Altered testicular cell type composition in males of two outbred mouse lines selected for high fertility

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Abstract

Background: Recently we described two outbred mouse lines which have been selected for high fertility. These mouse models doubled the number of offspring per litter.

Objectives: Although selected for a primarily female-trait of high fertility (increased litter size), we were interested whether also males of the fertility lines show differences within their reproductive organs.

Materials and methods: We investigated males from two outbred mouse lines which have been selected for the phenotype “high fertility” for more than 170 generations. In the present study, we analysed the testicular cell type composition by flow cytometry. We further investigated the weights of reproductive organs, histomorphometry of testis as well as studied sperm motility parameters using a thermal stress assay as well as a sperm hyperactivation assay.

Results: Here, we describe that males of the fertility line (FL) 1 show an increased percentage of diploid cells within the testis. Flow cytometric analysis identified this enlarged cell population as Leydig cells. Testis weights were unaffected whereas the weights of seminal vesicles of FL1 and FL2 were increased compared to Ctrl bucks. FL2 males show decreased diameter of tubulus seminiferi and an enhanced spermatid/Sertoli cell index. Sperm motility parameters of FL1 and Ctrl males are initially indistinguishable but FL1 spermatozoa show a better performance in a thermal stress experiment over a 5 hours observation period.

Discussion: These data indicate that although selected for a primarily female-trait of high fertility also males from the fertility lines are effected by defined alterations in their reproductive organs.

Conclusion: Some of these alterations are FL1-specific others are FL2-associated, indicating that different molecular strategies warrant the high-fertility phenotype on the female as well as on the male side.
1 | INTRODUCTION

Fertility research largely depends on informative animal models. Worldwide more than 1000 mouse models exist, which show a fertility phenotype. Among them, approximately 99% show a decreased fertility phenotype. By contrast, only ~1% of mouse models showing a fertility phenotype are associated with the annotations “enhanced fertility” and/or “increased litter size” (data extracted from Mouse Genome Informatics, MGI—www.informatics.jax.org).

Approximately a dozen transgenic or knockout inbred mouse models exist showing an increased fertility phenotype due to single gene alterations, most of them showing an increased female fertility. One example is the Bcl-2 knockout mouse. Due to the genetic inactivation of the Bcl-2 gene affected females show a decreased ovar -

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Two groups continuously have been performed to select for higher fertility due to outbreeding in different species including mice.4,5 Also in this case, higher litter sizes have been reported based on mating with CD46 knockout males. However, it should be noted that physiological consequences of increased litter sizes are moderate in those mouse models showing a single gene alteration described so far, regardless of whether the genetic modification is relevant for male or female fertility. Only moderately elevated litter sizes of 10%-20% have been reported.

In contrast to classical inbred mouse models, several attempts have been performed to select for higher fertility due to outbreeding in different species including mice.6,8 Two groups continuously developed outbred mouse models for more than 100 generations of selection. The group of Odd Vangen at the Agricultural University of Norway in Ås developed an outbred mouse model which has been selected for high fertility. These animals almost doubled the number of offspring per litter.9,10 Unfortunately, this line has been terminated in 2007 after 130 generations of selection. However, based on this genetic resource the inbred line QSiS has been developed which maintained an elevated litter size.11 Beside this Norwegian mouse line and its subsequent mouse line branch two outbred mouse models have been developed at the Leibniz Institute for Farm Animal Biology (FBN Dummerstorf).

