Phytoestrogen Effect on Maternal Nest Protection of the Rat

Kaylea Flater

University of Evansville

Supervisor:

Dr. Lora Becker

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Personal Relevance Preface

After I receive my degree from the University of Evansville, I plan to attend graduate school and obtain a doctorate in veterinary medicine with specializations in surgery and possibly research. With the specialization in research I will be able to delve into the realm of finding cures for animal diseases. As a veterinarian I will work with all types of animals, and after completing my clinical rotations in veterinarian school, I will be able to declare which classification of animals with which I would like to work.

Through experience working in the laboratory, I have gained the ability to infer meaning and relationships from my collected data; this is an invaluable tool to a veterinarian. Animals are not able to verbally express how they feel; therefore, a veterinarian must be able to look at symptoms and properly assess those symptoms to provide accurate treatment. Another benefit from conducting research is the hands-on experience working with the rats. I have learned how to properly administer food and water, how to clean cages, and how to maintain an ideal climate through the use of temperature control and light-dark cycles.

The purpose of my thesis is to analyze and compare the maternal nesting behaviors of rats on a phytoestrogen depleted diet and rats on normal rat chow. More specifically, I will be observing mother-pup interactions through techniques of direct observation, nest scattering, and ultrasonic vocalizations of the pups from each food condition.

Introduction

Prior to birth, rat embryos are dependent upon the maternal circulatory system to provide for nutritional, immunological, and excretory needs. More importantly, the fetus is dependent upon the mother to provide thermoregulation and protection (Robinson & Smotherman, 1992). After birth, the natural environment for rat pups consists of the nest area, its littermates, and mother (Shair, 1991). This indicates that rat pups are highly dependent upon maternal behavior as a source of nutrition, water and salt balance, heat, and protection (Alberts & Cramer, 1988).

For the first seventeen days after parturition the mother is the sole resource for fluid and food for the pups (Alberts & Cramer, 1988). For the first week after birth the pups wait for the dam to come to the nest in order to feed. Around their second week of age, and up until weaning, the pups will go in search of the dam when they are hungry. However, after approximately 21 days the needs of the pups exceeds what the dam can feasibly provide; therefore weaning begins. The dam will push her ventral side against the cage floor, forcing the pups to find nutrition elsewhere (Kristal, 2009).

Beyond the obvious necessities of food and fluids, the mother also provides a safe environment for her young. The nest of a pregnant female will be constructed just prior to parturition (Denenberg, Taylor, & Zarrow, 1969). The nest of a pregnant rat has steep slopes compared to the flat nest of a non-pregnant female (Kristal, 2009). The structure of the nest is designed to keep pups huddled together when the mother is absent. This huddle retains body heat, which aids in thermoregulation. The older the pups become the less time the dam spends in the nest, which means the pups must rely more heavily on each other to maintain their body heat (Alberts & Cramer, 1988). The initial reliance on the nest is essential in the survival of the pups. Denenberg, Zarrow, & Brandt (unpublished) found that dams forced to give birth on wire mesh screen with no nesting material yielded no surviving pups as compared to a 67% survival rate when the dams gave birth on wire mesh screen but were supplied with the desired nesting materials (Denenberg et al, 1969).

In infancy, the pup heavily relies on the nest, but more importantly, on the mother in order to survive. The moment the pup emerges from the birth canal it is licked by the dam. This accomplishes two necessary tasks: cleaning the pup and providing it with tactile stimulation, without which 40% of newborn pups will not survive (Alberts & Cramer, 1988). Throughout the first weeks of life the mother will alternate between bouts of nest attendance and foraging, resting, and grooming time. She is then brought back to the nest to provide thermal care and stimulation to her pups (Moore, 1992).

