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Effects of B chromosomes on egg fertility and clutch size in the grasshopper *Eyprepocnemis plorans*

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Abstract

We analyse here three components of reproductive success (egg fertility, egg clutch size and embryo clutch size) in several temporal samples from different Spanish and Moroccan populations of the grasshopper *Eyprepocnemis plorans*. The analysis of spatial and temporal variation suggests that egg clutch size, but neither embryo clutch size nor egg fertility, depends significantly on both year of sampling and population of origin. While the former effect could mainly be due to year-to-year variation in food availability (essential to egg production but not to hatching success), the spatial variation may also include population-dependent genetic factors. We also tested the effect of the presence of supernumerary (B) chromosomes carried by many individuals in most natural populations of this species. We found a slight but significant decrease in egg fertility associated with the presence of B chromosomes. We discuss possible causes of the observed variation for these three reproductive traits at both spatial and temporal levels, as well as the effect of B-chromosome presence as a parasitic element disturbing reproduction of carrier females.

Key words

B chromosomes, clutch size, fertility, grasshopper, *Eyprepocnemis plorans*, Orthoptera

Introduction

About 15% of eukaryote species carry supernumerary (B) chromosomes in addition to members of the standard chromosome complement (A chromosomes). Two models have been put forward to explain B-chromosome maintenance in natural populations: the parasitic (Östergren 1945) and the heterotic (Darlington 1958, White 1973) models. Both assume that B frequency may reach equilibrium as a consequence of the action of two opposite forces, *i.e.*, drive with harmful effects of the B chromosome in the parasitic model, and beneficial effects at low number of Bs, but harmful effects at high B numbers, in the heterotic model.

Most B chromosome systems where transmission and fitness effects have been sufficiently studied, fit the parasitic model (for a recent review, see Camacho 2005). A minority of Bs, however, show characteristics being compatible with the heterotic model, such as providing resistance to pathogens, *e.g.*, *Nectria haematococca* (Miao *et al.* 1991) (see other examples in Camacho 2005) or being beneficial for survival, as shown for the nondriving B chromosome in the plant *Allium schoenoprasum* (Plowman & Bougourd 1994).

A variant of the parasitic model not assuming equilibrium for B frequency, was built on the basis of population dynamics studies in the grasshopper *Eyprepocnemis plorans*. In this species, the first analyses of B-chromosome transmission showed an absence of drive for the three main B variants (López-León *et al.* 1992). Subsequent

experiments, however, found that one of these variants (B₂) showed significant drive when B-carrying females were crossed to males from a B-lacking population, thus suggesting that B drive was suppressed in the B-carrying population (Herrera *et al.* 1996). This led Camacho *et al.* (1997) to propose that the same B chromosome may show drive or not, depending on the population evolutionary stage, since parasitic B chromosomes may lose drive due to the evolution of modifier genes in the A chromosomes — a scenario previously suggested by Shaw (1984) and demonstrated by Shaw and Hewitt (1985) and Nur and Brett (1985, 1987).

Therefore, B chromosome frequency in natural populations is not necessarily at equilibrium, but may change as B chromosomes pass through several stages, *i.e.*, parasitic, drive-suppression and neutralized stages (Camacho *et al.* 1997). At any stage, the B chromosome can mutate to a new variant and when this generates a variant being able to drive, the near-neutral cycle restarts, thus prolonging the life of the B-chromosome polymorphism. Repeated generation of new variants and recovery of drive allow the polymorphism to persist in the populations. B chromosomes in *E. plorans* actually show high mutation rates (López-León *et al.* 1993, Bakkali & Camacho 2004), which putatively facilitate the substitution of neutralized Bs by derived driving variants. It is worth mentioning that this kind of polymorphism regeneration was evidenced in *E. plorans* (Zurita *et al.* 1998). Furthermore, the near-neutral model has been considered to explain the long-term evolution of other B-chromosome systems (González-Sánchez *et al.* 2003, Jones *et al.* 2008) and other selfish genetic elements (Johnson 1997); Frank (2000) highlighted its importance as a paradigm for the evolution of polymorphisms of attack-defence between parasitic elements and their hosts.

