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Structural and functional alterations in salivary gland chromosomes and enzyme activity of *Chironomus riparius* Mg. (Diptera, Chironomidae) from anthropogenically polluted sites in Bulgaria and Turkey

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The effect of environment contaminants on genome instability and changes in enzyme activity (acetylcholinesterase (AChE), glutathione S-transferase activities (GST), etoxyresorufin O-deethylase (EROD) and metallothionein (MT)) of *Chironomus riparius* Mg. from Bulgarian and Turkish stations over two years (2009, 2010) as well as laboratory reared larvae were studied. Physicochemical analysis of the sediments from the field stations indicated the presence of heavy metal pollutants (Cr, Cu, Mn, Pb, and Cd) whose concentrations were higher than the reference data. Genome instability was determined by somatic structural and functional alterations of the polytene chromosomes. In the field sites of both countries somatic aberrations occurred at a significantly higher frequency ($p < 0.001$) compared with control and laboratory material. *C. riparius* in sediments with higher concentrations of trace metals (Derincay River, Turkey and Chaya River, Bulgaria), was found to possess a high spectrum of somatic chromosome rearrangements with a somatic index of 2.53 and 3.25 respectively. Changes in functional activity included decreased activity of the Balbiani rings (BRs) and nucleolar organizer (NOR). The observed chromosome alterations agree with the high degree of trace metal pollution and high activity of the studied enzymes. However, no correlation between single somatic chromosome rearrangements and concentrations of specific metal ions was defined. The data are discussed in the light of the wide variety of interactions of metals in nature. The results show that the genome response and biochemical markers are sensitive markers of toxicity and provide early warning indicators of contaminants in the environment.

Keywords: Balbiani rings; Chironomidae; enzyme activity; nucleolar organizer; polytene chromosomes; somatic alterations

Introduction

The aquatic larvae of non-biting midges (Chironomidae, Diptera) are widely used in environmental monitoring and laboratory toxicity testing (Choi 2004). They possess a number of advantages for detecting and assessing the impact of contaminants in aquatic ecosystems. Several signs have been employed in monitoring using Chironomid larvae: potential indicator species for contamination of freshwaters (Rosenberg 1993), mouthpart deformities (Warwick 1988; Mergalli et al. 2002), and alterations in salivary gland chromosomes (Hudson and Ciborowski 1996; Michailova and Mettinen 2000; Michailova et al. 1998, Michailova, Ilkova et al. 2009; Michailova, Szarek-Gwiazda et al. 2009; Michailova 2011). Assessment of different chromosome rearrangements offers an effective bioindicator of sediment pollution and several studies have employed somatic chromosome alterations in salivary polytene chromosomes to monitor genotoxicity of pollutants (Michailova, Ilkova et al. 2009; Michailova, Szarek-Gwiazda et al. 2009; Michailova 2011). The genome instability of two sibling, homosequental species, *Chironomus piger* Strenzke and *Chironomus riparius* Mg., living in trace metal polluted stations was shown by many somatic structural and functional alterations in their chromosomes, with *C. riparius* showing a greater degree of change (Ilkova et al. 2007). The response of salivary gland chromosomes of *C. plumosus* L. and *C. anthracinus* Zett. to industrial and municipal contaminations was also shown in other studies (Michailova and Mettinen 2000). A high chromosome and genome polymorphism in *C. plumosus* and *C. annularius* Mg. from anthropogenically polluted stations provide these populations with a better chance of surviving extreme polluted conditions (Michailova et al. 2005). This polymorphism can be used as a biological system that very quickly records changes in the environment.

Bettinetti (1999), Michailova et al. (1998) and Michailova, Ilkova et al. (2009) detected inhibitions in

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the functional activity of key structures, specifically the Balbian rings (BRs – constantly active regions responsible for saliva protein synthesis) and nucleolar organizer (NOR – the region in which rRNA synthesis takes place) after exposure to toxicants. Moreover, when one of BRs was slightly or not expressed, a puff at the telomere of chromosome G was observed – a genome response which has been considered as a compensatory mechanism (Michailova, Ilkova et al. 2009).

Biomarkers that employ enzyme activity measurements detecting low levels of pollution are also used in ecological risk assessments of aquatic ecosystems. Acetylcholinesterase (AChE) inhibition or increased metallothionein (MT) content have been widely used in terrestrial and freshwater aquatic systems as an indicator of organophosphorous (OPs) and carbamate (CBs) exposure and heavy metal contamination (Kägi and Schäffer 1988). Measurement of etoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST) activities are used as the exposure index, thus enabling the identification of areas contaminated by industrial or domestic pollutants (Burgeot et al. 2001).

This study aims to make a better diagnosis of environmental quality by applying a multilevel approach involving somatic structure and functional alterations of salivary gland chromosomes and changes of enzymes activities of GST, EROD, AChE and MT. The widely distributed species *Chironomus riparius* provides a promising biological system to evaluate their use.

Materials and methods

Study area

The study was conducted at six field polluted stations: three from Bulgaria and three from Turkey, plus a laboratory culture of *C. riparius* originated from egg masses collected in Pazar (Turkey).

The three Bulgarian localities are near Plovdiv: the Maritsa and Chaya rivers, and a farm near Plovdiv. Based on data from the National Biomonitoring Program of Bulgaria (Peev and Gerassomov 1999), 16 unpolluted and 95 impacted (polluted) stations were examined depending on the type of ecosystems and the character of the pollutants: physical and chemical. However, no data about the concentrations of pollutants was available. Therefore we compared with reference data on sediment levels in the literature, specifically Forstner and Salomons 1980.

Based on the Bulgarian National Biomonitoring Program, the impacted region studied was on the Maritsa and Chaya rivers – industrial polluted sources containing Pb, Zn, SO_2, NO_2 and other toxicants. The contaminants originated from a factory producing different metals. Pollutants from the farm near Plovdiv include domestic sewage and animal waste.

Yeşilırmak River is one of the most prominent rivers in the northeast of Turkey. The drainage basin of Yeşilırmak River is 519 km long and covers an area of 38,000 km^2. Yeşilırmak River is subject to pollution from industrial waste water (sugar refinery, textiles, antimony mine and metals), municipal sewage and agricultural runoff. Derincay River is also polluted by factories (sugar refinery, lead, poultry and metals), agricultural runoff and domestic sewage. Corum Fountain was contaminated by domestic sewage.

