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Changes in maternal fecal corticosterone metabolites across lactation and in response to chronic stress

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ABSTRACT

Maternal exposure to stressors during lactation has previously been demonstrated to impact various aspects of milk synthesis and to have long-term physiological effects on offspring. Much of the current literature investigating the effects of stress during lactation has used acute stressors, and the studies investigating the effects of chronic stressors largely focus on neurological changes. Further, temporal variation in glucocorticoids across lactation in response to stressors has rarely been assessed. The present work uses a novel male intruder paradigm to model the effects of chronic stress on maternal fecal corticosterone metabolites (FCMs) in Sprague-Dawley rats across lactation. FCM levels were elevated in chronically-stressed mothers relative to the control group. Further, FCMs in the stress group were time-dependent either due to repeated exposure to the stressor or lactation stage. Together, this work demonstrates the efficacy of this established paradigm in increasing circulating glucocorticoids in lactating rats. These results highlight the need for repeated temporal sampling, as glucocorticoid levels in response to a chronic stressor may change across lactation.

1. Introduction

Milk synthesis is an adaptive feature of mammals and is accompanied by extensive changes to maternal physiology and morphology (Collier et al., 1984; Power and Schulkin, 2016). Of particular importance during lactation is the hypothalamic-pituitaryadrenal (HPA) axis, which controls circulating glucocorticoids that play a key regulatory role in metabolic homeostasis and the coordination of the vertebrate stress response (Sapolsky et al., 2000). During lactation, the HPA axis undergoes several changes to meet the increased metabolic demands placed on the mother. For example, basal HPA axis activity is increased due to suckling (Brunton et al., 2008) and the diurnal rhythm of glucocorticoid secretion is flattened such that there is a rise in the troughs accompanied by decreased peaks (Windle et al., 2013). Additionally, lactation has long been established as a hyporesponsive period marked by the attenuation of the HPA response to stressors (Brunton et al., 2008; Stern et al., 1973; Windle et al., 2013). Such changes may be adaptive and function to provide a more consistent level of glucocorticoids, thereby limiting downstream catabolic effects that accompany surges and allowing mothers to produce a more steady energy supply for their offspring (Windle et al., 2013). Further, as glucocorticoids have been known to freely enter milk, dampening these surges may serve to limit glucocorticoid exposure to neonates, which has been shown to have long-term effects on offspring physiology and behavior (Carini and Nephew, 2013; Casolini et al., 1997; Champagne and Meaney, 2006; Cottrell and Seckl, 2009; Hinde et al., 2015; Meaney, 2001; Murgatroyd and Nephew, 2013; Seckl and Meaney, 1993).

Given the importance of glucocorticoids during lactation, and the potential sequelae for offspring, numerous studies have focused on the impacts of stress during lactation on various aspects of physiology. It is well-established that stress suppresses lactation, though many of the studies using animal models have primarily used acute stressors to investigate this relationship (Lau and Simpson, 2004). More recently, emphasis has been placed on the development of ethologically-relevant stressors in rodent models so that the effects of chronic stress can be investigated (Carini et al., 2013; Carini and Nephew, 2013; Lau and Simpson, 2004). Such studies have found that chronic social stress decreases milk yield, impairs both dam and offspring growth, alters maternal behavior by attenuating maternal care, has transgenerational effects on maternal behavior of female offspring, and alters maternal neuroendocrinology (Carini and Nephew, 2013; Lau and Simpson, 2004; Murgatroyd and Nephew, 2013; Nephew and Bridges, 2011). This body of work, however, has not addressed the peripheral changes in levels of corticosterone, the primary glucocorticoid found in rodents (Piazza

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et al., 1993).

The analysis of fecal corticosterone metabolites (FCMs) offers a non-invasive technique to assess the physiological state of the individual while limiting the methodological concerns present when taking plasma corticosterone measurements (Touma et al., 2004). The reproductive status of a female significantly influences concentrations of circulating glucocorticoids, and in turn, concentrations of fecal glucocorticoid metabolites (Touma and Palme, 2005). Changes in fecal glucocorticoid metabolites during phases of female reproduction have been documented in many species including North American red squirrels (Dantzer et al., 2010), giant anteaters (Knott et al., 2013), snow leopards (Kinoshita et al., 2011), deer mice (Veitch et al., 2021), and laboratory rats (Cavigelli et al., 2005). However, much of the current literature comprises studies assessing changes in glucocorticoids during the estrous cycle or pregnancy with relatively little attention on lactation. Published studies where fecal glucocorticoid metabolites were measured during lactation (Gale et al., 2018), while informative, have not assessed changes across lactation stages (but see Dantzer et al., 2010), despite the fluctuating hormonal milieu during lactation (Canul-Medina and Fernandez-Mejia, 2019; Forsyth, 1983).

