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INBREEDING AND THERMAL ADAPTATION IN *DROSOPHILA SUBOBSCURA*

Goran Zivanovic[1], Conxita Arenas[2] and Francesc Mestres[3]

[1]Department of Genetics, Institute for Biological Research "Sinisa Stankovic". University of Belgrade, Serbia.

[2]Departament d'Estadística, Universitat de Barcelona, Barcelona, Spain.

[3]Departament de Genètica, Universitat de Barcelona, Barcelona, Spain.

Corresponding author:

Goran Zivanovic

Department of Genetics, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia

Phone: (38111) 2764422

FAX: (38111) 2761433

E-mail: goranziv@ibiss.bg.ac.rs

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Abstract: Using a well-adapted *Drosophila subobscura* population (Avala, Serbia), a drastic experiment of inbreeding was carried out to assess whether the expected level of homozygosity could be reached, or other evolutionary forces affected also the process. In general, no significant changes of inversion (or arrangement) frequencies were detected after twelve brother-sister mating generations. Furthermore, no significant differences were obtained between observed and expected (under the inbreeding model) karyotypic frequencies. Thus, these results seemed to indicate that the main evolutionary factor in the experiment was inbreeding. However, in G_{12} generation complete chromosomal fixation was reached only in two out of the eight final inbred lines. In these lines, the chromosomal compositions were difficult to interpret, but could be likely a consequence of adaptation to particular laboratory conditions (constant 18ºC, food, light period, etc.). Finally in a second experiment, the inbred lines presented higher fertility at 18ºC than at 13ºC. Also, there was a significant line effect on fertility: inbred line number 6 (A_1, J_1, $U_{\underline{1+2}}$; $U_{\underline{1+2+6}}$, E_8 and $O_{\underline{3+4}+\underline{7}}$) presented the higher values and maybe it was the result of an adaptation to laboratory environment. Thus, the results obtained in our experiments reflect the adaptive potential of *D. subobscura* inversions.

Key words: Drosophila subobscura, inbreeding, chromosomal inversions, thermal adaptation, fertility.

Introduction

Drosophila subobscura is a model species with a rich chromosomal polymorphism in most of its chromosomes: A (= X, the sex chromosome), E, J, O and U (Krimbas 1992, 1993; Powell 1997). It is generally accepted that this polymorphism is adaptive due to its geographic distribution pattern, although other explanations as historic factors could be also important (Krimbas and Loukas 1980; Sperlich and Pfriem 1986). The latitudinal clinal distribution of chromosomal inversions, both in North and South America, presenting the same pattern found in the Palearctic region was key evidence supporting their adaptive role (Prevosti et al. 1988, 1990; Menozzi and Krimbas 1992; Balanyà et al. 2003). Also, there are other observations supporting the adaptive role of inversions, for instance, their seasonal variation (Fontdevila et al. 1983; Rodriguez-Trelles et al. 1996; Zivanovic and Mestres 2010b) and long-term changes (Orengo and Prevosti 1996; Rodríguez-Trelles and Rodríguez 1998; Solé et al. 2002; Balanyà et al. 2004, 2006, 2009; Zivanovic and Mestres 2010a, 2011; Zivanovic et al. 2012), suggesting a response to climatic changes. Furthermore, in American populations of *D. subobscura*, the effect of selection has been measured in two chromosomal arrangements, O_5 and $O_{\underline{3+4+7}}$ (Mestres et al. 2001). Although strong gene flow has been observed between natural populations of *D. subobscura* (Latorre et al. 1992; Pascual et al. 2001; Zivanovic et al. 2007; Araúz et al. 2009; Pegueroles et al. 2013), natural selection acting on inversions is able to maintain their geographical differentiation.

This system of overlapped and non-overlapped inversions is a cornerstone of the adaptive and evolutionary potential of *D. subobscura*. One way to analyze this genomic architecture based on inversion is to disturb it, for example, by means of inbreeding experiments. In this species, the effect of inbreeding have been used to study the rate of development and fertility (Hollingsworth and Maynard Smith 1955), the pattern of

puffing activity in relation to chromosomal inversions (De Frutos et al. 1984), the genetic system of inversions present in American colonizing populations (Pegueroles et al. 1996) and to reveal interpopulation differences in inversion polymorphism (Rasic et al. 2008).

In the present study, our main aim is to assess whether the genetic architecture for inversions of a well-adapted population, in its optimum climatic conditions, could reach the expected homozygosity under a drastic inbreeding, or other evolutionary forces (as selection to laboratory conditions) could modify the expectations. For this purpose, we have chosen the Balkan population of Avala, a large and well established population, and collected the *D. subobscura* sample during the expansion peak of the species abundance. The inbreeding conditions have been a drastic 12 generations of brother-sister mating. A second objective is to use the resulting inbred lines (obtained in the previous inbreeding experiment) to study the adaptation of different chromosomal combinations to two different temperature conditions (13ºC and 18ºC).

Material and Methods

Population and samples

In this study, we analyzed a natural population of *Drosophila subobscura* collected from Avala Mountain (44º48´N 20º30'E), approximately 18 km south of Belgrade, Serbia. A detailed description of this locality can be found in Zivanovic and Mestres (2010a, b). *D. subobscura* individuals were sampled from a forest with polydominant communities of *Fagetum submontanum mixtum*, which is about 450 m a.s.l. Flies were collected exactly at the same site of previous sampling (Zivanovic and Mestres 2010a, b) from 30th of May to 5th of June 2011. These days were chosen to compare chromosomal polymorphism data with that from June 2004 (collected from the

