Infant cortisol and behavioral habituation to weekly maternal separations: Links with maternal prenatal cortisol and psychosocial stress

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Summary

Introduction: Our aim was to examine infants' behavioral and physiological stress responses to three weekly maternal separations, in relation to maternal prenatal psychosocial stress and cortisol. The hypothesis was that more prenatal stress and higher cortisol concentrations would predict smaller decreases in negative behavior and cortisol responses over the separations (i.e. less habituation).

Methods: General and pregnancy-related feelings of stress and anxiety, as well as circadian cortisol levels, were measured in 107 mothers in the third trimester of pregnancy. At 9 months of age, infants were subjected to three weekly 1-h maternal separations in their homes. Salivary cortisol was obtained from the infants prior to the separation and at 35, 75, and 90 min after the mother had left. For each separation, the area under the curve to the ground (AUCg) was calculated to measure the infants' cortisol response, and the sum of the time spent crying and fussing was calculated to measure the infants' behavioral response.

Results: Maternal pregnancy cortisol awakening response (CAR) significantly predicted infants' cortisol and behavioral responses. A lower CAR was related to a decreasing cortisol response, while a higher CAR was related to a stable cortisol response over all separations, as well as to less crying and fussing over all separations.

Conclusions: Increased maternal prenatal stress, as measured by the CAR, is related to altered behavioral and cortisol responses to a repeated stressor in the 9-month-old infant. These responses might result in prolonged periods with high cortisol levels that may affect the child's development.
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1. Introduction

Research on both animals and humans is providing more and more evidence that maternal prenatal factors can have long-term effects on offspring development. In humans, maternal prenatal stress and cortisol have been related to physical, cognitive, behavioral and temperamental postnatal child outcomes. This process is often referred to as fetal programming (Huizink et al., 2004a; Seckl and Meaney, 2004; Räikkönen et al., 2011), because the effects are often profound, long-term, and also seem to be transmitted from one generation to the next (de Weerth and Buitelaar, 2005b; Matthews and Phillips, 2012).

One mechanism through which maternal prenatal stress and cortisol might be related to later development is by altering the infants' behavioral and physiological stress systems. As a response to a new and stressful situation, infants often react with behavioral signs (e.g. crying and fussing) and physiological signs of distress (e.g. cortisol elevations, Jansen et al., 2010). The hormone cortisol is the end product of the hypothalamic-pituitary-adrenal axis (HPA-axis). Although the HPA-axis is essential for coping with stress, abnormal activity of the HPA-axis is a vulnerability factor for psychopathology later in life (Lupien et al., 2009; Guerry and Hastings, 2011; McCrory et al., 2012). Previous research already showed that prenatal stress and cortisol are related to altered infant cortisol responses upon a stressor (O'Connor et al., 2013; Tollenaar et al., 2011; Velders et al., 2012).

In animal models, exposure to a repeated stressor of the same type (homotypic stressor), such as restraint and maternal separation, is related to decreases in cortisol response (Barnum et al., 2007). This decrement in response that occurs with repeated exposure is often referred to as habituation. By contrast, repeated exposure to more severe homotypic stressors generally does not lead to habituation of the cortisol response, but to an increase, or sensitization, of the cortisol response (Barnum et al., 2007). In accordance with the animal literature, human newborn infants showed habituation of the cortisol response to a repeated discharge exam, while their cortisol response to a repeated heelstick tended to increase (Gunnar et al., 1989, 1991). Moreover, newborns that experienced more obstetric complications failed to show habituation of the cortisol response to the discharge exam, but responded equally to the repeated heelstick, compared to newborns with fewer obstetric complications (Gunnar et al., 1991).

Apparently, environmental conditions early in life can shape infants' cortisol habituation to repeated stress. In this line, environmental conditions during pregnancy, including maternal stress and cortisol concentrations, may also be related to difficulties habituating to stress. Prenatal maternal stress and cortisol may program the offspring's HPA-axis to continue to react to the same type of stressor and/or to habituate less quickly to a stressor, resulting in prolonged and continued physiological stress responses. In turn, these physiological responses may be intricately related to infant behavioral and temperamental difficulties, further shaping behavioral, neuroendocrine and immunological development in the long term (Guerry and Hastings, 2011; McCrory et al., 2012).

There are some indications from the animal literature that maternal prenatal stress is linked to offspring habituation to repeated stressors. In one study in prepubertal and adult rats, corticosterone habituation to a repeated stressor was slower in prenatally stressed rats, as compared to non-stressed controls (Fride et al., 1986). Another study in rats showed that prenatally stressed adult males, in contrast to control males, did not display corticosterone habituation to a repeated physical stressor (Bhatnagar et al., 2005). In female adult rats, the effects were less clear with some habituation in both the prenatally stressed and non-stressed groups. These results suggest that prenatal stress has long-lasting effects on animals' ability to respond to stress in adulthood. In a study of behavioral habituation to repeated tactile stimuli in young adult rhesus monkeys, individuals without prenatal maternal stress habituated across trials with different stimuli, whereas prenatally stressed individuals showed slight behavioral sensitization across trials (i.e. increased withdrawal; Schneider et al., 2008).

