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DIET EFFECTS ON MOUSE SPERM: A WARNING FOR RECOMBINATION STUDIES

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ABSTRACT

Meiotic recombination is a critical process for sexually reproducing organisms. This exchange of genetic information between homologous chromosomes during meiosis is important not only because it generates genetic diversity, but also because it is often required for proper chromosome segregation. Consequently, the frequency and distribution of crossovers are tightly controlled to ensure fertility and offspring viability. However, in many systems it has been shown that environmental factors can alter the frequency of crossover events. Two studies in flies and yeast point to nutritional status affecting the frequency of crossing over. However, this question remains unexplored in mammals. Here we test how crossover frequency varies in response to diet in *Mus musculus* males. We use immunohistochemistry to estimate crossover frequency in multiple genotypes under two diet treatments. Our results indicate that while crossover frequency was unaffected by diet in some strains, other strains were sensitive even to small composition changes between two common laboratory chows. Therefore, recombination is both resistant and sensitive to certain dietary changes in a strain-dependent manner and, hence, this response is genetically determined. Our study is the first to report a nutrition effect on genome-wide levels of recombination. Moreover, our work highlights the importance of controlling diet in recombination studies and may point to diet as a potential source of variability among studies, which is relevant for reproducibility.

INTRODUCTION

Meiotic recombination, or the exchange of genetic material between homologous chromosomes that occurs during meiosis, has been extensively studied since the early 20th century due to its important role in generating genetic variation and as an essential tool for genetic mapping; later, it was also found that recombination is required for proper chromosome segregation during meiosis in many organisms (Hassold and Hunt 2001; Morgan 1913). Alterations in the number or distribution of crossovers can result in chromosome missegregation and aneuploidy, with implications in fertility and offspring health (Hassold and Hunt 2001; Ottolini et al. 2015). This delicate balance between the selective benefits of genetic variation, proper chromosome segregation and reproductive success has been achieved through a tight regulation of the crossing over process, both at the genetic and the epigenetic levels.

Although the control of crossover number and distribution is a complex and yet not fully understood process, it results in three common observations: 1) each homolog must have at least one crossover (the obligate crossover or crossover assurance) (Dumont 2017; Mather 1937; Pardo-Manuel de Villena and Sapienza 2001); 2) crossovers are not independent, because the occurrence of one interferes with the occurrence of a second one nearby (positive crossover interference) (Muller 1916; Sturtevant 1915) (; 3) crossover numbers can be maintained in spite of variations in the number of double strand breaks (DSB) they originate from (crossover homeostasis) (Baier et al. 2014; Cole et al. 2012; Hunter 2015). Consequently, the recombination rate is

constrained within species, although considerable variation is observed between species (Dumont 2017; Dumont and Payseur 2008; Segura et al. 2013).

Nevertheless, recombination rate still can vary between individuals of the same species: for instance, up to 30% variation has been reported between mice of different strains and hence, of different genetic background (Baier et al. 2014; Dumont 2017; Dumont and Payseur 2011a; Koehler et al. 2002). In addition, crossover frequency is higher in females than in males in both human and mouse, and this difference has been associated to the length of the synaptonemal complex (SC), the proteinaceous scaffold that forms between chromosomes during meiotic prophase and mediates crossover formation (Petkov et al. 2007; Tease and Hulten 2004). A positive correlation between number of crossovers and SC length has also been observed between different inbred strains of mice (Baier et al. 2014; Lynn et al. 2002). These intraspecific studies suggest that genetic differences, as well as chromatin packaging changes, underlie differences in crossover frequency (Baier et al. 2014; de la Casa-Esperon 2012; de la Casa-Esperon and Sapienza 2003; Kleckner 2006; Kleckner et al. 2003). Among the few loci identified in mice that control recombination rate (Balcova et al. 2016; Baudat et al. 2010; de La Casa-Esperon et al. 2002; Myers et al. 2010; Parvanov et al. 2010), *Prdm9* codes for a histone methyltransferase that determines recombination hotspots. These and other studies in several species conclude that recombination is genetically and epigenetically controlled.

In spite of this control, certain external factors, such as temperature changes (Bomblies et al. 2015; Lloyd et al. 2018; Plough 1917), diet (Neel 1941), stress

(Belyaev and Borodin 1982), toxicants (Gely-Pernot et al. 2017; Susiarjo et al. 2007; Vrooman et al. 2015) and infections (Singh 2019), are capable of modifying the recombination rate. In mice, the best-studied case is that of bisphenol A (BPA) exposure, a component of epoxy resins and polycarbonate plastics used in a wide variety of consumer products. BPA is an endocrine disruptor capable of binding estrogen receptors (Alonso-Magdalena et al. 2012; Susiarjo et al. 2007). Accidental intake of BPA from damaged mouse cages led to the observation in female oocytes of abnormal increases in meiotic disturbances and aneuploidy (Hunt et al. 2003). Subsequent studies showed that BPA exposure also altered the levels of recombination in both female and male meiosis (Brieno-Enriquez et al. 2011; Susiarjo et al. 2007; Vrooman et al. 2015).

From the studies of BPA exposures in mice, we have learnt several lessons: First, BPA effects on recombination depend on sex and genetic background. BPA exposures resulted in increased recombination in C57BL/6 females, but not in males of the same inbred strain (Susiarjo et al. 2007; Vrooman et al. 2015). However, in males of the CD-1 outbred strain, BPA was able to induce the opposite effect (a reduction of crossover frequency) (Vrooman et al. 2015). Second, BPA also induces other alterations in the germline -e.g., in diverse processes during spermatogenesis, resulting in reduced sperm production (Liu et al. 2013; Wisniewski et al. 2015; Xie et al. 2016). Third, BPA is also capable of eliciting heritable changes: for instance, BPA and BPS (a common BPA replacement) from damaged cages induce significantly reductions in male meiotic recombination rate on two subsequent (and unexposed) generations (Horan et al. 2017). This and other inter- or transgenerational effects of BPA

and other endocrine disruptors have been associated to epigenetic modifications of the germline (Anway et al. 2005; Garcia-Arevalo et al. 2014; Guerrero-Bosagna et al. 2010; Manikkam et al. 2013; Rahman et al. 2020; Salian et al. 2009; Susiarjo et al. 2013; Susiarjo et al. 2015; Wolstenholme et al. 2013; Xin et al. 2015; Ziv-Gal et al. 2015).

The last major lesson learnt from germline studies is that BPA is not the only endocrine disruptor capable of inducing meiosis and recombination changes: other estrogenic substances, such as BPS and ethinyl estradiol, also do so(Horan et al. 2017; Horan et al. 2018; Vrooman et al. 2015). Moreover, the meiotic abnormalities caused by BPA on mouse oocytes can be prevented by a diet rich in phytoestrogens which, in turn, can elicit abnormalities in absence of BPA(Muhlhauser et al. 2009). These natural plant compounds, like the synthetic estrogenic substances, are capable of binding estrogen receptors; isoflavone phytoestrogens, mainly genistein and daidzein, are most abundant in soy and other legumes. Phytoestrogen-rich diets have shown to cause neuroendocrine, behavioral and reproductive defects (Assinder et al. 2007; Cederroth et al. 2010a; Eustache et al. 2009; Guerrero-Bosagna and Skinner 2014; Patisaul 2017; Thigpen et al. 2007); epigenetic effects have also been reported in maternal exposure studies, where such diets are capable of counteracting methylation changes induced by BPA (Dolinoy et al. 2007). The reproductive effects include fertility and spermatogenesis anomalies. However, the impact of phytoestrogens on meiotic recombination has not been explored yet.

Hence, we decided to examine if not only toxicants, but also diets could affect the recombination rate. Based on the lessons learnt from BPA studies, we chose diets that could modify the germline epigenome or differed in their

estrogenic content and tested their effects on recombination in diverse genetic backgrounds. From studies of paternal epigenetic inheritance, we found that several cases are elicited by dietary factors introduced before conception (Donkin and Barres 2018; Siddeek et al. 2018). Among the diets that induce heritable epigenetic changes in the male germline are low-protein, high fat or caloric restriction diets. For instance, in utero 50% caloric restriction can cause metabolic disturbances in the F1 and F2 generations in mice, as well as methylation changes in the transmitting sperm (Martínez et al. 2014; Radford et al. 2014). Male mouse undernourishment can reduce paternal sperm methylation and fertility and have a negative impact on the health of their offspring (Anderson et al. 2006; McPherson et al. 2016) . Hence, we decided to test whether paternal undernourishment could affect meiotic recombination rates in a mouse model. This treatment is of particular interest given its relevance to humans, where undernourishment is a burden for many.

We also wondered if common diets might affect the results of recombination studies. In our animal facility, two diets are regularly used, which differ in their protein, energy and phytoestrogen content (see Materials). As previously discussed, these three dietary factors have been shown to cause meiotic or epigenetic changes in the germline and, hence, we wondered if the content differences among common mouse diets may be capable of affecting recombination as well.

Therefore, we decided to analyze whether differences between common diets, as well as undernourishment, could affect recombination rates in adult males. We performed our study in diverse genetic backgrounds, given variability in crossover frequency, as well as variation in the response to

estrogenic substances (such as estradiol, phytoestrogens and BPA), reported between different mouse strains (Spearow et al. 1999; Thigpen et al. 2007; Vrooman et al. 2015). We observed that common diets can trigger recombination rate changes in adult male mice; unlike BPA, the diet with the highest phytoestrogen content increases crossover frequency. These changes are strain-specific and, hence, depend on the genetic background. In addition, these diets can elicit sperm motility changes. Therefore, we propose that recombination could be particularly sensitive to certain alterations, likely epigenetic, caused by diverse effectors such as diets, so that crossover analysis could reveal the impact of such effectors in the germline. Moreover, our data compellingly show that diet composition must be taken into account when performing recombination and sperm studies.

