Research

Male pheromone stimulates ovarian follicular development and body growth in juvenile female opossums (Monodelphis domestica)

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Abstract

Female opossums are induced into estrus by direct exposure to a non-volatile pheromone in male scentmarks. Juvenile females develop this responsiveness by 150 days of age (days), and earlier (130 days) if exposed to male pheromone beginning at 90 days. The goal of this study was to examine the effect of male pheromone on body growth and ovarian follicular development in young opossums. Females (n = 28) were weaned at 56 days and caged individually with *ad libitum* food and water from 84 days. Body mass was recorded 2–3 times weekly and the onset of estrus was monitored by urogenital sinus cytology. Exposed females were given continuous access to adult male scentmarks from 90 days and randomly selected for necropsy at 105 and 130 days. Unexposed females were isolated from direct contact with males and their scentmarks and randomly selected for necropsy at 90, 105, 130, and 150 days. Exposed females were larger (63.5 ± 1.1 g) than unexposed females (56.6 ± 1.1 g) at 130 days, and 4 of 5 had expressed estrus or proestrus. Uterine mass at 130 days was higher (P < 0.05) in exposed (129.8 ± 28.8 mg) than in unexposed (25.4 ± 6.7 mg) females, none of which expressed estrus by 150 days. The mean number of developing, antral follicles per female increased from 1.5 ± 1.5 at 90 days to 17.8 ± 5.2 at 130 days. Mean diameter of developing antral follicles at 130 days was larger in exposed (534 ± 54 μm) than in unexposed females (393 ± 4 μm). The results of this study demonstrate that pheromonal induction of first estrus in juvenile opossums is associated with an increased rate of body growth and follicular development.

Background

The rate of reproductive development and the onset of fertility are influenced by an array of environmental factors that can be broadly grouped as physical, nutritional, and social [1]. Chief among social cues in mammals are priming pheromones that can retard or accelerate sexual development within constraints of factors such as photoperiod and nutritional status of the individual. Subsequent to early studies that established the pheromonal qualities of male urine to synchronize estrous cycles of laboratory mice [2], sexual maturation in female mice was shown to be accelerated by male pheromones [3] and retarded by female pheromones [4]. Pheromonal induction of first estrus in juvenile females has been reported in a variety of other mammals including prairie voles [5], pigs [6], and opossums [7].

