Chapter 4

Response of the non-biting midge *Chironomus riparius* to multigeneration toxicant exposure

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Abstract

The ability of the non-biting midge *Chironomus riparius* to withstand long-term toxicant exposure has been attributed to genetic adaptation. Recently, however, evidence has arisen that supports phenotypic plasticity. Therefore, the present study aimed to investigate if *C. riparius* indeed copes with prolonged toxicant exposure through phenotypic plasticity. To this purpose, we performed a multigeneration experiment in which we exposed *C. riparius* laboratory cultures for nine consecutive generations to two exposure scenarios of, respectively, copper, cadmium and tributyltin. Total emergence and mean emergence time were monitored each generation, while the sensitivity of the cultures was assessed at least every 3rd generation using acute toxicity tests. We observed that the sublethally exposed cultures were hardly affected, while the cultures that were exposed to substantially higher toxicant concentrations after the 6th generation were severely affected in the 8th generation followed by signs of recovery. A marginal lowered sensitivity was only observed for the highly exposed cadmium culture, but this was lost again within one generation. We conclude that *C. riparius* can indeed withstand long-term sublethal toxicant exposure through phenotypic plasticity without genetic adaption.

Introduction

Biodiversity generally decreases with deteriorating environmental conditions (Crunkilton and Duchrow, 1990; Solà et al., 2004; Relyea, 2005). Some species, however, are able to maintain viable populations under conditions that are fatal to others. Non-biting midges belonging to the genus Chironomus (Insecta: Diptera) are such pollution tolerant and persistent species (Armitage et al., 1983; Gabriels et al., 2010). Indeed, larvae of *Chironomus riparius* can cope with low oxygen concentrations (Redecker and Zebe, 1988), increased salinities (Bervoets et al., 1996), wide ranges of pH (Jernelöv et al., 1981; Havas and Hutchinson, 1982) and are, above all, remarkably tolerant to organic (e.g. Gower and Buckland, 1978; Friberg et al., 2010) and heavy-metal pollution (e.g. Winner et al., 1980; Groenendijk et al., 1999). Studies that aimed to unravel the mechanism underlying their ability to withstand long-term pollution have been performed with *C. riparius* populations obtained from heavy-metal polluted environments (Postma et al., 1995a; 1995b), as well as with *C. riparius* laboratory cultures that were exposed for multiple generations to a single toxicant (Postma and Davids, 1995; Miller and Hendricks, 1996; Ristola et al., 2001; Vogt et al., 2007). Except Ristola et al. (2001), these studies reported a decreased sensitivity in the toxicant-exposed midges and suggested, to a greater or lesser extent, that this was due to genetic adaptation. Substantial evidence for genetic adaptation, i.e. heritability of decreased toxicant sensitivity in offspring reared under clean conditions (Morgan et al., 2007), was, however, only provided for historically exposed *C. riparius* field populations (Postma et al., 1995a; 1995b). The other multigeneration studies reported in literature either did not perform this check and could thus not rule out phenotypic plasticity, i.e. physiological acclimation and subsequent maternal effects (Postma and Davids, 1995; Vogt et al., 2007), or found evidence for both mechanisms (Miller and Hendricks, 1996). A recent multigeneration study performed with the closely related species *Chironomus plumosus* (Vedamanikam and Shazilli, 2008) has revitalized this discussion, as they obtained substantial evidence for phenotypic plasticity by showing that decreased metal sensitivity could be induced within six generations, but also lost again after two generations of rearing under clean conditions. Interestingly, the same process of gaining and subsequent losing of decreased toxicant sensitivity was also reported by Vogt et al. (2007) when culturing midges under continuous tributyltin pressure. Triggered by these latter observations, the aim of the present study was to verify if *Chironomus riparius* indeed copes with prolonged toxicant exposure via phenotypic plasticity. To this purpose, we performed a multigeneration experiment where *C. riparius* cultures were exposed for nine consecutive generations to three model toxicants that were previously shown to differently affect *C. riparius* during a single generation sediment toxicity test (Marinković et al., 2011). For each of these three toxicants, i.e. the essential metal copper, the non-essential metal cadmium and the organometal tributyltin, two exposure scenarios were designed. One in

which the sublethal concentration remained constant and one in which after the 6th generation the exposure concentration was increased for three more generations. Total emergence and mean emergence time were monitored during each generation for all cultures. To assess changes in sensitivity, 14-day sediment toxicity tests were conducted with the corresponding toxicant at the start of the multigeneration experiment and subsequently at least every third generation.

