FELINE ODOR-INDUCED ANXIETY IN POST-PARTUM FEMALE RATS

By

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A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN PHARMACOLOGY AND TOXICOLOGY

WASHINGTON STATE UNIVERSITY
Graduate Program in Pharmacology and Toxicology

May 2010

To the Faculty of Washington State University:

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FELINE ODOR-INDUCED ANXIETY IN POST-PARTUM FEMALE RATS

Abstract

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Post-partum mood disorders, including depression and anxiety, occur in 10-15% of mothers. It is suspected that reproductive hormones play a role in the severity of these disorders; for example, in women, correlations have been reported between low post-partum estrogen and progesterone levels and depressive symptoms. In this study, we tested anxiety levels in rats in the post-partum period using a feline odor stimulus. Rats innately respond to the presence of feline odor by displaying various anxiety-like behaviors. We hypothesized that post-partum rats – those that have been pregnant and those that have undergone a hormone-simulated pregnancy (HSP) – would display greater anxiety-like behaviors than control rats to feline odor. To generate previously pregnant rats, females were paired with a male, while control rats were paired with another control female, and separated on day 14. HSP rats were ovariectomized and given one of three injection regimens lasting the length of pregnancy (22 days) – an estradiol withdrawal model (HSP-E) which models a typical rat pregnancy, a progesterone withdrawal model (HSP-P), or vehicle treatment (control). Rats were habituated to the test chamber the day before testing, and then tested the next day (post-partum day 2, 4, or 7) with a piece of cat collar that had been worn by a cat. Post-partum rats showed significantly less anxiety-like behavior

than control females on post-partum days 2 and 4. In contrast, the HSP-E and -P groups did not display greater anxiety-like behavior than the control group. These results do not support the original hypothesis; rather than showing increased anxiety during the post-partum period, post-partum females have a blunted anxiety response to predator odor. Furthermore, the HSP results suggest that estradiol and progesterone withdrawal alone do not account for significant changes in anxiety during the early post-partum period.

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1. Introduction

Post-partum mood disorders, including depression and anxiety, occur in 10-15% of mothers (Cox et al., 1993). It is suspected that gestational stress and also hormone changes play a large role in the severity of these disorders. Post-partum mood disorders are serious, not just because of the negative impacts on the mother, but also because of the possible impairment of care to the infant, as well as lasting mood effects that may later be displayed in the infant (Smith et al., 2004).

1.1 Factors That May Influence Anxiety Levels Post-Partum

Many factors can influence anxiety and depression in the post-partum period. Stress during pregnancy can increase corticosterone levels via activation of the hypothalamic-pituitary-adrenal axis, which can both impair infant care and contribute to mood-related behavioral changes in rodents (Brummelte et al., 2006; Bosch et al., 2007). Even in the absence of stress, many changes occur within the mother's body during the early postnatal period. Several reproductive hormones are dramatically changing while the immune system is in effect resetting itself. Estradiol, the major estrogen in humans, which impacts reproductive functions as well as bone structure; as well as progesterone, the major progestogen in humans, which is involved in the menstrual cycle, pregnancy and embryogenesis, both dramatically decreases from child birth to the early post-partum period (Abou-Saleh et al., 1998; Bloch et al., 2003). In contrast, oxytocin and prolactin, peptide hormones associated with uterine contraction and lactation, respectively, increase dramatically during this same time period (Rhoades and Pflanzer, 2003). Finally, the immune system is modified during pregnancy to avoid an immune response provoked by the fetus, and the surge of proinflammatory cytokines such as IL-6 in the post-partum period has been linked to depression-like behaviors in mice (Chourbaji et al., 2006).

1.2 Effects of Post-partum Hormone Withdrawal on Depression and Anxiety

There is some evidence that changes in each of the reproductive hormones noted above can alter mood. For example, prolactin, which is high in nursing mothers and is a regulator of lactation and maternal behavior (Mann and Bridges, 2003), may decrease depression-like behavior in rodents (Neumann, 2003). In humans, multiparous breast-feeding mothers were less likely to develop depressive symptoms than mothers that bottle-fed with formula (Mezzacappa and Endicott, 2007), suggesting that continued increased levels of prolactin, which occur during the period when mothers are nursing, may decrease depressive symptoms.

In women, low post-partum estrogen and progesterone levels have been correlated with depressive symptoms (Ahokas et al., 2001; Harris et al., 1994). Additionally, women with a history of post-partum mood disorders experienced relapse after a pharmacologically-induced ovarian hormone withdrawal (Bloch et al., 2000). Progesterone has been shown to have anxiolytic effects caused by its metabolic derivative allopregnanolone, which is known to act as a GABA agonist (Nappi et al., 2001). Therefore, during the withdrawal from estradiol and progesterone that occurs post-partum, an increase in anxiety may result. For example, Lofengren and colleagues (2006) showed that rats undergoing progesterone withdrawal displayed an increase in anxiety-like behavior compared to control rats when tested in an open field. Estrogens have also been administered as a successful treatment in patients with post-partum depression (Gregoire et al., 1996), and estradiol administered systemically or directly to the amygdala has been shown to decrease anxiety-like behaviors in ovariectomized rats (Frye and Walf, 2004).

Changes in anxiety-like behaviors have been found during the post-partum period in rats; however, the evidence is conflicting: some studies report decreased anxiety-like behaviors in

post-partum rats (Lonstein, 2005; Pereira et al., 2005; Byrnes and Bridges, 2006), while other studies report increased anxiety-like behaviors during the post-partum period (Byrnes and Bridges, 2006; Lofgren et al., 2006). The discrepant findings may reflect differences in number of previous pregnancies, individual experiences during pregnancy and post-partum periods, different time points used in the post-partum testing, as well as many other variables. For example, Lonstein (2007) found that post-partum rats spent significantly less time freezing during an acoustic startle test only in the presence of their pups, suggesting that factors such as mother-infant interaction may play a role in post-partum anxiety. The precise role that each of the reproductive hormone changes plays in regulating anxiety during the post-partum period has not been determined.

1.3 Feline Odor-Induced Anxiety

Felines are a natural threat to rats. Even laboratory rats with no previous exposure to cats respond to feline odor as if it were threatening, showing specific physiological and behavioral changes described as defensive and anxiety-like, such as increases in blood pressure, decreases in exploration, and avoidance of the odor (Dielenberg and McGregor, 2001). The presence of cat odor also activates the immediate early gene c-FOS in the accessory olfactory bulb and its projection areas, which are associated with defensive behaviors and increased escape attempts (Staples et al., 2008). Repeated stressful experiences affect rats' responses to feline odor. For example, stressed rats exhibit blunted responses to feline odor when compared with non-stressed rats (Perrot-Sinal et al., 2004). Assuming that pregnancy itself is a stressful event, the feline odor test can be used to determine whether females' anxiety levels are altered during the post-partum period. The feline odor anxiety test entails a more ethologically relevant, specific threat than do other, albeit simpler anxiety tests such as the elevated plus maze or open field test. The

presence of feline odor simulates a direct predatory threat, perhaps especially significant to dams who must protect their pups in addition to themselves.

1.4 Purpose

The experiments described herein were designed to test the hypothesis that post-partum rats – those that have been pregnant and those that have undergone hormone-simulated pregnancy (HSP) – will display greater anxiety-like behaviors than control rats to feline odor. Such a result would suggest that anxiety-like behaviors are regulated by hormone state, as early post-partum females are undergoing rapid hormone changes. Gonadally intact post-partum females, two separate HSP groups, and control females were used. The gonadally intact post-partum females were used to characterize anxiety response to feline odor in previously pregnant ("normal") females. The HSP groups were used to detect differences in anxiety behavior specifically due to withdrawal from estradiol vs. progesterone, two ovarian hormones whose levels drop dramatically at parturition.

