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COMPARATIVE EFFECT OF HEAVY METALS ON THE POLYTENE CHROMOSOMES OF CHIRONOMIDAE, DIPTERA

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ABSTRACT. It was done the comparative analysis of the acute and chronic effect of some trace metals (Pb, Cr and Cu) on the structure and function of the polytene chromosomes. The analysis was performed on IVth instar larvae hatched from eggs exposed separately to different concentrations of Cu, Pb, and Cr. Chronic (with Cr and Pb iones) and acute (48 hours) (with Cu ions) treatments were performed. Common and specific reactions to the metals were evaluated. The response of *C. riparius* genome to the heavy metals is characterized by changes significantly the activity of BRs and NOR as well as by increasing the somatic aberrations. Significant differences in somatic aberrations (inversions, deletions, duplications) were found in chromosomes AB, CD, EF, G compared to unexposed *Chironomus riparius.* Deletions of chromosome G converted this chromosome to the so called "pompon" form. Chromosome breaks occur preferential at specific bands of the polytene chromosomes where copies of transposable elements or satellite DNA are present. The functional and somatic cytogenetic damage, together with the formation of "pompon" chromosomes are proposed as sensitive biomarkers at cytogenetical level, which can provide an early warning of adverse long-term effects of heavy metals in aquatic organisms.

KEY WORDS. Chironomidae, trace heavy metal, Balbiani ring, chromosome rearrangements, „pompon" chromosome

INTRODUCTION

Certain heavy metals are required as trace element for normal cellular functions. They presence in trace amounts is considered important for living

organisms, because it is an active site for a number of enzymes which are involved in oxidation-reduction reaction. However, heavy metals are toxic to cells once their levels exceed their low physiological values. The effect of heavy metals could be released in high concentrations for short periods, causing acute toxicity for the aquatic organisms, or in low concentrations but for long periods of time when it may cause chronic toxicity, leading to disorders in vital functions, such as changes in morphology, growth, maturation, reproduction, hatching, occurrence of deformities, which do not necessary result in early death (Aziz et al., 1991). It was described the binding of heavy metals to the phosphate, deoxyribose and heterocycle base residues of DNA. This interaction may induce the alteration of the primary as well as secondary structures of the DNA and result the mutation (Sahi et al., 1998).

Chironomids are the most widely distributed and abundant species in freshwater ecosystems. Their larva stage is the most critical, the most responsive to environmental stress and the metabolically active stage of its life. Chironomidae larvae (a stage which is exposed to contaminants) possess polytene chromosomes, which make Chironomids prospective subjects for cytogenetic monitoring. The following features of the polytene chromosomes are applicable for the purposes of cytogenetic monitoring: size, polyteny, somatic pairing, very good band pattern along the chromosomes (Michailova, 1989). On the other hand, individual larva represents the most basic unit of biological communities. They integrate into a single whole the lower levels of biological organisation (molecular, cellular and organ) and form the building blocks for the higher levels of organisation (population, communities and ecosystems). That's way larvae of wide distributed species could be used for tracing the mutagenous effects of a number of factors in environments, especially the influence of the heavy metals. The standard karyological characteristics (Michailova, 1989, Kiknadze et al., 1991) of these species can be employed as a basis to reveal the environmental mutagen monitoring by studying chromosome aberrations, appearance of heterochromatin and changes in the functional activity of the polytene chromosomes.

Species of different genera (Michailova, 1985) are cultured in the laboratory and can be used in dose response experiments established in field populations. This allows in experimental condition to trace the cytogenetical effects of some metals in various concentrations on the polytene chromosomes and to analyse the accumulation of heavy metals in larva body. So, the laboratory experiments will performed in or validate and future understand the field observations. Under laboratory conditions cytogenetic effects will be studied and typical chromosome rearrangements could be found as a signal for special contaminants.

In this study we present data on the effect of some trace heavy metals on the structure-functional organization of the polytene chromosomes of C. *riparius (syn. C. thummi).* It was chosen by us as a model species because this species has many advantages: it can be easily reared in the lab; has only four well banded polytene chromosomes (called AB CD EF G) whose standard banding pattern has been mapped by Hagele (1971) and Kiknadze et al (1991). It's chromosome G of

Chironomus riparius has very important and sencitive to different environmental factors structures (BRs and NOR).

Having in mind the advantages of Chironomids we inted to compare the acute and chronic effect of some trace heavy metals on the structure and function of the polytene chromosomes.