Materials and methods

Test organism and culturing conditions

The *C. riparius* larvae used in the present study originated from the University of Amsterdam's in-house laboratory culture. Regular exchange of egg-ropes with other laboratories and the maintenance of a large population size guaranteed a high level of genetic variation as previously reported by Nowak et al. (2007). The culture was maintained in aquaria containing quartz sand overlaid with Dutch Standard Water (deionised water with 200 mg/l $CaCl_2$*$2H_2O$, 180 mg/l $MgSO_4$*H_2O, 100 mg/l $NaHCO_3$ and 20 mg/l $KHCO_3$; hardness is 210 mg as $CaCO_3$/l and pH 8.2 ± 0.2) at 20 ± 1 ˚C, 65% humidity and a 16: 8 h light: dark photoperiod. The culture was fed a mixture of Trouvit® (Trouw, Fontaine-les-Vervins, France) and Tetraphyll® (Tetrawerke, Melle, Germany) in a weight ratio of 20:1. This mixture was also used as food for all subsequent experiments.

Experimental setup of the multigeneration study

Chironomus riparius test cultures were exposed for nine consecutive generations to artificial sediment spiked with either copper ($CuCl_2.2H_2O$, copper standard, Fluka), cadmium ($CdCl_2$, Titrisol®, Merck) or tributyltin (TBT-Cl, 96% purity, Aldrich). The artificial sediment had a pH of 7.0 ± 0.5, a moisture content of 50% and consisted for 75% of quartz sand (Sibelco® M34, Belgium), 20% of kaolin clay (WBB Vingerling, the Netherlands) and 5% of α-cellulose (Sigma). For each toxicant, two exposure scenarios were designed: In the first scenario, the sublethal test concentration remained constant over nine generations (tox cultures). Because the effects on emergence remained limited during the first six generations, a second scenario was designed where the test concentrations were increased from sublethal to partially lethal for three more generations (tox$^+$ cultures). The sublethal test concentrations were based on previously conducted one generation toxicity tests (Marinković et al., 2011) and aimed to reduce total emergence with 15%, while the partially lethal test concentrations were chosen because they caused substantial mortality in the sensitivity testing experiments in the present study. The nominal test concentrations

were for copper respectively 15 and 30 mg Cu/kg dw; cadmium: 2 and 8 mg Cd/kg dw; and tributyltin: 0.5 and 3 mg Sn/kg dw.

Each generation of the *C. riparius* test cultures consisted of two replicate 22 l aquaria (35*25*25cm) and two additional replicate 400 ml glass beakers that were used for chemical analysis at the start (day 0) and half-way a generation (day 14). A third chemical analysis was conducted at the end of the generation (day 28) by sampling sediment from one of the replicate aquaria. Spiking of the artificial sediment was conducted according to Marinković et al. (2011). In short, appropriate amounts of copper and cadmium stock solution were added to 3.2 kg wet sediment in glass 3 l bottles. To these sediment-metal mixtures 6.0 g of food, which corresponded to 0.25 mg food/larvae/day for the entire duration of a generation (28 days), was added and the bottles were placed for 24 hours on a roller bank (20 rpm). The homogenized food-metal-sediment mixtures were subsequently divided over the test vessels, i.e. 1.5 kg/ 22 l aquarium and 60 g/ 400 ml glass beaker. The test vessels were carefully topped up with Dutch Standard Water (6 l/aquarium; 250 ml/ beaker) and covered with plastic foil to prevent evaporation. After settling of the sediment gentle aeration was turned on. The test vessels were conditioned for one week, allowing the compounds to equilibrate with the water and sediment and a stable sediment layer to be formed. For the less water-soluble compound tributyltin, an additional pre-spiking step was performed where the acetone dissolved tributyltin was mixed through 10% of the total amount of dw sediment. The tributyltin-sediment mixture was allowed to dry in a fume board, after which deionized water and the remaining wet sediment were added. From here on the same mixing and equilibration procedures were followed as for copper and cadmium.

