Reproductive performance primarily depends on the female genotype in a two-factorial breeding experiment using high-fertility mouse lines

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Abstract

Mouse models showing an improved fertility phenotype are barely described in the literature. In the present study, we further characterized two outbred mouse models that have been selected for the phenotype ‘high fertility’ for more than 177 generations (fertility lines (FL) 1 and 2). In order to delineate the impact of males and females on fertility parameters, we performed a two-factorial breeding experiment by mating males and females of the three different genotypes (FL1, FL2, unselected control (Ctrl)) in all 9 possible combinations. Reproductive performance, such as number of offspring per litter or total birth weight of the entire pup, mainly depends on the female genotype. Although the reproductive performance of FL1 and FL2 is very similar, their phenotypes differ. FL2 animals of both genders are larger compared to FL1 and control animals. Females of the control line delivered offspring earlier compared to FL1 and FL2 dams. Males of FL1 are the lightest and the only ones who gained weight during the two weeks mating period. To address whether this effect is correlated with differing serum androgen levels, we measured the concentrations of testosterone, dehydroepiandrosterone, 4-androstenedione, androstanediol and dihydrotestosterone in males of all three lines by GC–MS. We measured serum testosterone between 5.0 and 6.4 ng/mL, whereas the concentrations of the other androgens were at least one order of magnitude lower, with no significant differences between the lines. Our data indicate that reproductive outcome largely depends on the genotype of the female in a two-factorial breeding experiment and supports previous findings that the phenotype ‘high fertility’ is warranted by using different physiological strategies.

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Introduction

Fertility research is largely supported by the use of informative animal models. Most of these animal models are mouse lines. Worldwide, there are more than 1000 transgenic or knockout mouse lines available showing a reproductive phenotype (Matzuk & Lamb 2008, Jamsai & O’Bryan 2011, Ogorevc et al. 2011). Almost all of them exhibit an infertile or at least subfertile phenotype. An infertile or subfertile phenotype of a novel mouse model is easily detectable in the context of standard animal care conditions. By contrast, a mouse model showing improved fertility is not obviously visible. Actually, animal models showing an improved fertility phenotype with an inherent illustration of the complex and biodiverse nature of fertility are highly informative. Up to now, approximately a dozen mouse models showing an improved fertility phenotype have been described (http://phenome.jax.org) and only few of them have been studied in more details (Holt et al. 2004, Wei et al. 2013, Langhammer et al. 2014).

To examine physiological and genetic responses to selection for high-fertility, long-term selection mouse lines have been established as models for farm animals at the Leibniz Institute for Farm Animal Biology (Dummerstorf). The initial population was a systematic crossbred of four inbred and four outbred founder mouse lines starting in the 1970s (Dietl et al. 2004). As selection criterion, a fecundity index was set up.
comprising both (i) number of offspring per litter and (ii) total birth weight of the entire litter (Dummerstorf fecundity index = 1.6 × number of offspring + birth weight of the entire litter). Animals born from the largest and heaviest litters were chosen for further breeding (Schüler & Bünger 1982). Whereas FL1 was untreated, the estrus of the females in FL2 was synchronized by application of the gestagen chlormadinone acetate up to the twenty-third generation. Thus, two independent mouse lines were developed. The resulting FLs (FL1 and FL2) were maintained with a population size of 60–100 breeding pairs per generation; consequently, these mouse lines are more heterogeneous and biodiverse in nature compared with classical inbred mouse lines. After more than 40 years and up to now 178 generations of breeding, litter size of first parturition increased from approximately ten animals per litter in the original founder population to 21.4 and 21.6 animals per litter in FL1 (generation 178) and FL2 (generation 177), respectively. The litter size of the unselected and randomly mated control line (Ctrl, generated from the same original founder pool of animals) remained mostly constant over the selection period.

Previous data suggest that the phenotype 'high fertility' warrants using different physiological, molecular and endocrine strategies, that is, males of the FL1 line are more explorative in an open field experiment and have a higher life expectancy compared with FL2 and Ctrl males (Michaelis et al. 2013, Langhammer et al. 2014). Endocrine investigations in females revealed higher progesterone concentrations during diestrus in FL2 compared to FL1 and Ctrl females (Spitschak et al. 2007). In an early work, the male impact on fecundity outcome has been analyzed in a two-factorial breeding experiment (Renne et al. 1995). To further address these questions, we performed a two-factorial breeding experiment by mating males and females of the three different genotypes (FL1, FL2, Ctrl) in all 9 possible combinations. Additionally, we performed an in-depth measurement of androgens in males of the three lines.

