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Does the tube of a benthic chironomid larva play a role in protecting its dweller against chemical toxicants?

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Abstract

A laboratory culture of an Israeli benthic midge, *Chironomus luridus*, was exposed to two chemicals: copper and monochloramine. The objective of this study was to determine the protective nature of *Chironomus'* larval tube. Three experimental conditions were tested: larva with sand tubes, with silt tubes and without tubes. Larvae without tubes were significantly more sensitive to copper and chloramine than larvae that had sand or silt as tube building substrate. The tubes protected the insects against chemicals throughout 14 days of exposure time. Silt tubes had higher protective value than sand tubes, especially when exposed to copper for a short period of time (LC_{50}/ 24 h, with silt, sand, or none: 80.0, 7.0 and 3.4 mg l^{-1} copper, respectively). *C. luridus* seemed to be better protected against copper than against chloramine (LC_{50}/ 24 h, with silt, sand, or none: 12.2, 6.4 and 3.7 mg l^{-1} chloramine, respectively). The acute toxicity of copper to chironomid larvae was investigated using a cytochemical method. Larva in silt tubes had significantly higher non-specific esterase activity in midgut cells than larvae without tubes. We conclude that, in addition to its role in feeding, respiration and anti-predation shelter, the *C. luridus* tube protects its inhabitant from toxic substances.

Introduction

Chironomids, 'non-biting midges' (Diptera; Chironomidae) are the most widely distributed and frequently the most abundant insect in freshwater. Chironomids species can tolerate and develop in polluted waters such as waste stabilization ponds where they become a dominant macroinvertebrate (Broza et al., 2000). In heavily metal polluted streams, they were found to compose up to 80% of the total fauna, whereas they constituted less than 10% in an unpolluted section of the same stream (Winner et al., 1980).

The larvae of *Chironomus* spp. live in tubes in the soft benthic sediments of freshwater habitats. The tube-dwelling behaviors of chironomids were originally coupled with feeding and respiratory behaviors (Walshe, 1951). Macchiusi & Baker (1992) showed that larvae responded very strongly to the presence of fish predators by remaining in their tubes longer when the percentage of time that fish are present increased. Hershey (1987) suggested that tubes evolved as anti-predator adaptations but were later modified in some chironomid groups to facilitate feeding and respiration.

Recently, chironomids occurred as pestiferous midges in Israel. Two chemicals that are currently used in water purification, monochloramine and copper sulfate, were examined to determine their efficacy in *Chironomus* control (Broza et al., 1998; Halpern et al., 1999). We used these trials to study whether the tubes of the chironomid larvae serve as chemical protectants apart from their role in feeding, respiration and anti-predation shelter.

Materials and methods

Experimental conditions and chemicals

All bioassay experiments were conducted on a laboratory culture of *Chironomus luridus* Strenzke that was collected from covered storage tanks of the water supply system at Shefar'am near Haifa, Israel. The laboratory culturing procedure was described in Halpern et al. (1999).

All experiments were conducted under 23±2 °C and a photoperiod of 14:10 h (L: D). Water chemical composition of the dechlorinated tap water used in all the experiments was presented in Halpern et al. (1999: 454, Table 1). Notice the high hardness (239 mg l^{-1} as $CaCO_3$) and conductivity (1069 μmho cm^{-1}, 25°C) values of the water. A stock solution of monochloramine of approximately 300 mg l^{-1} was prepared (at pH 8.2) by mixing sodium hypochlorite and ammonia in the ratio of 3:1 (w:w) of Cl_2–N (White, 1972) and stored up to 2 weeks at 4 °C in a dark bottle. Monochloramine residual concentration was determined before use of the chloramine stock solution by the DPD colorimetric method (APHA, 1992). In long-term bioassays, the chloramine concentration was readjusted every 24 h by removing and analyzing a 10 ml aliquot; chloramine was added as needed to restore the original concentration. A 1 g l^{-1} copper stock solution of $CuSO_4 \cdot 5H_20$ was prepared daily. Test concentrations were prepared by diluting appropriate aliquots of the stock solutions with test water. Residual concentration of copper was determined by 'Neve Ya'ar' extension service laboratory (Ministry of Agriculture), in an Atomic Absorption apparatus (Varian industry).

