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Evidence for B chromosome drive suppression in the grasshopper *Eyprepocnemis plorans*

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The grasshopper *Eyprepocnemis plorans* is polymorphic for both a B chromosome and a heterochromatic segment of chromatin on the smallest autosome. Females transmit these to their offspring more frequently after copulating with a male from a population without Bs than after copulating with a male from their own population. Paternity analyses using the heterochromatic segment as a marker showed that the effect of male on female transmission does not depend on fertilization because it occurs even when all the eggs are fertilized by sperm from another mating. The possible mechanisms include behavioural differences in mating and transfer of substances affecting female meiosis in male ejaculate. The data support the idea that the B chromosome is initially subject to meiotic drive in populations in which it has not previously existed, and that genes which suppress this drive are then selected.

Keywords: B chromosomes, drive suppression, *Eyprepocnemis plorans*, oogenesis, segregation.

Introduction

Eyprepocnemis plorans is an abundant species along the Mediterranean and south Atlantic coasts of the Iberian Peninsula, where it harbours a very widespread B chromosome polymorphism that is unusual in lacking detectable drive. This makes it hard to explain the origin, establishment and maintenance of the polymorphism (López-León *et al.*, 1992). Drive suppressor genes have been demonstrated in the standard genome of at least two animal species polymorphic for B chromosomes, i.e. *Myrmeleotettix maculatus* (Shaw & Hewitt, 1985; Shaw *et al.*, 1985) and *Pseudococcus affinis* (Nur & Brett, 1985, 1987, 1988). In addition, genetic control of B transmission has been shown in maize (Carlson, 1969), rye (Romera *et al.*, 1991) and *Aegilops speltoides* (Cebria *et al.*, 1994). It therefore seemed possible that the B chromosomes of *E. plorans* used to have drive which has been suppressed by the standard genome. The main objective of the present investigation was to test this hypothesis. Our strategy was to perform between-population crosses, especially between populations with and without Bs. We reasoned that populations without Bs might lack suppressor elements, so that B drive would be manifest. Remarkably, effects appeared in the parental generations of these crosses, rather than first in the hybrids as might be expected of genes controlling segregation. This implies that the genotype of the male to which a female is mated influences female segregation. We were able to use another polymorphism to show that this effect did not depend on the sperm themselves.

Materials and methods

We used animals from four populations with B chromosomes (Salobreña, Jete, Fuengirola and Tarragona) and one without (Albacete), collected between September and December for 1990–92. All females used for crosses were collected in the field when they were at the last nymphal instar to ensure virginity. Once adult, the grasshoppers were placed in culture cages to perform controlled crosses in a room regulated at 27°C with a 12 h light : 12 h dark-

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ness cycle. Cages were cleaned and fresh grass from the field was supplied daily. Once enough embryos had been obtained from a cross, the adults and the embryos were fixed for cytological analysis following methods described elsewhere (López-León *et al.*, 1993). Controlled crosses were performed according to several different procedures: C_1 to C_4 were simple crosses of a female from one population to a male from another; in C_5 two males from one population were simultaneously allowed access to a female from another population; in C_6 to C_{17} females were mated successively to two different males, the second being allowed access after an egg pod had been laid; and in C_{18} and C_{19} females were mated successively to three males, each being allowed access after a pod had been laid. Males were removed after one copulation, other than in cross C_5, and the days when eggs were laid were also noted. Males were analysed cytologically before mating by extracting some testis follicles through a small opening between the second and the third abdominal segments. In all crosses except C_{10} and C_{14} the female carried B chromosomes and the males lacked them. Except for the two males in the C_5 cross, all males mated once. Methods for cytological analysis were the same as those of López-León *et al.* (1992).

