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When idiopathic male infertility is rooted in maternal malnutrition during the perinatal period in mice

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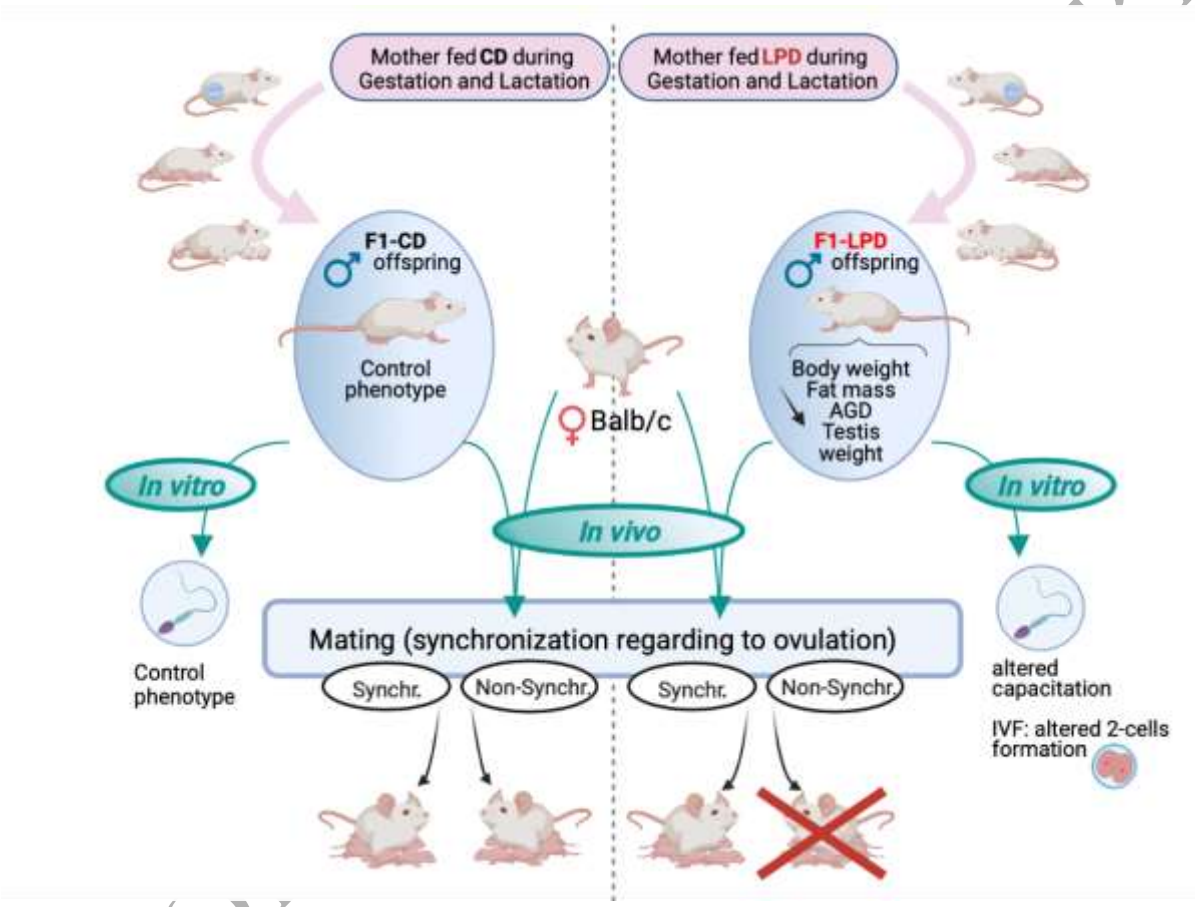
Title:

When idiopathic male infertility is rooted in maternal malnutrition during the perinatal period in mice

Graphical abstract



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Authors and affiliations

Yuki Muranishi [1, #a], Laurent Parry [1,] Mélanie Vachette-Dit-Martin [1], Fabrice Saez [2], Cécile Coudy-Gandilhon [1], Pierre Sauvanet [3,4], David H Volle [5], Jérémy Tournayre [1], Serge Bottari [6], Francesca Carpentiero [6,7], Guillaume Martinez [6,7,8], Jana Muroňová [6,7] Jessica Escoffier [6,7], Alain Bruhat [1], Anne-Catherine Maurin [1], Julien Averous [1], Christophe Arnoult [6,7], Pierre Fafournoux [1*], Céline Jousse [1*].

[1] Unité de Nutrition Humaine, INRAE, Université Clermont Auvergne, UMR1019, F-63000 Clermont-Ferrand, France.

[2] GReD Institute, Université Clermont Auvergne, Inserm, CNRS, Clermont-Ferrand, France

[3] UMR 1071 Inserm/Université Clermont Auvergne; USC-INRA 2018, Microbes, Intestin, Inflammation et Susceptibilité de l'Hôte (M2iSH), Clermont-Ferrand, France.

[4] Service de chirurgie et hépatobiliaire, CHU Clermont-Ferrand, Clermont-Ferrand, France

[5] Inserm U1103, CNRS UMR6293–Université Clermont Auvergne, Institute Genetic, Reproduction and Development, Team "Environment, Metabolism, Spermatogenesis and Pathophysiology & Inheritance", Clermont-Ferrand, France

[6] Université Grenoble Alpes, Grenoble, France

[7] Institute for Advanced Biosciences INSERM U1209, CNRS UMR5309, Grenoble, France

[8] Hôpital Couple-Enfant, Centre Hospitalier Universitaire de Grenoble, UM de Génétique Chromosomique, Grenoble, France

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[#a] Department of Life and Food Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

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* Corresponding Authors:

Email: celine.jousse@inrae.fr (CJ), pierre.fafournoux@inrae.fr (PF)

C. Jousse: https://orcid.org/0000-0002-3146-249X

P. Fafournoux : https://orcid.org/0000-0002-4900-4331

Unité de Nutrition Humaine, UMR1019, INRAE, Centre de Clermont-Ferrand Theix, site de Theix 63122 Saint Genès Champanelle, France.

Short title:

Maternal malnutrition and male offspring fertility

Summary Sentence:

Maternal nutritional stress has long-term consequences on the reproductive health of male progeny by affecting sperm physiology, especially capacitation, with no observable impact on spermatogenesis and classical sperm parameters.

Keywords:

nutritional programming, male fertility, sperm capacitation

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Abbreviations List

AGD	AnoGenital Distance
AGI	AnoGenital Index
BW	Body weight
CASA	Computer-Assisted Semen Analyzer
FACS	Flow Cytometry Cell Sorting
H&E	Hematoxylin and Eosin
IVF	Vitro Fertilization
pg-WAT	perigonadal-White Adipose Tissue
PI	Propidium Iodide

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Abstract

Infertility represents a growing burden worldwide, with one in seven couples presenting difficulties conceiving. Amongst these, 10-15% of the men have idiopathic infertility that does not correlate with any defect in the classical sperm parameters measured. In the present study, we used a mouse model to investigate the effects of maternal undernutrition on fertility in male progeny. Our results indicate that mothers fed on a low protein diet during gestation and lactation produce male offspring with normal sperm morphology, concentration and motility but exhibiting an overall decrease of fertility when they reach adulthood. Particularly, in contrast to control, sperm from these offspring show a remarkable lower capacity to fertilize oocytes when copulation occurs early in the estrus cycle relative to ovulation, due to an altered sperm capacitation. Our data demonstrate for the first time that maternal nutritional stress can have long-term consequences on the reproductive health of male progeny by affecting sperm physiology, especially capacitation, with no observable impact on spermatogenesis and classical quantitative and qualitative sperm parameters. Moreover, our experimental model could be of major interest to study, explain, and ultimately treat certain categories of infertilities.

