The effect of intrauterine position on precopulatory behaviors and morphological parameters in CF-1 male and female mice

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THE EFFECT OF INTRAUTERINE POSITION
ON PRECOPULATORY BEHAVIORS
AND MORPHOLOGICAL PARAMETERS
IN CF-1 MALE AND FEMALE MICE

by

Annette Stevenson

A Thesis
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of Lehigh University
in Candidacy for the Degree of
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in
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December 5, 1991
Date

Professor in Charge

Chairman of Department
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This thesis is dedicated to the memory of my mother,
Mary Bruce Stevenson.
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ABSTRACT

The intrauterine position phenomenon posits that the position of a fetus in utero, relative to its male littermates, can influence its prenatal hormonal environment and thus have bearing on many sexually-dimorphic traits expressed later in life. Animals identified in utero between two males (2M) or two females (OM) were compared in two sexually-dimorphic precopulatory behaviors (preference for urinary odors and ultrasonic vocalization) and morphological parameters (anogenital distance, seminal vesicle or uterine weight).

In Experiment 1, OM males showed greater preference toward female urine than 2M males. This effect was eliminated when the data were corrected for age and group size. In Experiment 2, OM and 2M males emitted similar levels of vocalization to OM and 2M females. The results of Experiment 3 indicated that OM and 2M males were similar in adulthood with respect to anogenital distance and seminal vesicle weight. In Experiment 4, 2M females showed slightly greater preference toward male urine than OM females.
However, in Experiment 5, adult OM and 2M females were similar with respect to anogenital distance and uterine weight.

Experiment 6 showed no difference in anogenital distance at delivery when OM and 2M animals of the same gender were compared.

Overall, the findings of this study did not strongly support the intrauterine position phenomenon.
CHAPTER 1

INTRODUCTION

In order to appreciate the research of this thesis, it is necessary to understand the process of sexual differentiation and the principles of hormonal regulation that mediate it. In Chapter 1, I will briefly discuss the Organizational/Activational Model of sexual differentiation and the relationship between degree of prenatal androgen exposure and its effect on subsequent behaviors in different rodent species. A discussion on heterotypical behaviors will follow which will logically lead to a discussion of the topic of this thesis: The Intrauterine Position Phenomenon. This phenomenon will be examined in light of current evidence.

In Chapter 2, six experiments which I conducted will be presented which examine the effects of intrauterine position on morphological and precopulatory behaviors in male and female CF-1 mice.

Finally in Chapter 3, I will discuss the results and the implications of my findings.
LITERATURE REVIEW

The Organization/Activation Model of Sexual Differentiation

In their now classic experiment, Phoenix, Goy, Gerall and Young (1959) provided the first evidence that androgen exposure during prenatal development established permanent, lasting effects on morphology and behavior. In this study, testosterone propionate administered to pregnant guinea pigs at specific times of gestation produced female offspring with external genitalia indistinguishable from their male siblings. Furthermore, when adults, these females were highly responsive to testosterone treatment (as shown by a high level of mounting behavior) but were generally unresponsive to estrogen and progesterone (expressed by the absence of, or inability to show appropriate lordosis behavior).

From these results, Phoenix and his associates concluded that during prenatal periods, exogenous testosterone administration 1) masculinized the physical characteristics of the female and 2)
permanently masculinized the female brain. Thus, male
typical (i.e. mounting) behavior was elicited in
response to testosterone administration in adulthood,
and concomittantly, female-typical (i.e. lordosis)
behavior was supressed in response to estrogen and
progesterone.

These conclusions led to the concept that sex
steroids have either organizational or activational
actions on the brain and subsequent behavior,
depending upon the time at which they exert their
influence. That is, hormones induce immediate and
reversible effects on behavior (an activational
action) when administered in adulthood. These same
hormones administered earlier in development act upon
the neural and peripheral tissues (an organizational
action) to permanently "hard-wire" and establish these
pathways in a specific male vs. female way.

Since the study by Phoenix and his associates was
published, the organization/activation concept has
been widely accepted among scientists examining sexual
behavior (for reviews, see Beach, 1981; Feder, 1984;
Gorski, 1979). However, this concept recently has
been challenged for over-simplifying steroid action (see Arnold & Breedlove, 1985; Williams, 1986).
This conceptual framework, nonetheless, remains the most coherent model for understanding the action of steroids on behavior.

It is important to understand the "organizational" action of steroids since this process underlies the intrauterine position effects (the topic if this thesis) which will be discussed shortly.

The Relationship Between Degree of Prenatal Androgen Exposure and Subsequent Behaviors: Comparison Between Rodent Species

Another important principle of sexual differentiation is that the degree of "masculine" behavior expressed in adulthood is relative to the amount of circulating androgens to which the fetus is exposed prenatally. In other words, the greater the exposure of the fetus to androgens, the more "masculinized" the system becomes, thus lowering the threshold for male-typical (e.g. mounting) behavior in adulthood. To illustrate this point more clearly, let us examine the variations in prenatal androgen
exposure across various species of rodents (Table 1), and then compare this to the behaviors observed in these species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sexual Differentiation (Testicular Activity Begins)</th>
<th>Behavioral Sexual Differentiation (Neural Tissue Influenced)</th>
<th>Birth</th>
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<tbody>
<tr>
<td>Mouse (a)</td>
<td>Day 13</td>
<td>Undetermined</td>
<td>Day 19</td>
</tr>
<tr>
<td>Rat (b)</td>
<td>Day 13 (b)-Day 15 (c)</td>
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<td>Day 25</td>
<td>Day 30</td>
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Note. From
a) vom Saal, 1984, p. 136.
c) Tobet, Dunlap and Gerall, 1982, p. 251.
d) Goy and McEwen, 1980, p. 16.

As shown in Table 1, a male rat fetus begins secreting androgens at approximately day 14 of
gestation. Thus, fetal rats are exposed to testicular androgens for approximately 9 days prior to birth. In comparison, a hamster fetus has a relatively shorter exposure period of only 4 days.

Furthermore, when androgen levels in amniotic fluid are compared, rats have significantly higher levels of circulating androgens than hamsters, with males having greater exposure than conspecific females (Vomachka & Lisk, 1986). Correlating with this interspecies difference, female rats readily show mounting in response to testosterone (Clemens, 1974) but female hamsters do not. Moreover, lordosis is more easily elicited in male hamsters than in male rats when primed with ovarian hormones (Vomachka & Lisk, 1986). Thus, it appears that there is a higher androgen threshold (i.e. less responsiveness to androgens) in hamsters than in rats; hamsters being less "masculinized" than rats, due to the lower level of androgen exposure prenatally.

These behavioral differences between rodent species, correlated with the degree of androgen exposure (both amount and duration of exposure) show a
clear correlational relationship between prenatal androgen exposure (or lack thereof) and subsequent sexual behaviors in adulthood. This is useful in making predictions about such behaviors based on the organizational action of steroids, and extrapolating this causal relationship to other species not yet studied.

Heterotypical Behaviors

Characteristically, males and females have certain behavioral patterns that are gender-typical. For example, in many rodent species, males are much more likely to display male-typical behaviors such as mounting, intromission, and ejaculation. Females are more likely to display female-typical behaviors which include solicitation of the male (expressed by darting, hopping, and ear-wiggling) and lordosis. Such behaviors are referred to as sexually-dimorphic and are adaptive for purposes of reproduction.

Interestingly, some mammalian species occasionally display opposite-sex, or heterotypical behaviors (cited in Gorski, 1979; Goy & McEwen, 1980; Meisel &
Ward, 1981; Schaeffer, Roos & Aron, 1990; Slob & Van der Schoot, 1981). The adaptive function is not clear. Vom Saal (1981) has alluded to these behaviors as being important in population regulation (discussed later in this section). In contrast, I propose that heterotypical behaviors are often "residual" effects of neural pathways which were once bipotential during prenatal development. Later in adulthood, these heterotypical pathways occasionally become activated causing the animal to respond in a heterotypical fashion.

As discussed in the previous section, the expression of heterotypical behavior varies between species. Even within members of the same sex and species, the degree to which a sexually-dimorphic trait is expressed varies greatly. Why do some females tend to be more aggressive than others? And why do some females display more mounting behaviors than their sisters? Why are some males more sexually active, or more prone to aggressive behavior than others? In other words, what accounts for individual differences in the display of certain behaviors? The
intrauterine position (IUP) phenomenon which is to be further examined in this thesis, has been proposed to account for some of the individual variability in sexually-dimorphic behaviors in polytocous (litter-bearing) mammals.

The Intrauterine Position Phenomenon

The intrauterine position theory was first described in 1974 by Clemens, citing preliminary work done in conjunction with Coniglio (Clemens & Coniglio, 1971), which showed a positive correlation between the number of female rats that displayed mounting behavior and the number of males in the litter. Following testosterone treatment in adulthood, female rats from litters with 3 or more males showed a higher tendency to exhibit male-typical mounting than females from litters with 2 or less males. Also, since anogenital (AG) distance (the distance between the genital papillae and anus) is a measurable index of androgen exposure (Clemens, 1974), anogenital distance was also examined. The presumption was that the amount of androgen exposure was directly related to the female's
proximity to her male littermates (i.e. the source of androgens were coming from the male fetuses). As might be expected from these considerations, when pups were delivered, the females located closer to males had longer AG distances than females located further from males.

These preliminary findings suggested that 1) variation in morphology and behavior varied as a function of the pup's exposure to prenatal androgens, and 2) that androgen exposure varied as a function of male proximity in utero; the closer to a male fetus, the greater the androgen exposure. Clemens' novel idea provided the basis for much research that was to follow.

Other studies (see Appendix 1) have challenged or extended these earlier findings. The intrauterine position model has been assessed in both sexes, across various species (mice, rats, guinea pigs, gerbils, hamsters, and pigs) and across various physiological, morphological, and behavioral parameters. Prior intrauterine position has been shown to influence various sex-trait characteristics including anogenital
distance (Gandelman, vom Saal & Reinisch, 1977; Meisel and Ward, 1981; Richmond & Sachs, 1984), body weight (Kinsley et al., 1986c), reproductive behavior (Tobet, Dunlap & Gerall, 1982; vom Saal & Bronson, 1978), aggression (Rines & vom Saal, 1984; vom Saal, Grant, McMullen & Laves, 1983) maternal behavior (Kinsley, Konen, Miele, Ghiraldi & Svare, 1986a), and acquisitional learning (Hauser & Gandelman, 1983).

**Intrauterine Position Classification Schemes**

The intrauterine position phenomenon suggests that the position of a fetus in utero, relative to its male littermates, can influence its hormonal environment prenatally and thus have bearing on many sexually dimorphic traits expressed later in life.

In order to identify animals of different intrauterine positions, vom Saal (1981) provided a "contiguity" classification scheme (Figure 1) which identifies the location of an animal relative to its proximity in utero to males. This classification scheme is based on the assumption that androgens are
produced by the male fetal testes, and are transported by diffusion across the fetal amniotic and chorionic membranes to neighboring fetuses (this proposed transport mechanism shall be discussed shortly). Therefore, animals located between two male fetuses in utero, termed 2M animals, have the greatest exposure to fetal testosterone during prenatal development. Animals located between two female fetuses during development, termed OM animals, are minimally exposed to testosterone. Animals located adjacent to only one male fetus, termed 1M, are intermediately exposed relative to their OM and 2M littermates. 1M animals are generally not used for comparison analyses since these animals have morphological and behavioral characteristics intermediate between OM and 2M animals (vom Saal, 1989).
Figure 1: Intrauterine position classification scheme, identifying animals between two males (2M), between two females (OM) and between a male and female (1M).

Much of the research examining the effects of intrauterine position is based on comparing same-gender 0M and 2M animals for various traits that normally show gender dimorphisms. This classification scheme will be the focus of this review and will be used in the subsequent set of experiments for this thesis.

However, another classification scheme (referred to as caudal-male) has also been used, based upon a different proposed mechanism of hormonal transport. This scheme (Meisel & Ward, 1981) classifies animals according to the number of males located caudally (i.e. toward to cervix) to the target animal in the same uterine horn. This scheme is based on the assumption that fetal testosterone is transported to other fetuses in the same uterine horn via maternal uterine blood flow. According to Meisel and Ward (1981) the maternal vascular system is set up such that "... both arterial and venous flow proceed[s] from the cervical end toward the ovary" (p.239), and therefore is unidirectional. Thus, fetal androgens are transported "downstream" (toward the ovarian end).
from male fetuses located further "upstream" from their littermates. Based on this model, OM would indicate that no males are located upstream to the animal, 1M indicating 1 male was upstream, and 2M indicating 2 or more males were located upstream to the animal (Meisel & Ward, 1981).

On the surface, these two (contiguity vs. caudal-male) classification systems appear similar, but in fact, they are conceptually different, based upon their proposed mechanisms of steroid transport. However, Houtsmuller and Slob (1990) are quick to point out that some "overlap" exists between these two systems even though they are conceptually different. In vom Saal's classification scheme, "2M" animals consist of 1 caudal male and 1 cephalic (or rostral) male to the animal of interest. Thus, 2M animals in vom Saal's scheme are synonymous to 1M animals (1 male caudal) in Meisel and Ward's classification. Therefore the contiguity and caudal-male references are not mutually exclusive coding schemes. Houtsmuller and Slob (1990) explain that the "results [supporting the contiguity hypothesis] need not be
contradictory to the caudal male hypothesis since females with two adjacent males have at least one male caudally" (p. 559). It is important to recognize that this confounding factor is present when evaluating the data using either of these two classification models.

More recently, two other classification systems have been developed; the first using a female referent in a study of male gerbils (Clark, Malenfant, Winter & Galef, 1990), such that 0F designated an animal adjacent to no females (i.e. being either a 2M classified by vom Saal, or a 1M animal located at the end of a uterine horn), and 2F identified animals adjacent to 2 females in utero.

The second classification model uses a cervical-male flanking ("CX") classification (Lephart, Fleming & Rhees, 1989), being similar to the caudal-male scheme. In this model, OMCX designates animals having no males between them and the cervical end of the uterine horn, 2MCX identifies animals that have 2 males between them and the cervical end of the uterine horn.

In the following discussion, the intrauterine
position data will be examined in the context of the various classification systems used [contiguity (vom Saal, 1981), caudal-male (Meisel & Ward, 1981), contiguous-female (Clark et al., 1990) and cervical-male flanking (Lephart et al., 1989)].