Sand and silt

Sand or silt was used for larvae's tube substrate. Silt was collected from the same storage tank's floor, from which the midges for culture were collected. It was washed, diluted (20:1 w:w water to silt), sterilized and stored at 4 °C. One ml of centrifuged silt (10 min at 1000 rpm) was added to the appropriate test cup. Sand was washed, sterilized and dried. Five grams of dry sand were added to the appropriate test cups. The dry weight of 1 ml silt was 0.11±0.01 g. The mechanical and organic composition of the sand and the silt was analyzed by 'Neve Ya'ar' service laboratory. Particle size was determined by hydrometric method (Black, 1986). Organic matter was determined by 6 h incubation at a temperature of 550 °C.

Bioassay procedure

Larval stage was determined according to Maier et al. (1990). Bioassays were conducted on the 2nd and 4th larval stage, with or without substrate. Sand or silt was used as the tubes' substrate. Ten larvae were placed in a 120 ml plastic cup containing 90 ml tap water, and allowed to acclimate and construct tubes for 24 h before experimentation. Test concentrations were prepared by diluting appropriate aliquots of the stock solution with tap water and adding 10 ml to each to yield a total of 100 ml.

Each bioassay consisted of 5 experimental concentrations and 1 control. Each concentration was repeated 5 times with 10 larvae in each cup, for a total of 300 larvae per each lethal or effective concentration value**.** These procedures were repeated with the following three substrates: sand, silt and no substrate. In bioassays lasting more than 24 h, larvae were fed by adding 10 mg of yeast extract to the appropriate cups every other day. Larvae were considered dead if they did not respond to gentle prodding or were missing.

The effect of the increasing amounts of sand and silt on the remainder concentration of monochloramine and copper sulfate after 24 h incubation was tested in plastic cups containing 100 ml test medium without larvae. The residual concentrations of chloramine, (15 mg l^{-1} at the start) were tested at 6 silt concentrations (0, 0.5, 1, 2, 3.5, 6 ml silt, prepared as described above, per cup test) and at 6 sand concentrations (0, 1, 2.5, 5, 10 and 25 g of dry sand per cup test) with 5 replicates for each concentration.

Microfluorometry procedures

Cytoplasmatic enzymes play a key role in the development of cellular injury and death. Injured or dead cells lose the activity of many cytoplasmatic enzymes. Fluorescein diacetate (FDA) was used for the determination of non-specific cytoplasmatic esterase. The fluorescence is due to fluorescein that by enzymatic hydrolysis is liberated from the non-fluorescent FDA through cytoplasmatic esterase. Fluorescense intensity of larval midgut cells was determined by a fluorescent microscopy in the vital contact microfluorometer. Sernetz & Thaer (1973) and Bresler et al. (1990) described this technique in detail. The fluorescein diacetate (FDA) was obtained from Serva (Germany). Four replicates of 4th instar larvae with no substrate, and with silt as a substrate, were exposed to copper concentrations (0, 25, 50, 100 mg l^{-1}) in 120 ml plastic cups. After 1.75 h the larvae were removed

Table 1. The susceptibility (The LC_{50} and LC_{90} calculated Values + 95% FL) of 2nd and 4th instar *Chironomus luridus* midges to chloramine or copper, exposed in different substrates; silt, sand and no substrate

Toxicant (exposure time)	Instar	Substrate	*n* mg l^{-1}	LC_{50} mg l^{-1}	(95% FL) mg l^{-1} *	LC_{90} mg l^{-1}	(95% FL) mg l^{-1} *
Chloramine (1 d)	4th						
		None	300	2.0	$(1.6–2.3)^a$	3.7	$(3.3–4.5)^a$
		Sand	300	6.4	$(5.5–7.1)^b$	11.5	$(10.3–13.6)^b$
		Silt	300	12.2	$(11.2–13)^c$	16.0	$(15.0–17.7)^c$
Copper (1 d)	4th						
		None	300	3.4	$(3.1–4.0)^a$	5.3	$(4.4–7.9)^a$
		Sand	300	7	$(6.8–7.6)^b$	8	$(7.4–10.8)^{ab}$
		Silt	300	80.5	$(55–107)^c$	336	$(240–570)^c$
Copper (14 d)	2nd						
		None	300	0.11	$(0.05–0.14)^a$	0.20	$(0.15–0.47)^a$
		Sand	300	0.83	$(0.52–1.10)^b$	1.60	$(1.20–2.30)^b$
		Silt	300	2.40	$(0.83–3.60)^b$	4.00	$(4.0–13.0)^c$