To our knowledge, there is only one human study that investigated the effects of maternal prenatal stress and cortisol on offspring cortisol habituation to a repeated stressor. In a small study, our group found that higher maternal pregnancy cortisol and pregnancy-related anxiety predicted higher cortisol on the first day of school after the summer vacation, and on a day a week later in 5-year old children (Gutteling et al., 2005). However, there was no difference between children of higher and lower pregnancy stress with respect to the cortisol habituation to school. This may be explained by the small size of the study ($N = 29$) and by the fact that the group as a whole showed no cortisol habituation over the first week at school.

The aim of the present prospective study was to examine if maternal prenatal stress and cortisol are related to infants' behavioral and cortisol habituation to a repeated stressor in a relatively large, healthy, non-clinical group. We included maternal reports on general stress as well as on specific pregnancy-related anxieties and hassles, as these have been found to be important contributors to infant HPA-axis development in past studies (Gutteling et al., 2005; Tollenaar et al., 2011; Buss et al., 2011). Furthermore, we included maternal circadian cortisol concentrations as a measure of physiological stress levels (Beijers et al., 2010; de Weerth and Buitelaar, 2005a; Pruessner et al., 2003). Behavioral and cortisol stress responses in the infants were measured in reaction to three weekly maternal home separations at 9 months of age. Maternal separations are an ecologically relevant stressor for infants of this age, as many infants are left for shorter or longer periods in the care of babysitters and in center-based childcare. Prolonged maternal separations (i.e. 30 min or longer) produce moderate cortisol elevations in infants at around 9 months of age (reviewed by Jansen et al., 2010). Moreover, the infants were left with an unfamiliar female caregiver, an unobtrusive, unfamiliar assistant that videotaped the session, and unfamiliar toys to play with, to ensure the novelty of the situation and to distinguish these maternal separations from other maternal separations with which the infant might be familiar. In animal models, exposure to mild or moderate stressors, including exposure to a repeated

Table 1 Descriptive statistics of the study variables (N = 88—107).

	Mean (SD)	Range
Demographics characteristics		
Maternal age (years)	31.27 (3.66)	20.00—40.00
Maternal educational level		
Primary education	5.70%	
Secondary education	24.50%	
College or university	69.80%	
Maternal marital status (living with partner)	95.30%	
Birth weight (grams)	3480.96 (500.34)	2470.00—4800.00
Length of gestation (days)	280.91 (9.14)	260.00—298.00
Apgar scores at 5 min	9.68 (0.61)	7.00—10.00
Infant sex (girls)	48.6%	
Additional confounders		
Pregnancy smoking	5.00%	
Duration of breastfeeding in weeks[a]	19.53 (12.48)	0.00—40.00
Non-parental care[a]	84.40%	
Attendance childcare center[a]	52.60%	
Number of weekly non-parental care hours[a]	5.96 (4.14)	0.00—23.00
Age of initiation of non-parental care (days)	91.94 (37.05)	70.00—273.00
Minutes since last sleep at the onset of 1st separation	89.34 (64.06)	0.00—330.00
Minutes since last sleep at the onset of 2nd separation	88.87 (64.86)	0.00—360.00
Minutes since last sleep at the onset of 3rd separation	82.96 (54.46)	10.00—240.00
Postnatal state anxiety (STAI)	14.90 (11.11)	1.00—61.00
Postnatal daily hassles (APL)	31.28 (8.23)	20.00—70.00
Prenatal cortisol levels (nmol/L)		
Cortisol awakening response (CAR)	8.17 (4.91)	−2.50 to 23.00
Cortisol decline over the day	10.09 (5.87)	−6.50 to 24.75
Area Under the Curve to the Ground (AUCg)	1879.93 (361.33)	1020.65—3236.15
Prenatal psychological stress and anxiety		
State anxiety (STAI)	30.62 (8.14)	20.00—58.00
Daily hassles (APL)	11.19 (9.50)	1.00—62.00
Fear of giving birth (PRAQ-R)	2.00 (.86)	1.00—4.67
Fear of bearing a handicapped child (PRAQ-R)	2.21 (.70)	1.00—4.25
Infant cortisol responses (nmol/L)		
Area under the curve to the ground (AUCg) 1st separation	771.95 (346.66)	291.00—3070.00
Area under the curve to the ground (AUCg) 2nd separation	756.62 (296.35)	344.75—2106.25
Area under the curve to the ground (AUCg) 3rd separation	697.22 (238.27)	371.25—1640.25
Infant behavioral responses (% observation periods)		
Crying and fussing 1st separation	24.65 (17.56)	0.00—73.53
Crying and fussing 2nd separation	27.03 (20.51)	0.00—96.22
Crying and fussing 3rd separation	22.78 (20.05)	0.00—77.27

[a] Within the first 9 months of life.

maternal separation, are related to decreases in cortisol responses (Barnum et al., 2007). In this line, we hypothesized to see decreasing infant cortisol and behavioral reactions over the separations. Moreover, we hypothesized that higher prenatal stress and cortisol would predict smaller decreases in infant negative behavior and cortisol responses over the separations (i.e. less habituation).

2. Methods

2.1. Participants

Pregnant primiparous women from the city of Utrecht and surroundings (The Netherlands) were invited to participate in a longitudinal study on the effects of prenatal

stress on infant development through folders distributed by their midwives. The local Medical Ethics Committee approved the study, and written informed consent was obtained from all the participants. Exclusion criteria were maternal health problems, medication use, severe pregnancy and/or childbirth complications, twinning, and prematurity. Of the 146 women that began the study in early pregnancy, 24 were excluded because of the exclusion criteria. A further 6 discontinued the study during pregnancy and 9 after delivery, due to lack of time or interest. These women did not differ from the remaining group in their demographic data. The remaining 107 subjects were healthy, and had normal, uncomplicated singleton pregnancies with term deliveries (>37 weeks) of healthy, first-born infants. Demographic characteristics of the mothers and infants are provided in Table 1.

