Original Article

Sexual Development

Sex Dev 2009;3:284–289
DOI: 10.1159/000253307

Received: September 8, 2009
Accepted: September 11, 2009
Published online: October 28, 2009

Abnormal Spermatid Formation in the Presence of the Parasitic B_{24} Chromosome in the Grasshopper *Eyprepocnemis plorans*

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Key Words
Aberrant spermatids · B chromosomes · *Eyprepocnemis* · Grasshopper

Abstract
Morphology and size of spermatids were analysed in the grasshopper *Eyprepocnemis plorans* by means of light and electron microscopy. At light microscopy, normal and abnormal (macro- and micro-) spermatids differed in size and number of centriolar adjuncts (CAs): 1 CA in normal spermatids and 2 or more CAs, depending on ploidy level, in macrospermatids. Males carrying the additional B_{24} chromosome showed significantly more macro- and microspermatids than 0B males. The frequency of macro- and microspermatids showed an odd-even pattern in respect to the number of B chromosomes, with a higher frequency of abnormal spermatids associated with odd B numbers. Transmission electron microscopy showed that macrospermatids carried more than one axoneme, depending on ploidy level: 2 for diploid, 3 for triploid, and 4 for tetraploid spermatids. In 0B males, the most frequent abnormal spermatids were diploid, whereas in 1B males they were the tetraploid spermatids and, to a lesser extent, triploid ones. This suggests that most macrospermatids derived from cytokinesis failure and nucleus restitution. The implications of aberrant spermatids on B chromosome transmission and male fertility are discussed.

About 15% of eukaryote genomes harbor supernumerary (B) chromosomes which, in most cases, behave parasitically, promoting their own transmission by means of a variety of drive mechanisms despite being harmful for carrier individuals [for recent reviews see Camacho, 2004, 2005]. B chromosome drive usually rests on a non-Mendelian behavior during mitosis and/or meiosis related to gamete formation. For instance, a frequent drive mechanism in plants is mitotic non-disjunction during pollen grain maturation [Jones, 1991]. In grasshoppers, mitotically unstable B chromosomes undergo drive during embryo development by the generation of between-cell variation in B number and the preferential destiny of cells with higher B number towards the germ line [Nur, 1969; Pardo et al., 1995]. Meiotic behavior of B chromosomes can also determine their chance to drive or elimination (drag). For instance, in the grasshopper *Myrmeleotettix maculatus*, B chromosomes show drive during oogenesis but drag during spermatogenesis [Hewitt, 1973a, b]. Female meiotic drive results from the preferential migration of B chromosomes towards the secondary oocyte, thus increasing the likelihood of going to the oocyte instead of the first polar body [Hewitt, 1976], whereas male meiotic drag is due to sperm dysfunction caused by B chromosomes [Hewitt et al., 1987].

The B_{24} chromosome in the grasshopper *Eyprepocnemis plorans* also shows drive through females, but there is no drag through males which transmit it at a Mendelian rate [Zurita et al., 1998]. Previous analysis by Suja

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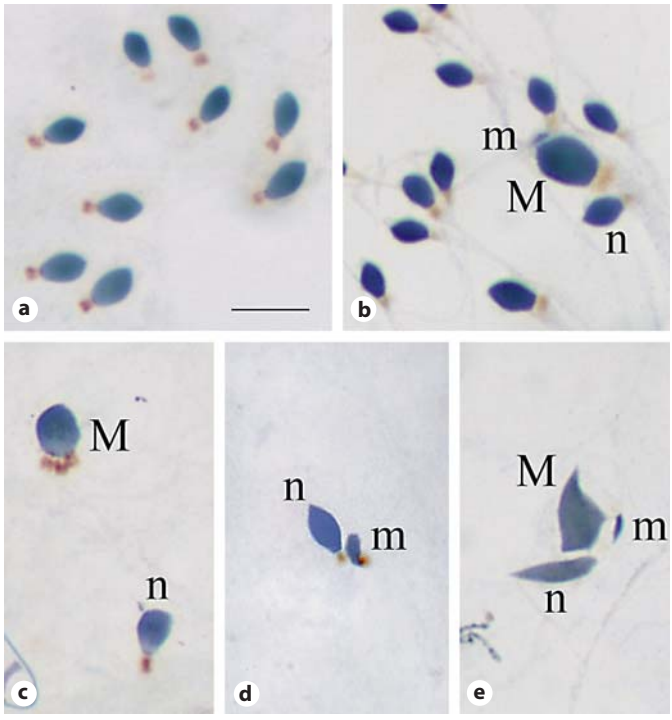