The mother stimulates her pups through anogenital licking. There are multiple benefits to anogenital stimulation in rat pups. This act helps aid in waste elimination and also helps the mother maintain her fluid balance (Kristal, 2009). Dams spend more time performing anogenital licking than body licking, specifically during the first two weeks of the pups lives when this stimulation is required in order for urination and defecation to occur (Moore & Morelli, 1979). Males receive more anogenital licks than do females during this critical period because of the testosterone secreted in the males urine (Moore, 1982). The chemical stimuli in urine are important for eliciting maternal behavior in particular pups within the litter. Male urine is more effective at eliciting these responses than female urine (Moore, 1992). This conclusion is supported by the fact that when one drop of male urine is placed on a female's skin she is licked more frequently. Also, when females are injected with testosterone at birth there is no significant difference in the amount of licking of males or females (Moore, 1981). A decrease in masculine behavior is observed in adult males that received a low level of anogenital stimulation as a pup (Moore, 1982).

Anogenital licking also contributes to less anxious individuals in adulthood. Higher levels of licking cause a rat to be less anxious than a rat receiving very little licking during infancy (Wöhr, 2008). Anxious pups are most likely to be retrieved when they emit high amounts of ultrasonic vocalizations after wandering away from the nest. Ultrasonic vocalizations are emitted above an audible frequency range around 50-70kHz and have a duration of less than 60ms. (Sánchez, 2003). The frequency of ultrasonic vocalizations steadily increase during the first 5-6 days after postpartum and peak around postnatal day 6 or 7, after which they steadily decrease and disappear around postpartum week two (Branchi, Santucci, Alleva, 2001). Ultrasonic vocalizations are produced by a whistle-like mechanism in the larynx by air passing outward against the closed glottis (Brudzynski, 2009).

Ultrasonic vocalizations are a way of measuring anxiety in both the pups and the dam (Wöhr, 2008). Ultrasonic vocalizations elicit maternal behaviors such as retrieval, anogenital licking, and milk letdown in lactating females (Hashimoto, Saito, Furudate, & Takahashi, 2001). When the pups emit ultrasonic vocalizations the mother will retrieve them by using her mouth. She grips them around the neck and the pups go into a relaxed hanging position in order to be transported back into the nest (Kristal, 2009).

Maternal behaviors are maintained by estrogen; however, the role of phytoestrogens on maternal behavior is not well documented. One prevalent family of phytoestroges is lignans. This family of phytoestrogens can be acquired through the consumption nuts, breads, legumes, fruits, vegetables, soy produces, processed foods, and many types of beverages (Thompson, L. U., B. A. Boucher, Z. Liu, M. Cotterchio, & N. Kreiger, 2006). Another prevalent family of dietary phytoestrogens is the isoflavonoids. This family is typically consumed in legumes, soybeans and soybean based products (Mazur, Wahala, Rasku, Salakka, Hase, & Adlercreutz 1998). Isoflavonoids is the family of phytoestrogens found in standard rat chow. Limited research indicates that phytoestroens are able to bind to estrogen receptors (Whitten & Patisaul, 2001), and that exposure to phytoestrogens are capable of altering developmental and basic reproductive processes (Sheehan, 1998). Depending on the concentration, phytoestrogens contain the potential to either enhance or suppress proliferation in estrogen-responsive cells (Whitten & Patisaul, 2001).

Circulating estrogen induces maternal behavior by increasing the number of estrogen receptor-α in the medial preoptic area (MPOA) of the hypothalamus. Throughout the progression of pregnancy the number of estrogen receptors in the MPOA increase (Champagne, Weaver, Diorio, Sharma, & Meaney, 2003). Estrogen increases oxytocin receptor binding (Champagne, Diorio, Sharma, & Meaney, 2001) by increasing the oxytocin receptor mRNA levels in the hypothalamus (Breton & Zing, 1997). Increases in oxytocin receptor levels increased maternal responsivity and increased the frequency of licking/grooming of the pup (Champagne et al, 2001). Differences displayed in maternal licking/grooming are transmitted to female offspring through tactile stimulation (Champagne, Weaver et al, 2003)).

There are naturally occurring variations in the maternal care provided by the dam. These naturally occurring variations impose consequences on offspring because offspring of high licking/grooming dams are themselves high licking/grooming mothers. Low licking/grooming dams are often fearful and in turn bear offspring that are fearful, anxious, and will be low licking/grooming mothers (Champagne, Francis, Mar, & Meaney, 2003). The high licking/grooming dams will reduce their licking/grooming frequency when placed under large amounts of gestational stress. This decrease in frequency can even affect the dam's subsequent litters (Champagne, Francis et al, 2003).