MATERIALS AND METHODS

Mouse strains and diets

C57BL/6J, PWK/PhJ and MOLF/EiJ mice were obtained from Jackson Laboratory through Charles River. All experimental procedures used in this study were approved by the Committee of Ethics in Animal Care of the University of Castilla-La Mancha. Mouse chow diets were provided by Harlan Laboratories and Capsumlab. Teklad Global 18% Protein Rodent Diet is designed to support gestation, lactation and growth of rodents and, therefore, fed to pregnant and nursing female mice; hence, it will be referred as the "breeding" diet from here on. Teklad Global 14% Protein Rodent Maintenance Diet (and its equivalent Capsumlab Maintenance Complete Chow, used only in the initial set of experiments) is designed to promote longevity and normal body weight in rodents and, therefore, the routinely "maintenance" diet used in many facilities like ours. Description of both diets can be found in Table S1.

We performed two studies: in the initial one, adult males from the three strains were analyzed for the effect of two diets on recombination (undernourishment and breeding diets) provided during 24 days relative to a control group kept *ad libitum* with maintenance diet. Animals switched to breeding diet had free access to the chow, but those of the "undernourishment" group were fed with 50% (2.25 g of maintenance diet) of the regular daily intake (4.5 g, according to Bachmanov *et al.* (2002). Each diet group had 3 adult mice, except the B6 control and B6 breeding groups, each with 2 mice (average 5.8 months). Health and weight of the animals were regularly monitored. The

second study was aimed to verify the differences observed between breeding and maintenance diets in B6 mice and expand the study to testes and sperm phenotypes. Hence, two groups of five B6 mice (average 5.9 months) were fed *ad libitum* during 24 days with each of the two diets.

Collaborative Cross (CC) founder mice were obtained from the Jackson Laboratory and reared on a different maintenance diet (Laboratory Rodent Diet 5001) in the Biological Research Facility at North Carolina State University. At 8 weeks of age, MLH1 immunohistochemistry analysis (see below) was performed in 3 animals per strain (2 in NZO/HILtj) and 25 spermatocytes per analyzed per mouse. All experimental protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Tissue collection and processing for histochemistry and sperm analyses

Dates for mouse euthanasia, sample collection and processing were randomized in order to avoid experimental artifacts and the diet group of the samples were blinded until all measurements were completed to avoid bias.

After the 24-day diet period, adult male mice were euthanized by cervical dislocation and weighed. After removing and weighing the testes, chromosome spreads for immunostaining were prepared from one testicle as described below. The other testicle was submerged in Bouin's solution and processed for histochemistry. Fixed and paraffin-embebed tissues were sectioned and stained with hematoxylin and eosin.

Mature spermatozoa were collected from the caudae epididymides in 500 µl modified TYH buffer (in mM: 135 NaCl, 4.7 KCl, 1.7 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5.6 glucose, 10 HEPES, pH 7.4 adjusted at 37°C with NaOH). Then, the sperm motility was assessed using a computer-aided sperm analyzer (Sperm Class Analyzer® CASA System, Microptic; Barcelona, Spain). Aliquots of 5 µl sperm/sample were placed on a pre-warmed (37°) Leja chamber and examined in a phase contrast microscope (Nikon Eclipse 80i, Tokyo; Japan) equipped with a warmed stage (37°) and a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany), which is connected to a computer by an IEEE 1394 interface. Evaluations were made at 10x magnification and at least ten fields or 200 spermatozoa were recorded for each sample. Settings were adjusted to mouse spermatozoa. Recorded parameters were total motility (%), progressive motility (%), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN; %), straightness (STR, %), wobble (WOB; %), lateral head displacement (ALH, µm) and beat cell frequency (BCF, Hz).

Sperm viability was assessed by mixing 5 µl of sperm diluted in THY buffer with 10 µl of eosin-nigrosin for 30 sec and spreading the mix on a slide .The percent of viable sperm was evaluated under the microscope, as eosin stains only the dead sperm, whereas live sperm remains white.

Chromatin stability was assessed using the Sperm Chromatin Structure Assay (SCSA), a flow cytometric test where sperm DNA breaks are evaluated indirectly by analyzing DNA denaturability (Evenson et al. 1980). The assay measures the susceptibility of sperm DNA to acid-induced DNA denaturation, detected by staining with the fluorescent dye acridine orange (AO). Samples

were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA; pH 7.4) at a final sperm concentration of 2 x 10^6 cells and mixed with 400 µl of an acid-detergent solution for 30 seconds. Then, 1.2 ml of AO was added, and samples were evaluated 2 minutes later with a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) AO was excited with a 488 nm argon laser. A total of 5,000 spermatozoa per sample were evaluated. We expressed the extent of DNA denaturation in terms of DNA fragmentation index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity, *i.e.,* the level of denatured DNA over the total DNA. The DFI value was calculated for each sperm cell in a sample, and the resulting DFI frequency profile was obtained. Total DNA fragmentation index (tDFI) was defined as the percentage of spermatozoa with a DFI value over 25. High DNA stainability (HDS), which offers a measure of the percentage of immature sperm cells, was defined as the percentage of spermatozoa with green fluorescence higher than channel 600 (of 1024 channels).

Immunostaining, microscopy and scoring

Chromosome spreads were prepared from spermatocytes as previously described (Anderson et al. 1999; de Boer et al. 2009; Milano et al. 2019). Briefly, one of the two testes was decapsulated in hypotonic extraction buffer (HEB: 30 mM Tris, pH 8.2, 50 mM sucrose, 17 mM trisodium citrate dihydrate, 5 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF). Seminiferous tubule fragments were minced in 100mM sucrose and then fixed onto slides with 1% paraformaldehide containing 0.15% Triton X-100 in a humidified chamber. Slides were washed in

1 × PBS with Photo-Flo 200 (Kodak), dried and processed for immunostaining, or stored at -80°C until use.

MLH1 immunostaining allows for identification of about 90% of mammalian crossover sites (Anderson et al. 1999; Cole et al. 2012). For immunostaining, chromosome spreads were washed in 1 x PBS with 0.4% Photo-Flo 200 (Kodak) and 1 x PBS with 0.1% Triton X-100. The slides were blocked in 10% antibody dilution buffer (ADB: 3% bovine serum albumin, 0.05% Triton, 10% goat serum in 1 x PBS). Then, they were incubated overnight at room temperature with primary antibodies: mouse anti-human MLH1 (BD Biosciences) diluted 1:100 and rabbit anti-SCP3 (Abcam) diluted 1:1000 in ADB. Slides were washed as previously and incubated for 1 h at 37° with secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG and goat anti-rabbit conjugated with Alexa Fluor 555 (Life Technologies) diluted 1:1000 and 1:2000 in ADB, respectively. Slides were washed in 0.4% Photo-Flo and mounted with Prolong Gold Antifade Reagent with DAPI (Life Technologies Limited)

All slides were imaged on a Zeiss LSM 710 confocal microscope and analyzed using Zeiss Zen lite software. Only pachytene-stage spermatocytes with fully synapsed autosomes and XY chromosomes were scored; cells with poor staining or other scoring difficulties were excluded. In the first study, 25 spermatocytes were analyzed per animal, while more (25-40, average 37.3) were examined per mice in the second. For each spermatocyte, we counted the number of foci localizing to the SC of the 19 autosomes. Total SC length was only measured in autosomes, because the appearance and disappearance of foci on the XY bivalent and on the autosomes are temporally uncoupled. Autosomal SC length was initially measured by manually tracing the length of

the SYCP3 signal. Given the large dataset of our second experiment, we developed an ImageJ Macro (named "Synaptonemal & CO analyzer") for SC semiautomatic measuring (J. Soriano, A. Belmonte and E. de la Casa-Esperon, in preparation). Distance between MLH1 foci was measured in bivalents with 2 or more foci. The diet group of the samples were blinded until after focus counts and measurements were determined.

Statistical analysis

Comparisons in the average numbers of foci between different strains and/or diets were tested by ANOVA or Student t-test analysis, pooling the results from multiple mice of each group; Welch ANOVA was applied when homogeneity of variances could not be assumed. These analyses have been successfully employed in comparable studies despite MLH1 foci not following a normal distribution, because of the robustness of ANOVA analysis (Baier et al. 2014; Dumont 2017). Similar conclusions about statistical significance were obtained if non-parametric tests were performed. For statistically significant differences ($P < 0.05$), a Tukey's post-hoc honestly significant difference (HSD) test was performed to infer which groups differed. A Chi-square test was used to determine significance in the number of bivalents classified according to their foci number (E0-E3) between diet groups. Weight, sperm count and SCSA data were analyzed by Student t-test. Total motility, progressive motile spermatozoa, VCL, VSL, VAP, LIN, ALH, BCF and sperm viability were evaluated by a factorial ANOVA in mice fed with different diets. When the variables were

significant ($P < 0.05$), *post hoc* comparisons with Bonferroni correction were carried out. Analyses were performed using SPSS Statistics software.

RESULTS

Recombination levels depend on the genetic background

Variation in crossover frequency, as well as variability in the effects of chemical exposures on recombination, have been observed among mouse strains (Baier et al. 2014; Dumont and Payseur 2011b; Koehler et al. 2002). In order to investigate if diets have an impact on recombination levels, we selected three mouse inbred strains of diverse genetic background: C57BL/6J (B6), PWK/PhJ (PWK) and MOLF/EiJ (MOLF). B6 is a classical inbred strain widely used in recombination studies (Baier et al. 2014; Dumont and Payseur 2011b; Koehler et al. 2002), which is mostly of *Mus musculus domesticus* origin (93% of autosomal sequences (Yang et al 2011)); PWK was derived from wild mice of *M. m. musculus* subspecies (Gregorova and Forejt 2000) (94% of autosomal sequences of *M. m. musculus* origin according to Yang et al. (2011); MOLF is representative of the Japanese *M. m. molossinus* subspecies, which is the result of the hybridization between *M. m. musculus* and *M. m. castaneus* (Silver 1995).