The gray short-tailed opossum (*Monodelphis domestica*), a small (60–150 g) marsupial native to scrub forests of southeastern Brazil, provides new opportunities for study
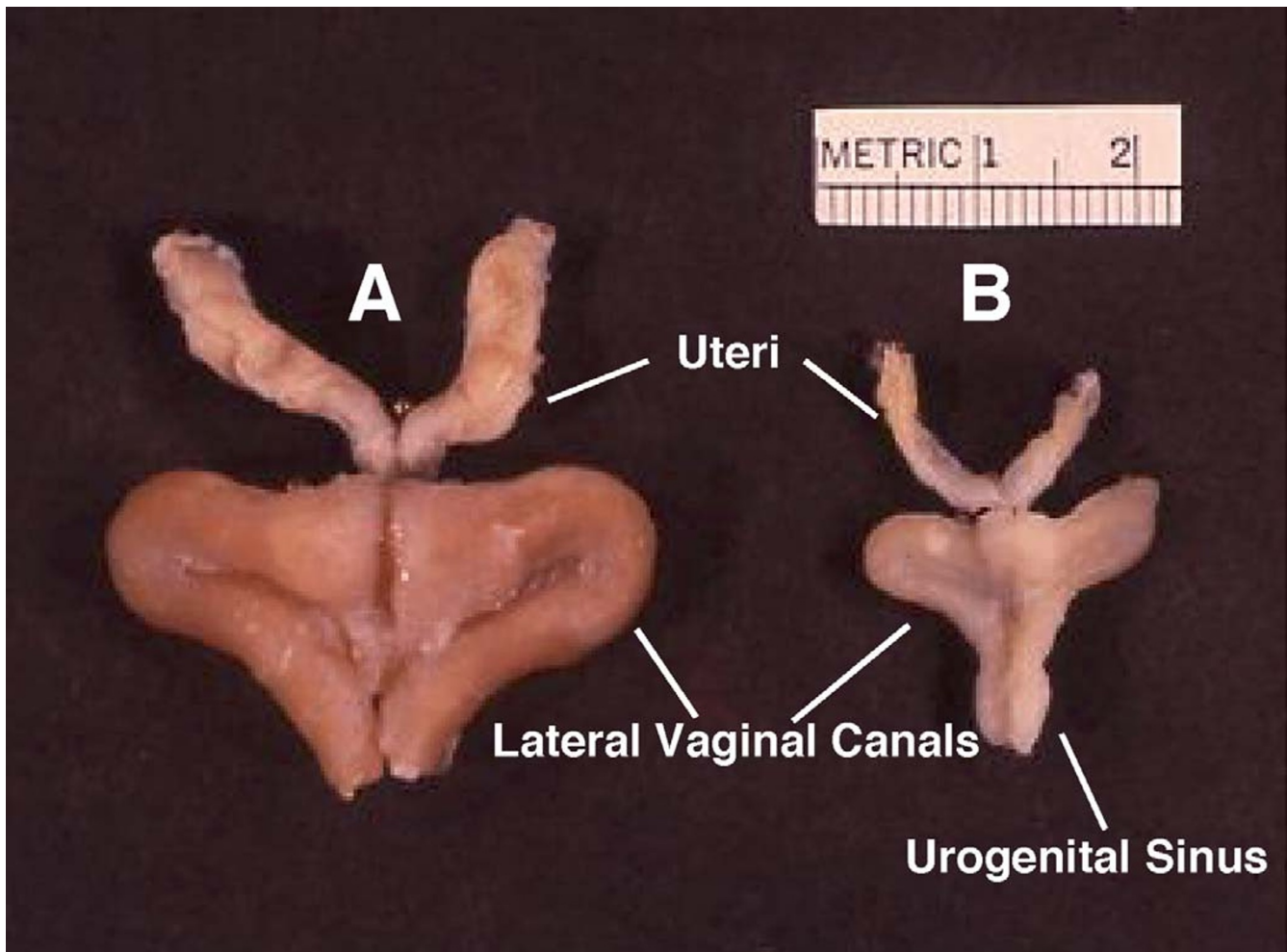
Figure 1
Reproductive tracts of estrous (A) and anestrous (B) female opossums showing relative sizes of uteri and lateral vaginal canals similar to that seen in pheromone exposed and unexposed females at 130 days. A portion of the urogenital sinus is evident in the caudal portion of tract B.

of olfactory communication and pheromonal activation of reproduction. Females lack an estrous cycle and anestrous adults are dependent upon direct contact with a non-volatile male pheromone for induction of estrus [8]. Hormonal control of scentmarking and sexual behavior of this species (hereinafter, opossum) has been described in some detail [9,10]. Estrus is induced when females nuzzle scentmarks from the androgen-dependent suprasternal (chest) gland [10,11]. Nuzzling delivers nonvolatile chemical signals to the vomeronasal organ (VNO) [12] and surgical removal of the VNO prevents induction of estrus by scentmarks alone [13], although cautery ablation of the VNO fails to prevent ovarian activation and ovulation in females that are caged with adult males [14]. While anestrous adults are reliant on male pheromone for repro-

ductive activation, spontaneous postlactational estrus occurs in the absence of direct contact with male pheromone [15].

Reproductive activation and the onset of estrus are monitored by cytology of the urogential sinus (UGS) [16,17], the sexual receptive organ in marsupials that lies caudal to paired lateral vaginal canals (LVC) (Fig. 1). UGS estrus (estrus, hereinafter) is evident after 5–10 days of pheromonal exposure. Estrous females will not ovulate unless paired with a male [7], but copulation is not required to stimulate ovulation in females caged with males [7,17]. Copulation typically occurs on the second night of paring [18]. Litters of about 8 young are born after a 15-day gestation, and young are weaned at 8 weeks of age.

