Negatively assorted gamete fertilization for supernumerary heterochromatin in two grasshopper species

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The analysis of controlled crosses and gravid females in two grasshopper species has shown nonrandom gamete fertilization, caused in both cases by a preference for ova and sperm to fuse with gametes carrying an allele different from their own. Such a fertilization bias was observed for a B chromosome in *Eyprepocnemis plorans* and a supernumerary chromosome segment on the M₇ autosome in *Chorthippus jacobsi*. In addition, a male-biased sex ratio was observed in *E. plorans* among the embryo offspring produced by 1B gravid females which had mated with a 1B male in the field, but no sex ratio distortion was apparent in the same type of crosses performed in the laboratory. The possible causes of these distortions, and the role which negatively assorted fertilization may play upon the maintenance of these polymorphisms in natural populations, are discussed.

Keywords: B chromosomes, grasshoppers, homogamy, Mendelian segregation, preferential fertilization.

Introduction

Random gamete fertilization is a general assumption for Mendelian inheritance, as random mating is for the Hardy–Weinberg law. Exceptions to random mating have been widely described and constitute the basis for sexual selection, as noted by Darwin (1871). Few exceptions to random gamete fertilization, however, have been reported up to now, especially in animals where sperm success in fertilization seems to depend little or not at all on the genotype (Muller & Settles, 1927; Zimmering *et al.*, 1970). Homogamy caused by preferential fertilization, however, has been reported in a few cases of hybridization between closely related animals (Hewitt *et al.*, 1987, 1989; Bella *et al.*, 1992; Gregory & Howard, 1994; Wade *et al.*, 1994). In plants, however, the pollen-tube growth velocity could play a significant role in competition between pollen grains to fertilize the macrospore. Unquestionable cases of preferential fertilization have been reported in maize, specifically for B chromosomes (Roman, 1948a,b). In recent years, the concept of animal sperm genic inactivity has been challenged because many genes showing postmeiotic activity have been observed in mammalian sperm (Erickson, 1990),

some of which could be relevant during fertilization, and whose variation could be the basis for nonrandomness of this process.

Supernumerary heterochromatin constitutes the most frequent chromosomal polymorphism in natural populations of grasshoppers. It may appear in the form of additional heterochromatic chromosome segments or else as independent entities, i.e. B chromosomes. Inheritance analyses for supernumerary segments in *Chorthippus jacobsi* have shown Mendelian inheritance for those located on the M₅ and M₆ chromosomes but accumulation mechanisms in both sexes for those on the S₈ chromosome (López-León *et al.*, 1992a). In contrast, the three most frequent types of B chromosome analysed in *Eyprepocnemis plorans* (B₁, B₂ and B₅) are transmitted in a Mendelian transmission ratio (López-León *et al.*, 1992b). The present paper reports nonrandom gamete fertilization related to the presence of a supernumerary heterochromatic segment on the M₇ autosome of *C. jacobsi* and the B₂ chromosome in *E. plorans*.

Materials and methods

Adult males and last instar nymph females of *E. plorans* were collected at Jete and Salobreña (Granada, Spain) to perform the controlled crosses

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which have been reported in previous papers (López-León *et al.*, 1992b, 1993a,b). These crosses constitute part of the material analysed in the present report. In addition, 106 gravid females from the same populations and the embryos they produced in the laboratory were analysed, 55 of which have been included in the present analyses. Methods for obtaining embryo offspring and cytological analysis have been described in detail previously (López-León *et al.*, 1992b, 1993a,b).

Chorthippus jacobsi specimens were collected from natural populations in the province of Granada, Spain: Los Corales (C) (between Granada and Almuñecar in the Cázulas Mountains), Beas de Granada (BG) and Vereda de la Estrella (VE) (Sierra Nevada Mountains). For methods see López-León *et al.* (1992a).

The strategy for analysis differed for the two species. The high number of crosses analysed in *E. plorans* permitted an analysis of global tendency in respect of sex ratio or the proportion of 1B offspring. Only two crosses between heterozygotes were studied in *C. jacobsi* and did not permit such an analysis; we simply tested Mendelian genotype proportions in each cross separately, by means of a simple χ^2 -test, and for the two crosses together, by a heterogeneity χ^2 -test.

