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ESTRUS CYCLE STATUS DEFINED BY VAGINAL CYTOLOGY DOES NOT CORRESPOND TO FLUCTUATIONS OF CIRCULATING ESTROGENS IN FEMALE MICE

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ABSTRACT—Gender-oriented studies in shock, trauma, and/or sepsis require accurate monitoring of hormonal fluctuations as estrogens may influence various end points. Yet, monitoring is challenging in small laboratory animals: e.g., despite its subjectivity, vaginal smears are the major method for determination of estrus cycle phases in mice. Using female mice of different age, we aimed to (*a*) characterize general age-related changes in systemic estrogens and (*b*) examine the utility of determination of the estrus cycle by vaginal smears and/or impedance simultaneously comparing them with oscillation of systemic estrogens. In this study, 3-, 15-, and 20-month-old mice underwent vaginal smear and impedance examination each morning for 22 days. Ten hours after each morning checkup, feces were collected, and a second vaginal smear performed. Blood was collected on days 15 and 22. In 3-month-old females, estrus (by smears) was three times more frequent than in older mice, but mean concentrations of plasma and fecal estrogens never decreased with age. Collectively (not individually) plotted fecal estrogens values increased in the proestrus/estrus interphase (by smears) in 3-month-old mice only. Impedance typically peaked (4.5 Ω in 3-month-old mice) in the estrus phase, and only the prediction of estrus (highest area under the curve = 0.87 in 3-month-old) but not of other phases was possible. Regardless of age, individual cycle phase (by smears) never correlated with corresponding fecal estrogens, and estrus could not be predicted. In conclusion, while the fecal estrogens oscillation and frequency of estrus phase were affected by age, the systemic hormone release persisted. In mice, vaginal cytology did not reflect changes of systemic (fecal) estrogens, whereas impedance accurately identified estrus. The flaws and advantages of the examined monitoring methods should be considered in the design of future shock studies.

KEYWORDS—Estrogens, animal disease models, vaginal cytology, feces, noninvasive impedance

Dissecting the role of gender in clinical sciences is of major importance because both male and female sex steroids have been shown to modulate various disease states and/or responses to treatment strategies (1, 2). Gender differences are especially pronounced in diseases with inflammatory background. For example, women have been found to display a higher prevalence of developing autoimmune disorders (3) such as rheumatoid arthritis (4), multiple sclerosis, and lupus erythematosus compared with their male counterparts (5, 6). Application of estrogens as therapeutics has shown promising results in some colonic cancers (7, 8) as well as in preclinical studies of acute brain injuries (9–12). The effects of hormones are no less important in acute diseases as men more frequently succumb to myocardial infarction and/or stroke compared with premenopausal women (13, 14). In preclinical studies, better survival of sexually mature female rodents (compared with males) after trauma/hemorrhage and sepsis has also been associated with the putative protective effects of circulating estrogens in proestrus (P) females (15, 16).

The previously mentioned findings emphasize the importance of accurate determination and/or protracted monitoring of changes in major sex hormones in both clinical and experimental studies. The wide variations in cyclic fluctuations of female hormones in available animal models require an appropriate tool enabling an effective and long-term hormonal surveillance. Mice are by far the most commonly used species in preclinical research on critical care diseases such as shock and sepsis (17), and this includes studies focused on gender idiosyncrasies (18, 19). Estrus cycle in the mouse, although short and simpler (e.g., mating-induced luteal phase), demonstrates a relative resemblance to the human cycling pattern (20), justifying the latter investigative direction.

In general, the blood remains the most accurate and common source for measurement of many mediators including circulating sex hormones. Yet, in small laboratory animals (such as the mouse), repeated blood sampling is relatively invasive and poses severe volume restrictions compared with larger animals. Also, stress due to restraining and the blood loss itself can affect/interrupt regular cycle physiology. This in turn may either alter studied end points and/or falsify subsequent conclusions (21). These potential pitfalls have instigated a search for alternative solutions, and a number of other, blood-independent methods have been used to assess concentration of sex steroids

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and/or define the cycle phase in small rodents. For example, steroids including corticosterone and numerous estrogen and androgen metabolites have been successfully measured in feces of species such as the mouse (22), rat (23), Syrian hamster (24), guinea pig (25), red squirrel (26), and quail (27), given that they adequately portray concentration of these hormones in the blood. Of note, fecal measurements, although shown to be very accurate, represent an average concentration of a circulating hormone over a longer period (23) compared with the snapshot values detected in the blood. In context of protracted and/or retrospective monitoring, however, this should be viewed as an important advantage: although transient hormone peaks can be easily missed by blood measurements, they will be always traceable in fecal samples (albeit reflected by lower concentrations). Yet, the by far most popular method for determination of cycle phase changes in small rodents is vaginal cytology: it is technically simple and relatively noninvasive and enables repeated monitoring of individuals over long periods (28). A newer alternative, i.e., the electrical resistance measurement of the vaginal mucous membrane (29), has been validated in different small animal species such as guinea pig and rat (30–32), but not in the mouse.

It is clear that reliable approaches for defining of hormonal status in female mice are needed to unequivocally identify the influence of sex hormones on various physiological/pathological states. Using 3-, 15-, and 20-month-old female mice, this study aimed to (*a*) characterize general age-related changes in systemic estrogens and (*b*) examine the experimental utility of determination of the estrus (E) cycle by vaginal smears and/or impedance simultaneously comparing them with oscillation of systemic estrogen concentrations measured in the feces and blood.

MATERIALS AND METHODS

Animals

For this study, 3- (n = 16; mean body weight = 30.6 g), 15- (n = 10; mean body weight = 45.5 g), and 20-month-old (n = 13; mean body weight = 40.6 g) female (also referred to as "young", middle-aged", and "mature" females, respectively), outbred, Hsd:ICR (CD-1) mice were used (Harlan Laboratories, Udine, Italy). All female mice were age matched and housed in groups of maximal five animals per cage (open wire top, type III, high). Animals were kept on a 12-h dark-light cycle under standard laboratory conditions (temperature 21°C ± 1°C; humidity: 50% ± 10%). Cages were enriched with houses, wood wool for nesting, and wooden boards, tunnels, and small blocks for gnawing (Abedd Lab & Vet Service, Vienna, Austria) to facilitate natural behavior prior to and throughout the experimentation. Food and water were available *ad libitum*. For the entire duration of experiments, all mice were fed with the same conventional mouse diet (V1534-000; Snniff Spezialdiäten GmbH, Soest, Germany) that may contain traces of phytoestrogens (according to manufacturer). All animals were allowed to habituate to housing for at least 7 days before the onset of experiments, and male mice in separate cages were present in the animal room. All animal procedures were approved by the Viennese (Austria) legislative committee and conducted according to the National Institutes of Health guidelines.