From a Turkish locality (Pazar) egg masses were collected and the larvae bred under laboratory conditions in the Cytotaxonomy and Evolution Laboratory at the Institute of Biodiversity and Ecosystem Research, Sofia. The experiment was carried out in an incubator with a photoperiod of 16 h light/8 h dark, temperature 20°C and constantly aerated water. In order to understand the effect of trace metals, the larvae were reared chronically in polluted sediment collected from a small pond near to an industrial region of Sofia. The laboratory culture was kept according to Michailova (1985). The pH was maintained about at 7.0 by adding 1 M HNO_3 or NaOH. The water was changed twice weekly.

Because *C. riparius* larvae were not found in unpolluted basins of either country, as a control site for cytogenetic analysis we used data from the literature, specifically at site in Corio, Italy (Sella et al. 2004) where concentration of trace metals were less than those of the unpolluted sediments.

Studies were carried during April–June 2009 and 2010. Sampling of water and sediments for physicochemical analysis and larvae of Chironomidae for cytogenetic analysis were collected.

Sampling and analyzing the sediment

Sediment samples were air dried overnight and then dried in an oven 60°C until constant weight. Dried sediments were ground and sieved. Digestion was carried out utilizing the Anton Paar Multiwave 3000 microwave system. Two different digestion procedures were applied for the accurate determination of the different elements.

Nitric acid, hydrochloric acid and hydrofluoric acid used to acidify the water samples following by treatment with acid and nitric acid, hydrogen peroxide digestion was also performed. Cr, Mn and Cu were determined with ICP-OES (Perkin Elmer Optoma 4300 DV); and ICP-MS (Perkin Elmer ELAN DRCII) was used for Cd and Pl. All measurements were performed for two sets of samples and in three replicates. Rh was used as internal standard in IPC-MS analysis.

Sampling Chironomid larvae

For cytogenetic studies Chironomidae larvae were collected by hand-net from a sediment surface layer of the stations and picked up using fine forceps. After cleaning, the largest larvae were separated and fixed for cytogenetic analysis.

Cytogenetic method

Chironomid larvae were fixed in alcohol: acetic acid (3:1). Fourth instars larvae, phase 6–7 (Wülker and Götz 1968) were used for preparing salivary gland chromosomes and external morphology of the mouth. Chromosome preparations were done according to Michailova (1989).Together with chromosome preparations from each larva, a preparation of the larval head capsule was performed. Different species of genera *Chronomus* and *Polypedilum* were found. *C. riparius*, an abundant species, was identified by the specific markers of the polytene chromosomes (Michailova 1989; Kiknadze et al. 1991). The *C. riparius* polytene chromosome maps of Hägele (1970) and Kiknadze et al. (1991) were used as standard. The numbers of studied specimens and cells from different localities are shown in Table 1.

Biochemical methods

C. riparius larvae for enzyme analysis were preserved in dry ice. Homogenization and preparation of cytosolic and microsomal fractions were performed on ice according to Schenkman and Cinti (1978). Aliquots of fractions were stored at –80°C for biomarker analysis. Protein concentrations of cytosolic and microsomal fractions were determined by the method of Lowry et al. (1951). Cytosolic AChE (acethylcholinesterase) activities were assayed according to Ellman et al. (1961) in a medium containing 0.6 mM 5,5′-dithiobis 2-nitrobenzoic acid (3,3′-6) (DTNB) and 7.5 mM ATC in 100 mM Tris-HCl buffer pH 8.5.

GST (glutathione S-transferase) activities using CDNB (1-chloro 2,4 dinitrobenzene) as a substrate were determined at room temperature spectrophotometrically at 340 nm. Enzyme activities were calculated using an extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$ (Habig et al. 1974).

Microsomal EROD (7-ethoxyresorufin-O-deethylase) activities were assayed using the methods of Burke and Mayer (1974), as optimized by Arinc and Sen (1994), using 7-ethoxyresorufin as a substrate. The fluorescence increase ($\lambda_{ex} = 535$ nm; $\lambda_{em} = 585$ nm) was recorded using a Cary Eclipse fluorometer for 120 seconds.

MT (metallothionein) content of *C. riparius* was determined as described by Viarengo et al. (1997) and as optimized by Ozdemir, Duran, Akyildiz et al. (2011), by evaluating the concentration of reduced sulphydryl by measuring the absorbance at 412 nm, utilizing reduced glutathione as a reference standard.

Statistical analysis

The established somatic structural alterations (paracentric and pericentric inversions, deletions, deficiencies and amplification) in all chromosomes were calculated as percentages. Chromosome aberrations were considered as somatic when they affected a few cells of the salivary gland of the studied individual (Sella et al. 2004).

Table 1. Years, localities, number of individuals and cells of *Chironomus riparius* collected from Bulgarian and Turkish stations.

	Bulgaria								Turkey							
	Corio – Italy (control site)		Kemera – Maritsa River		Asenovgrad – Chaya River		Plovdiv Farm		Corum Fountain		Derincay River		Amasya – Yeşilırmak River		Pazar – laboratory material	
Year-	*n*	*n′*	*n*	*n′*	*n*	*n′*	*n*	*n′*	*n*	*n′*	*n*	*n′*	*n*	*n′*	*n*	*n′*
2004	46	1349														
April–June 2009	—	—	13	394	14	347	15	439	17	512	18	378	12	248	31	662
April–June 2010	—	—	1	38	4	44	7	111	20	405	12	248	—	—	14	288

Abbreviations: *n* – number of individuals; *n′* – number of cells.

On the basis of the observed somatic chromosome rearrangements, in each locality the somatic index was calculated (Sella et al. 2004).

The level of functional activity was evaluated from the amount of puff activity of the key structures – Balbiani rings (BRs) and nucleolar organizer (NOR) – following Beermann's rule (1971): high activity (++/++), intermediate activity (+/+) and little or no activity (–/–). Somatic functional activities of BRc/BRb and NOR were calculated by Student's *t*-test. Levels of (BRc/BRb) and NOR activities were compared with standard activities of the 4th instar larva previously reported (Kiknadze 1978).

The somatic structural chromosome alterations were compared among individuals of Bulgarian and Turkish stations as well as between stations of each country by contingency G test (Sokal and Rohlf 1995). In all cases probabilities of $p < 0.001$, $p < 0.01$ and $p < 0.05$ were taken as the levels of significance.

The variations in the cytosolic AChE, GST and microsomal EROD activities and MT content between sampling sites and laboratory stocks were analyzed by the one-way analysis of multivariance test (one-way MANOVA) and the two samples test in Minitab 10.3.

Results

Sediment analysis

The sites under consideration in the present study were polluted by several trace metals (Table 2). At all of the stations the pH of the sediment was between 7.33 and 8.41 and the temperature were between 10.4 and 18.5°C. At all studied field stations the concentrations of Cr, Cu, Mn, Pb and Cd were higher than the reference data. For example, the concentration of Pb in the Bulgarian stations of both studied years was between 18 and 37 times that of fossil (reference unpolluted) sediment (Table 2). In turn, the sediment used in the laboratory cultures of egg masses from Pazar, Turkey contained considerably smaller amounts of these metals. In these cultures only the concentrations of Cu and Pb were 2–3 times higher than those in the fossil sediment.