2. Materials and Methods

2.1 Subjects

Female Sprague-Dawley rats (bred in-house from Taconic stock), 3-5 months old at the beginning of the study, served as subjects. Rats were housed in rooms maintained at 21.5 ± 2°C with a 12 hr light/12 hr dark cycle (lights on at 0600 hr). Access to food (Teklad rat chow) and water was available *ad libitum*, except during surgery and testing. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1986). Domesticated cats were used to obtain feline odor.

2.2 Apparatus

The apparatus consists of a Plexiglas enclosure (length = 60 cm x width = 29 cm x height = 36 cm) with an alligator clip at one end; this large enclosure contains within it an opaque "hide box" (length 24 cm x width 25 cm x height 23 cm), with a central opening for entering and leaving (5 cm x 5 cm); the "hide box" occupies the back ~1/3 of the large enclosure opposite the end with the alligator clip. The alligator clip holds a 1-cm piece of nylon cat collar.

2.3 Procedure

2.3.1 Experiment 1

To generate post-partum females, rats were first numbered and weighed. Half of the females were paired with a male ("mated"), and the other half of the females were paired with each other ("unmated" controls). Siblings were not paired together. All female rats were handled daily between 11 am-2 pm by grasping the base of the tail and lifting them up briefly while they were in their cage and the cage was on the rack (to avoid extra stress induced by carrying), until mated rats began to "bulge" (look definitively pregnant). This was usually observable approximately 2 weeks after pairing the rats. For any rat that did not look pregnant by 2 weeks, the male was left in for one more week. The date when the males were removed from the mated females was recorded; a pair of "unmated" females was separated on the same date so that post-partum and control females spent the same amount of time singly housed before testing. Handling of separated (and other) females continued daily between 11 am-2 pm. Once nesting behavior was observed, checks for pup births occurred 3 times/day, with handling once/day: 7-9 am (check but no handling), 11 am-2 pm (check and handling), and 5-7 pm (check but no handling). Unmated females were handled on the same schedule, to ensure equal experience. The date and time that pups were first observed was then recorded. Date of testing

was calculated as follows: "Day 2" was 40-56 hr (48 hr ± 8 hr) after pup birth; "Day 4" was 3.5 to 4.5 days (96 hr ± 12 hr) after pup birth; and "Day 7" was 6.5 to 7.5 days (168 ± 12 hr) after pup birth.

Non-pregnant control females as well as post-partum females were habituated and tested according to the protocol shown in Table 1.

Table 1. Treatment Groups for Experiment 1

Treatment Group	Days Post-Partum		
	Habituation	Test	
Control	1	2	N=8
	3	4	N=8
	6	7	N=8
Post-partum	1	2	N=13
	3	4	N=8
	6	7	N=8

2.3.2 Experiment 2

In Experiment 2, all rats were ovariectomized. Rats were anesthetized with Equithesin i.p. (active ingredients: sodium pentobarbital, 28.2 mg/kg and chloral hydrate, 123.3 mg/kg). Rats that did not reach a surgical plane of anesthesia after initial injection were given a "booster" injection of ~20% of their initial dose. Ovaries were located by palpation. Both sides of the back (in the thoracic-lumbar region) were shaved and scrubbed with chlorhexidine and 70% ethanol. A small incision (1-cm) was made through the skin, connective tissue and muscle layer. The ovary, associated fat pad, fallopian tube and upper uterine horn were exteriorized. The blood supply to the ovary was tied snugly with a 5-0 nylon suture. The ovary was cut and discarded, leaving the uterus intact. The muscle wall was closed with 3.0 chromic suture. Skin incisions were closed with 4-0 nylon suture and Nexaband liquid topical tissue adhesive (Veterinary

Medical Supply, Devore, CA). Estradiol-filled Silastic capsules were implanted s.c. between the shoulder blades in some rats.

Beginning the day after ovariectomy, rats were treated daily with safflower oil vehicle (V) or an HSP treatment regimen using estradiol benzoate (E) and progesterone (P) for 22 days. Then injections ceased, and feline odor anxiety was tested on "post-partum" days 2, 4, or 7. The hormone injection schedule is shown in Table 2. The HSP-E regimen models a typical rat pregnancy (McCarthy, 1997) and specifically tests the effect of estradiol withdrawal on anxiety during the post-partum period. The HSP-P regimen partially models a human pregnancy in which progesterone remains elevated throughout pregnancy (Austin and Short, 1984), and thus specifically tests the effect of progesterone withdrawal on anxiety during the post-partum period. These two separate hormone regimens were employed so that any variations in anxiety-like behaviors due to withdrawal from each of these hormones could be examined.

Table 2. Hormone Treatment Regimen for Experiment 2

Treatment Group	**Injection**	**Days 1-16**	**Days 17-22**
Control (Vehicle) (N=8/day)	Vehicle + Vehicle	0.1 ml 0.3 ml	0.1 ml --
	Or + Vehicle	1-mm blank capsule implant 0.3 ml	0.3 ml
HSP-Estradiol Withdrawal (HSP-E) (N=8/day)	Estradiol Benzoate + Progesterone	2.5 µg / 0.1 ml 4 mg / 0.3 ml	50 µg / 0.1 ml --
HSP-Progesterone Withdrawal (HSP-P) (N=8/day)	Estradiol Benzoate + Progesterone	1-mm E capsule implant 1 mg / 0.3 ml	4 mg / 0.3 ml

For rats in the HSP-E group, hormone replacement was achieved by daily E (2.5 µg on days 1-16 and 50 µg on days 17-22) plus P (4 mg on days 1-16) administered s.c. For the HSP-P group, an E capsule was implanted at the time of ovariectomy, and P injections (1 mg on days 1-

16 and 4 mg on days 17-22) were administered s.c. An E continuous release capsule was implanted s.c. in the HSP-P group to ensure that progesterone receptors, some of which are estradiol-dependent, were maintained (Leavitt and Blaha, 1972). The E capsule was not removed during the hormone withdrawal phase, so that only P was declining during this phase.

2.3.3 Behavioral Testing

Rats were observed for different anxiety-like behaviors with and without the presence of feline odor. A domesticated cat wore the test collar for 2 weeks; the control collar was not worn. The collars were then cut into 1-cm pieces, placed in a freezer for at least 2 days, and allowed to warm to room temperature for 1 hr prior to use. Each test day a new segment of collar from the freezer was used.

On the first day of testing (habituation), between approximately 12-3 pm, a control (no cat odor) collar piece was placed into the alligator clip. The rat was placed into the enclosure, facing the alligator clip/collar (face within 5 cm of the clip but not touching it), and the rat's behavior was videotaped for 15 min. After each rat was habituated, the apparatus was wiped down with 70% ethanol.

The next day at approximately the same time, a piece of test (cat odor) collar was placed into the alligator clip. The rat was put into the enclosure, facing the alligator clip/collar (face within 5 cm of the clip but not touching it), and the rat's behavior was again videotaped for 15 min. After testing each rat, the apparatus was wiped down with 70% ethanol.

2.4 Euthanasia and Tissue Collection

After behavioral testing was completed, rats were euthanized and vaginal cytology samples were collected. In Experiment 2, the uterus was also collected from all rats and placed in 10% formalin for at least two weeks. The uterus was then trimmed, blotted, and weighed, to

confirm that hormone treatments produced the expected effects. Vaginal cytology was evaluated under light microscopy to determine estrous cycle stage based on the predominant cell type (Freeman, 1988; Sell et al., 2000); this provided another way to compare/contrast hormonal differences among groups.

2.5 Data Analysis

Behaviors were scored blind to treatment group, from the videotaped tests to the nearest second in time spent: inside the hide box, in the "head-out" position, in contact with the collar, and grooming. Time spent inside the hide box (concealed) is defined as time during which the rat's body (all four paws) is completely inside the box, and stops when the rat's body has completely left the box. Time spent in the "head-out" position is included in time spent in the hide box. "Head-out" is defined as the nose protruding from the entry to at least the level of the eyes (not just the tip of the nose). Time spent in contact with the collar includes sniffing, licking, biting, and touching the collar. Time spent grooming includes grooming while inside or outside of the hide box.