Fig. 1. Light microscopy preparations of silver stained spermatids in *E. plorans*, counterstained with Giemsa. **a** Normal spermatids showing the centriolar adjunct (brown). **b** Normal spermatids (n), a macrospermatid (M), and a microspermatid (m). **c** A normal spermatid (n) with one centriolar adjunct and a triploid macrospermatid (M) with 3 centriolar adjuncts. **d** A microspermatid (m) and a normal spermatid (n). **e** A normal spermatid (n) along with a micro- (m) and a macrospermatid (M). Bar = 10 μm.

and Rufas [1989] showed that the presence of the B_2 chromosome in *E. plorans* is associated with an increase in the frequency of aberrant spermatids, especially macrospermatids, even though this B variant lacks drive or drag in both males and females [López-León et al., 1992]. It should thus be interesting to ascertain whether a driving B variant, i.e. B_{24}, is responsible for higher levels of aberrant spermatids in B-carrying males. In this paper we analyze the size and structure of spermatids under light and transmission electron microscopy in 0B and 1B males and perform quantitative analyses of the frequency of the different types of aberrant spermatids found.

Materials and Methods

More than a hundred males of the grasshopper *Eyprepocnemis plorans* were collected in Torrox (Málaga, Spain) in 2004. After determining their number of B chromosomes, 50 of them were chosen for the light microscopy study so that they would constitute similar sized groups with 0, 1, 2, and 3 B chromosomes. Testes were fixed in freshly prepared 3:1 ethanol-acetic acid and stored at 4°C. For light microscopy, squash preparations were submitted to the silver impregnation technique described in Rufas et al. [1982]. To facilitate distinction between nuclear material and centriolar adjunct (CA), slides were stained with 1% Giemsa for 1 min, yielding blue chromatin and brown CAs. 500 spermatids were scored in each male, and they were classified, on the basis of size and number of CAs, into normal spermatids (1 CA), macrospermatids (more than 1 CA), and microspermatids (smaller in size than normal spermatids). Images were captured on an Olympus microscope equipped with a cooled digital camera.

For transmission electron microscopy, we selected 4 additional males (2 lacking Bs and 2 with 1B) captured in the same population in 2005. To ascertain the number of Bs while alive, specimens were vivisected to extract several testis tubules which were immediately analysed cytologically to determine B chromosome number. For electron microscopy preparations, several testis tubules were immersed in a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH = 7.4). Ultrathin sections were performed at the Center for Scientific Instrumentation at the University of Granada following the protocol described in Megias and Renal [1998]. Preparations were observed in a JEM1011 JEOL transmission electron microscope, and photographs were captured with Megaview III (Soft Imaging Systems). Figures 1 and 3 were mounted with the Gimp freeware. Statistical tests (Student t test and one-way ANOVA) were performed with STATISTICA.

Table 1. Mean percentage (± standard error) of normal, macro-, and microspermatids in males of *E. plorans* with a different number of B_{24} chromosomes. 500 spermatids were scored in each male.

Number of B_{24} chromosomes	Number of males	Type of spermatid (%)		
		Normal	Macro	Micro
0	13	99.24 ± 0.21	0.54 ± 0.12	0.22 ± 0.10
1	13	96.76 ± 0.71	2.68 ± 0.65	0.57 ± 0.12
2	12	98.62 ± 0.33	1.07 ± 0.31	0.32 ± 0.06
3	12	96.08 ± 0.50	2.59 ± 0.52	1.33 ± 0.34

Results

At light microscopy, normal and abnormal spermatids differed in size and the number of centriolar adjuncts (CAs) (fig. 1). Normal spermatids showed a single CA, whereas macrospermatids showed 2 or more CAs depending on ploidy level (fig. 1). Microspermatids were smaller than normal spermatids and could also show sil-