Results

Table 1 shows the results of crossing 19 females to one or more males under controlled conditions. It is evident that the transmission ratio of the B chromosome varies dramatically between egg pods laid by the same female. There is no regular temporal trend in this and the phenomenon is seen in females from both Jete and Salobreña. Although the transmission frequency sometimes varies in pods laid without a further copulation (pods 2 and 3 in C_{12}; pods 1 and 2 in C_{18}), the transmission frequency seems to be linked to the particular copulation (e.g. pods 1–4 in C_5 or pods 1–3 in C_{19}). That is, the males are one factor influencing the B transmission ratio through the female. In 2B and 3B females, however, all within- and between-population egg pods showed Mendelian transmission ratios (Table 2), so that the male effect was not apparent.

Some females carried a supernumerary heterochromatic segment in the smallest autosome, which served as an additional marker. Previous within-population controlled crosses demonstrated that some females heterozygous (NS) for this marker and possessing 1B, and all NS females carrying 2B, transmit the segmented (S) chromosome significantly less often than expected (López-León *et al.*, 1991). In two of the six egg pods laid after mating with a local male, NS females undertransmitted the S chromosome (C_9 and C_{13}). In both these cases, however, egg pods laid after mating with a male from the Albacete population showed a Mendelian transmission ratio (k_S) of this chromosome (Table 1). Hence, supernumerary segment transmission, like that of the B, varies between egg pods, and may be influenced by the genotype of the most recently mated male.

The results in the nine females from Salobreña that were crossed to males from both Salobreña and Albacete populations were studied in more detail. The transmission ratios of the B chromosome (k_B) and the S chromosome (k_S) were significantly higher following copulation with an Albacete male than after copulation with a local male (Table 3). This strongly suggests that males can influence female transmission of both chromosomal markers.

The numbers of eggs and embryos per pod produced by these nine females did not differ significantly among the egg pods produced following mating with the males from the two localities (Table 3), so there was little differential egg or embryo mortality associated with copulation with an alien male. The figures in Table 3 were very close to those we have observed in naturally mated females from Salobreña laying in the laboratory. Therefore, the altered transmission ratio is not based on differential zygote survival and must result from changes in chromosome behaviour during female meiosis.

It is possible for males to influence chromosome behaviour during meiosis, because in *E. plorans* female meiotic segregation occurs after copulation. When the eggs are laid, the oocyte is arrested at metaphase I, and meiosis is continued outside the female's body, being completed by about 3–4 h later (Henriques-Gil *et al.*, 1986). As female meiosis is completed after laying, the spermatozoon has necessarily to be inside the egg when homologous chromosomes separate in anaphase I. This suggests that the effects might be produced by sperm post-meiotic gene expression. This hypothesis can be tested using the supernumerary segment polymorphism in males to determine which sperm fertilized the egg. Three crosses are suitable for this purpose. In the C_8 cross, the first mating was to a foreign NN male; Bs accumulated. The second mating was to a local SS male. The transmission ratio in the second pod was Mendelian; however, all 24 embryos analysed were NN and were therefore fertilized by the sperm of the first male. In both C_{14} and C_{19} crosses, one of the males used was heterozygous for the supernumerary segment, so inferences are

Table 1 Controlled crosses performed with individuals collected in five different Spanish populations of the grasshopper *Eyprepocnemis plorans*. Crosses were performed according to several different procedures: C_1 to C_4 were simple (interpopulational) crosses, C_5 was a simultaneous double (interpopulational) cross, C_6 and C_7 were double (interpopulational) crosses by substitution, C_8 to C_{17} were double (intra- and interpopulational) crosses by substitution, and C_{18} and C_{19} were triple (inter-, intra- and interpopulational) crosses by substitution. Whereas both males in the C_5 cross were present simultaneously, males in the remaining double and triple crosses were mated successively