Additionally, the number of rears inside and outside the hide box were counted. A rear was recorded each time the rat stood on its hind legs and its head reached the level of the top of the hide box.

When scoring the cat-odor collar tests, each behavior was scored after the initial contact with the collar (which usually lasted a few seconds), and then behaviors were compared between habituation and test sessions as percent time spent after the initial contact with the worn collar. The data were handled in this manner because there is no change in rat behavior in the presence of the worn collar until *after* the rat has contacted the collar.

Behavioral responses were compared between the absence vs. presence of feline odor (habituation vs. test sessions) in control vs. post-partum females, and in vehicle vs. HSP-E vs. HSP-P females. To more clearly depict changes in behavior from the "no-odor" to cat odor conditions, data were also plotted and analyzed as *change* in each behavior from the "no-odor" (habituation) to the cat-odor condition. Behaviors were compared among treatment groups using repeated measures ANOVA [Experiment 1: treatment group (2) x day (3) x session (2); Experiment 2: treatment group (3) x day (3) x session (2)]. The significance level for all ANOVAs was set at $p \leq 0.05$. Significant treatment group main effects or interactions were followed by examining differences between treatment groups on specific days using ANOVA within each post-partum day, and then Student's t-tests with the alpha level adjusted by the post-hoc Bonferroni method, or Dunnett's test for comparing multiple hormone groups to vehicle controls.

2.6 Hormones

Estradiol and progesterone (Steraloids, Newport, RI) for injection were dissolved in safflower oil, which served as the control vehicle. All hormone injections were given s.c. in a volume of 0.1 ml – 0.3 ml/rat. Blank or estradiol benzoate-filled 1-mm Silastic capsules were implanted s.c. at the time of ovariectomy, and remained in place throughout testing so that only progesterone was declining during the early "post-partum" period. We have previously shown that 1-mm estradiol capsules are sufficient to maintain normal uterine weight and sexual behavior out to 4-6 weeks after ovariectomy (Stoffel et al., 2003).

3. Results

In Experiment 1, data from one virgin control and one previously pregnant female on post-partum day 4 were dropped before analysis, because during testing these rats fell asleep and did not display any behaviors being measured. In Experiment 2, data from four rats were dropped before analysis: two vehicle control rats on post-partum day 2, because their ovaries were probably not entirely removed (these rats were in vaginal estrus at the time of testing, and had elevated uterine weights); one HSP-E rat on post-partum day 4, because this rat showed abnormal hindlimb locomotion after surgery; and one HSP-P rat on post-partum day 7, due to a bowel obstruction occurring after surgery.

3.1 Effect of Feline Odor on Anxiety-like Behaviors in Post-partum vs. Control Females (Experiment 1)

Figure 1 shows percent time engaged in various behaviors during the predator odor test, in post-partum and control female rats, without vs. with cat odor on post-partum days 2, 4, and 7. As shown in the top panels of Figure 1, both post-partum and control females showed increased anxiety-like behaviors when exposed to cat odor, as compared to the "no-odor" condition (habituation day), and these results were very similar across post-partum days 2-7. First, time spent in the hide box increased significantly from no-odor to cat odor test sessions (Session: $F(1,45)=90.70$, $p<0.001$). However, the magnitude of this change was smaller in the post-partum compared to the control group (Treatment Group x Session: $F(1,45)=9.45$, $p=0.004$). Time spent in the head-out position also increased significantly from the no-odor to the cat odor tests ($F(1,45)=11.11$, $p=0.002$) as did the time spent in contact with the collar ($F(1, 45)=64.95$, $p<0.001$), but there were no significant differences in these behaviors between post-partum and control females. In contrast, whereas time spent grooming was not significantly different

between the no-odor and cat-odor tests, post-partum females spent significantly more time grooming than the control females, regardless of odor condition (Treatment Group: $F(1,45)=61.68$, $p<0.001$).

As shown in the bottom panels of Figure 1, exploratory behavior (rearing) significantly decreased from the no-odor to cat-odor tests in both post-partum and control females, inside the hide box (Session: $F(1,45)=190.73$, $p<0.001$), outside the hide box ($F(1,45)=109.22$, $p<0.001$), and total rears per minute ($F(1,45)=199.22$, $p<0.001$). However, rearing decreased *less* in the post-partum group than in the control group (Treatment Group x Session: $F(1,45)=18.29$, $p<0.001$).

3.2 Feline Odor-Induced Behavioral Change in Post-partum vs. Control Females

To more clearly depict group differences in behavior in response to cat odor, data from Figure 1 were replotted in Figure 2 as *change* in each behavior from the "no-odor" (baseline) to the cat odor condition. In general, post-partum females showed a smaller increase in anxiety-like behaviors from the "no-odor" to the cat-odor condition, relative to control females. For example, although post-partum females increased time spent inside the hide box when exposed to cat odor, they did so to a lesser degree than the control females did (Treatment Group: $F(1,45)=9.45$, $p=0.004$). Additionally, when exposed to cat odor, post-partum females decreased rearing behavior significantly less than control females did (Treatment Group: $F(1,45)=18.29$, $p<0.001$). This group difference in rearing also significantly differed by day of testing in the post-partum period, with the greatest group differences occurring on post-partum day 4 (Treatment Group x Day: $F(2,45)=4.83$, $p=0.013$).

3.3 Effect of Feline Odor on Anxiety-like Behaviors in Ovariectomized, HSP vs. Vehicle-treated Females (Experiment 2)

Figure 3 shows percent time engaged in various anxiety-like behaviors during the predator odor test, in rats that were ovariectomized and treated with hormones for 22 days to simulate pregnancy. The behaviors of HSP-E, HSP-P, and vehicle-treated (control) females, without vs. with cat odor on "post-partum" (hormone withdrawal) days 2, 4, and 7 are shown. All groups of females showed increased anxiety-like behaviors when exposed to cat odor as compared to the "no-odor" control collar (habituation day). First, time spent in the hide box increased significantly from no-odor to cat odor tests (Session: $F(1,59)=93.06$, $p<0.001$). The magnitude of the increase differed slightly but not significantly between groups (Treatment Group x Session: $F(2,59)=2.67$, $p=0.077$). Time spent in the head-out position also increased significantly from the no-odor to the cat-odor tests (Session: $F(1,59)=24.28$, $p<0.001$). The magnitude of this change was significantly different between groups (Treatment Group x Session: $F(2,59)=4.13$, $p=0.021$), with the HSP-E group increasing time in the head-out position significantly more than the vehicle group. Time spent in contact with the collar also increased significantly from the no-odor to the cat-odor tests (Session: $F(1, 59)=24.89$, $p<0.001$), but there was no significant group difference. Overall, time spent grooming did not change significantly from the "no-odor" to cat-odor tests, nor did it differ significantly between vehicle and hormone-treated groups.

As shown in the bottom panels of Figure 3, exploratory behavior (rearing) significantly decreased from the no-odor to cat-odor tests in all groups, inside the hide box (Session: $F(1,59)=180.83$, $p<0.001$), outside the hide box ($F(1,59)=137.76$, $p<0.001$), and total rears per minute ($F(1,59)=256.87$, $p<0.001$). There were no significant group differences in rearing

behavior; however, total rears per minute increased significantly over days 2-7 post-partum (Day: $F(2, 59)=3.63$, $p=0.033$).