MATERIAL AND METHODS

Material and design of the experiments:

The stock of *C. riparius* used in these experiments originated from an egg mass kindly given to us by Dr. J.Diez (Spain) and Dr. H.Lamy (France).

Fertilized eggs of *C. riparius* were obtained from a lab.stock, which was bred in standard conditions: aeration, 16:8 light dark photoperiod and 20^{o}C supplied with power nettle leaves, mud and cellulose. The fertilized egg masses of *C. riparius* were exposed separately and chronically to the three different concentrations of Cr $(NO_3)_3$ (15, 2 mg/l, 152 mg/l, 304 mg/l) as well as to three different concentrations of Pb $(NO_3)_2$ (15,1 mg/l, 151 mg/l, 302 mg/l) (Michailova et al., 2001a, b). Also, acute treatment (48 hours) with three different concentrations of $CuCl_2$ (0,005 mg/l, 0,01 mg/l 0,05 mg/l) has been performed. The concentration of 152 mg/l Cr, 151 mg/l Pb and 0,01 mg/l Cu correspond to that present in polluted natural populations (Michailova et al., 1996, in press). Each experiment has a control lasted 25-30 days. The chronical treatments have been performed during the two generations while the acute treatment was done for one generation.

Cytogenetical analysis:

For cytogenetic analysis we used IVth larva stage (6-7 phase), determined by Wulker and Gotz (1968). Preparations of polytene chromosomes were obtained by means of squshed with aceto-orcein. The chromosome aberrations and functional activity of BRs and NOR after treatment with thee different concentrations of Cr $(NO_3)_3$, Pb $(NO_3)_2$ and CuCl2 are taken from Michailova et al (2001a, b, in press). In all cases the observed chromosome aberrations were compared with standard maps done by Hagele (1971) and Kiknadze etl (1991). Chromosome aberrations were considered to be somatic when the salivary glands contained nuclei both with and without chromosomal aberrations.

We evaluated the functional activity of BRs and NOR utilizing three levels of puffing: "++", high puff expansion,"+" intermediate puff expansion,"-", little or no puffing (Beermann, 1971).

For detecting the appearance of "C" bands we used the modified C band method (Michailova 1987).

In situ hybridization:

The location of repetitive DNA clusters (Alu and Hinf) was determined my means of FISH technique (Schmidt, 1992).

Morphological analysis:

The entomological preparation of the head for each larva capsule was performed in order to check the deformities of mandibeles, mentum, epipharyngeal pectin and antennae.

Chemical analysis:

Chemical analysis was done of tissues of treated and control larvae as well as of substrate using inductively-coupled plasma opical emission spectroscopy. (Michailova et al. 2001 a,b).

Statistical analysis:

In all types of treatments the functional activity of BRs and NOR was analysed by comparing the treatment groups with controls and between the treatment groups using Student Fisher test. Frequencies of aberrations were compared by G test. P< 0, 05 was considered as significant. We ckecked the co- localized break points of aberrations with repetitive DNA cluster applying the methodological approach by Bovero et al (2002).

RESULTS

1. Comparative chromosome functional reaction:

In all treatments: chronic with Cr and Pb ions and acute with Cu we observed that BRs (BRc/BRb) is very sensitive and it can be appeared in several states (Fig.1a, b, c). In F1 of both chronic and acute treatments states of BRc/BRb: ++/++ and ++/+ significant decreased in comparison with control, while state: -/+ significantly increased in treated materials (Fig.1a, b, c) and there is a dose dependence in comparison with different concentrations of both types of treatments (Michailova et al., 2001a,b). The state of BRc/BRrb (+/+) occurred in high frequency of both types of treatments but when the exposure was chronical there wasn't any significant differences in comparison with control. However, the acute exposure to three concentrations of Cu significantly (P<0, 05) increased the frequency of intermediate state of BRs activity. The high activity is observed after exposure to 0.005 mg/l (Michailova et a., in press). The state BRc/BRb (+/-) significantly decreased in comparison with control after chronic treatment (Fig.1b), while after acute ecposure it increased significantly both between treatments and control and between different concentrations. In F2 generations of chronic treatments with Cr and Pb ions we again observed the same tendence of responce reactions (Michailova et al., 2001a, b).

