Published in final edited form as:

Ethology. 2017 ; 123(6-7): 442–452. doi:10.1111/eth.12614.

Low temperatures during ontogeny increase fluctuating asymmetry and reduce maternal aggression in the house mouse, *Mus musculus*

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Abstract

Maternal aggression is behavior displayed by post-partum lactating female mice toward unfamiliar conspecifics, presumably as a defense against infanticide. A variety of perinatal stressors can impair maternal care in adulthood. Previous studies on associations between developmental perturbations and maternal aggression have produced mixed results. To address this issue, we employed a proxy for developmental instability, fluctuating asymmetry (FA) to further elucidate the relationship between low temperature stress and maternal aggression. FA, small, random deviations from perfect symmetry in bilateral characters is used as a quantitative measure of stress during ontogeny. Dams were either maintained in standard laboratory temperatures ($21 \pm 2^\circ\text{C}$), or cold temperatures ($8 \pm 2^\circ\text{C}$) during gestation. During lactation, their progeny either remained in the temperature condition in which they were gestated or were transferred to the other temperature condition. Four individual measures of FA, a composite of these measures, and three measures of maternal aggression were assessed in the female progeny in adulthood. Exposure to low temperatures during both pre- and early post-natal development increased composite FA and reduced all three measures of maternal aggression compared to controls. Exposure to low temperatures during the pre- or postnatal period alone did not induce either high FA or altered maternal aggression. Certain measures of FA and nest defense were negatively correlated. Our results suggest that low temperatures experienced during gestation and lactation may have important fitness costs. Low maternal aggression towards infanticidal conspecifics is likely to limit the number of offspring surviving into adulthood. Overall, FA appears to be a reliable indicator of chronic developmental stress with implications for fitness.

Keywords

Developmental instability; maternal behavior; glucocorticoids; cortisol; corticosterone; stress

Introduction

Maternal care in mammals comprises behaviors that contribute to the survival of offspring. These behaviors include, but are not limited to, provision of essential nutrients and shelter, retrieval of the young to the nest, and protection of the offspring from infanticide and predation. Infanticide and predation by conspecifics is exhibited in a variety of mammalian species ranging from primates to rodents under both laboratory and natural conditions (reviewed in Ebensperger 1998a). Maternal aggression is one strategy to defend the young against such threats (Wolff 1993; Ebensperger 1998a; Ebensperger 1998b), and is, presumably, an evolutionarily conserved trait.

Indeed, mammalian human and nonhuman mothers display increased aggressive behavior during the post-partum period. Women who exclusively breastfeed their infants are almost twice as aggressive as formula-feeding mothers and nulliparous adult females (Hahn-Holbrook 2011). Similarly, self-reported hostility increases among women after parturition (Mastrogriacomo et al. 1983; Ledesma de Luis et al. 1988). In nonhuman mammals, lactating rhesus macaques are more aggressive than non-lactating females (Maestripieri 1994). A similar increase in female aggressive behavior during lactation has been reported in lions (Grinnel & McComb 1996), domestic cats (Schneirla et al. 1963), deer (Smith 1987), sheep (Herscher et al. 1963), squirrels (Taylor 1966), rabbits (Ross et al. 1963), prairie voles (Yloen & Home 2002), hamsters (Giardono et al. 1984), rats, and mice (Lonstein & Gammie, 2002).

As in other mammals, aggressive and defensive behaviors towards intruders by the dam have important fitness consequences in mice (reviewed in Lonstein & Gammie 2002; Weber & Olson 2008). Nulliparous female mice do not generally engage in aggression (Masterpieiri et al. 1991; Lonstein & Gammie 2002; Martin-Sanchez et al. 2015a, 2015b). In contrast, most lactating dams show aggressive and defensive behaviors towards unfamiliar conspecifics in the nest (Gandelman 1972; Masterpieiri et al. 1991; Lonstein & Gammie 2002; Weber & Olson 2008; Heiming et al., 2013). A deficiency in the exhibition of maternal aggression by lactating rodents may indicate an underlying developmental condition that may adversely affect subsequent maternal care. Accordingly, the present study aimed to employ a proxy to quantify developmental stress, namely fluctuating asymmetry (FA), and investigated how FA may be associated with nest defense.

