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Emotion regulation and cortisol response to the still-face procedure in preterm and full-term infants

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ABSTRACT

In infancy, stress responses and emotion regulation are often coupled. Both are impacted by prematurity, though their relationship to one another in the case of infants born preterm is not fully understood. We investigated emotion regulation behaviours, cortisol reactivity and recovery and coupling between emotion regulation and cortisol reactivity to and recovery from a stressor in preterm infants. 53 preterm and 67 full-term infants with mean (range) gestational age at birth 29.4 (24.6–31.5) and 39.5 (36.2–42.8) weeks respectively were exposed to a socio-emotional stressor, the still-face (SF) paradigm, at 9 months of age (corrected for prematurity). The duration of negative affect and self-comforting behaviours exhibited in response to the SF, coded from a 10-minute video-taped interaction, were compared between groups. Saliva was collected from a subset (20 preterm, 24 term infants) at three timepoints: pre-SF and 20- and 30-minutes post SF. Cortisol concentrations at each timepoint were compared between groups. Associations between behavioural measures and cortisol concentrations were explored. There was no significant difference in duration of self-soothing behaviour between preterm and term infants. Preterm infants spent a significantly smaller proportion of time in a negative affective state compared to term infants (0.18 vs 0.25 s, p = 0.03). Salivary cortisol concentration was significantly higher in the preterm compared to the term group 30 min post SF (2.85 vs 1.77 nmol/L, p = 0.009), though findings were no longer significant after adjusting for time of day of sampling and socioeconomic deprivation. After controlling for time of day, greater negative affect was correlated with higher cortisol concentration 30 min post SF in the full-term (r = 0.58, p = 0.004) but not the preterm group (r = −0.01, p > 0.05). Our findings suggest altered response to an acute stressor in preterm infants, manifesting as a muted emotional response, and a lack of coupling between endocrine and behavioural stress response. Replication studies in larger samples would help to further understand biological stress reponse in preterm infants and its relationship to behaviour, time of day and deprivation.
1. Introduction

Early-life stress can impact the programming and functioning of the hypothalamic-pituitary-adrenal (HPA) axis, particularly if experienced during sensitive periods of brain development (Van Bodegom et al., 2017). Preterm birth (birth before 37 weeks of gestation) can be considered an early life physiological stressor due to co-exposures such as pain, maternal separation, sub-optimal nutrition, cardiorespiratory instability, and common co-morbidities (Boardman and Cournell, 2020). These exposures have been linked to altered programming and activity of the HPA axis and stress-related neural networks (Lammertink et al., 2021; Smith et al., 2011). Neonatal intensive care unit (NICU) related stress has been associated with increased internalising behaviours at 18 months and school age (Ranger et al., 2014; Vinall et al., 2013), and cortisol levels are dysregulated in preterm infants both while resting and in response to a stressor (Brummelte et al., 2015; Grunau et al., 2007; Stoye et al., 2021).

Preterm birth is associated with socioemotional difficulties (Johnson et al., 2018) and stress-related disorders including anxiety and depression (Johnson et al., 2010; Johnson and Marlow, 2016, 2011). Antecedents of these long-term outcomes may reside in atypical emotion regulation in infancy (Evrard et al., 2011; Jean and Stack, 2012; Provenzi et al., 2017; Yaari et al., 2018). Emotion regulation and stress coping are often closely coupled and rely on similar neural circuitry (Wang and Saudino, 2011). Maternal prenatal cortisol influences development of the neonatal amygdala, a key structure involved in infant emotion regulation (Stoye et al., 2020). Problems with emotion regulation have been associated with altered cortisol reactivity and internalising behaviours and disorders (Dickstein and Leibnul, 2006; Krkovic et al., 2018). Negative emotions at 15 months have been found to predict cortisol reactivity and recovery at 24 months following emotion eliciting tasks in term born infants (Wu and Feng, 2020). The authors concluded that infant affective emotion regulation precede the formation of a consistent cortisol reaction pattern. There is evidence that this relationship may be disrupted by preterm birth as indicated by a lack of association between emotion regulation and cortisol reactivity in preterm infants that is observed in their term born counterparts (Erickson, 2013). These relationships provide a potential opportunity to use early individual differences in both affective and endocrine stress responses and their relationship to one another as indicators of risk. Within group associations between affective and cortisol reactivity and recovery could be particularly fruitful in providing insights surrounding individual variability that could aid risk stratification.

A unique opportunity to study both emotion regulation and HPA axis activity in infants is provided by the still-face procedure (Tronick et al., 1978). Stress is induced by a period of non-responsiveness from the caregiver referred to as the “still-face episode”. This is preceded by a period of play and followed by another period of play referred to as the “reunion episode”. A meta-analytical review by Mesman, van IJzendoorn, and Bakermans-Kranenburg (2009) confirmed that the procedure produces replicable behavioural responses in typically developing infants including decreased positive affect, increased negative affect and increased regulatory behaviours during the still-face episode.

