



Effect of Cadmium on Salivary Gland Polytene Chromosomes of *Chironomus riparius* Meigen, 1804 (Diptera: Chironomidae)

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Abstract: The genotoxic effect of cadmium ions on the model species *Chironomus riparius* (Diptera: Chironomidae) was studied. The larvae were chronically treated in laboratory conditions with different concentrations of CdCl₂ (0.1 µg/ml, 1.1 µg/ml and 4.0 µg/ml). Numerous somatic chromosome aberrations were found in all four polytene chromosomes. They affected significantly more cells in the treated material compared to the control (P<0.001). On the basis of the observed somatic rearrangements, S-index was calculated; its values increased with increasing concentrations of CdCl₂. Alterations in activity of Balbiani Rings (BRs) and Nucleolar Organizer (NOR) as well as decondensation and appearance of new active puffs were also observed after treatment. In addition, a large number of individuals with morphological deformities affecting mouthparts were found (44–58 %). The induced changes at the cytogenetic and phenotypic level can be considered as a reaction of the individuals to the influence of Cd ions, which confirms their toxic and genotoxic effect.

Key words: *Chironomus riparius*, Cd, genotoxicity, chromosome aberrations, mouthpart deformities

Introduction

Environmental pollution is a global problem, primarily due to the ever-increasing anthropogenic impact. One of the main ubiquitous pollutants are the heavy metals. They are considered hazardous due to their three characteristics: persistence, bioaccumulation and toxicity (PBT) (LAGRANA et al. 2011, ALI et al. 2019). It is very important to assess and monitor the concentrations of potentially toxic heavy metals in different environmental segments as well as in the resident biota because the trophic transfer of these elements in food chains has important implications for wildlife and human health.

One of the non-essential metals with detrimental effects on different groups of organisms in

all aquatic and terrestrial communities is cadmium (WILLIAMS et al. 1986, ÜNYAYAR et al. 2006). Cadmium (Cd) is the seventh most toxic heavy metal as per ATSDR (Agency for Toxic Substance and Disease Registry) ranking (JAISHANKAR 2014), classified as a member of the carcinogenic group I by the International Agency for Research on Cancer (IARC) and as a likely human carcinogen (group B1) by the Environmental Protection Agency (EPA) (MERRILL et al. 2001). Cadmium is a toxic xenobiotic, persistent and widespread pollutant that accumulates in the environment from both anthropogenic and natural sources and can enter the atmosphere or freshwater environment by forming oxides and salts (WILLIAMS et al. 1986, SCULLOS et al. 2001). Cadmium and its compounds are high-

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ly water-soluble compared to other metals (JAISHANKAR 2014) and distributed in the environment, able to remain in soils and sediments for several decades.

Cadmium is a toxic metal and no uniform mechanism of its toxicity has been proposed so far (ÜNYAYAR et al 2006, JAISHANKAR et al. 2014). DEGREAVE (1981) has described that cadmium can modify the metabolism of nucleic acids either by direct reaction or by acting on their synthesis. Cd was found to induce chromosomal aberrations and micronucleus (MN) formation in plant root cells (ÜNYAYAR et al. 2006). *In vitro* experiments have shown that Cd ions have cytotoxic and genotoxic effects on mammalian bone marrow cells (ÇELİK et al. 2005). These ions induce a significant increase in DNA damage and the appearance of different types of chromosomal aberrations in oligochaete species of the family Tubificidae (KRAČUN-KOLAREVIĆ et al. 2015) and in the insect order Orthoptera (DEGREAVE 1981, YOUSEF et al. 2010).

The species of the family Chironomidae (Diptera) are increasingly used as test objects and bioindicators in ecological studies to assess the toxic impact in aquatic ecosystems (ROSENBERG 1992, SELLA et al. 2004, MICHAILOVA et al. 2012b). The genome of individual species, despite their different biology and phylogeny, is highly sensitive to anthropogenic pollution. Exposed to environmental stressors such as different metals ions, the genome responds through various chromosomal transformations at the structural and functional levels (MICHAILOVA et al. 2012b). The larvae of the aquatic midge *Chironomus riparius* Meigen are widely used in aquatic toxicology because of their association with sediments (OECD 2001). This species is considered as a model organism for cytogenetic studies for assessing the genotoxicity in aquatic ecosystems (MICHAILOVA et al. 2012b). Genotoxic effects of trace metals such as Cr, Pb, Al and Cu (MICHAILOVA et al. 2001ab, 2003, 2006) have been established in *C. riparius* larvae. A wide variety of chromosomal changes has been described and interpreted as a reaction of the genome to stressful conditions.

