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LETTER

Quantitative Analysis of Antennal Mosaic Generation in *Drosophila melanogaster* by the MARCM System

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Summary: Mosaics have been used in *Drosophila* to study development and to generate mutant structures when a mutant allele is homozygous lethal. New approaches of directed somatic recombination based on FRT/FLP methods, have increased mosaicism rates but likewise multiple clones in the same individual appeared more frequently. Production of single clones could be essential for developmental studies; however, for cell-autonomous gene function studies only the presence of homozygous cells for the target recessive allele is relevant. Herein, we report the number and extension of antennal mosaics generated by the MARCM system at different ages. This information is directed to obtain the appropriated mosaic type for the intended application. By applying heat shock at 10 different developmental stages from 0–12 h to 6–7 days after egg laying, more than 50% of mosaics were obtained from 5,028 adults. Single recombinant clones appeared mainly at early stages while massive recombinant areas were observed with late treatments. genesis 00:000–000, 2008. © 2008 Wiley-Liss, Inc.

Key words: antennal mosaics; olfaction; MARCM system; *Drosophila melanogaster*; quantitative analysis

Author Proof

Genetic mosaics appear spontaneously in nature proving the viability of genetically heterogeneous individuals. This knowledge has been largely applied in *Drosophila melanogaster* inducing mosaics to study developmental processes, gene function, and to generate mutant structures when homozygous mutant alleles were lethal for the whole individual (Homyk, 1977; Hotta and Benzer, 1970). Traditional methods involving X-radiation or unstable ring chromosomes have been followed by more modern techniques that increased dramatically the production of mosaic individuals. They included insertion of special DNA fragments (FRT) in *Drosophila*'s genome, which enabled directed somatic recombination (Golic and Lindquist, 1989; Stowers and Schwarz, 1999; Xu and Rubin, 1993) and labeling of recombinant cells (the MARCM system, Mosaic Analysis with a Repressible Cell Marker, Lee and Luo, 1999).

Different tissues and interaction between cell types in development have been addressed by these methods. The MARCM system has been directly used, for example, to determine the basis of the olfactory system assembly in *Drosophila* by means of single clones (Jefferis *et al.*, 2001, 2004). Antennae, the main olfactory receptor organs, can be selected as a tissue for testing genes related to neuronal function since some lethal mutant genotypes produce viable cells when restricted to the antennal organ (Acebes and Ferrus, 2001; Canal *et al.*, 1998). Antennal mosaics could be also applied to the study of genes affecting olfactory reception, which takes place at the third antennal segment. This structure contains a heterogeneous mixture of olfactory neurons according to the receptor molecules they express and their functional profile (Hallem *et al.*, 2004; Vosshall *et al.*, 1999). In mosaic antennae the effects of olfactory transduction mutations in a specific receptor neuron type could be easily compared with the corresponding homologous neuron that does not express the mutation if we have an appropriate marker, such as GFP.

In this report, we quantify the rate and type of antennal mosaics generated by heat shock-induced recombination. With these data we supply a useful tool for those scientists wanting to make clones in the antenna. Information about the best time window for heat shocking to obtain a specific type of clone is provided. We used the MARCM method (Lee and Luo, 1999, 2001) that combines the FLP/FRT, Gal-4/UAS, and Gal-80 systems to label uniquely mutant cells in mosaic flies. In the MARCM system, cells are initially heterozygous for a transgene encoding the GAL80 protein that inhibits the activity of the transcription factor GAL4. Following FLP/FRT-mediated mitotic recombination, the GAL80 transgene is removed from one of the daughter cells, thus allowing expression of a GAL4-driven reporter gene specifically in this daughter cell and its progeny. In our case, blue labeling was restricted to neuronal clones using the elav-Gal4 driver and a UAS-lacZ insert.

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Table 1
Frequency of Mosaic Flies for Either Second or Third Antennal Segments or Maxillary Palps

Age at the moment of heat shock[a] (h)	Number of flies	Number of mosaic flies	% of mosaic flies
0–12	372	171	45.96
12–24	606	196	32.34
24–36	406	124	30.54
36–48	545	328	60.18
48–60	799	459	57.44
60–72	758	389	51.32
72–96	485	368	75.88
96–120	245	199	81.22
120–144	581	460	79.17
144–168	231	108	46.75
	5,028	2,814	55.97

[a]Age refers to time after egg laying.