* Values in each column group (of mean separation) with same letter are not significantly different from each other (95% FL).

from the test cups, washed in clean water, and 2 μm FDA was added. Gut's cell fluorescence intensity was measured after 15 min of incubation.

Data analysis

Mortality in laboratory bioassays was corrected for control mortality, and the data were analyzed by log-dose-probit regression (U.S. Environmental Protection Agency, 1988). Failure of overlap in the 95% Fiduccial limit (95% FL) was used as a criterion for establishing a significant difference. Differences in the LC_{50} values of larvae in 3 conditions of substrate for tube building were tested using Fridman's method for randomized block (Sokal & Rohlf, 1998).

Results

The effects of two toxic chemicals: copper sulfate and monochloramine, on the survival of *Chironomus luridus* larvae in 3 experimental conditions were examined. The different conditions were: (a) no substrate, (b) sand, and (c) silt as substrate.

Fourth instar larvae

The LC_{50} values of naked 4th instar of *C. luridus* larvae, exposed to chloramine and copper for 24 h were significantly lower (failure of overlap in the 95% FL, $P<0.05$) than those of the same larvae within tubes made of sand or silt substrate (Table 1). Note that the LC_{50}values of the naked larvae were 6 times lower for chloramine and 23 times lower for copper than the LC_{50} values of larvae in silt tubes. Exposing larvae for 7 d to chloramine (Fig. 1a) or copper (Fig. 1b) resulted in a similar tendency ($P<0.05$), except for the difference between sand and silt in the chloramine exposure (Fig. 1a). The dosage of chloramine and copper that caused the mortality of half the naked larval population after 7 d of exposure was 9 and 17 times lower than the dosage for larvae within silt tubes. The LC_{90}'s of chloramine after 1 and 7 d of exposure within silt tubes were 16 and 10 mg l^{-1} respectively, while the LC_{90}'s of copper after 1 and 7 d of exposure in the same conditions were 336 and 8 mg l^{-1}, respectively. The relationships between those calculated LC_{90}'s reveal that the sensitivity of the larvae to copper after 7 d exposure increased dramatically, while the value for chloramine remained the same.

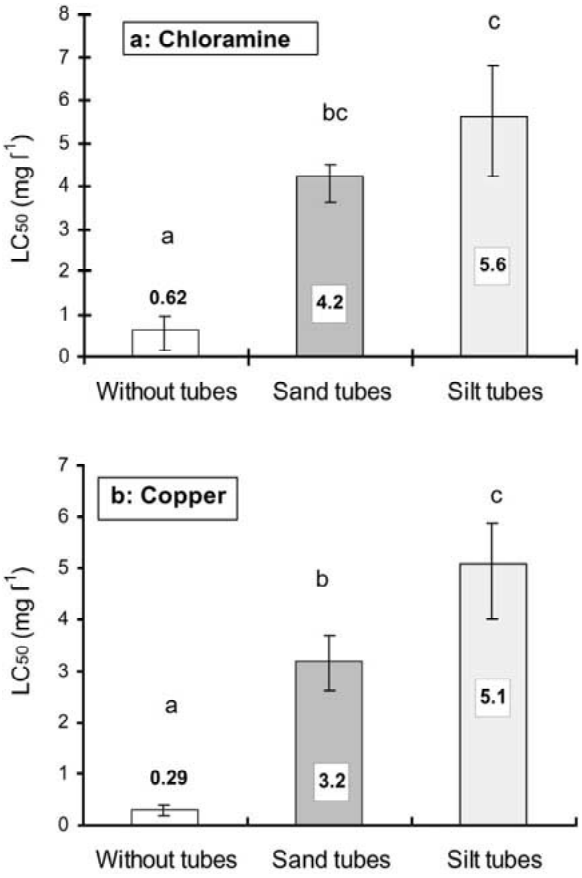

Figure 1. The $LC_{50} \pm 95\%$ FL (mg l^{-1}) values calculated for the effect of Chloramine (a) and Copper (b) on 4th instar larvae of *Chironomus luridus* after 7 d incubation in different substrates.

