Associations of gestational phthalate exposure and non-nutritive suck among infants from the Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) birth cohort study

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Abstract

\textbf{Background:} Infant non-nutritive suck (NNS), or sucking on a pacifier with no nutrients being delivered, has been used as an index of brain function and has been linked to subsequent neurodevelopment. Yet, no data are available connecting NNS to environmental exposures in utero. The goal of this study was to examine the relationship between gestational exposure to phthalates (a group of chemicals found in personal care products, PVC plastics, and other products) and NNS among infants in a birth cohort study in Puerto Rico.

\textbf{Methods:} Urinary phthalate metabolite levels were measured in women at up to three time points in pregnancy as a measure of in utero exposure to the child. We calculated the geometric mean of each metabolite for each woman as a measure of exposure across gestation. Infants had their NNS sampled using our custom research pacifier between 4–6 (± 2 weeks) weeks of age,
yielding the following NNS dependent measures: cycles/burst, frequency, amplitude, bursts/min, and cycles/min.

**Results:** Two hundred and eight mother-infant dyads completed this study. We used multiple linear regression to assess associations between individual phthalate metabolites and NNS measurements, adjusting for infant sex, birthweight, and urinary specific gravity. An interquartile range (IQR) increase in mono carboxyisononyl phthalate across pregnancy was associated with 3.5% (95%CI: −6.2, −0.8%) lower NNS frequency and 8.9% (0.6, 17.3%) higher NNS amplitude. Similarly, an IQR increase in mono-2-ethylhexyl phthalate was also associated with 3.4% (−6.5, −0.2%) lower NNS frequency, while an IQR increase in di-2-ethylhexyl terephthalate metabolites was associated with 11.2% (2.9, 19.5%) higher NNS amplitude. Gestational exposure to phthalates may alter NNS amplitude and frequency in full-term infants. These findings indicate that the infants may be increasing their NNS amplitude to compensate for their slower NNS frequency. These preliminary findings could have important clinical implications for earlier detection of exposure-related deficits in neurofunction as well as implications for subsequent neurodevelopment and related interventions.

1. **Introduction**

Sensitive tests of early neurofunction are essential as infancy is a critical period of brain growth laying the foundation for all areas of neurodevelopment. However, there remains a large gap in early, quantitative measures of neurodevelopment that can be administered in the first year of life in an effort to detect delays early. As a result, researchers are restricted to standardized tests that require significant training to administer, often underestimate delays, and are poor at predicting future neurodevelopment across preterm and full-term cohorts (Anderson et al. 2010; Anderson and Burnett 2017; Spencer-Smith et al. 2015). One such technology that could address this gap in early neurodevelopmental measures is non-nutritive sucking (NNS).

NNS is a suck pattern characterized by the absence of nutrients being delivered (e.g., sucking on a pacifier). Infant suck begins in utero at approximately 15 weeks’ gestational age (GA) (Humphrey 1970) and is stable and well-patterned by 34 weeks’ GA (Hack et al. 1985). Typically, NNS is assessed with a gloved finger by clinicians who feel the strength of the suck but more quantitative metrics exist that allow for in-depth NNS analyses. Wolff connected a pacifier to a polygraph system in 1968 and was the first to describe the stereotypical burst-pause pattern of NNS. He described bursts of sucking that consisted of approximately 6–12 suck cycles at a within burst frequency of 2 Hz separated by pause periods for respiration (Wolff 1968). In addition to frequency and counting suck cycles per burst, the strength of the suck can also be measured by examining the peak of the cycle also known as the amplitude (Fig. 1).

Most of the prior infant NNS research has surrounded preterm infants during their neonatal intensive care unit (NICU) stay (Bingham et al. 2010; da Costa et al. 2010; Pineda et al. 2019; Poore et al. 2008a) or full-term infants soon after birth (Capilouto et al. 2014). More recently, work from Martens and colleagues found that full-term infants significantly change their NNS duration, amplitude, burst number, cycles/burst and cycle number with no
significant changes present in NNS frequency between 3 and 12 months. More specifically, three-month-old infants produced a median of 4.50 suck bursts per minute that contained 9.60 cycles/burst, resulting in a burst duration of 4.74 s (Martens et al. 2020). The median NNS frequency was 2.09 Hz, with an average amplitude of 14.05 cmH20. At twelve months, these infants produced a median of 2.50 suck bursts that contained 3.75 cycles/burst, resulting in a burst duration of 1.67 s. The median NNS frequency was 2.11 Hz with an average amplitude of 19.75 cmH20. Knowledge of NNS emergence and maturation is imperative to better understand what is considered typical throughout development.

Assessment of NNS is important as delayed sucking and feeding have been reported in approximately 35–48% of infants with different types of neonatal brain injury (Slattery et al. 2012). Thus, NNS could be a sensitive indicator of disturbances in central nervous system (CNS) function in infants (Medoff-Cooper and Ray 1995). Furthermore, there is emerging retrospective data available that link infant NNS and feeding to subsequent development (Adams-Chapman et al. 2013; Malas et al. 2015; Malas et al. 2017; Wolthuis-Stigter et al. 2015; Wolthuis-Stigter et al. 2017). Wolthuis-Stigter and colleagues found that higher sucking scores in preterm infants between 42 and 50 weeks’ post-menstrual age (PMA), as measured on the Neonatal Oral-Motor Assessment Scale (NOMAS), were linked to higher test scores on the Movement Assessment Battery for Children, Wechsler Preschool and Primary Scale of Intelligence, and the Reynell Developmental Language Scales at age five. While this study used the NOMAS scale, which is based on clinician observation, to rate the rhythmicity of the jaw and tongue movements during sucking, more quantitative and physiological-based approaches are needed to further link infant NNS to subsequent development.

