High-fertility phenotypes: two outbred mouse models exhibit substantially different molecular and physiological strategies warranting improved fertility

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Abstract

Animal models are valuable tools in fertility research. Worldwide, there are more than 400 transgenic or knockout mouse models available showing a reproductive phenotype; almost all of them exhibit an infertile or at least subfertile phenotype. By contrast, animal models revealing an improved fertility phenotype are barely described. This article summarizes data on two outbred mouse models exhibiting a 'high-fertility' phenotype. These mouse lines were generated via selection over a time period of more than 40 years and 161 generations. During this selection period, the number of offspring per litter and the total birth weight of the entire litter nearly doubled. Concomitantly with the increased fertility phenotype, several endocrine parameters (e.g. serum testosterone concentrations in male animals), physiological parameters (e.g. body weight, accelerated puberty, and life expectancy), and behavioral parameters (e.g. behavior in an open field and endurance fitness on a treadmill) were altered. We demonstrate that the two independently bred high-fertility mouse lines warranted their improved fertility phenotype using different molecular and physiological strategies. The fertility lines display female- as well as male-specific characteristics. These genetically heterogeneous mouse models provide new insights into molecular and cellular mechanisms that enhance fertility. In view of decreasing fertility in men, these models will therefore be a precious information source for human reproductive medicine.

Translated abstract

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Introduction

Animal models for fertility research

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 400 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. The infertile or rather subfertile characteristics of a yet unelucidated novel mouse model are readily detectable in the context of standard animal care conditions due to obvious difficulties in breeding these animals. In general, mouse models revealing reduced fertility are unquestionably helpful in identifying crucial proteins that

interfere with any critical step necessary for proper fertility in man and/or animal both in males and in females. Furthermore, these mouse lines are undoubtedly helpful in detecting and dissecting fertility-relevant pathways.

However, infertility in man appears to be more complex and as such does not affect only one single gene but rather a combination of multiple genes. Additionally, it might be an oversimplification to divide all fertility phenotypes into only two categories, i.e. fertile and infertile. A more subtle differentiation might be more appropriate to reflect the diverse fertility phenomena. Furthermore, it might be valuable to involve and pursue more comprehensive parameters for analyzing fertility (e.g. partner, environment, and endocrine disruptors). To this end, animal models showing an improved fertility phenotype with an inherent illustration of the complex and biodiverse fertility nature might become highly informative.

Animal models with a high-fertility phenotype

In contrast to animal models revealing diminished fertility, the reciprocal phenotype (high fertility) is not easily identified during standard animal care conditions. To open up this bottleneck, breeding protocols have been set up to select for high-fertility traits. Such protocols have been conducted in several animal species so far, e.g. mouse, pig, and rabbit (Johnson *et al.* 1999, Holt *et al.* 2004, Su *et al.* 2007, Ziadi *et al.* 2013).

Particularly, establishing a high-fertility model for pigs is considerably relevant for farm animal biology. This is pointed out by the circumstance that breeding over the last several 1000 years has led to a rise in the average litter size in pigs from two to eight piglets in wild boars to more than 15 piglets in contemporary domestic pigs (Su *et al.* 2007, Beaulieu *et al.* 2010). Although this litter size is economically desirable, its generation by breeding is currently associated with major problems resulting from highly unbalanced birth weights of piglets ranging from <1 kg (very small piglets) to >1.8 kg (very large piglets). Hence, this heterogeneous piglet population leads to higher and unintended follow-up breeding costs (Rehfeldt *et al.* 2008, Beaulieu *et al.* 2010). This can be avoided by achieving homogeneous litters with a size of more than 15 piglets and a balanced birth weight of ~1.3–1.5 kg/piglet. As our high-fertility mouse models generate homogeneous litters despite high offspring numbers, they might be of great value to elucidate physiological mechanisms and target genes responsible for this desired property.

Mouse models for high-fertility traits

To examine physiological and genetic responses to selection for fertility traits, long-term selection mouse lines were established as models for farm animals at the Leibniz Institute for Farm Animal Biology (Dummerstorf),

which were selected for the phenotype 'high fertility'. All the animal experiments were approved by local authorities (Landkreis Bad Doberan, Veterinär- und Lebensmittelüberwachungsamt, Mecklenburg-Vorpommern, Germany). The initial population was a systematic crossbreed 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 = $1.6 \times \text{number of offspring} + \text{birth weight of the entire litter}$). Animals born from the largest and heaviest litters were chosen for further breeding (Schüler & Bünger 1982). Whereas fertility line 1 (FL1) was treated, 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 animals per generation; consequently, these outbred mouse lines are more heterogeneous and biodiverse in nature compared with classical inbred mouse lines. Females were exposed to males at an age of 63 days with a mating ratio of 1:1. After more than 40 years and 161 generations of breeding, the litter size increased from approximately ten animals per litter in the original founder population to 17.6 ± 3.3 and 20.2 ± 2.0 animals per litter in FL1 and FL2 respectively. The litter size of the unselected and randomly mated control line (Ctrl) animals remained largely constant over the selection period. The breeding success during the whole selection proceeding of more than 40 years with regard to the number of born pups per litter is summarized in Fig. 1.

