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Genotoxic effects of chromium onpolytene chromosomes of *Chironomus riparius* Meigen 1804 (Diptera, Chironomidae)

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Abstract — Genotoxic effect as tested of chronic exposure to three different concentrations of Chromium (III) on polytene chromosomes of larvae of *Chironomus riparius* (syn. *Chironomus thummi)* from the embryonic stage to the IV larval instar for two successive generations. In chromosomes AB, CD, EF and G significant differences of chromosome aberrations were found between exposed and control larvae as well as changes in functional activity (induction of novel puffs not corresponding to those induced during normal larval development in arms A, B, C and E, telomeric and centromeric decondensations especially at telomeres of chromosome G and arm C.). No significant differences were found between the effects of the three treatments nor between the two generations. In chromosome G the Balbiani Ring system (where some permanently active regions are involved in transcription of salivary proteins) appeared as a model for studying the response of the genome to Cr (III) treatment. In approximately one-third of the cells of the exposed larvae, the activity of the Balbiani rings BRc and BRb was reversed. In 10% of the cells of both generations of treated larvae, deletion or collapse of BRc was observed. Pompon-like G chromosomes were present in 6% of the cells. This type of chromoosme appeared either in a very decondesed or in a highly condensed state. In 5 % of the treated larvae the apical region of the chromosome G folded back so that the nucleolar organizer region appeared as if it was at the end of the chromosome. These structural and functional chromosomal changes are interpreted as a reaction of the genome to stressful rearing conditions.

Key words: *Chironomus riparius* (syn. *Chironomus thummi),* Chromium genotoxicity, polytene chromosomes, pompon chromosomes, somatic aberrations.

INTRODUCTION

Cells for their normal functions require some heavy metals (e.g., Cu, Zn, Co, Fe, Mg, etc.); for this reason, they are called essential metals. However, when they exceed normal physiological levels, they have toxic effects. Chronic toxicity occurs when the metals influence metabolism for a long period of time at a higher than normal concentration. In contrast, acute toxicity is observed when heavy metals affect metabolism for a short time, but at a very high concentration.

In trace amounts, Cr is an essential component of animal nutrition, functioning mainly in glucose metabolism and possibly in fat metabolism (Losi *et al.* 1994). The toxic effects of Cr are mainly due to its hexavalent salts (LEONARD and LAUWERYS 1980; DE FLORA *et al.* 1990), which are very unstable and rapidly reduced to a trivalent form. Trivalent salts of Cr(III) repre-

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sent the most common state of this metal in water and sediments. In this state, they are poorly absorbed by organisms because they do not easily cross cell membranes. However, according to DE FLORA *et al.* (1990), genotoxic effects of Cr(III) can be observed when this heavy metal is directly assayed on purified nucleic acids or on cell nuclei. In positive studies, the potency of Cr(III) compounds is several orders of magnitude lower than that of Cr(VI) compounds tested in the same system (DE FLORA *et al.* 1990). The strong binding of trivalent cromium to DNA may contribute to possible mutations and chromosome damage (BANCHI *et al.* 1983). Also, according to BIANCHI *et al.* (1983) and DE FLORA *et al.* (1990), genotoxic effects of Cr(III) may result from a variety of factors, including non-specific effects at very high doses of Cr(III), unchecked contamination with Cr(VI) traces, or penetration by endocytosis following long-lasting *in vitro* exposure. Endocytosis is commonly performed by hindgut cells in Chi-ronomids as well as in other insects, such as mosquito larvae (WIGGLESWORTH 1950; VIARENGO 1989).

Larvae of *Chironomus riparius,* (syn. *Chironomus thummi),* have been used for toxicity testing (WARWICK 1988; TIMMERMANS *et al.* 1989) because they are widely distributed, highly responsive to environmental stress and represent the most metabolically active stage in the species' life cycle (HOPKIN 1989; McCAHON and PASCOE 1990). However, until now they have never been used for genotoxicity testing, at the chromosomal level even though they have polytene chromosomes with a clear banding pattern. In salivary glands of *C. riparius* larvae at phase 6-7 of the IV instar, the polytene chromosomes are known to have DNA strands replicated 10-11 times (KIKNADZE *et al.* 1975). This feature allows us to observe every small structural rearrangement in detail under light microscopy.

C. riparius has *2n* = 8: AB and CD (metacentric), EF (submetacentric) and G (acrocentric). In chromosome G, there are three Balbiani rings (BRa, BRb, BRc) and a nucleolar organizer region (NOR). Balbiani rings are constantly active sections involved in the synthesis of salivary proteins. Therefore, variation in chromosomal functional activity can be observed more easily on chromosome G than on the other chromosomes.

Recently, in the industrial area near Turin larvae of *C. riparius* were found in sediments containing some heavy metals (MICHAILOVA *et al.* 1996) Among them, Cr had a concentration of 150-283 (Ag/g than that found in a control sediment of a mountain lake of the same region, i.e., 21 ± 1.6 µg/g (MICHAILOVA *et al.* 1998). Sixty % of *C. riparius* larvae from two stations in this area showed either somatic chromosomal rearrangements or functional alterations (MICHAILOVA *et al.* 1996; SELLA *et al.* 1997). In contrast a laboratory stock of *C. riparius* reared on unpolluted sediments showed only 1% of larvae with somatic aberrations (HAGELE 1984).

To investigate whether the high concentration of Cr in sediments around Torino could be among the causes of the observed high genome mobilization, we analyzed the effect of chronic exposure to different concentrations of Cr on polytene chromosomes of salivary glands and on head morphology of larvae in a laboratory stock of *C. riparius*. We assessed the genotoxic effects of Cr(III) by testing three different concentrations of Cr(NO3)3: the average Cr concentration in a polluted sediment near Turin (152 g/ml) (ACCOMASSO *et al.* 1983), a concentration ten times lower (15.2 g/ml) and a concentration two times higher (304 g/ml). Two successive generations of larvae were reared in order to check for the appearance of inherited aberrations in the second generation.