The influence of Cd ions to different developmental stages in chironomid species were analysed first and significant impairment was observed for survival and growth, developmental rate, time to emergence and egg production (POSTMA & DAVIS 1995, SILDANCHANDRA & CRANE 2000, CHOI & HA 2009). Morphological abnormalities such as induction of mouthpart deformities in *Chironomus* have been found as result of Cd exposure (JANSSENS DE

BISTHOVEN et al. 2001, MARTINEZ et al. 2003). Subsequently, alterations in the enzyme systems and the expression of some genes after Cd impact were found in *C. riparius* (LEE et al. 2006, CHOI & HA 2009, PLANELLÓ et al. 2010, NAIR & CHOI 2011, ZHENG et al. 2017, MARTÍN-FOLGAR & MARTÍNEZ-GUITARTE 2017), thus providing a sensitive biomarker of cadmium exposure.

There are no data about the effect of cadmium ions at cytogenetic level on polytene chromosomes of chironomids. This determined the purpose of our study: (1) to trace the genotoxic effect of Cd ions on the structural and functional organisation of salivary gland polytene chromosomes of *C. riparius* larvae treated in laboratory conditions with three different concentrations of CdCl₂ and (2) to analyse external morphological features of treated larvae.

Materials and Methods

Animals and experimental procedure

Laboratory stock of *Chironomus riparius* Meigen reared in standard conditions (MICHAILOVA 1985) was used to obtain fertile egg masses. One egg mass was used for control (without treatment) and three others for treatments with three concentrations of Cd ions. The larvae in all experiments were reared in constant laboratory conditions at 20°C, constant aeration and photoperiod 16 h light : 8 h dark. The concentrations of Cd ions were obtained by diluting stock solution of cadmium chloride (CdCl₂): 0.1 µg/ml; 1.1 µg/ml and 4.0 µg/ml. The concentration of 0.2 µg/ml has been found in fossil uncontaminated sediment (FÖRSTNER & SALOMONS 1980). For our experiment, we have chosen a concentration 2 times lower (0.1 µg/ml), 5 times higher (1.1 µg/ml) and 20 times higher (4.0 µg/ml). The numbers of individuals and cells studied in each treatment are given in Table 1.

Cytogenetic analysis

IV instar (6-7 phase) larvae of *C. riparius* were used and fixed in alcohol : acetic acid 3:1 for cytogenetic analysis. The permanent microscope preparations of salivary gland polytene chromosomes were made following the aceto-orsein staining (MICHAILOVA 1989). Standard chromosome maps of the species (HAGELE 1970, KIKNADZE et al. 1991) were used for determination and localisation of chromosomal aberrations. In the present study, somatic aberrations were considered only (they present only in few cells of an individual). The level of “puffing” activity of BRs and NOR were described according BEERMANN (1971) and three levels of puffing were considered: ++ high, +/+ intermediate and -/- low/no activity.

Table 1. Number (N) of individuals and cells examined in the control and treated material, number of somatic aberrations, S-index and percentage of individuals with malformations.

	No. of studied individuals	No. of studied cells	No of somatic aberrations	S-index	% individuals with deformities
Control	22	361	16	0.727	13.63 %
0.1 µg/ml	18	380	54	3.000	44.44 %
1.1 µg/ml	14	221	47	3.357	57.14 %
4.0 µg/ml	19	500	91	4.789	57.90 %

