Spermatogenesis in house mouse in a Robertsonian polymorphism zone

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Abstract

Robertsonian (Rb) translocations can be important in speciation as a mechanism of postzygotic isolation between populations. Meiotic non-disjunction, gametogenic impairment, and association of impaired autosomal segments with sex chromosomes have been postulated as mechanisms responsible for reducing fertility in Rb mice. Quantitative histological studies needed to understand the role of Rb fusions in gametogenic impairment are scarce. Most research on Rb mice has analyzed meiotic non-disjunction of laboratory and wild-derived strains, which have complex or simple structural heterozygosity with large numbers of fusions. Using histological multilevel sampling, we examined spermatogenesis in mice from the Rb polymorphism area of Barcelona. We studied four chromosomal groups having: a) one Rb heterozygote fusion and $2n=39$, b) one Rb heterozygote fusion and $2n=31$, c) three Rb heterozygote fusions without monobrachial homology and with diploid number ranging from $2n=29$ to $2n=37$, and d) only Rb homozygote fusions with diploid number ranging from $2n=28$ to $2n=30$. Standard mice from the area surrounding the Rb zone were used as control. We analyzed morphological variables of the testes, relative frequency of stages in the seminiferous epithelium cycle, the 'round spermatids:primary spermatocytes' ratio, and other derived parameters. Our results reveal that structural homozygote mice and simple heterozygote mice having as few as one to three Rb fusions undergo greater germ cell death (GCD) than standard mice, suggesting that Rb fusions are related to increased GCD (in both the heterozygous and homozygous state) and may be the main cause of decreased gene flow between mice populations from this area.

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Introduction

Chromosome rearrangements can play an important role in speciation by acting as a mechanism of postzygotic isolation between chromosomally differentiated populations, either preventing the formation of hybrids or producing hybrids with reduced viability or with partial or complete sterility (King 1993, Spirito 2000, Rieseberg 2001). The Robertsonian (Rb) translocation, i.e. a centric fusion between two telocentric chromosomes to form a metacentric one, is a chromosomal rearrangement widely found between and within species. It has a high spontaneous mutation rate (estimated accurately only in humans: 1.0×10^{-4} per gamete per generation; Jacobs 1981) and is present in high frequency in some mammal populations (e.g. Baker & Bickham 1986, Piálek *et al.* 2005, Basset *et al.* 2008, Mao *et al.* 2008, Robinson *et al.* 2008). This, together with its effects in the reduction of both fertility and recombination

(especially in animals with Rb translocations in heterozygosis), suggests that Rb fusions may play an important role in speciation (e.g. Rieseberg 2001, Capanna & Castiglia 2004). Moreover, several studies have supported the idea that Rb fusions cause reproductive isolation in a number of mammal species (Theiler & Blanco 1996, De Oliveira *et al.* 2000, Rogatcheva *et al.* 2000, Fernández-Donoso *et al.* 2001, Jadwiszczak & Banaszek 2006, Lanzone *et al.* 2007, Franchini *et al.* 2008).

The western house mouse (*Mus musculus domesticus*, also referred to as *M. domesticus*) has extremely high karyotypic diversity. Its standard karyotype is 40 telocentric chromosomes, but the presence of Rb fusions can lead to a diploid number ranging between 22 and 39 chromosomes (Piálek *et al.* 2005, Sans-Fuentes *et al.* 2007, Solano *et al.* 2007, Burt *et al.* 2009). Heterozygotes for Rb translocations can occur either at hybrid zones between karyotypic races (for definition of race, see Hausser *et al.* (1994)) or in areas of Rb polymorphism

(Gündüz *et al.* 2001, Sans-Fuentes *et al.* 2007), and they can act as isolating barriers between populations. Meiotic non-disjunction (e.g. Gropp *et al.* 1982), meiotic failure (Miklos 1974, Burgoyne & Baker 1984, Manterola *et al.* 2009), and association of impaired autosomal segments with sex chromosomes (Johannisson & Winking 1994) have been postulated as mechanisms responsible for the reduction in fertility in carriers of heterozygous Rb fusions. They may, therefore, act as postmating isolating mechanisms. To date, two major conclusions can be drawn from the research on fertility of Rb mice: 1) reduction in fertility is more severe in complex heterozygotes characterized by one or more metacentrics with monobrachial arm homology than in simple heterozygotes characterized by one or multiple heterozygotes each one complemented by its homologous telocentrics (Redi & Capanna 1988, Hauffe & Searle 1998); 2) simple heterozygotes have a significant reduction in fertility associated with the presence of seven to nine heterozygous Rb fusions (Hauffe & Searle 1998, Castiglia & Capanna 2000, Wallace *et al.* 2002, Merico *et al.* 2003, Manterola *et al.* 2009), while when the number Rb fusions is low (from 1 to 3), less important alterations in reproduction have been detected (Britton-Davidian *et al.* 1990, Wallace *et al.* 1992, Hauffe & Searle 1998, Wallace 2003). These conclusions are based mainly on a number of analyses of meiotic non-disjunction of multivalents in structural heterozygotes (e.g. Baker & Bickham 1986, King 1993, Castiglia & Capanna 2000) and on a few histological studies conducted mainly in both laboratory and wild-derived strains (e.g. Redi & Capanna 1988 and references therein, Garagna *et al.* 2001, Wallace *et al.* 2002, Merico *et al.* 2003, 2008). There is little literature on histological studies of wild populations of mice, and these have focused mainly on the variation of the 'round spermatids:primary spermatocytes' ratio in mice with a high number of heterozygous Rb fusions (Wallace *et al.* 1992, Hauffe & Searle 1998, Wallace *et al.* 2002). Studies on this subject are important for several reasons. On the one hand, quantitative histological data are necessary to develop a proper understanding of the relationship between Rb fusions and gametogenic impairment. On the other hand, studies in wild populations may reveal how genetic isolation between Rb populations is proceeding. Hybrid zones between Rb races are especially relevant, since these zones may represent a barrier to gene flow (Panithanarak *et al.* 2004, Franchini *et al.* 2008), and their structure and extension are partially determined by the degree of selection against hybrids. Moreover, as these populations have been through several generations of natural selection, a reduction in meiotic aberrations in Rb heterozygotes is expected, when compared with hybrids from laboratory crosses between wild-derived strains, between laboratory strains or between wild-derived and laboratory strains.

