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Mouthpart Deformities and Nucleolus Activity in Field-Collected *Chironomus riparius* Larvae

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Abstract. Chironomid mouthpart deformities and aberrations of their polytenic chromosomes are sublethal responses to toxic stress. These endpoints have been used in several cases as bioindications for sediment pollution. In the present study we aimed to establish whether there was an association between mouthpart deformities and nucleolus activity in the polytenic chromosomes. Such information could be useful to gain insight into the mechanisms involved in the occurrence of mouthpart deformities and their consequences on the larvae. Third-instar larvae of *Chironomus riparius* were collected at a site downstream of a sewage treatment plant mostly contaminated by pesticides. Larvae were then raised in the laboratory in aquaria containing sediment and water from the study location. During a 16-day period, larvae ready to molt to the fourth instar were reared individually. Within a few hours of their molt, the larvae were preserved. The presence of mouthpart deformities (mentum, mandibles, and pecten epipharyngis) and the percentage of active nucleoli were assessed. Those larvae presenting mentum deformities had a significantly higher incidence of active nucleoli in their polytenic chromosomes than nondeformed larvae. Because a high number of active nucleoli generally indicates increased rRNA synthesis, deformed larvae seemed to exhibit a higher protein synthesis than normal individuals. The synthesis of additional proteins may increase deformed larva tolerance to toxicants.

Monitoring toxicity levels in specific environmental compartments is necessary to evaluate the risks to which all living beings are exposed. It is impossible to perform chemical analyses to monitor all toxicants in the environment. Moreover, the mere presence of a pollutant does not indicate an impact on living beings, as its bioavailability is influenced by many factors (*e.g.*, organic matter content and pH; Landrum and Robbins 1990) and measuring the concentration is often insufficient to predict the impact. Additionally, the simple presence of chemicals does not allow us to predict the effects of their mixtures.

Organisms representative of the environment of interest can provide more complete information on the impacts of the toxicants present than chemical analyses (Vermeulen 1995). The use of organisms as bioindicators is advantageous because it is likely that such organisms reflect the exposure to pollutants, and the integrated effect of all (known and unknown) chemicals present is determined in a cost-effective way.

Chironomids are ubiquitous in almost every type of aquatic environment (Warwick 1990), and their benthic pre-imago stages are therefore commonly used as bioindicators of freshwater environments (Rosenberg 1992). *Chironomus riparius*, in particular, is a species often used in toxicology (Rosenberg 1992).

Several endpoints have been employed in monitoring studies using chironomids, such as species occurrence (Rosenberg 1992), mouthpart deformities (Hamilton and Sæther 1971; Köhn and Frank 1980; Warwick 1980; Janssens de Bisthoven *et al.* 1992, 1995, 1998; Bird 1994; Vermeulen *et al.* 1998) and aberrations in the polytenic chromosomes (Michailova and Belcheva 1990; Aziz *et al.* 1991; Bentivegna and Cooper 1993; Hudson and Ciborowski 1996; Michailova *et al.* 1996, 1998; Bettinetti 1999; Michailova and Mettinen 2000). Investigations on the presence/absence of species by means of biotic indexes can give indications on the (physical and chemical) habitat quality (Rosenberg 1992). In the case of chironomids, the presence/absence of species is investigated also to reconstruct past environmental conditions by means of subfossil head capsules collected from lake sediments (Brodersen 1998). Occurrence of mouthpart deformities in chironomids is a sublethal effect of larval exposure to toxicants (Vermeulen 1995). Assessment of these abnormal morphological features offers an effective bioindication of sediment pollution (Vermeulen 1995). In several field studies a relation has been established between deformities and xenobiotics in the sediment (Hamilton and Sæther 1971; Köhn and Frank 1980; Warwick 1980; Janssens de Bisthoven *et al.* 1992, 1995, 1998; Bird 1994; Vermeulen *et al.* 1998). Finally, various authors employed polytenic chromosomes of the salivary glands of chironomid larvae

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to monitor genotoxicity. The presence of structural and functional anomalies in the chromosomes has been generally associated with exposure to pollutants (Michailova and Belcheva 1990; Michailova *et al.* 1996, 1998; Michailova and Mettinen 2000). Other authors focused on specific parts of the chromosomes, such as the Balbiani rings (constantly active regions responsible for saliva protein synthesis; Aziz *et al.* 1991; Bentivegna and Cooper 1993; Bettinetti 1999) or the nucleolus (region of the nucleus in which rRNA synthesis takes place, also known as nucleolar organizing region [NOR]; Aziz *et al.* 1991; Hudson and Ciborowski 1996; Bettinetti 1999) and detected inhibitions of the activity of these structures after exposure to toxicants.

