Effects of Population Density on Stress and Maternal Care in a Wild Rodent (*Peromyscus maniculatus*)

by

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ABSTRACT

EFFECTS OF POPULATION DENSITY ON STRESS AND MATERNAL CARE IN A WILD RODENT
(PEROMYSCUS MANICULATUS)

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Laboratory studies have demonstrated that variation in maternal care is linked to differences of offspring stress axis dynamics. Offspring who receive a low level nursing face increased exposure to elevated CORT compared to high nursing offspring which, according to the CORT-fitness hypothesis, leads to a reduced fitness. There is a growing body of empirical data which suggests that this relationship between CORT and fitness may depend on the environmental conditions. In the wild, if a female has access to environmental cues, it may be advantageous for a female to program her offspring’s stress axis to match the environment they will experience upon independence. Here, I tested the effects of population density on maternal behaviour by bringing wild-caught Peromyscus maniculatus into captivity to measure their frequency of nursing while experimentally manipulating perceived density cues through the presence of soiled bedding. I predicted the mechanism for this behavioural response to environmental cues to be related to a change in maternal glucocorticoids. As population density increased over the breeding season the frequency of nursing decreased by approximately 27% however, olfactory cues alone do not experimentally alter nursing. We report a U-shaped relationship between population density and maternal stress levels that can be explained by a significant interaction between population density and time of breeding. This suggests that CORT is not the mechanistic link between maternal behaviour and population density but rather may be related to the level of maternal care the female received as a pup. Wild mice face this mismatch of environments where the density in which they were born can be very different from the density in which they breed. These complex relationships between natal and breeding environments might require complex systems for appropriately programming offspring for the current environmental conditions. Matching behavioural phenotypes to a changing environment could be the driver responsible for maintaining variation in maternal behaviour in a wild environment.
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1.0 Introduction

Maternal care plays a critical role in an offspring’s growth and development. This is especially true in mammals as the extended period of mother-infant contact is not only essential for survival, but the behaviour of the mother can be highly influential on the long term wellbeing of her offspring (Myers et al., 1989; Meaney, 2001; Champagne et al., 2003). Elegant laboratory studies have identified individual variation in the maternal care of laboratory rodents, such as the variation in the frequency of nursing over the first seven days post partum (Myers et al., 1989; Champagne et al., 2003; Champagne et al., 2007). These studies have traditionally categorized females as being either high- or low-nursing mothers (Liu et al., 1997; Meaney, 2001; Champagne et al., 2003; Champagne et al., 2007), but this classification is based on an artificially constructed dichotomy as the frequency of nursing is a normally distributed variable (Champagne et al., 2003). In addition to providing essential nutrition to the offspring during the early life environment, the frequency of nursing has been shown to affect long-term aspects of the offspring’s physiological and psychological health including cardiovascular functions, cognitive abilities, immune response and the development of the stress axis (Liu et al., 1997; Meaney, 2001; Champagne et al., 2003; Champagne et al., 2007).

The stress response of an individual is controlled by the Hypothalamic-Pituitary-Adrenal (HPA) axis where glucocorticoids (GCs) are maintained at basal levels through negative feedback (Smith and Vale, 2006). When an individual is presented with a stressor, levels of circulating corticosterone (CORT), the primary GC in rodents, increase (Meaney, 2001; Smith and Vale, 2006). Elevated CORT mobilizes glucose and lipid reserves thereby diverting energy towards physiological functions essential for survival (i.e. increased cardiac function, attentional processes, learning and memory) and activating the ‘fight or flight response’ (Meaney, 2001). A large body of literature on laboratory rodents has shown that variation in the frequency of nursing is linked to differences in offspring stress axis dynamics. Offspring who receive a low level of nursing have a reduced number of glucocorticoid receptors (GRs) in the brain compared to high nursing offspring. During a stress response, a fewer number of GRs ultimately leads to prolonged CORT elevation as the negative feedback cannot respond as quickly (Liu et al., 1997; Weaver et al.,
Cross-fostering studies provide evidence for a nongenomic transmission of individual differences in stress reactivity and maternal behaviour (Meaney, 2001). Female offspring birthed by low-nursing mothers but reared by high-nursing mothers will experience reduced exposure to elevated CORT as a result of an increased number of GRs and will also subsequently exhibit a high level of nursing towards their offspring (Meaney, 2001). These laboratory studies have been fundamental in our understanding of the consequences of maternal care for physiological and psychological development, but the ultimate question of why variation in maternal behaviour persists remains unanswered.

It is commonly assumed, especially in the biomedical literature, that an individual or population with a slower HPA negative feedback has reduced fitness (Bonier et al., 2009; Boonstra, 2012). This has been termed the CORT-Fitness hypothesis and is based on the observation that individuals exposed to elevated CORT for a prolonged period of time are at increased risk of heart disease, diabetes, anxiety disorders and depression (Meaney, 2001; Bonier et al., 2009). However, if offspring who receive a low level of nursing always have lower fitness when compared to offspring that received more nursing, the low maternal behaviour phenotype should be eliminated from the population through natural selection, thereby reducing and/or eliminating any variation in the frequency of nursing. As this is not the case, there must be a mechanism present in the natural world that maintains this variation in nursing frequency. In contrast to the ‘stress as pathology’ paradigm, there has been a recently growing body of empirical data that suggest the relationship between GCs and fitness is not static, but might vary with changing environmental conditions (Bonier et al., 2009; Boonstra, 2012). In stressful environments, an earlier and longer activation of the fight or flight response may outweigh the cost of increased exposure to elevated CORT (Meaney, 2001; Harris et al., 2012). Laboratory studies with rodents have demonstrated that maternal behaviour is altered by stressors experienced during gestation; females who experience high CORT during the prenatal environment exhibit reduced maternal behaviour towards their pups compared to pregnant females in non stressful environments (Moore and Power, 1986; Patin et al., 2002; Smith et al., 2004; Champagne and Meaney, 2006; Baker et al., 2008; Brummelte and Galea, 2010). Furthermore,
experimental manipulations of maternal stress during gestation have been found to affect the CORT levels of the pups indicating that maternal stress hormones can affect the stress levels of the offspring (Weinstock 2001, 2008; Smith et al., 2004; Champagne and Meaney, 2006; Baker et al., 2008). In stressful environments, low maternal care would result in offspring with increased exposure to elevated CORT that would display behaviours essential for survival (i.e. increased cardiac function, attentional processes, learning and memory) faster and for a longer period of time than offspring who experienced high levels of maternal care with reduced exposure to elevated CORT (Meaney, 2001; Harris et al., 2012). In a wild environment, where gestational stressors vary in space and time, if females have access to cues of the environment that offspring will experience then it might be advantageous for a mother to program her offspring’s stress axis to match the environment they will experience upon independence (Uller, 2008; Dantzer et al., 2013). Specifically, a female might program an HPA axis with a reduced number of GRs in her offspring when offspring are likely to experience consistent or repeatedly stressful environments.