Table 2 Pearson correlations for maternal prenatal stress and cortisol variables, and infant cortisol and behavioral measures.

	CAR	Cortisol decline	AUCg	APL	STAI	PRAQ-R birth	PRAQ-R handicapped	AUCg1	AUCg2	AUCg3	Cry/fuss 1	Cry/fuss 2	Cry/fuss 3
CAR													
Cortisol decline	−.23*												
AUCg	.03	−.02											
APL	.04	.03	−.01										
STAI	.18†	.06	.07	.46**									
PRAQ-R birth	−.04	.01	−.10	.29*	.23*								
PRAQ-R handicapped	.04	.04	−.02	.19†	.42**	.31**							
AUCg separation 1	−.07	−.07	−.09	.02	−.06	−.17†	−.22*						
AUCg separation 2	.06	.06	.13	.05	−.08	−.15	−.11	.32**					
AUCg separation 3	.15	−.11	.02	.13	.00	.02	−.20†	.23*	.46**				
Cry/fuss separation 1	−.10	−.14	.01	−.01	−.21*	−.19†	−.20*	.36**	.02	.10			
Cry/fuss separation 2	−.14	−.10	.12	.05	−.17	−.13	−.18†	.17*	.16	.08	.63**		
Cry/fuss separation 3	−.07	−.06	.00	−.00	−.07	−.12	−.02	.01	−.05	.01	.58**	.60**	

Note: CAR = cortisol awakening response, AUCg = area under the curve to the ground, APL = daily hassles, STAI = state anxiety, PRAQ-R$_{birth}$ = fear of giving birth, PRAQ-R$_{handicapped}$ = fear of bearing a handicapped child.
  * $p < .05$.
  ** $p < .01$.
  † $p < .10$.

2.2. Procedure

In the third month of pregnancy, the mothers collected circadian samples of saliva for two consecutive days (M = 32.8, SD = 1.1 weeks of gestation) and filled in questionnaires on general and pregnancy-related feelings of stress and anxiety (M = 37.2, SD = 1.1 weeks of gestation). At 9 months of age (age first separation: M = 8 months and 26 days, SD = 7.7 days), their infants were subjected to three weekly 1-h maternal separations. Salivary cortisol was obtained from the infants prior to the separation (T1: baseline concentration) and at 35 (T2), 75 (T3), and 90 min (T4) after the mother had left. Because the cortisol measured in saliva represents the saliva production of approximately 25 min before (Dickerson and Kemeny, 2004), these samples correspond to 10 (T2) and 50 min (T3) post maternal departure, and 5 min (T4) post reunion with the mother. Furthermore, the sum of crying and fussing vocalizations were scored for each separation. Demographic information of the mother and her infant was obtained with questionnaires filled in during pregnancy or after birth. To control for maternal postnatal stress, stress-related questionnaires were filled in at three and nine months postpartum.

The three weekly maternal separations were carried out in the infants' homes, in the same manner and at the same time of day each week (separation 1: M = 11:39 h, SD = 2:26 h, separation 2: M = 11:43 h, SD = 2:28 h, separation 3: M = 11:40 h, SD = 2:26 h). The separation was designed to emulate a regular babysitting situation, and mothers could choose between remaining in another room and actually leaving the house (e.g. for shopping). The mothers were asked to have the infant fed and rested before the visit started (at least 40 min awake from last sleep). The time of day of the visit was chosen in order to accommodate this in each individual infant's regular feeding and sleeping schedule. The infants were left with an unfamiliar female caregiver and an unobtrusive assistant that videotaped the session. The caregiver was the same female research assistant each week. The separations always started with the infant being seated on the floor in front of a play mat with ten age-appropriate toys (provided by the researchers). The caregiver and the mother sat on the floor on each side of the infant. If needed, the infant was stimulated to engage with the toys. After 5 min the mother said good-bye to the infant and left the room, initiating in this way the 60-min separation period. The caregiver remained next to the infant for 2 min and then sat on a chair or sofa for the rest of the separation. She responded sensitively to infant distress (i.e. following a standard soothing protocol), but read a magazine and generally ignored the infant when non-distressed. The mother was called back into the room at the end of the separation period. The caregiver left the room once the mother was reunited with her infant, and the mother then played with or cuddled her infant for 5 min. If during the separation the infant cried and was very difficult to sooth for a period of 5 min, then the mother was called back and the separation was ended before the 60-min period had elapsed.