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Introduction

Infertility is increasing worldwide, and it currently affects about one in seven couples attempting to conceive [1]. In about 50% of cases, infertility is due to the male partner [2]. Meta-analyses [3] indicate that there has been a considerable decline in sperm quality since the 1940s. However, a significant proportion of male infertility is idiopathic and not related to defect in spermatogenesis. Whatever the case, the reason for this infertility is not well understood, and it probably has multifactorial causes. Some authors suggest that reproductive disorders may have a developmental origin [4]. Indeed, a few studies report that, in sheep and rodents, maternal dietary restriction has been linked to impaired reproductive performance in female and male offspring [5,6]. However, the underlying causes are unknown.

The "developmental origins of health and disease" (DOHaD) hypothesis proposes that maternal nutritional status during pregnancy and lactation plays a major role in offspring development and postnatal growth, determining long-term health status [7]. This process, termed nutritional programming, allows specific adaptations to be made that permanently change the phenotype of the organism. These changes persist even in the absence of the early nutritional stress that initiated them, leading to lifelong health consequences.

In humans, a considerable amount of evidence indicates that maternal malnutrition during gestation can confer an increased risk of metabolic dysfunction in offspring [8,9]. In mice, several studies have also shown that maternal nutritional programming has long lasting effect on offspring metabolism [10,11]. However, even though it is now clear that the range of altered phenotypes in the offspring is broader than just metabolic dysfunction [12], a limited number of studies have explored the effect of nutritional programming on reproductive function. Indeed, some studies report that, in

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sheep and rodent, maternal dietary restriction impairs the reproductive performance of female offspring [5,6]. Regarding male testicular development and reproductive performance, it has been shown that in utero protein restriction reduces Sertoli cell number [13] and impairs reproductive capacity [14]. However, the causes of this defect in fertility are not known.

Amongst all the models of nutritional programming available in rodent, we work with a specific model of offspring born from mothers fed an isocaloric Low Protein Diet (LPD) during gestation and lactation. We have previously shown that male offspring have an altered metabolic status in adulthood, displaying lower body weight, lower fat mass and hypermetabolism [15,16]. Here, using the same maternal nutritional model, we investigated the effects of maternal undernutrition on the offspring's fertility and in particular, assessed the hypothesis that fertilization capacity of F1-LPD sperm might be affected. Our results indicate that LPD intake by dams is associated with reduced fertility in male offspring due to altered sperm fitness.

Materials and Methods

Ethics statements

Maintenance of the mice and all experiments were approved by our institutional-animal care and use committee in conformance with French and European Union laws (permission to experiment on mice B63-150, local ethic committee CEMEAA18-13, animal facilities agreement C6334514, project agreement APAFIS#13710-2016122214418591 v7).

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Animal housing and nutrition

All mice were housed individually in polycarbonate, standard mouse cages (31l*20w*15h cm3) containing wood chips bedding and a plastic tube for enrichment and were changed to clean cages every two weeks. The ventilated housing room was maintained at a 12:12 hour light-dark cycle beginning at 8 am, at an ambient temperature of 22 ± 1°C and 60 ± 5% humidity. All mice were fed ad libitum and given free access to water. Experimental diets were manufactured in our institute facilities (INRAE de Jouy-en-Josas, Sciences de l'Animal et de l'Aliment de Jouy – Régimes à Façon, Jouy-en-Josas, France): Control Diet (CD) contains 22% protein and Low Protein Diet (LPD) contains 10% protein as shown Table S1 and previously described in [15]. After weaning at 4 weeks of age, the F1-CD and F1-LPD offspring, born respectively to CD or LPD-fed mothers were single-housed and were given standard chow diet (A03; Safe, Augy, France) throughout life.

Experimental Design

Mating, gestation and lactation: Pairs of 10-week-old Balb/c virgin female mice from Janvier Labs (53941 Saint-Berthevin, France) were mated with a single male. At detection of vaginal plugs, females were randomly allocated into 2 groups and fed experimental diets (either CD or LPD) throughout gestation and lactation.

Design of experimental groups: Litters of different sizes were obtained from each group (CD or LPD) of pregnant female. Since the litter size is important in the offspring life trajectory, our strategy was to erase all possible confounding factors coming from litter size and the allotting protocol was as follow: (1) mice from litter of 3 pups or less or from litter of more than 10 pups were excluded to minimize the variability of each parameter known to be impacted by litter size and we thus considered only litters that have a total number of pups comprised between 4 and 10

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to avoid extreme litter size, (2) at the indicated age, gross anatomy measurements (body weight and anogenital distance) were done on all the offspring obtained for a given mating (F1 animals), (3) for all experiments requiring sacrifice (tissue collection and sperm analysis) and for fertility assessment we constituted subgroups of male offspring from each experimental group (F1-CD and F1-LPD). Males were allotted in subgroups taking into account the following criteria. (i) each of the subgroups has the same representativeness of litter size, (ii) we also took care of having, for each subgroup, a mean body weight representative of the whole experimental group: all subgroups of F1-CD had similar mean body weight in all experiments and all subgroups of F1-LPD had similar mean body weight in all experiments.

Body weight and anogenital distance measurement

Body weight and anogenital distance (AGD) of all offspring were measured at 5, 15, 20 days and 6 months of age for at least 6 individuals as specified in the figures' legends. Body weight was measured using a standard top-loading laboratory balance. AGD (expressed in mm) was measured with a digital caliper from the center of the anus to posterior edge of the genital papilla, by a single investigator in a blind experiment in order to reduce inter-individual variability and experimental bias. Pups were gently restrained, and the anogenital area was subjected to slight tension to impart tautness to the region AGD measurements were corrected by cubic root of body weight (Anogenital index = AGI= $AGD/(BW)^{1/3}$) [17].

Tissue collection for tissue weight measurement and histological analysis

Testis were collected and weighted at 5, 15, 20 days and 6 months of age for at least 6 individuals euthanized by cervical dislocation under isoflurane anesthesia. Perigonadal white adipose tissue, epididymis and seminal vesicles were collected

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and weighted from 6-month-old animals (n=6 per group), euthanized by cervical dislocation under isoflurane anesthesia.

To analyze testicular integrity, testes from 3 animals of 2 months of age were fixed by immersion in 4% paraformaldehyde (PFA) overnight at 4° C, embedded in paraffin, and sectioned (5 µm). For histological analysis, after being deparaffinized slides were stained with hematoxylin and eosin. The colored sections were digitized at ×40 magnification through an Axioscope microscope (Zeiss, Germany) equipped with a motorized X-Y-sensitive stage. In order to evaluate spermatogenesis, the whole area and the lumen area of round cross sections were measured and the % of lumen area/total area was calculated.

Sperm morphology analysis.

Sperm were collected from 8-month-old males (n=3 per group) by pressure on cauda epididymis in 1.0 mL of non-capacitating WH medium (no-capa-WH, as described below) and then counted using a Malassez hemocytometer. Sperm were then washed twice in PBS and displayed over slides, dried at room temperature, and then fixed in 75% ethanol for Harris–Shorr staining. At least 100 sperm cells were analyzed per sample.

Fertility evaluation

F1 Male fertility with non-synchronized females. Adult F1-CD (n=7) and F1-LPD (n=9) male mice aged 4 months were caged with females from the animal supplier (Janvier Labs) at a 1:2 male to female sex ratio for 5 days. Plugs were checked every day and plugged females were removed and housed individually until parturition. The total number of females was 28 females for F1-CD males and 33 females for F1-LPD males distributed in 2 rounds of mating. Plugged and pregnant females were counted

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to calculate the frequency of copulatory plug and frequency of conception. Offspring were counted for litter size determination only for the first round of mating (11 litters from F1-CD males and 8 litters from F1-LPD males).

F1 Female fertility. Adult F1-CD (n=16) and F1-LPD (n=12) 4-month-old virgin female mice were caged with males from the animal supplier (Janvier Labs) at a 1:2 male to female sex ratio for 5 days. Plugs were checked every day and plugged females were removed and housed individually until parturition. The number of total females, plugged females, litters and offspring was counted to calculate the frequency of copulatory plug and frequency of conception.

