EFFECT OF TRACE METALS AT ACID AND NEUTRAL CONDITIONS ON THE STRUCTURE AND FUNCTION OF POLYTENE CHROMOSOMES IN *CHIRONOMUS* SPECIES

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With 1 figure and 1 table

ABSTRACT. Changes in the genome of *Chironomus acidophilus* from the environment and *C. riparius* in the laboratory in response to trace metals at, respectively, acidic and neutral pH were examined. Marked somatic structural and functional aberrations, a new Balbiani ring and specific 'puffs' were observed in the salivary gland polytene chromosomes of both species. The reasons for these alterations are discussed in relation to acidity and metal contamination.

RESUMO. Alterações no genoma do *Chironomus acidophilus* a partir do ambiente e do *C. riparius* em laboratório foram examinadas em resposta aos metais vestigiais, em ph ácido e pH neutro, respectivamente. Aberrações estruturais e funcionais somáticas marcadas, um novo anel de Balbiani e "puffs" específicos foram observados nos cromossomas de polytene das glândulas salivares de ambas as espécies. São discutidas as razões para estas alterações relativamente à acidez e contaminação por metal.

KEY WORDS: Chironomidae, polytene chromosomes, chromosome rearrangements, trace metals, acid mine drainage

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INTRODUCTION

Aquatic ecosystems are recipients of various pollutants, most of which have potential genotoxic effects and thus may result in ecological damage. Genotoxic effects of environmental pollutants can be indicated by gene mutation, chromosome and DNA damage (BRUSIK, 1980). Chironomid larvae possess salivary gland polytene (giant) chromosomes that are very sensitive to contaminants, especially trace metals (SELLA *et al*., 2004). These chromosomes have a well characterized band structure that allow precise cytogenetic analysis (MICHAILOVA, 1989), so facilitating the use of chironomids as indicators of environmental trace metal genotoxicity (MICHAILOVA et al., 2003).

This paper reports on the genotoxic effect of trace metals at neutral and acid pH on two species of *Chironomus*. *C. acidophilus* Keyl was collected from the Afon Goch a UK river subject to long term (approx. 250 years) trace metal pollution from acid mine drainage (AMD) (BOULT *et al*., 1994). *C. riparius* Mg was exposed to aluminium (Al) at neutral pH under controlled laboratory conditions. We selected Al, and at neutral pH, as the genotoxicity of Al under these conditions have been poorly studied despite earlier work showing that the metal is bioavailable and toxic to a range of benthic invertebrates (see refs in MICHAILOVA *et al*., 2003). *C. acidophilus* is only the species present in the highly acidic Afon Goch. Its karyotype (2n=8 with chromosome arm combination AE, CD, FB G) indicate that *C. acidophilus* belongs to the "*pseudothummi*" cytocomplex (Keyl & Keyl, 1959; Keyl, 1962). The Balbiani ring (BR) and nucleolar organizer (NOR) are located on chromosome G. *C. riparius* is a widely distributed species and belongs to *"thummi"* cytocomplex (2n = 8, with chromosome arm combinations AB CD EF G). Chromosome G has the three Balbiani rings (BRa, BRb, BRc) and a nucleolar organizer (NOR) (MICHAILOVA, 1989; KIKNADZE *et al.*, 1991).

MATERIAL AND METHODS

C. riparius was reared in the laboratory from original stock collected from an unpolluted pond near Sofia, Bulgaria. Three generations (egg masses and larvae) were exposed to 500 μg L^{-1} Al at neutral pH under controlled laboratory conditions (16 hr light/ 8 hr dark cycle; 20°C) using 'standard snail water' of known composition (MICHAILOVA *et al*., 2003) and constantly aerated. The exposure concentration reflect the amount of Al present in acid mine drainage (BOULT *et al*., 1994). For each experiment, controls containing no added Al were ran in parallel. The number of individuals/salivary gland

cells examined was as follows - F1: 21/288; control: 19/112, F2: 19/165; control: 19/101, F3: 19/214; control: 19/238.