Parents						Days between				Embryo offspring				
Female			Male								Supernumerary segment			
Karyotype	Population†	Cross	Code†	Karyotype	Pod no.	Successive matings	Mating and laying	Embryos	Eggs	B-chromosome k_B‡	NN	NS	SS	k_S§
1BNN	SA	C_3	AB-2	0BNN	1	—	7	27	49	1.000*	27	—	—	—
1BNN	JE	C_5¶	FG-2	0BNN	1	—	5	22	35	0.277*	22	—	—	—
			FG-1	0BNN	2	8	2	44	44	0.932*	44	—	—	—
			FG-2	0BNN	3	3	6	36	45	0.500	36	—	—	—
			FG-1	0BNN	4	10	5	34	36	0.914*	34	—	—	—
1BNN	JE	C_6	TA-1	0BNN	1	—	5	33	34	0.515	33	—	—	—
			SA-11	0BNN	2	—††	24	29	29	0.586	29	—	—	—
1BNN	JE	C_7	SA-9	0BNN	1	—	25	35	35	0.743*	35	—	—	—
			AB-10	0BNN	2	32	4	23	27	0.870*	23	—	—	—
1BNN	SA	C_8	AB-4	0BNN	1	—	40	51	53	0.647*	51	—	—	—
			SA-1	0BSS	2	45	2	24	29	0.417	24	—	—	0
1BNN	SA	C_{14}	SA-8	1BNS	1	—	39	51	51	0.451	24	27	—	0.530
			AB-10	0BNN	2	39	3	41	44	0.695*‡‡	22	19	—	0.464
1BNN	SA	C_{15}	TA-1	0BNN	1	—	16	46	47	0.630	46	—	—	—
			SA-9	0BNN	2	20	9	17	32	0.176*	17	—	—	—
1BNN	SA	C_{17}	TA-1	0BNN	1	—	2	35	41	0.657	35	—	—	—
			SA-10	0BNN	2	2	6	31	36	0.710*	31	—	—	—
1BNN	SA	C_{18}	TA-2	0BNN	1	—	2	33	39	0.394	33	—	—	—
					2	—	6	37	47	0.730*	37	—	—	—
			SA-12	0BNN	3	7	6	50	51	0.560	50	—	—	—
			AB-11	0BNN	4	8	3	46	47	0.848*	46	—	—	—
1BNS	SA	C_1	AB-1	0BNN	1	—	3	46	51	0.087*	29	17	—	0.370
1BNS	SA	C_{12}	AB-8	0BNN	1	—	3	35	38	0.600	16	19	—	0.542
			SA-6	0BNN	2	8	2	49	49	0.735*	25	24	—	0.490
					3	8	9	38	39	0.526	24	14	—	0.368
1BNS	SA	C_{13}	AB-9	0BNN	1	—	3	40	42	0.725*	18	22	—	0.550
			SA-7	0BNN	2	5	17	27	39	0.481	22	5	—	0.186*
1BNS	SA	C_{16}	TA-1	0BNN	1	—	10	18	31	0.500	11	7	—	0.388
			SA-9	0BNN	2	11	15	16	35	0.688	7	9	—	0.562
1BNS	SA	C_{19}	JE-1	0BNN	1	—	12	49	50	0.714*	19	30	—	0.612
			SA-13	0BNS	2	15	3	50	57	0.480	18	24	8	0.400
			AB-10	0BNN	3	7	8	50	57	0.771*	12	28	8	0.458

Table 1 *Continued*

Parents										Embryo offspring				
Female			Male			Days between					Supernumerary segment			
Karyotype	Population†	Cross	Code†	Karyotype	Pod no.	Successive matings	Mating and laying	Embryos	Eggs	B-chromosome k_B‡	NN	NS	SS	k_S§
2BNN	SA	C_2	AB-1	0BNN	1	—	10	50	53	0.530	50	—	—	—
2BNN	SA	C_{10}	SA-3	1BNS	1	—	7	39	50	0.487	19	20	—	0.513
			AB-6	0BNN	2	11	2	37	41	0.500	37	—	—	—
2BNS	SA	C_9	AB-5	0BNN	1	—	22	27	38	0.500	12	15	—	0.556
			SA-2	0BNN	2	22	10	26	39	0.400	20	6	—	0.231*
2BNS	SA	C_{11}	AB-6	0BNN	1	—	5	19	51	0.395	10	9	—	0.474
			SA-4	0BNN	2	6	17	32	38	0.438	17	15	—	0.468
3BNS	SA	C_4	AB-3	0BNN	1	—	9	42	47	0.429	19	23	—	0.548

*$P<0.05$ compared with Mendelian ratio. Statistical tests employed were goodness of fit χ^2-test, except for k_B in C_4 and C_{10-1} which was tested against a normal distribution using the binomial variance based on the number of embryos examined.