The onset of puberty in female opossums is strongly influenced by pheromonal and, perhaps, other social cues. An earlier study found that juvenile females isolated from all adult stimuli from 90 days of age grew to adult size (60 g) but failed to express first estrus by 180 days [7]. In contrast, all females exposed directly to male pheromones by cage switching reached first estrus at an average age of 127 days, and more than half of females individually caged (i.e., isolated from male scentmarks) and housed in a mixed sex colony expressed first estrus at 162 days, on average. This suggests that age at first estrus varied from 109 to 162 days, depending on pheromone exposure [7]. However, body mass at first estrus was remarkably close to 60 g in all females, indicating that puberty in opossums, as in most mammals, is dependent on attainment of a threshold body mass. More importantly, advancing the age of first estrus by pheromone exposure was accompanied by an apparent acceleration of somatic growth such that this critical body mass was reached at a younger age.

In the present study, we compared body (somatic) growth and ovarian activity in females given daily, nuzzling contact with male scentmarks (exposed) with that in females individually caged in a mixed sex colony but isolated from direct contact with male scentmarks (unexposed). The study tested the hypothesis that females exposed to male scentmarks will have greater somatic growth and their ovaries will contain developing antral follicles in greater numbers and larger in size than those found in ovaries from females of the same age exposed only to the ambient social stimuli of the colony. Circulating levels of estradiol and mass of reproductive organs were also measured as evidence of reproductive activation.

Materials and Methods
Animals
Juvenile female opossums (n = 28) were weaned at 56 days and individually housed at 84 days in polycarbonate cages (30 × 30 × 15 cm) in a mixed-sex colony in an American Association for Accreditation of Laboratory Animal Care-approved facility in the Botany and Zoology Building at The Ohio State University. The animal room was maintained on a 14L:10D cycle, and room temperature was 25–28°C; food (Fox Reproduction Food; Milk Specialties Co., New Holstein, WI) and acidified water (pH= 4.2) were provided *ad libitum*. Males (n = 6 proven breeders) and females were caged individually and held on different shelving racks, separated by at least 2 m. Incidental transfer of pheromones in male scentmarks to females was avoided by handling females and their cages prior to handling males or male cages. All procedures relating to care and use of opossums in this study were described in protocols approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Experimental Design
This study utilized three timepoints identified in previous studies as key stages in pheromonal induction of first estrus in juvenile females:

109 days – the youngest age recorded for pheromonal induction of estrus

127 days – the average age of first estrus in juveniles exposed to male pheromone

150 days – the recognized age for first use in routine breeding and litter production, i.e. the age at which most females will respond to male pheromone with expression of estrus

Unexposed females were isolated from direct contact with males and male scentmarks and randomly selected for necropsy at 90 (n = 4), 105 (n = 4), 130 (n = 5), and 150 days (n = 5). Exposed females were given continuous access to adult male scentmarks from 90 days and randomly selected for necropsy at 105 (n = 5) and 130 days (n = 5). The 90 day old group provided baseline data for assessment of development in both unexposed and exposed animals. Data from unexposed females killed at 105, 130, and 150 days provided a basis for evaluating the effects of male pheromone on somatic growth and reproductive activation during pheromonal induction of first estrus in exposed females. Exposed females were only examined through 130 days because we expected they would have attained sexual maturity and expressed first estrus by then [7], making them developmentally comparable to unexposed females at 150 days.

Scentmarks, containing estrus-inducing pheromone, were collected by rubbing 4 sides of a 7-ml glass vial on the suprasternal gland of an unrelated male. The vial was then inverted and screwed into a stand and placed daily in each female's cage [15]. Females actively nuzzle the scent-marked vials, particularly during the first 5 minutes of exposure [11]. UGS smears were collected with a saline-moistened cotton swab every 1–3 days [15]. Body mass was recorded when smears were collected and growth rates (g/week) were calculated for each age and treatment group as the mass gained between the beginning of exposure (day 90) and necropsy.

Blood and Organ Collection and Analyses
Blood samples were collected by cardiac puncture at necropsy and the plasma stored at -20°C. Final body mass and total body length were also recorded at this time. Ovaries, uteri, and LVC were removed, trimmed of connective tissue, weighed, and fixed in phosphate buffered formalin. Fixed ovaries were embedded in Paraplast[R], cut

Table 1: Summary of the effects of male pheromone on somatic and follicular growth in juvenile opossums. Different superscripts indicate age-related differences in unexposed females. Asterisks indicate differences between exposed and unexposed females within an age group (P < 0.05).