F1 Male fertility with synchronized/superovulated females. Two-months-old Balb/c females from the animal supplier (Janvier Labs) were synchronized and superovulated using intraperitoneal injection of 5 units of Pregnant Mare Serum Gonadotrophin (PMSG) at 3 pm on day 1, followed by 5 units of human Chorionic Gonadotropin (hCG) 48 h later (at 3 pm on day 3). Ovulation occurs 12h following the hCG injection (at 3 am on day 4). Protocol (A): Adult (4 months of age) F1-CD and F1-LPD male mice (n=5 per group) were caged with synchronized/superovulated females at a 1:1 male to female sex ratio for 15 hours the night from day 1 to day 2 (from 6 pm day 1 to 9 am day 2, i.e. from 42 h to 57 h before ovulation). Protocol (B): Adult (4 months of age) F1-CD and F1-LPD male mice (n=10 per group) were caged with synchronized/superovulated females at a 1:2 male to female sex ratio for 15 hours the night from day 3 to day 4 (from 6 pm day 3 to 9 am day 4, i.e. from 9 h before ovulation to 6 h after ovulation). In both cases, plugs were checked the morning after and plugged females were removed and housed individually until sacrifice 14 days later.

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Sperm capacitation analysis

Capacitation media. The basic medium used throughout these studies for sperm preparation and in vitro capacitation was a modified Krebs Ringer medium (Whitten's-HEPES buffer (no-capa-WH) containing 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 1 mM pyruvic acid, 4.8 mM lactic acid, 20 mM HEPES, pH 7.4). Capacitation medium (capa-WH) contained 5 mg/mL fatty-acid-free BSA (Sigma-Aldrich), 2 mM $CaCl_2$ and 20 mM $NaHCO_3$ in WH.

Sperm collection. For all sperm analysis experiments, sperm were collected from 4- to 8-month-old males by pressure on cauda epididymis in 1.0 mL of no-capa-WH medium and then counted using a Malassez hemocytometer

Analysis of capacitation by Western blot. Sperm collected from 3 males (4-month old) per group were incubated in capacitation medium at the final concentration of 15×10^6 cells/mL during 0, 10, 30, 60, 90 or 120 min at 37°C, 5% CO_2. At each time point, 0.75×10^6 cells were collected, centrifuged at 500 g for five minutes, washed with 300 µL of phosphate buffered saline (PBS), centrifuged at 500 g for five minutes and then resuspended in Laemli buffer without ß-Mercaptoethanol and boiled for five minutes. After a centrifugation at 7000 g for five minutes, the supernatant was collected, boiled in the presence of 5% ß-Mercaptoethanol (Sigma) for five minutes and then subjected to SDS-PAGE and transferred on nitrocellulose membrane (GE Healthcare #RPN3032D). Blots were blocked with Tris Buffered Saline (50 mM Tris, 15 0mM NaCl) containing 0.1% v/v Tween 20 and 10% w/v low-fat dried milk. Membranes were probed overnight at 4°C with anti-phosphotyrosine antibody (1/1000, 05-321, clone 4G10, Merck Millipore) in blocking solution. After washing, membranes were incubated with the following secondary antibodies: anti-mouse horseradish peroxidase-conjugated (HRP) (Cell Signaling Technology # 7076S,

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1/5000). Detection was performed using the Immobilon Western Chemiluminescent HRP substrate (Millipore Immobilon Crescendo Western HRP substrate # WBLUR0500) on Syngene G:BOX camera, and densitometric analyses were carried out using ImageJ software. Normalization was performed by reprobing the blots using anti-Tubulin antibody (Cell Signaling Technology # 2144S, 1/1000), in blocking solution. After washing, membranes were incubated with the following secondary antibodies: anti-rabbit horseradish peroxidase-conjugated (HRP) (Cell Signaling Technology # 7074S, 1/5000).

Sperm Analysis by Flow Cytometry

Sperm from cauda epididymis of 8-months-old males (n=3 per group) were allowed to swim in no-capa-WH buffer (see above) for 10 min at 37°C.

These sperm were then incubated in capa-WH for 1, 2 or 4 hours at 37°C, 5%-CO_2. Before assaying the sperm by flow cytometry, sperm suspensions were filtered through a 100 µm nylon mesh and 2.1 µM of propium iodide (PI) was added. Analyses were conducted using a MACSQuant® Analyzer 10 (Miltenyi Biotec). PI was excited using a 488 nm argon excitation laser. Nonviable cells became PI positive, and their red fluorescent signal detected as fluorescence of wavelength 670. Non-sperm events were gated out from analyses by judging the events' scatter properties, as detected in the forward-scatter (FSC) and sideways-scatter (SSC) detector [18]. Recording of scatter and fluorescent properties of all events stopped when 50 000 events were reached. Two-dimensional plots of SSC and FSC properties, as well as of PI were obtained using MACSQuantify™ software and used to calculate the percentage of PI positive spem cells.

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Sperm motility analysis and In Vitro Fertilization (IVF)

Sperm collection. For all sperm analysis experiments described below, mouse sperm, obtained from 4-month-old males by manual trituration of cauda epididymis in 1 mL M2 were allowed to swim out for 10 min at 37 °C then counted using a Malassez hemocytometer. Sperm were then capacitated in M16 medium containing 2% fatty acid-free bovine serum albumin (BSA) at 37 °C in a 5% CO_2 incubator at a concentration of $2*10^6$ sperm cells /mL for either 1 h (for motility analysis) or 12 h (for IVF)

Sperm motility analysis. Before capacitation or after 1 h or 12 h capacitation, sperm suspensions from F1-CD and F1-LPD (n≥4) males were loaded on a 20 µm chamber slide (Leja Slide, Spectrum Technologies) and placed on a microscope stage at 37 °C. Sperm parameters such as sperm count, progressive motility and hyperactivated sperm, VAP (Average Path Velocity= average track distance of sperm head per unit time), VSL (Straight Line Velocity= total gain of space by the sperm head per unit time), VCL (Curvilinear Velocity= the total distance travelled by the sperm head per unit time), ALH (Lateral Amplitude of Head displacement= width of sperm head movement), STR (Straightness= VSL/VAP) and LIN (Linearity= VSL/VCL) were examined using a computer-assisted semen analysis (CASA) system (Hamilton Thorne Research, Beverly, MA).

In vitro fertilization and embryo development. Oocytes were collected from mature Balb/c females, synchronized by exposure to 5 units of pregnant mare serum gonadotropin (PMSG) and 5 units of human chorionic Gonadotropin (hCG). After 12 h capacitation, sperm collected from F1-CD (n=4) and F1-LPD (n=4) males were washed with M16 then introduced into droplets containing >10 oocytes. Oocytes were incubated with 0.5×10^6 capacitated sperm (100 µL) (37° C, 5% CO_2) in M16

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medium, and unbound sperm were washed away after 4 h incubation. Oocytes were washed and transferred to potassium simplex medium (KSOM containing NaCl, 5.55 g/l ; KCl, 0.19 g/l ; KH_2PO_4, 0.05 g/l ; $MgSO_4 \cdot 7H_2O$, 0.05 g/l ;Glucose, 0.04 g/l ; Penicillin G, 0.06 g/l ; Streptomycin, 0.05 g/l ; Sodium lactate, 1.12 g/l ; $NaHCO_3$, 2.1 g/l ; Sodium pyruvate, 0.02 g/l ; $CaCl_2 \cdot 2H_2O$, 0.25 g/l ; EDTA, 0.004 g/l ; L-glutamine, 0.146 g/l ; Bovine serum albumin, 1 g/l) for culture to later stages. The percentage of the 2-cell embryos examined 24–30 h later was regarded as an indication of successful fertilization.