An important aspect of the research on parasitic B chromosomes is to ascertain whether they impose some load on host fitness. At the cytological level, B chromosomes of Spanish *E. plorans* populations were found to increase chiasma frequency (Camacho *et al.* 1980, Camacho *et al.* 2002), and the number of active nucleolus organizer regions (NOR) (Cabrero *et al.* 1987, López-León *et al.* 1995), although the relationship between these traits and fitness is unknown. Analyses of several exophenotypic traits, however, failed to show any effect of B chromosomes (Camacho *et al.* 1980, Martín-Alganza *et al.* 1997). However, B chromosomes in *E. plorans* have been reported to decrease egg fertility (Muñoz *et al.* 1998, Zurita *et al.* 1998) and to increase the formation of abnormal spermatids (Suja & Rufas 1989, Teruel *et al.* 2009), although it is not clear that this latter effect significantly decreases male fertility. In the grasshopper *Myrmeleotettix maculatus*, sperm dysfunction in B-carrying males seems to be associated with a poor transmission of B chromosomes (Hewitt 1973a,b; Hewitt *et al.* 1987). In high parallelism with Spanish Bs, both parasitic (driving) and neutral-

Table 1. Percentage of B-carrying females, egg fertility and egg and embryo clutch size in eight populations of the grasshopper *E. plorans*. N= Number of females. SP= Spain, MO= Morocco, SD= Standard deviation.

Population	N	% B-carrying	Egg fertility		Egg clutch size		Embryo clutch size	
			Mean	SD	Mean	SD	Mean	SD
Jete	88	67.05	0.95	0.1	39.92	10.06	38.03	10.61
Salobreña	69	60.87	0.86	0.18	46.02	14.65	39.34	14.27
Algarrobo	87	37.93	0.85	0.2	39.39	10.94	33.5	13.21
Nerja	23	39.13	0.71	0.24	32.69	7.95	23.7	11.16
Torrox	39	87.18	0.74	0.19	38.72	9.98	28.64	10.87
Smir	44	40.91	0.88	0.11	34.96	6.77	30.89	7.54
SO.DE.A	38	31.58	0.82	0.19	29.79	8.83	24.66	9.99
Mechra	55	41.82	0.85	0.16	36.49	8.35	30.96	9.82
Total	443	51.92	0.85	0.18	38.5	11.26	33.04	12.41

ized (nondriving) B chromosomes have been found in different Moroccan populations of *E. plorans* (Bakkali *et al.* 2002). In addition, Moroccan B chromosomes are made up of the same two types of repetitive DNA, *i.e.*, ribosomal DNA and a 180 bp tandem repeat DNA (Cabrero *et al.* 1999), and also influence chiasma frequency (Camacho *et al.* 2002) and NOR activity (Bakkali *et al.* 2001).

In the grasshopper *Dichroplus elongatus*, it has recently been shown that B chromosomes tend to decrease body size and enhance female reproductive potential, since their presence was significantly associated with increased numbers of ovarioles and embryos per clutch (Rosetti *et al.* 2007). In *E. plorans*, B chromosome effects on egg fertility have been found in some populations, but no attempt has hitherto been made to ascertain whether they influence egg and embryo clutch size. In this paper we analyse B-chromosome effects on egg fertility and clutch size in five Spanish and three Moroccan populations of *E. plorans*. The fact that some of these populations were sampled in several years permitted us to analyze spatial as well as temporal variation for these traits.

Materials and methods

We scored the number of egg-pods per female, and the number of eggs and embryos per pod in 443 progeny analyses (controlled crosses and gravid females) from eight natural populations of the grasshopper *Eyprepocnemis plorans* (Table 1). For each egg pod, we obtained three variables: egg fertility (the proportion of eggs carrying an embryo), egg clutch size (the number of eggs per pod) and embryo clutch size (the number of embryos per pod).

Results on egg fertility had previously been reported in seven of these populations: Jete and Salobreña (Granada, Spain) (López-León *et al.* 1992, 1993), Algarrobo (Málaga, Spain) (Manrique-Poyato *et al.* 2006), Torrox (Zurita *et al.* 1998, Perfectti *et al.* 2004), and Smir, SO.DE.A and Mechra (Morocco) (Bakkali *et al.* 2002, Bakkali & Camacho 2004). Here we include data on a new population (Nerja, Málaga, Spain) and add new samples of the Algarrobo population (Málaga, Spain). B chromosomes in *E. plorans* are mitotically stable, which means that all cells within the same individual carry the same number of B chromosomes. In addition to egg fertility, we analyse egg and embryo clutch size, as well as the effects of B chromosome presence on these traits. A simple relationship exists between the three reproductive traits, since embryo clutch size is the product of egg clutch size and egg fertility. Methods for these new samples were similar to those described in the above references. We had data for two or more sampling years in four of the five Spanish populations, which allowed us to investigate temporal variation. The statistical analyses of the data were based on one-way and two-way ANOVA, and Student *t* test, performed with the Statistica program.