One biotic factor that fluctuates and could have important implications for the appropriateness of a hyper- or hypo-responsive stress axis is population density. High population density has been hypothesized to be a stressful environment for mouse populations as a result of the positive associations between population density and food competition, negative social interactions and predation (Kaiser and Sachser, 2005; Creel et al., 2012). This hypothesis is based on laboratory and field studies that have shown a decrease in reproductive output at high population density (laboratory studies: Christian 1950, 1956, 1961; Terman 1965, 1968, 1969; Rogers and Beauchamp, 1976; Lidicker, 1976; Lombardi and Whitsett, 1980; Creigh and Terman, 1988; field studies: Morris, 1989; Lusk and Millar, 1989; Montogery, 1989; Drost and Fellers, 1991; Terman, 1993). This reduction in reproductive output has been attributed to the reduced size of reproductive organs, decreases in fertility and the inhibition of juvenile maturation (Christian 1950, 1956, 1961; Terman, 1969; Rogers and Beauchamp, 1976; Lidicker, 1976; Lombardi and Whitsett, 1980; Creigh and Terman, 1988). Laboratory studies working with *Mus, Acromys* and *Peromyscus* show a positive relationship between the number of individuals housed in each cage and
CORT levels suggesting that stress may be the possible mechanism responsible for the reduction in reproductive output observed at high population densities in the wild (Hunt and Hambly, 2006; Novákova et al., 2008; Peng et al., 2009; Thomason et al., 2013). To date only one field study has investigated the relationship between CORT and population density in wild mice; Harper and Austad (2004) documented that CORT increased with rising population density in a deer mouse (*Peromyscus maniculatus*) population.

Many small mammal species, including deer mice (*Peromyscus maniculatus*), exhibit large seasonal and annual fluctuations in population density (Fryxell et al., 1998; Falls et al., 2007). In addition to reproductive output, population density has been previously shown to affect immune function (Pedersen and Greives, 2008; Thomason et al., 2013) behaviour (Wolff, 1985) and home range size (Taitt, 1981; Wolff, 1985; Boutin et al., 1990) in wild deer mice. It is unclear what social cues deer mice use to assess population density, but these could be tactile, auditory, olfactory or visual (Terman, 1979, 1980). Scent appears to be the most likely cue for assessing population density as reproductive inhibition and the delay of sexual maturation in juveniles, two phenomena observed under high population density, can be mediated through olfactory signals in the absence of other social cues (Champlin, 1970; Drickamer, 1974; Ma et al., 1998). These findings are derived from laboratory experiments in which the effect of multiple individuals in a cage was simulated by exposing singly housed females to the urine or soiled bedding of conspecifics (*Mus musculus*: Champlin, 1970; Drickamer, 1974; Ma et al., 1998; *Peromyscus maniculatus bairdii*: Lawton and Whitsett, 1979; Lombardi and Whitsett, 1980; Creigh and Terman, 1988; *Peromyscus leucopus*: Rogers and Beauchamp, 1976; Terman, 1984). In the wild, as population density increases there is greater home range overlap between individuals (Wolff, 1985; Boutin et al., 1990), which presumably results in an increase in the number of unique deer mouse scents or scent marking within an individual's home range. Unfortunately, it is virtually unknown how scent marking is used by wild mice (Wolff, 2003).
While numerous laboratory studies have examined variation in maternal care in mice (Myers et al., 1989; Liu et al., 1997; Meaney, 2001; Champagne et al., 2003 Champagne et al., 2007), only one previous study has applied these laboratory measures of maternal behaviour to a wild rodent (Stewart and McAdam, 2014). Stewart and McAdam (2014) used standard laboratory rodent protocols (Myers et al., 1989; Champagne et al., 2003; Champagne et al., 2007) to measure the maternal behaviour of wild-caught female deer mice. The maternal behaviour of deer mice was found to be repeatable but changed plasticly over the summer as population density increased (Stewart and McAdam, 2014). Females at the beginning of the breeding season spent twice as much time nursing as females that gave birth at the end of the breeding season when population density was much higher (Stewart and McAdam, 2014). This plasticity differs from previous studies of laboratory rodents, which have found maternal behaviour to be very consistent within an individual (Meaney, 2001), and suggests that mother’s might adjust their behaviour toward offspring depending on cues of population density. This one field study, however, was observational and could, therefore, not definitively attribute the changes in maternal behaviour to changes in population density since population density was confounded with seasonality (Stewart and McAdam, 2014). Experiments are, therefore, needed to test the effects of population density on maternal behaviour, and identify the modality by which density is assessed. Furthermore, although laboratory studies have shown CORT to affect maternal behaviour and field studies have documented increases in CORT levels with increasing population density the role of maternal stress in mediating the linkage between seasonal changes in density and maternal behaviour has not yet been explored.