3.4 Feline Odor-Induced Behavioral Change in Ovariectomized, HSP vs. Vehicle-treated Females

To more clearly depict group differences in behavior in response to cat odor, data from Figure 3 were replotted in Figure 4 as *change* in each behavior from the "no-odor" (habituation) to the cat odor condition for vehicle-treated (control), HSP-E, and HSP-P rats. Figure 4 shows that change in time spent inside the hide box differed slightly but not significantly between groups (Treatment Group: $F(2,59)=2.67$, $p=0.077$). Time spent in the head-out position increased more in the hormone-withdrawal groups than in the vehicle control group (Treatment Group: $F(2,59)=4.13$, $p=0.021$). Specifically, HSP-E females increased head-out time to a greater degree than vehicle females, when data from all three days were pooled ($F(1,39)=8.21$, $p=0.007$); however, within each day there were no significant group differences. Grooming tended to decrease less in the hormone groups than in the vehicle group, but this difference was not statistically significant (Treatment Group: $F(2,59)=2.75$, $p=0.072$). Finally, as shown on the bottom panels of Figure 4, change in rearing behavior from "no-odor" to cat-odor tests was very similar in all groups.

3.5 Uterine Weights

Figure 5 shows that uterine weights differed among hormone treatment groups in Experiment 2, with rats in both HSP groups having significantly larger uteruses than the vehicle controls (Treatment Group: $F(2,59)=264.14$, $p<0.001$). Uterine weights also significantly changed over days post-partum, depending on the group (Treatment Group x Day: $F(4,59)=6.08$, $p<0.001$); specifically, uterine weights in the HSP-E group declined during post-partum days 2-7

while remaining stable in the other groups. Thus, uterine weight in the HSP-E group was greater than uterine weight in the HSP-P group only on post-partum day 2.

3.6 Vaginal Cytology

Table 3 shows the estrous stage of rats in each treatment group at the end of behavioral testing. In Experiment 1, the virgin controls were mostly in diestrus, with some in proestrus, and one in estrus, while the previously pregnant females were all in diestrus. In Experiment 2, all ovariectomized, vehicle control females were in diestrus, while the HSP-E females were predominantly in proestrus and estrus. In contrast, the HSP-P females shifted over the post-partum period, from predominantly in diestrus on post-partum day 2 to mostly in proestrus and estrus on post-partum day 7.

4. Discussion

4.1 Experiment 1: Anxiety-like Behaviors in Post-partum vs. Control Females

Overall, the presence of cat odor elicited many of the expected behavioral changes in the virgin control and previously pregnant (post-partum) rats. From the "no-odor" to cat-odor conditions, rats increased time spent in contact with the collar, increased time spent in the hide box and in the head-out position, and decreased rearing. These behavioral changes can be interpreted as an overall increase in defensive/anxiety-like behaviors (Dielenberg and McGregor, 2001). One expected behavioral change that was not observed in this experiment was a significant decrease in grooming from the "no-odor" to cat-odor conditions (Dielenberg and McGregor, 2001), perhaps because (control) rats in the present experiment spent very little time engaged in this behavior.

Regarding differences between control and post-partum females, post-partum females showed less anxiety-like behavior than controls did. Specifically, post-partum females showed significantly smaller increases in time spent in the hide box (days 2 and 4) and significantly smaller decreases in rearing behavior (day 4) than controls did. Thus, the results of Experiment 1 do not support the hypothesis that post-partum females display greater anxiety-like behaviors than control rats, but do corroborate the results of several previous studies suggesting that post-partum females show *blunted* anxiety-like behaviors in the early post-partum period (Lonstein, 2005; Pereira et al., 2005; Byrnes and Bridges, 2006). As noted in the introduction, the literature regarding post-partum anxiety in the rat has been equivocal, with some studies reporting less and others reporting more anxiety-like behavior in post-partum females compared to controls. This may be due to many factors, such as the anxiety test employed as well as the post-partum hormone model used. When looking at the elevated plus maze, post-partum females spent significantly more time in the open arms than virgin controls (Lonstein, 2007), which is typically interpreted as decreased anxiety-like behavior. However, an increase in anxiety-like behavior was observed by Lofgren et al. (2006) using a progesterone withdrawal model of post-partum anxiety; specifically, rats in the early stages of progesterone withdrawal avoided open areas in the open field test. Although it can be argued that the predator odor test used in the present experiments is more ethologically relevant than many other anxiety tests currently in use (Dielenberg and McGregor, 2001), the results in this test can be more complex to interpret as there are multiple behavioral changes that may indicate changes in anxiety. In the present study there were several behavioral changes that differed between post-partum and control females; taken together these changes suggest blunted anxiety in females during the early post-partum period.

One unexpected result in the present study was that post-partum females spent more time grooming than control females did, regardless of the odor condition. Grooming is not typically considered a defensive behavior (Dielenberg and McGregor, 2001), although it has occasionally been associated with increased anxiety in the rodent. For example, i.c.v. administration of corticotrophin releasing factor increased grooming in rats (Colbern et al., 1978; Dunn et al., 1981). Although it is hard to determine what motivates each rat to groom, group differences in grooming behavior may also have been related to pup care. Post-partum females are actively caring for their pups, and their only time away from their pups was during testing periods; therefore, perhaps they found the testing period a "break from pup care" (i.e., time for self-care). However, elevated plus maze experiments conducted in our laboratory did not show more grooming in post-partum females compared to virgin controls (unpublished data), so this phenomenon does not appear to generalize across anxiety tests. At any rate, there were no significant *changes* in grooming behavior from the "no-odor" to cat-odor conditions in either control or post-partum females, so this behavior cannot be used to examine group differences in anxiety.

4.2 Experiment 2: Anxiety-like Behaviors in HSP vs. Vehicle-treated Controls

In Experiment 2, two different Hormone-Simulated Pregnancy (HSP) models were used to examine withdrawal from estradiol and progesterone separately. The HSP-E (estradiol withdrawal) group reflects the basic hormone profile of a rat pregnancy, in which progesterone is declining beginning from approximately day 17 of pregnancy, and estradiol begins declining at parturition (McCarthy, 1997). In contrast, humans go through both estradiol and progesterone withdrawal during the early post-partum period (Parker, 1993). Thus, the HSP-P group was used to examine the effect of progesterone withdrawal in the post-partum period. Estradiol was held

constant in the HSP-P model so that any effects of progesterone withdrawal at parturition could be isolated from effects due to estradiol withdrawal.

In Experiment 2, changes in anxiety-like/defensive behaviors from the "no-odor" to cat-odor conditions were similar to those seen in Experiment 1. That is, rats in all groups increased time spent in the hide box and in the head-out position, and decreased rearing behavior, indicating increased anxiety. The HSP groups differed from the vehicle control group in only one case, and it was only the HSP-E group that showed any significant difference from the control group. Specifically, from the "no-odor" to cat-odor conditions, the HSP-E rats tended to show less of an increase in time spent inside the hide box; and a significantly larger increase in time spent in the head-out position than the vehicle rats. The fact that the HSP-E group showed a smaller increase in time spent in the hide box may suggest a blunted response to the cat odor; however, the HSP-E group also increased time in the head-out position more than the vehicle rats (and head-out time is included in the "in-hide-box" time), so these changes could also be interpreted as an *increase* in risk-assessing behavior (i.e., more anxiety: Dielenberg and McGregor, 2001). Thus, the original hypothesis was not clearly supported: the HSP groups did not display greater anxiety-like behavior than vehicle controls when looking at the largest changes in behavior from no-odor to cat odor conditions (time spent in the hide box, and also rears per minute). In contrast to the original hypothesis, the results of Experiment 2 suggest that withdrawing from either estradiol or progesterone alone does not significantly elevate anxiety in the predator odor anxiety test.