Because the total number of embryos analysed in *E. plorans* differed among crosses, we worked with relative frequencies per cross instead of absolute numbers in this species, so that all crosses were given the same weight independently of sample size. The variables used for statistical analysis were the proportion of embryos possessing 1B (*IB*) and the

proportion of male embryos (*sex ratio*). Mendelian segregation implies an expected value of 0.5 for these two variables in the three types of cross used for analysis, i.e. ♀1B × ♂0B, ♀0B × ♂1B and ♀1B × ♂1B, and this will be the theoretical value employed for one-sample statistical tests. In gravid females, the proportion of eggs containing an embryo (*fertil*) was also analysed. A preliminary analysis using the Shapiro–Wilks test for normality showed that *IB* and *sex ratio* fitted a normal distribution in gravid females, as did *sex ratio* in controlled crosses, but *IB* in controlled crosses and *fertil* in gravid females were not normally distributed even though many transformations were attempted (in the latter case non-normality was probably because of the fact that *fertil* = 1 in the majority of females). Hence, both parametric and nonparametric tests were applied to all variables, but as their results were never contradictory for those variables which showed a normal distribution, the results for the nonparametric tests will be shown in all cases for the sake of simplicity. All statistical tests in *E. plorans* were performed using the BMDP statistical software (Dixon, 1990).

Results

Controlled crosses in *Eyrepocnemis plorans*

The one-sample Wilcoxon signed rank test showed a significant excess of 1B embryos (in respect of the expected 0.5) in ♀1B × ♂1B crosses but not in the crosses in which the B was carried by one parent only (Table 1). *Sex ratio*, however, did not differ

Table 1 Comparison of *sex ratio* and the proportion of 1B embryos (*IB*) with the values expected under Mendelian inheritance (0.5), by means of one-sample Wilcoxon signed rank tests, in 53 controlled crosses performed in the laboratory with *Eyrepocnemis plorans* specimens

Cross type	Variable	Mean	SE	<i>n</i>	Wilcoxon signed rank test	
					<i>T</i> ⁺	<i>P</i>
1B♀ × 0B♂	<i>IB</i>	0.497	0.023	15	51.0	0.925
	<i>sex ratio</i>	0.513	0.017	15	46.0	0.427
0B♀ × 1B♂	<i>IB</i>	0.501	0.018	11	32.0	0.929
	<i>sex ratio</i>	0.532	0.022	11	16.0	0.131
1B♀ × 1B♂	<i>IB</i>	0.604	0.029	27	47.5	<0.002
	<i>sex ratio</i>	0.492	0.012	27	93.0	0.434

Significant results are shown in bold.

n = no. of crosses.

significantly from 0.5 in any of the three types of cross.

Gravid females in *Eyprepocnemis plorans*

Mann–Whitney tests showed significant differences between the Jete and Salobreña populations for *fertil* but not for the two other variables (Table 2). A comparison of the *1B* and *sex ratio* variables with the theoretical value expected from Mendelian transmission (0.5 in both cases) by means of the one-sample Wilcoxon signed-rank test did not show any signi-

ficant differences for these variables in crosses involving a single B-carrier, but they were significantly higher than 0.5 in $1B \times 1B$ crosses (Table 3). A comparison performed in $1B \times 1B$ crosses between the frequencies of $0B$ and $2B^+$ embryos showed no difference between them in both controlled crosses and gravid females, which suggests that the excess of $1B$ embryos is achieved at the expense of both $0B$ and $2B$ ones (Table 4).