Here, I tested the effects of population density on maternal stress and maternal behaviour, by bringing pregnant, wild-caught Peromyscus maniculatus gracilis into captivity to measure their maternal behaviours following standard protocols under controlled conditions (Myers et al., 1989; Champagne et al., 2003; Champagne et al., 2007). In the laboratory, I experimentally manipulated cues of perceived density and collected fecal samples from the female to measure CORT levels. I hypothesized that female deer mice would adjust their maternal behaviour in response to population density and that this would be mediated by maternal stress levels. I therefore, predicted that as deer mouse population density
increased as a result of natural seasonal fluctuations in the wild, the frequency of nursing would decrease and maternal CORT levels would increase. I further predicted that female deer mice exposed to the scent of unknown deer mice in the lab would have a lower frequency of nursing and higher maternal CORT levels than control females not exposed to conspecific scent.

2.0 Methods

Study Location and Trapping

Research was conducted at the Wildlife Research Station (WRS) in Algonquin Provincial Park, Ontario, Canada (45°37’N, 78°21’W) from April to August 2013. Deer mice were trapped in Longworth live traps (Roger’s Manufacturing, British Columbia Canada) on 11 transects that were each 100m long with two traps placed every 10m along each transect. Traps were baited with water soaked sunflower seeds (Helianthus spp) and freeze-killed mealworms (Tenebrio molitor) were provided to reduce accidental shrew mortality (Soricomorpha: Soricidae; Do et al., 2013). Cotton bedding was provided in the traps for warmth. Traps were set three consecutive nights per week at dusk and checked at dawn. Upon capture, deer mice were removed from the trap, placed in a large handling bag and weighed with a Pesola spring balance (Pesola® Switzerland) to the nearest 0.5g. Deer mice were tagged in both ears with metal ear tags (National Brand and Tag Co.) for identification. Age of the individual was determined based on dorsal pelage where juveniles are grey, sub-adults are a mixture of grey and brown and adults are brown (Schug et al., 1991). All females who were not perforate were assumed to be pregnant and were then placed in a clean Sherman trap (H. B. Sherman Inc., Tallahassee Florida USA) and transported to the animal care facility at the WRS.

Laboratory Procedures

Animal care

Females were housed in an animal proof laboratory with natural diel light hours. Windows in the laboratory were left open so that temperature and humidity conditions were as close to the current
weather conditions as possible. When external temperatures dropped below 5°C, the heat was turned on and maintained at 15°C to prevent dam or pup mortality. Individuals were housed in Plexiglas cages (35 cm x 15 cm x 17 cm) with ad lib supply of food (Rodent Chow 14% protein) and water. The cages contained Corn Cob bedding (Harlan Corn Cob Bedding), paper (Andersons CN Crink-'n'nest™) and additional cotton bedding (Ancare Nestlets) as well as a cardboard tube for environmental enrichment. All bedding was replaced every three days when the female’s cage was cleaned except when pups were present. During the cage cleaning, the female was placed in a clean handling bag and weighed to the nearest 0.5g with a Pesola spring balance. The female usually produced a fecal sample while inside the handling bag. If the female did not defecate the mouse was held until a fecal sample was produced, so all feces collected were known to be fresh. Fecal samples were collected in a clean, labelled 1.5mL microcentrifuge tube and frozen until extraction. Cages were thoroughly cleaned with a 10% bleach solution between occupants.

Experimental density manipulation

Upon arrival in captivity, females were randomly assigned to either control (n = 28) or treatment (n = 26) groups. Treatment females were exposed to four cotton ball sized pieces of frozen soiled bedding from four different adult deer mice; two males and two females. Control females were exposed to four similar sized pieces of clean, frozen cotton bedding. Soiled bedding was collected from live traps that contained adult deer mice on live trapping transects used to assess the density of various small mammal species in Algonquin Park (Fryxell et al., 1998; Falls et al., 2007). The transects were located well outside the home range of the females studied in the laboratory, so individuals from which the soiled bedding was collected were presumably unfamiliar. Soiled bedding was collected directly from the trap upon release of the captured mouse and placed in a clean plastic bag that was labelled and sealed. Upon return to the laboratory, the cotton bedding was cut into cotton ball sized pieces and immediately frozen. The bedding pieces were replaced every three days when the females’ cage was cleaned unless pups were present. By cutting the soiled bedding into pieces at the time of collection, I was able to expose each treatment female to the scent of the same four unknown individuals over the course of her time in the laboratory.
Maternal observation

Females were checked at least every 12 hours in order to determine the day of birth, which was designated as postnatal day (PND) 0. When females gave birth, pups were weighed to the nearest 0.1g using an electronic balance (Ohaus Corporation, New Jersey USA) and females were weighed to the nearest 0.5g with a Pesola spring balance. A fecal sample was also collected from the female at this time. Females who gave birth prior to July 3rd were defined as early breeders while females who gave birth on or after July 3rd were defined as late breeders. Early breeders were likely over-wintered (OW) animals born in the previous fall since the young-of-the-year (YY) in this study and a concurrent long-term study of small mammals in the area (Fryxell et al., 1998; Falls et al., 2007) were first captured on May 29th, 2013. Based on an age of sexual maturation of one month and a gestation period between 22.4 and 25.5 days (Millar, 1982; Millar, 1985; Kirkland and Layne, 1989) known YY could not have given birth before July 3rd, 2013. Females defined as late-breeders could have been either OW or YY mice. The female and pups were then left undisturbed until PND3. During this three-day period maternal behaviour was observed by videotaping the female with her pups under infrared conditions from dusk to dawn, when deer mice are most active (Champagne et al., 2007).

Maternal behaviour was quantified following the methods of Myers et al. (1989) and Champagne et al. (2003) during four predetermined 1-hour focal periods within each night; 1 hour after sunset, 23:30, 1:30 and 2 hours before sunrise. All recordings were made using a SONY HANDYCAM (MODEL #DCR-SR68) video camera. Maternal behaviour was scored every 3 minutes within each 1-hour focal period (21 observations per 1-hour focal × 4 focal periods per night × 3 post natal nights = 252 observations per female) with the observer blind to conditions. At each 3-minute interval maternal behaviour was first scored based on the location of the female 1) “in nest” when the female was in physical contact with the nesting material or 2) “out of nest” when the female was not in contact with nesting maternal. The female’s behaviour was then classified from the following list in which behaviours were not mutually exclusive 1) nursing, 2) licking/grooming pups, 3) manipulating nest shavings, 4) manipulating non nest shavings 5)
self grooming, 6) eating, 7) drinking, 8) rearing/climbing, 9) resting, 10) carrying pup, 11) out of sight (Table 1). Nursing postures are often concealed by bedding material rendering them difficult to classify (Stewart and McAdam, 2014). Therefore, for the purpose of this study all nursing postures were combined and simply described as nursing.