Unlike C57BL/6, crossover frequencies by MLH1 immunostaining have never been described for PWK and MOLF wild-derived mice. Hence, our first goal was to determine the baseline crossover frequencies of the three strains with regular maintenance diet. Our analysis reveals a significant effect of strain on MLH1 focus count ($P << 0.0001$, ANOVA). We observed that while C57BL/6 males have 23.54±1.97 MLH1 foci per spermatocyte (mean±SD), MOLF males have significantly more foci per spermatocyte (24.85±1.97; $P = 0.015$, HSD). MLH1

focus counts are substantially higher in PWK males than both C57BL/6 and MOLF males (29.15±2.87; *P* << 0,0001, both comparisons HSD) (Figure 1A and Table 1). Hence, genetic differences between the selected strains have an impact on the levels of recombination.

Recombination variability in genetically diverse mice: the Collaborative Cross and Diversity Outbred stock founder strains.

We observed significant differences in male recombination levels between the three strains selected for our investigation, but given the implications of our results for recombination and recombination-dependent studies (such as genetic mapping), we wondered whether these strains were representative of the recombination variability present in *Mus musculus*. Several studies have reported crossover frequencies by MLH1 immunohistochemistry analysis of diverse mouse strains (Baier et al. 2014; Dumont and Payseur 2011b; Koehler et al. 2002). However, the potential effects of differences in diets or other environmental factors may hamper comparisons between their recombination results and ours. Hence, we extended our analysis to six additional strains: A/J, 129S1/SvImJ, NOD/LtJ, NZO/HlLtJ, CAST/EiJ and WSB/EiJ, compared to PWK/PhJ and C57BL/6J (representing the extreme values in our study), all housed under the same conditions and diet. These 8 strains capture most of the genetic diversity present in *Mus musculus* and, hence, were chosen as the founder strains of the Collaborative Cross (CC) and Diversity Outbred (DO) population, both important resources for mouse genetic studies (Chesler et al. 2008; Churchill et al. 2004; Collaborative Cross Consortium 2012; Roberts et al.

2007; Svenson et al. 2012; Threadgill et al. 2011). Indeed, analysis of CC mice has allowed to characterize several loci and mechanisms that control recombination (Liu et al. 2014). But the baseline meiotic crossover rate of all the founder lines had not been described yet. Therefore, we aimed to provide comparable data for these mice, as samples were collected under the same developmental and environmental conditions.

We observe 22.21±1.86 MLH1 foci per spermatocyte in CAST/EiJ mice, 22.80±2.22 in NZO/HlLtJ, 22.89±2.60 in C57BL/6J, 23.40±2.35 in A/J, 23.75±2.61 in 129S1/SvImJ, 24.19±23.36 in WSB/EiJ, 25.48±2.40 in NOD/LtJ, 28.11±3.83 in PWK/PhJ (Figure 2). There are significant differences in the MLH1 foci per spermatocyte between the strains (P << 0.0001, ANOVA) although, as shown in Figure 2, the majority has frequencies similar to that of B6. CAST/EiJ has the least MLH1 foci count per spermatocyte, but not significantly lower than B6. At the other extreme, PWK values are again significantly higher than any of the other strains (P < 0,0001 in all cases). Intermediate values are observed in NOD/LtJ (also significantly higher than B6 (P < 0,0001)) and WSB/EiJ. Prior studies reported up to 30% variability in crossover frequency between mice of different strains (Baier et al. 2014; Dumont and Payseur 2011b; Koehler et al. 2002), a range of variation corroborated by our results.

Comparing these with our previous data, we observe that B6 and PWK results are not significantly different than those obtained in our study for the same strains fed with control maintenance diet (P = 0.26 and P = 0.06, respectively, *t*-test). Hence, the strains selected in our study (B6, MOLF and PWK) are representative of the low, medium and high levels of recombination

present in mouse males, respectively. Our results confirm previous observations of the impact of the genetic background on recombination frequency (Liu et al. 2014) and provide new data about the crossover rate of the CC and DO founder strains.

Changes in synaptonemal complex length or interference may underlay recombination differences between strains.

Crossover distribution and frequency is limited by crossover interference, because the presence of a meiotic crossover interferes with the occurrence of a second one nearby. Hence, interference establishment and distance depends on the physical length of the chromosomes during meiosis (measured as μm of immunostained synaptonemal complex (SC)). Because interference limits the occurrence of multiple recombination events, chromosomes with short SC can only undergo one crossover in mouse spermatocytes (Lawrie et al. 1995; Petkov et al. 2007; Sym and Roeder 1994; Tease and Hulten 2004). Indeed, a positive correlation between crossover frequency and SC length has been observed (Dumont and Payseur 2011a; Froenicke et al. 2002; Kleckner et al. 2003; Lynn et al. 2002). Our data indicate a significant effect of strain on SC length ($P << 0.0001$, ANOVA). When we compare the total length of the SC of the autosomes per cell (in μm, Table 1 and Figure 1B), we observe significantly longer SC in PWK (183.3±19.4) compared to those observed in B6 (162.2±17.1) and MOLF spermatocytes (156.6±16.0; $P << 0.0001$ in both cases, HSD). Hence, the larger SC in PWK may explain the higher crossover frequency observed in this strain respect to B6. We found no significant

difference in SC length between MOLF and B6 spermatocytes (P = 0.20, HSD). This is interesting because these two strains have significant differences in the number of MLH1 foci as reported above. This suggests that factors other than SC length may account for the differences in recombination levels observed between these two strains.

As SC length cannot explain the increase of MLH1 foci in MOLF respect to B6 spermatocytes, we wonder if variation in interference strength could be the cause. Analysis of variance indicates a significant effect of strain on intercrossover distance (P << 0.0001, ANOVA; similar results were obtained with non-parametric tests). Post hoc tests reveal that the average interfocus distance in MOLF spermatocytes is significantly shorter than in B6 and PWK spermatocytes (P << 0.0001, both comparisons, HSD; Table 1 and Figure 1C). Hence, weaker interference could explain why MOLF spermatocytes, in spite of having similar or shorter SC, could accumulate more crossovers per cell than B6 cells.

Finally, it was previously suggested the relationship between SC length and the number of MLH1 foci would be almost constant in mouse spermatocytes (Lynn et al. 2002). We therefore analyzed the amount of SC per MLH1 focus (μm SC/MLH1 foci; Table 1). This value is 6.9 μm in B6, while the strains with higher crossover rates have lower SC length per MLH1 foci: 6.3 μm in both, despite of the differences in recombination between PWK and MOLF. This further supports the suggestion that SC length is only one factor that affects recombination rate in male mice.

Undernourishment may influence recombination levels in a strain-dependent manner

Previous studies showed that *in utero* 50% caloric restriction has an effect on adult male germline methylation (Martínez et al. 2014; Radford et al. 2014). Adult sperm quality and epigenome have also been shown to be rapidly susceptible to dietary changes or restriction (McPherson et al. 2016; Nätt et al. 2019). Because crossover frequency and distribution is under genetic and epigenetic control, we decided to explore whether a 50% restriction to food access in adult males could also have an impact on meiotic recombination. Based on data from previous studies we decided to feed adult male mice of each of the three strains with 50% of their regular daily intake of maintenance chow for 24 days, while controls had access to the same maintenance diet *ad libitum*. At the end of the 24-day period, animals were euthanized and testes were processed for crossover analysis. Analysis of the data by a univariate generalized linear model shows that, besides strain ($P < 0.0001$), diet also significantly affects the MLH1 foci frequency ($P_{diet} = 0{,}035$).

Neither B6, nor MOLF spermatocytes showed significant changes in MLH1 focus count between *ad libitum* and 50% restricted diets by *post hoc* tests (Table 2), suggesting an efficient control of the recombination levels when adult males of these two strains face undernutrition. In contrast, PWK spermatocytes, already with high crossover frequency, had a small but significant increase ($P = 0.033$) in MLH1 foci number when food intake was restricted to 50% (Table 2). Therefore, our results suggest that, in adult male mice, undernourishment affects recombination levels in particular genetic backgrounds.

Diet composition can affect recombination levels in a strain-dependent manner

We wondered if common laboratory diets could have an effect on recombination and, consequently, may confound the results of recombination studies. Two chows are routinely used in our and many other animal facilities, depending on the purpose: animal maintenance or breeding (see Materials and Methods and Table S1). The breeding diet is aimed to support gestation, lactation and growth, and has 2% more protein and 8% additional energy density than the maintenance chow, devised to promote longevity and normal body weight. In addition, phytoestrogens are present in the breeding diet (150-250 mg isoflavones/kg diet), while avoided in the maintenance one. As previously discussed, these components have been linked to epigenetic or developmental changes in the germline. Hence, we decided to test if crossover frequency could vary in mouse spermatocytes depending on the diet of choice.

Adult mice are routinely fed with maintenance diet. We separated animals of the three strains and provided them with *ad libitum* access to the breeding diet during 24 days, while others were kept with the maintenance diet. After the 24-day period, we obtained spermatocytes spreads of both diet groups and analyzed recombination by immunohistochemistry. In order to examine which factors are associated with recombination variability, we used a univariate generalized linear model. Our results indicate that diet significantly affects the MLH1 focus frequency (P_{diet} = 0,042). We used post hoc tests to determine which diet comparisons were of particular note statistically. We observed no

significant effect of the diets on MLH1 foci frequency in MOLF and PWK mice (Table 2). However, a significant increase was observed in B6 mice fed with breeding diet (24.70±2.21) compared to maintenance chow (23.54±1.97, P = 0.008, Table 2). Our results also indicate that strain significantly affects MLH1 frequency (P << 0.0001), and there is a significant strain by diet interaction effect (P = 0,010) as well. These data therefore indicate that, in addition to genetic differences in recombination frequency, diet composition can affect crossover frequency in adult male mice in a strain-dependent manner.