All test cultures, including control and solvent control cultures, were started simultaneously from a batch of 50 egg-ropes that originated from our *C. riparius* laboratory culture. Using a stereo microscope and a blunt glass Pasteur pipette, 400 randomly chosen larvae (< 24 hours) were introduced in each of the two replicate 22 l aquaria (35*25*25cm) per treatment. To the two replicate 400 ml glass beakers that were used for chemical analysis, 10 larvae were added from the same batch. To allow settlement of the introduced larvae on the sediment, aeration in the test vessels was temporarily switched off and restarted 4 hours later. Two additional feedings of 700 mg food/aquarium and 17.5 mg food/beaker, corresponding to 0.25 mg food/larvae/day for a period of one week, were administered during each generation at days 7 and 14. On day 14 the plastic cover was replaced by a cage to prevent emerging midges from escaping during exhausting and from this day on, the aquaria were inspected daily for emerged midges until termination of the exposure period (day 28, in case of strongly delayed emergence day 33). Emerged midges were caught with an exhauster, sexed and transferred to a mating cage where they were allowed to swarm and lay egg-ropes. Egg ropes were daily removed from the meeting cage

and stored in Dutch Standard Water for up to three days at 4 ˚C. Typically the 30 first laid egg-ropes were hatched at 20˚ C in Dutch Standard Water and the larvae (< 24 hours) were used to start up the next generation in freshly prepared test vessels. This was possible for all cultures except for the TBT^{+} culture where due to the strongly reduced emergence in the 8th generation only 21 good quality egg ropes could be used to start the next generation.

Sensitivity testing experiments.

To determine the sensitivity of the test cultures during the multigeneration experiment, 14-day sediment toxicity experiments were conducted with the corresponding toxicant at the start of the multigeneration experiment and subsequently every 3rd generation. The control culture was tested with all three compounds. When decreased toxicant sensitivity was observed in a test culture, additional sensitivity testing was conducted with the respective test culture during the following generation to determine the stability of the decreased sensitivity. The sensitivity testing experiments were based on OECD guideline 218 (OECD, 2004) and were performed with the following nominal test concentrations of copper: 10, 20, 30, 40 and 50 mg Cu/kg dw; cadmium: 0.5, 1, 2, 4, and 8 mg Cd/kg dw; and tributyltin: 0.5, 1, 2, 3 and 4 mg Sn/kg dw. Controls and solvent controls were included. Spiking of the artificial sediment was performed the same way as previously described for the multigeneration experiment, with the only difference being that the initial feeding corresponded to 0.5 mg food/larvae/day for a period of two weeks (70 mg food/beaker). Each treatment consisted of seven replicate 400 ml glass beakers of which two were sacrificed for chemical analysis at the start (day 0) and halfway (day 7) the experiment. A third chemical analysis was conducted by sampling sediment from one of the remaining five replicates upon termination of the experiment (day 14). Ten larvae (< 24 hours) that were obtained from a batch of at least five hatched egg ropes per test culture were added to each test beaker. After 7 days an additional feeding of 17.5 mg food/beaker, corresponding to 0.25 mg food/larvae/day for a period of one week, was administered. On day 14 the experiments were terminated, the sediment was sieved through a 350 µm sieve and surviving larvae were counted.

Quality criteria and chemical analyses

The quality of the overlying water and the actual toxicant concentrations in the sediment were determined by sacrificing one replicate per treatment at the start, half-way and at the end of both the multigeneration experiment and the sensitivity testing experiments. The actual toxicant concentrations in the sediment were determined as previously described in Marinković et al. (2011). In short, sediment was centrifuged at 3000 rpm for 15 minutes and stored at -20 °C until analysis. For the copper and cadmium samples, the frozen sediment was oven-dried and duplicate 130 mg subsamples were digested for 7 hours at 140 °C in 2 ml of a 4:1 mixture of nitric acid (65% p.a.; Sigma-Aldrich) and hydrochloric

acid (37% p.a., Sigma-Aldrich). The digested samples were diluted with deionised water and analysed by flame atomic absorption spectrophotometry (Perkin Elmer AAnalyst 100, Germany). Certified reference material ISE 989 Riverclay (Wageningen Agricultural University, The Netherlands) was included for quality assurance and the measured metal test concentrations were corrected for copper (86%) and cadmium (88%) recovery. The actual metal concentrations in the sediment were calculated as time-weighted means of the three measurements per test concentration according to OECD guideline 211 (1998).

