Human Milk Cortisol Concentration Predicts Experimentally-Induced Infant Fear Reactivity: Moderation by Infant Sex

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Abbreviated title: Milk cortisol and infant fear reactivity

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Highlights:

- Human milk cortisol concentration and infant fear reactivity was studied.
- Milk cortisol positively predicted fear reactivity in a laboratory setting at 8 months in infant girls but not in boys.
- There was no association between milk cortisol and mother-reported infant fearfulness at 6 months.
- The results suggest that exposure to stress hormones via breast milk might be involved in girls' early emotional development.

List of figure legends:

Figure 1. The association between breast milk cortisol and infant fear reactivity in a laboratory setting (standardized values) in boys and girls separately using the mask type (the stimulus intensity) as a grouping variable

Abstract

Little consideration has been given to the possibility of human infant development being shaped via lactocrine programming, and by breast milk cortisol levels specifically. Despite animal models indicating that glucocorticoid (GC) exposure via lactation might modify brain development and behavior, only one study has reported that milk cortisol levels were positively associated with infant negative affectivity, especially fearfulness and sadness – early emerging risk factors for internalizing difficulties, such as anxiety. The aim of the current study was to investigate whether human milk cortisol is associated with mother-reported fearfulness and experimentally-induced infant fear reactivity. Mother-infant dyads (n = 65) enrolled in the FinnBrain Cohort Study participated. Breast milk samples were obtained 2.5 months postpartum, and milk cortisol concentrations were ascertained using validated luminescence immunoassay methodology. Infant fear reactivity was assessed using maternal reports 6 months postpartum and in a laboratory 8 months postpartum. There was a significant interaction between infant sex and milk cortisol such that higher milk cortisol was related to higher infant fear reactivity in a laboratory setting in girls (β = 0.36, p = 0.04) but not in boys (β = –0.15, p = 0.40). Milk cortisol was not associated with mother-reported infant fearfulness. Results suggest that higher human milk cortisol concentrations are associated with elevated experimentally-induced fear in infancy. Findings support lactocrine programming, and suggest that mothers may "communicate" vital information about stressful environments via cortisol contained in breast milk, shaping girls' early emotional reactivity.

Keywords: Cortisol, Human milk, Stress, Fear reactivity, Infancy

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Early life glucocorticoid (GC) exposure appears to affect childhood development (Sandman, Davis, Buss, & Glynn, 2012). GC programming refers to the role of GC exposure as a way of preparing offspring during the prenatal period for postnatal environmental demands, potentially placing them at greater susceptibility to later adverse health outcomes (Moisiadis & Matthews, 2014) but in some instances, also promoting resilience (Daskalakis, Bagot, Parker, Vinkers, & de Kloet, 2013). Recently, the possibility of such programming extending into the postnatal period via infant exposure to breast milk GCs has been introduced. Human breast milk represents one of the key early biological exposures for the developing child, including hormones like cortisol, oxytocin, leptin, epidermal growth factor (EGF), thyroid stimulating hormone (TSH), and prostaglandin. In light of the growing understanding of the nutritional role of human milk, the term lactocrine signaling/programming was established by Bartol et al. (2008). Lactocrine programming refers to the transmission of bioactive factors from mothers to offspring via breastfeeding. Currently, the mechanisms underlying lactocrine programming are not known in detail, but the findings that human milk contains biologically active factors that are absorbed by infant epithelial cells (Melnik et al., 2016) supports the notion that human milk may modify infant development and behavior (Donovan & Odle, 1994) and the view that milk GCs specifically may independently program behavior[1].

Along with research on early life programming more generally, there is growing interest in potential outcomes associated with lactocrine programming specifically (Hahn-Holbrook, Le, Chung, Davis, & Glynn, 2016). Early childhood negative emotional reactivity is one such plausible outcome because of its frequently reported associations with prenatal cortisol (Bergman, Glover, Sarkar, Abbott, & O'Connor, 2010; De Weerth, Van Hees, & Buitelaar, 2003), and later psychopathology, especially internalizing symptoms in childhood (De Pauw & Mervielde, 2010; Sayal, Heron, Maughan, Rowe, & Ramchandani, 2014). Moreover, fear, a specific aspect of negative emotional reactivity, is frequently emphasized, given its potential to predict later anxiety disorders (Baker,

[1] For additional information on milk cortisol, see Patacchioli et al. (1992) and van der Voorn et al. (2016).

