Research Notes

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Transcripts involved in spermatogenesis are not qualitatively underexpressed in hybrids of *Drosophila simulans* and *D. mauritiana.*

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Introduction

Several recent lines of evidence suggest that disruptions in gene expression may contribute to hybrid sterility and other hybrid dysfunctions. First, the overexpression of *Xmrk-2* (*Xiphophorus melanoma receptor kinase*) causes hybrid lethality in crosses between two *Xiphophorus* species (Orr and Presgraves, 2000; Schartl *et al*., 1999). Second, hybrid anomalies and inviability in crosses of *Drosophila melanogaster* and *D. simulans* are at least sometimes associated with gene regulation. For example, bristle loss in these hybrids is due to misregulation of *Achaete-Scute* (Skaer and Simpson, 2000), and hybrid inviability is sometimes associated with a transposition into *cyclinE* (Sawamura *et al*., 2000), which is involved in cell-cycle regulation. Third, regulatory changes have been implicated in a wide variety of striking differences between species, including *Drosophila* species (*e.g*., Doebley *et al*., 1997; Ludwig *et al.*, 1998; Stern, 1998; Sucena and Stern, 2000). Finally, a recent theoretical model suggests that binding strength of regulator proteins with promoter regions can provide biologically plausible and powerful postmating isolation, such as hybrid sterility (Johnson and Porter, 2000).

Some failures in gene expression have been documented in species hybrids (*e.g*., Reiland and Noor, 2002), but no one has performed a directed effort to evaluate if genes known to be involved in spermatogenesis (*e.g*., Fuller, 1998) are under- or overexpressed in sterile male hybrids relative to pure-species males. As such, the role of such gene expression failures in causing hybrid sterility is still only hypothetical. Here, we investigate whether qualitative (*e.g*., 10-fold or greater) misexpression of spermatogenesis-related transcripts occurs in adult sterile male hybrids of *Drosophila simulans* and *D. mauritiana* relative to pure species males.

Methods

The *D. simulans* Florida City and *D. mauritiana* Synthetic strains were used in this study. RNA was isolated from 1-day post-eclosion males (20-50 individuals for each preparation) from each test group (*D. simulans*, *D. mauritiana*, and F_1 hybrid males from the cross *D. simulans* females × *D. mauritiana* males) using the Qiagen RNeasy kit. The RNA was treated with DNase and quantified by UV spectrophotometry. From each sample, we reverse transcribed 1µg total RNA and 0.1µg total RNA (see conditions in Bertucci and Noor, 2001), and the resultant cDNA was amplified via PCR using primers specifically designed for each gene tested. PCRs were terminated such that the exponential phase was captured, and differences in intensity between the 1µg RNA and 0.1µg RNA could be visualized. This follows the semi-quantitative PCR assay of Dallman and Porter (1993) and can identify qualitative differences in gene expression. Negative controls were used for the RT and PCR reactions, and positive controls (genomic DNA) were included for the PCR reactions. Whenever possible, these primers were designed from *D. melanogaster* to replicate a segment which spanned an intron, so contamination by genomic DNA could have been detected.

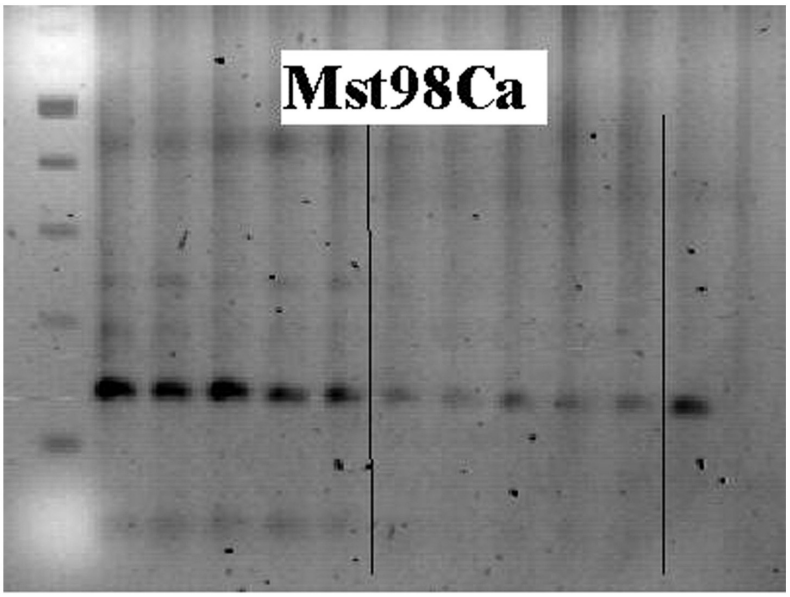

Figure 1.

PCR products were visualized on 2% TBE agarose gels stained with ethidium bromide under UV light. Differences in intensity between the samples would indicate differences in starting concentrations of the transcript. Intensity of fluorescence of sample bands was quantified both visually and using Intelligent Quantifier software.

Results

We assayed 10 genes putatively involved in spermatogenesis using the semi-quantitative PCR assay: *always early, cannonball, OdysseusH, schnurri, twine, gonadal, don juan, janus B, Mst87F,* and *Mst98Ca*. In the figure, all PCRs from 1µg RNA produced a more intense band than PCRs from 0.1µg RNA. This suggests that any differences in expression between these 1-day posteclosion pure species and F_1 hybrids are likely to be less than 10-fold.

Discussion

Similar product amplifications from RT-PCRs of pure-species and F_1 hybrid males suggest that, if differences in gene expression contribute to the sterility of these hybrids, 1) few spermatogenesis-related genes are involved, 2) the misexpression is limited to a particular life cycle stage, 3) differences in expression are highly tissue-specific, and/or 4) differences in expression are quantitative. Data from our laboratory suggest that the fourth option is likely to be correct: we have identified some transcripts underexpressed in hybrids, but the differences are typically quantitative (Reiland and Noor, 2002; Michalak and Noor, 2003).

Research on the genetics of hybrid sterility over the past 70 years has only identified one gene putatively involved (Ting *et al*., 1998), and even this case is not certain (Orr and Presgraves, 2000). As research has focused primarily on the use of mapping techniques to date, we suggest that a "reverse genetics" approach may be a useful complement. Further analyses of genes that may be under- or overexpressed in sterile species hybrids relative to pure species may yield novel insights into this important contributor to the speciation process.

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The spindle poisons demecolcine and vinblastine induce mitotic recombination in the somatic *w/w+* assay of *Drosophila melanogaster*.

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Abstract. The genotoxicity of the spindle poisons demecolcine (DEM) and vinblatine (VBL) were evaluated by means of the *in vivo* eye *w/w+* somatic assay of *Drosophila melanogaster*. We used the standard insecticide-susceptible Leiden strain (LS) with normal bioactivation and the insecticide-resistant Hague-79 (HG-R) strain with increased cytochrome P450-dependent biotransformation capacity. The compounds were administrated chronically. Our experiments showed that the both compounds are active in this test system although in different strains. The agent demecolcine (DEM) was positive in the ST strain, while vinblastine (VBL) was active only in the HG-R strain. Both alkaloids showed to be cytotoxic: DEM in the standard strain produced larval death at the beginning of the pupal stage; VBL induced several malformations in the eye-antennal imaginal disc thus the compound was also tested by acute treatment. No malformations were observed in the acute series, the alkaloid was active in ST at the highest (LD_{50}) concentration assayed and in addition showed to be positive in the HG-R cross. The combined application of strains ST and HG-R detect somatic cell recombinagens in the *w/w+* eye assay of *Drosophila*. Further studies are needed to clarify whether these anticancer drugs are capable of inducing besides homologous mitotic recombination structural chromosome aberrations.