Results

In four Spanish populations (Jete, Salobreña, Algarrobo and Torrox), we had information on egg fertility and clutch size for two or more samples collected in different years (Fig. 1), which permitted the analysis of temporal variation for the variables analyzed. Two way ANOVA per population, with egg fertility or clutch size (both for eggs and embryos separately) as dependent variable, and year of sampling and B chromosome presence in the female as factors, revealed significant temporal variation for the three dependent variables in the four populations, with the only exception of egg-clutch size in Torrox (Table 2 and Fig. 1). On the contrary, B chromosome effects were observed only in Salobreña, namely for embryo clutch size (Fig. 1). In Torrox, a significant year x B interaction was apparent in egg fertility and embryo clutch size. When the effect of the presence of the B chromosome was tested for each year separately, a significant decrease in egg fertility, egg clutch size and embryo clutch size was apparent in B-carrying females collected in 1992 (Table 3), but no effect was observed in females collected six years later, *i.e.*, in 1998 (not shown).

In Nerja and the three Moroccan populations however, we had samples from a single year, for which reason we only tested for B chromosome presence. One-way ANOVA showed the absence of significant effects of B presence on any of the three variables analyzed (Table 2).

Spatial variation among populations for the three variables analyzed (egg fertility, egg clutch size and embryo clutch size), (see Table 1) was tested by two-way ANOVA, with population and B presence as independent variables. We found significant differences among populations for egg fertility, egg clutch size and embryo clutch size (Table 4). The highest egg fertility was observed in Jete, and the lowest in Nerja and Torrox (Fig. 2A). The highest egg clutch size, however, was observed in Salobreña, and the lowest in Nerja and SO.DE.A., whereas embryo clutch size was highest in Jete and Salobreña and lowest in Nerja and SO.DE.A. (Fig. 2B,C). In the analysis of egg fertility, a significant effect of B chromosome presence was also apparent (Table 4), due to the lower values shown by B-carrying females in Salobreña, Torrox and SO.DE.A. (see Fig. 2A).

Since data collection was carried out over a wide temporal range (from 1984 in Salobreña to 2005 in Algarrobo), with scarce temporal coincidence among populations, the possibility exists that the spatial variation observed is a by-product of having sampled the populations in different years. A way to test this possibility is by analysing population differences in those cases where two populations were sampled in the same year. This could be done in the Jete and Salobreña populations (samples collected in 1986,