With this study, we aimed to establish a relation between the presence of mouthpart deformities and the corresponding degree of NOR activity in field-sampled *C. riparius* larvae. Such information can be helpful to shed more light on the mechanisms behind the occurrence of mouthpart deformities and their consequences for the larvae.

Table 1. Percentages of organic matter and clay and concentrations of metals, PAHs, PCBs, organochlorine pesticides (OCPs), extractable organically bound halogens (EOXs), and ApHs in the sediment of the sampled site on the River Grote Gete (Tienen, Belgium)

Organic matter (%)	1.8
Clay (%)	4
Cr (mg/kg)	22
Pb (mg/kg)	54
As (mg/kg)	2.6
Cd (mg/kg)	0.3
Cu (mg/kg)	28
Hg (mg/kg)	0.2
Ni (mg/kg)	10
Zn (mg/kg)	212
ApHs (mg/kg)	115
EOXs (mg/kg)	19
OCPs (μg/kg)	92
PCBs (μg/kg)	23
PAHs (μg/kg)	4.5

Materials and Methods

Third-instar larvae of *C. riparius* were sampled from one location on the River Grote Gete (Tienen, Belgium) on July 7, 1998, about 600 m downstream of a sewage treatment plant. Sediment characteristics and the concentrations of specific categories of pollutants were analyzed by a Flemish Governmental Environment Agency (AMINAL) during a general survey in 1997 and are presented in Table 1. Within the survey the data collected at each site are compared to Flemish reference stations (De Cooman *et al.* 1998) to estimate the environmental quality. The site, under consideration in the present study, was mainly polluted by pesticides, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and apolar hydrocarbons (ApHs) (De Cooman *et al.* 1998). The porewater of this location had a serious acute impact on the alga *Selenastrum capricornutum* and on the anostracan *Thamnocephalus platyurus* (De Cooman *et al.* 1998). Finally, a low biotic index value and a high percentage of chironomid mentum deformities (*i.e.*, $>$ 40%) indicated, in general, very low biological quality (De Cooman *et al.* 1998).

At the site, the water temperature (18.2°C), pH (7.9), and O_2 saturation (55%) were measured. Water (15 L) and sediment (1 L) from the location were transported to the laboratory. In the laboratory, the benthic fauna were first removed from the sediment using a 250-μm sieve. The experiment was conducted at a temperature of 15 ± 1°C. An aquarium tank (dimensions: 34 × 19 × 19 cm) was prepared with the sediment and 4 L of water from the site. Aeration was provided by means of a glass pipette.

Using a dissection microscope, it was determined which larvae were ready to molt to the fourth instar. Eyes of larvae close to molting display a characteristic backward position (Vermeulen *et al.* 2000a). Such larvae were isolated in small glass containers. These containers were filled with water from the River Grote Gete, and a gentle aeration was provided. Larvae that had a normal eye position were put back in the aquarium. Every 24 h, during a 16-day period, the molted larvae were collected and preserved in alcohol/acetic acid (3:1), necessary for chromosome investigation. The remaining larvae were examined daily for eye position and placed in small containers when close to molting.

Polytenic chromosome slides were mounted adopting the method described by Keyl and Keyl (1959) and from Michailova *et al.* (1998). The whole larvae were first stained in an orcein solution (1 g in 100 ml acetic acid 50%) at 50°C for 1 h. After immersion in lactic acid 88%, the salivary glands were removed from the body and mounted on microscope slides. Each gland cell was carefully opened and the membrane and cytoplasm were eliminated. The slides were immersed in liquid nitrogen and then dehydrated in ethanol and buthanol. Finally they were mounted in Eukitt® (Carlo Erba, Italy). The number of cells in the two salivary glands of the larvae, for which the cells could be still distinguished after preparation of the slides (seven), was counted, and the mean number of cells for one larva was determined to be 57. Such value was then used to calculate the percentage of active nucleoli. Active nucleoli—in *C. riparius* present on the chromosome G—were distinguished from nonactive ones for the presence of specifically localized puffs (swellings) around the chromosome (Figure 1).

The head capsule of each larva was mounted next to its polytenic chromosomes. The head capsules were first cleared in KOH (10%) on a hot plate (temperature = 90°C), then dehydrated in ethanol and finally mounted in Euparal. Deformities in the mentum, mandibles and pecten epipharyngis were assessed according to Vermeulen *et al.* (1998, 2000b).