Treatment: and Age	n	Body Length (cm)	Body Mass (g)	Growth Rate (g/week)	Number of Follicles	Follicle Diameter (μm)
Unexposed: 90 days	4	19.6 ± 0.2 a	45.3 ± 2.7 a		1.5 ± 2.8 a ‡	354 ± 46 ab
Unexposed: 105 days	4	20.4 ± 0.5 a	46.4 ± 1.3 a	2.2 ± 0.5 a	10.8 ± 1.7 b	353 ± 12 a
Exposed: 105 days	5	21.2 ± 0.2	52.3 ± 2.1 *	4.3 ± 0.7 *	13.2 ± 2.0	388 ± 7 *
Unexposed: 130 days	5	21.8 ± 0.1 b	56.6 ± 1.1 b	1.8 ± 0.3 a	17.2 ± 2.6 b	393 ± 4 b
Exposed: 130 days	5 #	21.9 ± 0.1	63.5 ± 1.1 *	3.8 ± 0.1 *	17.8 ± 5.2	534 ± 54 *
Unexposed: 150 days	5	21.9 ± 0.4 b	58.2 ± 5.6 ab	1.3 ± 0.4 a	14.4 ± 1.8 b	394 ± 6 b

‡ 6 follicles in one of four females. # Ovarian histology available for four of five females.

into 8 μm serial sections, mounted on slides, and stained with Lillie's Allochrome stain.

All histological sections of both ovaries collected at necropsy were examined for antral follicles, which were recognized by the presence of one or more fluid-filled cavities lined with granulosa cells. Atretic follicles were identified by the presence of 5 or more pyknotic nuclei in granulosa cells, leukocytes in the antrum, and loosening or degeneration of the mural layer of granulosa cells. Antral follicles not exhibiting these obvious indications of atresia were classified as developing. The proportion of total antral follicles per female that was atretic was used as an index of atresia (atresia, hereinafter). The diameter of each antral follicle was recorded as the average of 2 perpendicular diameters measured at 10X with an ocular reticle in the section containing the oocyte nucleolus or the largest oocyte nucleus. Mean diameters of developing and atretic follicles per individual animal were the basis for comparison between groups.

Estradiol concentrations in plasma were measured with a RIA validated for female opossum plasma in our laboratory [19]. The antiserum, a gift from R.L. Butcher, West Virginia University, Morgantown, was raised in a ewe against 1,3,5 (10)-estratrien-3,17β-diol 3 hemisuccinate-human serum albumin. Cross reactivity of the antiserum was limited to 12% with estrone, and 3% with estriol [20]. Sensitivity of the assay is 2 pg/200 μl; intra- and interassay coefficients of variation averaged 6.9% and 13.7%, respectively.

Variation in body length; body mass; gain in body mass; number, size, and atresia of antral follicles; plasma estradiol concentrations; and organ mass were analyzed using mixed models method [21]: $Y_{ij} = \mu + T_i = e_{ij}$ where Y_{ij} is the dependent variable, μ is the overall mean, T_i is the fixed effect of the ith treatment (i = 1,..,6), and e_{ij} is the residual error. Heterogeneity of subclass error variance was as-

sessed using a likelihood ratio test [21]. The model was fitted with Proc Mixed of SAS (V8.1). A mixed model allows for the direct representation of heterogeneity of variance (hence the name Generalized Least-Squares – GLS) and, therefore, does not require transformation of the dependent variable to restore homogeneity of variance, as is necessary for ordinary least-squares methods. Linear *a priori* contrasts were used for comparing: (1) unexposed vs. exposed at 105 days; (2) unexposed vs. exposed at 130 days; (3) unexposed at 90 vs. unexposed at the three older ages, (4) unexposed at 105 vs. unexposed at the two older ages, and (5) unexposed at 130 vs. 150 days. Differences in the size distribution of follicles were tested with a Chi-squared test. For all analyses, the level of significance was P < 0.05. Mean values are expressed ± standard error of the mean (SEM).