FA is defined as small, random deviations from perfect symmetry in paired traits, such as hand width and ankle circumference. The term “fluctuating” refers to non-directional deviations, because the size difference could favor either the left or right side of the bilateral character. The development of each side of a paired trait is presumably determined by a common set of genetic instructions. Deviations from perfect symmetry may thus reflect how individual organisms have coped with developmental stress. (Clarke & McKenzie 1992; Canovas et al. 2015). For example, perinatal oxidative, audiogenic, and cold stress (Siegel & Doyle 1975; Siegel et al. 1977; Sciulli et al. 1979; Mooney et al. 1985; Gest et al. 1986; Lavin et al., 2015; Breno et al., 2013), inbreeding, (Mather 1953; Thoday 1953), or harmful mutations (McKenzie & Clarke 1988) elevate FA in the offspring. Also, exposure to chemical pollution (Valentine et al. 1973; Ames et al. 1979; Hoffmann and Parsons 1989),

temperature extremes (Siegel & Doyle 1975; Mooney et al. 1985; Gest et al. 1986), and high population density (Zakharov et al. 1991) yield high FA. In addition, FA is positively associated with mortality (Cote & Festa-Bianchet 2001), morbidity (Waynforth 1998), and several neurological disorders (Naugler & Ludman 1996; Reilly et al. 2001; Burton et al. 2002). Accordingly, FA is often used as a proxy to quantify developmental stress and its effects on individuals' health, fitness, and behavior (De Coster et al. 2013; Beasley et al. 2013).

Similarly, unfavorable conditions during ontogeny, such as exposure to gestational stress can have adverse consequences for maternal care and nest defense. For example, some reports indicate that maternal aggression against intruders decreases under these circumstances in mice (Politch and Herrenkohl 1979; Maestripieri et al. 1991, but see Kinsley & Svare 1988; Meek et al. 2001). Increased maternal aggression is in turn attributed to decreased fear and anxiety in lactating rodents (Maestripieri et al. 1991; Parmigiani et al. 1999; Lonstein & Gammie 2002). Lactating females are usually less fearful and less anxious than sexually naïve females on a variety of indices, including acoustic startle, open-field test, elevated plus-maze, and light-dark choice (reviewed in Lonstein & Gammie 2002). The neurochemical basis underlying decreased fear and anxiety in dams is not completely understood. However, the neuroendocrine function of the hypothalamic-pituitary-adrenal (HPA) axis appears to play a significant role (Schulkin et al. 1994; Gammie et al. 2004). Indeed, stress during critical periods of development alters HPA function and its response to fear- and anxiety-provoking stimuli in both dams and their offspring (Barbazanges et al. 1996; Francis et al. 1999). Experimental manipulations of neuroendocrine function in the HPA axis also affect attack behaviors and nest defense in lactating rodents (Lonstein & Gammie, 2002; Klamfl et al. 2013).

HPA axis function is, in turn, controlled by several negative feedback mechanisms in which glucocorticoids -including the stress hormones, -cortisol and corticosterone, play a major role in the organism's timely return to the equilibrium state. High glucocorticoids over extended periods can disrupt negative feedback (Viau 2002; Kudielka & Kirschbaum 2005). It follows that as stress during ontogeny increases FA, extended exposure to glucocorticoids in developing organisms should also yield high FA. There is supporting evidence for this proposition. Both corticosterone and cortisol treatments in vertebrate embryos increase FA in the offspring (Satterlee et al. 2008; Gagliano & McCormick 2009). Moreover, vertebrates genetically selected for a high plasma corticosterone response to stress have significantly greater FA than those selected for a low physiological response to the same stressor (Satterlee et al. 2000; Satterlee et al. 2008).

Accordingly, we hypothesized that increased FA would be associated with reduced maternal aggression, because attack behaviors and nest defense in lactating rodents are affected by the neuroendocrine function of the HPA axis, and, prenatal exposure to a key component of HPA, namely glucocorticoids, increases FA. We also hypothesized that both pre- and early post-natal developmental stress would yield high FA. Low temperature during ontogeny was used as a natural stressor, because it is relevant to behavioral ecology and has been found to raise circulating stress hormone concentrations (Dronjak et al. 2004) and yields high FA in rodents (Siegel & Doyle 1975; Mooney et al. 1985; Gest et al. 1986).

Methods

Animals and Housing

Twenty-four, 60-day-old female F_0 mice (CD-1) were procured from an outbred stock at Charles River Laboratories. They were mated after a 10-day-long acclimation period with age-matched F_0 male mice of the same strain at The Ohio State University facilities. These animals were part of a larger study that examined perinatal stress and maternal function, reproductive status and outcome, including litter composition (Benderlioglu et al. 2006), food intake, total activity, anxiety- and, depressive-like behaviors, and partner preference (Benderlioglu & Nelson unpublished data). The results reported here pertain to maternal aggression and FA in female progeny (F_1) of the 24 dams (F_0). All procedures were conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. The Institutional Animal Care and Use Committee at The Ohio State University approved the study protocol. Maternal aggressive encounters were observed by the attending veterinarian and found acceptable for use in the study.