The tributyltin samples were analysed by RWS-Waterdienst, Lelystad, according to their in-house developed method accredited by the Dutch Accreditation Council. Two 1 gram subsamples of freeze-dried sediment were extracted by adding 15 ml methanol (J.T.Baker), 1.5 ml acetic acid (99.9%, J.T.Baker) and 7 ml hexane (J.T.Baker). After 5 minutes of mixing, 3 ml of 4M sodium acetate (J.T.Baker) and 4 ml 5% sodium tetraethylborate were added, and the samples were incubated for 22 min. The reactions were stopped with 5 ml 10M NaOH. The ethylated organotins were concentrated in hexane using an AlOx column (10% moisture, MP Ecochrom), after which they were transferred into iso-octane using a Kuderna-Danish solvent evaporator and a gentle stream of nitrogen. These extracts were analyzed with a gas chromatography mass selective detector GC-MSD (GC 6890 Series, Agilent Technologies; MSD: 5973 inert MSD, Agilent Technologies; HP Network Hewlett Packard). The quality control included reference sediment (Wadden sediment) and an internal reference consisting of monopropyltin (111%) and tripropyltin (102%). Tributyltin was measured in generation F1 and F9 of the multigeneration experiment at the start (day 0) and at the end (day 28). The measured tributyltin test concentrations were corrected for extraction losses of the reference sediment (91% recovery), and ranged between 69% and 80% of the nominal values at the start of the experiment. Using the time-weighted averages of the measured tributyltin test concentrations a correction factor was calculated allowing extrapolation of the remaining actual exposure concentrations.

Data analyses

All statistical analyses were performed at a 5% significance level in SPSS® 17 for windows. Emergence data from the multigeneration experiment was used to compare the exposed cultures with the (solvent) control cultures within and over the generations. Total emergence data was arcsine-sqrt transformed and tested for homogeneity of variance with Levene's test. A t test was used to compare total emergence of the exposed and the control cultures over the generations and per generation. The EmT50, i.e. the day at which 50% emergence occurred during a generation, was calculated for each culture according to Haanstra et al. (1985) by fitting a logistic curve ($y = c / (1+ e^{b*(\log(x) - \log(a))})$) through the cumulative number of emerged midges against time. In this equation a is the EmT50, b is the slope of the logistic curve, c is the average total emergence per replicate and x is the day

at which emergence was recorded. Since *C. riparius* has a bimodal emergence pattern, where males emerge prior to females (Watts and Pascoe, 2000), EmT50 values were calculated for all emerged midges as well as males and females separately. The EmT50 values of the exposed cultures were subsequently compared to the (solvent) control culture EmT50 value using generalised likelihood ratio tests (van Gestel and Hensbergen, 1997).

Larval survival data from the sensitivity testing experiments were used to calculate the concentrations of the test compounds that caused 50% mortality (LC50). The LC50 values were calculated with the actual toxicant concentrations using the same logistic response model (Haanstra et al., 1985) and were compared to the control culture LC50 values using generalised likelihood ratio tests (van Gestel and Hensbergen, 1997).

Results

Quality criteria and actual toxicant concentrations in the sediment

All experiments met the OECD guideline 218 validity criteria regarding water quality (OECD, 2004). The pH was 7.5 ± 0.4, ammonium concentrations remained 0 mg NH_4^+/l, conductivity was 665 ± 46 µS/cm, dissolved oxygen levels were above 70% air saturation and water temperature was 20 ± 1° C. The total emergence of the control culture ranged during the multigeneration experiment between 72% and 92% for all generations except the 6th generation where it amounted to 65% ± 0.5%. While the control emergence in the 6th generation was just below the required 70%, the multigeneration experiment was considered valid with an average (n=9) control culture emergence of 80 ± 8.8% over the nine generations.