Statistics

Statistical analyses were performed using GraphPad Prism software (version 9; San Diego, CA). Data were represented as the mean ± SEM. Student's t-test was used for comparison between 2 groups. P-values ≤ 0.05 (*), 0.01 (**), or 0.001 (***) were considered to indicate statistically significant differences. (^{ns}) indicates a p-value > 0.05 (non-significant). For Fig 1B, 4 and S2, statistical significance was calculated using a Khi-squared test.

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Results

Maternal undernutrition alters fertility in offspring

Female Balb/c mice were fed either a control diet (CD) or a Low Protein Diet (LPD) throughout pregnancy and lactation. After weaning, the F1-CD and F1-LPD offspring – born and suckled from CD- or LPD-fed mothers, respectively – were fed a standard chow diet. At the age of six months, both male and female F1-LPD exhibited a lower body weight than their F1-CD counterparts (Fig 1A). As previously published [15], this weight difference is observed throughout life (Fig S1A). It is also noteworthy that, the body weight difference is observed for each litter size into the range of litter size considered (From 4 to 10 F1 pups per litter) (Fig S1B). Moreover, male F1-LPD offspring had a significantly lower perigonadal-White Adipose Tissue weight (pg-WAT). Even though a similar pg-WAT difference was observed in F1-LPD females, due to greater interindividual variability in females, the difference was not significant (Fig 1A). This gross anatomical phenotype was also associated with a shorter AnoGenital Distance (AGD) in F1-LPD compared to F1-CD males. AGD is a marker of differentiation of male and female external genitalia, and a lower AGD is commonly used as a predictive indicator of diminished reproductive function in the mature male [19,20] and female [21]. To adjust AGD to compensate for differences in weight between animals, Gallavan et al. [17] reported that the cube root of body weight would be an appropriate standard to use. This corrected factor called AGI (AnoGenital Index), was also significantly reduced in F1-LPD. Interestingly, maternal undernutrition affected neither AGD nor AGI in F1-LPD females (Fig 1A).

In light of the observation of indicators of potential fertility problems, we compared fertility rates for both F1-CD and F1-LPD animals. Firstly, adult F1-CD or F1-LPD

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males were mated with control females. We observed no difference in the number of plugged females relative to the total number of mated females between the two groups. Thus, copulation occurred at the same rate for F1-LPD and F1-CD males. However, the fertility rate recorded (calculated as the percentage of plugged females that went on to develop a pregnancy) was significantly reduced for F1-LPD males (63%) compared to F1-CD males (92%) (Fig 1B). Interestingly, F2 litter size was not significantly different between the two groups (Fig 1C). Based on these results, we can conclude that the reduced fertility observed for the F1-LPD males was not the result of a lack of copulation.

Secondly, given that maternal nutritional stress may affect fertility in female offspring through several mechanisms (such as gonad development alteration and/or metabolic/endocrine alterations that could produce an inadequate metabolic environment for optimal pregnancy), adult F1-CD or F1-LPD females were mated with control males. Surprisingly, fertility in F1-LPD females was only slightly lower than in F1-CD females, and the difference was not statistically significant (Fig S2). In absence of significant difference in female fertility, we decided to focus our investigations on the causes of the decline in male fertility only, for the remainder of this study

Maternal protein malnutrition effects on male sexual organ development and sperm count

To assess whether maternal undernutrition alters the male reproductive system, we measured the mass of sexual organs in adult mice. Fig 2A shows that the masses of testis and epididymis from adult F1-LPD were significantly lower than those from F1-CD. On the other hand, the weight of the seminal vesicles was not strongly affected. It is generally accepted that testes weight varies little between individuals

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from the same species [22]. However, to determine whether the significantly lower testis weight recorded for F1-LPD compared to F1-CD could be linked to the lower body weight observed, we calculated the testis/body weight ratio for Balb/c mice. Fig S3A (left panel) shows that, across a large group of mice raised without nutritional stress, there was no correlation between the weight of the animal and the testis weight whereas when comparing F1-CD and F1-LPD males, testis mass is correlated with body weight (Fig S3B, left panel). These data suggest that the smaller testis weight recorded for F1-LPD is not due to their reduced overall stature, but is a consequence of the nutritional stress experienced during early life. As a counter example, our data revealed a strong correlation between the animals' weight and that of their epidydimal adipose tissue (Fig S3A and B, right panel).

Based on these findings, we explored the hypothesis that the gross anatomy defect observed (i.e. testis mass and AGD differences) occurred during the period of nutritional stress. Indeed, in males, testicular cell lines differentiate, proliferate, and mature during postnatal life, particularly during suckling. These processes are crucial in establishing future reproductive capacity. Stresses experienced during suckling could thus impair fertility in adult animals [23]. Fig 2B shows that body weight was significantly lower in F1-LPD animals from 5 days after birth. However, significant differences in AGD (Fig 2B) and testis weight (Fig 2C) were only observed after 15 days. These observations suggest that the lactation period could be critical in determining the impact of maternal nutrition on the future reproductive capacity of male offspring.

Because we observed weight differences for F1-LPD testes, we assessed the spermatogenesis of F1 males by semen analysis and by observing the structure of seminiferous tubules by histology. Sperm collected from the cauda epididymis had

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similar cell morphology (Fig 2E), were present in equivalent numbers (Fig 2D) and presented a similar percentage of abnormal cells (F1-CD: 50.33%; F1-LPD 50.67%) between F1-CD and F1-LPD males. Moreover, we did not notice the presence of round cells in the epididymis extracts, likely suggesting the lack of spermatogenesis distress. These results were reinforced by histological analysis of testis sections, showing no differences between the two groups of mice (Fig 3). We focused our attention on different parameters including the area of tubule, the % of lumen /total area (Fig 3F) and the presence of tubule sections at different stages, and in particular the presence of different organized layers of spermatocytes, round spermatids (RS) and elongated spermatids (Spt) and finally the presence of testicular sperm within the lumen of the tubule (Spz) as a witness of the spermatogenesis progress (Fig 3A-D). Overall, based on histological studies and semen analysis, spermatogenesis is not altered in F1-LPD males.

Maternal protein malnutrition reduces the duration of sperm fertility in the female reproductive tract

Given that the reduced fertility observed for the F1-LPD males seemed to be neither the result of a capacity to copulate, nor of a problem with sperm production, it is reasonable to hypothesize that sperm functions might be affected. For a given species, the duration of sperm cells capacity to fertilize while in the female reproductive tract is adapted to the interval between the onset of estrus and ovulation, allowing sperm to survive in a fertilization-competent form until ovulation occurs [24]. In mice (as it is the case in human), males mount females regardless of their estrus state [25]. We therefore considered whether the timing of copulation, relative to the female's estrus cycle, could be a determinant factor in the reduced fertility observed for F1-LPD males. We speculated that the fertilizing capacity of the

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F1-LPD male semen might be affected by the interval between copulation and ovulation. To test this hypothesis, we synchronized ovulation in control females by gonadotropins treatment. In a first experimental procedure (Fig 4, protocol A), males were mated with females overnight (i.e., over a short time window) to allow copulation only at the time of the hormonally-stimulated ovulation (indicated by a red lightning bolt). In this set-up, no differences in the rate of female pregnancy were observed between F1-CD and F1-LPD males, thus sperm produced by F1-LPD males can fertilize oocytes. In contrast, in a second experimental procedure (Fig 4, protocol B), males were mated with females in a copulation window of 57 h to 42 h before ovulation (ovulation occurring at T0). In these conditions, the F1-CD males could still successfully fertilize the females, but the capacity of the F1-LPD males to do so was severely compromised. These data show that F1-LPD sperm could no longer fertilize oocytes if they remained in the female reproductive tract for too long before ovulation indicating that the fertilizing capacity of the sperm cells produced by F1-LPD males decreased rapidly once in the female reproductive tract.