Proposed Mechanisms of Hormonal Transport: Caudal Male Versus Contiguity Hypothesis

The exact mechanism of androgen transport between fetuses is still unknown. However, two theories have been proposed, 1) the contiguity hypothesis and 2) the caudal-male hypothesis, which is the basis for the classification schemes previously described.

Contiguity Hypothesis

As mentioned earlier, the contiguity hypothesis (Clemens, 1974; Gandelman et al., 1977; vom Saal, 1981) proposes that fetal androgens, produced by the male testes, are transported by diffusion across the amniotic and chorionic membranes.

To examine this possibility (vom Saal, 1989), radio-labelled testosterone ([3H] T) capsules were
implanted into the amniotic fluid of designated rat fetuses and the amount of tracer radioactive hydrogen (tritium) was measured in adjacent fetuses. Tritium was found in the amniotic fluid of contiguous fetuses on both sides of the T-implanted fetus (supporting the contiguity argument). More interestingly, the percent of recovered tritium in the fetus located on the cervical side was three times greater than the amount of tritium recovered in the fetus located on the ovarian side, which is opposite to predictions from the caudal-male theory of uterine blood flow.

A second experiment in rats (vom Saal, 1989) utilized the injection of pontamine blue dye into the uterine artery and vein of each horn to examine the direction of its movement. Injections into these vessels were made mid-way between the fetuses, so that approximately half of the fetuses were located caudally and rostrally to the injection site. Results showed that the dye spread in both caudal and rostral directions from the injection site, indicating bidirectional arterial and venous blood flow (not unidirectional as Meisel and Ward had suggested).
Furthermore, when the artery was clamped at particular points along the uterus, "the dye moved through the artery up to the clamp from both directions" (vom Saal, 1989, p. 1836), thus providing evidence that in rats, uterine blood flow is bidirectional, again in contradiction to the caudal-male theory of androgen transport.

This mechanism of transport has been further supported by studies in rats (Tobet et al., 1982), mice (Gandelman et al., 1977; Kinsley, Miele, Konen, Ghiraldi & Svare, 1986b; vom Saal & Bronson, 1978), and gerbils (Clark, Bone & Galef, 1989; Clark & Galef, 1988), but not in hamsters (Vomachka & Lisk, 1986) or pigs (Rohde Parfet et al., 1990).

Caudal-Male Hypothesis

An alternative hypothesis proposed by Meisel and Ward (1981) suggested that androgens from male fetuses are transported to neighboring fetuses via the maternal blood. The authors cite earlier work from Del Campo and Ginther (1972) which indicated that uterine blood in rats "drained in the cranial
direction" (p. 2575), from the cervical to the ovarian end of the uterine horn. Meisel and Ward (1981) proposed that the close proximity of uterine veins and arteries enable substances (such as fetal androgens) in the venous blood to transfer into arterial blood via a countercurrent mechanism, which then feeds fetuses located further downstream.

Applying this theory, Meisel and Ward were able to show that female rats located downstream from 1 or 2 caudal males (1M and 2M, respectively) in the uterine horn, displayed significantly more mounting behavior (in both percentage, 1M = 75%, 2M = 67%, and frequency, 1M: \( M = 22 \pm 9.4 \) mounts; 2M: \( M = 24.4 \pm 7.4 \) mounts) after testosterone priming, than females with no caudal males (32%; \( M = 7.9 \pm 3 \) mounts). Moreover, 1 caudal male appeared to be just as effective as 2 caudal males in accentuating female mounting behavior. When these same animals were classified according to the contiguity hypothesis, Meisel and Ward found a significantly higher percentage of 2M females that mounted (86%) than 0M females (33%), however, the frequency of mounting between groups was not
significantly different.

When anogenital distance/ body weight ratios were examined (Meisel & Ward, 1981), females with no caudal males had significantly smaller ratios than females with 1 or 2 caudal males. However, contiguity to males had no effect on anogenital distance/ body weight ratios between 0M and 2M animals (strangely, the intermediate 1M females had significantly greater ratios than 0M or 2M females classified this way).

Further analysis of the 1M females (contiguity scheme) examined mounting behavior based on whether the male was on the caudal or ovarian side. 1M females with males on the caudal side displayed more mounting ($M = 33.3 \pm 13.7$) than females with the male on the ovarian side ($M = 10.5 \pm 4.9$), again supporting the caudal-male model of androgen transport. In addition, the importance of the caudal-male has also been supported in hamsters (Vomachka & Lisk, 1986).

To determine which transport mechanism is best supported by the data, 2 studies in female rats (in addition to Meisel and Ward's study) have utilized both the contiguity and caudal male classification
schemes in the same test design (Houtsmuller & Slob, 1990; Richmond & Sachs, 1984).

In the first of these two studies, Richmond and Sachs (1984) assessed anogenital distance in female rats as a function of their location in the uterus via both classification schemes. Findings revealed that 2M females had longer anogenital distances than both 0M females and females with a male positioned rostrally to it. But 2M females did not differ from females with a male located caudally to it, indicating that the masculinizing effect in these females was attributed to the male located caudally. Furthermore, the presence of 2 or more caudal males had no difference on the masculinizing effect on anogenital distance of these females than did 1 caudal male, supporting the earlier findings of Meisel and Ward (1981).

More recently, lordosis and mounting behaviors in female rats were analyzed according to both classification methods (Houtsmuller & Slob, 1990). After appropriate hormone treatment, females classified by the contiguity scheme failed to show any
difference in lordosis (mean lordosis quotients were not significantly different). However, females of the caudal-male groups showed significant differences in lordosis; females with caudal males had significantly lower lordosis quotients (i.e. more defeminized) than females with no caudal males present. In mounting behavior, females classified as having caudal-males showed significant differences; females with caudal males displayed higher levels of mounting (i.e. more masculinized) than females with no caudal males. These differences were not seen between females classified in the different groups of the contiguity method. Further analysis revealed that caudal males did not have to be adjacent to the female in order to exert masculinizing effects; mounting levels were similar in female groups that had adjacent caudal males or non-adjacent caudal males.

The findings of these two studies unanimously support the caudal-male theory and not the contiguity hypothesis in rats. Furthermore, they suggest that 1) only 1 caudal male is sufficient to exert its masculinizing effects on the female, and 2) that these
effects of caudal males are independent of their proximity (i.e. they don’t have to be adjacent to exert their effects). These findings, however, are limited to rat data and therefore caution is warranted in drawing definitive conclusions about the mechanism of transport in other species.

In guinea pigs, Gandelman (1986) suggested that both contiguity and caudal position of the male (i.e. a male must be both adjacent and upstream) are important factors in masculinizing the female to display mounting. When precopulatory behaviors (hip swaying and genital sniffing) and copulatory behavior (mounting) were assessed in female guinea pigs, intrauterine position had no effect upon precopulatory behaviors, but did affect mounting.

The apparent lack of intrauterine position effects observed in swine (Rohde Parfet et al., 1990) based upon the contiguity scheme, may in part be attributed to the nature of the uterine vascular system. vom Saal (1989) points out that unlike in rats and mice, the fetal membranes of pigs do not contact one another. Because of this inter-fetal space, diffusion of
androgens across fetal amniotic membranes may not be possible. To date, no studies have examined intrauterine position effects in pigs based on the caudal-male method.

Finally, a pivotal issue concerning the contiguity vs. caudal-male argument centers on the confounding factor of 2M animals (based upon the contiguity scheme) being equal to the 1M (1 male caudal) animals based upon the caudal-male scheme (discussed earlier). This design of identifying the caudal and rostral position of males relative to the animal of interest is currently being investigated in our lab (Jubilan & Nyby, unpublished).

Clearly, these studies indicate that the apparent mechanism of transport may be contingent on the anatomical structure of the uterine environment which differs between species.

Hormonal Differences in Animals of Different Intrauterine Positions

Mice

vom Saal and Bronson (1980a) examined
concentrations of Testosterone (T), Estradiol (E2), and Progesterone (P) in fetal amniotic fluid and blood in CF-1 mice, identified by intrauterine position on Day 17 of gestation. As expected, blood testosterone concentrations were three times greater in males than in females. However when females were examined, 2M females had significantly higher concentrations of testosterone in their amniotic fluid and blood serum than OM females. E2 and P concentrations were not different between males and females, nor between OM and 2M females. When serum T concentrations were assessed in adult OM and 2M females, no differences were found. Furthermore, to rule out the maternal circulation transporting the androgens, two groups of pregnant females were sacrificed prior to delivery (day 17 of gestation). Each group consisted of ten mothers. One group of mothers carried litters consisting of 9 males and 3 females. The other group carried litters consisting of the reverse ratio (9 females and 3 males). When the maternal blood was examined from each group of mothers, no differences in T, E2 or P concentrations were found, suggesting that
differences in testosterone levels in female fetuses could be attributed directly to inter-fetal transport and not through maternal circulation.

A more recent study examining these same parameters on day 18 of gestation (vom Saal et al., 1990) support the earlier findings, showing that 2M females had significantly higher T concentrations in their plasma than OM females. But contrary to the previous study, this study showed that estradiol levels varied as a function of intrauterine position as well. In addition to having higher T concentrations, 2M females had significantly lower levels of estradiol than OM females, suggesting that the intrauterine position phenomenon may be mediated by "an interaction between testosterone and estradiol rather than by testosterone alone" (as was suggested in the previous report) (vom Saal et al., 1990, p. 757).

In male CF-1 mice, estradiol, testosterone and progesterone concentrations were also assessed (vom Saal et al., 1983) on day 17 of gestation (Day 19= birth). As expected, female fetuses had higher levels of estradiol in their amniotic fluid than did males.
When males were compared on day 17 of gestation, males located in utero between 2 females (i.e. OM males) were found to have higher concentrations of estradiol in their amniotic fluid than their 2M counterparts. No differences were found in the concentrations of testosterone or progesterone in amniotic fluid or blood levels of OM vs. 2M males.

However, when these same parameters were examined on day 18 of gestation (1 day later than was previously studied), significant differences in both T and E2 plasma concentrations between OM and 2M males were observed (vom Saal, 1989). 2M males were found to have higher T while at the same time lower E2 levels in plasma than OM males.

From these studies it appeared that on day 17 of gestation, T varied in females and E2 varied in males. When these same levels were determined on day 18 2M males and 2M females had higher plasma concentrations of testosterone and lower concentrations of estradiol than their same-sex OM counterpart.

As a result, vom Saal (1989) suggested that sex differences are mediated by both testosterone and
estrogen. Tissue receptors specific to androgens are enhanced in the 2M animal because of their greater exposure to testosterone prenatally (when the tissues are organized). On the contrary, tissue receptors specific to estrogens are enhanced in the OM animal due to the fact that these animals are exposed to greater levels of E2 prenatally. vom Saal and his colleagues (vom Saal et al., 1983) further suggested that elevated concentrations of estradiol mediate sexual and aggressive activity in male mice. However, this suggestion seems inconsistent with current evidence concerning the aromatization hypothesis and also discounts the role of alphafetoprotein.

Nonetheless, the findings of these four previous studies do indicate that steroid levels vary as a function of intrauterine position for both sexes; animals located between 2 males (2M animals) have elevated concentrations of testosterone and decreased levels of estradiol when compared to animals adjacent to no males (OM animals). Furthermore, these studies suggest a sensitive period for intrauterine position effects, since concentration differences of both
steroids (T and E2) were apparent at a specific gestational period (i.e. after day 18) which were not apparent previously.

Hamsters

In a study examining hamsters, Vomachka and Lisk (1986) found expected gender differences in plasma testosterone (males significantly more than females) but not in estradiol levels (males and females were equivalent) on day 14 and 15 of gestation (Day 16 = birth). When amniotic fluid concentrations were evaluated using a female reference classification scheme, males with 2 or more caudal (upstream) females (i.e. 2F males) had significantly higher levels of estradiol and lower levels of androgen, than males with no caudal females (i.e. OF males). Moreover, hormone levels (both T and E2) were not different between OF and 2F males when compared according to the number of females located cephalically (i.e. downstream) nor by the proximity to females according to the contiguous classification scheme. Interestingly, using the normal "male" reference.
(i.e. OM vs. 2M ) in either caudal-male or contiguous classification schemes failed to show differences in hormone levels (T and E2) in females or in males. Thus, the findings of this study failed to support the notion that male fetuses in utero (either proximally or caudally) influence the hormonal milieu of female (or male) siblings prenatally. Rather, these results suggest the importance of the female's proximity in hamsters, and support the caudal (transvascular) mechanism of steroid transport. Based on these findings, the authors suggest that the prenatal steroids may be operating by a "feedback suppression" mechanism in hamsters (p. 189) whereby E2 from female siblings could suppress the androgen exposure of males downstream. This transport mechanism is consistent with the literature (see Goy & McEwen, 1980, p.16) which show that male hamsters are less responsive to androgens in adulthood (being incompletely masculinized) than most other rodent species (refer to Table 1) due to the fact that they are exposed to less androgens prenatally.
Rats

In contrast to the positive findings above, a recent study (Baum, Woutersen & Slob, 1991) examined whole-body androgen concentrations (testosterone + 5 alpha-dihydrotestosterone) in male and female rats on fetal days 16-22. Results showed expected sex differences in fetal androgen concentration on day 18 and 19; males had significantly greater androgen concentration than females. However, the proximity to males had no effect upon whole-body androgen concentrations in females (inconsistent with the findings of vom Saal & Bronson, 1980a). Androgen concentrations were not significantly different between 0M, 1M and 2M females classified by either the caudal-male or contiguity models. Moreover, these findings provided no evidence that the origin of the prenatal androgens were from male littermates.

Ferrets

A similar study in ferrets (Krohmer & Baum, 1989) examined whole-body androgen concentrations in females using both classification methods. Unlike the negative
findings in rats, female ferrets with 2 caudal males had significantly higher concentrations of testosterone than females with 1 or less caudal males. However, there was no significant difference in androgen concentrations between females classified by the contiguity method.

Species Differences

Collectively, these studies point to the differences in hormonal concentrations that could exist because of differences in 1) species 2) hormone secretion on specific days of gestation and 3) the hormonal transport mechanism.