During the multigeneration experiment, the actual toxicant concentrations of the different treatments hardly varied over the generations (Supporting Table S1), resulting in the following average actual toxicant concentrations for the tox (n=9) and tox$^+$ (n=3) cultures for, respectively, copper: 18.9 and 34.7 mg Cu/ kg dw sediment; cadmium: 1.9 and 7.0 mg Cd/kg dw sediment; and tributyltin: 0.3 and 2.1 mg Sn/kg dw sediment. Also the actual toxicant concentrations measured in the sensitivity testing experiments did not show strong deviations from the nominal concentrations (Supporting Table S2) and averaged for, respectively, copper (n=4): 4.7, 14.6, 23.7, 33.2, 43.1 and 54.9 mg Cu/kg dw sediment; cadmium (n=5): <0.01, 0.4, 1.0, 1.9, 3.4 and 7.1 mg Cd/kg dw sediment; and tributyltin (n=4): <0.001, 0.3, 0.7, 1.4, 2.1 and 2.7 mg Sn/kg dw sediment. For the actual concentrations in the water we refer to Marinković et al. (2011) who performed toxicity tests with the same toxicants and under identical experimental conditions, and reported the actual toxicant concentrations in the sediment, overlaying water and pore water.

Multigeneration exposure of C. riparius

Average (± st.dev) total emergence of the control culture (n=9) was 80 ± 8.8% during the multigeneration experiment (Figure 1a-b). There were no significant differences between the total emergence of the control culture and the solvent control culture which showed an average (n=9) total emergence of 82 ± 9.1% during the multigeneration experiment (Figure 1c). Exposure of *C. riparius* cultures to sublethal copper, cadmium and tributyltin concentrations resulted for the respective cultures in a significant emergence reduction of 12%, 12% and 33% during the 1st generation (Figure 1a-c). Over the nine