Maternal protein malnutrition alters capacitation process

Sperm are maintained inactive in the epididymis for relatively long periods of time (1-2 weeks, depending on the species). When sperm are released into the female reproductive tract, the change in environment acts as a physiological trigger, and sperm start the capacitation process. This process, necessary to allow oocyte fertilization [26,27], involves remodeling of lipids and proteins [28], resulting in alterations to membrane fluidity [29]. Capacitation is also associated with an increase in oxidation levels that may affect the sperm's lifespan (DNA and lipid damage lead to a loss of sperm motility and a commitment to cell death) [30,31].

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To understand the underlying causes of the observed alteration in fitness of F1-LPD sperm, we first used a Computer-Assisted Semen Analyzer (CASA) to assess physiological parameters in sperm isolated from the cauda epididymis. As shown in Fig 5A, after 1 h of in vitro capacitation, the percentage of motile F1-LPD sperm cells was significantly decreased compared to F1-CD samples whereas no such difference was observed before capacitation. Moreover, the percentage of hyperactivated sperm cells was also significantly decreased in F1-LPD compared to F1-CD. None of the others kinetics parameters measured were affected (Fig S4). These data led us to conclude that, sperm cell motility is no different between the two groups before the start of capacitation, but that differences appear in response to capacitation signals. However, this difference is abolished after long capacitation process (12h). From these observations, we hypothesized that in vitro capacitation process either (i) leads to a premature commitment of F1-LPD sperm to cell death and/or (ii) is less efficient in F1-LPD sperm compared to F1-CD. In order to explore this hypothesis, we accessed sperm cell viability following in vitro capacitation. Viability of sperm cells was performed by Flow cytometry analysis following a kinetic of 0, 1 h, 2 h and 4 h of in vitro capacitation. The percentage of PI positive sperm cells (Fig S5) did not show any significant differences between groups.

For assessing capacitation, we measured and analyzed the level of protein tyrosine phosphorylation (a hallmark of capacitation) from F1-CD and F1-LPD sperm. Fig 5B shows that the intensity of the bands representing tyrosine-phosphorylated proteins in F1-CD sperm extracts was maximal after 60 min incubation in capacitating medium. Interestingly, although the kinetic profile for tyrosine phosphorylation was no different for F1-LPD sperm extracts, the phosphorylation intensity was significantly

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lower after 60 min. These results indicate that the capacitation process was strongly affected in F1-LPD sperm cells.

Capacitation is essential to successful oocyte fertilization, and any defects in this process compromise the fertilization competence of sperm. Overall, our results strongly suggest that F1-LPD sperm are less fit, thus their capacity to fertilize may be reduced in challenging conditions. To test this hypothesis, we determined how many two-cell embryos were obtained upon In Vitro Fertilization (IVF) of oocytes with either F1-CD or F1-LPD sperm. In these experiments, the percentage of 2-cell embryos is regarded as the in vitro fertilization rate. We chose to incubate sperm in a capacitation medium for a long challenging time (12 h) before mixing gametes as a simulation of what occurs in the genital female tract in vivo when copulation takes place a long time before ovulation. Fig 5C shows that after 12 h of in vitro capacitation, the in vitro fertilization rate was strongly reduced for F1-LPD compared to F1-CD sperm. Thus, after a long in vitro capacitation step, F1-LPD sperm were less capable of fertilizing oocytes.

Overall, our data show that a low maternal dietary protein intake during gestation and lactation is associated with altered sperm fitness in the offspring, resulting in a lower capacity to successfully fertilize oocytes when copulation takes place early before ovulation.

Discussion

Our results indicate that maternal nutritional stress during gestation and lactation decreased fertility in male offspring. First, we have shown that F1-LPD male offspring exhibit a shorter AGD associated with a smaller testis weight. AGD is commonly presented as a marker of early life hormonal environment suggesting either that

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offspring may have been exposed to an altered maternal hormonal environment due to LPD feeding or that their own hormonal levels are affected. AGD is mostly regulated by testosterone from the fetal testes. Several studies, mainly from rats and sheep suggest that testosterone level can be affected by maternal malnutrition [6,14,32] but complex results, that appear to be contradictory, make it difficult to interpret. For example, Genovese et al. [33] have shown, in rats, that perinatal nutritional stress leads to a small number of Sertoli cells, whose development is dependent upon testosterone level. Toledo et al. [13] have shown a slight reduction in serum testosterone but without any statistically significant reduction whereas for Leonhardt et al. [34], maternal food restriction during the perinatal period in rat did not affect testosterone concentrations. It is thus not clearly established whether or not testosterone level is affected in models of maternal nutritional stress. Interestingly, in our model, we did not observe any effect on testis structure (no difference in H&E staining of testis slice as shown Fig 3). This strongly suggests that testosterone concentration is as a minimum sufficient for normal spermatogenesis.

Sperm capacitation is altered in F1-LPD sperm

Most importantly, we show that sperm fertilizing competence is altered due to an important capacitation defect. The capacitation process corresponds to the various changes that are required for sperm cells to become capable of fertilizing oocytes. It occurs as sperm pass through the female reproductive tract. As sperm are transcriptionally and translationally inactive, all the changes leading to capacitation are controlled by complex signaling cascades involving post-translational modifications of proteins (phosphorylation) and redox processes [35]. In sperm from F1-LPD males, both the overall intensity of tyrosine phosphorylation and the proportion of hyperactivated sperm cells following in vitro capacitation were

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decreased. These observations suggest a defect in the sperm's response to the capacitation signal, which may be caused by several factors, acting in isolation or in combination.

Deregulation of the redox balance: whereas physiological levels of ROS are vital for optimal sperm function, when present at excess levels, ROS adversely affect sperm quality and function, ultimately resulting in infertility [30,31]. Interestingly, our previous studies indicated that mitochondrial function is affected in F1-LPD animals. Indeed, they present increased mitochondrial function in skeletal muscle and increased mitochondrial density in White Adipose Tissue [16]. Whether this increase in mitochondrial function also occurs in F1-LPD sperm cells (and participate to oxidative stress) has yet to be addressed. Gene expression data (not shown) indicate that antioxidant enzymes such as *Gpx1*, *Gpx3* and *Sod3* are slightly increased (around 1.3-fold each, with p-value = 0.03; 0.08; 0.03 respectively) in the epididymis of F1-LPD animals. This increased expression could be due to a defensive response to oxidative stress. As mentioned above, oxidative stress and excessive ROS production may affect sperm parameters such as DNA damage, motility or commitment toward apoptosis (see [36,37] for review). DNA damage in sperm is a defect that is mostly associated with an increased incidence of miscarriage due to developmental defects in the offspring [38]. Nevertheless, in our model, we did not observe any major developmental defect since 1) F2 offspring number is not affected, 2) IVF results show that the defect lies at the fertilization step since the 2-cells stage is impaired. Oxidative stress could also lead to premature commitment toward apoptosis [30]; however, we did not observe any difference in cell viability between F1-CD and F1-LPD sperm (Fig S5). On the other hand, motility, which is affected by oxidative stress [39] is altered in F1-LPD sperm following in vitro

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capacitation (Fig 5A), suggesting that sperm from F1-LPD males may suffer of increased oxidative stress. Nevertheless, whether this alteration is the result of excess oxidative stress remains to be demonstrated.

Low leptin expression: The adipokine leptin, a well-characterized metabolic actor, has also been shown to play an important role in several aspect of the control of fertility. We have previously shown that in our model, the leptin plasmatic concentration is strongly decreased in F1-LPD animals [15]. First, leptin plays a crucial role on the onset of puberty. Indeed, it has been shown that leptin signaling may be involved in puberty delay observed in pups of both sexes from mothers exposed to food restriction during both gestation and lactation [34]. However, our experiments were realized far after puberty (between 4 to 8 months) and whether this phenomenon occurs in our model should not impact our results.

Moreover, leptin has been shown to influence sperm capacitation by enhancing cholesterol efflux and protein tyrosine phosphorylation [40]. It could thus be hypothesized that leptin could affect sperm capacitation through its effect on lipid metabolism. Nevertheless, leptin may only act as an aggravating in vivo factor, since capacitation defects were also observed in vitro, where leptin is absent.