MATERIALS AND METHODS

Material

The stock of *C. riparius* used in this experiment originated from an egg mass kindly given to us by Dr. *J.* Diez (University of Madrid) from his lab stock. The culture of the stock was further extended in the Institute of Zoology of the Bulgarian Academy of Sciences and reared in standard conditions according to MICHAILOVA (1985).

Design of the experiment

All experiments were carried out in an incubator at 20°C and with a photoperiod of 16 h light. The fertilized egg masses were exposed to three dilutions of Cr(NO3)3 (15.2 fig/ml, 152 µg/ml and 304 (µg/ml). These dilutions were obtained from a stock solution

of Cr(NO3)3 . 9H2O (1000 mg/L) Merck (Darm-stad, Germany). The lack of Cr(VI) contamination in the stock solution was checked colorimetrically by reaction with diphenylcarbazide in acid-solution. The reaction is very sensitive, the absorption based on Cr being about 40.000 L g^{-1} cm^{-1} at 540 nm. We added 60 g of unpolluted sediment to each of the three Cr(III) (w/v) dilutions in 2 L of dechlorinated water. The content of Cr(III) in the unpolluted sediment was 2.6 mg/g and water containing nominal concentrations of Cr(III) was changed twice a week. Previously the unpolluted sediment was sterilized.

Two successive generations of fertilized egg masses were exposed to the three treatments for the entire larval development period (approximately 20 days). In each generation and for each tested dilution of Cr(III), together with the treated material we reared an untreated stock of larvae as a control. Egg masses used for control experiments were obtained from the same stock as the egg masses for treated material.

Chemical analysis

Chemical analysis of the Cr load in tissues of treated and control larvae was performed by atomic absorption spectrophotometry on a sample of 50 IV install larvae for each group. A Perkin Elmer 500 spectrophotometer was used with a background correction with a deuterium lamp. Analyses were performed according to REGOLI and ORLANDO (1994). Larvae had been starved for three days before analysis in order to analyze only the Cr(III) which had penetrated into the tissues.

Karyological analysis

For each treatment and each generation, only larvae that developed up to the phase 6-7 of the IV instar were studied. The phase was determined according to WULKER and GOTZ (1968). Preparations of polytene chromosomes were obtained by means of squashes with aceto-orcein. All nuclei of salivary glands were analyzed, both those of cells belonging to the so-called special lobe and those from the main lobe (GUNDERINA *et al.* 1984).

From the first generation material, we analyzed 27 control larvae, 24 larvae exposed to the concentration of 15.2 µg/ml Cr, 22 larvae exposed to 152 Hg/ml Cr and 18 larvae exposed to 304 j,g/ml Cr. From the second generation material, we analyzed 16 control larvae, 17 larvae exposed to 15.2 µ/ml Cr, 28 exposed to 15 µg/ml Cr and 28 exposed to 304 (xg/ml Cr. Table 1 reports the sample sizes of salivary gland nuclei from which we analyzed chromosome G. Sample sizes of nuclei we used for karyological analysis of the other chromosomes are reported in Table 2.

To identify chromosomal rearrangements, we used the standard chromosome maps of HAGELE (1971) and KIKNADZE *et al.* (1991). To detect activity of puffs and the BR system in the control and treated larvae, we used the standard map of puffs and BR activity of KIKNADZE (1976). To describe levels of puffing activity of BRs, NOR and the telomere of chromosome G (E2de region), according to BEERMANN (1971), we scored three levels of puffing (high = ++, intermediate = +, low or nil = —) for each of the two homologue. BRs were named according to KIKNADZE *et al.* (1985).

Chromosomal aberrations were considered to be of somatic origin when the salivary glands contained nuclei both with and without chromosomal aberrations.

Morphological analysis

To check for deformities of the sclerotized mandibles, mentum, epipharyngeal pecten, premandibles and antennae, an entomological preparation of the head capsule was performed for each larva.

Statistical analysis

Values of the Cr content in treated and control larvae were compared with the non-parametric Cruskal-Wallis ANOVA and frequencies of chromosomal aberrations in treated and control larvae were compared with G tests (SOKAL and RHOLF 1981).

Table 1 — Number of cells studied for analysis of different functional activities and somatic aberrations in chromosome G

	F1 Treatments				F2 Treatments			
	Control	15.2 µg/ml	152 µg/ml	304 µg/ml	Control	15.2 µg/ml	152 µg/ml	304 µg/ml
BRc/BRb activity	443	429	503	309	207	277	373	549
NOR and E2de sections' activity	269	148	177	100	31	388	386	609
somatic aberrations	438	524	591	380	207	388	449	589

Table 2 — Number of somatic chromosome aberrations in A,B,C,D,E and F arms in Fl and F2 generations of control and treated larvae of *Chironomus riparius.*
inv. = inversion; het = heterozygous. All inversions are in heterozygous state.

Arm	Map of Aberrations	F1 Generation		F2 Generation	
		Control (443 cells)	treated (1041cell)	Control (207 cells)	treated (1169)
A	inv. B4a-C2g	-	-	-	2
	inv. C1c-2g	-	1	-	-
	inv. C2a-i	-	2	-	-
	inv. C4c-D1c	-	-	-	2
B	inv. D3g-E2j	-	3	3	4
	inv. E1a-3f	-	1	-	-
	inv. E3c-i	1	-	-	-
	inv. E3f-F1h	-	2	-	-
	inv. F1a-h	1	-	-	-
	inv. F4a-h	-	1	-	1
C	break B3e-h	-	-	-	3
	inv. B3g-5k	-	-	-	1
	inv. B5a-C2m	-	1	-	-
	inv. C2a-i	-	-	-	3
D	Deficiency	-	-	-	4
	inv. C4c-5d	-	-	-	3
	inv. C6a-e	-	5	-	-
E	Amplification A4f-g	-	23	1	35
	Amplification A5e-g	-	29	1	36
	inv. B1c-q	2	-	-	5
	Pericentric inv. B2e-p	1	-	-	6
F	inv. C1a-4g	1	-		-
	inv. B3I-o	3	-	-	2
	Amplification B3h	4	86	3	111
	Total number of aberrations	13	154	8	232
	% frequency	2.9	14.8	3.9	19.8