For the considered mouthparts, one-way analyses of variance (ANOVAs) were calculated using Statistica 5.1 (StatSoft) to determine whether the normal and deformed larvae had a statistically different percentage of active nucleoli in their chromosome G. Head capsule status (normal/deformed; a discrete variable) was used as an independent variable, whereas the percentage of active nucleoli represented the dependent variable. The data met the assumptions (homogeneity of variances and normality of the distribution of the dependent variable) for this analysis. Results were considered significant at $p \leq 0.05$.

Results

From a total of 100 larvae, the chromosomes were clearly visible in 63 larvae. Table 2 gives the mean and the 95% confidence interval of the frequency of deformities in the three mouthparts and the percentage of active nucleoli. The deformation data follow a binomial distribution, and the active nucleolus data a normal distribution.

All the larvae were divided in normal and deformed in any of the mouthparts. The mean percentage of active nucleoli was significantly higher in deformed in comparison with normal larvae ($F_{1,59}$* = 5.73, p = 0.02; Figure 2).

Larvae displaying deformities in their mentum had a signif-

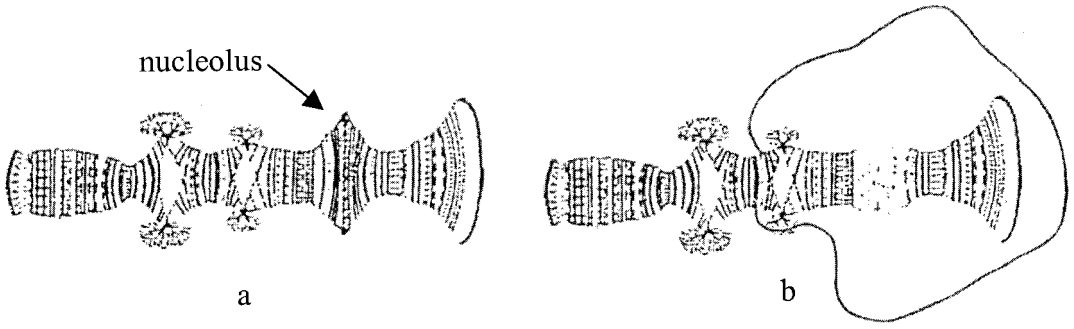

Fig. 1. Schematic representation of nonactive (a) and active (b) nucleoli on the chromosome G

Table 2. Number of analyzed larvae, mean and 95% confidence interval (CI) of the frequencies of mentum, mandible, and pecten deformities and the percentage of active nucleoli

	n	Mean ± CI
Mentum deformities	63	0.38 ± 0.12
Mandible deformities	63	0.06 ± 0.06
Pecten deformities	61	0.64 ± 0.12
Active nucleoli	63	51.88 ± 4.77

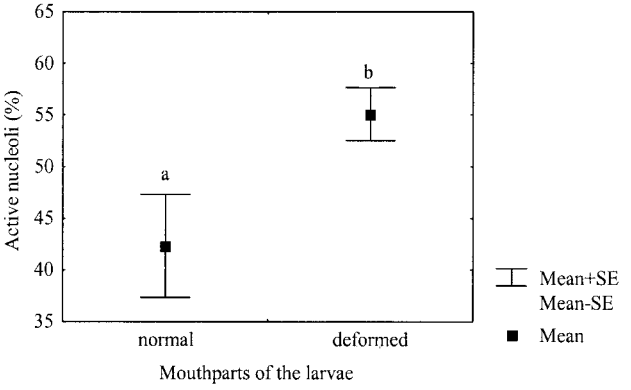

Fig. 2. Mean and SE of the percentage of active nucleoli in normal and deformed (in any of the structures) larvae. Symbols followed by different letters are significantly different ($p \leq 0.05$)

icantly higher mean percentage of active nucleoli than normal ones ($F_{1,61}$* = 4.33, p = 0.04; Table 3).

The mean percentage of active nucleoli was not statistically different in normal and deformed larvae when mandibles ($F_{1,61}$ = 0.007, p = 0.94) or pecten ($F_{1,59}$ = 2.05, p = 0.16) were considered, although in all cases deformed larvae displayed a higher number of active nucleoli than their normal counterparts (Table 3).

