Variation in Ano-Genital Distance in Spontaneously Cycling Female Mice

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Contents

A recently observed developmental instability of the ano-genital distance (AGD) in female mice indicates that natural prenatal androgens do not have such a robust effect on female genital morphology as has been generally assumed. Part of this instability might be caused by oestrous cyclicity. To check this assumption, we examined the effect of the stage of the oestrous cycle on the AGD in adult (61–75 days old) female mice. Consistent with our assumption, the female AGD (1) varied during the oestrous cycle (p < 0.05), indicating thus rapid changes in morphology of female external genitalia, and (2) showed good repeatability (>0.66) in each stage of the oestrous cycle, suggesting that female genital morphology systematically varied within the oestrous cycle. Therefore, the stage of the oestrous cycle should be considered when assessing prenatal masculinization in adult female mice.

Introduction

In laboratory rodents, including the house mouse (Mus musculus), it has been well documented that prenatal exposure of genetic females to exogenous androgens results in a longer ano-genital distance (AGD), that is, the distance between the anus and the posterior aspect of the genital papilla (Gandelman et al. 1977; Clemens et al. 1978; Clark and Galef 1998). The AGD thus has become widely used as a marker of prenatal female masculinization (Ryan and Vandenbergh 2002).

However, because some studies (Simon and Cologner-Clifford 1991; Nagao et al. 2004; Hurd et al. 2008) did not find any relation between the AGD and the levels of natural prenatal androgens, it is possible that natural prenatal androgens do not have such a robust effect on female genital morphology as has been generally assumed (Baum et al. 1991). We have recently supported these doubts showing that the AGD of female mice is not developmentally stable morphometric measurement either during pre- (days 1–21) or post-weaning (days 21–61) periods (Dušek et al. 2010). As the vaginal appearance of female mice changes during the oestrous cycle (Allen 1922; Champlin et al. 1973), we assumed that oestrous cyclicity could be partly responsible for the developmental instability of the AGD during the post-weaning period (Dušek et al. 2010).

To check this assumption, we examined the effect of the stage of the oestrous cycle on the AGD in female mice. Because heavier animals tend to have longer AGD than lighter ones (e.g. Graham and Gandelman 1986; Vandenbergh and Huggett 1995; Kerin et al. 2003), we also examined the effect of the stage of the oestrous cycle on the body weight (BW). In addition, to check whether the AGD systematically varied within the oestrous cycle, we examined repeatability of the AGD. Presuming that the stage of the oestrous cycle will affect the AGD variation, we hypothesized that the AGD will show good repeatability in each stage of the oestrous cycle.

Materials and Methods

Animals and data collection

The AGD and the BW measurements were taken from 21 adult (61–75 days old) nulliparous female CD-1 (ICR) mice (Velaz, s.r.o., Praha, Czech Republic). The advantage of this strain is that it represents a multipurpose research animal model and can be used for testing the effects of prenatal androgens on postnatal phenotypic variation, including the female AGD (see e.g. Vandenbergh and Huggett 1994; Nagao et al. 2004). The AGD was measured from the base of the genital papilla to the proximal end of the anal opening, using a digital calliper, with the female being slightly anesthetized with CO2 (as recommended by Suckow et al. 2000). The AGD (mm) and the BW (g) were measured accurately to 0.01 by the same observer (A.D.) throughout the experiment. The accuracy of the data was improved by using the means of three consecutive measurements.

The stages of the oestrous cycle were determined by examining vaginal smears of cycling mice (Allen 1922). The vaginal smears were obtained by a vaginal lavage with 10 µl of sterile phosphate-buffered saline. The vaginal lavage sample was smeared onto a clear glass microscope slide and stained by the Pappenheim method (May-Gruwald, Giemsa). The microscope preparations were analysed under a light microscope. The slides were viewed under a high power (40×) magnification for qualitative and quantitative examination of cell types (leucocytes, nucleated epithelial cells and cornified epithelial cells). Based upon cytological examination, the vaginal smears were classified into the four basic stages of the oestrous cycle (Green 1966): prooestrus (P), oestrus (O), metoestrus (M), and dioestrus (D). As we did not detect the prooestrus or oestrus stages during each oestrous cycle, the prooestrus and oestrus stages were assumed to last for <24 h (e.g. Silver 1995; Grasso et al. 1998) and were, therefore, merged together as (PO). Both the measurements and the vaginal lavages began at postnatal day 61, were performed daily in the early afternoon (starting always at 12:00 AM) and lasted for a period of 15 consecutive days to cover at least three oestrous cycles.

The mice were maintained in a temperature-controlled (24 ± 1°C) room with a 14 : 10 light/dark cycle starting at 5:00 AM and provided with standard grain-based pellet diet (ST-I, Velas, a.s., Hrabanov, Lysá nad Labem, Czech Republic) and water ad libitum. All animals were individually housed in a plastic cage
(22 × 36 × 14 cm). The animals were cared for in accordance with the principles of the ‘Guide for the Care and Use of Laboratory Animals’ of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996), and the project (Ref. No. MZe 1146) was approved by the Central Commission for Animal Welfare of the Czech Republic.