All F_0 females, and their F_1 progeny, were kept throughout the study in propylene cages (27.8×7.5×13 cm) with corn cob bedding. Except where otherwise noted, they were kept at standard laboratory temperatures ($21 \pm 2^\circ\text{C}$, $50\% \pm \text{RH} \pm 0.5 \text{ SE}$). They were provided with *ad libitum* access to water and food (Harlan Tekland 8640 rodent diet, Indianapolis, IN) and given 16 hours of light and 8 hours of dark per day (lights illuminated at 24:00 hours, Eastern Standard Time). The cages and water bottles were changed weekly and there were no other disturbances. The females were allowed to make nests and given equivalent amount of cotton nesting material. On day 16 of pairing, all males were removed from the females' cages. After Day 17, females were monitored daily for the presence of pups. If pups were present during weekly cleaning schedule, the cages were not cleaned until pups were one-week old.

Experimental Conditions

F_0 females and their mates were randomly allocated to either a low temperature ($n=12$) or a standard temperature ($n=12$) condition one week after mating. The cages containing the F_0 low temperature group animals were placed in refrigerated chambers at $8 \pm 2^\circ\text{C}$, with $50\% \text{ RH} \pm 0.5 \text{ SE}$. We selected this temperature level to induce stress, while also minimizing pup loss as gestating female rodents frequently abort at more extreme temperatures (Schneider and Wade 1991). Also, many temperate zone rodents are exposed to such temperatures during breeding season. Moreover, temperatures varying from 8 to 10°C have been reported to induce FA in rodents (Siegel & Doyle 1975; Mooney et al. 1985; Gest et al. 1986). F_0 animals in the standard temperature group were maintained at $21 \pm 2^\circ\text{C}$, with $50\% \text{ RH} \pm 0.5 \text{ SE}$. This temperature range was standard for maintaining mice in our laboratory, and typical of laboratory studies of *Mus musculus*.

The day of parturition was considered as Day 0 of age for the offspring. On Day 2 after birth, a total of 64 female F_1 pups was assigned to 4 experimental conditions until weaning as follows:

1. F₁ mice were maintained in standard temperatures (21 ± 2 °C) throughout gestation and lactation (n=20; group [CONTROL]);
2. F₁ mice underwent gestation in low temperatures (8 ± 2 °C) and lactation in standard temperatures (n=20; group [G]);
3. F₁ mice underwent gestation in standard temperatures and lactation in low temperatures (n=12; group [L]);
4. F₁ mice underwent both gestation and lactation in low temperatures (n=12; group [GL]).

Cross-fostering (Day 2)

Half of the F₁ control females were raised by F₀ dams exposed to low temperatures during gestation (n=10) and half of the F₁ G females that were exposed to low temperatures during gestation were raised by F₀ control mothers (n=10) (Figure. 1, Table. 1). This cross-fostering procedure was used to partially control for the potential effect of low temperatures on maternal care by F₀ dams (see Benderlioglu et al. 2006). GL and L mothers were maintained in low temperatures during lactation as part of the experimental design, precluding any cross-fostering of their pups to “standard temperature” dams on Day 2. Testing of all F₀ dams for maternal behavior on Day 4 post-partum revealed no differences between groups in the latency and number of pups retrieved (Benderlioglu et al. 2006). Therefore, we pooled our data across cross-fostering groups for analyses pertaining to F₁ maternal aggression and FA. Experimental manipulations and timelines are depicted in Figure. 1 and Table. 1.

Maternal Aggression (Nest Defense)

After weaning, F₁ females were randomly assigned to groups of four and housed to adulthood in the standard laboratory temperatures and housing conditions described above. At 5 months of age, each female was paired with an age-matched non-sibling male. The breeding procedure was the same as for the F₀ generation, except that all animals were maintained throughout pregnancy and lactation under standard temperatures (21 ± 2 °C). On Day 6 after parturition, F₁ dams with viable litters were tested for aggressive behavior towards an adult, unfamiliar male intruder. Previous studies have indicated that maternal aggression is expressed consistently starting at Day 6 post-partum (D’Anna et al. 2005; D’Anna & Gammie, 2009). Some F₁ females produced no offspring or lost their litters before the maternal aggression tests, reducing sample sizes for the aggression tests ($N_{G=}$ 12, $N_{L=}$ 8, $N_{GL=}$ 7, $N_{CONTROL=}$ 12; total F₁ aggression tests $N=39$).