Third and second instar larvae

Exposing 3rd instar to chloramine and copper for 7 d resulted in LC_{50} values of 0.35 and 0.3 mg l^{-1}, respectively. These values were 3.5 and 10 times, respectively, lower than that of the sand tubes larval population and 8 and 23 times lower than the silt tubes larval population. The LC_{50}value of 14 d exposure of 2nd instar to copper was significantly lower in naked larvae ($P<0.05$) than in larvae with sand or silt tubes (Table 1).

The LC_{50} values from the different exposure times, 3 larval instars and 3 substrate conditions, were compared using Fridman's method for randomized block. Significant differences were found between LC_{50} values of naked larvae, larvae with sand tubes and larvae with silt tubes that were exposed to chloramine (X^2=6, df=2, $P<0.05$) and to copper (X^2=8, df=2, $P<0.05$). The results described here indicate that naked larvae are much more sensitive to chloramine and copper in comparison to larvae with tubes, especially silt tubes.

Behavioral response

Chloramine significantly affected the behavior of 4th instar larvae within silt tubes in a different way than the copper and untreated control (Fig. 2, one-way ANOVA, for 4 h incubation, $F_{(2,1)}$=22, $P<0.001$). A short exposure of larvae in silt tubes to high dosage of chloramine (=20 mg l^{-1}) resulted in tube desertion, followed by larval color change from red to white. The higher the chloramine dosage was, a higher desertion occurred and in shorter time. Exposing larvae in silt tubes to copper (up to 500 mg l^{-1}, 4 h) did not result in tube desertion or mortality (Fig. 2).

Non-specific esterase activity

Silt tubes had a significant effect on esterase activity in larval midgut's cells when larvae were exposed to copper (two-way ANOVA, $F_{(1,24)}$=4.35, $P<0.05$). Naked larvae showed a distinct decrease in enzymatic activity (Fig. 3). They retained only 8% of its original enzyme activity at 100 mg l^{-1} copper after 1.75 h. Silt tube dwelling was not affected at the two lower concentrations and retained about 60% of the non-specific esterase activity at 100 mg l^{-1} copper (note that LC_{50} for 24 h was 80.5 mg, see Table 1).

The properties of sand and silt used in these experiments

Sand was mainly (99.9%) inorganic matter whereas silt consisted of 22.5% organic matter. Ninety-six percent of sand particles size were 200–2000 μm and 4% was between 2 and 20 μm. Silt particles were composed of: 54.5% 200–2000 μm, 28.9% 20–200 μm and 16.7% 2–20 μm.

The remainder concentration of 15 mg l^{-1} monochloramine in test water after 24 h was 32% (4.8 ± 0.2 mg l^{-1}). Significant differences in the residual chloramine concentrations were found when different amounts of silt were added to the water (Table 2, one-way ANOVA $F_{(5,23)} = 303$, $P \ll 0.001$). The more silt was added the less concentration of chloramine remained $Y = -0.684X + 3.877$ ($P<0.001$, $n = 28$, $R^2 = 0.81$). Sand had no effect on the residual concentration of 15 mg l^{-1} monochloramine ($F_{(5,24)} = 2.18$, $P>0.05$).

