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Stress reactivity in preschool-aged children: Evaluation of a social stress paradigm and investigation of the impact of prenatal maternal stress

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ABSTRACT

Prenatal maternal stress is an established risk factor for somatic and psychological health of the offspring. A dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis in offspring has been suggested as an important mechanism. However, the impact of prenatal stress on stress reactivity in preschool-aged children is not yet well understood. This is partly due to the fact that for this age group there is no stress test as well established as for older children and adults.

In the present work a previously published stress test (Kryski et al., 2011) was evaluated in a large sample of 45-month-old children (n = 339). Furthermore, the relation between measures of prenatal maternal stress and cortisol reactivity was investigated. Prenatal stress was defined as psychopathology (self-report available for n = 339; expert-rating available for a subsample of n = 246) and perceived stress (n = 244) during pregnancy.

The stress paradigm elicited significant increases in salivary cortisol 30 and 40 min after the test, and 60.8% of the children were classified as responders. Lower cortisol levels after the stress test were observed in the group of children with prenatal stress defined as maternal psychopathology (both self-reported and expert-rated). Maternal perceived stress as a continuous measure was not significantly associated with cortisol levels. However, when comparing children in the highest quartile of maternal perceived stress to all other children, significantly lower cortisol values were observed in the prenatally stressed group.

The present study confirms the paradigm by Kryski et al. as an effective stress test for preschool-aged children. Moreover, it provides further evidence that prenatal stress impacts HPA axis reactivity. Future studies should target the timing, nature, and intensity of prenatal stressors and their effect on the stress response in offspring at different developmental stages.

1. Introduction

In animals and humans, prenatal maternal stress (PS) has impact on birth outcomes, development and stress regulation as well as disease in the offspring (e.g., Stein et al., 2014; Van den Bergh et al., 2017). The intrauterine environment contributes to long-term consequences particularly via epigenetic modifications—a process referred to as prenatal programming (Sosnowski et al., 2018). While the importance of PS is well-established, its precise mechanisms and the timing of its effects are still within the scope of current research. One important system involved in prenatal programming is the hypothalamic-pituitary-adrenal (HPA) axis (Beijers et al., 2014). In response to stressful situations the final product of the HPA axis—the steroid hormone cortisol—is secreted from the adrenal cortex, setting off a range of physiological changes with the aim to re-establish homeostasis. During pregnancy, elevated levels of cortisol can affect the developing fetus by crossing the placental barrier (Jansson and Powell, 2007) and shape the functioning of the developing HPA axis of the offspring (e.g., Maccari et al., 2003;

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Van den Bergh et al., 2017; Zijlmans et al., 2015). Subsequent HPA axis alterations, often described as hyper- and hypo(re)activity, can persist into adulthood (e.g., Carpenter et al., 2017; Kumsta et al., 2017; Pesonen et al., 2010). HPA axis dysregulation is often observed in psychiatric patients, and has been suggested as a mechanism of premorbid vulnerability (Handwerger, 2009). Furthermore, subjectively perceived stress is considered a risk factor and a trigger for psychopathology, and perceived stress is increased during episodes of a psychiatric disorder (McEwen, 2004; Myin-Germeys et al., 2009). Therefore, the presence of a maternal psychiatric disorder during pregnancy can be conceptualized as a specific prenatal stressor.

In rodent models of PS, an altered response to stressful stimuli is a common finding in the offspring—e.g., mainly higher corticosterone responses to acute stressors have been reported (Maccari et al., 2003; van Bodegom et al., 2017). However, the effects of stress during the postnatal period are less consistent and seem to be specific regarding the stressor, its timing and the age at which the stress response is assessed (van Bodegom et al., 2017).

In humans, PS has been associated with altered cortisol reactivity in the offspring as well, although the direction of the effects is less clear (Pearson et al., 2015; Van den Bergh et al., 2017). Most studies report a hyperreactive HPA axis in the offspring after exposure to PS (Entringer et al., 2009; Martinez-Torteya et al., 2016; Stroud et al., 2016; Yong Ping et al., 2015). These studies include different stressors during pregnancy (e.g., severe critical life events, maternal psychopathology), different stress paradigms (e.g., short maternal separation, vaccination) and different ages at assessment, from newborns to adults. However, some studies show no relation (Braithwaite et al., 2016) or mixed results (Fernandes et al., 2015; Tollenaar et al., 2011). The existing literature suggests complex effects of PS on cortisol reactivity in children, depending on the type and severity of the prenatal stressor, the children's age at assessment as well as the stress paradigm. Early postnatal stress in humans is mainly associated with a blunted cortisol response to social stress, although the literature is not consistent (Bunea et al., 2017; Fogelman and Canli, 2018). Some studies suggest that early adversity results in increased cortisol secretion during infancy, which is followed by reduced cortisol secretion later in life (McLaughlin et al., 2015; Sanchez, 2006).

Especially in preschool-aged children, the effects of prenatal and postnatal stress on cortisol reactivity have not been evaluated in many studies (Van den Bergh et al., 2017). One major obstacle for research with this age group is that—in contrast to adults and older children—to date no established stress test reliably eliciting an HPA axis response is available (reviewed by Gunnar et al., 2009b). For adults, the Trier Social Stress Test (TSST; Kirschbaum et al., 1993) can be considered the 'gold standard' for laboratory stress tests, and the Trier Social Stress Test for Children (TSST-C; Buske-Kirschbaum et al., 1997), used for children eight years and older, is also a well-established and widely recognized stress paradigm. However, for preschool-aged children, a similarly reliable method is still missing.