Cytogenetic characteristics

Chironomus riparius has a chromosome set $2n = 8$. It belongs to the "thummi" cytocomplex (Keyl 1962) with chromosome arm combinations of AB CD EF and G. Chromosomes AB and CD are metacentric, chromosome EF is submetacentric and chromosome G is acrocentric (Figures 1a, b, c, d). Chromosome G has three Balbiani rings (BRa, BRb, and BRc) and one nucleolar organizer (NOR). BRa is active in four cells of the salivary gland cells only. The karyotype of the species does not differ from the standard described by Michailova (1989) and Kiknadze et al. (1991).

Somatic structural chromosome aberrations

At all stations in both years we found several types of somatic rearrangements: pericentric and paracentric heterozygous inversions distributed in all chromosomes (Figures 2a, b), deletions of BRc/BRb in chromosome G (Figure 3c) and deficiencies in all chromosomes as well as amplifications in chromosome EF (Tables 3 and 4). However, in all individuals these rearrangements appeared at a low frequency and affected only few cells and a small region of the polytene chromosomes.

In both years the somatic aberrations from all Bulgarian and Turkish stations appeared at a statistically higher frequency ($p < 0.001$) in comparison with those of the control data (Corio, Italy; Sella et al. 2004). The somatic index based on the observed somatic chromosome rearrangements occurred at a higher value in Derincay River (Turkey) (2009) and Chaya River (Bulgaria) (2010) (Table 3 and Table 4).

Somatic structural chromosome aberrations, 2009

Deletions of BRc/BRb of chromosome G in *C. riparius* sampled in 2009 significantly increased at Bulgarian (Kemera – Maritsa River, $G = 43.609$, $df = 1$, $p < 0.001$; Asenovgrad – Chaya River, $G = 33.081$, $df = 1$, $p < 0.001$; Plovdiv $G = 40.834$, $df = 1$, $p < 0.001$) and Turkish stations (Corum Fountain, $G = 61.062$, $df = 1$, $p < 0.001$; Derincay River, $G = 22.088$, $df = 1$, $p < 0.001$ and Amasya – Yeşilırmak River, $G = 4.41$, $df = 1$, $p < 0.05$) compared to reared laboratory material (Pazar). Also, pericentric (Kemera – Maritsa River ($G = 12.105$, $df = 1$, $p < 0.001$) and paracentric heterozygous inversions (Asenovgrad – Chaya River ($G = 11.46$, $df = 1$, $p < 0.001$), Plovdiv Farm ($G = 9.67$, $df = 1$, $p < 0.01$) (Bulgaria) and paracentric and pericentric inversions in the Derincay River (Turkey) ($G = 40.31$, $df = 1$; $G = 47.29$, $df = 1$, $p < 0.001$) occurred at a significantly higher frequency than in reared laboratory material.

Some differences of chromosome aberrations were found among the studied stations in both countries. For instance: the pericentric inversions in Kemera – Maritsa River, Bulgaria appeared at a significantly higher frequency compared with that from Plovdiv Farm ($G = 9.79$, $df = 1$, $p < 0.01$). In the same year in Turkish stations the pericentric and paracentric inversions in Derincay River (14.8% and 17.2% respectively) occurred at a significantly higher frequency compared with Corum Fountain ($G = 38.34$, $df = 1$; $G = 19.02$, $df = 1$, $p < 0.001$), and Amasya – Yeşilırmak River stations ($G = 30.45$, $df = 1$; $G = 18.13$, $df = 1$, $p < 0.001$). Deletions of BRc/BRb of chromosome G of *C. riparius* from Corum Fountain (Turkey) significantly increased ($p < 0.05$) by up to 11.13% (Table 3).

Cytogenetic damage (pericentric and paracentric inversion) in *C. riparius* from Bulgarian and Turkish stations significantly increased in the Derincay River

Table 2. Concentrations of trace metals (μg g^{-1}) and some physicochemical results of Bulgarian and Turkish stations.

Trace metals, pH and t°C	Corio, Italy (control site)	Fossil sediments	Bulgaria, April–June 2009			Turkey, April–June 2009			
			Kemera – Maritsa River	Asenovgrad – Chaya River	Plovdiv Farm Corum Fountain	Derincay River	Amasya – Yeşilırmak River	Pazar – laboratory material	
Cr	35	59.00	45.19 ± 0.8	70.4 ± 0.97	1302.4 ± 15.6	173.3 ± 1.6	191.1 ± 1.4	424.7 ± 3.1	0.08 ± 0.001
Cu	20	25.00	120.6 ± 0.4	314.3 ± 2.2	86.42 ± 1.7	27.7 ± 1.4	56.1 ± 2.1	44.04 ± 0.6	46.53 ± 1.52
Mn	0	406.00	1885.4 ± 14.7	434.7 ± 3.2	73.76 ± 1.05	1442.2 ± 7.0	725.6 ± 6.2	932.0 ± 4.8	59.67 ± 1.29
Pb	2.4	16.00	388.2 ± 4.7	585.9 ± 1.6	339.0 ± 1.5	19.78 ± 0.11	16.5 ± 0.11	13.68 ± 0.3	50.87 ± 0.56
Cd	0	0.20	16.83 ± 0.35	7.34 ± 0.09	4.73 ± 0.08	0.00	0.19 ± 0.01	0.1 ± 0.007	0.23 ± 0.02
pH			8.81 ± 0.01	7.58 ± 0.002	8.92 ± 0.002	7.35 ± 0.001	7.33 ± 0.02	8.41 ± 0.01	6.9 ± 0.01
Temperature			12.4 ± 0.2	10.4 ± 0.1	13.9 ± 0.3	13.8 ± 0.2	13.2 ± 0.1	11.5 ± 0.1	10.6 ± 0.1
			Bulgaria, April–June 2010			Turkey, April–June 2010			
			Kemera – Maritsa River	Asenovgrad – Chaya River	Plovdiv Farm	Corum Fountain	Derincay River	Amasya – Yeşilırmak River	Pazar – laboratory material
Cr	35	59.00	0.17 ± 0.001	0.11 ± 0.001	0.13 ± 0.001	0.06 ± 0.001	776.3 ± 7.2	543.6 ± 4.3	0.11 ± 0.001
Cu	20	25.00	144.8 ± 1.3	213.8 ± 2.5	66.44 ± 1.2	34.91 ± 0.3	39.79 ± 1.5	48.34 ± 0.5	49.63 ± 0.7
Mn	0	406.00	63.8 ± 0.9	60.21 ± 1.0	74.05 ± 1.9	150.4 ± 0.4	333.9 ± 2.6	836.0 ± 6.2	44.51 ± 0.7
Pb	2.4	16.00	559.78 ± 7.6	536.75 ± 3.6	293.07 ± 0.4	10.8 ± 0.09	28.7 ± 0.38	11.21 ± 0.2	57.2 ± 0.4
Cd	0	0.20	18.81 ± 0.2	2.86 ± 0.1	2.84 ± 0.05	0.00	3.36 ± 0.09	0.1 ± 0.002	0.21 ± 0.01
pH			8.24 ± 0.01	7.78 ± 0.00	8.42 ± 0.01	7.8 ± 0.001	8.26 ± 0.02	8.29 ± 0.001	7.1 ± 0.01
Temperature			13.6 ± 0.2	11.3 ± 0.1	14.5 ± 0.2	14.7 ± 0.3	18.5 ± 0.4	17.3 ± 0.4	11.2 ± 0.2

