The Journal of Experimental Biology 215, 2795-2805
© 2012. Published by The Company of Biologists Ltd
doi:10.1242/jeb.069211

RESEARCH ARTICLE

Transgenerational effects of microcystin-LR on *Daphnia magna*

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SUMMARY

Anthropogenic and climate factors increase the frequency of problematic cyanobacterial blooms in freshwater. Among other toxins, some cyanobacteria produce microcystins (MCs), which inhibit phosphatases type 1 and type 2A and provokes oxidative stress. Toxic cyanobacteria affect the growth, survival and reproduction of zooplankton, particularly those from the genus *Daphnia*, which have a central position in pelagic food webs. However, one possibility to ameliorate effects is to biotransform MC *via* glutathione S transferase (GST) to a less toxic glutathione conjugate. This process was hypothesised to underlie the ability of *Daphnia* to withstand MC and to explain the enhanced tolerance of the offspring from mothers exposed to toxic cyanobacteria. Thus we conducted multigenerational experiments with *D. magna*, exposing the parental generation to MC for 1 or 7 days and determining the enzyme-mediated tolerance to MC in their offspring by assessing the acute effect of MC on biotransformation and antioxidant and metabolism enzymes, and through 21 day chronic tests on toxicity and growth. Seven days of exposure of the parental generation to MC induced higher activity of GST and malate dehydrogenase in the offspring and enabled them to increment the catalase activity when challenged with MC, whereas 1 day of exposure of the parental generation did not. Offspring from non-exposed and 1-day-exposed mothers suffered decreased survival when exposed to MC compared with offspring from 7-day-exposed mothers; survival was correlated with the elevated activity of GST, malate dehydrogenase and catalase, suggesting maternal transfer of activation factors. However, increased survival occurred at the expense of individual growth. These results suggest that transgenerational effects are provoked by MC in *D. magna*, which may explain the observed acquirement of enhanced tolerance over generations.

Key words: microcystin, cyanobacterial toxin, transgenerational effect, *Daphnia*, zooplankton.

Received 8 December 2011; Accepted 19 April 2012

INTRODUCTION

Eutrophication of waters from urban, agricultural and industrial sources has promoted cyanobacteria blooms for decades (Codd et al., 2005; Paerl and Fulton, 2006). In addition, the interaction between eutrophication, global warming and rising atmospheric CO_2 is likely to increase phytoplankton productivity and consequentially the frequency of blooms (Ibelings and Maberly, 1998; Schippers et al., 2004; Granéli et al., 2008; Paul, 2008; Paerl and Huisman, 2008; Paerl and Huisman, 2009; Rouco et al., 2011). Blooms of cyanobacteria are considered an important problem, they are difficult to remediate (Scheffer et al., 1997) and are hazardous to the aquatic ecosystem and human health because of their capability of producing bioactive or toxic compounds. The most frequently found toxins in blooms from fresh and brackish waters are the cyclic heptapeptides microcystins (MCs), which are hepatotoxic to vertebrates (Sivonen and Jones, 1999; Dittmann and Wiegand, 2006). They occur in more than 80 variants, differing in changes of side groups of the amino acids, but mainly in two variable amino acids, e.g. leucine and arginine for MC-LR, which is one of the most frequent and toxic, and hence the most studied, variant (Botes et al., 1985; Welker and von Döhren, 2006). Microcystins inhibit serine/threonine protein phosphatases type 1 and type 2A by introducing the amino acids ADDA into the active gap of the enzymes; the complex is further stabilized by binding *via* the methylene group of *N*-methyldehydroalanine to the phosphatases (MacKintosh et al., 1990; MacKintosh et al., 1995; Goldberg et al., 1995; Demott and Dhawale, 1995). Inhibition of protein phosphatases 1 and 2A causes hyperphosphorylation of various proteins in the cell, including those in the cytoskeleton, leading to cell death (Eriksson et al., 1989). In addition, oxidative stress *via* formation of reactive oxygen species (ROS) and lipid peroxidation is a toxicological consequence of the exposure to MCs in aquatic animals (Amado and Montserrat, 2010).