Experimental design

The exact experimental design time line is presented in Figure 1. In the morning of day 1, each mouse was briefly restrained, and vaginal impedance was recorded, followed by collection of a vaginal cytology sample. The time of sampling was recorded (start 7 AM), and a second vaginal smear and fecal sample were obtained 10 h later from the respective mice (start at 5 PM). These procedures were then repeated daily until day 22. In addition, on days 15 and 22, a 300 μL blood sample was collected by facial vein puncture (immediately after the morning impedance and cytology assessment). To rule out a potential impact of blood sampling on the continuity of the E cycle, the head-to-head comparison of studied parameters was separated into two time blocks: one before (days 1–15, block 1) and one after (days 16–22, block 2) the first blood sampling (on day 15). A separate group of 24-month-old females was monitored for E cycle frequency by vaginal cytology (See Figure, Supplemental Digital Content 1 at http://links.lww.com/SHK/A190) but not for other parameters. All mice were habituated to vaginal smears, impedance measurement, and fecal sample collection (but not to the blood sampling) before the study.

Vaginal cytology

Vaginal smears were collected twice per day for 22 consecutive days and were used as the reference assessment for comparison of other tested methods. The vagina was flushed with a pipette 10 times with 10 μL of NaCl to suspend epithelial and immune cells from the vaginal membranes. The retrieved liquid was then placed onto a glass slide and allowed to dry. Cells were stained with Diff Quick (Medion Diagnostics, Düdingen, Switzerland). Cell morphology was microscopically evaluated and E cycle stage determined according to the published literature (19, 33, 34). All slides were analyzed by two independent evaluators. The following cycle phases and interphases were defined in the study: P, proestrus/estrus (PE), E, estrus/metestrus (EM), metestrus (M), metestrus/diestrus (MD), diestrus (D), diestrus/proestrus (DP).

Vaginal impedance

Vaginal impedance was measured daily in the morning (at 8 AM) with a vaginal impedance checker MK-11 device (Muromachi Kikai Co, Ltd, Tokyo, Japan) immediately before collection of the vaginal cytology sample. This device optionally includes a mouse probe, but the measurement applicability in mice has been neither guaranteed nor verified by the manufacturer (www.muromachi.com/english/).

Blood sampling

Animal welfare guidelines recommend the collection of maximum 10% of an animal's total blood volume (TBV) for a single blood draw every 2 to 3 weeks without resuscitation (www.gv-solas.de/auss/tie/tie_blutentnahme09.pdf). The removal of larger volumes needs a proper scientific justification, and adequate fluid resuscitation must be performed. The above guidelines severely limit the allowable blood sampling volumes and collection frequency in the mouse. For an effective EIA, between 100 to 200 μL of plasma is necessary (22, 35–37). To obtain the needed volume of plasma, we collected 300 μL of blood via the

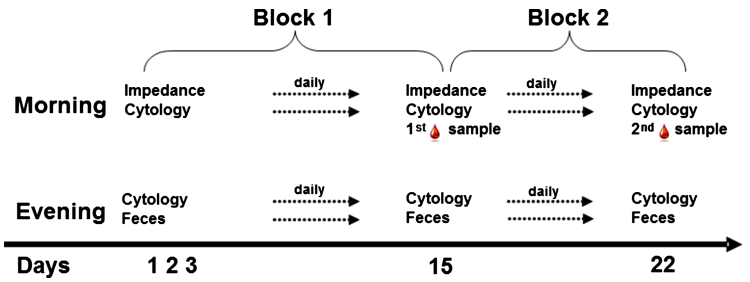

FIG. 1. **Schematic of time points for collection of samples from 3-, 15-, and 20-month-old mice**. Vaginal cytology (both morning and evening), impedance measurements (morning only), and fecal samples (evening only; collected 10 h after vaginal cytology of the same day) were collected daily. Blood samples (300 μL) were collected on days 15 and 22 (end of the study). The observation period was divided into two time periods: block 1 (days 1–15) and block 2 (days 16–22) to examine the influence of the blood sampling/loss–related stress. For comparison/correlation of fecal estrogens and vaginal impedance with E cycle phases, only the morning vaginal cytology was matched with either fecal samples collected on the same time 10 h later or morning impedance values, respectively. Both morning and evening smears were used for protracted trajectories of E cycle phases by vaginal cytology.

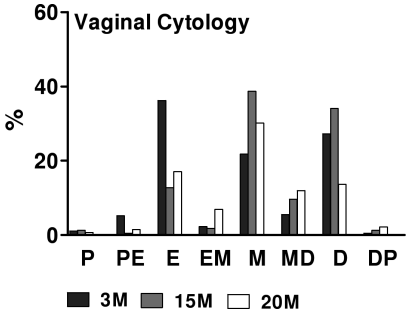

FIG. 2. **Distribution of the different E cycle phases evaluated by vaginal cytology from days 1 to 22 in 3-, 15-, and 20-month-old mice**. All vaginal smears collected over the 22-day observation period were pooled and used for comparison. The number of measurements in the 3-/15-/20-month-old age groups is as follows: P: 7/5/4; PE: 32/2/8; E: 224/50/94; EM: 14/7/38; M: 135/152/166; MD: 34/38/66; D: 169/134/75; DP: 3/5/12. Slides with excessive mucus content and/or a limited amount of cells were excluded from analysis.

facial vein sampling method (38) on days 15 and 22 (the second sample taken at the end of the observation time) from each mouse. As the 300-μL volume slightly exceeded the recommended 10% of the 3-month-old mouse's TBV (6%–8% of body weight), all mice received an adequate fluid substitution (0.5 mL) with Ringer's solution administered subcutaneously. In addition, baseline plasma samples from a separate cohort of animals (n = 35) were included for the analysis of total plasma estrogens. The blood was centrifuged (3,000*g*, 5 min), and plasma was removed and stored at −80°C for further analysis.

Feces sampling

To compare fecal estrogens with vaginal smears, fecal samples were collected from each mouse precisely 10 h after the morning vaginal cytology assessment for 22 consecutive days. The 10 h delay was selected based on studies that evaluated the delay time of excreted corticosterone metabolites into the feces of the mouse (22, 33). Animals were briefly separated into individual cages (for approximately 10–15 min) until they defecated. Fecal samples were collected without further handling the animals and stored at −20°C for analysis with an estrogen enzyme immunoassay (EIA). All mice remained healthy in the course of the study and collected fecal samples were of a similar macroscopic quality (i.e., there were no cases of diarrhea).