Laboratory stressors that reliably elicit HPA axis responses in adults are characterized by the combination of an uncontrollable situation and a social-evaluative threat (Dickerson and Kemeny, 2004). Similarly, Gunnar et al. (2009b) conclude that for children, the clearest HPA axis responses are yielded by stress tasks threatening their social self. Those principles have been followed by a stress test for 5- to 6-year-old children which yielded good responder rates (de Weerth et al., 2013). Another very promising paradigm for preschool-aged children was developed by Kryski et al. (2011), who evaluated their test with 42-month-old children in a home-setting. This stress test has recently been replicated and validated in a small sample in a laboratory setting by Roos et al. (2017) who, using a control condition, showed significant differences in mean HPA axis activation between the stress and the control condition. The same stress test was used in a laboratory setting by Tolep and Dougherty (2014), who found significant differences between pre- and post-stressor measures when taking the inter-individual peak of cortisol responses after the test into account. However, in their sample the stress test did not elicit a robust mean HPA axis response.

The aims of this study were (1) to evaluate this stress test in a large sample of 45-month-old children by assessing whether it elicits a reliable cortisol response, (2a) to evaluate the relation between PS, defined as self-reported psychopathology during pregnancy, and the children's cortisol reactivity, (2b) to confirm this finding in a subsample with objectively diagnosed psychiatric disorders during the third trimester of pregnancy, and (3) to evaluate the relation between PS, defined as perceived stress during pregnancy, and the children's cortisol reactivity.

2. Materials and methods

2.1. Study design and sample

The present work is part of an ongoing longitudinal study focusing on PS and offspring postnatal development and health (Pre-, Peri-, and Postnatal Stress: Epigenetic impact on Depression; POSEIDON; see also Dukal et al., 2015; Nieratschker et al., 2014; Send et al., 2017; Wolf et al., 2017, 2018). A total of 410 pregnant women were recruited for the POSEIDON cohort about 4–8 weeks prior to delivery. The women were included if they were 16–45 years old, German-speaking and presumably the child's main caregiver. They were excluded if they were positive for hepatitis B, hepatitis C or human immunodeficiency virus, if they were diagnosed with any current psychiatric disorder requiring inpatient treatment, if they had any history or current diagnosis of schizophrenia or psychotic disorder or if they had any substance dependency other than nicotine during pregnancy. At birth, they were excluded if the child was born before 30 weeks of pregnancy, if the child weighed less than 1,500 g or in case of a multiple birth. Furthermore, they were excluded in case of any congenital disease, malformation, deformation or chromosomal abnormality.

Data assessment was conducted in four waves: during third trimester of pregnancy (T1), within a few days after childbirth (T2), six months postpartum (T3) and 45 months postpartum (T4).

The sample, the recruiting procedure, and the phenotypic assessment of mothers and newborns from T1 to T3 have been described in more detail by Send et al. (2017) and Wolf et al. (2017).

The latest wave (T4) took place between August 2014 and January 2017 when the children were 45 months old. A total of 382 children who were still part of the POSEIDON cohort at T3 were invited to participate. Of these 382 children, 302 participated again, equalling a retention rate of 79.1%. Dropouts were replaced by children of the same age as the original POSEIDON cohort (45 months). For this purpose, information about eligible new participants was obtained from the local registries of residents (birth year of the child, parents' names and address). The eligible new participants were informed about the study by mail and completed a short questionnaire screening for inclusion and exclusion criteria listed above. Additionally, they were included if their child was German-speaking and Caucasian-decent (the latter for genetic analyses, not reported here). All interested families meeting the study criteria were invited to participate. Of those, 101 families participated in the study. While multiple birth was an exclusion criterion in the original POSEIDON cohort, it was not in the new subsample. Thus, 4 twin pairs participated in the new cohort. Data about pregnancy, birth and the early postnatal period were collected retrospectively for the new sample at T4. In total, the complete sample at T4 consisted of 403 children, 302 from the POSEIDON cohort and 101 from the new cohort. The study was approved by the Ethics Committee of the Medical Faculty Mannheim of the University of Heidelberg, and all families provided written consent.

2.2. Definition of prenatal stress

At T1 the mothers-to-be were asked for their subjective evaluation of whether or not they were suffering from a psychiatric disorder during

their third trimester of pregnancy. We will refer to the mothers who reported a prenatal psychiatric disorder as the group with "self-reported psychopathology". In addition, structured interviews were conducted, and the women were screened for current and lifetime psychopathology using the structured Mini International Neuropsychiatric Interview (MINI; German version 5.0.0; Sheehan et al., 1998). We considered a MINI diagnosis at T1 as a proxy for PS. A lifetime diagnosis (without a current diagnosis at T1) was not considered PS. We will refer to the mothers with a MINI diagnosis at T1 as the group with "expert-rated psychopathology". Details regarding the definitions of maternal psychopathology are shown in Supplementary Tables 1, 2.

For the subsample recruited at T4 only retrospectively reported measures of PS could be included in the analyses. Mothers were asked for their subjective evaluation of whether or not they were suffering from a psychiatric disorder throughout their pregnancy. The trimester was not specified.

For the POSEIDON subsample, in the third trimester of pregnancy perceived stress was assessed using the Perceived Stress Scale (PSS; Cohen et al., 1983). The PSS is a 14-item self-report questionnaire measuring the experienced level of stress during the last month. Higher values regarding the sum score of all items indicate higher perceived stress. We refer to PS assessed with this questionnaire as "perceived stress".