Baibazarova, Ktistaki, Shelton, & van Goozen, 2012; Buss, 2011; Buss & McDoniel, 2016). To our knowledge, only one human study has reported a positive association between milk cortisol and aspects of infant negative emotional reactivity, especially fearfulness and sadness (Grey, Davis, Sandman, & Glynn, 2013). Though milk cortisol was not directly measured, another study showed a positive association between maternal cortisol and infant fearfulness only in breastfed, but not in formula-fed, infants (Glynn et al., 2007), suggesting that breastfeeding may be involved in postnatal programming of infant emotional reactivity. Likewise, links between milk cortisol and higher negative emotional reactivity in rhesus monkey offspring have been reported (Hinde, Skibiel, Foster, Rosso, & Sally, 2015).

From an evolutionary perspective, heightened emotional reactivity may provide survival advantages in stressful environments, increasing alertness and avoidance of threat, but on the other hand, predispose an individual to later psychopathology (Glover, 2011). However, even though early life stress (ELS) exposure is suggested to be a risk factor, it has also been considered as an accelerator of development (DiPietro, Novak, Costigan, Atella, & Reusing, 2006; Ellman et al., 2008). In line with this view, GCs are generally accepted as agents that aid the maturation of tissues during prenatal development (Moisiadis & Matthews, 2014; Seckl & Meaney, 2004). Although no human research exists on GC exposure affecting maturation, some studies report associations between postnatal GC exposure and enhanced learning in rodents (Casolini et al., 1997; Catalani et al., 2002). Moreover, the brain activation patterns and structural connectivity of children with severe ELS resemble those of adults (Gee et al., 2013), which is potentially indicative of more rapid neural development. Infant fear reactivity reportedly correlates with higher cognitive skills, also potentially reflecting earlier neural maturation (Graham et al., 2015). Thus, it can be hypothesized that early GC exposure has programming influences that might result in elevated risk, but also earlier development of certain, age-specific functions, such as fear to novel stimuli.

Beyond the potential main effects of programming, stress hormone effects may be dependent upon child sex (McEwen & Morrison, 2013; Sandman, Glynn, & Davis, 2013). For instance, in females, ELS exposure is linked with higher fearfulness, risk of anxiety and depression, or larger amygdala volumes, a possible mechanism underlying such heightened reactivity (Braithwaite et al.,

2017; Buss et al., 2012; Quarini et al., 2016). From an evolutionary perspective, it may be that females and males have biologically different strategies to adapt to environmental stresses, and females might be more susceptible to elevated emotional reactivity in the face of stress in comparison to males (Glover & Hill, 2012; Sandman et al., 2013). Providing support for these findings in the context of lactocrine programming, Grey et al. (2013) reported an association between milk cortisol and fearfulness/sadness only in girls. Similarly, sex-specific associations between milk cortisol and behavior in both rhesus macaques (Dettmer et al., 2017; Hinde et al., 2015; Sullivan, Hinde, Mendoza, & Capitanio, 2002) or postnatal GC exposure and behavior in rodents (Catalani et al., 2002) have been reported.

The aim of this study was to extend the current research on the effects of milk cortisol on infant fearfulness, a facet of negative emotional reactivity, by considering relations between milk cortisol and experimentally-induced infant fear reactivity in addition to mother-reported fearfulness. We expected that higher milk cortisol concentrations would predict both elevated fearfulness at 6 months and experimentally-induced infant fear reactivity at 8 months. In line with the results of a previous study on milk cortisol effects (Grey et al., 2013), we anticipated that the association between milk cortisol and fear reactivity would be stronger in girls.

