The Anogenital Distance Index, a Predictor of the Intrauterine Position Effects on Reproduction in Female House Mice

John G. Vandenbergh and Cynthia L. Huggett

Abstract

The anogenital distances (AGD) of newborn female house mice vary as a function of prior intrauterine position. Females with long AGDs are more likely to be derived from intrauterine positions adjacent to males (2M) than females not adjacent to males (OM). Females with a male on one side (1M) show intermediate AGDs. Hence the AGD reflects the degree of androgenization experienced by the female in utero and correlates with a number of androgen-dependent anatomical, physiological, and behavioral events in adulthood. This experiment tested the usefulness of AGD measurements of female house mice taken at weaning rather than at birth as an index of prior androgenization. The AGD was normalized for body weight at weaning to yield an anogenital distance index (AGDI). Intrauterine position (IUP) was determined by caesarian section. pups were marked and reared by foster mothers. Comparison of AGDI showed that OM females had a significantly lower mean AGDI score than 2M females and 1M females were intermediate. This confirmed that AGDI reflects prior IUP and can be used as an index of prenatal androgenization. While testing for responsiveness to male urine, a stimulus known to accelerate puberty, only females from the 1M and 2M positions differed from controls indicating that OM females had already attained puberty. Choosing females from unknown IUPs with short-AGDI, mid-AGDI, and long AGDI and testing these with either urine or saline on the nose for the 4 yr after weaning yielded the same response indicating that AGDI can also be used to preselect females sensitive to factors influencing puberty. These results demonstrate that some of the variability known to be related to intrauterine position can be predicted by AGDI, a relatively easy measure to acquire at weaning commonly used laboratory rodents. Such preselection could reduce variability of experimental results in the subje of studies related to rodent reproduction and may reduce the number of animals needed without loss of predictive ability.

The anogenital distance (AGD) of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position (IUP) of the animal (1). Longer AGD is associated with the presence of males on either-side of the developing fetus in utero, and shorter AGD is associated with the absence of males on either side the developing female fetus. Females with a male fetus only one side are intermediate. The neighboring males have a masculinizing effect on their sisters due to androgens transferred to adjacent fetuses either through amniotic fluid and membranes (4, 5) or via the maternal uterine circulation (6-7). The result of this intrauterine communication is that the amniotic fluid and blood of female fetuses developing between two males have higher concentrations of testosterone and lower concentrations of estradiol than fetuses developing between two females (8). Testosterone serves as a priming pheromone in utero because it is a natural product of the same species through the environment, and has developmental and physiologic consequences on the recipient (9).

Differences in AGD between the sexes and among females from different IUP sets to be most pronounced at birth. Thus AGD measurements have usually been taken on newborn pups. By using measures of prior androgenization only on newborn pups, investigators are limited to using laboratory-bred mice so that the pups AGD can be measured at birth. Here we investigated whether AGD at weaning, adjusted for body weight, reflects prior androgenization of female mice. If this AGD index (AGDI) correlated closely with IUP, it could be used at weaning as a convenient measure of prenatal androgenization in females. A preliminary description of the AGDI was presented in a study showing a correlation between AGDI and the later sex ratio of litters produced (10), but more complete details on the use of AGDI are described here.

The ability to determine conveniently the level of prenatal androgenization, whether derived from the IUP phenomenon or from other androgenizing factors such as environmental agents, would permit reduction in experimental variance through the selection of animal subjects or
influences
Vol 45, No 5
Laboratory Animal Science
October 1995

Although females have received more attention, IUP also

weaning, can be used to predict several reproductive char­

teristics of the mouse in accordance with the NIH Guide for the Care and Use

of Laboratory Animals and were inspected by the Institutional

Animal Care and Use Committee. They were maintained

a 14:10-h light/dark cycle and provided with Prolab 3000

food (Agway Prolab RMH 3000, Raleigh, N.C.) and water.

At the age of 60 to 90 days, females were mated

with stud males, and the date of vaginal plug, considered

by(IUP, 4), the size of androgen-sensitive, sexually dimorphic

areas in the hypothalamus of the brain (11), age at onset of

puberty (3, 10, 12), ovarian cyclicity (13, 14), mating behav­

ior (2), the sex ratio of litters produced in gerbils (15) and

mice (16), are all partially influenced by

whether there were alterations in the hypothalamic-pitu­

itary-adrenal axis as a result of IUP.