2.3. Testing procedure and data collection

Testing took place in a dedicated room of the Central Institute of Mental Health in Mannheim. Most children attended with their mother, some with their father or both parents. Children were told they would be attending a playtime and were promised a gift, which they obtained at the end of testing. The mother was in the same room but seated behind the child and involved in an interview with another researcher at a second table. Mother and child were not able to see each other or interact during the tasks since they were seated with their backs to each other. The parental written consent and child assent (verbally by the investigator) were (re)established at T4. The testing session started with a playful test of the children's cognitive development (Wechsler Preschool and Primary Scale of Intelligence - III, not reported here; Petermann, 2014). In some cases where children needed time to feel comfortable about the new situation, the testing session was started by reading a children's book together. Since children are more familiar with female caregivers—e.g., kindergarten teachers—the testing was done by female researchers only. The testing sessions started at either 10 a.m. or 2 p.m., and the cognitive testing took about one hour, after which the first cortisol sample was obtained (baseline). The stress test was then conducted as described below. To assess cortisol secretion following the stress test, three more samples were taken 10, 30 and 40 min (+10, +30, +40) after finishing the test.

To evaluate subjective stress, the researcher rated how stressed the child appeared on a 4-point Likert scale immediately after the test. Furthermore, she asked the children before and after the stress test how "happy or sad" they felt. To facilitate and quantify this self-evaluation, we asked the children to select one of four smileys with different facial expressions from happy to sad each time. After the stress test, the children were encouraged to stay seated and play with coloring books, puzzles and children's books together with the researcher to minimize the effect of physical activity on cortisol secretion.

An overview of the measures and biosamples included in the present work is provided in Table 1.

2.4. Stress test

All children participated in a stress test adapted by Kryski et al. (2011) from Lewis and Ramsay (2002). At the beginning of the test, children were asked which one of several stickers they liked the best. They were instructed that this sticker would be at stake in the following game, and that they would only win it if they did well in this game. The children received a box filled with magnets of two colors, and the researcher explained that they should attach the magnets to the corresponding animals on a game board. An example on the game board indicated which color corresponded to which animal in the game. The children were further instructed that the color of a remote-controlled stoplight indicated the time for the game: The switch from green to yellow indicated that there was only little time left. The switch from yellow to red was accompanied by a loud buzzing noise indicating that time was up. The color of the stoplight was switched by the researcher using the remote control according to the child's speed in the game, so that all children received too little time and failed. After the first attempt the children were encouraged to try for a second and then for a third time, but every time the stoplight was switched to red before the children could finish successfully. The researcher used non-reinforcing language and provided negative feedback about the child's failure repeatedly after each attempt. After three unsuccessful attempts (or earlier in case the child refused to complete three attempts) the researcher debriefed the child and explained that the researcher made a mistake and that the time was too short to finish the game. The child received the sticker and the possibility to finish the game with enough time.

To further enhance uncontrollability and negative social evaluation, small changes to Kryski's original protocol were made: Adapting the available time to each child's individual speed strengthened the uncontrollability of the task. Similar to adaptions of the paradigm described by Roos et al. (2017) the researcher used non-reinforcing language and provided negative feedback about the child's failure repeatedly after each attempt which emphasized the negative social evaluation aspect of the stress test.

2.5. Description of salivary cortisol assay

All cortisol samples were obtained using salivettes (Sarstedt, Nümbrecht, Germany). After thawing, salivettes were centrifuged at 3000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Until analysis, saliva samples were frozen and stored at -80 ° C. Salivary concentrations were measured using a commercially available chemiluminescence immunoassay with high sensitivity (IBL International, Hamburg, Germany). Sample and reagent handling was semi-automated using a liquid handling robot (Genesis, Tecan, Switzerland) and quality control samples of low, medium and high cortisol concentrations were run on each microtiter plate assayed. The intra- and interassay coefficients of variation for cortisol were both below 8%. Saliva samples were analyzed in singlet assays, and three control samples with increasing cortisol concentrations were assayed in duplicates on each microtiter plate.

2.6. Statistical analysis

T-tests and X^2-tests were calculated to compare the children who provided all four saliva samples to the children who participated in the appointment but did not provide all saliva samples and to control the distribution of subgroups regarding morning and afternoon testing sessions.

Cortisol data were log 10 transformed to reduce skewness. Children were classified as responders to the stress test if one of their post-stress cortisol values increased at least twice the average intra- and interassay coefficient of variation (i.e., 16% or more) with regard to the baseline value (Levendosky et al., 2016; Tolep and Dougherty, 2014). This definition is also close to the criterion of a baseline-to-peak-increase in cortisol of 15.5% suggested by Miller et al. (2013). General Linear Models (GLM) for repeated measures were computed to test the effect of the *time course* on cortisol responses (aim 1), the effect of *PS*, defined as psychopathology, on cortisol responses (aims 2a and 2b), and the effect of *PS*, defined as perceived stress, on cortisol responses (aim 3). The variable *time course* represents the time points for the repeated

Table 1
Summary of all study measures included in the present work.