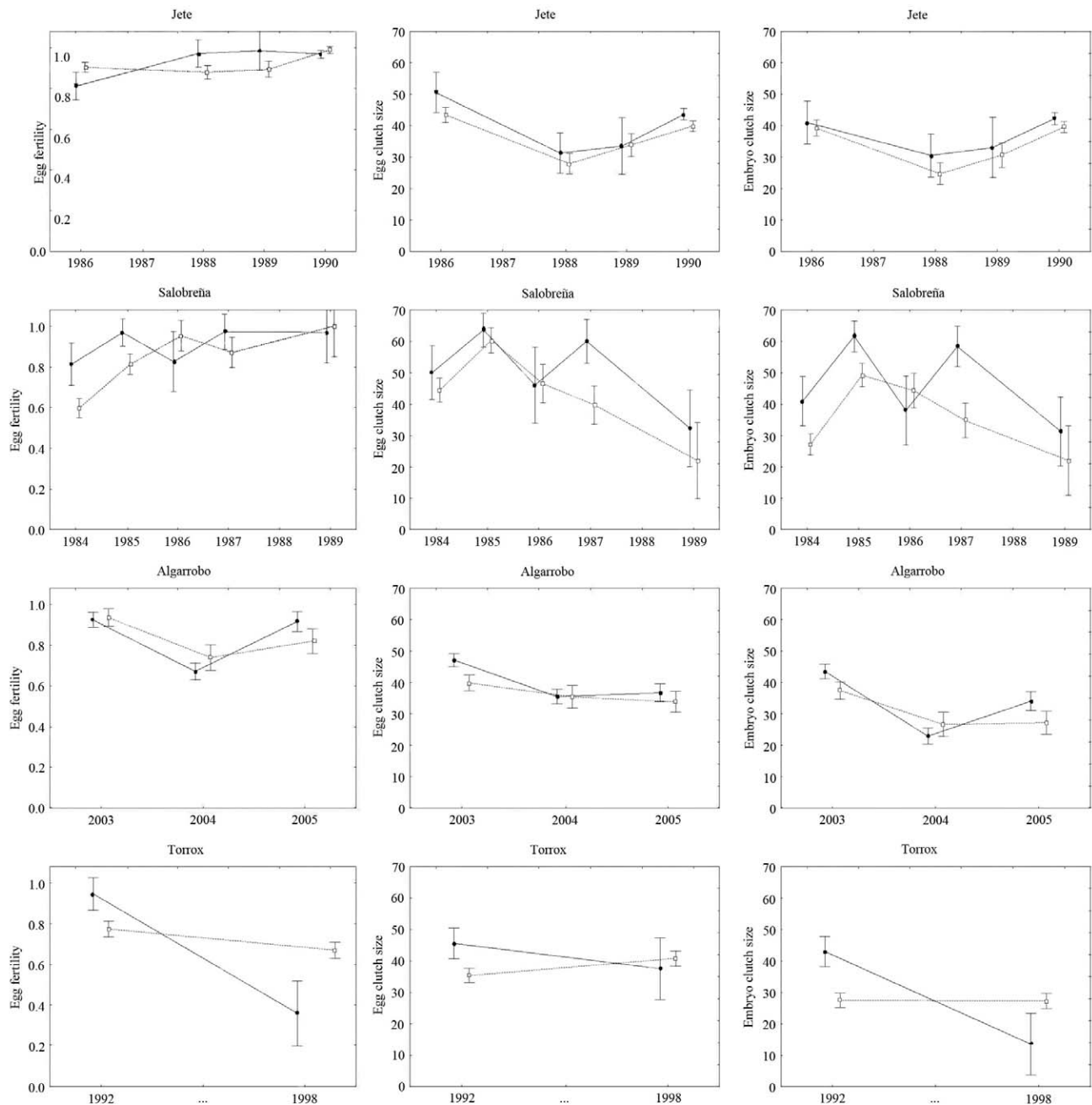


Fig. 1. Temporal dynamics for three reproductive traits in four populations of the grasshopper *E. plorans*. Means (\pm standard errors) are shown for B-chromosome carrying females (open squares) and OB females (black-filled circles).

1989 and 1990) and also in Algarrobo and Nerja (samples collected in 2004). Two-way ANOVA comparing the samples from Jete and Salobreña collected in the three above-mentioned years, failed to show significant differences between populations for egg fertility, egg clutch size and embryo clutch size, but there were significant differences among years for the latter two reproductive traits (Table 5). The Algarrobo and Nerja populations were cosampled only in 2004. In this case, one-way ANOVA failed to show significant difference for any of the three dependent reproductive traits, between the 2004 samples from both populations. The results of these analyses suggest that part of the variation observed among the eight populations (see Table 4 and Fig. 2) could be due to differences between the years of sampling.

Discussion

B chromosomes have been shown to decrease fertility of B-carrying individuals in many species (for review, see Jones & Rees 1982, Camacho 2005, Jones *et al.* 2008). In *E. plorans*, a reduction of egg fertility has only been observed for the parasitic B_{24} variant in the Spanish population of Torrox (Zurita *et al.* 1998) and in some controlled crosses involving the Spanish variant B_2 and mating frequency limitation (Muñoz *et al.* 1998). Our present analysis has revealed significant among-year differences in egg fertility and egg and embryo clutch size in the four populations where temporal variation could be tested (see Table 2). This same analysis

Table 2. Effect of B chromosome presence and year of sampling on egg fertility and clutch size. Four Spanish populations were analysed by two-way ANOVA; Nerja and the three Moroccan populations were only tested for B chromosome presence. Significant tests are highlighted in bold-type.