Studies exploring stress response to the still-face procedure in preterm infants (using corrected gestational age) have returned inconsistent results. Findings include no differences in negative affect but less positive affect during play and less recovery of positive affect during reunion in preterm infants compared to term infants at 4 months (Yaari et al., 2018). Conversely, Hsu and Jeng (2008) found similar patterns of positive affect between groups at 2 months, but preterm infants became distressed faster and stayed in a negative state for longer. Montirosso et al. (2010) reported no differences in positive or negative affect between groups at 6–9 months. Similarly, 3 month old term and preterm infants did not differ in negative affect (Provenzi et al., 2017). Differences in regulatory behaviours have also been described, with a small majority of studies reporting reduced evidence of self-regulation in the preterm group (Jean and Stack, 2012; Provenzi et al., 2017). Yaari et al. (2018) did not find group differences, though noted differences in the pattern of change between episodes where the very preterm group (gestational age 24–32 weeks) engaged in less self-comforting behaviours across episodes. One study reported similar levels of self-comforting in infants born preterm at 6–9 months (Montirosso et al., 2010).

Cortisol can be measured from saliva as a reliable indicator of HPA axis activity (Kirschbaum and Hellhammer, 1994) which can be acquired from infants using relatively non-invasive methods. In infants, salivary cortisol concentrations typically increase in response to stress with peak cortisol levels being reached approximately 20–25 min post-stressor followed by decrease over time (Ramsay and Lewis, 2003). The still-face procedure stimulates such a cortisol reactivity and the neuroendocrine response is stronger when using the double-exposure version of this observational paradigm (DiCorcia et al., 2016). A meta-analytical review concluded that a repeated still-face design (inclusion of a second non-responsive episode and reunion episode, for a total of five episodes) produced larger effect sizes compared to the classic three-episode design (Provenzi et al., 2016b; Puhakka and Peltona, 2020). The behavioural still-face effect is maintained using the repeated design (DiCorcia et al., 2016; Haley and Stansbury, 2003).

Only a handful of studies have measured cortisol response to the still-face procedure in preterm infants. Provenzi and colleagues (2019) found that 3-month-old term infants’ cortisol levels increased in response to the still-face while levels decreased in the preterm group. In addition, cortisol levels decreased from 20 to 30 min post stressor in the term group, while they increased in the preterm group. Using a similar design in 22 4-month-old preterm infants and 28 term controls, another study found that after adjustment of time of day of sampling, preterm infants had a blunted cortisol response from baseline to 20 min post still-face (Erickson et al., 2019). In an earlier study, Erickson, and colleagues (2013) reported no difference in cortisol reactivity to the still-face between 29 preterm and 24 term aged 6–8 months, though they did find a significant correlation between behavioural and cortisol reactivity in the term group which was absent in the preterm group. This finding is of particular interest as it suggests a lack of coordination between emotion regulation and stress response in preterm infants. In another study, preterm infants were classified into low and high pain groups based on the median number of NICU procedures experienced. Cortisol reactivity to the still-face at 3 months of age was higher in the high pain group compared to the low pain group and to term controls, which did not differ (Provenzi et al., 2016a).

Attempting to resolve some of the contradictions in the literature, we compared emotion regulation and cortisol reactivity and recovery following the still-face procedure in 9-month-old preterm infants versus term born controls and investigated coupling between affective and cortisol response. Based on studies including infants of similar age using similar cortisol sample timings, we hypothesized that negative affect would not differ between groups but that there would be less self-regulatory behaviours in the preterm group. We expected no difference in cortisol reactivity, but that cortisol recovery would be delayed in the preterm group. We expected a lack of coupling between behavioural and cortisol response in preterm infants.

2. Methodology

2.1. Participants

Participants were recruited to a prospective longitudinal cohort study Theirworld Edinburgh Birth Cohort (TTEB (Boardman et al., 2020)) between November 2016 and June 2019. The study includes 58 extremely / very preterm infants (<33 weeks of gestation) and 70 term born infants (>37 weeks of gestation) born at the Royal Infirmary of Edinburgh, UK. Infants with congenital anomalies (e.g., metabolic disorders) were excluded. Ethical approval was obtained from the National Research Ethics Service (NRES), South East Scotland Research Ethics Committee.
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Committee 01 (16/SS/0154), and NHS Lothian Research and Development (2016/0255) and parents provided written informed consent.

Demographic information including gestational age (GA), birthweight and infant sex were collected from infant medical records. Scottish Index of Multiple Deprivation 2016 (SIMD) ranking was generated from postcode information collected via parental questionnaire. SIMD rank is a multidimensional measure of deprivation encompassing local income, employment, health, education, geographic access to services, crime, and housing. All infants contributed behavioural data and after securing additional funding and the approval of an ethical amendment for the introduction of pre- and post-stressor saliva sampling to the protocol, a subset (n = 48) contributed saliva samples.

Participants attended a follow up appointment 9 months after the infant’s expected date of birth at a University of Edinburgh site at the Royal Edinburgh Hospital, Edinburgh, UK. 9 months corrected age was selected as an appropriate time point within the first year of life for assessing neurodevelopmental milestones across a breadth of domains, that did not coincide with routine clinical appointments for preterm infants in the UK. Visit duration was approximately 2.5 h and comprised a comprehensive assessment battery including the still-face procedure with pre and post saliva sampling (see Boardman et al., 2020 for the full study protocol). Assessments were carried out in the following order: anthropometry and bio-sampling, eye-tracking, parent child play, visual acuity, pre stressor saliva sample, still-face paradigm, parent interview, post stressor saliva samples.