Here, we compare spermatogenesis in wild mice from the Rb polymorphism area of Barcelona, where only simple heterozygotes have been found (for a detailed description of this zone, see Gündüz *et al.* (2001) and Sans-Fuentes *et al.* (2007)). Although this area is not a typical hybrid zone between Rb races, a reduction in gene flow between populations is occurring, since both morphological (Muñoz-Muñoz *et al.* 2003, 2006, Sans-Fuentes *et al.* 2009) and ethological (Sans-Fuentes *et al.* 2005) differentiation have been detected between animals differing in karyotypes. Specifically, we studied spermatogenesis by a histological multilevel sampling of standard mice (40ST), considered as a control group since a normal progression of spermatogenesis is expected in these animals, and four Rb groups having: a) one Rb heterozygote fusion and $2n=39$ (heterozygotes I-39, HTI-39), b) one Rb heterozygote fusion and $2n=31$ (heterozygotes I-31, HTI-31), c) three Rb heterozygote fusions without monobrachial homology and with a diploid number ranging from $2n=29$ to $2n=37$ (heterozygotes III, HTIII), and d) only Rb homozygote fusions with a diploid number ranging from $2n=28$ to $2n=30$ (HH). This study represents the first time that mice (within a polymorphic Rb area) having only one fusion, which is in heterozygosis, are compared with Rb heterozygotes mice with many Rb fusions in homozygosis, and to Rb homozygous mice. Moreover, most previous studies on wild mice from Rb zones have focused on multiple and complex heterozygotes, while spermatogenesis studies conducted on simple heterozygotes and on structural homozygotes are scarce (Redi & Capanna 1988, Wallace *et al.* 1992, Hauffe & Searle 1998). Therefore, the results obtained here contribute to completing the information on Rb metacentric effects in germ cell death (GCD), especially when the number of fusions in heterozygosis is low.

Results

Standard mice have bigger testes than Rb mice

As a first approximation to study a possible reduction in spermatogenic cells in Rb mice, we analyzed the volume of the testis and the length of the seminal vesicle (space Ω_0 ; Fig. 1A) in a sample of 61 mice ($n_{40ST}=17$; $n_{HH}=11$; $n_{HTI-31}=10$; $n_{HTI-39}=10$; and $n_{HTIII}=13$). The relative testis volume (testis volume in mm³/body mass in g) was different between the chromosomal groups ($F=4.76$, $P<0.01$). Although 40ST mice had the higher mean ($\bar{X}_{40ST}=0.00488\pm0.00021$; $\bar{X}_{HH}=0.00435\pm0.00016$; $\bar{X}_{HTI-31}=0.00352\pm0.00027$; $\bar{X}_{HTI-39}=0.00437\pm0.00029$; and $\bar{X}_{HTIII}=0.00389\pm0.00027$), a *post-hoc* Tukey's test revealed that only the groups HTI-31 and HTIII had significantly smaller means than the group 40ST ($P<0.01$ and $P<0.05$ respectively). The mean values of relative vesicle size did not differ significantly between the chromosomal groups.

Figure 1 Multiphasic hierarchical sampling. (A) Testis (Ω_0 space) with the central section highlighted. (B) Cross-section of the testis at $40\times$ (Ω_1 space) projected, using a *camera lucida* (Olympus BH2-DA), on a $0.5\times 0.5\text{-cm}^2$ grid; the crosses show the selected squares (one out of every five inside the grid limited by the cross-section). (C) Cross-section of the testis observed at $100\times$. One out of every five squares was selected, starting in the top left of the projection and following systematically from left to right and top to bottom. The exclusion limits are highlighted in red. (D) Cross-section of a seminiferous tubule at $400\times$ projected in a coherent grid of equidistant points; the points counted for the estimation of the areas are shown in red. (E) Cross-section of a seminiferous tubule at $400\times$ projected, with a *camera lucida*, onto a radial grid of 12 sectors with a nominal angle of 30° ; every other sector was selected for counting; the exclusion line is highlighted in red, i.e. the cells that touch this line are not taken into account.

The seminiferous epithelium cycle proceeds according to the expected pattern in Rb mice

To determine whether the succession of maturation stages is altered in Rb mice, we calculated, in space Ω_1 (Fig. 1B and C), the relative frequency of stages in the seminiferous epithelium cycle. To this end, as for the rest of the histological analysis, we used a subsample of 25 mice (five from each chromosomal group; Table 1). We did not detect significant differences between the chromosomal groups in the relative length of the epithelium maturation stages. The length values described in our study were consistent with those described in other studies on laboratory mice strains (Table 2; Clermont & Trott 1969). This suggests that the succession of maturation stages proceeds according to the expected pattern.

Reduction in spermatogenic activity in Rb mice

Spermatogenic activity and spermatogenic state of the tubules were studied by measuring the diameter of the tubule and the volume density of the epithelium respectively (space Ω_2 ; Fig. 1D). The minor diameter of the tubular cross-section showed significant differences between the chromosomal groups ($F=4.54$, $P<0.01$) and animals ($F=10.28$, $P<0.001$). The group 40ST had

significant higher mean diameter than the groups HTI-39 and HTIII ($P<0.05$); no significant differences were found between the groups HH, HTI-31, HTI-39, and HTIII (Fig. 2A). All groups except HH showed differences between animals. Despite high individual variance, 40ST mice had a higher frequency of tubules with diameter $>150\text{ }\mu\text{m}$, whereas in Rb mice, most tubules had diameters smaller than $150\text{ }\mu\text{m}$. The volume density of the epithelium showed significant differences between the chromosomal groups ($F=20.12$, $P<0.001$) and between animals ($F=7.55$; $P<0.001$). The groups HH and HTIII had less epithelium than the groups 40ST ($P<0.05$, $P<0.001$ respectively), HTI-31 ($P<0.01$, $P<0.001$ respectively), and HTI-39 ($P<0.001$ in both the cases; Figs 2B and 3A and B). No significant differences were observed between the groups 40ST, HTI-31, and HTI-39. Differences between individual means of the volume density of the epithelium were significant in all the groups.

Higher GCD during meiosis in Rb mice correlated positively with the number of Rb translocations

Also in space Ω_2 , the number of round spermatids (β) and pachytene spermatocytes (α) were counted to calculate several informative variables of cell population in the

Table 1 Biometrical and karyological characteristics of the specimens analyzed.