Item	Population	Year			B			Year x B		
		F	df	P	F	df	P	F	df	P
Egg fertility	Jete	3.82	3, 80	0.012937	0.22	1, 80	0.636937	1.44	3, 80	0.237252
	Salobreña	2.64	5, 57	0.032624	0.79	1, 57	0.377070	1.40	5, 57	0.238425
	Algarrobo	11.27	2, 81	0.000048	0.02	1, 81	0.892126	1.21	2, 81	0.304217
	Torrox	13.76	1, 35	0.000718	0.56	1, 35	0.460584	6.71	1, 35	0.013881
	Nerja				0.17	1, 21	0.680807			
	Smir				0.19	1, 42	0.665144			
	SO.DE.A				2.88	1, 36	0.098598			
	Mechra				0.07	1, 53	0.795485			
Egg clutch size	Jete	5.30	3, 80	0.002213	0.96	1, 80	0.330445	0.14	3, 80	0.936439
	Salobreña	7.23	5, 57	0.000026	2.16	1, 57	0.146697	0.79	5, 57	0.561172
	Algarrobo	6.71	2, 81	0.002007	2.23	1, 81	0.139051	1.01	2, 81	0.368175
	Torrox	0.06	1, 35	0.814922	0.37	1, 35	0.547046	1.42	1, 35	0.241843
	Nerja				0.69	1, 21	0.414464			
	Smir				0.63	1, 42	0.430601			
	SO.DE.A				3.10	1, 36	0.086856			
	Mechra				0.28	1, 53	0.600449			
Embryo clutch size	Jete	4.38	3, 80	0.006579	0.71	1, 80	0.400546	0.06	3, 80	0.980711
	Salobreña	7.41	5, 57	0.000021	4.53	1, 57	0.037671	2.04	5, 57	0.086513
	Algarrobo	15.48	2, 81	0.000002	1.52	1, 81	0.220517	1.72	2, 81	0.184855
	Torrox	6.70	1, 35	0.013945	0.02	1, 35	0.881518	6.47	1, 35	0.015576
	Nerja				0.24	1, 21	0.628935			
	Smir				0.27	1, 42	0.604665			
	SO.DE.A				0.11	1, 36	0.740578			
	Mechra				0.48	1, 53	0.491881			

also revealed that B chromosome presence does not influence egg fertility in most populations, the only exception being the Torrox population, where the year \times B interaction was significant for egg fertility and embryo clutch size.

The analysis of B chromosome effect per year revealed a significant decrease of the three reproductive variables in B-carrying females collected in 1992, in agreement with the results of Zurita *et al.* (1998), but not in those females collected in 1998, in coincidence with the observations by Perfectti *et al.* (2004). As discussed by these latter authors, this seems to suggest a rapid neutralization of the harmful effects of this parasitic-B chromosome in this population. In fact, the Nerja population, located toward the east of Torrox, is currently experiencing invasion by the B_{24} variant and shows the lowest values for the three reproductive parameters measured here (see Fig. 2). The Algarrobo population is also being invaded by B_{24} but does not seem to show such reproductive decrease. Although it is difficult to pinpoint the ultimate causes of this difference from the Nerja population, we may speculate that the B_{24} chromosome arrived at Algarrobo earlier than at Nerja, so the modifier genes on the A chromosomes have had more time to neutralize its harmful effects in Algarrobo. The higher frequency of B_{24} in Algarrobo than Nerja (Manrique-Poyato, unpub.) would give some support to this hypothesis, as it could suggest that the B chromosome actually had

more time to spread and reach higher frequency in Algarrobo than Nerja. Alternatively, and given the high variation in the three reproductive traits observed among years and among populations (see Tables 2 and 4), environmental conditions in Algarrobo might be more favorable for female reproduction. However, our comparison of both populations in 2004 does not give support to this possibility. Still, it could be that 2004 was a less favorable year than 2003 and 2005, when we sampled Algarrobo, as all reproductive values were lower in 2004 (not shown).

Our analysis of the spatial variation in egg fertility and clutch size showed significant differences among populations (see Table 4 and Fig. 2). In addition, this same analysis showed a slightly significant decrease for these traits in B-carrying females (see Fig. 2). The intensity of this effect is slight, due to a mixture of factors, among which we want to highlight the interpopulation variation in B chromosome evolutionary stage, which determines that B chromosome's drive and harmful effects can vary both spatially and temporally (Camacho *et al.* 1997). This explains why, in only six years, the two Torrox samples showed significant differences for egg fertility and embryo clutch size. It also explains why the same B chromosome drives in one Moroccan population (Mechra, the southern, where the B chromosome seems to have arrived later), but not in others (Smir and SO.DE.A., the northern and central, where the B chromosome seems to have arrived earlier) (Bakkali *et al.* 1999, 2002).