In this study I examined the maternal behaviors of dams as a result of being placed on normal rat chow or phytoestrogen minus rat chow at the time of breeding. The experimental group in this study was fed soy-free rat chow low in phytoestrogens while the control was fed normal soy rat chow. I examined nest building, retrieval behaviors, and mother-pup interactions. The results suggest differences in maternal behaviors as a result of a low phytoestrogen diet; these differences are most notably observed by the retrieval behaviors of the two dietary groups.

Methods

Subjects

A total of 8 female and 8 male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) were used to produce 8 litters using time-mated pairs in the Behavioral Neuroscience Laboratory at the University of Evansville. The subjects were housed under constant room temperature (25-26ºC), constant humidity (30-50%), and on a 12-hour light: 12-hour dark cycle (lights off at 1000 hours) throughout the experiment. All breeding rats were tattooed prior to the experiment. For breeding, the adult rats were housed in groups of two females and one male in a plastic cage measuring 36 X 23 X 20 cm for five days. Vaginal smears were collected each day, and the first day of detectable sperm designated embryonic day 1. Each female was housed individually and litters were kept with the dam until weaning on postnatal day (PD) 1.

All litters were culled to 10 pups, 5 males and 5 females, on PD 1 to ensure equal availability of nutrients for all pups. Culling the pups involved decapitation and then storage of the carcass in the freezer until they were sent to the incinerator at the Evansville Animal Shelter. The pups were weaned on PD 21. At the end of testing, adult rats were euthanized by CO_2 and stored in the freezer until they were sent to the incinerator at the Evansville Animal Shelter

Materials and Procedures

dietary exposure.

Pregnant females (total =8) were placed on either normal rat chow or rat chow with very low levels of phytoestrogens (Harlan Teklad Global Diet Program, Madison, WI) at day 1 of breeding and continued on this diet throughout the entire research period. All subjects were supplied with unlimited supplies of food and water.

nest dimensions.

The dams were placed in a new cage five days and one day prior to delivering her pups. The nest she made in a 25 minute time period was compared to the nest of her original cage. These dimensions were also used to compare nests of the two different food groups.

retrieval behavior.

On days 1, 3, and 7, during the dark cycle in a separate lit room, the dam was removed from the home cage and the pups were scattered around the floor of the cage, each placed in the same position every time (Figure 1). The mom was placed in the nest and was timed for 10 minutes for the collection period. The behaviors of the dam were video recorded and the tapes were analyzed at a later date looking for differences in food groups, differences between the days for moving pups back to the nest, and differences in her collecting behavior toward male and female pups. Differences in grooming and smelling were also analyzed between the different food groups and for the different days.

observable mother-pup interactions.

On days 2, 4, and 6 all litters were observed for 15 minutes under a red light and using a video recording device for observing. This was done in a location other than the colony room. The videotapes were later analyzed looking for differences between food groups and differences between days in pup interactions, eating/drinking, and grooming.

statistical analysis.

In order to analyze retrieval behaviors, histograms were constructed for postnatal days 1, 3, and 7 showing pup retrieval as a function of time. Basic statistical comparisons illustrated mother-pup interactions on postnatal days 2, 4, and 6, for dams on normal chow and low phytoestrogen chow using a multivariate ANOVA .

Results

nest dimensions

No difference was observed in nest building for the two treatment conditions. None of the females, regardless of their dietary condition, built a nest on prenatal day 5 or prenatal day 1.

retrieval behavior

There were no significant differences between the two dietary conditions for retrieval of male and female pups; however, retrieval behavior differed between the two treatment conditions and also differed based on the day of the scatter. Overall, dams on normal chow collected all of their pups in 22% of the trials while dams on low phytoestrogen chow collected all of their pups in 47% of the trials. On day 1 the dams on normal chow collected 53% of their pups while the dams on phytoestrogen minus chow collected 40% of their pups. Day 1 also indicated a preference for collecting pups at the last half of the 10-minute time period (Figure 2). On day 3 the dams on normal chow collected 60% of their pups while the dams on phytoestrogen minus chow collected 58% of their pups. On day 3 the dams, regardless of their dietary level, gathered their pups fairly evenly throughout the entire 10-minute time period (Figure 3). On day 7 the dams on normal chow collected 13% of their pups while dams on phytoestrogen minus chow collected 30% of their pups. On day 7 very few dams, independent of their dietary level, collected even 1 pup during the allotted 10-minute interval (Figure 4).