Also, there was a difference of state of activity of BRc/BRb (++/- and -/-) between chronic and acute treatments. In acute treatment there were significant differences between treated materials and control of state: BRc/BRb (++/-) as well as between different treatments (Fig.1c).In state -/- of BRc/BRb significant differences were observed between control and treatments (Fig.1c).

The other structure - the nucleolar organizer (NOR) is also very sensitive to these different types of treatments. This structure significantly change it's activity after chronic and acute treatment: the high activity of NOR descreased due to shift to the intermediate state (Michailova et al., 2001a,b, in press).

Compared to the controls, functional abnormalities increased in both types of treatments: chronic and acute treated larvae. The polytene chromosomes showed decondensed centromeres in all chromosomes, more often in chromosomes CD and EF. This process increased with increasing concentrations of chronic tretamnets (Michailova et al., 2001.a, b). A dose dependent relationship between Cu oncentration and frequency of centromere decondensations was observed ($P<0.001$).

2. Comparative chromosome structural reaction

On the whole the polytene chromosomes of *C. riparius* reacted to the effect of Cr, Pb and Cu ions in a sensitive way: occurrence of the somatic rearrangements in all chromosomes. In all treatments no specimens with standard morphology of the polytene chromosomes were detected. The most pronounced chromosome aberrations were heterozygous para and pericentric inversions followed by duplications, deletions and deficiencies. Highly significant differences were found between frequencies of aberrations of chronic treatements with Cr and Pb and control larvae (Michailova et al.2001a, b). Also, dose dependet relationship between Cu concentrations and the frequency of chromosomal aberrations was detected (Michailova et al. in press). Heterozygous inversions in varying lengths were induced in chromosome arms A, B, C, D, E, F. The somatic inversions were in a low frequency (0,14%-6,00%). It is important to underline that in most cases the trace metals (acute and chronic exposure) affected one and the same site of the chromosomes AB, CD, EF and G. Even more, in one and the same subsection we observed breaks either of chronoc or acute treatments. Some breakpoints were common to larvae exposed to acute Cu, and to larvae treated chronically with Pb (For instance: for chromosome AB there are: B2a, B2h, C4a, D3e, D3d, E1a,b, E2o; for chromosome CD – B4o, B5a, B5o, C2l, C5e; for chromosome EF- B1a, B3a, B3o, B3h, C3d) (Scheme 1). Common break points were estableished after treatment with Cu (acute treatment) and Cr (chronic treatment): chromosome AB – B4a, E1a, F1h; chromosome CD – B3g, B5a, C4bc, C6a; Chromosome EF: A5g, B1c, B3o, B3h, B3i (Scheme 1). The frequency of amplification observed in section B3h of arm F increased significantly after treatment with Cu ions ($P<0.005$) (Michailova et al. in press). Especially very interesting is the chromosome G. After chronical exposure with different concentrations of Cr and Pb ions in both generations we observed significantly increasing the homozygous deletions of BRc in comparison with control (Fig.2 a). Dose dependence of these aberrations is established in the F2 generations (Fig. 2 a) (Michailova et al., 2001a, b). Also, the deletions of BRc + BRb are found after treatment with these heavy metals which frequency significantly increased in comparison with controls and in F2 increased with increasing the concentrations of ions (Fig.2 a). Deletion of this chromosome was also observed after acute treatment, however it is significantly higher after treatment with 0,05 mg/l than that of 0,01 mg/l (Michailova et al. in press). Chromosome G with deletion of BRc or both deletions of BRc and BRb changed it's morphology and appeared very often as “pompon”. This “pompon” chromosome has two different states: decondensed (Fig.2 b, c, d) and very condensed.

Together with common reactions of *C. riparius* genome there is also some specific response to different ions. In F1 generations of *C. riparius* treated with Pb, a puff was observed in chromosome arm D, near to the centromere regions. It's frequency significantly increased after treatment with 151 mg/l (15,7%) in comparison with the lowest exposure concentrations (3,2%) (Michailova et al., 2001a).

In both generations of Cr treated larvae the telomeres of arms A, C, D and G are decondensed looking liked as Balbiani ring (Michailova et al., 2001b).

Only in material treated acutely with 0.01 mg/l Cu we observed a partial structural deletion in chromosome G, including BRc, BRb and NOR (Michailova et al., in press).

In all studied materials the "C" heterochromatin appears in centromere regions of the chromosomes as well as on both sides of the centromeres.