Methods

Study Design and Participants

Sixty-five healthy Finnish breastfed infants and their mothers participated. Participants were a subsample of families participating the larger FinnBrain Cohort Study that took part in both breast milk sample collection 2.5 months postpartum and infant fear reactivity assessment at 6 and 8 months. Initial data included 76 families with both measures available and of these, 65 mother-infant pairs had complete data that were used in the final analyses. Mothers were recruited to the study during their first trimester ultrasound visit by a research nurse. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland and all the participants provided written informed consent for their participation. Infants of mothers taking medication that could affect GC levels during the postpartum period were excluded from the sample. The characteristics of mother-

infant dyads, and the associations between background factors and infant fear reactivity are presented in Table 1.

[Table 1]

[Table 2]

Infant Fear Reactivity

At 6.5 months, fearfulness was assessed using Infant Behavior Questionnaire-Revised Short Form (IBQ-R-SF; Putnam, Helbig, Gartstein, Rothbart, & Leerkes, 2014) that was completed by mothers at home. The IBQ-R-SF is a widely used and validated measure of infant temperament that has 91 items asking about infant behavior during the past one or two weeks. The measure comprises of 14 subscales that form three main dimensions. The subscale Fear, which loads onto the dimension of Negative Affectivity, consisting of 6 items (Cronbach's $\alpha = .86$), was used.

At 8 months, assessment of infant fear reactivity was obtained in the laboratory using the Masks episode from Laboratory Temperament Assessment Battery Prelocomotor (Lab-TAB; Goldsmith & Rothbart, 1999), a standardized battery for the laboratory-based assessment of infant temperament. Infants were exposed to four masks, ranging from lower to higher in fear-eliciting intensity, presented for 10 seconds each and always in the same order. Three indicators of fear, infant facial (0–3) and bodily fear (0–3) and fearful vocalizations (0–5) were coded in response to presentation of each mask. The composite of the indicators of fear reactivity showed high internal consistency ($\alpha = .88$) and inter-rater reliability (Mean Cohen's Kappa = .79, inter-rater $r = .95–.98$). The indicators of fear ($r = .63–.84$) and fear reactivity in each of the Masks ($r = .51–.85$) were highly correlated. Caregiver interference during observation was assessed (0 = severe, 1 = mild, 2 = no interference) to control for parent behavior effects on fear reactivity. See Table 2 for descriptive statistics of fear reactivity.

Milk Cortisol Concentration

Breast milk samples (one per mother) were collected in the presence of a study nurse during visits to the FinnBrain Research Center. To avoid error caused by the circadian variation in cortisol levels (van der Voorn et al., 2016), sample collection time was controlled for in analyses. Mothers were instructed to feed their infant from their right breast 1.5–2 hours prior to study visit, but breastfeeding from the left breast was allowed based on the infant needs during the same day. While

wearing latex gloves, mothers expressed 10 ml of front milk, into a sterile cup, from their right breast using manual expression (Pundir et al., 2017). Breast milk was immediately transferred into tubes, transported to the laboratory, and stored in a –70 °C freezer. Prior to processing, thawed milk was gently mixed for 1 minute. Cortisol was extracted using dichloromethane and analyzed by using validated luminescence immunoassay method (IBL International, product RE62111) (Grey et al. 2013). Assays were carried out at the Finnish Institute of Occupational Health.

Maternal Postnatal Psychological Distress

Within two to four weeks from the milk sample collection (3 months postpartum), maternal psychological distress was assessed using Edinburgh Postnatal Depression Scale (EPDS, Cox, Holden, & Sagovsky, 1987) and Symptom Checklist-90 anxiety subscale (SCL-90, Derogatis, Lipman, & Covi, 1973). As these two measures of psychological distress were highly correlated (r = .63, $p < .001$), a sum variable labeled "Postnatal psychological distress" was calculated and used in analyses as a continuous variable to control for the possible association between maternal symptoms and infant fear reactivity (see e.g. Grey et al., 2013).

Statistical Analyses

The distribution of milk cortisol concentrations was transformed using the natural logarithm transformation. First, zero-order associations between background factors, milk cortisol concentrations and mother-reported and experimentally-induced infant fear reactivity in the entire sample, and for females and males separately, were examined using Pearson correlation coefficients and partial correlations. Next, a linear mixed effect model was fit using R and nlme package (Pinheiro et al., 2016; R Core Team, 2016) to test whether milk cortisol or the interaction between milk cortisol and infant sex predicted infant fear reactivity after accounting for covariates. Mixed modeling was used to take into account the variability in responses to different masks. Consequently, mask type was used as a grouping variable with random effects for sex and the sex-milk cortisol interaction. In the final model, the effect of infant sex and milk cortisol, and the interaction between them, were analyzed after controlling for sample collection time, caregiver behavior during the observation and maternal psychological distress. Further, the significance of the interactions was examined with simple slope analyses.