The male-contiguity model is best supported by hormone data from mice. However, the evidence on steroid concentrations in rats does not support either the caudal-male or contiguity models. A third profile was apparent in hamsters. In this species, contiguity to females rather than males was responsible for differences in steroid concentrations. In ferrets, intrauterine position effects upon hormone levels were apparent when animals were classified by the caudal-
The Effect of Intrauterine Position on Morphological Parameters

Morphological considerations have entered into all attempts to understand the mediation of sexually dimorphic behaviors. It has been argued that the effects of perinatal hormones on behavior can be better accounted for by the changes in peripheral organs than by modification of the central nervous system (Goy & McEwen, 1980, p.17).

In the previous section we have seen that differences in hormonal concentrations exist between animals of different intrauterine positions. But does prior intrauterine position (and the differences in steroid exposure that this phenomenon implies) also affect the anatomical structures of these animals? This section will examine this issue in light of the research that supports it.

Anogenital Distance

Anogenital distance (AGD; the distance between the anus and the genital papilla), is a sensitive and
reliable measure of prenatal androgen exposure (Clemens, 1974; Meisel & Ward, 1981; Tobet et al., 1982). Characteristically, males have longer AGD's than females (Zielinski, Vandenbergh & Montano, 1991), due to the masculinizing effects of prenatal androgens. Thus, AGD has been used as an index of androgen exposure in many intrauterine position studies.

Females.

Preliminary work by Clemens (1974), and later by Clemens, Gladue and Coniglio (1978), demonstrated an inverse relationship between AGD in female rats and the proximity to males in utero. Thus the variation in AGD was proportional to the degree of androgen exposure prenatally. Interestingly, these intrauterine position effects were eliminated when antiandrogens (flutamide) were administered to pregnant mothers during the latter half of gestation; AGD in the delivered females from all intrauterine positions were nearly identical (being similar to that of OM females). Thus, prenatal androgens appeared
responsible for the masculinizing effects upon AGD in female rats.

Significant differences in female rat pups were also reported (Meisel & Ward, 1981) using the caudal-male classification scheme. Since AGD co-varied as a function of body weight (BW) in this study, AGD/BW ratios were used to control for this confounding factor. Females with 1 or more caudal males had greater AGD/BW ratios than did females with no caudal-males in utero. Similar findings were reported by Richmond and Sachs (1984); female rat pups with 1 or more caudal-males had greater AGD/BW scores than females with no caudal-males. However, when AGD was examined again at day 22 of age, these intrauterine position effects had disappeared, with all female groups having similar AGD/BW scores.

Female rats classified by the contiguity method were also found to differ on AGD at birth (Tobet et al., 1982); 2M females having longer AGD's than their OM counterparts.

Similar findings were reported in CF-1 female mice; 2M females having longer AGD's than their OM counterparts.
counterparts (Gandelman et al., 1977; vom Saal & Bronson, 1978; vom Saal et al., 1990). It should be noted that AGD co-varied as a function of body weight in only 1 of these 3 studies (vom Saal et al., 1990). However, when this was controlled (using analysis of covariance), AGD still varied as a function of intrauterine position; 2M females having longer AGD's than OM females. In adulthood, these intrauterine position effects in CF-1 females (2M > OM) were still apparent (vom Saal & Bronson, 1978) suggesting that these characteristics are independent of the activational effects of steroid hormones in adulthood.

In wild house mice (Zielinski et al., 1991), the data is consistent with the previously reported findings; 2M females had significantly longer AGD's at delivery than OM females. Moreover, in this strain, (unlike in rats) the variance in AGD was not attributed to the number of cervical-flanking males, but rather to the contiguity of males.

The general findings in female AGD appear consistent between species and strains and according to the caudal-male and-contiguity methods; 2M females
had longer AGD's than OM females and caudal-male females had longer AGD's than non-caudal-male females.

Males.

The previous studies have provided fairly conclusive evidence that masculinization of female rodents appears to be due to the intrauterine position of these females with respect to male littermates. However, the data on AGD in males is not as conclusive.

In wild mice (Zielinski et al., 1991), no differences were found in AGD between OM and 2M male pups at delivery. However, in a study of rat pups at day 18 of gestation (Lephart et al., 1989), males with 2 cervical-flanking males (2MCX) had significantly longer AGD than males with no cervical-flanking males (OMCX). However, these differences were not found when the pups were measured 2 days later (day 20). This short-term effect would suggest the occurrence of an androgen surge. The authors propose that prior to the peak in T levels (i.e. days 18 and 19), male fetuses are sensitive to the circulating levels of
androgens produced by male littermates. This would explain why AGD differences were found at day 18. However, these differences in AGD are eliminated on day 20 due to the T surge in all male fetuses (i.e. T threshold increases, and thus sensitivity to T decreases).

In adult Mongolian gerbils (Clark et al., 1990) using a female reference scheme, males located next to females had shorter anogenital distances than males located next to males. These findings are congruent with the findings reported in female mice (vom Saal & Bronson, 1978) using the male-contiguity scheme.

Contrary to the previous findings in rats, mice and gerbils, no correlation was found between intrauterine position and anogenital distance in male or female pigs at birth or in adulthood (Rohde Parfet et al., 1990), again indicating that intrauterine position effects may be species-specific.

Looking at these studies collectively, AGD appears to be a more reliable indicator of intrauterine position in females than in males. This may be due to the fact that males, which are exposed to their own
androgens, are not as responsive to the subtle variations in androgen levels contributed by other male fetuses. Females, on the other hand, may be more responsive because they have much lower baselines of androgenic stimulation. These findings also point to the species differences in the intrauterine position phenomenon.

**Body Weight**

Body weight is another dimorphic trait modulated by androgens, with males tending to gain more weight than females (Kinsley et al., 1986c, p. 202). Body weights of IUP-identified male and female R-S mice were measured during early development (birth-day 25) and again during pubertal and adult development (day 25-120) (Kinsley et al., 1986c). As expected, males were heavier than females throughout. Intrauterine position significantly affected body weight in early development in IUP-identified females with 2M females being heavier than their OM counterparts. Moreover, 2M females were similar in weight to OM and 2M males. These effects were also consistent throughout
adulthood.

Males in this study showed a similar trend (although not-significant) with 2M males being heavier during early development than OM males. In adulthood, males showed a similar weight pattern as the female groups (i.e. 2M > OM), although this effect was not as consistent as the female groups (i.e. weight differences were found only on days 30-100, day 115). Furthermore, no relationship was found between food intake levels and intrauterine position during puberty and adulthood in male and female groups. Thus, weight differences in IUP-identified animals appear to be attributed to the organizational effects of androgens prenatally.

The general findings of this study indicate that 1) 2M animals are heavier than OM animals, and 2) intrauterine position effects are more consistently observed in females than in males.

Seminal Vesicle, Prostate and Semen Characteristics in Males

Seminal vesicle weight is often used as a
morphological index of testosterone sensitivity. OM and 2M male CF-1 mice (vom Saal et al., 1983) received 35 days of testosterone treatment in adulthood and were later sacrificed to remove and weigh their seminal vesicles. 2M males had significantly heavier seminal vesicles than their OM counterparts, thereby suggesting a higher sensitivity to T in 2M males than in OM males. A similar study in CF-1 males (Wechman, Brown & Hilton, 1985) did not support these earlier findings; seminal vesicle weights were similar in OM, 1M and 2M adult males.

Contrary to expected predictions, prostate glands in gonadally intact adult male mice were heavier in OM males than in 2M males (vom Saal, 1989). This apparent contradiction (that 2M's have heavier seminal vesicles, but lighter prostates than OM's) is explained by vom Saal (1989) who suggests that the organization of these tissues is regulated by specific binding of estrogen and/or androgens receptors:

Tissues that have androgen receptors, but not estrogen receptors [e.g. seminal vesicles], are enhanced in 2M male fetuses to elevated concentrations of testosterone. In contrast, in tissues with estrogen receptors [e.g. prostates], fetal
organization is correlated positively with circulating estradiol concentrations (OM > 2M). However, estradiol alone does not lead to masculinization of tissues that have both androgen and estrogen receptors [as in prostates]. These tissues required binding of both androgen and estrogen to specific receptors to show normal development. (vom Saal, 1989, p. 1835).

In male rats (Lephart et al., 1989) classified by the male-flanking method, there were no reliable differences in testis weights between OMCX males and 2MCX males when body weight was taken into account.

In a related study in gerbils, using a female reference classification scheme (Clark et al., 1990), testes weight and ventral gland size were measured between 100-110 days of age. Males located in utero between 2 females (2F) had significantly lighter testes and smaller ventral gland pads than males not contiguous to females (OF) in utero.

No significant morphological differences were observed in male pigs (Rohde Parfet et al., 1990). Testes weights and semen characteristics (i.e. volume, sperm conc., sperm/ejaculate, % motility, % of abnormalities) were measured in adulthood in OM, 1M and 2M males. Overall findings showed similar testes
weights and semen characteristics between groups.

Collectively, the morphological findings in males are inconsistent. The 2 studies examining seminal vesicle weight in mice are contradictory (vom Saal et al., 1983 vs. Wechman et al., 1985). While intrauterine position influenced testes weights in gerbils, it was not influential in rats or pigs. It is clear that further research examining these morphological characteristics in male rodents is warranted.

The Effect of Intrauterine Position on Reproduction

Reproduction in rodents is highly complex and is intimately linked with neuroendocrine events within the animal and communication (primarily pheromonal via the olfactory system) with other conspecifics. Thus, the ovaries (or testes), pituitary and hypothalamus interact with one another; the secretions from one gland stimulating the secretions of the others. As illustrated in Figure 2, these hormonal interactions in turn, relay information to higher centers of the
brain which modulate appropriate behaviors in response to internal events.

Figure 2: Highly schematic representation of two levels of involvement of the brain in the regulation of reproduction: 1) intrinsic regulatory processes which include the reciprocal interactions between brain, pituitary (P) and gonad and 2) extrinsic regulatory processes which involve an interaction between two individuals either at the level of special sensory input (2a, e.g. olfaction, vision) or general afferent stimuli (2b, e.g. genital stimulation). Abbreviations: A, adrenal; O, ovary; T, testis; U, uterus. Note. From "The neuroendocrinology of reproduction: An overview" by R. A. Gorski, 1979. Biology of Reproduction, 20, p. 112.
Concomitantly, sensory information from external stimuli (transmitted via the olfactory system) is processed in the brain and interacts with these intrinsic mechanisms. Thus, the animal's transmission of, and response to, social cues is regulated by this hormonal feedback system.

This section will describe the effects that intrauterine position has on these intrinsic mechanisms in adulthood.

Females

Estrous cycle.

It is well-established that the estrous cycle of the female rodent can be facilitated by the presence of a male (particularly by pheromonal cues) whereas the presence of another female has exactly the opposite effect of prolonging and sometimes inhibiting the cycle (Johns, 1980; Novotny et al., 1980; Wysocki, 1979). Thus, regulation of the estrous cycle (an intrinsic mechanism) is strongly influenced by pheromonal cues (extrinsic factors) in the environment.
vom Saal and Bronson (1978) compared 0M and 2M females under individual and group-housing conditions to examine the effects of both intrauterine position and housing conditions on age of puberty. 0M and 2M females, individually-housed with a stud male, showed no difference in their time to mating; all females in both groups mated, (0M group: $M = 5.5 \pm 0.5$ days, 2M group: $M = 6.5 \pm 0.9$ days). When females were group-housed according to IUP-type and exposed to a stud male, a significant delay in time to mate was found. However, the 0M group took longer to mate ($M = 14.8 \pm 0.9$ days) than did the 2M group ($M = 11.2 \pm 0.8$ days), suggesting that 0M females have a greater inhibitory effect on the male's priming cues than 2M females.

In a related study using similar housing (individual vs. group) conditions (vom Saal & Bronson, 1980b), the length of the estrous cycle was compared for 0M and 2M females raised either in rooms containing males or rooms that were male-free. As expected, group-housing in the absence of a male significantly lengthened estrous cycles in females regardless of intrauterine position. However, 0M
females exhibited shorter estrous cycles than 2M females regardless of housing condition or male presence. This consistent finding suggests that prior intrauterine position can affect the intrinsic control of reproduction. Similar findings have been reported by vom Saal, Pryor and Bronson (1981).

**Age of vaginal opening.**

Age of vaginal opening is another marker of sexual development that, like the estrous cycle, is intrinsically regulated, and is a reliable index of subsequent reproductive events. Clark and Galef (1988) examined this parameter in Mongolian gerbils and found that early vaginal opening correlated with the characteristics of early-maturing (E-M) females, which tended to be more reproductively successful than late-maturing (L-M) females. OM, 1M and 2M females in this study were categorized as either E-M (vaginal opening prior to day 25) or L-M (vaginal opening after day 25) females. Age of vaginal opening was highly correlated ($p < 0.001$) with intrauterine position; a greater percentage of OM females (96%) exhibited early vaginal
opening than did 1M (58%) and 2M (41%) females. The mean age of vaginal opening for 0M, 1M and 2M females was 18, 26 and 29 days, respectively.

Extending from these previous findings (Tobet et al., 1982), premature sterility was examined in 0M and 2M female rats that had been administered a low dose of testosterone propionate on Day 3 to induce sterility. Overall, 2M females became anovulatory earlier ($M = 65.9 \pm 5.2$) than 0M females ($M = 81.1 \pm 5.76$), however these differences did not reach statistical significance.

Collectively, these findings indicated that intrauterine position relative to male fetuses can modulate intrinsic, reproductive events later in life.

**Attractiveness to males and pheromonal cues.**

Vom Saal and Bronson (1978) examined preference by adult males to 0M and 2M females. Females were in diestrus when presented, and a preference apparatus with 2 goal boxes allowed the male to make a choice between either type of female simultaneously. Of the males that made a discrimination, significantly more
males chose a OM female (84%) than a 2M female (16%). These findings were replicated in two later studies (Rines & vom Saal, 1984; vom Saal & Bronson, 1980a).

In a related experiment (vom Saal & Bronson, 1978) examining behavioral sensitivity of OM and 2M females primed with estrogens, no differences in lordosis measures were found between either type of female. However, males mounted the OM females significantly more than they did the 2M females, regardless of the dose of estrogens that the female received. Thus, OM females were more sexually arousing to males than 2M females. These findings also suggest that the effect of intrauterine position is independent of the female's adult hormonal state, thus being an organizational effect.

In support of these earlier findings, Rines and vom Saal (1984) found that when males were placed with a sexually receptive OM and 2M female, they inseminated the OM female before inseminating the 2M female.