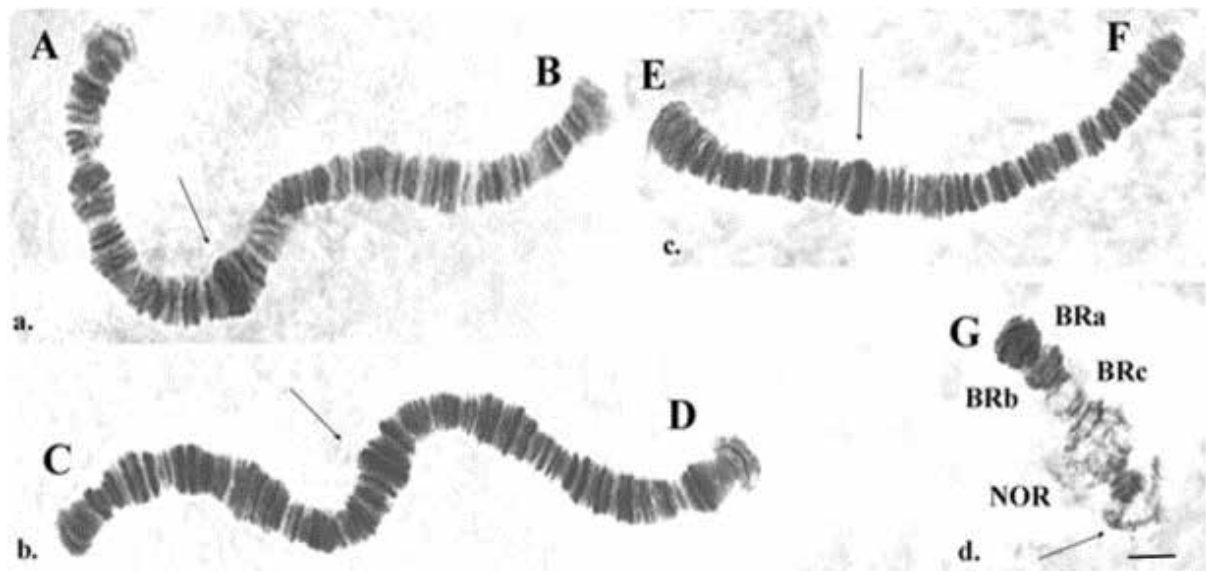


Fig. 1. Polytene chromosomes of *C. riparius* – standard karyotype: a. AB chromosome; b. CD chromosome; c. EF chromosome; d. chromosome G; BRa, BRb, BRc – Balbiani Rings, NOR – Nucleolar Organizer. Arrows indicate centromere regions. Scale-bar 10 µm.

Morphological analysis

To detect any malformations in the larvae, preparations of body and head capsule of each studied cytogenetically individual were made (SAETHER 1979).

Statistical analysis

The frequency of chromosome aberrations was calculated as percentage of the total examined cells and for the comparison between treated and control larvae G-test was used (SOKAL & RHOLF 1995). Student's t-test was used to analyse and compare the results of BRs and NOR activity. Somatic index (S) was calculated based on the observed somatic chromosome aberrations (SELLA et al. 2004). Significant levels were determined at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Results

Polytene chromosomes of *C. riparius* of untreated control

Chironomus riparius has chromosome set $2n=8$, with chromosomes AB, CD, EF and G. AB and CD

chromosomes are metacentric, EF is submetacentric and G is telocentric. The polytene chromosomes of *C. riparius* of the control had the same banding pattern as those of standard maps (HAGELE 1970, KIKNADZE et al. 1991) (Fig. 1). Three Balbiani Rings (BRa, BRb, BRc) and a Nucleolar Organizer (NOR) are localised in chromosome G (Fig. 1d).

In control individuals, 15 somatic heterozygous inversions were found in all chromosomes (Fig. 2a). A deletion of one Balbiani ring BRc (0.2 %) was also observed in chromosome G (Fig. 2b). The S-index was 0.727 (Table 1).

Concerning functional activity, six states of activity of BRs were observed: ++/++, +/+ , ++/-, +/+, -/- and +/- . The state +/- dominated with significant frequency (48.95 %) compared to other levels of activity ($P < 0.05$) (Fig. 2b). Overall, the normal high BRc activity (++) occurred in 19.75 % of the cells tested. NOR in the control was occurring at four levels of activity: +/+, ++/++, +/-, ++/+ (Fig. 2c). The state of high activity, i.e. ++/++ (57.56 %), was the most frequent and significantly higher than

+/- and +++ (P<0.05). The state of low NOR activity (++) was also significantly more frequent (40.97 %) than +/- and +++ (P<0.05). Cells, in which NOR was inactive (-/-), were not detected in the control.