The Dummerstorf mouse lines FL1 and FL2 represent – to our knowledge – a worldwide unique animal recourse. Admittedly, a similar long-term breeding approach in mice has been followed by Odd Vangen *et al.* from the Agricultural University of Norway in Ås

Figure 1 Selection success concerning the mean number of born offspring per litter in FL1 and FL2 during 161 generations of fertility index selection compared with the unselected control line (Ctrl). Data points represent the mean of at least 50 individual litters per generation and mouse line.

with high-fertility mice selected for more than 110 generations (Holt *et al.* 2004, 2005). However, this line was terminated in 2007 after 130 generations of selection (Odd Vangen 2013, personal communication). Moreover, reports can be found about experimental attempts to increase fecundity in mice on the basis of inbred lines. One of these inbred models is the QSi5 mouse line created by Peter Williamson *et al.* from Sydney, Australia (Wei *et al.* 2013). These mice are distinguished by an average litter size of 13.4 animals.

Breeding success and fecundity indices

Our Dummerstorf mouse lines were selected according to a fecundity index corresponding to $1.6 \times \text{litter size} + \text{litter weight on the day of birth}$. This fertility index was selected to avoid a selection for only high litter sizes with perhaps simultaneously reduced individual (and possibly unhealthy) body weight of the offspring. Thus, this fertility index reflects both parameters: size and birth weight of the entire litter. Other commonly used mouse fecundity indices integrate the following fertility-relevant parameters: i) litter size; ii) litters born per dam (lifetime fecundity); and iii) successful mating rate (Silver 1995). As lifetime fecundity in our mouse lines has – unfortunately – not been determined so far, it is somewhat difficult to draw comparisons with these lines on the basis of the Silver fecundity index. However, restricted to the parameters of litter size and successful mating rate, comparisons of FL1/FL2 opposed to QSi5 and other inbred mouse lines revealed similar or actually higher values (Table 1).

It is quite noteworthy that unselected outbred mouse lines (as those employed in our model as the Ctrl) exhibit a significantly higher fecundity with an average litter size of around ten animals (Bowman & Falconer 1960, Bradford 1979) compared with today's inbred mouse lines (see Table 1). Inbred mouse lines have to be generally considered as subfertile if compared with unselected outbred mouse lines. Thus, it is important to take into account that the majority of investigations of genetically modified mouse models that have been back-crossed to C57BL/6J deal with subfertile control

animals. Even those inbred lines that have been thought of as being relatively highly fertile are indeed subfertile in comparison with outbred mice; in particular, even C57BL/6J mice are subfertile (see Table 1). Moreover, it is not clear which reproductive and physiological parameters are altered by inbreeding. This means that inbred mouse lines already exhibit decreased reproductive physiology and might not constitute the best model for investigations regarding proper fertility.

Bias during breeding protocol

Every selection protocol is prone to select additionally for undesired or initially overlooked side effects. An unintended side effect was observed during the last 115 generations of breeding. The body weight for both genders of the FLs was increased at the time of mating – an effect especially pronounced in FL2 females (Fig. 2A). Additionally, it is worth noting that the common sex dimorphism in body weight inverts in FL2 animals in favor of the females. FL1 females at least reach the same body masses as the male animals. As the selection aimed toward large litters, it can be suggested that an overall growth in body weight of the dams might be helpful for proper delivery. A rise in dam body weight during the selection period has also been noticed in the Norwegian high-FL (Holt *et al.* 2004).

Furthermore, a shift in growth development and accelerated puberty of FL1 and FL2 animals has been detected. Whereas the time point of vaginal opening did not differ between the Ctrl, FL1, and FL2 animals, we observed an earlier entry into the first estrus for the FLs in contrast to the Ctrl (Fig. 2B). Thus, it could be assumed that the developmental program is accelerated in high-FLs.