While NNS is an important early marker of CNS integrity and has associations with subsequent development, to our knowledge there are no prior studies available linking NNS to potentially neurotoxic environmental exposures. More specifically, given that NNS is an early emerging reflex in infancy, it begs the question: can NNS sampled soon after birth detect the effects of environmental exposures experienced in utero? Gestational exposure to a range of environmental chemicals has been associated with increased risk of adverse cognitive and behavioral outcomes in childhood (Braun et al. 2009; Ejaredar et al. 2015; Yolton et al. 2011). For example, in utero exposure to phthalates, a group of chemicals widely used in personal care products, PVC plastics, and other consumer products, has been associated with lower IQ, increased attention problems and hyperactivity, and poorer social communication (Ejaredar et al. 2015). However, many of these effects are not observed until later in childhood, minimizing options for early interventions. Identification of NNS as a sensitive indicator of neurotoxicity would provide a tool for early detection of exposure-related changes in neurodevelopment. Therefore, the purpose of this exploratory study was to examine the relation between gestational phthalate exposure and infant NNS. We hypothesized that higher phthalate exposures would be associated with NNS characterized by reduced amplitudes and slower frequencies.
2. **Materials and methods**

2.1. **Study population and design**

Participants in this study were mother-infant pairs from the Northern Karst aquifer region of Puerto Rico, an underserved, low-income population with significant health disparities. Puerto Rico also has substantial environmental contamination, including 18 Superfund sites and over 200 hazardous waste sites. Mothers were recruited into the Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) birth cohort study early in pregnancy (targeted < 20 weeks’ gestation) from two hospitals and five nearby clinics (Ferguson et al. 2019) between 2016 and 2019. Exclusion criteria were: maternal age < 18 or > 40 years; residence outside of the Northern Karst aquifer region; use of oral contraceptives within the three months prior to pregnancy; use of in vitro fertilization to become pregnant; or any major pre-existing medical conditions (e.g., diabetes) (Ferguson et al. 2019). From 2017 to 2019, children born to PROTECT mothers were then recruited into the Center for Research on Early Childhood Exposure and Development in Puerto Rico, or CRECE study (Manjourides et al. 2020). In order to be included in the present analysis, infants were required to be born full-term (≥37 weeks’ gestation), and have both an NNS measurement sampled in infancy and maternal phthalate metabolite concentrations from at least one urine sample collected during pregnancy.

At an initial screening, participants provided basic demographic information, reported the first day of their last menstrual period, and provided written informed consent prior to participation in the study. At three subsequent prenatal study visits (18 ± 2 weeks’, 22 ± 2 weeks’, and 26 ± 2 weeks’ gestation) participants provided spot urine samples and we collected questionnaire information on demographic and pregnancy characteristics, including maternal age, educational attainment, family income, marital status, pre-pregnancy body mass index, employment status, current and former tobacco use, current and former alcohol use, and number of children (parity). CRECE infants born to PROTECT mothers then attended a follow-up study visit at 4–6 weeks of age for measurement of NNS.

2.2. **Ethical clearance**

The research protocol was approved by the Ethics and Research Committees of the University of Puerto Rico (IRB # A8570110), participating clinics, and Northeastern University (IRB # 15–06–29). The involvement of the Centers for Disease Control and Prevention (CDC) laboratory was determined not to constitute engagement in human subject research.

2.3. **Maternal urinary phthalate metabolite measurements**

Maternal urine samples were collected at each prenatal study visit using procedures, supplies, and processing methods according to Centers for Disease Control and Prevention (CDC) protocols. Sample aliquots were frozen at 80 °C and shipped overnight on dry ice to the CDC and then analyzed for phthalate and phthalate replacement metabolites using online solid phase extraction high-performance liquid chromatography–isotope dilution tandem mass spectrometry as described in detail elsewhere (Silva et al. 2007). The following phthalate metabolites were measured: mono-ethyl phthalate (MEP); mono-n-
butyl phthalate (MBP); mono-isobutyl phthalate (MiBP); mono-2-ethylhexyl phthalate (MEHP); mono-2-ethyl-5-carboxypentyl phthalate (MECPP); mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP); mono-2-ethyl-5-oxohexyl phthalate (MEOHP); mono-benzyl phthalate (MBzP); mono-3-carboxypropyl phthalate (MCPP); mono carboxyisooctyl phthalate (MCOP); mono carboxyisooctyl phthalate (MCNP); mono oxoisononyl phthalate (MONP); mono-hydroxybutyl phthalate (mHBP); mono-hydroxyisobutyl phthalate (mHiBP); and the di-2-ethylhexyl terephthalate (DEHTP) metabolites mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP) and mono-2-ethyl-5-hydro-hexyl terephthalate (MEHHTP).