One of the groups most affected by toxic cyanobacteria and cyanotoxins is planktivorous zooplankton. The toxicity, mechanical interference and nutritional inadequacy of cyanobacteria are the factors governing the detrimental effect on zooplankton populations (Rohrlack et al., 2001; von Elert et al., 2004). The most important zooplanktonic herbivores in freshwater are crustaceans, especially cladocerans of the genus *Daphnia*, which inhabit a central position in pelagic food webs connecting primary producers with consumers (Lampert and Sommer, 2007). The high ecological value and wide geographic distribution – in addition to ease of handling in the laboratory and parthenogenetic direct reproduction, which provides genetic uniformity in test organisms – has allowed *Daphnia* to be the best represented genus in (cyanobacterial) toxicity studies related to zooplankton (Lampert, 2006). In addition, the recent publication of the *Daphnia* genome will prompt an even wider research effort on this genus. The negative effects of toxic

cyanobacteria on *Daphnia* are reflected by, for example, reduced growth, survival and reproduction (Lampert, 1981; Demott et al., 1991; Hietala et al., 1995; Nogueira et al., 2004; Trubetskova and Haney, 2006; Dao et al., 2010). However, one possible way to reduce toxic effects is to biotransform the toxin MC-LR by conjugation to the tripeptide glutathione (GSH) *via* the enzyme glutathione S transferase (GST) (Pflugmacher et al., 1998). MCs are conjugated to GSH through the terminal methylene of *N*-methyldehydroalanine (Kondo, 1992), which is the moiety that binds to the active center of protein phosphatases (MacKintosh, 1995). The resulting conjugate is less toxic (Metcalf et al., 2000) and more hydrophilic, for easier transportation or excretion (Pflugmacher et al., 2001). This MC-LR detoxification process has been described in several aquatic organisms ranging from plants to invertebrates (including *Daphnia magna*) to fish (Pflugmacher et al., 1998; Wiegand and Pflugmacher, 2005). Three major families of proteins that are widely distributed in nature exhibit GST activity: the first two families comprise soluble GST (cytosolic and mitochondrial GST) (sGST) and the third and smallest family comprises membrane-associated (microsomal) GST (mGST) (Jakobsson et al., 1999; Hayes et al., 2005). mGST and sGST have completely different three-dimensional structures, despite the fact that they catalyse similar reactions and display overlapping substrate specificity (Holm et al., 1992). sGST consists of several isoenzymes; seven sGST isoenzymes have been isolated from *D. magna* (LeBlanc and Cochrane, 1987). Up to certain MC-LR concentrations, *Daphnia* have been shown to respond with increasing enzyme activity of sGST (Wiegand et al., 2002; Ortiz-Rodríguez and Wiegand, 2010). Furthermore, antioxidant enzymes such as catalase (CAT) have been shown to diminish oxidative stress in *Daphnia* within a certain concentration range of the toxin (Wiegand et al., 2002; Ortiz-Rodríguez and Wiegand, 2010).

Increased biotransformation and antioxidant enzyme activity requires additional energy. The enzyme lactate dehydrogenase (LDH; converting pyruvate to lactate and back) is involved in the supply or storage of energy from carbohydrates. As both elevation and inhibition as a result of exposure to cyanobacterial toxins have been observed in *D. magna* (Chen et al., 2005; Ortiz-Rodríguez and Wiegand, 2010), further investigation is required to assess energy consumption due to the exposure to cyanobacterial toxins. Malate dehydrogenase (MDH) is an enzyme involved in the citric acid cycle and its activity can be used to estimate the metabolic rate, as a measure for energy allocation.

Additional energy used for biotransformation and antioxidant processes is lacking for other functions, hence consequences in life history parameters such as growth and survival can be expected, considering that energy in an organism is distributed into maintenance, reproduction and growth (Calow et al., 1991).