2.2. Procedure

2.2.1. Still-Face

The infant was seated in a highchair with the caregiver seated approximately 50 cm away. A Panasonic HC-W580 video camera positioned on a tripod recorded the infant. A second identical camera recorded the mother. A repeated still-face design was used consisting of 5 episodes, each with a duration of 2 min. For the play and reunion episodes, the caregiver was instructed to play and interact with the infant as they would at home, without the use of toys. For the still-face episodes, the caregiver was instructed to adopt a neutral facial expression, to cease physical contact with the infant and to avoid looking directly at the infant, while still facing towards them. The researcher remained in the room, concealed behind a partition screen. The researcher verbally signalled to the caregiver when it was time to transition between episodes. Parents were given the option to terminate the procedure if the infant became distressed. A random subset of infants (n = 21, 9 preterm, 12 term) wore motor sensors on their wrists, ankles, and torso. These were concealed within specially designed clothing. This was to pilot the addition of motor sensors to the protocol. Chi-squared tests were conducted to compare the frequency of wearing of motor sensors between preterm and term groups. As this did not differ significantly between groups, participants who wore motor sensors were included in the analyses.

2.2.2. Behavioural coding scheme

Infant behaviour was coded using a revised version of the Infant Caregiver Engagement Phases (ICEP) (Reck, Noe, Cenciotti, Tronick and Weinberg, 2009). The ICEP includes separate sets of mutually exclusive states for both infant and caregiver engagement behaviours, along with infant regulatory codes. Only infant behaviour was coded. States are assessed via a combination of facial expressions, direction of gaze, vocalisations, and activity levels (see supplementary Table 1 for a description of each behavioural state and coding criteria). Negative affect and self-comforting were selected for investigation in the current study (Gianino and Tronick, 1988; Provenzi et al., 2017).

Coding was conducted using ELAN (EUDICO Linguistic Annotator) video coding software, Version 5.7-FX (Wittenburg et al., 2006). All five episodes were coded, but in order to retain the largest possible sample, and because early termination was most common in episodes 4–5 due to infant distress, behavioural data for episodes 1–3 only were included in the analyses. Videos were coded first for engagement states followed by a second viewing when self-comforting states were coded. A variety of procedural violations were observed during the paradigm. Each video was assigned one or more violation codes (supplementary table 2). A randomly selected 10% of videos were coded by a second coder for inter-rater reliability. The first coder was not completely blind to group as they conducted the research visits and parents often disclosed the infant’s history. The second coder was blind to term vs preterm status. ICC was calculated using a two-way mixed, consistency, single measures ICC (Halgren, 2012). ICC was 0.98 for negative affect and 0.81 for self-comforting.

2.2.3. Salivary cortisol

Saliva was collected at three timepoints to obtain baseline, reactivity, and recovery measurements of neuroendocrine responses to the still-face procedure. Sample one was collected immediately prior to the still-face procedure (between 1 and 5 min approximately) to give a baseline measure. Sample two was collected 20 min after the end of the first still-face episode (episode 2). Sample three was collected 30 min after the completion of episode 2. Saliva was collected using SalivaBio Children’s Swab. The swab was held in the infant’s mouth until saturated (approximately 30 s) then transferred to a labelled Sarstedt swab storage tube. Samples were stored in a fridge at 4 °C for a maximum of one week. Samples were centrifuged at 3500 rpm for 15 min before storage at −80 °C prior to analysis.

Targeted analysis of cortisol (F) was carried out by automated supported liquid extraction (SLE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in multiple reaction mode (MRM). Calibration standards were prepared in water (F 0.25–100 ng/mL). Samples were defrosted, 100 µL of each aliquotted into 96-well deep well plate, enriched (1 ng; 4 ng) as internal standard. Samples and calibrants were diluted on an Extrahera liquid extraction robot (Biotage, Uppsala, Sweden) with 0.1% formic acid (100 µL; aq. 0.1% v/v), on an SLE200 plate, followed by elution with dichloromethane/isopropanol (98:2; 1000 µL), reduction to dryness, resuspension in water/methanol (100 µL; 70:30 (v/v) water/methanol), the plate sealed with a zone-free 96 well plate sealing film (Sigma-Aldrich, Gillingham, UK) prior to LC-MS/ MS analysis.