However, it is unlikely that B chromosome presence is the only explanation for the spatial variation observed in these reproductive traits, since remarkable variation was observed also at the temporal level. In fact, when we compared the three year values between the Jete and Salobreña populations, no significant differences appeared among populations, but there were significant differences among

Table 3. Comparison of three fitness components in standard (OB) and B-carrying (B+) females from Torrox collected in 1992.

Item	OB		B+		t	df	P
	Mean	SD	Mean	SD			
Egg fertility	0.95	0.05	0.77	0.14	2.46	20	0.0231
Egg clutch size	45.63	7.11	35.38	6.30	2.88	20	0.0092
Embryo clutch size	43.00	5.10	27.52	7.45	3.92	20	0.0009

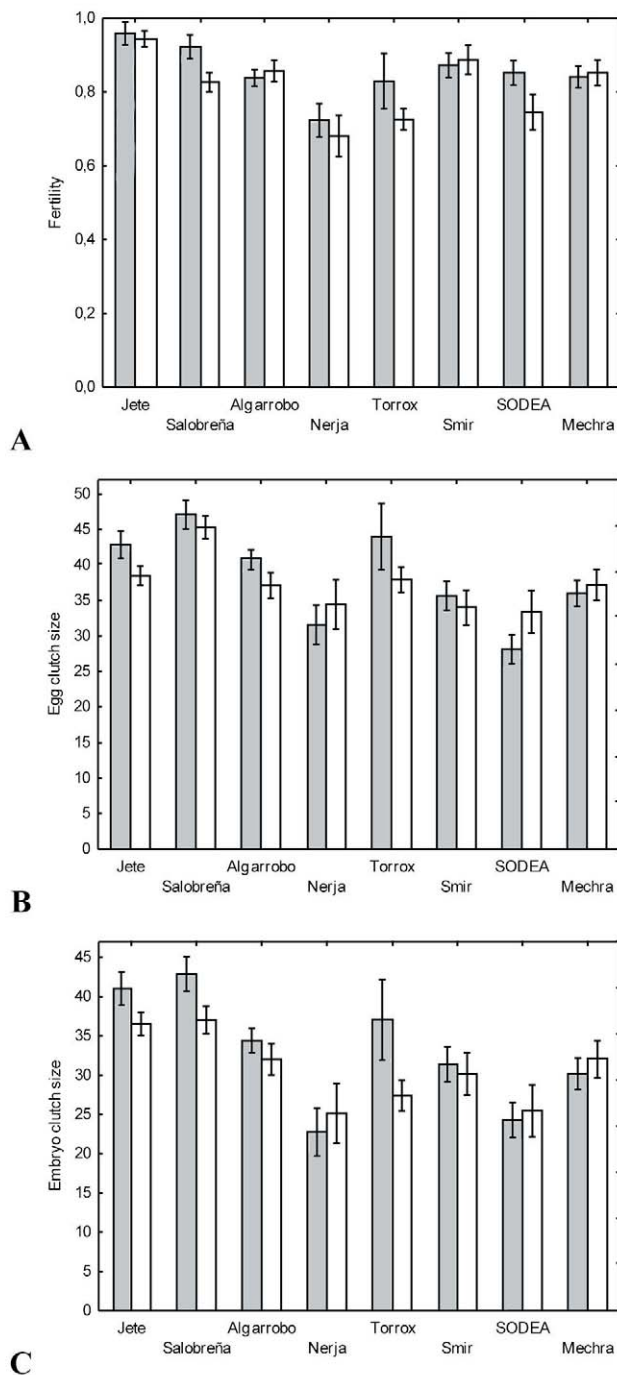


Fig. 2. Differences between *E. plorans* females with B chromosomes (empty columns) and without B chromosomes (filled columns) in eight populations for three reproductive variables: egg fertility (A), egg clutch size (B), and embryo clutch size (C). Whiskers indicate \pm one standard error.

years for egg and embryo clutch size. This indicates that clutch size seems to be more dependent on year to year environmental changes than fertility does (which did not show temporal changes in these two populations). Indeed, this is a logical result since egg fertility mostly depends on sperm supply and, although *E. plorans* females can lay fertilized eggs during their entire life with just a single mating (López-León *et al.* 1994), it is also true that this species' females lay more eggs after multiply mating (Pardo *et al.* 1995a). Since *E.*

Table 4. Two-way ANOVA analysis of the spatial variation in egg fertility and clutch size of pods laid by females of the grasshopper *E. plorans*.