Phthalate concentrations with instrument-reported values below the limit of detection (LOD) were used as reported, while concentrations below the LOD that were not reported with a numerical value were imputed with the LOD divided by the square root of 2. We calculated the molar sum of di-2-ethylhexyl phthalate (DEHP) metabolites (∑DEHP = MEHP, MEHHP, MEOHP, MECPP) by dividing concentrations of each metabolite (ng/mL) by its molecular weight (g/mol), summing the metabolites, and then multiplying by the molecular weight of MEHP for unit comparability (ng/mL) as in the following equation.

$$\sum_{DEHP} = \left(\frac{MEHP}{278.34}\right) + \left(\frac{MEHPP}{294.34}\right) + \left(\frac{MEOHP}{292.33}\right) + \left(\frac{MECPP}{308.33}\right) \times 278.34$$

Similarly, we created molar sums for di-n-butyl phthalate (∑DBP = MBP, MHBP), di isobutyl phthalate (∑DiBP = MiBP, MHiBP), and DEHTP (∑DEHTP = MECPTP, MEHHTP).

In order to create more stable estimates of individual exposure over the course of pregnancy, we created subject-specific geometric mean concentrations across pregnancy for each phthalate metabolite and phthalate summary measure using all available prenatal measurements (75% of participants had phthalate measurements from all three study visits, 18% had 2 measurements, 5% had 1 measurement). We measured urinary specific gravity (SG) using a digital handheld refractometer (ATAGO Co., Ltd., Tokyo, Japan) at the time of urine sample aliquoting to assess urinary dilution.

2.4. Infant Non-Nutritive Suck

Infant NNS was collected between 4 and 6 (±2 weeks) weeks of life in our clinic in Manatí, Puerto Rico. To assess NNS, we utilized our custom research pacifier, which yields quantitative NNS data in real-time (Fig. 1). This user-friendly device includes a Soothie pacifier (Philips Avent) attached to a handle, connected to a pressure transducer that transmits information to a data acquisition system (Power Lab, ADInstruments, Dunedin, New Zealand). The data acquisition system then connects to a laptop with LabChart software (ADInstruments). Calibration was completed before every session. In order to calibrate, a range of pressure measurements from the system were recorded simultaneously from both the internal, uncalibrated pressure transducer, as well as an external pressure calibrator. This information was used to produce a linear calibration curve for the NNS system, and these values were then updated in the ADInstruments software. Once the device was set-up and calibrated to ensure pressure readings were accurate, the parents were
instructed to cradle hold the infant and to offer the pacifier to their child, as seen in Fig. 1, for approximately five minutes. The same instructions were given to all participants.

After the visit was concluded, all data were analyzed using LabChart software. Trained researchers manually selected NNS bursts using the following criteria: bursts must contain two or more suck cycles, each suck cycle’s amplitude must be over 1 cmH20, and a cycle is considered a new burst if there is a break of >1,000 ms between cycles. These criteria are the same as previous studies examining NNS in young infants (Barlow et al. 2012; Estep et al. 2008; Poore et al., 2008a,b). Once NNS bursts were manually selected for each suck sample, they were entered into a custom NNS Burst Macro, which allows for quick processing of the following burst variables: amplitude (cmH20), cycles/burst, frequency (cycles per second within a burst measured in Hz), number of cycles, and bursts. Next, the two consecutive minutes with the highest cycle count were used for the final analyses and minute rate averages were attained from the two-minute sample (cycles/minute). This process has been utilized in prior work examining NNS (Barlow et al. 2012; Estep et al. 2008; Martens et al. 2020; Poore et al. 2008b) as well as allows investigators to get the most active two minutes of NNS for analyses. Further, it also allows us to have the same NNS time sample across infants.

2.5. Statistical analysis

We summarized distributions of phthalate metabolite concentrations across pregnancy, calculating geometric means, standard deviations, and select percentiles. Phthalate metabolite concentrations were log-normally distributed, and therefore natural log-transformed prior to data analysis. We also calculated distributions of NNS outcome measures for all term infants (n = 207). NNS measures were approximately normally distributed, and thus not transformed prior to analyses.

We used multiple linear regression to estimate associations between individual phthalate metabolite concentrations across pregnancy and continuous NNS variables. Basic models were adjusted for SG to account for urinary dilution, and fully adjusted models were adjusted for SG, infant sex, and birthweight. These covariates were chosen based on prior knowledge, influence on the phthalate effect estimate, and informed by construction of a directed acyclic graph (Supplemental Material, Figure S1). Effect estimates are calculated as the percent change in NNS outcome measure compared to the population median per interquartile range (IQR) increase in urinary phthalate metabolite concentration using the following equation:

\[
\% \Delta_{\text{per IQR}} = \left( \frac{(\beta \times \ln(75th\ percentile)) - (\beta \times \ln(25th\ percentile))}{\text{median outcome}} \right) \times 100
\]

To assess linearity of associations, we created tertiles for each phthalate metabolite and phthalate summary measure for use in regression models. To create tertiles, we first corrected individual phthalate metabolite concentrations for urinary dilution using SG and the following formula: \( P_{c} = P \times (1.019 - 1)/(SG - 1) \). \( P_{c} \) is the SG-adjusted phthalate metabolite concentration (ng/mL), \( P \) is the observed phthalate metabolite concentration, 1.019 is the population median SG, and SG is the specific gravity of the urine sample. We then
calculated geometric means of SG-corrected concentrations of each phthalate metabolite for each individual across pregnancy. Tertiles were then created based on distributions of the SG-corrected concentrations and entered into regression models as categorical exposure variables. P-values for linear trend were calculated by entering phthalate tertiles into regression models as continuous variables.