RESULTS

Total Cr content in larvae

Average concentration (in µg/g) of total Cr was 5.85 + 0.49 in Fl and F2 control larvae, 12.85 ± 2.89 in larvae treated with 15.2µg/ ml of nominal concentration of Cr, 14.4 ± 10.41 in larvae treated with 152 µg / ml of Cr and 18.42 ± 13.75 in larvae treated with 304 µg /ml of Cr. Differences in total Cr contents among the three groups of treated larvae were not significant (Cruskal-Wallis ANOVA; H = 0.28, df = 2 and P > 0.10). This is the reason to compare the control with all treated material, not separately with three different concentrations.

Karyological analysis

The karyotype of *C. riparius* from control larvae is shown in Fig. 1. The polytene chromosomes of the control larvae do not differ from those of the standard chromosomal map done by of HAGELE (1971) and KIKNADZE *et al.* (1991).

Functional chromosomal alterations

Compared to control, functional abnormaties increased in the polytene chromosomes of the treated larvae. The polytene chromosomes showed decondensed centromeres in all chromosomes, but more often in chromosomes CD and EF.Telomere regions often appeared in grain structure. In Fl of treated larvae, 10% of the telomeres of arms C, G and A were decondensed , looking like a Balbiani ring (Fig. 2a, b). In F2 of treated larvae, frequency of decondensed telomeres of arms C, G and A was approximately 6% In treated larvae, novel puffs (i.e., not corresponding to those induced during normal larval development) were observed in section B2a-h of arm A, in section F2a-k of arm

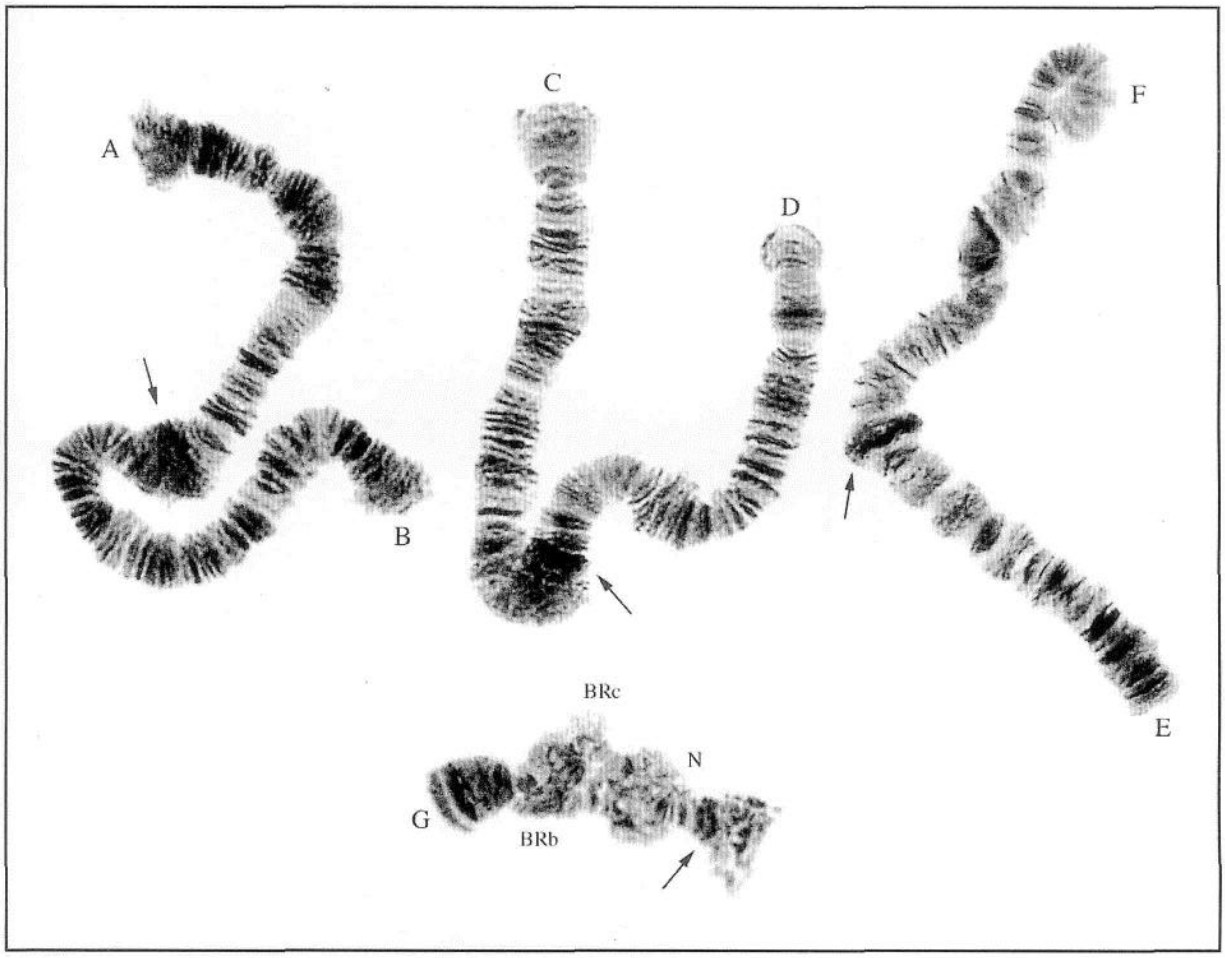


Fig. 1 — The standard chromosomes of *Chironomus riparius* from the control stock used in the experiment. Chromosomes AB, CD, EF and G; — Balbiani ring; N-nuclear organizer; arrow —centromere region.