Diet effects on recombination levels in C57BL/6 mice are reproducible

We were surprised to find that common chows could affect crossover frequency in B6 mice, while not in the two other strains. In order to find if this was a fortuitous or a consistent observation, we designed a larger experiment (see Materials and Methods) that also allowed us to collect additional information about the sperm. Animals were also subject to maintenance or breeding diet for 24 days and spermatocytes were prepared for analysis immediately after. As shown in Table 3 and Figure 3A, B6 mice kept in maintenance diet had similar MLH1 foci number (23.50±2.17) to those previously observed, and the breeding diet also elicited a significant increase in crossover frequency (24.24±2.31, two-sided Student t-test P = 0.001). This increase could not be explained by significant changes in SC total length (Table 3).

The studies of BPA effects on recombination stemmed from the observation of an increment in chromosome missegregation and aneuploidy caused by this

endocrine disruptor (Hunt et al. 2003). In mouse as in other species, proper chromosome segregation requires a minimum of one crossover per chromosome; otherwise, crossover failure often results in aneuploidy (Hassold and Hunt 2001). Hence, we decided to explore if the observed diet effect on recombination had any impact on the frequency of non-recombinant bivalents (E0) and, hence on the risk of aneuploidy. We compared the number of bivalents with zero, one, two or three MLH1 foci (E0, E1, E2 and E3, respectively) of each diet group. In both cases, 95% of the spermatocytes had between 20 and 28 MLH1 foci (Figures 3A and 3B) mainly located in E1 and E2 bivalents; those with no crossovers were very rare, as were bivalents with three crossovers, representing 0.5% or less each (Table 3). A significant change was observed between the two diet groups, mainly due to an increase of E2 bivalents at the expense of E1 in animals fed with breeding diet respect to those kept in maintenance diet (χ^2 = 13.2; *P* = 0.004, Table 3 and Figure 3B). Therefore, we conclude that, compared to the maintenance diet, the breeding chow induces an increase in MLH1 frequency through a shift of E1 to E2 bivalents, without substantial changes in E0 and the associated risk of aneuploidy.

Diet effects on C57BL/6 sperm: motility is also affected by diet.

We wondered if the observed diet effect on the male germline was not restricted to recombination. Hence, we explored if diet could also affect other aspects of spermatogenesis that resulted in changes in the quality and number of sperm, as observed in several diet and estrogenic exposure studies

(Assinder et al. 2007; Horan et al. 2017; Horan et al. 2018; Meena et al. 2017; Nassan et al. 2018; Nätt et al. 2019; Spearow et al. 1999). The breeding diet is more caloric than the maintenance diet (Table S1) and, accordingly, we observed a slight but not significant increase in body and testis weight in animals fed with breeding chow respect to those kept in maintenance diet (Table S2). This small difference disappears when testis weight is corrected for body weight (Table S2). We examined if testes histology was affected by diet, as observed in animals treated with natural or synthetic estrogens (Horan et al. 2017; Spearow et al. 1999). We did not observe noticeable changes in the number and cell composition of seminiferous tubules between the two diet groups (Figure S1). When sperm isolated from the cauda epididymis was analyzed, no differences were detected in sperm count between the two diet groups. Sperm viability was neither significantly affected (Table S2).

Sperm acquires progressive motility in the epididymis, characterized by high velocities and symmetrical, low-amplitude flagellar bends. By computer-assisted sperm analysis (CASA), we assessed several parameters of epididymal sperm motility: percentage of total motility and of progressively motile spermatozoa, VAP, VCL and VSL, LIN, STR, WOB, ALH and BCF (see Methods) (Boyers 1989; Mortimer 1997). None of them showed significant differences between the two diet groups, except the proportion of sperm with progressive motility, which was significantly reduced in the breeding diet (18.8±2.0) respect to the maintenance diet (25.8±2.4, mean±SE; P = 0.038) (Table S2). Though not significantly, sperm velocity also appeared to decrease in the breeding diet group (measured as VAP, VCL and VSL). The proportion of motile sperm has been associated with fertilization success (Davis et al. 1991).

In addition, we evaluated whether diet had an effect on sperm DNA integrity by SCSA (Evenson et al. 1980). We analyzed both the DNA fragmentation index (average and total, see Material and Methods), as well as the high DNA stainability; the latter offers a measure of the condensation degree of the sperm chromatin and the percentage of immature sperm cells, because this high stainability is considered to be the result of a lack of full protamination and, thus, an increased histone retention (Evenson et al. 2000). None of these parameters showed significant differences between sperm of mice fed with maintenance *vs.* breeding diets (Table S2). Therefore, these diets had no significant effect on sperm DNA damage or condensation, as measured by SCSA.

DISCUSSION:

Sperm quality has declined over the last decades among healthy men (Carlsen et al. 1992; Levine et al. 2017; Sengupta et al. 2017; Splingart et al. 2012). This decline has been associated to chemical exposures and life-style changes, including diets and the increase of diet-related diseases such as obesity (Nassan et al. 2018; Nordkap et al. 2012). Changes in sperm count and motility have a direct impact on fertility. But changes in other underrated sperm features, such as meiotic recombination, are also observed in infertile men (Ferguson et al. 2009; Ren et al. 2016). Alterations of crossover number or distribution increase the risk of chromosome missegregation and aneuploidy (Hassold and Hunt 2001). Consequently, crossover frequency and distribution are tightly controlled in order to ensure proper chromosome disjunction during meiosis (Cole et al. 2012; Coop and Przeworski 2007).

In spite of this control, several studies have shown that environmental factors such as temperature changes (Bomblies et al. 2015; Lloyd et al. 2018; Plough 1917), stress (Belyaev and Borodin 1982), endocrine disruptors (Susiarjo et al. 2007; Vrooman et al. 2015) and infections (Singh 2019) are able to modify the recombination rate. Even if these recombination changes may not be large enough to compromise fertility, they could have important consequences in the transmission and evolution of traits, as well as in genetic mapping studies (Dumont and Payseur 2008; Krzywinska et al. 2016; Pardo-Manuel de Villena et al. 2000; Ritz et al. 2017). To date, only one analysis in flies has reported an effect of nutrition on crossover rate (Mostoufi 2021; Neel 1941), an effect that

has also been suggested in yeast (Abdullah and Borts 2001). Hence, we decided to explore whether diet could not only affect sperm features, but also recombination in mammalian spermatocytes.

Recombination rate variation among mouse inbred strains: crossover frequency is regulated by different mechanisms

Because both crossover rate and the effect of environmental exposures on recombination depend on the genetic background (Baier et al. 2014; Dumont and Payseur 2011b; Koehler et al. 2002; Vrooman et al. 2015), we selected for our study genetically diverse strains representative of three *Mus musculus* subspecies: C57BL/6J (B6) for *M. m. domesticus*, PWK/PhJ (PWK) for *M. m. musculus* and MOLF/EiJ (MOLF) for *M. m. molossinus* (Gregorova and Forejt 2000; Silver 1995; Yang et al. 2011). We found that crossover frequency, measured by MLH1 immunostaining, was significantly different among them. B6 values were similar to those obtained in previous studies (Baier et al. 2014; Balcova et al. 2016; Vrooman et al. 2014). But MLH1 foci frequencies for PWK and MOLF wild-derived mice had never been described before. We observed maximum values in PWK mice, only comparable to those of the PWD/PhJ strain (29.92±2.51 (Dumont and Payseur 2011b); 29.58 (95%CI 28.6630.56) (Balcova et al. 2016)), also of *M. m. domesticus* origin (Gregorova and Forejt 2000).

For a broader view of recombination variability in mouse, we expanded our analysis to characterize the crossover frequencies of the 8 founder strains of the Collaborative Cross (CC) and Diversity Outbred (DO) stock, because they capture nearly 90% of the known variation present in laboratory mice (Churchill

et al. 2004; Roberts et al. 2007). These are very important resources designed for the characterization and mapping of complex traits and diseases with complex etiologies (Chesler et al. 2008; Churchill et al. 2004; Collaborative Cross Consortium 2012; Svenson et al. 2012; Threadgill et al. 2011). Analysis of CC mice has also proven to be a valuable resource for unveiling the loci and mechanisms that control recombination (Liu et al. 2014). However, the baseline meiotic crossover rate of all the founder lines had not been characterized, although other sperm and testis phenotypes were reported (Odet et al. 2015). Again, our results showed that PWK spermatocytes have the highest crossover frequency, while CAST/EiJ mice are at the opposite extreme, in agreement with the low recombination levels previously detected in this strain (Baier et al. 2014). Our observations confirm the importance of the genetic background on the levels of recombination (Baier et al. 2014; Dumont 2017; Dumont and Payseur 2011b; Koehler et al. 2002; Liu et al. 2014), and provide new data about the relative crossover rate of the CC and DO founder strains, which are relevant for future mapping and recombination studies in mice.

These results also revealed that the three strains selected for our study represent the low (B6), medium (MOLF) and high (PWK) levels of recombination present in *Mus musculus*. Because crossover distribution and frequency depend on crossover interference and chromosome physical length (measured as synaptonemal complex (SC) length) (Dumont and Payseur 2011a; Froenicke et al. 2002; Kleckner et al. 2003; Lynn et al. 2002; Petkov et al. 2007; Tease and Hulten 2004), we explored which of these two factors was involved in the recombination differences observed between strains. Moreover, as interference limits the proximity between crossovers, the number of these

can increase by either reducing interference or expanding the SC length and we found that both possibilities occurred in the strains under study.