The previous studies suggest that a pheromonal component may play a role in the preference profiles of males toward OM females. But what specific cue-
eliciting properties do OM females possess over 2M females? To investigate this issue Wechman, Brown and Hilton (1985) assayed beta-glucuronidase activity from the preputial glands of OM and 2M females. Beta-glucuronidase has been proposed to be the enzyme responsible for the release of aggressive-promoting pheromones in urine (Wechman, Brown & Hilton, 1985). The findings revealed that 2M females possess higher levels of beta-glucuronidase in their preputial glands than do OM females, thereby making urine from 2M females less attractive to males than urine from OM females.

Taken together, the findings on reproductive-related characteristics in the female points to more "favorable" reproductive conditions for OM females than for 2M females. This reproductive advantage in OM females however, has no bearing on a female's basic capacity to reproduce and raise healthy young (vom Saal & Bronson, 1978). In other words, after conception, OM and 2M females do not differ in the number of pups they deliver nor in the weight of their pups (vom Saal & Bronson, 1978). vom Saal points out
that "the basic ability to mate and successfully produce and rear young in a small cage in a laboratory is not influenced by [intra]uterine position" (vom Saal & Bronson, 1978, p.852). Such similarities between OM and 2M females in rearing their young were also supported by Kinsley et al., (1986a).
Males

As seen in the previous section, the intrauterine position phenomenon has focused on sexually dimorphic behaviors of the female. However, relatively little data has examined sexually-dimorphic traits in the male. A plausible reason was that males, normally being exposed to their own testosterone production in utero, should not be influenced by circulating androgens from other male fetuses. Therefore, the data on the intrauterine position phenomenon in the male is limited. However, as we shall see in this section, some studies have shown that male reproductive behaviors are modulated by intrauterine position.

Male sexual behavior.

Preliminary findings in CF-1 male mice (vom Saal et al., 1983) reported that after being primed with testosterone, OM males showed significantly more mounts and intromissions with a receptive female than did 2M males. Thus it was concluded that OM males
were more "sexually active" than their 2M counterparts. What is interesting and perhaps misleading is the author's interpretation of the data and the definition of "sexually active". While this study did demonstrate more mounts and intromissions by OM males, it did not take into account ejaculation frequencies (which signifies copulatory success), or the ratio of mounts or intromissions to ejaculation.

More recent findings in male gerbils (Clark et al., 1990) proposed quite a different conclusion. A female reference scheme was used to classify the male subjects (2F designating males contiguous to 2 female fetuses, and OF designating males contiguous to no female fetuses in utero). Thus this classification was somewhat comparable to vom Saal's scheme (2F animals being identical to the OM classification, and OF animals being similar to either 2M animals or 1M animals located at the end of the uterine horn, per vom Saal's scheme). The results of this study demonstrated that while 2F (OM) males exhibited more mounts and intromissions than OF (2M) males (replicating the earlier findings of vom Saal et
al.,[1983] in mice), 2F (OM) males were actually less efficient. It took the 2F (OM) males longer (latency to reach ejaculation) and required more mounts and intromissions to reach ejaculation. From this data it appears that 2M males may actually be more reproductively capable and efficient than OM males. However, whether this finding is species-specific (gerbils vs. mice) has yet to be determined.

Aggressive and Competitive Behaviors

Male and female rodents exhibit different aggression profiles. Typically, male mice show aggressive behavior toward other males in territorial situations in order to establish dominance (referred to as intermale aggression). Female mice on the other hand, rarely exhibit social aggression toward other females (Goy & McEwen, 1980; vom Saal, 1984), although spontaneous aggression has been observed in mice (vom Saal & Bronson, 1978). Females are however actively aggressive during pregnancy and lactation (Svare & Mann, 1983) as a response to protecting their young (termed maternal aggression).
As in reproduction, aggressive behavior is also regulated by the organizational and activational effects of androgens (Barfield, 1984). In this section, we shall examine whether prenatal androgen exposure (via male littermates) also influences aggressive behavior.

**Females**

Adult OM, 1M and 2M female mice, were gonadectomized at birth, primed with testosterone, and singly-housed prior to testing. At testing these females were presented with a bulbectomized male (bulbectomy assured that the male would not initiate attacks or retaliate). Findings revealed that the 2M females required a shorter period of testosterone exposure ($M = 15$ d) to display fighting behaviors than OM females ($M = 25$ d). 1M females were intermediate ($M = 21$ d) to the other 2 groups. Moreover, the delay in fighting was inversely related to the intrauterine distance from a male (i.e. the further away from the male fetus, the longer period of T exposure was required to fight). Thus, it can be concluded that 2M
females by virtue of their greater exposure to androgens prenatally, were more masculinized and showed a higher responsiveness to testosterone than their OM counterparts.

Similar findings were reported when OM and 2M diestrous females were placed in direct competition. More 2M females initiated spontaneous aggression (86%) than did OM females (14%) (vom Saal & Bronson, 1978). In a related study (Quadagno et al., 1987), 2M females showed more aggressive behavior (77% of the time) than OM females (22% of the time) when paired with females of the same IUP.

Postpartum aggression was also examined (vom Saal & Bronson, 1978) and again, the findings are consistent. When presented with a male intruder, all females from both groups attacked with the same intensity level. However, when presented with a female intruder, more 2M females attacked (83%) than OM females (74%) and the total duration of fighting was much higher in 2M females (M = 39.9 ± 7.5 seconds) than OM females (M = 16.3 ± 3.7 seconds). Consistent with these earlier findings (Kinsley et al., 1986a), 2M females were
found to be display greater levels of pregnancy-induced and postpartum aggression than OM females both in terms of the number of days of fighting and in the number of lunges and attacks toward the intruder.

An interesting study which examined aggressive behavior as a function of intrauterine position and age was conducted by Rines and vom Saal (1984). Ovariectomized young (9 months) and old (21 months) females were primed with testosterone prior to testing. OM females were then paired with 2M females of the same age. OM and 2M females did not show any difference in their frequency to attack their same-age counterpart in either age group. However, as age increased, only the OM females showed a significant increase in the tendency to fight. This was not true of the 2M females. Rines and vom Saal concluded that "OM females became more like 2M females in their behavioral response to testosterone as they aged" (p. 124). Thus, as OM females aged, their sensitivity to the activational effects of testosterone increased. 2M females, on the other hand, already sensitive to the organizational effects of testosterone by virtue of
their intrauterine position, did not show this change in sensitivity.

A different profile was shown when OM and 2M females were challenged for food in limited area (Quadagno et al., 1987). Based upon these earlier findings on aggressive behavior, it was expected that 2M females would "out-compete" the OM females and gain control of the food source. Interestingly, just the opposite occurred. When food-deprived OM and 2M females were paired and placed in a chamber in which a single food pellet was dropped, the OM females gained control of the food pellet significantly more times ($M = 11.9 \pm 3.39$) than the 2M female ($M = 6.9 \pm 2.67$), and OM females were in control of the pellet longer (19% of the time) than 2M females were (14.2% of the time). Thus, these findings are incongruent with the previous findings on aggressive behavior in females.

Males

Ninety day-old OM and 2M adult males, castrated at birth and administered testosterone, were tested for aggressive behavior toward bulbectomized 1M males over
a 16-day period. During this time, 2M males were more likely to attack an intruder (70%) than OM males (40%) (vom Saal et al., 1983). This same experiment was replicated in another group of males that were 200 days old. Again, the findings were consistent: more 2M males (45%) displayed aggressive attacks toward an intruder than OM males (15%), although the overall number of animals to attack in both groups decreased (vom Saal et al., 1983).

This decrease in behavioral responsiveness is believed to reflect a decrease in sensitivity to testosterone as a function of the male’s age. In contrast to the age-related findings in females of Rines and vom Saal (1984) which indicated that females with the least exposure to androgens (i.e. OM’s) prenatally, increase their sensitivity to testosterone over time, males on the other hand (with the greatest exposure to prenatal androgens) lose their sensitivity to testosterone over time. This is an important issue since it suggests that organizational and activational effects of steroids may not be as dichotomous as previously thought, and points to the fact that the
effects of steroids may be more "transient" throughout the animal's lifespan.

However, the overall effect of intrauterine position on aggression appears consistent in males and females. With the exception of food competition in females, 2M animals display more aggressive behavior than OM animals under similar conditions (i.e. initiate more attacks and attack longer). OM females, however, appear to out-compete 2M females for a food source, suggesting that some but not all aggressive behaviors may be modulated by the prenatal effects of hormones.

Activity Patterns

Previous work on levels of activity in rodents has shown that females tend to display higher levels of motor activity (e.g. wheel running) than do males and that this behavior is modulated by the organizational effects of testosterone (Goy & McEwen, 1980; Kinsley et al., 1986b).

Using a photobeam interruption apparatus (Kinsley et al., 1986b), OM and 2M male and female mice were
measured for locomotor activity over a 6-day period. Findings revealed significant differences in activity levels, being highest to lowest in the following order: OM females-> 2M females-> OM males-> 2M males. Thus 2M animals had lower levels of activity than their same-sex OM counterparts with females being more active than males. The authors concluded that male contiguity in utero masculinized to an extent (i.e. not completely) the activity patterns of both males and females in adulthood.

**Learned Behaviors**

As seen in previous sections, reproductive behaviors of OM and 2M animals are often quantitatively different, due to the differences in hormone exposure prenatally. However, the behaviors described so far are largely innate and do not require higher (learning) centers in the brain. The question arises, could prior positioning in utero also influence acquired (learned) behaviors as well? In order to address this issue, it is first necessary to identify whether there are any sex differences in
learning processes.

Avoidance responding, a conditioned, learned behavior, was assessed in adult male and female mice, identified by prior intrauterine position (Hauser & Gandelman, 1983). An aversive stimulus (electrical shock) was presented concomitantly with a "warning" stimulus (light) such that the shock could be terminated by pressing a lever (an avoidance response). OM females learned avoidance responding better than did the other 3 groups (2M females behaving similarly to the males). These findings suggest that prenatal androgen exposure decreases the potential for avoidance learning in mice.

In a related study (Babine & Smotherman, 1984), conditioned taste aversions were evaluated in female rats using the caudal-male classification scheme (MF = 1 male caudal to the female, FF = no caudal male). The presence (or absence) of a caudal male had no effect on rates of acquisition and extinction of the taste aversion in the female groups; MF and FF females exhibited similar profiles. However, contrary to the previous findings in mice, male rats were faster in
acquiring the taste aversion, and maintained this learned response longer during extinction than either female group. Although a sex difference was apparent with males being faster at acquiring this aversion and in maintaining the aversion during extinction, no differences were seen between the 2 female (MF and FF) groups.

Thus, the effects of intrauterine position on sexually-dimorphic learned behaviors appears inconsistent, as the above two studies have shown.

The Effects of Stress on Fetuses: Implications For Population Density Control

Preliminary work by vom Saal (1984) in CF-1 mice showed that prenatal stress masculinizes both morphology and behavior; OM and 1M females of stressed mothers had anogenital distances and estrous cycles that were similar to control (i.e. unstressed) 2M females. In addition, stressed male and female fetuses showed marked elevations in serum testosterone levels which spiked on day 17 of gestation. Furthermore, prenatal stress eliminated the effects of
intrauterine position, such that OM and 1M animals were morphologically and behaviorally similar to 2M animals.

Three more recent studies have examined the influence of prenatal stress on the intrauterine position phenomenon and how it affects postnatal sex-trait characteristics.

In the first study (Lephart et al., 1989), prenatally-stressed male rats (i.e. mothers receiving light/heat, or ACTH injections for 6 days prior to sacrifice) were compared to non-stressed male rats on four different morphological measurements, using the cervical-male flanking ("CX") classification scheme described previously (being similar to the caudal-male model). OMCX designated animals with no males between them and the cervical end of the uterine horn, 2MCX identified males that had 2 males between them and the cervical end of the uterine horn. Findings on day 20 of gestation revealed significant decreases in body and testis weights (but not in AGD or adrenal weight) in all pre-stressed male groups when compared to the same CX classification in controls. Expected
incremental differences (i.e. OM < 1M < 2M) in AGD, adrenal, testes and body weights were observed between OMCX, 1MCX and 2MCX controls. However, no consistent pattern emerged between these male groups under the stressed conditions. Thus, prenatal stress appeared to 1) reduce body and testis weights and 2) eliminate the "cervical-male flanking effect in male rats.

In another study (vom Saal et al., 1990), female mice from different intrauterine positions were compared from stressed mothers (via. light exposure) and from unstressed mothers (control). Estrogen concentrations in female fetuses from stressed and unstressed conditions were unaffected. However, females from stressed mothers showed significant increases in testosterone concentrations compared to females of unstressed mothers. In addition, maternal stress eliminated the effect of intrauterine position (i.e. OM, 1M and 2M females all showed similarly high levels of T conc.).

It was expected that all females from stressed mothers would exhibit postnatal traits (AGD and length of estrous cycle) similar to 2M control females (since
all 3 IUP types from the stressed group were exposed to similar levels of T prenatally). However, when postnatal traits were assessed, all females in the stressed condition had significantly lower body weights at birth than unstressed females (consistent with the data of Lephart et al.,[1989]). Only OM females from the stressed group showed differences from OM controls that made them characteristically more "2M-like"(i.e. longer AGD at birth, longer estrous cycles). Thus, while testosterone increased in all female fetuses, only OM females were responsive to the stress-induced T surge.

Effects of crowding (low, moderate and high density housing conditions on mother) and intrauterine position were assessed in male and female wild mice (Zielinski et al., 1991). Anogenital distances were marginally longer in females born to mothers in the high density conditions than other female groups. However, due to the large variance of the dam effect, attaining meaningful data concerning density on intrauterine position in females was not possible. In males, anogenital distances were unaffected by either
crowding condition or intrauterine position. In both sexes, birth weight was also unaffected by the effects of prior intrauterine position and crowding condition.

The general findings of these studies suggest that prenatal stress can alter the prenatal hormonal environment by increasing testosterone concentrations in all fetuses, thereby eliminating the intrauterine position effects normally seen. The increase in androgen exposure imposes 2M trait characteristics on OM and 1M individuals.