A multiple regression analysis on the 18 $\text{♀}1B \times \text{♂}1B$ gravid females, using *sex ratio* as the dependent variable and *1B* and *fertil* as independent

Table 2 Between-population comparison for the three variables analysed in the three types of cross studied from gravid females of *Eyprepocnemis plorans* which had mated in the field

Cross type	Variable	Jete			Salobreña			Mann–Whitney test	
		Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	<i>U</i>	<i>P</i>
$1B\text{♀} \times 0B\text{♂}$	<i>1B</i>	0.427	0.058	7	0.467	0.049	10	33.0	0.845
	<i>sex ratio</i>	0.556	0.033	7	0.495	0.020	10	48.5	0.187
	<i>fertil</i>	1.000	0	7	0.945	0.021	10	59.5	<0.008
$0B\text{♀} \times 1B\text{♂}$	<i>1B</i>	0.458	0.028	7	0.470	0.043	13	42.5	0.812
	<i>sex ratio</i>	0.505	0.031	7	0.515	0.014	13	43.5	0.874
	<i>fertil</i>	1.000	0	7	0.937	0.031	13	66.5	<0.05
$1B\text{♀} \times 1B\text{♂}$	<i>1B</i>	0.578	0.030	8	0.566	0.040	10	45.0	0.657
	<i>sex ratio</i>	0.573	0.017	8	0.535	0.036	10	51.0	0.328
	<i>fertil</i>	1.000	0	8	0.956	0.014	10	64.0	<0.012

Significant results are shown in bold.

n = no. of crosses.

Table 3 Comparison of *sex ratio* and the proportion of $1B$ embryos (*1B*) with the values expected under Mendelian inheritance (0.5), by means of one-sample Wilcoxon signed rank tests, in 55 gravid *Eyprepocnemis plorans* females from Jete and Salobreña which had been mated in the field

Cross type	Variable	Mean	SE	<i>n</i>	Wilcoxon signed rank test	
					<i>T</i> ⁺	<i>P</i>
$1B\text{♀} \times 0B\text{♂}$	<i>1B</i>	0.451	0.036	17	47.0	0.163
	<i>sex ratio</i>	0.520	0.019	17	43.0	0.334
$0B\text{♀} \times 1B\text{♂}$	<i>1B</i>	0.466	0.029	20	74.5	0.255
	<i>sex ratio</i>	0.512	0.014	20	63.0	0.327
$1B\text{♀} \times 1B\text{♂}$	<i>1B</i>	0.571	0.025	18	30.0	<0.016
	<i>sex ratio</i>	0.552	0.021	18	31.0	<0.032

Significant results are shown in bold.

n = no. of crosses.

Table 4 Comparison between the frequencies of 0B and 2B offspring from 1B × 1B crosses in *Eyprepocnemis plorans* by means of two-sample Wilcoxon signed rank tests

Type of females	<i>n</i>	Sex	Frequency of 0B embryos		Frequency of 2B embryos		Wilcoxon signed rank test	
			Mean	SE	Mean	SE	<i>T</i> ⁺	<i>P</i>
Controlled crosses	27	♀	0.194	0.019	0.201	0.022	109.0	0.821
		♂	0.206	0.023	0.191	0.024	126.5	0.726
		Total	0.200	0.019	0.196	0.021	178.5	0.801
Gravid females	18	♀	0.233	0.024	0.186	0.026	47.5	0.169
		♂	0.225	0.024	0.214	0.020	63.5	0.816
		Total	0.229	0.021	0.200	0.019	59.0	0.407

n = no. of crosses.

Table 5 Transmission analysis for karyotypic frequencies among the progeny of two crosses between parents heterozygous (*Ns*) for the *M*₇ chromosome in *Chorthippus jacobsi*

Cross	No. of embryos				Chromosome frequencies <i>H</i> ₀ = 1:1				Karyotype frequencies <i>H</i> ₀ = 1:2:1		
	<i>NN</i>	<i>Ns</i>	<i>ss</i>	Total	<i>N</i>	<i>s</i>	χ^2_1	<i>P</i>	χ^2	d.f.	<i>P</i>
C2	11	39	8	58	61	55	0.31	0.58	7.21	2	<0.05
BG2	13	36	6	55	62	48	1.78	0.18	7.04	2	<0.05
Total observed	24	75	14	113	123	103	1.77	0.18	χ^2_4 total = 14.25, <i>P</i> < 0.05		
									χ^2_2 accumulated = 13.89, <i>P</i> < 0.001		
									χ^2_2 heterogeneity = 0.36, <i>P</i> > 0.05		
Total expected (1:2:1)	28.25	56.5	28.25								

Significant results are shown in bold.

variables, failed to show any significant dependence among them (ANOVA for regression: $F_{2,15} = 0.19$ $P = 0.827$). Finally, a correlation analysis between *fertil* and the frequency of 1B embryos showed no significant relationship ($r_s = -0.16$, d.f. = 16, $P = 0.52$).