As previous studies of gestational phthalate exposure have found sex-specific effects, in sensitivity analyses we explored whether associations between phthalate metabolite levels and NNS measures differed by infant sex. We first included phthalate*sex interaction terms in regression models and then stratified analyses by sex. Previous work within this cohort has demonstrated that women pregnant during the immediate aftermath of Hurricane Maria, which occurred in September 2017 and within the timeframe of this study, had increased urinary levels of several phthalate metabolites (Watkins et al. 2020). In previous studies, experiencing a traumatic natural disaster during pregnancy has also been related to subsequent delays in neurodevelopment (Buekens et al. 2006; Sotomayor 2013). To address potential confounding by Hurricane Maria, we performed a sensitivity analysis first evaluating associations between the timing of pregnancy relative to the hurricane (gave birth before, pregnant during, or became pregnant after the storm; referred to here as “hurricane status”) and NNS measurements, and then evaluated hurricane status as a covariate in our adjusted models of phthalates and NNS.

3. Results

NNS measurements were available for 207 term infants, with means and standard deviations (SD) according to sociodemographic variables and hurricane status shown in Table 1. On average, infants from the cohort had an NNS frequency of 1.92 Hz, an amplitude of 16.7 CmH\textsubscript{2}O with 6.27 bursts/minute, 60.3 cycles/minute, and 11.4 cycles/burst. The mean birthweight was 3.33 kg (SD = 0.43) among 108 male and 99 female infants.

Prenatal urinary phthalate and phthalate replacement metabolite concentrations from the subset of PROTECT women whose infants had NNS measurements are presented in Table 2. Concentrations of most phthalate metabolites in this subset of participants were either similar to or lower than concentrations reported in the full PROTECT cohort (Rodríguez-Carmona et al. 2020). However, metabolites of the phthalate replacement DEHTP were higher. Associations between sociodemographic variables and phthalate metabolite concentrations within the PROTECT cohort have been previously published (Rodríguez-Carmona et al. 2020).

In linear regression models, an IQR increase in MCNP concentrations across pregnancy was associated with 3.5% lower NNS frequency (95% confidence interval (CI): −6.2, −0.8) and 8.9% higher NNS amplitude (95%CI: 0.6, 17.3) after adjustment for infant sex, birthweight, and urinary SG (Table 3). Results from models adjusted only for SG were similar to findings from the fully adjusted analysis (Supplemental Material, Table S1). An IQR increase in MEHP was also associated with 3.4% lower NNS frequency (95% CI: −6.5, −0.2), while an IQR increase in ZDEHTP was associated with 11.2% higher NNS amplitude (95%CI: 2.9, 19.5). Although not statistically significant, all phthalate metabolites except MEP
and $\Sigma$DEHTP were also associated with fewer NNS cycles/minute. Gestational phthalate and phthalate replacement metabolite concentrations were not associated with NNS bursts/minute or cycles/burst.

Models regressing NNS measures on categorical phthalate variables confirmed that the observed associations from models with continuous exposure variables were linear. However, we did observe non-linear associations between $\Sigma$DEHTP and MEP with cycles/minute, as well as with MEP and NNS frequency. Specifically, the middle $\Sigma$DEHTP tertile had 26% higher cycles/minute compared to the reference group (tertile 1), whereas tertile 3 only had 15% higher cycles/minute compared to the reference group (Fig. 2, Supplemental Material, Table S2). Similarly, the middle MEP tertile had significantly fewer cycles/minute compared to tertile 1 (~29%), while the highest and lowest MEP tertiles were similar (Fig. 3, Supplemental Material, Table S2). A similar pattern was also observed for tertiles of MEP and lower NNS frequency.

When we explored interactions between the effects of gestational phthalate levels and infant sex on NNS measures, we found no significant differences between male and female infants (p-value > 0.1 for all phthalate*infant sex interaction terms; data not shown). In analyses stratified by infant sex, associations between phthalates and NNS outcomes were similar among males and females (data not shown). In sensitivity analyses exploring Hurricane Maria as a potential confounder, with the exception of higher amplitude among infants born to women pregnant during the hurricane, hurricane status was not associated with NNS measurements. When hurricane status was added to the fully adjusted model, there were few meaningful changes in our results. Specifically, associations of $\Sigma$DEHTP with cycles/burst became stronger and significant, while associations with amplitude were slightly attenuated.

4. Discussion

In this study we found relationships between in utero phthalate exposure and aspects of infant NNS among participants in PROTECT, an ongoing birth cohort in Northern Puerto Rico. More specifically, higher levels of MEHP and MCNP were associated with significantly slower NNS frequencies, while higher levels of MCNP and $\Sigma$DEHTP metabolites were associated with significantly higher NNS amplitudes. These novel findings suggest that infant NNS could be sensitive to exposure-related changes in neurodevelopment and a useful assessment for infants in environmental epidemiology studies.