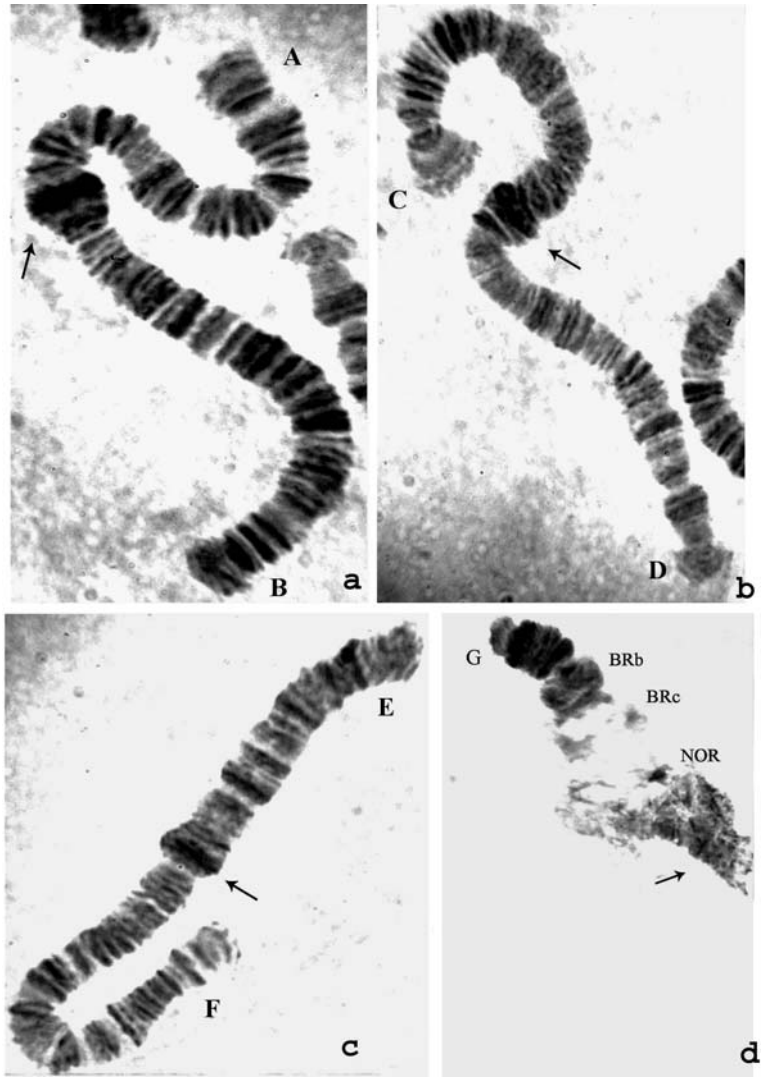

Figure 1. Standard polytene chromosomes of *Chironomus riparius*. (a) Chromosome AB; (b) chromosome CD; (c) chromosome EF; (d) chromosome G. Arrows indicate the centromere region of each chromosome. Balbiani rings and nucleolar organizer are shown as BRb, BRc and NOR. Scale bar = 100 μm.

(14.8%; 17.2%) compared with Asenovgrad – Chaya River (6.05%, G = 15.10, df = 1, $p < 0.001$; 9.80%, G = 8.50, df = 1, $p < 0.01$), Plovdiv Farm (2.24%, G = 32.48, df = 1, $p < 0.001$; 6.06%, G = 12.59, df = 1, $p < 0.001$) and Kemera – Maritsa River (8.82%, G = 6.72, df = 1, $p < 0.01$; 4.28%, G = 35.74, df = 1, $p < 0.001$).

Somatic structural chromosome aberrations, 2010

The frequency of BRc/BRb deletions in chromosome G is shown in Table 4. There is a significant difference between field and laboratory material (Asenovgrad – Chaya River (13.64%) (G = 9.93, df = 1, $p < 0.01$); Kemera – Maritsa River (13.16%) (G = 8.15, df = 1, $p < 0.001$); Plovdiv Farm (8.11%) (G = 8.00, df = 1, $p < 0.01$); Derincay River (10.89%) (G = 20.95, df = 1, $p < 0.001$); Corum Fountain (G = 9.54, df = 1, $p < 0.01$) The frequencies of paracentric inversions in Bulgarian stations (Asenovgrad – Chaya River (29.54%), Kemera – Maritsa River (21.10%) and Plovdiv Farm (13.51%)) increased significantly compared with those of Turkish stations (Corum Fountain (4.20%, G = 24.40, df = 1, $p < 0.001$; G = 11.22, df = 1, $p < 0.001$, G = 10.64, df = 1, $p < 0.01$), Derincay River (6.05%, G = 17.10, df = 17.28, $p < 0.001$; G = 7.28, df = 1, $p < 0.01$, G = 5.08, df = 1, $p < 0.05$)) and Paraz – reared material (2.08%, G = 31.70, df = 1, $p < 0.001$; G = 16.38, df = 1, $p < 0.001$, G = 17.73, df = 1, $p < 0.001$). The pericentric inversions showed no statistical difference between *C. riparius* from Bulgarian and Turkish stations ($p > 0.1$) (Table 4).