Behaviorally, OM females are the more sensitive to this stress effect, showing longer estrous cycles, similar to 2M control females. However, males do not appear as sensitive, most likely due to the fact that they are more fully masculinized already. Interestingly, if stress conditions impose a shift toward 2M characteristics, we would also expect to see morphological findings in this same (2M) direction. However, body weight of animals from stressed mothers was shown to decrease in both sexes, and testis weight was also less than that of control males. Therefore, the findings of the behavioral and
morphological data appear inconsistent in this respect.

Adaptive Significance of Intrauterine Position: Population Density Control

The previous studies showed that stressing a pregnant mother significantly alters the hormonal milieu of the fetus (increasing testosterone levels). These findings are important, since they imply that external, environmental forces (such as stress and overcrowding) can affect the intrinsic, reproductive characteristics of the unborn animal.

vom Saal (1983b; 1984) proposes that changes in the proportion of animals from different intrauterine positions can directly alter the dynamics of that population. vom Saal's theory is based on the Chitty-Krebs and Christian hypotheses (vom Saal, 1984), which respectively suggest that population dynamics are mediated by 1) genotype and 2) endocrine functions (ACTH and glucocorticoid secretions) of the individual, which in turn, alters the physiology and behavior of the individual, and ultimately affects
reproductive success and survival rate. vom Saal's (1984) theory is summarized in the following excerpt:

The intrauterine hypothesis proposes that shifts in the proportions of OM, 1M and 2M animals within a population as population density increases, due to the aggressive 2M males and 2M females dispersing the less aggressive 1M and OM animals, will lead to a general increase in aggressive interactions among the 2M animals that remain in the home environment. A high proportion of 2M animals in an environment may thus precipitate a decline in population size, since 2M animals are both aggressive and exhibit a decrement in sexual performance (p. 162).

Thus, the adaptive significance of the intrauterine position phenomenon is that it provides intrinsic feedback control for population fluctuations. When population density is low, OM females would have a reproductive advantage over 2M females (being more attractive and sexually arousing to males (vom Saal & Bronson, 1978), reaching puberty earlier (vom Saal & Bronson, 1978), and having shorter estrus cycles (vom Saal & Bronson, 1980b), thus ensuring the most favorable factors for reproduction, and thereby increasing the population size. On the other hand, when the population density becomes too high, the
competition for limited space and food increases. The 2M animals become more adaptive in this case, by virtue of their more aggressive tendencies (Gandelman et al., 1977), and would have the reproductive advantage over their OM counterparts, driving them out of the group. Furthermore, 2M females, having the opposite sexual attraction profile of OM females, would be less likely to reproduce at the same rate as OM females, thereby decreasing the population density.

The findings of the stress studies are consistent with this theory. Prenatal stress (vom Saal et al., 1990) was shown to influence the reproductive traits of the OM female (lengthening the estrous cycle) thus shifting its behavior toward characteristics associated with 2M females.

Thus, the intrauterine position phenomenon homeostatically regulates population size by accelerating or decreasing the rate of reproduction, which in turn, shifts the ratio of 2M and OM animals in the population.
Intrauterine Position Summary

As we have seen from the previous discussion, intrauterine position, and the exposure to organizational effects of prenatal androgens that this theory implies, has been shown to influence reproductive, social and activity level behaviors as well as morphology in both sexes of various rodent species. While controversy still exists concerning the mechanism of hormonal transport, it appears dependent on the species studied, which may be due to uterine vasculature differences within these species. The male-contiguity model was best supported by data in mice, while the caudal-male scheme appears most consistent in studies on rats and ferrets. The limited data on hamsters however, suggests that contiguity to females, rather than males, may play a role in organizing behavior in this species. Finally, pigs do not appear to be responsive to prenatal effects of hormones according to the contiguity hypothesis.

Regardless of the classification used, the data presented in this thesis has shown that the
variability in many sexually-dimorphic behaviors are mediated by the exposure to prenatal hormones and has provided valuable insight in the process of sexual differentiation.
Sexual Dimorphism in Two Precopulatory Behaviors:
Preference For Urinary Odors
and Ultrasonic Vocalization;
A Background

The previous review has examined the variations that exist in sexually dimorphic behaviors in males and females as a result of prenatal androgen exposure from male littermates. In this thesis, I will examine the effect of intrauterine position on two sexually-dimorphic behaviors which are of current interest in our lab, and which have not been previously examined in intrauterine position studies. The two behaviors to be examined are 1) preference for urinary odors and 2) ultrasonic vocalization. These precopulatory behaviors can be used to assess the animal's level of attraction.

Preference for Urinary Odors

Males

The preference profile of male mice has shown that males are attracted to urine of conspecific females but are indifferent to urine of conspecific males (Nyby et al., 1985; Bean, Nyby, Kerchner and Dahinden,
In a series of experiments (Nyby et al., 1985), adult hybrid (C57BL/6J x AKR/J) socially-experienced and inexperienced male mice were tested for odor preference using a two-choice apparatus. During testing, animals were presented with an odor stimulus at one end of the apparatus, while a control (or baseline) stimulus was presented simultaneously at the other end. Findings revealed that males preferred the odor of female urine over male urine. Furthermore, prior experience with a female did not affect the male's attraction to female urine, since both socially-experienced and naive males showed similar preference profiles.

In a related study (Bean et al., 1986), male mice were castrated or sham-operated upon reaching adulthood, and then measured for odor preference to female urine, using the same two-choice odor preference apparatus previously described. Sham-operated males showed a greater preference toward female urine than did castrated males. Both sham and castrated males were similarly indifferent to the odor of male urine. Thus, castration lowered the preference
toward female urine but did not affect the overall low preference levels (or indifference) toward male urine.

Moreover, when castrated adult males in the same study were treated for 22 days with testosterone or estradiol (via Silastic capsule-implantation), attraction levels toward female urine were comparable to the attraction levels of intact males. Thus, preference levels could be reinstated with adult hormone treatment of testosterone or estradiol.

Females

In contrast to the odor preference profile of males, females tested in our lab show a strong preference toward male urine, but are generally indifferent to the odor of female urine (Nyby, unpublished data). Moreover, the magnitude of this behavior is generally lower than that seen in males. Unlike that of males, the preference profile of the female is relatively unchanged after gonadectomy; ovariectomized and sham-operated females showed similar preference profiles to that of intact females (Nyby, unpublished data). However, when
ovariectomized females were treated with testosterone propionate, they showed a high attraction to the odors of male urine (a female-typical response) but also showed a high attraction to female urine (a male-typical response) (Nyby, unpublished data).

In both male and female preference profiles, such sexually-dimorphic behavior was not completely reversed by manipulation of adult sex hormones. In other words, castrating adult males (with and without E2 treatment) did not produce expected female-typical behaviors (i.e. indifference to female urine and high preference to male urine). Nor did TP treatment to adult ovariectomized females produce expected male-typical behaviors (i.e. an indifference to male urine and high preference to female urine). Thus, because we do not see a complete "sex-reversal" of males behaving like females, or females behaving like males, it is reasonable to conclude that the activational effects of hormones are not completely responsible for the sex differences observed in preference behavior. Rather, these findings suggest that the organizational effects of hormones may play a role in mediating odor
preference behavior.

**Ultrasonic Vocalization**

As demonstrated in the preference profiles of male and female mice, clear sex differences were evident in preference to urinary odors in mice. Sexual dimorphisms have also been observed in ultrasonic vocalization in mice.

Males typically emit ultrasonic vocalizations towards conspecific females (Nyby, Wysocki, Whitney and Dizinno, 1977). Females, on the other hand, do not typically emit vocalizations to other females nor in response to males (Nyby et al., 1977).

These sex differences were demonstrated in the following (Nyby et al., 1977) study. Adult female mice were randomly assigned to one of three treatment groups: ovariectomized females receiving TP (OVX-TP), ovariectomized females receiving oil (OVX-OIL) and sham-ovariectomized females receiving oil (SHAM-OIL). Intact males receiving an oil vehicle (MALE-OIL) were used as a baseline comparison. Test animals were presented with an intact female as a stimulus.
Findings revealed that SHAM-OIL and OVX-OIL females had responses that were close to zero, and which were not significantly different from each other. However, the OVX-TP females emitted significantly more vocalizations than did SHAM-OIL or OVX-OIL females. Yet when the OVX-TP females were compared to the male group, the males emitted significantly more ultrasound than the females treated with TP. These findings suggest that while the activational effects of TP increased the level of vocalizations in females, it failed to reach ultrasound levels of males.

Subsequent experiments in the same (Nyby et al., 1977) study employed TP replacement therapy of longer duration to gonadectomized males and females. Findings of these experiments revealed that males attained higher levels of vocalization more quickly than did females. However, as TP treatment continued over time, females reached similar vocalization levels as males. When TP treatment was discontinued (through 6 weeks post-therapy), both males and females showed a similar rate of decline in vocalization response. Collectively, these findings suggest that
vocalization behavior is dependent upon the activational effects of androgens, and that females, under conditions of prolonged TP treatment, can display similar levels of ultrasound vocalizations to that of males. Similar findings supporting the activational effects of androgens in ultrasonic vocalization were also reported by Dizinno and Whitney (1977), and Bean et al. (1986).

In summary, these two dimorphic behaviors show that in males and females, there is a cross-gender attraction but a general indifference to same gender conspecifics. Moreover, odor preference appears to be more dependent upon the organizational effects of hormones, whereas ultrasounds appear to be dependent upon the activational effects of hormones. Thus, within the context of the intrauterine position theory (being a strictly organizational effect), intrauterine position would be expected to have greater effects upon odor preference than in ultrasonic vocalization.
CHAPTER 2

RESEARCH CONDUCTED

Study Rationale

In the present study, in addition to examining morphological parameters, we will also examine 1) attraction to urinary odors of conspecifics in males and females and 2) male ultrasonic courtship vocalizations to female conspecifics. Odor preference will serve as a bioassay to measure attraction in males and females to same and cross-gender conspecifics. Vocalization will also serve as an assay in males to measure attraction toward conspecific females.

As shown in Appendix 1, previous studies examining the intrauterine position phenomenon assessed its effects primarily in females, but not in males. Of these 37 studies cited, only 11 focused on intrauterine position effects in males.

Furthermore, the behavioral parameters used focused primarily on copulatory (i.e. mounting) behaviors in
intrauterine position-identified animals. Only one study to date (Gandelman, 1986) has examined this phenomenon in precopulatory behaviors.

Moreover, test animals used in intrauterine position studies were usually gonadectomized and primed with exogenous hormones prior to testing for the purpose of eliciting the desired response. Thus, the normal hormonal environment of these test animals was altered at the outset, and we cannot be sure if the behaviors elicited were the result of the hormonal manipulation, or were directly attributable to the effect of intrauterine position. By the design of such experiments, findings become confounded, making interpretation of the data difficult.

Finally, no published study to date has examined if an interaction effect can be demonstrated by testing IUP-identified animals with IUP-identified stimuli. Would we tend to see heightened responses (i.e. greater differences) when testing an IUP-identified animal from one extreme position (e.g. 2M male) using an IUP-identified stimulus from the opposite position (e.g. OM female)? While this seems inherent in the
intrauterine position model, it seems strange that this issue has never been examined.

Previous studies have left many unanswered questions which leads to the purpose of the present study. This study will extend previous research focusing on the four issues described above.

Specifically, IUP-identified males will be tested in addition to females. Precopulatory behaviors (odor preference and ultrasonic vocalization) will be examined, in addition to morphological parameters. No exogenous hormones will be administered to the test animals. Therefore, any behavioral differences observed in these animals should be attributed to their intrauterine position, if all other variables are held constant. The stimulus presented (animals and urine) will also be identified by intrauterine position, making an interaction assessment (between IUP test subjects and IUP stimuli) possible.

With these specific goals in mind, the overall objective of this study will be to test the integrity of the intrauterine position theory.
General Method

Animals

CF-1 males and females were obtained from Charles River Breeding Laboratories (Wilmington, MA) at approximately 40 days of age. Breeding groups were established and placed in opaque plastic cages (12.5 cm x 17 cm x 28 cm) with wood chips for bedding. Animals were maintained on a 12-hour light/dark cycle with food (Purina Mouse Chow) and water available ad libitum.

Females were time-mated and checked each morning for the presence of a copulatory plug. A copulatory plug was evidence that insemination had successfully occurred. If a plug was discovered, then the female was removed and housed separately.

On the 18th day of gestation (1 day prior to normal parturition), the pregnant female was sacrificed by cervical dislocation and pups removed following cesarian section. Upon delivery, pups were swabbed at the mouth with a cotton swab to initiate a breathing reflex and placed on a wood chip bedding in the order
of their location within the uterine horns. A light 4 inches above the pups provided warmth.

The sex of each pup was determined by anogenital distance measurements using Vernier calipers. Anogenital distance was defined as the distance between the base of the genital papillae and the center of the anus. Characteristically, females have shorter anogenital distances than males. Animals located in utero between 2 males (2M) and those located between 2 females (OM) were identified according to the Contiguity classification system (vom Saal, 1981), and their anogenital distance measured and recorded. Animals were then toe-clipped to permanently identify their intrauterine position.

All animals were then cross-fostered to a lactating CF-1 mother (usually 4-5 same-sex animals per mother) for 21 days following delivery. After 21 days, the pups were weaned and group housed (usually 2-4 to a group) according to their IUP type.

A total of 104 2M male, 57 OM male, 61 2M female and 37 OM female pups were delivered over an 11-month period from a total of 52 cesarian deliveries.
these IUP-delivered animals, 28 2M males, 8 OM males, 23 2M females and 23 OM females were raised to adulthood to be used as test subjects or stimulus donors. The remaining IUP-delivered animals either did not survive cross-fostering or were placed in the breeding colony room. The test animals consisted of 20 2M males, 8 OM males, 15 2M females and 15 OM females. Stimulus donors consisted of 8 2M males, 8 2M females and 8 OM females. The 8 OM males used as test animals also served as stimulus donors after behavioral testing for that animal had been completed. Due to a high incidence of infanticide, particularly of the OM male pups (see Discussion section), equal numbers of test animals in each group was not possible.

**Apparatus**

Two behaviors were examined for the male test animals; 1) ultrasonic vocalization and 2) preference for urinary odors. Since adult females rarely emit ultrasonic vocalizations in response to males (Nyby & Whitney, 1978; Whitney, Coble, Stockton & Tilson,
1973), females were tested for odor preference only.