Materials and methods

Animals

All procedures were performed in accordance with national and international guidelines and approved by our own institutional board (Animal Protection Board from the Leibniz Institute for Farm Animal Biology). The animals were maintained in a specific pathogen-free (SPF) environment with defined hygienic conditions at a temperature of 22.5°C, at least 40% humidity and a controlled light regime with a 12:12-h light–darkness cycle. The mice were kept in polysulfone cages of 267 × 207 × 140 mm (H-Temp PSU, Type II, Eurostandard, Tecniplast, Germany) and had free access to pellet concentrate and water. A standard breeding diet with 22% crude protein, 34% starch, 5% sugar, 4.5% crude fat, 3.9% crude fiber, 50.1% N free extracts and 3.2% mineral mixture (ssniff M-Z autoclavable, Soest, Germany) were fed ad libitum. FL1 and FL2 had been selected for an index (Dummerstorf fecundity index = 1.6 × number of offspring + birth weight of the entire litter given in gram) in primiparous females for 162 generations, followed by 9 generations of BLUP (Best Linear Unbiased Prediction) breeding value estimation, focusing only for the number of pups in primiparous females, before starting the present study. After the 171st generation, animals were breed according to the number of offspring. The raising inbreeding coefficient in the selection lines was controlled by the method of Meuwissen (Meuwissen 1997).

Design of the two-factorial breeding experiment

The basic design was a 3 × 3 line cross, including males and females of the two fertility lines, FL1 and FL2, and a randomly selected control line (unselected control line: Ctrl). The different genotypes were cross-classified in all 9 possible combinations. The mating groups in one replicate of the experiment consisted of 10 unrelated males and 30 females per genotype. In each case, 3 full-sisters of 10 different offspring groups were assigned randomly between the male genotypes in order to consider the dam origin effect. Every male was caged with three females of the three different genotypes for two weeks, after which the 90 females were kept singly. The age at mating ranged from 10 to 12 weeks both in males and females. The cross-classification in 9 combinations was repeated in five successive experiments at intervals of three months (subsequently indicated as replicates). In summary, 50 males and 150 females of each genotype FL1, FL2 and Ctrl were included in this study.

To consider the influence of body mass and age during the mating procedure, body mass was recorded at the beginning of mating in dams and sires and additionally in males after the two weeks mating period. The percentage of successful fertilizations was calculated as number of females that gave birth to living offspring divided by the total number of mating. The number of days from exposure to a male to delivery was measured. Litter size and litter weight at parturition (LS0), litter weight at weaning (LS21) and at weaning (LS21, LW21) were included in this study. The quotient between litter weight and litter size of an individual litter was used to produce the trait mean birth capacity. The quotient between litter weight and litter size of an individual litter was used to produce the mean body weight gain in the postnatal rearing period. In addition sex ratio of the born litter and the individual birth weights of the pups were recorded.

Data analysis and statistics of the two-factorial breeding experiment

Data were evaluated using software package SAS© (version 9.4, SAS Inst. Inc., Cary, NC, USA, 2012). Results are reported as least squares means (LSM) ± standard error. The reproductive performance data were analyzed by Proc GLIMMIX or Proc MIXED depending on distribution of traits. Both procedures used a model with the fixed factors.
High-fertility mouse lines

Table 1 Successful mating rates have been calculated by the number of females who give birth to living offspring divided by the total number of mating in %.

