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The Selective Environment: genetic adaptation of the midge *Chironomus riparius* to metal pollution

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The aim of the present study was to provide conclusive evidence for either genetic adaptation or phenotypic plasticity of the midge *Chironomus riparius* in response to metal pollution. To this purpose the genetic structure and copper sensitivity of *C. riparius* populations from metal-polluted and reference sites was compared. Microsatellite analysis demonstrated that populations from metal-polluted sites were genetically different from the population of a nearby reference site. In addition, midges from a metal-polluted site were less sensitive to copper than the laboratory culture. By combining the population genetic structure of *C. riparius* and copper sensitivity of the F1 larvae we concluded that metal tolerance in *C. riparius* is most likely due to genetic adaptation.

Keywords: Chironomus riparius, metal contamination, genetic adaptation, copper sensitivity, microsatellite analysis.

In metal-polluted environments high abundances of *Chironomus riparius* have been reported (de Haas *et al*. 2005) and this species is considered tolerant to metal pollution (Postma *et al*. 1995, Groenendijk *et al*. 2002, Airas *et al*. 2008). However, the mechanism by which its tolerance is achieved still remains uncertain. Groenendijk *et al*. (2002) argued that a decreased sensitivity to cadmium of F1 offspring from midge larvae originating from metal-polluted sites and their elevated mortality under control conditions are indicators of genetic adaptation to metals. Several studies provided evidence supporting genetic metal adaptation (van Straalen *et al*. 2005, Buchwalter *et al*. 2008). But there is also evidence in conflict with genetic adaptation: in laboratory-reared hybrid offspring metal adaptation was quickly lost (Groenendijk *et al*. 2002). Also Nowak *et al*. (2009)

found no evidence in favor of genetic adaptation in a midge population that had been exposed to tributiltin for 12 generations.

An alternative hypothesis to genetic adaptation is phenotypic plasticity, which involves phenotypic changes in response to environmental changes. Phenotypic plasticity occurs especially when environmental conditions are variable, which is common in polluted ecosystems (Brown *et al*. 1982, Roesijiad 1992, de Witt 1998, Patrick *et al*. 2002, Whitman & Agrawal 2009). Therefore, phenotypic plasticity could also play a role in the tolerance of *C. riparius* to metal pollution. Hence, it is not yet known whether the metal tolerance of this species is based on genetic adaptation or phenotypic plasticity (Manguette 2009). Therefore, the aim of this study was to provide conclusive evidence for either genetic adaptation or phenotypic plasticity of *C. riparius* in response to metal pollution. To this end *C. riparius* larvae were sampled at reference and polluted sites. To determine the population genetic structure of these *C. riparius* populations, microsatellite analysis was used and it was determined whether midge assemblages at different field locations were in panmixis or not. To determine the possible heritability of copper tolerance, the sensitivity to copper of first-generation (F1) larvae from the population from a polluted site was compared to the sensitivity to copper of the F1 from a laboratory culture.

MATERIAL AND METHODS

Site description

Chironomus riparius larvae from three streams in Belgium, differing in metal pollution, were sampled in March and May 2009 (Fig. 1). Site selection was based on metal concentrations measured by the Flemish Environment Agency (= Vlaamse Milieumaatschappij, VMM): copper, nickel, cadmium and zinc (VMM 2009). The two polluted sites showed concentrations exceeding the Maximum Permissable Risk level (= maximaal toelaatbaar risico, MTR) for at least one metal.

River water characteristics, sampling and analysis

At each sampling site, the following characteristics were measured: stream velocity, temperature, pH and conductivity. Also at each site, a 1-l water sample was taken. These samples were acidified using a 10 ml l^{-1} HNO_3 Ultrex solution (65%, Sigma Chemie BV, Zwijndrecht, The Netherlands). Samples were analysed for copper, cadmium, nickel and zinc. Analysis was performed using Inductively Coupled Plasma (Perkin Elmer 3000XL, ICP-OES). Chloride analysis was performed by titration using 0.1 M $AgNO_3$ and the program TiNet 2.5 (Metrohm AG, Herisau, Switzerland).