Liquid chromatographic separation was achieved by injection (20 µL) on to an Acquity I-Class UPLC (Waters, UK) using a Kinetic C18 (150 x 2.1 mm; 2.6 µm; Phenomenex, UK) column, protected by a Kinetex KrudKatcher® (Phenomenex, UK) at 40 °C. The mobile phase consisted of 0.05 mM ammonium fluoride in water (A) and 0.05 mM ammonium fluoride in methanol (B) at a flow rate of 0.3 mL/min. Gradient elution was achieved with a total run time of 16 min from 55% to 100% B. The analytes were detected on a QTrap 6500+ mass spectrometer (AB Sciex, UK) operated in positive electrospray mode (600 °C, 5.5 kV). The steroids and internal standards cortisol, cortisone, d4F eluted from the column at 3.45, 2.94, and 3.40 mins respectively, demonstrating temporal separation of F and E, necessary to exclude contribution of cortisone to the F signal. MRM transitions monitored for were F m/z 363.1 → 121.2, 91.0 at 31 and 83 V, cortisone m/z 361.1 → 163.1, 77.0 at 31 and 107 V and d4-F m/z 367.2 → 121.0, 29 V. Linear regression analysis (1/x for F) was applied to the ratio of the peak area of F to the internal standards d4-F using MultiQuant 3.0.3 software. The amount of cortisol in the samples was calculated from the peak area ratio using the linear regression analysis equation. The method was verified against Certified reference material (cortisol and cortisone, saliva Level II, Chromsystems). Lower limit of detection (LLOD) was 0.25 ng/mL (inter assay variation 8.3%; intra-assay variation 6.7%).

MS grade methanol and water (Fisher Scientific, Loughborough, UK) and formic acid were from SigmaAldrich (Gillingham, UK). Ammonium fluoride (338869–25 G), Cortisol (C-106, 1 mg/mL (certified)), cortisone (C-130, 100 µg/mL in methanol (certified)) and d4-cortisol (C-113, 100 µg/mL in methanol (certified)) were from Cerilliant, Sigma-Aldrich
Dorset, UK. D8-Cortisone (C900170, powder) was from Sigma-Aldrich. Certified reference material (Cortisol, Cortisone Saliva Level II – (0350, Lot 0921, ChromSystems Instruments and Chemicals GmbH, Graefeling, Germany).

2.3. Analysis methods

2.3.1. Data processing

2.3.1.1. Still-face. Raw duration scores for each state were converted to proportional duration scores by dividing the time spent in each state by the total available time per episode. Duration of time spent in an unscorable state (the infant was concealed from view of the camera and no behavioural state could be assigned) per episode was subtracted from the total available time per episode prior to calculation of the proportional scores. Participants were excluded (n = 3) if they spent more than 20% of any episode in an unscorable state. Negative behaviour codes (negative, protesting and withdrawn) were combined to give one overall measure of negative affect. Self-comforting codes (oral self-comforting and self-clasp) were combined to give an overall measure of self-comforting. Chi-squared tests were conducted to compare the frequency of violations between preterm and term groups. Where frequencies were significantly different, participants assigned that violation code were excluded from the analysis.

2.3.1.2. Salivary cortisol. Where one or two samples were missing from one individual (due to infant non-compliance), values were imputed with the group (term / preterm) mean sample value for that timepoint (n = 11; 5 preterm, 6 term). In the preterm group, a total of 5 and 3 samples were missing at the 20- and 30-minute timepoints respectively. In the term group 3, 2 and 3 samples were missing at the pre stressor, 20 and 30 min timepoints respectively. Cortisol values that were below the lower limit of detection (<LOD) were replaced with the value of the minimum detected value (0.25 ng/mL) (n = 13). Outliers that were more than 3 times the IQR below the first or above the third quartile were examined. Visual inspection of the outlying values revealed a cluster of very high values that may represent sample contamination or interference. These samples with values between 13.54 ng/mL and 123.13 ng/mL were excluded (n = 4). A second cluster of samples with values between 2.93 ng/mL and 6.4 ng/mL were retained and winsorized (n = 3) using the Winsorize function from the R package DescTools (Signorell, 2021).

2.4. Statistical analysis

Normality was assessed via visual inspection of histograms and QQ plots and Shapiro Wilks tests. In cases where data were not normally distributed, transformations were applied. Where transformations were unsuccessful in improving normality, parametric tests were used. In these cases, data were reported as mean and standard deviation. Where transformations failed to improve normality and no appropriate non-parametric tests were available, parametric tests were carried out and the violation of normality was reported. Where non-parametric alternatives were available these were carried out and median and interquartile range was reported. Untransformed values are reported in tables and figures for ease of interpretation. A significance threshold of p < 0.05 was used. Analyses were conducted in R version 4.0.1 (R Core Team, 2020).

Data for negative affect and self-comforting were positively skewed and an arcsine transformation for proportional data did not substantially improve normality. As the F-test has been found to be robust to violations of normality, untransformed data were used in the analysis (Blanca et al., 2017). A sensitivity analysis revealed there were no differences in the overall results when using the transformed compared to the untransformed values. Results from analyses conducted on the untransformed data are reported here, results from analyses conducted using the transformed data are available in supplement 1. The data satisfied the assumption of homogeneity of variance as assessed by Levene’s test (p > 0.05). There was homogeneity of covariance as assessed by Box’s test of equality of covariance matrices (p > 0.001).

Cortisol data were positively skewed, and log 10 transformed values were used in the analysis. Raw values are reported in tables for ease of interpretation. The data satisfied the assumption of homogeneity of variance as assessed by Box’s test of equality of covariance matrices (p > 0.001).