97 2nd to 9th of June). The 2011 collection was sampled about 2 days at average earlier,
98 because spring/summer has advanced an average of 2.5 days per decade in Europe
99 (Menzel et al. 2006). Furthermore, *D. subobscura* presents the highest peak of
100 expansion during spring (Krimbas 1993; Argemí et al. 1999; Araúz et al. 2009).
101 Meteorological data for Avala Mountain (maximum, minimum and mean temperatures,
102 and rainfall) were recorded from Republic Hydrometeorological Service (Serbia). The
103 average values of these meteorological parameters during trapping days were:
104 maximum T = 27.9 Cº, minimum T = 17.0 Cº, mean T = 22.6 Cº and rainfall = 0.98
105 mm. Detailed weather information for all collecting days is shown in Table S1.
106
107 **Inbreeding crosses and chromosomal preparation**
108 We started the experiment with 28 isofemale lines from females sampled in
109 Avala Mountain. Each one was put in an individual vial with 25ml standard corn-meal-
110 sugar-agar-yeast medium, at 18ºC, 60% relative humidity under a 12h/12h light/dark
111 cycle. A detailed description of inbreeding procedure can be found in Pegueroles et al.
112 (1996). Couples (using virgin females) of offspring from the wild females were chosen
113 as parents of the first generation of sib mating. For each line and in every generation,
114 three or four (but in many cases even more) brother-sister pairs were mated in
115 individual vials. Among those that produce offspring, one of them was chosen to
116 continue the experiment. However, after 12 generations of a long systematic inbreeding
117 process, many consequences were evident: delay of development time, reduction in
118 viability and even many lines were lost. The number of surviving lines (in brackets) per
119 generation was the following: G_4 (15), G_6 (11), G_8 (11), G_{10} (8) and G_{12} (8).
120 For studying the effect of inbreeding on chromosomal inversion polymorphism,
121 third instar larvae were analyzed in the initial sample (G_0) and also in G_4, G_6, G_8, G_{10}

and G_{12} generations. For the initial sample, only one son of each wild female was crossed with virgin females of the Küsnacht strain, which is homokaryotypic for standard chromosomal inversions in all five chromosomes. The polytene chromosomes were stained and squashed in aceto-orcein solution. At least eight larvae from the progeny of each cross were examined. For the cytological analysis of chromosomal arrangements, the Kunze-Mühl and Müller (1958) chromosome map was used. The designation of inversions and chromosomal arrangements followed that of Kunze-Mühl and Sperlich (1955) and Krimbas (1993). The same procedure (only one male per line was used) was repeated for the chromosomal studies in G_4, G_6, G_8, G_{10} and G_{12} generations. Finally, the degree of chromosomal inversion polymorphism in the initial and inbred lines was assessed using the index of free recombination (IFR) computed according to Carson (1955).

Development time and fertility of inbred lines at different temperatures

After 12 generations of inbreeding process we selected the surviving lines (a total of 8) to carry out a study of fertility. For each line, three replicates were founded using 3 males and 3 virgin females and putting them in individual vials. These vials were left, at the same time, in a chamber with an optimal temperature (18ºC). The temperature was not changed, and after 7 days the parents were eliminated. Then, each day at the same hour, the number of arising males and females was counted for each vial. This procedure was repeated each day until the medium was exhausted. A similar procedure was simultaneously carried out at rather cold temperature (13ºC), but in this case the parents were eliminated after 10 days of crossings.

147 **Mathematical and statistical methods**

148 The theoretical value of the inbreeding coefficient *F* was calculated according to
149 Wright (1921, 1969), and it follows the nonhomogeneous second order difference
150 equation $F_t = \frac{1}{4}(1 + 2F_{t-1} + F_{t-2})$, where F_t represents the inbreeding coefficient in the t^{th}
151 generation. In a given generation *t*, the expected karyotypic frequencies for the different
152 combinations of inversions (or arrangements) per chromosome were obtained using
153 inversion (or arrangements) frequencies (*p*, *q*, *r*, *s*, etc.), considering each one as a single
154 allele, and using the corresponding *F* value of the given generation (Wright 1931; Hartl
155 and Clark 1989; Hedrick 2000). Thus, for a homozygote the mathematic expression
156 would be: $P = p^2(1-F) + pF$, and for a heterozygote $H = 2pq(1-F)$.
157 For each chromosome, the differences between the observed and expected genotypic
158 frequencies in the G_4, G_8 and G_{12} generations of inbreeding were determined by two-
159 sided Fisher's exact test (statistically significant *p-value* < 0.05). The same statistical
160 procedure was also carried out to compare the observed chromosomal frequencies
161 between G_0 and G_{12}. This test has been utilized because it is more accurate than chi-
162 squared test when the expected frequencies are small. Using the bootstrap procedure
163 (100000 runs) the corresponding *p-values* were obtained. These computations were
164 carried out with R package (http://CRAN.R-project.org).
165 To compare the beginning of fly emergence and the period of emergence (in
166 days) at the two development temperatures (13ºC and 18º), a Mann-Whitney test was
167 computed. Also, to study fertility a factorial ANOVA, with fixed factors being
168 temperature, chromosomal line and replicates was computed. For a fixed 18 ºC of
169 temperature, a factorial ANOVA with fixed factors being chromosomal line and
170 replicates was applied. When significances were detected, a pairwise Tukey analysis
171 was carried out. In the case of 13ºC, as very few individuals were born, a non-

parametric one-way ANOVA (Kruskal-Wallis) was computed to ascertain the effect of chromosomal line factor. All these analyses were carried out with R package (http://CRAN.R-project.org).

Results

Variation of inversion polymorphism during inbreeding

The chromosomal polymorphism frequencies in the first generation (G_0) and in the inbred after four (G_4), six (G_6), eight (G_8), ten (G_{10}) and twelve (G_{12}) generations are presented in Table 1. During the sib-mating process a reduction of viability in some strains and a loss of lines were observed. In Fig. 1, variations in the inversions and chromosomal arrangements frequencies during inbreeding generations are shown. For most common inversions and arrangements, frequencies stabilization seems to begin in G_8 and confirm in G_{10}. Likely, it is due to inbreeding coefficients values, which present few changes from G_8 (0.826) to G_{12} (0.926). In the chromosomal polymorphism comparison between G_0 and G_{12}, there are no significant differences for any of the chromosomes: A (Fisher's exact test, *p-value* = 0.8784), J (*p-value* = 1), U (*p-value* = 0.4059), E (*p-value* = 0.8297) and O (*p-value* = 0.2245).