At the FBN Dummerstorf, two high-fertility mouse lines have been developed (Fertility Lines 1 and 2, FL1 and FL2). Starting from an initial population of four inbred and four outbred founder mouse lines starting in the 1970s,12 two long-term outbred selection lines have been developed. The animals were selected according to the following selection criteria: (a) the number of pups per litter and (b) total birth weight of the first litter.13 These selection criteria prevent any signs of developmental failures in newborn puppies. After > 40 years and 170 generations of selection these mouse lines almost doubled the amount of offspring per litter up to 20.5 and 21.1, respectively.14,15

In a two-factorial breeding experiment, we recently described that the fertility phenotype mainly depends on the female gender.16 The rationale of the present study was to clarify whether long-term selection of a primarily female-derived trait of high fertility (increased litter size) also leads to alterations on the male side. We described differences in physiological and behavioural parameters as well as in endocrine and molecular markers between males of the FLs compared to control bucks.14,15,17 Since we noticed elevated Leydig cell-specific gene transcription in FL1 bucks18 but neither in FL2 nor in control (Ctrl) males, we were interested to address this question in more details. In the present study, we further wanted to investigate the cell type composition in testis as well as analysing sperm motility parameters under stress conditions in order to pre-cede the characterization of the male phenotype of mice selected for high fertility.

2 | MATERIALS AND METHODS

2.1 | Animals

All procedures were performed in accordance to national and international guidelines and approved by our 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-dark cycle. The mice were kept in Polysulfone cages of 267 × 207 × 140 mm (H-Temp PSU, Type II, Eurostandard, Tecniplast) 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 fibre, 50.1% N free extracts and 3.2% mineral mixture (ssniff® M-Z autoclavable) 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 at first delivery) in primiparous females for 162 generations, followed by the selection procedure of BLUP (Best Linear Unbiased Prediction) breeding value estimation, focusing only for the number of born pups in primiparous females, before starting the present study. The raising inbreeding coefficient in the selection lines was controlled by the method “Optimal Genetic Contributions to the Next Generations” of Meuwissen.19

2.2 | Flow cytometry

For flow cytometric experiments animals with an age of 12 to 15 weeks were euthanized by CO₂ inhalation (n = 6-10 per line). Testes were cleaned from fat tissue, the tunica albuginea was removed, and parenchyma was dissociated in 5 mL 1 × PBS/1% BSA using GentleMACS dissociator (C Tubes/gentleMACS, Miltenyi Biotec). The suspension was filtered (100 µm mesh, BD Falcon) and centrifuged (60 g, 5 minutes, 4°C) to remove cell debris. The pellet was re-suspended in 2 mL PBS until by pipetting until a single cell suspension was achieved. For fixation, 600 µL of this cell suspension was added dropwise either in ice-cooled (~20°C) 70% ethanol or in 600 µL ice-cooled (~20°C) methanol. For total cell cytological evaluation the cells of the ethanol fixation have been treated with RNase A (1 mg/mL), loaded with propidium iodide (75 µM) and applied to a flow cytometer (Gallios, Beckman Coulter) as previously described.20 For immunoreaction, methanol fixed cells were washed two times in PBS and re-suspended in RNA digestion solution (1 mg/mL RNase A in PBS pH 7.4, 30 min, 37°C) to remove cellular RNA. After digestion, cells were washed (300 g, 5 minutes, RT) in PBS and re-suspended in staining buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.4). Cells were incubated overnight (4°C) with polyclonal rabbit anti-bovine CYP17 antibody (1:5000, kind gift of Alan J. Conley, University of California-Davis, USA).20 Unbound antibody was removed by washing and cells were re-suspended in staining buffer. Afterwards, cells were incubated for 1 hour at 4°C with goat anti-rabbit AlexaFluor488 (2 mg/mL, A11034, ThermoFisher Scientific) followed by counterstaining with propidium iodide (75 µM, 30 minutes, RT). After washing and re-suspension in PBS, cells were analysed by flow cytometer (Gallios, Beckman Coulter). Statistical analysis was performed with one way ANOVA followed by Dunnett’s multiple comparison test for each cell type.