Introduction

Antineoplastic agents have play a vital role in the curative and palliate treatment of cancers, among them, the antimicrotubule agents have been widely used in cancer chemotherapy for more than 30 years (Rowinsky and Donehaver, 1991; Zou and Ramani, 1992). The alkaloids demecolcine (DEM) and vinblastine (VBL) have been used for the treatment of gout and several types of cancer (breast, lung, and leukemia) (Maral *et al*., 1984), respectively, are spindle poisons. DEM and VBL bind specifically to cytosolic tubulins and microtubules, prevent assembly of tubulin subunits into filaments and depolymerized microtubules; thus, they exert its action by inhibiting mitosis in metaphase (Belisario, 1965; Wallin *et al*., 1988; Wallin and Hartley-Asp, 1993; Jordan *et al*., 1991). DEM exerts a selectively destructive action on rodent (basal cell) carcinomas, solar keratoses, Bowen's disease, and keratoacanthomas, while sparing surrounding normal tissues (Belisario, 1965). Vinblastine (VBL) induces mutations in the mouse lymphoma assay (Honma *et al*., 1999), was classified as a weakly active genotoxin in the standard *w/w+* somatic assay of *Drosophila* (Vogel and Nivard, 1993) and positive in the wing somatic mutation and recombination test (Tiburi *et al*., 2002).

Table 1. Summary of results obtained in the *Drosophila w/w+* eye assay [a].

Genotype	Compound and Concen.	N° of eyes	Number of spots scored								Spots per 100 eyes [b]			Average clone size	Clones per 10^4 cells [c]	Activity [d]
			1-2	3-4	5-8	9-16	17-32	33-64	>64	Total	Small 1-8	Large >8	Total			
DEMECOLCINE - DEM																
ST	Control	500	20	4	6	2				32	6	0.4	6.4	3.31	5.34	
	0.025[e]	228	27	6	5	1				39	16.67	0.44	17.11	2.92	12.49	+
	0.05[e]	70	7	1	1					9	12.86	0	12.86	3.33	10.7	-
	0.075[e]	120	14	6	2	3	1			26	18.33	3.33	21.66	4.85	26.27	+++
HG-R	Control	486	41	4	5	4		1		55	10.73	1.07	11.8	3.84	11.33	
	0.025	398	40	7	1		1			49	12.06	0.25	12.31	2.61	8.03	-
	0.05	612	53	13	5	1	3			75	11.6	0.65	12.25	3.24	9.93	-
	0.075[e]	258	28	5	2	2		1		38	13.57	1.16	14.73	3.97	14.62	-
VINBLASTINE - VBL																
Chronic																
ST	Control	478	12	5	3	1	1	1	23	33	4.18	0.63	4.81	4.75	6.2	
	0.025	508	17	4	1			2		24	4.33	0.39	4.72	6.46	7.63	-
	0.05[e]	260	11	2	1	1	1	1		17	5.38	1.15	6.53	2.06	3.37	-
HG-R	Control	580	48	16	8	4	3	3		82	12.41	1.72	14.13	5.27	18.63	
	0.025[e]	25	9	2						11	44	0	44	2.27	24.97	+++
	0.05[e]	14	8	3	3	2		1		18	100	28.6	128.57	6.16	198.02	+++
Acute																
ST	Control	500	19	3	2	2				26	4.6	0.6	5.2	3.19	4.02	
	0.025	500	19	4	1	2	1			27	4.8	0.6	5.4	3.04	4.1	-
	0.05	500	139	51	36	5	2			111	20.8	1.4	22.2	2.9	30.45	+++
HG-R	Control	500	33	11	3	2	1			50	9.4	0.6	10	3.32	8.3	
	0.025	500	88	8	5	5	4	1		100	18	2	20	4.11	20.55	+
	0.05	500	154	24	16	1	9	1		178	33.4	2.2	35.6	4.29	38.18	+++

[a] Chronic (or acute) treatment with anticancer drugs. Genotypes tested standard (ST) and Hagg-R (HG-R)
[b] Size classes: Small 1-8 ommatidia affected; Large >8 ommatidia; T, total spots.
[c] Calculated according to the formula f = 2nm/NC
[d] Activity: + positive, +++ positive (no statistics required), - inactive
[e] Cytotoxic

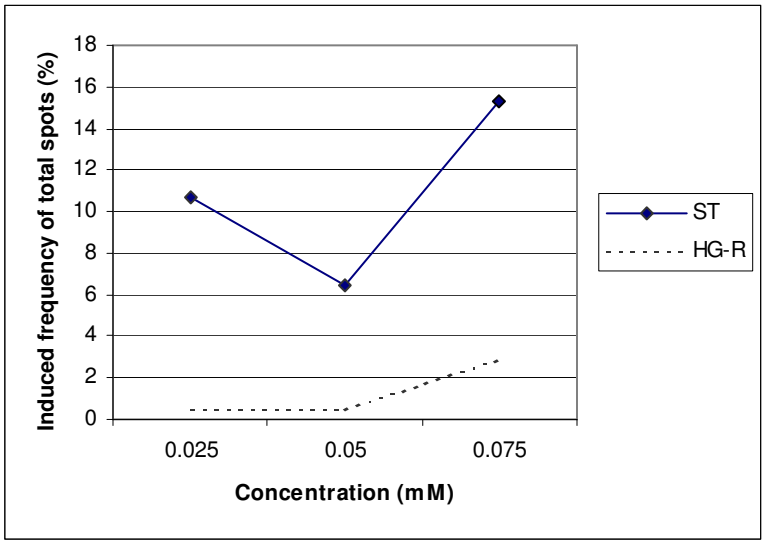


Figure 1. Induced frequency of total spots by DEM in the *w*/*w*$^+$ assay of *Drosophila.*