Correlations between biological and behavioural stress response measures and potentially confounding demographic variables were conducted. These variables included SIMD rank and GA. Here we considered GA as a proxy measure for individual differences in early life stress exposures as earlier GA necessitates a longer NICU stay which is associated with multiple stressors (Newnham et al., 2009). Socioeconomic deprivation has been associated with cortisol dysregulation in infants and children (Cleerfield et al., 2014; Lupien et al., 2001). Variables that were significantly correlated with negative affect, self-comforting or cortisol concentration were included as covariates in the relevant hypothesis tests. SIMD rank was included a covariate in the analysis of self-comforting and GA was included as a covariate in the analysis of negative affect. SIMD was included as a covariate in the analysis of cortisol concentration. Independent t-tests were carried out to test for differences in cortisol concentration at each sample timepoint between infants whose visit took place in the morning compared to the afternoon and time of day was included as a covariate in the analysis of cortisol.

2.4.1. Still-face

To determine the effect of group and episode on duration of time spent in each state, separate two (group: term/preterm) by three (episode: play/still-face/reunion) way mixed ANOVAs were conducted for negative affect and self-comforting, with proportional duration as the dependent variable, group as the within-subjects independent variable and episode as the between-subjects independent variable. Where main effects were significant, post hoc pairwise comparisons with Bonferroni corrections were conducted.

2.4.2. Salivary cortisol

To determine the effect of group and saliva collection timepoint on cortisol concentration, a two (group: term/preterm) by three (saliva collection timepoint: baseline/response/recovery) way repeated measures mixed ANOVA was conducted with cortisol concentration as the dependent variable, group as the between-subjects independent variable and saliva collection timepoint as the within-subjects independent variable. Where main effects were significant, post hoc pairwise comparisons with Bonferroni corrections were conducted. As previous studies have found that greater cortisol reactivity is stimulated in response to a cluster of very high values that may represent sample contamination or interference. These samples with values between 13.54 ng/mL and 123.13 ng/mL were excluded (n = 4). A second cluster of samples with values between 2.93 ng/mL and 6.4 ng/mL were retained and winsorized (n = 3) using the Winsorize function from the R package DescTools (Signorell, 2021).

2.4.3. Biological-behavioural coupling

Partial pairwise correlations were conducted to assess the relationships between biological (cortisol concentrations) and behavioural (negative affect and self-comforting) stress response while adjusting for time of day. Episodes 2 and 3 were selected to represent behavioural stress response and recovery respectively, while 20 min and 30-minute cortisol concentrations were selected to represent biological stress response and recovery respectively.

G-Power software was used to conduct a post-hoc power analysis for the given sample size. For F-tests the behavioural sample provides 99% power to detect a medium effect size (0.5) at an alpha-level of 0.05. The
X
nificant associations between group and frequency of early termination
of cortisol sample had 82% power to detect a medium effect (0.42).

3. Results

3.1. Behavioural analyses

Chi-squared tests of independence showed that there were no
significant associations between group and frequency of early termination
X\(^2\) (1, 120) = 0.49, p = 0.49, frequency of procedural violations X\(^2\) (1,
120) = 0.54, p = 0.46 or frequency of wearing motor sensors X\(^2\) (1, 120)
= 0.74, p = 0.39, and so no participants were excluded for this reason.
In order to retain the largest possible sample, and because early termina-
tion was most common in episodes 4–5, behavioural data for episodes
1–3 only were included in the main analyses. The majority of infants
(75%) included in the analysis of cortisol did complete 5 full episodes
and so the effects of the extended still-face design on cortisol are
maintained.

The final dataset after exclusions included 53 preterm (30 male) and
67 term (38 male) infants (see Fig. 1). Demographic characteristics of
this full sample and the subset who completed saliva collections are
displayed in Table 1. Mean gestational age at birth (SD) for the preterm
group was 29. ± 1.74 weeks and mean birthweight was 1312 ± 344 g.
The preterm group had a mean SIMD ranking of 4003 ± 2167 and the
term group had a mean ranking of 4715 ± 1863 (higher scores indicate
less deprivation). Demographic characteristics of the cortisol subset
were not significantly different from the main sample, and there were no
significant differences in behaviour between these subsets (supplemen-
tary Table 3).

3.1.1. Covariates

Appointments took place in the morning for 60% of preterm infants
and 33% of term participants. Mean proportional duration of negative
affect did not differ between infants whose appointment took place in
the morning compared to the afternoon for play t (29.12)
= 1.04, p = 0.15, p = 0.89 episodes. Mean proportional duration of self-
comforting did not differ between infants whose appointment took
place in the morning compared to the afternoon for play t (29.12)
= 1.04, p = 0.31, still-face t (26.70) = 1.40, p = 0.17 or reunion t
(25.18) = 2.08, p = 0.05 episodes.

Lower SIMD rank (i.e., greater deprivation) was associated with
greater self-comforting during episode 2 in the preterm group (r (38)
= −0.4, p = 0.02). Earlier gestational age was associated with decreased
negative affect during episodes 2 (r (65) = −0.39, p = 0.001) and 3 (r
(65) = 0.34, p = 0.006) in the term group. All other correlations were
not significant, and results are reported in supplementary Table 4.