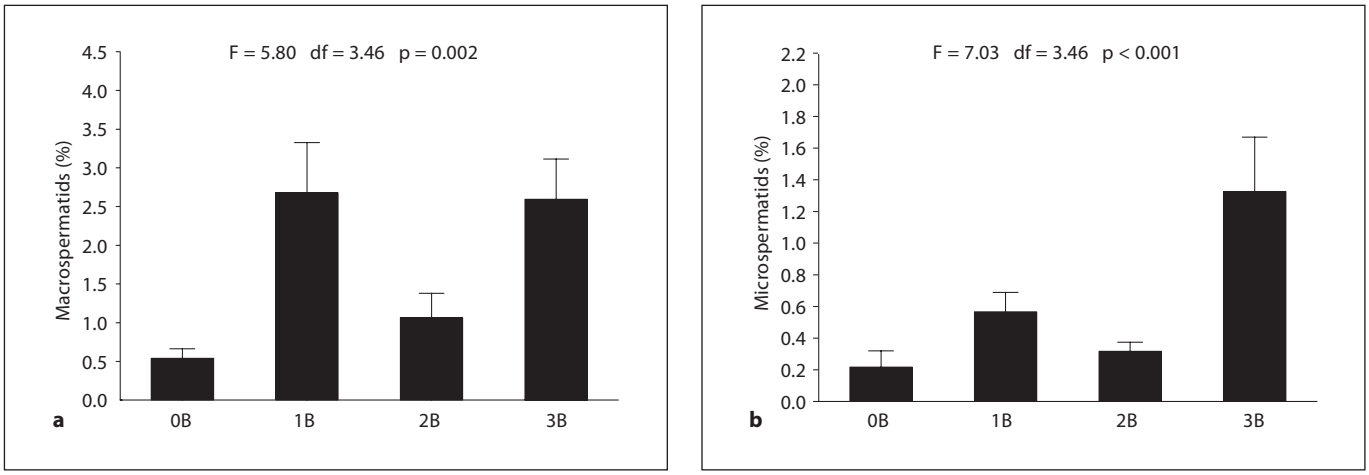

Fig. 2. Odd-even effect of B_{24} chromosome on the frequency of macrospermatids (**a**) and microspermatids (**b**). Bars correspond to ±1 standard error.

Table 2. Frequency of macrospermatids with different ploidy levels, deduced from the number of axonemes visualized at electron microscopy (one per haploid chromosome set) in 2 males with 0B and 2 males with $1B_{24}$

Type of male	Ploidy level			Total
	2C	3C	4C	
0B	39	1	7	47
$1B_{24}$	27	9	45	81

ver deposits in the CA region (see fig. 1d). A score of the mean percentage of the 3 types of spermatids observed, i.e., macro-, micro-, and normal spermatids, in males with and without B chromosomes (table 1) showed that B-carrying males formed significantly more macrospermatids ($t = 2.91$, $df = 48$, $p = 0.005$) and microspermatids ($t = 2.14$, $df = 48$, $p = 0.037$) than 0B males. When B chromosome number was used as a classification factor, an odd-even pattern was apparent, with higher percentage of abnormal spermatids associated with odd B numbers (fig. 2). A comparison of the percentage of abnormal spermatids between individuals with odd (grouping 1B and 3B classes) and even (2B) number of B chromosomes showed a higher percentage in the former (macrospermatids: odd = 2.64%, even = 1.07%, $t = 2.47$, $df = 35$, $p = 0.019$; microspermatids: odd = 0.93%, even = 0.32%, $t = 2.21$, $df = 35$, $p = 0.034$).

At electron microscopy, testis tubules in 0B and 1B males showed that most spermatids were normal and arranged within sperm packages (fig. 3a). However, macrospermatids were also present with larger nucleus size (fig. 3a) and the presence of more than one axoneme, depending on ploidy level: 1 for haploid, 2 for diploid, 3 for triploid, and 4 for tetraploid spermatids (fig. 3b, c). No higher ploidy levels were found, i.e., macrospermatids with more than 4 axonemes, as it was described in the grasshopper *Myrmeleotettix maculatus* [Hewitt et al., 1987]. In 0B males the most frequent macrospermatids were diploid, whereas in 1B males they were the tetraploid ones and, to a lesser extent, triploid ones, which were rarely observed in 0B males (table 2). A Montecarlo approached χ^2 test with 10,000 permutations indicated significant differences between 0B and 1B males for the frequency of the different types of macrospermatids ($\chi^2 = 29.40$, $p < 0.0001$).

Discussion

Under light microscopy, microspermatids and macrospermatids are easily recognizable because of their characteristic size and the presence of more than 1 centriolar adjunct in macrospermatids. The observation of thin sections at transmission electron microscopy only allowed recognizing the macrospermatids by the presence of 2 or more axonemes (1 per haploid chromosome set) in addition to their larger size.