In situ hybridization performed by us with clone of repetative DNA (Alu and Hinf) allowed to map 22 Alu and 28 Hinf repeats on the chromosomes AB, CD, EF. In chromosome G we established Hinf elements only (Michailova et al., 2001b), (Bovero et al, 2002). Like the breakpoints, repetitative DNA clusters appeared to be significantly more abundant in regions of constitutive heterochromatin like the pericentromeric regions, rare or absent in distal sections of chromosomal arms.

3. Morphological deformities

Only few larvae showed some deformities of head capsule (Michailova et al. 2001a, in press).

4. Amount of lead and chrom associated in larval tissue

Concentrations of trace metals (Cr and Pb) were not related to exposure concentrations (Michailova et al., 2001a, b). Also, these authors found that there was not significant differences in amounts of Cr and Pb ions accumulated by two generations of treated materials.

DISCUSSION

The response of *C. riparius* genome to the heavy metal ions is characterized by changes in gene expression and increase in somatic aberrations. Our study showed that different types of treatment (acute and chronic) are effective in producing structural and functional chromosome aberrations in *C. riparius.* This result occurred because the both treatments of the trace metals act on the polytene chromosomes at the structural and functional level from the very beginning of the polytenization process.

Normal, the puff activity of BRc is relatively stable in fourth instar larvae but occurs in fully expanded state up to the 6-7 phases of the IVth larva stage (Diez et al., 1990). BRb is very active in young larvae, while in larva phase (6-7) it shows slight activity or completely inactive (Diez et al., 1990). However, after treatment with trace metals we observed a specific case of alterations of BRc and BRb. BRc shows a slight activity or collabses, while BRb is activated, or both BRs are collapsed. So, Balbiani ring system appeared as a model for studying the response of the genome to trace heavy metals treatment. These are sites of intensive transcription

of genes encoding silk proteins (Wieslander, 1994) and used by the Chironomus species in the construction of the tube in which the larva live. Such reactions have been established after chronic treatment with Cu ion (Aziz et al., 1991) and Al (Micahilova et al., 2003). These authors suggested that the suppression of the BRs may be due to increased the transcription activity resulting from the production of a heat shock proteins (Todorova et al. 2001) or metalthionein like protein detoxifying potentially toxic trace metals.

Other very sensitive structure is the nucleolar organizer (NOR). Reduction of the size of the NOR is indicative of overall decreased of RNA synthesis which implies general impairment of metabolic function (Hudson, Ciborowski, 1996). Hevay metalsions may be inhibited RNA polymerize I which involved in the synthesis of nucleolar RNA (Horgen and Griffin, 1971). Acute and chronic exposure disturbed the process of heterochromatin condensation in the centromere regions and very often they appeared as pseudopuff. Presudopuffing could result from a structural modification of centromere heterochromatin following exposure to different agents. In might be possible these agents to have some inhibiting effects on the synthesis of proteins involved in chromatin condenzation.

Bovero et al. (2002) postulated that some chromosome breaks following stress are non random and are concentrated at sites rich in repetitive DNA clusters (Alu and Hinf). They postulated that the frequency of co-localization between common breakpoints and repetitive DNA hybridization signal was significantly higher than expected by chance. Our comparative analysis between distribution of chromosome breaks of aberrations induced by chronic and acute treatments and location of DNA clusters showed that twenty of breakpoints occurred at sites of Alu and Hinf elements. The distribution of common breakpoints along the chromosomes AB, CD Ef is not random: in each chromosome the majority of common breakpoints are located in the pericentric regions, where the constitutive heterochromatin is present. Common breakpoints were established in every chromosome arm. For instance: arm A – C4a, arm B – D3d, arm C – B5a, C2l, arm D – C4b, C5e, and arm F – B3o, B3h and C3d. They are mapped to the sites where Alu and Hinf repeat are located as well as some tramsposable elements (MEC) were detected (Kiknadze et al., 1987). These data confirmed once again the idea that breaks might be occurred more frequently in sections containing blocks of repetitive DNA or transposable elements and the observed chromosome instability depend on structural feature of the genome.

The many structural and functional chromosome alterations observed in *C. riparius* tretaded with different heavy metals showed the genotoxicity effect of these metals. Some authors (Bianchi et al., 1983, Sahi et al., 1998) postulated the strong binding of the Cr, Pb and Cu ions to DNA, which might be possible causation of mutation and observed chromosome damage. Although these metals was genotoxic, relatively little heavy metal ions was accumulated by *C. riparius* and tissue concentrations were lower than the observed in many other aquatic invertebrates (Philips, Rainbow, 1993). This different reaction of Chironomids could be explaine by their ability to tolerate elevated trace metal be due to excrete some amounts of

them or by synthesis of metalthionein like proteins, which are involved in detoxication of the larvae.