On PND3, pups were sexed and weighed with an electronic balance to the nearest 0.1g. Ano-genital distance (AGD; distance from the pups genital papilla to their anus in mm; Ryan and Vandenbergh, 2002), and body length (distance from the tip of the nose to the base of the tail in mm) measurements were also taken. Adult females were weighed to the nearest 0.5g with a Pesola spring balance and a fecal sample was collected. Females and their offspring were then placed in a nest box (12.5 cm x 12.5 cm x 15 cm) with the bedding from their nest, an additional nestlet and approximately 50 sunflower seeds. Nest boxes were hung on a tree at the site of their original capture location at approximately chest height and entry holes were exposed so that the female could depart the nest box with her pups.

*Measuring stress response*

Fecal samples were used to assess stress levels as they are a non-invasive, integrated measure of an individual’s basal GC profile. Fecal samples are also not as sensitive to handling disturbances compared to blood sampling as they represent GC levels over a number of hours instead of a specific instance in time (Harper and Austad, 2000; Palme, 2005). GCs in the blood are broken down by the liver into species-specific GC metabolites and then excreted as bile into the small intestine (Palme, 2005). In *Peromyscus*, the GC metabolites can then be measured in the fecal samples to determine the stress level of the individual 4-6 hours prior to the sample being produced (Harper and Austad, 2000; Harper and Austad, 2004; Palme, 2005).
**Fecal sample extraction**

Prior to extraction, all fecal samples were freeze dried to remove variation in water content (Wasser et al., 1993). For the extraction, all samples were weighed on an electronic balance to the microgram and then transferred to individually labelled 16x100mm glass test tubes. A small amount of 100% methanol was then added to the test tube and samples were homogenized with a glass rod. Samples were then topped up to 2mL with 100% methanol. Samples were vortexed for 20 seconds, placed in a fume hood, covered and shaken for 2 hours in a room temperature water bath. After 2 hours, samples were centrifuged for 10 minutes at 2500xg and the supernatant was poured into a clean 12x75mm test tube. Samples were then dried in a fume hood and frozen at -80°C until analysis.

To quantify CORT, we used a double-antibody I-125 radioimmunoassay (MP Biomedicals LLC, Orangeburg NY; catalog no. 07120103) that was modified following (Newman et al., 2008). Briefly, manufacturer's directions were followed except all reagents were halved and extracts were diluted to between 0.004-0.006g per 50µL of assay buffer (0.004-0.006 g per tube). CORT concentrations are expressed as nanogram of hormone per gram of fecal sample. Further dilution of a sample was completed if the initial results were beyond the extrapolation potential of the standard curve. We tested for parallelism between a serially diluted pool of fecal samples and the standard curve using an analysis of covariance in order to determine the biochemical validity of the assay (ANCOVA: Main effect of Sample Type (standard or feces), F_{1,11} = 717.0, p < 0.001; Main effect of Sample Dose (nanograms of CORT or milligrams of feces), F_{1,11} = 634.27, p < 0.001; Interaction between Sample Type and Dose, F_{1,11} = 0.25, p = 0.63). The lack of a significant interaction between serial dilution and standard curve indicates that the line slopes did not differ.

**ETHICAL NOTE**

All laboratory and field procedures were approved by the University of Guelph Animal Care Committee (AUP #1418).
**Statistical Analysis**

*Measuring population density of wild Peromyscus*

Nearby 100m transects, located less than 300m away from the 100m transects used in this study, were used to assess the local density the female was experiencing at the time she was brought into the lab. These transects are part of a long-term small mammal study in Algonquin Park (Fryxell et al., 1998; Falls et al., 2007). These density lines were trapped biweekly throughout the summer, with two traps placed at 10m intervals and there were 2-3 density transects in each of the three habitat types. This created eight discrete density time periods for each habitat type. The number of captures for each two week period within one habitat type was combined and density was measured as the number of captures of deer mice per 100 trap nights. Females were brought into the laboratory every week, so the density measurement for each female represented the population density on the nearby density lines in the same habitat type either the week she was brought into the lab or the week before. In order to test for a seasonal change in population density, I regressed total density and the density in each habitat type for each two-week period against date (measured in Julian dates).

*Natural density*

The numbers of nursing observations were summed over the three days maternal behaviour was measured and divided by the total number of observations scored for that female in order to calculate the proportion of time spent nursing by that female for that litter. This resulted in a single nursing value for each female. Two females were brought into captivity more than once for successive litters, but only the first litter was used in the analysis. I tested whether natural seasonal fluctuations in density was a significant predictor of maternal behaviour using linear mixed-effect models where the proportion of time spent nursing was modeled by the population density at the time of capture (captures/100 trap nights). Despite being a proportion, the proportion of time spent nursing was normally distributed so general linear models of nursing were used. Time period was included as a random effect but a likelihood ratio test indicated that the inclusion of this random effect of time period did not significantly improve the fit of the model (likelihood ratio test: $\chi^2 = 3.17 \times 10^{-9}$, df = 1, p = 1), so it was excluded.
Only PND3 fecal samples were used in the analysis in order to remove any variability in CORT resulting from the female’s stage of gestation when she entered the laboratory (Pawluski et al., 2009). All of the fecal pellets collected from the female on PND3 were used in the analysis. CORT concentrations were log-transformed to obtain a normal distribution. There was a significant negative relationship between CORT concentrations and the mass of the fecal sample collected ($\beta = -438.25 \pm 84.42, t_{23} = - 5.19, p < 0.0001$), so the residuals of this model were used in subsequent analyses. These residuals reflect the CORT concentration after correcting for the mass of the original fecal sample collected from the female and are referred to as corrected-CORT measures. I used a general linear model to test for an effect of natural population density at the time of capture on corrected-CORT three days after parturition in captivity with time of breeding (i.e. early or late) included in the model as a covariate.