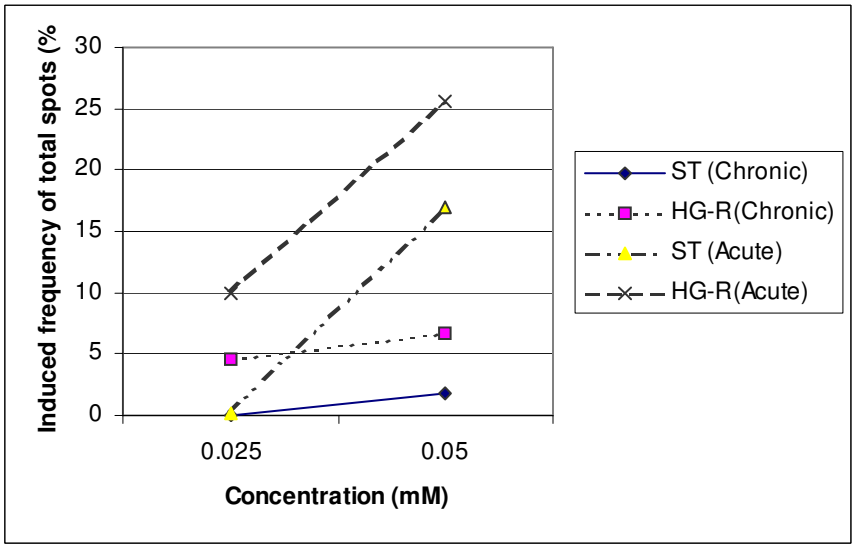
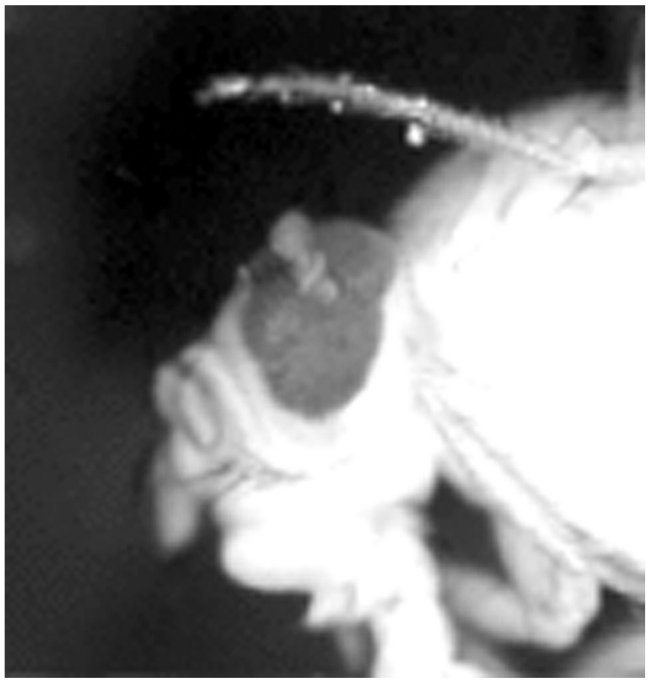
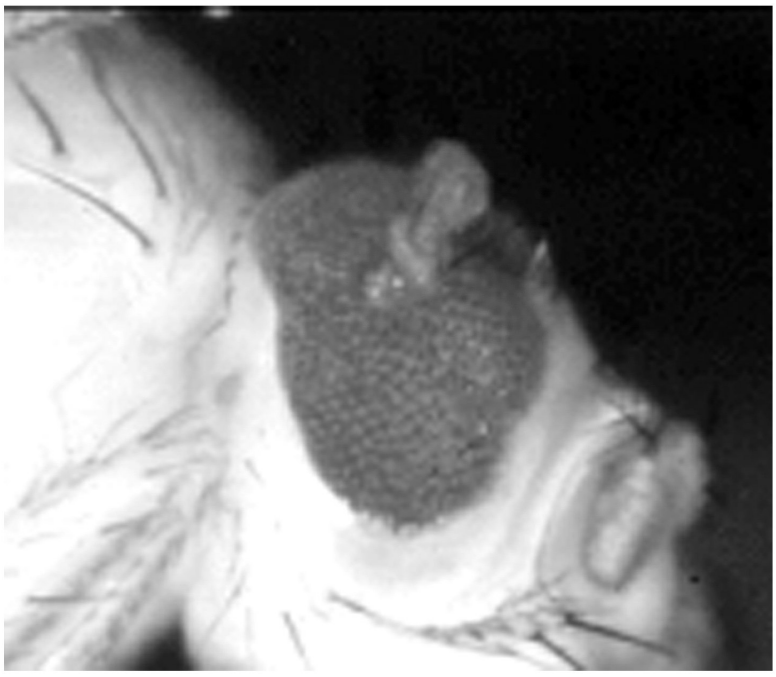


Figure 2. Induced frequency of total spots by VBL in the *w*/*w*$^+$ assay of *Drosophila*. Chronic and acute treatments.

Somatic cells are an indispensable target for the study of loss of heterozygosity (LOH) produced by several mechanisms, including large deletions, mitotic recombination, and chromosome loss (Lasko *et al*., 1991). LOH by mitotic recombination, an important cancer-prone mechanism, can be detected in *Drosophila* by several systems, such as the *multiple wing hair/flare wing* spot system (Graf *et al*., 1984) and the *white/white+* (*w*/*w*+) eye mosaic test (Vogel and Zijilstra, 1987). The basic principle of the *w*/*w*+ assay is the detection of phenotypically visible light spots in the red eyes of adult females, resulting from LOH and the expression of the reporter gene *white* in female genotypes heterozygous for this marker (Vogel and Nivard, 1993). The use of multiple inverted X-chromosome, which in XX cells suppresses recombination between the two Xs, identified homologous (inter-chromosomal) mitotic recombination as the pre-dominant cause generating loss of the *white*+ reporter gene (Vogel and Szakmary, 1991). It has been shown a strong intraspecies variability for the metabolic conversion of several progenotoxins, activation that involve oxidative metabolism by P450 enzymes (Rodriguez-Arnaiz *et al*., 1993).

This study focused on the ability of the spindle poisons DEM and VBL to induce loss of heterozygosity in the *in vivo white/white+* eye somatic assay of *Drosophila melanogaster* using a standard (ST) strain with normal bioactivation as well as the resistant Hague-R (HG-R) strain characterized by an increase constitutive overexpression of cytochrome P450-dependent biotransformation.





Figures 3 (above) and 4 (below). Mutant phenotypes induced by VBL in the eye-antennal imaginal disc.

Materials and Methods

Chemical compounds and concentrations tested

Demecolcine (DEM, CAS # 477-30-5) and vinblastine sulfate (VBL, CAS # 143-67-9) were purchased from Sigma (St. Louis, MO, USA). The chemicals were dissolved in a mixture of 1% Tween-80 and 3% ethanol immediately prior to use. The final concentration of this solvent mix was 4%. The higher exposure dose was determined as the LD_{50}; two additional lower doses were tested.

Somatic assay

Two *Drosophila* strains were used to perform the assay, the Leiden Standard insecticide susceptible strain (ST) and the insecticide resistant Hague-79 (HG-R) strain. Crosses were done by mass-mating virgin homozygous *w* (white) females with *w+* (red) males. Chemicals were administrated by chronic exposure. And in addition vinblastine sulfate was tested by acute treatment. For chronic treatment fifteen pairs of flies were permitted to lay eggs in bottles for three days on food supplemented with the test substance dissolved in the 4% mixture solvent. Growing cultures were exposed to each compound during all three instar stages of larval development. For acute treatment larvae of 72 ± 3 hours were collected by washing them out with an aqueous solution of 20% sucrose and exposed during 6 hours to the different concentrations of the alkaloid. Newly-hatched females were transferred to fresh medium and scored 1-5 days later. Adult females are heterozygous for *white* and were inspected for the occurrence of white in their compound eyes. More details about the test procedure are given in Vogel and Nivard (1993). We conduct at least two separate experiments with each single chemical at the same exposure dose. We score, when possible, at least 250 eyes per dose group. For each experiment a concurrent control was run, where larvae were treated with the solvent mixture alone. For evaluation of the genotoxic effects recorded, the total frequency of spots of the treated series was compared to its concurrent negative control series. These statistical comparisons were done using the Kastenbaum-Bowman test for proportions and followed by the double decision test of Selby-Olson according to Frei and Würgler (1995).