In Experiment 2, an unexpected effect was that total rears per minute increased significantly over days 2-7 post-partum in all groups. This change may have been due to the increasing number of days being handled. After the last "pregnancy period" injection ceased,

handling was continued throughout the experiment; perhaps the extra days of handling without injections lowered the overall anxiety of the later post-partum testing groups. To control for this issue, in future experiments, during the "post-partum" period, a continuation of injection containing vehicle only may be used to keep each group's handling more alike. Also unlike Experiment 1, in Experiment 2 there were no differences in time spent grooming between groups. Because none of the groups in Experiment 2 had pups to care for, this result may be interpreted to support the hypothesis that in Experiment 1, pup care may have led to the greater time spent grooming in the post-partum group compared to the control group, regardless of odor condition.

Comparing data from Experiments 1 and 2, the HSP-E group looked the most similar to the previously pregnant females. However, the HSP-E group increased time in the head-out position whereas the post-partum group showed no change to a slight decrease in this measure when exposed to cat odor. Thus, the HSP-E group spent more time engaged in risk-assessing behaviors than both their vehicle controls as well as previously pregnant females. The HSP-E group also looks similar to the stressed female group in the Perrot-Sinal et al. (2004) experiment, in which the stressed females increased time spent in the head-out position more than the non-stressed control females, a behavioral change not seen in the actual previously pregnant females. Therefore, estradiol withdrawal alone may be comparable to a chronic stressful experience, insofar as its ability to produce similar results to chronic stress as measured in the predator odor test.

Overall neither HSP group displayed a clearly blunted anxiety response to cat odor when compared to the vehicle controls. Although this could theoretically be attributed to a lower anxiety response in the vehicle control group, the vehicle controls in Experiment 2 did respond

similarly to the virgin controls in Experiment 1. Thus, the HSP groups did not appear to respond to cat odor in the same way as the actual previously pregnant females, and it can be concluded that modulation of the levels of one ovarian hormone alone do not significantly change overall anxiety response in the predator odor test.

4.3 Physiological Mechanisms of Post-Partum Changes in Anxiety

The apparently blunted anxiety response to cat odor in the post-partum group could be attributed to various pregnancy-induced changes in physiology. For example, the post-partum group may have shown less anxiety due to a blunted Hypothalamic-Pituitary-Adrenal (HPA) axis (Heinrichs et al., 2001). The HPA axis is a neuroendocrine negative feedback network that regulates the adrenal gland's hormonal activities. By measuring corticosterone levels, Doornbos et al. (2008) demonstrated that there is a significant pregnancy-induced blunting of the HPA axis. While Jolley et al. (2007) found that women with post-partum mood disorders had significantly lower levels of cortisol and higher levels of adrenocorticotropic hormone (ACTH), with no relationship between their ACTH (which stimulates synthesis and secretion of corticosteroids) and cortisol levels, when compared to a nondepressed control group at both 6 and 12 weeks post-partum. This significant difference in cortisol levels, in women with post-partum mood disorders, indicates that alterations in HPA axis activity likely play a role in post-partum mood disorders. In rodents, a pregnancy-induced blunting of the HPA axis can lead to a decrease in corticosterone levels following exposure to a stressor when compared to virgin controls, in prenatally unstressed females (Bosch et al., 2007). Therefore changes in anxiety like behaviors following predator odor exposure, in the post-partum period, are most likely due to a combination of several factors including ovarian hormone withdrawal and alterations in cotricosterone levels.

4.4 Physiological Measures of Ovarian Hormone State

Assuming that anxiety is affected by ovarian hormones, as suggested by previous research (Bloch et al., 2003), the vaginal cytology results suggest at least one reason why neither of the HSP groups behaved similarly to the previously pregnant group when tested post-partum. Whereas previously pregnant females were all in diestrus during testing, HSP-E females were predominantly in proestrus or estrus, while the HSP-P females shifted from estrus and diestrus on days 2 and 4 to estrus and proestrus on day 7 post-partum. These results indicate that hormonally, the three groups were quite different, with blood levels of estradiol and progesterone likely already quite low by post-partum day 2 in the previously pregnant group, but not in the HSP groups. Elevated uterine weights in the HSP groups relative to vehicle controls support this hypothesis; it has been shown previously that uterine weight is a sensitive indicator of circulating ovarian hormone levels (e.g., Nandi et al., 2006). Therefore, the HSP groups likely had considerably higher ovarian hormone levels than the previously pregnant group during the early post-partum period.

Another indicator that the HSP-P model might not have been a good model of the post-partum period in rats was the failure of this group to display late pregnancy nesting behavior that was observed in the post-partum group (unpublished observation). Their vaginal cytology suggested that the HSP-P rats were cycling from diestrous to proestrous (when estradiol is at its peak) throughout days 2-7 in the post-partum period. This may have been due to withdrawal of progesterone while estradiol levels remained constant, such that estradiol was the predominant ovarian hormone by "post-partum" day 7, inducing the rats to go through a progesterone withdrawal-stimulated estrous cycle. While nesting behavior was usually observed during approximately the last 2-5 days of pregnancy in actual pregnant females and the HSP-E group,

nesting was not observed in the HSP-P group above and beyond the nesting observed in the vehicle control group, until approximately post-partum day 2 (unpublished observation), indicating that progesterone withdrawal is important for the initiation of nesting behavior during a rat pregnancy. In fact, maternal behavior in the rat has been observed to begin during the decrease in progesterone and rise in estradiol that occurs during the last week of pregnancy, before parturition (Rosenblatt, 1975). Therefore, late nesting behavior observed in the HSP-P group confirms that withdrawal from progesterone during the rat "post-partum" period did not model a rat pregnancy in terms of maternal behavior.

The significantly larger uterine size in the HSP rats compared to vehicle controls was expected as previously demonstrated in this laboratory (Navarre et al., in preparation). The decrease in uterine size over time post-partum seen in the HSP-E rats was also expected. However, uterine size in the HSP-P rats did not change over days 2-7 in the post-partum period, which is not comparable to previously pregnant rats that do show a dramatic decrease in uterine weight over the post-partum period (Navarre et al., in preparation). Presumably uterine size in the HSP-P group was maintained by their extended-release estradiol capsule implants, which were not removed before testing.

It should be noted that another vehicle control group implanted with an estradiol capsule, rather than just a blank capsule, may have served as a more compatible control group for the HSP-P group. The HSP-P group had constant levels of estradiol with only progesterone levels declining in the "post-partum" period. Thus using a vehicle treatment group implanted with an estradiol capsule could control for the physiological effects attributed to a continuous administration of low-dose estradiol.

4.5 Human Post-Partum Mood Disorder

In the human post-partum period, many factors such as socioeconomic status, relationship status, education, and overall health can affect the development of post-partum mood disorder and the severity of symptoms (Kurtz Landy et al., 2008). Using an animal model of post-partum mood disorders can be helpful to isolate single contributing factors such as hormone withdrawal; however, in humans, the number of contributing factors are not only numerous, but vary between each individual. In the future, the analysis of hormone levels and hormone treatments, with current drug and therapy treatments, may lead to a decrease in the time period to reach full therapeutic effect, and an increase in the number of patients that are successfully treated.

4.6 Future Directions

Focusing on not only estradiol and progesterone but also other hormones that fluctuate during the post-partum period (oxytocin, prolactin, and corticosterone) may lead to further insight regarding the role of hormones in post-partum mood disorder. For example, oxytocin and prolactin, hormones associated with uterine contraction and lactation, respectively, are both increasing during the post-partum period, and have been shown to have anxiolytic properties (Neumann, 2003; Rhoades and Pflanzer, 2003). And corticosterone levels, which are directly related to stress and the activity of the HPA axis, may be blunted during the post-partum period (Heinrichs et al., 2001).

Post-partum rats are not a perfect model for post-partum humans. For example, rats do not withdraw from both estradiol and progesterone concurrently in the early post-partum period. Although the HSP models used in this study were useful for manipulating one hormone at a time, to model a human pregnancy more accurately, withdrawal from both hormones at the same time

would have mirrored the human post-partum period more closely. In this predator-odor anxiety experiment, the HSP models used did not produce an overall change in anxiety-like behaviors, suggesting that hormonal fluctuations in the post-partum period other than progesterone and estradiol may play greater roles in post-partum anxiety.