Results
Three of four exposed females expressed estrus by 130 days; a fourth was in proestrus, and the fifth remained anestrous. The mean age at estrus was 127.7 ± 1.4 days, and exposed females weighed more than unexposed females at both 105 days and 130 days, (P < 0.05, Table 1). None of the unexposed females expressed estrus during the study, even though 2 weighed more than 60 g at 150 days.

Body mass and total body length increased with age in both exposed and unexposed females, but growth was faster in females exposed to male pheromone from 90 days. Exposed females gained more body mass than unexposed females (4.3 + 0.7 vs. 2.2 + 0.5 g/week at 105 days, P < 0.05) (Table 1). Growth curves for the 10 females monitored until necropsy at 130 days revealed more rapid growth in exposed than in unexposed females after 115 days, with differences in body mass evident (P < 0.05) by 123 days (Fig. 2).

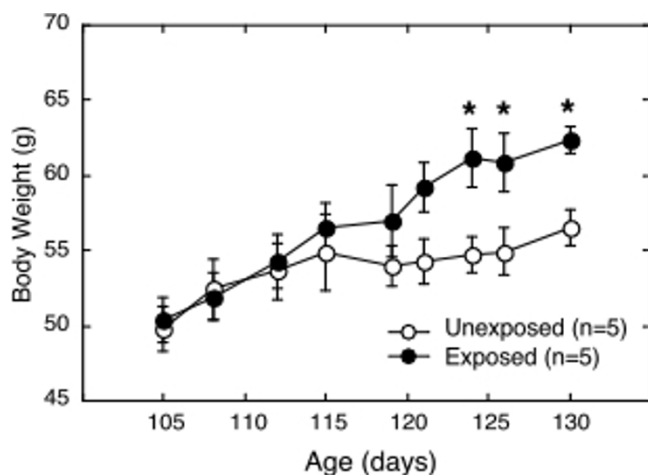The number of developing antral follicles per female exceeded those that were atretic in all but three females in

Figure 2
Growth curves of female opossums monitored from 105 to 130 days. Asterisks indicate significant differences (t-test, P < 0.05) between mean values for pheromone exposed and unexposed females.

the study. The average diameter of developing and atretic follicles within groups was similar (data not shown). Overall, follicular atresia averaged 0.37 ± 0.02 and did not differ with age or pheromone exposure (P > 0.05). Therefore, data are reported for developing antral follicles only. Follicles apparently first reach the antral stage in unexposed juvenile females near 90 days, because antral follicles were observed in only two of the four females examined at this age. One of these females had 3 atretic follicles and the other had 6 developing follicles. The average number of developing antral follicles in unexposed females increased to 10.8 ± 1.7 at 105 days (P < 0.05) and to 17.7 ± 2.6 at 130 days but did not differ by pheromone exposure within these age groups (Table 1).

Mean follicular diameter in unexposed females increased between 105 and 130 days (P < 0.05), but overall (90 to 150 days), the age-related increase in size was relatively small. Pheromone exposure was associated with increased follicle size at both 105 and 130 days (P < 0.05, Table 1). Also, the size distribution of follicles in exposed females at 130 days was markedly different from that observed in other groups (Fig. 3); 27% were 550 μm or larger. Plasma estradiol concentrations were low (< 15 pg/ml) in most females. Mean concentrations per group varied from 4.4 ± 2.0 to 11.3 ± 3.4 pg/ml but did not differ by age or pheromone exposure. The highest concentration (21.7 pg/ml) was measured in plasma from an estrous female with 6 large (> 650 μm), developing follicles.

The effect of male pheromone on sexual maturation was most apparent in the increased size of uteri and LVC collected from exposed females. Mean uterine mass was higher in exposed than unexposed females at 130 days (P < 0.05), and the LVC of exposed females were larger at both 105 and 130 days (Figs. 1, 4).

Discussion
This study demonstrates that exposure of juvenile females to pheromone from a single source, the male suprasternal gland, accelerates ovarian follicular development and first estrus. One of the more interesting findings of this study is that the size distribution of developing follicles in unexposed females remained relatively stable from 105 through 150 days, seldom exceeding 450 μm in diameter (Fig. 3). The pattern was distinctly different in exposed females at 130 days wherein nearly half (34/71) of the follicles exceeded 500 μm in diameter and 10% were larger than 600 μm, similar to the largest antral follicles (0.67 mm) in superovulated opossums [22]. This increased size of developing follicles in exposed opossums is similar in magnitude to that seen in the pre-Graafian follicles of rats between diestrus and proestrus [23]. Prepubertal pine voles exposed to male urine also showed an increase in the number of largest follicles, but with no change in atresia [24], as in our study.