For the collection of midge larvae, nets (mesh size 300 µm) were used to scrape off the sediments and for kick sampling. In the field, the sediment was sieved (three sieves used sequentially, with mesh sizes of 4.75, 2.24 and 0.85

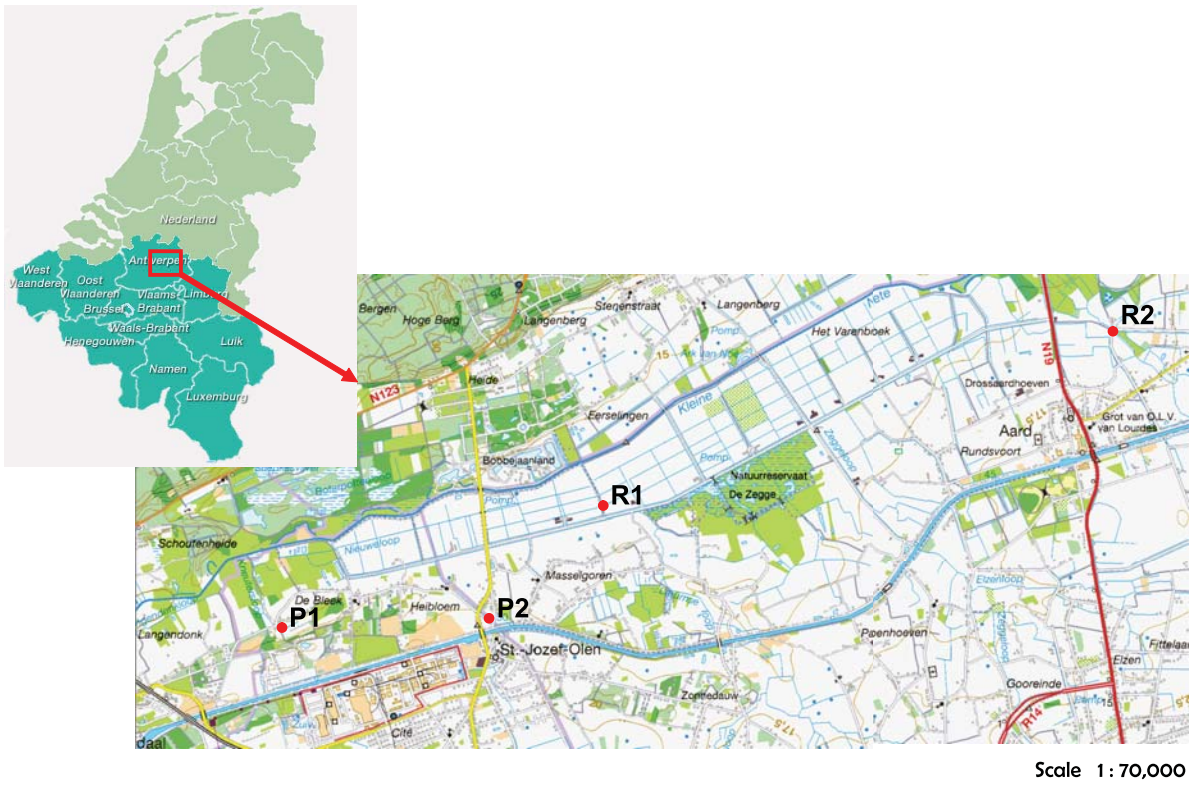

Figure 1. Sample sites in Belgium. P1: Kneutersloop (VMM code 303600), P2: Steenhovenloop (VMMcode 303800), R1: Larumseloop (VMM code 303840), R2: Dalemansloop (VMM code 303900).

mm), and midge larvae were collected. The sampled individuals were placed in buckets and transported to the laboratory on the same day. Within 24 h after sampling the individuals were selected for either microsatellite analysis or to set up a culture. Only the P2 sample contained enough individuals to set up a culture.