Polytene chromosomes of *C. riparius* treated with CdCl₂

Structural chromosome aberrations: Somatic structural chromosomal aberrations were found in all analysed individuals. Large number of heterozygous paracentric and pericentric inversions on chromosomes AB, CD, EF and G were observed after treatment with the three Cd concentrations and they were significantly more compared to untreated control (P<0.001) (Table 1, Fig. 2a, 3). In addition to the inversions, deletions in the G chromosome were reported, affecting either one Balbiani ring (BRc or BRb) or both (BRc/BRb). After treatment with 1.1 µg/ml and 4 µg/ml CdCl₂, the total number of deletions were significantly more frequent than the control (P<0.001) (Fig. 2b) and after 4 µg/ml, BRc deletions increased significantly compared to the two lower concentrations (P<0.001). The analysis of the results showed that the cells with chromosome aberrations found in the treated material were significantly more than the control: at 0.1 µg/ml CdCl₂ (G=74.328, P<0.001), at 1.1 µg/ml CdCl₂ (G=77.719, P<0.001) and at 4 µg/ml CdCl₂ (G=80.361, P<0.001). The values of calculated S-index increased with increasing concentration of CdCl₂ (from 3.0 to 4.789) (Table 1).

Functional changes: Changes in the functional activity of the Balbiani rings (BRs) and the Nucleolar organizer (NOR) were observed, which resulted in a reduction in their normal high activity (++/++). The Balbiani rings BRc/BRb occurred in six different states of activity after treatment (Fig. 2b). Compared to the control, the three Cd concentrations significantly increased the frequency of cells, in which BRc/BRb were completely suppressed (-/-) (P<0.05). There was a tendency to decrease the frequency of total high activity (++) of BRc at 0.1 µg/ml and 1.1 µg/ml Cd compared to the control but, at 4 µg/ml Cd, it increased again (Fig. 2b). The frequency of low activity (++) of BRc/BRb was significantly reduced at 0.1 µg/ml and 1.1 µg/ml Cd compared to the control (P<0.05) but increased again after the highest concentration.

Cd ions also affected the NOR. The frequency of high activity ++/++ of NOR significantly decreased after treatments compared to the control (P<0.05) while, at the same time, the states of low activity +/+, heterozygous NOR +/- and lack of activity -/- significantly increased (P<0.05) (Fig. 2c).

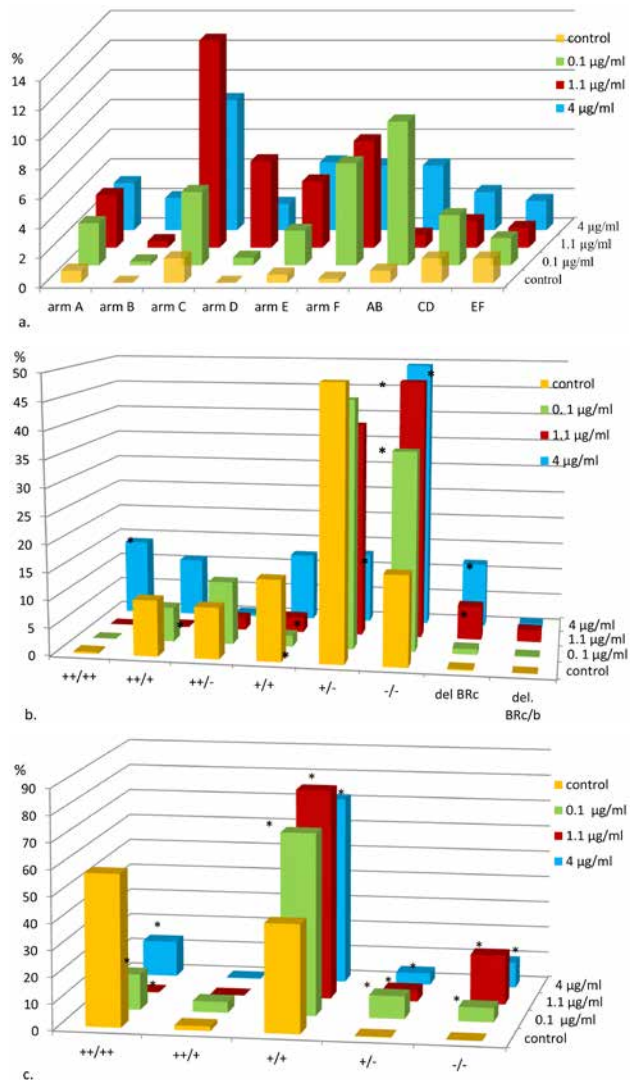


Fig. 2. Frequency of somatic heterozygous inversions, deletions and states of BRs and NOR activity in in polytene chromosomes of *C. riparius*. Scale bar 10 µm; * - significant difference compare with the control (P<0.05); a. Somatic heterozygous inversions; b. Deletions and activity of BRc/BRb; c. NOR activity.