B, in sections B2a-i, B3e-i, B4a-c and B5 of arm C, and in section B3j-n of arm E. In 2% of cells of exposed larvae, a puff in the heterozygous state was observed in region DC of chromosome G (Fig. 3).

Ectopic contacts were found more often in treated larvae than in controls and they occurred more often between telomeres of chromosomes than between intercalary regions. The treated larvae, in mosaic state had polytene chromosomes showing a grain structure (Fig. 4).

As expected, in approximately 70% of cells of control larvae, chromosome G was characterized by an activity of BRc higher than (++/+) or similar to (++/++ and +/+) the activity of BRb. Only 15% of cells of treated larvae showed this normal situation. In contrast, in treated larvae, approximately 37% of the cells of Fl and 21% of cells of F2 showed a regression of the activity of BRc together with an increase of the activity of BRb (+/++, -/++ and -/+) (Fig. 5), while this situation was observed in only 2-10% of cells of control larvae.

There was no evident difference in the activity of the nucleolar organizer region between treated and control larvae of both generations.

In the cells of control larvae of both generations, region E2de of chromosome G showed a normal or very high activity on both homologues. However, in 39% of cells of treated Fl larvae and in 5 % of cells of treated F2 larvae, this region showed a low level of decondensation including some cases in which it completely collapsed.

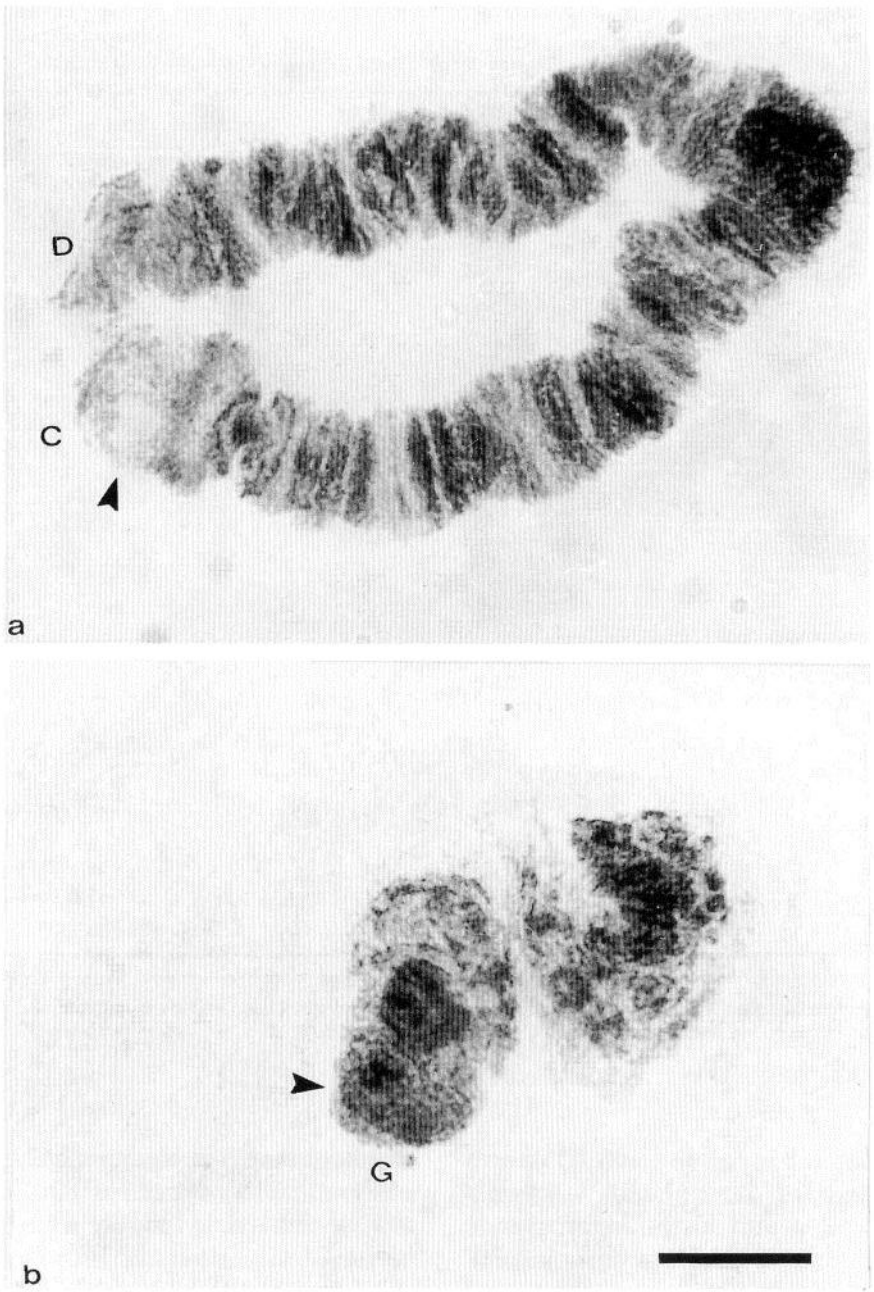
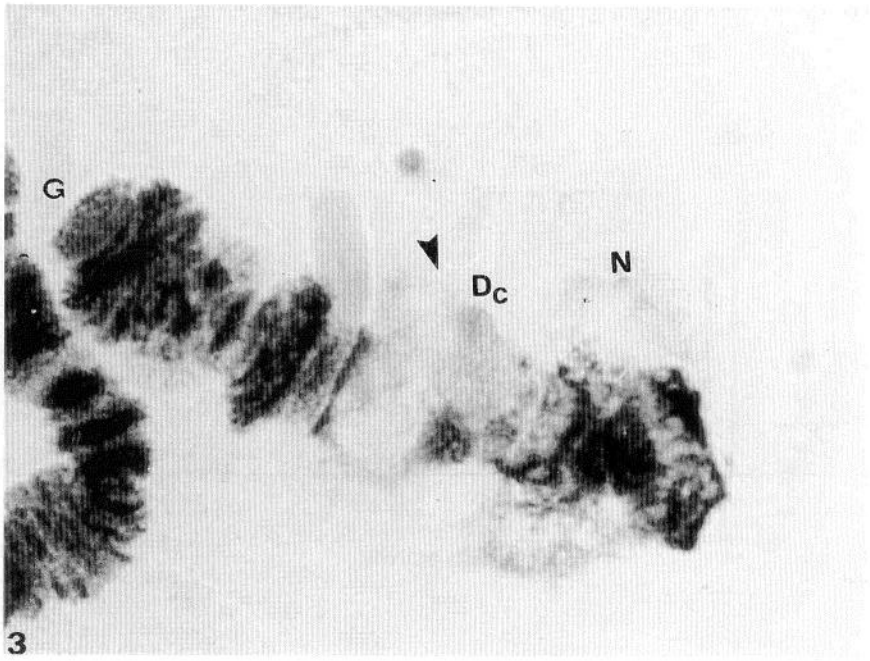