Genetic structure of *Chironomus riparius* populations

From the individuals for microsatellite analysis DNA was isolated using the CHELEX method (Walsh *et al.* 1991). Individual larvae were stored in 1.5-ml propylene tubes containing 0.2 ml zirconium beats and 400 µl 5% chelex (Sigma). Five µl proteinase-K (Sigma) was added to each tube after which samples were crushed using a precellys 24 (6500 rpm, 2× 30 s). Thereafter, the samples were incubated at 56 °C for 1 h, after which a second incubation took place at 98 °C for 10 min to denature the proteinase-K (Sigma). Thereafter, the samples were stored at -20 °C.

Microsatellite analysis was conducted on four loci: msco2, msco4 (Nowak 2006), locus04 and locus06 (IBED). The analysis was performed on 20 individuals from five populations each (P1, P2, R1, R2 and Laboratory culture). For the analysis, DNA samples were diluted 5× with milli-Q water. A PCR-reaction mix was made with a total volume of 10 µl, containing 4.15 µl ddH_2O, 1 µl 10×

PCR buffer (SpheroQ), 0.2 µl 10 mg ml^{-1} BSA (Sigma), 2 µl 1 mM of each dNTP (Fermentas, Burlington, Canada), 0.3 µl 10 µM primer forward (Biolegio), 0.3 µl 10 µM primer reverse (Biolegio) and 0.05 µl 5 U $µl^{-1}$ Super taq (SpheroQ) per sample. For loci MSC02 and Msc04 the following PCR-cycles were conducted: 94 °C for 2 min, 34× the following steps: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final step of 72 °C for 7 min. For loci locus04 and locus06 the following PCR-cycles were carried out: 94 °C for 3 min, 34× the following steps: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and a final step of 72 °C for 5 min. The samples were stored at -20 °C until further analysis.

A mixture of 5 µl loading dye (20 mM EDTA, 0.08% Bromophenolblue in deionised formamide), 4 µl ddH_2O and 1 µl of PCR product was made. From this mixture 0.3 µl was loaded on a 6.5% polyacrylamide gel in a Li-cor 4200 series machine. Run conditions were 1,400 V, 48 °C for approximately 45 min. Length of the visualised bands was scored. Population genetic analysis was conducted using the software package Arlequin v.3.1 (Excoffier *et al.* 2005) to calculate the genetic difference between sites and the Hardy-Weinberg equilibria per site.

Copper sensitivity of *Chironomus riparius* populations

From P2 enough individuals were collected to start a culture. P2 larvae were transplanted to three plastic aquariums (32 × 17 × 18 cm) containing per aquarium 4.5 l Dutch Standard Water [DSW, containing per litre demineralised water 100 mg $NaHCO_3$ (Merck), 20 mg $KHCO_3$ (Merck), 200 mg $CaCl_2.2H_2O$ (Sigma) and 180 mg $MgSO_4.7H_2O$ (Merck)] and a layer of 2 cm of clean fine sand. Evaporated water was replaced with demineralised water. A flight cage was set on top of each aquarium and air supply was added to the water. Midges were fed every 3 days with half a teaspoon of Trouvit (Trouw, Fontaine-les-Vervins, France): tetraphyl (20:1). The culture was placed in a climate room at 20 ± 1 °C and a light:dark regime of 16:8 h, with a twilight zone of 30 min between these periods. On a daily basis, emerged adults were transported to a cage for mating. A dish containing DSW was placed in the cage for the females to deposit their eggs. Every day, egg ropes were removed from the dish and placed in a 20-ml cuvet containing DSW. After 3 days newly hatched larvae (<24 h) were used in toxicity tests.

To determine the copper sensitivity of the P2 population a copper toxicity test was performed. The laboratory culture was used as a reference. The laboratory culture was started in 1986 using larvae originating from a small experimental pond from the University of Amsterdam. Egg masses are regularly exchanged with other laboratory cultures, maintained in The Netherlands, to reduce the effects of inbreeding. In addition, the culture is constantly maintained at a large population size.