2.3. Independent variables

2.3.1. Prenatal maternal cortisol

The mothers collected four salivary samples on two consecutive days. The mothers collected saliva by direct spitting and were instructed to fill in a form indicating exact sampling times. The women took the first daily sample upon awakening (07:30 h ± 01:06 h; range: 05:00–11:00 h), and the second after remaining in a recumbent position for 30 min. Additional samples for a daytime profile of cortisol production were taken at midday (12:00 h) and in the evening (21:00 h). Noon samples were taken before lunch, and between 11:30 and 13:30 h, and evening samples were taken at least 1.5 h after dinner, and between 20:00 and 23:00 h (de Weerth and Buitelaar, 2005a). The samples were kept at −20 °C until the moment of analysis (see below). First, the cortisol awakening response (CAR, 30 min after waking minus waking; de Weerth and Buitelaar, 2005a) was calculated for day 1 and day 2. The CAR between day 1 and day 2 correlated significantly (r = .27, $p < .01$), and the mean CAR of the two days was used. Second, because of positive inter-correlations (r's ranging from 0.42 to 0.62, all p-values <0.001), mean cortisol concentrations over the two days were calculated for each sampling time. The variables that were derived from these data were the area under the curve to the ground (AUCg; Pruessner et al., 2003) and the cortisol decline over the day (waking minus evening; Beijers et al., 2010). The CAR, AUCg and the cortisol decline represent the activity of the HPA axis upon awakening, the general diurnal production of cortisol, and the slope of the diurnal curve, respectively. As such, they reflect different aspects of cortisol physiology. Moreover, these measures are not or weakly intercorrelated (see Table 2).

2.3.2. Prenatal maternal psychological stress and anxiety

Anxiety was assessed by means of the State-Trait Anxiety Inventory (STAI; Spielberger, 1983; Van der Ploeg et al., 1981). This self-report questionnaire consists of two subscales each containing 20 items, scored on a 4-point scale. Higher scores indicate a higher intensity of anxiety. Only the state anxiety subscale was included in this study. The state anxiety subscale measures anxiety at the moment of scoring, and is conceptualized as an unpleasant negative emotion experienced in response to a threatening stimulus.

Stress was assessed by means of a Dutch questionnaire which measures the rate of occurrence and intensity of daily hassles in the past two months (APL; Vingerhoets et al., 1989). Respondents have to check 49 items describing situations that have occurred in their personal life, and to rate how much each situation bothered them on a 4-point scale. Examples of items are 'you could not find important belongings' and 'you had a conflict with your colleagues'. Scoring was based on the mean intensity rating: the sum of how much daily hassles bothered the respondent divided by the frequency of daily hassles. Higher values indicate that the participant experienced more negativity as a result of her daily hassles.

Pregnancy-specific anxiety was assessed by means of the Pregnancy-specific anxieties questionnaire − revised (PRAQ-R; Huizink et al., 2004b). This questionnaire consists of 10

items that fit a three-factor-model: fear of giving birth, fear of bearing a physically or mentally handicapped child, and concern about one's appearance. We used two subscales in the present study, namely fear of giving birth and fear of bearing a physically or mentally handicapped child. The items were answered on a 5-point scale, ranging from 'never' to 'very often'. Higher values on the subscales therefore indicated higher pregnancy-related anxieties.

2.4. Dependent variables

2.4.1. Infant cortisol response

The infant's saliva was collected by swabbing the child's mouth with a cotton dentist roll for a few minutes. The roll was then placed in a disposable 5 ml syringe, closed with a small plastic cap. The samples were kept at $-20\,^{\circ}$C until the moment of analysis. In order to avoid the loss of data due to insufficient saliva, the samples were analyzed in duplo using an extraction method specially designed for small quantities of saliva (de Weerth et al., 2003a). To control for interassay variation, the cortisol assays were performed with all samples of the same infant in the same batch. Cortisol was determined with an in house competitive RIA (see below),

which is insensitive to breast milk and formula contamination (de Weerth et al., 2003a). For every separation, the area under the curve to the ground (AUCg) was calculated to measure the infants' cortisol response (Pruessner et al., 2003).

2.4.2. Infant crying and fussing

Each separation was observed and coded for infant vocalizations with one-zero sampling using 30-s intervals. For each interval one of the following four categories was scored: no vocalization, positive or neutral vocalization, fussing vocalization (discrete squeaks, fretting, whining, and whimpering), or crying vocalization (weeping; higher arousal level than when fussing, more muscle tension, often accompanied by intense facial coloring). When more than one of these vocalizations took place during the 30-s period, the behavior with the highest degree of negative emotion was scored. Three research assistants who were blind to the maternal prenatal stress status, scored the videotapes after reaching an adequate inter-observer reliability with the first author (mean Cohen's kappa over 10 infants was 0.81). The sum of the periods with crying and fussing was calculated, and expressed as the percentage of the total number of 30-s
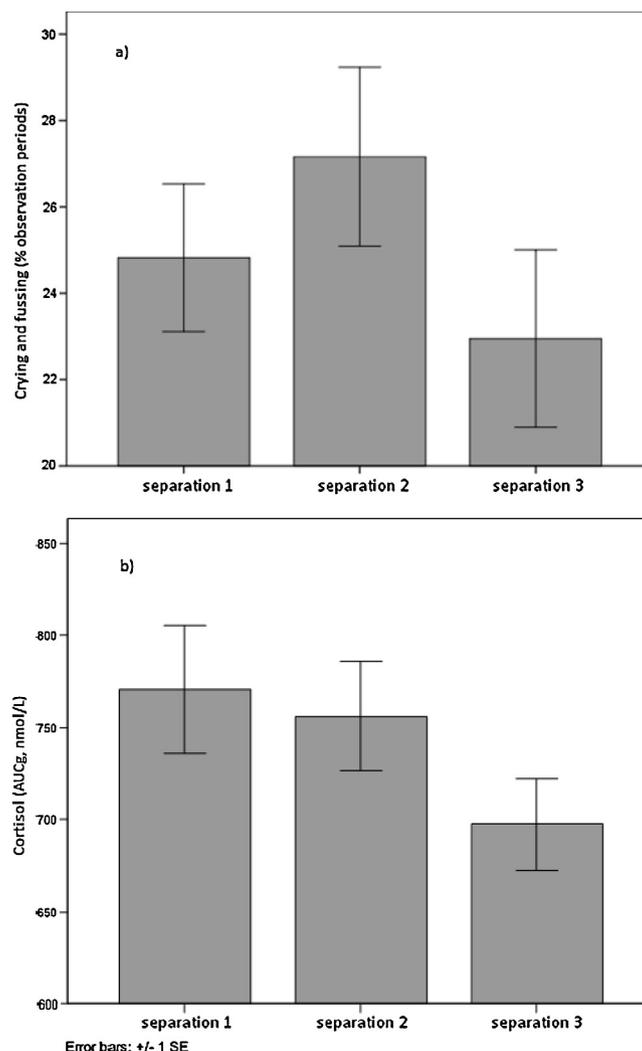