Trait	Item	F	df	P
Egg fertility	Population	7.51	7, 427	<0.000001
	B	4.14	1, 427	0.042533
	Population x B	1.21	7, 427	0.293558
Egg clutch size	Population	10.23	7, 427	<0.000001
	B	0.72	1, 427	0.395665
	Population x B	1.22	7, 427	0.289740
Embryo clutch size	Population	10.98	7, 427	<0.000001
	B	2.95	1, 427	0.086662
	Population x B	1.10	7, 427	0.363069

plorans is very abundant in Jete and Salobreña, it is unlikely that male availability (*i.e.*, mating frequency) may represent a limiting factor for female fertility in these populations. Previous studies have shown that insect reproductive development and the number of eggs per clutch show plastic responses, depending on food availability (Moehrlin & Juliano 1998, Hatle *et al.* 2000). It is therefore logically possible that food quality and quantity changing between years could condition the reproductive response of these grasshoppers. For instance, part of the variation observed in egg clutch size could be due to the fact that female grasshoppers resorb some developing oocytes when stressed (Stauffer & Whitman 1997, Sundberg *et al.* 2001).

Another possible source of error in our data could be that, although all crosses were carried out within just one generation, all egg-pods in *E. plorans* were obtained in the laboratory and might not be representative of field laying: it has been shown in other grasshopper species that egg clutch size is significantly lower in the field than in the laboratory (Stauffer & Whitman 2007). In addition, that egg production in phytophagous insects is generally protein limited (Nijhout 1994), and this appears to apply to grasshoppers (Waskey *et al.* 2002). Therefore, although our laboratory conditions are largely homogenous between populations and years and, hence, should have a rather homogenizing effect, the possibility exists that laboratory culture might imply an additional source of variation in egg clutch size, due to potentially limited access to food selected, *i.e.*, preferred, by grasshoppers in their different natural environments.

In all cases where we observed B-chromosome effects on egg fertility and clutch size, in *E. plorans*, B-carrying females showed lower values than 0B females, in consistency with the parasitic nature of these B chromosomes. In the grasshopper *Dichroplus*

Table 5. Two-way ANOVA comparison of the spatial and temporal variation in egg fertility and clutch size between the Jete and Salobreña populations of *E. plorans*.

Trait	Item	F	df	P
Egg fertility	Population	0.377	1, 108	0.540578
	Year	1.271	2, 108	0.284772
	Population \times Year	2.363	2, 108	0.098984
Egg clutch size	Population	0.406	1, 108	0.525558
	Year	4.428	2, 108	0.014177
	Population \times Year	0.426	2, 108	0.654307
Embryo clutch size	Population	0.425	1, 108	0.515786
	Year	3.783	2, 108	0.025824
	Population \times Year	1.388	2, 108	0.254060

elongatus, B-carrying females displayed higher number of ovarioles and embryo clutch size than B-lacking females (Rosetti *et al.* 2007), with no significant differences among years. The beneficial effect of the B chromosome on *D. elongatus* females contrasts with its harmful effect on male mating success, through a decreased body size (Rosetti *et al.* 2007).

These sexually antagonistic effects of B chromosomes might have something to do with B chromosome transmission, with Bs being more detrimental in the sex where they show drive. In *D. elongatus*, B chromosomes are mitotically unstable, *i.e.*, their number varies among cells within the same individual. It has been shown that mitotic instability of B chromosomes during embryo development leads to B-chromosome drive in males of other grasshopper species (Nur 1963, 1969, Kayano 1971, Viseras *et al.* 1990, Pardo *et al.* 1995b). It would be highly interesting to ascertain whether mitotic instability of B chromosomes in *D. elongatus* leads to B drive in males, but not in females, to test for a possible relationship between B transmission and effects in this species. In the case of *E. plorans*, scarce effects have been found in males at the level of formation of a low proportion of aberrant spermatids (Teruel *et al.* 2009). However, B chromosomes in this species are more detrimental in females, decreasing egg fertility, especially when B chromosomes are in a driving stage (see Zurita *et al.* 1998).

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