Materials and Methods

Experiment 1: Charles River CD-1 albino mice derived

from our breeding colony were used. Animals were cared for

in accordance with the NIH Guide for the Care and Use

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Results

Experiment 1: A significant correlation existed between AGD and the body weight of female mice at weaning within each of the three IUP categories (Figure 2). Mean ± SEM body weight for OM females was 12.3 ± 0.27 g, for 1M females was 12.1 ± 0.27 g, and for 2M females was 11.4 ± 0.38 g. Mean body weight of the weanlings from 2M females was significantly (P < 0.05) lower than that of the weanlings derived from 0M IUP. Body weight of 1M females was not significantly different from that of OM or 2M females.

Because a relationship exists between body weight and AGD at weaning, AGD was adjusted for body weight to yield AGDI for females from each known IUP (Figure 3). The mean AGDI for OM, 1M, and 2M females was 23.5 ± 0.32, 26.6 ± 0.32, and 29.7 ± 0.56, respectively. An overall analysis of variance of AGDI values across the three IUP indicated significant differences (P < 0.0001). By the Tukey-Kramer test, the mean AGDI for females from each of the three IUP significantly (P < 0.05) differed from each other.

The overall reproductive performance of female mice from different IUP is presented in Table 1. The age at first estrus was later in 2M than in OM females, and that in 1M females was intermediate. When mated for the first time at 60 to 90 days of age, 2M females took 1.7 days longer to conceive, a difference almost reaching significance at P < 0.058. A higher proportion of 2M females produced litters with a higher proportion of males than did OM females. The alteration in sex ratio is presented more fully elsewhere (10). Litter size and mean pup weight at birth did not vary with IUP. At the second mating, mean latency to conception was not significantly longer in 2M than in OM females, litter size

Figure 3. Mean AGDI of 21-day-old female mice derived from 0M, 1M, and 2M adjacent IUP.
Table 1. Reproductive performance in the first and second matings of female mice derived from known intrauterine positions

<table>
<thead>
<tr>
<th>Variable</th>
<th>1M</th>
<th>2M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First mating</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of females mated</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td>Number of litters born</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Age at first estrus (days)</td>
<td>29.70 ± 1.04</td>
<td>30.30 ± 1.00</td>
</tr>
<tr>
<td>Mean latency to conception (days)</td>
<td>3.60 ± 0.75</td>
<td>3.70 ± 0.50</td>
</tr>
<tr>
<td>Percentage pregnant</td>
<td>72.30</td>
<td>75.60</td>
</tr>
<tr>
<td>Litter size</td>
<td>12.60 ± 0.32</td>
<td>12.89 ± 0.28</td>
</tr>
<tr>
<td>Mean pup weight (g)</td>
<td>1.66 ± 0.04</td>
<td>1.67 ± 0.00</td>
</tr>
<tr>
<td>Sex ratio of litter</td>
<td>41.90 ± 2.40</td>
<td>51.40 ± 2.00</td>
</tr>
<tr>
<td><strong>Second mating</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of females mated</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Mean latency to conception (days)</td>
<td>4.44 ± 0.74</td>
<td>5.30 ± 1.35</td>
</tr>
<tr>
<td>Percentage pregnant</td>
<td>77.30</td>
<td>85.70*</td>
</tr>
<tr>
<td>Litter size</td>
<td>12.60 ± 0.64</td>
<td>14.60 ± 0.76</td>
</tr>
<tr>
<td>Sex ratio of litter</td>
<td>45.80 ± 2.21</td>
<td>53.10 ± 2.85*</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with OM female results

Table 2. Number and percentage of females delivered by cesarean section from each intrauterine position. Losses were attributable to pup mortality during surgery, cross-fostering, and exclusion due to body weight at weaning being outside the range of 6 to 18 g

<table>
<thead>
<tr>
<th>Variable</th>
<th>OM</th>
<th>IM</th>
<th>2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivered by cesarean section</td>
<td>78</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Loss</td>
<td>29</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Available at weaning</td>
<td>49</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>

AGDI

Figure 4. Number of 21-day-old female mice at each of three AGDI intervals shown.

was significantly larger for 2M females, and the sex ratio of the litter of 2M females continued to favor males.