Figure 1   The infant behavioral (a) and cortisol (b) responses to the three maternal home separations.

Table 3 Estimates for the best fitting multilevel models for the infant cortisol (AUCg) and behavioral (crying and fussing) reactions to a repeated maternal separation.

	Infant cortisol AUCg			Infant crying and fussing		
	Estimate	SE	p	Estimate	SE	p
Fixed effects						
Intercept	793.46	179.19	.001	29.89	24.90	.235
Time linear	−27.91	21.01	.188	16.41	8.35	.054[†]
Time quadratic				−4.69	2.07	.026[*]
Pregnancy smoking	400.63	142.46	.008[**]	−.72	17.42	.967
Maternal educational level	19.00	15.15	.216	3.22	1.73	.069[†]
Birth weight				−.01	.00	.223
Duration of breastfeeding in weeks				−.21	.19	.252
Attendance childcare center	−.19	41.98	.996	−10.41	4.64	.029[*]
Minutes since last sleep	−.39	.34	.255	.01	.02	.750
Cortisol awakening response (CAR)	−14.43	9.23	.121	−.99	.44	.030[*]
Cortisol decline over the day	1.13	3.51	.750	−.28	.42	.501
Area under the curve to the ground (AUCg)	−.03	.07	.681	.00	.01	.663
Daily hassles (APL)	2.71	2.23	.233	.00	.00	.871
Time by Cortisol Awakening Response (CAR)	10.03	4.11	.017[*]			
Deviance	1549.523			1046.217		

Notes: Additional multilevel analyses using the highest infant cortisol peak value of T2/T3, and controlling for the lowest baseline sample of T1/T2, are comparable to the results in Table 3 and also show that the 'Time by Cortisol Awakening Response' is significantly related to infant cortisol peak.

[†] $p < .10$.
[*] $p < .05$.
[**] $p < 01$.

periods for each separation. This variable represents the infants' negative vocalizations during the maternal separations.

2.5. Cortisol analysis

Both mother and infant cortisol samples were stored at −20 °C until analysis. Saliva samples were analyzed in duplo at the Laboratory of Endocrinology of the University Medical Center Utrecht. Cortisol was measured without extraction using an in house competitive radio-immunoassay employing a polyclonal anticortisol-antibody (K7348). [1.2-3H(N)]-Hydrocortisone (NET185,NEN − DUPONT, Dreiech, Germany) was used as a tracer following chromatografic verification of its purity. The lower limit of detection of the assay was

0.5 nmol/l. For the maternal samples the inter-assay variation was 10.0, 6.4, and 6.0% at 5.1, 11.9, and 20.6 nmol/l respectively; for the infant samples the inter-assay variation was 9% at 4 nmol/L and 5% at 10 nmol/L. For both mother and infant samples the intra-assay variation was 4% at 10 nmol/L.

2.6. Confounders

The following potential confounders were included: maternal educational level, pregnancy smoking (yes or no), length of gestation, birth weight, infant sex, and duration of breastfeeding in weeks. With regard to non-parental care, we included four confounders: non-parental care within the first 9 months of life (yes or no), mean number of weekly non-parental care hours within the first 9 months of life, age of initiation of non-parental care, and attending center-based care within the first 9 months of life (yes or no). To control for maternal postnatal anxiety and stress, state anxiety (STAI) and daily hassles (APL) were assessed postnatally at 3 and 9 months. Because of moderate to strong intercorrelations ($r = .43$ and $r = .72$ respectively, p-values <0.01), mean anxiety and daily hassles scores were calculated. Furthermore, we controlled for the time of day, and time since the last infant sleep at the onset of the three maternal separations.

2.7. Statistical analyses

Data preparation included checking the variables for violations of normality, and for outliers. No skewed data was found. One outlier was detected for daily hassles (APL), two for the infants' cortisol response to the first maternal
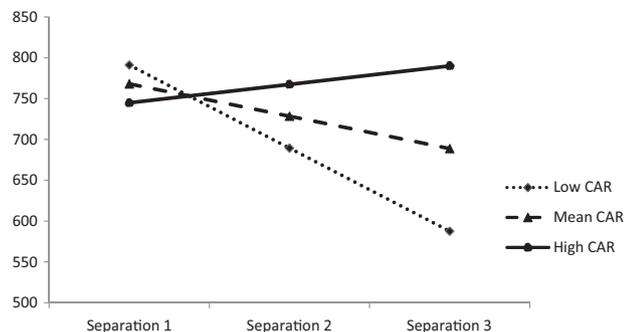
Figure 2 Interaction effect time × maternal cortisol awakening response (CAR) on infant cortisol reactions (AUCg) to the repeated maternal separation.

separation, and two for the infants' cortisol response to the second maternal separation. These outliers disappeared after log transformation, except for one. This outlier for the infants' cortisol response to the first separation was removed from the data for the analyses. Repeated measures analyses of variance (ANOVA) were conducted to examine the time course of infant cortisol and behavioral responses over the three maternal separations.