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6. Figure and Table Legends

Figure 1. Percent time engaged in anxiety-like behaviors (mean ± S.E.M.) in previously pregnant and control female rats, without and with cat odor, on post-partum days 2, 4, and 7. N=7-13 rats/treatment group/day. *****Significantly different from "no-odor" condition, P<0.05. +Significantly different from control females, P<0.05.

Figure 2. Change in anxiety-like behaviors from baseline ("no-odor") to cat odor conditions (mean ± S.E.M.) in previously pregnant and control female rats on post-partum days 2, 4, and 7. N=7-13 rats/treatment group/day. *****Significantly different from control females, P<0.05.

Figure 3. Percent time engaged in anxiety-like behaviors (mean ± S.E.M.) in HSP-E, HSP-P, and vehicle control female rats, without and with cat odor, on postpartum days 2, 4, and 7. N=6-8 rats/treatment group/day. *****Significantly different from "no-odor" condition, P<0.05.

Figure 4. Change in anxiety-like behaviors from baseline ("no-odor") to cat odor conditions (mean ± S.E.M.) in HSP-E, HSP-P, and vehicle control female rats on postpartum days 2, 4, and 7. N=6-8 rats/treatment group/day.

Figure 5. Uterine weights (mean ± S.E.M.) in HSP-E, HSP-P, and vehicle control female rats on post-partum days 2, 4, and 7. N=6-8 rats/treatment group/day. *****Significantly greater than vehicle control females, P<0.05. +Significantly greater than HSP-P group, P<0.05.

Table 3. Vaginal cytology was sampled immediately after cat odor testing in virgin controls and previously pregnant rats (Experiment 1), and in vehicle controls, HSP-E, and HSP-P groups (Experiment 2) on post-partum days 2, 4, and 7. Estrus includes rats in between proestrus and estrus, and diestrus includes diestrus-day 1 (metestrus) and diestrus-day 2. N=6-13 rats/treatment group/day.

Figure 1

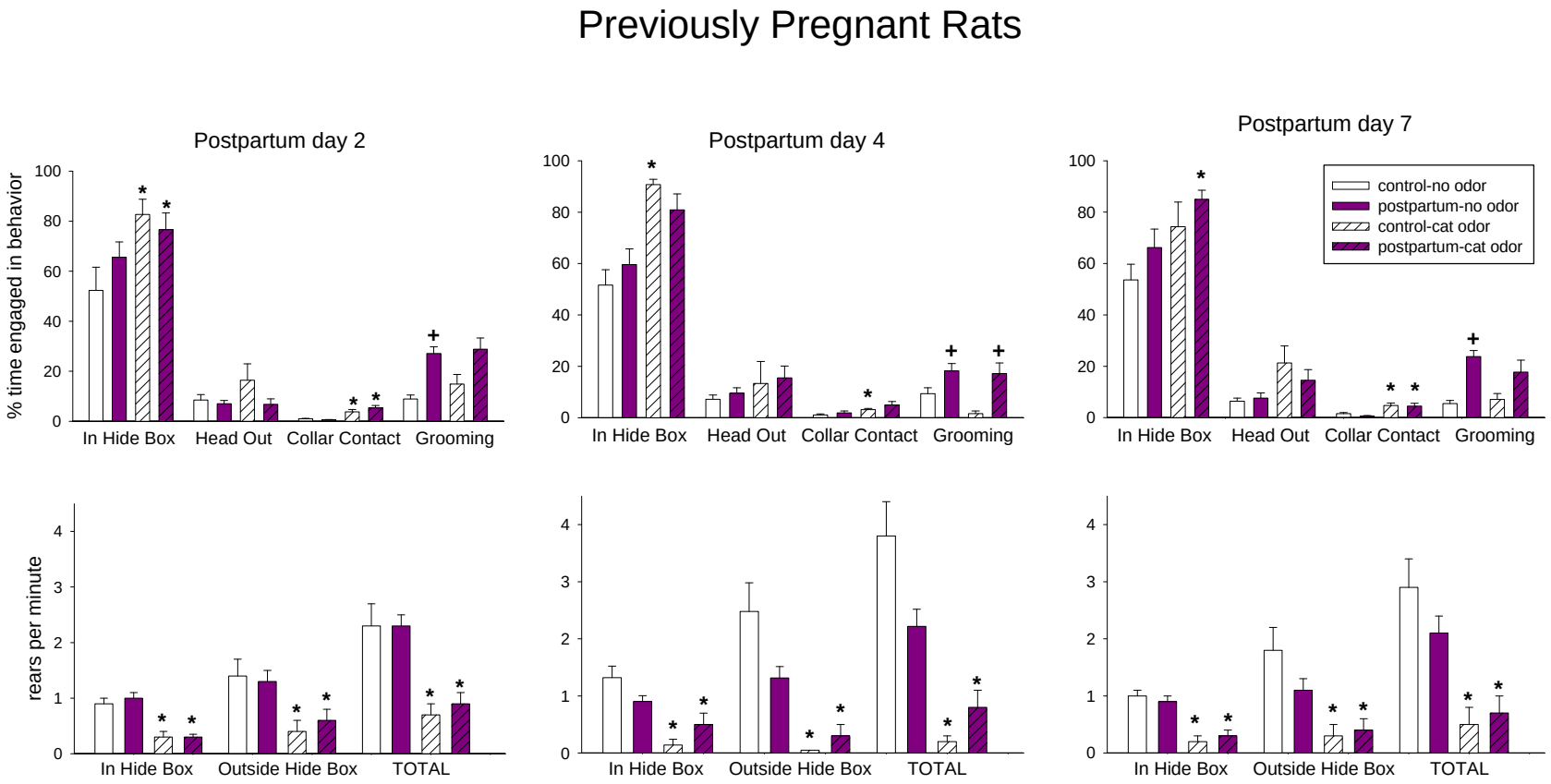
Previously Pregnant Rats
Postpartum day 2
Postpartum day 4
Postpartum day 7
% time engaged in behavior
rears per minute
control-no odor
postpartum-no odor
control-cat odor
postpartum-cat odor
In Hide Box
Head Out
Collar Contact
Grooming
Outside Hide Box
TOTAL