Duration of Sperm fertility of F1-LPD males is compromised

For a given species, the duration of sperm capacity to fertilize correlates directly with the interval between the onset of estrus and ovulation. In normal conditions, successful fertilization relies on heterogeneity among sperm cells ("sperm heterogeneity"). Since capacitation is a time-limited process, various sperm subpopulations coexist and capacitate at different times [41,42] (Illustrated by the theoretical scheme shown Fig 6). This particularity responds to a physiological need to ensure that sperm will remain fertilization-competent until ovulation takes place.

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We have clearly shown that F1-LPD males were unable to fertilize females if copulation was not timed to coincide with ovulation. Similarly, IVF experiments showed that the percentage of sperm cells that lose their fertilizing competence following a long capacitation (12 h) is higher for F1-LPD than F1-CD sperm.

Altogether, these results demonstrate that maternal nutritional stress affects sperm physiology and particularly the in vivo duration of sperm capacity to fertilize, but has no effect on spermatogenesis per se. We can hypothesize that the altered sperm capacity to fertilize may be due to (i) defective capacitation once in the female reproductive tract and/or (ii) decreased "sperm heterogeneity" due to a lack of the subpopulations of sperm cells showing delayed capacitation (a theorical representation of the "sperm heterogeneity" is given Fig 6).

Seminal vesicle secretions role has been proposed as a key player in supporting sperm fertilizing ability (as reviewed in [43]). However, it does not seem to be the case in our model since (i) F1-LPD males did not show copulatory plug defects and (ii) the subfertility phenotype is observed even in an in vitro protocol after collecting sperm in the epididymis and in absence of any seminal vesicle secretion. It can thus be hypothesized that the sperm cell by itself is at least in part the support of the defect that is observed.

Whether or not the capacitation defect observed in F1 animals is transmitted to F2 males is yet to be explored and if so, the mechanism involved has to be identify. Among the epigenetic mechanism that may be involved, the role of DNA methylation on sperm epigenetic inheritance is still controversial [44]. However evidences exist for a role of miRNA in transmission of metabolic phenotype [45].

In humans, it is important to note that 10 to 15% of men who consult for difficulties conceiving present an idiopathic infertility, without defects in the classical sperm

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parameters measured, including sperm morphology and concentration, DNA compaction and DNA breaks and sperm motility [46]. Sperm heterogeneity and sperm physiology are very rarely assessed and in particular sperm capacitation is never measured. However, any defects affecting such parameters (as it is the case in our experimental model) could play a role in idiopathic infertility.

Interestingly, Hua et al. [47] have identified in human undergoing IVF treatment that sperm sample presenting normal classical semen parameters could generate high rate or low rate of good quality embryo. Moreover, they identified small non coding RNA (sncRNA) that are differentially expressed in sperm leading to high-rate vs low-rate of good quality embryo. These sncRNA may be potential clinical biomarkers for the assessment of sperm quality in IVF [47]. In that context, the use of our mouse model would be a tool of choice for the identification of molecular mechanisms involved in the establishment and maintenance of the imprint leading to idiopathic infertility for example, through sncRNA rescue experiments. Ultimately this model could allow to study these categories of infertilities in humans.

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Conflict of interest

The authors declare that they have no conflict of interests

Authors' contributions

YM, CA, PF, CJ contributed to the conception and design of the study. JE, GM, FC, YM, LP, MVDM, FS, CCG, PS, CJ, JM performed the experiments. JT and SB provided reagent, materials or analysis tools. Data were analyzed by YM, CA, PF, CJ, FS, DV, JE, GM, JM. The article was written by PF and CJ and critically revised by CA, AB, ACM, JA.

Ethics approval

Maintenance of the mice and all experiments were approved by our institutional-animal care and use committee in conformance with French and European Union laws (permission to experiment on mice B63-150, local ethic committee CEMEAA18-13, animal facilities agreement C6334514, project agreement APAFIS#13710-2016122214418591 v7).

Data Availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Figure's legends

Fig 1. Reproductive characterization of F1 offspring. (A) Phenotypic characterization of F1 offspring. Body weight, peri-gonadal White Adipose Tissue mass (pg-WAT), AnoGenital Distance (AGD) and AnoGenital Index (AGI) are given for F1-CD and F1-LPD adult males and females at 6 months of age. AGI is defined as $AGD/(BW)^{1/3}$. Results are expressed as means +/- SEM for at least 6 individuals per group and student's t-test was used to test the significance (** p-value < 0.01, * p-value < 0.05, ns= non-significant). For non-invasive measures (BW, AGD and AGI) similar data obtained from a large number of individuals have not been included in the figure. **(B)** Fertility evaluation of F1-CD and F1-LPD males when mated with Balb/c females. F1-CD (n=7) and F1-LPD (n=9) males (blue bars) were caged with non-synchronized Balb/c females (pink bars) at Day 1 (D1) at a 1:2 male to female ratio for 4 days (until D5). Vaginal plugs were checked every day (●) and plugged females were removed and isolated until delivery (◖). For each group, the number of males tested, total females, plugged females and pregnant females were indicated in the table. Fertility rates are calculated as the percentage of plugged females that are pregnant. Statistical significance was calculated using a Khi-squared test. * indicates a p-value < 0.05, ns= non-significant. **(C)** Litter size of offspring obtained after mating of F1-CD and F1-LPD males with non-synchronized Balb/c females. Each dot represents the litter size of offspring born to one Balb/c Female mated with either F1-CD or F1-LPD males. Results are expressed as mean +/- SEM and student's t-test was used to test the significance (ns= non-significant).

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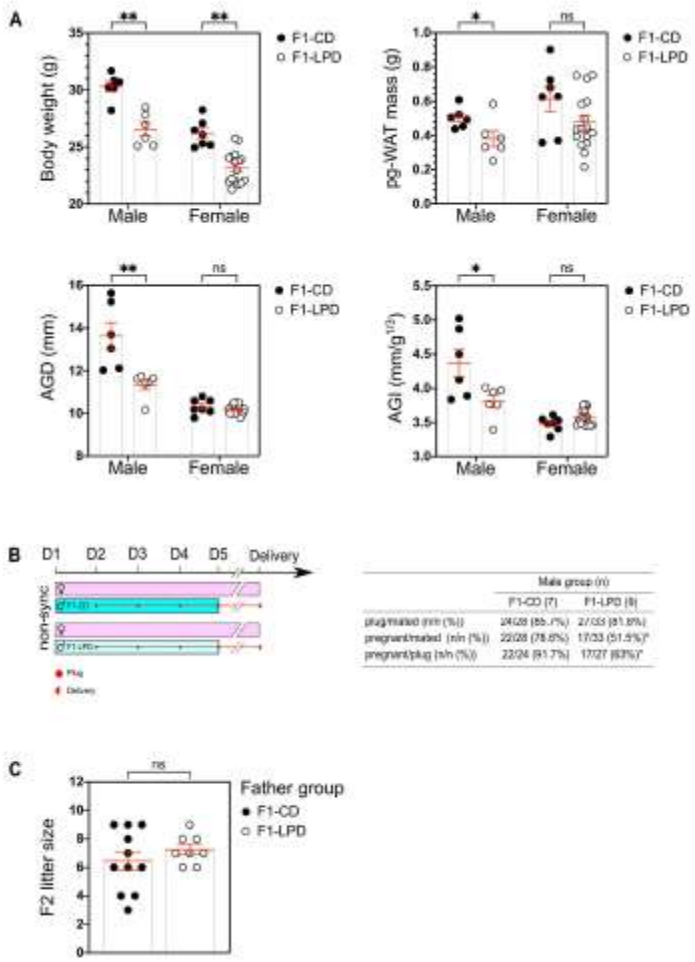

Fig 2. Reproductive organs analysis. (A) Reproductive organ masses of F1 adult males. Testis, Epididymis (Epid.) and Seminal Vesicles (Sem.Ves) masses are given for F1-CD and F1-LPD males at 6 months of age. Results are expressed as means +/- SEM for at least 6 individuals per group and. **(B)** Body weight (g) and AnoGenital Distance (AGD, mm) of F1-CD and F1-LPD males during suckling period. Body weight and AGD are given for F1-CD and F1-LPD males at 5, 15 and 20 post-natal day. Results are expressed as means +/- SEM for at least 6 individuals per group **(C)** Testis mass of F1-CD and F1-LPD males during suckling period. Testis mass are given for F1-CD and F1-LPD males at 1, 15 and 20 post-natal day. Results are expressed as means +/- SEM for at least 6 individuals per group and **(D)** Sperm count. Number of sperm cells following isolation from the cauda epididymis of 2-

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month old mice are expressed as millions of sperm cells per mL (M/mL). Results are expressed as means +/- SEM for 6 individuals per group. **(E)** Harris-Shorr staining of sperm from F1-CD and F1-LPD males. Scale bar = 10 µm. **(A-D)** student's t-test was used to test the significance (** p-value < 0.01, * p-value < 0.05, ns= non-significant).