<table>
<thead>
<tr>
<th>Male</th>
<th>Ctrl</th>
<th>FL1</th>
<th>FL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>92.9±3.8</td>
<td>92.9±3.5</td>
<td>96.5±2.6</td>
</tr>
<tr>
<td>FL1</td>
<td>94.7±3.3</td>
<td>91.0±3.9</td>
<td>94.7±3.2</td>
</tr>
<tr>
<td>FL2</td>
<td>92.9±3.8</td>
<td>83.4±5.2</td>
<td>92.9±3.6</td>
</tr>
</tbody>
</table>

‘replicates’ (5 levels), ‘male-line’ (3 levels), ‘female-line’ (3 levels), and interactions between male- and female-lines and also interactions with replicates. Because 3 full-sisters of 10 different offspring groups are randomly assigned between the male genotypes a random factor ‘female full-sib group’ was included. Therefore variation in litter size, which is due to maternal is accounted for by this random effect. Because every male was kept in a single cage together with three females, each one of the three different mouse lines, ‘cage’ was used as repeated factor. The corresponding covariances were modeled as ‘unstructured’. The non-normal trait ‘rate of successful fertilization’ was evaluated by Proc GLIMMIX with Poisson distribution. The weight data for males and females at mating were modeled by Proc MIXED with fixed factor ‘replicate’ (5 levels) and ‘male’- or ‘female-line’. Furthermore, we modeled the weight difference of male mice (weight after mating minus weight before mating) by Proc MIXED with factors ‘replicate’ and ‘male-line’ and corresponding interactions. Post-hoc comparisons were made using the Tukey–Kramer correction for multiple testing. The significance level was set at \( p = 0.05 \). All presented LS-means are adjusted for all interactions and replicates.

**Steroid measurement by gas chromatography–mass spectrometry (GC–MS)**

Serum was prepared from males of all three lines (FL1, FL2, Ctrl) from \( n = 15 \) animals at age of 12 weeks. Up to 0.2 mL serum was spiked with a cocktail of internal standards containing (16,16,17-2H3) testosterone (d3-T), (7,7-2H2) 4-androstenedione (d2-4A), (16,16,17-2H3) 5α-androstane-3β-ol-17β (d3-AD), (7,7-2H2) dehydroepiandrosterone (d2-DHEA), (16,16,17-2H3) dihydrotestosterone (d3-DHT), (11,11,12,12-2H4) 17-hydroxyprogesterone (d4-17OHP), (2,2,4,4,21,21,21-2H7) 17-hydroxyprogrenolone (d7-17PE), (1α,2α-2H2) 11-deoxycorticisol (d2-S), (2,2,4,6,6,17α,21,21,21-2H9) progesterone (d9-Prog) and (2,2,4,6,6,17α,21,21,21-2H8) corticosterone (d8-B). Then the samples were allowed to equilibrate overnight at 37°C. After extraction with ethylacetate (three times), the combined organic extracts were evaporated and thereafter purified by gel chromatography on Sephadex LH-20 mini columns (50 mm × 5 mm I.D.). Cyclohexane (LGC Standards, Wesel, Germany)/ethanol (Sigma-Aldrich) (90:10 v/v) was used to elute the steroids. For derivatization, heptafluorobutyric anhydride (Sigma-Aldrich) was utilized. Gas chromatography was performed on an Optima 1-MS capillary column (25 m × 0.2 mm I.D., df 0.1 µm, Macherey-Nagel, Düren, Germany) housed in an Agilent 6890N GC with an Agilent 7683B Series injector (split/splitless automatic liquid sampler) coupled to Agilent 5975 inert XL Mass Selective Detector. Helium was used as carrier gas at 1.0 mL/min. The injector temperature was 270°C and the initial column temperature was set at 80°C. The steroids of interest eluted at a rate of 3°C/min until the column temperature reached 250°C. Quantification was performed in the selected ion mode using the peak area ratio between analyte and internal standard. The following \( m/z \) ratios were measured for the analytes and their corresponding internal standards: \( m/z \) 600/602.4 for T/d3-T, \( m/z \) 462.3/464.3 for 4A/d2-4A, \( m/z \) 470.3/473.3 for AD/d3-AD, \( m/z \) 270.2/272.2 for DHEA/d2-DHEA, \( m/z \) 414.3/417.3 for DHT/d3-DHT, \( m/z \) 465.4/469.4 for 17OH/ d4-17OHP, \( m/z \) 467.4/471.4 for 17PE/d4-17PE, \( m/z \) 465.2/467.2 for S/d2-S and \( m/z \) 510.3/518.3 for Prog/d9-Prog and 720.4/726.4 for B/d8-B (Sanchez-Guijo et al. 2013). Statistical analysis was carried out using the software package GraphPad Prism (Version 5.01). Significances were tested employing one-way ANOVA post-tested by Tukey’s multiple comparison tests.