To test whether prenatal stress and cortisol uniquely predicted infants' negative vocalizations and cortisol stress responses to three repeated maternal separations, longitudinal regression analyses using mixed-model (multilevel) designs in SPSS 19.0 were conducted. An advantage of multilevel analyses over repeated measures analyses is the potential to include infants with missing data at one or two of the time points. With this technique, all valid data points can be included in the analyses. The physiological or behavioral responses to the three maternal separations were introduced at level 1 and nested within the infants at level 2. First, the intraclass correlation was calculated using a null model, to examine whether the nested structure is needed for the analyses. The intraclass correlation was 36.1% for the infants' cortisol AUCg, so 36.1% of the variability in infants' cortisol responses to the repeated maternal separation was associated with differences between infants, and multilevel analyses were appropriate. For infants' crying and fussing, the intraclass correlation was 60.4%, also indicating that multilevel analyses were appropriate. Second, a build-up strategy was used, adding predictors into the model one by one and examining their deviance on the −2 log likelihood ratio scale after generalized least square estimation. Linear time and quadratic time were first entered into the model. Linear time was considered a random factor. The time model which improved the model fit the best was retained, and thereafter the covariates were entered one by one. We then added the prenatal stress measures (self-report and circadian cortisol). In the last models we also included the interactions between the prenatal stress measures and linear and quadratic time variables (calculated by multiplying the centered prenatal stress measure by the time variable) to examine the effect of prenatal stress on infant behavioral and cortisol responses over the separations (i.e. habituation). The best fitting models are presented in the results.

3. Results

3.1. Descriptive analyses

Crying and fussing data were available for 105 infants at separation 1, 97 infants at separation 2, and 95 infants at separation 3. Drop-out was due to problems scheduling the remaining separations or to the mother wishing to discontinue the separations. Furthermore, cortisol data was available for 98 infants at separation 1, 96 infants at separation 2 and 90 infants at separation 3. Missing cortisol samples were due to lack of sufficient saliva for analysis, or to contamination of the samples.

Separations were ended before time due to the infant being distressed and difficult to sooth for 9 infants at separation 1, 7 infants at separation 2, and 7 infants at separation 2. Independent samples T-tests showed no significant differences between these infants and the infants that completed the separations on demographic characteristics or the prenatal stress measures.

Descriptive statistics are presented in Table 1 (untransformed data). The behavioral and cortisol responses of the infants to the three repeated maternal separations are presented in Fig. 1. A repeated measures ANOVA revealed a significant quadratic time effect for crying and fussing (F (1, 91) = 3.87, $p < 0.05$, $\eta^2 = 0.04$), showing that the infants' behavioral reactions to the repeated maternal separations followed a quadratic trajectory, with the highest levels of crying and fussing during the 2nd separation. A second repeated measures ANOVA revealed a significant linear time effect for the cortisol AUCg (F (1, 86) = 4.05, $p < 0.05$, $\eta^2 = 0.05$), showing that the infants' cortisol reactions to the repeated maternal separations decreased linearly over the separations.

Table 2 presents the correlations for the prenatal stress and cortisol predictors with the infant outcomes. The psychological anxiety and stress predictors were all (marginally) intercorrelated (r's ranging from .19 to .46, $p < .10$). Regarding the cortisol predictors, only the CAR and the cortisol decline were intercorrelated ($r = -.23$, $p < .05$). A higher CAR was related to a less steep cortisol decline over the day. Furthermore, the cortisol and psychological variables were not correlated, except for one marginally significant correlation between the CAR and state anxiety ($r = .18$, $p < .10$). A higher CAR tended to be related to higher reported anxiety.

Regarding the infant outcomes, the AUCg for the different separations were all intercorrelated (r's ranging from .23 to .46, $p < .05$), and the same goes for crying and fussing (r's ranging from .58 to .63, $p < .01$). Hence, the infants displayed a certain stability in behavioral and cortisol responses over the three separations. Infant cortisol reactions were related to crying and fussing only for the first maternal separation ($r = .31$, $p < .01$). There were no significant correlations between the infant cortisol and behavioral reactions to the other two separations.

3.2. Main analyses

The final longitudinal regression models are summarized in Table 3. For the infant cortisol AUCg, the analyses showed that a higher CAR in pregnant women predicted the interindividual pattern of infant cortisol responses over time (time by cortisol awakening response, $p = .017$). Fig. 2 depicts this interaction effect. A lower CAR was related to a decreasing cortisol response, while a higher CAR was related to a stable cortisol response over the separations (i.e. no habituation). Of the confounding variables, smoking during pregnancy was related to higher infant cortisol reactions (AUCg, $p = .008$).