Somatic functional alterations in 2009 and 2010 samples

BRs and NOR are the chromosomal landmarks that are very sensitive to trace metal contamination. In all studied stations in both years three types of BR activity were observed: high activity (++); intermediate activity (+); and low or no activity (–) (Figures 3a, b). In *C. riparius*

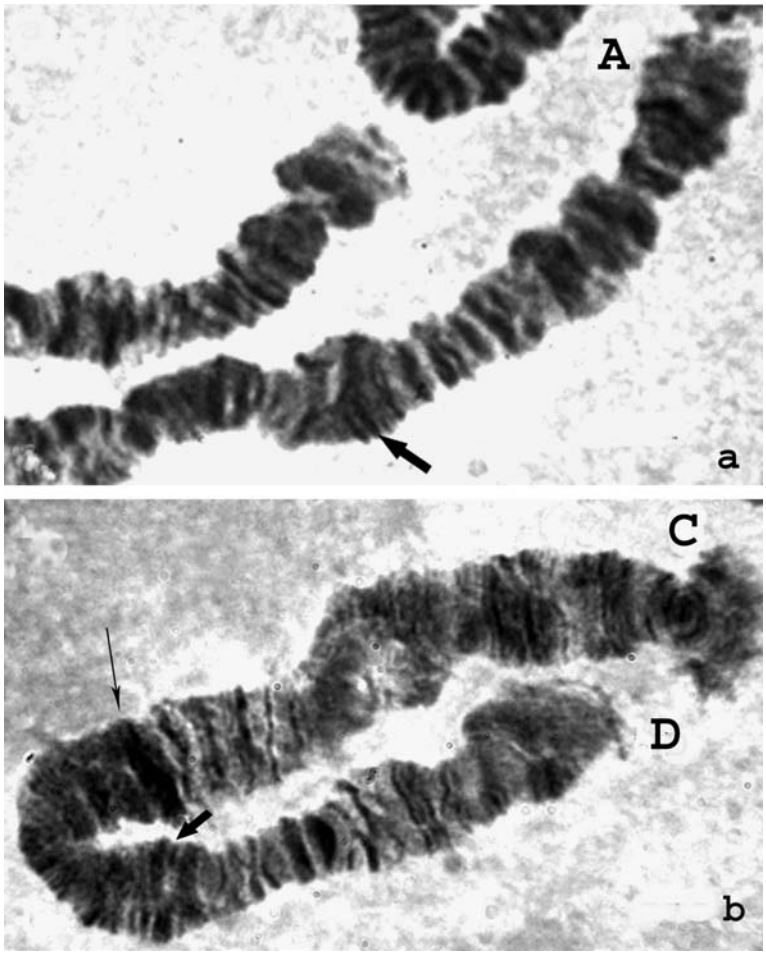

Figure 2. Somatic aberrations in polytene chromosomes of *Chironomus riparius*. (a) Heterozygous pericentric inversion in chromosome AB; (b) heterozygous paracentric inversion in chromosome CD, arm D. The longer arrow indicates the centromere region; the shorter arrow indicates the inversion. Scale bar = 100 μm.

reared under constant laboratory conditions the standard activity was observed: BRc was very expanded, BRb occurred in an intermediate activity ($p < 0.05$ or $p < 0.001$) (Figures 4a, b).

In most stations in 2009 the frequency of BRc/BRb with intermediate activity level (+/+) significantly increased ($p < 0.001$) in larvae from Kemera – Maritsa River (G = 336.651. df = 1), from Asenovgrad – Chaya River (G = 29.723, df = 1), from Corum Fountain (G = 62.253, df = 1), and from Amasya – Yeşilırmak River (G = 26.416, df = 1) compared with high activity level (++/++) (Figure 4a). Moreover, the activity of BRc/BRb of *C. riparius* from Plovdiv Farm and Derincay River was changed to a situation of slight activity or collapsed (respectively G = 213.527, df = 1 and G = 687.36, df = 1, $p < 0.001$).

From field stations sampled in 2010, larvae of *C. riparius* significantly ($p < 0.001$) increased the frequency of intermediate state of BRc/BRb (Kemera – Maritsa River G = 77.391. df = 1, Asenovgrad – Chaya River G = 30.751, df – 1, Plovdiv Farm, G = 112.574, df = 1, Corum Fountain, G = 84.39, df = 1, Derincay River, G = 141.583, df = 1) compared with high activity level (+ +/++) (Figure 4b).

The NOR of *C. riparius* of all studied stations in both years showed a change in its activity from a very high activity of both homologues to a heterozygous activity of one homologous or intermediate activity of both homologues (Figures 4c, d). The frequency of NOR with intermediate activity level (+/+) significantly increased to 88% and 92% respectively ($p < 0.001$) in larvae sampled in both 2009 and 2010: Kemera – Maritsa River (G = 7.342, df = 1), Asenovgrad – Chaya River (G = 11.698, df = 1), Plovdiv Farm (G = 150.147, df = 1), Corum Fountain (G = 41.89, df = 1) and Amasya – Yeşilırmak River (G = 364.33, df = 1) (2009); Maritsa River (G = 45.279, df = 1), Chaya River (G = 77.409, df = 1), Plovdiv Farm (G = 131.114, df = 1), Corum Fountain (G = 80.079, df = 1), Derincay River (G = 204.668, df = 1) (2010). The intermediate activity of NOR of reared *C. riparius* material was significantly greater ($p < 0.001$) than its high activity (Figures 4c, d).

A decondenzation of telomere and centromere regions of all chromosomes was established in polytene

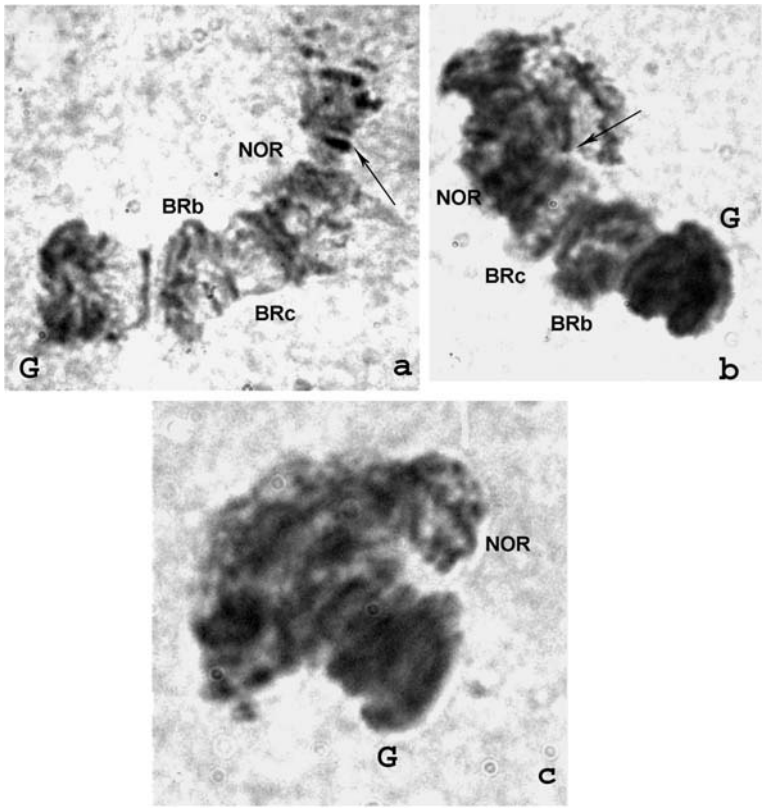

Figure 3. Chromosome G of *Chironomus riparius*. (a) Balbiani rings with different activity (BRc/BRb –/+); nucleolar organizer (NOR) with intermediate activity (+/+); (b) Balbiani rings with intermediate activity (BRc/BRb +/+); NOR with a low activity (–/–). Deletions of BRb/BRc. Arrow indicates the centromere region. Bar – 100 μm.