Results and Discussion

Detection of the recombinogenic effect induced by the spindle poisons was assayed in at least two chronic (or acute) and independent experiments. The data of each experiment were compared and analyzed before being pooled for statistical testing by the H-test of Kruskall and Wallis ($P < 0.05$) (Statistica 5.0). Table 1 shows the obtained results with the compounds in the somatic *w/w+* assay of *Drosophila melanogaster*. It can be noted that the antineoplastic drugs produced overall a statistical increase in the frequency of total spots, which means that they are genotoxic in this assay system. The recombinogenic effect induced by the alkaloids is related to increases in the frequencies of small spots (1-8 ommatidia affected) in almost all concentrations assayed. Large spots (more than 8 ommatidia affected) were by far less frequent and showed a statistical increase above control series almost only at the highest concentration tested with the compounds.

Regarding the strains the figure is quite different while the demecolcine showed to be positive in the insecticide-susceptible standard cross, vinblastine sulfate was active only in the insecticide-resistant Hague 79 strain. In addition when VBL was tested in acute treatment the alkaloid showed to be genotoxic in the ST cross only at the highest concentration and again active in the HG-R cross in all concentrations assayed.

Demecolcine showed to be toxic in the assay, especially in the insecticide susceptible strain where larvae died when they reach the pupal stage. DEM a typical aneugen increased in our experiments the frequency of total spots in the ST strain, and was inactive in the HG-R cross (Figure 1). Results that are in agreement with a recent study that has shown that demecolcine had no effect in any studied P450 isoform while colchicine caused an increase of CYP2E1 protein content in primary cultures of human hepatocytes (Dvorak *et al*., 2000).

Vinblastine was the only compound tested by two exposure routes. In the chronic series the compound induced frequent irregularities of the compound eye, reduced survival of HG-R larvae and several malformations (see below). The compound was classified as negative in the ST strain, because it gave values very near to the control series in all concentrations assayed. On the contrary, the chemical was classified as positive in the HG-R strain where values of spots increased significantly above control series. In the acute experiments the cytotoxic effects were diminished although still some irregularities of the compound eye were observed. The compound showed to be clearly positive in both strains at the highest LD_{50} concentration while at the lower concentration probed to be negative in the standard strain (Figure 2), results that are in agreement with a previous study (Vogel and Nivard, 1993). Vinblastine induced somatic recombination in the wing somatic assay while the vinca analogs vincristine and vinorelbine showed to induce mutagenic and recombinagenic events almost equally (Tiburi *et al*., 2002).

Clone induction as a measure of a genotoxic and recombinogenic effect was around 1 for DEM in both strains. In the experiments with VBL no clone induction was observed for the ST cross. For HG-R it varies between one and one order of magnitude in the chronic experiments. In the acute experiments VBL induced clones 7 times in ST and between 2 and 4 times in HG-R.

In the chronic experiments VBL induced mutant phenotypes in the eye-antennal imaginal disc primordium (Figures 3 and 4). During development the head capsule derives from portions of the disc surrounding both eye and antenna (Haynie, 1986). Head involution folds the ectodermal reserved for eye and antenna development internally to form the eye-antennal disc. The disc remains a simple epithelial sac throughout the first and second instars, the eye-antennal disc grows by cell division increasing in number from about 130 at the end of the first instar to 1,300-1,600 at the beginning of the third (Becker, 1976). Proliferative cell divisions continue in the third instar and increase the 1,300 cells to the 9,700 cells used to seed the full complement of ommatidial precursors (≈13 cells/ommatidium × 750) (Cohen, 1993). VBL in this study interfere with the developmental pathways of embryonic segment primordial and their larval and adult derivatives.

Frequent irregularities of the structure of the compound eye are associated with cytotoxicity effect that was observed with both spindle poisons supporting the idea that these agents may produce numerical chromosome aberrations.

In summary from the results obtained in this study, it has been shown that by the combined application of strains ST (IS) and HG-R (IR) the two spindle poisons are active in the *w/w+* eye somatic assay. Also, further studies are needed to clarify whether these anticancer drugs are capable of inducing besides homologous mitotic recombination structural chromosome aberrations.

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The effects of Aflatoxin-B_1 on some development stages and phenotypic abnormalities in *Drosophila melanogaster.*

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Abstract

In this research, the effects of AFB_1 on some developmental stages of *Drosophila melanogaster* were investigated. Different concentrations of AFB_1 were fed during the developmental period (egg, larva and pupa). When F_1 and F_2 generations of control and applications groups are compared with each other, AFB_1 was found to have extended the process of metamorphosis and decreased the total number of offspring, especially in F_1. Furthermore, in the application groups, malformed individuals were also observed.

The possible mechanisms of the negative effects of AFB_1 on the development of *Drosophila melanogaster* is discussed.

Introduction

Mycotoxins are toxic metabolites which they are produced by a lot of toxic fungi species and when they have been received by animals and humans they caused chronic and acute intoxications (Uysal and Sisman, 2002). Approximately 300 mold species produce mycotoxins and those of 20 mold species have got high toxicity for humans and animals (Steyn, 1995). According to previous experimental studies, aflatoxins are the most dangerous of these the fungal secondary metabolites.

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus paraziticus* and they have carcinogenic, mutagenic, hepatotoxic and teratogenic effects (Davis and Diener, 1978; Wogan, 1975). One of the secondary metabolites is Aflatoxin B_1 and it is present at high concentrations in contaminated feed and agricultural crops. It is found that aflatoxins have carcinogenic influences on rat and rainbow trout (Lancaster *et al.*, 1961), various domestic animals (Alleroft, 1965), and monkeys (Adamson *et al.*, 1973) when they are nourished by the feed and crops. In the studies by Adamson *et al.* (1973), when the feed including 800 mg/kg aflatoxin was eaten by a female monkey during two weeks and three times in a day, then the female became jaundiced, inactivated, and liver carcinoma was observed. But the LD_{50} of AFB_1 causing toxic effect changes with respect to age, sex, species, and body weight in different animal groups (Davis and Diener, 1978).

According to some investigators, aflatoxins cause osteoarthrosis, defeat of bone development, lesions of skin and liver degenerations in humans (Stoloff, 1977; Wilson, 1978). Also aflatoxins adduct DNA, RNA, histone and nuclear proteins, and they exhibit synthesis of DNA, RNA and protein (Bressac *et al.*, 1991; Chih *et al.*, 1991; Harris, 1991; Hsu *et al.*, 1991; Nishiyama and Krebe, 1981). In addition mutagenic effects and chromosome abnormalites appear (Schlember *et al.*, 1991; Turchi *et al.*, 1987; Dunn *et al.*, 1982; Leonard *et al.*, 1975).