Fig. 2 — Chromosomes CD and G. a. Chromosome CD with decondenzation of the telomere region of arm C. b. Chromosome G with well pronounced functional activity in section Ala of arm G.

Structural chromosomal alterations

Location and frequency of somatic rearrangements in arms A, B, C, D, E, and F are shown in Table 2.

No significant differences were found in total frequencies of aberrations among the three treatments, in either the comparison between the two generations (heterogeneity G test; G2 = 4.95; P > 0.05) or the comparison among the treatments (heterogeneity G test, G2 = 2.95; P > 0.05). Therefore, we pooled together the aberrations of all treated larvae irrespective of treatment and compared them with frequencies of aberrations in control larvae. Highly significant differences were found between treated and control larvae both in Fl (G test; G = 53.92; P < 0.001) and F2 (G test; G = 11.82; P < 0.01). The type of aberration by far the most frequent in treated larvae was amplification of



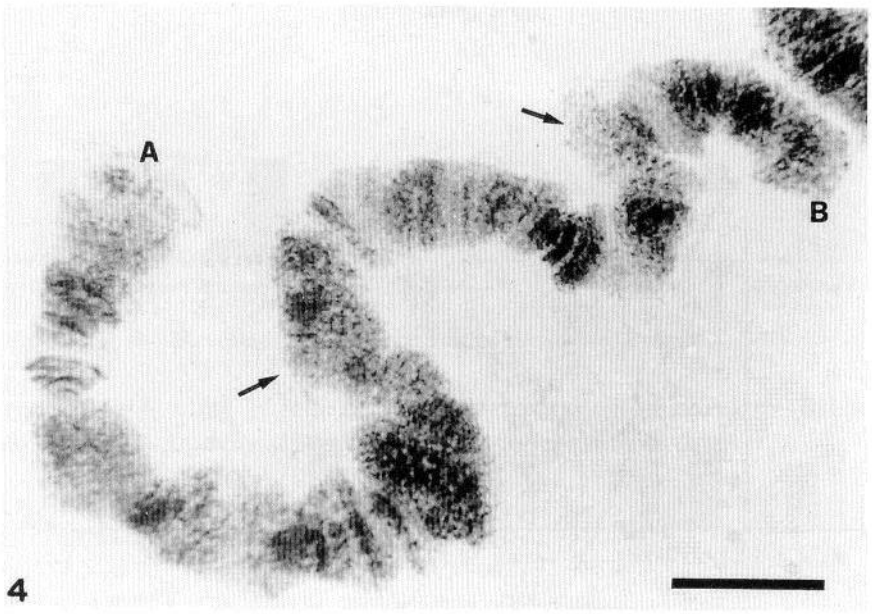


Fig. 3 — Chromosome G with a heterozygous puff in section DC. Fig. 4 — Chromosome AB with a grain structure.

three sections of chromosome EF which in control larvae had the same narrow size they show in the standard map.

In treated larvae only one inherited heterozygous inversion (i.e. a rearrangement affecting all cells of a salivary gland) was found in 4 individuals of the first generation and in 6 individuals of the second generation (arm B:F3a-C2m).Since this inversion has been found in the first generation, it might be expected this incevsion to exist in low frequincy in the control. No new heritable aberration was detected in treated larvae from the first to the second generation.

Location and frequencies of aberrations in chromosome G are shown in Fig. 6. There was a significant difference between treated and control larvae in the total frequencies of somatic rearrangements in chromosome G, both in F1 (G test; G3 = 155.6; P<.001) and F2 (G test; G3 =