A large number of studies have tried to elucidate the transgenerational effects of environmental factors and toxicants on *D. magna* through multigenerational exposures investigating at the molecular, biochemical and population level. At the population level, the biological importance of the biochemical and molecular responses can be integrated, particularly when parameters such as survival, growth or intrinsic rate of population growth are used. The main factors or stressors considered in previous studies are: metals (Muyssen and Janssen, 2004; Vandegehuchte et al., 2010), organic compounds (Villarroel et al., 2000; Brausch and Salice, 2011), food availability and quality (Naylor et al., 1992; Gorbi et al., 2011), and natural toxins (Hairston et al., 2001; Gustafsson and Hansson, 2004; Gustafsson et al., 2005).

These studies demonstrated effects from the parental up to the sixth generation with different degrees of resistance, tolerance or magnified toxic effects of the toxicant through the generations. In most of these experimental setups the progeny was exposed while developing in the brood chamber, reflecting the ecologically relevant situation. Few studies tried to isolate the influence of the toxicant on the parental generation from the potential inheritance of the physiological response to the progeny, searching for underlying toxicological mechanisms. To understand the processes governing the potential tolerance, resistance or magnification of toxic effects, both exposure scenarios should be considered.

Tolerance transfer from the parental generation F0 to the offspring generation F1 after exposure to toxic cyanobacteria has been demonstrated by Gustafsson et al. (Gustafsson et al., 2005), who exposed the F0 of *D. magna* to a mixed diet of green algae and toxic *Microcystis*. Fitness increased in the second generation of *D. magna* compared with the previously exposed mothers but no difference between the second and third generations was found. The authors assumed that the second generation was born with constitutively higher amounts of expressed detoxifying mechanisms, and thus improved performance in toxic environments (Gustafsson et al., 2005). Such stimulated tolerance may have a prolonged impact on overall population survival and could be a common mechanism for sustaining herbivore production and availability, therefore stabilizing material and energy transfer to higher trophic levels (Gustafsson et al., 2005).

The aim of this study was to determine the transgenerational effects of MC-LR on the response of detoxification-, antioxidant- and energy-related enzymes in *D. magna* and to connect these effects to survival and growth of the animals under toxic stress. We hypothesize that, depending on the exposure of the parental generation to MC-LR, the offspring will express a different enzymatic and physiological response if exposed to the same toxin, influencing their toxin tolerance. To accomplish this, the parental generation was exposed for 1 or 7 days to different concentrations of MC-LR, and the offspring were exposed for 1 day (for antioxidant, biotransformation and metabolic enzyme analysis) or 21 days (for survival and mass determination). The offspring in this study were not exposed during embryonic development.

MATERIALS AND METHODS

Test organisms

Daphnia magna Straus 1820 were collected from Lake Müggelsee, Germany, and were cultured for >3 years under laboratory conditions at the Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Individuals used in this experimental setup originate from a single female to eliminate variation among clones. *Daphnia magna* cultivation medium contained 2 mmol l^{-1} $CaCl_2$, 0.08 mmol l^{-1} KCl, 1 mmol l^{-1} $MgSO_4$ and 6 mmol l^{-1} $NaHCO_3$ dissolved in reverse osmosis water. *Daphnia magna* were cultivated at constant conditions of temperature (20±2°C) under a light:dark cycle of 14 h:10 h. They were fed daily *ad libitum* with *Scenedesmus* sp. cultured in aerated columns in Z8 medium (Kotai, 1972) under the same photoperiod as *D. magna*.

Test chemicals

All reagents are from the purest grade commercially available and were purchased from Sigma-Aldrich (Germany). MC-LR was purchased from Axxora (Lörrach, Germany). It was dissolved in methanol and this stock was used to prepare the following concentrations in *D. magna* cultivation medium: 0, 50 and 100 µg l^{-1}. Control organisms were exposed to methanol at the same concentrations of the exposed groups to ensure that the carrier did not affect the variables measured. These sub-lethal concentrations