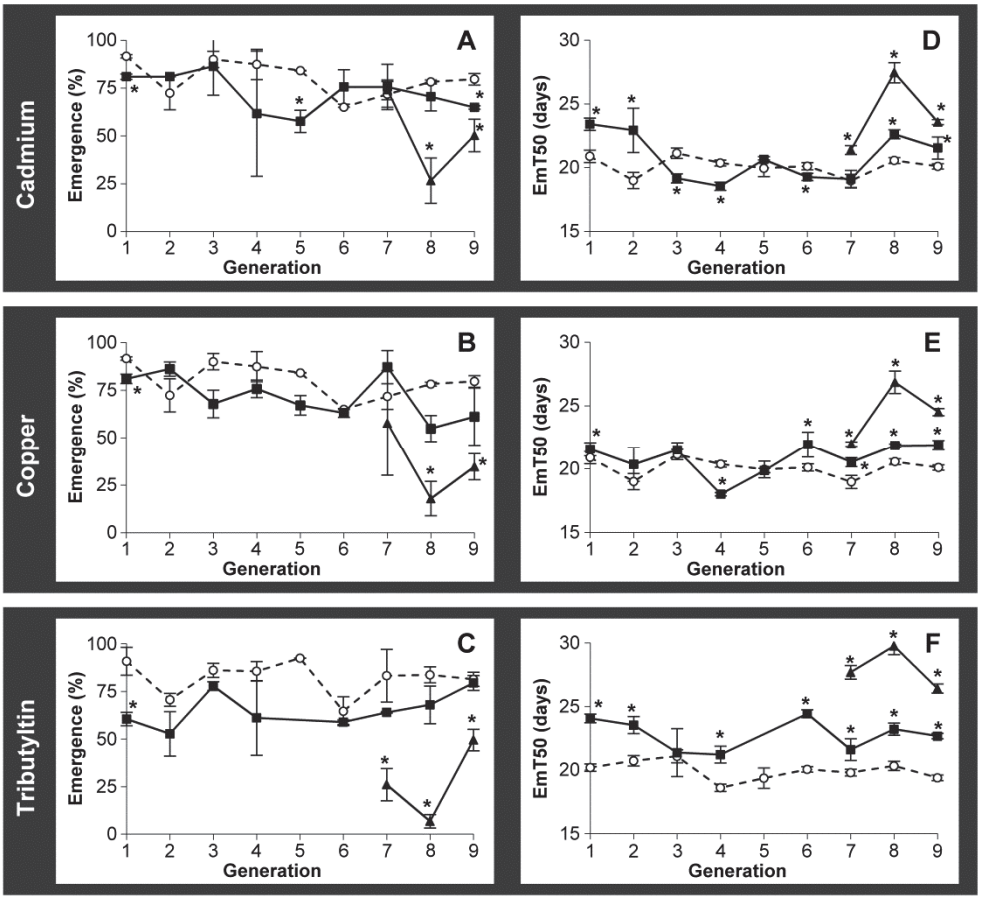

Figure 1: Effects of the three toxicants on *C. riparius* total emergence and the mean emergence time (EmT50) during nine consecutive generations of exposure. Fig. 1a-c: Total emergence (average % ± st.dev) per generation. Fig. 1d-f: EmT50 (day ± 95% C.I) values per generation. The open circles represent the control culture (for tributyltin the solvent control culture), the black squares the tox culture and the grey triangles the tox+ culture. * indicates values significantly differing from the (solvent) control culture ($p<0.05$).

generations only the tributyltin culture had a significantly lower total emergence which averaged (n=8) 65 ± 9.3% (Figure 1c). As shown in Figure 1c, the 5th generation of the tributyltin culture was excluded from the calculations. This was because the concerning aquaria suffered from a two-day power failure that resulted in unreliable emergence data for the tributyltin culture. Nevertheless, sufficient good quality egg ropes were obtained to start the 6th generation. The tox^{+} cultures, which were exposed to substantially higher toxicant concentrations after the 6th generation, were much stronger impacted (Figure 1a-c). The tributyltin^{+} culture was immediately significantly impaired with a total emergence of 26% ± 8.5% in the 7th generation, while it took a generation longer to see a comparable significant reduction in the total emergence of the copper^{+} and cadmium^{+} cultures. In the 9th generation, total emergence of the three tox^{+} cultures remained significantly impaired, however, obvious signs of recovery were observed (Figure 1a-c). The average total emergence (n=3) of the cadmium^{+}, copper^{+} and tributyltin^{+} cultures was significantly lower than that of the (solvent)control culture and amounted to, respectively, 50 ± 24%, 37 ± 20% and 27 ± 21%.

The mean emergence time (EmT50) of the control culture ranged between 19.0 and 21.1 days and averaged 20.1 ± 0.8 days over the nine generations (Figure 1d-e). A similar pattern was observed for the solvent control culture with an average EmT50 of 20.0 ± 0.8 days and a range of 18.6 to 21.1 days (Figure 1f). The tox cultures showed EmT50's that deviated in most generations significantly, though limitedly from the (solvent) control culture EmT50's. While the copper and cadmium cultures had an average emergence delay of 0.7 days over the nine generations and on occasions emerged even faster than the control culture, the midges exposed to the sublethal tributyltin concentration were always delayed compared to the solvent control with an average delay (n=8) of 2.8 days. After the increase of the toxic pressure, all three tox^{+} cultures were much stronger delayed (Figure 1d-f). The strongest delay was observed in the 8th generation and amounted for the cadmium^{+}, copper+ and tributyltin^{+} cultures, respectively, 6.8, 6.3 and 9.2 days in comparison to the (solvent) control culture. In the 9th generation a recovery was observed as the emergence delay of the respective tox^{+} cultures was limited to 3.0, 3.9, and 5.8 days. To assess if males and females were equally affected we also calculated the EmT50 values for males and females separately (Supporting Figure S1). The observed emergence patterns showed that even though the females emerged consistently later than the males, both sexes were equally affected.

Sensitivity testing of exposed cultures

To assess the sensitivity of the test cultures, 14-day toxicity tests were conducted with the corresponding toxicant at the start of the multigeneration experiment and subsequently every 3rd generation (Figure 2). During each of these sensitivity testing experiments, the control culture was tested with all three toxicants. The LC50 values demonstrate that the

sensitivity of the control culture showed no distinct development over the generations and that the tox cultures remained as sensitive as the control culture throughout the multigeneration experiment. The increase of the toxicant concentration after the 6th generation resulted only for the cadmium+ culture in a significantly higher LC50 value in the 9th generation compared to the control culture. However, one generation later this decreased cadmium sensitivity was not observed anymore.