2.3 | Histomorphometry

The testes have been fixed in Bouin’s solution over night at 4°C, dehydrated in an ascending ethanol series within a carousel tissue processor (MTP Slee, Mainz, Germany) and embedded in paraffin by standard protocol. For histological evaluation testis were sliced in approximately 3 µm thick cross-sections and stained with haematoxylin and eosin (H&E). Slides were assessed using a Zeiss image-analysing system (Carl Zeiss AG) and 400-fold magnification. Five sections per animal deriving from different testis segments were examined for Sertoli cell nucleolus (SC) amounts and round spermatid (St) counts per tubulus seminiferi (approximately stages 2-6) with corresponding Sertoli cell index (St/SC ratio) as well as for tubule diameter. For each section 4 circular tubules were chosen (n = 20 tubules/testis) and either directly explored under microscope or picture based by AxioVision (total: n = 200 tubules/line). For quantification, at least 10 randomly chosen frames were analysed for the proportions of the tubular and interstitial compartment.

2.4 | Tissue weight

Organ weights have been determined right after extraction from the animal. Paired organs were weighed together. Statistical differences have been analysed by Dunnett’s multiple comparison test.

2.5 | Sperm motility analysis – Sperm motility stress test

The sperm motility analysis was accomplished by CASA (computer-assisted sperm analysis) using SpermVision (Minitube) as described previously.14 Prior to a 5-hour thermal stress procedure along with CASA measurements, 12-week-old males (n = 10 per line) were euthanized by CO₂ inhalation. To obtain spermatozoa the cauda epididymis was extracted, cleaned and minced (five cuts) in freshly prepared 300 µL sperm-suitable M199 media (M7528, Sigma-Aldrich) and incubated for 5 minutes at 37°C for sperm release. To exclude tissue remnants the suspension was filtered using 30 µm mesh. For generating stress conditions, the sperm suspension was continuously exposed to 37°C for a 5 hours thermal stress period. Every hour the current sperm motility characteristics were analysed by applying a fraction of 3 µl to 37°C tempered chamber slides (20 micron, Leja). Sperm motility parameters represent an average of 8 defined chamber partitions were viewed and measured by CASA system (Minitube). For hyperactivation experiment spermatozoa were solved in PBS and hyperactivation has been induced by PBS containing 2 mM CaCl₂, 25 mM NaHCO₃ and 3 mg/mL BSA and measured at t = 0 min and t = 30 min. Statistical analysis was carried out by two-way ANOVA using the software package GraphPrism 5.01 (GraphPad Software).

3 | RESULTS

3.1 | Elevated Leydig cell population in FL1

We recently described the characterization of two mouse lines (FL1 and FL2) which have been selected for high fertility. Although these mice have been primarily selected for a female-derived trait of fertility (increased litter size and elevated birth weight), we noticed distinct alterations also on the male side. Beside altered testicular gene expression patterns and endocrine variations, we also...
observed distinct physiological characteristics eg in behavioural assays.17,18 Since we have previously detected an increased diploid cell population in males of the FL1 line18 we performed flow cytometry analysis in Ctrl, FL1 and FL2 of testicular tissue in order to examine the cell distribution and to identify different cell types. As shown in Figure 1 bucks of FL1 showed decreased portions of haploid cell populations. These cell types include elongated spermatids (peak 1C' in Figure 1) and round spermatids (peak 1C). Additionally, FL1 males exhibited increased concentrations of spermatogonia which synthesis DNA (peak S-Ph) and FL1 and FL2 revealed enhanced tetraploid cells (secondary spermatocytes, peak 4C). However, the most pronounced observation in this flow cytometric analysis is a significantly elevated percentage of diploid cells (peak 2C) in bucks of FL1 which is neither observed in Ctrl nor in FL2 males (Figure 1). We investigated different ages of males, proven fertile males as well as randomly selected males from the population and received very similar results. Especially, the augmented diploid cell population in FL1 bucks is highly significant and reproducible in all populations (Figure 1 and data not shown).