Table 2 Number of offspring per litter at parturition after mating males and females of the fertility lines FL1 and FL2 plus an unselected control line (Ctrl). LS-means are adjusted for all interactions and replicates (LSM±s.e.).

<table>
<thead>
<tr>
<th>Male</th>
<th>Ctrl</th>
<th>FL1</th>
<th>FL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>11.4±0.4</td>
<td>21.1±0.6</td>
<td>20.7±0.6</td>
</tr>
<tr>
<td>FL1</td>
<td>11.9±0.5</td>
<td>22.0±0.6</td>
<td>21.7±0.6</td>
</tr>
<tr>
<td>FL2</td>
<td>12.4±0.5</td>
<td>22.6±0.6</td>
<td>21.0±0.6</td>
</tr>
</tbody>
</table>

Table 3 Number of offspring per litter at day 10 after mating males and females of the fertility lines FL1 and FL2 plus an unselected control line (Ctrl). LS-means are adjusted for all interactions and replicates (LSM±s.e.).

<table>
<thead>
<tr>
<th>Male</th>
<th>Ctrl</th>
<th>FL1</th>
<th>FL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>10.9±0.5</td>
<td>18.7±0.6</td>
<td>17.1±0.6</td>
</tr>
<tr>
<td>FL1</td>
<td>11.2±0.5</td>
<td>19.9±0.6</td>
<td>18.4±0.6</td>
</tr>
<tr>
<td>FL2</td>
<td>11.2±0.5</td>
<td>19.3±0.7</td>
<td>18.3±0.6</td>
</tr>
</tbody>
</table>

Table 4 Number of offspring per litter at day 21 after mating males and females of the fertility lines FL1 and FL2 plus an unselected control line (Ctrl). LS-means are adjusted for all interactions and replicates (LSM±s.e.).

<table>
<thead>
<tr>
<th>Male</th>
<th>Ctrl</th>
<th>FL1</th>
<th>FL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>10.5±0.5</td>
<td>17.8±0.6</td>
<td>16.6±0.6</td>
</tr>
<tr>
<td>FL1</td>
<td>11.0±0.5</td>
<td>18.7±0.7</td>
<td>17.9±0.6</td>
</tr>
<tr>
<td>FL2</td>
<td>11.1±0.5</td>
<td>18.4±0.7</td>
<td>17.7±0.6</td>
</tr>
</tbody>
</table>
Results

Two-factorial breeding experiment

In order to analyze the impact of male- and female-derived factors on fertility outcome of the FLs we performed a complete 3 × 3 two-factorial breeding experiments. As shown in Table 1, we received successful mating rates between 83.4% and 96.5%, which is very similar to the mating rates when breeding a male to female with a ratio of 1:1 as in our classical breeding program (Langhammer et al. 2014). A summary of the two-factorial breeding experiment is shown in Table 2. Mating a female Ctrl mouse resulted in 11.4–12.4 offspring per litter depending on which males have been used for breeding with no significant differences. Mating a female FL1 resulted in 21.1–22.6 offspring per litter and mating a female FL2 resulted in 20.7–21.7 offspring per litter. Development of litter size during the suckling period in all mated groups is shown in Tables 3 and 4. At all given time points litter size was significantly higher in mated combinations with females from the FL1 and FL2 compared to the female Ctrl line. When the male genotypes were modeled as fixed effects, no statistically significant influences could be detected (P = 0.08, Table 2) depending on whether the males are derived from the FL1, FL2 or Ctrl line. However, female-line effects were significant (P < 0.0001). These findings could be confirmed for litter size and litter weights at all considered ages. Thus, these data indicate that the selection criteria ‘offspring per litter’ in P0 animals almost exclusively depends on the female genotype.

Since we mated one particular male together with three female animals from all three different lines in a single cage, we took advantage of comparing the parturition outcome between these three females. Since we mated with the same male, a possible influence of one particular buck remains constant in this experimental setting. We noticed a slight increase in litter size by mating male FL2 with female FL1 (22.6 animals per litter) compared to Ctrl male × female FL2 (20.7 offspring per litter) (Table 2). However, this LSM difference of +1.9 animals per litter is statistically not significant.