With regard to karyotypic frequencies, the observed and expected (under inbreeding model) values are presented in Table 2. For J chromosome there were not significant differences in these frequencies for G_4 (*p-value* = 1), G_8 (*p-value* = 1) and G_{12} (*p-value* = 1) generations. In the case of U chromosome, only G_8 was significant: G_4 (*p-value* = 0.3104), G_8 (*p-value* = 0.0240) and G_{12} (*p-value* = 0.0534). Differences in E chromosome were not significant: G_4 (*p-value* = 0.7872), G_8 (*p-value* = 1) and G_{12} (*p-value* = 1). Finally, for the O chromosome, differences were neither significant: G_4 (*p-value* = 0.9213), G_8 (*p-value* = 0.9066) and G_{12} (*p-value* = 1). During the inbreeding

process an increment in IFR values can be observed (Table 2). This result is expected because a consequence of inbreeding is an increase of homozygotes. Finally, it is worth to study the chromosomal composition of the remaining inbred lines in G_{12} (Table 3). In six of them (lines 1, 2, 5, 6, 7 and 8), one chromosome of the karyotype still segregated. For the O chromosome, lines 1 and 2 presented the inversion or arrangements O_{3+4+1}; O_{3+4+22} and O_{st}; O_{3+4+6}, respectively, whereas the other lines (5, 6, 7 and 8) segregated for the U chromosome, all with the combination U_{1+2}; U_{1+2+6}. For the A, J and E chromosomes all lines were homokaryotypic. Thus, the percentages of fixation were 100% for the A, J and E chromosomes, but 75% and 50% for the O and U chromosomes, respectively. According to Schäfer (1937), after 12 generations of brother-sister mating the expected percentage of fixation would be 85.9%, if all initial crosses were due to heterologous heterozygotes (A_1A_2 x A_3A_4). This percentage could be even larger if initially there were less different alleles (Haldane 1955).

Development time and fertility

The number of flies emerged at both temperatures are presented in Table S2 (13ºC) and Table S3 (18ºC). For development time, the difference in the beginning of fly emergence was significant between 13ºC and 18ºC (Mann-Whitney test, W = 40.0; *p-value* = 0.004), whereas the period of emergence (in days) was no significant (W = 20.0; *p-value* = 0.941).

When comparing 13ºC and 18ºC, significant differences in the number of flies were obtained for temperature and chromosomal lines, but replicates were no significant (Table 4). In all pairwise comparisons, differences between chromosomal lines were due to line number 6. When analyzing only 18ºC, the effect of chromosomal lines was significant, but replicates factor was no significant (Table 5). As in the previous

analysis, pairwise comparisons were significant for all cases where chromosomal line 6 was involved, with the exception of the comparison between lines 3 and 6. For the 13ºC, chromosomal lines factor was no significant (Kruskal-Wallis = 2.965; *p-value* = 0.564).

Finally, a malformation resembling *club* mutation of *D. melanogaster* (Lindsley and Zimm 1992) was observed in different lines, mainly those reared at 13ºC. It has been observed that many *Drosophila* genus mutants tend to slightly increase their viability at lower temperatures (Dobzhansky 1982; Lindsley and Zimm 1992; Ashburner et al. 2005). However, flies which emerged with their wings drastically reduced died soon.

Discussion

In the G_0, the inversions (or chromosomal arrangements) and karyotypes found are characteristic of Avala mountain population (Zivanovic and Mestres 2010a) and also of the Balkan region (Zivanovic et al. 1995, 2002; Zivanovic 2007; Rasic et al. 2008; Stamenkovic-Radak et al. 2008; Kenig et al. 2010; Zivanovic and Mestres 2011; Jelic et al. 2012). No significant changes between G_0 and G_{12} were observed for the inversion and arrangement frequencies. In inbreeding experiments using *D. subobscura*, other authors (although analyzing fewer inbreeding generations) found similar results (Pegueroles et al. 1996; Rasic et al. 2008). Furthermore, most inversions (or arrangements) seem to reach stabilization around G_8 (Fig. 1). In general, no significant differences were neither detected when comparing the observed and expected (under inbreeding model) karyotypes. For all these reasons, it seems that inbreeding is the leading factor acting on inversions (or arrangements) and karyotypes, whereas the effects other evolutionary forces appear to be secondary. Also, values of IFR along generations changed accordingly with the increasing levels of inbreeding.

However, there were many exceptions: for instance, significant differences for the U chromosome were found in G_8. Likely, these are due to an increase of U_{1+2}/U_{1+2+6} and a decrease of U_{1+2+6}/U_{1+2+6} karyotypes (Table 2). It is worth to point out that U_{1+2}; U_{1+2+6} arrangements were found still segregating in four lines at the end of the inbreeding experiment (G_{12}) (Table 3). In this last generation, this was not the only case of segregating arrangemets: two lines had no fixation for the O chromosome, presenting the O_{3+4+1}; O_{3+4+22} and O_{st}; O_{3+4+6} constitution (Table 3). Thus, considering all lines together, six out eight lines presented segregation for at least one chromosome. U_{1+2} seems to be an ancient arrangement (Krimbas 1993) and "warm" adapted (Menozzi and Krimbas 1999; Solé et al. 2002) and it was fixed in four of the final inbred lines and still segregated with U_{1+2+6} in the remaining four. This latter arrangement presents a typical Balkan distribution, and in this region it could confer an adaptive advantage to its carriers. The inbreeding experiment was carried out at constant 18ºC, and in general, it is considered a good temperature for the species (Buzzati-Traverso 1942; Rocha-Pité 1980; Krimbas 1993; Santos et al. 2004, 2005). With regard to O chromosome, the segregating arrangements O_{3+4+1}; O_{3+4+22} (Line 1) was a surprising combination (Table 3), because they are not the most frequent O chromosome arrangements in Avala population (Table 1). The first arrangement presents rather high frequencies in Balkan populations (Krimbas 1993), depending on the population and date of the sample (ranging from 5.0 to 27.0%) (Zivanovic et al. 1995, 2002; Zivanovic 2007; Zivanovic and Mestres 2010a, 2011; Zivanovic et al. 2012). The second one (O_{3+4+22}) is also common in the Balkans (Krimbas 1993), and can be found in frequencies ranging from 6.5 to 16.7% (Zivanovic et al. 2002; Zivanovic 2007; Zivanovic and Mestres 2010a, 2011; Zivanovic et al. 2012). Maybe despite the inbreeding, they were segregating due to lethal genes trapped inside the O_1 and O_{22} inversions, because they are small and

recombination inside them is dramatically reduced (Albornoz and Dominguez 1994; Chang and Lin 1995; Chang et al. 1996; Yang et al. 2002; Mestres et al. 2009). However, this possibility is only speculative. The O arrangements segregating in Line 2, O_{st} and O_{3+4+6}, (Table 3) is also surprising, because the latter is infrequent in the Balkans (Krimbas 1993), where it has been seldom detected and presenting very low frequencies (Zivanovic et al. 2002; Zivanovic and Mestres 2010a; Zivanovic et al. 2012). However, under laboratory conditions O_{3+4+6} had a higher segregation than expected in the heterokaryotypes (Pegueroles et al. 2010).