Chromosomal groups ^a	Month of capture	Locality	2n	Body mass (g)	Body length (mm)	Testis' sagittal diameter (mm)	Testis' transversal diameter (mm)	Relative testis mass (%) ^b	Relative seminal vesicle size (mm/g) ^c	Metacentric chromosomes	
										Homozygous	Heterozygous
<i>40S/T</i>											
313	September	Fulleda	40	18.00	88.00	8.15	4.60	0.481	0.517	–	–
316	September	Fulleda	40	18.00	88.00	7.82	4.40	0.426	0.541	–	–
317	September	Fulleda	40	19.50	87.00	7.76	5.35	0.562	0.464	–	–
318	September	Fulleda	40	19.00	93.00	7.63	4.29	0.377	0.506	–	–
319	September	Fulleda	40	20.00	88.00	7.60	4.96	0.466	0.553	–	–
Group mean ^d			40±0.00	18.90±0.40	88.80±1.07	7.79±0.10	4.72±0.19	0.462±0.031	0.516±0.015		
<i>HH</i>											
194	May	Sant Pau d'Ordal	28	22.50	90.00	7.70	4.50	0.350	0.416	3.8, 4.14, 5.15, 6.10, 9.11, 12.13	–
224	June	Sant Pau d'Ordal	30	18.50	87.00	6.99	4.70	0.422	0.444	4.14, 5.15, 6.10, 9.11, 12.13	–
250	June	Sant Pau d'Ordal	28	13.00	79.00	7.58	3.92	0.465	0.489	3.8, 4.14, 5.15, 6.10, 9.11, 12.13	–
255	June	Garraf	28	14.50	81.00	5.95	4.06	0.358	0.687	3.8, 4.14, 5.15, 6.10, 9.11, 12.13	–
288	August	Garraf	28	21.00	94.00	8.48	4.94	0.488	0.537	3.8, 4.14, 5.15, 6.10, 9.11, 12.13	–
Group mean ^d			28.40±0.40	17.90±1.83	86.20±2.72	7.34±0.42	4.42±0.19	0.417±0.028	0.515±0.048		
<i>HTI-31</i>											
189	May	Sant Pau d'Ordal	31	24.00	94.00	7.00	4.00	0.243	0.396	4.14, 5.15, 9.11, 12.13	3.8
195	May	Sant Pau d'Ordal	31	21.50	94.00	7.50	3.80	0.264	0.442	4.14, 5.15, 6.10, 12.13	9.11
218	May	La Granada	31	17.00	83.00	7.97	4.44	0.467	0.575	4.14, 5.15, 9.11, 12.13	6.10
241	June	Sant Pau d'Ordal	31	20.00	90.00	6.26	3.44	0.204	0.554	4.14, 5.15, 9.11, 12.13	3.8
279	July	Vilanova	31	16.25	83.00	7.43	4.39	0.449	0.657	4.14, 5.15, 9.11, 12.13	3.8
Group mean ^d			31.00±0.00	19.75±1.43	88.80±2.48	7.23±0.29	4.01±0.19	0.325±0.055	0.525±0.047		
<i>HTI-39</i>											
168	April	Les Pobles	39	16.00	87.00	6.72	3.65	0.300	0.481	–	12.13
335	September	Bellaterra (UAB)	39	21.00	86.00	7.14	4.61	0.366	0.467	–	5.15
336	September	Bellaterra (UAB)	39	23.00	90.00	8.12	4.96	0.431	0.456	–	4.14
349	September	Bellaterra (UAB)	39	19.50	81.00	7.11	4.57	0.387	0.352	–	4.14
351	September	Bellaterra (UAB)	39	19.00	81.00	7.39	4.26	0.362	0.488	–	5.15
Group mean ^d			39.00±0.00	19.70±1.16	85.00±1.76	7.30±0.23	4.41±0.22	0.369±0.021	0.449±0.025		
<i>HTIII</i>											
166	April	Les Pobles	37	18.00	93.00	6.60	4.47	0.376	0.513	–	4.14, 5.15, 12.13
191	May	Sant Pau d'Ordal	31	20.00	86.00	6.50	4.00	0.274	0.480	4.14, 5.15, 12.13	3.8, 6.10, 9.11
204	May	Calafell	35	15.00	83.00	6.66	4.10	0.389	0.539	12.13	4.14, 5.15, 9.11
221	May	Calafell	35	19.00	84.00	7.16	4.00	0.314	0.432	12.13	4.14, 5.15, 9.11
284	August	Garraf	29	15.00	75.00	7.30	4.70	0.542	0.627	3.8, 5.15, 9.11, 12.13	4.14, 6.10, 7.17
Group mean ^d			33.40±1.47	17.40±1.03	84.20±2.89	6.84±0.16	4.25±0.14	0.379±0.046	0.518±0.032		

^aFor abbreviations of the chromosomal groups, see Materials and Methods section. In each group the animal identifier is shown. ^bRelative testis mass calculated as 'testis mass (mg)/body mass (mg)', expressed as percentage. ^cRelative seminal vesicle size calculated as 'seminal vesicle length (mm)/body mass (g)'. ^dValues reported are mean±s.e.m.

Table 2 Relative mean length of the stages of the seminiferous epithelium cycle in each chromosomal group analyzed. Values reported are mean $\pm$ S.E.M.

Stage	Relative mean length (%)						Swiss albino mice
	40ST	HH	HTI-31	HTI-39	HTIII	C3H	
I	12.08 $\pm$ 3.93	12.36 $\pm$ 7.72	17.8 $\pm$ 1.99	15.98 $\pm$ 7.12	8.58 $\pm$ 3.80	11.12	11.73
II–III	10.78 $\pm$ 2.74	6.12 $\pm$ 1.78	8.72 $\pm$ 2.21	10 $\pm$ 4.19	11.88 $\pm$ 5.13	8.85	7.91
IV	6.14 $\pm$ 2.33	6.48 $\pm$ 2.30	5.9 $\pm$ 2.79	7.24 $\pm$ 2.83	6.5 $\pm$ 2.87	9.11	10.26
V	4.68 $\pm$ 2.89	3.76 $\pm$ 1.45	4.06 $\pm$ 1.32	4.7 $\pm$ 1.08	7.94 $\pm$ 3.24	8.77	8.58
VI	7.78 $\pm$ 2.93	9.12 $\pm$ 1.29	5.26 $\pm$ 3.62	8.64 $\pm$ 3.31	6.76 $\pm$ 2.86	8.85	6.98
VII	18.7 $\pm$ 1.39	16.32 $\pm$ 3.20	18.82 $\pm$ 3.80	19.42 $\pm$ 3.28	19.14 $\pm$ 5.45	14.38	15.16
VIII	3.56 $\pm$ 2.63	6.58 $\pm$ 3.62	4.66 $\pm$ 2.48	4.9 $\pm$ 3.33	4.58 $\pm$ 1.90	6.85	8.39
IX	12.1 $\pm$ 4.96	13 $\pm$ 2.66	13.48 $\pm$ 2.03	10.06 $\pm$ 2.47	11.44 $\pm$ 2.19	8.26	9.59
X	4.66 $\pm$ 2.53	5.02 $\pm$ 2.61	5.04 $\pm$ 2.46	3.46 $\pm$ 1.31	5.52 $\pm$ 2.30	3.66	4.16
XI	6.98 $\pm$ 2.90	6.78 $\pm$ 4.52	6.2 $\pm$ 0.97	5.82 $\pm$ 2.91	4.6 $\pm$ 1.73	9.87	8.45
XII	12.66 $\pm$ 3.36	14.36 $\pm$ 5.66	10.06 $\pm$ 4.48	9.76 $\pm$ 2.94	13.12 $\pm$ 4.82	10.51	8.86

The last two columns summarize the values obtained by Clermont & Trott (1969) in C3H and Swiss albino mice.