Here, we aimed to characterize FCMs in chronically-stressed and control rats during different stages of lactation (i.e., early, mid, and late lactation). We predicted that chronic stress would elevate FCMs and that this impact would change over the course of lactation. Given that the metabolic demands of milk synthesis increase towards peak lactation (Gittleman and Thompson, 1988), approximately 15 days after parturition in laboratory rats (Barber et al., 1997), we predicted that FCMs between chronically-stressed and control rats would be particularly divergent at this time.

2. Materials and methods

2.1. Animals and experimental design

Fifty-six nulliparous adult female Sprague-Dawley rats (Envigo, Indianapolis, IN) aged 12–17 weeks on arrival were housed from July to September 2020 at the University of Idaho's Lab Animal Research Facility under the approval of the University of Idaho's IACUC (PRN #2020-39). Sprague-Dawley rats are an excellent strain of *Rattus norvegicus* to study mechanisms underlying the impact of stress during lactation because they are a widely used research model and have previously been used in protocols to model chronic stress during this period. Females were time-mated, arrived between days 14–16 of gestation, and were acclimated approximately 7 days prior to the start of the experiment. All females and their offspring were housed individually in standard polypropylene rodent boxes (~29.2×19.0×12.7 cm^3) and given *ad libitum* access to water and standard rodent chow (Teklad Global Rodent Diet, Envigo, Indianapolis, IN). The rats were maintained on a 12:12 light:dark cycle beginning at 0600 at approximately 24 °C. Cages were regularly monitored for the presence of new pups around the expected parturition date. Date of parturition was designated as post-natal day (PND) 1. Litters were culled to 8 pups on PND2; in 7 cases, mothers cannibalized their offspring before the start of the experiment, resulting in litters of 6 or 7 pups (control = 4, stress = 3 cases of reduced litter sizes).

Female rats were randomly assigned to either the chronic stress (n = 28) or control (n = 28) treatment group. Stress was induced using a novel male intruder paradigm following methods detailed in Carini and Nephew, 2013, Lau and Simpson, 2004, Murgatroyd and Nephew, 2013, and Nephew and Bridges, 2011. Every day, on PND 4 through PND14, females and their offspring were placed in a clean cage and were allowed to acclimate for 15 min before a novel male rat intruder was placed in the cage for one hour. Females and their offspring were separated from the males using a mesh barrier in order to prevent infanticide and injuries. Control females were similarly placed with their offspring in a clean cage with a clean mesh barrier for the same amount of time. Fourteen adult male rats were used in this experiment and were pair-housed in a separate room from the females. Females did not interact with the same intruder more than one time during the study, though the same males were used for multiple females. After the intruder challenge, females and their offspring remained in the cage for 15 min before they were placed in their home cages. Interactions occurred at random times during the light cycle in order for the stressor to be unpredictable.

2.2. Fecal steroid extraction and analysis

Feces from the mothers were collected into clean tubes while the mother was weighed on PNDs 4, 9, and 14 (representing early, mid, and late lactation, respectively). Feces visibly contaminated with urine or bedding were not collected. These data were collected from 56 individuals (control = 28, stress = 28). Most females were sampled only at one time point (control = 15, stress = 17), though some females were sampled at two (control = 11, stress = 8) or all three (control = 2, stress = 3) time points. All samples were collected between 11:00–16:00. Samples were initially stored at room temperature for up to one month before they were refrigerated at 4 °C. To measure the concentration of fecal corticosterone metabolites (FCMs), we used the DetectX ELISA kit from Arbor Assays (#K014, Ann Arbor, Michigan, USA) following the manufacturer's instructions.