After treatment with the highest concentration of 4 µg/ml, a slight increase in the frequency of active NOR (++/++) was observed (Fig. 2c).

Additional changes in chromosomes of treated larvae were found. Decondensation in the centromeric regions of chromosomes were detected in 3.4 % of the cells after 0.1 µg/ml, 2 % (after 1.1 µg/ml) and 7.8 % (after 4 µg/ml). Cadmium exposure induced also heterozygous state of the centromeric region in the G chromosome with a frequency of 2.1 % (at 0.1 µg/ml Cd), 1.35 % (after 1.1 µg/ml) and 3.4 % (after 4 µg/ml), which were not observed in the control.

New puffs appeared in the chromosomes of *C. riparius* after treatment with 0.1 µg/ml Cd (19.47 %) and 1.1 µg/ml Cd in 35.74 % of the cells. The

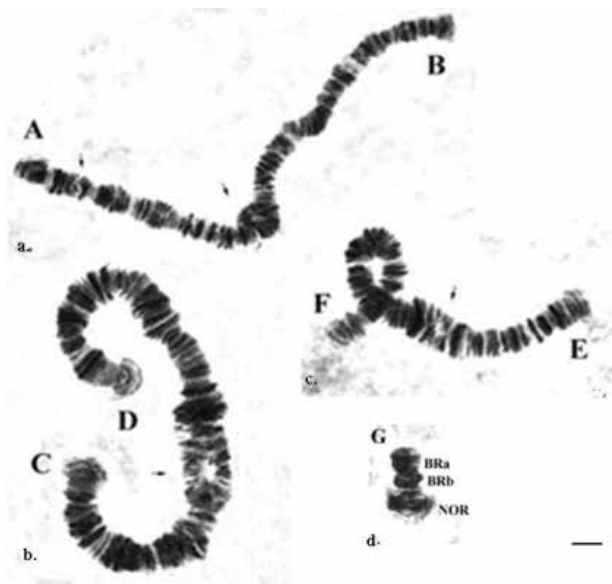


Fig. 3. Somatic aberrations in polytene chromosomes and of *C. riparius*: a. Heterozygous inversion in arm A and centromere region of AB chromosome after 0.1 µg/ml CdCl₂; b. Heterozygous inversion in arm C after 1.1 µg/ml CdCl₂; c. Heterozygous inversion in arm E after 4 µg/ml Cd; d. Deletion of BRC after 0.1 µg/ml CdCl₂. Scale-bar 10 µm.

lowest concentration of 0.1 µg/ml induced puffs in arm B (sections F4, G1 and G2), in arm C (section B4), in arm E (sections A3, A5) and arm F (sections B3, B4) with frequencies of 1 % to 11 %. The puffs in treated with 1.1 µg/ml Cd individuals were localised in arm A (section A2), arm B (sections B4, E1, G1), in arm C (in sections B4, B5), in arm E (sections A3, A4, A5, D1) and arm F (sections C4, D1) with frequencies of 1 % to 21 %.

Morphological deformations: Cd ions induced changes in the phenotype of *C. riparius* expressed by the appearance of morphological deformations in mandibles and (or) mentum. Changes in the mandibles and in the mentum were also found in control in three individuals only (13 %). However, their frequency increased after exposure in a dose-dependent manner and reach up to more than 57 % after exposure to 1.1 µg/ml and 4 µg/ml CdCl₂ (Table 1, Fig. 4).