chromosomes of *C. riparius* from Corum Fountain in 2010: 0.5% and 10.37% respectively. These functional alterations were observed in Amasya – Yeşilırmak River (2009) in 10.88% and 8.66% respectively.

Enzyme activity

We measured cytosolic GST and AChE activities and microsomal EROD activity together with total MT levels in *C. riparius* samples collected from Bulgaria and Turkey. GST activities of the specimens from the Maritsa River and Plovdiv Farm in the year 2009 occurred at a significantly higher frequency compared with reared laboratory material (Figure 5a). Similarly, GST activity of *C. riparius* collected from Derincay River and Amasya – Yeşilırmak River significantly increased ($p < 0.001$) (Figure 5a). In addition to GST activity, the activity of EROD indicated PAH (polycyclic aromatic hydrocarbon) contamination was higher in Turkish than Bulgarian samples. Samples from the Derincay and Yeşilırmak rivers in spring 2009 showed 3-fold and 2.30-fold higher microsomal EROD activities than reared material (Figure 5b). As shown in Figure 5c, samples of Corum Fountain and Yeşilırmak Rivers (spring 2009), Derincay River and Amasya – Yeşilırmak Rivers (spring 2010) demonstrate approximately 30–50% cytosolic AChE inhibition of *C. riparius* reared in the laboratory. Total MT content of *C. riparius* collected from Bulgaria and Turkey showed significant induction in the Chaya and Derincay rivers (2009) and the Plovdiv Farm site in 2010 ($p < 0.001$) (Figure 5d).

Discussion

In this study genome instability and changes of selected enzyme activities in *C. riparius* collected from trace metal polluted stations in Bulgaria and Turkey have been analyzed. The results reveal that the genome of *C. riparius* was very sensitive to metal stress as every individual showed either structural or functional alterations. The structural chromosome aberrations observed in *C. riparius* from all stations appeared at a significantly higher frequency than those of a control station (Corio, Italy) examined by Sella et al. (2004). Also, it is interesting to note that *C. riparius* reared in the laboratory under standard conditions but in sediment containing less but still elevated amounts of trace metals showed the same somatic aberrations as those in the field. This is a good indicator that the trace metals did induce the observed chromosome damage. However, the laboratory sediment ions of two metals (Cu, and Pb) occurred in lower concentrations than in the field stations but higher than the reference data (Forstner and Salomons 1980).

Table 3. Somatic rearrangements of *Chironomus riparius* collected in 2009 (April and June) from Bulgarian and Turkish stations.

Material studied (localities)	Number of individuals with aberrations/%	Number of cells with aberrations/%	Number of cells with pericentric inversions/%	Number of cells with paracentric inversions/%	Number of cells with deletions/%	Number of cells with deficiencies and amplifications/%	Number of somatic aberrations	Somatic index
Corio, Italy (control site)	6/13.04	6/0.446	—	6/0.446	—	—	6	0.130
Kemera – Maritsa River	12/92.31	90/22.84	35/8.88	17/4.31	38/9.64	—	13	1.00
Asenovgrad – Chaya River	14/100	87/25.07	21/6.05	34/9.80	29/8.36	3/0.86	22	1.57
Plovdiv Farm	12/80	94/21.41	16/3.64	15/3.42	8/1.82	—	38	2.53
Corum Fountain	16/94.12	114/22.27	17/3.32	39/7.62	57/11.13	1/0.2	30	1.76
Derincay River	17/94.44	145/38.36	56/14.81	65/17.2	24/6.35	—	47	2.61
Amasya – Yeşilırmak River	7/58.33	29/11.69	6/2.42	15/6.05	8/3.23	—	16	1.33
Pazar – laboratory material	22/70.97	59/8.91	24/3.63	28/4.23	7/1.06	—	15	1.07

Table 4. Somatic rearrangements of *Chironomus riparius* collected in 2010 (April and June) from Bulgarian and Turkish stations.

Material studied (localities)	Number of individuals with aberrations/%	Number of cells with aberrations/%	Number of cells with pericentric inversions/%	Number of cells with paracentric inversions/%	Number of cells with deletions/%	Number of cells with deficiencies and amplifications/%	Number of somatic aberrations	Somatic index
Corio, Italy (control site)	6/13.04	6/0.446	—	6/0.446	—	—	6	0.130
Kemera – Maritsa River	1/100	14/36.84	5/13.16	8/21.05	5/13.16	—	7	7
Asenovgrad – Chaya River	4/100	16/36.36	2/4.54	13/29.54	6/13.64	—	13	3.25
Plovdiv Farm	7/100	26/23.42	11/9.91	15/13.51	9/8.11	—	16	2.28
Corum Fountain	16/80	40/9.88	26/6.42	17/4.20	26/6.42	—	20	1
Derincay River	12/100	42/16.94	12/4.84	15/6.05	27/10.89	1/0.40	19	1.58
Pazar – laboratory material	9/64.28	21/7.29	10/3.47	6/2.08	5/1.74	—	8	0.57