Steroids were first extracted following the protocol for solid steroid extraction provided by the kit manufacturer. Briefly, pellets were dried at 37 °C before being homogenized. The dried, homogenized samples were resuspended in ethanol at a final concentration of 0.1 g feces/1mL ethanol and the mixture was shaken at room temperature for 1 h. The mixture was then centrifuged at 5000 rpm for 15 min at 4 °C before the supernatant was extracted. The ethanol was then evaporated and the remaining sample was reconstituted with assay buffer and briefly stored at −20 °C for later analysis. All samples were run in duplicate, and intra-plate coefficient of variation was <15% for all samples. Extraction efficiency was 93.2%. Inter-plate reliability was 91.3%. Concentrations of FCMs are given relative to grams of dried feces used in the analyses (ng of FCM/g dried feces).

2.3. Statistical analysis

Data were analyzed using R Studio version 1.2 (Team R.C., 2013). FCM levels were first analyzed using a linear mixed effects model with treatment, post-natal day, and their interaction as fixed effects and maternal ID as a random effect. Maternal ID accounted for a small proportion (0.5%) of the variance, as only 5 individuals were sampled on all three occasions. Thus, a two-way ANOVA was used to model the data instead. Differences between groups were determined using Tukey's HSD. Unless otherwise noted, data are presented as mean ± standard error.

3. Results

Maternal body mass did not differ between groups at any of the time points measured (Supplemental Table 1). FCM levels were significantly impacted by treatment ($F_{1,82} = 33.41$, $p < 0.0001$), post-natal day ($F_{2,82} = 9.71$, $p = 0.0002$), and their interaction ($F_{2,82} = 13.07$, $p < 0.0001$) (Fig. 1, Table 1). Within the control group, FCM concentration was similar across lactation. However, within the stress group, FCM levels were increased at the late lactation time point compared to the two earlier time points. There were no significant differences in FCM levels between the two treatment groups during early lactation, though chronically-stressed mothers had higher FCM levels compared to the control group during mid and late lactation.

4. Discussion and conclusion

Our primary goal was to characterize FCMs in chronically-stressed

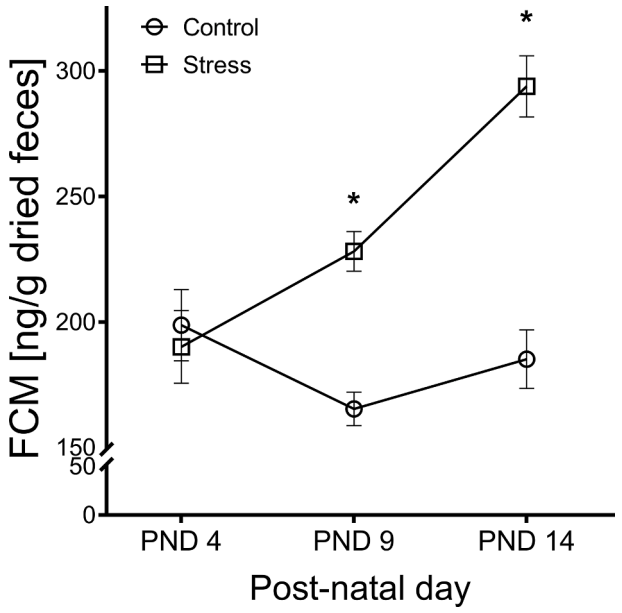

Fig. 1. Fecal corticosterone metabolites (FCMs) across lactation in feces of control and chronically-stressed rats. FCM levels were measured at three time points, post-natal day (PND) 4, 9, and 14, representing early, mid, and late lactation, respectively. The chronically-stressed group was subject to daily introduction of a novel male intruder between PND 4–14. Data were analyzed by ANOVA. FCM concentration varied by treatment ($F_{1,82} = 33.41$, $P < 0.0001$), post-natal day ($F_{2,82} = 9.71$, $P = 0.0002$), and a treatment × day interaction ($F_{2,82} = 13.07$, $P < 0.0001$). Asterisks indicate significant differences between groups (PND 9, $P = 0.0026$; PND 14, $P < 0.0001$). Within the chronically-stressed group, FCM was significantly different between PND 4 and 14 ($P < 0.0001$) and PND 9 and 14 ($P = 0.0014$), indicating an effect of time.