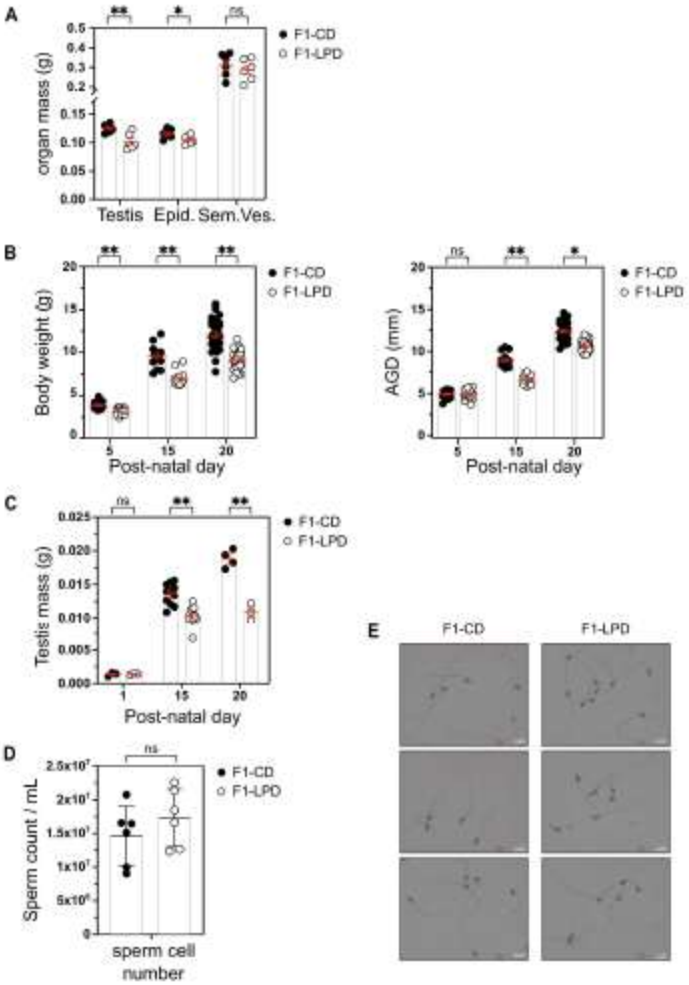

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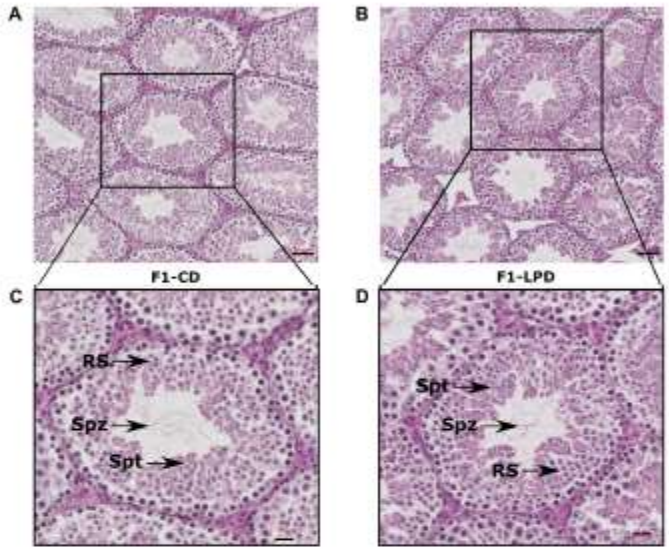
Fig 3. Histological analyses of F1-CD and F1-LPD's testis sections. (**A**) Representative testis sections from F1-CD and (**B**) F1-LPD males stained with hematoxylin/eosin. (**C, D**) Enlargements of squares drawn in A and B, respectively. Spermatogenic cells (RS for round spermatids and Spt for elongated spermatids) and mature sperm (Spz) are indicated. Scales bars for each enlargement are A, B=50µm, C, D= 20µm. (**E**) Seminiferous tubules area (µm^2) and % of lumen area/total area were measured for round cross sections of n>20 tubules per testis section. Data are expressed in the table as mean +/- SEM for n=4 and n=3 testis section respectively for F1-CD and F1-LPD. Student's t-test was used to test the significance.



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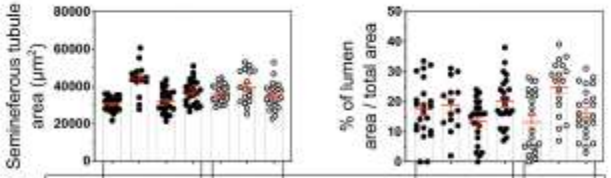

	F1-CD	F1-LPD		F1-CD	F1-LPD
Mean	35861	36948	Mean	17%	18%
SEM	±2976	±1396	SEM	±1%	±4%
p-value		0.8	p-value		0.8

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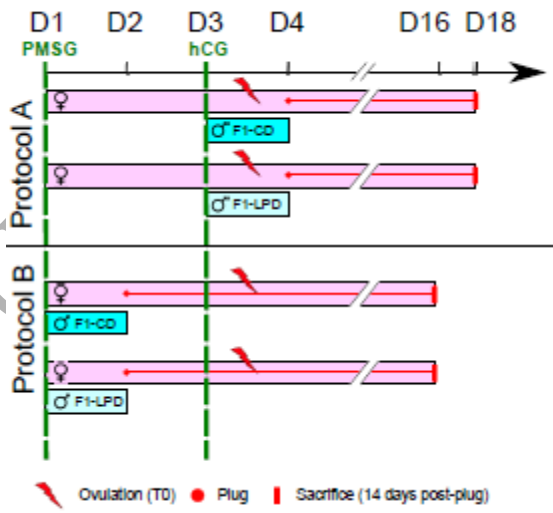
Fig 4. Fertilizing capacity evaluation of F1-CD and F1-LPD males mated with synchronized Balb/c females. F1-CD and F1-LPD males (blue bars) were caged with Balb/c females (pink bars) at a 1:1 (Protocol A) or 1:2 (Protocol B) male to female ratio for 1 day according to 2 different protocols. In both cases, females were hyper-ovulated and synchronized by intraperitoneal injection of PMSG (at D1, green dashed line) followed by hCG 48 h later (at D3, green dashed line). Ovulation takes place the night following hCG injection, i.e. between D3 and D4, characterized by a red lightning bolt. In protocol A, males and females were caged together during the ovulation period, between D3 and D4. In protocol B, males and females are caged together between D1 and D2, about 48 h before ovulation. For both protocols, vaginal plugs were checked (●) at the end of the copulatory period and plugged females were isolated for 14 days. Females were then sacrificed (❙) in order to evaluate pregnancy. For each group and each protocol, the number of males tested, total females, plugged females and pregnant females were indicated in the table. Fertility rates are calculated as the percentage of plugged females that are pregnant. Statistical significance was calculated using a Khi-squared test. * indicates a p-value < 0.05.