There was no effect of silt or sand on the residual concentration of 20 mg l^{-1} copper after 24 h. The

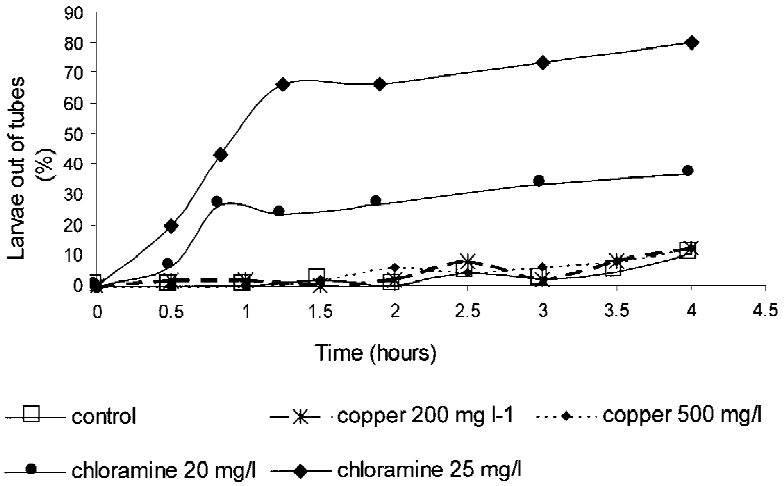

Figure 2. Getting out of silt tubes – Comparison of the proportion of larvae that deserted their silt tubes in response to exposure to elevated copper or chloramine concentration (n=50 larvae for each concentration).

Table 2. The effect of silt concentrations on the 24 h residual concentration of chloramine (SD denotes one standard deviation from the mean)

Silt volume	Silt dry weight	Chloramine residual concentration (mg l^{-1})		Chloramine, % of initial concentration	
(ml)	(mg l^{-1})	Mean	SD	Mean (%)	SD
0	0	4.8	0.2	32.1	1.3
0.5	0.055	3.7	0.3	24.7	2.1
1	0.11	2.6	0.2	17.8	1.5
2	0.22	1.7	0.2	11.5	1.5
3.5	0.39	1.8	0.04	7.8	0.3
6	0.66	0.4	0.05	2.8	0.3

residual concentration of copper in the control water was 0.94 (±0.03) and in silt and sand 0.97 (±0.07) mg l^{-1} copper. The high pH (8.1) of tap water used in all the experiments seemed to restrict the copper solubility. No significant difference was found (two-way ANOVA) in the residual concentration of 10, 20, 40, 100 and 200 mg l^{-1} copper between test water, sand and silt after 24 h incubation ($F_{(2,56)}$=3.05, P>0.05).

Discussion

The larvae of the midge C. *luridus* that remain outside their burrow are much more sensitive to chloramine and copper than those dwelling in tubes. Because larval sensitivity depends upon the material from which its tube is made (sand or silt), the defense maybe connected with the tube itself. Three elements may be involved in the protective nature of the *Chironomus* 'house' against chemicals: the protein matrix of the tubes, the foreign particles adhering to the tube protein, and the behavioral response of the larvae.

Larvae within sand tubes are more sensitive to the chemicals examined than those living in silt tubes (Table 1, Fig. 1). In *C. luridus*, as in many other species, the tube consists of foreign particles embedded within and around the larva's own protein secretion (Edgar & Meadows, 1969). Sand particles are bigger than silt's at least in one order of magnitude, and its organic matter content is lower in two-magnitude (Burton & Rodgers, 1994). Similar findings were found in our study. Rui-de-xue & Ali (1997) showed that particle size affected case-making behavior of larval *Glyptotendipes paripes*. The larvae preferred smaller sized sand (<0.84-mm-diam) to make longer tubes. These observations emphasize a behavioral choice that could influence the physical property of silt versus sand tubes, the first being tighter and more protective. Silt had a significant effect in the residual concentration of chloramine (Table 2), while there were no significant differences on the residual concentrations of copper among water, silt and sand. These findings assess the hypothesis that the tube itself is the larval defender. The differences found between larvae's sensitivity to copper (in the 3 substrate situations) are due to the protection that the tube gives its resident and not because silt or sand for themselves reduce the effective concentration of the toxicant. Although