Controlled crosses in *Chorthippus jacobsi*

Normal *M*₇ chromosomes (*N*) showed a very small distal C-band, which was larger in segmented *M*₇ chromosomes (*s*) (see Cabrero *et al.*, 1987). In the VE1 cross the female was heterozygous (*Ns*) but the male lacked the extra segment (*NN*). In C2 and BG2 crosses both parents were heterozygous. In the VE1 cross, 12 out of 26 embryo offspring analysed were *Ns* and the remaining 14 were *NN*, which was not significantly different from the 1:1 ratio expected under Mendelian segregation ($\chi^2_1 = 0.15$, $P = 0.69$). The results for the two crosses between heterozygotes showed that whereas chromosome frequencies

did not differ from the 1:1 transmission ratio, the analysis of karyotype frequencies revealed a significant excess of heterozygous embryo progeny (Table 5).

Discussion

The excess of 1B embryos in 1B × 1B crosses in *E. plorans* cannot be related to differential egg resorption in females because in grasshoppers meiotic segregation occurs after egg laying (see López-León *et al.*, 1992a). Neither is it the result of segregation distortion favouring the B in one sex but not in the other, because the frequencies of 1B embryos yielded by crosses in which the B was carried by a single parent did not differ from those expected under Mendelian segregation (López-León *et al.*, 1992b; this report). The surfeit of 1B embryos is also not attributable to preferential death of 0B and 2B zygotes during early development because the frequency of 1B embryos is not correlated with *fertil*,

which was very high in most gravid females. In *C. jacobsi*, the low proportion of infertile eggs (10 per cent in the C2 cross and 5 per cent in the BG2 cross) runs against selective zygotic death of homozygous embryos as an explanation for the significant excess of heterozygotes yielded by $Ns \times Ns$ crosses, because the observed deficit in homozygote frequency would imply about 25 per cent egg mortality. [Note in Table 5 that 75 heterozygotes were obtained in the two crosses. Assuming that survival was 100 per cent for heterozygotes, about 75 eggs should have contained homozygotes of which only 38 survived. This would imply a mortality rate equal to $(75 - 38)/150 = 0.25$.] The possibility that the *s* chromosome showed accumulation during gamete formation in one sex but elimination in the other (so that the resulting pooled transmission ratio for both sexes would be Mendelian, but the excess of heterozygotes would derive from random fertilization of distorted gametic frequencies in both sexes) cannot be completely ruled out. However, the observation that the *Ns* female in the VE1 cross (mated to an *NN* male) yielded the two M_7 chromosome types in Mendelian ratios gives little support to this hypothesis.

The most plausible hypothesis in both species, with the available data, is nonrandom gamete fertilization, and specifically, a kind of negative assortative fertilization favouring the fusion of male and female gametes differing for the markers used, i.e. the B_2 chromosome in *E. plorans* and the supernumerary segment on the M_7 chromosome in *C. jacobsi*. Such a process could be favoured by polyspermy, which is a relatively frequent phenomenon in hemimetabolan insects (Anderson, 1972) and has been shown to be plausible in *C. jacobsi* (Cabrero & Camacho, 1985) (formerly mistaken for *C. brunneus*; see López-León *et al.*, 1992a).

An alternative hypothesis, which does not necessarily depend on polyspermy, emerges from the fact that the sperm pronucleus is present in the egg when it engages in meiotic segregation, so that it is conceivable that the sperm genotype could influence meiotic segregation in the egg, e.g. favouring migration of the marker it carries towards the first polar body. The male effects on transmission ratio of the B_2 chromosome through 1B females (Herrera *et al.*, 1996) that we have recently observed in *E. plorans* suggest that meiotic segregation cannot be entirely under female control, thus leaving some scope for sperm effects.