The B6 total autosomal SC length we observe is similar to that previously reported (Vranis et al. 2010). But in PWK, our data suggest that high levels of recombination could be a consequence of the longer SC respect to the other two strains. A correlation between total SC length and recombination rate has also been observed by others in mouse and other animals (Baier et al. 2014; Lynn et al. 2002; Ruiz-Herrera et al. 2017), likely as a result of differences in the degree of chromatin compaction and interaction between homologues. Interestingly, a recent study has identified several loci that affect SC length and some of them also modulate recombination rate (Wang et al. 2019). A previous study proposed a simple linear relationship between crossover rate and total SC length, so that the ratio between SC length and the number of MLH1 foci would be almost constant in mouse spermatocytes (Lynn et al. 2002). The values observed in our B6 animals coincide with those reported in that study (6.9 μm). However, lower values are observed for MOLF and PWK (6.3 μm), which have higher crossover rates. Wang et al. (2019) and others (Vranis et al. 2010) also found the length of SC per MLH1 focus varies among mouse strains and proposed that it could be a consequence of interference variation, but our PWK data suggest this ratio can change independently of interference fluctuations.

In contrast, differences in total SC length cannot explain the intermediate level of recombination found in MOLF spermatocytes. In this case, the shorter intercrossover distance suggests that, compared to B6, a weaker positive interference in MOLF spermatocytes could be the cause of their higher

crossover rate. An inverse correlation between interference strength and recombination rate has also been observed in other mammals (Segura et al. 2013) and a locus that affects both interference and recombination levels has been identified in cattle (Wang et al. 2016). Therefore, our results suggest that diverse and, at least to some extent, independent mechanisms determine the breadth of recombination levels present in mice.

Recombination rate is both sensitive and resistant to diets: genetic background determines crossover frequency, even under stressful nutritional conditions.

Next, we explored if diets can alter recombination levels and whether this effect can be modulated by the genetic background. Based on the findings of the best-studied environmental effects on recombination in mice, those of BPA exposure (Susiarjo et al. 2007; Vrooman et al. 2015), we looked for candidate diets that could also affect the male germline epigenome or compromise sperm function (Manikkam et al. 2013; Rahman et al. 2015; Rahman et al. 2020; Salian et al. 2009; Susiarjo et al. 2013; Xin et al. 2015). Among them, we found that in utero 50% dietary restriction could affect sperm methylation and offspring health (Martínez et al. 2014; Radford et al. 2014). Sperm function and offspring health were also altered by direct undernutrition of adult mouse males (McPherson et al. 2016). When we analyzed if the spermatocytes of males temporarily subjected to undernutrition experienced recombination changes, we found a strain-dependent diet effect.

On the one hand, PWK spermatocytes recombination rate, already high, increased even more when food intake was limited to 50%. Nutritional deficit also causes an increase in crossover frequency in *D. melanogaster* and *S. cerevisiae* (Abdullah and Borts 2001; Neel 1941). However, we cannot conclude that a reduction in nutrients availability was directly responsible for the observed effect on recombination. Although diverse dietary restrictions have been reported to improve life span (Fontana et al. 2010; McCay et al. 1935), not all mouse strains respond the same; on the contrary, health deterioration and life shortening occurs in some (Liao et al. 2010; Mitchell et al. 2016; Radford et al. 2014). Similarly, we found that while B6 and MOLF animals performed well under reduced food intake, 2 of the 3 PWK animals had to be euthanized before the end of the 24-day undernutrition period due to severe weight loss (20% of body weight). Hence, undernutrition may have generated extreme physiological or metabolic disturbances in PWK males capable of altering the control of the recombination levels. Indeed, diverse types of stress have been reported to affect recombination rate in mouse and other organisms (Belyaev and Borodin 1982; Modliszewski and Copenhaver 2017). Though it is possible that meiosis and recombination might have evolved to be able adapt to environmental challenges (Bomblies et al. 2015; Modliszewski and Copenhaver 2017), the adaptive significance of the environment-induced changes in recombination remains unclear. In Arabidopsis, studies on temperature effects on recombination often report U-shaped response curves which suggest that, in optimal conditions, organisms generally have lower recombination rates than under extreme and stressful ones (*e.g.* Lloyd *et al.,* 2018); previous reports of increased recombination rate in stressed mice (Belyaev and Borodin 1982) and

our observations are consistent with this prediction. Future studies with different dietary restriction regimes or deprivation of specific nutrients (Ideraabdullah and Zeisel 2018) will be able to discriminate whether food intake reduction or undernourishment-induced stress and health deterioration are responsible for the observed effect on crossing over frequency.

On the other hand, recombination rate in the other two strains (MOLF and B6) was not significantly affected by 50% dietary restriction. While B6 mice often get overweight and might resist well brief periods of food shortage, such is not the case of the MOLF animals, a wild-derived strain of lean mice as PWK; in fact, MOLF mice lost 5-7% body weight in 11 days during the treatment, while it was reduced 9-20% in PWK. Hence, we conclude that, in certain genetic backgrounds, recombination levels are tightly controlled even under stressful conditions such as undernutrition or, as reported in other studies, infections (Dumont et al. 2015). As many organisms, including (unfortunately) many humans, face short, seasonal or long periods of nutrients deprivation, understanding the effects of nutritional changes on recombination, a critically meiotic important process, is important. Given how fundamental diet is to organismal fitness and function, understanding the effect to which diet-induced changes in recombination persist across generations is important as well. Moreover, given that the effects of diet are genotype-specific, more work is needed to comprehend the genetic basis of this interaction.

Common diets can affect male recombination rate in a strain-dependent manner: recombination in mice is more sensitive to environmental exposures than previously expected.

We decided to test whether not just toxicants or stressful exposures, but also small differences between common diets, can alter crossover frequencies in adult male mice. Hence, we temporarily fed adult males of the three selected strains with the two chows routinely used in our facility: one specific for breeding periods, which is richer in proteins and energy density than the regular one, used for mice maintenance, in which phytoestrogen-rich plants are absent in order to avoid estrogenic effects that might interfere with some studies. Although they also differ in other nutrients, their compositions are not markedly dissimilar. Hence, we were surprised to find that males of one of the three strains, B6, showed higher recombination rates when fed with the breeding chow than when kept in the maintenance diet. Again, we observed that the diet effect on recombination was strain-dependent, but now affected to a different strain than undernutrition (PWK), demonstrating the importance of genetic differences in the variable response to diverse diets.

Although a genetic background-dependent response to environmental effects on recombination has been reported in diverse species (Vrooman et al. 2015), we were surprised to find that B6, a strain that is insensitive to the effect BPA and other estrogenic substances on male recombination, was precisely the one responsive to our diets differences (Vrooman et al. 2015). However, those results were also unexpected in view of the high estrogenic sensitivity of B6 testes (Spearow et al. 1999) and previous results reporting a B6 female recombination response to BPA (Susiarjo et al. 2007). Moreover, our results

(increased recombination in the breeding diet group, which contains phytoestrogens) were in the opposite direction to the reduced crossover frequency observed in CD-1 mouse males after exposure to synthetic estrogenic substances (Vrooman et al. 2015). Hence, we decided to test whether our observation was a spurious result by providing the same dietary regime to an independent group of B6 adult mice. Our results confirmed that crossover frequency is sensitive to small and apparently healthy diet changes.

These results have important implications, especially for recombination studies, as the chows selected for our study are just a small example of the composition variability found among common rodent diets (Ruhlen et al. 2011). We also wondered if the diet-induced recombination changes could also affect meiotic chromosome segregation and aneuploidy studies, as an increase in achiasmatic chromosomes could compromise meiotic disjunction and elevate the aneuploidy rate, as observed with exposures to BPA and other estrogenic compounds (Hassold and Hunt 2001; Hunt et al. 2003). Our studies showed that the occurrence of achyasmate chromosomes was similarly low in both diet groups and the breeding chow induced an increase recombination rate by elevating the frequency of double recombinants at the expense of single recombinant chromosomes (triple recombinants are rare in B6 mice). Hence, our results predict that aneuploidy rate and subsequent fertility should not be affected by changes in common chows composition in B6 mice, although we cannot exclude effects with other diets or genetic backgrounds.

We wondered which mechanisms could explain this diet-induced recombination rate change and if this may be associated to total SC length or interference variation, as we observed for strain-dependent diversity in

crossover frequencies. Unfortunately, we were unable to discriminate if interference was affected by diet and could explain our observations. While the diet effect on recombination we detect, though reproducible, is small, intercrossover distances are quite variable; consequently, interference analyses by this method are only possible when relatively large effects on recombination are examined, as those observed by strain effects in our study or by mutations in other reports ((Roig et al. 2010)). We also interrogated if the diet effect we observe on recombination could be associated to total SC length variation, as described in recombination changes induced by environmental exposures such as temperature in plants (Lloyd et al. 2018; Modliszewski and Copenhaver 2017; Phillips et al. 2015). However, we did not see significant differences in autosomal SC length between the animals subject to breeding vs. maintenance diets. Similarly, Vrooman *et al.* (2015) did not detect SC length changes associated to crossover frequency variation caused by BPA or ethynil estradiol in adult mice, neither SC length could explain all the temperature effects on recombination in Arabidopsis (Lloyd et al. 2018).

Diet effects go beyond recombination to affect sperm motility.

In view of the results, three questions emerge: which diet components caused the observed effect on recombination? and how? Are other aspects of meiosis or spermatogenesis affected? To provide an answer to the last one, we examined the testes and sperm of the same mice studied for recombination.

Previous studies had reported changes in spermatogenesis progression, sperm count or motility caused by diets differing in fat, protein or phytoestrogen

contents among others (Assinder et al. 2007; Cederroth et al. 2010b; Eustache et al. 2009; Matuszewska et al. 2020; Morgan et al. 2020; Tavares et al. 2016). Although the breeding chow provides more energy than the maintenance one, the composition differences were insufficient for the breeding diet to cause a significant increase in body or testis weight during the time of exposure. Histological analysis of the testes did not reveal any apparent changes in the morphology or cell content of the seminiferous tubules between the treatment groups, suggesting diets changes did not affect spermatogenesis.