Ten groups of elav-Gal4, hs-FLP; FRTG13/FRTG13-Gal80; UAS-lacZ-individuals were aged for different time periods, from 0–12 h to 6–7 days after egg laying (AEL), and then heat shocked, promoting recombination during this time. The frequency of recombinant cells was analyzed in adult flies. On average 22 tubes were measured for each condition. The experimental classes, total number of analyzed flies, number and percentage of mosaics obtained for each class is presented in Table 1.

Complete heads were analyzed under the microscope for the presence of LacZ expression in the antennae and maxillary palps. Second antennal segment and maxillary palps were only examined for presence or absence of recombinant clones. For the third antennal segment, flies were classified according to the staining level of each side antennae. Figure 1 shows the three types of third antennal segment mosaics that were considered (for a complete description see the methods section).

A single individual is considered mosaic for each structure if at least one of them, at the left or the right side, showed any staining.

Figure 2 illustrates the percentage of mosaics over the total number of analyzed adult flies for each of the three structures that were studied; second and third antennal segments and maxillary palps. Each point was calculated from the different replicate tubes.

It has to be noted that the mosaic values described along the report are relative frequencies and there could be underestimations of the real frequencies. The crossing scheme that was followed (see the method section) generated also a second type of individuals (elav-Gal4, hs-FLP; +/FRTG13-Gal80; UAS-lacZ) that do not undergo somatic recombination but cannot be distinguished from the experimental ones by external markers. However, direct mathematical correction of the data cannot be applied since we find mosaic frequencies higher than the expected maximum of 50% if all the experimental flies become mosaics. Deviation of the theoretical expected proportions for different genotypes in the offspring is usual, especially for complex genotypes involving several inserts and submitted to the heat shock treatment, which may affect differently their viability. Thus,

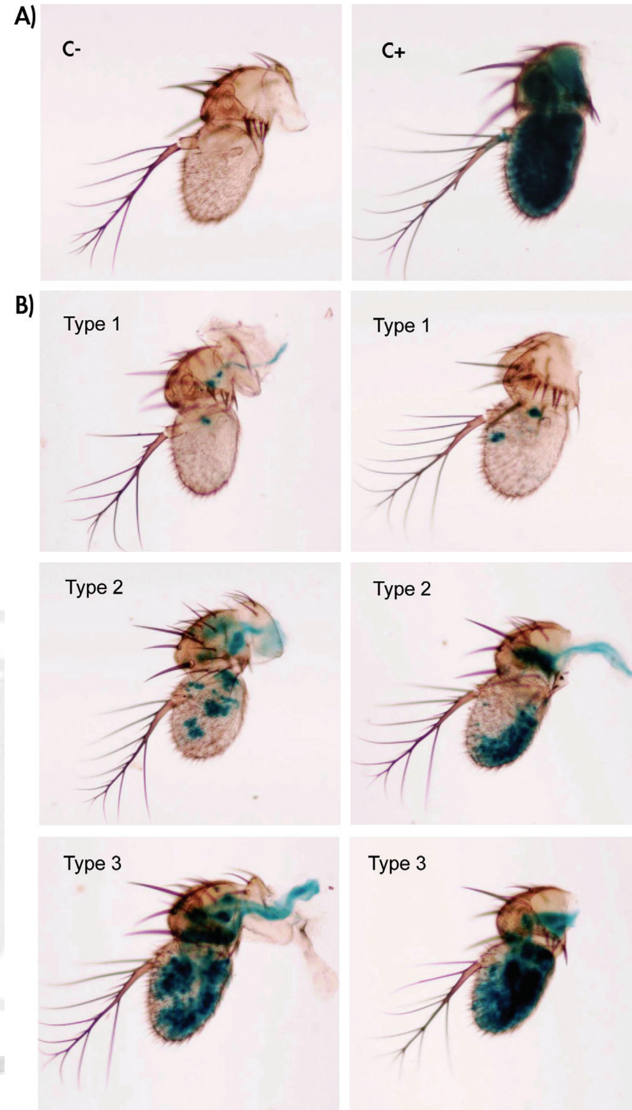

FIG. 1. Different types of antennal mosaics. (**a**) Positive and negative control antennae of sibling flies of the experimental individuals. Flies C− were Gal4-elav, hs-FLP; FRT_{G13}/FRT_{G13}-Gal80; Gl^1/+, and C+ genotype was Gal4-elav, hs-FLP; FRT-G13/CyO; UAS-lacZ/+. (**b**) Type 1, 2, and 3 mosaics, corresponding respectively to, one or two small spots, more than two spots to 50% of the third antennal segment surface and, more than 50% of the surface stained.

in a similar experiment to the one described in this report, but where the FRTG13 chromosome was substituted by a FRTG13-UAS.mCD8::GFP chromosome to evaluate absolute frequencies of the experimental group, we also found significant deviations of the expected 1:1 segregation. In this case, only 16.67% of flies corresponded to the experimental group, with no obvious differences depending on the age at heat shock.