**Ultrasonic Vocalizations**

Male vocalizations were monitored using a QMC ultrasonic bat detector (model S-100) which transduces inaudible, high frequency vocalizations (70 kHz) into audible sounds. The microphone was placed approximately 25 cm above the animal's cage and the detector tuned to 70 kHz.

**Odor Preference**

A 2-choice odor preference apparatus (Figure 3) was used to measure odor preference behavior. The apparatus consisted of a glass sheet (33 cm x 24 cm x 0.5 cm) designed to fit over a standard mouse cage (29 cm x 18 cm x 13 cm). When a vacuum pump was turned on, air flow passed down the two tubes and exited through the center opening.
Figure 3: Odor preference testing apparatus.

Stimuli

Urine (for odor preference testing).

One to four hours prior to testing, urine was obtained by manual stimulation (palpating the bladder) of animals of known IUP and collected in glass vials. Except for OM males, all urine donors came from animals of known IUP, group-housed according to their IUP-type. Because of the paucity of OM males (N=8), the same males were used for both behavioral testing and as urine donors. However, OM males were used as urine donors only after completing behavioral testing. "Pooled male" urine, used as a control stimulus for the male test animals, was collected from approximately 6 to 8 males of unknown IUP. Urine was drawn from collection vials into 1.0 ml plastic syringes. A cotton-tipped applicator was impregnated with urine and presented to the test animal within one minute prior to behavioral testing.

Females (for vocalization testing).

OM and 2M females, group-housed according to IUP-
type were used as stimulus females in the vocalization testing of males. These females were the same animals that were urine donors in the preference testing. ("Stimulus" females should not be confused with the "test" females described in Experiments 4 and 5, which were individually-housed at time of weaning).

Procedure

Prior to Testing

Individual housing.

After 55 days of age, all test animals were individually-housed in clear lucite cages for at least 8 days, prior to the social experience procedure (described below).

Social experience.

After being individually-housed, each test animal encountered a CF-1 male and female (from a foster population group). This procedure (described in detail; see Nyby & Whitney, 1980) involved presenting a socially-experienced male and female separately in
the test animal's home cage for a 3-minute period each
day for 8 consecutive days. The order of the male and
female presentation to the test animal was reversed
each day.

The reason for using the social experience regimen
was that previous research in mice (Dizinno, Whitney &
Nyby, 1978) and more recently prairie voles (Lepri,
Theodorides & Wysocki, 1988) has shown that naive
males given social experience with male and female
conspecifics prior to testing, will emit ultrasonic
vocalizations more frequently than naive males given
no prior social experience. Moreover, such experience
helps to familiarize males with conspecific odors and
encourages preference behavior (i.e. sniffing).

Apparatus experience.

Four days prior to the first test, males were
familiarized with the odor preference apparatus for
two consecutive days (on days 7 and 8 of the social
experience procedure). The male was placed in the
odor chamber for a 3-min. period with the vacuum pump
turned on. This procedure was carried out to minimize
the novelty of the testing situation.

Female subjects, however, were familiarized with the apparatus for 8 consecutive days, simultaneously with social experience. This additional exposure was employed to enhance overall preference behavior, since females normally display lower levels of preference for urinary odors than males.

For all test animals, forty-eight hours elapsed from the last day of apparatus familiarization to the first testing trial.

Testing

During all tests, animals were removed from the colony room and tested in their home cage in an adjacent room.

Ultrasonic vocalization.

Before stimulus presentation, males were habituated to the ultrasound apparatus for 1 min. If vocalizations occurred during this period, the test animal was habituated until 2 minutes had elapsed without a vocalization.
Each male was presented with 1 of 2 stimulus conditions (either a OM or 2M female), and 48 hours later, the other. Subject order and stimulus order were randomized.

When the stimulus female (either OM or 2M) was placed into the male's home cage, behavioral testing commenced. Vocalizations were time-sampled during 36 5-second intervals for a total time of three minutes. The amount of ultrasound was quantified by summing the number of intervals in which a vocalization occurred. Possible scores ranged from 0-36.

Odor preference.

a) male test subjects

Three stimulus conditions (OM female urine, 2M female urine and "pooled" male urine) were presented to each male subject over three separate testing trials, each separated by 48 hours. Subject order, stimulus order (either OM female, 2M female or "pooled" male urine) and stimulus position (left or right) were randomly assigned. Before stimulus presentation, the subject was habituated to the
apparatus for one minute to minimize novelty. During this time, the stimulus was prepared by injecting the urine (0.1 ml) into a clean cotton swab. To begin a test, the stimulus was mounted in one of the odor tubes and a clean cotton swab was placed in the other odor tube. A second experimenter, "blind" to testing conditions, recorded the time the subject sniffed the two odor tubes for a 2-min. period. "Sniffing" was measured by the amount of time (in seconds) that the subject spent sniffing with its nose inside of or within 1 cm in of the tube opening. Time was measured in 0.1 seconds, using two stop watches (1 for each preference tube).

After each test, the preference apparatus was cleaned with a clean cotton ball soaked in 70% ethanol held by a hemostat. Attempts were made to minimize handling and touching of the apparatus. The apparatus was then dried using a hot-air gun for 2-3 minutes and allowed to cool to room temperature prior to the next test.

Two measures of odor preference were calculated; absolute and relative. Absolute preference quantified
the actual time the animal spent sniffing the odor of interest. Absolute preference was determined by calculating the difference in "sniffing" times between the two preference tubes: "urine-stimulus" tube minus "blank" tube. Relative (or percent) preference took into account the time that the animal sniffed at the odorous tube in proportion to the total time the animal sniffed at both tubes. Relative (percent) preference was calculated by measuring the sniffing time at the "urine-stimulus" tube divided by the total sniffing time (time spent at both tubes), multiplied by 100. Each trial was separated by 48 hours.

Conceptually, each of these two measurements reflect somewhat different components of sniffing behavior. Absolute preference better reflects the magnitude of the behavior, whereas relative preference better reflects the direction of the animal's attraction. However, the responses are nonetheless expected to be highly correlated.

b) female test subjects

Odor preference tests for females followed the same
format as that for males, except that females were presented with only two stimulus conditions (OM and 2M male urine) presented over 2 separate tests. No control stimulus (i.e. "pooled" female urine) was presented as this experiment was executed for purposes of assessing feasibility.

 Statistical Tests

Analysis of Variance (ANOVA) was used to test for significance in all experiments. Selected contrasts utilized orthogonal comparisons, if ANOVA revealed statistical significance within each test. Additional statistical tests will be described in the results section of specific experiments. Data are presented as group mean (±SE).
Experiment 1:
Odor Preference Behavior In IUP Males

The purpose of Experiment 1 was to examine the odor preferences of OM and 2M male CF-1 mice for conspecific OM and 2M female urine. Based upon the findings that OM males were more sexually active than 2M males (vom Saal et al., 1983) and that OM females were found more attractive to males than 2M females (vom Saal & Bronson, 1978), I predicted that 1) OM males would show greater preference for female urine than 2M males and 2) males would be more attracted to OM female urine than 2M female urine. This experiment was designed to test these 2 hypotheses.

**Method**

**Animals**

Twenty 2M and 8 OM males were tested between 72 and 134 days of age on the first preference test. OM and 2M females, group-housed according to IUP-type, provided the urine used as stimuli for the male
preference testing. (These females are not to be confused with the test females which were individually housed and tested for female preference described in Experiment #4). Male urine, used as a control stimulus, was collected from group-housed CF-1 males of unknown IUP.

Procedure

Prior to the first test, animals were given social experience (8 days) and apparatus experience (2 days). On the day of each test, stimulus urine was collected within 4 hours of presentation. Each test was separated by 48 hours. The details of these preparatory procedures and each testing trial are described in the General Methods section.

Results

The odor preference profiles of OM and 2M males are shown in Figures 4 and 5.
Figure 4: Absolute odor preference (mean ± SE) of OM and 2M male CF-1 mice for urine from OM females, 2M females, and males. Note. Refer to the general methods section for a complete description of the absolute preference measurement.

For absolute preference, a 2X3 ANOVA revealed that OM males showed greater preference for female urine.
than did 2M males, $F(1, 78) = 6.79, p < .05$.

The stimulus effect was also significant, $F(2, 78) = 4.05, p < .05$. Orthogonal comparisons revealed that the 2 female urine conditions irrespective of their IUP-type, were preferred over the male urine by both male groups (2M group: $F(1, 57) = 5.29, p < .03$; OM group: $F(1, 21) = 2.90, p < .10$; both groups combined: $F(1, 81) = 7.81, p < .008$). However, the group by stimulus interaction effect was non-significant, $F(2, 78) = 0.11, p = \text{ns}$.) indicating that both groups had similar response profiles to male and female urine.

For relative (percent) preference, both groups responded similarly, $F(1, 78) = 0.98, p = \text{ns}$. However, the stimulus effect was again significant, $F(2, 78) = 3.16, p < .05$. 

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Figure 5: Relative (percent) odor preference (mean ± SE) of OM and 2M male CF-1 mice for urine from OM females, 2M females, and males. Note. Refer to the general methods section for a complete description of the relative preference measurement.

Orthogonal comparison again indicated that the female urine regardless of the IUP-type it came from, was
more attractive to both male groups than male urine (2M group: \( F(1, 57) = 4.37, p < .05 \); 0M group: \( F(1, 21) = 5.79, p < .03 \); both groups combined: \( F(1, 81) = 7.79, p < .008 \)). Again, no interaction was found between groups and urine stimuli, \( F(2, 78) = 0.08, p = \text{ns} \).

Further analysis revealed a significant age difference in the 0M (\( M = 100.63 \pm 8.16 \) days) and 2M (\( M = 75.7 \pm 0.79 \) days) groups on the first testing trial, \( F(1, 26) = 23.31, p < .001 \). This difference was attributed to three subject "outliers" in the 0M group (mouse # M31, M42, M43 whose ages were much older than the other test animals in that group, being 121 d, 134 d and 127 d, respectively). When these 3 outlier animals were excluded and the remaining animals from both groups were age-matched (5 per group), the ages for the 0M (\( M = 84.6 \pm 3.3 \) days) and 2M (\( M = 78.8 \pm 2.31 \) days) groups were much closer.

To determine if age was a contributing factor, the 2M males were divided equally into two groups of young vs. old test animals (10 per group) using the median age of 75 days of age as the dividing point. A one-way ANOVA compared young vs. old 2M males for
preference differences and found no significant difference on any of these parameters (see Appendix II). Thus, young and old 2M subjects behaved similarly. OM males were also divided into 2 equal groups (4 per group), using the median age of 89 as the dividing point. A one-way ANOVA again revealed no significant difference in any preference parameter (see Appendix II), indicating that young and old OM males behaved similarly. Thus, any behavioral differences found between these two groups would appear to be attributed to their intrauterine position and not to their difference in age.

Since unequal group size was also an inherent problem in the first analysis, animals were age-matched from each group (mouse # M11, M14, M15, M27, M29 from the 2M group were respectively matched with mouse # M12, M13, M57, M58, M59 from the OM group). This design allowed for an equal (albeit small) number of subjects per group which were also comparable by age. A 2X3 ANOVA for this subset revealed no significant difference between the two groups on absolute preference, $F(1, 24) = 0.108, p = \text{ns}$, or
percent preference, $F(1, 24) = 0.028, p = \text{ns}$. The effect of the urine stimulus condition was significant in absolute, $F(2, 24) = 3.773, p < .04$, but not in relative preference, $F(2, 24) = 1.125, p = \text{ns}$. Further analysis (by orthogonal comparison) of the absolute preference data indicated that the significant difference was found in the 2 female urine conditions vs. the male urine, $F(1, 24) = 6.794, p < .006$, and not between the 0M male vs. 2M male urine stimulus, $F(1, 24) = 0.752, p = \text{ns}$. No group by stimulus interaction was found significant in either absolute, $F(2, 24) = 0.793, p = \text{ns}$, or relative preference, $F(2, 24) = 0.899, p = \text{ns}$.

The findings of these age-matched data indicate that the two groups did not differ in preference behavior (absolute or relative) when age was controlled and group size was equal. However, a stimulus effect was significant; both female urine types were found more attractive than the male urine, but only when absolute behavior (not relative behavior) was assessed.

Appendix III summarizes the results of the
absolute and relative preference profiles with respect to unmatched and matched data.

It appears then, that when age and number of subjects per group were not controlled, OM males showed greater preference than 2M males. This difference was apparent in absolute (i.e. actual odor sniffing time) but not in relative preference behavior (i.e. the proportion of the total sniffing time that the animal actually sniffed at the stimulus). Further analysis demonstrated that the variation of preference behavior shown in the two male groups was not attributed to their difference in age, but rather to their intrauterine position. Female urine was found more attractive than male urine in both absolute and relative profiles. However, there was no distinguishable preference between either 0M or 2M female urine conditions; the male groups were attracted equally to both.

When age and group size were controlled, OM and 2M male groups were comparable in preference behavior (absolute and relative). The effect of urine conditions was only significant when assessing
absolute preference behavior, indicating that female urine (OM and 2M equally) was more attractive than male urine.

Overall, these findings did not support the 2 proposed hypotheses. While the absolute preference behavior from unmatched animal data did indicate that OM males showed greater preference than 2M males overall (supporting vom Saal et al., 1983), this effect was eliminated in relative preference behavior, and also disappeared when age and group size were controlled for. Furthermore, while the effect of urine stimulus was significant in three of the four analyses (see Appendix III), it appeared to be due to the variation in male vs. female urine conditions, rather than due to the differences in intrauterine position of the females.
Experiment 2: Vocalization Behavior in IUP Males

As discussed previously, ultrasonic vocalizations are emitted by males during courtship and reflect the male's level of sexual arousal. The purpose of this experiment was to examine ultrasonic vocalizations in OM and 2M males toward OM and 2M female conspecifics.

Based upon the findings that OM male mice are more sexually active than their 2M counterparts (vom Saal et al., 1983), it is predicted that OM males should emit a greater amounts of ultrasonic vocalizations toward females than 2M males.

A second hypothesis is that OM females would elicit more vocalizations than would 2M females, based upon OM females being more attractive to males than their 2M counterparts (vom Saal, 1989; vom Saal & Bronson, 1978). This experiment was designed to examine these hypotheses.

Method

Animals

Twenty 2M and 8 OM males were tested between 97 and
155 days of age on the first vocalization trial. OM and 2M females group-housed according to IUP were used as the stimulus conditions.