testis. As a measurement of GCD during meiosis, we calculated the $\beta:\alpha$ ratio. Chromosomal groups and animals differed significantly for this ratio ($F=41.28$, $P<0.001$ and $F=4.74$, $P<0.001$ respectively; Table 3). *A posteriori post-hoc* Tukey's test did not reveal significant differences between the groups HH, HTI-31, and HTI-39. These groups showed intermediate $\beta:\alpha$ values, with lower values than the group 40ST ($P<0.001$) and higher values than the group HTIII ($P<0.001$; Table 3, Fig. 2C). The percentages of GCD were 19.51% for 40ST and 39.61% for HTIII groups (Table 3). Further analysis using Student's *t*-test corrected by the method of Bonferroni (Rice 1989) revealed that some groups were significantly heterogeneous (Table 3). The distribution of cell death among tubules was

estimated as the percentage of defective tubules per animal (% DT). For the criterion % DT < 3 (i.e. percentage of tubules with $\beta:\alpha$ ratio lower than 3:1), significant differences between the groups were detected ($F=8.71$, $P<0.001$). The group 40ST had the lowest mean ($P<0.05$ when compared with the groups HH and HTI-39; $P<0.01$ when compared with the group HTI-31; $P<0.001$ when compared with the group HTIII). No differences were detected between the Rb groups (Table 3, Fig. 2D). Under the criterion % DT < 2, significant differences were also detected between the groups ($F=3.48$, $P<0.05$); the group 40ST had a lower mean value than the group HTIII ($P<0.05$). For criterion % DT < 1, only two animals – belonging to the group HTIII – had $\beta:\alpha < 1:1$ (Table 3).

Figure 2 (A) Seminiferous tubule diameter. (B) Volume density of the epithelium. (C) Spermatids:spermatocytes ratio ($\beta:\alpha$). (D) Percentage of defective tubules under the criteria $\beta:\alpha < 3:1$. (E) Numeric density of spermatids (filled symbols) and spermatocytes (empty symbols). (F) Total number of spermatids (filled symbols) and spermatocytes (empty symbols) in the testis. Mean $\pm$ 2 S.E.M. is represented.

Table 3 Derived parameters from spermatocytes and spermatids counts in the seminiferous tubules. Values reported are mean $\pm$ s.e.m.

Chromosomal group	Animal number	2n	Number of analyzed tubules	Mean GCD $\pm$ s.e.m. (%)	Mean $\alpha:\beta \pm$ s.e.m.	Mean Nv α $(10^5 \text{ cel./mm}^3) \pm$ s.e.m.	Mean Nv β $(10^5 \text{ cel./mm}^3) \pm$ s.e.m.	Mean TN α $(10^6 \text{ cel./test.}) \pm$ s.e.m.	Mean TN β $(10^6 \text{ cel./test.}) \pm$ s.e.m.	% DT < 1	% DT < 2	% DT < 3
40ST	313	40	29	16.77 $\pm$ 2.87	1:3.33 $\pm$ 0.10	3.38 $\pm$ 0.01	11.10 $\pm$ 0.29	28.09 $\pm$ 0.82	92.33 $\pm$ 2.43	0.00	0.00	20.70
	316	40	27	17.83 $\pm$ 4.11	1:3.29 $\pm$ 0.16	3.61 $\pm$ 0.17	12.10 $\pm$ 0.49	27.34 $\pm$ 1.37	89.05 $\pm$ 3.60	0.00	3.70	37.00
	317	40	24	22.79 $\pm$ 3.20	1:3.09 $\pm$ 0.13	2.80 $\pm$ 0.15	8.85 $\pm$ 0.35	28.64 $\pm$ 1.33	91.24 $\pm$ 3.16	0.00	4.20	41.70
	318	40	33	18.84 $\pm$ 2.27	1:3.25 $\pm$ 0.09	4.17 $\pm$ 0.13	13.79 $\pm$ 0.34	29.81 $\pm$ 1.02	98.01 $\pm$ 2.73	0.00	0.00	24.20
	319	40	40	21.20 $\pm$ 2.37	1:3.15 $\pm$ 0.09	3.54 $\pm$ 0.14	10.72 $\pm$ 0.28	30.80 $\pm$ 1.10	96.20 $\pm$ 2.49	0.00	0.00	37.50
	Group mean		153	19.51 $\pm$ 1.27	1:3.22 $\pm$ 0.05	3.54 $\pm$ 0.07	11.38 $\pm$ 0.20	29.14 $\pm$ 0.51	93.94 $\pm$ 1.29	0.00	7.30	32.00
HH	194	28	38	25.73 $\pm$ 2.56	1:2.97 $\pm$ 0.10	3.54 $\pm$ 0.12	10.60 $\pm$ 0.34	27.87 $\pm$ 1.14	80.34 $\pm$ 2.55	0.00	5.30	47.40
	224	30	35	25.86 $\pm$ 1.99	1:2.96 $\pm$ 0.08	3.68 $\pm$ 0.13	10.65 $\pm$ 0.35	28.57 $\pm$ 1.11	82.63 $\pm$ 3.08	0.00	0.00	54.30
	250	28	30	22.68 $\pm$ 2.82	1:3.09 $\pm$ 0.11	3.48 $\pm$ 0.12	10.61 $\pm$ 0.25	20.23 $\pm$ 0.68	61.75 $\pm$ 1.48	0.00	0.00	56.70
	255	28	37	24.00 $\pm$ 2.67	1:3.04 $\pm$ 0.11	3.38 $\pm$ 0.01	9.54 $\pm$ 0.29	16.62 $\pm$ 0.52	49.62 $\pm$ 1.95	0.00	2.70	48.60
	288	28	34	32.88 $\pm$ 3.02	1:2.68 $\pm$ 0.12	3.44 $\pm$ 0.12	8.77 $\pm$ 0.28	33.37 $\pm$ 1.30	84.84 $\pm$ 3.16	0.00	14.70	73.50
	Group mean		174	26.26 $\pm$ 1.20	1:2.95 $\pm$ 0.05	3.50 $\pm$ 0.05	10.04 $\pm$ 0.15	25.41 $\pm$ 0.64	71.76 $\pm$ 1.55	0.00	4.60	55.70
HTI-31	189	31	33	35.73 $\pm$ 2.08	1:2.57 $\pm$ 0.08	3.34 $\pm$ 0.11	8.74 $\pm$ 0.31	18.76 $\pm$ 0.64	49.10 $\pm$ 1.72	0.00	12.10	81.80
	195	31	36	21.04 $\pm$ 3.10	1:3.16 $\pm$ 0.12	3.41 $\pm$ 0.15	10.35 $\pm$ 0.36	18.62 $\pm$ 0.79	56.42 $\pm$ 1.95	0.00	5.60	38.90
	218	31	40	30.09 $\pm$ 3.01	1:2.80 $\pm$ 0.12	4.28 $\pm$ 0.17	11.09 $\pm$ 0.35	32.71 $\pm$ 1.32	85.94 $\pm$ 2.89	0.00	10.00	62.50
	241	31	32	25.68 $\pm$ 2.32	1:2.98 $\pm$ 0.09	3.24 $\pm$ 0.12	9.26 $\pm$ 0.36	12.72 $\pm$ 0.46	35.56 $\pm$ 1.56	0.00	0.00	50.00
	279	31	45	36.97 $\pm$ 1.81	1:2.52 $\pm$ 0.07	4.60 $\pm$ 0.13	11.09 $\pm$ 0.36	32.24 $\pm$ 0.89	80.63 $\pm$ 2.84	0.00	6.70	80.00
	Group mean		186	30.25 $\pm$ 1.20	1:2.79 $\pm$ 0.05	3.83 $\pm$ 0.07	10.17 $\pm$ 0.17	23.78 $\pm$ 0.71	63.17 $\pm$ 1.74	0.00	7.00	63.44
HTI-39	168	39	32	31.38 $\pm$ 1.70	1:2.75 $\pm$ 0.07	4.31 $\pm$ 0.15	12.24 $\pm$ 0.45	21.85 $\pm$ 0.93	59.26 $\pm$ 2.43	0.00	0.00	75.00
	335	39	30	24.45 $\pm$ 2.61	1:3.02 $\pm$ 0.10	3.10 $\pm$ 0.14	9.28 $\pm$ 0.37	22.92 $\pm$ 1.05	68.60 $\pm$ 2.77	0.00	3.30	56.70
	336	39	52	28.26 $\pm$ 1.97	1:2.87 $\pm$ 0.08	3.56 $\pm$ 0.13	10.02 $\pm$ 0.29	31.75 $\pm$ 1.03	94.75 $\pm$ 2.71	0.00	7.70	59.60
	349	39	60	31.58 $\pm$ 2.32	1:2.74 $\pm$ 0.09	2.88 $\pm$ 0.10	7.67 $\pm$ 0.19	20.88 $\pm$ 0.76	55.61 $\pm$ 1.42	0.00	18.30	65.00
	351	39	48	25.12 $\pm$ 1.90	1:2.99 $\pm$ 0.08	3.86 $\pm$ 0.12	11.70 $\pm$ 0.40	25.52 $\pm$ 0.77	78.19 $\pm$ 2.75	0.00	2.10	47.90
	Group mean		222	28.41 $\pm$ 0.99	1:2.75 $\pm$ 0.07	3.47 $\pm$ 0.06	9.93 $\pm$ 0.18	24.52 $\pm$ 0.48	71.66 $\pm$ 1.46	0.00	7.70	60.40
HTIII	166	37	42	48.82 $\pm$ 4.06	1:2.05 $\pm$ 0.16	4.09 $\pm$ 0.17	7.88 $\pm$ 0.44	27.06 $\pm$ 1.14	52.34 $\pm$ 2.69	7.10	54.80	76.20
	191	31	29	35.45 $\pm$ 2.24	1:2.58 $\pm$ 0.09	3.63 $\pm$ 0.14	9.22 $\pm$ 0.32	19.08 $\pm$ 0.72	48.48 $\pm$ 1.98	0.00	10.30	75.90
	204	35	39	32.11 $\pm$ 2.64	1:2.72 $\pm$ 0.11	3.11 $\pm$ 0.13	8.40 $\pm$ 0.38	17.49 $\pm$ 0.72	47.19 $\pm$ 1.82	2.60	12.80	64.10
	221	35	28	32.43 $\pm$ 2.60	1:2.70 $\pm$ 0.10	3.28 $\pm$ 0.15	8.82 $\pm$ 0.36	19.38 $\pm$ 1.03	50.55 $\pm$ 2.08	0.00	7.10	75.00
	284	29	39	45.43 $\pm$ 1.67	1:2.18 $\pm$ 0.07	3.69 $\pm$ 0.15	8.22 $\pm$ 0.30	28.87 $\pm$ 1.16	61.37 $\pm$ 1.75	0.00	30.80	94.90
	Group mean		177	39.61 $\pm$ 1.40	1:2.42 $\pm$ 0.06	3.57 $\pm$ 0.07	8.45 $\pm$ 0.16	22.77 $\pm$ 0.57	52.25 $\pm$ 1.01	1.30	25.40	77.40