So, on the basis of these data we can conclude:

- Chironomids can be used as an indicator of genotoxic concentrations of pollutants in aquatic ecosystems;
- Very important structures in the polytene chromosomes such as BR system and NOR might be considered as an important model for studying the response of the genome to the heavy metal pollution;
- The functional alterations plus somatic cytogenetic damages (heterozygous inversions, deletions, deficiencies, formation of "pompon" chromosome G) are particularly suitable as biomarkers as these cytogenetic changes are easily identified and provide early warning signals of asverse long effects in organisms;
- Most of somatic chromosome rearrangements are not randomly distributed but occur more frequently in specific sections of the chromosomes composed either by satellite DNA or by transposable elements;
- Analysis of cytogenetic responses is therefore potentially a powerful tool in preventing the long-term effects of anthropogenic stress at the populations and community level.

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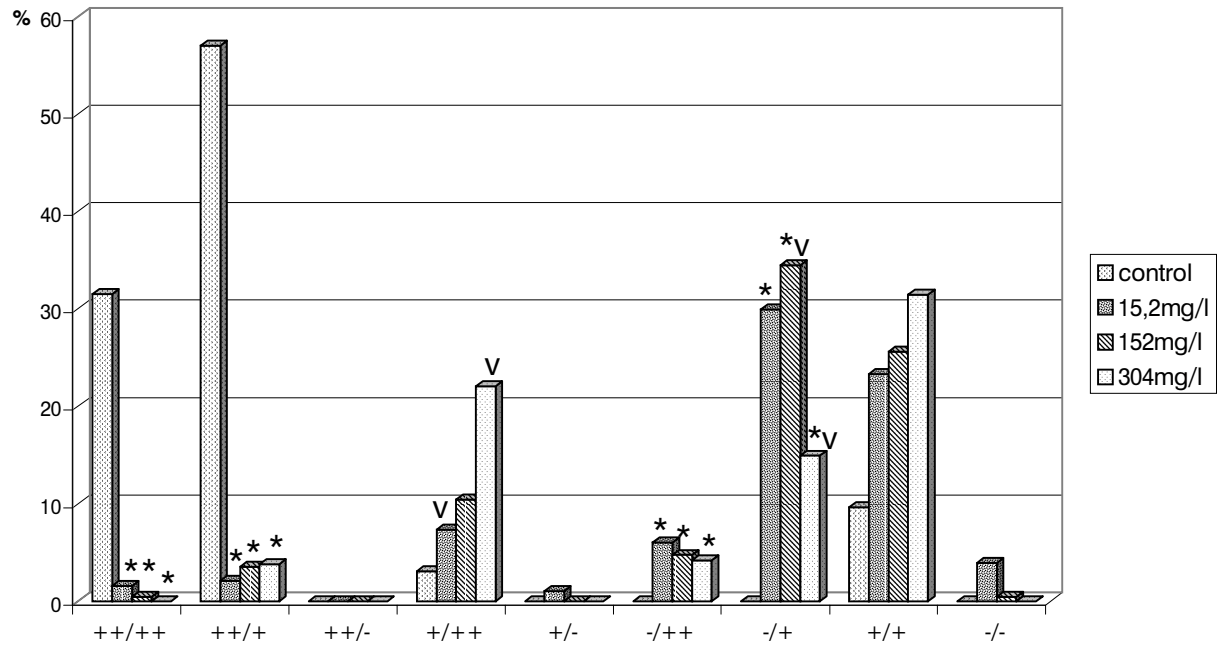
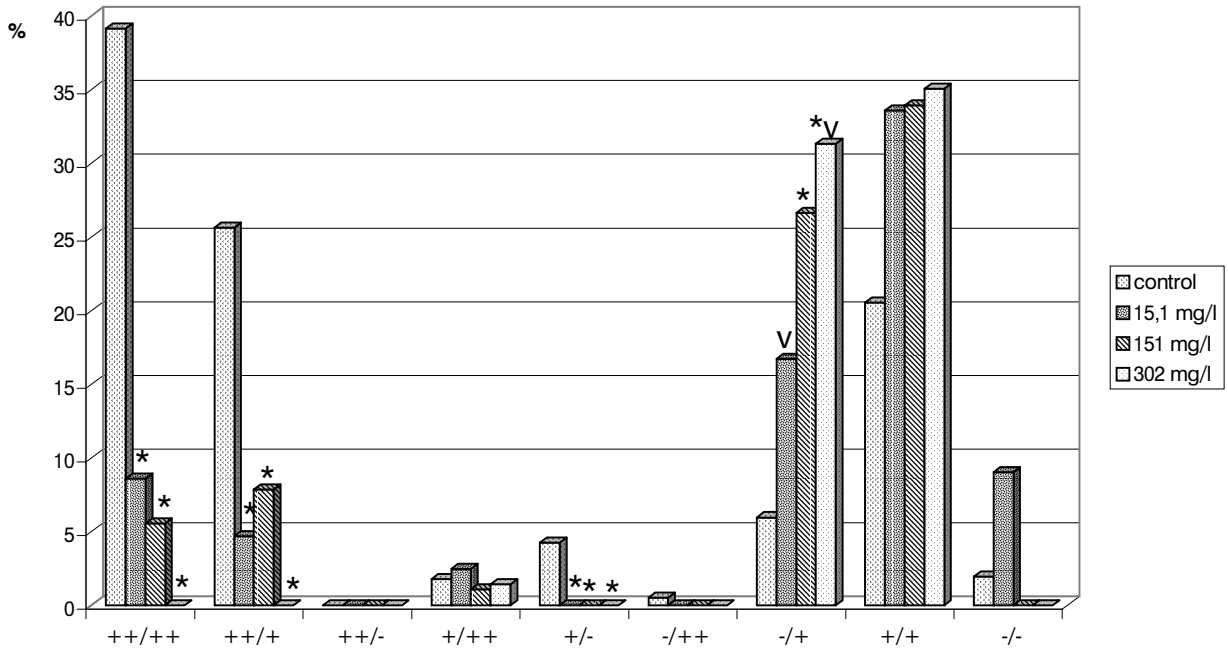
BR activity - F1 (Cr)



