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EFFECT OF LEAD ON POLYTENIC CHROMOSOMES FROM SALIVARY GLANDS OF *CHIRONOMUS PLUMOSUS* L. AND *GLYPTOTENDIPES GLAUCUS* MG. (DIPTERA, CHIRONOMIDAE)

© **Yu.V. Belonogova, N.A. Durnova, A.S. Sheremetyeva**

Saratov State Medical University named after Razumovsky, Saratov, Russia

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❀ **Background.** Experimental conditions allow to determine the structural and functional changes of polytene chromosomes under the influence of free ions of an individual metal. **Materials and methods.** *C. plumosus* (L.) and *G. glaucus* (Mg) larvae were placed in solutions of lead nitrate: 0.01, 0.02, 0.1 and 0.5 mg/l. Exposure — 12 h. Analysis of preparations of polytene chromosomes was carried out using the Carl Zeiss PrimoStar microscope. The functional activity factor of the nucleolus organizer (NOR), the coefficient of genetic activity of the Balbiani ring (BRR) was calculated. **Results.** Equations of the dependence of the change in the coefficients: NOR = 5,187−0,01 lnC for *C. plumosus* and NOR = 2,11−0,03 lnC for *G. glaucus*; BRR = 1,504−0,04 lnC for *C. plumosus* and BRR = 2,018 + 0,03 lnC for *G. glaucus*. **Conclusion.** With an increase in the concentration of lead in both *C. plumosus* and *G. glaucus* decreases NOR, which implies a decrease in the intensity of protein biosynthesis processes. BRR decreases in *C. plumosus* and increases in *G. glaucus*. The different genome reactions of the two species indicate the existence of different mechanisms of adaptation to lead ions

❀ **Keywords:** chironomids; polytene chromosome; nucleolus organizer; Balbiani ring; heavy metals; lead nitrate.

ВЛИЯНИЕ СВИНЦА НА ПОЛИТЕННЫЕ ХРОМОСОМЫ СЛЮННЫХ ЖЕЛЕЗ *CHIRONOMUS PLUMOSUS* L. И *GLYPTOTENDIPES GLAUCUS* MG. (DIPTERA, CHIRONOMIDAE)

© **Ю.В. Белоногова, Н.А. Дурнова, А.С. Шереметьева**

ФГБОУ ВО «Саратовский ГМУ им. В.И. Разумовского» Минздрава России, Саратов

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❀ Представлены результаты экспериментальной работы по изучению влияния нитрата свинца различных концентраций при 12 ч экспозиции на структурно-функциональные характеристики политенных хромосом клеток слюнных желез двух видов хирономид: *Chironomus plumosus* (Linnaeus, 1758) и *Glyptotendipes glaucus* (Meigen, 1818). В качестве критерия функционального состояния политенных хромосом были использованы количественные показатели относительных размеров постоянно генетически активных районов — ядрышкового организатора и кольца Бальбиани (коэффициенты NOR и BRR соответственно). Впервые получены уравнения зависимости изменения коэффициента функциональной активности ядрышкового организатора от концентрации ионов свинца в среде NOR = 5,187−0,01 lnC для *C. plumosus* и NOR = 2,11−0,03 lnC для *G. glaucus* и коэффициента функциональной активности кольца Бальбиани: BRR = 1,504−0,04 lnC для *C. plumosus* и BRR = 2,018 + 0,03 lnC для *G. glaucus*. Полученные зависимости позволили провести сравнительный анализ морфологических показателей, отражающих интенсивность транскрипции генов политенных хромосом в присутствии ионов свинца. С увеличением концентрации ионов свинца в среде активность ядрышкового организатора и кольца Бальбиани политенных хромосом *C. plumosus* снижалась. При тех же условиях активность ядрышкового организатора политенных хромосом *G. glaucus* снижалась, а кольца Бальбиани повышалась. С увеличением концентрации свинца значения NOR снижались у *C. plumosus* и *G. glaucus*, что предполагает снижение интенсивности процессов биосинтеза белка. Значения BRR уменьшались у *C. plumosus* и увеличивались у *G. glaucus*. Различия в реакции геномов двух видов указывают на существование у них различных механизмов адаптации к повышенным концентрациям свинца.