4.1. NNS within the PROTECT cohort

NNS characteristics within the PROTECT cohort were slightly different from recent findings published by Martens, Hines and Zimmerman using the same NNS device from a full-term infant cohort in the northeast US at 3-months (Martens et al. 2020). Infants from the PROTECT cohort had a slower average NNS frequency (1.92 Hz compared to 2.09 Hz), higher amplitudes (16.60 cmH$_2$O compared to 14.05 cmH$_2$O), more NNS bursts (6.25 compared to 4.50) and more cycles per burst (11.50 compared to 9.60). The differences in NNS outcomes between studies are likely due to differences in the age of the participants, with infants in the Martens and colleagues’ study being older (approximately 3 months compared to 4–6 weeks), which likely results in a more mature NNS pattern. In addition,
future studies with infants the same age and with different exposures levels could help to determine if these differences are due to exposures or other factors.

4.2. Phthalates and NNS

Multiple phthalate and phthalate replacement metabolites were associated with aspects of NNS. MCNP, which was associated with both NNS frequency and amplitude, is a metabolite of di-isodecyl phthalate (DiDP), a commonly used plasticizer in polyvinyl chloride (PVC). MEP, a metabolite of the diethyl phthalate (DEP) commonly used in personal care products was non-linearly associated with lower NNS frequency. Metabolites of DEHTP, a replacement plasticizer for the phthalate DEHP, were associated with higher NNS amplitude. Associations between commonly used and replacement (and presumably safer) chemicals in consumer products with NNS measures is concerning, given the widespread exposure and implications for early neurodevelopment. Investigators are still learning about what is considered typical NNS across the first year of life and across patient populations. While it remains unclear what a slower NNS frequency or a higher NNS amplitude indicate for brain development, the increases evident in NNS amplitude in this cohort could be a compensatory mechanism that the infant uses in response to the slower NNS frequency. This needs to be investigated in more detail in subsequent studies.

Data surrounding in utero phthalate exposure and neurodevelopment are mixed, as described by two recent review articles by Zhang et al. (2019) and Radke et al. (2020). Zhang et al. (2019) examined the relationship between in utero exposure to various phthalates and subsequent cognition and behavioral development in children (0–12 years) by examining 26 birth cohort studies, concluding that exposure to DEHP, butyl benzyl phthalate (BBzP), DEP, and DBP during this sensitive period influenced cognition and behavior development. Exposure to phthalates had similar adverse effects on cognitive development in boys and girls; however, boys were more likely to have behavioral problems as a result of phthalate exposure than girls. However, the review by Radke et al. (2020) showed no clear pattern between prenatal phthalate exposure and neurodevelopment. The authors describe exposure measurement error or misclassification, periods of heightened susceptibility, sex-specific effects, and a lack of consideration for chemical mixtures as possible explanations as to why there were no consistently clear associations observed.

Many of the difficulties in investigating the long-term effects of phthalate exposure on neurodevelopment are related to the various testing options available to measure neurodevelopment. As mentioned in the introduction, many tests of neurodevelopment, particularly in the first year of life, tend to over- or under- estimate delays (Anderson et al. 2010; Anderson and Burnett 2017; Spencer-Smith et al. 2015). Sampling NNS soon after birth is important particularly if the goal is to relate these outcomes to the intrauterine environment. As aforementioned, NNS starts in the womb at approximately 15 wks’ GA (Humphrey 1970) and remains reflexive until approximately 6 months when this reflex is suppressed as the frontal lobe develops. Measuring infant NNS while it is still a reflex is important as this is when it serves as a gauge of neurologic maturity or integrity in the developing infant. In this way, the NNS reflex is a form of motor behavior that lays foundations for voluntary motor control (Kondraciuket al. 2014; Miller 2002).
together, these findings highlight the importance of an assessment tool, like NNS, that can be used *early* in development with links to subsequent neurodevelopment.

### 4.3. NNS as measure of neurotoxicity

These data point to the possibility of using NNS as an early measure of exposure-related changes in gestational neurodevelopment. This finding is important for several reasons. NNS is an early measure that can be sampled soon after birth in all infants. Knowledge of altered NNS patterning in relation to environmental exposures could help to guide treatment in early infancy and childhood to reduce subsequent developmental delays. This is particularly important given the emerging data available linking sucking and feeding patterns to subsequent neurodevelopment (Adams-Chapman et al. 2013; Malas et al. 2015; Malas et al. 2017; Wolthuis-Stigter et al. 2015; Wolthuis-Stigter et al. 2017). More specifically, sucking scores, sampled between 37 and 50 weeks’ PMA, were linked to total motor skills, balance, total intelligence, verbal intelligence, performance intelligence, and language at age five. There is overlap between the neurobehavioral domains associated with gestational phthalate exposure and those indicated in studies of NNS and subsequent neurobehavior. For instance, higher gestational exposure to specific phthalates was associated with lower motor function among 11-year old girls while higher postnatal exposure to DEHP metabolites was associated with lower motor scores among boys (Balalian et al. 2019). Data is also available linking gestational phthalate exposure and lower scores in language development in boys, with a doubling in prenatal urinary MEP and DEHP metabolites associated with increased odds of vocabulary scores below the 15th percentile (Olesen et al. 2018). Another study also showed that gestational exposure to DBP and BBzP was significantly associated with language delay in children at 30 and 37 months (Bornehag et al. 2018). Taken together, these shared outcomes potentially point to shared relations between gestational phthalate exposure, infant NNS, and subsequent motor and language development; however, more studies are needed to examine these associations in more detail.