Measurement of total estrogens by EIA

For the EIA, each fecal sample was dried (80°C) and thoroughly homogenized (mortar and pestle), and an aliquot of 0.05 g was extracted by adding 1 mL of 80% methanol and shaking on a vortex for 30 min (Vortex Evaporator; Labconco, Kansas City, Mo). After centrifugation (2,500*g*, 15 min), 100 μL of supernatant was removed (22) and stored at −30°C for further analysis by a total estrogen EIA as described previously (37, 39). The antisera used in the assay cross-reacted with relevant steroids (Steraloids, Wilton, NH) as follows: estrone (100%), estradiol-17α (19%), estradiol (70%), estriol (129%), 1,3,5,(10),7-estratetraen-3, 17β-diol (20%), 1,3,5,(10),7-estratetraen-3-ol-17-one (87%). The same EIA (37) was used for measurements of plasma estrogens. The stored plasma samples were thawed; the volume measured and filled up with distilled water to reach a total of 0.5 mL. Afterward, steroids were extracted with 5 mL of diethylether. Following evaporation of the ether, assay buffer was added, and an aliquot measured in the EIA (37, 39).

Statistical analysis

Fisher exact test was used to analyze frequency of specific cycle phases (by vaginal cytology) in the different age groups (Fig. 2). For the analysis of total estrogen concentration in the blood plasma and feces across age (Fig. 3), all sampling time points were pooled. Given that estrogen concentrations in the feces and blood were not normally distributed, Kruskal-Wallis test followed by Dunn multiple comparison procedure was applied.

For remaining comparison and/or correlation analyses, morning E cycle phases (defined by vaginal cytology—the reference measurement) of each mouse were compared with the vaginal impedance value and evening fecal estrogen concentration from the same day.

Correlation between vaginal cytology and feces was calculated using Spearman correlation coefficient. Receiver operating characteristic (ROC) curve was calculated to identify the optimal impedance cutoff value for each age group, which was chosen where the Youden index reached its maximum. Sensitivity, specificity, and positive and negative predictive values were calculated. Logistic regression was performed to estimate the probability of being in PE/E according to the cutoff value. All statistical analyses were carried out with either Graph Pad Prism 5 software (San Diego, CA) or IBM SPSS Statistics version 20 (Armonk, NY) with statistical significance set at $\alpha = 0.05$.

RESULTS

Vaginal cytology—frequency and protracted characterization of the E cycle phases

The E cycle phase of each mouse was determined by vaginal cytology twice daily (morning and evening) for 22 consecutive days (see Fig. 1 for study design). The prevalence of each phase in examined age groups is depicted in Figure 2. Vaginal smear examination demonstrated that, in 3-month-old mice, E phase (pooled values) occurred up to three times more frequently (42% of all collected smears) than in older females, whereas M was least frequent in 3-month-old. The E phase in 15- and 20-month-old was comparable (12% and 17% of all collected smears). Diestrus was two times more frequent in 3- and 15-month-old mice compared with 20-month-old, and P was least frequent. In addition, a clear-cut phase allocation was obscured by repeated smears with an "in-between phases" characteristic (i.e., PE, EM, MD, and DP) detected at all cycle stages and age groups. The overall rate of measurements defined as "indeterminable" was 15% in 3-month-old, 12% in 15-month-old, and 2% in 20-month-old mice. The cycle phase prevalence in 24-month-old females (the only end point assessed in this age group) is provided in (See Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A190).

We also examined accuracy of repeated vaginal cytology examination in monitoring of the E cycle oscillation over time in individual mice. Vaginal smears taken over 22 days failed to display a regular cycle phase oscillation. For example, the same phase was repeatedly recorded at several consecutive

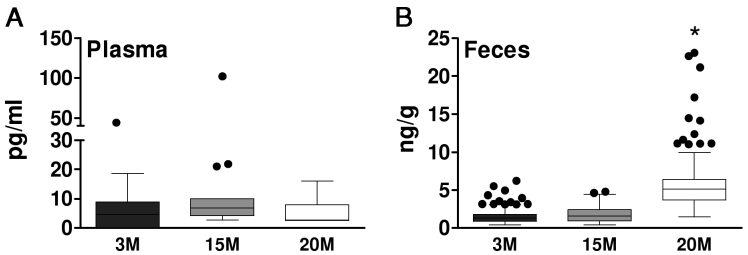

FIG. 3. **Concentration of total estrogens in the blood and feces from 3-, 15-, and 20-month-old mice**. A, Total estrogen concentration in the blood. Both days 15 and 22 sampling time points were pooled for comparison. In addition, baseline samples from a separate cohort of 3-, 15-, and 20-month-old mice were added to the two groups. For 3-month-old mice, total n = 32; for 15-month-old mice, n = 20; and for 20-month-old mice, n = 24. B, Total estrogen concentration in feces. All the daily fecal samples collected over the 22-day observation period were pooled and used for comparison. For 3-month-old mice, total n = 363; for 15-month-old mice, n = 168; and for 20-month-old mice, n = 242. *$P < 0.05$ for 20- vs. 3- and 15-month-old.

sampling time points, and this was true in all age groups (See Table, Supplemental Digital Content 2, at http://links.lww.com/SHK/A191).

Comparison of total estrogens in plasma and feces across different age groups

All estrogen values measured from plasma and feces were pooled from all sampling time points (days 15 and 22 for plasma, days 1–22 for fecal samples) and compared among the age groups (Fig. 3). There was no significant difference in the total circulating plasma estrogen concentrations between 3-, 15-, and 20-month-old groups (Fig. 3A). The total fecal estrogens were similar between 3- and 15-month-old females, whereas their concentration in 20-month-old was 31% and 25% higher compared with 3- and 15-month-old females ($P < 0.05$).

Simultaneous profiling of vaginal cytology, fecal estrogens, and vaginal impedance in different age groups and sampling blocks

3-month-old females—In block 1, concentration of fecal estrogens was highest in the PE phase (average 2,075 pg/g): it was 33% higher compared with E (average 1,399 pg/g) and 29% higher than M (average 1,465 pg/g; all $P < 0.05$; Fig. 4A, upper panel). The vaginal impedance was most pronounced in 3-month-old and peaked in E (average 4.5 Ω) and PE (average 3.0 Ω) compared with mice in the P, M, and D phases combined (average 1.3 Ω; $P < 0.05$; Fig. 4A, lower panel). Other phases displayed low/negligible membrane resistance.

In block 2, P, PE, and E phases were detectable by vaginal cytology, yet the peak of fecal estrogens in PE disappeared, and all phases displayed comparable hormone concentrations in feces (Fig. 4B, upper panel). The average vaginal impedance increased in PE (2.4 Ω) and remained highest in PE and E (4.5 Ω) compared with M and D phases combined ($P < 0.05$; Fig. 4B, lower panel).