were chosen because they have been shown to exert a measurable effect on physiological processes in *D. magna* in acute and chronic toxicity tests (Wiegand et al., 2002; Dao et al., 2010; Ortiz-Rodríguez and Wiegand, 2010). The MC-LR concentration was determined in the exposure media according to Dahlmann et al. (Dahlmann et al., 2003) using an HPLC Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) and the MS/MS System 3200 QTrap (AB Sciex, Framingham, MA, USA). The chromatographic separation was performed on an Agilent Eclipse Plus C18 (RP18) column (4.6 mm diameter × 50 mm length, 5 µm particle size) and an Agilent Guard Eclipse XDB-C8 column (2.1 diameter × 12.5 mm length, 5 µm particle size). The injection volume of the samples was 10 µl with a column temperature of 40°C; the gradient had a flow of 200 µl min^{-1}. Two solvents were used: (A) acetonitrile + 0.1% trifluoroacetic acid (TFA) and (B) H_2O + 0.1% TFA. The gradient program began with a linear increase over 12.1 min from 15 to 100% of solvent A. The conditions were maintained for 2 min, followed by a decrease of solvent A to 15% within 0.1 min, followed by a second decrease of solvent A to 5% within 5.9 min. The measurements were performed in the positive multiple reaction monitoring mode, with the mass transitions of (*m*/*z*) for MC-LR from 995.5 to 135.1. The substance-specific MS/MS conditions and ionization and fragmentation settings were optimized by direct injection of standard solutions to the MS/MS system. The method validation was achieved over a linear range from 10 to 250 µg l^{-1} MC-LR. The MC-LR solutions for the standard curve were prepared in methanol. All data were analysed using Analyst 1.4.2 (Applied Biosystems, Carlsbad, CA, USA).

Exposure scenarios

To test the transgenerational effects of the exposure of the parental generation (F0) on the biochemical parameters of the offspring (F1), groups of neonate *D. magna* were selected and considered as F0. These individuals were healthy, age synchronized (1–2 days old) and originated from a parental generation from a high food environment where all mothers were from the third brood and onwards. Three groups of F0 were exposed to 0 (control), 50 and 100 µg l^{-1} MC-LR for 7 days and three groups of F0 were only exposed the last day (of the 7 days) to the same concentrations. This procedure ensured the same age of the F0 at the end of the exposure period. After exposure, they were transferred to medium without toxin. They were monitored every following day and all the neonates produced up to the third day after the end of the exposure were discarded, as they may have been in contact with the toxin during their embryonic development. Groups of neonates (F1) produced from the fourth to the seventh day after the exposure of F0 were collected and exposed to 0, 50 or 100 µg l^{-1} MC-LR for 1 day (for enzyme analysis) or 21 days (for survival and growth determination). At the end of the exposure period of the neonates (1 day) for enzyme analysis, samples were collected with a plankton mesh, briefly rinsed with toxin-free medium and immediately frozen in liquid N_2 and stored at –80°C. For the 21 day exposure, each treatment (18) consisted of 10 to 30 *D. magna* (depending on the reproduction of the F0) placed individually in medium with 0, 50 or 100 µg l^{-1} MC-LR. Individuals were monitored every day and death was assessed under a stereomicroscope (determined as the time at which the heart stopped beating). At the end of the 21 day exposure, surviving individuals were collected, dried at >60°C for 48 h and weighed on an electronic microscale (Supermicro S4, Sartorius, Goettingen, Germany). During all exposure scenarios, the same conditions of light and temperature as in the original culture were maintained. The organisms from the parental generation were not fed during the 1 day exposure but were fed before and afterwards *ad libitum* with *Scenedesmus* sp. During the 7 and 21 day exposures, they were fed every other day with a concentration equivalent to 3 mg C l^{-1}. To prevent dilution of the concentration of MC-LR in the exposure units (beakers), algae suspensions were prepared within the corresponding test solutions.