Perceived density experiment

I compared the proportion of time spent nursing and corrected-CORT between treatment and control groups using separate general linear models to test whether manipulation of perceived density affected maternal behaviour and maternal CORT. There was no significant interaction between the treatment and natural density ($\beta = 0.0018 \pm 0.005, t_{36} = 0.37, p = 0.71$), so the interaction was excluded from subsequent models however, natural density remained as a covariate. Finally, I tested whether there was an effect of corrected-CORT on maternal behaviour using a general linear model.

All statistical analyses were performed in R version 2.15.1 (R Development Core Team, 2012). Linear mixed-effect models used the nlme package (Pinheiro et al., 2011) with a Gaussian error distribution. All values are presented as means ± s.e.m. unless otherwise stated.
3.0 Results

During the 2013 breeding season in Algonquin Provincial Park, natural population density measurements of wild deer mice ranged from 0 to 26.7 captures/100 trap nights. The number of deer mice increased over the course of the breeding season overall ($\beta = 1.92\times 10^{-2} \pm 0.33$, $t_6 = 5.78$, $p = 0.001$; Figure 1) and within each of the three habitat types (cut over mixed woods: $\beta = 2.19\times 10^{-2} \pm 0.053$, $t_6 = 4.12$, $p = 0.006$; dense mixed woods: $\beta = 2.21\times 10^{-2} \pm 0.053$, $t_6 = 4.18$, $p = 0.006$; and white pine and white spruce: $\beta = 1.21\times 10^{-2} \pm 0.02$, $t_6 = 5.94$, $p = 0.001$).

Effects of natural population density

Thirty mothers spent an average of 48.3 ± 25.7% of their time nursing their litters. Females who experienced greater population densities in the field at the time of capture spent significantly less time nursing their pups as predicted ($\beta = -0.005 \pm 0.002$, $t_{22} = -2.18$, $p = 0.038$; Figure 2).

The twenty-five mothers that produced a fecal sample on PND3 had an average CORT level of 58.2 ± 221.78ng/g. There was a significant nonlinear effect of the natural density of deer mice at the time of initial capture on corrected-CORT values on PND3. Overall, there was a negative effect of density on corrected-CORT (linear: $\beta = -0.1 \pm 0.036$, $t_{22} = -2.75$, $p = 0.012$) but there was also a significant positive quadratic relationship (quadratic: $\beta = 0.004 \pm 0.001$, $t_{22} = 2.91$, $p = 0.008$; Cook’s Distance <0.05). Females that experienced very low and very high population densities at the time of capture had the highest corrected-CORT levels, whereas those that experienced moderate densities had the lowest corrected-CORT levels (Figure 3). This quadratic relationship is consistent with a significant interaction between timing of breeding and population density ($\beta = 0.087 \pm 0.041$, $t_{21} = 2.13$, $p = 0.045$; Figure 4). There was a positive effect of population density on corrected-CORT for late breeders ($\beta = 0.077 \pm 0.023$, $t_{11} = 3.40$, $p = 0.006$) but no effect of density on corrected-CORT for early breeders ($\beta = -0.102 \pm 0.035$, $t_{10} = -0.29$, $p = 0.778$). Finally, nursing was not negatively affected by CORT as we had predicted. Instead, there was a significant positive relationship between maternal stress hormones and maternal
behaviour as females with elevated corrected-CORT levels spent significantly more time nursing their pups than those with lower corrected-CORT levels ($\beta = 0.085 \pm 0.039$, $t_{23} = 2.20$, $p = 0.038$).

**Effects of experimentally manipulated density**

There was no effect of the experimental manipulation of perceived density on the proportion of time spent nursing ($\beta = 0.066 \pm 0.034$, $t_{27} = 1.77$, $p = 0.094$; Figure 5) or corrected-CORT levels ($\beta = -0.281 \pm 0.186$, $t_{21} = -1.51$, $p = 0.146$; Figure 6). Control females spent on average 44.6 ± 22% of their time nursing with an average CORT measurement of 59.5 ± 220.6 ng/g. Conversely, treatment females spent on average 51.6 ± 16.3% with an average CORT measurement of 56.9 ± 198.9 ng/g. Furthermore, the presence of the soiled bedding did not result in any significant differences between control and treatment females for any other variable measured (date into lab, natural density, lab density, length of gestation, female weight, litter size, pup weight, percent male, AGD, body size: $t_{28} < 1.98$, $p > 0.05$).

**4.0 Discussion**

*Relationship between maternal behaviour and population density in wild mice*

In contrast to previous laboratory studies that report maternal behaviour in captive mice to be repeatable within an individual, I found that maternal behaviour in wild-caught mice was plastic; specifically, the time spent nursing drastically decreased over the course of the breeding season as population density increased. This result supports the only other study on maternal behaviour in mice from the natural environment (Stewart and McAdam, 2014), and highlights the importance of studying and understanding behaviour in a natural context. Interestingly, Stewart and McAdam (2014) studied this same deer mouse population in 2011, a year in which densities increased from 5 to 45 deer mouse captures per 100 trap nights while nursing behaviour declined by almost 50%. In comparison, density peaked in the current study (in 2013) at 27 captures per 100 trap nights and nursing behaviour declined by 27% (Figure 7B). While it is possible that another seasonal variable is responsible for the change in nursing behaviour, I would then expect a consistent seasonal decline in nursing behaviour across years, independent of peak
population density (Figure 7A). The similarity in the quantitative response of nursing behaviour to population density between years of high versus low density suggests a causal relationship. Nonetheless, experimental density manipulations in the wild are needed to conclusively demonstrate the effect of population density on maternal behaviour.