The mean values obtained by the chromosomal group are shown in italics. GCD, germ cell death; α , number of spermatocytes; β , number of spermatids; Nv α and TN α , numeric density and total number of spermatocytes respectively; Nv β and TN β , numeric density and total number of spermatids respectively; % DT, percentage of defective tubules.

Figure 3 (A and B) Histological section of the seminiferous tubules (100 $\times$) of specimens 313 (40ST) and 166 (HTIII) respectively. (C) Binucleated spermatid (1000 $\times$) observed in the cross-section of a seminiferous tubule at stage VI of the specimen 224 (HH). (D) Symplastic spermatids (1000 $\times$) observed in the cross-section of a seminiferous tubule at stage V of specimen 284 (HTIII). (E) Apoptotic cell (1000 $\times$) found in a seminiferous tubule in stages II–III of specimen 218 (HTI-31). (F) Apoptotic cell (1000 $\times$) found in a seminiferous tubule in stage VI of specimen 166 (HTIII). The scale bar represents 100 μ m in A and B and 10 μ m in C–F. The histological cross-section was stained with periodic acid-Schiff (P.A.S.) and contrasted with hematoxylin.

Light microscopic observation on testis cross-sections revealed that apoptotic spermatids and abnormal multinucleated syncytia (symplast) were prevalent in HTIII mice. Binucleated spermatids were also frequently observed in HH, HTI-31, and HTI-39 mice (Fig. 3C–F).

Reduction in the number of primary spermatocytes per testis in Rb mice

Because the number of round spermatids and primary spermatocytes provide additional information on meiotic and pre-meiotic GCD respectively, we calculated the numeric densities and the total number of cells for round spermatids (Nv β and TN β) and primary spermatocytes (Nv α and TN α).

Nv β and Nv α were significantly different between the chromosomal groups (Nv β : $F=36.91$, $P<0.001$; Nv α : $F=3.59$, $P<0.05$) and animals (Nv β : $F=13.91$, $P<0.001$; Nv α : $F=12.35$, $P<0.001$). The *post-hoc* Tukey test for Nv β revealed the same pattern found for β : α ; the group 40ST had the highest average ($P<0.001$), and the group HTIII had the lowest, while no significant differences were observed between the groups HH, HTI-31, and HTI-39 ($P<0.001$; Table 3, Fig. 2E). This result

confirms GCD during meiosis. All groups, except HTIII, showed significant differences for Nv β between animals (Table 3). Nevertheless, for Nv α only significant differences between the groups HTI-31 and 40ST were found, with HTI-31 showing the highest value ($P<0.01$; Fig. 2E). Significant differences between animals were also detected for Nv α , in all groups except in HH (Table 3).