Toxicity experiments with the laboratory culture consisted of five replicates of 20 larvae per copper concentration (in a test volume of 100 ml), whereas experiments with P2 larvae consisted of four replicates. The following nominal

copper concentrations were tested: 0, 250, 500, 1000, 1750 and 2500 µg l^{-1} (10 g l^{-1} copper concentrate, Sigma). At the start of the experiment 2.0 mg per larva Trouvit (Trouw):tetraphyl (20:1) was fed. To prevent evaporation, the jars were covered with transparent foil. After 96 h, surviving larvae were counted. Toxicity tests were performed under the same conditions as the culture. At the beginning and at the end of the experiment 2-ml water samples were taken in duplicate from each test concentration and acidified using 20 µl 69-70% HNO_3 Ultrex (Sigma). Samples were stored at room temperature until further analysis. To determine the actual copper concentrations of the treatments, the water samples were analysed using Perkin AAnalist 100 Atomic Absorption Spectrophotometer. Survival of the larvae was expressed as a percentage of the corresponding control and plotted against the actual copper concentrations in the water.

From the dose-response curves obtained in the toxicity tests the LC_{50} values were calculated using the logistic response model of Haanstra *et al.* (1985). A log-likelihood test was performed to determine whether the LC_{50} values differed significantly. Statistical analyses were performed using the software SPSS version 16.0.

RESULTS

River water characteristics

All sites contained copper concentrations above the MTR set by the RIVM (= National Institute for Public Health and the Environment, Bilthoven, The Netherlands), P1 being extremely copper polluted (1273 µg l^{-1}) (Table 1). Nickel and zinc concentrations were above the MTR at the two polluted sites and, although less elevated, at the reference site R2. Cadmium concentrations only exceeded the MTR at P1. P1 and P2 show a high conductivity.

Table 1. Water characteristics and chemical composition (µg l^{-1}) of the sampled streams. The values given in bold exceed the MTR (maximum permissible risk level) set by the RIVM.

	Heavy metals				Other metals	Other	Physical parameters				
	Cu	Cd	Ni	Zn	Ba	Cl^-	Width (m)	Temp (°C)	Cond (µ cm^{-1})	pH	Vel (km h^{-1})
MTR	2.0	2000	5.1	40.0	230.0	200000					
P1	**1273.0**	**3000**	**95.0**	**207.0**	49.0	36800	1.5	9.4	**685.0**	6.63	3.57
P2	**15.0**	<2.0	**53.0**	**44.0**	15.0	**1917300**	1.8	16.1	**8760.0**	6.78	5.38
R1	**4.0**	<2.0	3.0	23.0	34.0	18700	3.1	11.4	402.0	6.21	2.60
R2	**41.0**	<2.0	**12.0**	**61.0**	49.0	41700	6.3	11.6	420.0	6.47	2.55

Temp, temperature; Cond, conductivity; Vel, velocity

Genetic structure of *Chironomus riparius* populations

Only individuals showing a complete genotype (four loci) were included for analysis, because a positive outcome on all microsatellite loci is an indication that the individual belongs to the species *C. riparius* (Manguette 2009). Too few individuals from the site R1 met this criterion and therefore R1 was excluded from the analysis. Sample sizes of the P1, P2, R2 and laboratory culture were 8, 13, 6 and 26, respectively. An overview of the genetic structure, including Fst-values is given in Fig. 2. Sites P1 and P2 showed no significant difference in Fst-values (0.029, P = 0.35), and are therefore in panmixis. Site R2, as well as the laboratory culture, differed significantly from all other sites (P<0.01) and are therefore genetically different from both polluted sites. All field sites were in close proximity of each other with a maximum distance of 9 km. Hence, differences in genetic structure between field populations could not be caused by distance.