Sample preparation

Enzyme extracts were prepared according to Wiegand et al. (Wiegand et al., 2000); samples were homogenized in ice-cooled buffer (0.1 mol l^{-1} sodium phosphate buffer pH 6.5 containing 20% glycerol, 1 mmol l^{-1} EDTA and 1.4 mmol l^{-1} dithioerythriol) followed by centrifugation to clear cell debris. The supernatant was centrifuged again (105,000 ***g***) to separate the soluble and microsomal fractions. The proteins from the soluble fraction were concentrated by precipitation with ammonium sulphate and centrifugation, after which the pellet was suspended in 20 mmol l^{-1} sodium phosphate buffer pH 7.0, desalted, frozen in liquid N_2 and stored at –80°C. The microsomal fraction was homogenized in ice-cooled 20 mmol l^{-1} sodium phosphate buffer pH 7.0 containing 20% v/v glycerol, frozen in liquid N_2 and stored at –80°C until enzyme analysis.

Enzyme analysis

GST was assayed at 340 nm in both soluble/cytosolic (sGST) and microsomal (mGST) fractions using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). Catalase (CAT) activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm (Chang and Kao, 1997). LDH and MDH activity were assayed according to Diamantino et al. (Diamantino et al., 2001) and Morgado and Soares (Morgado and Soares, 1995) by measuring the rate of disappearance of NADH at 340 nm after adding pyruvate or oxaloacetate, respectively. All enzyme activities are related to the protein concentration in the sample, determined using the Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO, USA) according to Bradford (Bradford, 1976). The concentration was calculated using a standard curve of bovine serum albumin.

Statistical analysis

All data are presented as means ± s.d. Enzyme activities of only the progeny are presented. We performed *t*-tests to determine the difference between exposed *D. magna* and controls (non-exposed progeny from a non-exposed parental generation). To test the difference in enzyme activities between the exposure treatments, a three-way main effects ANOVA was performed using the following factors: (1) exposure concentration of the progeny F1 (0, 50 and 100 µg l^{-1} MC-LR), (2) exposure concentration of the parental generation F0 (0, 50 and 100 µg l^{-1} MC-LR) and (3) the period of exposure of the parental generation (1 or 7 days). *Post hoc* tests (Tukey's tests) were conducted when factors were statistically different. All analyses were performed with STATISTICA 8.0 (StatSoft, Tulsa, OK, USA) and the significance limit was set at $P<0.05$.

RESULTS

Exposure conditions

Analysis of MC-LR concentration in the exposure medium at the start of the exposures was found to be >74% and >62% of the nominal concentration for the exposures of 50 and 100 µg l^{-1}, respectively. As such, the nominal concentrations are mentioned in text and figures, despite the fact that measured concentrations diverged to some extent.

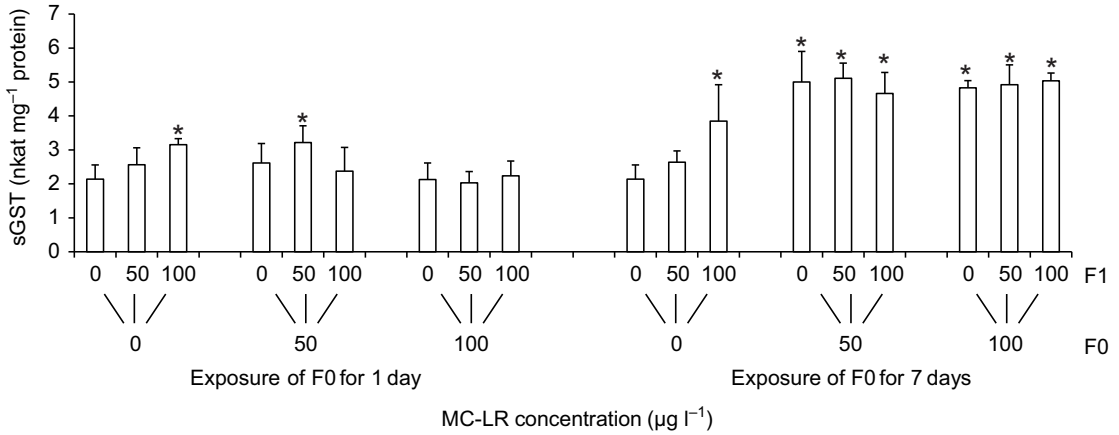

Fig. 1. Enzymatic response of soluble glutathione S transferase (sGST) of *Daphnia magna* neonates (F1) after exposure to increasing concentrations of microcystin-LR (MC-LR) (0, 50 and 100 μg l^{-1}) for 1 day. The parental generation F0 that originated these neonates was previously exposed to 0, 50 or 100 μg l^{-1} for 1 or 7 days. Asterisks denote significant differences to control (*t*-test, **P*<0.05).