	Synchronization	Male group	
		F1-CD	F1-LPD
Protocol A	Male tested (n)	5	5
	plug/mated (n/n (%))	5/5 (100%)	5/5 (100%)
	pregnant/mated (n/n (%))	5/5 (100%)	5/5 (100%)
	pregnant/plug (n/n (%))	5/5 (100%)	5/5 (100%)
Protocol B	Male tested (n)	10	10
	plug/mated (n/n (%))	13/20 (65%)	14/20 (70%)
	pregnant/mated (n/n (%))	13/20 (65%)	5/20 (25%)*
	pregnant/plug (n/n (%))	13/13 (100%)	5/14 (35.7%)*

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Fig 5. Sperm cell parameters and functionality. **(A)** Sperm cell parameters (motility and activation). Motility and activation parameters (% of hyperactivated) evaluated by CASA (Computer Aided Sperm Analysis) are shown for sperm cells before capacitation and after 1 or 12 h of capacitation. Mean +/- SEM for each parameter is shown for n≥4 for each group. **(B)** Capacitation efficiency determined with anti-phosphotyrosine Western blot. Identical numbers of sperm cells, isolated from cauda epididymis of 3 F1-CD and 3 F1-LPD mice, were incubated 0, 10, 30, 60, 90, 120 min in classical capacitation medium for in vitro capacitation kinetics. Protein extracts were analyzed by Western bloting analysis of protein Tyrosine phosphorylation using anti-phosphotyrosine antibody. Protein loads were controlled with TGX stain free precast gels and a quantification was realized using ImageJ software. Mean +/- SEM is shown for n=3 in each group. Student's t-test was used for comparison between groups. The blot given is representative of semen from three separate individuals of each group. **(C)** Fertilization competence of sperm isolated from F1-CD and F1-LPD males using in vitro fertilization assay. Sperm were isolated from 4 males of each group and incubated in capacitating medium for 12 h. Oocytes were isolated from Balb/c hyperovulated females. Fertilization competence was measured by determining the percentage of two-cell embryos obtained 24 h after fertilization of at least 10 oocytes per male sample. (A-C) Student's t-test was used for comparison between groups. Statistical significance is indicated on the graphs: * p-value < 0.05, ** p-value < 0.01, ns= non-significant).

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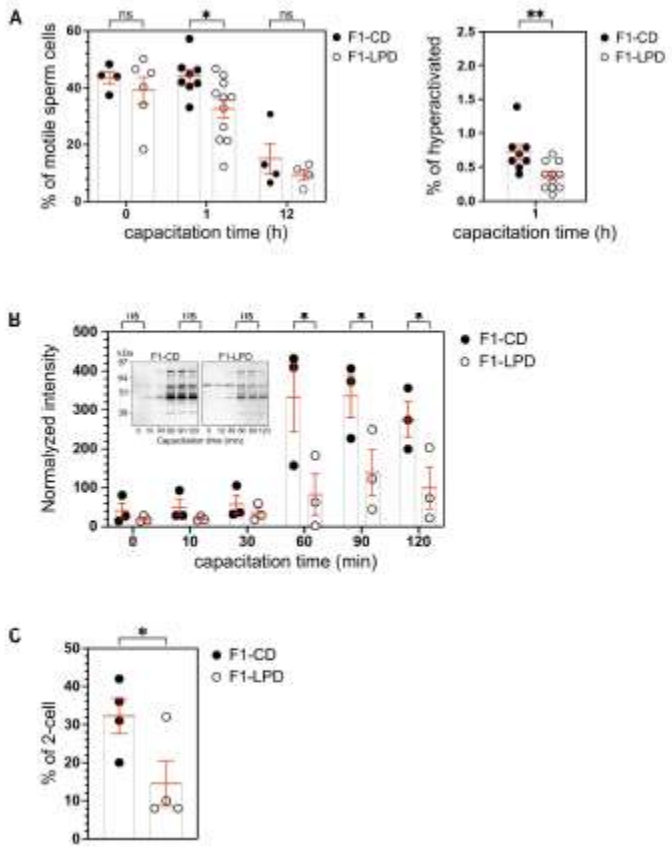

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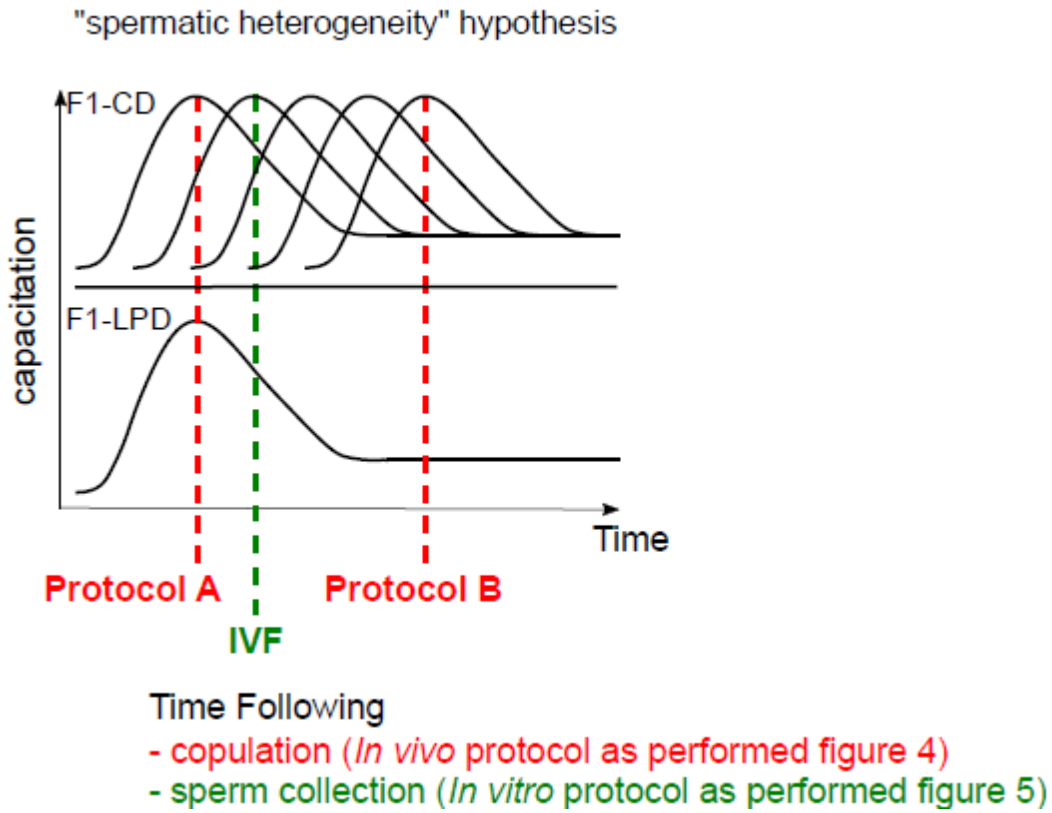
Fig 6. Schematic representation of the spermatic heterogeneity hypothesis.

In normal conditions, as it is the case for F1-CD males (upper panel): successful fertilization relies on heterogeneity among sperm cells. Since capacitation is a time-limited process, various sperm subpopulations coexist and capacitate at different time, allowing sperm to remain able to fertilize until ovulation [41,42]. Red dashed-lines indicate the period at which ovulation takes place: early after copulation as in protocol A or late after copulation as in protocol B (see Fig 4). Green dashed-line indicate the period at which IVF is performed following capacitation process (as described for Fig 5C).

For sperm from F1-LPD animals (lower panel): one of our hypotheses is based on the possibility that sperm heterogeneity is decreased due to a lack of subpopulations of sperm cells showing delayed capacitation. As a consequence, sperm is not able to fertilize when ovulation occurs late after copulation as in Protocol B (see Fig 4).



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