Because the mutagenic properties can easily be observed in *Drosophila melanogaster,* this organism has been often used in genetic experiments. What are the teratogenic effects caused by aflatoxins? How do aflatoxins influence some developmental stages of *Drosophila melanogaster*? What are the mechanisms of these effects? The main aim of this study is to find the proper answers to above questions.

Material and Methods

For all of the applications, the Oregon (OR) wild type (w.t.) culture of *Drosophila melanogaster* was used as parents. The flies were kept constantly at 25 ± 1°C on Standard *Drosophila* Medium (SDM) composed of maize, flour, agar, sucrose, dried yeast and a mold inhibitor (propionic acid). The humidity of the experimental cabinets was 40-60% r.h. The flies were always in darkness except during transfers onto fresh medium when they were in light.

All the females used in the experiments were virgins. The flies with the same age were used for experiments and seven males and seven females were mated. The culture vials containing only the SDM and SDM + DMSO were used as control (untreated). The developmental stages were followed daily. After pupa formation, the parental individuals were removed. Offspring were counted every day from the first day of eclosion; sexes and phenotypic abnormality were noted. Examination and counting of flies on the control and treated groupings was also done for two successive generations. The F_1 generations obtained from the parents fed on different concentrations of the AFB_1 were raised on normal feeding medium separately and the F_2 generations obtained. The experiments were replicated three times for each concentration of AFB_1. Differences between control and experimental groups were verified using Duncan's Multiple Range Test.

Preparation and Application of AFB_1: Crystalline AFB_1 (Acros Organics, No: 227340100, New Jersey, USA) was dissolved in 10 ml of 10% DMSO and mixed into standard medium to produce a 10 ppm stock solution of AFB_1. Control + DMSO group was made as a different control group. Experimental concentrations of 0.2, 0.5, 0.8, 1.1, 1.4 and 1.7 ppm AFB_1 were made by appropriate dilutions of the stock solution. An equivalent quantity of DMSO was added to the control medium (0.0 ppm AFB_1).

Results and Discussion

The effect of the AFB_1 on the development stages of *D. melanogaster* is presented in Table 1. Developmental time was followed from day of egg deposition to day the adult eclosed.

In the laboratory at 25 ± 1°C the life cycle (egg-adult) is 9 days. In control and experimental groups (0.2 - 1.7 ppm) laid eggs were observed on the second day from mating. Then, in the control group, first, second, third instar larvae, prepupa, pupa and adult individuals were formed, respectively. The first adult was observed at the 9^{th} days after mating. But, at the applications of 0.2, 0.5 and 0.8 ppm, egg-adult development time increased with increasing concentrations of AFB_1 (Table 1). Furthermore, at the applications of 1.1, 1.4 and 1.7 ppm of AFB_1, developmental stages after egg, second and third instar larvae was found to be stopped and no offspring formed.

Table 1. The effect of AFB_1 on egg-adult developmental stages of *Drosophila melanogaster.*

Developmental Stages	Days of stages at control and applications						
		($F_1 - F_2$)			(Only F_1*)		
	Control	0.2	0.5	0.8	1.1	1.4	1.7
Mating	1-1	1-1	1-1	1-1	1	1	1
Egg	2-2	2-2	2-2	2-2	2	2	2
1st instar larvae	3-3	4-3	4-4	5	5	6	-
2nd instar larvae	4-4	5-4	6-5	7	6	9	-
3rd instar larvae	5-5	6-5	7-6	9	8	-	-
Prepupa	6-6	7-6	8-8	10	-	-	-
Pupa	7-7	8-7	10-9	11	-	-	-
Adult	9-9	10-9	11-10	13	-	-	-

*At the concentrations of 1.1, 1.4 and 1.7 ppm F_1 generation did not form adult individuals and so F_2 generations were not observed.

Table 2. Comparison of the numbers of total and abnormal offsprings of *D. melanogaster* exposed to the AFB_1 at different concentrations.

Concentration(ppm)	F_1	Abnormal	%	F_2	Abnormal	%
Control	2305 a*	9	0.39	2358 a	11	0.46
C+ DMSO	2218 a	7	0.32	2275 a	8	0.35
0.2	1120 b	73	65.17	1522 b	52	34.16
0.5	924 c	75	81.16	1137 c	47	41.33
0.8	39 d	3	76.92	–	–	–

* The means were compared within blocks. Values with the same letter are not significantly different according to Duncan's Multiple Range Test ($P< 0.01$ for F_1 generations; $P< 0.05$ for F_2 generations).

We could follow only the development stages of 0.2 and 0.5 ppm application groups in the F_2 generation. The development stages of these groups were completed in a short time with respect to control groups. But, development stages after egg phase were not completed in 0.8 ppm. Similar larval and pupal toxic effects caused by AFB_1 have also been demonstrated by various authors on *Musca domestica* (Beard and Walton, 1971) and *Drosophila melanogaster* (Kirk *et al.*, 1971). Again, Lalor *et al.* (1976) found that growth on AFB_1 media of *Drosophila melanogaster* caused significant increases in egg- to- adult developmental time. According to these researchers there was a direct relationship between concentration of aflatoxin and degree of reduction of pupal and larval case.

On the other hand it was observed that the total number of offspring obtained from F_1 and F_2 generations were also decreased (Table 2). As seen in Table 2, the numbers of offspring of 0.2, 0.5 and 0.8 ppm application groups decreased from 1120 to 39. When these results were compared with control (2305) and DMSO + control group (2218), the difference between the groups is statistically important ($P < 0.01$). As a result of $F_1 \times F_1$ crosses it was observed that the number of offspring was increased at the applications of 0.2 and 0.5 ppm (1522 and 1137, respectively). The mean values are low in respect of control groups (2358), ($P < 0.05$). The offspring which belong to F_2 generation did not exist in the 0.8 ppm application group.

According to us, one of the following mechanisms can play a role on the decreasing of the number of offspring. *i*) Both the reduction in fertility of Aflatoxin B_1-treated females, *ii)* and reduced motility sperm count and the increased number of non-viable spermatozoa of males. Matsumura and Knight (1967), using a 0.005% mixture of aflatoxins fed to *D. melanogaster* adults, noted a decrease

in egg production. The same effects were also seen on mice exposed *to* aflatoxin (Verma and Nair, 2001). According to Chinnici *et al.* (1976), too, the females of *D. melanogaster* fed a 674 ppb aflatoxin B_1-contaminated medium showed a significant reduction in fertility, *iii*) Besides, this effect may be associated with a very sensitive larvae- pupae and their death at the higher concentrations (Llewellyn and Chinnici, 1978; Kirk *et al.*, 1971).