❀ **Ключевые слова:** хирономиды; политенные хромосомы; ядрышковый организатор; кольцо Бальбиани; тяжелые металлы; нитрат свинца.

INTRODUCTION

Heavy metals enter freshwater ecosystems through industrial effluent and aerosol emissions and have toxic effects to both ecosystems as well as wildlife in relatively low concentrations [1–3]. Besides their effect on the ecology of populations of hydrobionts and on the morphological and physiological features of individual organisms, heavy metals can cause structural and functional changes in genetic material [4–7]. To study the effect of heavy metals on genetic material, chironomid larvae (Diptera, Chironomidae) can be used. These larvae have a wide range and dominate in number within the aquatic invertebrates; they are known to bioaccumulate xenobiotics, giving them significant value in ecological and toxicological tests for biomonitoring of freshwater ecosystems [8]. The availability of polytene chromosomes in the interphase cells of the salivary glands of larvae allows cytogenetic analysis which is impossible for other species of invertebrate hydrobionts. By means of large polytene chromosomes with clear species-specific disk patterns, it is possible to identify each in karyotype, and to detect natural and induced genome variability of both individuals and the entire population [9, 10].

The main criteria for the assessment of structural and functional variability in polytene chromosomes are an increase in frequency of chromosomic rearrangements, changes in dimension of the nucleolar organizer, Balbiani rings, puffs, and the occurrence of puffs *de novo*. Contamination of water reservoirs with heavy metals increases the frequency of structural rearrangements of polytene chromosomes in *Chironomus riparius* larvae (heterozygous paracentric and pericentric inversions; deletions; deficiencies; heterozygosis in disks, including centromeric heterozygosis; increase in the thickness of some intercalary disks; and asynapsis of homologues), as well as occurrence of somatic mosaics by paracentric and pericentric inversions was detected [11]. Occurrence of pompom-like chromosome IV (G) is proposed as a cytogenetic marker of water reservoir contamination with heavy metals [12]. When studying the effect of lead acetate on polytene chromosomes in *Glyptotendipes barbipes*, different structural and functional deviations were detected. Pericentric heterozygous inversions and asynapsis of homologues were detected in chromosome I; in chromosome II, under-replication of the centromeric area and pairing in the centromeric area was found [13]. It was determined for chromosomes in *Chironomus bernensis* that only the nucleolar organizer (NOR_2) in the E arm responds to contamination with heavy metals; changes in NOR_1 and BR_s do not occur [14].

However, it is almost impossible to determine the specificity of the effect of individual metals on the structural and functional properties of genetic material in the complex matrix of contaminants penetrating the water reservoirs. Univocal dependence between the total metal concentration in the surface water and the response of the genome has not been detected; indicators can vary due to the sex and age composition of the population. Metals are also constantly rearranged in freshwater ecosystems, as they undergo chemical and biochemical transformations between the components of the ecosystem: water, bottom sediments, and hydrobionts. These processes have different intensities, and depend on the hydrochemical properties of the environment, dynamics of accumulation in the bottom sediments, and bioaccumulation and migration in the food chains. These factors explain the co-existence of different forms of metals in freshwater ecosystems [15–18]. For example, lead is more frequently found in freshwater reservoirs in the form of fulvate and hydro complexes or in adsorbed form on the surface of the bottom sediments and suspended matters [19]. These compounds demonstrate different chemical activity and bioavailability, which also complicates the assessment of metal toxicity. Experimental conditions can avoid such obstacles, where it is possible to then assess the effect of free ions of the individual metal at set concentrations and exposures. Such examinations are required for determining possible regularities of response of the genetic apparatus to the presence of metal compounds in the medium.

Well-dissolved lead nitrate was used as a toxicant for the study of the presence of free metal ions in solution; the larvae of chironomids *Chironomus plumosus* and *Glyptotendipes glaucus* were used as model organisms. Both have wild areas, but the ecological features are typical for each; larvae of *C. plumosus* are included in infauna, while larvae of *G. glaucus* are attributed to phytophilous species [20, 21]. Comparative analysis of the response of the active areas of polytene chromosomes to the presence of heavy metals in the medium was not conducted for these species.