However, although we cannot present absolute frequencies of mosaics obtained from the experimental group, if viability differences among genotypes were independent of age at treatment, comparisons among classes remain valid.

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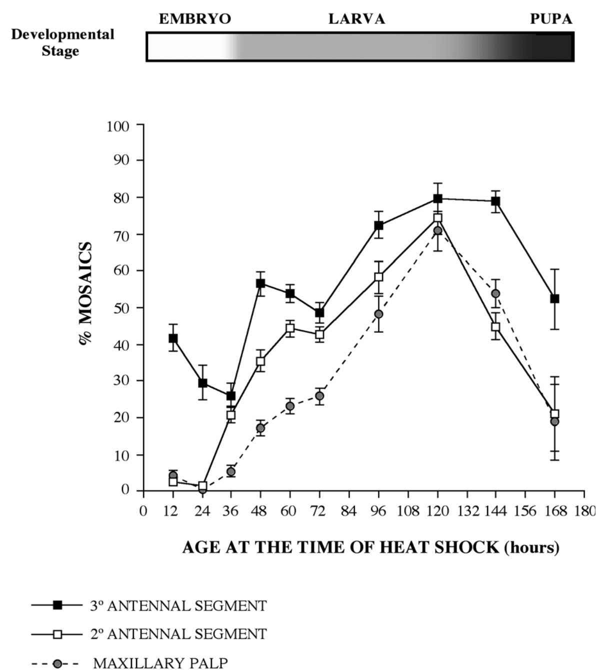

FIG. 2. Percentage of flies with clones in the second or third antennal segments or the maxillary palps (either in the right or the left antennae or both), as a function of the age at the time of treatment.

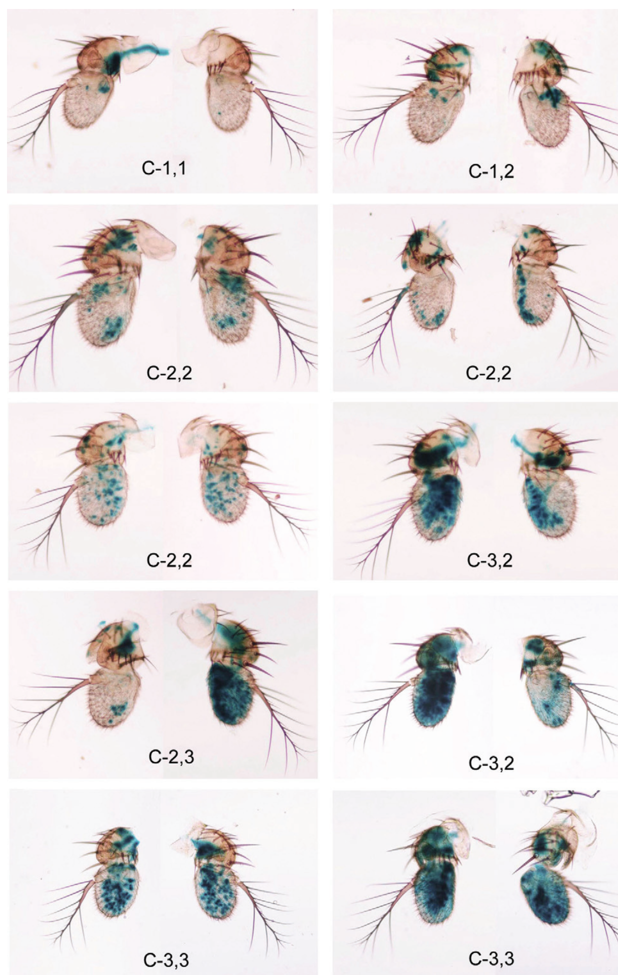

FIG. 3. Different combinations of left and right side antennae for several mosaic flies.

COLOR

Figure 2 does not show homogeneous mosaic rates throughout all the groups. Frequencies ranged from 25 to 80% depending on the age of treatment. Three different peaks of mosaic generation, coincident with the most active mitotic periods, could be observed when heat shock was applied at the embryo, first instar and third instar larvae-early pupa, corresponding to 44%, 55%, and 80% of mosaic flies, respectively.