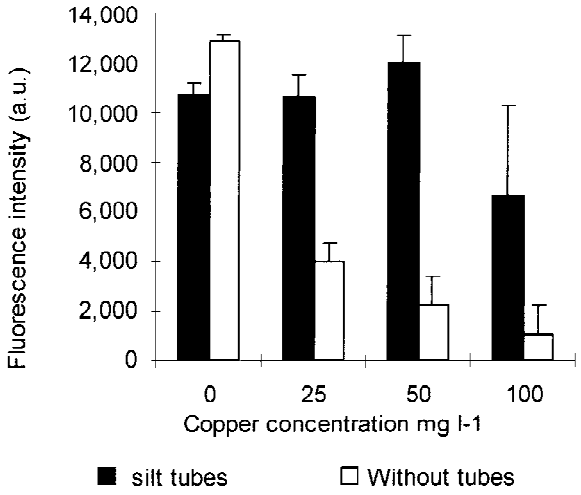

Figure 3. Effect of exposure of *Chironomus luridus* 4th instar larvae (with and without tubes) to different copper concentrations on the non-specific esterase activity (expressed as fluorescence intensity, mean a.u. ± SD) in midgut. Larvae with silt tubes were exposed to copper while within tubes then removed from tubes before applying FDA (n=4 larvae for each concentration).

it might be that the slope of the curve at which the copper concentrations is decreasing (from 20 mg l^{-1} in 24h) is not the same in all 3 substrate situations. Further work is needed.

The sensitivity (LC_{90}) of 4th instar to copper after 1 d exposure was 42 times lower than those for 7 d. Thus, we suggest that in the short term the larvae may increase their protection by controlling the rate of water exchange. Such a mechanism no longer holds at long exposure. Even so, at long exposure a larva that owns a tube is better protected than a tubeless one. An alternative explanation can be that chloramine is an acute toxicant that does not need to be accumulated, while copper should be bio-accumulated before it can become toxic. Basically the same tube protects the larvae from chloramine as well as from copper, although it is likely that the 2 chemicals have a different mode of action.

Using the microfluorometric technique (Fig. 3) enabled us to identify non-lethal stress caused by copper. The non-specific esterase plays a role in the development of cellular injury. The naked larvae showed a 'dose-response' reaction, with the gradual decrease of non-specific esterase activity. On the other hand, the larvae within the silt tube were indifferent to the lower concentrations, as a result of the tube protection and responded only to a dose higher than LC_{50} /24h (100 mg l^{-1}). This assay supports our observation regarding the role of the tubes as a barrier against the chemicals. When *C. luridus* was exposed to a lethal concentration of chloramine the larvae escaped from the tubes (Fig. 2). Bay et al. (1966) reported that when oxygen is depleted, some chironomid larvae leave the bottom and are carried about by water currents. They return to the bottom only when the oxygen supply has been restored. Dimond (1967) concluded that horizontal movement in lakes is related to conditions such as crowding and unfavorable environment. These phenomena may indicate that larvae abandon their tubes because the tubes can no longer supply protection. As a result the midges larvae move away to settle in a new unpolluted location. Different researchers used different types of substrates, such as sand (e.g. Ali & Nayar, 1986; Alexander et al., 1997) paper or cellulose (e.g. Gauss et al. 1985; Postma & Davis, 1995), no substrate at all (Kondo et al. 1995), or food as substrate (e.g. Hatakeyama & Yasuno, 1981; Koswalt & Knight, 1987; Postma et al., 1995). Our results suggest that the type of substrate used in bioassay with chironomid larva is critical and there is a need for a better standardization even if we limit ourselves to examination of heavy metals, pesticides and biological pesticides.

Sediment provides a habitat for various aquatic organisms and at the same time is a major repository for many of the more persistent chemicals that are introduced into surface water (Ingersoll et al., 1995). To such habitats Chironominae species are well adapted (Winner et al., 1980). In studying the defense mechanism of the chironomid tubes, we refer to copper and chloramine as a model for tube-chemical interaction. We suggest that the structure and function of *Chironomus* tubes, play a role in chironomid's adaptation to polluted habitats, aside of their physiological tolerance as was demonstrated for example, by Krantzberg & Stokes (1989). They found that *Chironomus* spp. could regulate the body concentration of copper via metallothioneine-like protein.

Benke (1998) claimed recently that the annual production/biomass (*P/B*) values of larval chironomid assemblage are among the highest estimated for aquatic metazoans and are similar to or exceed turnover rates of microbes in many aquatic ecosystems. The approach presented in the current study may give a better understanding of the adaptation of tube-dwelling chironomids to aquatic systems.

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