The goal of this study was to determine the mathematical dependence of the change of factors involved in the functional activity of the nucleolar organizer NOR and Balbiani rings BRR of polytene chromosomes of *C. plumosus* and *G. glaucus* on the concentration of lead ions as well as compare species-specific structural and functional variability of the polytene chromosomes.

MATERIALS AND METHODS

Larvae of chironomids of the summer generation of IV age of *Chironomus plumosus* ($2n = 8$) and *Glyptotendipes glaucus* ($2n = 8$) were collected from the population of lake Sazanka, located within the boundaries of Engels of Saratov region (51°29′14″N, 46°04′52″E). *C. plumosus* usually inhabit the silt bottom of water reservoirs; therefore, the bottom sampler DAK-250 with a gripping area 1/40 m^2 was used. Larvae of *G. glaucus* inhabited the semi-aquatic plants, and any embedded substrates for the major part of the year were collected from stems of the common reed embedded in water. Advantages of *G. glaucus* as the test organism for toxicological examinations were justified earlier. For example, larvae of this species (in contrast to *C. plumosus*) are available for mass collection almost all year round [22]. Specific identification of larvae was conducted by karyotype using cytophotomap of chironomids [23]. For the purpose of acclimation, larvae were held in laboratory conditions for 24 h in dechlorinated water at 19 °C–23 °C.

According to regulations, the maximum allowable concentrations (MAC) of harmful substances in water reservoirs of fisheries (Order of the Ministry of Agriculture of Russia dated 12.13.16 No. 552) for lead is 0.01 mg/L. Working solutions were prepared before beginning the experiment by dilution of 1 M standard solution of lead nitrate to concentrations of 0.01, 0.02, 0.1, and 0.5 mg/L. The set of experimental solutions with increased concentrations of toxicant relates to the values stated in the regulations at 1 MAC, 2 MAC, 10 MAC, and 50 MAC, and aims to show the different degrees of effect these concentrations have on the structural and functional indicators of polytene chromosomes of the experimental larvae. Exposure of 12 h was used as it was previously demonstrated that tissues of larvae accumulate the lead ions most actively at a 12-h exposure [24]. The experiment was conducted without changing the medium; a minimum of three replicants were used at equal volumes for the experimental and reference groups (per 6–10 species). Chromosomes of 10 cells of salivary glands were examined for each species. Upon completion of experiment, the larvae were dried for 1 min on filtration paper and fixed in a mixture of ethanol and acetic acid in the ratio of 3:1. Preparations from cells of salivary glands of larvae were made according to the aceto-orcein technique that allows simulataneous fixing and painting of chromosomes [25]. Micropreparations were analyzed using microscopy (Carl Zeiss Primo Star and video eyeglass CMOS3, 1 MP with magnification 15 × 40). Morphometric analysis was conducted using a micrometer eyepiece.

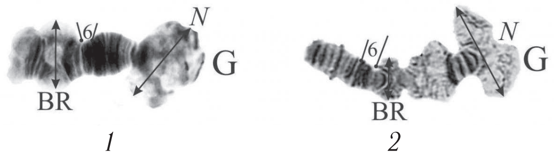

Fig. 1. Measured sites of chromosome IV (G): *1* — *C. plumosus*; *2* — *G. glaucus*

Quantitative indicators of the relative dimensions of the permanently genetically-active areas of the nucleolar organizer and Balbiani rings (factors NOR and BRR, respectively) were used as criteria to assess the functional state of polytene chromosomes. It has been demonstrated that these factors are effective morphological indicators that show the intensity of gene transcription of polytene chromosomes [26, 29]. Factors independent from the absolute dimensions of chromosomes permitted comparative analysis of functional activity of the genomes of two types of chironomids (untreated controls and those treated with different concentrations of toxicant). Factors were determined by the following calculations: NOR is the ratio of the maximum diameter of nucleolar organizer (N) and the width of intact area of the 6th chromosome IV (G); BRR is the ratio of maximum diameter of Balbiani rings to the width of intact area of the 6th chromosome IV (G) (Fig. 1).