*C. acidophilu*s was collected from the Afon Goch in June 2004. Although mining has ceased, weathering of exposed rocks and spoil still releases acidic drainage (pH < 3.0) to the Afon Goch resulting in high concentrations of copper, zinc, iron, manganese and aluminium (BOULT *et al.*, 1994). Thus the *C. acidophilus* had been exposed to elevated environmental trace metals through numerous generations. Cytogenetical analysis of the *C. acidophilus* genome was performed on 705 salivary gland cells from 35 individuals.

Cytogenetic analysis was carried out on the IVth larva stage (6-7 phase) of both species of chironomid. Larvae were fixed in alcohol:acetic acid (3:1) and salivary gland chromosome preparations were done using the acet-orcein method as described by MICHAILOVA (1989). Standard chromosome maps of *C. riparius* **(**KIKNADZE *et al*., 1991) and *C. acidophilus* (KEYL, KEYL, 1959; KEYL, 1962) were used for the detection of chromosome rearrangements.

Chromosome rearrangements (paracentric/pericentric inversions, deletions, deficiencies) were considered somatic if only a few cells of one individual were affected. Functional alterations of BRs and NOR were assessed following MICHAILOVA *at el*. (2003). Methods of analysis of trace metal concentrations in sediment and water are as outlined in MICHAILOVA *et al.* (2003). Functional activity of BRs and NOR were compared using a Student's t-test. The frequency of rearrangements in the chromosomes was compared by the G test. A probability of $P < 0.05$ was taken as significant in all cases.

Analysis of the water and sediments collected from the Afon Goch (*C. acidophilus*) and in the experimental containers (*C. riparius*) was carried out as described in MICHAILOVA *et al*. (2003).

RESULTS

Metal concentrations

Concentrations of aluminium in the experiments with *C. riparius* in which 0.5 mg L^{-1} Al was added at the start of the experiment (see MICHAILOVA *et al.*, 2003) ranged from 0.18 to 0.74 mgL^{-1} compared to 0.07 and 0.14 mg L^{-1} in controls (Table 1). The pH was maintained at 7.0. Analysis of pH, Al, copper and zinc in the water and sediments of the Afon Goch confirm that the *C. acidophilus* collected from this site were exposed to very acidic (pH 2.3-2.7) highly metal-contaminated sediment and water compared to an adjacent unpolluted site (which did not contain *C. acidophilus* as it was not acidic: Table 1), and to other UK rivers (BOULT *et al*., 1994).

TABLE 1. pH and concentration of aluminium, copper and zinc in the water column (mg L-1) and sediments (mg g dry wt-1) in the laboratory experiments utilising C. riparius and the polluted site on the Afon Goch containing C. acidophilus. An unpolluted site adjacent to the Afon Goch is included as an indicator of the degree of metal contamination in the Afon Goch. * pH maintained at 7.0

Species	Treatment	pH	Medium	Metal concentration		
				Al	Cu	Zn
C. riparius	ExperimentalWater (added Al)	7.0*	0.18-0.74 Sediment	0.055 17.07	0.087 -	-
	ControlWater (no added Al)	7.0*	0.07-0.14 Sediment	0.055 16.4	0.087 -	-
C. acidophilus	Polluted site	2.2-2.7	Water	21.6-23.3	3.5-8.0	1.0-4.7
			Sediment	21.6-23.3	2.5-3.1	0.5-3.8
	Adjacent control site	6.3-6.5	Water	0.06-0.23	0.02-0.08	0.02-0.18
			Sediment	0.4-1.2	0.1-0.4	0.2-0.8

Cytogenetic characteristics of C. acidophilus and C. riparius

The chromosome set and band sequences of both *C.acidophilus* and *C.riparius* did not differ from those considered as standard for both species (KEYL AND KEYL, 1959; KEYL, 1962; MICHAILOVA ,1989; KIKNADZE *et al*., 1991).

Structural and functional alterations in C. acidophilus and C. riparius

Previous studies have shown that the presence of chromosomal aberrations in somatic cells can be used as an indicator of genotoxicty (LAGADIC & CAQUET, 1998). As no *C. acidophilus* were found in unpolluted areas we therefore used the frequency of somatic chromosome rearrangements in the salivary gland chromosomes as an measure of the genotoxicity of the trace metals in the Afon Goch In the studies using *C. riparius*, chromosomal rearrangements were compared with unexposed controls (MICHAILOVA et al., 2003).