Figure 2

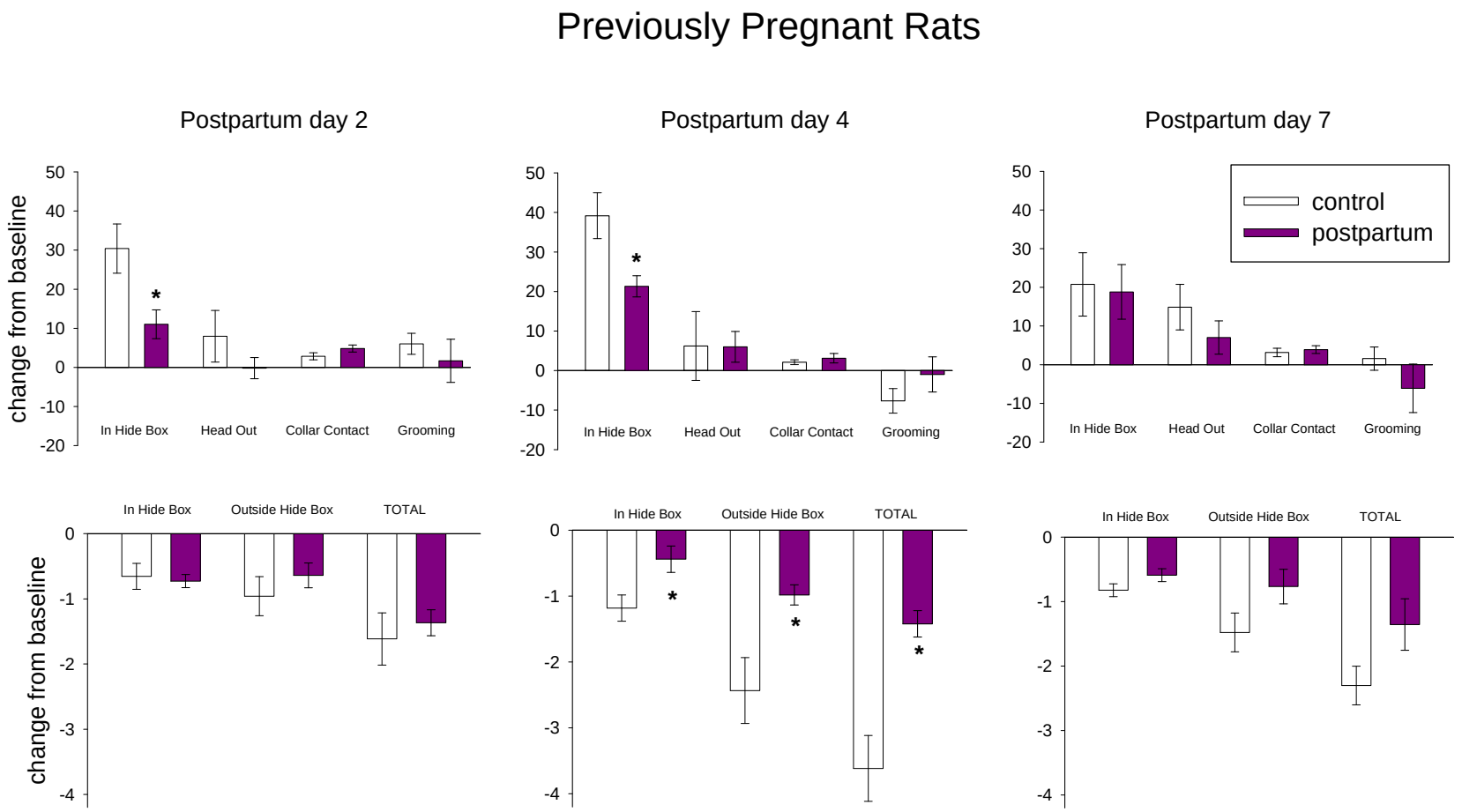
Previously Pregnant Rats
Postpartum day 2
Postpartum day 4
Postpartum day 7
change from baseline
control
postpartum
In Hide Box
Head Out
Collar Contact
Grooming
Outside Hide Box
TOTAL
*

Figure 3

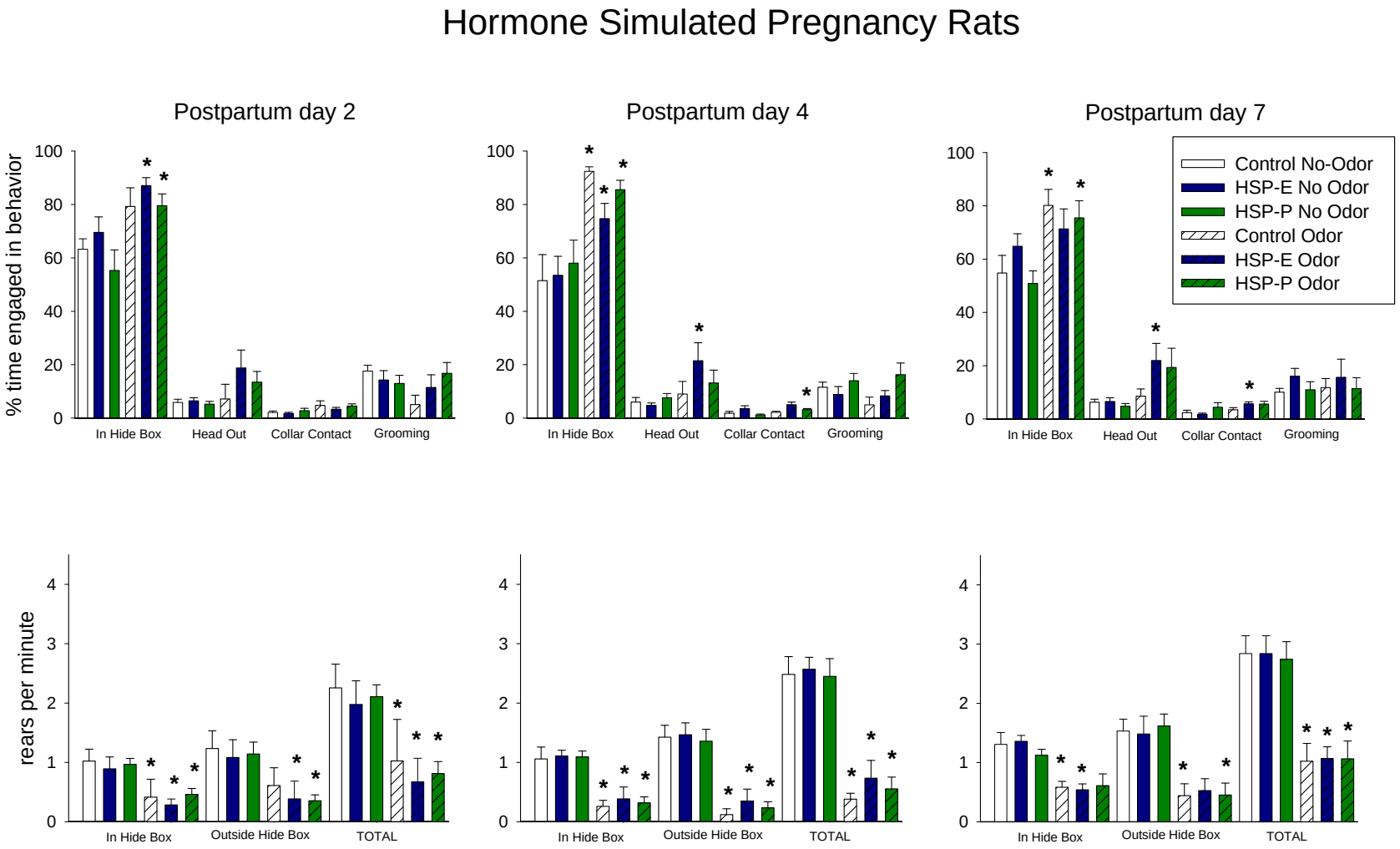
Hormone Simulated Pregnancy Rats
Postpartum day 2
Postpartum day 4
Postpartum day 7
% time engaged in behavior
rears per minute
In Hide Box
Head Out
Collar Contact
Grooming
Outside Hide Box
TOTAL
Control No-Odor
HSP-E No Odor
HSP-P No Odor
Control Odor
HSP-E Odor
HSP-P Odor