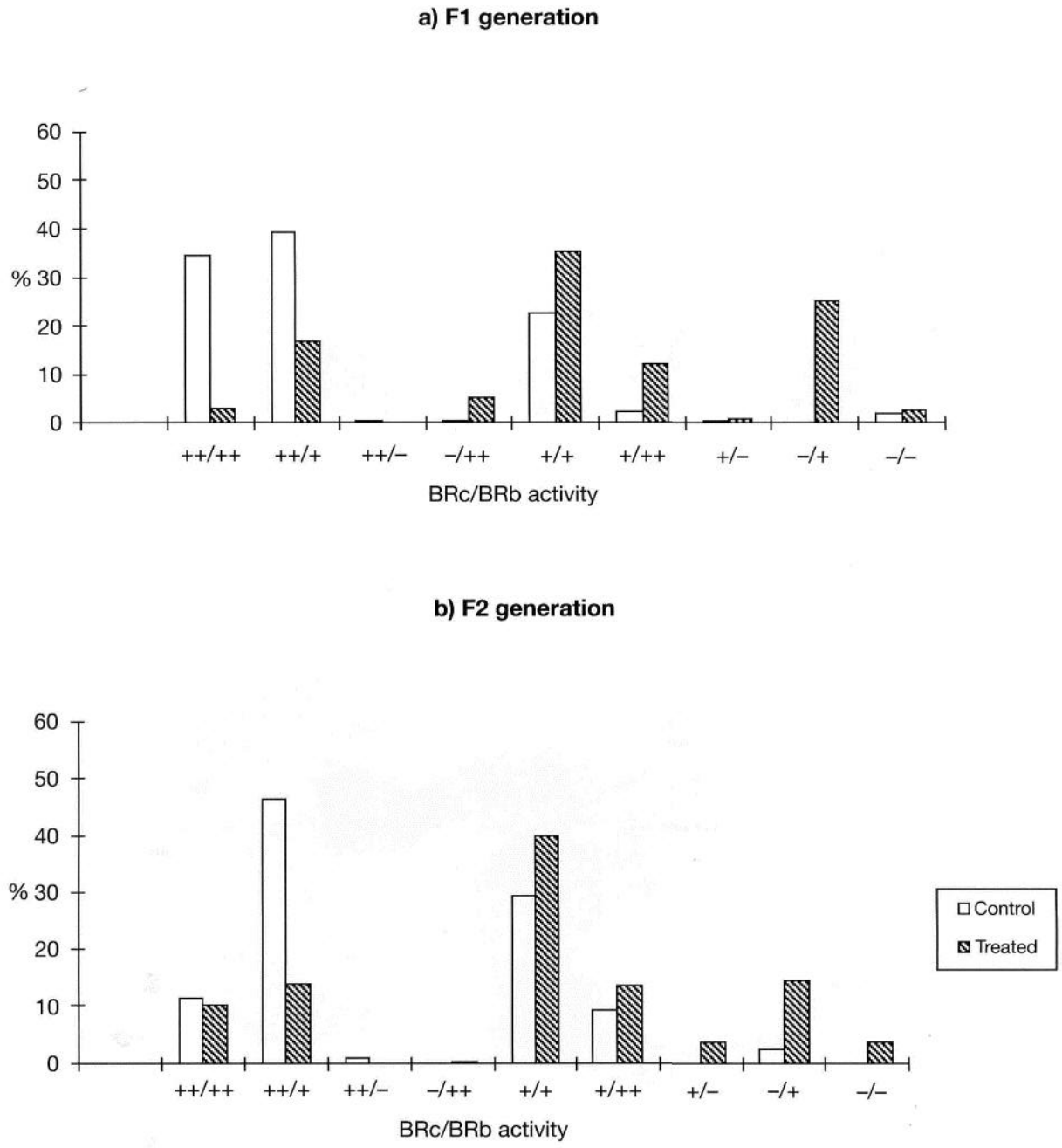


Fig. 5 — Chromosome G- percentage frequency of the different levels of BRc/BRb puffing in cells of control and treated larvae. Levels of activity are scored as: ++, high puffing; +, intermediate puffing; — low or no puffing.

42.6; P<.001). Frequencies of aberrations between the three treatments were significantly different, in either the comparison between the two generations (heterogeneity G-test; G2 = 3.074; P > 0.10) or the comparison among the treatments (heterogeneity G test; G2 = 3.4; P > 0.05).

In treated larvae a deletion of BRc was observed in both generations with the frequency of 10 and 14% respectively (Figs. 7a,c). A deletion of both BRs (BRb and BRc) was established in a low frequency from the first to the second generation (Fig. 7b). In 6% of cells of treated larvae, chromosome G with both BRs deleted appeared as pompons, as described by MICHAILOVA *et al.* (1996). These pompon chromosomes had two different aspects. In cells located in the main lobe of salivary glands, they