We also measured several physiological parameters before and after the breeding period. As described earlier (Langhammer et al. 2014), females of the FL2 line

Table 5  Body weight gain of offspring per litter from birth to day 21. The weight gain has been calculated by litter weight/litter size at the 21st day minus litter weight/litter size at parturition in g. LS-means are adjusted for all interactions and replicates (LSM ± s.e.).

<table>
<thead>
<tr>
<th>Female</th>
<th>Ctrl</th>
<th>FL1</th>
<th>FL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>12.39 ±0.44</td>
<td>6.69 ±0.42</td>
<td>8.68 ±0.41</td>
</tr>
<tr>
<td>FL1</td>
<td>12.23 ±0.42</td>
<td>6.13 ±0.42</td>
<td>8.83 ±0.41</td>
</tr>
<tr>
<td>FL2</td>
<td>12.54 ±0.42</td>
<td>7.07 ±0.45</td>
<td>8.26 ±0.41</td>
</tr>
</tbody>
</table>

Figure 1 Body weight of females (A) and males (B) of the indicated genotypes (FL1, FL2, Ctrl) before mating at 10–12 weeks of age. Ctrl: light gray, FL1: dark gray, FL2: black bars. (C) Gain or loss of body weight in males of the indicated genotype after two weeks of breeding. Statistically significant differences (P < 0.05) are indicated by asterisks.
exhibit the highest body weight compared to females of the FL1 and the control line at time of mating (Fig. 1A) with a body mass minimum to maximum range of 26.4–39.7 g in Ctrl, 29.5–44.7 g in FL1 and 28.6–50.9 g in FL2, respectively. This has also been observed for males of the FL2 line (Fig. 1B). Males of the FL1 line were the lightest of the three lines at the beginning of breeding (Fig. 1A). Interestingly, males of the control line and especially FL1 males gained weight during the 2 weeks mating period. This phenomenon was not observed for males of the FL2 line, further underscoring the biodiversity between the two FLs (Fig. 1C).

In addition, we also phenotyped offspring resulting from the different mating conditions. We did not observe any differences in gender distribution or an altered distribution of body weight of individual animals at day of birth as well as at day 21 post-partum (data not shown). Thus, it can be assumed, that increased numbers of offspring per litter in crosses with a female high-fertility background (FL1 and FL2) will not result in a higher variability of individual birth weight of the newborn pups. During the suckling period the increase in body weight of an individual animal was lower in huge pups despite an increased total rearing capacity of the FLs (Table 5). For these traits the interactions between lines were not significant. Interactions between lines and replicate were also included, but were also not significant.

As we mated three different females of the three different mouse lines together with one particular male in a single cage (see above), we could also compare the time of parturition of the different females. We noticed that females of the control line delivered offspring significantly earlier compared to FL1 and FL2 females (Fig. 2). This effect is not due to different gestation periods which remained constant at 19 days in all three genotypes. The male genotype does not influence the mating disposition. Thus, it could be assumed that Ctrl females are inseminated earlier compared to FL1 and FL2 dams.

**Endocrinology**

Complementary to the previous description of physiological and molecular alterations in males of the FL1 and FL2 lines compared to control males, we analyzed the concentrations of several steroid hormones in these animals. To this end, we prepared serum of 12-week-old males and analyzed the serum steroid profiles by stable isotope dilution/GC–MS. As shown in Fig. 3A, males of all three lines contain between 5.0 and 6.4 ng/mL testosterone showing no significant differences between the lines. These data are in conflict with previous data using an ELISA technique for testosterone.

![Figure 2](https://www.reproduction-online.org)

**Figure 2** Females time to delivery of the indicated genotypes. Time points have been calculated relative to the time point males and females have been caged together. Statistically significant differences ($P < 0.05$) are indicated by asterisks. Ctrl: light gray, FL1: dark gray, FL2: black bars.

![Figure 3](https://www.reproduction-online.org)

**Figure 3** Serum steroid hormone concentrations in males of the indicated genotypes. Data are shown for testosterone, dehydroepiandrosterone (DHEA), 4-androstenedione, androstanediol, dihydrotestosterone (DHT) and corticosterone. Statistically significant differences ($P < 0.05$) are indicated by asterisks. Ctrl: light gray, FL1: dark gray, FL2: black bars.