Figure 4

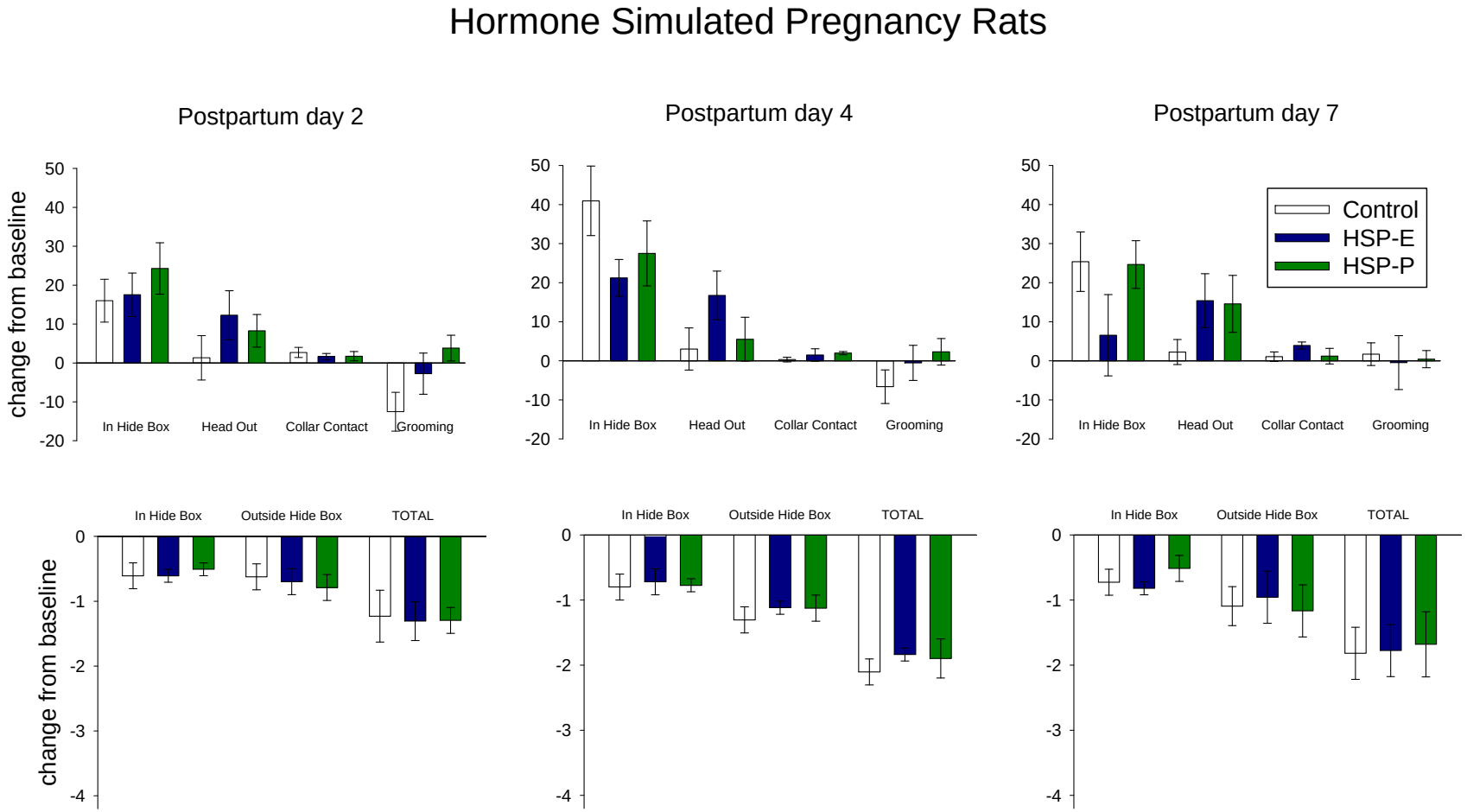
Hormone Simulated Pregnancy Rats
Postpartum day 2
Postpartum day 4
Postpartum day 7
change from baseline
50
40
30
20
10
0
-10
-20
In Hide Box
Head Out
Collar Contact
Grooming
Control
HSP-E
HSP-P
In Hide Box
Outside Hide Box
TOTAL
change from baseline
0
-1
-2
-3
-4

Figure 5

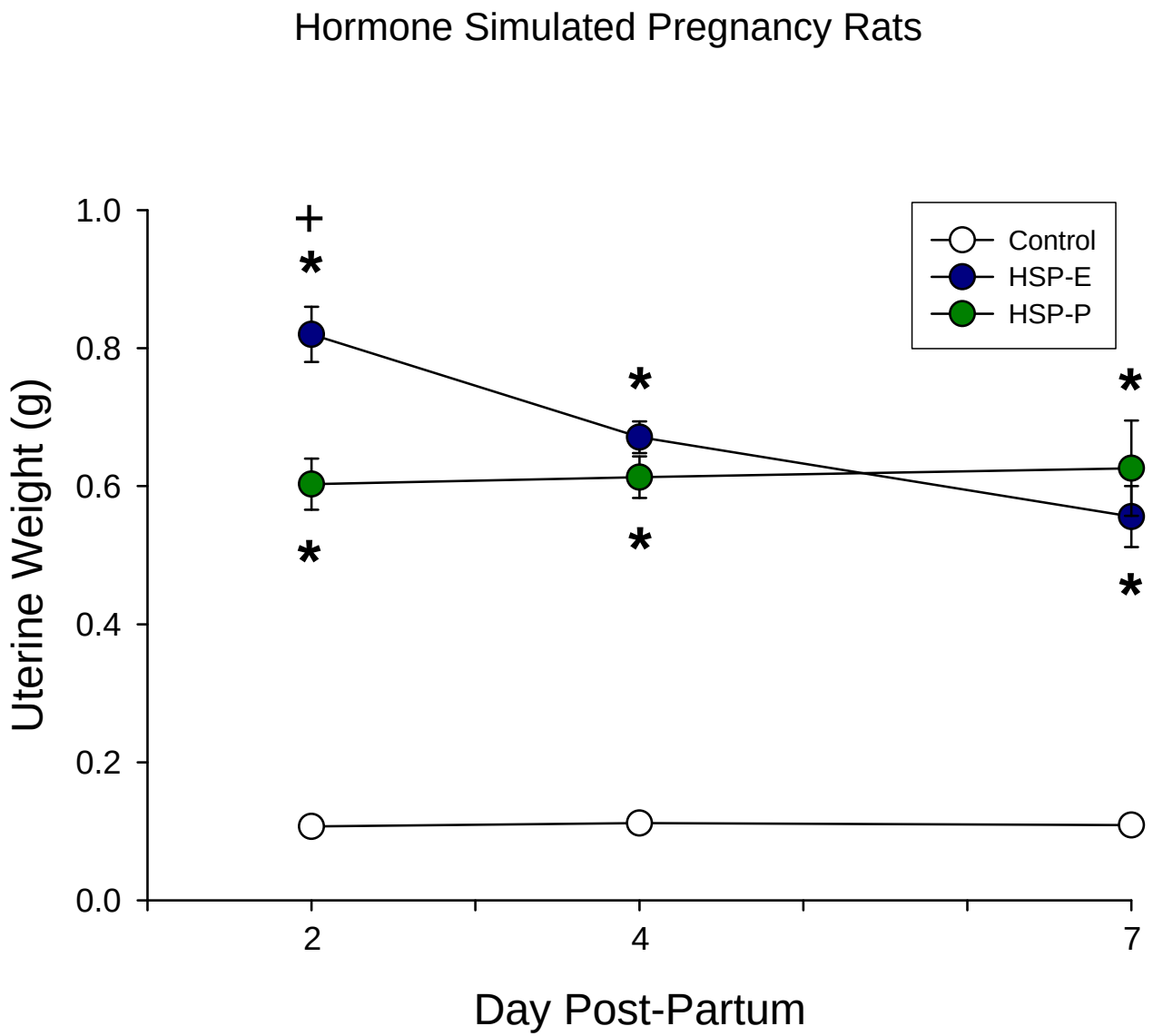
Hormone Simulated Pregnancy Rats
Uterine Weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
2
4
7
Day Post-Partum
Control
HSP-E
HSP-P
+
*
*
*
*
*

Table 3

Treatment Group	Post-Partum Day 2			Post-Partum Day 4			Post-Partum Day 7		
	Proestrus	Estrus	Diestrus	Proestrus	Estrus	Diestrus	Proestrus	Estrus	Diestrus
Virgin Controls	2	1	5	2	0	5	2	0	6
Previously Pregnant	0	0	13	0	0	7	0	0	8
Vehicle Controls	0	0	6	0	0	8	0	0	8
HSP-E	4	2	2	5	2	0	5	3	0
HSP-P	0	2	6	0	3	5	4	2	1