Epididymal sperm count, DNA fragmentation and viability were not significantly affected, as they were not most of the sperm kinematic parameters analyzed. But interestingly, the percentage of progressively motile spermatozoa decreased after mice transferring to breeding diet, suggesting this diet might be optimal during pregnancy or nursing, but not for male fertility (Davis et al. 1991). Our results are in agreement with those of Nätt *et al.* (2019) and others (Assinder et al. 2007; Nassan et al. 2018; Salas-Huetos et al. 2018), suggesting sperm is capable of rapidly responding to diet, even to small changes.

Indeed, the composition differences between the chows under our study are relatively small compared with those analyzed in studies of high-fat or low-protein diets effects in testes (Crisostomo et al. 2019; Matuszewska et al. 2020; Morgan et al. 2020). However, they are comparable to other diet studies such as the one performed by Assinder *et al.* (2007), who analyzed the effects of low- and high-phytoestrogen diets fed to adult Wistar rats during 24 days on testes, finding, among others, reduced sperm count and increased apoptosis during spermatogenesis. However, the level of isoflavones of our breeding diet (150-250 µg/g) is intermediate between the high- (465 µg/g) and low-

phytoestrogen (112 µg/g) diets of that study. Nevertheless, it also suggests that particular components of common diets, such as phytoestrogens, could have an impact on spermatogenesis and sperm quality.

Our findings raise the question as to whether the two observed diet-induced effects (on recombination levels in pachytene spermatocytes and on epididymal sperm motility) are related or not. BPA studies have revealed effects not only on recombination, but also on sperm motility (Rahman et al. 2015; Vrooman et al. 2015). Moreover, studies on mice have shown that BPA fertility effects arise at multiple stages of spermatogenesis, being spermatocytes and mid-spermatids the most sensitive to this endocrine disruptor (Tiwari and Vanage 2013). Hence, it remains to be determined whether the diet effect we observe during meiosis is also affecting other germline features that result in changes in the percentage of progressively motile sperm; alternatively, these changes could be the result of an independent later-stage diet effect. In this case, it could be exerted directly on the germline or indirectly through the surrounding somatic cells or fluids, and by the same or a different diet component from the one (or ones) affecting recombination.

5. What are the causes? Implications for recombination studies.

What is the mechanism that links diet with recombination? It is tempting to speculate that epigenetic changes could be involved in the diet effect on recombination rate for several reasons. First, crossover frequency and distribution depend on the chromatin architecture and epigenetic marks of the chromosomes (de la Casa-Esperon and Sapienza 2003; Zelkowski et al. 2019).

As we discussed before, crossover frequency and distribution differences between individuals of the same species depend on interference strength and SC length variation, which in turn reflect chromatin compaction during meiosis (Kleckner et al. 2003; Ruiz-Herrera et al. 2017). In addition, specific epigenetic marks are characteristic of the heterochromatic regions depleted of recombination (Termolino et al. 2016; Underwood et al. 2018) as well as the recombination hotspots: in mice these are specified by the histone methyltransferase PRDM9, which also modifies the recombination rate (Balcova et al. 2016; Buard et al. 2009; Imai et al. 2020; Spruce et al. 2020). Second, environmental exposures that affect recombination rate, can also affect the germline epigenetic marks, as it is the case of BPA or atrazine in mice (Gely-Pernot et al. 2017; Manikkam et al. 2013; Modliszewski and Copenhaver 2017; Xin et al. 2015). Third, dietary changes, such as changes in energy, protein or phytoestrogen content, can also elicit epigenetic changes in the germline (Donkin and Barres 2018; Siddeek et al. 2018); like endocrine disruptors as BPA, some of these diets can induce transgenerational effects mediated by epigenetic changes transmitted by the germline (Carone et al. 2010; de Castro Barbosa et al. 2016). Hence, recombination rate could vary as a consequence of the germline epigenetic response to differences in diet composition, as suggested for other environmental exposures (Modliszewski and Copenhaver 2017). Moreover, epigenetic modifications may also be the underlying cause of the sperm motility differences observed between the two diet groups, as other diets have been reported to elicit both sperm epigenome and motility changes (Nätt et al. 2019; Siddeek et al. 2018).

Although the SCSA results did not suggest large sperm chromatin alterations due to diets, this is not surprising in view of the moderate differences between the diets under study and the reproductive success of the animals fed with them in facilities throughout the world. Future studies will have to determine whether epigenetic changes mediate diet effects on recombination and, if so, what is their nature. This may be a complicated enterprise, as diet-induced epigenetic changes in the male germline have been shown to be heterogeneous among studies (Donkin and Barres 2018; Sharma and Rando 2017; Siddeek et al. 2018); for instance, the nature of the epigenetic marks that result in transgenerational inheritance has been questioned, as they are either of small magnitude, variable sort or even undetectable in some generations (Sharma and Rando 2017; Shea et al. 2015; Xue et al. 2016). Hence, the analysis of the sperm epigenome might turn out to be very complicated or even insufficient to become a marker for environmental exposures and lifestyle, as previously proposed (Siddeek et al. 2018). In contrast, our results show that crossover rate is sensitive not only to disrupting toxicants (Horan et al. 2018), but also to small changes in diet and could potentially be used as an indicator of environmentally-induced perturbations in the germline.

Our results also show that this recombination sensitivity depends on the genetic background, which is also true for many other responses to diverse exposures, including diets (Latchney et al. 2018; Spearow et al. 1999; Thigpen et al. 2007; Vrooman et al. 2015). Nowadays, it is unavoidable for studies about the effects of environmental factors to explore their impact in genetically diverse strains, such as the founders of the CC and DO mice. Disparate results can also result from variability in the doses, timing and duration of exposure, among

others. For instance, the effects of BPA on recombination are developmental stage, sex and strain dependent (Susiarjo et al. 2007; Vrooman et al. 2015). We now add and further factor to control in recombination studies: diet.

Future studies will determine which component of the chows (or combination of them) is responsible for the observed changes in recombination, as well as the effective doses. Phytoestrogens are attractive candidates, as they can elicit germline epigenetic as well as sperm motility changes, have estrogenic properties like BPA and can even modulate the effects of this compound in mice (Atanassova et al. 2000; Dolinoy et al. 2007; Muhlhauser et al. 2009; Patisaul 2017). But changes in energy content or even minor components of the diets have also shown to affect the germline and constitute interesting candidates (Crisostomo et al. 2019; Nassan et al. 2018; Ruhlen et al. 2011; Salas-Huetos et al. 2018; Siddeek et al. 2018).

Finally, while BPA and other estrogenic compounds affect recombination when provided to female embryos or neonatal males (Gely-Pernot et al. 2017; Susiarjo et al. 2007; Vrooman et al. 2015), our results demonstrate that recombination in adult male mice is sensitive to diet influences. It will be interesting to explore whether earlier developmental stages, particularly those in which the germline epigenetic reprogramming takes place and are particularly vulnerable to exposures such as endocrine disruptors (Ly et al. 2015; McCarrey 2014), as well as female recombination, are also susceptible to diet effects.

In conclusion, our study in mice shows that male recombination rate is sensitive to dietary changes, and this sensitivity depends on the genetic background. This is the first report of a diet effect on genome-wide levels of

recombination. Our results send a cautionary note for recombination studies, as diet constitutes a new factor that should be taken into account.

Data availability: The data underlying this article will be shared on reasonable request to the corresponding author.

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The authors declare that there is no conflict of interest.

Table 1 Strain effects on the number of autosomal MLH1 foci and total autosomal SC length per pachytene spermatocyte, and on the length of the SC between MLH1 foci

Strain	B6	MOLF	PWK
MLH1 foci:	23.54±1.97[a]	24.85±2.57[b]	29.15±2.87[c]
SC length (µm):	162.2±17.1[d]	156.6±16.0 [d]	183.3±19.4[e]
Interfocus distance (µm):	6,63±2.00[f]	5.43±1.57 [g]	6.83±2.06[f]
µmSC/MLH1 foci	6.9	6.3	6.3

Comparison of the three strains fed with control maintenance diet shows significant differences in the average number of MLH1 foci ($F = 81.5$, $P << 0.0001$), SC length ($F = 45.4$, $P << 0.0001$) and intercrossover distance ($F = 73.4$, $P << 0.0001$). Post-hoc analysis reveals significant differences in MLH1 foci frequency between the three strains (ab, $P=0.015$; bc, $P<10^{-9}$; ac, $P<10^{-9}$). In addition to displaying the highest crossover frequency, PWK has the longest SC length (de, $P <10^{-8}$ when compared to any of the two other strains). In contrast, MOLF has the shortest intercrossover distance (fg, $P<10^{-8}$ when compared to any of the two other strains). Analyses were performed by ANOVA and significant differences between groups were assessed using Tukey's post-hoc tests. Values are shown as mean ± SD.

Table 2 Diet effects on MLH1 foci number per spermatocyte.

Strain	B6	MOLF	PWK
Maintenance diet	23.54±1.97[a]	24.85±2.57[b]	29.15±2.87[c]
50% diet	23.79±1.63[a]	25.01±2.08	30.13±1.95[e]
Breeding diet	24.70±2.21[d]	24.35±1.62	29.95±2.41[c]

Comparison of the three types of diets within strains reveals significant differences in MLH foci number in B6 and PWK mice (*P*=0,006 and *P*=0.033, respectively, with ANOVA tests). In the B6 strain, significant differences are observed between the breeding diet and both the maintenance and 50% diet (*P*=0.008 and *P*=0.026, respectively, by Tukey's tests), but not between these two. In the PWK strain, significant differences are only observed between 50% and maintenance diets (*P*=0.033, Tukey's test, see text for discussion). Values are shown as mean±SD. Overall, a significant interaction between strain and diet effects is observed (*P*=0.005).

Table 3 Diet effects on crossover frequency in C57BL/6 male mice: analyses per spermatocyte and per bivalent.