In both species we assessed somatic chromosome rearrangements (inversions, deletions, deficiencies and asynapsis of both chromosome homologues) and as well as functional alterations in the BRs and NOR. Somatic paracentric heterozygous inversions in *C. riparius* affected the whole genome, while in *C. acidophilus* such aberrations were confined to arms A, B, E, F, D.

In all generations of *C. riparius* exposed to Al, somatic paracentric inversions

occurred at a significantly higher frequency than in controls (G = 35.263, P<0.001). A comparison of both species revealed that, in general, the somatic paracentric inversions occurred at a significantly higher level in *C. riparius* (G = 33.246, P<0.001) than in *C. acidophilus.* The number of somatic inversions in *C. riparius* was significantly higher (G-60.311, P<0.001) in the third generation of exposure to Al compared to pre-exposed *C. acidophilus*. No difference was however observed in first and second generations (F1: G-10.187, P<0.01; F2: G-0.109, P<0.1 respectively).

The pericentric inversions observed in both species affected the large chromosomes AB, CD, EF of *C. riparius* and AE, BF and CD of *C. acidophilus*. The pericentric inversions of chromosome AE of *C. acidophilus* (2.12%) occurred at a significantly higher frequency in comparison with those of BF (0.28%) (G test - 4.735, P<0.05) and CD (0.14%) (G test = 6.66, P<0.05). The pericentric inversions in *C. riparius* exposed to Al occurred in all generations in chromosome AB (2.24 %), CD (2.54 %) and EF (2.54%). No significant differences were detected between different chromosomes, that is between AB and CD and EF (G test 0,127, P<0.1); however, by the third generation the heterozygous pericentric inversion in chromosome EF occurred at a higher frequency (5.14%).

Comparative analysis of the frequency of the pericentric inversions revealed a significantly higher level in *C. riparius* treated with Al than in *C. acidophilus* collected from an acidic river (G = 17.502, P<0.001).

Deletions in chromosome G occurred in both species. However, in *C. riparius* the deletion in chromosome G occurred in the homozygous state and affected either BRc or both BRs (BRc and BRb) (F1- BRc – 8.08%; BRc+Brb – 10.2%; F2 – BRc+BRb – 1.05%; F3 – BRc- 13.6% and BRc+BRb – 6.88%). Deletions of the BRs were more frequent in the F1 and F3 generations than in the F2 generation (P<0.05). Chromosome G of *C. riparius* carrying this aberration was converted to the so called "pompon" form (MICHAILOVA *et al*., 2003). However, in *C. acidophilus* the deletion in chromosome G is in the heterozygous state and this aberration appeared at a very low frequency (<1%). In both species the deficiency affected only a small number of single cells (<1%).

Asynapsis in *C. acidophilus* occurred in all chromosomes (AE, 8.09%; BF – 5.25%; CD-4.11%; G-12.77%). In all generations of *C. riparius* treated with Al, asynapsis appeared at a high frequency only in chromosome G (F1 – 13.19%; F2-42.10%; F3 - 30.4%). Asynapsis was observed in chromosome arms A and E but at a very low frequency (<1%).

BR_1 is a characteristic feature of *C. acidophilus* (KEYL, KEYL, 1959), but a second, BR_2, was detected for the first time in specimens from the Afon Goch (Fig.1a). A "puff" at the telomere region of chromosome G was observed (Fig.1b) when the new BR_2 was slightly active or inactive (Fig.1b).