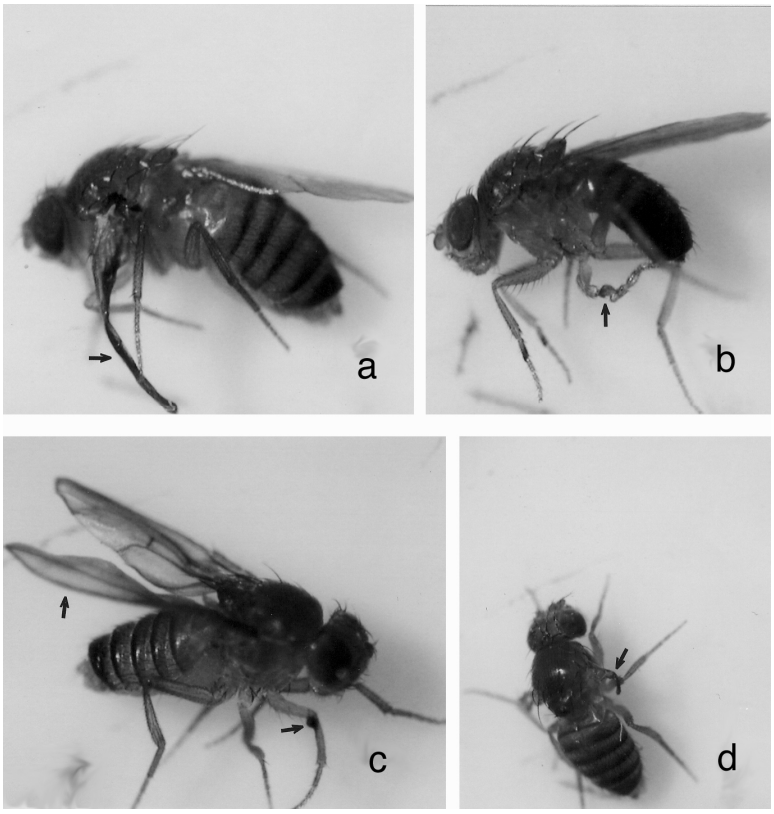

Figure 1 – a) Arrow shaped left wing, b) Unformed leg segments c) Malformed wing and tumor legs, d) Unformed and reduced wings.

In addition to the findings mentioned above, phenotypic abnormalities in the F_1 and F_2 generations were also observed. It was found that in all concentrations, AFB_1 induced various abnormalities in wings, legs, and thorax (Figure 1a-d).

The results obtained are shown in Table 2. The ratio of abnormal individuals for control and applications groups of F_1 were 0.39 - 76.92%, respectively. While the ratio of abnormal offspring at the control group of F_2 is 0.46%, this ratio changes between 34.16% (for 0.2 ppm) and 41.33% (for 0.5 ppm) at application groups. According to these results, the increasing concentrations of AFB_1 increased the ratio of abnormal individuals (Table 2). But, as a result of intermating between the malformed individuals, normal individuals were obtained. According to this result, we considered that concentrations used in our experiments (0.2, 0.5 and 0.8 ppm) do not cause the mutations. The occurrence of the malformation both in male and female individuals showed that these abnormalities are not related with sex.

In the previous studies, similar results have also been reported. For example, teratogenic effects of AFB_1 were observed on fetuses of hamster, mice, rat and cow by Aleksandrowicz and

Smyk (1973). These teratogenic effects were as follows; decreases in body size (Chinnici *et al.*, 1976) and wing length (Lalor *et al.*, 1976), the formation of tumors on different body parts (Sidorov *et al.*, 2001), hepatocarcinogen effects in vertebrates (Pier, 1981). These findings are in accord with our results (Figure 1a-d).

The teratogenic effects caused by AFB_1 may be the results of following reasons: Some faults during transcription of developmental genes and defects in homeotic genes which affect the final conditions of imaginal discs (Wallace *et al.*, 1991), the inhibition of replication and induction of apoptosis by AFB_1 in normal cells due to DNA damage (Shi *et al.*, 1994; Hsieh *et al.*, 1992). In addition the gene p53 was inhibited and so in the cells which do not have the p53 protein the cell cycle does not stop and the damage was not repaired (Klug and Cummings, 2000; Riede, 2000).

The chromosomal aberrations caused by AFB_1 can form the phenotypic abnormalities. The studies on the possible source of these aberrations are continuing.

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The influence of metopren on puffing in *Drosophila melanogaster* chromosomes in experiments *in vitro*.

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The problem of regulation of gene expression in eukaryotes is of great importance in modern genetics and covers a wide spectrum of factors affecting the nuclear apparatus of cells. One of them is the factor of hormonal induction. This work deals with the mechanisms of hormonal regulation of the genetic program of ontogenesis in insects. It is known that an important part in cyclic postembryonic development of insects is played by two hormones: ecdysone (20-OH-ecdysterone) and juvenile hormone (JH). Ecdysone stimulates molt and metamorphosis, induces the development of imaginal disks and apoptosis of larval tissues. JH regulates the development of larvae in intermolt periods and prevents metamorphosis. The presence or absence of JH determines the molt pattern (larval-larval or larval-pupal and pupal). In addition, JH controls the maturation of eggs and the reproductive function in females and is also an important component in the mechanism of insect protection against stress factors [1-4]. The cyclicity of development of insects is correlated in time with periodical changes of the puffing pattern and the role of hormones in these processes has also been experimentally confirmed [1-3].

The practical aspect of the study is related to the possibility of using hormonal preparations in the control of insect development and in pest control. Various synthetic analogs of ecdysone and JH are currently used for these purposes. Metopren (juvemone) studied in this work is an analog of JH. The aim of this work is the study of the action of metopren on the puffing activity in *Drosophila melanogaster* polytene chromosomes in experiments *in vitro*.

Materials and methods

The work has been performed using a classical object in genetic experiments - fruit fly *Drosophila melanogaster* Meig, non-selective line Oregon-R. The flies were grown in a standard sugar-yeast medium at 24^{o}C. The polytene chromosomes were examined on squash aceto-orcein salivary gland preparations [5].

The influence of a juvenile hormone analog, metopren, at a concentration of 0.1 mkg/ml and 1 mkg/ml on the puffing activity during *Drosophila* ontogenesis was studied. Metopren (juvemone) is a hormonal insecticide suppressing the transportation of larvae or nymphs of insects in viable adult flies and having an ovicidal action. By its chemical nature metopren is isopropyl ether of 2E,4E-11-metoxy-3,7,11-trimethyl-2,4-dodecadiene acid.

Salivary glands were incubated *in vitro* at 24^{o}C in metopren solution for 30 min. The experiment consisted of several stages. In control 1 the puffing in polytene chromosomes of salivary glands was studied at the stage of 0-hour prepupa with their staining immediately after removal from the medium. In control 2 the salivary glands were incubated in 1% DMSO (dimethylsulfoxide) solution for 30 min. In experiment 1 and in experiment 2 the salivary glands were incubated in 1% DMSO supplemented with metopren at concentrations of 0.1 mkg/ml and 1 mkg/ml, respectively. DMSO was used as a solvent for metopren which is insoluble in water.

The study was based on the analysis of puffs in loci activated in the intermolt period at the prepupal stage in the presence of JH: 43E, 58DE, 60B (chromosome 2R), 85D, 100DE (chromosome 3R) [6]. The regression of late ecdysone puffs 63E (chromosome 3L) and 82EF (chromosome 3R) was also studied. The puffing activity in polytene chromosomes was evaluated by the ratio of cross dimensions of puffs to the chromosome width in the region of a neighboring disk non-involved in the puffing process (puff-disk ratio): 43E/44A, 58DE/58A, 60B/60C, 85D/85B, 100DE/100D, 63E/64B, 82EF/84D. Measurements were made with an ocular micrometer.