*www.reproduction-online.org*
measurement (Langhammer et al. 2014). To further address this issue in more detail we analyzed further androgens from the same serum samples. Concentrations of 4-androstenedione and DHEA were close to the detection limit ranging between undetectable levels (in FL1 males) up to 0.06 ng/mL (4-androstenedione, 4-A) and between undetectable (FL1) concentrations and 0.31 ng/mL (DHEA), respectively. The concentrations of androstanediol ranged from 0.14 to 0.39 ng/mL and those of dihydrotestosterone (DHT) ranged from 0.08 to 0.19 ng/mL. DHEA and androstanediol levels were detectable in all three lines whereas 4-androstenedione and dehydroepiandrosterone were below the limit of detection in FL1 males but detectable in FL2 and Ctrl animals. All androgens were at least 10-fold lower in concentration than testosterone. None of the androgen concentrations reached statistically significant differences between the three mouse lines (Fig. 3). The concentrations of progesterone, 17α-hydroxy-pregnenolone, 17α-hydroxy-progesterone and 11-deoxycortisol were below the limit of detection in this assay (not shown).

In contrast to the concentrations of androgens and progestagens, we observed different serum concentrations of corticosterone in the males of the three lines. We measured elevated mean levels in male FL1 animals (49.7 ng/mL) compared to FL2 (30.8 ng/mL) and Ctrl animals (26.7 ng/mL) (Fig. 3F).

**Discussion**

Recent data from our laboratory questioned the impact of males in a two-factorial breeding experiment. These data have shown elevated pub size by mating a female of the fertility line FL1 together with a male from a control line (Renne et al. 1995). This has been expected and is supported by earlier data (Falconer 1960). Unexpectedly, an increased number of offspring per litter has also been observed in the reciprocal breeding approach when mating a FL1 male together with a control female (Renne et al. 1995). In order to verify this previous observation we repeated the two-factorial breeding experiment from the 1990s with a higher number of individuals for a better statistical substantiation. Surprisingly, we failed to reproduce the earlier findings. In the present breeding experiment the litter size solely depended on the genetic background of the female in P0 animals. Breeding results did not differ on whether a male from one of the FLs or from the control line has been used for mating together with a given female (Table 2). The high level of reproduction rate as well as number of born and reared offspring after line crossing were demonstrated in five replicates on a basis of 150 male and 450 female animals and we checked this experiment for all possible impact factors (see experimental design). All mothers and fathers are purebred animals and thus heterosis can only occur in the pups, which are crossbreds. Clearly heterosis of embryos can in principle have an effect on their survival (e.g. Pomp et al. 1989) and thus also the number of born pups per litter. In our analyses such effects, if they exist, are part of the interaction effects between lines, which we found to be not significant. Litter size is known to be affected by the maternal environment of females who will later become mothers themselves. These maternal effects therefore are attributed to the grandmothers of the litters in our experiment. Mothers of the same line are grouped into trios of full-sibs, each of them sharing the same maternal environment. Therefore variation in litter size, which is due to maternal is accounted for by the random effects for common litter environment of the mothers. Several basic points have been changed over the past 20 years; that is, animals of generation 73 have been used for analyses in the 1990s whereas we currently analyzed mouse generations 171/172. These might be a genetic drift over 100 generations. The inbreeding level has increased during the long selection period. As population sizes and mating schemes were fairly constant over time it was however possible to extrapolate the average inbreeding level in each line by using average increases of inbreeding per generation in later (i.e. pedigreed) generations. The respective values are 0.175, 0.926 and 0.977 for Ctrl, FL1 and FL2. Thus, there are huge differences in the average inbreeding coefficients between lines. Within lines, however, such differences do almost not exist. Obviously high levels of inbreeding are not prohibitive for high litter sizes in the described selection lines. Additionally, the health status has been changed from the 1990s compared to the SPF conditions in our modern mouse facility. The current two-factorial breeding experiment included the combination male FL1 × female FL2 and also the opposite combination. However, cross mating of both FLs did not induce additional litter gain. Based on these findings we assume even though both FLs used different molecular and physiological pathways in order to warrant high-fertility phenotypes (Langhammer et al. 2014) these strategies are not additive.