Table 1
Fecal corticosterone metabolites (FCMs) across lactation in control and chronically-stressed rats. Results from post-hoc analyses (Tukey's HSD). FCMs were extracted and analyzed using feces from control and chronically-stressed females during lactation. The chronically-stressed group was subject to daily introduction to a novel male intruder between PND 4–14. Feces were collected from individuals in both groups on PND 4, 9, and 14. Sample sizes for each time point are given in parentheses. Significance ($P \leq 0.05$) is indicated in boldface.

	Mean FCM [ng/g dried feces] ± SE		Comparisons
	Control	Stress	Control v. Stress
PND 4	198.68 ± 14.19 (15)	190.04 ± 14.48 (14)	$P = 0.9949$
PND 9	165.34 ± 6.65 (15)	228. 07 ± 7.90 (15)	$\mathbf{P = 0.0026}$
PND 14	185.19 ± 11.65 (14)	293.80 ± 12.17 (15)	$\mathbf{P < 0.0001}$
Within-group comparisons			
PND 4 v. 9	$P = 0.31$	$P = 0.20$	
PND 4 v. 14	$P = 0.96$	$\mathbf{P < 0.0001}$	
PND 9 v. 14	$P = 0.83$	$\mathbf{P = 0.0014}$	

and control rats across lactation (i.e., early, mid, and late lactation). Consistent with our hypothesis, our results demonstrate that chronic stress during lactation impacts FCM levels in a time and treatment-dependent manner, potentially due to physiological changes across lactation and/or repeated exposure to the same stressor. Specifically, FCM levels increased towards late lactation in chronically-stressed mothers and were higher in stressed mothers relative to those in the control group.

We chose the novel male intruder paradigm to model chronic social stress, as this stimulus represents an ethologically-relevant stressor for the mother and because a considerable body of literature indicates this stimulus is a potent stressor in rats (Carini and Nephew, 2013; Lau and Simpson, 2004; Murgatroyd and Nephew, 2013; Nephew and Bridges, 2011). This past work has revealed neuroendocrinological and behavioral differences between control and stressed mothers. Specifically, exposure to chronic stress has been found to decrease both maternal and offspring growth, increase maternal self-grooming and aggressive behaviors, and decrease maternal milk release (Carini et al., 2013; Lau and Simpson, 2004; Nephew and Bridges, 2011). Further, chronic stress has long-term impacts on maternal behavior and neuroendocrinology of maternal care (i.e., prolactin and oxytocin release during lactation) in female offspring of chronically-stressed mothers (Carini and Nephew, 2013; Murgatroyd and Nephew, 2013; Nephew and Bridges, 2011). For offspring, stress encountered during critical windows of development during the early life period has the potential to program HPA activity during adulthood (Maniam et al., 2014; Meaney et al., 1996; Murgatroyd and Spengler, 2011; Schmidt et al., 2014; Zimmer and Spencer, 2014).

Lactation is a period of substantial metabolic demand that requires tissue remodeling and numerous physiological adaptations coordinated across tissues (Canul-Medina and Fernandez-Mejia, 2019; Forsyth, 1983; Hammond and Diamond, 1997), many of which have an endocrinological basis (Collier et al., 1984). The HPA axis plays a role in determining both basal levels of glucocorticoids that are associated with metabolic regulation (i.e., energy acquisition, deposition, and mobilization) as well as the vertebrate stress response that results in acute surges of glucocorticoids that ultimately lead to the termination of the stress response (Sapolsky et al., 2000). Stress responses in lactating mammals are attenuated, which is thought to serve an adaptive role as chronic exposure to high levels of glucocorticoids is damaging, particularly for developing offspring (Brunton et al., 2008). Limiting fluctuations in these hormones may contribute to the conservation of maternal energy stores (Douglas, 2005). For these reasons, basal levels of glucocorticoids were not expected to change over the course of lactation in absence of a stressor, which is consistent with our finding that FCMs in our control group did not change over time. These results contrast those found by Dantzer and colleagues (2010), who reported a decrease in FCMs across lactation in free-ranging red squirrels. The difference in results may be due to a number of factors, such as species differences, artificial selection, or captivity.

To our knowledge, the peripheral glucocorticoid response in mothers exposed to chronic stressors during lactation has not been measured outside of the present work. Further, much of the work assessing physiological changes associated with stress during lactation relies on samples collected at one time point (but see Dantzer et al., 2010), thereby making the implicit assumption that lactation is a monolithic state. On the contrary, lactation is a dynamic process associated with physiological and behavioral changes across lactation (Canul-Medina and Fernandez-Mejia, 2019; Fleming and Rosenblatt, 1974; Forsyth, 1983); as such, temporal sampling is needed and may yield different results than cross-sectional sampling (Josefson et al., 2020).