If we focus in the inversions (or arrangements) fixed, there was variability for the A chromosome, because A_1, A_2 and A_{st} were in homozygous condition in three, one and four lines, respectively (Table 3). It is an expected result due to the initial composition of the sample (Table 1). For the J chromosome, two and six lines were fixed for the J_{st} and J_1 inversions, respectively. This result is also compatible with the initial sample composition (Table 1). However, U chromosome presented interesting results, because four lines had the U_{1+2} arrangement fixed, whereas in the remaining lines the U_{1+2}; U_{1+2+6} combination was segregating. This situation has been previously commented, but it is worth noting that no inbred line showed the U_{1+2+6} arrangement fixed at the end of the process. This arrangement is not lethal *per se*, because homozygotes for it were recorded in the initial sample of flies. With regard to the E chromosome, all inbred lines reached a fixation status: E_{st}, E_8 and E_{1+2+9} were fixed in three, two and three lines, respectively. This result could be considered as expected, because they presented the highest frequencies at the beginning of the inbreeding process. However, E_{st}/E_{st} was recorded at a higher frequency than expected (Table 2), although it is considered a "cold" adapted inversion and the inbreeding experiment was developed at constant 18ºC. This is not the case for the other two arrangements: for the

297 E_{1+2+9}/E_{1+2+9} the opposite tendency was detected and for the E_8/E_8 karyotypes, observed
298 and expected values were very similar. Finally the O chromosome, as previously
299 commented, presented two inbred lines where arrangements are still segregating, but six
300 were fixed for the same arrangements: O_{st} (two lines), O_{3+4} (three lines) and O_{3+4+7}
301 (only one line). Although O_{st} is considered a "cold" adapted arrangement and
302 inbreeding conditions seem *a priori* not favor it, its frequency was increasing along the
303 inbreeding experiment (Table 1) until reaching a plateau (Fig. 1). The result observed
304 for the O_{3+4} could be considered as expected, but its frequency had a tendency to
305 decrease. However, the inbred line with the fixed arrangement O_{3+4+7} was really
306 surprising. This arrangement is very uncommon in the Balkan region (Krimbas 1993;
307 Zivanovic et al. 1995, 2002; Zivanovic 2007; Zivanovic and Mestres 2010a, 2011;
308 Zivanovic et al. 2012), but it is frequent in the Western Mediterranean (Prevosti et al.
309 1984; Krimbas 1993; Orengo and Prevosti 1996; Solé et al. 2002; Araúz et al. 2009) and
310 American colonizing populations (Prevosti et al. 1988, 1990; Balanyà et al. 2003;
311 Mestres et al. 2009; Castañeda et al. 2013). It was also described in Asia Minor and
312 Israel, but data available from these regions are not recent (Goldschmidt 1956; Götz
313 1967; Malogolowkin-Cohen and Sperlich 1981). In the present study, its initial
314 frequency was so small that it was undetected in G_0 (Table 1). In G_4, O_{3+4+7}/O_{3+4+7}
315 homozygotes were detected for the first time and their frequency was increasing until
316 G_{12} (Table 2). It could be interpreted that this combination was adaptive for the
317 particular laboratory rearing conditions. Furthermore, this adaptation to laboratory
318 conditions seems to be supported by an experiment of Pegueroles et al. (2010): in
319 O_{3+4}/O_{3+4+7} individuals, a significant deviation of random segregation was observed,
320 being the O_{3+4+7} gametes in higher proportion than expected.

Although the inbreeding process until G_{12} was very drastic for *D. subobscura* individuals, valuable information on development time and fertility was gathered using the inbred lines. It is well know that species of *Drosophila* genus develop faster at higher temperatures (for a review see Kuntz and Eisen 2014) and *D. subobscura* is not an exception (Krimbas 1993). It seems that developing time is adaptive to climate, but the relative timing of main events in *Drosophila* embryogenesis seem to be constant (Kuntz and Eisen 2014). In the present research, we have found significant differences in the beginning time of fly emergence, but not in the period of emergence. When comparing the inbred lines reared at 13ºC and 18ºC, significant differences were observed in the number of flies obtained. According to this result, Santos (2007) found also lower fitness at 13ºC than at 22ºC. With regard to the number of flies, we also found significant differences between chromosomal lines. This effect is mainly due to inbred line number 6, in which the infrequent arrangement in the Balkans O_{3+4+7} was fixed. However, as previously commented, this arrangement could be adaptive to laboratory rearing conditions. Analyzing the whole karyotype of this inbred line and using the classification of inversions and arrangements in "cold" and "warm" according to Rego et al. (2010), based on Menozzi and Krimbas (1992), then inbred line 6 is: A_1 (cold), J_1 (warm), U_{1+2} (warm); U_{1+2+6}, E_8 and O_{3+4+7}. Thus, not a clear pattern of 'thermal adaptation' is observed in this inbred line.

In summary, we hypothesize that the main evolutionary force in the present research was inbreeding, but during the experimental process flies seem also to adapt in some way to laboratory conditions. Inversions seem to adapt not only to temperature, but to other environmental factors. Thus, results obtained in laboratory experiments reflect the adaptive potential of *D. subobscura* inversions, even after a severe disturbance produced by inbreeding.

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620 **FIGURE LEGENDS**

621 **Fig 1.** Variations of inversions and chromosomal arrangements frequencies (in
622 percentage) during the inbreeding experiment (from G_0 to G_{12}). (A) Chromosome A,
623 (B) Chromosome J, (C) Chromosome U, (D) Chromosome E and (E) Chromosome O.

Table 1. Frequencies of *D. subobscura* chromosomal inversions and arrangements from the initial generation (G_0) and in the inbreed lines after four (G_4), six (G_6), eight (G_8), ten (G_{10}) and twelve (G_{12}) generations. We have used the nomenclature of Kunze-Mühl and Sperlich (1955) and Krimbas (1993).