Since our gene expression data indicate elevated Leydig cell-specific gene transcripts (eg Cyp11, Cyp17) from testes of FL1 compared to Ctrl and FL2 bucks,15,18 we tested the hypothesis whether these increased diploid cell population might be Leydig cells. To this end, we incubated testicular cell populations from Ctrl, FL1 and FL2 with an antibody directed against Cyp17 and counter stained the entire cell population by propidium iodide. We measured 12% and 11% of all testicular cells to be positive for Leydig cell marker Cyp17 in Ctrl and FL1 and FL2 revealed enhanced areas of Leydig cells almost doubled in FL1 males the Cyp17 protein load per cell is slightly decreased (Figure 2C).

3.2 | Size of gonads

To test whether these alterations at the single cell level might also be reflected by different sizes of reproductive organs, we measured the weights of testis, prostate (ventral and lateral) and seminal vesicles. As shown in Figure 3A we did not notice any differences in testis weight (sum of weights of left and right testis). The prostate weight showed only a tendency of being slightly increased in males of the fertility lines (FL1: +28%, FL2: +21%, Figure 3B). However, we detected significantly elevated weights of seminal vesicles in bucks of FL1 and FL2 (FL1: +51%, FL2: +44%) compared to Ctrl males (Figure 3C).

3.3 | Histomorphometry

To further analyse morphological characteristics of testis, we measured the diameter of tubulus seminiferi, the area of interstitium as well as the area of tubulus seminiferi from Ctrl, FL1 and FL2 males. We did not notice significant differences between FL1 and Ctrl bucks (Figure 4). By contrast, we found alterations in FL2 compared to Ctrl males. Diameter of tubulus is reduced, area of tubulus is increased whereas area of interstitium is decreased in FL2 compared to Ctrl males (Figure 4A-C). We also counted the abundance of round spermatids (St) and Sertoli cells (SC) in all three lines. We noticed slightly elevated round spermatid numbers in FL2 compared to FL1 and Ctrl (Figure 4D). Since the number of Sertoli cells remained unaffected the Sertoli cell index (St/SC ratio) was increased in FL2 (Figure 4D,E).

3.4 | CASA

Previous sperm motility analysis did not reveal significant differences between lines at t = 0 minute at standard conditions; however, we observed a significant decrease in thermal stability in FL2 spermatozoa compared to Ctrl spermatozoa over a 5 hours observation window.14 To test whether this phenomenon is also true for FL1 spermatozoa, we applied the thermal stress experiment for FL1 spermatozoa. Again, we noticed no difference in sperm motility parameter between Ctrl and FL1 bucks at t = 0 minutes (Figure 5) in agreement with previous data.18 However, after thermal stress we noticed a significant better performance of FL1 spermatozoa compared to Ctrl spermatozoa. This effect is obvious in percentages of motile spermatozoa (+10% in FL1 compared to Ctrl at t = 5 hours, Figure 5A,B) as well as in various sperm velocity parameters (eg VCL: +10 µm/s elevated pace compared to Ctrl at t = 5 hours, Figure 5D). We also performed a hyperactivation assay by investigating spermatozoa of all three lines at t = 0 minute as well as t = 30 minutes after induction of hyperactivation by a bicarbonate/BSA buffer. As shown in

![Figure 1](image-url)
Figure 5G, the percentage of spermatozoa classified as hyperactive according to the CASAnova classification rose from 4.8% to 7.9% in Ctrl spermatozoa. In contrast, this sperm population is already enhanced in FL1 (6.4%) and FL2 spermatozoa (9.7%), however, this proportion of spermatozoa could not be further increased by the bicarbonate treatment Figure 5G.