BR activity- F1 (Pb)



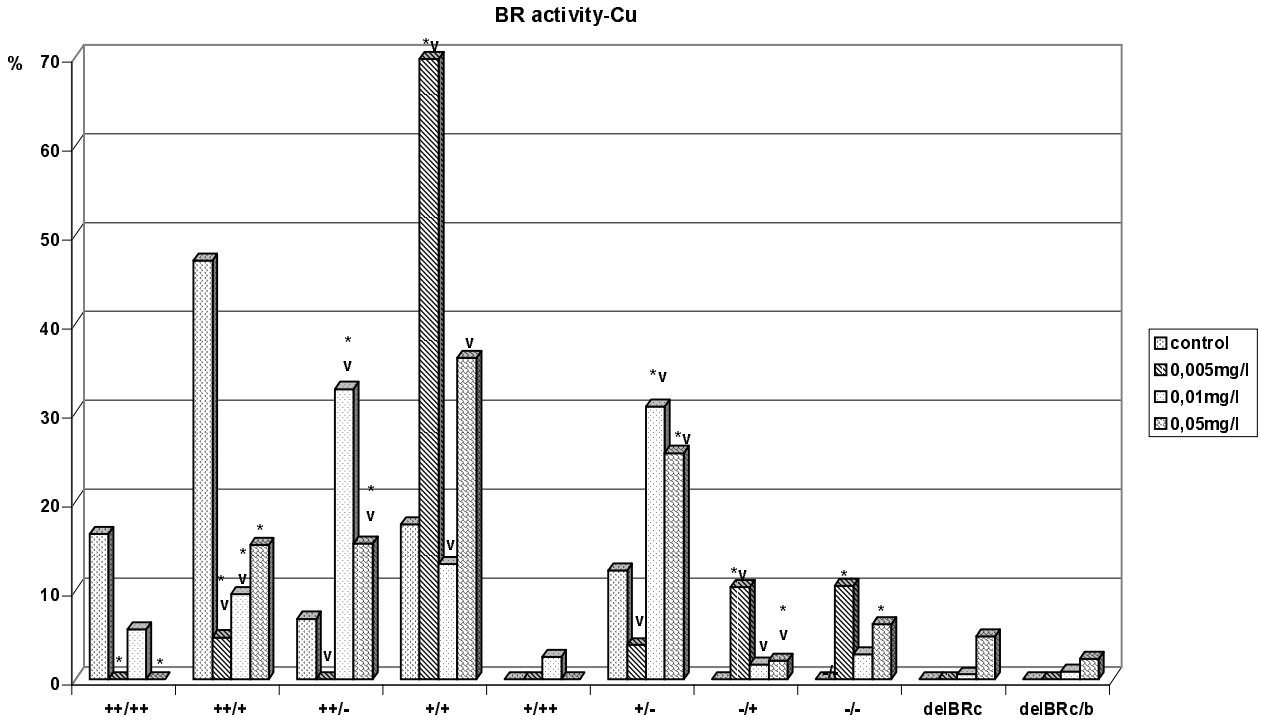


Fig. 1. Change the activity of Balbiani ring system (BRc/BRb)
a. Chronical treatments with Cr ions;
b. Chronical treatments with Pb ions;
c. Acute treatments with Cu ions.
"V" - significant differences between concentrations; "*" significant differences between control and treated materials.
++/++ - high activity of both BRs: BRc and BRb respectively;
+/+ - intermidate activity of both BRs: BRc and BRb respectively;
-/- - low or no activity of both BRs: BRc and BRb respectively;