3.1.2. Groupwise analysis of negative affect

There was a significant interaction between group and episode while
controlling for gestational age, F (1, 234) = 4.3, p = 0.01. There was a
significant difference in the proportion of time preterm versus term in-
fants spent in a negative affective state, F (1, 117) = 4.76, p = 0.03.
Preterm infants spent significantly less time in a negative state (M
= 0.18 s) compared to term (M = 0.25 s) infants during the still-face
episode (p = 0.006, \(\eta^2_p = 0.06\)). This difference was significant at a
Bonferroni adjusted alpha level of 0.02. Both groups spent a similar
amount of time in a negative state during the play (p = 0.51) and
reunion (p = 0.08) episodes. There was a significant change in negative
affect across episodes F (1, 234) = 5.37, p = 0.005. Post hoc tests
revealed that regardless of group membership, negative affect signifi-
cantly increased between play and still-face episodes (p adjusted
<0.001, d = 0.69) and between play and reunion episodes (p adjusted
<0.001, d = 0.76) but not between still-face and reunion episodes (p
adjusted = 0.48). These results indicate that the still-face episode elic-
itied an increase in negative affect in both groups, but that preterm
infants displayed significantly less negative affect than the term group
during the still-face episode.

3.1.3. Groupwise analysis of self-comforting

There was no significant difference in the proportion of time preterm
compared to term infants spent engaged in self-comforting behaviours,
F (1, 95) = 1.33, p = 0.25. There was no change in duration of self-
comforting across episodes, F (1.83, 173.67) = 1.88, p = 0.16. There
was no interaction between group and episode, F (1.83, 173.67) = 0.34,
p = 0.71. Fig. 2.

3.2. Salivary cortisol analyses

The final sample after exclusions comprised 20 preterm and 24 term
participants (see Fig. 1).

![Flowchart](https://via.placeholder.com/150)

**Fig. 1.** Sample and sub-sample inclusion and exclusion flowchart.
negative correlation between SIMD rank and 30-minute cortisol concentration in the preterm group (r (16) = -0.40, p = 0.001). There was a significant difference in cortisol concentration compared to the afternoon at both the baseline (t (38.34) = 3.24, p = 0.003) and 30-minute sample timepoint (t (38.83) = 3.24, p = 0.003).

3.2.1. Covariates
Appointments took place in the morning for 60% of preterm infants and 33% of term participants. Mean cortisol concentration was significantly higher for infants whose appointment took place in the morning compared to the afternoon at both the baseline t (38.34) = 3.12, p = 0.003 and 30-minute sample timepoint, t (38.83) = 2.24, p = 0.03.

Gestational age was not associated with cortisol concentration at any sample timepoint (supplementary Table 5). There was a significant negative correlation between SIMD rank and 30-minute cortisol concentration in the preterm group (r (16) = -0.64, p = 0.004) such that greater deprivation was associated with higher cortisol concentration. All other correlations were non-significant, and results are reported in supplementary Table 5.

3.2.2. Groupwise analysis of cortisol concentration
Cortisol concentration was significantly higher in the preterm compared to the term group F (1, 42) = 5.95, p = 0.02, (Table 2). Post-hoc tests confirmed that cortisol concentration was significantly higher in the preterm group at 30 min post stressor (p adjusted =0.009, generalized eta squared (n² G) = 0.15) but not for the pre-stressor (p adjusted = 0.09) or 20-minute post-stressor samples (p adjusted = 0.10). However, this group difference did not remain significant when SIMD and time of day were controlled for F (1, 34) = 1.51, p = 0.23, n² G = 0.03. Effect sizes (generalised eta squared) for time of day and SIMD rank were 0.07 and 0.06 respectively. There was no significant change in cortisol concentration across sample timepoints F (2, 68) = 2.8, p = 0.07 and no interaction between group and timepoint F (2, 68) = 0.13, p = 0.87.

Findings from the sub-analysis including infants who participated in 5 still-face episodes did not differ from those presented above for infants who participated in the classic 3-episode still-face procedure and are reported in the supplementary materials along with the demographic characteristics of this sub-sample and mean cortisol values (supplementary Tables 6 and 7 respectively).

3.2.3. Association between affective and cortisol response adjusted for time of day
Negative affect was not significantly correlated with cortisol concentration in the preterm group at 30 min post stressor (p adjusted = 0.009).