In this study we performed cesarean sections on 34 dams that produced 237 female pups. Losses at surgery, cross-fostering, pup mortality, and elimination due to low or high body weight at weaning (<6 g or >18 g) reduced this number to 153 weanlings. The percentage of female pups produced and used in the analysis from each IUP is shown in Table 2. There were no significant differences in percentage loss among the three IUP categories. In a population of weanling females of known IUP, 22% were OM, 46% were IM, and 44% were 2M.
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Table 5. Mean (± SEM) first day of estrus and body weight for 0M, 1M, and 2M females treated with saline or urine

<table>
<thead>
<tr>
<th>Group</th>
<th>Estrus (days)</th>
<th>Body weight (g)</th>
<th>Estrus (days)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0M</td>
<td>23.1 ± 1.03</td>
<td>28.0 ± 0.30</td>
<td>23.1 ± 1.04</td>
<td>28.0 ± 0.30</td>
</tr>
<tr>
<td>1M</td>
<td>34.3 ± 1.23</td>
<td>28.0 ± 0.30</td>
<td>34.3 ± 1.25</td>
<td>28.0 ± 0.30</td>
</tr>
<tr>
<td>2M</td>
<td>28.1 ± 0.41</td>
<td>30.3 ± 0.50</td>
<td>28.1 ± 0.42</td>
<td>30.3 ± 0.50</td>
</tr>
</tbody>
</table>

Kruskal test, the 0M and 1M females attained first estrus significantly earlier than did the 2M females.

There were no significant differences in first estrus among the females from each IUP when exposed to male urine (Table 5). There was also no difference between saline- and urine-exposed 0M females; however, 1M and 2M females differed between treatments (IUP = 0.03 and P < 0.005 respectively).

**Experiment 2:** We allotted 175 weanling females of unknown IUP to three groups on the basis of presumed prenatal androgen exposure: low, mid-level, or high. Females were assigned to this new designation on the basis of AGDI categories shown in Figure 4, with the cut-off percentages for low, mid-level, and high prenatal androgen females differing significantly (P < 0.005) among groups (Figure 5).

The females with low prenatal androgen had the highest mean uterine weight, and the females with high prenatal androgen had the lowest. Only the saline-treated mid-level and high prenatal androgen females differed significantly (P < 0.05) from the urine-treated females.

**Discussion**

Several reproductive parameters of female mice varied as a function of IUP. Female mice from the 0M position attained puberty earlier than did 2M females, confirming the findings of vom Saal (12). Latency to conception when they were paired with a male tended to be longer among females derived from the 2M position, and they produced a higher ratio of males in their first two litters than did 0M females. Measures for females derived from the 1M position were intermediate. This finding of sex ratio differences related to IUP was similar to that in the Mongolian gerbil (15). The mechanism for such alteration is not yet known, but the finding indicates that factors related to the proximity of males to females in utero are likely to be involved. The 15% of mice with highest AGDI scores below this value and of these, 22 were from the lower end of the AGDI range, the 1M females are in the highest range, and the 2M females are in the highest range. From these data a conservative cut-off point for AGDI values at weaning would be 20, 65, and 15% for 0M, 1M, and 2M females respectively. Conservative values were chosen to reduce the threshold score overlap into the range for the 1M mice. These cut-off values could be set at different points depending on the circumstances of the study.

A frequency histogram of the number of animals per AGDI interval (Figure 4) indicates that 0M females cluster at the lower end of the AGDI range, the 1M females are in the middle range, and the 2M females are at the higher end. Among these data a conservative cut-off point for AGDI values at weaning would be 20, 65, and 15% for 0M, 1M, and 2M females respectively. Conservative values were chosen to reduce the AGDI score overlap into the range for the 1M mice. These cut-off values could be set at different points depending on the circumstances of the study. The 0% of mice with the lowest AGDI scores yielded an IUP score of 23.5 as an upper limit for females with low AGDI values. Thirty-one females, 20% of the total, AGDI scores below this value and of these, 28 were from the lower end of the AGDI range, the 1M females are in the highest range, and the 2M females are in the middle range. From these data a conservative cut-off point for AGDI values at weaning would be 20, 65, and 15% for 0M, 1M, and 2M females respectively. The mice were treated with either saline or male mouse urine to test for puberty acceleration by the uterine weight assay (21). Uterine weight at 26 to 28 days of age, after 4 to 6 days of exposure to saline, differed significantly (P < 0.005) among the females from each IUP.

There were no significant differences in first estrus among the females from each IUP when exposed to male urine (Table 5). There was also no difference between saline- and urine-exposed 0M females; however, 1M and 2M females differed between treatments (IUP = 0.03 and P < 0.005 respectively).