Mosaic information for the third antennal segment was further analyzed to establish the best age for the treatment depending on the preferred type of mosaics to be obtained. Figure 3 illustrates different mosaic flies analyzed at the present report. F3

Figure 4 displays for each treatment class and each mosaic type, the percentage of third antennal segment mosaics obtained from the total number of flies analyzed for each class. A clear distribution of different mosaic types depending on the moment of somatic recombination was observed. From the different studied classes, small clones appeared preferentially when the heat shock was applied at early developmental stages, classes 0–12 h and 12–24 h. As the age of treatment progressed, for classes 36–48 h and 48–60 h, antennae with small spots still appeared but large stained areas (C-2,2 and C-2,3) were becoming more frequent. For treatments applied at third instar larvae and early pupa stages, classes 72–96 h, 96–120 h, and 120–144 h, the number of extensively stained antennae increased significantly. F4

To get some quantitative estimation of the expected mosaic types within each treatment class, mosaic type frequency is presented for each class in Table 2. With treatments at 0–12 h and 12–24 h of age around 25–30% of flies would be mosaics with one to two small spots at one or both antennae. However, if we are interested in getting more extensive recombinant areas, as much as 60% of the analyzed flies will be mosaics of the C-2,2, C-2,3, and C-3,3 types if the treatment is applied between 72 and 144 h of age. T2

Theoretically, one would expect that early somatic recombination will produce extensive recombinant areas in the adult antenna due to the low number of precursor cells in the imaginal discs. Likewise, recombination at late larval or early pupal stages should give smaller clones due to the lower number of mitoses that these cells undergo. However, our results did not comply with this expectation.

The number and size of the clones obtained in a mosaic depends on the degree of somatic recombination induced by the treatment and the moment of treatment, which should be more effective in very active mitotic periods or at G2-M arrested cells.

Postlethwait and Schneiderman (1971) showed that the antenna arises from about nine cells that remain

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undivided until the first 24 h AEL. This means that the embryonary stage is not a very active mitotic period. Moreover, it has been reported that some neuroblast precursor cells arrest at G1-S transition (Selleck *et al.*, 1992). This could also be the case for olfactory receptor neuroblasts. Considering the previous work published in the development of the olfactory system (Javheri and Rodrigues, 2000; Jefferis *et al.*, 2004; Lee and Luo, 1999; Stocker *et al.*, 1995; Tissot *et al.*, 1997), adult olfactory receptor neurons develop from the eye-antennal imaginal disc during metamorphosis. The first neurons are recognized in the antennal disc 3 h after puparium formation (Tissot *et al.*, 1997). Neuroblast cells (Nb) undergo asymmetric division giving a ganglion mother cell (GMC), which will divide into two neurons, and another Nb. Therefore, the moment of mitotic recombination will have different consequences. In fact, Lee and Luo (1999) reported an important bias of Nb clones, which produced extended marked areas, over two cell clones derived of GMC in the mushroom body lineage.

Also, the amplification ability of recombinant cells and their duplication rate compared to those that contain the original genotype has to be taken into account.

Finally, we have to remember that we are not labeling all the mutant cells resulting from somatic recombination but only the neurons.

The results presented here could be directed to obtain the preferred antennal mosaic type in a double manner. Either selecting the best age to apply heat shock, when it is used for driving flipase expression or, in those cases where a gene promotor-flipase construct is used instead, choosing the appropriate promotor that should correspond to a gene expressed in the desired antennal cells at the selected developmental stage.

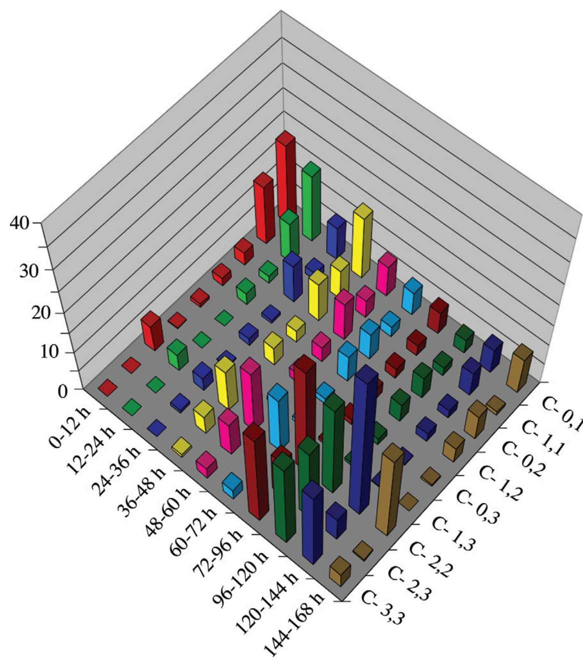

FIG. 4. Ratio of different combinations of mosaic flies for the third antennal segment measured in each class as a percentage of the total number of studied flies. For each combination there is an indication of the mosaic type for both antennae.