†Codes indicate the population (SA = Salobreña, AB = Albacete, JE = Jete, FG = Fuengirola, TA = Tarragona). In males this is followed by a number denoting the individual.

‡Transmission ratio for the B chromosome: mean number of Bs per embryo/number of Bs in the parents.

§Transmision ratio for the segmented (S) chromosome.

¶C_5 was a double interpopulational cross with both males introduced simultaneously. To distinguish matings, the pronotum of one of the males was marked with nail varnish. The male specified for each pod is the male to mate last before laying.

††The SA-11 male was not seen mating.

‡‡The supernumerary segment shows that the embryos in this cross were sired by male SA-8, not AB-10. k_B was calculated assuming that the B frequency in the sperm was the same for both egg pods, and equal in male and female in the first cross.

Table 2 A summary of B chromosome transmission in the 36 crosses of *Eyprepochemis plorans*

No. Bs in female	Type of cross	Type of B transmission ratio (no. crosses)		
		Elimination	Mendelian	Accumulation
1B	Within-population	1	6	2
	Between-population	2	6	11
2B†	Within-population	0	3	0
	Between-population	0	5	0

†One of these crosses involved a 3B female.

Table 3 Comparison of clutch size and transmission ratios for the B chromosome (k_B) and the supernumerary segment (k_S) in nine Salobreña females each crossed to a male from the same population (within-population crosses) and to a male from the non-B population (between-population crosses)

Item	Within-population crosses			Between-population crosses		
	Mean	SE	*n*	Mean	SE	*n*
Eggs per pod	44.20	2.90	9	45.70	2.30	9
Embryos per pod	38.10	3.70	9	38.40	3.50	9
Embryos/eggs	0.85	0.04	9	0.85	0.06	9
k_B	0.48	0.02	9	0.63*	0.05	9
k_S	0.34	0.06	5	0.52*	0.02	5

*$P<0.05$ (paired t-test) compared with corresponding data for within-population crosses.

weaker and must be based on the N:S ratio of the offspring. In the second pod of the C_{14} cross and the third pod of the C_{19} cross, eggs had an excess of B chromosomes following copulation with a foreign male, even though the sperm from this foreign male did not fertilize enough to disturb the ratio of NN:NS embryos (see Table 1).

Discussion

The fact that foreign male effects are only detectable on 1B females, and not on 2B and 3B ones, in which a large proportion of the Bs form bivalents, suggests that they depend on the presence of B univalents in metaphase I, and most likely act on the first meiotic division where 75 per cent of B univalents in 1B females divide reductionally (López-León *et al.*, 1992). Thus preferential migration of B univalents (to the secondary oocyte in cases of B accumulation or to the first polar body in cases of B elimination) could explain the observed male effects. In the case of the supernumerary segment system, the male effects determine that N and S chromosomes migrate randomly becoming independent of the presence of B chromosomes.

It is possible that the effects need a minimum time between mating and laying to become apparent. This is suggested by the C_{18} and C_{12} crosses. In C_{18} the first mating was to a foreign male. The B transmission ratio in the first egg pod (laid two days after mating, see Table 1) was Mendelian, but there was accumulation in the second, laid six days after mating. In the C_{12} cross, the B transmission ratio was Mendelian in the first egg pod, laid only three days after mating with a foreign male. However, the next pod, laid two days after mating with a local male, showed B accumulation ($k_B = 0.74$). An additional pod, laid nine days after mating with the local male, showed the k_B expected from a within-population cross (0.53). In the C_{13} cross, however, the B accumulated in a pod laid only three days after mating with a foreign male. It is possible, therefore, that the minimum time for effects is between two and three days.