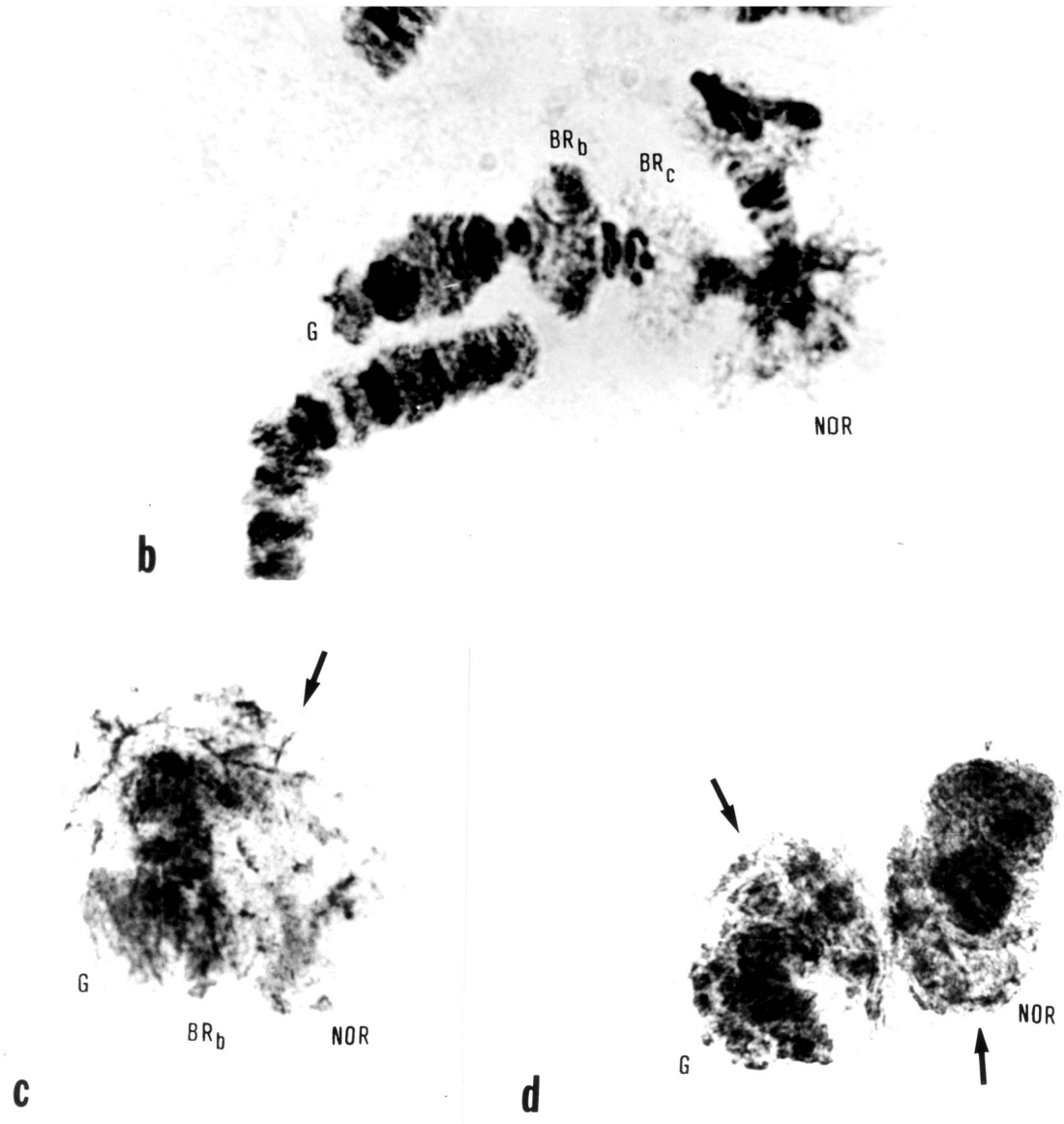


Fig.2. Chromosome G
a. Deletions of chromosome G after treatment with Pb (Two generations);
b. Normal G chromosome ;
c. Chromosome G - "pompon", exposure to Cr ions (15, 2 mg/l);
d. Chromosome G - "pompon", exposure to Cr ions (152 mg/l);
Scheme 1. Schematic map of *Chironomus riparius* with sites of localization of repetitative DNA elements, common breaks after chronic and acute treatments.