	Inbreeding generation											
	G_0		G_4		G_6		G_8		G_{10}		G_{12}	
Chrom. arrangements	n	%	n	%	n	%	n	%	n	%	n	%
A_{st}	13	46.4	5	33.3	5	45.5	6	54.5	4	50.0	4	50.0
A_1	8	28.6	5	33.3	5	45.5	4	36.4	3	37.5	3	37.5
A_2	7	25.0	5	33.3	1	9.0	1	9.1	1	12.5	1	12.5
Total	28		15		11		11		8		8	
J_{st}	15	26.8	9	30.0	7	31.8	7	31.8	4	25.0	4	25.0
J_1	41	73.2	21	70.0	15	68.2	15	68.2	12	75.0	12	75.0
Total	56		30		22		22		16		16	
U_{st}	6	10.7	/	/	/	/	/	/	/	/	/	/
$U_{\underline{1+2}}$	33	58.9	18	60.0	15	68.2	17	77.3	12	75.0	12	75.0
$U_{\underline{1+2+6}}$	12	21.4	10	33.3	7	31.8	5	22.7	4	25.0	4	25.0
$U_{\underline{1+8+2}}$	5	8.9	2	6.7	/	/	/	/	/	/	/	/
Total	56		30		22		22		16		16	
E_{st}	15	26.8	7	23.3	7	31.8	5	22.7	6	37.5	6	37.5
$E_{\underline{1+2}}$	1	1.8	/	/	/	/	/	/	/	/	/	/
$E_{\underline{1+2+9}}$	25	44.6	15	50.0	6	27.3	9	40.9	6	37.5	6	37.5

$E_{1+2+9+12}$	1	1.8	1	3.3	/	/	/	/	/	/	/	/
E_8	14	25.0	7	23.3	9	40.9	8	36.4	4	25.0	4	25.0
Total	56		30		22		22		16		16	
O_{st}	12	21.4	7	23.3	6	27.3	7	31.8	5	31.2	5	31.2
O_{3+4}	26	46.4	11	36.7	7	31.8	8	36.4	6	37.5	6	37.5
O_{3+4+1}	8	14.3	4	13.3	2	9.1	1	4.5	1	6.3	1	6.3
O_{3+4+5}	1	1.8	/	/	/	/	/	/	/	/	/	/
O_{3+4+6}	1	1.8	2	6.7	1	4.5	/	/	1	6.3	1	6.3
O_{3+4+7}	/	/	2	6.7	2	9.1	2	9.1	2	12.5	2	12.5
O_{3+4+8}	2	3.6	1	3.3	2	9.1	1	4.5	/	/	/	/
O_{3+4+22}	6	10.7	3	10.0	2	9.1	3	13.6	1	6.3	1	6.3
Total	56		30		22		22		16		16	

Table 2. Observed and expected frequencies of chromosomal karyotypes in the first generation (G_0), and in G_4, G_8 and G_{12} generations of inbreeding. IFR values for each inbreeding generation are also presented. OBS. and EXP. mean observed and expected, respectively.

	Generations						
	G_0	G_4		G_8		G_{12}	
Karyotypes	OBS.	OBS.	EXP.	OBS.	EXP.	OBS.	EXP.
J_{st}/J_{st}	0.036	0.267	0.189	0.273	0.234	0.250	0.253
J_{st}/J_1	0.464	0.067	0.159	0.091	0.068	/	0.029
J_1/J_1	0.500	0.666	0.652	0.636	0.698	0.750	0.717
U_{st}/U_{st}	0.036	/	0.068	/	0.090	/	0.100
$U_{st}/U_{\underline{1+2}}$	0.072	/	0.051	/	0.022	/	0.009
$U_{st}/U_{\underline{1+2+6}}$	0.036	/	0.019	/	0.008	/	0.003

$U_{st}/U_{\underline{1+8}+\underline{2}}$	0.036	/	0.008	/	0.003	/	0.001
$U_{\underline{1}+\underline{2}}/U_{\underline{1}+\underline{2}}$	0.357	0.333	0.491	0.545	0.547	0.500	0.571
$U_{\underline{1}+\underline{2}}/U_{\underline{1+2+6}}$	0.321	0.466	0.102	0.455	0.044	0.500	0.019
$U_{\underline{1}+\underline{2}}/U_{\underline{1+8}+\underline{2}}$	0.072	0.067	0.043	/	0.018	/	0.008
$U_{\underline{1+2+6}}/U_{\underline{1+2+6}}$	0.036	0.067	0.146	/	0.188	/	0.201
$U_{\underline{1+2+6}}/U_{\underline{1+8}+\underline{2}}$	/	0.067	0.015	/	0.018	/	0.003
$U_{\underline{1+8}+\underline{2}}/U_{\underline{1+8}+\underline{2}}$	0.036	/	0.056	/	0.075	/	0.083
E_{st}/E_{st}	/	0.133	0.188	0.182	0.234	0.375	0.253
$E_{st}/E_{\underline{1+2+9}}$	0.321	0.067	0.101	/	0.043	/	0.019
E_{st}/E_{8}	0.214	0.133	0.054	0.091	0.023	/	0.010
$E_{\underline{1}+\underline{2}}/E_{8}$	0.036	/	0,003	/	0.002	/	<0.001
$E_{\underline{1+2+9}}/E_{\underline{1+2+9}}$	0.214	0.333	0.363	0.363	0.421	0.375	0.445
$E_{\underline{1+2+9}}/E_{\underline{1+2+9+12}}$	/	0.067	0.007	/	0.003	/	0.001
$E_{\underline{1+2+9}}/E_{8}$	0.143	0.200	0.094	0.091	0.040	/	0.017