Study wave	Sample	Study measure
T1	POSEIDON cohort	• Mini International Neuropsychiatric Interview (MINI) • Self-report on psychiatric disorders during third trimester of pregnancy • Perceived Stress Scale (PSS)
T4	New subsample	• Retrospective self-report on psychiatric disorders during pregnancy
	Complete sample	• Salivary cortisol at baseline and 10, 30 and 40 minutes after the stress test

measurements. Time points of measurement are Baseline, 10 min post stress test, 30 min post stress test, and 40 min post stress test. For aims 2a and 2b *PS* was entered as a between-subjects effect. For aim 3, *PS* was entered as a covariate instead. To explore effects of more extreme manifestations of perceived stress, the analysis for aim 3 was repeated comparing the 25% with the highest values of perceived stress to the rest of the sample. In all analyses, the confounding variables *sex* and *daytime* were controlled for. The continuous variable *daytime* was defined as the time at which the baseline measure was taken.

The corresponding interactions were included in the model: for aim 1 *time course* * *sex* and *time course* * *daytime*; additionally for aims 2 and 3 *time course* * *PS*, *time course* * *sex* * *PS* and *sex* * *PS*.

To test the influence of possible confounders we repeated the analyses regarding PS including further control variables: 1. the corresponding measure of postnatal stress (for definition of postnatal stress see Supplementary Table 3), 2. measures of socioeconomic status, i.e., household income, maternal education, and single parent status, and 3. the duration of the stress test.

Greenhouse-Geisser corrected tests are reported for all analyses. η^2_p-values are given as an effect size measure. Post hoc group comparisons were conducted based on estimated marginal means using Bonferroni-adjusted alpha levels of $p < .0125$ for the number of tested time points ($\alpha = 0.05/4 = 0.0125$).

All statistical analyses were carried out with IBM SPSS Statistics (Version 24).

3. Results

3.1. Descriptives

403 children (age 45.0 months ± 1.0) and their parents participated in the wave T4. Some children who participated in T4 were not included in the following analyses: 13 children participated in all study parts except the appointment for testing; 31 children refused to provide the baseline measure, in which case the stress test and the other three saliva samples were skipped; 1 child refused to participate in the test; 4 children did not understand the instructions of the stress test; 11 children refused to provide one or more saliva samples after the test; the samples of 1 child were lost due to irregularities in processing and storage; 3 children provided samples with insufficient saliva to measure cortisol. Thus, 339 children completed the stress test and provided all four saliva samples.

Percentages for all variables of interest and control variables are shown in Table 2. Descriptive statistics for cortisol values are displayed by groups in Table 3.

More children with PS defined as psychopathology refused the stress test or at least one of the saliva samples (see Supplementary Table 4): This difference was marginally significant for PS defined as self-reported psychopathology and significant for PS defined as expert-rated psychopathology. No differences between the groups were observed for PS defined as perceived stress. Regarding the control variables, no difference for daytime was observed, but a difference for sex: Boys refused to take part in the test or to provide saliva samples marginally significant more often than girls.

Table 2
Descriptive statistics for maternal psychopathology and control variables.

Variable	Percentage
Maternal self-reported psychiatric disorder during pregnancy[a]	11.8%
Maternal expert-rated psychiatric disorder during third trimester of pregnancy[b]	10.6%
Children's sex (girls)[a]	55.8%
Time of testing (morning testing sessions)[a]	49.6%

SD = standard deviation.
[a] With respect to the complete sample ($n = 339$ children).
[b] With respect to the POSEIDON subsample ($n = 246$ children); psychiatric disorder assessed with the Mini International Neuropsychiatric Interview.

All children were randomly distributed to morning and afternoon testing sessions. No significant differences were observed regarding the distribution of children with and without PS to the different times of testing (see Supplementary Table 5).

The different definitions of PS were highly significant correlated (self-reported and expert-rated psychopathology $r = 0.76$, $p < .001$; self-reported psychopathology and perceived stress $r = 0.25$, $p < .001$; expert-rated psychopathology and perceived stress $r = 0.27$, $p < .001$).

3.2. Aim 1: Validation of the stress test (complete sample; n = 339)

Of the participating children, 60.8% were classified as responders and 39.2% were classified as non-responders.

A marginally significant positive correlation between the researchers' subjective stress rating and the children's stress rating after the test was observed ($r = 0.11$, $p = .06$, $n = 284$).

A GLM for repeated measures with cortisol as the dependent variable showed that the stress test led to significant increases in cortisol (main effect *time course*: $F_{(1.80;603.54)} = 6.21$, $p = .003$, $\eta^2_p = 0.018$). Post hoc tests demonstrated significantly higher cortisol levels at +30 and +40 compared to baseline and +10 (all $p < .001$). Fig. 1 displays the time course of cortisol concentrations before the stress test (baseline) and 10, 30 and 40 min after the stress test.

Furthermore, a significant interaction between *time course* and *daytime* as well as a significant main effect of *daytime* was observed. The effects indicate a steeper increase as well as a higher intercept for children tested during the morning compared to children tested during the afternoon. Besides, the results revealed a marginally significant interaction between *time course* and *sex* with a descriptively stronger increase in females, but no significant main effect of *sex*. Details regarding the control variables *daytime* and *sex* are shown in Supplementary Table 6.

3.3. Aim 2a: Effects of PS, defined as self-reported psychopathology during pregnancy (complete sample; n = 339)

A GLM for repeated measures revealed a significant main effect of *time course* ($F_{(1.80;602.41)} = 4.36$, $p = .016$, $\eta^2_p = 0.013$) on the children's cortisol.

Table 3
Descriptive statistics for raw cortisol values and scores on the Perceived Stress Scale (PSS) in the complete sample and groups with or without prenatal stress, defined as psychopathology.