COLOR

METHODS

Drosophila Stocks

Experimental individuals submitted to heat shock were generated by the MARCM method (Lee and Luo, 1999) by crossing females of the 5145 line of the Bloomington stock Center (donated by L. Luo) P{w[+mw.hs] = GawB} elav[C155], P{ry[t7.2] = hsFLP}1, w[*]; P{w[+mw.hs] = FRT (w[hs])} G13, P{w[+mC] = tub P-Gal 80}LL2/CyO with males w[*]; P{w[+mw.hs] = FRT (w[hs])}G13/+; Gl[1]/ P{w[+mC] = UAS-lacZ.B} Bg4-2-4b (see the crossing scheme). These males proceed of successive crosses among the following stocks of the Bloomington stock Center, males of stock 504 (donated by T. Wright), amd[21] Bl[1]/CyO; Gl[1]/TM3, Sb[1] Ser[1] and females of stock 1956, w[*]; P{w[+mw.hs] = FRT (w[hs])} G13, (both donated by N. Perrimon). In the next generation, males w[*]; P{w [+mw.hs] = FRT (w[hs])} G13/CyO; Gl[1]/+ were crossed to females of stock 1777, w[*]; P{w [+mC] = UAS-lacZ.B} Bg4-2-4b.

The crossing scheme shows the genotype of experimental flies, both males and females.

Table 2

% of Mosaics Depending on Type and Age at the Time of Heat-Shock Treatment

Class	C-0,1	C-1,1	C-0,2	C-1,2	C-0,3	C-1,3	C-2,2	C-2,3	C-3,3	% of mosaics
0–12 h	18.28	13.71	2.96	1.88	0.81	0.27	6.45	0.00	0.00	44.35
12–24 h	15.51	9.41	1.82	2.81	0.00	0.00	4.13	0.00	0.17	33.83
24–36 h	7.64	1.72	8.87	0.74	1.72	0.25	3.20	0.74	0.00	24.88
36–48 h	14.50	6.97	9.17	2.94	4.40	2.02	10.28	4.59	0.73	55.60
48–60 h	7.26	4.26	9.01	3.63	2.50	0.88	15.27	8.14	2.75	53.69
60–72 h	5.54	2.51	6.33	6.46	1.45	1.06	13.98	5.80	2.64	45.78
72–96 h	5.36	2.68	2.47	2.68	1.24	0.82	26.80	9.69	22.89	74.64
96–120 h	3.27	2.04	5.31	4.49	1.63	0.82	22.86	18.37	21.22	80.00
120–144 h	5.85	5.85	2.07	2.24	0.17	0.17	35.28	5.85	19.79	77.28
144–168 h	8.66	0.87	6.06	3.90	0.43	0.00	21.65	0.43	3.90	45.89

Most frequent mosaic type for each Class.
Second most frequent mosaic type for each Class.