Juvenile females in this study were exposed to male scent-marks from the age of 90 days, but they did not express first estrus until 130 days, much longer than the average of 7 days required for pheromonal induction of estrus in adults. In other respects, however, first estrus in juveniles is similar to pheromone-induced estrus in adults. Plasma concentrations of estradiol in opossums are low (<15 pg/ml) during anestrus and are elevated (>20 pg/ml) only during estrus [15]. Mean plasma estradiol concentrations as high as 58 pg/ml have been reported in estrous females [25], but a gradual increase in circulating estradiol during pheromonal induction of estrus in opossums has not been described. Therefore, it is not surprising that estradiol concentrations measured in single samples collected at necropsy did not correlate with reproductive status in this experiment. The one female killed at estrus, however, did have the highest plasma estradiol concentration as well as the heaviest reproductive organs.

Evidence of the stimulatory effect of male pheromone on sexual maturation of juvenile females was seen most clearly in the large increase in mass of the LVC and uteri of exposed females at 130 days, and although not reflected in plasma estradiol levels, classic estrogenic effects of male pheromone exposure are evident in the increased mass of these reproductive organs. Juvenile and adult prairie voles exhibit a similar response to male stimuli. For example, a one-hour exposure to an adult male stimulated, within 48
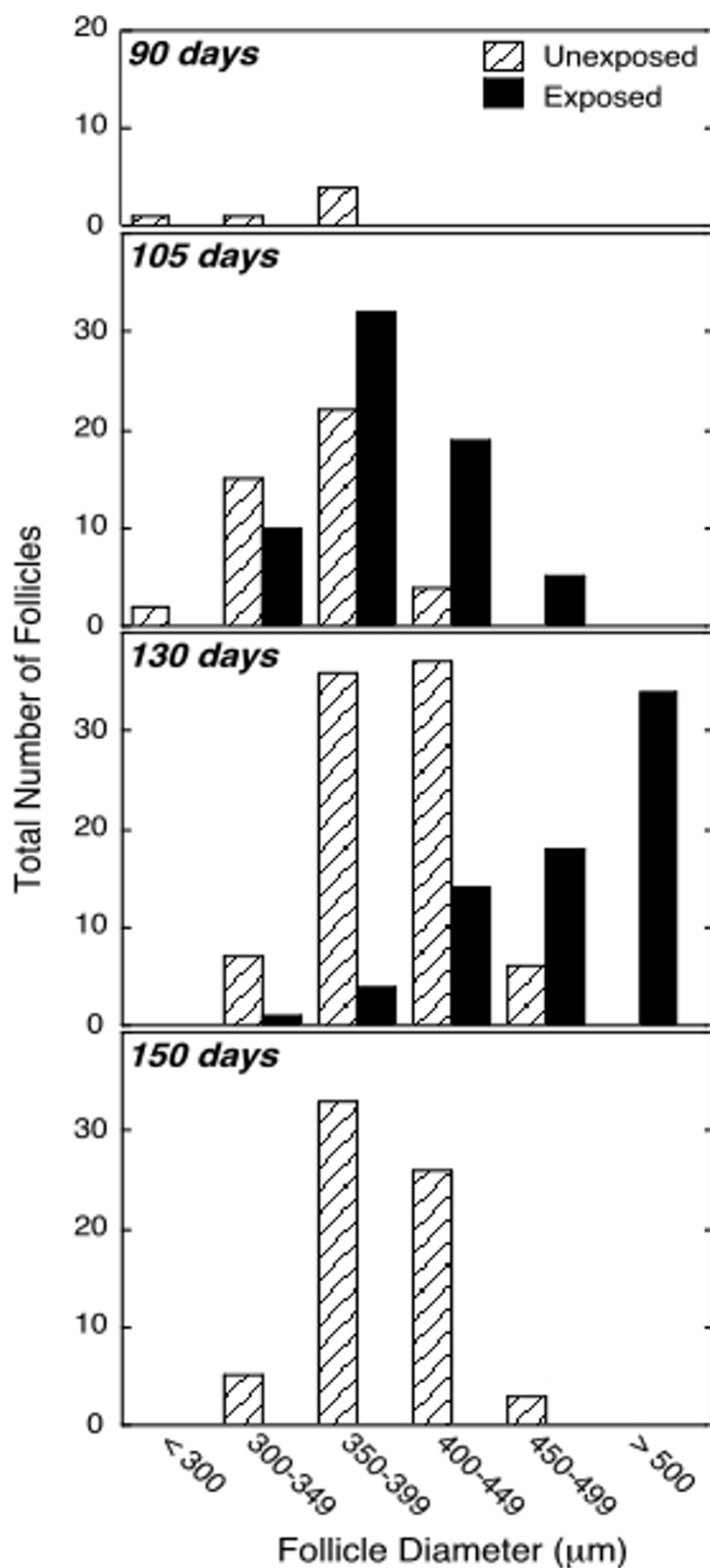
Figure 3
Distribution of antral follicle diameters in pheromone exposed and unexposed female opossums at 90, 105, 130, and 150 days (n = 4 or 5 for each age and treatment group). A significant shift (P < 0.05) in numbers of developing antral follicles to larger size classes was observed in exposed females at 105 and 130 days.
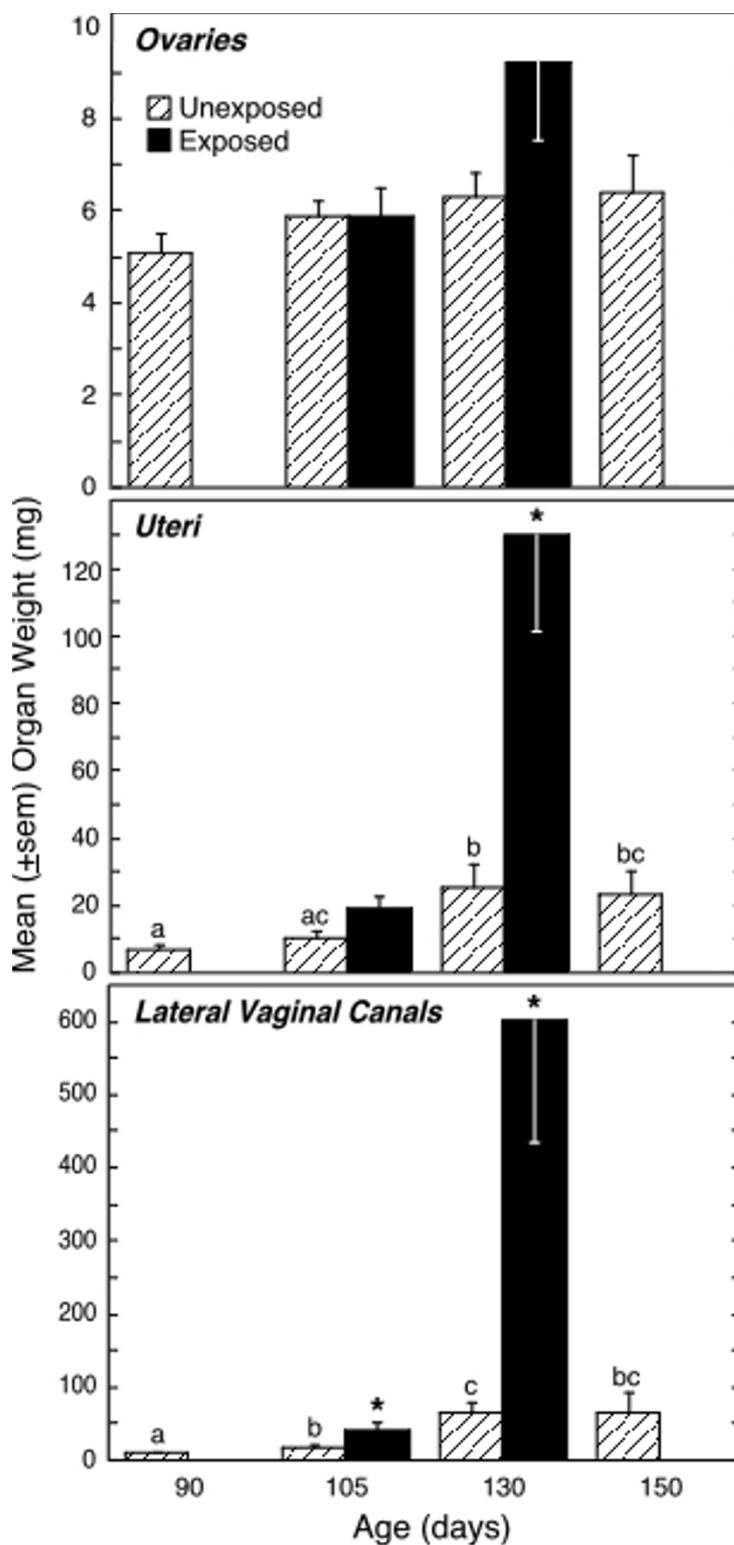
Figure 4
Reproductive organ weights in pheromone exposed and unexposed female opossums. Mean (± SEM) mass of ovaries (top), uteri (middle) and lateral vaginal canals (bottom) in exposed and unexposed females from 90 through 150 days of age (n = 4 or 5 for each age and treatment group). Different superscripts denote age-related differences for unexposed females. Asterisks indicate differences between exposed and unexposed females within an age group (P < 0.05).