The results of the experiments were processed by the methods of biometric statistics. The significance of differences in the expression of puffs was determined by the Student's t-criterion.

Results and discussion

The results of the study of the influence of metopren on puffing are presented in Figure 1. In control 1 we studied puffing in polytene chromosomes of *Drosophila* at the stage of 0-hour prepupa which is characterized by an increased titer of ecdysone and a decreased level of juvenile hormone. No puffing was observed in intermolt loci at this stage in the control. A considerable activity of the late ecdysone puffs, 63E and 82EF, was established. The sizes of these puffs were estimated by the marks 1.82 and 2.13, respectively.

In control 2 (30-min incubation of salivary glands in 1% DMSO solution) no formation of intermolt puffs was detected. A slight increase in the size of ecdysone puffs 63E and 82EF was observed in control 2 as compared to control 1. Differences for puff 63E constituted 16.48% ($P < 0.001$), puff 82EF grew in size by 10.13% ($P < 0.001$) in control 2 as compared to control 1. The visible differences in the size of the puffs can be explained by the fact that during incubation of salivary glands in the given solution the chromosomes are loosened due to a decrease of ionic strength in the solution and probably under the action of the organic component of the solution. According to Gruzdev [7], puffing of chromosomes occurs under such conditions due to the enhancement of electrostatic interactions between homologous charged groups inside chromosomes.

Experiment 1 (metopren at a concentration of 0.1 mkg/ml in 1% DMSO solution) was characterized by an activation of two intermolt puffs 85D and 43E. In experiment 2 (metopren at a concentration of 1 mkg/ml in 1% DMSO solution) an induction of all five intermolt puffs studied in this work was observed and the most active puffs were at the loci 58DE, 60B, 43E (chromosome 2R) and 85D (chromosome 3R). As for puff 100DE, its activity under the given conditions was insignificant and, as a rule, did not exceed the mark 1. The size of puff 100DE in experiment 2 increased by 60.67 % ($P < 0.001$) as compared to that in experiment 1.

The size differences for puff 60B in experiment 2 constituted 43.33% ($P < 0.001$) as compared to experiment 1. Puff 58DE grew in size by 21.8% ($P < 0.001$). Differences in the size of puffs 85D and 43E in experiments 1 and 2 were insignificant. Microphotographs of the puffs induced by metopren are presented in Figure 2.

Ecdysone puffs 63E and 82EF significantly regressed under the action of metopren. As compared to control 1, the size of puff 63E reduced by 12.6% ($P < 0.05$) in experiment 1 and by 8.2% ($P < 0.05$) in experiment 2. In respect to control 2, the size differences made up 25.0% ($P < 0.001$) and 21.2% ($P < 0.001$) in experiment 1 and in experiment 2, respectively. The size of puff 82EF reduced due to regression by 14.6% ($P < 0.05$) in experiment 1 and by 13.6% ($P < 0.05$) in experiment 2 as compared to control 1. With respect to control 2, the size of this puff decreased by 23.2% ($P < 0.05$) in experiment 1 and by 21.9% ($P < 0.05$) in experiment 2. Thus, it follows that regression of puffs 63E and 82EF in experiments 1 and 2 is not merely a result of washing of ecdysone but it occurs under the active action of metopren.

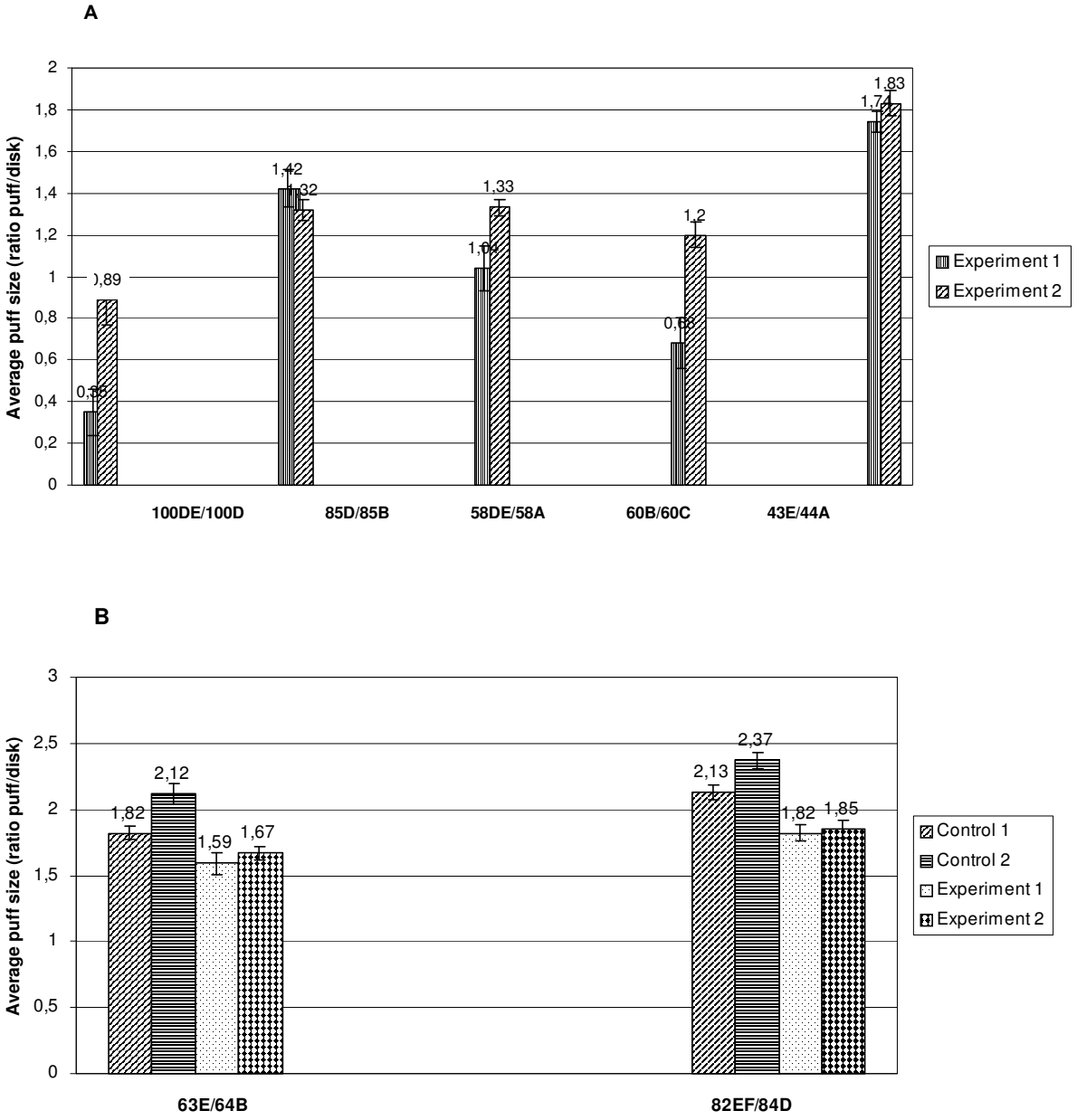

Figure 1. Influence of metopren on puffing activity in polytene chromosomes of 0 h prepupae of *Drosophila melanogaster*: A – intermolt puffs, B – late ecdysone puffs.