Characterization of the FLs FL1 and FL2 on the female side

The two Dummerstorf FLs FL1 and FL2 were initially analyzed in more detail on the female side. In this context, it became clear that different physiological parameters are altered in both mouse lines. While FL2

Table 1 Fecundity index according to Silver (1995). Summary of litter size, litters born per dam, and successful mating rates in different outbred and inbred mouse lines (for details see text).

Strains	Litter size (n)	Litters born per dam	Successful mating rate	Fecundity index	References
FL1	17.6	ND	0.92	ND	This study
FL2	20.2	ND	0.88	ND	This study
Ctrl (Dummerstorf)	10.8	ND	0.84	ND	This study
WT	8.3	ND	0.98	ND	Bradford (1979)
Norwegian line	21.6	ND	0.80	ND	Holt <i>et al.</i> (2004, 2005)
QSi5	13.4	5.0	0.97	65.0	Wei <i>et al.</i> (2013)
C57BL/6J	7.0	4.0	0.84	23.5	Silver (1995)
DBA/2J	5.4	3.9	0.75	15.8	Silver (1995)
A/J	6.3	2.9	0.65	11.9	Silver (1995)
BALB/cJ	5.2	3.8	0.47	9.3	Silver (1995)

ND, not determined.

Figure 2 (A) Female body weight at the time of mating in the fertility lines (FLs) FL1 and FL2 compared with the unselected Ctrl. Female animals were measured at 9 weeks of life over the selection period. (B) Time point of first estrus in the FLs FL1 and FL2 as well as in unselected control animals (Ctrl). Data points represent means $\pm$ s.d. of at least 1300 individual animals per mouse line. Different letters indicate statistically significant differences between lines (Student's *t*-test, $P < 0.05$).

females displayed increased total plasma progesterone concentrations during the cycle, this phenomenon could not be observed in FL1 mice (Fig. 3A; Spitschak *et al.* 2007). In turn, FL1 females exhibited multiple (up to seven) oocytes per follicle (Alm *et al.* 2010). Again, this finding is specific for only one FL (FL1) and thus could not be demonstrated for FL2 or unselected control mice.

Additionally, analysis of embryonic survival rates revealed increased gestational losses only in FL1 animals. FL1 females release an average of ~ 28 oocytes per cycle, compared with ~ 12 oocytes released by Ctrl females (Spitschak *et al.* 2007). Among this high number of ovulated oocytes, ~ 18 develop into living offspring (compare with Table 1). Thus, pregnancy losses are $\sim 1/3$ calculated relative to the originally ovulated population. This gestational loss rate is higher compared with that observed in the Ctrl ($\sim 11\%$; corresponds to ~ 11 offspring per litter – delivered out of ~ 12 ovulated oocytes) and FL2 ($\sim 13\%$; ~ 20 offspring – ~ 23 ovulated oocytes). Nevertheless, independent of the fertility strategy selected by the particular mouse line, the improved fecundity lines deliver a substantially higher number of offspring per litter in comparison with Ctrl animals (Table 1). Interestingly, a significantly increased rate of gestational losses (as observed for FL1) has also been described for the Norwegian high-FL (Holt *et al.* 2004). Moreover, the majority of gestational losses in FL1 animals occur during the first 4 days of pregnancy (Spitschak *et al.* 2007). It is therefore tempting to speculate that not all of the multiple oocytes that have been ovulated by FL1 females from only one follicle are properly pregnable. As our selection protocol has only focused on first delivery, we are currently forced to speculate whether this 'wasting' phenotype has negative effects on lifetime fecundity performance of our FLs. Furthermore, up to now, we also have no data on further health of the mothers. However, from today's perspective, it is suggested that future health of the females might be impaired due to the strain of huge and heavy litters at

first delivery. To clarify these highly crucial issues, further experiments remain to be conducted.

Characterization of the FLs FL1 and FL2 on the male side

Diallelic breeding experiments indicate a significant contribution of the male side to higher fertility rates. After breeding of male animals from FL1 together with female animals from the Ctrl, significant improved fertility has been observed (Michaelis *et al.* 2013). Thus, high-fertility male contribution seems to largely determine the reproductive outcome. This obviously suggests that not only female ovarian function but also sperm quality of the males and/or heterosis effects of the embryo carry major weight as the limiting factors. This discovered male-only contribution triggered further investigations of FL bucks, which have just started recently.