4 | DISCUSSION

The cell-type composition in testis is highly dynamic during development due to a complete lack of mature germ-cell stages prior to the onset of puberty.23-25 Also in adults within one species cell-type composition might differ in cases where dominant and philopatric males live together in one mouse colony.26 Surprisingly, we noticed differences in testicular cell type composition in (single caged) males of FL1 compared to males of Ctrl and FL2 (Figure 1). We observed an elevated percentage of diploid cells in FL1 bucks. Further analysis identified these elevated cell population as Leydig cells due to the expression of Leydig cell marker Cyp17 (Figure 2A,B). Approximately 1/4 of the diploid cell population within the testis correspond to Leydig cells in rodents which is also the order of magnitude in Ctrl and FL2 (27% and 24%, respectively, Figure 2B). By contrast, this cell portion is increased to 51% in FL1 males (Figure 2B). Of note, the amount of Cyp17 load per positive cell is decreased in FL1 compared to Ctrl and FL2 (Figure 2C). Elevated numbers of Leydig cells in FL1 are not accompanied by significantly increased intestinal area (Figure 4B) and are not associated with Leydig cell hyperplasia and decreased fertility as it has been described for several knockout mouse models.27-29 Accelerated gene transcription of Leydig cell markers in FL1 has already been reported in previous work.15,18 Based on this observation, one might suggest elevated testosterone concentrations in FL1 males compared to Ctrl and FL2 males. However, we did neither observe enhanced testosterone, 4-androstenedione, 3α-androstanediol, DHEA nor increased DHT concentrations in FL1 males.16 Thus, a raised Leydig cell number in conjunction with augmented expression of Leydig cell markers relative to total testicular transcripts (or total testis weight, respectively) did not reflect increased serum androgen concentrations in FL1 bucks. Since we noticed slightly elevated progesterone and corticosterone levels in FL1,16 the effect of unaffected steady-state androgen concentrations might be explained by an accelerated metabolic turnover rate in these animals. In line with this assumption, we noticed altered steady-state transcript levels of steroid hormone clearance enzymes (such as Sult1e1) in holistic gene expression analysis from FL1 testis.15

Beside the responsibility for sperm production, the testis is as an endocrine organ and the hormonal demand influences its weight. An elevated androgen activity would point towards an increased testicular weight, which we did not observe. Testis weights were shown to remain unaffected in FL1 compared to FL2 and Ctrl males. However, we could detect the weight of seminal vesicles.
to be increased by around 50% and the weight of the prostate to be slightly enhanced by ~20% in males of the two fertility lines compared to Ctrl bucks (Figure 3B,C). Several publications analysed the effect of elevated or dampened androgen levels. It is long known that size and/or weight of accessory sex glands are more or less directly related to androgens levels.\cite{30,31} Hence, according to our study findings, we cannot exclude elevated androgen activities in FL bucks. However, the testes weights of the Dummerstorfer outbred lines are in the upper range compared to different tissue weights, which were described in a systematic study of eight different inbred lines (Figure 3A) and.\cite{32} Furthermore, analysis of tissue weights might be biased by differing body weights of the animals at the time point of analysis. As we described recently males of the FL2 lines are generally heavier compared to Ctrl and FL1 males\cite{16} as well as compared to most inbred mice.\cite{32} Thus normalized to body weight, FL2 males appear to have rather smaller testis compared to FL1.

**FIGURE 4** Histomorphometry of testis. Diameter of tubuli seminiferi (A), area of interstitium (B), area of tubuli seminiferi (C), Sertoli cell index (St/SC ratio) (D) and number of spermatids (St) and Sertoli cells (SC) (E) from Ctrl, FL1 and FL2. Ctrl: green bars; FL1: red bars; FL2: blue bars. Statistical analysis was performed with Dunnett's multiple comparison test (n = 8-10 per group; mean SD; **P < .01, ***P < .001)