Maternal aggression tests were performed during the dark portion of the daily light-dark cycle in a quiet testing room under red light. Immediately after the dam was removed from her cage, the pups were transferred into a clean unit, which was not used for any other litter. Removal of pups from a dam just before an aggression test does not decrease attacks towards an intruder (Svare et al. 1981; Lonstein & Gammie 2002). The dam was then returned to her original cage and the male intruder was introduced into the cage. The intruder was an unrelated adult of the same strain (CD-1) procured from Charles River laboratories. Intruders were only used once to avoid pseudoreplication. The interaction between the dam and the intruder was recorded on videotape for 10 minutes. The following

aggressive behaviors by the dams were scored by a single observer using The Observer version 5.0 (Noldus Corp., Leesburg, VA): (1) latency to first attack, (2) frequency of attacks, and (3) total duration of attacks. The attacks included aggressive behaviors, in which the dam engaged in boxing, kicking, biting, and rapid thrusts towards the intruder. If no aggression was displayed by the dam, then the latency to first attack was scored as 600 seconds. The rater was uninformed about the conditions of the experiment.

FA Measurements

FA measurements of F₁ females were taken at 7 months of age, 2 months after the aggression tests. We conducted several other behavioral experiments concerning the effect of low temperature stress on offspring during this 2-month gap (Benderlioglu et al. 2006; Benderlioglu & Nelson unpublished data) before F₁ animals were euthanized and FA measurements were made.

Four bilateral traits were measured with a digital caliper to the nearest 0.01 mm: (1) the width of the joint between the tibia and tarsal bones (ankle); (2) the length corresponding to the calcaneus and metatarsal bones (foot); (3) the length corresponding to the ulna, and (4) ear width. Previous studies on FA and mechanical demands on feet, ankle, and ulna for aggressive attacks constituted the basis for trait selection (see Siegel et al. 1977; Doyle et al. 1977; Manning & Wood, 1998).

All measurements were taken on the skin surface of the animals. Each animal was measured twice by the same investigator. The characters were measured in random order alternating between animals and without reference to the prior data. There was about a 30-minute lag between the two measurements of the same trait. A second investigator recorded the values for each trait in a spreadsheet that was not accessible to the first investigator.

Preliminary Statistical Analyses for FA measurements

Because the size difference between two sides of a bilateral trait is very small and can be confounded by measurement error, we aimed to assess this error by employing a mixed model two-way ANOVA (side $\times$ animal) with repeated measurements on each side (Palmer 2004). Results showed that the side $\times$ animal interaction was significant, thus suggesting that the measurement error was negligible for all four traits (ankle: $F_{[1,42]} = 2.55$; $P < 0.0001$; foot: $F_{[1,42]} = 3.89$; $P < 0.0001$; ulna: $F_{[1,42]} = 4.01$; $P < 0.0001$; ear: $F_{[1,42]} = 3$; $P < 0.0001$). We then averaged the two replicate values for each trait to further reduce the measurement error (Graham et al. 2003). To investigate whether the traits were size-dependent or not, we regressed absolute symmetry ($|R-L|$) of each trait on the average size of that particular trait $[(R+L)/2]$ (Palmer & Strobeck 1986). We did not find any indication of size dependency (all P values > 0.05). We thus summed and averaged absolute R-L values for each individual to calculate a composite FA score from the four measured FA traits.

We performed statistical analyses using SAS version 9.3 (SAS Institute, Cary, NC). The main effects of temperature stress on rank-transformed aggression and FA scores were evaluated in PROC GLM as a one-factor ANCOVA with F₁ female body weight and litter size as covariates. *Post hoc* comparisons among group means were performed in PROC GLM using the Tukey-Kramer test for differences in adjusted least squares means for

unbalanced data. Correlational analyses between composite and trait FA and maternal aggression measures were performed on rank transformed data using PROC CORR with partial function controlling for body weight and litter size where appropriate. Results were considered statistically significant when P values were less than or equal to 0.05. The use of rank transformed data in PROC GLM and PROC CORR was appropriate for our small sample and its distributional properties (see Conover & Iman 1981; Chilko & Hobbs 1981; Iman & Conover 1980). PROC GLM in our statistical tests performed a rank analog of the ANCOVA and pairwise comparisons between least square means (Chilko & Hobbs 1981; Iman & Conover 1980).