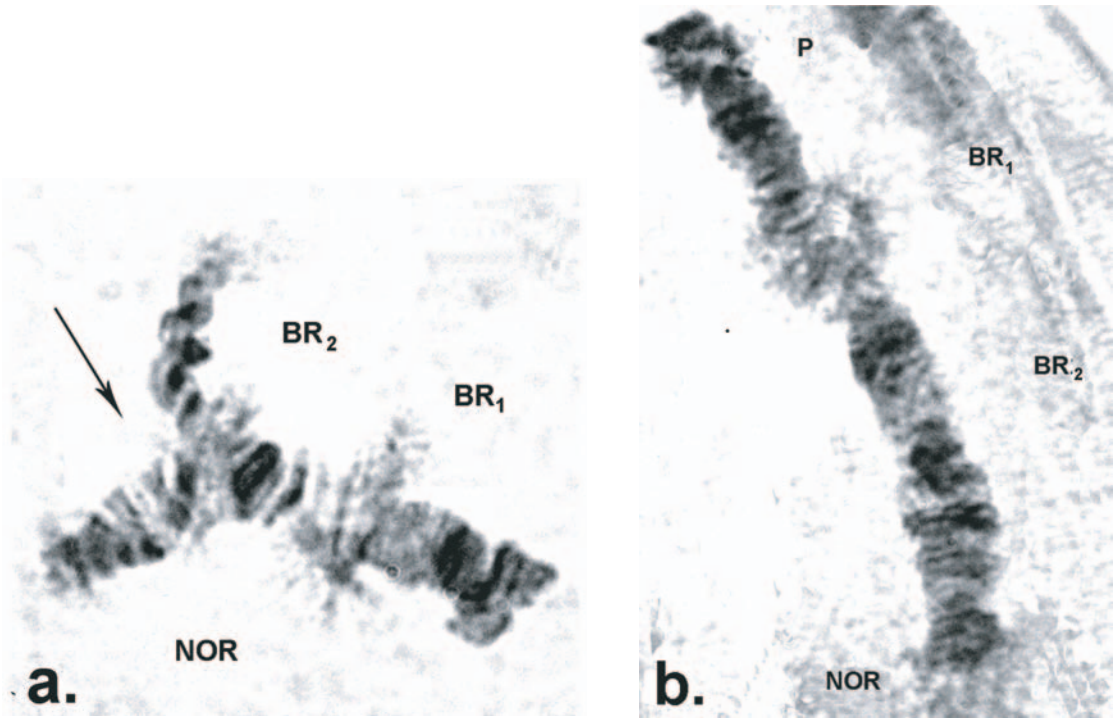

Fig. 1. Polytene chromosome G in *Chironomus acidophilus* Keyl from the Afon Goch, UK. A. Example of chromosome G with asynapsis (arrow) and BR1/BR2 showing intermediate activity. B. Example of chromosome G with a puff (P) at the telomere and BR2 not active. BR, Balbiani ring; NOR, nucleolar organizer.

Changes in the functional activity of BRs and NOR in both species were observed in response to trace metal pollution. The intermediate states of BRc/BRb (+/+) in *C. riparius* (MICHAILOVA *et al.*, 2003) and the state (+/-) of BR1/BR2 in *C. acidophilus* were both significantly higher ($P<0.05$). The intermediate state of NOR (+/+) in both species was also significantly ($P<0.05$) higher.

DISCUSSION

The *C. acidophilus* population has been subjected to acidic (pH about 2.5, see also Boult *et al.,* 1994) metal-rich water for numerous generations although the gene pool is likely to be influenced by populations from elsewhere. In contrast, *C. riparius* was exposed to chronic levels Al at neutral pH in our experiments.

In both species we observed a large number of somatic chromosome rearrangements and changes in functional activity of key structures such as the BRs and NOR which indicate the genotoxic effect of Al under laboratory conditions and trace metals, including Al, in the environment. However, both chironomids displayed species-specific genome responses. This study and that of MICHAILOVA *et al.* (2003) indicate that the *C. riparius* genome is more sensitive to trace metals than *C. acidophilus* as all somatic somatic aberrations occurred at a significantly higher frequency in *C. riparius*.

Other studies have also noted that the *C. riparius* genome is very sensitive to other trace metals such as copper and chromium (SELLA *et al.*, 2004).

GANROT (1986) notes an affinity of Al for DNA, which is probably a result of binding to the phosphate groups. KARLIK *et al*. (1980) found that Al forms different complexes depending on pH and it is thus possible that the chromosome alterations in exposed *C. riparius* larvae observed in this study are a result of binding of Al to DNA.