Hardy-Weinberg Equilibrium (HWE) was calculated (Table 2), since deviations from HWE suggest disturbances. A lack of heterozygosity at one or more loci was observed in populations P1, P2 and in the laboratory culture. The two

Table 2. Observed (Ho) and expected (He) heterozygosity in the analyzed populations. Significant differences (P<0.05) are indicated in bold.

		Msc2			Msc4			Locus4			Locus6		
	n	Ho	He	P	Ho	He	P	Ho	He	P	Ho	He	P
P1	8	0.38	0.79	**0.01**	0.00	0.43	**<0.01**	0.75	0.92	0.19	0.25	0.77	**<0.01**
P2	13	0.69	0.83	0.12	0.31	0.58	**0.01**	0.77	0.85	0.42	0.46	0.82	0.08
R2	6	0.67	0.79	0.29	0.67	0.62	1.00	0.17	0.44	0.09	0.67	0.77	0.53
Lab	26	0.19	0.50	**<0.01**	0.35	0.43	**0.03**	0.38	0.40	1.00	0.69	0.62	0.96

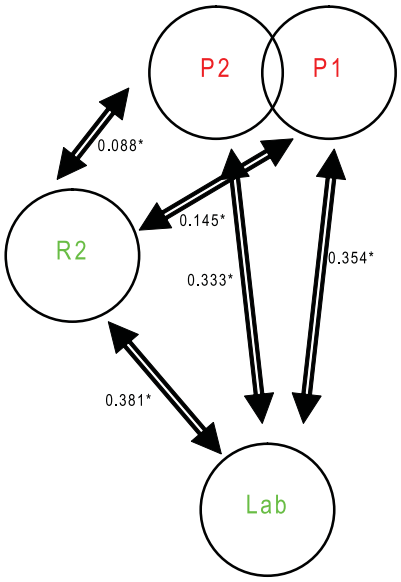

Figure 2. Comparison of population genetic structure of *Chironomus riparius* from P1 (n = 8), P2 (n = 13), R2 (n = 6), and the laboratory culture (n = 26) indicated as 'lab'. Circles represent sample sites. * indicates a significant difference in Fst values between sites (P<0.01). When there was no significant difference circles are clustered together.

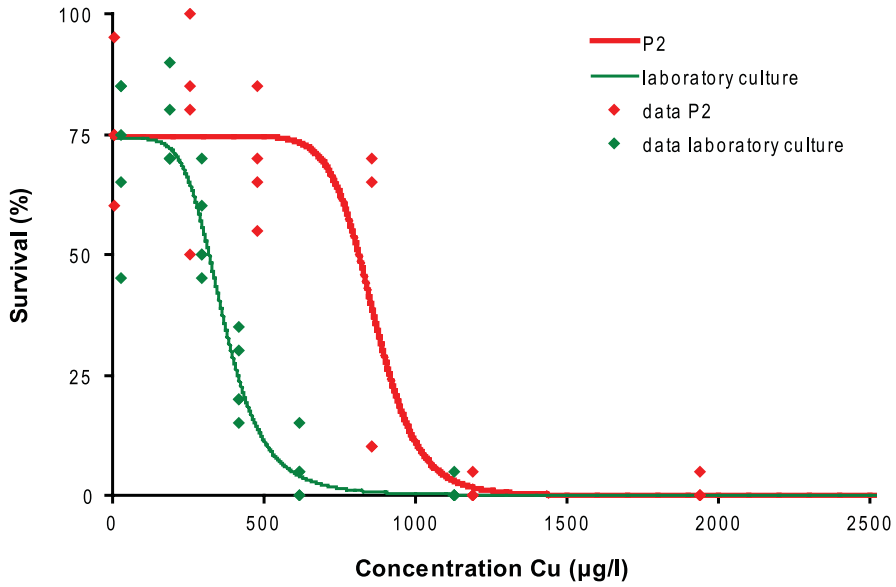

Figure 3. Survival (%) of *Chironomus riparius* first instars after 96 h of exposure to copper. Dots indicate data points, solid lines indicate the logistic response model of Haanstra *et al.* (1985).

polluted sites showed a deviation from HWE at least at one locus. In the reference population R2, no deviation from HWE was observed. The laboratory culture showed deviations from HWE at two loci.