Discussion

High sensitivity of the *C. riparius* genome to exposure of Cd ions was established. The genome instability was expressed by large number of somatic structural chromosomal aberrations affecting all chromosomes and various functional changes in all analysed individuals. Somatic aberrations occurred significantly more frequent in treated material compared to con-

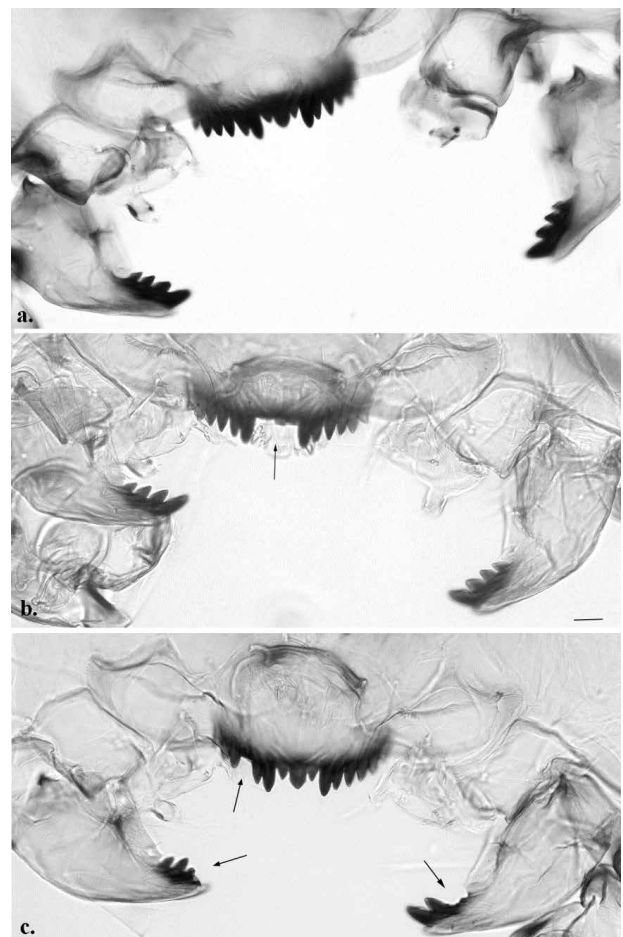


Fig. 4. Malformations in *C. riparius* larvae after treatment with Cd: a. Normal view (control); b. Mentum deformities after 1.1 µg/ml CdCl₂; c. Mentum and mandibles deformities after 0.1 µg/ml CdCl₂. Scale-bar 10 µm.

trol ($P < 0.001$) and increased with increasing cadmium concentration. Generally, the somatic inversions are the most common aberrations as a result of stress on the species genome, indicating a genotoxicity in the environment (SELLA et al. 2004, MICHAILOVA et al. 2012b, PETROVA & MICHAILOVA 2021). Very sensitive to cadmium exposure was the smallest G chromosome and, in addition to the somatic inversions, deletions of Balbiani Rings were found. Loss of genetic material, expressed by somatic deletions, has also been found in *C. riparius* as genotoxic effect of metal ions (Pb, Cr, Al, Cu) (MICHAILOVA et al. 2001ab, 2003, 2006) as well as in individuals inhabiting anthropogenically polluted natural water bodies (PETROVA et al. 2004, SELLA et al. 2004, MICHAILOVA et al. 2012a). Together with somatic inversions, they are considered as one of the proper biomarkers of environmental stress. The calculated S-index also showed increased values with increasing concentration of CdCl₂. The S-index was several times higher in chironomids of the genera *Chironomus* and *Pro-*

diamesa inhabiting heavily polluted biotopes than in unpolluted (SELLA et al. 2004, ILKOVA et al. 2017, MICHAILOVA et al. 2012a, 2015). The increase in the values of the S-index indicates increased sensitivity and genome instability of the species in stressful conditions. Therefore, it can be considered as a good marker for estimate genotoxicity and the pollution in the freshwater biotopes.

The genome of *C. riparius* reacted to the Cd exposure at the functional level also by chromosome changes. Very important chromosome structures in G chromosome as BRs and NOR were affected and their activity was suppressed to varying degrees. Cd suppressed the normal high BRs and NOR activity and cells with no active BRs significantly increased with dose dependent manner. In contrast, PLANELLO et al. (2007) have described that Cd reduces NOR activity but does not affect BRs activity. Regarding to NOR, it is clearly visible significantly decreasing of high activity of NOR after Cd treatment and rising cases of intermediate (+/+; +/-) or no activity (-/-) also. The species of the family Chironomidae show a similar response to different stress agents, expressed by a change in the activity of BRs and NOR, which was observed after treatment with Pb (MICHAILOVA et al. 2001a), Cu (MICHAILOVA et al. 2006), Cr (MICHAILOVA et al. 2001b) and Al (MICHAILOVA et al. 2003). These structures (BRs and NOR) are the most transcriptional active region in the genome and support homeostasis in the cells. Their response to stress factors can serve as a cell activity indicator (GUSEV et al. 2012). Our results confirm that changes in their functioning can be considered as a biomarker of genotoxicity in biotopes (PLANELLO et al., 2007, GUSEV et al. 2012, MICHAILOVA et al. 2012b).