observable mother-pup interactions

There were no differences in grooming, nutritional consumption, or mother-pup interactions between the two dietary conditions. There were also no notable differences in these behaviors between the different days of testing. The dam spent equivalent amounts of time carrying out these behaviors on days 2, 4, and 6.

Discussion

Results of the present study suggest that changes in phytoestrogen content of the dam's diet can alter maternal behaviors associated with pup retrieval. Dams placed on rat chow low in phytoestrogens collected their entire litters more frequently than did the dams on normal rat chow. The two dietary groups were, however, statistically equivalent in the total percentage of pups retrieved. The total percentage of pups retrieved incorporated the retrieval behaviors of all dams, not just those that collected all of their pups.

The difference in retrieval behavior between the two dietary conditions may be contributed to anxiety. Estrogen has been shown to decrease anxiety in rats (Walf & Frye, 2007). The dams placed on normal chow are considerably less anxious than those on low phytoestrogen chow. The dams on normal rat chow did not collect all of their pups as frequently as the dams on the low phytoestrogen chow. This retrieval behavior can also be attributed to the pup's behavior. Pups placed on low phytoestrogen chow prior to birth produce high numbers of ultrasonic vocalizations when isolated from the dam (Becker, Kunkel, Brown, Ball, & Williams, 2005).

The retrieval behaviors, independent of the dietary variable, were dependent upon the day of the scatter. Only 3 of the dams collected their pups on postnatal day 1. The mothers were also more likely to interact with their pups in the last five minutes of the ten-minute time period. A possible explanation is that the dam is not heavily invested in her pups at this point. She would not lose an abundance of time and resources if a predator were to consume all of her offspring on postnatal day 1. On postnatal day 3, 4 of the dams collected all of their pups. Day 3 also indicated a high level of mother-pup interactions. The dams were likely to collect their pups throughout the entire 10-minute time period because of a significant amount of resource and time investment. On postnatal day 7, only 2 dams collected all of their pups. This day also showed low levels for a dam retrieving even one of her pups. This result is likely contributed to the increase in activity of the pups. By day 7 the pups are able to crawl out of the nest on their own; therefore, the scatter was probably not anxiety inducing because of its normalcy. Wöhr (2008) found similar results; the retrieval rate of pups was highest around postnatal day 4 when 5 out of 6 dams retrieved all of their pups and on postnatal day 10 the retrieval rate was significantly lower, only 2 out of 6 dams collected all of their pups.

This study found that maternal behavior between the two dietary conditions does differ in stressful situations. During the 10-minute retrieval period the dam did not collect her male pups before collecting her female pups. Moore (1979) found that a dam spends more time licking and grooming her male pups due to the testosterone found in their urine. This higher level of interaction with male pups led to the hypothesis that the dam would collect the males before collecting her female pups in a stressful situation.

Analysis of the 15-minute observational periods does not indicate that the phytoestrogen consumption by the dam alters interactions with her pups. Champagne, Weaver, Diorio, Sharma, and Meaney (2003) concluded that estrogen increases maternal behaviors in the dam. This finding supported the hypothesis that dams on normal rat chow would interact more frequently with their pups than dams on low phytoestrogen chow. This study found that the dams, regardless of their dietary condition, interacted with their pups, groomed, and met nutritional needs equally. Her interactions with male and female pups were not analyzed during this observational period. There is a possibility, however, that the dams on low phytoestrogen chow interacted with her pups an equivalent amount of time as those dams on normal rat chow; however, the phytoestrogen minus dams may not have provided as much tactile stimulation as those dams on normal rat chow.