Results

Zero-order associations are displayed in Table 3. When both sexes were included in the analyses, milk cortisol concentration was not significantly correlated with mother-reported fearfulness or experimentally-induced infant fear reactivity. However, when examined separately by infant sex, a positive correlation between milk cortisol and experimentally-induced fear reactivity was present for girls, but not for boys. After adjusting for sample collection time, the association between milk cortisol and fear reactivity became non-significant; however, this was mainly explained by a negative association between sample collection time and fear reactivity and not by an association between milk cortisol and sample collection time. Milk cortisol was not significantly correlated with maternal postnatal psychological distress, but caregiver interference was associated with higher fear reactivity to masks 3 and 4 ($F [2, 63] = 2.56–3.60$, $p = .03–.09$). Experimentally-induced and parent-reported fear reactivity were not related.

[Table 3]

In the regression analysis (see Table 4), after controlling for caregiver behavior and milk sample collection time, the main effect of infant sex and maternal psychological distress at 3 months, there was an interaction between infant sex and milk cortisol at 2.5 months in predicting experimentally-induced fear reactivity at 8 months. Milk cortisol positively predicted fear reactivity in girls (simple slope for overall fear reactivity of $\beta = 0.36$, $p = .04$) but not in boys ($\beta = –0.15$, $p = .40$) (Figure 1). There was no significant milk cortisol by infant sex interaction in predicting mother-reported fearfulness.

[Table 4]

[Figure 1]

Discussion

The aim of the present study was to investigate the associations between milk cortisol and mother-reported and experimentally-induced infant fear reactivity. In line with results reported by Grey et al. (2013), we found that after controlling for maternal postnatal psychological state, milk cortisol concentration was positively related to infant fear reactivity in a laboratory setting, but only for girls. However, in contrast to the earlier study, milk cortisol was not associated with mother-

reported fearfulness. To our knowledge, this study is the first to provide evidence for a link between human milk cortisol and experimentally-induced infant fear reactivity.

The results of the current study suggest that breast milk GCs may be linked to infant experimentally-induced emotional reactivity, and could be considered as an example of lactocrine programming. Lactocrine programming (Bartol et al., 2008) can be understood as an extension of prenatal programming, or a way of readjusting prenatal programming after birth to adapt infants to the current environmental setting. Importantly, it must be noted that there are several secondary routes to lactocrine GC programming. The effects of infant breastfeeding are shown to be two-fold, also affecting the hormonal profile of the lactating mother (Dawood, Khan-Dawood, Wahi, & Fuchs, 1981) and milk cortisol has been positively associated with maternal hostility (Hart et al., 2004), which in turn may affect caregiving behavior. As we could not control for environmental factors influencing infant fearfulness, such as the quality of mother-infant interaction, future studies should address these questions. Moreover, even though earlier studies suggest that postnatal GCs independently affect offspring development and behavior (Catalani et al., 2000), it cannot be inferred from our findings that fear differences were entirely due to postnatal influences and not to prenatal cortisol. To investigate the interactions between prenatal and postnatal GC programming, future studies could include both pre- and postnatal cortisol measurements.

Several previous studies suggest that the effects of early life programming may be sex-specific (Braithwaite et al., 2017; Hahn-Holbrook et al., 2016; Sandman et al., 2013). The evolutionary explanation of ELS programming and sex differences suggests that females would adapt to stress exposure with heightened reactivity to threat, whereas for males, stress exposure might result in gross developmental problems at the cost of viability, or possible "benefits" of more confidence and aggression. Our finding along with the previous findings in humans (Grey et al., 2013) and in animals (Catalani et al., 2002; Dettmer et al., 2017; Hinde et al., 2015), suggest, at least in part, the presence of a pattern of sex differences similar to prenatal programming studies. That is, both prenatal and milk GC exposure might be related to females' heightened fear reactivity. However, there is evidence that infant sex also modifies milk composition. For instance, infant sex and its interaction with maternal socioeconomic status reportedly affect milk energy density in macaque and human

mothers (Fujita et al., 2012; Hinde, 2009; Powe, Knott, & Conklin-Brittain, 2010). In turn, the energy density of milk seems to modify offspring behavior (Hinde & Capitanio, 2010), indicating that interactions between milk composition and sex should be among the foci of future research.