Results

Maternal Aggression and Cold Stress

Cold stress significantly affected the latency to attack ($F_{[3, 37]}=2.75$, $P=0.056$), frequency of attacks ($F_{[3, 38]}=3.18$, $P=0.035$), and duration of attacks ($F_{[3, 38]}=3.69$, $P=0.012$). GL females showed reduced maternal aggression based on all three measures (Figures 2A–C). Post-hoc results indicated that the latency to attack was significantly higher in GL females than in G and control females ($t_{37}=-2.80$, $P=0.009$; $t_{37}=3.27$, $P=0.03$, respectively). The frequency of attacks was lower in GL animals than both G and control females ($t_{37}=2.53$, $P=0.016$; $t_{37}=-2.53$, $P=0.017$, respectively). GL animals also engaged in lower duration of attacks compared to any other group ($t_{37}=2.79$, $P=0.009$ for GL vs. G; $t_{37}=-2.17$, $P=0.04$ for GL vs. L; $t_{37}=-2.83$, $P=0.008$ for GL vs. control). G, L, and control animals did not differ from each other in any of the aggression scores. Body weight and litter size did not have any significant effects on the aggression scores (P values >0.05).

Composite/Trait FA and Cold Stress

The main effect of cold stress on composite FA was significant ($F_{[3, 57]}=2.86$, $P=0.045$). Post-hoc comparisons indicated that GL females had higher composite FA than those in any other group (Figure. 3). This was statistically significant in comparison to G and control females (Tukey-Kramer adjusted means; $t_{37}=2.10$, $P=0.04$; $t_{37}=2.28$, $P=0.03$, respectively). The difference between GL and L groups was marginally statistically insignificant ($t_{37}=1.94$, $P=0.06$). G and L groups did not differ from the control group in composite FA. None of the individual FA traits were significantly affected by cold stress (rank transformed mean_{ankle}= 34.46 ± 4.77 SEM; mean_{foot}= 27.96 ± 4.96 SEM; mean_{ulna}= 28.23 ± 4.66 SEM; mean_{earwidth}= 30.88 ± 5.07 SEM) and nor were FA scores significantly affected by body weight or litter size (P values >0.05).

Composite/Trait FA and Aggression

Composite FA did not correlate with any of the measures of maternal aggression. However, latency to attack was positively correlated with increased FA of the foot (*partial* $r=0.43$, $P=0.004$ controlling for body weight and litter size). Foot FA was negatively correlated with frequency of attacks (*partial* $r=-0.32$, $P=0.05$). Ankle FA was also negatively correlated with duration of attacks (*partial* $r=-0.30$, $P=0.05$).

Discussion

The present study examined the relationship between developmental stress and FA of four morphological traits, as well as nest defense in female mice. We hypothesized that suboptimal temperatures during development would induce high FA in both pre- and postnatally stressed offspring. Moreover, we hypothesized that this effect would be mirrored by differences in nest defense in adulthood resulting in negative correlations between FA and maternal aggression scores. The results partially support our hypotheses.

High FA was associated with low maternal aggression in the female offspring by some measures. Specifically, lactating females with high FA of the foot took a longer time to attack an unfamiliar intruder and did so less frequently compared to those with low FA of the same trait. Also, female offspring with high FA of the ankle engaged in shorter attacks than those with low ankle FA. Composite FA was not significantly correlated with any nest defense measures.

Exposure to low temperatures during both pre- and postnatal development was associated with high composite FA compared to the control group. There were no significant differences among control, only pre-, and only postnatally-stressed females with regard to composite FA. These findings were mirrored by maternal aggression. Nest defense in both pre- and postnatally-stressed females (GL) was reduced in all three measures of aggression compared to non-stressed females. There were no statistically significant differences among G, L, and control animals in latency to attack or frequency of attacks. Duration of attacks was significantly reduced in GL females compared to any other group. Body and litter size did not have any significant effect on either the aggression or FA scores in any of the experimental groups.

There is a disagreement among researchers regarding the use of single trait FA as an indicator of negative health and fitness outcomes. Some authors assert that FA is an organism-wide property (Leamy 1994; Dufur & Weatherhead 1996; Leung & Forbes, 1997; Thornhill et al. 1999; Leung et al. 2000), rendering the composite FA score a more appropriate and reliable measure of stress, whereas others argue that FAs are trait-specific (Soule & Cuzin-Roudy 1982), with single trait FA just as important and reliable. Examining correlations among FA of various traits may shed light on these assertions. When significant correlations are observed, traits are developmentally (Leamy 1994; Livshits et al. 1998) and functionally (Livshits et al. 1998) related, such as the bones of the human hand involved in grasping. Nest defense and attack behaviors in our study involved kicking, chasing, and running, relying on proper functioning, and presumably, symmetry of foot and ankle, two closely related traits. Indeed, one study indicated that high ankle FA was associated with reduced self-reported physical aggression in boys (Manning & Wood 1998). Perhaps because maternal aggression relied on foot, ankle, and in part, ulna symmetry, we found no association between nest defense and composite asymmetry, which included ear width.