Transgenerational effect of MC-LR exposure on *D. magna* progeny through enzyme analysis

Soluble glutathione S transferase

sGST activity of neonates (F1) from non-exposed mothers (F0) increased directly in relation to MC-LR concentration (Fig. 1). sGST activity significantly increased in neonates from mothers exposed for 1 day to 50 μg l^{-1} MC-LR when neonates were exposed to 50 μg l^{-1} MC-LR (*t*-test, $P<0.05$) but it did not increase when they were exposed to 100 μg l^{-1} MC-LR. Those neonates from mothers exposed to 100 μg l^{-1} for 1 day demonstrated no change in the activity of this enzyme.

In contrast, sGST increased significantly in neonates from mothers exposed to 50 and 100 μg l^{-1} MC-LR for 7 days (*t*-test, $P<0.05$) compared with non-exposed neonates from non-exposed mothers, independent of the concentration at which both the mothers and the progeny were exposed (0, 50 or 100 μg l^{-1} MC-LR, *t*-test, $P<0.05$). Even in the non-exposed neonates (0 μg l^{-1} MC-LR), the sGST activity was elevated, indicating a constitutive higher level as a result of the mothers' exposure for 7 days.

According to the three-way main effects ANOVA, concentration and duration of exposure of the mothers led to significantly different responses in the neonates ($P<0.05$). According to the *post hoc* Tukey's test, neonates from mothers exposed for 7 days to 50 and 100 μg l^{-1} MC-LR had significantly higher activity compared with all other groups of neonates ($P<0.001$; Fig. 1).

Microsomal glutathione S transferase

mGST activity of neonates from non-exposed mothers (1 and 7 days) showed no reaction when exposed to 50 μg l^{-1} or 100 μg l^{-1} MC-LR. The non-exposed neonates from mothers previously exposed to 100 μg l^{-1} MC-LR for 1 day had a significantly higher activity than controls and all other groups (*t*-test, $P<0.05$). Exposure of the F0 to higher concentrations for 7 days caused a significant reduction in mGST activity in the neonates, as can be seen in neonates exposed to 100 μg l^{-1} MC-LR originating from mothers exposed to 50 μg l^{-1} MC-LR and in all the neonates (exposed to 0, 50 and 100 μg l^{-1} MC-LR) from mothers exposed to 100 μg l^{-1} MC-LR (*t*-test, $P<0.05$). Even those neonates that were not exposed (0 μg l^{-1} MC-LR) suffered from the reduction in mGST activity (Fig. 2).

Catalase

CAT activity of neonates (F1) from non-exposed mothers (F0) significantly increased with MC-LR concentration (*t*-test, $P<0.05$). It