The main outcome of this study was infant fear reactivity, which starts to develop in late infancy (from 6 to 12 months) (Bridgett, Burt, Edwards, & Deater-Deckard, 2015). High fear reactivity, and especially dysregulated fear is reportedly one of the risk factors for later anxiety disorders (Buss, 2011; Gartstein et al., 2010). On the other hand, as fear reactivity to novel stimuli is a normative developmental trait emerging in late infancy, it might not be persistent but rather reflect a transient period in some infants. Fear is also suggested to be a marker of early maturation, or advanced cognitive development, in infancy (Graham et al., 2015). Correspondingly, it is known that GCs play a role in the maturation of fetal nervous system (Seckl & Meaney, 2004). Given that the female brain develops more rapidly (Giedd et al., 2014), and that the window of sensitivity might be at younger age for females (Hinde et al., 2015), it is possible that the association between milk cortisol and fear reactivity might be present in girls earlier than in boys. However, as there is no previous research on the potential of milk GCs to accelerate emergence of fear, this possibility should be tested in future studies.

The present study has several strengths, including the measurement of fear reactivity in two different contexts, and the use of standardized, well-recognized measures of biological and psychological stress. Fear reactivity was measured at 6 and 8 months when fear is considered to emerge as a more observable trait (Gartstein et al., 2010). However, in contrast to the study by Grey et al. (2013), we did not find any correlation between milk cortisol and mother-reported infant fearfulness. In Grey et al. (2013), mother-reported fearfulness was assessed simultaneously with milk cortisol at 3 months, and the long version of the IBQ-R was used to measure fearfulness, possibly capturing a larger variety of maternal observed fearful behaviors. Thus, the differences in measurement and assessment timing might explain the inconsistency of the results between the current study and findings reported by Grey et al. In addition, maternal reports and laboratory assessment of fearfulness might at least partially reflect different aspects of fear. Specifically, the laboratory assessment that was employed only assesses infant fear in reaction to novelty, whereas the

IBQ-R assesses fearfulness in both novel and non-novel contexts. Thus, multiple measurements and more frequent follow-ups of both milk cortisol and fearfulness may have provided more insights to the inconsistency between this and the prior research, as well as whether elevated fearfulness is indicative of later risk or earlier maturation. Finally, as earlier studies suggest associations with outcomes other than fear reactivity (Grey et al., 2013; Sullivan et al., 2002), future research should assess milk cortisol effects on multiple facets of emotional reactivity.

Conclusion

The present study suggests that breast milk cortisol concentration is associated with elevated infant fear reactivity in a laboratory setting, and is the first to report such an association in humans. This association was only found for girls, suggesting that girls may be more susceptible to GC programming influences, especially with regard to their reactivity to fear-eliciting stimuli in a novel environment. However, more research, building on the current study by addressing its limitations, is needed to consider the potential of milk GCs to affect infant emotional and behavioral development.

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Table 1. The sample characteristics of the mother-infant dyads (N = 65)

	Mother-infant dyad
Maternal age	31.3 (23-45)
Education	
High school or vocational	12%
University degree	88%
Monthly income	
< 1500 euros	42%
> 1500 euros	58%
Parity (primiparous)	55%
Infant sex (boy)	57%
Gestational age at birth (range)	40.0 (36–42)
Birth weight (range)	3600 (2500–4900)
Infant age at IBQ-R return (SD) [a]	6.5 (0.52)
Infant age at observation, months (SD)[a]	8.1 (0.22)
Breast milk cortisol, nmol/l, mean (SD)	4.5 (2.86)
Postnatal psychological distress (SD)	3.1 (3.19)
Maternal depressive symptoms, mean (range)[b]	4.0 (3.65)
Maternal anxiety symptoms, mean (range)[c]	2.4 (3.42)