Statistical and graphic analyses were conducted using specialized program packages for Excel, LaTeX, and Statgraphics. Regression analysis and approximation of experimental data were used [27, 28]. Mean arithmetic and standard deviations were used to analyze characteristics of selections. Statistical significance of differences between the values of the control selection and selections under effect of different concentrations of toxicant were assessed by means of one-factor dispersion analysis (Student's *t*test). Differences were considered significant at $p < 0.05$.

Table 1

Coefficient of functional activity of nucleolar organizer (NOR) for *C. plumosus* and *G. glaucus*

Toxicant concentration, mg/l	Values NOR $M \pm m, p$	
	C. plumosus	*G. glaucus*
Control	3,45 ± 0,13	2,24 ± 0,07
0.01	5.16 ± 0.14, <0.001	2.48 ± 0.11, <0,05
0.02	4.94 ± 0.22, <0.001	2.03 ± 0.11, >0.05
0.1	5.14 ± 0.10, <0.001	2.04 ± 0.07, <0.01
0.5	5.05 ± 0.09, <0.001	2.07 ± 0.10, >0.05

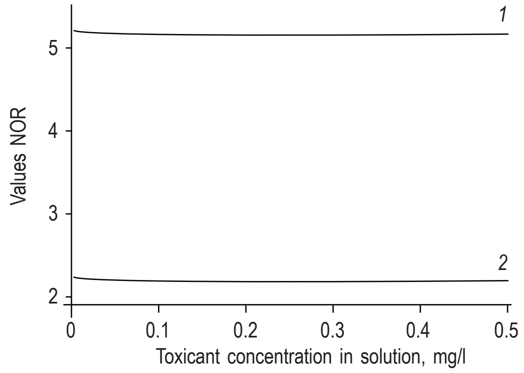

Fig. 2. Dependence of the activity of the nucleolar organizer (NOR) on the concentration of lead ions, exposure time 12 h: *1 — C. plumosus*, *2 — G. glaucus*

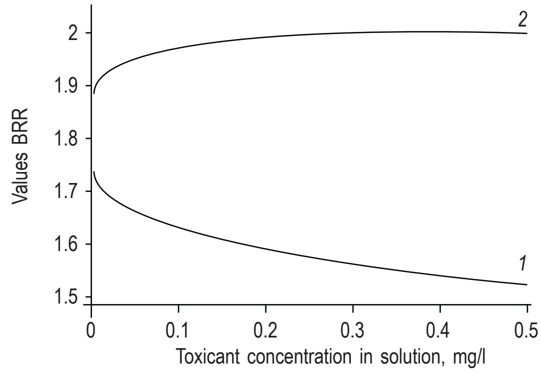

Fig. 3. Dependence of the activity coefficient of the Balbiani ring (BRR) on the concentration of lead ions, exposure time 12 h: *1 — C. plumosus*, *2 — G. glaucus*

Table 2

Coefficient of functional activity of the Balbiani ring (BRR) for *C. plumosus* and *G. glaucus*

Toxicant concentration, mg/l	Values BRR $M \pm m$, p	
	C. plumosus	*G. glaucus*
Control	1.52 ± 0.03	1.69 ± 0.09
0.01	1.80 ± 0.05, < 0.001	1.85 ± 0.07, < 0.001
0.02	1.50 ± 0.03, < 0.001	2.03 ± 0.07, < 0.01
0.1	1.67 ± 0.03, < 0.001	1.80 ± 0.08, < 0.001
0.5	1.51 ± 0.02, < 0.001	1.98 ± 0.10, < 0.01

RESULTS

Values of NOR factors for polytene chromosomes of *C. plumosus* and *G. glaucus* are presented in Table 1. After 12 h of exposure, *C. plumosus* in solutions at concentrations 0.01, 0.02, 0.1, and 0.5 mg/L of toxicant had significantly different NOR values from the control group ($p < 0.001$). Under the same experimental conditions, NOR values of treated *G. glaucus* significantly differed from the control group at toxicant concentrations of 0.01 mg/L ($p < 0.05$) and 0.1 mg/L ($p < 0.01$).