The action of JH on puffing of polytene chromosomes is yet poorly known and the available data are rather contradictory [2, 3]. Most of the studies concerned the modifying effect of JH on the induction of ecdysone puffs. The size of early ecdysone puffs was found to be decreased under the influence of JH in *Drosophila virilis* [8]. In experiments with *Drosophila melanogaster* JH did not affect early and late ecdysone puffs in larvae and had the inhibiting effect on ecdysone prepupal puffs [2]. Upon incubation of *Drosophila virilis* salivary glands in a medium containing JH and ES changes in the activity of 70 puffs were observed: 57 loci responded to ES and 41 loci responded to JH [9].

An inhibitory effect of JH on Balbiani rings 1 and ecdysone puff 1-18C and an activation of the ecdysone repressed puff 1-19A in *Chironomus tentants* [10] were revealed.

The mechanisms of JH action on genome are also unclear. Well understood is the regulation of a cascade of puffs under the influence of ecdysone. Ashburner suggested a trigger scheme which has presently been further developed and improved by Richards [11]. It explains the mechanism of interaction between the hormone-receptor complex and early, early-late and late ecdysone loci. As far as JH is concerned, more or less certain information is available on cytophysiological changes brought about by this hormone, on its influence on bioelectric potentials and ionic permeability of cellular and nuclear membranes [12-15]. A number of hypotheses explaining the influence of JH on genome are known. According to the hypotheses of indirect influence, the action of juvenile hormone leads to depolarization of membranes and to an increase of their permeability to Na^+ ions. As a result, the intracellular and intranuclear content of Na^+ increases. The action of ecdysone is opposite: it increases the membrane potential and membrane permeability to K^+ ions. As a result, the concentration of this cation in the cytoplasm and nucleus increases. In accordance with the character of action of the hormones on ionic permeability of membranes in ontogenesis of dipterans, changes in the hormonal situation (juvenile hormone-ecdysone ratio) occur in parallel with changes in the Na^+/K^+ ratio [10,15]. The hypothesis of direct hormonal action on genome suggests a direct hormonal influence on the cell genetic apparatus.

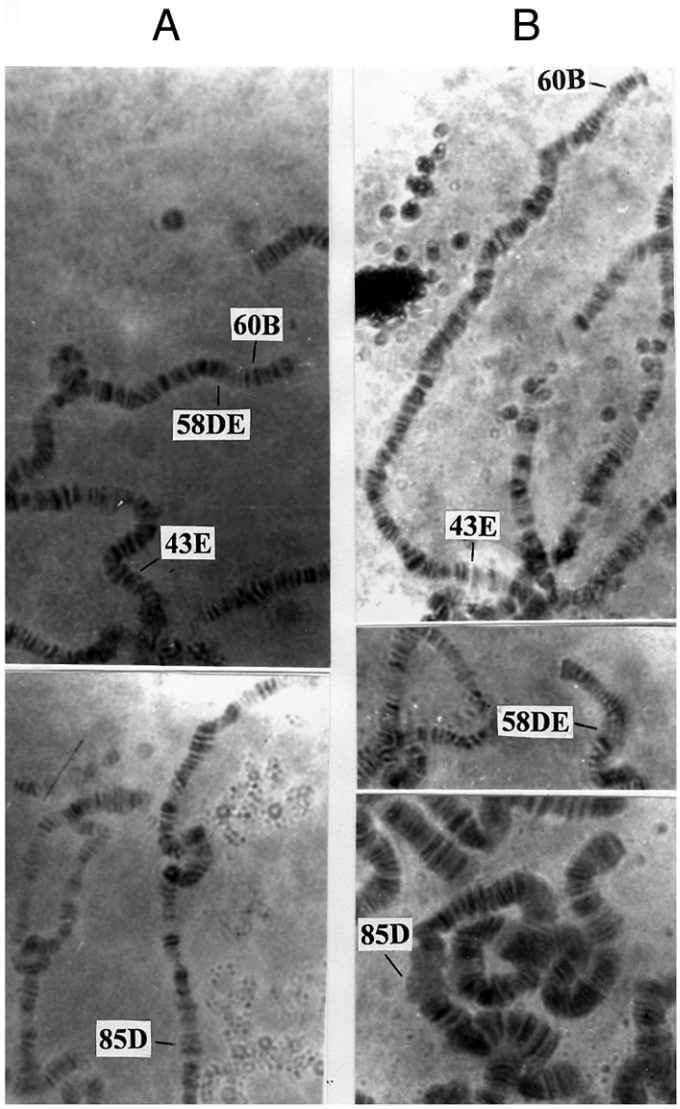

Figure 2. Puffs induced by metopren in polytene chromosomes of 0 h prepupae of *Drosophila melanogaster*. A – control, B – influence of metopren at concentration of 1 mkg/ml, exposure 30 sec.

The existence of the hypotheses of direct and indirect action of hormones is quite explicable taking into consideration the existence of several levels of regulation of gene activity. One of them is associated with the conformational state of chromatin, the degree of its diffusion. Structural

chromatin changes permit the fine mechanisms of regulation of gene activity involving enzymes and regulatory proteins to be triggered.

The both approaches have recently been combined in the ionic model of hormonal induction of puffs in accordance to which the interaction of hormones with the nuclear genome proceeds in several stages including changes in ionic balance, decondensation of certain loci of chromosomes and the interaction of the hormone-receptor complex with the cellular genetic apparatus [16, 17]. The same point of view is also discussed by us in one of our works [18].

In earlier experiments *in vitro* we demonstrated a sharp fall of the electrokinetic (ς-) potential of cell nuclei of *Drosophila* salivary glands under the action of metopren at a concentration of 1 mkg/ml [19]. This result is in agreement with the data on the alteration of the electrokinetic characteristics of cell nuclei in the intermolt periods of ontogenesis in larval and prepupal of *Drosophila* [20] and is opposite to the results obtained for ecdysone.

Along with the induction of a number of intermolt puffs the present work has demonstrated an active role of JH in regression of late ecdysone puffs. This result complements the trigger scheme of regulation of a cascade of ecdysone puffs developed by Ashburner and Richards in which the given aspect is not considered.

Conclusions

1. A number of puffs specifically activated by metopren (43E, 58DE, 60B, 85D) have been identified.
2. A decrease has been established in the size of late ecdysone puffs 63E and 82 EF under the action of this hormone suggesting the involvement of JH in regression of late ecdysone puffs.
3. The dependence of effect on hormone concentration has been demonstrated: at a concentration of 0.1 mkg/ml the number of activated puffs is smaller than at a concentration of 1 mkg/ml. 0.1 mkg/ml has been found to be a threshold concentration of metopren for puffs 85D and 43E. The concentration of 1 mkg/ml is sufficient to activate puffing at all loci under study.

The present investigation gives additional support to the current views on the mechanisms of hormonal regulation of insect development.

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