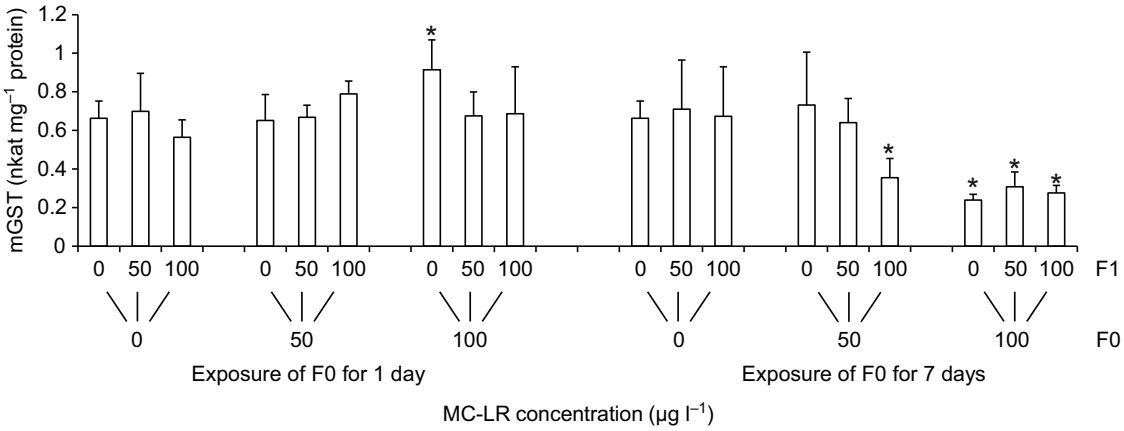

Fig. 2. Enzymatic response of microsomal GST (mGST) of *Daphnia magna* neonates (F1) after exposure to increasing concentrations of MC-LR (0, 50 and 100 μg l^{-1}) for 1 day. The parental generation F0 that originated these neonates was previously exposed to 0, 50 or 100 μg l^{-1} for 1 or 7 days. Asterisks denote significant differences to control (*t*-test, **P*<0.05).

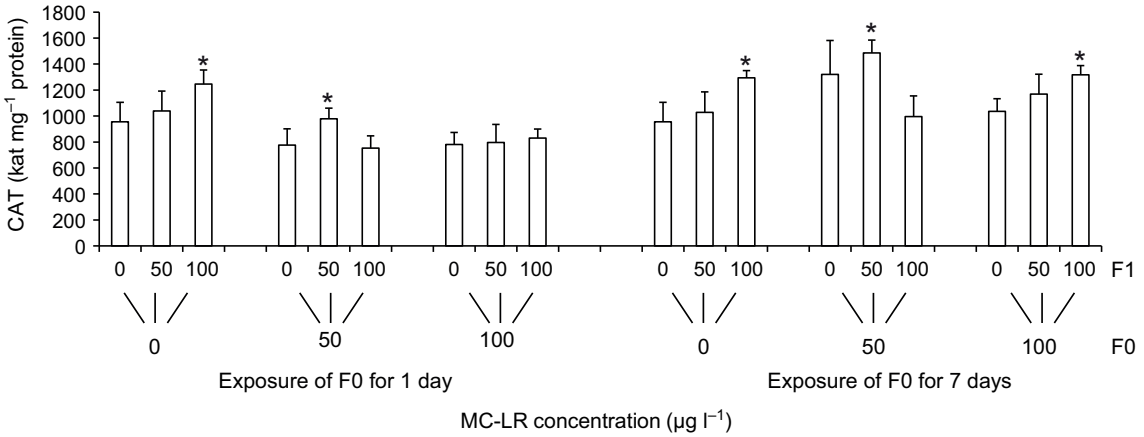

Fig. 3. Enzymatic response of catalase (CAT) of *Daphnia magna* neonates (F1) after exposure to increasing concentrations of MC-LR (0, 50 and 100 μg l^{-1}) for 1 day. The parental generation F0 that originated these neonates was previously exposed to 0, 50 or 100 μg l^{-1} for 1 or 7 days. Asterisks denote significant differences to control (*t*-test, **P*<0.05).

also increased when neonates from mothers exposed to 50 μg l^{-1} MC-LR for 1 day were exposed to 50 μg l^{-1} MC-LR (*t*-test, $P<0.05$) but it did not increase when neonates were exposed to 100 μg l^{-1} MC-LR. Those neonates from mothers exposed to 100 μg l^{-1} MC-LR for 1 day had no change in the activity of this enzyme. The CAT activity of neonates from mothers exposed to 50 and 100 μg l^{-1} MC-LR for 1 day had slightly lower activity than neonates from non-exposed mothers. CAT significantly increased in neonates from mothers exposed to 50 μg l^{-1} MC-LR for 7 days when they were exposed to 50 μg l^{-1} MC-LR (*t*-test, $P<0.05$) but decreased slightly when they were exposed to 100 μg l^{-1} MC-LR. The CAT activity of the neonates from mothers exposed to 100 μg l^{-1} MC-LR for 7 days increased directly with MC-LR concentration, similar to the CAT activity of neonates from non-exposed mothers (Fig. 3). According to the three-way main effects ANOVA, the concentration of exposure of the progeny and duration of exposure of the mothers led to statistically different CAT responses in the neonates ($P<0.05$). According to the Tukey's test ($P<0.05$), CAT activity in neonates exposed to 50 and 100 μg l^{-1} was significantly higher than in non-exposed neonates, as was the enzyme activity of the progeny from 7-day-exposed mothers compared with the progeny from 1-day-exposed mothers.