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variations even if IUP is unknown. The absolute values for AGDI may vary because of measurement differences between investigators, as long as a sufficient range of measurements is available, the percentage cut-off figures chosen should yield mice from different levels of prenatal androgenization. For our data on CD-1 female mice we chose 20, 60, and 100% cut-offs to identify OHI, 1M, and 2M females. Jubilant and Nyby (23) had mixed success in relating IUP with measurements of AGD at birth and adulthood in three strains of mice. They found that IUP did not have a consistent effect on urinary odor preferences, ultrasonic mating vocalizations, or AGD. The inability to find a consistent relationship between IUP and AGD may be due to the effects of the estrous cycle on the perineum of adult females, to procedural differences used to measure AGD, or to strain differences. We have observed that AGD measurements on cycling females vary widely and are useless as an index of masculinization. The AGD measurements by Jubilant and Nyby yielded means with small SEM, suggesting that differences among the strains were detectable but not found. Strain differences seem to be a likely reason for the discrepancy between our findings and theirs. Thus AGDI may be a better index for some genotypes than for others.

Further refinement of AGDI may be possible. In this study we adjusted AGD for body weight; however, because body weight may reflect increased size in all dimensions, body length may be a more appropriate parameter for adjusting AGD. We chose body weight because it is the easy, commonly used measure of the mass of a rodent.

Intrauterine position is a source of variability in the production of priming pheromones that affect puberty and in the response to these pheromones by female mice (12). Intrauterine position affected the onset of puberty in our study, with 0H females attaining estrus earlier than 3M females, a finding similar to that of vom Saal (12). Our finding further substantiates that 2M females are masculinized because puberty onset in male mice is typically later than in females (24). Interestingly, when male urine was used to accelerate puberty (25) only females from the 1M and 2M positions differed from saline-exposed controls. The absence of a difference in first estrus between saline- and urine-exposed 0H females was probably due to the three rapid sexual transitions of the females from the 0H position. Thus the use of first estrus to detect puberty acceleration or inhibition could be made more sensitive by excluding females from the 0H position. Responsiveness of the uterine weight response of puberty to the puberty-accelerating pheromone also correlated with AGD (Figure 5). Thus AGDI, which can be obtained at weaning, permits exclusion of females with short AGDI, reducing the variance in this assay. From these data it is clear that the use of female mice from known IUP or known AGDI can significantly decrease the variance of criteria for puberty and thus increase their accuracy.

The uterine weight response of the immature laboratory mouse as first detected by Evens et al. (26) and standardized by Theggen et al. (27) is the most commonly used homolog for detecting estrogenic substances. In developing mammalian bioassays, Thigpen et al. (27) fed diet contain­ ing diethylstilbestrol to female CD-1 mice weaned at 15 days of age. Because of heterogeneity in the data, the included controls from the data set and performed analysis of variance on uterine weight measured daily in subsets of mice. Even with these measures taken to reduce the variance, the mean uterine weight of the central females had an SEM of 24% (10.8 ± 2.63) and the females fed diethylstilbestrol for 6 days had an SEM of 29% uterine weight (16.7 ± 3.73). These data indicate that a great deal of variance in present in the weight of the mouse uterus.

Our results suggest that a substantial portion of this variance may be due to IUP of the test females. Our results indicate that some of the variability known to be related to IUP can be predicted by AGDI. Because AGDI is a relatively easy measure to acquire from female mice at weaning, a common age for acquiring animals from research vendors, it can be used to assign subjects to experimental protocols. By AGDI it should be possible to preselect genotypes, perhaps other rodents, that are most suitable for experiments involving manipulations of gonadal hormones. Such preselection could reduce variability in experimental results, thus reducing the number of animals needed with a loss of predictive ability.

Changes in female AGD at birth result from testoster­ on transfer from adjacent males in utero (8) and from the normal endocrine changes due to social stress applied to the mother during days 12 to 16 of gestation (28). Because AGDI has been documented to be an important correlate of female reproductive characteristics in rats and rodents (3, 11), it is probable that AGDI for these species and perhaps other polytalous mammals could be developed; it would be more useful for the latter because their large litters would mean lower availability from each IUP. However, the finding that AGDI is influenced by maternal stress (28) suggests that AGDI might also be useful to detect prenatal androgenization effects in males with small litters or single births such as ungulates or primates.

References