Nest defense with heightened levels of aggression in lactating dams is considered a strategy employed against infanticidal conspecifics (Wolff 1993; Ebensperger 1998a; 1998b). Infanticide in mammals may provide the “perpetrator” with nutrition through

cannibalization, or access to limited resources, including other food sources, nest sites, and breeding females (reviewed in Ebensperger, 1998a). Moreover, infanticide may be employed by conspecifics to avoid the provision of parental care to unrelated young or may be a pathological or neutral behavior (Ebensperger, 1998a). Infanticide in house mouse, however, appears to be a form of intraspecific aggression. Laboratory experiments conducted with these species show that neurohumoral bases of inter-male aggression and pup-killing behavior are similar, but differ from those modulating prey killing (Parmigiani & Palanza 1991). The aggression tests in our study did not include the presence of pups. Therefore, we cannot provide a direct comparison between pup killing behavior, potential breeding opportunities, and intraspecific aggression. However, the type of agonistic behavior exhibited by intruder males in the current study was very similar to intermale aggression in previous tests conducted in our laboratory (Benderlioglu & Nelson unpublished data).

Curiously, lactating house mice are frequently unable to protect their infants from intruders in laboratory experiments (Ebensperger 1998b). It is possible that an isolated laboratory setting does not provide a natural physical and social environment to adequately test the efficiency of nest defense against male intruders. Indeed, female house mice are communal breeders and they are frequently successful in fending off attacks by male conspecifics in communal nests (Maestripieri & Rossi-Arnaud 1991; Parmigiani 1986; Wilkinson & Baker 1988). Supporting evidence shows that infanticide is more commonly observed in single-dam nests compared to communal nests (Manning et al. 1995). Furthermore, dams are more successful in defending their young when they are assisted by their mates against male intruders (Palanza et al. 1996). Our study employed only single-dam nests, therefore, we cannot assess the efficiency of maternal defense of the young according to the nest type and presence of the mates. However, regardless of the outcome, suckling stimulation after parturition triggers a hormonal response that elicits maternal aggression in mice (Svare & Gandelman 1976) and female aggression and territoriality appear to be associated with a threat of infanticide (reviewed in Wolff 1993).

Increased maternal aggression is generally attributed to decreased fear and anxiety and proper functioning of the HPA axis in lactating female rodents (Maestripieri et al. 1991; Parmigiani et al. 1999; Lonstein & Gammie 2002). Stress during critical periods of development impairs HPA function and alters its response to fear- and anxiety- eliciting stimuli (Barbazanges et al. 1996; Francis et al. 1999). Specifically, activation of HPA in response to a stressor results in the secretion of corticotrophin-releasing hormone (CRH) from the hypothalamus. CRH provokes the release of adrenocorticotrophic hormone (ACTH) from the pituitary. Low CRH is necessary for attack behaviors to occur in lactating female rodents (Gammie et al. 2004; Klamfl et al. 2013), and, injection of CRH significantly inhibits maternal aggression (Gammie et al. 2004). Increased CRH induces fear and anxiety-like behaviors (Lonstein & Gammie 2002). High concentrations of ACTH also decreases attack behaviors in lactating mice (Lonstein & Gammie, 2002). It is possible that the combined pre- and postnatal developmental stress in our study may have altered the HPA response to the fear- and anxiety-provoking stimulus- an unfamiliar, intruding male in the nest, and, significantly decreased attack behaviors in GL females compared to the control group.