$E_{\underline{1+2+9+12}}/E_8$	0.036	/	0.004	/	0.002	/	<0.001
E_8/E_8	0.036	0.067	0.174	0.273	0.217	0.250	0.236
O_{st}/O_{st}	/	0.067	0.146	0.273	0.185	0.250	0.201
$O_{st}/O_{\underline{3+4}}$	0.214	0.200	0.081	0.091	0.036	/	0.015
$O_{st}/O_{\underline{3+4+1}}$	0.071	0.067	0.025	/	0.011	/	0.005
$O_{st}/O_{\underline{3+4+6}}$	0.036	0.067	0.003	/	0.001	0.125	<0.001
$O_{st}/O_{\underline{3+4+22}}$	0.107	/	0.019	/	0.008	/	0.003
$O_{\underline{3+4}}/O_{\underline{3+4}}$	0.214	0.200	0.363	0.273	0.421	0.375	0.445
$O_{\underline{3+4}}/O_{\underline{3+4+1}}$	0.214	0.067	0.054	/	0.023	/	0.010
$O_{\underline{3+4}}/O_{\underline{3+4+8}}$	/	0.067	0.014	0.091	0.006	/	0.003
$O_{\underline{3+4}}/O_{\underline{3+4+22}}$	0.071	/	0.040	/	0.017	/	0.007
$O_{\underline{3+4+1}}/O_{\underline{3+4+6}}$	/	0.067	0.002	/	<0.001	/	<0.001
$O_{\underline{3+4+1}}/O_{\underline{3+4+22}}$	/	0.067	0.012	0.091	0.005	0.125	0.002
$O_{\underline{3+4+5}}/O_{\underline{3+4+8}}$	0.036	/	<0.001	/	<0.001	/	<0.001

O_{3+4+7}/O_{3+4+7}	/	0.067	<0.001	0.091	<0.001	0.125	<0.001
O_{3+4+8}/O_{3+4+8}	/	/	0.023	/	0.032	/	0.035
O_{3+4+8}/O_{3+4+22}	0.036	/	0.003	/	<0.001	/	<0.001
O_{3+4+22}/O_{3+4+22}	/	0.067	0.068	0.091	0.090	/	0.100
IFR	80.36±1.62	86.36±1.78		93.55±2.35		94.15±1.58	

Table 3. Chromosomal inversions and arrangements observed in the remaining lines of inbreeding process (G_{12}).

Inbred chromosomal lines	Chromosomes				
	A	J	U	E	O
1	A_1	J_{st}	$U_{\underline{1+2}}$	$E_{\underline{1+2+9}}$	$O_{\underline{3+4}+\underline{1}}$; $O_{\underline{3+4}+\underline{22}}$
2	A_1	J_{st}	$U_{\underline{1+2}}$	E_{st}	O_{st}; $O_{\underline{3+4+6}}$
3	A_{st}	J_1	$U_{\underline{1+2}}$	E_{st}	O_{st}
4	A_{st}	J_1	$U_{\underline{1+2}}$	E_8	$O_{\underline{3+4}}$
5	A_2	J_1	$U_{\underline{1+2}}$; $U_{\underline{1+2+6}}$	$E_{\underline{1+2+9}}$	$O_{\underline{3+4}}$
6	A_1	J_1	$U_{\underline{1+2}}$; $U_{\underline{1+2+6}}$	E_8	$O_{\underline{3+4}+\underline{7}}$
7	A_{st}	J_1	$U_{\underline{1+2}}$; $U_{\underline{1+2+6}}$	$E_{\underline{1+2+9}}$	O_{st}
8	A_{st}	J_1	$U_{\underline{1+2}}$; $U_{\underline{1+2+6}}$	E_{st}	$O_{\underline{3+4}}$

Segregating arrangements are denoted by ";" symbol.

Table 4. ANOVA analysis for fertility (number of arisen flies). Fixed factors are: temperature, chromosomal line and replicates. Bold *p-values* are significant.

Source of variation	d.f.	MS	F	*P*-value
Temperature	1	258.133	29.900	**0.000**
Chrom. Line	4	51.283	5.940	**0.002**
Replicates	2	10.133	1.170	0.328
Error	22	8.633		

Table 5. ANOVA analysis for fertility (number of arisen flies) at 18ºC. Fixed factors are: chromosomal line and replicates. Bold *p-values* are significant.

Source of variation	d.f.	MS	F	*P*-value
Chrom. Line	7	47.333	5.890	**0.002**
Replicates	2	10.792	1.340	0.293
Error	14	8.030		