		Subgroups			
	Complete sample (n = 339)	Self-reported psychiatric disorder during pregnancy[a] Yes (n = 40)	No (n = 299)	Expert-rated psychiatric disorder during third trimester of pregnancy[b] Yes (n = 26)	No (n = 220)
Cortisol values					
Baseline	1.90 ± 1.74	1.93 ± 1.09	1.89 ± 1.81	1.77 ± 1.01	1.81 ± 1.30
10 minutes post stress test	2.07 ± 1.88	1.89 ± 1.47	2.10 ± 1.92	1.69 ± 1.27	2.04 ± 1.64
30 minutes post stress test	2.37 ± 2.00	1.81 ± 1.25	2.45 ± 2.07	1.43 ± 0.84	2.42 ± 2.03
40 minutes post stress test	2.54 ± 2.49	1.99 ± 1.37	2.61 ± 2.59	1.55 ± 0.79	2.50 ± 2.13
PSS Score[c]	21.0 ± 8.4	26.5 ± 8.0	20.2 ± 8.1	27.8 ± 7.0	20.2 ± 8.2

Cortisol mean values (in nmol/l) and standard deviations are depicted.
[a] With respect to the complete sample (n = 339 children).
[b] With respect to the POSEIDON subsample (n = 246 children); psychiatric disorder assessed with the Mini International Neuropsychiatric Interview.
[c] With respect to the POSEIDON subsample (n = 244 children).

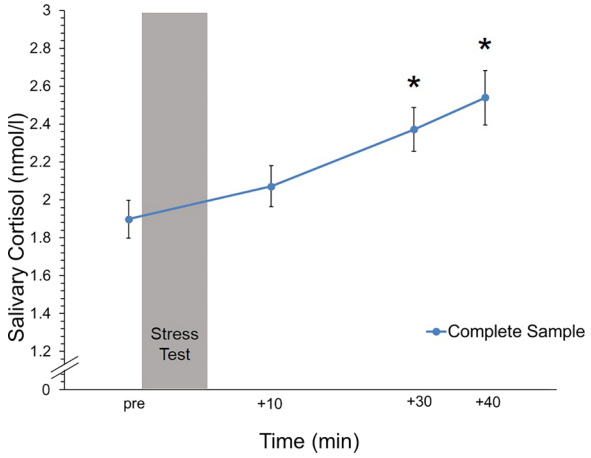

Fig. 1. Mean levels of salivary cortisol (nmol/l) over the course of time for the complete sample.
* Significant differences to baseline and +10 in post hoc tests. Depicted data are not log-transformed. Error bars represent the standard error of the mean.

A significant interaction between *time course* and *PS*, defined as self-reported psychopathology ($F_{(1.80;602.41)}$ = 3.94, p = .024, η^2_p = 0.012), but no main effect of *PS* ($F_{(1;334)}$ = 0.18, p = .67, η^2_p = 0.001) was observed. As depicted in Fig. 2a, the groups of children with and without maternal self-reported psychopathology are comparable regarding baseline cortisol values. After the stress test, children without maternal self-reported psychopathology showed the generally expected increase in cortisol levels, especially 30 and 40 min after the test, while the group of children with maternal self-reported psychopathology showed no cortisol response. However, post hoc tests demonstrated no significant differences in cortisol levels between the groups (all p > .14). Details regarding the control variables *daytime* and *sex* are shown in Supplementary Table 6.

3.4. Aim 2b: Effects of PS, defined as expert-rated psychopathology during third trimester of pregnancy (POSEIDON subsample; n = 246)

A GLM for repeated measures showed no significant main effect of *time course* for this subsample ($F_{(1.72;415.26)}$ = 1.75, p = .18, η^2_p = 0.007*)*.

As for self-reported psychopathology, a significant interaction between *time course* and *PS*, defined as expert-rated psychopathology, was observed ($F_{(1.72;415.26)}$ = 5.42, p = .007, η^2_p = 0.022*)*. Moreover, the analysis revealed a significant main effect of *PS* ($F_{(1;241)}$ = 4.79, p = .03, η^2_p = 0.019). As depicted in Fig. 2b, baseline values of the two groups are similar. Likewise, the groups differ after the stress test: The group of children without maternal expert-rated psychopathology showed increased cortisol levels, especially 30 and 40 min after the test, while for the group of children with maternal expert-rated