To disentangle the effects of population density and season, I attempted to experimentally increase the perceived local density in my wild-caught female mice using olfactory cues. I predicted that exposure to the scents of unknown individuals would increase perceived density and cause a reduction in nursing. Furthermore, I predicted that the mechanism for this behavioural response to environmental cues would be an increase in maternal CORT levels, similar to Dantzer et al. (2013). Surprisingly, despite notable odour differences between the treatment and control bedding, there was no significant difference in maternal behaviour or fecal CORT levels between the two groups. There are two possible explanations for why the soiled bedding did not elicit the predicted response. First, it is possible that olfaction is not the correct social cue modality. Despite the considerable evidence of conspecific scent decreasing reproductive output and retarding sexual maturation in laboratory mice, including Peromyscus (Mus musculus): Champlin, 1970; Drickamer, 1974; Ma et al., 1998; Peromyscus maniculatus bairdii: Lawton and Whitsett, 1979; Lombardi and Whitsett, 1980; Creigh and Terman, 1988; Peromyscus leucopus: Rogers and Beauchamp, 1976; Terman, 1984), the results from this study clearly demonstrate that scent alone does not cause wild female deer mice to respond to local density conditions. Some studies suggest that tactile communication may also be required to elicit the physiological responses demonstrated at high population density in Peromyscus maniculatus bairdii (Terman, 1979, 1980; Lombardo and Terman, 1980). These laboratory studies show that individuals who remain in a colony without any tactile cues (i.e. kept in a "no contact" box) do not show a decline in reproductive output at high population density (Terman, 1979, 1980; Lombardo and Terman, 1980). Future studies are needed to isolate individual and/or combinations of social cues i.e. olfactory, tactile, visual and/or auditory in order to identify what cues are required to elicit a response in wild female deer mice as a result of fluctuations in population density.
Alternatively, it is possible that despite my evidence for a causal relationship between population density and maternal behaviour, maternal behaviour might also be related to another seasonally changing variable. Previous studies have identified that population density is often confounded with food availability in wild mice (Taitt et al., 1981; Wolff, 1985). To address this possibility, I conducted a food supplementation experiment during the 2013 breeding season in a subset of our population where one section of our study site received additional food over the course of 12 weeks and another section did not (Appendix 1). The food supplementation had no effect on density, thus females in the supplemented section had access to more food resources than control females. Despite an increase in food resources, there was no effect of this additional food on the proportion of time spent nursing (Appendix 1), suggesting that food resources, which might otherwise be confounded with population density, do not affect maternal behaviour in deer mice.

Another variable that changed seasonally was female mass at parturition (PND 0) and three days post parturition (PND 3) where female mass decreased significantly over the breeding season (female weight PND0: $\beta = -6.51 \pm 2.59$, $t_{28} = -2.52$, $p = 0.018$; PND3: $\beta = -6.36 \pm 3.04$, $t_{28} = -2.09$, $p = 0.045$). Female mass could also explain a significant proportion of the variation in the frequency of nursing (female weight PND0: $\beta = 0.024 \pm 0.008$, $t_{28} = 2.87$, $p = 0.008$; PND3: $\beta = 0.034 \pm 0.009$, $t_{28} = 3.87$, $p = 0.0006$) notably however, maternal weight is not considered an accurate predictor of maternal condition in Peromyscus (Millar and Teferi, 1992), but it is possible that body mass is related to time spent nursing. Finally, laboratory density might also be a seasonally changing variable responsible for the decline in maternal behaviour. As pregnant females were opportunistically brought into captivity, the number of females housed in the laboratory was also correlated with natural density (PND0: $R^2 = 0.44$, $p = 3.58 \times 10^{-5}$; PND3: $R^2 = 0.20$, $p = 0.008$), and as such increased over the course of the summer (laboratory density PND0: $\beta = 4.4 \pm 0.95$, $t_{28} = 4.61$, $p = 8.04 \times 10^{-5}$; PND3: $\beta = 3.17 \pm 1.07$, $t_{28} = 2.95$, $p = 0.006$). The number of females housed in the lab was a weak predictor of the proportion of time spent nursing (PND0: $\beta = -0.009 \pm 0.004$, $t_{28} = -2.34$, $p = 0.027$; PND3: $\beta = -0.008 \pm 0.004$, $t_{28} = -1.94$, $p = 0.062$). Future studies should
control for or manipulate housing density (number of individually housed mice in a single room) to test for the possible effects on maternal behaviour.

**Maternal behaviour, fecal corticosterone and population density**

A positive relationship between population density and stress hormone levels has been documented in the majority of vertebrate studies in both the laboratory and field (Newman et al., *submitted*) including the one other field study to investigate the relationship between population density and CORT in wild *Peromyscus* (Harper and Austad, 2004). This relationship has been attributed to the positive association between population density and the number of predators, resource competition and negative social interactions (Creel et al., 2012). Here, I report a U-shaped relationship between post-natal maternal CORT in the laboratory and natural population density at the time the female was brought into the laboratory; females captured under high and low density conditions experienced higher stress levels than females under moderate density conditions. When population densities were greater than 12 deer mouse captures per 100 trap nights there was a positive relationship between CORT and population density; stress levels increased with increasing population density. Interestingly however, when population densities were low, there was the negative relationship between population density and CORT. It is known that many social interactions induce stress in wild female deer mice (Harper and Austad, 2004; Creel et al., 2012) but it is possible that too few interactions also increase CORT. Laboratory studies reveal that laboratory mice that are removed from a colonial environment and placed into isolation experience higher stress levels than individuals who remain in the colony (Guidotti et al., 2001; Matsumoto et al., 2007), however, the data are inconsistent and currently unresolved (Brian, 1975; Hunt and Hambly, 2006; Arndt et al., 2009).

CORT was measured in fecal samples collected from females three days after parturition, but because females were brought into the laboratory at various stages of pregnancy, pregnant mice were housed in the laboratory anywhere between 1 to 20 days before giving birth (mean: 9.13 days). The CORT levels measured in the fecal samples of wild deer mice are an integrated measure of the individual's GC profile
4-6 hours prior to the sample being produced (Harper and Austad, 2000), therefore, our CORT measurements are not likely to reflect a direct response to natural population density at the time of capture. A more plausible explanation is that the U-shaped relationship between CORT and population density may be explained by the early-life environment. Early breeders in 2013 are those females that were born at the end of the 2012 breeding season, under high-density conditions, and over-wintered (OW) to reproduce the following spring (Millar, 1979). Conversely, late breeders are probably young of the year (YY), but it is also possible that some could have been long-lived OW females that were not captured until late summer (Millar, 1979; Havelka and Millar, 2004). YY would have been born in early spring 2013 under low-density conditions. Early-born Peromyscus mature and breed during the summer of their birth and typically do not survive over the winter (Millar, 1979). In contrast to OW females, YY are born at low density but tend to breed at high density. It is possible that natal density interacts with current density resulting in distinct behavioural and physiological phenotypes and that the high CORT phenotype of late summer carries over in the OW female’s physiology, but not their nursing behaviour.