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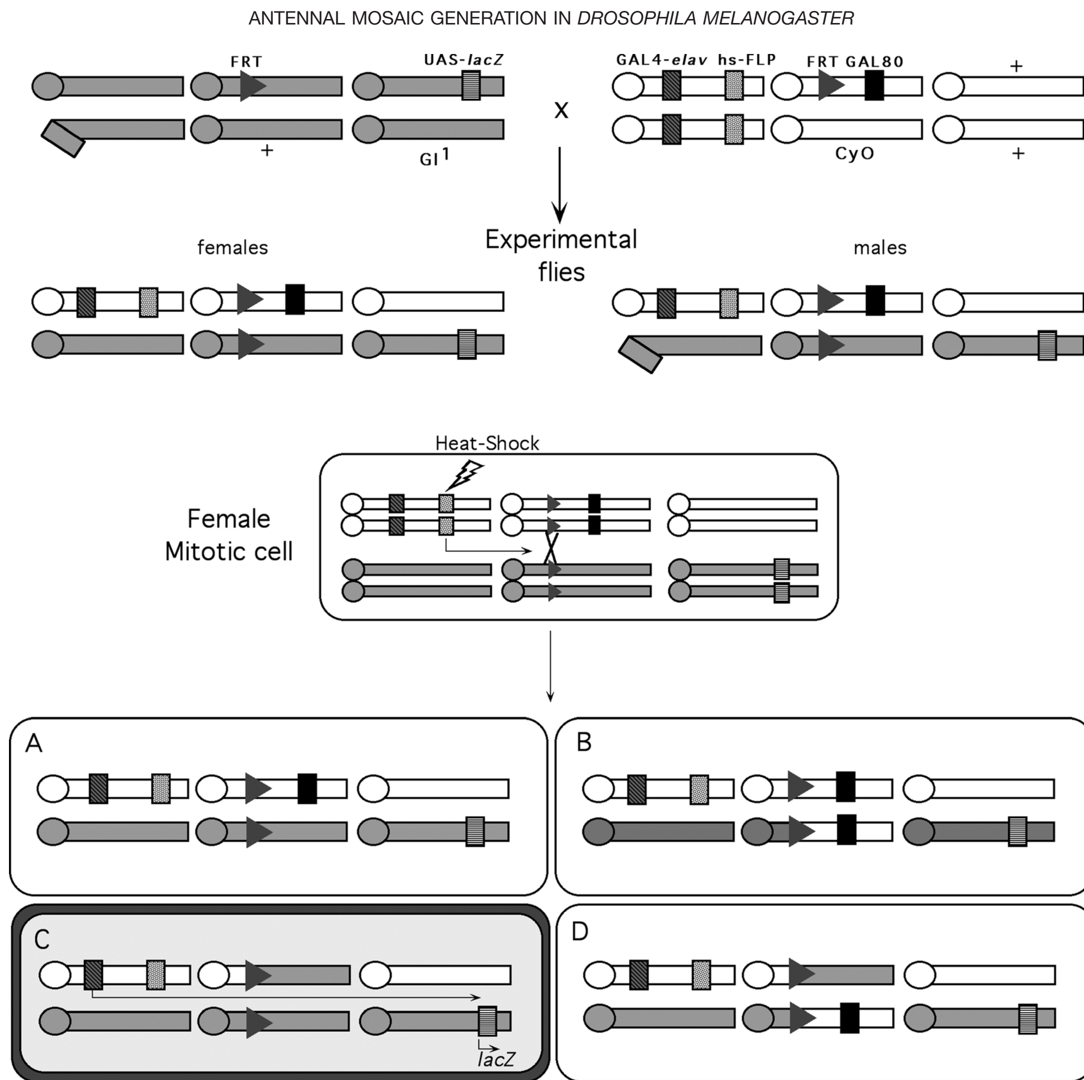

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It has to be noted that males of the experimental group should show higher expression of Gal-4 and flipase than females because of the genetic dose compensation for the X chromosome. In fact, it has been observed an increased percentage of mosaics in males than females. However, the same pattern of relative mosaic frequencies depending on the age at treatment can be observed inside each sex group. Because of this and the approximate 1:1 distribution of males and females for each age class, data of both sexes have been pooled together.

Heat-Shock Treatment

Somatic recombination was induced by heat shock at different ages, always expressed as hours AEL. Flies that were grown at 24°C ± 1°C, were submitted during 1 h to a water bath at 37°C and returned to 24°C. Adult flies were prepared to analyze β-gal expression.

To extend the results presented in this report to flies raised at different temperatures, see the graphs on duration of developmental stages depending on temperature in Ashburner (1989).

Mosaic Analysis

Complete heads were examined under the microscope for blue staining in the antennae and maxillary palps. For the third antennal segment flies were classified according to the staining level of each side antennae. Type 0 represents no staining, type 1 corresponds to only one or two small points, type 2 ranges from more than two small spots to the staining of 50% of the surface (distributed either in one o more staining areas), and type 3 accounts for staining in more than 50% of the area. Since statistical data analysis showed no frequency differences depending on the right or left side location of a certain clone type, information was grouped. Thus, group C-0,1 reunited both 0,1 (left antenna of type 0 and right antenna of type 1) and 1,0 situations (type 1 for the left antenna and 0 for the right sided antenna), group C-1,3 indicates 1,3 and 3,1 data and so on.

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