In our study, FCMs were elevated in stressed individuals during mid and late lactation, in contrast with previous work in rats that reported greater adrenocorticotropic hormone (ACTH) levels in response to an acute stressor during early lactation compared to late lactation (Deschamps et al., 2003; Walker et al., 1995). Despite these differences in ACTH responses, no difference in basal nor stress-induced plasma corticosterone was reported between the control and stress treatment groups during either early or late lactation (Deschamps et al., 2003). It is possible that the discrepancy between the findings from this work and ours is due to our use of a chronic stressor. The differential effects in response to acute and chronic stressors have long been noted in the context of many aspects of physiology, though the exact mechanisms and scope of the response are not yet known (Wada, 2019), especially during lactation. Stress responsiveness during lactation is also dependent on the specific type of stressor to which lactating mothers are exposed (Maestripieri et al., 2008; Ralph and Tilbrook, 2016). For example, the presence of pups during exposure to a stressor has been found to mediate the maternal stress response (Deschamps et al., 2003).

Our finding that FCMs increased across lactation in chronically-stressed mothers may be due to several non-mutually exclusive biological mechanisms. Lactation is a hyporesponsive period during which maternal responses to stressors are attenuated (Brunton et al., 2008; Stern et al., 1973; Windle et al., 2013). As mothers approach weaning, the hyporesponsive effects are dampened, and mothers may be more able to respond to stressors. Additionally, this increase in FCMs may be due to hormonal shifts associated with mammary involution towards the end of lactation. The increase in FCMs over the course of lactation may also be due to a greater number of exposures to the chronic stressor challenge, as it is not known whether exposures to a novel intruder is cumulative. The cumulative effects of stress are not fully understood and are often investigated within the context of exposure to multiple, concurrent stressors or of repeated acute surges of glucocorticoids (Barnum et al., 2007; Busch et al., 2008; McCormick et al., 2015; Ottenweller et al., 1992).

Stressed and control groups differed in FCM levels at mid and late lactation, but not early lactation (PND 4), indicating that both our control and stress groups had similar FCM levels initially. No effects of treatment were expected at this timepoint, as our chronic stress paradigm began just a few hours before feces were collected from both groups on PND 4. Previous work testing the gut passage time in laboratory rats after individuals were given intravenous radiolabeled corticosterone reports that for females, radiolabeled FCMs began to appear at about 6 h and peaked at about 15 h after administration (Lepschy et al., 2007). Similarly, when these rats were given an ACTH challenge, FCM levels rose 4–12 h later and remained elevated for 4–10 h (Lepschy et al., 2007).

Traditionally, glucocorticoid concentration is determined from blood samples, which is likely to be artificially high as blood sample collection is itself an acute stressor. Measuring FCMs is becoming an increasingly popular non-invasive alternative to blood samples because this technique is more integrated and less prone to acute changes in response to stressors (Palme, 2019; Touma and Palme, 2005). As with any technique, pitfalls of using FCMs (e.g., the influence of diet and other environmental effects) have been pointed out in several reviews (Goymann, 2012; Palme, 2019), though they are expected to be minimized when the experiment is conducted in a controlled laboratory setting as in our study. It is possible that the differences in FCMs that we observed may be due to individual differences in metabolism and/or stress reactivity among females that impact the excretion of FCMs and/or defecation frequency. However, we do not expect this to be the case due to similar litter sizes, parity, and body mass between treatment groups.

This study demonstrates that chronic social stress during lactation impacts FCM concentrations in a time and treatment-dependent manner such that FCM concentrations were highest in late lactation compared to earlier time points. These changes were not seen in the control group who were not subjected to a stressor. This change in FCM concentrations may be due to the stage of lactation that the chronically-stressed mothers are in or may be due to cumulative effects of repeated exposure to the same stressor. More research is required in order to parse out the nature of these time-dependent changes in FCMs. These results highlight the need for repeated sampling within the same reproductive period (i.e., lactation) rather than sampling at one point in order to gain a more holistic perspective on the impacts of stress.

CRediT authorship contribution statement

Chloe C. Josefson: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Amy L. Skibiel:** Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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