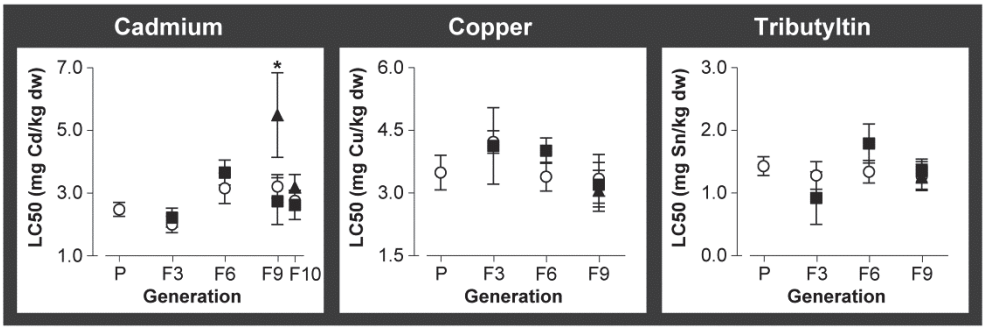

Figure 2: Sensitivity of the *C. riparius* cultures was assessed at the start of the multigeneration experiment (P) and subsequently after the 3rd, 6th and 9th generation (i.e., F3, F6 and F9) with the appropriate toxicant using 14-day toxicity tests. An additional sensitivity testing experiment was performed with cadmium after the tenth generation (F10). The figures show the LC50 (conc. ± 95% C.I) values obtained for the cultures exposed to, respectively, cadmium, copper and tributytlin. In each figure the open circles represent the control culture (for tributyltin the solvent control culture), the black squares the tox culture and the grey triangles the tox+ culture. * indicates values significantly differing from the (solvent) control culture ($p<0.05$).

Discussion

In the present study we postulated that phenotypic plasticity, i.e. the capacity of a single genotype to exhibit a range of phenotypes in response to different environmental conditions (Whitman and Agrawal, 2009), underlies *C. riparius*' well documented ability to cope with a wide variety of stressors (e.g. Redecker and Zebe, 1988; Bervoets et al., 1996; Jernelöv et al., 1981; Havas and Hutchinson, 1982; Gower and Buckland, 1978; Friberg et al., 2010; Winner et al., 1980). To verify this postulate we exposed *C. riparius* laboratory cultures for nine consecutive generations to three model compounds that were previously shown to differently affect *C. riparius* during its life-cycle (Marinković et al., 2011). We observed that long-term exposure to sublethal concentrations of cadmium and copper had an impact on the *C. riparius* cultures, yet the total emergence and the mean emergence time (EmT50) of these cultures oscillated around the control culture values over the nine generations. The tributyltin culture was stronger affected, resulting in a significantly lower total emergence

during the nine generations. Interestingly, while previous *C. riparius* multigeneration studies showed that effects worsened with increasing number of exposed generations (Postma and Davids, 1995), we did not observe such a trend. In fact, the sensitivity of the sublethally exposed cultures was not significantly affected during the nine generations of consecutive exposure, as was clearly demonstrated by the sensitivity testing experiments. It might be argued that the chosen sublethal concentrations were too low. However, previous multigeneration studies where *C. riparius* laboratory cultures were exposed to sublethal concentrations of cadmium (Postma and Davids, 1995), zinc (Miller and Hendricks, 1996), and tributyltin (Vogt et al., 2007) showed that the presently tested sublethal concentrations of these compounds can lead to decreased toxicant sensitivity as well as to extinction. Possible explanations for the discrepancies between these studies and the present study might be both the toxic pressure which might be lower in the present study due to the binding capacity of the artificial sediment, as well as the genetic variability of our *C. riparius* laboratory culture that might have been higher and thus effectively prevented extinction.