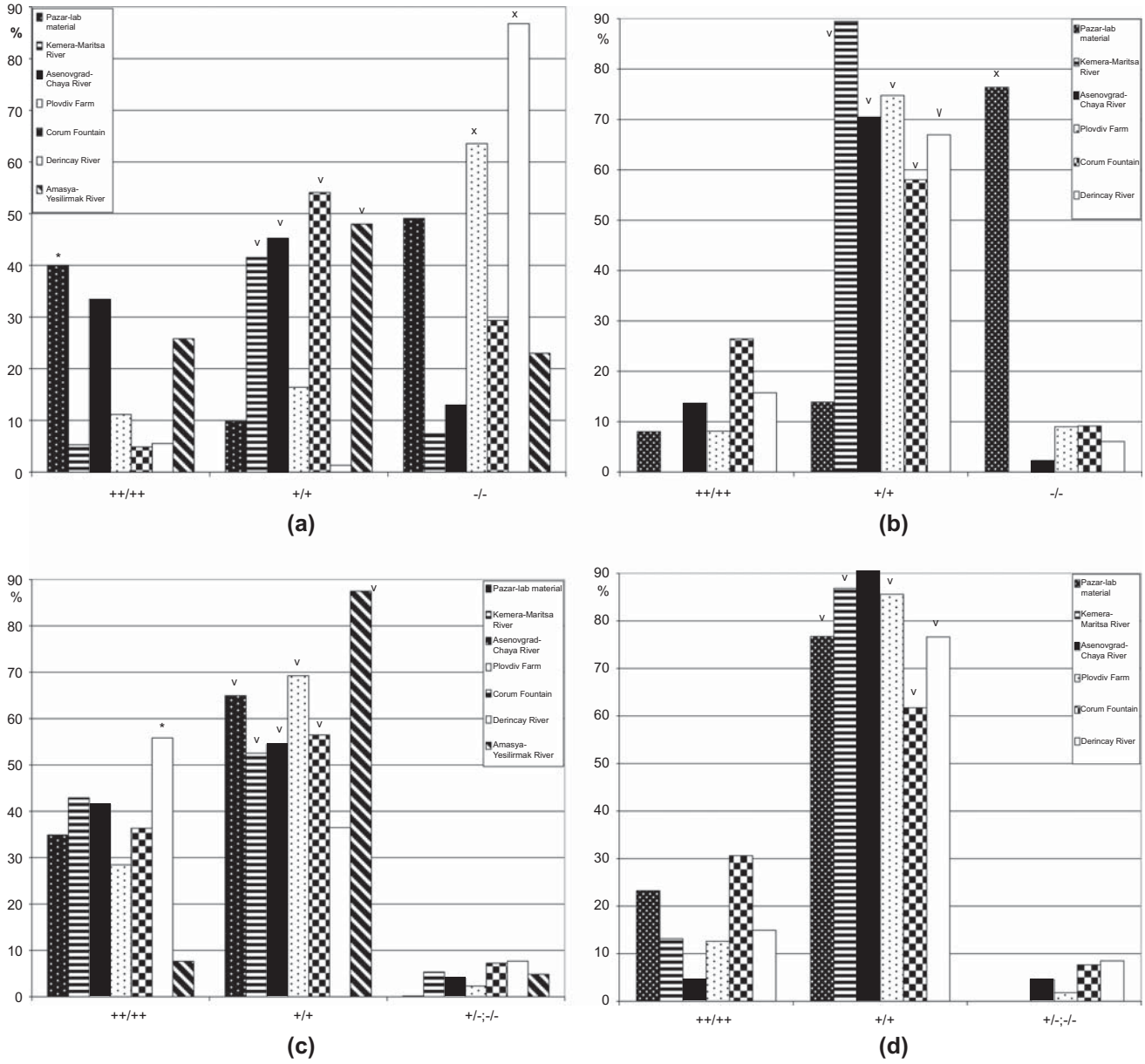

Figure 4. BRc/BRb and NOR activity of *Chironomus riparius* from Bulgarian and Turkish stations. (a) Levels of BRc/BRb activity on polytene chromosomes of *Chironomus riparius* collected from Bulgaria and Turkey in 2009. (b) Levels of BRc/BRb activity on polytene chromosomes of *Chironomus riparius* collected from Bulgaria and Turkey in 2010. Superscript letters a and b indicate statistically significant difference ($p < 0.05$ or $p < 0.001$). (c) Levels of NOR activity on polytene chromosomes of *Chironomus riparius* collected from Bulgaria and Turkey in 2009. (d) Levels of NOR activity on polytene chromosomes of *Chironomus riparius* collected from Bulgaria and Turkey in 2010. Superscript letters c and d indicate statistically significant difference ($p < 0.001$). ++/++ indicates high activity; +/+ intermediate activity; –/– no activity; and –/+ indicates that one homologous is not active and, the other has intermediate activity.

Moreover, the somatic rearrangements of *C. riparius* from the laboratory material appeared in lower frequencies than those from the field stations. For instance, the deletions of BRc/BRb in chromosome G found in the field stations occurred at a significantly higher frequency compared with *C. riparius* reared in the laboratory.

The results obtained showed that there is not a correlation between single trace metals and a specific type of chromosome alteration. For instance, in Bulgarian stations with high concentrations of Pb ions in both 2009 and 2010, the frequency of paracentric inversions in *C. riparius* genome was not high. This type of inversion was at the highest frequency in Derincay River (2009) where the concentrations of this ion were not particularly high. This peculiar response can be explained by the fact that pollutants in nature mix together and a wide variety of interactions (antagonistic or synergistic) (Baršiene 2003) is possible. It is therefore not possible to predict the effect of such combinations in nature. Individual chemicals rapidly change their characteristics in the aqua-