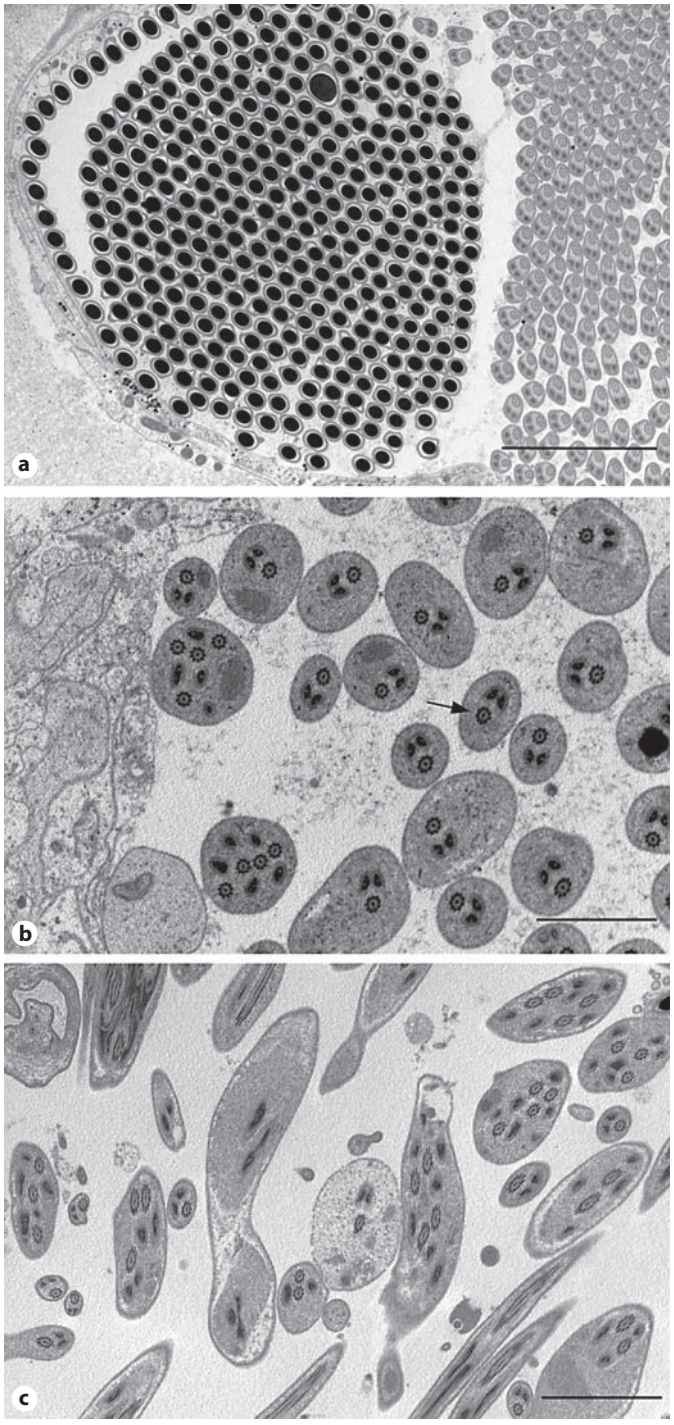

Fig. 3. Spermatids at transmission electron microscopy. **a** A cyst of haploid spermatids showing the nucleus (highly electron-dense), including one macrospermatid. **b** Normal spermatids with 1 axoneme (arrow) and tetraploid macrospermatids with 4 axonemes. **c** Spermatids with several ploidy levels deduced from the number of axonemes: haploid (1 axoneme), diploid (2), triploid (3), and tetraploid (4). Bar = 5 μm in (**a**) and 2 μm in (**b**, **c**).

The presence of the parasitic B_{24} chromosome in *E. plorans* males seems to negatively influence spermatogenesis by significantly increasing the frequency of abnormal spermatids, especially tetraploid macrospermatids. This increased proportion of macrospermatids is undoubtedly in detriment of the proportion of normal haploid sperm, although its impact on male fertility might actually be low (see below). This same effect has previously been reported in other grasshopper species carrying mitotically stable [Hewitt et al., 1987] or mitotically unstable [Camacho et al., 2004] B chromosomes. In *E. plorans*, Suja and Rufas [1989] reported an increase in macrospermatids associated with the presence of B_2, a neutralized B chromosome variant [López-León et al., 1992].