NOR values of the two types of chironomid larvae in the presence of toxicant in the medium exceeded those of the controls. However, after the lead concentration in the experimental solutions was increased from 0.01 to 0.5 mg/L, a reduction in NOR values was observed (though not significantly). Dependence of NOR on the lead concentration in solution can be described with the following logarithmic curve equations:

NOR = 5.187 − 0.01 lnC for *C. plumosus*
NOR = 2.11 − 0.03 lnC for *G. glaucus*

Where C is the concentration of lead ions in the medium (mg/L) and NOR is the value of the factor of functional activity of the nucleolar organizer.

In the equations above, the factors with arguments −0.01 and −0.03 indicate the tendency of reduction of NOR values with the increase in toxicant concentration. Relative dimensions of the nucleolar organizer of polytene chromosomes of *C. plumosus* and *G. glaucus* were reduced with increased toxicant concentration, indicating a one-type response of the genome of both species to the presence of toxicant in the medium (Fig. 2).

BRR values of polytene chromosomes of *C. plumosus* and *G. glaucus* are presented in Table 2. In experimental solutions at concentrations of 0.01, 0.02, 0.1, and 0.5 mg/L, the BRR value of polytene chromosomes of both species significantly differed from the controls for *C. plumosus* ($p < 0.001$) and for *G. glaucus* ($p < 0.001$, $p < 0.01$).

Dynamics of changes in BRR values are described with equations of dependence of the factor of functional activity of the Balbiani ring on the concentration of lead ions:

BRR = 1.504 − 0.04 lnC for *C. Plumosus*
BRR = 2.018 + 0.03 lnC for *G. glaucus*

Where C is the concentration of lead ions in the medium (mg/L); BRR is the value of the factor of functional activity of the Balbiani ring.

In the provided equations, the factors have different signs (−0.04 and +0.03) accordingly, reflecting the oppositely-directed dynamics of changes in the relative size of Balbiani rings in the two types of chironomids. As long as the lead ion concentration is increased, the values of BRR of the polytene chromosomes of *C. plumosus* are reduced; this parameter is increased in polytene chromosomes of *G. glaucus* (Fig. 3).

DISCUSSION

An overview of previously published results on the effects of different factors on the structural and functional variability of polytenic chromosomes of chironomids indicates a wide range of detected cytogenetic effects. These include the activity and depression of puffs available in karyotype, the occurrence of puffs *de novo*, condensation—decondensation of chromosomes, modification of disk structure, activation of pretelomeric side, and the occurrence of chromosome aberrations. Permanent transcriptionally-active areas of polytene chromosomes, the nucleolar organizer and Balbiani ringss, are the most sensitive loci to environmental factors [29]. Morphometric parameters of these areas are changed due to the condensation—decondensation of chromatin, which is considered an indicator of the cell functional genome activity [30, 31].

It is known that the nucleolar organizer contains copies of rRNA genes, and the subunits of Palade's granules are formed in the same area. This is the basis for the nucleolar organizer to be considered as an indicator of the "common functions" of the genome [30, 31]. We have detected that under 12-h of exposure to an increase in concentration of lead ions from 0.01 to 0.5 mg/L is insufficient to generate significant changes, but it does naturally reduce the transcriptional activity of genes of the nucleolar organizer of polytene chromosomes in *C. plumosus* and *G. glaucus*, making it possible to assume that there is also a reduction in intensity of the biosynthesis of general-purpose proteins in the cells of salivary glands.

Balbiani ringss, as a tissue-specific puff, contain permanently-expressed genes encoding unique proteins of salivary gland secretions of choromonides [25]. Examination of the effect of lead on the functional activity of this area of polytene chromosomes of the two types of chiromonides allowed detection of certain differences in their responses. In case of 12-h exposure, the increase of lead ions in the medium seems to suppress transcriptional activity of the tissue-specific genes of the Balbiani rings in *C. plumosus* and stimulate those of *G. glaucus*.