The effects of the male on chromosome segregation in the female appear to be dependent on copulation but independent of fertilization. A possible explanation is that they are produced by some substance(s) contained in the ejaculate. It is known that males of many insects contribute proteinaceous nutrients with the ejaculate (Markow & Ankney, 1984; Pitnick *et al.*, 1991). We have recently observed transfer of nutrients from ejaculate into eggs of *E. plorans* using radioactive labelling (Pardo *et al.*, 1995). Interestingly, transfer of nutrients from ejaculate was low in eggs from pods laid promptly after mating but high in subsequent pods, which clearly supports the existence of a minimum time for male effects on female meiotic segregation.

Insect ejaculate contains a variety of substances, mainly produced in the accessory gland, that influence female reproductive behaviour and physiology (Leopold, 1976; Gromko *et al.*, 1984). As examples, we may mention an anti-aphrodisiac pheromone (Jallon *et al.*, 1981), or the enzyme esterase-6, which influences the time until a female remates, the rate of sperm usage, productivity, copulation duration and pairing rate (Richmond *et al.*, 1980; Gilbert *et al.*, 1981; Gilbert & Richmond, 1982a,b). Another enzyme, glucose oxidase, may function as a bactericide and fungicide (Cavener, 1980), and other substances stimulate oviposition (Baumann, 1974a,b). Perhaps the most interesting ejaculate component, however, bearing in mind that male effects are exerted on chromosome behaviour during the first meiotic division in the female, was found in *Drosophila*, i.e. a series of filamentous structures with a size and morphology identical to the microtubules (Bairati, 1966; Perotti, 1971). The function of microtubules in the seminal fluid is completely unknown, although some authors have suggested that they could help sperm to move toward storage organs (Bairati, 1966), or that they could serve as a sperm nutrient during storage (Perotti, 1971).

In looking for a cause of male effects on female meiotic segregation, perhaps microtubule proteins might regularly be transferred from males and used in female meiosis. Interestingly, it has recently been demonstrated that in the apical diverticulum of the *E. plorans* spermatheca there is a progressive dismantling of the sperm flagellum, so that accessory tubules separate from the axoneme and the microtubule doublets scatter throughout the cytoplasm and depolymerize (Longo *et al.*, 1993). This might constitute a source for microtubule components entering the eggs and influencing meiotic segregation in the oocytes which, when the eggs are laid, are arrested at metaphase I (Henriques-Gil *et al.*, 1986).

Paternal contribution of microtubules has been described in ferns, in which it was reported to be important in growth and development (Bell, 1979). This kind of contribution could also be an explanation for male effects on the rate of cleavage of zygotes resulting from inter-strain crosses, observed a long time ago in rabbits (Castle & Gregory, 1929; Gregory & Castle, 1931; Castle, 1941).

Finally, we would like to comment on the implications the present results have for the population biology of B chromosomes in *E. plorans*, which was our initial objective. Crosses between populations demonstrate that these B chromosomes can be driven in matings with males from populations without Bs. This suggests the existence of genes suppressing drive in the genome of populations possessing Bs. These genes could act by influencing the amount of male-derived microtubule components entering the eggs. The fact that most males from the non-B population induce accumulation of Bs suggests that these suppressor genes are scarce in this population. This would be expected if Bs have never been present in this population, or they have been absent for a long time period. We suggest the hypothesis that B chromosomes in *E. plorans* possessed drive when they originated, allowing the present widespread polymorphism to be established, but that this drive has now been suppressed by the host genome. This is consistent with the standard selfish theory about B chromosomes (Shaw & Hewitt, 1990), but *E. plorans* is a very dramatic example of the coevolution of A and B chromosomes.

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