15-month-old females—In block 1, only three P and one PE incidences (out of 136) were identified by vaginal cytology. No oscillation of fecal estrogen concentrations (allocated based on the vaginal cytology) was observed (Fig. 4C, upper panel). The overall impedance was lower in 15-month-old when compared with 3-month-old females, but it was always higher in the E phase (average 2.1 Ω) compared with M and D phases combined ($P < 0.05$; Fig. 4C, lower panel).

In block 2, there was no oscillation of fecal estrogens (no P or PE phases were identified), whereas impedance characteristic was similar to the one observed in block 1 (Fig. 4D).

20-month-old females—No clear separation in fecal estrogen concentration was detectable in either block (Fig. 4, E and F, upper panels). Block 1 vaginal impedance in E was higher compared with block 2 (2.4 vs. 1.3 Ω), but both were always significantly higher compared with M and D combined ($P < 0.05$; Fig. 4, E and F, lower panels). (See Table, Supplemental Digital Content 3, at http://links.lww.com/SHK/A192) for the exact cycle phase/mouse distribution.

Correlation of vaginal cytology with fecal estrogens and vaginal impedance changes

Next, we aimed to investigate whether the E cycling (defined by vaginal smears) is associated with systemic fluctuations of estrogens (measured in feces) and whether it is accurately reflected by vaginal impedance measurements.

First, daily fecal estrogen concentration and impedance value of exemplary mice from each age group were compared with the corresponding (same day) vaginal cytology (Fig. 5). Regardless of the age, fluctuations of fecal estrogens failed to match the E phases: high/peak fecal estrogen levels were frequently detected in E phases other than PE and E. In contrast, impedance values were always markedly higher in PE/E compared with other phases. This effect was evident in 3- and 15-month-old females but negligible in 20-month-old females. In addition, the oscillation was not interrupted by the blood collection on day 15.

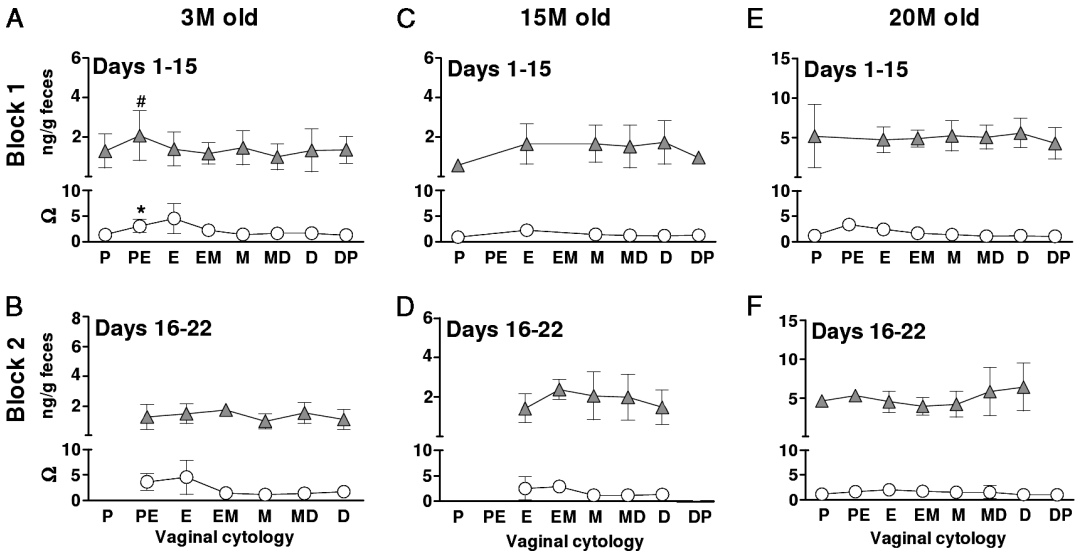

FIG. 4. **Comparison of vaginal cytology with matching vaginal impedance measurements and fecal estrogen concentrations in block 1 (days 1–15) and block 2 (days 15–22) in 3-, 15-, and 20-month-old mice**. Each morning, the E phase cycle was determined by vaginal cytology and compared with the corresponding morning impedance value and evening (10 h after cytology) fecal estrogen concentration of the same day. A, Three-month-old mice, block 1. B, Three-month-old mice, block 2. C, Fifteen-month-old mice, block 1. D, Fifteen-month-old mice, block 2. E: Twenty-month-old mice, block 1. F, Twenty-month-old mice, block 2. All mice and all time points from both phases of observation were pooled for analysis. x = indeterminable. $^{\#}P < 0.05$ for fecal estrogen PE vs. E, and $^{*}P < 0.05$ for impedance PE vs. P, M, D. Detailed n/group distribution is listed in (See Table, Supplemental Digital Content 3, at http://links.lww.com/SHK/A192).