The negative correlation between certain FA and maternal aggression scores may be due in part to shared pathways in the development of anatomical characters and neuroendocrine functions. The development of the central nervous system is concurrent with the development of bilateral morphological traits that show minor deviations from symmetry. If a return to equilibrium after a stressful event is not achieved due the chronic nature of the stressor, then both anatomical and neuroendocrine pathways are presumably altered. The HPA axis function is controlled by negative feedback mechanisms in which glucocorticoids affect the organism's timely return to the equilibrium state. Specifically, ACTH secretion during stress triggers the release of glucocorticoids, with cortisol predominant in most primates and corticosterone in most rodents, avian species, and reptiles. Communication between the central nervous system and endocrine function may affect not only immediate survival, but future growth, health, and reproductive behaviors, all implicated in FA (reviewed in Benderlioglu 2010). If environmental perturbations during ontogeny adversely affect bilateral symmetry (Beasley et al. 2013), and the individual cannot effectively cope with these stressors, elevated concentrations of glucocorticoids should be associated with asymmetric development. Indeed, prenatal exposure to corticosterone and cortisol increase FA in the offspring (Satterlee et al. 2008; Gagliano & McCormick 2009). Moreover, an elevated corticosterone response to stress is associated with FA in vertebrates (Satterlee et al. 2000; Satterlee et al. 2008). We did not measure stress hormones and, therefore, we cannot conclusively state that low maternal aggression in our study is due to increased glucocorticoids in the female offspring. Future research will benefit from direct physiological and morphological measures, and genetic manipulations involving perinatal stress, FA, and reproductive behaviors.

The fact that pre- or postnatal stress alone did not result in reduced nest defense and increased FA compared to control animals warrants further attention. Previous studies indicate that low temperatures, audiogenic, nutritional and heat stress administered during both pre- and postnatal development, or pre-, or postnatal period alone induce high dental FA in rats and mice (Siegel & Smookler 1973; Siegel & Doyle 1975a, 1975b; Siegel et al. 1977; Doyle et al. 1977; Sciulli et al. 1979). When limbs are considered, however, the relationship between stress and FA remains inconclusive. For example, FA in the ulna, humerus, tibia, or femur did not increase in rats that underwent gestation or lactation in conditions of heat or audiogenic stress (Siegel et al. 1977; Doyle et al. 1977), whereas the same stressors administered during the gestational period increased rat femoral FA (Gest et al. 1986). Because the age of animals at the time of FA measurements differs across studies, i.e., at birth (Gest et al. 1986), weaning (Siegel et al. 1977; Doyle et al. 1977), or in adulthood (the current study), these contradictory findings may be attributed to the age-dependent functional alteration of long bones. The length of long bones is highly responsive to functional demands involved in locomotion that would likely change with age (Arkin & Katz 1956; Reisenfeld 1966; Doyle 1976). It is also possible that the choice of strains and species in different studies leads to variant results, as well as use of different methods of measurement. We also controlled for litter size and composition (i.e., sex), and applied uniform animal care to all groups. These details are not available in the studies above.

Our sample size was relatively small for FA studies, because some of the animals did not survive long enough to be included in the FA sample, although we had an adequate number

of individuals comparable to previous work with mice on maternal aggression (e.g., Lonstein & Gammie 2002; Gammie et al. 2004). Although low sample size certainly constitutes a limitation, in the current study, we detected two concomitant consequences for animals exposed to cold stress during both the pre- and early postnatal developmental period. That is, increased FA in the GL group mirrored reduced maternal aggression compared to the non-stressed control group. Accordingly, our results are unlikely to be spurious.

Our previous research with F₁ females indicated that postnatal low temperature stress had less deleterious effects on reproductive function in female offspring than combined pre- and postnatal low temperature stress (Benderlioglu et al. 2006). We found that, although mean litter sizes and weights of all F₁ groups exposed to low temperatures were significantly reduced compared to the control group, infant mortality was highest in the progeny of combined pre- and postnatally stressed F₁ females, indicating a greater reaction to longer-term low temperature stress. Moreover, gestational stress, including exposure to teratogenic substances and extreme temperatures has been shown to have adverse consequences on physical development, physiology, and behavior of rodents in other studies (Benderlioglu et al. 2006; Breno et al. 2013; Aminabadi et al. 2016). Some of these adverse consequences of prenatal treatments may be reversed or ameliorated by exposure to postnatal manipulations, such as “handling”, that is, separation of pups from the mother for a limited time. For example, postnatal handling reduces fearfulness and freezing behaviors, and also increases exploration in novel environments in adult rats and mice (Bodnoff et al. 1987; Meaney et al. 1991; Washlak & Weinstock 1990). It may be that animals must be exposed to low temperature conditions during both pre- and neonatal development to affect fear and anxiety in adulthood thereby reducing maternal aggression. Similarly, combined gestational and lactational low temperature stress is presumably a more potent instigator for asymmetrical developmental trajectory than gestational or lactational stress alone.