Discussion

We established a significant relation between the presence of mouthpart deformities and increase of the nucleolus activity of *C. riparius* larvae collected from a polluted site. The incidence of mentum deformities is already employed as a sign of polluted sediments in monitoring programs (De Cooman *et al.* 1998). We now also detected that larvae deformed in their mentum showed a significantly higher percentage of active nucleoli than normal ones. In a similar study, Hudson and Ciborowski (1996) examined the influence of mixtures of contaminated field sediment on mentum deformity occurrence and reduction of nucleolus diameter of *Chironomus salinarius* and found a positive relation between the proportion of contaminated sediment and both the incidence of mentum deformities and the reduction of nucleolus diameter. These authors, however, did not determine the developmental stage of the individuals, as they calculated the "relative (to the chromosome diameter) nucleolus diameter," which is independent of the larval age. In our study, all specimens were of the fourth larval instar, which represents almost one-third of the complete *C. riparius* life cycle (Holloway 1983). Metabolism, and hence nucleolus activity, undergo relevant physiological variations during this long instar. By killing the larvae at the same developmental moment (just after the molt to the fourth stage), the chromosomes were presumably expressed in a similar way (*i.e.*, coding proteins for an analogous physiological stage), making nucleolus activities comparable. Deformities seen in the fourth stage are induced during molting of instar III larvae (Vermeulen *et al.* 2000a) and are probably fully expressed at the molts (Janssens de Bisthoven *et al.* 1992). As for all arthropods, chironomids' exoskeleton hardens soon after the molt and its structure remains basically unchanged (and subject to abrasion only) until the successive molt event. Therefore, during a given stage, deformities remain unaffected, even if the larvae are continuously exposed to the contaminants. Metabolism and coding genes' expression are, on the contrary, constantly influenced by a prolonged exposure to toxicants. The larvae were killed just after the molt to the fourth instar to fix the chromosomes soon after the formation of possible deformities.

Further evidences from laboratory bioassays show that exposure of the chironomid *Glyptotendipes barbipes* to lead acetate leads to aberrations and replication defects in the polytene chromosomes (Michailova and Belcheva 1990). It is also observed that different *Chironomus* species exposed to copper, benzo[*a*]pyrene, actinomycin D, dimethylnitrosamine, and 4-nonylphenol displayed activity reductions in the Balbiani rings and the nucleolus (Aziz *et al.* 1991; Bentivegna and Cooper 1993; Bettinetti 1999). Michailova *et al.* (1996) investigated the polytene chromosomes of

Table 3. Mean and SE of the percentages of active nucleoli for larvae showing normal and deformed mentum, mandibles, and pectens

Mouthpart Structures	Active nucleoli (%)					
	Normal larvae			Deformed larvae		
	n	Mean	SE	n	Mean	SE
Mentum*	39	48.09	2.93	24	58.04	3.80
Mandibles	59	51.83	2.52	4	52.63	6.56
Pectens	22	47.45	4.51	39	54.48	2.68

n: sample size.
* The two groups are significantly different ($p \leq 0.05$).

field-collected *C. riparius* larvae sampled from a trace metal–polluted station and found a great number of structural and functional aberrations. Michailova *et al.* (1998) studied the chromosome G of *C. riparius* larvae sampled in a heavy metal–polluted area and determined a clear reversed activity between the Balbiani rings (BRb and BRc), a change in the activity of the NOR, and an activation of the telomere region. Michailova and Mettinen (2000) detected structural and functional alterations in the chromosomes of *Chironomus plumosus* and *Chironomus anthracinus* sampled in industrial and municipal polluted areas. Assessment of aberrations in polytene chromosomes is very interesting to determine possible genotoxic effects of pollutants, but this technique, like the estimation of the nucleolus diameter, is very laborious for monitoring purposes. In the case of the nucleolus diameter, for instance, the assessed diameter depends on the place and direction of the measurement, as active nucleoli are seldom perfectly spherical. Moreover, mounting and flattening of the chromosomes on microscope slides can increase confounding factors for this type of estimation. Verifying whether nucleoli are active, on the other hand, represents an easy and unequivocal method, and as such it could be helpful in monitoring studies.

The relation between deformed chironomids and their corresponding higher percentage of active nucleoli suggests the possibility of using both endpoints as bioindications of pollution. Nucleoli are the site of rRNA synthesis. A high number of active nucleoli likely indicates an enhanced rRNA synthesis, which would denote an increased protein synthesis. It is possible that these larvae produce proteins to increase their tolerance to toxicants. As chromatin decondensation may not always coincide with DNA transcription, future studies should detect the presence of rRNA in the NOR to prove whether our hypothesis is correct. If this is the case, further investigations could run in parallel with physiological analyses to understand the function of increased rRNA synthesis.

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