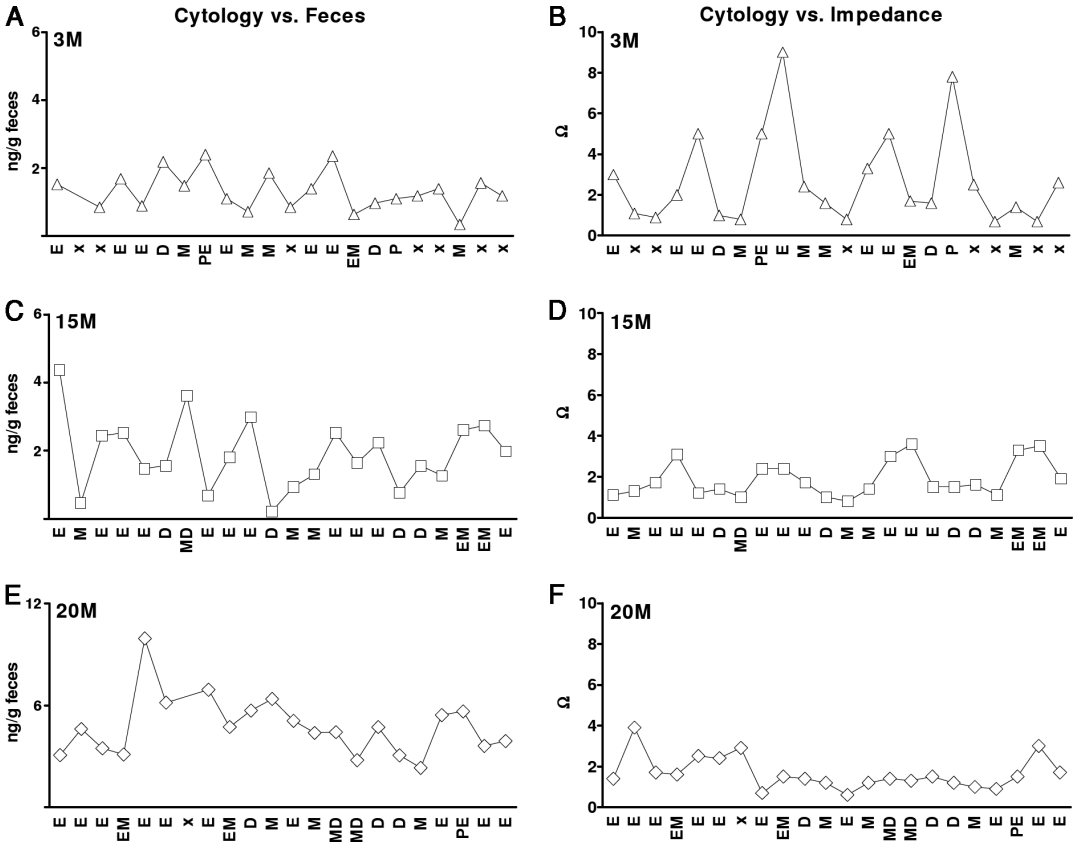

FIG. 5. **Fecal estrogen concentrations (left column) and vaginal impedance (right column) values matched with corresponding vaginal cytology for three individual mice of different age over 22 consecutive days**. A and B, Three-month-old mice. C and D, Fifteen-month-old mice. E and F, Twenty-month-old mice.

Second, using the entire data set, we performed a correlation analysis between cycle phases and fecal estrogen and impedance measurements. Regardless of the age group, no meaningful correlation was observed between systemic estrogens measured in the feces and cycle phases defined by smears ($r = -0.117$ [$P = 0.047$] in 3-month-old; $r = 0.026$ in 15-month-old and $r = 0.112$ in 20-month-old). This was reconfirmed by a nonsignificant ROC analysis for prediction of E by fecal estrogens ($0.5 \leq$ area under the curve ≤ 0.55 in all age groups).

In contrast, a very strong association was detected between the E cycle phase (by smears) and vaginal impedance (Table 1). The correlation was strongest in the 3-month-old females and gradually declined with age. Receiver operating characteristic curve demonstrated that vaginal impedance had a very high capacity and accuracy to predict E but not any other cycle phase. The prediction accuracy for E was highest in 3-month-old and poorest in 20-month-old females. In addition, combining PE and E as a joint prediction group further improved the predictive accuracy (area under the curve = 0.87 vs. 0.84 in 3-month-old). Of note, the blood collection on day 15 did not interrupt the predictive capacity of vaginal impedance (data not shown).

Oscillation of fecal estrogens in different age groups

Given the lack of correlation of fecal estrogens with individual E cycle phases (by smears), we wanted to determine whether any (age-dependent) oscillation can be detected based on the

TABLE 1. **Statistical evaluation of E cycle phase prediction via vaginal impedance measurements**

Mice age, mo	ROC	Youden index	Cutoff, Ω	Sensitivity, %	Specificity, %	*P*(E), %	PPV, %	NPV, %
3								
E	0.84	0.54	2.85	64	90	41	79	82
PE + E	0.87	0.60	1.75	82	78	30	74	86
PE + E	0.84	0.55	2.85	61	94	42	79	82
15								
E	0.79	0.45	1.65	57	88	15	43	93
PE + E	0.77	0.43	1.65	55	88	15	38	92
20								
E	0.82	0.52	1.65	71	82	16	39	92
PE + E	0.83	0.54	1.65	71	83	20	50	92

NPV indicates negative predictive value; *P*(E), probability to be in E based on logistic regression; PPV, Positive predictive value.

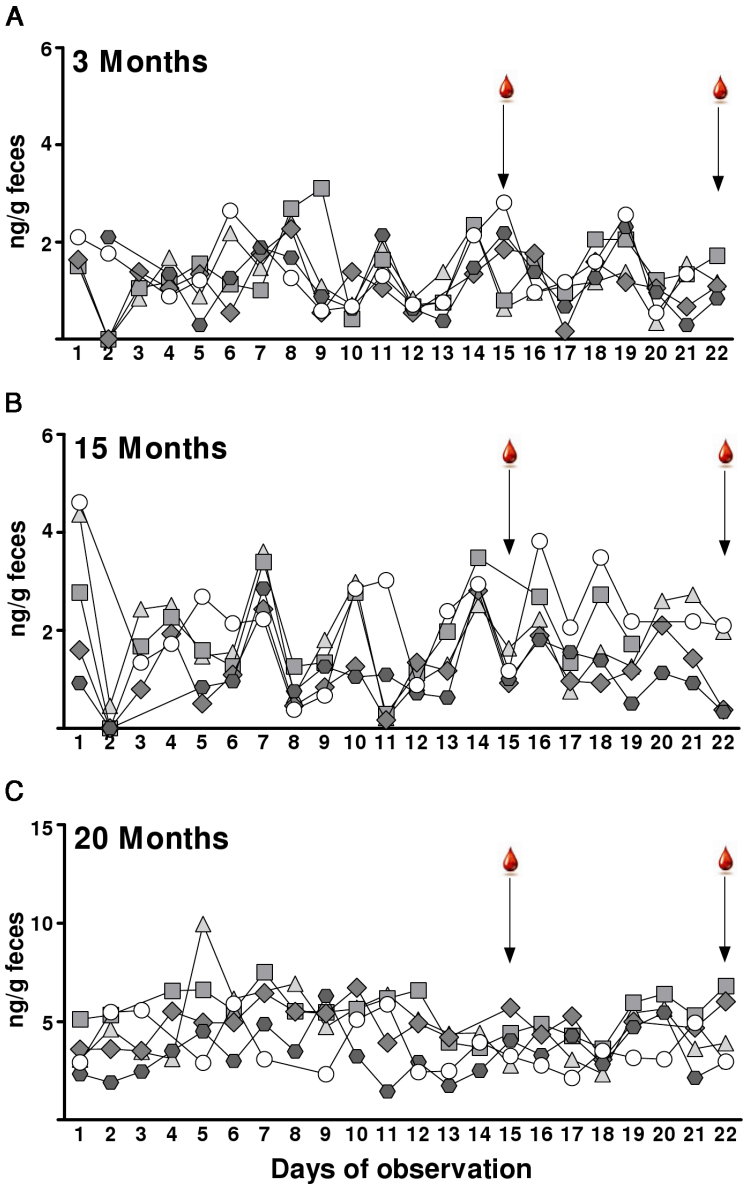

FIG. 6. **Fluctuations of fecal estrogens in individual mice over 22 consecutive days**. Concentration of total estrogen was measured in feces collected from five mice of the same age housed in the same cage. Blood drops indicate blood sampling time points. A, Three-month-old mice. B, Fifteen-month-old mice. C, Twenty-month-old mice.