hours, increases in uterine mass that lasted for 10 days or more [5]. Uterine mass also remained elevated 3–4 days after estrus in the two metestrous females in this study, even though their plasma estradiol concentrations were low at the time of necropsy.

The mean age at estrus, 127.7 ± 1.4 days, although based on a small sample (n = 3), is very similar to that recorded (126.7 ± 2.5) in our previous study [7] in which females were exposed to multiple potential sources of male pheromone, e.g. urine, feces, and scentmarks, through cage switching. The increased growth rate in females suggested in our previous experiment was confirmed in the present study. Some of the increase in body mass might be due to estrogen-stimulated edema, as adult females experience a 4–6% gain in body mass during induced estrus [15]. However, an increased growth rate (g/wk) was evident as early as 105 days in exposed females who would not be expected to express first estrus or the associated changes in body composition until approximately 130 days. (Table 1).

Sixty grams is confirmed as a critical body mass for induction of estrus in juvenile female opossums as identified in our previous study [7]. Somatic growth and attainment of a threshold body mass or energy reserves are known requisites for puberty in many mammals including the tammar wallaby [26], sheep [27,28], and several species of rodents [1], but these factors seldom act independently of environmental factors such as photoperiod [26,27] or social cues [3,29]. The opossum is unusual in this regard, because social cues in the form of male pheromone appear to stimulate both somatic growth and ovarian activation. Moreover, this growth response marks a difference between pheromonal acceleration of puberty in opossums and that which is well-documented in mice, wherein females paired with males ovulate at a lower body mass (< 20 g) than that of isolated females (> 20 g) [30,31].

Our findings provide the critical descriptive groundwork for further investigations into the physiological pathways and underlying mechanisms involved in pheromonal regulation of somatic and sexual maturation in opossums. The fact that pheromonal stimulation of both follicular and somatic growth was evident as early as 105 days makes it difficult to say, with any certainty, whether pheromones affect these phenomena by independent or interacting pathways. Pheromone exposure activates the hypothalamo-pituitary-gonadal (HPG) axis as evidenced in increased plasma LH in opossums [32] and voles [33], and ovarian activation and subsequent steroid hormone production could stimulate somatic growth indirectly by effects on growth hormone and fat deposition, or alterations in metabolism and appetite. Alternatively, in addition to stimulating the HPG axis, male pheromones could act centrally to alter neuroendocrine control of

growth and metabolism (e.g. via growth hormone releasing hormone, thyroid hormone releasing hormone, or corticotrophin releasing hormone). Future studies that control for estrogenic effects of ovarian activation, food availability, and body growth during the relatively protracted period of pheromonal induction of first estrus offer promise for extending our understanding of the role of social and environmental signals on body growth and sexual maturation.

Acknowledgments

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