For infant crying and fussing, the analyses showed that a higher maternal prenatal CAR was related to less crying and fussing in general as a reaction to the maternal separations (cortisol awakening response, $p = .030$). Also, attending a childcare center was related to less crying and fussing ($p = .029$), while maternal educational level tended to be positively related to crying and fussing in general as a reaction to the separations ($p = .069$).

4. Discussion

In the present study we investigated infant cortisol and behavioral reactions of 9-month-old infants to three weekly maternal separations, and the possible links with maternal late pregnancy psychological stress and cortisol. For the group as a whole, our data showed that the infants habituated to the repeated separations in their crying and fussing behavior, and in their cortisol concentrations; both decreased over time. Furthermore, higher infant cortisol reactions were related to more crying and fussing, but only for the first maternal separation; there were no significant correlations between the infant cortisol and behavioral reactions in the second or third separations. The results also showed that, as hypothesized, maternal prenatal stress was related to less habituation in the infants. The maternal cortisol awakening response (CAR) was related to distinct patterns of infant responses to a repeated maternal separation. A lower CAR was related to a decreasing cortisol response, while a higher CAR was related to a stable cortisol response over the separations (i.e. no habituation), as well as to less crying and fussing over all separations. In earlier studies an elevated maternal CAR during pregnancy has been related to shorter gestational length (Buss et al., 2009) and lower birth weight (Bolten et al., 2011). To our knowledge the present study is the first to relate the maternal prenatal CAR to later developmental outcomes in infants.

Our results provide important information about infant cortisol and behavioral habituation to an ecologically valid and stressful situation (maternal separation). The ability to habituate is one of the most basic forms of learning and part of normal development. Failure to habituate might have important implications for later development, including the risk for hyper-reactivity to stimuli and other types of psychopathology (Guiraud et al., 2011). Our finding that higher maternal CAR is related to less habituation to a repeated stressor is in line with previous findings in animal models (Bhatnagar et al., 2005; Fride et al., 1986; Schneider et al., 2008), but they do not support those of the only human study. Gutteling et al. (2005) found no links between maternal prenatal stress and habituation in 5-year-old children. As mentioned before, their lack of findings could be due to the small sample size as well as to the fact that the group as a whole did not habituate to the stressor in the study period. In the present study the sample size was more than three times larger, and the group as a whole did show habituation to the repeated maternal separation. Other explanatory factors for these differences in results could be the much younger age of our participants, the fact that we also assessed the CAR while Gutteling et al. (2005) assessed early morning cortisol, and finally, that we assessed maternal stress in late pregnancy while Gutteling et al. (2005) assessed maternal stress in early pregnancy. The hypothesized effects of maternal prenatal stress on the fetus are often found to be different according to when they occur during pregnancy (e.g. Dancause et al., 2011; Davis et al., 2011)

Of the seven self-report and cortisol prenatal stress variables we investigated in this paper, the CAR was the only variable that showed a link to both cortisol as well as behavioral responses to a repeated stressor in the 9-month-old offspring. The CAR is closely related to the process

of awakening (Wilhelm et al., 2007; Kudielka and Wüst, 2010). It characterizes the brisk increase in the release of cortisol into the blood stream ($\pm 38-70\%$) upon awakening in the morning, reaching its maximum at around 30 min after awakening (Pruessner et al., 1997; Fries et al., 2009). Although the exact function of the CAR remains to be determined, recent ideas are that it plays a role in orienting the individual in time and space, preparing the individual for the demands of the upcoming day (Fries et al., 2009). This is supported by the fact that the CAR apparently depends more on situational factors than on trait factors (Hellhammer et al., 2007) and that the CAR is increased in subjects facing elevated burden. For example, Rohleder et al. (2007) found that participants of a competitive ballroom dance tournament had an increased CAR on the day of the competition, whereas their CAR on a regular, non-competition day was much lower. However, in our sample, the CAR was mostly unrelated to the maternal psychological functioning during late pregnancy, except for a marginally significant correlation with state anxiety ($r = .18$, $p < .010$).

The question remains what the underlying mechanism is that links the CAR of primiparae during late pregnancy to their infants' habituation to stressful situations in the first year of life. There is evidence from animal models and human studies that elevated cortisol levels in the mother may play an important role in programming effects on the development of the offspring's brain (e.g. Charil et al., 2010). It is important to note that during the second half of pregnancy basal cortisol levels, and especially morning levels, increase to levels that toward the end of pregnancy are more than two-fold the non-pregnant levels (de Weerth and Buitelaar, 2005b), and that the CAR declines as pregnancy progresses (Entringer et al., 2010). This means that a daily elevated CAR would represent a lack of this normal attenuation process, and result in high absolute concentrations of cortisol in maternal blood. In turn, these high levels of maternal cortisol may have programming effects on the offspring's capacities to habituate to stressors.

Note that an alternative explanation for the association between maternal prenatal CAR and the infants' lack of cortisol habituation is hereditary transmission. It is possible that shared genetic makeup between the mother and the offspring explains both the elevated CAR and the lack of cortisol habituation to the repeated maternal separation.