In the present study, we also measured the concentrations of testosterone and other steroids from serum samples of the three different mouse lines. The analyses were carried out by using stable isotope dilution/GC–MS, a technique providing highest specificity. It therefore currently represents the gold standard for steroid analysis. In contrast to immunoassays, GC–MS further allows the simultaneous determination of multiple steroids from a single sample (‘profiling’). To the best of our knowledge, no comprehensive analysis describing the concentrations of five different androgens in parallel has been published from mouse serum samples so far. We observed average concentrations between 5.0 and 6.4 ng/mL of testosterone with no significant differences between lines. Analysis of different individual animals...
of one particular line revealed a huge fluctuation of concentrations ranging from 0.3 up to 20 ng/mL of testosterone (Fig. 3). This has previously been described and seems to be more pronounced in mice than in humans (Bartke et al. 1973). Published average concentrations of mouse serum testosterone differ between 8.2 ng/mL using mass spectrometry (Nilsson et al. 2015) and 1 and 8.5 ng/mL using different antibody-based ELISA or RIA assays (Overpeck et al. 1978). Thus, we have to consider the concentrations, measured by mass spectrometry in the present study, as reference values in mice.

The synthesis of androgens (C19 steroids) requires hydroxylation in position C-17 as well as cleavage of the side chain between C-17 and C-20. Both reactions are performed by a single enzyme (17-hydroxylase/17,20 lyase). In humans, both adrenals and gonads possess 17-hydroxylase/17,20 lyase activity. Human dehydroepiandrosterone is almost exclusively secreted by the adrenals. Furthermore, in humans, both adrenals and gonads secrete androgens such as 4-androstenedione or testosterone. However, the mouse adrenal lacks 17-hydroxylase/17,20 lyase activity and thus cannot contribute to gonadal androgen synthesis (Rodriguez et al. 1997). Thus, in the mouse, all androgens reflect gonadal activity. The concentrations of androgens such as 4-androstenedione, dehydroepiandrosterone, androstanediol and dihydrotestosterone are at least one to two orders of magnitude lower compared to testosterone (Fig. 3). In a recent study in BL6 inbred mice, Nilsson et al. have described values of ~0.17 ng/mL of DHT which have been very similar to the concentrations measured in our assay. By contrast, 4-androstenedione concentrations have been higher, whereas DHEA have been below the limit of detection in the Nilsson study (Nilsson et al. 2015). Taken together, due to the lack of adrenal 17-hydroxylase activity, the androgen concentrations in mice appear to be generally lower than in humans, taking further into consideration that values additionally differ depending on the age of the individual (Overpeck et al. 1978, Soeborg et al. 2013).

In a previous report, we described enhanced testosterone values from mice of the FL1 line (but not FL2 and Ctrl lines) using a commercial testosterone ELISA optimized for human samples. This could not be reproduced using mass spectrometry in the current study and also could not be reproduced using an independent ELISA assay in biological replicate samples. Thus, we have to conclude that our previous testosterone measurements might be biased by cross-reaction to other steroids, possibly by cross-reactivity of the antibody utilized in this assay. Indeed, we measured elevated corticosterone in FL1 males (see below). Another reason for elevated testosterone concentrations in our previous study might be due to different health status in the old mouse facility compared to our today’s modern SPF mouse house.

The lack of adrenal 17-hydroxylase activity in mice is further reflected by the fact that the 17-deoxy steroid corticosterone is by far the dominating C21 steroid. Unlike in human, where cortisol is the prevailing glucocorticoid, corticosterone constitutes the prevailing glucocorticoid in the mouse and thus reflects exclusively an adrenal marker. Likewise, the corticosterone concentrations presented in this study can be used as reference values. We measured elevated corticosterone concentrations in FL1 males but not in males of the FL2 and Ctrl lines (Fig. 3F). However, these concentrations are still within the normal physiological range (Gong et al. 2015).

Taken together, these data indicate that high fertility based on the selection criteria ‘number of offspring per litter’ and ‘total litter weight’ mainly depend on the genetic background of the female. Male’s contribution appears to be marginal, although high-fertility males show different physiological and endocrine phenotypes compared to Ctrl males. Our data further strengthen the interpretation that different phenotypes guarantee the strain ‘high fertility’ and further underscore the biodiversity of the Dummerstorf high-fertility mouse lines. Furthermore, we provide reference values for various marker steroids of adrenal and gonadal origin that were determined by reference methodology (GC–MS).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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