This study does not support the idea that phytoestrogens affect mother-pup interactions and it also does not support the idea that phytoestrogen consumption by the dam alters her ability to build a sufficient nest. Regardless of her condition, no dam built a nest on prenatal day 5 or prenatal day 1. A pregnant rat does not construct her nest until right before parturition (Denenberg, 1969). There is a significant possibility that on prenatal day 1 the testing was still not close enough to the time of parturition for the dam to build her nest during the given 25-minute time period. Due to lack of time for completing this study, only half of the dams underwent the nest dimension testing. The main reason for lack of time was that too many pups in a litter were dying, making the litter unusable. Also, the dams were not having their pups on day 21 after insemination like expected. Because of this, data for nest scattering for all dams was not able to be collected.

In this study, the high number of pup deaths is most likely explained by the anxiety of the dam. The first four dams that were used in this study, two of which were not used for data collection because of pup death, were placed in a room on a different light/dark cycle than the breeding room. Abrupt and severe changes to light-dark cycles causes a high level of stress (Van der Meer, Van Loo1 & V. Baumans, 2004), which causes a decrease in licking and grooming behaviors performed by the dam (Champagne, Francis et al, 2003). Without adequate levels of tactile stimulation 40% of pups will not survive (Alberts & Cramer, 1988). For the entire duration of this study the cages of the rats were being changed every 7-10 days. The effects of changing the environment this frequently has been shown to cause large amounts of stress to the mother. Research indicates that changing cages every 14 days to every 21 days is less stressful on the dam and will therefore cause a decrease in pup death. By decreasing the frequency of cage changes, pup death decreased from 3.4 ± 0.7 to 1.3 ± 0.4, a statistically significant difference (Reeb-Whitaker et al, 2000). The use of clear cages for this study provided additional stressors for the dam. Having direct exposure to humans and no visual covering increased her anxiety levels. However, for this study the use of clear cages was essential to for videotaping mother-pup interactions on days 2, 4, and 6. A possibility for future research is to partially cover the cages when testing is not occurring.

In conclusion, this study found that phytoestrogen consumption as a result of the dam's diet alters maternal behavior. When placed in a stressful situation, dams on a low-phytoestrogen diet were more likely to retrieve their pups than dams placed on normal rat chow. The anxiety level of the dam explains this difference. Phytoestrogens bind to estrogen receptors and are naturally calming to the dam; therefore, in a stressful situation the dams on normal rat chow are less likely to become anxious. The correct balance of maternal behaviors is essential to pup survival because without the necessary amount of anxiety, tactile stimulation, and maternal responsiveness the pups will not reach a viable age.

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1		3		nest
	8		10	
				9
	2		4	
6		7		5

Figure 1. Pattern used for nest scatter in order to observe retrieval behavior on postnatal days 1, 3, and 7.

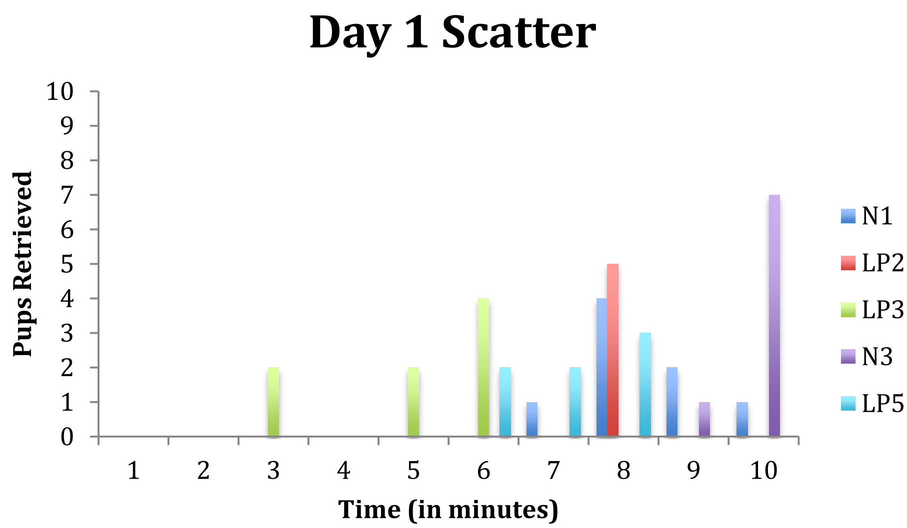

Figure 2. Number of pups retrieved in a ten minute time period on postnatal day 1 by dams on normal rat chow and dams on phytoestrogen minus rat chow. Dams on normal rat chow collected 53% of their pups while dams on phytoestrogen minus rat chow collected 40% of their pups.