According to the analysis carried out so far, we could find, on the one hand, a reduced percentage of motile sperm and, on the other hand, increased sperm velocity parameters (such as velocity straight line and velocity average path, among others) compared with control animals (Michaelis *et al.* 2013). These observations are in contradiction to other findings that typically indicate a higher percentage of motile sperm in fertile characterized animals (Schradin *et al.* 2012). Furthermore, morphological alterations have been detected in FL1 bucks using a flow cytometric assay with propidium iodide staining (Weitzel *et al.* 2013). In the testicular parenchyma of these animals, the percentage of haploid cells is decreased with a concomitantly increased percentage of diploid cells (Michaelis *et al.* 2013). Interestingly, examination of testicular cell-type composition in free-living African striped mice has actually disclosed contrary findings. Corresponding to these data, published by Karin Müller *et al.*, male-dominant and reproductively active African striped mice, which usually live together in colonies with several philopatric, as such reproductively inactive males, exhibit not only

Figure 3 (A) Plasma progesterone concentrations during diestrus in female animals and (B) serum testosterone concentrations in male animals of the fertility lines (FL1 and FL2) and the control line (Ctrl). Data are extracted from the work of Spitschak *et al.* (2007) and Michaelis *et al.* (2013) respectively. Different letters indicate statistically significant differences between lines (Student's *t*-test, $P < 0.05$).

Figure 4 (A) Locomotor activity and (B) latency period in open-field assays for males in both fertility lines (FL1, $n=1013$ and FL2, $n=1033$) and in the control line (Ctrl, $n=819$). Male animals were analyzed on day 70 of life in different generations during the selection period. (A) Distances covered within 3 min were measured in an unknown round surrounding. (B) Initial latency period (in seconds) was analyzed in another open field. (C) Running performance on a treadmill was measured for males of the FLs FL1 ($n=828$) and FL2 ($n=835$) as well as the unselected Ctrl ($n=796$). The endurance fitness was recorded as running distance in meters. Bars represent means $\pm$ S.D. Different letters indicate statistically significant differences between lines (Student's t -test, $P < 0.05$).

an increase in haploid cells as well as in motile sperm concentrations, but also an accompanying decline of the diploid cell portion (Raynaud *et al.* 2012). It might be that different mechanisms are separately and specifically acting to decrease fertility (as in the philopatric mice), to maintain proper fertility (as in the dominant mice), or to induce increased fertility (as in our high-fertility mice).

Additional endocrine blood analyses on FL1 and FL2 bucks have demonstrated dramatically increased total serum testosterone concentrations in FL1 bucks (20.8 ng/ml; sevenfold higher compared with control animals), whereas FL2 bucks exhibited only slightly raised values (4.4 ng/ml) (Fig. 3B). In line with this observation, Leydig cell markers have been found to be elevated in the testes of FL1 bucks (Michaelis *et al.* 2013).

Endocrine and behavioral characteristics

High testosterone concentrations are often correlated with a higher explorative phenotype (Berenbaum & Beltz 2011, Eisenegger *et al.* 2011). Indeed, for our improved FLs, a higher locomotor activity in an open-field experiment could be shown (Renne & Langhammer 2000). Whereas male control animals covered distances

of ~ 41 m within 3 min of analysis, the distance traveled by FL1 animals accounted for ~ 67 m in the same time. FL2 bucks, exhibiting only slightly elevated testosterone concentrations compared with control animals, traveled a distance of ~ 57 m in this assay (Fig. 4A). In a second open-field study, we measured the latency time in an unknown surrounding. This assay revealed a dramatically reduced latency time for FL1 bucks (4.8 s) in comparison with FL2 (9.7 s) as well as control (11.9 s) bucks (Fig. 4B). Thus, male FL1 animals are distinguished by a higher explorative behavior in contrast to FL2 and Ctrl animals.

In another locomotor assay, we tested the endurance fitness of the FL as well as Ctrl males. The running performance, determined on a treadmill (Brenmoehl *et al.* 2013), was lowered in FL animals compared with control animals (Fig. 4C). Additionally, we noticed that the running performance of all the three lines dropped during the selection process (data not shown). We can currently only speculate about the reasons for this observation. Admittedly, testosterone concentrations have been described to increase during endurance training (Santtila *et al.* 2009); however, the inverse correlation appears to be unsustainable. Possibly, the decreased running performance reflects an undesired bias due to simultaneously increasing body weight over the selection period (Fig. 2A and data not shown).

Finally, we examined the life expectancy of female and male animals of the FLs in relation to the unselected Ctrl animals. In this assay, females of FL1 did not show any alteration in life expectancy compared with those of the Ctrl, whereas FL2 females exhibited a reduced life span (Fig. 5A). As changes had occurred to a small extent, females of the FLs in general appear to be properly healthy. However, analyses have been carried out on virgin females. Thus, we cannot predict possible negative effects on health and consequently on life expectancy due to the delivery of extremely large and heavy litters.