Results from this study show altered NNS linked to gestational neurotoxic exposures, which is important for clinicians to know when assessing early feeding. For instance, if clinicians know to expect an increase in amplitude and a reduction in frequency, then they can work on alternative and more targeted strategies to better support the infant. Prior work shows that sensory stimulation (e.g., olfactory cues and visual, auditory and oral-tactile stimuli) is capable of altering infant NNS (Bingham et al. 2007; Zimmerman and Barlow 2008; Zimmerman and Foran 2017; Zimmerman et al. 2017) and this might be one way in which to support NNS development. Further, mothers who are high risk for exposure could be counseled on the connections between phthalate exposure *in utero* and subsequent child outcome. Therefore, NNS could be a promising avenue in which to detect *in utero* neurotoxicity.

Currently, the mechanisms by which phthalate exposures might influence NNS are unknown. However, previous studies have shown that prenatal phthalate exposure is associated with changes in thyroid and reproductive hormone levels (Cathey et al. 2019; Johns et al. 2015; Johns et al. 2016; Romano et al. 2018; Sathyanarayana et al. 2014; Sathyanarayana et al. 2017) and increased oxidative stress during pregnancy (Ferguson
et al. 2014; Ferguson et al. 2015), which in turn may influence fetal neurodevelopment. These findings indicate that environmental exposures could indeed affect infant NNS, but additional research is needed to determine biological mechanisms.

5. Strengths and limitations

This is the first study to investigate the sensitivity of NNS to environmental exposures, and it benefitted from highly sensitive biomarkers of exposure measured at multiple times during pregnancy. Another strength of the study was that it also featured a quantitatively physiological device to measure infant NNS, and this is the first time such a device has been used in environmental health research. While our findings are novel, this work must also be replicated in subsequent studies including other known neurotoxicants and with other populations. This study was limited by the relatively modest sample size and involved a vulnerable population of pregnant women in Puerto Rico, potentially making generalizing these findings to other locations and populations more challenging. We also made a number of statistical comparisons, increasing the possibility of chance findings. NNS was only sampled at one time point and additional measures across development would be advantageous, given the aforementioned findings by Martens et al. (2020) that showed significant changes in infant NNS between 3 and 12 months of age. Additionally, we are unable to rule out potential confounding from unmeasured factors such as postnatal phthalate exposure, although the relevant time period between birth and NNS measurement was relatively short (4–6 weeks). Further, it is currently unclear if other neurotoxic chemicals, or mixtures of chemicals, influence infant NNS or may have confounded the observed associations between phthalates and NNS. Subsequent studies must examine these questions in more detail.

In conclusion these findings revealed significant associations between various markers of phthalate exposure across gestation with lower NNS frequency and higher NNS amplitude in infancy. This study is the first to prospectively investigate the sensitivity of NNS as a quantitative index of altered neurodevelopment related to in utero environmental exposures. This is important, as NNS can be measured soon after birth, while most previous exposure studies link gestational exposure to outcomes measured later in infancy or early childhood. Next steps include examining NNS outcomes in a larger sample size, across patient populations (e.g., preterm and full-term), and across geographical regions (e.g., mainland US and Puerto Rico), as well as examining exposure to a range of potential neurotoxicants, both individually and as exposure mixtures. Results from this work could have important clinical implications, as early detection of adverse neurodevelopment is critical to improving long-term neurobehavioral function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.  
Left: A picture of a mother giving her infant the NNS assessment (photo used with permission). Right: An image of 35 s of NNS activity. Three NNS bursts are present with pause periods for respiration. Frequency is measured by examining the cycles per second within a burst. The black dot indicates the NNS cycle within the peak. The amplitude is measured in CmH$_2$O, see y-axis.
Fig. 2.
Percent difference in infant NNS features associated with increasing tertiles of urinary DEHTP metabolites during pregnancy compared to the lowest tertile. Diamond markers indicate average difference and lines indicate 95% confidence intervals.
**Fig. 3.**
Percent difference in infant NNS features associated with increasing tertiles of urinary MEP during pregnancy compared to the lowest tertile. Diamond markers indicate average difference and lines indicate 95% confidence intervals.
### Table 1

NNS measurements among CRECE infants according to sociodemographic characteristics (n = 207).