### Statistical analysis

A general linear mixed model (GLMM, PROC MIXED, SAS System, V 9.2, 2002–2008; SAS Institute Inc., Cary, NC, USA), with random effect ‘identity of the female’ (for accounting the repeated measurements on the same subject), was applied to test factors affecting (i) ‘female AGD’ and (ii) ‘female BW’. The tested fixed effects were the class variable ‘oestrous cycle stage’ (PO, M, D: i, ii), and the continuous variables ‘female age’ (days 61–75: i, ii), ‘female BW’ (i), and ‘cube root of female BW’ (i), normalizing the allometric relation between the AGD and the BW (as recommended by Gallavan et al. 1999). The interaction terms were tested, but were not reported unless statistically significant. To check whether the female AGD systematically varied with the female BW within the oestrous cycle, the dependent variable ‘female AGD’ was explicitly tested for the interaction between (1) the ‘female BW’ and the ‘oestrous cycle stage’, and (2) the ‘cube root of female BW’ and the ‘oestrous cycle stage’. The within-class differences were indicated by model-adjusted least-squares means with standard errors (±SE) to account for unbalanced numbers of oestrous cycle stages. Differences within the class were tested by a t-test. When a significant difference was detected, the least-squares means with standard errors (±SE) to account for unbalanced numbers of oestrous cycle stages. Differences within the class were tested by a t-test. When a significant difference was detected, the least-squares means were compared by the Tukey–Kramer adjustment for multiple comparisons.

The repeatability of the AGD was tested using the intraclass correlation coefficient – ICC (Koch 1982). The ICC is an overall index of repeatability ranging between 0 and 1. A value of 1 indicates perfect repeatability of the measurement, and a value of 0 indicates no repeatability of the measurement. The ICC is commonly interpreted as follows (Fleiss 1986): ICC < 0.4 indicates poor repeatability; 0.4 < ICC < 0.75 indicates fair-to-good repeatability; and ICC > 0.75 indicates good-to-excellent repeatability. For each stage of the oestrous cycle, the ICC of the AGD was derived from the GLMM model tested for the fixed effects ‘female BW’ and ‘cube root of female BW’, with the REPEATED statement ‘female age’. All cited p-values were two-tailed, with a significance level set at 0.05.

The length of the oestrous cycle was assessed using the mean (±SE) of all complete cycles detected.

### Results

The factors affecting the AGD (i) and the BW (ii) of the female are listed in Table 1. In accordance with our assumptions, the AGD of the female (1) varied depending on the stage of the oestrous cycle (Table 2) and (2) showed good repeatability in each stage of the oestrous cycle (Table 3). The average length of the oestrous cycle was 5.77 ± 0.26 days.

### Discussion

The variation in the AGD observed in this study was generally consistent with the variation in the vaginal appearance observed in previous studies (Allen 1922; Champlin et al. 1973). Also, the observed length of the oestrous cycle was in accordance with the previously reported range of 4–6 days (van der Lee and Boot 1955, 1956; Silver 1995). As the female AGD showed good repeatability in each stage of the oestrous cycle (Table 3), it seems that this morphometric measurement systematically varied within the oestrous cycle.

Although the female BW contributed to the variation in the female AGD (Table 1) and the oestrous cycle stage affected the female BW (Tables 1 and 2), the female AGD was not affected either by (1) the interaction between the female BW and the oestrous cycle...
stage, or (2) the interaction between the cuneal root of female BW and the oestrous cycle stage (see Table 1). Hence, it seems that within the oestrous cycle, the AGD varied owing to not only the variation in the BW, but also the sex steroid–mediated remodelling in the genital tissues (Wood et al. 2007). These cyclical changes in the sex steroids could also be responsible for the variation in the female BW (Asarian and Geary 2006).

The present results imply rapid changes in the morphology of female external genitalia during a very short period of time, indicating that the oestrous cyclicity could be partly responsible for the developmental instability of the AGD in post-weaning female mice. However, it is important to note that the AGD showed developmental instability also during the pre-weaning period (Dusék et al. 2010). Therefore, our previous conclusion that the AGD is generally not a reliable marker of prenatal female masculinization seems to be still valid.

In addition to these cyclical changes in the genital tissues, the applicability of the AGD as a marker of prenatal female masculinization is complicated by a number of factors affecting the oestrous cycle alone. One of these factors is prenatal female masculinization in itself (vom Saal 1989; vom Saal et al. 1999). vom Saal et al. (1990) reported that adult female mice prolonged their oestrous cycles as a consequence of natural prenatal masculinization. Moreover, this effect varied depending on the age of the female (vom Saal et al. 1991) and the maternal stress (vom Saal et al. 1991).

In conclusion, to our knowledge, the present study is the first to show that the stage of the oestrous cycle contributed to the variation in the AGD of adult female mice. Therefore, the stage of the oestrous cycle should be considered when assessing prenatal masculinization in adult female mice. Also, to account for genetic background and environmental variables, future research should focus on testing the effect of the oestrous cycle stage on the female AGD in various mouse and rat (Rattus norvegicus) strains under different experimental conditions.

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Conflict of interest
None of the authors have any conflict of interest to declare.

Author contributions
A. Dušek contributed to the design of the study, the data collection, the statistical analysis and the writing of the article. L. Bartoš contributed to the design of the study, the statistical analysis and the writing of the article.

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