Note: There were no significant differences between girls and boys in given background variables.
[a]From expected due date
[b]Measured with EPDS
[c]Measured with SCL-90

Table 2. The descriptive statistics of experimentally-induced and parent-reported fear reactivity

Infant fear reactivity (theoretical range)	Overall, mean (range)	Means by stimuli			
		Mask 1	Mask 2	Mask 3	Mask 4
Fear composite	3.11 (0-7.00)[a]	1.53	2.01	3.12	4.73
Bodily (0-3)	1.19 (0-2.75)	0.71	0.89	1.55	1.63
Facial (0-3)	1.10 (0-2.25)	0.57	0.72	1.45	1.66
Vocal (0-5)	0.82 (0-2.50)	0.25	0.40	1.12	1.44
Parent-reported (1-7)	2.42 (1.00-5.17)				

[a]A non-standardized composite score of fear indicators

Table 3. The zero-order associations between milk cortisol concentration and fear reactivity and the confounding factors in the whole sample

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Fear reactivity (IBQ)															
2. Fear reactivity (lab)	.07														
3. Mask 1	.07	.70**													
4. Mask 2	.16	.80**	.62**												
5. Mask 3	–.03	.91**	.51**	.59**											
6. Mask 4	.07	.91**	.48**	.60**	.82**										
7. Milk cortisol[a]	.08	.15	.18	.15	.08	.09									
In girls	–.05	.44*	.32	.43*	.20	.39*									
In boys	.19	–.13	.02	–.19	–.04	–.18									
8. Sample coll. time	–.18	–.32*	–.23†	–.35**	–.25*	–.26*	–.22†								
9. Postnatal psych.	.25†	–.12	–.08	–.09	–.10	–.12	–.06	–.00							
10. Depressive	.27*	–.13	.00	–.06	–.14	–.14	–.13	.05	.91**						
11. Anxiety	.19	–.10	–.14	–.10	–.04	–.08	.02	–.05	.90**	.63*					
12. Maternal age	–.06	–.08	–.14	.07	–.00	–.10	–.11	.16	–.12	–.02	–.20				
13. Gestational weeks	.14	–.00	.05	.10	–.03	–.01	–.10	.05	.05	.08	.01	–.10			
14. Birth weight	.12	.13	.14	.13	.12	.09	–.15	.09	.07	.05	.08	–.01	.45**		
15. Infant age (IBQ)	.15	–.20	–.10	–.03	–.20	–.23†	–.06	.06	.02	.14	–.12	.13	.59**	.11	
16. Infant age (lab)	.01	.10	.15	.13	.03	.03	.11	.05	.10	.17	–.00	–.02	.08	.19	.18
The partial correlations between milk cortisol and fear after adjusting for sample collection time															
Milk cortisol	.06	.08	.09	.08	.13	.09									
In girls	–.07	.29	.16	.32	.01	.28									
In boys	.17	–.15	.02	–.23	–.05	–.20									

** *p* < .01, * < .05, † *p*<.10

Table 4. The linear mixed effect model for milk cortisol and standardized infant fear reactivity in a laboratory setting: moderation by infant sex (N = 65)

		Effect sizes (β) for the overall and stimulus-related standardized infant fear reactivity[a]				
	T value[b]	Overall	Mask 1	Mask 2	Mask 3	Mask 4
Intercept	–3.85***					
Caregiver behavior (in contrast to: severe interference)						
Mild interference	–1.43	–0.15				
No interference	–2.17*	–0.18				
Sample collection time	–4.08***	–0.02				
Maternal psychological distress	–1.54	–0.00				
Infant sex (girl)	–3.34***	–0.20				
Milk cortisol	–1.77†	–0.05	–0.03	–0.04	–0.06	–0.08
Milk cortisol by sex (girl)	3.83***	0.16	0.12	0.15	0.18	0.20

*** $p < 0.001$, *< 0.05, † $p < .10$

[a]Effect sizes are shown for the standardized overall and stimulus-related infant fear reactivity and log-transformed milk cortisol along with the parameter estimates

[b]T value is shown for the overall effect across the stimuli