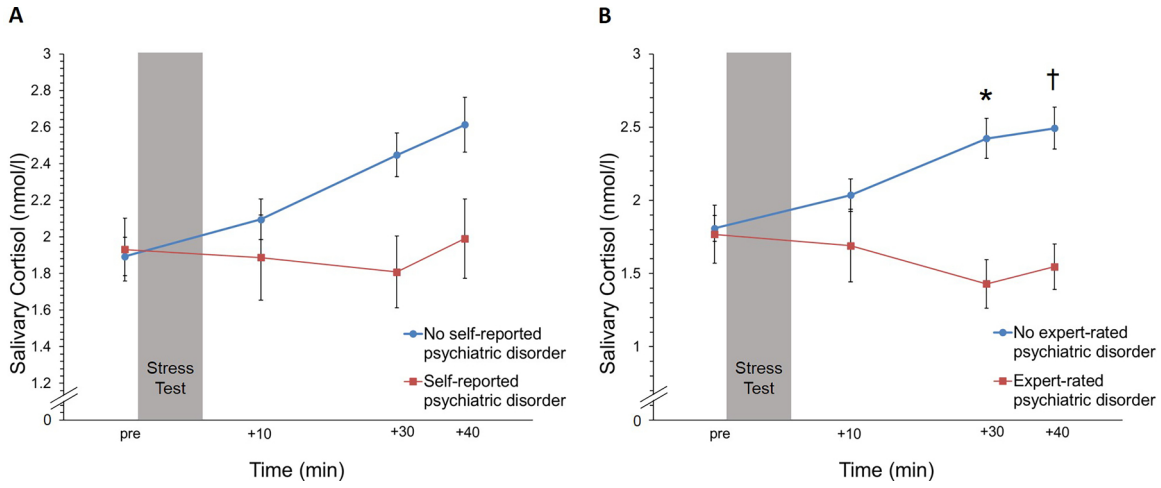

Fig. 2. Mean levels of salivary cortisol (nmol/l) over the course of time for the groups with and without prenatal maternal stress, defined as (A) self-reported psychopathology during pregnancy and (B) expert-rated psychopathology during third trimester of pregnancy.
* Significant group differences in post hoc tests. † Marginally significant group differences in post hoc tests. Depicted data are not log-transformed. Error bars represent the standard error of the mean.

psychopathology no cortisol response was observed. In contrast to the analysis for aim 2a, post hoc tests demonstrated a significant difference in cortisol levels between the groups at +30 ($p = .002$) and a marginally significant difference at +40 ($p = .013$) with respect to the Bonferroni-adjusted alpha level of $p < .0125$. Details regarding the control variables *daytime* and *sex* are shown in Supplementary Table 6.

3.5. Aim 3: Effects of PS, defined as perceived stress during third trimester of pregnancy (POSEIDON subsample; n = 244)

A GLM for repeated measures showed a marginally significant main effect of *time course* for the POSEIDON subsample ($F_{(1.72;409.97)} = 3.13$, $p = .053$, $\eta^2_p = 0.013$).

No significant interaction between *time course* and *PS*, defined as perceived stress, was observed ($F_{(1.72;\ 409.97)} = 1.78$, $p = .18$, $\eta^2_p = 0.007$). The main effect of *PS* was non-significant as well ($F_{(1;239)} = .86$, $p = .36$, $\eta^2_p = 0.004$). Details regarding the control variables *daytime* and *sex* are shown in Supplementary Table 6.

The exploratory analysis splitting the sample at the top 25% of perceived stress and comparing the children's cortisol levels of this quartile to all other children's cortisol levels showed a non-significant interaction between *time course* and *PS* ($F_{(1.70;407.36)} = 1.53$, $p = .22$, $\eta^2_p = 0.006$). However, it revealed a significant main effect of *PS* ($F_{(1;239)} = 4.37$, $p = .038$, $\eta^2_p = 0.018$): Children of highly stressed mothers showed lower levels of cortisol compared to children of low-to-medium stressed mothers. Post hoc tests revealed a significant difference in cortisol responses between the groups at +30 ($p = 0.008$) with respect to the Bonferroni-adjusted alpha level of $p < 0.0125$. This is depicted in Supplementary Fig. 1.

3.6. Exploratory analyses with additional covariates

In additional analyses including postnatal stress as a control variable no significant effects of postnatal stress could be observed for aims 2a, 2b and 3. Adding postnatal stress to the model did not alter the reported effects regarding *PS* for these aims (see Supplementary Table 7). Adding postnatal stress to the exploratory analysis regarding aim 3 (comparison of top quartile of perceived stress with the remaining sample) changed the effect size for the main effect of PS from $\eta^2_p = 0.018$ ($p = .038$) to $\eta^2_p = 0.014$ ($p = .068$).

Adding socioeconomic variables, i.e., household income and maternal education, to the models did not result in major changes regarding the variables of interest. For details see Supplementary Tables 8, 9, and 10. Adding single parent status to the analyses changed the effects for one of the definitions of PS (PS defined as self-reported psychopathology) on cortisol reactivity from $\eta^2_p = 0.012$ ($p = .024$) to $\eta^2_p = 0.009$ ($p = .064$). Indeed, self-reported psychopathology was more prevalent in mothers raising the child alone (32.4%) compared to mothers living together with a partner (9.5%). For details see Supplementary Table 11.

For all reported aims, the duration of the test was not significant. All effects of PS remained significant. For details see Supplementary Table 12.

4. Discussion

This study validates a stress test for preschool-aged children in a large sample (aim 1): The task adapted from Kryski et al. (2011) elicited a mean increase in cortisol, especially 30 and 40 min after completing the stress test in a laboratory setting. Aspects known to increase cortisol responses are realized within this stress test—especially uncontrollability of the situation and negative social evaluation (Dickerson and Kemeny, 2004; Gunnar et al., 2009b).

The observed responder rates of over 60% are close to responder rates of over 70% usually found for the TSST (Kudielka et al., 2007). Moreover, they are very similar to responder rates of another successful stress test for slightly older children (5–6 years; de Weerth et al., 2013). In line with previous findings the present work confirms the paradigm as a valid stress test for preschool-aged children in the largest sample so far (Kryski et al., 2011; Roos et al., 2017; Tolep and Dougherty, 2014). It has to be noted that in comparison to studies using the TSST, a lower absolute increase of cortisol was observed. However, given the lower baseline values in young children and the special challenges with stress tests for this age group (Gunnar et al., 2009b), we consider the observed responder rates as good. The stress test by Kryski et al. has shown evidence of being applicable in home (Kryski et al., 2011) and laboratory settings (Roos et al., 2017; Tolep and Dougherty, 2014). While home visits might increase willingness to participate and adherence, a laboratory research setting facilitates standardization over all participants and reduces the influence of important confounders.