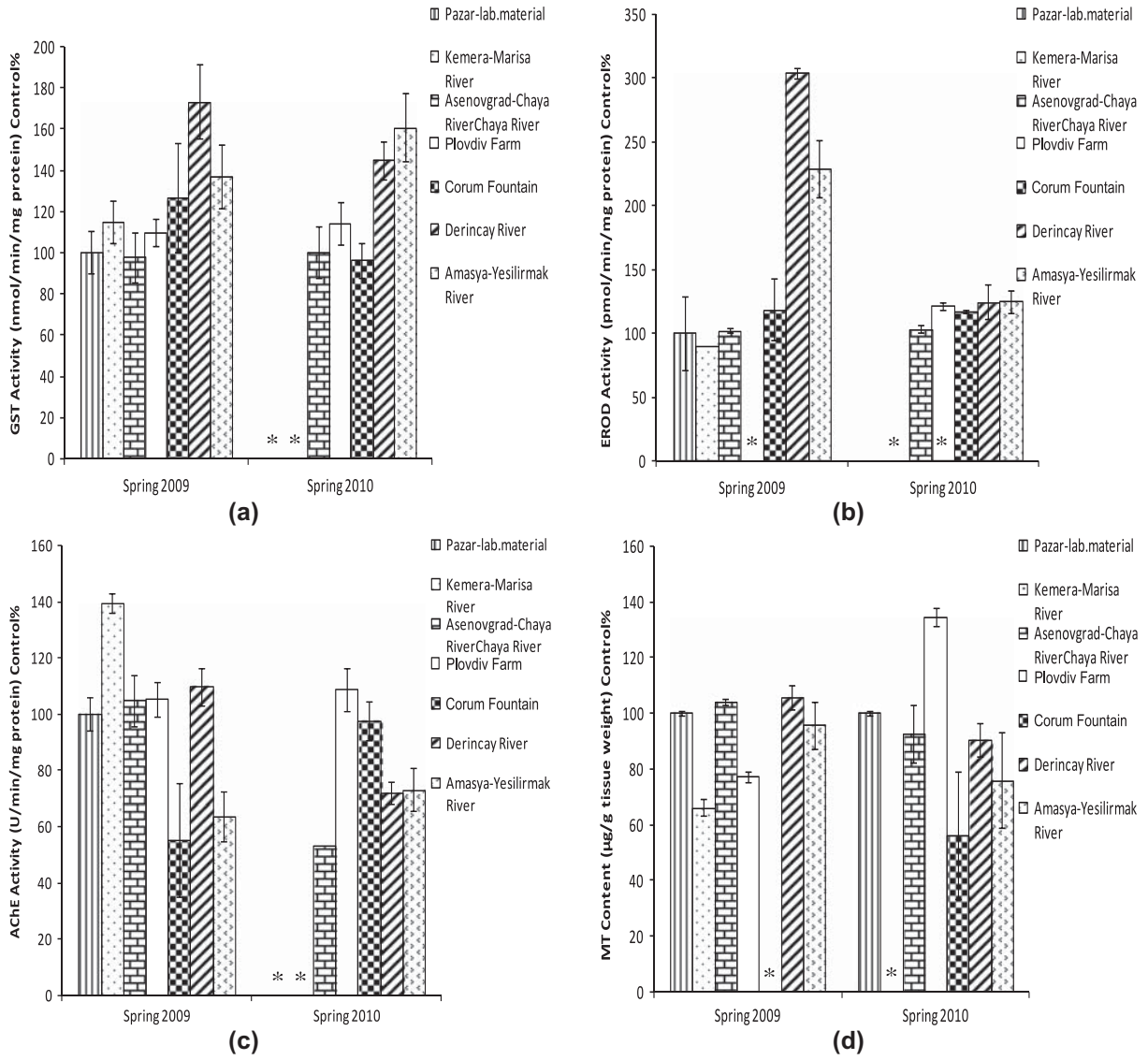

Figure 5. Biomarker enzyme activities of *C. riparius* collected from Bulgaria and Turkey. Activities are calculated against Pazar – laboratory material activities and given as% of control in figures. Data are presented as the mean ± SD of at least three sets of triplicate measurements. *Data were not included as they were not sampled enough and not measured. (a) Cytosolic GST activity (% of control); (b) microsomal EROD activity (% of control); (c) cytosolic AChE activitiy (% of control); (d) total MT contents (% of control).

tic environment as they are modified and integrated through physical, chemical and biological processes in the environment. It is for this reason that we are not able to identify which trace metals induce specific chromosome rearrangements. However, laboratory experiments revealed that even at a low concentration, Pb and Cr induce a variety of somatic rearrangements (Michailova, Ilkova et al. 2001; Michailova, Petrova et al. 2001), which supports the hypothesis that the observed genotoxic effects of trace metals established in the sediments of Bulgarian and Turkish stations are due to trace metal contaminations. Such somatic structural aberrations in polytene chromosomes are indicative of a mutagenic response to trace metals. Moreover, Sella et al. (2004) showed that somatic inversions increased with increasing levels of heavy metal pollution. Also, in other aquatic organisms such as Mollusca, positive correlations between different contaminants, including heavy metals, and the chromosome set were determined (Baršiene 1994).

The highest somatic index in Derincay River (in 2009) corroborates with high concentrations of trace metals and the highest enzyme activities (GST, EROD, AChE and MT (see Figures 5a, b, c, d). It is well known that MTs have a high affinity to some heavy metals (e.g. Cu and Cd) (Kägi and Schaffer1988; Mourgaud et al. 2002; Amiard et al. 2006) and serve as protection against toxicity. Similar to our results, several studies have shown that MT content is increased when the gradient of metal (Cd, Cu, Zn) pollution is increased (Mourgaud et al. 2002; Ross et al. 2002; Amiard et al. 2006). MTs play a major role in the homeostasis of essential metals (such as Zn and Cu) and also in detoxification of nones-

sential metals such as Ag, Hg and Cd (Amiard et al. 2006). A high metal concentration in a cell induces an increase in MT concentration, and the use of MTs as biomarkers of Ag, Cd, Cu and Hg contamination has been evaluated by several authors for different animal species (Amiard et al. 2006).

Some samples of *C. riparius* collected in 2010 showed an increase the activity of AChE. It is quite possible the pesticide contaminants in these studied areas occurred because the activity of this enzyme is a good biomarker not only of trace metal exposure but also of pesticides (Ozdemir, Duran, Akyildiz et al. 2011; Ozdemir, Duran and Sen 2011).

In additional to somatic structural alterations and changes of enzyme activities, in all studied localities there was a decrease in the activity of the key chromosome structures, specifically the BRs and NOR. It is known that BRs are very important for normal development of the larvae as they are sites of intensive transcription of genes encoding for the silk proteins (Wieslander 1994) used in the construction of larval tubes. The nucleolar organizer (NOR) is responsible for the synthesis of ribosomal RNA and hence is important for normal cell function. Because similar changes in the puffing activity of BRs and suppression of NOR was observed in *C. riparius* from other trace metal polluted regions (Michailova et al. 1998; Petrova et al. 2004) and treated materials (Planello et al. 2007) as well as in exposure experiments to different concentrations of Cr, Pb, and Al (Michailova, Ilkova et al. 2001; Michailova, Petrova et al. 2001; Michailova et al. 2003) it is possible to consider this effect as a general stress response of BRs and NOR. The results revealed that important chromosomal structures such as BRs and NOR are the target of the trace metal pollution, and so monitoring their activity may contribute to work relating to development of risk assessment strategies and biomarkers.

In summary, our data confirm the hypothesis of Steinberg et al. (2008) that responses to pollutants involve interactions between genes, proteins and metabolism. The present study shows a clear relationship between chromosome rearrangements, functional alterations, enzyme activity and trace metal pollution. Therefore genome instability together with changes in enzyme activities can be used in ecological risk assessment of aquatic systems. They are good biomarkers that have the potential to identify the incidence of exposure and effects caused by contaminants. The results obtained in this study showed that the response of the genome at the cytogenetic and biochemical level is a sensitive biomarker and can serve as an early warning indicator of the environmental damaging effects of chemicals. Environmental diagnoses by a multilevel approach will a permit better understanding of the impact of pollutants on organisms and could be successful if implemented in environmental monitoring procedures.

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