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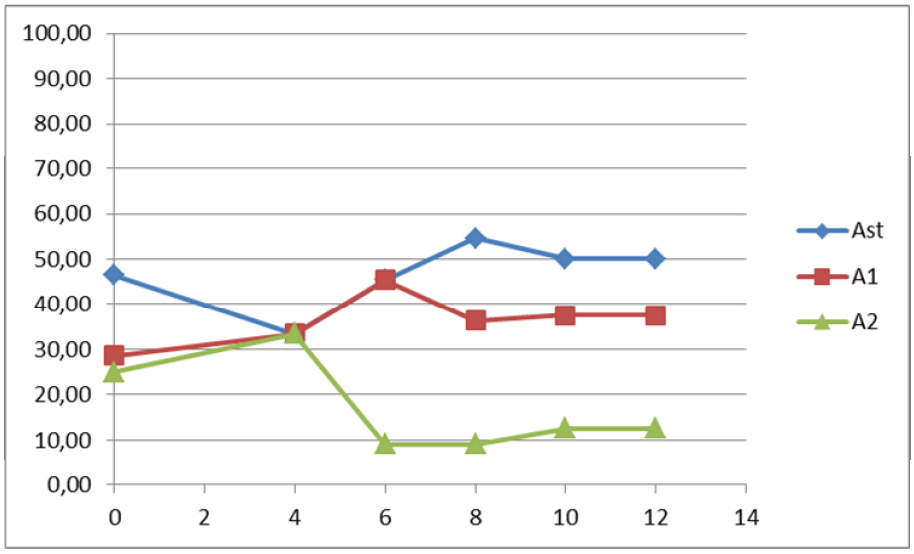
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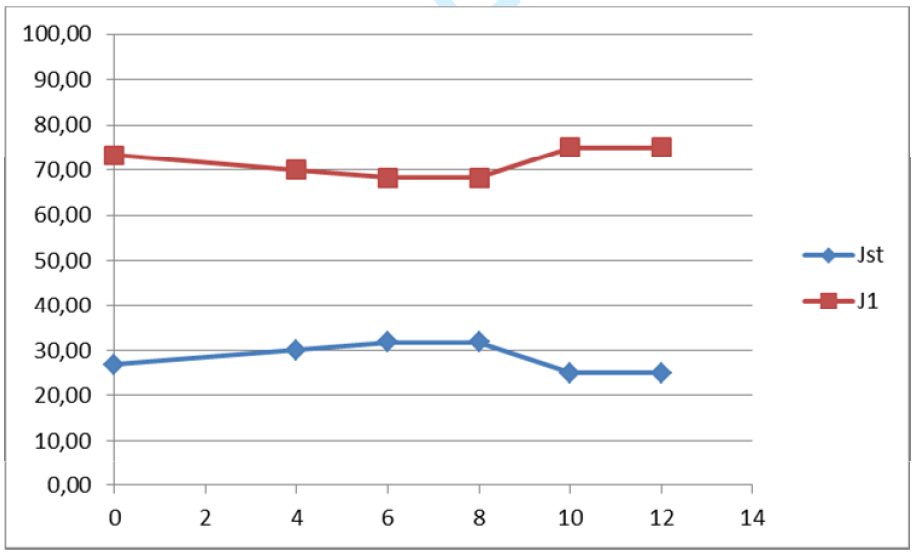
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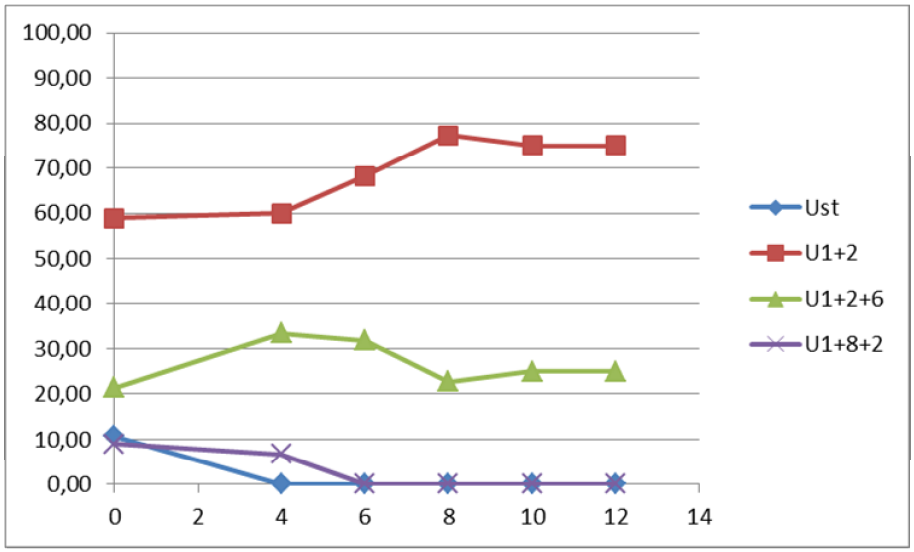
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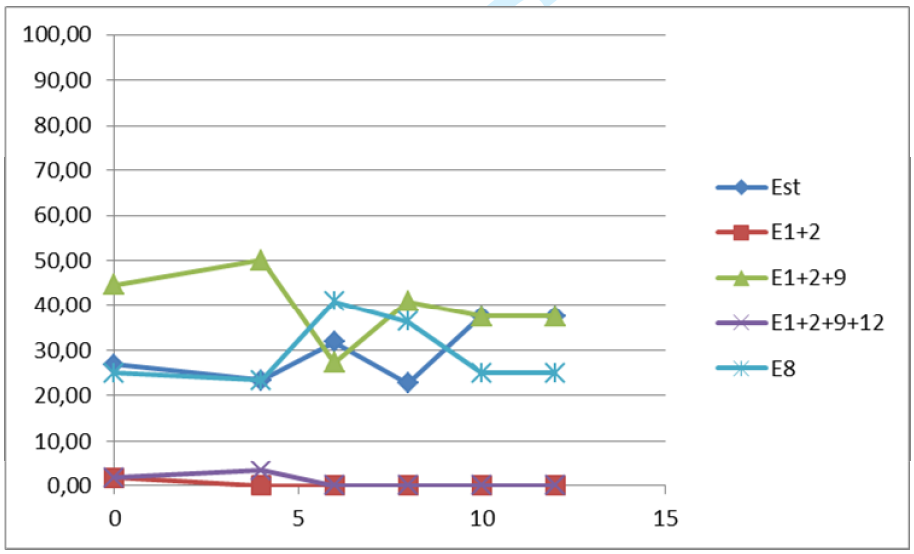
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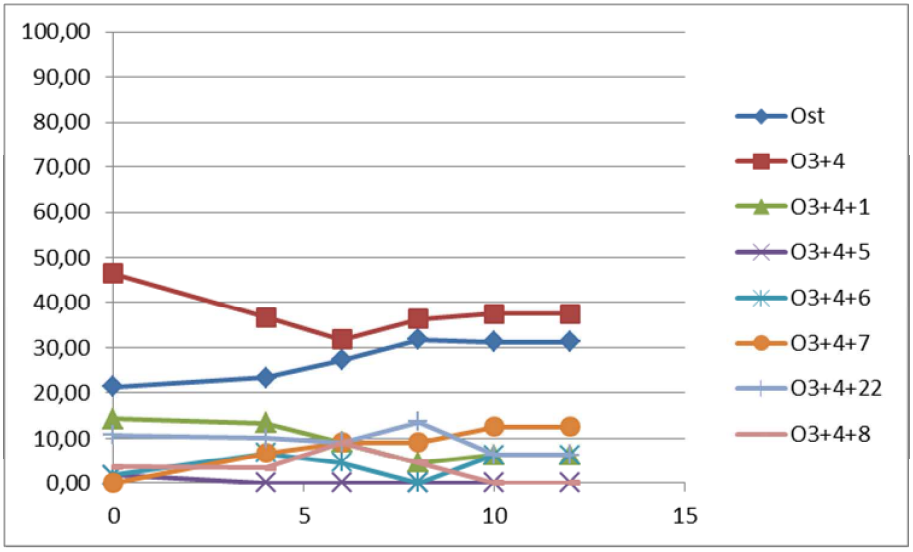
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O3+4+8

Supplementary Table S1. Meteorological data for the Avala Mountain for days 30th of May to 5th of June 2011.

Days	Max. T (ºC)	Min. T (ºC)	Mean T (ºC)	Rainfall (mm)
30. 05. 2011	26.1	14.0	21.7	0.3
31. 05. 2011	29.0	18.3	23.0	/
01. 06. 2011	28.3	17.0	22.2	/
02. 06. 2011	26.7	16.7	19.8	/
03. 06. 2011	26.3	17.3	22.3	6.6
04. 06. 2011	29.1	17.0	23.9	/
05. 06. 2011	29.8	19.0	25.5	/

Max. T and Min. T stand for maximum and minimum temperatures, respectively.