Besides their similar effect on the Chironomidae species genome, the metal ions can demonstrate also specific impact. Decondensation in the centromeric regions of *C. riparius* chromosomes was detected after Cd exposure but with low and insignificant frequencies. In contrast, other studies of *C. riparius* have shown that Al, Pb and Cu induce asynapsis and chromosome decondensation but with much more significant frequencies (MICHAILOVA et al. 2001a, 2003, 2006). It has been suggested that the inhibition of synthesis of proteins involved in condensation of heterochromatin occurred in extreme conditions.

The results indicate the appearance of new specific puffs in the chromosomes of *C. riparius* after treated with 0.1 µg/ml CdCl₂ and 1.1 µg/ml CdCl₂. No such active puffs were observed either in controls or in individuals exposed to the high-

est concentration. Moreover, treatment with Pb and Cr also resulted in specific “puff” activity in *C. riparius* (MICHAILOVA et al. 2001ab), which was absent both in controls or after exposure to Al or Cu (MICHAILOVA et al. 2003, 2006). The appearance of new puffs can be considered as a specific reaction of the genome to the harmful impact of some environmental factors. This is probably a protective mechanism of synthesis of additional proteins necessary for the survival of individuals in specific environmental conditions (MIREJI et al. 2006).

Results of our experiment showing a slight increase and recovery of the normal high activity of NOR and BRs after exposure to the highest Cd concentration (4 µg/ml) were very interesting. The restoration of the activity of BRc and NOR were observed also in *C. riparius* after treatment with Pb and Al (MICHAILOVA et al. 2001a, 2003). The recovery of normal functional activity in these cases can be associated with the so-called “metal resistance” found in some chironomid species (POSTMA et al. 1994). It is expressed by the development of mechanisms increasing the species’ resistance to the corresponding stress factor and protect the cell from damage. It is possible to induce the synthesis of some additional proteins and metallothioneins (MTs), which are involved in the process of detoxification of nonessential metals such as Ag, Hg and Cd (AMIARD et al. 2006). Moreover, an increase in the amount of MT proteins was found in *C. riparius* after laboratory treatment with various concentrations of Cd (GILLIS et al. 2002, TOUSOVA et al. 2016).

In addition to chromosome alterations at the cytogenetic level, Cd ions induced also changes at the phenotypic level and some morphological deformations affecting mentum and mandibles of *C. riparius* larvae were found. Morphological abnormalities such as the induction of deformities in mouth-parts of chironomid larvae are considered an evidence for the teratogenic effect of cadmium (JANSSENS DE BISTHOVEN et al. 2001, MARTINEZ et al. 2003, DI VEROLI et al. 2014). Pathological changes in mouthparts of chironomid larvae have been repeatedly observed in environments polluted with heavy metals (JANSSENS DE BISTHOVEN et al. 1998, DI VEROLI et al. 2014, ILKOVA et al. 2017). Such changes have also been demonstrated in laboratory experiments after chronic treatment with Cr (MICHAILOVA et al. 2001b), Pb and Hg (VERMEULEN et al. 2000), Zn (MARTINEZ et al. 2001) and Cd (WATTS & PASCOE 2000). They have been considered as a potential tool for the biomonitoring of the effects of metals such as cadmium (JANSSENS DE BISTHOVEN et al. 2001, MARTINEZ et al. 2003).

Conclusions

The stress factors such as cadmium ions result in high genome instability in *C. riparius* individuals. The genotoxic effect of Cd ions was established and it was expressed by the appearance of large number of somatic structural aberrations in the polytene chromosomes with significantly higher frequencies as well as a wide range of functional changes. Cd ions induced also malformations in mentum and mandibles in *C. riparius*. The results indicate that the induced changes at the genomic and phenotypic level can be considered as a reaction of the treated individuals to the impact of Cd, confirming its toxic and genotoxic effect on organisms. The results support and expand data from previous studies of chironomids proving their successful use to assess the presence of genotoxicity in water ecosystems.

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