Procedure

The preparation and testing of the animals are described in detail in General Methods section. It should be noted that awake, non-anesthetized females were presented as stimuli to males (for rationale, see Warburton, Sales & Milligan, 1989).

Results

Age differences were not significantly different ($F(1, 26) = 1.88, p = ns.$) between OM males ($M = 119.25 \pm 8.75 \text{ d}$) and 2M males ($M = 134.45 \pm 6.07 \text{ d}$). Vocalizations toward OM and 2M females by both male groups are shown in Figure 6.
Figure 6: Amount of ultrasound (mean ± SE) emitted from OM and 2M CF-1 males in response to OM and 2M CF-1 females. Ultrasound scores were calculated by the number of 5-second blocks containing vocalizations.

A 2x2 ANOVA revealed no significant differences in group, \( F(1, 52) = 0.42, p = \text{ns.} \), stimulus, \( F(1, 52) = 0.58, p = \text{ns.} \), or interaction effects, \( F(1, 52) = 0.01, p = \text{ns.} \), indicating that OM and 2M males showed similar amounts and patterns of vocalizations to OM and 2M females.

Similar results were shown when the groups were
analyzed to account for the unequal number of subjects in each group. A subset from each group were again age-matched with 8 animals per group \((M = 119.4 \pm 10.1\) d for 2M males, \(M = 119.25 \pm 8.75\) d for OM males). A 2X2 ANOVA revealed no significant difference between OM and 2M males, \(F(1, 28) = 0.664, p = \text{ns.}\), between the stimulus value of OM and 2M females, \(F(1, 28) = 1.295, p = \text{ns.}\), nor did these components of variance interact, \(F(1, 28) = 0.393, p = \text{ns.}\).

These results indicate that vocalizations did not vary as a function of intrauterine position, and in fact, the animals from both groups showed nearly the same level of vocalization to both types of females. However, the animals from both groups appeared near the upper limit of responsiveness possible. This "ceiling effect" may have masked possible differences which might have emerged had the study utilized less optimal conditions (e.g. used anesthetized females as stimuli).

Nonetheless, the findings of this study do not support the hypotheses that intrauterine position affects courtship vocalizations in male mice.
Experiment 3: Morphological Parameters in IUP Males

Experiment 3 examined whether OM and 2M males differed in morphology as a function of their intrauterine position. Three variables examined in adulthood were: 1) anogenital distance (AGD), 2) AGD/body weight ratio and 3) seminal vesicle weight (as percentage of body weight).

Three studies to date have examined anogenital distance in adulthood as a function of intrauterine position in males. A study in adult gerbils (Clark et al., 1990), found that males located next to females had shorter anogenital distances than males not located next to females. However in two recent studies in adult wild house mice and pigs, intrauterine position had no effect on anogenital distance in males (Rohde Parfet et al., 1990; Zielinski et al., 1991). While this characteristic appears evident in gerbils, it is not apparent in wild mice or in swine, and raises the question as to
whether species or strain differences exist in this phenomenon. Hence, the purpose of the first (AGD) and second (AGD/Body Weight) subtests was to assess if intrauterine position effects are present in CF-1 adult male mice.

Another study in mice (vom Saal et al., 1983) has shown 2M males to have heavier seminal vesicles than OM males (but see also Wechman et al., 1985, for negative findings). In a related study (Clark et al., 1990), males prenatally residing next to other males had significantly larger ventral gland pads and heavier testes than males located next to females. The purpose of the third measure was to see if these general findings could be replicated.

Method

Animals

Twenty 2M and 8 OM males between 188 and 272 days of age were measured and weighed to provide data for the parameters described above.
Procedure

Measurements were obtained after the completion of vocalization testing and after these test animals had provided urine stimulus for the female odor preference testing. All test animals were sacrificed immediately prior to measurement. Anogenital distance was measured using Vernier calipers. An average of two AGD measurements were recorded for each animal. Body weights were then measured and recorded. Following these measurements, seminal vesicles were removed from each animal and their weights recorded.

Results

Anogenital Distance

Mean anogenital distance measurements of both groups are shown in Figure 7. A one-way ANOVA found no significant difference between OM and 2M males in their mean anogenital distance, F(1, 26) < 0.01, p = ns.
Figure 7: Anogenital distance (mm) of OM and 2M CF-1 males (mean ± SE) in adulthood.

Anogenital Distance/ Body Weight Ratio

Anogenital distance has been shown to correlate significantly with body weight in mice (Graham & Gandelman, 1986) and rats (Meisel & Ward, 1981). Therefore, the absolute AGD measure (in the previous section) may be confounded by the weight of the animal.Erroneously, we might be examining data that
reflect other variable(s) (e.g. food intake) that contribute to body weight, and therefore AGD.

To control for this confounding factor, a transformation score was used to assess AGD as a function of intrauterine position: absolute AGD/ body weight, as replicated in the study by Graham and Gandelman (1986). As shown in Figure 8, the group means for AGD/body weight scores were not significantly different, $F(1, 26) = 1.84$, $p = \text{ns}$.

![Figure 8: Anogenital distance as a percentage of body weight (mean ± SE) in 0M and 2M adult CF-1 males.](image-url)
Thus, neither AGD nor AGD relative to body weight was affected by intrauterine position in males.

**Seminal Vesicle Weight (as percent of body weight)**

Seminal vesicle weights were calculated with respect to the animal's total body weight. The relative seminal vesicle weights for OM and 2M male groups are shown in Figure 9. A one-way ANOVA revealed no significant differences between these two groups, $F(1, 26) = 0.15, p = \text{ns}$.

![Graph showing seminal vesicle weight as a percentage of body weight](image)

**Figure 9:** Seminal vesicle weight as a percentage of body weight (mean ± SE) on OM and 2M adult CF-1 males.
The ages at which morphological measurements were taken were found to be significantly different \((F(1, 26) = 25.76, p < .05)\) between OM males \((M = 228.5 \pm 13.22\) d) and 2M males \((M = 270.15 \pm 0.76\) d). This difference was accounted for by 4 "outlier" subjects in the OM group \((M43, M57, M58, M59,\) being 226, 188, 188 and 188 days, respectively). Thus there is some question as to whether the significant difference in age between the OM and 2M groups may have affected the "non-results" seen in the morphological parameters \((i.e.\) perhaps morphological differences may have been seen between the 2 groups had the animals been comparable by age).

To determine if age was a contributing factor in the results, animals were again age-matched \((5\) per group), \(F(1, 8) = 1.70, p = \text{ns}.\) One-way ANOVA's run on each independent parameter showed no significant differences between matched groups in AGD, \(F(1, 8) = 0.43, p = \text{ns}.\), AGD/body weight, \(F(1, 8) = 1.01, p = \text{ns}.\), or seminal vesicle weight/body weight ratios, \(F(1, 8) = 0.02, p = \text{ns}.\) A second method \((a\) within-group, young vs. old comparison) similarly used in
the preference data, showed no differences between young vs. old mice within each group on any morphological parameter (see Appendix IV), indicating that young and old mice within each group were similar with respect to genital morphology. Thus, findings (or in this case, lack of findings) of the morphological data would appear to be attributed to the animal's prior intrauterine position and not to its age.

Collectively, the results of the three morphological measures indicated that OM and 2M males were similar with respect to anogenital distance and seminal vesicle weight, suggesting that prior intrauterine position, and hence, the variation in prenatal exposure to androgens, does not affect morphology in males in adulthood.

The 2 experiments (#4 and #5) that follow were originally designed as pilot studies to assess 1) IUP-identified female preference behavior toward IUP-identified males, and 2) to determine if any morphological differences exist between these two IUP female types.
Experiment 4: Odor Preference In IUP Females

The purpose of experiment 4 was to examine the odor preferences of OM and 2M females for OM and 2M male conspecific urine. Based upon the findings described earlier (Rines & vom Saal, 1984; vom Saal & Bronson, 1980b; vom Saal, Pryor and Bronson, 1981), it would be predicted that OM females might show greater preference for male conspecific odors than would 2M females. This experiment was designed to test this hypothesis.

Method

Animals

Fifteen OM and 15 2M females, between 65 and 121 days of age were tested for odor preference using OM and 2M male urine as stimulus.

Stimulus

Urine was collected from OM and 2M male donors.
within 4 hours prior to presentation. Since the OM males were in short supply, the same OM males previously used for behavioral testing were later used as urine donors.

Procedure

Females were given concomitant social and apparatus experience for 8 consecutive days prior to testing. Two days elapsed from the last day of social/apparatus experience to the first testing trial. Complete details of the study procedures are described in General Methods.

Results

The age of OM females \( (M = 92.53 \pm 3.95 \text{ d}) \) did not differ significantly from 2M females \( (M=95.6 \pm 4.26 \text{ d}) \) at preference testing, \( F(1, 28) = 0.28, p = \text{ ns} \). Odor preference profiles are shown in Figure 10 (absolute) and Figure 11 (relative).
Figure 10: Absolute odor preference (mean ± SE) of 0M and 2M female CF-1 mice for urine from 0M males and 2M males. Note. Refer to general methods for a complete description of the absolute preference measurement.

A 2x2 ANOVA revealed a marginally significant group effect for absolute preference, $F(1, 56) = 3.077$, p
\[ F(1, 56) = 3.830, \ p < .06, \] indicating that 2M females spent slightly more time sniffing (i.e. greater preference for) male urine (of either IUP-type) than did OM females.

![Bar graph](image)

**Figure 11:** Relative (percent) odor preference (mean ± SE) of OM and 2M female CF-1 mice for urine from OM males and 2M males. **Note:** Refer to general methods for a complete description of the relative preference measurement.
While it appeared that OM male urine was preferred over the 2M male urine by both female groups, due to the large variance, statistical significance of a stimulus effect was not achieved in absolute preference $F(1, 56) = 1.566, p = ns.$, or relative preference, $F(1, 56) = 0.877, p = ns.$ A group by stimulus interaction was also non-significant in absolute preference, $F(1, 56) = 0.218, p = ns.$, and relative preference, $F(1, 56) = 0.013, p = ns.$

Contrary to the proposed hypothesis, these data suggest that a slightly greater preference exists for male urine by the 2M females when compared to the OM females. However, neither the particular male urine presented (OM or 2M), nor the interaction of group by stimulus was significant.

Thus it appeared that intrauterine position might possibly have affected the general preference behavior of certain females (i.e. 2M females being more investigatory) rather than a preference for a particular (OM vs. 2M male) stimulus odor. Since female urine was not presented as a stimulus, it cannot be determined if females preferred male over
female urine. Therefore, interpretation of these data is limited only to preference toward male stimuli.

Females displayed much lower levels of investigatory behavior overall than did males (replicating previous findings in our lab). The mean amount of time spent investigating an odor stimulus ranged from 0.38 seconds to 6.56 seconds for females. However, the mean amount of time for males was considerably higher, ranging from 6.465 to 20.075 seconds.

In summary, there was no difference (at .05 significance level) between the two female groups in odor preference behavior. However, 2M females were slightly more attracted to male urine than were OM females, contradicting the proposed hypothesis.
Experiment 5:
Morphological Parameters In IUP Females

The purpose of this experiment was to determine if OM and 2M females differed on morphological parameters as a function of their intrauterine position. Three variables were examined in adulthood; 1) AGD, 2) AGD/Body Weight Ratio, and 3) Uterine Weight / Body Weight Ratio.

Based upon previous findings (vom Saal & Bronson, 1978; vom Saal et al., 1990), it would be predicted that 2M females would have longer anogenital distances than their OM counterparts. While no studies have examined intrauterine position effects on uterine weight, would differences in uterine/ body weight exist between 2M and OM females? This experiment was designed to test these parameters.

**Method**

**Animals**

Twelve OM and 10 2M females between 188 and 277
days of age were measured and weighed to provide data for the parameters described above. (No data was available for the other 3 OM or 5 2M test females.)

Procedure

Measurements were obtained after all animals had completed the behavioral testing. Preparation and measurement procedures are identical to that for males (see Experiment 3). Following AGD measurements, the uterus was removed from each animal and their weight recorded.

Results

Mean ages of the IUP female groups were not significantly different at time of measurement ($F(1, 20) = 1.56, p = ns.$), being $248.08 \pm 3.57$ d and $228.4 \pm 12.93$ d for the OM and 2M female groups, respectively.

Anogenital Distance

Mean anogenital distances are shown in Figure 12. One-way ANOVA revealed no significant difference ($F(1,$
between the anogenital distance of OM females (M = 4.358 ± 0.188 mm) and 2M females (M = 4.133 ± 0.194 mm).

![Figure 12: Anogenital distance (mm) of OM and 2M CF-1 females (mean ± SE) in adulthood.](image)

**Anogenital Distance/ Body Weight**

Anogenital distance relative to body weight is presented in Figure 13. No significant difference in AGD/ body weight was found in OM females when compared to 2M females, F(1, 20) = 1.13, p = ns.
Figure 13: Anogenital distance as a percentage of body weight (mean ± SE) in OM and 2M adult CF-1 females.

**Uterine Weight (as percentage of body weight)**

Relative uterine weights are shown in Figure 14. While uterine weights appeared slightly heavier in the 2M group than in the OM group, these results were non-significant, $F(1, 20) = 1.52, p = ns$. 

-130-
Figure 14: Uterine weight as a percentage of body weight (mean ± SE) in OM and 2M adult CF-1 females.

As shown in these parameters, OM and 2M females were similar with respect to anogenital distance and uterine weight, suggesting that these morphological characteristics are not influenced by prior intrauterine position.
Experiment 6:
Anogenital Distance At Birth
in IUP Males And Females

The purpose of this experiment was to determine if OM animals were different from 2M animals of the same gender in anogenital distance at birth.

Method

Animals

One-hundred and four 2M males were compared to 57 OM males at delivery. Sixty-one 2M females were compared to 37 OM females at delivery.

Procedure

Anogenital distance was measured at the time of delivery from IUP-identified animals delivered from 52 cesarian sections. The average of 2 AGD measurements was recorded for each animal.
Results

As shown in Figure 15, the anogenital distance of 2M males ($M = 2.63 \pm 0.025$ mm) at delivery did not differ significantly from the anogenital distance of OM males ($M = 2.588 \pm 0.0397$ mm) at delivery, $F(1, 159) = 0.93$, $p = ns$.