Tissue-specific puffs of larvae *C. plumosus* are the most active in accumulation of lead ions under 12-h of exposure [24]. Because of this, it is possible to explain the obtained equations of dependence reflecting coordinated and simultaneous reduction of activity in the nucleolar organizer and Balbiani rings of *C. plumosus*. The detected tendency will likely be maintained with an increase in exposure, though this remains to be demonstrated by further research. The genome of *G. Glaucus* ambiguously responds to changes of concentration of the lead ions under the same exposure: expression of genes of the Balbiani rings is increased on the background of reduction of activity of nucleolar organizer. Such a combination of indicators of the structural and functional variability of polytene chromosomes was also observed in testing the effect of phenol on the two types of chiromonides larvae, *Camptochironomus tentans* and *Prodiamesa olivacea* [32]. Different genome responses of chiromonides larvae of the species obtained in this study (*C. plumosus* and *G. glaucus*) likely indicate the availability of different adaptation mechanisms to increasing concentration of lead ions. These are likely based on the activation of the product of the secretory material (tissue-specific proteins) of eurybiontic *G. glaucus* under control of genes of the Balbiani rings. Activity of these genes in cells of salivary glands controls several regulatory systems, one of which is autonomous, and functions according to the principle of feedback depending on the degree of filling of the salivary glands ducts with secretion [25]. Increase of lead concentration likely stimulates release of the secretions of the salivary gland ducts and as a result, activates the genes of the Balbiani rings. Larvae of chironomids *C. plumosus* likely do not have such a mechanism, or it is suppressed in the case of an increase of lead ion concentration.

Adaptive responses of the organism ensuring homeostasis and survival are based on gene expression modification. The obtained data in this study demonstrates the availability of different strategies of homeostatic support in chironomids based on modification of gene expression that ensures their adaptation and survival in contaminated water reservoirs. Determined species-specific features of modifications of the indicators of structural and functional lability of the nucleolar organizer and Balbiani rings that were dependent on the concentration of the lead ions in the medium define the competitiveness of the studied species. Presence of lead ions in the medium suppresses functional activity of the genome of *C. plumosus*; the response of the genome of *G. glaucus* is ambiguous, likely speaking to the higher ecological plasticity of the latter. Experimental research resulted in mathematical dependencies describing variability at the level of the cell genome dynamics that can be taken into account to make models of the system of genetic homeostasis of chironomid larvae populations. Increase in concentration of exposure in future experiments will likely allow more detailed examination of the mechanisms of adaptation of the individual organisms as well as the overall population and, therefore, of the biological consequences of water reservoir contamination with lead compounds. Experimental research on the chironomid genome response to heavy metals in the medium has given perspectives for the resolution of the biological issues of environmental protection.

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❀ Information about the authors

Yulia V. Belonogova – Candidate of Biology, Senior Lecturer, Department of General Biology, Pharmacognosy and Botany. Saratov State Medical University named after V.I. Razumovskiy, Saratov, Russia. SPIN: 3658-8797. E-mail: belonogova.u@gmail.com.

Natalia A. Durnova – Doctor of Biology, Associate Professor, Head of Department, Department of General Biology, Pharmacognosy and Botany. Saratov State Medical University named after V.I. Razumovskiy, Saratov, Russia. SPIN: 3348-2957. E-mail: ndurnova@mail.ru.

Anna S. Sheremetyeva – Assistant, Department of General Biology, Pharmacognosy and Botany. Saratov State Medical University named after V.I. Razumovskiy, Saratov, Russia. SPIN: 3755-4410. E-mail: anna-sheremetyewa@yandex.ru.

❀ Информация об авторах

Юлия Владимировна Белоногова — канд. биол. наук, старший преподаватель, кафедра общей биологии, фармакогнозии и ботаники. ФГБОУ ВО «Саратовский государственный медицинский университет им. В.И. Разумовского» Минздрава России, Саратов. SPIN: 3658-8797. E-mail: belonogova.u@gmail.com.

Наталья Анатольевна Дурнова — д-р биол. наук, доцент, заведущая кафедрой, кафедра общей биологии, фармакогнозии и ботаники. ФГБОУ ВО «Саратовский государственный медицинский университет им. В.И. Разумовского» Минздрава России, Саратов. SPIN: 3348-2957. E-mail: ndurnova@mail.ru.

Анна Сергеевна Шереметьева — ассистент, кафедра общей биологии, фармакогнозии и ботаники. ФГБОУ ВО «Саратовский государственный медицинский университет им. В.И. Разумовского» Минздрава России, Саратов. SPIN: 3755-4410. E-mail: anna-sheremetyewa@yandex.ru.