Supplementary Table S2. Numbers of arising males and females from the different inbred line (using three replicates) reared at 13ºC.

	LINE 1 Replicate 1		LINE 1 Replicate 2		LINE 1 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
39	1	/	/	/	/	/
40	/	/	/	/	/	/
41	/	/	/	/	/	/
42	/	/	/	/	/	/
43	/	/	/	/	/	/
44	/	/	/	/	/	1
45	/	/	/	/	/	/
46	/	/	2	/	/	/
47	/	1	/	/	/	/
48	1	1	/	/	/	/
49	/	/	1	/	/	/
Total	2	2	3	0	0	1

	LINE 3 Replicate 1		LINE 3 Replicate 2		LINE 3 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
42	/	1	/	/	/	/
43	/	/	/	1	/	/
44	/	/	/	/	/	/
45	/	/	/	/	/	/
46	/	/	/	/	/	/
47	/	/	/	/	/	/
48	/	/	/	/	/	/
49	/	/	/	/	/	/
50	/	/	/	/	1	/
51-53	/	/	/	/	/	/
54	/	/	/	/	/	1
Total	0	1	0	1	1	1

	LINE 4 Replicate 1		LINE 4 Replicate 2		LINE 4 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
45	1	1	/	/	/	/
46	/	/	/	/	/	/
47	1	/	/	/	/	/
48	/	1	/	/	/	/
49	/	/	/	/	/	/
50	/	/	/	/	/	/
51	/	/	/	/	/	/
52	/	/	/	1	/	/
53	/	/	/	/	/	1
Total	2	2	0	1	0	1

	LINE 5 Replicate 1		LINE 5 Replicate 2		LINE 5 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
42	1	/	/	/	/	/
43	/	/	/	1	/	/
44	/	/	/	/	/	/
45	/	/	/	/	/	/
46	/	/	/	/	/	/
47	1	/	/	/	/	/
Total	2	0	0	1	0	0

	LINE 6 Replicate 1		LINE 6 Replicate 2		LINE 6 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
47	3	/	/	/	/	/
48	1	1	/	/	/	/
49	1	/	1	/	/	/
50	1	1	/	/	/	1
51	1	3	/	/	/	/
52	1	/	/	/	/	/
53	/	1	/	/	/	/
54	/	/	/	1	/	/
55	/	/	/	/	/	/
56	/	1	/	/	/	/
Total	8	7	1	1	0	1

Supplementary Table S3. Numbers of arising males and females from the different inbred line (using three replicates) reared at 18ºC.

	LINE 1 Replicate 1		LINE 1 Replicate 2		LINE 1 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
25	1	/	/	/	1	/
26	3	/	1	1	2	/
27	/	1	1	1	/	/
28	/	/	1	1	1	3
29	/	/	/	/	/	/
30	/	/	1	/	/	/
31	/	/	1	/	/	/
32	/	1	/	/	/	/
Total	4	2	5	3	4	3

	LINE 2 Replicate 1		LINE 2 Replicate 2		LINE 2 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
26	/	/	/	1	/	/
27	/	/	3	2	1	/
28	/	/	3	3	1	1
29	/	/	/	/	1	/
30	/	/	/	/	/	1
31	/	/	/	/	/	1
32	1	/	/	/	/	/
33	/	1	/	/	/	/
Total	1	1	6	6	3	3

	LINE 3 Replicate 1		LINE 3 Replicate 2		LINE 3 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
24	/	/	1	/	/	/
25	/	/	/	/	/	/
26	/	/	/	/	/	1
27	1	/	1	/	1	/
28	4	1	/	1	1	/
29	/	2	1	1	1	1
30	/	1	/	/	1	/
31	/	/	/	/	/	/
32	/	/	/	/	/	1
33	/	/	/	/	/	/
34	/	/	/	/	/	/
35	/	/	/	/	/	/
36	/	/	/	/	/	/
37	/	/	/	/	/	/
38	/	/	/	/	/	1
Total	5	4	3	2	4	4

	LINE 4 Replicate 1		LINE 4 Replicate 2		LINE 4 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
25	1	/	/	/	/	/
26	/	/	1	/	/	/
27	/	/	1	/	/	2
28	/	/	1	2	1	/
29	/	1	/	/	/	/
30	2	3	/	1	1	/
31	/	1	/	/	/	1
32	/	/	/	/	/	/
33	/	/	/	/	1	/
34	/	/	/	/	/	/
35	/	/	/	/	/	1
Total	3	5	3	3	3	4

	LINE 5 Replicate 1		LINE 5 Replicate 2		LINE 5 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
26	/	/	1	/	/	/
27	1	1	3	1	/	/
28	1	/	/	/	2	/
29	/	1	/	/	1	2
30	1	/	1	/	/	/
31	/	/	/	1	/	/
Total	3	2	5	2	3	2

	LINE 6 Replicate 1		LINE 6 Replicate 2		LINE 6 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
27	1	1	/	/	/	/
28	1	/	1	1	/	/
29	1	2	5	2	2	1
30	1	2	7	3	2	1
31	1	1	/	/	3	/
32	1	/	/	/	1	3
33	/	/	/	1	/	/
34 – 36	/	/	/	/	/	/
37	/	/	/	/	1	/
Total	6	6	13	7	9	5

Days after initial crosses	LINE 7 Replicate 1		LINE 7 Replicate 1		LINE 7 Replicate 1	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
25	/	/	/	1	/	/
26	/	/	/	/	/	/
27	/	/	/	/	/	/
28	2	/	2	/	/	/
29	2	/	/	/	/	/
30	2	/	/	/	/	/
31	/	/	/	/	/	/
32	/	/	1	1	/	/
33	/	/	/	1	/	/
34 - 36	/	/	/	/	/	/
37	/	1	/	/	/	/
Total	6	1	3	3	0	0

	LINE 8 Replicate 1		LINE 8 Replicate 2		LINE 8 Replicate 3	
Days after initial crosses	number of arising males	number of arising females	number of arising males	number of arising females	number of arising males	number of arising females
26	/	3	/	/	/	/
27	/	/	/	/	/	/
28	/	/	/	/	/	/
29	/	/	/	/	/	/
30	/	/	/	/	/	/
31	/	/	/	/	/	/
32	/	/	/	1	/	/
Total	0	3	0	1	0	0