There is considerable evidence for the long term consequences of early life programming on the HPA axis; maternal behaviour experienced during early life is significantly correlated with the magnitude of a stress induced CORT response as an adult (Liu et al., 1997; Caldji et al., 1998; Weaver et al., 2004; Meaney and Szyf, 2005; Hellstrom et al., 2012). Within Rodentia, laboratory studies show that the frequency of tactile stimulation through licking/grooming and nursing alters the methylation pattern in the promoter region of the glucocorticoid receptor (GR) gene within the offspring (Liu et al., 1997; Weaver et al., 2004; Hellstrom et al., 2012). The consequence of these epigenetic changes is that individuals who received high maternal care, have greater GR expression, and thus have a rapidly responding HPA axis, ultimately resulting in lower overall amounts of GC release (Liu et al., 1997; Caldji et al., 1998; Weaver et al., 2004; Meaney and Szyf, 2005; Hellstrom et al., 2012). However, in laboratory studies, the environment is intentionally kept constant, thus interpreting the link between hormone-behaviour relationships in a natural context is difficult. I propose that variation in maternal CORT is indeed related to the level of maternal care females received when they were pups, but CORT is not the mechanistic link
driving future maternal behaviour; rather maternal behaviour is related to the current and anticipated environmental conditions rather than early-life programming of the HPA axis. My data support this hypothesis as OW females born under high density conditions at the end of the previous season, who received less maternal care and were programmed with a “high CORT” phenotype, exhibited high levels of nursing to their own offspring born under low density conditions the following spring. Further, early born YY, born under low density conditions, would have received higher maternal care and had a “low CORT” phenotype, but as density increased over the breeding season, later born YY would have received correspondingly less maternal care, accounting for the positive relationship between population density and CORT later in the season. Interestingly this would also explain the positive relationship reported in the one previous field study to investigate the relationship between CORT and population density in *Peromyscus* as the study only took place over a portion of the breeding season (late July – mid October) when the majority of the samples were most likely from YY.

The adaptive nature of these physiological and behavioural phenotypes is evident when I consider the variation in environmental conditions experienced within and among individuals’ life-history. In the beginning of the breeding season, under low-density conditions, early-born offspring may have greater fitness with a “low CORT” phenotype, as there is little advantage of the more reactive “high CORT” phenotype (i.e. increased cardiac function, attentional processes, consumptive behaviour; Bonier et al., 2012; Boonstra et al., 2012). Conversely, offspring born at high population density, where they face an increased number of both predators and competitors, may have increased fitness with a “high CORT” reactive behavioural phenotype (Meaney, 2001; Bonier et al., 2012; Boonstra, 2012; Creel et al., 2012). Matching physiological and behavioural phenotypes to a changing environment could be the driver responsible for maintaining variation in maternal behaviour in a wild population. Future field experiments should investigate how maternal behaviour shapes offspring HPA axis in wild mice and quantify the fitness consequences of variation in the HPA axis.
5.0 Conclusion

In conclusion, in wild deer mice maternal behaviour is a plastic phenotype as the frequency of nursing drastically decreased over the course of the breeding season as population density increased. Despite the substantial laboratory evidence demonstrating mice responding to the scent of conspecifics, I have shown that olfactory cues alone do not cause wild female deer mice to respond to local density conditions. Future studies are needed to disentangle the effects of population density and seasonality on maternal behaviour and to investigate what environmental cues deer mice use to assess local density conditions.

Maternal CORT is not the mechanistic link between population density and maternal behaviour but rather the stress phenotype of the female may be related to the level of maternal care the female received in the early life environment as a pup. Alternatively, a female’s maternal behaviour is related to the current or anticipated environmental conditions her offspring will experience. The relationships reported in this study exemplify the ways in which wild mice are different from laboratory mice. Wild mice face this mismatch of environments where the density in which they were born can be very different from the density in which they breed. These complex relationships between natal and breeding environments might require complex systems for appropriately programming offspring for the current environmental conditions.

This project provided a rare opportunity to incorporate proven laboratory techniques with a wild population to demonstrate how females alter in maternal behaviour in response to a changing environment, a phenomena not observed in a laboratory environment. Laboratory studies have identified significant consequences of a reduced number of GRs, but little work has been done in the natural environment. The results from this study suggest that prolonged exposure to elevated CORT might not always result in a reduced fitness, as often assumed by the biomedical literature, but that programming hyper-responsive offspring might be adaptive under high-density conditions. Future studies are needed to determine how maternal behaviour affects HPA axis development in wild rodents and the fitness trade-off between HPA axis sensitivity and behavioural response under various population density conditions.
6.0 Literature Cited


Physiology & Behavior, 95(1-2), 187–93.


### 7.0 Tables

**Table 1:** Description of the behaviours and locations used to classify a female’s maternal care. Location and behaviour were categorized every three minutes during four one-hour focals for the first three nights postnatal. Behaviours were not mutually exclusive as a female could be performing multiple behaviours at a time.