The three tox^{+} cultures that were exposed to substantially higher toxicant concentrations after the 6th generation, were, as expected, much stronger impacted and showed a significantly reduced total emergence in the 8th generation. Even though the differences remained significant in the 9th generation, all three tox^{+} cultures showed a marked recovery. The recovery of these cultures was confirmed by the subsequent sensitivity testing experiments where the obtained LC50 values clearly showed that the copper^{+} and tributyltin^{+} cultures remained equally sensitive as the controls, while a significantly lower sensitivity was only observed for the cadmium^{+} culture. On first glance, the latter observation seemed to be in good agreement with Postma and Davids (1995), who demonstrated that nine consecutive generations of sublethal cadmium exposure decreased the cadmium sensitivity of *C. riparius*. While these authors did not continue to monitor the cadmium sensitivity of their *C. riparius* culture after the 9th generation, we did and observed that even though the cadmium pressure was retained, the reduced cadmium sensitivity of the cadmium^{+} culture was already lost in the next generation. Similarly, Vogt et al. (2007) observed that a *C. riparius* culture that was exposed for eleven consecutive generations to tributyltin was significantly less sensitive to tributyltin in the 9th and 10th generations, while in the last generation of the multigeneration experiment the sensitivity of the exposed culture was again similar to that of the control culture. This pattern has been observed in other multigeneration studies too (Miller and Hendricks, 1996, Vedamanikam and Shazilli, 2008), however, since in those studies the tolerant midge cultures were reared for two generations under clean conditions, the authors concluded that the lower toxicant sensitivity was lost due to a lack of toxic pressure and/ or cost of tolerance which can be the result of between-environment trade-offs (Shirley and Sibly, 1999). Hence, when we consider our results as well as the above discussed observations from other *C. riparius*

multigeneration studies, it appears that response of *C. riparius* to long-term toxicant exposure is rather plastic, i.e. surviving cultures do not always develop a decreased toxicant sensitivity, and, more so, when they do it may be rapidly lost again regardless of the presence/ absence of toxic pressure. We therefore conclude that *C. riparius* laboratory cultures can indeed withstand long-term sublethal toxicant exposure without getting genetically adapted due to phenotypic plasticity. This plasticity is apparently responsible for fluctuating responses between successive generations.

With the demonstrated plasticity in mind, we reassessed the studies that were conducted with historically metal exposed *C. riparius* field populations in order to verify if phenotypic plasticity could also explain these field observations. The strongest evidence for genetic adaptation was provided by Postma et al. (1995a; 1995b) who showed that first-generation clean water laboratory reared offspring of historically metal exposed *C. riparius* field populations was less cadmium sensitive than offspring of nearby sampled reference populations. Interestingly, these two studies also showed that one of the tested metal-exposed field populations lost its decreased metal sensitivity in the interval between two successive sampling campaigns. Similarly, Groenendijk et al. (1999) reported that the level of metal sensitivity of several historically metal exposed *C. riparius* populations varied considerably during a five month field sampling campaign. Since these historically metal exposed *C. riparius* field populations were periodically replenished with non-exposed larvae from clean upstream river reaches as well as egg-ropes deposited by non-exposed females, it remains, however, impossible to determine to what extent the observed variability was due to gene flow, to the loss of tolerance or to both. Groenendijk et al. (2002) also showed that crossing midges from polluted and reference sites yielded offspring with intermediate levels of metal sensitivity. They advocated a major genetic component, however, a slight maternal effect was also observable, therefore phenotypic plasticity cannot be ruled out. Finally, it should be noted that although the above discussed field studies showed heritability in first-generation clean water laboratory reared offspring, a prerequisite to postulate genetic adaptation as the responsible mechanism (Morgan et al., 2007), this does not exclude other heritable mechanisms that are not mediated through alterations at the DNA sequence level and that can be reversible, i.e. epigenetic changes (Vandegehuchte et al., 2011) and that are in fact part of the broader term ‘maternal effects’. This is especially worth nothing, since Groenendijk et al. (2002) observed that the decreased metal sensitivity was rapidly lost in the hybrid offspring what might indicate a transgenerational epigenetic effect (Ho and Burggren, 2010). Epigenetic changes, particularly those mediated through DNA methylation, have been suggested to play a key role in the regulation of phenotypic plasticity (Zhang and Meaney, 2010) and insects have been proposed as model organisms for studying this phenomenon (Glastad et al., 2011). Thus, phenotypic plasticity is most likely the mechanism that underlies *C. riparius* ability

to cope with long-term sublethal toxicant exposure, but due to the complexity of field studies it can only be demonstrated under controlled laboratory conditions.

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Supporting information available: Tables S1-S2 and Figure S1. This information is available free of charge via the Internet at http://pubs.acs.org.