Another interesting result of this study is the finding that the infants' behavioral and cortisol reactions appear to dissociate over the maternal separations. In the first separation, cortisol and behavior were moderately positively correlated, indicating that the infants that cried the most were also those with the highest cortisol response. However, this association between cortisol and behavior was not present in the second and third separations. This type of dissociation among stress systems has been observed before in both animal as human research. Gunnar et al. (1991) found, for example, that the newborn infant cortisol response readily habituates, while exposure to a repeated stressor did not affect the behavioral distress response (i.e. fussing and crying). It is not clear why behavioral and physiological responses dissociate during repeated stress. However, two stress models can be used to explain the development of the relations between cortisol and behavior during infancy. Selye's 'General Adaptation Syndrome' (1952) explains the

development of the relations between cortisol and behavior over sustained or repeated stressors. During the first stage, stressful events cause a general stress response, resulting both in behavioral and physiological signs of stress, including cortisol elevations, to provide instant energy to deal with the stress (the alarm stage). When shifting into the second stage (the resistance stage), cortisol levels return to normal. Nevertheless, if in this stage an individual still perceives the situation as stressful, he or she can remain in a state of arousal that is accompanied by behavioral signs of stress.

According to the 'coping model' (Spangler and Schieche, 1998), stressful situations only elicit physiological stress responses when appropriate behavioral regulation strategies are not available. When being exposed to a stressor for the first time, infants have not yet had the opportunity to master the appropriate stress regulation strategies and will show a general stress response accompanied by both behavioral and physiological signs of stress. However, repeated experiences with stressful situations will permit infants to learn to cope with the stressor, i.e. adaptation to the situation by learning behavioral strategies to respond to and cope with the stress. Specifically, crying may be used as a strategy as it will elicit care and support from others, which constitutes a source of external regulation for an infant. In this way crying may help infants regulate their emotions before their HPA-axis is overly activated. On the other hand, some infants may be slower or even unable to adapt behaviorally with a repeated stressor. These infants might show a physiological stress response, accompanied by less effective behavioral coping strategies (e.g. reduced crying), or continue showing a general stress response, accompanied by both behavioral and physiological signs of stress.

This primary association, followed by a later disassociation between behavior and cortisol, has important implications. Caregivers of young infants rely heavily on behavioral signs such as crying and fussing to monitor the infant's well-being. However, infant behavior and cortisol apparently dissociate over time when confronted with a repeated stressor such as a maternal separation. In turn, caregivers may erroneously interpret negative vocalizations as a sign of infant overall stress, or the absence of negative vocalizations as a sign of overall well-being. Our findings with weekly maternal separations in the form of 1-h 'babysitter' situations in the infant's own home brings questions about how habituation to other real-life repeated maternal separations, such as center-based childcare, actually proceeds on a physiological level. Prolonged maternal separations of up to 8 or 9 h, with multiple caregivers and in settings outside the home (e.g. in a childcare center or hospital), could theoretically be expected to be more challenging and stressful for the infant, possibly producing stronger results. Previous research has already shown that infants exhibit higher concentrations of the stress hormone cortisol on days when they are at center-based childcare compared with days when they are at home (e.g. Ahnert et al., 2004; Watamura et al., 2010). We do not know, however, whether and how cortisol in center-based childcare is related to behaviors representative of arousal and stress in infants and toddlers. This question is extremely relevant given that we know that repeated elevations of cortisol can lead to chronic physiological stress, that in turn may affect the child's later development and functioning, and even be related to increased chances of developing psychopathology (Guerry and Hastings, 2011; Lupien et al., 2009; McCrory et al., 2012). If certain infants display no overt signs of distress while at the same time are producing high concentrations of cortisol, caregivers may be led to erroneously concluding that the infant is well-adapted and non-stressed in childcare. Future longitudinal research with multiple sampling over longer periods of time is needed to investigate this important issue.

A major strength of the current study is that, to our knowledge, it constitutes the first study to investigate the relations between maternal prenatal stress and habituation to a repeated stressor during infancy. The study was prospective in nature, carried out in a relatively large, non-clinical population, and used maternal separations as an ecologically valid stressor for young infants. Furthermore, by controlling for important confounders, including attendance to center-based childcare and postnatal maternal stress and anxiety, we ruled out many alternative factors that could have influenced the results.

Despite its strengths, the study also has limitations that need to be addressed. First, the mothers sampled their pregnancy cortisol by themselves in their home, making the samples subject to measurement (timing) errors. CAR samples taken at home have been found to be comparable to CAR samples taken under strictly controlled conditions in a sleep lab (Wilhelm et al., 2007). Nevertheless, future research should consider using electronic monitoring devices (e.g. MEMS TrackCap; Aardex Ltd., Zug, Switzerland) to provide objective maternal compliance data with collection times. Second, our maternal samples were limited to the third trimester, and our stressor to a maternal separation, making it impossible to make predictions about other periods of pregnancy and other types of stressors. Finally, all mothers had healthy pregnancies, and most were highly educated and lived together with their partner, compromising the generalizability of the study. Including a more high-risk sample would increase the possibility of severe stress during pregnancy, and might show stronger or different associations with infant habituation to maternal separations.

Follow-up studies are needed to draw conclusions about habituation in the long-run. Studies over the course of several months including more repeated measurements are necessary to investigate whether the relations found change with time. Also, longitudinal studies linking maternal prenatal stress and cortisol to habituation to ecologically valid stressors later in a child's life are much needed to be able to understand these results in a developmental perspective. Knowing that important HPA axis set-points are established during pregnancy, and further development and fine-tuning occur both in uterus and during the first year of life (de Weerth et al., 2003b; Tollenaar et al., 2010), it is important to investigate how the HPA-axis in children will further adapt and change according to the environmental challenges encountered by the individual.

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Conflicts of interest

There are no conflicts of interest, financial or otherwise to declare.

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