based studies. *European Journal of Epidemiology*, *26*, 511-525.
- Greaves-Lord, K., Ferdinand, R.F., Oldehinkel, A.J., Sondejker, F.E., Ormel, J., & Verhulst, F.C. (2007). Higher cortisol awakening response in young adolescents with persistent anxiety problems. *Acta Psychiatrica Scandinavica*, *116*, 137-144.
- Herman, J.P., Ostrander, M.M., Mueller, N.K., & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *29*, 1201-1213.

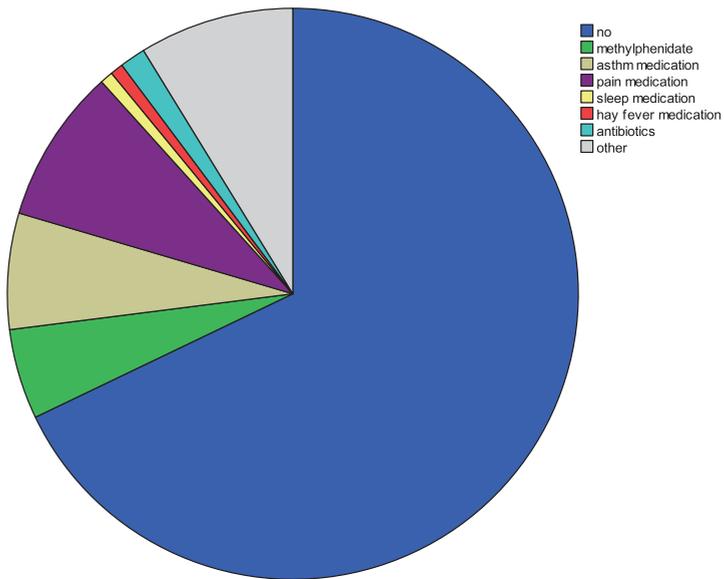
- Hettema, J.M., Prescott, C.A., Myers, J.M., Neale, M.C., & Kendler, K.S. (2005). The structure of genetic and environmental risk factors for anxiety disorders in men and women. *Archives of General Psychiatry*, *62*, 182-189.
- Hoehn-Saric, R., & McLeod, D.R. (2000). Anxiety and arousal: physiological changes and their perception. *Journal of Affective Disorders*, *61*, 217-224.
- Kallen, V.L., Tulen, J.H., Utens, E.M., Treffers, P.D., De Jong, F.H., & Ferdinand, R.F. (2008). Associations between HPA axis functioning and level of anxiety in children and adolescents with an anxiety disorder. *Depression and Anxiety*, *25*, 131-141.
- Kamath, M.V., & Fallen, E.L. (1993). Power spectral analysis of heart rate variability: a noninvasive signature of cardiac autonomic function. *Critical Reviews in Biomedical Engineering*, *21*, 245-311.
- Kendall, P.C., Compton, S.N., Walkup, J.T., Birmaher, B., Albano, A.M., Sherrill, J., Ginsburg, G., Rynn, M., McCracken, J., Gosch, E., Keeton, C., Bergman, L., Sakolsky, D., Suveg, C., Iyengar, S., March, J., & Piacentini, J. (2010). Clinical characteristics of anxiety disordered youth. *Journal of Anxiety Disorders*, *24*, 360-365.
- Kirschbaum, C., Pirke, K.M., & Hellhammer, D.H. (1993). The 'Trier Social Stress Test'-a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology*, *28*, 76-81.
- Kossowsky, J., Wilhelm, F.H., Roth, W.T., & Schneider, S. (2012). Separation anxiety disorder in children: disorder-specific responses to experimental separation from the mother. *Journal of Child Psychology and Psychiatry*, *53*, 178-187.
- Kovacs, M. (1981). Rating scales to assess depression in school-aged children. *Acta Paedopsychiatrica*, *46*, 305-315.
- Liber, J.M., Van Widenfelt, B.M., Utens, E.M.W.J., Ferdinand, R.F., Van der Leeden, A.J.M., Van Gastel, W., & Treffers, P.D.A. (2008). No differences between group versus individual treatment of childhood anxiety disorders in a randomised clinical trial. *Journal of Child Psychology and Psychiatry*, *49*, 886-993.
- March, J.S., Parker, J.D., Sullivan, K., Stallings, P., & Conners, C.K. (1997). The Multidimensional Anxiety Scale for Children (MASC): factor structure, reliability, and validity. *Journal of the American Academy of Child and Adolescent Psychiatry*, *36*, 554-565.
- Mulder, L.J.M., Van Dellen, H.J., Van der Meulen, P., & Opheikens, B. (1988). *CARSPAN: a spectral analysis program for cardiovascular time series*. Lisse: Swets & Zeitlinger.
- Pervanidou, P. (2008). Biology of post-traumatic stress disorder in childhood and adolescence. *Journal of Neuroendocrinology*, *20*, 632-638.
- Pine, D.S., Cohen, P., Gurley, D., Brook, J., & Ma, Y. (1998). The risk for early-adulthood anxiety and depressive disorders in adolescents with anxiety and depressive disorders. *Archives of General Psychiatry*, *55*, 56-64.
- Pruessner, M., Hellhammer, D.H., Pruessner, J.C., & Lupien, S.J. (2003). Self-reported depressive symptoms and stress levels in healthy young men: associations with the cortisol response to awakening. *Psychosomatic Medicine*, *65*, 92-99.
- Radley, J.J., Kabbaj, M., Jacobson, L., Heydendaal, W., Yehuda, R., & Herman, J.P. (2011). Stress risk factors and stress-related pathology: neuroplasticity, epigenetics and endophenotypes. *Stress*, *14*, 481-497.
- Rosmalen, J.G., Oldehinkel, A.J., Ormel, J., De Winter, A.F., Buitelaar, J.K., & Verhulst, F.C. (2005). Determinants of salivary cortisol levels in 10-12 year old children; a population-based study of individual differences. *Psychoneuroendocrinology*, *30*, 483-495.
- Rotenberg, S., McGrath, J.J., Roy-Gagnon, M.H., & Tu, M.T. (2012). Stability of the diurnal cortisol profile in children and adolescents. *Psychoneuroendocrinology*, *37*, 1981-1989.
- Sapolsky, R.M. (2002). Endocrinology of the Stress-Response. In J.B. Becker, S.M. Breedlove, D. Crews & M.M. McCarthy (Eds.), *Behavioral Endocrinology* (pp. 409-451). Cambridge: The MIT Press.