### Table 1
Demographic characteristics split by sample subset, and by group.

|                           | Preterm (n = 53) | Term (n = 67) | Mean comparison | Preterm (n = 20) | Term (n = 24) | Mean comparison |
|---------------------------|------------------|--------------|-----------------|------------------|--------------|----------------|
| **Gestational age at birth/weeks, median (IQR)** | 30.0 (28.4–30.6) | 39.4 (39.6–40.2) | 3564 < 29.3 (27.5–30.6) | 40.3 (39.2–41.2) | 480 < 0.001 |
| Birthweight / grams, mean (SD) | 1312.93 (344) | 3520 (506) | -28.36 < 1294 (410) | 3544 (433) | -17.69 < 0.001 |
| Age at testing / months, median (IQR) | 8.91 (8.6–9.2) | 8.96 (8.6–9.3) | 1880.5 .01 | 8.41 (8.49–9.15) | 8.94 (8.83–9.40) | 308.5 0.11 |
| Sex M: F (%) | 30:23 (43%) | 38:29 (43%) | 0.002 .99 | 11:9 (45%) | 10:14 (58%) | 0.33 0.56 |
| SIMD rank, median (IQR) | 4353 (5360) | 3245-6396 | 1407 .09 | 5396 | 251 0.04 |
| Time of day of testing AM: PM (%)| NA | NA | NA | 12:8 (30%) | 8:16 (66%) | 2.14 0.13 |

SIMD = Scottish Index of Multiple Deprivation 2016, SD = Standard Deviation, IQR = Interquartile range, acorrected age for preterm group, btime of day data were only collected for the cortisol subset. Student’s t-test was used for group comparison of birthweight, Wilcoxon rank sum tests were used for group comparison of gestational age at birth, SIMD 2016, and age at testing.

**Fig. 2.** Changes in mean proportional duration of negative affect (A) and self-comforting (B) behaviours across still-face episodes, **= p < 0.01.** Error bars represent standard deviation.

**Table 2**
Cortisol concentration in nmol/L at each sample timepoint, per group.

| Sample Timepoint | Preterm (n = 20) | Term (n = 24) | Mean comparison |
|------------------|------------------|--------------|----------------|
| Pre stressor     | 2.43 (1.26–3.74) | 1.61 (0.63–2.46) |                |
| 20 min post stressor | 3.0 (1.41–3.58) | 1.67 (0.85–2.17) |                |
| 30 min post stressor | 2.56 (1.79–3.54) | 1.11 (0.63–2.1) ** |

**= p < 0.01.”
concentration at 20 min post stressor (i.e., reactivity) in either group (Table 3). Higher cortisol concentration 30 min post stressor (i.e., recovery) was associated with a greater proportion of time spent in a negative affective state during the still-face (r_2 (22) = 0.65 p < 0.001) and reunion (r_2 (22) = 0.53, p = 0.01) episodes in the term group while 30-minute cortisol concentration was not associated with negative affect in the preterm group (Table 3 and Fig. 3). Self-comforting was not associated with cortisol concentration at 20- or 30-minutes post stressor during the still-face or reunion episodes in either group (Table 3).

4. Discussion

Here, we report both affective and cortisol response to the still-face procedure in late infancy for children born preterm and controls born at term. The data show that affective stress response, and the coupling between affective and cortisol responses are altered in preterm infants. However, there is evidence that these findings may be at least partially influenced by socioeconomic deprivation and time of day of saliva sample collection. This is one of few studies to assess both affective and neuroendocrine response to the still-face in preterm infants and only the second to explore associations between affective and cortisol stress response.

Preterm infants spent significantly less time in a negative affective state during the still-face episode compared their term born peers, though the difference was small and was only detected during the still-face episode. As expected, negative affect durations were very small for both groups (0.06 s vs 0.07 s) during the play episode. The lack of significant difference in the reunion episode suggests that preterm infant’s emotion regulation following a stressor may be bolstered by maternal support while preterm infant’s struggle to independently regulate emotions while their caregiver is unresponsive during the still-face episode. In the current study, to account for individual differences in early life stress-exposures, we controlled for gestational age in the analysis because lower gestational age is a proxy for burden of early life stress (Newham, 2009). Previous studies did not control for degree of early life stress exposure using gestational age or otherwise which may explain why previous studies did not report group differences in negative affect (Montiroso et al., 2010; Provenzi et al., 2017; Segal et al., 2016; Yaari et al., 2018). Early life experiences associated with earlier gestational age may also disrupt preterm infants’ emotion regulation abilities. In this study, term and preterm infants exhibited similar levels of self-comforting behaviours and the still-face procedure did not elicit a significant change in self-comforting behaviours in either group. The observed lack of group differences in self-comforting contradicts some previous findings which indicate less self-comforting behaviours in preterm infants (Jean and Stack, 2012; Provenzi et al., 2017). Some others have reported no group difference (Montiroso et al., 2010; Yaari et al., 2018) though Yaari et al. (2018) do report a difference in the pattern of self-comforting behaviours between groups with self-comforting behaviours decreasing across episodes in the preterm group but not the term group. In the current study, greater socioeconomic deprivation was significantly associated with greater self-comforting behaviours in the preterm group and was controlled for in the groupwise analysis of self-comforting. Previous studies did not control for socioeconomic status and findings may have been confounded by this.