<table>
<thead>
<tr>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Nest</td>
<td>Female was in physical contact with nesting material</td>
</tr>
<tr>
<td>Out of Nest</td>
<td>Female not in physical contact with nesting material</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nursing</td>
<td>It is difficult to visualize the female nursing, so anytime the female was in contact with her pups the behaviour was classified as nursing. Previous studies have found mother-pup contact and nursing to be highly correlated behaviours (Champagne et al., 2007). In addition to this, laboratory studies have determined that females are always active when in contact with their pups (Myers et al., 1989; Champagne et al., 2003; Champagne et al., 2007).</td>
</tr>
<tr>
<td>Licking/Grooming</td>
<td>Female was licking/grooming pups.</td>
</tr>
<tr>
<td>Manipulating Nest Shavings</td>
<td>The female was either retrieving nesting material or moving around existing nesting paternal with her front paws or snout.</td>
</tr>
<tr>
<td>Manipulating Non Nest Shavings</td>
<td>The female was moving around material in the cage but not retrieving the material to the nest.</td>
</tr>
<tr>
<td>Self Grooming</td>
<td>Female was licking herself.</td>
</tr>
<tr>
<td>Eating</td>
<td>Female was eating either the rodent chow or the sunflower seeds that were provided in the cage.</td>
</tr>
<tr>
<td>Drinking</td>
<td>Female was drinking from the water bottle provided.</td>
</tr>
<tr>
<td>Smelling</td>
<td>Female was smelling the air or an object in the cage.</td>
</tr>
<tr>
<td>Rearing/Climbing</td>
<td>Female was on her hind legs or climbing and biting at metal bars on the lid of the cage.</td>
</tr>
<tr>
<td>Resting</td>
<td>Female did not appear to be doing anything. Only occurred when the female was outside of the</td>
</tr>
</tbody>
</table>
nest.

Carrying Pup  Female was carrying a pup either in her paws or mouth.
Out of Sight  Female was not visible.

8.0 Figures

Figure 1: The number of wild *P. maniculatus* captures significantly increased over the course of the 2013 breeding season (Julian Date). Mouse population density was measured as the total number of deer
mouse captures per 100 trap nights on nearby 100m transects within three habitat types at the time of initial capture.

Figure 2: The proportion of time spent nursing by *P. maniculatus* females significantly declined with increasing population density (measured as captures/100 trap nights; *n*=30). The population density measurement for each female represents the local density the female was experiencing at the time she was brought into the lab.
Figure 3: There was a significant positive quadratic relationship between corrected-CORT on PND3 and population density at the time of the female’s capture. There are fewer females with corrected-CORT values as not all females produced a fecal sample on PND3 (n=25).
Figure 4: Maternal CORT levels show an inconsistent response to population density. Early breeders show no effect of population density on maternal CORT (closed circles; n=12) while late breeders show the predicted positive relationship between population density and maternal CORT (open circles; n=13).
**Figure 5:** There was no significant difference in the proportion of time spent nursing between control females (n=14) and treatment females (n=16) who were exposed to the soiled bedding of four unknown individuals. Females were exposed to the bedding during their pregnancy up until PND3.
Figure 6: There was not a significant difference in corrected-CORT values on PND3 between control females (n=13) and treatment females (n=12) who were exposed to the soiled bedding of four unknown individuals.
Figure 7: The proportion of time spent nursing decreased over the course of the season in both 2013 (solid line) and 2011 (dotted line; A). In 2011 (dotted line) the proportion of time spent nursing declined almost 50% from the beginning to the end of the breeding season compared to just over 27% in 2013 (solid line). In contrast, in both 2011 and 2013 there were remarkably consistent declines in nursing in response to a seasonal increase in population density, despite overall densities being lower in 2013 than 2011 (B).
9.0 Appendix

Effect of food supplementation on maternal behaviour

Population density is often confounded with the availability of food resources (Taitt, 1981; Wolff, 1985), so studies disentangling these two factors experimentally can identify the independent effects of each of these important variables separately (Dantzer et al., 2013). I conducted a food supplementation experiment during the 2013 breeding season as a second way to attempt to experimentally increase population density in order to disentangle the effects of density and seasonality on maternal behaviour. Starting the first week of June, 2013 I broadcasted a total of 26kg of sunflower seeds over 7.4 ha of a 20ha study grid weekly for a total of 12 weeks. A 7.8 ha control area was located adjacent to the food-supplemented grid with a buffer zone in between. Beginning May 1st, the grid was trapped biweekly with Longworth live traps placed at 20m intervals on transects that were 20m apart. Traps were baited with water soaked sunflower seeds, freeze-killed mealworms and cotton bedding for warmth. Traps were set at dusk and check the next morning at dawn. Upon capture, deer mice were removed from the trap, placed in a large handling bag and weighed with a Pesola spring balance to the nearest 0.5g. Deer mice were tagged in both ears with metal ear tags for identification. Age of the individual was determined based on dorsal pelage where juveniles are grey, sub-adults are a mixture of grey and brown and adults are brown (Schug et al., 1991). Pregnant females (>22g or obvious pear shape) from control and food supplemented populations were brought into the lab to measure their maternal behaviour. Prior to the beginning of the food supplementation, females trapped on either side of the grid prior were designated as control. After this, females were categorized based on their trapping location i.e. food supplementation or control.

Previous field studies working with wild Peromyscus have demonstrated that population density will increase with increased food availability (Taitt, 1981; Wolff, 1985), but I found no effect of the food supplementation on the local population density around each female’s home range ($\beta = 0.32 \pm 1.32$, $t_{32} = -0.243$, $p=0.81$). As a result, females on the food-supplemented population had more per capita access to food resources than females on the control population. The home range of a female was defined as the
eight traps surrounding the individual’s home trap (trap most frequently visited) three weeks prior to coming into the lab. Density was measured as the number of deer mouse captures per 100 trap nights, within those nine traps, over the three-week period. A density measurement was only calculated for females who were captured on two or more occasions on the grid in order to correctly identify a home trap.

Animals were cared for and maternal behaviour was measured using the same methods as those already described above with the exceptions that females were not exposed to any unknown scent and fecal samples were not collected.

There was no effect of this supplemental food on the frequency of nursing so maternal behaviour does not appear to depend on food resources experienced in the field during gestation prior to coming into captivity ($F_{32} = 0.009, p = 0.93$). There was also no significant difference between food supplementation and control females for any of the variables measured (days in lab, lab density, number of pups, pup weight, AGD, body length, female enter laboratory weight, parturition weight, PND3 weight: $t_{35} = <1.66, p = > 0.05$)