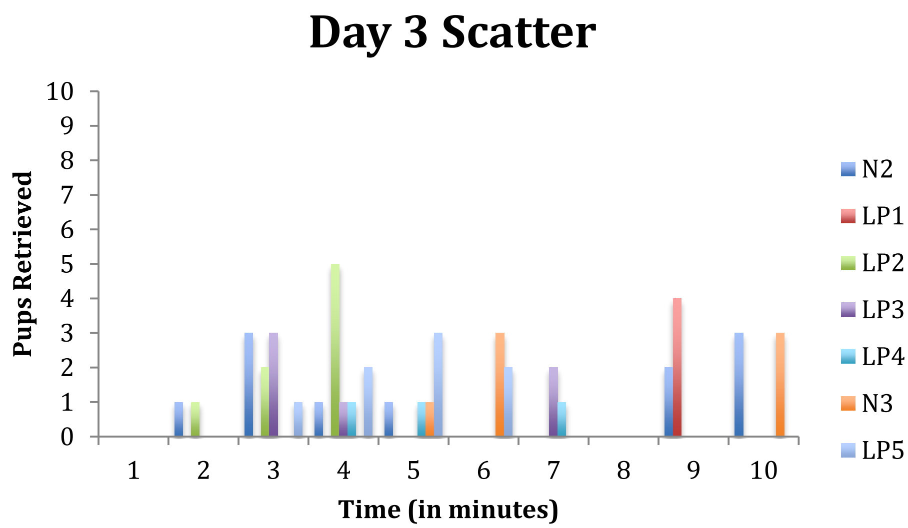

Figure 3. Number of pups retrieved in a ten minute time period on postnatal day 3 by dams on normal rat chow and dams on phytoestrogen minus rat chow. Dams on normal rat chow collected 60% of their pups while dams on phytoestrogen minus rat chow collected 58% of their pups.

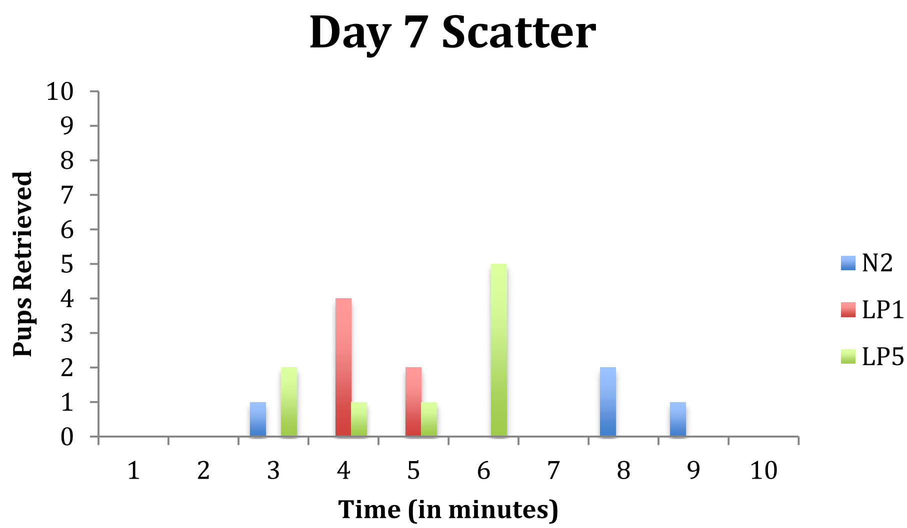

Figure 4. Number of pups retrieved by dams on postnatal day 7 by dams on normal rat chow and dams on phytoestrogen minus rat chow. Dams on normal rat chow collected 13% of their pups while dams on phytoestrogen minus rat chow collected 30% of their pups.