**FIGURE 5** Changes in sperm motility in thermal stress and hyperactivation experiments. Sperm motility changes after continuous incubation at 37°C: average motility (A), progressive motility (B), average path velocity (VAP) (C), curvilinear velocity (VCL) (D), beat cross frequency (BCF) (E) and linearity (LIN) (F). For this analysis, cauda epididymis spermatozoa with similar sperm concentration of FL1 (red) and Ctrl mouse line (green) (n = 5-8 per group) were extracted and incubated at 37°C for 5 hours. Each point represents the mean of eight defined chamber partitions viewed and measured using a computer-assisted sperm analysis system (CASA). (G) Percentages of hyperactivated spermatozoa grouped according the CASAnova classification without (open bars) and with bicarbonate induction (closed bars). The experiment has been statistically evaluated for the two factors time and mouse line effect using two-way ANOVA (GraphPad Prism) as indicated in the graph
between FL1 and Ctrl bucks (Figure 4). Since we also observed an elevated Sertoli cell index (St/SC ratio) in FL2 (Figure 4D), which might point towards a higher Sertoli cell efficiency and a higher spermatogonic efficiency in FL2 compared to FL1.35 St/SC ratios identified in our mouse lines (between 6 and 8, Figure 4D) are rather smaller compared to reports from other mouse strains (between 8 and 11) as well as compared to most other mammals.34,36 Unfortunately, we cannot correlate spermatid/Sertoli cell indices from our lines with sperm production rates since we prepared epididymal spermatozoa in the present study; however, a negative correlation between tubulus diameter and spermatid/Sertoli cell index (as observed for FL2, Figure 4A,D) has also been described in cats.37

We recently described a decreased successful mating rate of 82% in FL2,14 which is relatively low compared to wild type mice (98%38 as well as compared to FL1 and Ctrl bucks (94% and 95%, respectively).15 In this context it should be noted that successful mating rates of commonly used inbred mouse strains such as C57BL/6J, CBA/J, A/J and BALB/cJ are with 55%-85% much lower.39 Detailed sperm motility analysis in FL2 detected relatively subtle alterations under standard condition, although some velocity parameters already reached statistically significant differences (eg VCL).16 More pronounced effects occurred by analysing sperm motility parameters on elevated temperature of 37°C during a stress period of up to 5 hours. Since FL2 spermatozoa demonstrated decreased motility in this heat stress experiment, we suggested that males of FL2 show a rather decreased fertility compared to females of the same line, which clearly show enhanced fertility (higher ovulation rate, increased litter size).16 To test whether this hypothesis might also be true for FL1 bucks we performed a heat stress experiment using FL1 spermatozoa. Interestingly, we noticed the opposite effect in FL1 compared to FL2 bucks. FL1 spermatozoa remain more mobile with improved velocity compared to Ctrl spermatozoa (Figure 5) and compared to FL2 spermatozoa.14 In a hyperactivation assay of spermatozoa from all three lines, we noticed an elevated percentage of hyperactivated spermatozoa already in uninduced spermatozoa from FL1 and FL2 compared to Ctrl animals. However, this already elevated proportion of hyperactivated spermatozoa could not be increased further by the bicarbonate/BSA treatment (Figure 5G). We recently described that the fertility phenotype of the mice is mainly depending on the female gender in a two-factorial breeding experiment using pure-bred animals.16 In the present study as well as in previous studies, we demonstrated that selection for a primarily female-derived trait of high fertility also leads to complex alterations on the male side. Some of these (subtle) changes seem to be beneficial for adequate fertility, while others tend to have opposite effects (see above). Therefore, we cannot claim from these data that male fertility is generally compromised by selection for a primary female feature of increased fertility, as suggested recently.40

Taken together, this data indicates that males as well as females of the two high fertile mouse lines—FL1 and FL2—developed different strategies in order to maintain a high-fertility phenotype. Furthermore, there seems to be no direct link between fertility of both sexes. High fertility on female side does not necessarily mean elevated fertility in males within the same line. Further experiments will focus on the identification of pathways that are differentially regulated in females of the high-fertility lines and their possible impact in defining the male phenotype.

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CONFLICT OF INTEREST
All authors declare that they have no conflict of interests.

AUTHORS’ CONTRIBUTIONS
MM: conceived study, performed experiments, analysed data, carried out statistical analysis; AS, CL, HM: performed experiments, analysed data, carried out statistical analysis; ML: conducted animal breeding, analysed data; J5: conceived study, interpreted data; JW: conceived study, analysed data, interpreted data, drafted the manuscript. All authors have read and approved the manuscript.

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