The total number of cells was different between the groups (TN β : $F=142.78$, $P<0.001$; TN α : $F=34.17$, $P<0.001$) and between animals (TN β : $F=30.11$, $P<0.001$; TN α : $F=43.04$, $P<0.001$). Further analyses using the *post-hoc* Tukey test for TN β showed that the group 40ST had the highest value, and the group HTIII had the lowest. The groups HH, HTI-31, and HTI-39 had intermediate mean values, with HTI-31 showing lower values than HH and HTI-39. All these differences were significant at $P<0.001$ (Table 3, Fig. 2F). For TN α values, the group 40ST had the highest mean value ($P<0.001$), and the group HTIII had the lowest ($P<0.001$, when compared with the groups 40ST and HH; $P<0.05$, when compared with the group HTI-39). The groups HTI-31 and HTIII were not significantly different (Table 3, Fig. 2F). All groups, except 40ST, showed differences between specimens for TN β and TN α (Table 3).

Discussion

Our results on GCD and spermatogenic activity-related parameters indicate a reduction in spermatozoid production in mice from the Rb zone of Barcelona, especially in those with three heterozygous fusions. Since a positive correlation between the mass of the testis and the number of spermatogenic cells has been reported in the house mouse (Searle & Beechey 1974, Meistrich 1982, Sinha Hikim *et al.* 1989), we analyzed the size of the reproductive organs in animals from different chromosomal groups. We found a tendency for standard animals to have higher values of relative testis size than Rb mice. This corroborates previous results obtained in other Rb zones where differences in testis mass between standard and Rb mice were detected (Wallace *et al.* 1992, Hauffe & Searle 1998, Chatti *et al.* 2005).

Unlike other studies, we did not find spermatogenic arrest or delay in the maturation of the seminiferous epithelium. However, Redi *et al.* (1985) and Eaker *et al.* (2001) observed a delay in spermatogenesis at stage XII with an increase of apoptotic cells, suggesting the loss of spermatocytes during the first meiotic division. This inconsistency between the results could be due to differences between the number and type of metacentrics carried by the specimens. According to Hauffe & Searle (1998), an increase in the number of heterozygous fusions and especially the presence of fusions with monobrachial homology can have drastic effects during spermatogenesis. It should be borne in mind that, whilst our study analyzed mice with a maximum number of three Rb fusions in heterozygosis, animals in the study by Eaker *et al.* (2001) were structural heterozygotes for four Rb translocations. Likewise, Redi *et al.* (1985) focused their research on animals carrying two or more fusions but with monobrachial homology. Moreover, the animals in their studies were hybrids from crosses between different laboratory strains or wild-derived strains; thus, genetic background incompatibility can be an additional factor in spermatogenic arrest.

Mice from the Rb polymorphism area of Barcelona had significantly smaller diameters of the seminiferous tubules than the standard mice. A positive relationship between the tubular diameter and the spermatogenic activity of testis has been reported (Sinha Hikim *et al.* 1989), with the tubular diameter being a better indicator than the testis mass for discriminating between different levels of spermatogenic alterations (Russell *et al.* 1990). Therefore, these results indicate lower spermatogenic activity in Rb mice (heterozygotes and homozygotes) than in 40ST ones. Previous studies also reported a reduction in the tubular diameter in simple Rb heterozygote mice carrying nine fusions and in backcrosses of these hybrids with the parental races (Said *et al.* 1993, Chatti *et al.* 2005).

Our results on the volume density of the epithelium indicate that HH mice, and especially HTIII mice, have a smaller fraction of the tubule occupied by cells. This suggests that the cell population in these two groups is substantially smaller than that in 40ST mice. To our knowledge, other authors studying fertility in Rb mice did not quantify the fraction of the seminiferous tubules filled by cells. However, qualitative histological observations reveal that subfertile and sterile Rb mice can show bigger areas of lumen than the standard mice (e.g. Fig. 5 in Capanna *et al.* (1976), Fig. 2 in Redi *et al.* (1985), Fig. 1d in Said *et al.* (1993), Fig. 3 in Garagna *et al.* (2001); Fig. 1c in Merico *et al.* (2003)).

The smaller testis sizes, lower spermatogenic activity, and smaller volume density in mice from the Rb polymorphism area of Barcelona can be partially explained by a decrease in the number of spermatids. Mice from the Rb area (especially those from HTIII) showed lower $\beta:\alpha$, $Nv\beta$, and $TN\beta$ values, and higher percentages of GCD than the standard mice, suggesting a reduction in the spermatid population when Rb translocations are present. The decrease in spermatid numbers was greater in mice with three Rb heterozygote fusions. This result was also supported by direct observations of the cross-sections under the light microscope, since symplastic and apoptotic spermatids were frequently observed in Rb mice (Fig. 3C–F). It is worth noting that GCD is not homogeneous among tubules. Whereas the % DT with $\beta:\alpha < 3:1$ barely exceeded 40% in 40ST specimens, it oscillated between 39 and 95% in the Rb groups. The highest % DT under the criterion $\beta:\alpha < 2:1$ was observed in the HTIII group; and only in this group were animals meeting the criterion $\beta:\alpha < 1:1$ found.

GCD values obtained in mice from the Rb polymorphism area of Barcelona show that the main factor responsible for the positive association between Rb translocation and GCD is the number of fusions in heterozygosis. This is probably because the greater number of trivalent configurations in the spermatocytes increases the probability of an association with the XY bivalent, impairing the survival of the spermatocytes (e.g. Johannisson & Winking 1994, Merico *et al.* 2003, Manterola *et al.* 2009). Our results are in accordance with those reported by other investigations of wild mice (Wallace *et al.* 1992, Hauffe & Searle 1998) and hybrids from laboratory crosses between Rb races of mice from geographically distant populations (Wallace *et al.* 2002). Interestingly, HH mice showed lower mean $\beta:\alpha$ ratio values than 40ST mice and similar to those obtained for HTI-31 and HTI-39 mice. Previous studies, using wild mice or wild-derived strains, did not find differences between Rb homozygotes and the standard mice (e.g. Wallace *et al.* 1992, Hauffe & Searle 1998, Merico *et al.* 2003). Unlike in those studies, HH mice from the Rb area of Barcelona are not a race (Sans-Fuentes *et al.* 2007), but are hybrids derived either from crosses

between HH mice with equal karyotype or from crosses between mice with the same set of heterozygous Rb fusions. It is important to note that a) spermatogenesis is a very complex process that can be impaired by both structural and genetic incompatibilities and b) some different allelic variants could be linked to different metacentrics due to the suppression of recombination in Rb translocations (Davisson & Akeson 1993, Dumas & Britton-Davidian 2002). Therefore, HH mice from the Rb polymorphism area of Barcelona are likely to present a larger number of negative epistatic interactions than an established chromosomal race, since the latter has been under more generations of natural selection, and therefore allele combinations that disrupt spermatogenesis could have been eliminated from the population. It must also be pointed out that, although not statistically different, the mean $\beta:\alpha$ ratio values for HTI-31 mice were slightly lower than HTI-39 mice. This could be due to a combined effect of structural heterozygosity and genetic incompatibilities similar to those discussed previously for HH mice. Other authors have suggested the relation of GCD with genetic incompatibilities in Rb mice (Said *et al.* 1993, Everett *et al.* 1996, Wallace *et al.* 2002). Even though different histological studies cannot be compared directly, especially if different methods for cell number correction are used (see Mouton (2002a)), the results obtained by Everett *et al.* (1996) are worth mentioning; they report high percentages of GCD (36.2–57.3%) for heterozygote and homozygote animals for only one Rb fusion. In this case, the remarkably high GCD values could be due to incompatibilities in the genetic background, since these animals belong to a laboratory strain in which wild Rb fusions were introduced.