Figure 15: Anogenital distance (mm) of OM and 2M CF-1 males (mean $\pm$ SE) at delivery.
For females (Figure 16), the anogenital distance at delivery were also similar between 2M ($M = 1.852 \pm 0.027$ mm) and OM ($M = 1.795 \pm 0.027$ mm) animals, $F(1, 96) = 1.90, p = \text{ns}$.

Figure 16: Anogenital distance (mm) of OM and 2M CF-1 females (mean \(\pm\) SE) at delivery.
In a cross-gender comparison (Figure 17), males had significantly longer anogenital distance ($M = 2.616 \pm 0.021$ mm) than females ($M = 1.830 \pm 0.020$ mm), $F(1, 257) = 639.34, p < .0001$), as expected.

![Graph showing anogenital distance comparison between males and females](image)

**Figure 17:** Anogenital distance (mm) at delivery; Gender comparison of CF-1 males and CF-1 females.
The results of Experiment 6 indicate that OM animals were morphologically similar to 2M animals of the same-sex with respect to their anogenital distance at delivery, suggesting that intrauterine position (i.e. the variation of androgen exposure prenatally) had no effect upon external morphological characteristics at birth.

These findings then, are inconsistent with the previous studies which have supported this phenomenon in mice (Gandelman et al, 1977; vom Saal & Bronson, 1978) and rats (Richmond & Sachs, 1984; Tobet et al., 1982), but did coincide with the negative results found in a study of piglets (Rohde Parfet et al., 1990).
CHAPTER 3
DISCUSSION

In general, the overall results of these six experiments did not strongly support the intrauterine position phenomenon. In Experiment 1, OM males did show greater absolute preference for (and attraction toward) female urine than did 2M males. These results are similar to findings that OM males are more sexually active than their 2M counterparts (vom Saal et al., 1983). However, this difference was eliminated when group size was equal (i.e. when subjects were age-matched, 5 per group). This may have been due to the fact that a smaller group of subjects were analyzed, therefore achieving statistical significance was not possible.

Furthermore, urine collected from the two female conditions (OM vs. 2M) failed to elicit a differential response from either male group, which is inconsistent with previous findings that males preferred OM females over 2M females (Rines & vom Saal, 1984; vom Saal & Bronson, 1978). The findings of Experiment 1 did reaffirm a basic principle in pheromonal
communication, namely, that in males, urine from female conspecifics is more attractive than from conspecific males (Nyby, 1983; Nyby et al., 1985; Nyby et al., 1977).

In Experiment 2, OM and 2M males emitted similar levels of vocalization to OM and 2M females. These results were inconsistent with earlier findings that OM males are more sexually active than 2M males (vom Saal et al., 1983).

Moreover, the two stimulus conditions (OM and 2M females) did not elicit a differential response in the males (similar to the findings in Experiment 1); males vocalized equally to both OM and 2M females. This was inconsistent with the hypothesis that OM females are more attractive to males than their 2M counterparts (vom Saal, 1989; vom Saal & Bronson, 1978). Also, a "ceiling effect" was observed as both groups were near maximal levels of responsiveness. Perhaps if anesthetized females had been used as the stimuli, the overall level of response may have been decreased to a point at which group differences could be seen. However, because preliminary testing revealed very low
and inconsistent levels of vocalization when anesthetized females were used, it was decided to use awake females which would elicit higher levels of vocalization and prompt a more "real-life" interaction. This rationale was further supported by the findings of two studies (Nyby et al., 1977—see experiment # 2; Warburton, Sales & Milligan, 1989).

Findings of Experiment 3 indicated that OM and 2M males were similar in adulthood with respect to anogenital distance (supporting the findings of Zielinski et al., 1991; and Rohde Parfet et al., 1990) and seminal vesicle weight (supported by Wechman et al., 1985), suggesting that prior intrauterine position and the variation of androgen exposure prenatally, does not influence genital morphology in adult males.

Experiment 4 suggested that 2M females were only slightly more attracted to male urine than were OM females, which is opposite to the proposed hypothesis that OM females would show greater preference toward male urine than 2M females. These findings are also inconsistent with the findings that OM females are
more sexually receptive than 2M females (Rines & vom Saal, 1984), but are similar to the negative findings of a recent study which found that a female's sexual preference for a male was not related to the female's prior intrauterine position (Quadagno et al., 1987).

Similarly, no differences between OM and 2M females were evident in morphological parameters, as shown in Experiment 5. Both female groups were similar in adulthood with respect to anogenital distance and uterine weight. Results of this experiment are inconsistent with the findings of 2 previous studies that showed that in adult mice, 2M females had longer anogenital distances than OM females (vom Saal & Bronson, 1978; vom Saal et al., 1990). However, the findings of this study did support a recent study in swine (Rohde Parfet et al., 1990) indicating that intrauterine position had no effect on anogenital distance in adulthood.

In the final experiment (#6), the anogenital distance of same-gender IUP-identified animals were compared at delivery. Intrauterine position had no effect on anogenital distance between OM and 2M males.
or females, thus contradicting previous studies in mice (Gandelman et al., 1977; vom Saal & Bronson, 1978) and rats (Richmond & Sachs, 1984; Tobet et al., 1982), but supporting the negative findings in a study of piglets (Rohde Parfet et al., 1990). Thus, the variation in androgen exposure prenatally had no effect upon external morphological characteristics at birth.

One of the major problems in this study was obtaining a sufficient number of OM males for testing. This was difficult for two reasons: 1) OM males were less frequently delivered than 2M males, and 2) once delivered, OM males were less likely to survive to adulthood (observation).

Of 52 cesarian deliveries performed over an 11-month period, 104 2M males, 57 OM males, 61 2M females and 37 OM females were obtained. Thus, OM animals were not as frequent in the litters as 2M animals.

Secondly, a high degree of infanticide occurred, particularly of the OM male pups, even though all IUP-identified animals were fostered in the same manner. The number of 2M males needed for testing (n=20) and
stimulus (n=8) conditions were unaffected by the infanticide due to the large number of 2M males delivered. On the other hand, most (49/57, 86%) of the OM males never survived to weaning.

While it is known that infanticide is a response to overcrowding (Gandelman, 1983), what is not clear is why the foster mothers selectively cannibalized the males and not females, and why OM males were more prone to this than 2M males.

Previous research examining infanticide has focused on the conditions (e.g. genetic strain differences, stress during pregnancy, prior pup experience) of the animal committing the infanticide (see McCarthy, 1990; Miley, Blaustein & Kennedy, 1982; Svare, Kinsley, Mann & Broida, 1984), rather than on the condition (e.g. sex, IUP-type, etc.) of animal that was killed. Perhaps this is where further research on this phenomenon should be focused.

Disappointingly, the findings from these 6 experiments did not strongly support the intrauterine position phenomenon. This may have been due to the problems inherent in the collection of test animals.
and methods of testing as previously described. Another factor may be that the behavioral and morphological indices were insensitive and thus the subtle differences that might exist between 0M and 2M animals could not be detected. Perhaps differences would have been evident had copulatory (rather than precopulatory) behaviors been studied (apparent in the study by Gandelman, 1986). Unlike most of the previous studies examining intrauterine position effects, the animals in this study were intact, and not primed with hormones prior to testing. Behavioral differences between 0M and 2M animals may have been precipitated had the test animals been primed prior to testing. A final factor to consider may be in the strain and species used for testing. While CF-1 mice were used in several positive studies by vom Saal and his associates, it is possible that CF-1 mice only show discernable differences in certain behaviors (i.e. copulatory rather than precopulatory behavior). These suggestions are aimed at providing some direction for further research examining the intrauterine position phenomenon.
CONCLUSIONS

* OM males showed greater absolute preference toward female urine than 2M males. This effect was eliminated when age and group size were controlled for.

* Vocalization profiles were similar in OM and 2M males.

* OM and 2M males were similar with respect to morphological characteristics, at delivery and in adulthood.

* 2M females showed only slightly greater preference toward male urine than did OM females.

* OM and 2M females were similar with respect to morphological characteristics, at delivery and in adulthood.

* Overall, the findings of this study did not strongly support the intrauterine position phenomenon.


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APPENDIX 1
INTRAUTERINE POSITION STUDIES
(Chronological Listing by Year)

<table>
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APPENDIX II

Within-Group "Young" Versus "Old" Subjects; Comparison of Odor Preference Parameters

I. 2M Males - "Young" vs. "Old" Comparison on Odor Preference Parameters

(10 young vs. 10 old subjects, using day 75 as dividing point)

A. One-way ANOVA used to determine whether there was a significant difference between young and old 2M males on each variable.

1. Absolute Preference for OM Female Urine  
   \( F(1, 18) = 0.85, p = .369 \)

2. Absolute Preference for 2M Female Urine  
   \( F(1, 18) = 1.63, p = .217 \)

3. Absolute Preference for Male Urine  
   \( F(1, 18) = 0.07, p = .788 \)

4. Percent Preference for OM Female Urine  
   \( F(1, 18) = 0.05, p = .830 \)

5. Percent Preference for 2M Female Urine  
   \( F(1, 18) = 1.48, p = .239 \)

6. Percent Preference for Male Urine  
   \( F(1, 18) = 0.08, p = .784 \)

II. OM Males - "Young" vs. "Old" Comparison on Odor Preference Parameters

(4 young vs. 4 old subjects, using day 89 as dividing point)
A. One-way ANOVA used to determine whether there was a significant difference between young and old OM males on each variable.

1. Absolute Preference for OM Female Urine
   $F(1, 6) = 0.01, p = .931$

2. Absolute Preference for 2M Female Urine
   $F(1, 6) = 0.05, p = .823$

3. Absolute Preference for Male Urine
   $F(1, 6) = 2.20, p = .189$

4. Percent Preference for OM Female Urine
   $F(1, 6) = 0.19, p = .680$

5. Percent Preference for 2M Female Urine
   $F(1, 6) = 0.05, p = .828$

6. Percent Preference for Male Urine
   $F(1, 6) = 2.28, p = .182$
APPENDIX III

SUMMARY OF ODOR PREFERENCE BEHAVIOR
IN OM AND 2M MALES

1) **Unmatched Analysis**: (20 vs. 8 Ss)

Absolute Behavior: Group effect: OM > 2M SIG.
Stimulus effect: Fem. > Male SIG.
Interaction: none N.S.

Relative Behavior: Group effect: none N.S.
Stimulus effect: Fem. > Male SIG.
Interaction: none N.S.

2) **Matched Analysis**: (Age-Matched; 5 per group)

Absolute Behavior: Group effect: none N.S.
Stimulus effect: Fem. > Male SIG.
Interaction: none N.S.

Relative Behavior: Group effect: none N.S.
Stimulus effect: none N.S.
Interaction: none N.S.

*(Note. Significant effects at p < .05)*
APPENDIX IV

Within-Group "Young" Versus "Old" Subjects; Comparison of Morphological Parameters

I. 2M Males - "Young" vs. "Old" Comparison on Morphological Parameters

(7 young vs. 13 old subjects, using day 270 as dividing point)

A. One-way ANOVA used to determine whether there was a significant difference between young and old 2M males on each variable.

1. Anogenital Distance
   $F(1, 18) = 2.22, p = .154$

2. Seminal Vesicle Weight
   $F(1, 18) = 0.04, p = .847$

3. AGD/ Body Weight Ratio
   $F(1, 18) = 1.94, p = .181$

II. OM Males - "Young" vs. "Old" Comparison on Morphological Parameters

(4 young vs. 4 old subjects, using day 230 as dividing point)

A. One-way ANOVA used to determine whether there was a significant difference between young and old OM males on each variable.

1. Anogenital Distance
   $F(1, 6) = 3.06, p = .131$

2. Seminal Vesicle Weight
   $F(1, 6) = 0.72, p = .429$

3. AGD/ Body Weight Ratio
   $F(1, 6) = 0.11, p = .750$

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VITA

ANNETTE PARKE STEVENSON

EDUCATION
Lehigh University, Bethlehem, PA
M.S. Psychology, January 1992

Pine Manor College, Chestnut Hill, MA
B.A. Biological Psychology, Developmental Psychology
Summa Cum Laude, May 1984

HONORS
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Awards for Distinction in Biological Psychology, Developmental Psychology and Chemistry; Dean's Award, May 1984

New England Psychological Association Honorary Undergraduate Fellowship, October 1983

EXPERIENCE
Rhone-Poulenc Rorer Pharmaceutical Corporation, Division of Clinical Research, Collegeville, PA
Clinical Research Specialist since 10/83
Clinical Research Associate 4/83-9/83
Clinical Research Assistant 1/87-3/88

Concentration in cardiovascular studies: congestive heart failure, hypertension and hyperlipidemia. Responsible for development of study protocols, field-monitoring of clinical trials, management of clinical data, writing of study progress reports and final medical reports of trial results. Also responsible for project tracking status, negotiation of study budgets with study contractors.
Lehigh University, Bethlehem, PA
Graduate Researcher Fall 1984-Fall 1986

Conducted research examining prenatal hormonal effects as a function of intrauterine position, on precopulatory behaviors and morphological parameters in CF-1 mice.

Teaching Assistant Fall 1984-Spring 1986

Assisted professors in undergraduate psychology courses; involved in preparation of course material, examinations, grading and lecturing.

National Institute of Mental Health, Division of Special Mental Health Research, Saint Elizabeth's Hospital, Washington, D.C.
Research Assistant Summer 1983

Assistant to neuroscientist in experimental laboratory. Involved in a study examining various drugs' effects on seizure-induced behavior in rats. Collected and analyzed data, interpreted results and wrote final research summary. Also assisted in a brain graft study examining the effects of neural tissue grafts in animal-model Parkinson's disease.

Children's Hospital Medical Center, Learning Disabilities Clinic, Department of Neurology, Boston, MA
Research Assistant Spring '83

Assisted neuropsychologist in a clinical setting. Designed coding system for a study correlating learning disability and neurological impairment in children. Collected, managed, interpreted and coded
data in preparation for analysis. Also prepared a case study evaluation from multi-disciplinary clinical assessments.

Harvard Medical School, Laboratory in Social Psychiatry, Boston, MA
Research Assistant Fall 1981-Spring 1983

Assistant in longitudinal study of adolescent and family development. Responsible for individual and family interviewing, administering psychological measures, data management, coding and data entry. Supervised and trained new co-workers in coding and computer usage.
END
OF
TITLE