The *C. acidophilus* genome was however exposed to long-term metal contamination not only with Al but also copper, iron and manganese at low pH. All these trace metals may be implicated in the chromosomal rearrangements observed in the natural population from the polluted environment. Especially interesting is the appearance of a new BR (BR_2) in chromosome G of *C. acidophilus* which may be linked to the synthesis of specific proteins important for the development and survival of the species in the acidic metal-rich environment of the Afon Goch. Moreover, when BR_2 in *C. acidophilus* was only slightly or not expressed, a puff at the telomere of chromosome G was observed which we suggest is a compensatory mechanism in response to metal stress. Further molecular investigations are therefore required to identify and sequences the genes up and down regulated in response to trace metals.

In conclusion, trace metals result in genotoxic effects at both acidic and neutral pH to *C. acidophilus* and *C. riparius* respectively, as indicated by structural and functional alterations in the salivary gland polytene chromosomes. Both *C. acidophilus* and *C. riparius* showed species-specific responses. *C. riparius* displayed chromosome rearrangements (homozygous deletions in chromosome G which is converted to a "pompon" form; specific somatic peri- and paracentric inversions) and change the functional activity of BRs and NOR. In contrast, metal stress in *C. acidophilus* resulted in a new Balbiani ring (BR_2) of variable activity and a new puff at the telomere region when BR_2 is not expressed. These data has reinforced our earlier suggestion that chironomid are good indicators of trace metal genotoxicity in both neutral and acidic freshwater (MICHAILOVA *et al.,* 2003).

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BIBLIOGRAPHY

BOULT, S, D.N. COLLINS, K. N. WHITE & C.D. CURTIS:
1994. Metal transport in a stream polluted by acid mine Drainage – the Afon Goch, Anglesey,

UK. *Environmental Pollution,* **84:** 279-284.

BRUSICK, D:

1980. Principles of genetic toxicology. Plenum Press, New York.

GANROT, P.O.:

1986. Metabolism and Possible Health Effects of Aluminium. *Environmental Health Perspectives,* **65:** 363-441.

S.J.KARLIK, G.L.EICHHORN & D.R.MCLACHLAN CRAPPER:

1980. Molecular interactions of aluminium with DNA. *Neurotoxicology*, **1:** 83-88.

KEYL, H.:

1962. Chromosomenevolution bei *Chironomus*. II. Chromosomenumbauten und Phylogenetische Beziehungen der Arten. *Chromosoma*, **13**: 496-451.

KEYL, H. & I. KEYL:

1959. Die cytologische Diagnostik der Chironomiden I. Bestimmungstabelle fur die Gattung *Chironomus* auf Grund der Speicheldrusen – Chromosomen. *Archives Hydrobiologia,* **56**: 43-57.

KIKNADZE,I.I., A.SHILOVA, I.KERKIS, N.SHOBANOV, N.ZELENZOV, F. GREBENUJUK A.ISTOMINA & B.PRASLOV:

1991. Karyotype and morphology of larvae in Chironomini. Atlas. Novosibirsk, 117 pp.

LAGADIC, L& T.CAQUET:

1989.Invertebrates in Testing of Environmental Chemicals. Are they alternatives?.*Environmental Health Perspectives,* **106,** 2**:** 593- 603**.**

MICHAILOVA,P.:

1989.The polytene chromosomes and their significance to the systematics of the family Chironomidae, Diptera. *Acta Zool. Fennica*, **189**:1-107.

MICHAILOVA, P., J. ILKOVA & K. WHITE:

2003. Functional and Structural rearrangements of salivary gland chromosomes of *Chironomus riparius* Mg. (Diptera, Chironomidae) in response to freshly neutralized aluminium. *Environmental Pollution,* **123**: 193-207.

SELLA,G., S. BOVERO , M. GINEPRO , P. MICHAILOVA , N. PETROVA , C ROBOTTI & V. ZELANO:

2004. Inherited and somatic cytogenetic variability in Palearctic populations of *Chironomus riparius* Meigen 1804 (Diptera, Chironomidae). *Genome,* **47**: 332-344.

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