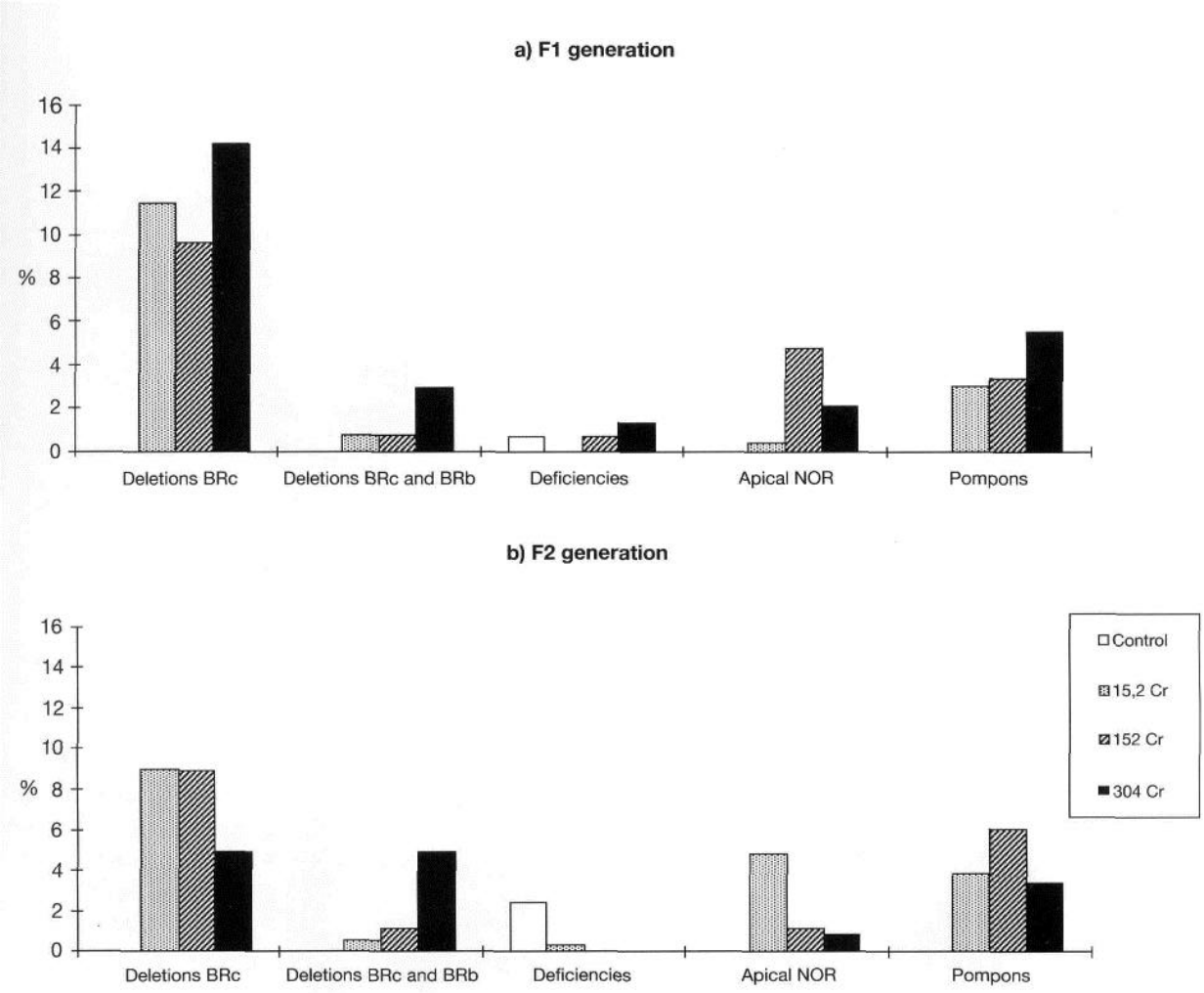


Fig. 6 — Percentage frequency of structural aberrations of chromosome G.

looked very decondensed, while in cells of the special lobe they were in a condensed state (Fig. 7b).

Another very interesting situation in chromosome G was observed in treated larvae only: in approximately 2.5% of the cells of treated larvae of both generations, the apical region folded back so that the nucleolus appeared as if it was at the end of the chromosome (Fig. 8). Thus the morphological appearance of this chromosome changed without involving any rearrangement of genetic material. In a few cases (2.1% of the cells of Fl and 1.3% of the cells of F2), the apical position of the nucleolus could possibly be interpreted as the result of a somatic homozygous pericentric inversion.

Morphological analysis

Three malformations of submentum were observed in two treated larvae (lack of mid teeth of mentum, weak medial deformity, an additional tooth of mentum).

DISCUSSION

Many authors using Chironomids as bioindicators have shown a relationship between acute toxicity of different polluting agents and larval mortality (HUDSON and CIBOROWSKY 1996).

Our results demonstrate a relationship between chronic exposure (20 days on average) to high doses of Cr and a genotoxic effect that was not observed in control larvae (e.g., deletions of Brb and BRc, decondensation of telomeres of chromosomes C, A and G, formation of pompon-like chromosomes G, significantly higher frequency of somatic amplifications of three

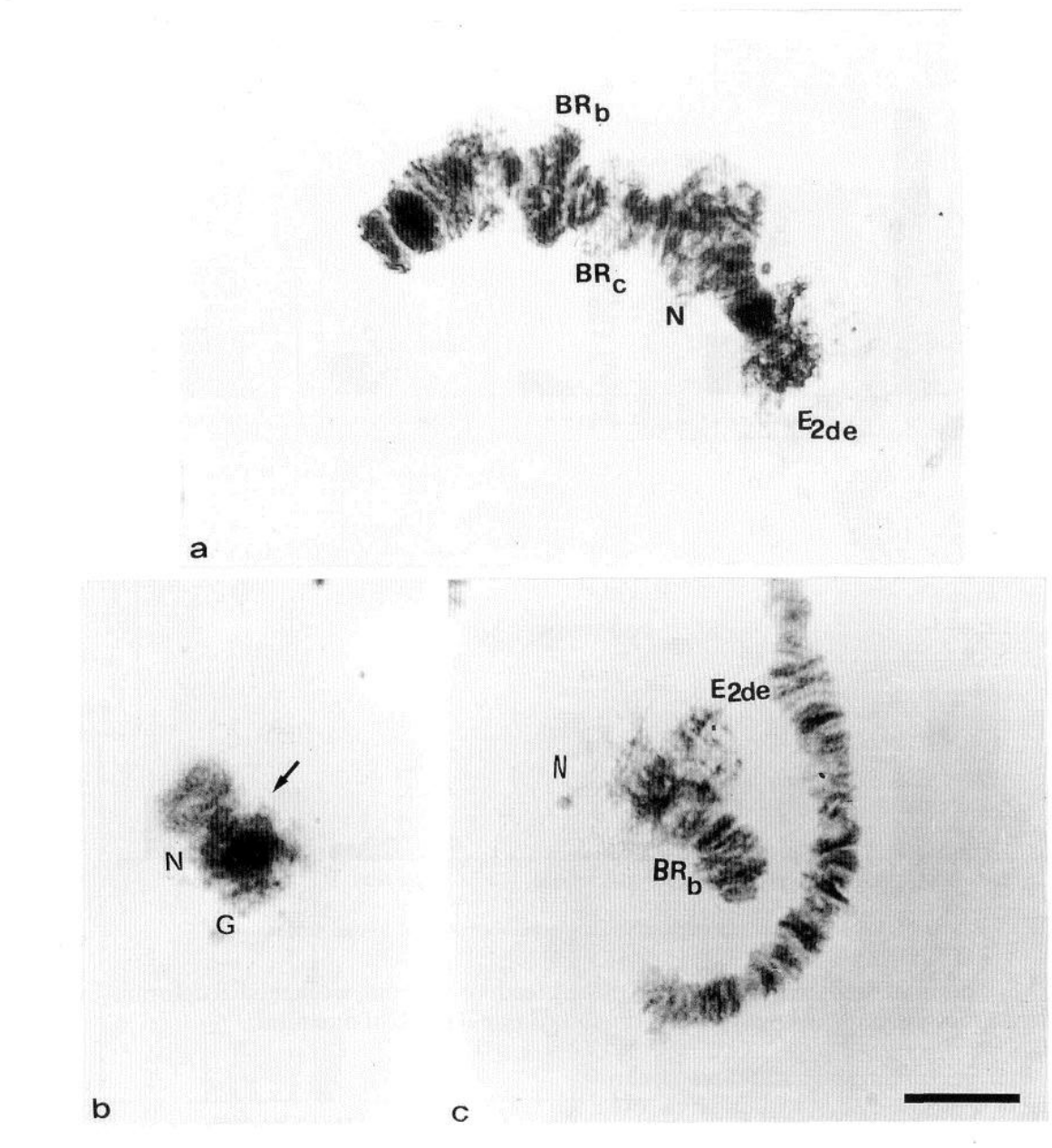


Fig. 7 — Chromosome G. a.BRc — a slight activity, b. A deletion ofBRb and Brc. c. A deletion ofBRc.