|                        | Frequency (Hz) | Amplitude (CmH₂O) | Bursts/Minute | Cycles/Minute | Cycles/Burst |
|------------------------|----------------|-------------------|---------------|---------------|-------------|
|                        | N (%), Mean, SD | N (%), Mean, SD   | N (%), Mean, SD | N (%), Mean, SD | N (%), Mean, SD |
| All Infants            | 207 (100) 1.92 0.25 16.7 6.59 6.27 2.48 60.3 20.7 11.4 6.28 |
| Income (USD)           |               |                   |               |               |              |
| < $20,000              | 79 (38) 1.91 0.23 17.6 6.96 6.70 2.41 60.4 18.5 10.2 4.57 |
| $20,000 to < $50,000   | 73 (35) 1.95 0.26 15.5 5.85 6.04 2.61 62.3 21.3 12.9 7.67 |
| > $50,000              | 33 (16) 1.92 0.25 17.0 6.69 5.83 2.21 59.6 22.1 11.7 6.39 |
| Missing                | 22 (11) 1.84 0.26 16.9 7.24 6.11 2.65 54.4 23.8 10.7 5.70 |
| Maternal Education     |               |                   |               |               |              |
| ≤High School           | 31 (15) 1.92 0.22 16.6 7.25 6.34 2.33 61.5 20.6 11.8 5.96 |
| Some College           | 60 (29) 1.87 0.25 17.3 6.52 6.57 2.25 59.8 16.7 10.8 6.29 |
| Bachelor's Degree      | 78 (38) 1.91 0.25 16.8 6.67 6.14 2.63 59.0 23.5 10.9 5.82 |
| Graduate Degree        | 36 (17) 2.00 0.25 15.3 5.89 5.85 2.57 62.5 20.7 13.5 7.38 |
| Missing                | 2 (1) n/a     |                   |               |               |              |
| Marital Status         |               |                   |               |               |              |
| Married/Cohabitating   | 171 (83) 1.91 0.25 16.5 6.42 6.14 2.44 60.0 21.1 11.6 6.53 |
| Single                 | 34 (16) 1.95 0.21 17.7 7.56 6.96 2.70 61.4 19.4 10.3 4.98 |
| Missing                | 2 (1) n/a     |                   |               |               |              |
| Parity                 |               |                   |               |               |              |
| Nulliparous            | 50 (24) 1.94 0.26 14.9 6.54 6.76 2.59 63.4 18.5 11.5 7.14 |
| 1 or more children     | 98 (47) 1.91 0.24 16.5 6.06 6.02 2.32 58.0 21.1 11.3 5.80 |
| Missing                | 59 (29) 1.90 0.25 18.5 7.10 6.26 2.63 61.5 21.5 11.6 6.39 |
| Maternal Age (years)   |               |                   |               |               |              |
| ≤28*                   | 111 (54) 1.90 0.24 17.1 7.00 6.42 2.53 61.9 20.7 11.3 6.16 |
| >28                    | 96 (46) 1.93 0.26 16.2 6.08 6.08 2.43 58.4 20.5 11.5 6.45 |
| Infant Sex             |               |                   |               |               |              |
| Male                    | 108 (52) 1.91 0.25 16.8 6.30 6.42 2.59 60.5 20.8 11.0 5.59 |
| Female                 | 99 (48) 1.92 0.24 16.5 6.92 6.10 2.37 60.0 20.6 11.9 6.96 |
| Hurricane Maria Status |               |                   |               |               |              |
|                                | N (%) | Frequency (Hz) | Amplitude (CmH$_2$O) | Bursts/Minute | Cycles/Minute | Cycles/Burst |
|--------------------------------|-------|----------------|----------------------|---------------|---------------|--------------|
|                                |       | Mean           | SD           | Mean           | SD          | Mean          | SD            | Mean          | SD            |
| Gave birth before              | 22 (11)| 1.94           | 0.26       | 14.7         | 7.60          | 5.52          | 1.92          | 63.4         | 22.7          | 13.7          | 7.40          |
| Pregnant during                | 53 (25)| 1.89           | 0.28       | 19.1         | 5.29          | 6.52          | 2.25          | 59.4         | 20.2          | 10.4          | 5.68          |
| Became pregnant after          | 132 (64)| 1.92           | 0.23       | 16.0         | 6.67          | 6.29          | 2.64          | 60.1         | 20.6          | 11.5          | 6.27          |

SD: standard deviation; n/a: not calculated due to small number of participants.

*median maternal age was 28 years.
Table 2

Distributions of geometric mean urinary phthalate and phthalate replacement metabolite concentrations (ng/mL) across pregnancy (n = 207).