We thus recommend the test for future studies investigating stress reactivity in preschool-aged children. It is important that an approved stress paradigm is available for every age group. As the vast amount of literature on the TSST shows, the benefit regarding the comparability and replicability of research results based on the same stress paradigm is considerable and cannot be emphasized enough.

Moreover, the present study links maternal psychopathology during pregnancy to a reduced HPA axis response after a laboratory stressor (aim 2). This was shown for self-reported psychopathology in the complete sample of $n = 339$ (aim 2a). The finding was confirmed in the subsample that was objectively screened for psychiatric disorders during the third trimester of pregnancy ($n = 246$; aim 2b). The present work demonstrates that suffering from a psychiatric disorder during pregnancy may lower the reactivity of the HPA axis in 45-month-old offspring. By contrast, most animal studies point to elevated HPA axis reactivity following PS (Maccari et al., 2003). While some human studies point in the same direction, the evidence to date is still inconclusive (Fernandes et al., 2015; Pearson et al., 2015; Stroud et al., 2016; Yong Ping et al., 2015). One aspect which might underlie contradicting results is that PS effects may result in differential and potentially opposing effects throughout the lifespan of the offspring. This is well reflected by the study of Tollenaar et al. (2011) which focused on pregnancy-specific anxiety as PS and assessed infants' cortisol reactivity in the same sample throughout the first year of life. In this study, pregnancy-specific anxiety was associated with elevated cortisol reactivity at 5 weeks of age and with reduced cortisol reactivity at 8 weeks and 12 months, whereas no association was found at 5 months. Although PS-induced alterations of HPA axis function may be long-lasting, the exact relation between PS and cortisol reactivity may differ depending on the children's age at which cortisol reactivity is assessed. Findings from research with humans and nonhuman primates suggest that early adverse caregiving environments can lead to elevated cortisol secretion during the first year of life and lower cortisol secretion afterwards (Sanchez, 2006; McLaughlin et al., 2015). This may be related to down-regulation of pituitary corticotropin-releasing hormone receptors or to increased negative feedback to cortisol on a hippocampal level.

Descriptively, the association between PS and a reduced HPA axis response to the stress test was observed for all definitions of PS. For PS defined as perceived stress (aim 3), this observation was only significant, when comparing the smaller group of children with stressed mothers (top quartile) to the larger group of children with less stressed mothers (remaining 75% of the sample). A considerable overlap between the stress concepts "psychiatric disorder" and "perceived stress" can be assumed: psychiatric disorders co-occur with increased perceived stress, which in the present study is also reflected in the correlation between the mothers' mental health status during pregnancy and the corresponding PSS value. However, psychiatric disorders can be considered a specific type of stress, since subjects with high perceived stress do not necessarily suffer from a psychiatric disorder, and not all affected subjects might subjectively perceive increased levels of stress. Therefore, the concepts "psychiatric disorder" and "perceived stress" might to some degree affect different biological pathways. Moreover, a

psychiatric diagnosis draws a line between individuals with several problems (or fulfilled diagnostic criteria) and individuals without such problems or fewer fulfilled diagnostic criteria. In the present study, the observed associations are consistent with a threshold model, i.e., alterations in HPA axis reactivity are only prevalent in offspring of mothers exposed to more severe stress.

In the field of stress research, nature and intensity of stressors need to be considered. Several studies provide evidence that the severity of PS influences various outcomes in children (e.g., Dipietro, 2012; Laplante et al., 2017; Sandman et al., 2012). Also, non-linear U-shaped relationships between pre- and postnatal stress and the offspring's HPA axis regulation have been reported (Fernandes et al., 2015; Gunnar et al., 2009a) and theoretical models have been developed to integrate these partially divergent findings (Del Giudice, 2014). The prenatally stressed children in the present study were not exposed to extreme maternal stress in the womb since severe psychiatric disorders requiring inpatient treatment were an exclusion criterion and extreme stressors such as exposure to natural catastrophes did not occur. The results of the present study indicate that a certain severity of PS is necessary to result in alterations of the HPA axis regulation in the offspring. However, we are not able to draw conclusions regarding the effects of severe traumatic events during pregnancy.

In summary, our findings indicate that PS is associated with reduced HPA axis reactivity in preschool-aged children. This is in line with other studies showing associations between measures of PS and HPA axis dysregulation in the offspring. However, specifics of the precise definition of PS and the assessment of stress responses in the offspring—including the age at which stress responses are assessed—might contribute to the inconsistencies of effects between studies.

Since not only the prenatal but also the postnatal environment may exert influence on children's stress reactivity, we analyzed the effects of postnatal stress in our models as well. Interestingly, no significant effects of postnatal stress could be observed. The effects of PS remained largely unchanged if postnatal stress was included in the models. Thus, the present study underlines the importance of the prenatal period. However, we cannot rule out that further stress-related characteristics of the children's postnatal environment might impact the reactivity of their HPA axes.

Of the potentially confounding socioeconomic variables at the time of testing, only single parent status slightly influenced the effects of PS, and here only of PS defined as self-reported psychopathology. We therefore conclude that the observed effects of PS are largely independent of socioeconomic status. However, psychiatric disorders undoubtedly result in multiple adverse social and psychological consequences and psychopathology during pregnancy and single parent status at any time point may be related. Other samples including more single parents are needed to derive conclusions regarding the relations between PS, single parent status and cortisol reactivity.