- *- Localization of Alu elements;
- o – Localization of Hinf elements;
- + - Localization of breaks after Pb treatments;
- v - Localization after Cr treatments;
- x - Localization after Cu treatments.

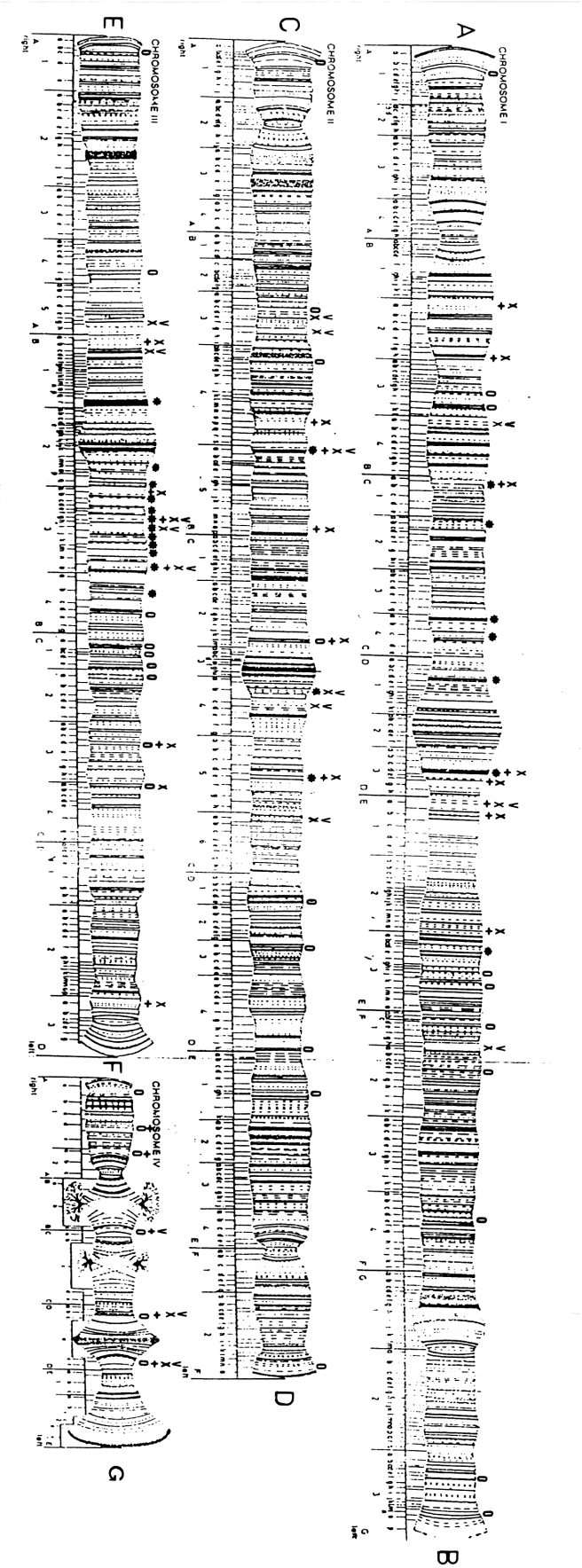


Figure 1. Chromosome maps of the salivary gland chromosomes of Chironomus thummi thummi Kieffer.