| Metabolite | LOD | % > LOD | GM   | GSD  | 25th | 50th | 75th | 95th |
|------------|-----|---------|------|------|------|------|------|------|
| MEHP       | 0.8 | 84.5    | 1.88 | 2.37 | 1.01 | 1.85 | 3.31 | 10.8 |
| MEHHP      | 0.4 | 100     | 5.07 | 2.10 | 3.10 | 5.06 | 8.41 | 17.8 |
| MEOHP      | 0.2 | 100     | 4.76 | 2.17 | 2.94 | 4.85 | 7.77 | 17.6 |
| MECPP      | 0.4 | 100     | 9.09 | 2.02 | 5.55 | 9.51 | 14.5 | 28.5 |
| MBzP       | 0.3 | 96.1    | 1.84 | 2.87 | 0.88 | 1.83 | 3.50 | 10.7 |
| MCP       | 0.4 | 85.0    | 0.91 | 2.25 | 0.52 | 0.90 | 1.60 | 3.32 |
| MCNP      | 0.2 | 99.0    | 1.11 | 1.87 | 0.78 | 1.13 | 1.64 | 2.50 |
| MCOP       | 0.3 | 100     | 5.45 | 2.24 | 3.36 | 5.08 | 9.29 | 20.6 |
| MONP*      | 0.4 | 97.1    | 2.05 | 2.35 | 1.19 | 1.97 | 3.70 | 8.13 |
| MEP        | 1.2 | 100     | 27.4 | 3.98 | 9.35 | 24.4 | 63.7 | 319  |
| MBP        | 0.4 | 100     | 13.1 | 2.44 | 7.22 | 13.4 | 21.2 | 70.2 |
| MBzP       | 0.4 | 88.9    | 1.35 | 2.58 | 0.76 | 1.34 | 2.28 | 7.36 |
| MiBP       | 0.8 | 100     | 7.50 | 2.20 | 4.36 | 8.18 | 12.6 | 26.3 |
| MHbP       | 0.4 | 97.6    | 3.00 | 2.29 | 1.90 | 3.20 | 4.88 | 10.7 |
| MECPTP*    | 0.2 | 100     | 31.7 | 2.62 | 16.1 | 27.9 | 57.1 | 235  |
| MEENHTP*   | 0.4 | 100     | 5.29 | 2.57 | 2.90 | 4.99 | 9.52 | 27.9 |
| ΣDBP       |     |         | 14.4 | 2.44 | 7.73 | 14.9 | 23.8 | 78.2 |
| ΣDiBP      |     |         | 10.4 | 2.20 | 6.23 | 11.4 | 17.0 | 36.6 |
| ΣDEHP      |     |         | 19.9 | 2.04 | 12.5 | 20.3 | 30.5 | 70.9 |
| ΣDEHTP     |     |         | 36.2 | 2.57 | 19.0 | 31.2 | 63.0 | 252  |

LOD: limit of detection; GM: geometric mean; GSD: geometric standard deviation.

* n = 206 for MONP, MECPTP, MEENHTP

ΣDBP: MBP + MHbP
ΣDiBP: MiBP + MHbP
ΣDEHP: MEHP + MEENHP + MEOHP + MECPP
ΣDEHTP: MECPTP + MEENHTP
Table 3

Percent change in infant NNS measures per IQR increase in maternal urinary phthalate or phthalate replacement metabolite concentrations across pregnancy, adjusted for urinary specific gravity, infant sex, and birthweight (n = 189).

| Metabolite | Frequency (Hz) | Amplitude (CmH₂O) | Bursts/Minute | Cycles/Minute | Cycles/Burst |
|------------|----------------|-------------------|---------------|---------------|--------------|
| MEHP       | −3.4 (−6.5, −0.2) * | 3.2 (−6.5, 12.9) | 0.1 (−10.1, 10.3) | −5.5 (−13.3, 2.2) | −4.7 (−21.1, 11.7) |
| MBzP       | −1.3 (−4.1, 1.6)  | −0.6 (−9.4, 8.2)  | 0.9 (−8.4, 10.1) | −3.7 (−10.7, 3.4) | −7.1 (−22.7, 7.7)  |
| MCPP       | −1.7 (−5, 1.7)    | 9.2 (−1, 19.5)    | 2.7 (−8.1, 13.6) | −2.6 (−10.9, 5.7) | −5.7 (−23.1, 11.8) |
| MCNP       | −3.5 (−6.2, −0.8) * | 8.9 (0.6, 17.3) * | −4.2 (−13, 4.7)  | −0.2 (−7, 6.6)  | 6.5 (−7.8, 20.7)  |
| MCOP       | −2 (−4.9, 0.9)    | 5.1 (−3.8, 14.1)  | −1.2 (−10.6, 8.3) | −0.6 (−7.8, 6.6) | 3.4 (−11.8, 18.5) |
| MONP       | 0.1 (−3.1, 3.3)   | 5.4 (−4.3, 15.1)  | −1.4 (−11.6, 8.8) | −2.3 (−10.1, 5.5) | 3.3 (−13.1, 19.7) |
| MEP        | 0.9 (−2, 3.8)     | 0.9 (−8, 9.8)     | 0.4 (−8.9, 9.8)  | 1.2 (−5.9, 8.4)  | 6.4 (−8.6, 21.4)  |
| ΣDBP       | −0.1 (−3.2, 3)    | −4.7 (−14.2, 4.8) | 1.7 (−8.3, 11.7) | −4.2 (−11.8, 3.4) | −7.2 (−23.2, 8.9) |
| ΣDiBP      | 0 (−2.7, 2.8)     | −2.4 (−10.8, 6.1) | 0.8 (−8.1, 9.7)  | −2.3 (−9.1, 4.5) | −4.4 (−18.7, 9.9) |
| ΣDEHP      | −2.3 (−5.3, 0.7)  | 7.7 (−1.5, 16.9)  | 3.2 (−6.5, 13)   | −4 (−11.5, 3.4)  | −8.1 (−23.8, 7.5) |
| ΣDEHTP     | 0 (−2.7, 2.7)     | 11.2 (2.9, 19.5) * | −1.4 (−10.2, 7.5) | 3.6 (−3.2, 10.3) | 11.7 (−2.4, 25.7) |

IQR: interquartile range increase; CI: confidence interval.
*p-value < 0.05.
ΣDBP: MBP + MHBP
ΣDiBP: MiBP + MHiBP
ΣDEHP: MEHP + MEHHP + MEOHP + MECPP
ΣDEHTP: MECPT + MEHNTP