The odd-even effect of the B_{24} chromosome on the frequency of micro- and macrospermatids reported here suggests that this B chromosome causes some nuclear instability during spermatogenesis and that B chromosomes are more harmful in odd numbers, as was pointed out by Jones and Rees [1982]. This is most likely due to the presence of B univalents during meiosis [Camacho et al., 2004] since they are more frequent in males with an odd B number. The odd-even effect has been reported in B chromosome systems of many plants and animals for traits such as chiasma frequency, number of nucleoli per cell, frequency of aberrant spermatids, etc. [for review see Camacho, 2005]. In grasshoppers, extra autosomes have been reported in some species in a polysomic state and, in this case, an odd-even pattern has also been found for the frequency of microspermatids [Talavera et al., 1990; Camacho et al., 2004].

Nur [1969] suggested that most microspermatids derive from lost B chromosomes. In maize it has been shown, using DNA probes specific to B chromosomes and DNA probes specific to some A chromosomes, that micronuclei formed during male meiosis can include both A and B chromosomes [Chiavarino et al., 2000]. It is thus conceivable that a proportion of the observed microspermatids in *E. plorans* include A chromosomes, with the consequent formation of other apparently normal spermatids but being aneuploid for an A chromosome [Camacho et al., 2004]. However, the low proportion of microspermatids in 0B males (0.22%) compared to that in B-carrying males (0.73%) suggests that most microspermatids presumably include B chromosomes.

Macrospermatids show higher ploidy level than the normal haploid ones. Nur [1969] suggested 3 possible explanations for the presence of macrospermatids in B-carrying males: (1) they can derive from polyploid spermato-

gonia, (2) they can form as a consequence of failures in the cytokinesis in one or both meiotic divisions, thus forming restitution nuclei and giving rise to diploid or tetraploid macrospermatids, respectively, and (3) cell or nuclear fusion, which would explain the existence of macrospermatids in odd ploidy numbers (e.g., triploid). In our present study, we have not detected the presence of polyploid spermatogonia, implying that the most likely explanation for the observed macrospermatids in *E. plorans* is cytokinesis failure or cell fusion. Nucleus restitution has been suggested as an explanation for macrospermatid formation in the grasshoppers *Sphingonotus coerulans* [Gosálvez et al., 1985] and *Metalaptea brevicornis* [Bidau, 1986]. However, the finding of macrospermatids with up to 10 and 6 centriolar adjuncts in the grasshoppers *Aiolopus strepens* [Suja et al., 1987] and *Myrmeleotettix maculatus* [Hewitt et al., 1987], respectively, suggests that cell fusion is also a frequent phenomenon in some cases, giving rise to spermatids with a ploidy level higher than 4 [Nur, 1969].

Camacho et al. [2004] suggested that the presence of B chromosome univalents generates an extra dose of instability to the dividing spermatocytes thus favoring cytokinesis failure and the formation of restitution nuclei. In fact, whereas B bivalents remain oriented and static during metaphase I, B univalents move from pole to pole during this stage in *E. plorans* [Rebollo et al., 1998]. This behavior could produce additional complications to meiotic division leading to cytokinesis failure.

The consequences of the production of micro- and macrospermatids for B chromosome transmission are expected to be different. If microspermatids include B chromosomes more frequently than A chromosomes, then a decrease in B transmission rate should be expected. Macrospermatids, however, imply the simultaneous elimination of the B chromosome and the A chromosome sets accompanying it, so that no relative gaining or loss is expected in B transmission rate. In *E. plorans*, the B_{24} chromosome shows Mendelian transmission rates through males [Zurita et al., 1998; Manrique-Poyato et al., 2006], and the same has been observed for other B variants [López-León et al., 1992]. Therefore, the formation of aberrant spermatids, despite being more frequent in presence of B chromosomes, does not seem to imply a significant decrease in B transmission rate. This is in high contrast with the case of the grasshopper *Myrmeleotettix maculatus*, where male transmission of B chromosomes is significantly lower than 0.5 [Hewitt, 1973a, b] and results from the dysfunction of spermatids carrying B chromosomes [Hewitt et al., 1987]. In any case, male grasshoppers produce sperm in excess to fertilize all the eggs in an egg-pod laid by a female, and a single mating is enough for females to fertilize the eggs laid over their entire life [López-León et al., 1994], so that a low impact of aberrant sperm formation on male fertility is expected in *E. plorans*.

Acknowledgements

We thank Tatiana López for technical assistance and David Martínez for English corrections. This study was supported by grants from the Spanish Ministerio de Ciencia y Tecnología (CGL2006-06307) and Plan Andaluz de Investigación (CVI-1664). This research was partially financed by FEDER funds.

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