A) B6 spermatocytes:	Maintenance diet	Breeding diet
MLH1 foci:	23.50±2,17[a]	24.24±2.31[b]
SC length (µm):	169,0±21,2	164,6±17,9
B) Number and type of bivalents:		
E0:	19 (0.5%)	16 (0.5%)
E1:	2808 (75.4%)	2417 (71.8%)
E2:	895 (24.0%)	927 (27.5%)
E3:	2 (0.1%)	5 (0.1%)

A) After analyzing an independent group of B6 mice, a significant increase in MLH1 foci count per spermatocyte was again observed in animals fed with breeding diet respect to those kept in maintenance diet (ab, t-student test, P = 0.001), while no significant differences in SC length were detected (P = 0,20, Mann-Whitney U test). B) When bivalents were classified according to the number of crossovers (E0-E3), a significant change was also observed (P = 0.004, χ^2 test). This change was mainly due to an increase of E2 bivalents at the expense of E1 in the breeding group, respect to the maintenance group.

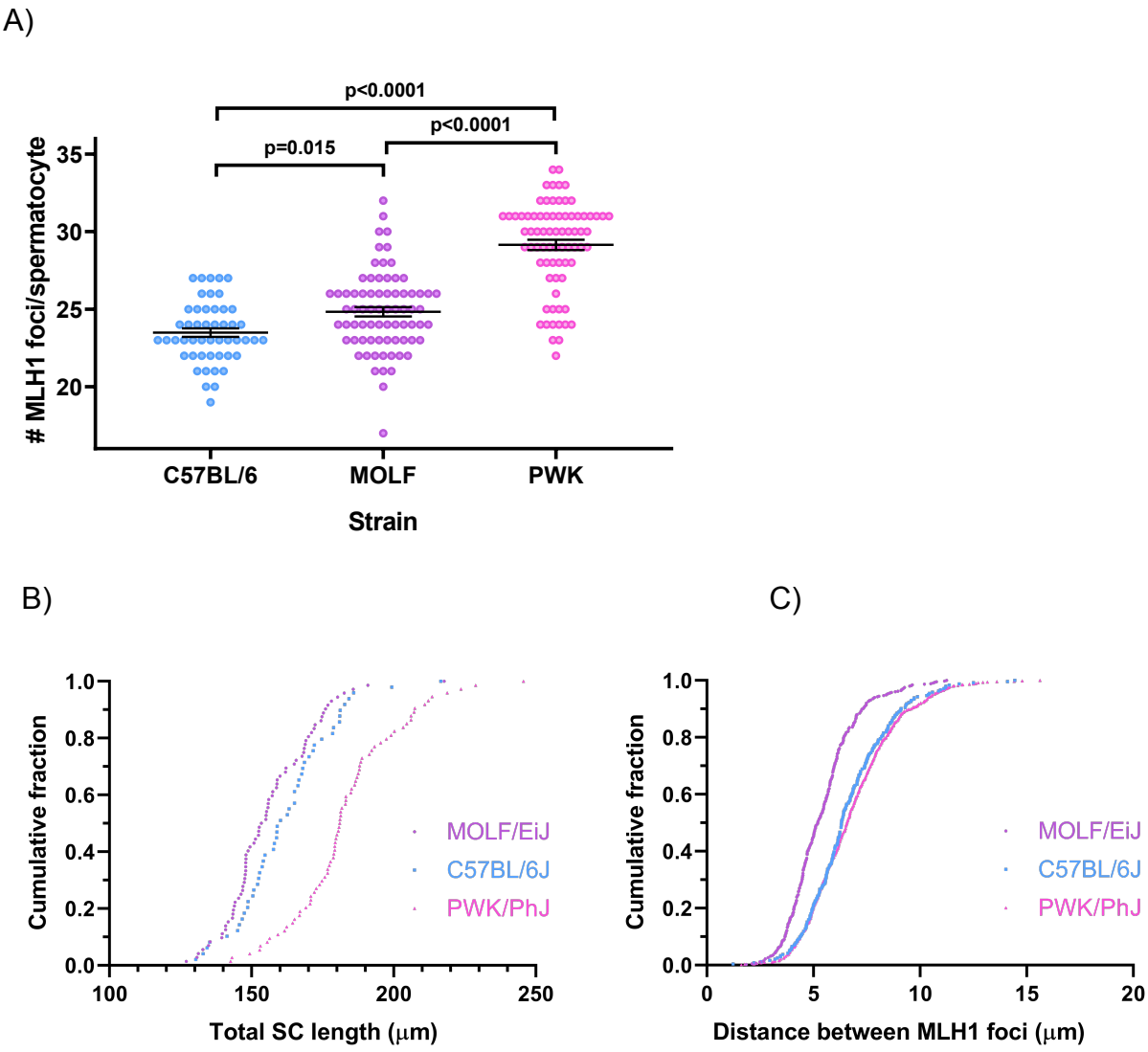


Figure 1 Strain effects on recombination rates, SC length and interference. Recombination levels depend on the genetic background. Autosomal MLH1 foci counts in mid-pachytene spermatocytes are shown in A for mice of the 3 strains fed *ad libitum* with maintenance diet. Significant differences were observed between the three strains, as explained in Table 1. Each dot represents the focus count of a single nucleus. Black bars represent means ± SEM. The cumulative fraction of the total autosomal SC length and the inter-crossover distances measured in micrometers are represented in B and C, respectively. PWK spermatocytes have significantly longer SC, while MLH1 interfocus

distances in MOLF are significantly shorter than those of the other two strains (Table 1).

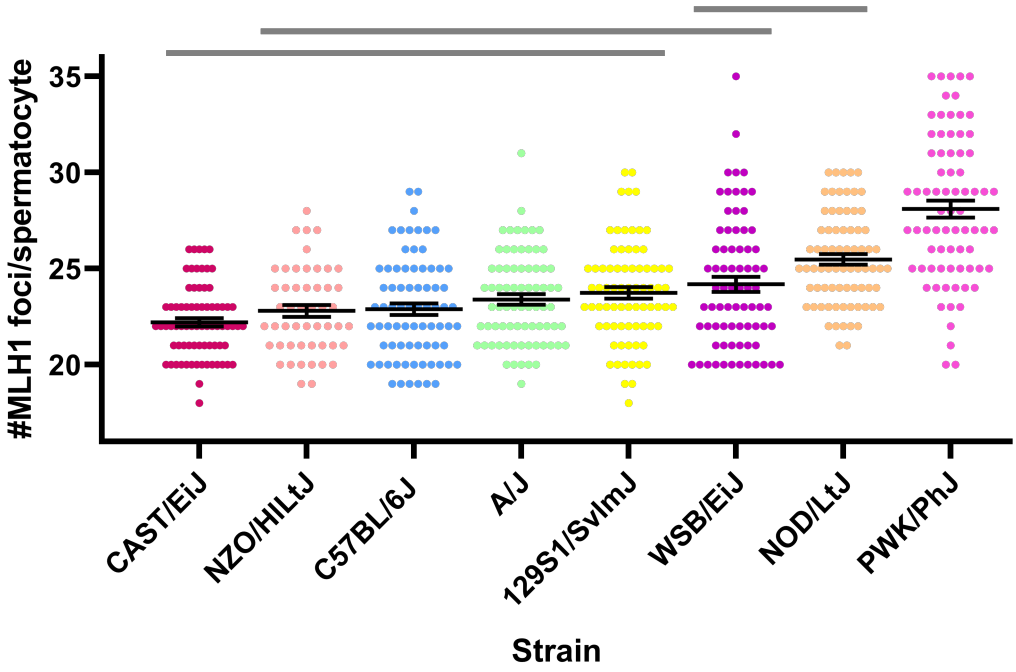
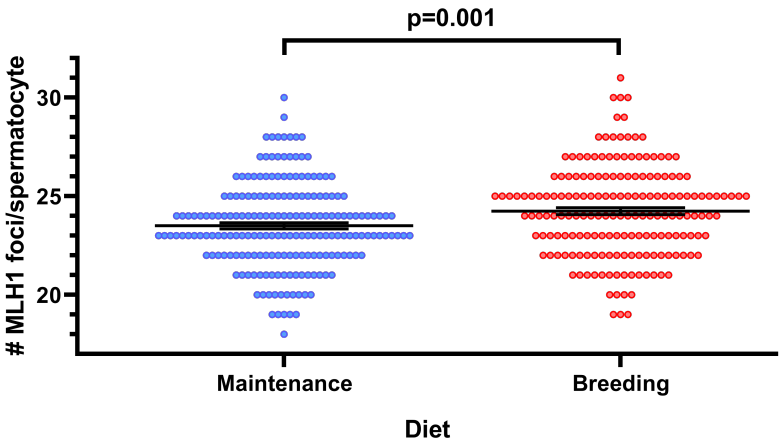


Figure 2 Recombination rate variability among CC founder strains. Autosomal MLH1 foci counts in mid-pachytene spermatocytes are shown for 8 genetically diverse mouse inbred strains, mostly of *M. m. domesticus* origin, except CAST/EiJ and PWK/PhJ, which are *M. m. castaneus* and *M. m. muculus* subspecies, respectively (Yang 2011). Each dot represents the focus count of a single nucleus. Black bars symbolize means ± SEM . Horizontal upper lines represent homogeneous subsets from post hoc comparisons using the Tukey test (α=0.01).