measurements of fecal estrogens alone (not matched with cycle phases and/or impedance). To examine this, we longitudinally plotted daily fecal estrogens values for five individual mice from the same cage and of the respective age group over the entire observation time (Fig. 6, A–C). Protracted trajectories demonstrated a distinct regular oscillation in fecal estrogens in both 3- and 15-month-old females (Fig. 6, A and B) that disappeared in the 20-month-old group (Fig. 6C). Interestingly, the day 15 blood sampling appeared to deregulate hormone oscillation in 15-month-old but not in 3-month-old females. This reconfirms utility of fecal estrogen measurements given that estrogen peaks were not missed by this assay and that typical hormonal fluctuations were recorded.

DISCUSSION

Given that gender appears to be a key factor in critical diseases research, this study set out to analyze three methods for the characterization of the reproductive cycle phase in an outbred laboratory mouse strain. We were interested in resolving three main questions: (*a*) defining the age span in which female CD-1 mice transition into a low estrogen phase (i.e., persistent D (19), (*b*) whether it is feasible to use vaginal cytology smears as a surrogate for defining concentration of circulating estrogens, and (*c*) whether the use of vaginal impedance method for detection of E cycle phases is applicable in mice.

To comprehensively address the above aims, healthy 3-, 15-, and 20-month-old mice were enrolled. The CD-1 strain reaches fertility at approximately 3 to 4 weeks of age. Thus, 3-month-old mice represented human females at the peak reproductive age, 15-month-old represented "middle-aged" females, whereas 20-month-old mice aimed at representing mature female population with a distinct decline in the E cycle oscillation and/or fertility (i.e., entering menopause). Specifically, the age of the second age group was selected to cover the transition time in

which aging begins to impact fertility, yet without presence of a distinct senescent phenotype in these mice; i.e., in the CD-1 strain, the 15- to-20-month-old age gap is the specific period in which a distinct rise in age-dependent mortality begins (40). In addition, Felicio et al. (19) demonstrated that, in C57BL/6J mice, the age of cycle cessation varies between 11 and 16 months. Hence, the chosen age groups constituted an optimal age period for the assessment of menopause-like changes using profiles of circulating sex hormones. Although fertility, reproduction, and survival may differ among specific strains (in particular between in- and outbred) (18), the overall findings of our study are likely applicable to the majority of available mouse strains. The most caution is needed in translation of the age-specific findings—this element should be individually adjusted for each strain based on its specific fertility and age-mortality profiles.

The classic E cycle in the mouse lasts approximately 4 to 5 days and has four main phases: (*a*) P, (*b*) E, (*c*) M, and (*d*) D (33, 41). In our study, we also recorded the number of intermediate phases (i.e., PE, EM, MD, and DP) analogous to those described previously (34, 41). We identified the vast majority of P, PE, and E in 3-month-old mice, but they were also recorded in 15- and 20-month-old albeit at much lower frequency (24-month-old mice shown in (See Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A190). This shows that cycle intervals in older mice were prolonged/erratic, but cycling itself had not yet fully ceased. Yet, P and PE were overall the least frequent cycle phases in any age group. This could be partly related to our smear collection timing: it has been demonstrated that P mainly occurs at night, the peak of biological activity of the mouse (42). Overall, rapid progression of the murine E cycle, frequent interphase, and/or indeterminable measurements hinder a clear-cut identification of specific cycle phases by vaginal cytology. Hence, this method does not appear as a fully reliable monitoring tool.

Interestingly, despite the age-related steep decline in P, PE, and E incidences (synonymous with declining fertility), estrogen concentrations measured both in the blood and feces were very similar in all three age groups (Fig. 3). Moreover, hormonal profiling based on the corresponding vaginal cytology (Fig. 4) revealed overlapping fecal estrogen concentrations irrespective of the cycle phases. This implies that weakening fertility in the mouse does not correspond with the decline of systemic estrogens. Such a lasting persistence of estrogens corroborates the findings of our recent study in which CD-1 females with posttraumatic sepsis exhibited a consistently better survival (vs. males) that was independent of their age and the E cycle (43), a phenomenon typically explained by the protective effects of high estrogens in young mice (15, 44). It is suggestive that in the mouse, similar to other species, the presence of E cycling is driven by dynamic fluctuations of circulating estrogens rather than their sheer systemic presence.

Evaluation of smears in individual mice revealed yet another inaccuracy of cytological assessment: in all age groups, there were frequent incidences of detecting the same cycle phases on several consecutive days, and similar observations were reported before (33). Measurements of fecal estrogens offer a further and new insight. Although our measurements showed a slight estrogen peak in pooled fecal samples from 3-month-old mice that matched the PE phase, this association was gone as soon as estrogen levels were compared against vaginal smear profiles in individual and/or older mice. Moreover, we frequently observed strongly varying fecal hormone concentrations in several subsequent E phases in the same mouse (see estrogens profile in an exemplary mouse identified as E on three successive days: Fig. 5E). This is indicative of dynamic estrogen oscillation also between individual E phases, and this effect cannot be attributed to a snap-shot detection given that fecal measurements reflect a pooled period and are less fluctuation-prone compared with episodic and erratic blood samples. A robust analysis of the entire data set reconfirmed our initial observation: there was a complete lack of correlation between increases of systemic estrogen measured in feces and the E phase identified by vaginal cytology. In other words, E—regardless of its frequency and/or the age group—was never accurately predicted by fluctuations of systemic estrogens. This is an important finding as it points out that detection of E (identified by smears) in the mouse cannot be automatically associated with any defined (i.e., high) level of total systemic estrogens. The above findings partly contrast earlier reports by Jarrar et al. (34) and Yu et al. (35) that demonstrated a significant increase in circulating 17β-estradiol in rat females in the P phase (by vaginal smears) compared with other phases. This discrepancy may be species/strain specific. Alternatively, estrogen peaks may be also present in mice, but the large variation in the baseline estrogen values among individual mice obscures detection of such a transient increase. Repeated estrogen measurements in individual mice are required to verify it, but application of such a protocol in the mouse is problematic (see below).