Maintaining an ideal developmental trajectory may be energetically costly in winter-like low temperature conditions. Restricted food, extreme temperatures, and the prevalence of infectious agents vary with seasons, which can be predicted by changes in the environment, including photoperiod and ambient temperatures. By responding to predictive environmental cues, non-human animals generally cease reproduction in winter and allocate appropriate energy to other important physiological functions, such as enhanced immune function (Nelson et al. 2002). Although our cold treatments did not prevent reproduction, it is possible that the extended exposure to low temperatures in the GL treatment rendered it necessary for F₁ females to trade off growth and reproductive output with other functions crucial for survival. High infant mortality and reduced litter size in our previous study with cold-stressed F₁ females support this proposition (Benderlioglu et al. 2006). Also, we previously found that winter and spring births in humans are associated with elevated FA, lending support for this conjecture (Benderlioglu & Nelson 2004).

In sum, developmental insults appear to jointly contribute to the imprecise expression of symmetrical phenotypes and limited response to intruders. The increased FA and reduced maternal aggression in animals exposed to both pre- and postnatal stress support this hypothesis as low maternal attack and defense behaviors are attributed to altered functioning of the HPA axis (i.e., Barbazanges et al. 1996; Francis et al. 1999). To our knowledge, this is

also the first study that links FA and maternal aggression in human and nonhuman animals. Our results also suggest that low temperatures experienced by many temperate zone rodents during the breeding season may have important fitness costs. It appears that these stressors have more adverse consequences if experienced during both the pre- and postnatal growth periods compared to the pre- or postnatal period alone. Inadequate maternal responsiveness towards the young in nursing dams could limit the number of surviving offspring into adulthood. The relative ease with which FA is measured makes it a powerful tool to quantify developmental stress. Our study suggests that, FA is a reliable indicator of early life stress with important fitness implications.

Acknowledgments

We thank Randy J. Nelson for allowing us to use his laboratory resources to conduct this study, Leah Pyter, Randy J. Nelson, Lynn B Martin II, and two anonymous reviewers for their insightful comments, Zach Weil for help with the experimental procedures, and Trish Uhor and her staff for expert animal care. This research was supported by NIH grant MH 57760 to RJN.

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Figure 1. Illustration of experimental and cross-fostering conditions for F_0 dams (Total N=24 of which 12 went through pregnancy in cold chambers) and their female offspring (F_1) during gestation and lactation

CONTROL= F_1 raised in standard laboratory temperatures during both gestation and lactation; G= F_1 exposed to low temperatures during gestation; L= F_1 exposed to low temperatures during lactation; GL= F_1 exposed to low temperatures during both gestation and lactation.

Figure. 2A

Figure. 2B

Figure. 2C

FIGURE 2. Maternal aggression by lactating females toward a male intruder (rank transformed Mean ± SEM)

(A) Latency to attack. (B) Frequency of attacks. (C) Duration of attacks. Group means with different letters are significantly different ($P < 0.05$). *CONTROL* = females raised in standard

laboratory temperatures ($21 \pm 2^{\circ}\text{C}$) throughout ontogeny; G = females exposed to low temperatures ($8 \pm 2^{\circ}\text{C}$) during gestation; L = females exposed to low temperatures during lactation; GL = females exposed to low temperatures during both gestation and lactation.

FIGURE 3. Mean ($\pm$ SEM) composite FA scores (rank transformed)

Group means with different letters are significantly different ($P < 0.05$; $N_G = 15$; $N_L = 10$; $N_{GL} = 9$; $N_{CONTROL} = 15$; total F_1 FA measures $N = 49$). Other conventions and symbols are as in Figure 2.

Table 1
Timeline for developmental stress in F_1 females

CONTROL= F_1 raised in standard laboratory temperatures during both gestation and lactation; G= F_1 exposed to low temperatures during gestation; L= F_1 exposed to low temperatures during lactation; GL= F_1 exposed to low temperatures during both gestation and lactation. GL and L pups were reared by their natural birth mother during both pre- and postnatal periods.

gestational period			neonatal period			
week1	week2	week3	week1	week2	week3	
	low temperatures			low temperatures		GL=12
			half raised by birth mothers F_0 G half cross-fostered by F_0 CONTROL day2			
week1	week2	week3	week1	week2	week3	
	low temperatures			standard temperatures		G=20
week1	week2	week3	week1	week2	week3	
	standard temperatures			low temperatures		L=12
			half raised by birth mothers F_0 CONTROL half cross-fostered by F_0 G day2			
week1	week2	week3	week1	week2	week3	
	standard temperatures			standard temperatures		CONTROL=20