It must be noted that, in contrast to Kryski's original protocol, we adapted the available time not only for children who were very good at the task, but for all children. In consequence, the overall duration of the paradigm differed between children as the time lengths of the attempts were adjusted to the individual performance of each child. However, in future adaptations the overall length of the paradigm instead of the number of attempts (as in the current study) could be held constant, so that within this constant time frame (e.g., 10 min) the children can try to solve the tasks as many times as they are able to. To realize uncontrollability of the task, it should be ensured that in each attempt the stoplight turns red before the child reaches the goal.

According to the stress paradigm by Kryski et al. (2011), the mother was in the same room at the time of the stress test. This can be considered a social buffer, i.e., the paradigm would have been even more stressful without the mother in the room. However, it would not have been feasible to let 45-month-old children participate in a laboratory visit without a parent. Nonetheless, in future studies with somewhat older children, it should be considered whether to perform the stress paradigm with or without a parent in the room.

Regarding the present study, it cannot be ruled out completely that prenatally exposed children perceived the test as less stressful, and therefore showed a lower HPA axis response. However, neither the children's nor the researchers' subjective ratings after the test were related to the mothers' mental health status during pregnancy (see Supplementary Table 13). Thus, children with and without exposure to PS rated themselves as equally stressed and they appeared equally stressed in the researchers' evaluation. This underpins the interpretation that the children felt and acted stressed in terms of their observable behavior regardless of PS. However, the corresponding endocrine reaction was largely missing for children with PS.

Alterations of the regulation of the HPA axis are observed in various psychiatric disorders (Handwerger, 2009). It is possible that HPA axis dysregulation at a young age represents an early risk factor for the subsequent development of psychiatric disorders. However, the plasticity of an organism can be considered an adaptive mechanism regarding the environmental stimuli it is exposed to and it has been postulated that prenatal programming prepares the offspring for the environment outside the womb (van Bodegom et al., 2017). In the case of a diminished stress response the protective effect may comprise the prevention of permanently elevated cortisol levels in a stressor-rich environment. However, in a safer environment a diminished HPA responsivity can be maladaptive since the organism may lack necessary physiological adaptations in response to acute stress. A mismatch between altered HPA axis reactivity and environmental characteristics might predispose the individual to develop mental disorders. As the reported data is part of an ongoing longitudinal study, assessment in future phases can be used to address whether the observed changes in HPA axis functioning are stable throughout childhood development, and whether associations with the children's mental health will emerge.

Blunted HPA axis reactivity may partly be an inherited risk factor for the development of a psychiatric disorder later in life, and the observed association might also reflect the shared genetic variation between the mothers and their children. Given the heritability of HPA axis reactivity (Steptoe et al., 2009), we cannot exclude that the observed hyporesponsive cortisol pattern may be partly explained by genetic factors and the interaction between PS and genotype, as reported by Buchmann et al. (2014). Further, experimental animal studies have suggested that epigenetic modifications mediate the effects of PS (Beijers et al., 2014). Future studies should include (epi-)genetic information from parents and children to assess how these factors contribute to the association of PS and changes in HPA axis regulation.

There are several limitations in the design of this study: First, the used definitions of PS exclusively focused on psychopathology and perceived stress. Second, it must be noted that in our sample we observed, on average, an unusually late peak in cortisol levels at +40 min post stress test. We do not assume that the nature of the laboratory visit itself led to this cortisol increase since the children had already been in the laboratory for approximately one hour when the stress test started. However, we cannot rule out that the laboratory environment is associated with a different recovery pattern compared to a familiar home environment. Further cortisol samples at a later time point would have been necessary to cover the full recovery phase in our participants. However, this was not possible to implement in our protocol due to a time limit for the total visit. Third, a control condition for the stress test could not be implemented in the study. Such a condition would have enabled us to better differentiate between the possible stress of the laboratory situation and the stress elicited by the stress test itself. We thus recommend the implementation of a control condition for future applications of the stress test whenever feasible. Fourth, new subjects were included in the study at T4, and the expert-rated psychiatric diagnosis assessed in the third trimester of pregnancy was only available for the POSEIDON cohort, not for the subsample recruited at T4. For this new subsample, retrospectively reported psychopathology was included in the analyses. To facilitate maternal recall of mental health

status during pregnancy, the retrospective self-report comprised psychiatric disorders throughout the entire pregnancy. The self-report in the original sample comprised the mental health status at T1 (i.e., during the third trimester of pregnancy). Consequently, conclusions about the timing of PS are limited. Fifth, performing the stress test in the morning and in the afternoon constitutes a limitation of this study. It would have doubtless been best to test all children at the same time point, preferably in the afternoon. However, this was not possible in our study. Therefore, we statistically controlled for the daytime at which testing took place.

To sum up, the present work further contributes to the emerging evidence that the evaluated stress test is a successful laboratory stressor for preschool-aged children. We therefore recommend its use for future studies investigating HPA axis reactivity in this age group. Furthermore, this is the largest longitudinal study on moderate PS and cortisol response to a laboratory stressor in preschool-aged children. In the context of the existing literature on PS and cortisol reactivity, our results suggest that PS may go along with a blunted HPA axis response to acute stress. At present it is unclear whether the blunted cortisol response in prenatally stressed children constitutes a protective factor or a risk factor for psychiatric disorders. Subsequent waves of our study, in addition to other prospective studies, which take the severity of stressors and age into account, will be able to address this research question as well as the impact beyond childhood.

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Declarations of interest

None.

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Appendix A. Supplementary data

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