In contrast to the easily measurable fecal estrogens, their detection in murine blood plasma is challenging. In our study, concentration of circulating estrogens was overall very low and frequently under detection limit (regardless of the age and/or sampling day). Such a technical drawback can be, at least partially, overcome by using large blood volumes. Yet, in mice (and other small rodents) studies in which sex hormone profiling is a secondary (supportive) end point, the blood is an unlikely source of choice. This is especially true in studies using protracted monitoring where a small TBV drastically restricts the allowable sample volume(s) and/or frequency (45). Hence, it was beyond our technical capacity to effectively examine the direct (and daily) correlation between cycle phases and blood estrogens. Because a blood loss can be a stressor deregulating hormonal homeostasis, we separated the pooled comparisons of fecal estrogens, impedance, and vaginal cytology into two sampling time blocks. The total fecal estrogen concentration was not altered by blood sampling, but the estrogen peak recorded in 3-month-old PE/E females (Fig. 4, A and B) disappeared after the blood collection even though vaginal cytology was unaltered. We speculate that the blood loss/sampling stress was too transient to diminish the accumulative concentration of fecal estrogens but strong enough to interfere with signaling of the hypothalamic-pituitary-gonadal axis, thereby eliminating the preovulatory hormonal peak.

Measurements of vaginal membrane resistance have been effectively used for optimizing reproduction in large species

(46, 47) years before it was first used in small laboratory animals (48). Trans-specimen properties of this method rely on the sex hormone–dependent changes of vaginal membrane composition leading to alterations in the membrane's electrical resistance; i.e., cycle phases are identified by characteristic oscillation of impedance values. In our study, vaginal impedance showed high correlation but only with selected cycle phases (by smears); i.e., it very accurately identified E and/or PE/E but failed to discriminate any other phase. This effect was highly age-dependent: the highest predictive power for E detection was obvious in young females but declined in older mice (Table 1). Our data corroborate the findings of Agrawal et al. (49), who detected the highest impedance peaks during both the PE and E phases in cycling mice. In contrast, an increase in impedance was typical in the P rather than E phase in rats and guinea pigs indicating its high species specificity (31, 32). Interestingly, Singletary et al. (30) used impedance to correlate circulating levels of estrogens and progesterone with the P phase in rats but found no association. In our hands, vaginal impedance was the most reliable and stable method for detection of the PE/E phases in young female mice.

Summarizing, vaginal cytology was never correlated with the oscillation of circulating estrogens measured in the feces; hence, an estrogen peak cannot be reflexively assumed in mice in the PE/E phases. In contrast, vaginal impedance reliably identified PE/E, yet without further discrimination of other cycle phases. In addition, CD-1 mice up to 20 months did not manifest a clear postmenopausal anestrus as evidenced by the consistently high concentration of circulating estrogens in all age groups. Furthermore, blood sampling for EIA should be avoided as it deregulates the cycling homeostasis and may lead to misinterpretation of findings. Conversely, measurement of fecal estrogens, although relatively laborious, constitutes a practical retrospective, noninvasive profiling method that can be used in any well-powered, short- and long-term shock/sepsis models and beyond.

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REFERENCES

1. Niv Y: Gender medicine in gastroenterology. *Isr Med Assoc J* 13(4):244–246, 2011.
2. Regitz-Zagrosek V, Oertelt-Prigione S, Seeland U, Hetzer R: Sex and gender differences in myocardial hypertrophy and heart failure. *Circ J* 74(7): 1265–1273, 2010.
3. Quintero OL, Mador-Patarroyo MJ, Montoya-Ortiz G, Rojas-Villarraga A, Anaya JM: Autoimmune disease and gender: plausible mechanisms for the female predominance of autoimmunity. *J Autoimmun* 38(2–3):J109–J119, 2012.
4. Islander U, Jochems C, Lagerquist MK, Forsblad-d'Elia H, Carlsten H: Estrogens in rheumatoid arthritis; the immune system and bone. *Mol Cell Endocrinol* 335(1):14–29, 2011.
5. Nussinovitch U, Shoenfeld Y: The role of gender and organ specific autoimmunity. *Autoimmun Rev* 11(6–7):A377–A385, 2012.
6. Greer JM, McCombe PA: Role of gender in multiple sclerosis: clinical effects and potential molecular mechanisms. *J Neuroimmunol* 234(1–2):7–18, 2011.
7. Parks M, Tillhon M, Dona F, Prosperi E, Scovassi AI: 2-Methoxyestradiol: new perspectives in colon carcinoma treatment. *Mol Cell Endocrinol* 331(1): 119–128, 2011.
8. Schleipen B, Hertrampf T, Fritzemeier KH, Kluxen FM, Lorenz A, Molzberger A, Velders M, Diel P: ERbeta-specific agonists and genistein inhibit proliferation and induce apoptosis in the large and small intestine. *Carcinogenesis* 32(11):1675–1683, 2011.
9. Liu M, Kelley MH, Herson PS, Hurn PD: Neuroprotection of sex steroids. *Minerva Endocrinol* 35(2):127–143, 2010.
10. Shahrokhi N, Khaksari M, Soltani Z, Mahmoodi M, Nakhaee N: Effect of sex steroid hormones on brain edema, intracranial pressure, and neurologic outcomes after traumatic brain injury. *Can J Physiol Pharmacol* 88(4):414–421, 2010.
11. Herson PS, Koerner IP, Hurn PD: Sex, sex steroids, and brain injury. *Semin Reprod Med*. 27(3):229–239, 2009.
12. Vagnerova K, Koerner IP, Hurn PD: Gender and the injured brain. *Anesth Analg* 107(1):201–214, 2008.
13. Murphy SJ, McCullough LD, Smith JM: Stroke in the female: role of biological sex and estrogen. *ILAR J* 45(2):147–159, 2004.
14. Kannel WB: The Framingham Study: historical insight on the impact of cardiovascular risk factors in men versus women. *J Gend Specif Med* 5(2):27–37, 2002.
15. Knoferl MW, Angele MK, Diodato MD, Schwacha MG, Ayala A, Cioffi WG, Bland KI, Chaudry IH: Female sex hormones regulate macrophage function after trauma-hemorrhage and prevent increased death rate from subsequent sepsis. *Ann Surg* 235(1):105–112, 2002.
16. Raju R, Bland KI, Chaudry IH: Estrogen: a novel therapeutic adjunct for the treatment of trauma-hemorrhage–induced immunological alterations. *Mol Med* 14(3–4):213–221, 2008.
17. Deitch EA: Animal models of sepsis: a review and lessons learned. *Shock* 9(1):1–11,1998.
18. Angele MK, Schwacha MG, Ayala A, Chaudry IH: Effect of gender and sex hormones n immune responses following shock. *Shock* 14(2):81–90, 2000.
19. Felicio LS, Nelson JF, Finch CE: Longitudinal studies of estrous cyclicity in aging C57BL/6J mice: II. Cessation of cyclicity and the duration of persistent vaginal cornification. *Biol Reprod* 31(3):446–453, 1984.
20. vom Saal FS, Finch CE, Nelson JF: Natural history and mechanisms of reproductive aging in humans, laboratory rodents, and other selected vertebrates. In: Knobil E, Neill J (eds). *The Physiology of Reproduction*. New York: Raven Press, pp. 861–1010, 1994.
21. McGuill MW, Rowan AN: Perspectives on animal use. *ILAR J* V31(4), 1989.
22. Touma C, Sachser N, Mostl E, Palme R: Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *Gen Comp Endocrinol* 130(3):267–278, 2003.
23. Lepschy M, Touma C, Hruby R, Palme R: Non-invasive measurement of adrenocortical activity in male and female rats. *Lab Anim* 41(3):372–387, 2007.
24. Chelini MO, Palme R, Otta E: Social stress and reproductive success in the female Syrian hamster: endocrine and behavioral correlates. *Physiol Behav* 104(5):948–954, 2011.
25. Bauer B, Palme R, Machatschke IH, Dittami J, Huber S: Non-invasive measurement of adrenocortical and gonadal activity in male and female guinea pigs (*Cavia aperea f. porcellus*). *Gen Comp Endocrinol* 156(3):482–489, 2008.
26. Dantzer B, McAdam AG, Palme R, Humphries MM, Boutin S, Boonstra R: Maternal androgens and behavior in free-ranging North American Red Squirrels. *Anim Behav* 81:469–479, 2011.
27. Busso J, Dominchin MF, Marin RH, Palme R: Cloacal gland, endocrine testicular, and adrenocortical photoresponsiveness in male Japanese quail exposed to short days. *Dom Anim Endocrinol* 44(3):151–156, 2013.
28. Evans HM, Long JA: Characteristic effects upon growth, oestrus and ovulation induced by the intraperitoneal administration of fresh anterior hypophyseal substance. *Proc Natl Acad Sci U S A* 8(3):38–39, 1922.
29. Ramos SD, Lee JM, Peuler JD: An inexpensive meter to measure differences in electrical resistance in the rat vagina during the ovarian cycle. *J Appl Physiol* 91(2):667–670, 2001.
30. Singletary SJ, Kirsch AJ, Watson J, Karim BO, Huso DL, Hurn PD, Murphy SJ: Lack of correlation of vaginal impedance measurements with hormone levels in the rat. *Contemp Top Lab Anim Sci* 44(6):37–42, 2005.
31. Bartos L, Sedlacek J: Vaginal impedance measurement used for mating in the guinea-pig. *Lab Anim* 11(1):57–58, 1977.
32. Bartos L: Vaginal impedance measurement used for mating in the rat. *Lab Anim* 11(1):53–55, 1977.
33. Caligioni, CS: Assessing Reproductive Status/Stages in Mice. *Curr Protoc Neurosci*. 48:A.4I.1–A.4I.8, 2009.
34. Jarrar D, Wang P, Cioffi WG, Bland KI, Chaudry IH: The female reproductive cycle is an important variable in the response to trauma/hemorrhage. *Am J Physiol Heart Circ Physiol* 279(3):H1015–H1021, 2000.
35. Yu HP, Yang S, Hsieh YC, Choudry MA, Bland KI, Chaudry IH: Maintenance of lung myeloperoxidase activity in proestrus females after trauma-hemorrhage: upregulation of heme oxygenase-1. *Am J Physiol Lung Cell Mol* 291(3): L400–L406, 2006.
36. Walmer DK, Wrona MA, Hughes CL, Nelson KG: Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone. *Endocrinology* 131(3):1458–1466, 1992.