The male-biased sex ratio shown by embryo offspring from 1B gravid females presumably mated to 1B males in the field was neither caused by differ-

ential zygote mortality nor related to the excess of 1B offspring (as was indicated by a multiple regression analysis). The most likely hypothesis is preferential fertilization by male-determining sperm, as in the case of the grasshopper *Chorthippus parallelus* (Bella *et al.*, 1992). In the latter case a preference for homogamic fertilizations was also noted in crosses between two subspecies, a fact that had also been reported in the case of the hybrid zone in the Alpes Maritimes for other grasshopper species, e.g. *Podisma pedestris* (Hewitt *et al.*, 1987, 1989). Remarkably, homogamic fertilization has been found to be responsible for postmating prezygotic reproductive isolation between closely related species in two insects, *Tribolium* (Wade *et al.*, 1994) and *Allonemobius* (Gregory & Howard, 1994).

In two other insect species, the ladybird *Exochomus quadripustulatus* and the parasitic wasp *Nasonia vitripennis*, a relationship between B chromosomes and sex ratio distortion has been reported. In *E. quadripustulatus* an analysis of 14 populations showed that the proportion of males was negatively correlated with the frequency of B chromosomes (Henderson, 1988). However, it was suggested that the Bs were not responsible in themselves for the sex ratio distortion (because it was also apparent in related species lacking Bs) but that both B and male frequencies in natural populations were affected by the same factors (Henderson, 1988). The B chromosome in *N. vitripennis* (known as Paternal Sex Ratio or PSR), on the other hand, is inherently associated with a male-biased sex ratio distortion. This forms the basis of a powerful accumulation mechanism by converting diploid (female) eggs into haploid eggs which develop into B carrying males, thus constituting an extreme example of a parasitic B chromosome (see Beukeboom & Werren, 1993 for a review).

The most intriguing feature of *E. plorans* is that sex ratio distortion was apparent in gravid females but not in controlled crosses. Perhaps the excess of male embryos was caused by differential sperm ageing favouring male determining ones, which was facilitated in gravid females because they were maintained isolated from males in the laboratory until laying, whereas the females in controlled crosses always had an available male with which to mate, and so were continuously obtaining fresh sperm.

Although in plants it is commonly accepted that skewed segregation can arise from male gametophytic selection (see Wendel *et al.*, 1987 for references), male gamete selection in animals is controversial because most evidence hitherto suggests genetic inactivity of spermatozoa and that

their success in fertilization depends exclusively on the diploid genotype of the male (Zimmering *et al.*, 1970). However, recent studies have demonstrated the existence of a number of postmeiotically expressed genes in sperm (Erickson, 1990), which opens the possibility that gamete fertilization can depend on the interaction of the genotypes of gametes.

The results in the present report, combined with those on *P. pedestris* (Hewitt *et al.*, 1987, 1989) and *C. parallelus* (Bella *et al.*, 1992) suggest that grasshopper gametes may combine in a nonrandom manner, which could be accomplished simply on the basis of genetically induced changes in the chemistry of fertilization. This subject clearly demands thorough investigation not only in grasshoppers but also in other insects, because nonrandom fertilization in animals might not be as rare as has been hitherto thought. Thus, for example, selective fertilization could account for the absence of certain karyotypes in natural populations of the Californian bark weevil *Pissodes terminalis* showing polymorphism for the C chromosome (Smith, 1962; Smith & Takenouchi, 1962).

As a final comment, the negatively assorted gamete fertilization related to the presence of the B₂ chromosome in *E. plorans* and the s chromosome in *C. jacobsi* could help maintain these polymorphisms in natural populations by stabilizing their frequencies. This is especially interesting in *E. plorans* where the three most widespread B chromosome variants (B₁, B₂ and B₅) lack any drive helping their maintenance in natural populations. Perhaps the excess of 1B offspring yielded in 1B × 1B crosses could produce a slight buffering effect on B frequency, which may depend on the intensity of B effects on carrier fitness and random variation caused by small population size.

Acknowledgements

We are indebted to M. W. Shaw and an anonymous referee for perceptive criticism. This study was supported by grants from the Spanish D.G.I.C.Y.T. (PB93–1108) and Plan Andaluz de Investigación (Grupo no. 3122).

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