Copper sensitivity of *Chironomus riparius* populations

Average control survival of the laboratory culture and P2 larvae was 74%. Clear dose-response relationships were observed for both P2 and the laboratory culture (Fig. 3). The LC_{50} of 863 µg l^{-1} (95% confidence limits: 792-936) obtained for P2 was significantly higher ($P<0.01$) than that of the laboratory culture (364 µg l^{-1}, 95% CL: 332-397) (Fig. 3).

DISCUSSION

River water characteristics

At P1, the copper concentration exceeded the LC_{50} for *C. riparius* (de Haas *et al.* 2004, Milani *et al.* 2003, Manguette 2009) and can therefore be considered as a selective force. R2 was more polluted than expected based on the VMM measurements. However, the copper concentration did not exceed the LC_{50} of aquatic invertebrates, whereas it exceeded the LC_{50} of one invertebrate species at P2 and of 10 invertebrates at P1 (van der Geest *et al.* 1999, Roman *et al.* 2007). Sediments of the reference sites were also of good quality (VMM 2005) and a high biodiversity was observed compared to the polluted sites (van der Wiele 2009). Therefore it is concluded that the elevated metal levels at the polluted sites may exert selection pressure on the present biota, in contrast to the reference sites.

Genetic structure of *Chironomus riparius* populations

The population genetic structure of *C. riparius* at P1 and P2 did not differ significantly, suggesting panmixis. In contrast, the genetic structure of R2 differed from the polluted sites, although the locations were nearby (7 and 9 km) and there was no physical barrier to migration. Panmixis within populations more than 40 km apart has been observed (Manguette 2009), so the distance between sites in the present study cannot explain the observed genetic differences. The results are opposite of the complete panmixis found by Manguette (2009), probably due to a high temporal variation in genetic structure or to the small sample size. The different genetic composition of the laboratory culture compared to all field populations is probably due to years of isolation.

In addition to differences in population genetic structure, deviations from HWE were also observed in populations at the polluted sites. This suggests the presence of disturbing influences like non-random mating, selection, small population size or random drift (Futuyma 2005). At P2, the midge population was quite large. Therefore, only non-random mating and selection due to metal pollution are left as possible causes.

The reference and polluted sites are genetically different and therefore it is concluded that gene flow in the field is reduced. This reduction is possibly due to selection for metal-tolerant individuals at polluted sites. The reduced gene flow between polluted and reference sites, as well as the deviations from HWE, suggest that polluted sites are subjected to selection, causing the populations to diverge. These observations are expected to be found when genetic adaptation is the mechanism of metal tolerance in *C. riparius*.

Copper sensitivity of *Chironomus riparius* populations

The LC_{50} for the P2 population was twice as high as that of the laboratory culture, indicating a consistently lower sensitivity of the population from this site compared to the laboratory culture. Unfortunately, no reference population from the field was successfully cultured due to shortage of *C. riparius* larvae. The genetic composition of the laboratory culture differed from all the field sites and therefore limits this comparison. However, the laboratory culture could be considered as an non-adapted reference population. The results of the present study and of Manguette's (2009) showed that a population is able to obtain a heritable, lowered sensitivity to copper when exposed to it for several generations, indicating genetic adaptation of the population.

Mechanism of metal tolerance in *Chironomus riparius*

The aim of this study was to provide conclusive evidence for how metal tolerance in *C. riparius* is achieved: by genetic adaptation or by phenotypic plasticity. The lower sensitivity to copper of F1 larvae from a polluted site compared to the laboratory culture suggests that tolerance is heritable. Moreover, both polluted sites were in panmixis, but separated from the nearby located reference site, sug-

gesting restricted gene flow. It is therefore concluded that genetic adaptation is the dominant mechanism leading to metal tolerance in *C. riparius*.

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