A)



B)

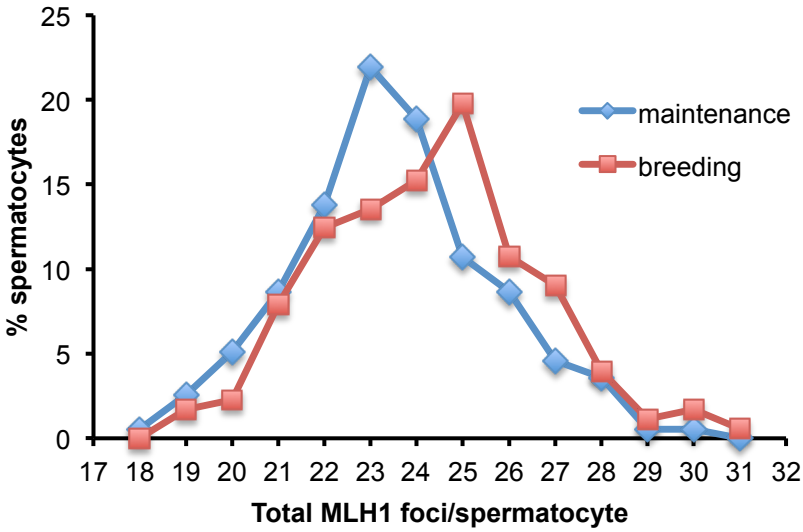


Figure 3 Diet effects on recombination rates. A) Diet effects on recombination rates are strain-dependent. Only C57BL/6 mice show significant differences in recombination rates when fed with maintenance *vs.* breeding diets (see Table 3). Each dot represents the focus count of a single nucleus. Black bars represent means ± SEM. B) Spermatocyte distribution according to the total number of MLH1 foci in C57BL/6 males subject to maintenance and breeding diets.

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Table S1: Summary of diets composition

Chow	Maintenance: Teklad 2014	Breeding: Harlan Teklad 2018	LabDiet 5001
Isoflavones	0-20 mg/kg	150-250 mg/kg	high*
Protein	14.3%	18.6%	23.9%
Fat extract	4.0%	6.2%	5.0%
Carbohydrate (available)	48.0%	44.2%	n/a
Crude fiber	4.1%	3.5%	5.1%
Neutral detergent fiber	18.0%	14.7%	15.6%
Ash	4.7%	5.3%	7.0
Energy density	2.9 kcal/g	3.1 kcal/g	3.4 kcal/g
Calories from protein	20%	24%	28.5%
Calories from fat	13%	18%	13.5%
Calories from carbohydrate	67%	58%	58.0%

2014: Teklad Global 14% Protein Rodent Maintenance Diet (Harlan Laboratories), designed to promote longevity and normal body weight in rodents. It does not contain alfalfa or soybean meal in order to minimize the occurrence of natural phytoestrogens. Capsumlab Maintenance Complete Chow (Capsumlab, Spain) has the same composition.

2018: Teklad Global 18% Protein Rodent Diet (Harlan Laboratories), formulated to support gestation, lactation and growth of rodents. It contains soy, but not alfalfa in order to limit the amount of phytoestrogens.

Laboratory Rodent Diet 5001 (LabDiet 5001 or Purina 5001): a classical chow designed for life-cycle nutrition (maintenance), but not for maximizing production in mouse breeding colonies. It contains soy and alfalfa.

Mineral and vitamin content, as well as other detailed information about the composition are also available from the manufacturers websites.

*Thigpen et al. (2013) reported that Purina 5001 contains 484±113 mg/kg daidzein+genistein (810±10 mg/kg according to Brown and Setchell (2001), mostly daidzin and genistein (72%), plus smaller amounts of other conjugates

and aglycones). The isoflavone contents reported by Harlan Laboratories refer to daidzein plus genistein aglycone equivalents.

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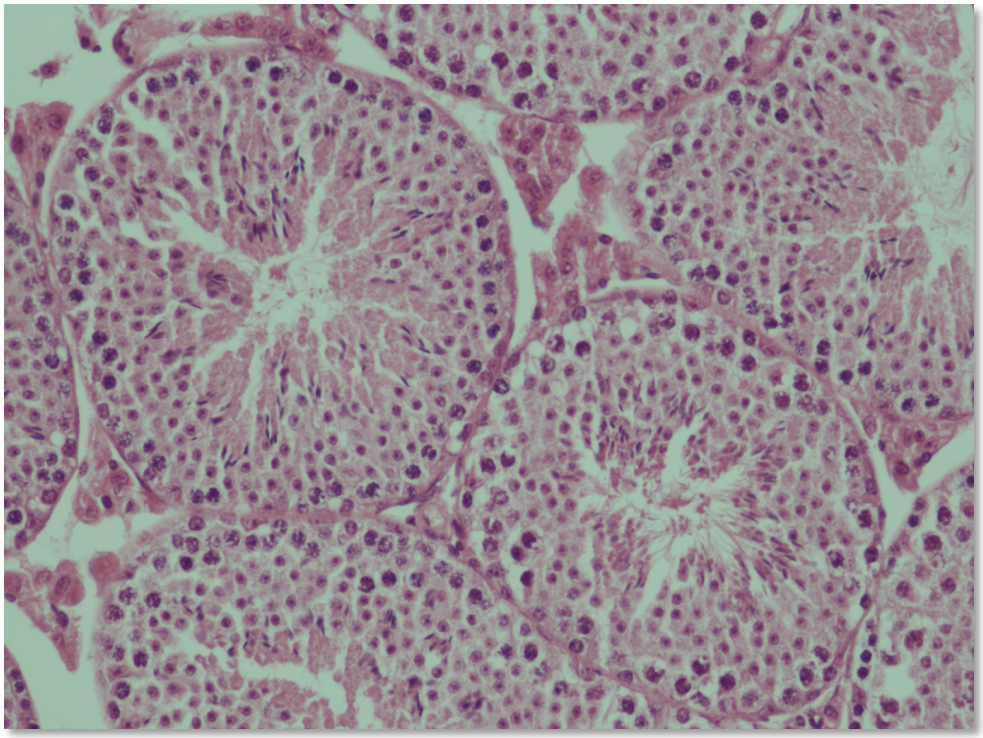
Table S2: Diet effects on body and testis weight, sperm kinematic parameters and SCSA measurements.

C57BL/6 mice diet:	Maintenance	Breeding	*P*-value
Body weight (g)	31.5±1.4	32.3±1.1	0.410
Testis weight (g)	0.105±0.003	0.108±0.002	0.410
Testis/body weight (%)	0.338±0.017	0.334±0.006	0.799
Sperm count/ml	$2.27 \cdot 10^7 \pm 0.55 \cdot 10^7$	$2.27 \cdot 10^7 \pm 0.31 \cdot 10^7$	0.566
Sperm viability (%)	59.2±4.1	57.3±1.9	0.655
tDFI (%)	4.55±1.07	5.71±1.24	0.760
HDS (%)	13.3±5.3	12.1±5.6	0.759
Progressive motility (%)	25.8±2.4	18.8±2.0	0.038*
Total motility (%)	60.5±3.6	57.8±3.1	0.580
VCL (μm/s)	88.8±6.3	71.3±5.4	0.061
VSL (μm/s)	47.3±4.3	36.3±3.6	0.064
VAP (μm/s)	61.5±5.0	48.7±4.2	0.062
LIN (%)	51.0±2.3	47.5±2.0	0.266
STR (%)	72.2±1.9	68.5±1.6	0.140
WOB (%)	67.8±1.7	66.5±1.5	0.563
ALH (μm)	3.36±0.20	2.84±0.17	0.055
BCF (μm)	6.35±0.28	5.76±0.23	0.120

Testis/body weight: testis weight fraction of body weight. tDFI, total DNA fragmentation index; HDS, high DNA stainability; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, lateral head displacement; BCF, beat cell frequency. Body, testis, sperm and SCSA data were analyzed by two-sided Student t-test; values are shown as mean ± SEM. Kinematic parameters were analyzed by factorial ANOVA and values are shown as LS mean ± SE. * $P < 0.05$

A)



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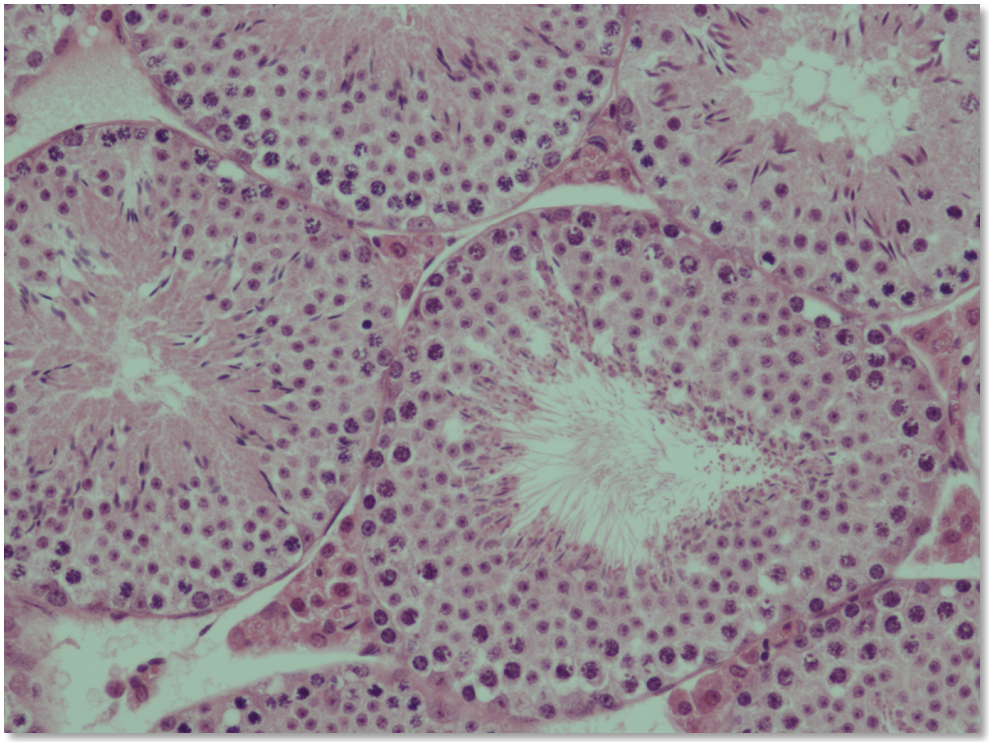

Figure S1: Histological analysis does not reveal any diet effect on seminiferous tubules cell-type composition. A) Example of testis section of a mouse fed with maintenance diet and B) of breeding diet-treated male, both stained with hematoxylin and eosin.