- Schlotz, W., Kumsta, R., Layes, I., Entringer, S., Jones, A., & Wüst, S. (2008). Covariance between psychological and endocrine responses to pharmacological challenge and psychosocial stress: a question of timing. *Psychosomatic Medicine*, *70*, 787-796.
- Schmitz, J., Krämer, M., Tuschen-Caffier, B., Heinrichs, N., & Blechert, J. (2011). Restricted autonomic flexibility in children with social phobia. *Journal of Child Psychology and Psychiatry*, *52*, 1203-1211.
- Shaffer, D., Fisher, P., & Lucas, C. (1998). *NIHM DISC-IV. Diagnostic interview schedule for children, parent-informant*. New York, NY: Columbia University.
- Shaffer, D., Fisher, P., Lucas, C.P., Dulcan, M.K., & Schwab Stone, M.E. (2000). NIMH Diagnostic Interview Schedule for Children Version IV (NIMH DISC-IV): Description, differences from previous versions, and reliability of some common diagnoses. *Journal of the American Academy of Child and Adolescent Psychiatry*, *39*, 28-38.
- Silverman, W.K., & Albano, A.M. (1996). *Anxiety Disorders Interview Schedule for DSM-IV – Child Version: The Clinician Manual*. San Antonio, TX: The Psychological Corporation.
- Silverman, W.K., Saavedra, L.M., & Pina, A.A. (2001). Test-retest reliability of anxiety symptoms and diagnoses with the Anxiety Disorders Interview Schedule for DSM-IV: child and parent versions. *Journal of the American Academy of Child and Adolescent Psychiatry*, *40*, 937-944.
- Soravia, L.M., Heinrichs, M., Aerni, A., Maroni, C., Schelling, G., Ehlert, U., Roozendaal, B., & De Quervain, D.J. (2006). Glucocorticoids reduce phobic fear in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 5585-5590.
- Steutde, S., Stalder, T., Dettenborn, L., Klumbies, E., Foley, P., Beesdo-Baum, K., & Kirschbaum, C. (2011). Decreased hair cortisol concentrations in generalised anxiety disorder. *Psychiatry Research*, *186*, 310-314.
- Stratakis, C.A., & Chrousos, G.P. (1995). Neuroendocrinology and pathophysiology of the stress system. *Annals of the New York Academy of Sciences*, *771*, 1-18.
- Terleph, T.A., Klein, R.G., Roberson-Nay, R., Mannuzza, S., Moulton, J.L., 3rd, Woldehawariat, G., Guardino, M., & Pine, D.S. (2006). Stress responsivity and HPA axis activity in juveniles: results from a home-based CO₂ inhalation study. *American Journal of Psychiatry*, *163*, 738-740.
- Tick, N.T., Van der Ende, J., & Verhulst, F.C. (2007). Twenty-year trends of emotional and behavioral problems in Dutch children in a changing society. *Acta Psychiatrica Scandinavica*, *116*, 473-482.
- Van Steenis, H.G., Tulen, J.H., & Mulder, L.J. (1994). Heart rate variability spectra based on non-equidistant sampling: the spectrum of counts and the instantaneous heart rate spectrum. *Medical Engineering & Physics*, *16*, 355-362.
- Verhulst, F.C., & Van der Ende, J. (2013). *Handleiding ASEBA Vragenlijsten voor leeftijden 6 tot en met 18 jaar*. Rotterdam: ASEBA Nederland.
- Woodward, L.J., & Fergusson, D.M. (2001). Life course outcomes of young people with anxiety disorders in adolescence. *Journal of the American Academy of Child and Adolescent Psychiatry*, *40*, 1086-1093.
- Wust, S., Wolf, J., Hellhammer, D.H., Federenko, I., Schommer, N., & Kirschbaum, C. (2000). The cortisol awakening response – normal values and confounds. *Noise and Health*, *2*, 79-88.

SUPPLEMENTARY FIGURES



General population sample



Anxiety disorder sample

Figure 1. Medication use during last two months