By contrast, male animals exhibited a different life expectancy with opposing trends in FL1 and FL2.

Figure 5 Survival rates of male (A) and female (B) animals of the fertility lines (FL1 and FL2) compared with unselected control animals (Ctrl) shown as Kaplan-Meier plots. The experiment initially started with $n=76$ (FL1), $n=97$ (FL2), and $n=104$ (Ctrl) female mice and $n=79$ (FL1), $n=94$ (FL2), and $n=104$ (Ctrl) male mice. Statistically significant differences for FL1 and FL2 animals relative to Ctrl animals are shown as inset (Student's t -test). Animals were housed under standard care conditions.

Interestingly, FL1 bucks exhibited an increased average life span of about 17%, whereas FL2 males exhibited a life expectancy decrease of around 18% relative to control animals (Fig. 5B). Obvious explanations for these findings are unfortunately elusive, but it is noteworthy that extremely high testosterone concentrations in FL1 have not elicited any negative effect on life expectancy. By contrast, high testosterone concentrations seem to contribute to an extended life span. Consistently, investigations in humans indicate that higher testosterone concentrations quite correlate with reduced mortality and therefore improved life expectancy (Araujo *et al.* 2011). Nevertheless, variations in testosterone concentrations in the human study have been subtle alterations within the normal range, whereas FL1 bucks exhibited a sevenfold increased testosterone concentration (Fig. 3B).

Genetic alterations

Our breeding program over 161 generations implies that multiple genetic alterations might have occurred over this time period. Although we have not addressed this issue experimentally so far, it can be assumed that these genomic modifications are rather polygenetic than monogenetic. This hypothesis is stressed by a recent study comparing seven independent outbred mouse lines selected for high body weight. The breeding protocol of these mice is comparable to our study in spite of differing selection criteria. The analysis indicates that a total of 67 genomic regions have been altered in different mouse lines during selection proceeding (Chan *et al.* 2012). Additionally, Diethard Tautz *et al.* could show that the phenotype 'high body weight' based on a smaller subset of genetic variations that had been derived from the pool of 67 genomic regions. Furthermore, it has been striking that each of the seven different mouse lines used another defined subset out of this pool to construct their particular, line-individual genotype (Chan *et al.* 2012). Thus, the same phenotype (high body weight) is warranted using different genotypes (different genetic alterations). Moreover, another recent genetic screening of an ethnic group referred to as 'Hutterites', distinguished by proscribing contraception and large family sizes, allows the suggestion that 41 genomic regions have been selected for this ethnicity as opposed to an unselected control group (Kosova *et al.* 2012). Even though both examinations mentioned above are not easy to transfer to our high-fertility mouse models, we nevertheless would suppose that roughly 50–100 genomic regions have been selected and hence modified in our mice.

Presumably, an alteration in every predisposing genomic region is not required to generate, as well as sustain, the improved fertility phenotype. As demonstrated for FL1 and FL2, high fertility is warranted by substantially different molecular strategies in these

Table 2 Overview of different endocrine, physiological, and behavioral phenotypes of the fertility lines FL1 and FL2.

	FL1		FL2	
	Male	Female	Male	Female
Progesterone		→		↑
Testosterone	↑↑		↗	
Number of ovulated oocytes per cycle		↑↑		↑
Multiple oocytes per follicle		↑↑		→
Embryonic losses		↑		→
Accelerated puberty		↑		↑↑
Body weight	↑	↑	↑	↑↑
Locomotor activity (open field)	↑↑		↑	
Running performance	↘		↘	
Life expectancy	↑	→	↓	↓

Data are shown relative to the control line animals with the corresponding same gender.

two lines. Consequently, it is reasonable to assume that these lines have been created by a variable combination of different genetic alterations deriving from a pool of predisposing and fertility-relevant genomic regions.

Heterogeneity and biodiversity

The several physiological, behavioral, and endocrine varieties in the FLs FL1 and FL2 are summarized in Table 2. On the basis of the preceding considerations for the Dummerstorf long-term selection, high-fertility mouse lines, it has become obvious that the advantages of these mouse models are unquestionably their heterogeneity and biodiversity. Thus, the sophisticated genetic background of these outbred mouse lines can shed light on genetic alterations that are associated with highly fertile phenotypes. In contrast to our heterogenetically enhanced FLs, conventional transgenic or knockout mouse models, which merely employ a single-gene variation, constitute a homogenetic basis. Hence, due to their heterogenetic, apparently realistic nature of fertility, our high-fecundity mouse lines offer valuable cues, also for human reproductive medicine.

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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