37. Touma C, Palme R, Sachser N: Different types of oestrous cycle in two closely related South American rodents (*Cavia aperea* and *Galea musteloides*) with different social and mating systems. *Reproduction* 121(5):791–801, 2001.
38. Weixelbaumer KM, Raeven P, Redl H, van Griensven M, Bahrami S, Osuchowski MF: Repetitive low-volume blood sampling method as a feasible monitoring tool in a mouse model of sepsis. *Shock* 34(4):420–426, 2010.
39. Palme R, Möstl E: Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. In: Görög S (ed.): *Advances of Steroid Analysis* '93. Budapest, Hungary: Akadèmiai Kiadó, pp 111–117, 1994.
40. Eaton GJ, Johnson FN, Custer RP, Crane AR: The Icr:Ha(ICR) mouse: a current account of breeding, mutations, diseases and mortality. *Lab Anim* 14(1):17–24, 1980.
41. Nelson JF, Felicio LS, Randall PK, Sims C, Finch CE: A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. *Biol Reprod* 27(2):327–339, 1982.
42. Parkening TA, Collins TJ, Smith ER: Plasma and pituitary concentrations of LH, FSH, and prolactin in aging C57BL/6 mice at various times of the estrous cycle. *Neurobiol Aging* 3(1):31–35, 1982.
43. Drechsler S, Weixelbaumer K, Raeven P, Jafarmadar M, Khadem A, van Griensven M, Bahrami S, Osuchowski MF: Relationship between age/gender-induced survival changes and the magnitude of inflammatory activation and organ dysfunction in post-traumatic sepsis. *PLoS One* 7(12):e51457, 2012.
44. Diodato MD, Knoferl MW, Schwacha MG, Bland KI, Chaudry IH: Gender differences in the inflammatory response and survival following hemorrhage and subsequent sepsis. *Cytokine* 14(3):162–169, 2001.
45. Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, van de Vorstenbosch C: A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol* 21(1):15–23, 2001.
46. Yamauchi S, Nakamura S, Yoshimoto T, Nakada T, Ashizawa K, Tatemoto H: Prediction of the estrous cycle and optimal insemination time by monitoring vaginal electrical resistance (VER) in order to improve the reproductive efficiency of the Okinawan native Agu pig. *Anim Reprod Sci* 113(1–4):311–316, 2009.
47. Kitwood SE, Phillips CJ, Weise M: Use of a vaginal mucus impedance meter to detect estrus in the cow. *Theriogenology* 40(3):559–569, 1993.
48. Jaramillo LM, Balcazar IB, Duran C: Using vaginal wall impedance to determine estrous cycle phase in Lewis rats. *Lab Anim (NY)* 41(5):122–128, 2012.
49. Agrawal V, Jaiswal MK, Chaturvedi MM, Tiwari DC, Jaiswal YK: Lipopolysaccharide alters the vaginal electrical resistance in cycling and pregnant mice. *Am J Reprod Immunol* 61(2):158–166, 2009.