sections in chromosome EF). So, the toxicity effect of Chromium on the polytene chromosomes might be resulted from the fixation of trivalent chromium in the larvae. This can be inferred by the fact that the content of Cr in the treated larvae of both generations was higher than that of the control. In the literature there are data of the toxicity of Cr (III) on the cell division *of Allmm cepa*, resulted from the fixation of trivalent chromium in the plant tissues (SAHI *et al.* 1998).

Cr was given as Cr(III); Cr(VI) was not detected with the diphenylcarbazide method in the Cr(III) compound we used. Therefore, the positive genotoxic effects observed should be ascribed to Cr(III). Its toxic effect may result from the fixation of the Cr by the tissues and disturbance the osmotic relationships. This restricted the transport of Ca (2) across the membrane into the cytoplasma (LIU *et al.* 1992). The level of Ca (2) in the cells becomes very low, this disturbs the physiological activity of the cells,

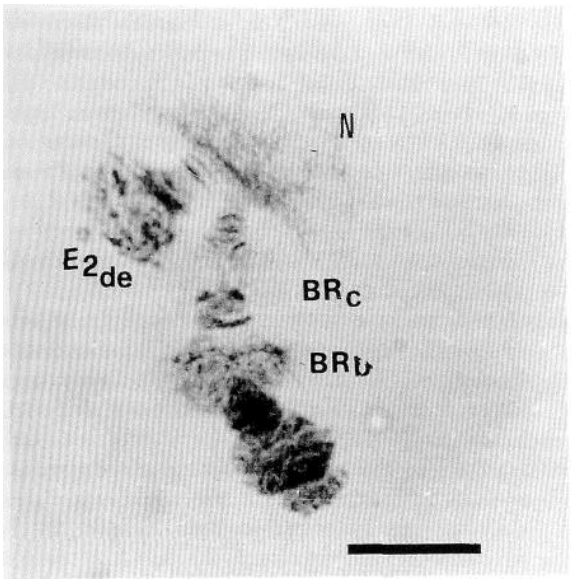


Fig. 8 — A folded chromosome G. N-nuclear organizer, BRb, BRc - Balbiani rings. E2de -decondenzation section of chromosome G.

especially the Camodulin, (CaM) which on its site has the ability to activate a number of key enzymes (LIU *et al.* 1992). On an other hand, the strong binding to DNA could be reason for the reduction of Cr (III) solubility stabilization of the double strand structure of DNA and possible causation of muttation and chromosome damage (BIANCHI *et al.* 1983).

It is difficult to explain absence of a significant correlation between the concentrations of Cr given to treated animals and total Cr content in tissues of treated larvae. Two hypotheses can be proposed: either much of the Cr(III) precipitated (LOSI *et al.* 1994) or the ability to incorporate Cr compounds through endocytosis is limited. However, at present, nothing is known about these topics in midges.

The main chromosomal aberration observed in treated larvae was the occurrence of size variation in sections A4f-g , A5e-g of arm E and B3h of arm F . Size increase of these sections has been observed also in larvae from the heavy metal polluted area near Turin (MICHAILOVA *et al.* 1996; SELLA *et al.* 1997). The three sections are known to contain repetitive DNA (KIKNADZE *et al.* 1987; BOVERO 1999) where misalignments can occasionally occur and yield reciprocal products i.e. sections with amplified and reduced size. Cr treatment could have enhanced the phenomenon of unequal crossover, which has been demonstrated in a variety of organisms (SMITH 1976). These chromosomal amplifications occurred during embryonic development prior to the polytenization process.

As already stated in the Introduction, responses of chromosome G to environmental changes can be detected easily. Cr treatment induced changes in BRs puffing that are similar to those observed in larvae of *C, riparius* from the heavy metal polluted areas near Turin (MICHAILOVA *et al.* 1998). Similar changes in the puffing activity of BRs have been reported in the literature: heat shocks (BARETTINO *et al.* 1988), galactose (DIEZ *et al.* 1990) and sugar treatment (BEERMANN 1973) can induce a regression of BRc activity and an increase in that of BRb. Thus, it can be hypothesized that the BR system reacts in the same way (i.e., with a regression of BRc and an increase of BRb activity) to different stressful conditions.

In some chromosomes G of treated larvae, we observed that the nucleolus appeared to be located at the end of the chromosome as a result of a folding back of the section immediately following BRc. The same phenomenon has been observed in several chromosomes G of *C. riparius* larvae living at a heavy metal polluted site near Turin (MICHAILOVA *et al.* 1998). Together with the formation of pompon-like chromosomes G, this folded-back appearance of the apical region of the chromosome can be considered a specific cytogenetic reaction to stressful conditions.

On the whole polytene chromosomes of *C. riparius* reacted to the genotoxic action of the Cr treatment in a sensitive way (occurrence of amplified sections, of pompon-like G chromosomes, repression of BRc), thus indicating that the genome of *C. riparius* represents a very sensitive system to easily track genomic changes induced by a stressful environment. The strong binding of trivalent chromium to DNA might be the reasuns of the mutations and chromosome alterations . The chromosome assay on the salivary gland cells of *C. riparius* can also be employed for in situ monitoring of water containing a high cromium content.

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