For the number of spermatocytes, the highest values of $Nv\alpha$ were observed in the group HTI-31, mainly due to the high $Nv\alpha$ of two specimens (218 and 279, Table 1). These two animals have high values of GCD in their group and have $Nv\alpha$ values higher than 40ST animals. This trend of high levels of spermatid death and high numbers of spermatocytes can be observed in other mice from the Rb polymorphism area of Barcelona, especially in multiple heterozygotes; for instance, mice 166 and 284 showed the lowest $\beta:\alpha$ ratio but had the highest $Nv\alpha$ in their group (HTIII). This could be related to a mechanism to buffer cell death during meiosis by increasing the number of proliferative spermatogonia that start differentiating when cell death is significant. This phenomenon has been described for the case of complex Rb heterozygote mice with 2 and 16 metacentrics, in which the number of proliferative spermatogonia initiating differentiation was higher than in the standard mice (Redi *et al.* 1983, 1985).

The total number of spermatocytes ($TN\alpha$) and spermatids ($TN\beta$) in the testis showed a similar pattern to those found for the ratio $\beta:\alpha$. The group 40ST had the

highest average, and the group HTIII had the lowest. While the pattern observed for $TN\beta$ was consistent with the results obtained for $Nv\beta$, $TN\alpha$ and $Nv\alpha$ showed different patterns. The results for TN should be interpreted with care, since they can be driven by testis volume, as ' $TN = Nv \times \text{testis volume}$ '. Assuming that Nv is homogeneous throughout the testis, and that the size of the testis is mainly determined by germ cell numbers (Yang *et al.* 1997), we may conclude that Rb mice produce a smaller total number of germ cells. If these assumptions are correct, the results obtained for TN suggest that GCD also occurs before the first meiotic division, either among the primary spermatocyte population or in the previous stages, for example, during the mitotic divisions that maintain the spermatogonia population. In fact, the existence of one pachytene checkpoint that arrests meiosis and induces cell elimination if the recombination or synapsis is altered has been postulated (Roeder & Bailis 2000, Merico *et al.* 2003, 2008). Moreover, it has been observed that primary spermatocytes of Rb structural heterozygotes have alterations in the regulation of enzymatic activity (Hotta & Chandley 1982), qualitative differences in the protein patterns (Forejt 1982, Merico *et al.* 2008), and longer prophase I (Redi *et al.* 1985).

In addition to the differences between chromosomal groups for the spermatogenic parameters analyzed, a high variability within the groups was detected. This suggests that other factors, such as metacentric composition of the karyotypes (Gropp & Winking 1981, Redi & Capanna 1988, Searle 1993) and genetic incompatibilities (Said *et al.* 1993, Wallace *et al.* 2002), may also be playing an important role in the degree of spermatogenesis impairment.

The results of this study suggest that Rb translocations affect spermatogenesis by increasing GCD, especially when three heterozygous fusions are present. Nevertheless, we cannot discard other factors such as genetic incompatibilities or the metacentric composition of the karyotype. If this increase in GCD results in a reduction in male fertility, it could be a major cause of the reduction in gene flow between populations found previously in this area (Muñoz-Muñoz *et al.* 2003, 2006, Sans-Fuentes *et al.* 2005, 2009). Moreover, other selective pressures related to reproduction, such as the impairment of female gametogenesis and embryonic loss in crosses between animals with fusions, could play an important role in the reduction in gene flow between populations (Cattanach & Moseley 1973, Epstein & Travis 1979, Marchetti *et al.* 1999). Therefore, more detailed studies that examine, for example, female fertility and crosses in the laboratory between animals with different karyotypes should be conducted to evaluate the degree of isolation between populations of the Rb polymorphism area from Barcelona due to potentially negatively heterotic Rb rearrangements.

Materials and Methods

Animals

The analysis of male reproductive organ size and the histology study of spermatogenesis were conducted on four chromosomal groups of mice from the Rb polymorphism area of Barcelona (Gündüz *et al.* 2001, Sans-Fuentes *et al.* 2007): homozygotes (HH), animals with all the Rb fusions in structural homozygosis and with a diploid number ranging from $2n=28$ to $2n=30$; HTI-31, specimens with one Rb fusion in heterozygosis and four in homozygosis, and with $2n=31$; HTI-39, specimens with one fusion in heterozygosis and $2n=39$; and HTIII, mice with three heterozygous fusions and from zero to four fusions in homozygosis, and with a diploid number ranging from $2n=29$ to $2n=37$. These groups were established for chromosome number and structural heterozygosity–homozygosity. As a control, we used standard mice (40ST) from the area surrounding the Rb zone. It should be noted that both HTI-31 and HTI-39 have one Rb fusion in heterozygosis but differ in the number of homozygous metacentrics. This allows us to analyze whether there is an added effect on reproduction produced by the number of homozygous metacentrics, apart from the effect produced by structural heterozygosity.

For each category, adult mice were selected, i.e. sexually active animals with body mass higher than 12 g and body length longer than 75 mm. An animal was classified as sexually active when smears from both right testis and right epididymis had spermatozooids (Gosálbez *et al.* 1979). Size of reproductive organs (analysis of Ω_0 space, see sampling strategy section; Fig. 1A) was studied in 61 mice ($n_{40ST}=17$; $n_{HH}=11$; $n_{HTI-31}=10$; $n_{HTI-39}=10$; and $n_{HTIII}=13$) captured between March and September of 1996–2000. The histological study of spermatogenesis (analysis of Ω_1 and Ω_2 spaces, see sampling strategy section Fig. 1B–E) was conducted on a subset of the previous sample consisting of 25 mice (5 for each chromosomal group) captured between April and September of 1998. When selecting the animals of this subsample, we tried to minimize the heterogeneity of the following factors: sampling locality, Rb chromosome composition per chromosomal group, and reproductive season and year (Table 1).