Findings related to cortisol concentration must be carefully considered in the context of demographic and confounding factors, as the significant group difference in cortisol concentration observed at 30 min post stressor did not survive correction for time of day of sampling and socioeconomic status. In the preterm group, gestational age was not correlated with cortisol concentration, while SIMD was, such that higher levels of deprivation were associated with higher 30-minute cortisol concentration, suggesting that deprivation rather than prematurity or degree of early life stress had a greater effect on cortisol recovery in this sample. The SIMD is a multidimensional measure with over 6000 rankings so that categorisation is carried out on a fine-grained basis. Future studies capturing deprivation in similarly multifaceted, additive, and detailed ways would be of interest in determining whether the development of the stress-response system in preterm infants is vulnerable to the effects of deprivation, and which aspects of deprivation score are responsible for this relationship. Cortisol concentrations typically peak in the morning and decline throughout the day. By chance, significantly more preterm compared to term infants had visits in the morning and so the higher cortisol concentrations seen in the preterm group prior to correction for time of day and deprivation may also reflect this.

The finding that negative affect was positively associated with cortisol concentration in term but not preterm infants is in partial agreement with one other study that tested this relationship. Erickson et al. (2013) reported a negative association between positive affect and cortisol reactivity (change from baseline to 20 min post stressor) to the still-face in 6–8-month-old term born infants that was not present in the very low birth weight group. Though the relationship was not significant for negative affect the authors reported a qualitative difference in the direction of the relationship between cortisol and negative affect between term and preterm infants that indicated a difference in the way affective and biological response are coordinated.

4.1. Strengths, limitations, and future directions

Sample collection timings, based on previous studies measuring salivary cortisol reactivity and recovery following the still-face (Crockett et al., 2013; Haley et al., 2006; Müller et al., 2015; Provenzi et al., 2016b) and literature investigating timing of infant peak cortisol reactivity and recovery following a stressor (Ramsay and Lewis, 2003), are likely to be good representations of peak stress response and recovery respectively. However, there are also some methodological limitations around the fact that cortisol has a known diurnal rhythm, such that cortisol levels are highest in the morning and gradually decline throughout the day. This pattern is observed from as young as three months of age (Price et al., 1983). We observed a group difference in the number of morning versus afternoon appointments and when this variable was controlled for along with other potential confounders group differences in cortisol concentrations were no longer significant. Time of day data was only available for the subset of infants who provided saliva samples. Time of day may also influence behaviour as infants may be more or less fussy in the morning versus afternoon and so the behavioural results may be limited by the inability to correct for time of day. In addition, while the assessment schedule remained fixed as far as possible, flexibility in the schedule was allowed depending on the infants’ feeds, naps, or fussiness; importantly, there was no group difference in negative affect in the baseline episode specifically. A further limitation arising from practical constraints was that the first coder was not blind to participant group. However, the second coder was blinded and the excellent interrater reliability scores indicate low risk of bias. In

Table 3
Partial spearman correlation coefficients for the associations between cortisol concentration and negative affect and self-comforting during episodes 2 (still-face 1) and 3 (reunion 1).

|                | Preterm Cortisol + 20 | Preterm Cortisol + 30 | Term Cortisol + 20 | Term Cortisol + 30 |
|----------------|-----------------------|-----------------------|-------------------|-------------------|
| **Negative Affect** |                       |                       |                   |                   |
| Still-face 1     | 0.14                  | 0.03                  | 0.37              | 0.65***           |
| Reunion 1        | -0.04                 | -0.18                 | 0.25              | 0.53**            |
| Self-comforting  |                       |                       |                   |                   |
| Still-face 1     | -0.19                 | 0.13                  | -0.16             | 0.13              |
| Reunion 1        | 0.09                  | 0.14                  | 0.10              | 0.05              |

*p < 0.01, **p < 0.05, ***p < 0.001
an effort to retain the maximum sample, missing cortisol values were imputed where appropriate. Imputation may have been non-random in cases where samples were difficult to collect due to infant non-compliance in infants who became most distressed by the procedure. This may have resulted in an underestimation of associations between stress and behaviour. Finally, interpretations are limited by the size of cortisol sample, and replication studies are required to confirm the exploratory findings reported here.

Emotion regulation and stress response are complex processes that may be influenced by additional factors not measured here, most notably maternal behaviour. Mesman et al. (2009) conducted a meta-analysis of 8 studies concluding that there is a medium overall effect for the influence of maternal behaviour on positive infant affect but a non-significant influence of maternal behaviour on infant negative affect. In terms of cortisol stress response however, several studies report an association between greater maternal sensitivity and cortisol reactivity (Bosquet Enlow et al., 2014; Erickson et al., 2019; Müller et al., 2015) suggesting that future studies that take maternal behaviours into consideration when assessing the relationship between prematurity and stress response would be of value.

5. Conclusions

The data indicate that there may be some differences in the way preterm versus term infants respond to the still-face stressor. Preterm infants displayed reduced negative affect, and demonstrated a lack of coupling between affective and cortisol responses that was present in term infants. Future research in larger samples and controlling for confounding factors may be of value for understanding whether early indicators of emotion regulation and stress response elicited by the still-face may be of value for stratification of infants at risk for poorer outcome.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2022.105760.

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Declaration of Competing Interest

None.

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Fig. 3. Change in mean cortisol concentration across timepoints (A) and correlations between mean cortisol concentration 30 min post-stressor and mean negative affect (B) for still-face (i) and reunion (ii) episodes. Spread is represented as standard deviation (A) and 95% confidence intervals (B). * = p-value < 0.01.
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