Sampling strategy, parameters calculations, and statistical analyses

Reproductive data were obtained from multilevel sampling (Cruz-Orive & Weibel 1981, Browne *et al.* 1995). Three levels of organization were analyzed as follows (Fig. 1): testis and seminal vesicle (Ω_0 space), where the size of testis and seminal vesicle were measured; cross-section of the testis examined at $40\times$ (Ω_1 space) to calculate the relative frequency of stages in the seminiferous epithelium cycle; and cross-section of the seminiferous tubule examined at $400\times$ (Ω_2 space) to conduct a morphometric study of the seminiferous tubules and to quantify the cell population of the testis. In the space Ω_0 , the data correspond with measurements of the left testis and seminal vesicle that were taken right after the animal was killed. To analyze the spaces Ω_1 and Ω_2 , left testes were fixed in 4% formaldehyde solution. Then, the samples were treated by

standard histological techniques (Durfort 1995), embedded in paraffin, cut into $5\mu\text{m}$ serial cross-sections using a rotation microtome (MICROM HM 330), and stained with periodic acid-Schiff and hematoxylin (Leblond & Clermont 1952). Since distal areas of testis are rich in longitudinal sections of seminiferous tubules due to the great number of encirclements, the serial histological cross-sections were obtained from the third central part of the testis.

In Ω_0 space, the size of left testis and left seminal vesicle were analyzed. Testis weight data were not available for all the animals. Nevertheless, we calculated the volume of the testis from the sagittal and transversal diameter ($4/3\pi(1/2\times\text{sagittal diameter})(1/2\times\text{transversal diameter})^2$), which was used as a measurement of testis size. It has been reported that the specific mass of the testes is very close to 1 mg/mm^3 (Russell *et al.* 1990); thus, testis volume in cubic millimeters is an approximation of testis mass in milligrams. The size of the seminal vesicle was the length from the junction of the two lobules to the external left end. Since the size of the testis and seminal vesicle can be related to body mass, these values were corrected by body mass (relative testis volume and relative seminal vesicle size respectively). Differences between the chromosomal groups for these variables were studied by one-way ANOVA.

In Ω_1 space, the relative frequency of stages in the seminiferous epithelium cycle was calculated (Eaker *et al.* 2001) to determine whether the succession of maturation stages is altered in Rb mice. Two cross-sections were analyzed, the first was randomly selected in the central part of the testis and the second $50\mu\text{m}$ from the first one. These two cross-sections correspond to one cross-section of each of the two dissectors used for cell count (see below). The seminiferous tubules studied in this space were selected by random systematic sampling (Fig. 1B and C; Gundersen & Jensen 1987). Seminiferous tubules were classified for the stage of maturation of the epithelium, following Oakberg (1956), and stage frequencies were estimated for each cross-section. Mean frequency values of both cross-sections were calculated, and χ^2 -tests were used to analyze differences between the chromosomal groups.

In the space Ω_2 , two morphometric variables were measured on the same cross-sections used in the space Ω_1 : the diameter of the tubule and the volume density of the epithelium. The former provides information about spermatogenic activity since there is a positive correlation between tubular diameter and spermatogenic activity (Sinha Hikim *et al.* 1989). The latter reveals information about the spermatogenic state of the tubules (Russell *et al.* 1990) since cell death during spermatogenesis can result in a thinner epithelium. The diameter of the tubule was obtained with a calibrated eyepiece micrometer. Only the minor axis of the tubule was measured since it does not vary if the section of the tubule taken is oblique. The volume density of the epithelium, i.e. the fraction of the seminiferous tubule occupied by the epithelium, was estimated following Cruz-Orive (1987). The areas were calculated by means of a coherent grid of equidistant points superimposed on the projection of the cross-section of the seminiferous tubule obtained by the *camera lucida*.

The number of points captured for the lumen and the epithelium of the seminiferous tubule were counted (Fig. 1D).

Moreover, also in the space Ω_2 , the number of round spermatids (β) and pachytene spermatocytes (α) were counted to calculate several informative variables of cell population in the testis, such as GCD, % DT per animal, $Nv\beta$ and $Nv\alpha$, and $TN\beta$ and $TN\alpha$. These estimates were obtained in the tubules selected in the previous space, but only stages I through VIII of maturation of the epithelium were used, since round spermatids can only be found in these stages. The cell numbers were quantified by the disector principle, an unbiased method for calculating the total number of objects per unit of volume (for details, see Sterio (1984) and Mouton (2002b)). For each animal, two independent physical dissectors were used, the first was randomly selected in the central part of the testis and the second located 50 μ m from the first one. Fifty micrometers is a safe distance, at which the same cell cannot be sampled in the two cross-sections. Therefore, in the data analyses, the second disector was considered a replica of the first one. In each disector, the number of β and α in each seminiferous tubule were obtained by radial sampling (angular intervals; Fig. 1E). The $\beta:\alpha$ ratio was calculated to quantify GCD. The expected ratio is 4:1 if no GCD occurs or 0:1 if complete GCD occurs. The percentage of GCD was calculated as $100 \times (1 - \beta/(4\alpha))$. The distribution of cell death among tubules was estimated as the % DT per animal, with the tubule considered as defective when the ratio $\beta:\alpha$ was lower than 3:1, i.e. that the number of spermatids divided by the number of spermatocytes was lower than 3 (Garagna *et al.* 1989). We calculated the percentage of tubules with $\beta:\alpha$ lower than 3:1 (% DT < 3), 2:1 (% DT < 2), and 1:1 (% DT < 1). These percentages were obtained by pooling both the cross-sections.

$Nv\beta$ and $Nv\alpha$ were calculated for each seminiferous tubule, following Sterio (1984). $TN\beta$ and $TN\alpha$ were obtained by multiplying numeric densities by testis volume (Zirkin & Ewing 1987). The volume of the testis of this set of animals was estimated from the mass of the testis, using as a specific mass 1.04 g/ml (Russell *et al.* 1990).

Significant differences between groups of all the parameters obtained in this space, except for the % DT, were studied by three-way nested ANOVA for non-balanced data. The fixed factors were the chromosomal group and animal; and the random factor, either the histological cross-section for the morphometric variables or the disector for the variables derived from the cell counts. The chromosomal group was the main factor, the animal was nested in the chromosomal group, and the cross-section was nested in both the animal and chromosomal group. When the chromosomal group factor was significant, the Tukey test for multiple comparisons was performed. To further explore the differences between animals inside the chromosomal group, Student's *t*-test was performed between the mice with the Bonferroni correction applied (Rice 1989). To study the differences between chromosomal groups for the % DT, one-way ANOVA was used, and then the *post-hoc* Tukey test was performed when ANOVA was significant. All the analyses were conducted by SAS (1999–2001; SAS Institute Inc., Cary, NC, USA). Data were reported as mean $\pm$ S.E.M.

The normality of data was studied by the Kolmogorov–Smirnov test, and the homogeneity of variance was studied by Levene's test. Outliers, defined as lying beyond three times the interquartile amplitude, were eliminated from the analysis.

Declaration of interest

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

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