Lactate dehydrogenase

LDH activity in the progeny was not significantly affected by the time or concentration of exposure of the parental generation (Fig. 4). There were no differences in LDH activity between groups.

Malate dehydrogenase

The activity of MDH showed no significant changes after exposure to 50 or 100 μg l^{-1} MC-LR in neonates originated from non-exposed mothers (1 and 7 days) or mothers exposed to 50 μg l^{-1} MC-LR for 1 day. Those neonates from mothers exposed for 1 day to 100 μg l^{-1} MC-LR overall had significantly higher activities (at all concentrations of neonate exposure; *t*-test, $P<0.05$). Neonates originated from mothers exposed to 50 μg l^{-1} MC-LR for 7 days (when exposed to 50 and 100 μg l^{-1} MC-LR) had significantly higher MDH activity than the other neonates (*t*-test, $P<0.05$). The neonates originating from mothers exposed to 100 μg l^{-1} MC-LR for 7 days had, like those from the 1-day-exposed mothers, an overall significantly higher MDH activity (*t*-test, $P<0.05$). The activity of this enzyme increases in neonates as a consequence of either: (1) exposure of mothers to high concentrations of MC-LR, as can be seen in neonates from mothers exposed to 100 μg l^{-1} MC-LR for 1 and 7 days or (2) time of exposure

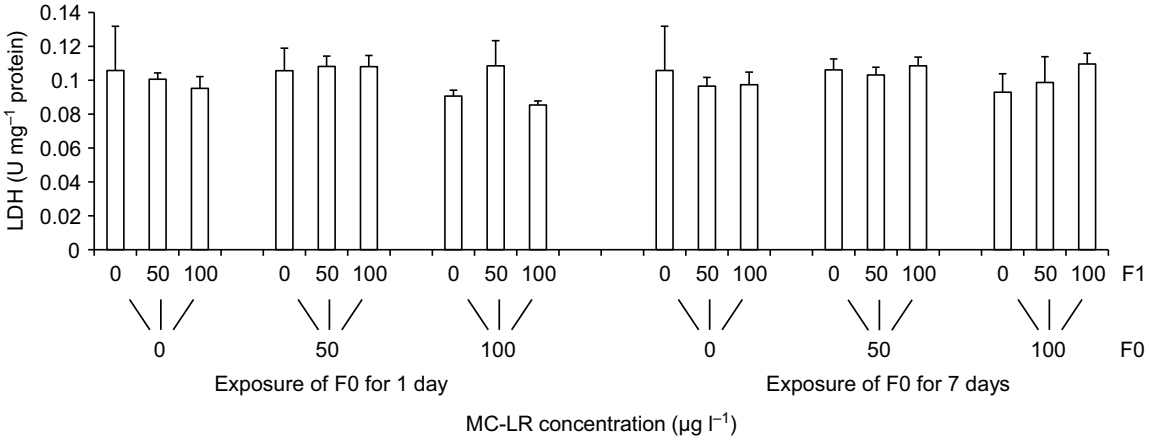

Fig. 4. Enzymatic response of lactate dehydrogenase (LDH) of *Daphnia magna* neonates (F1) after exposure to increasing concentrations of MC-LR (0, 50 and 100 μg l^{-1}) for 1 day. The parental generation F0 that originated these neonates was previously exposed to 0, 50 and 100 μg l^{-1} for 1 day.

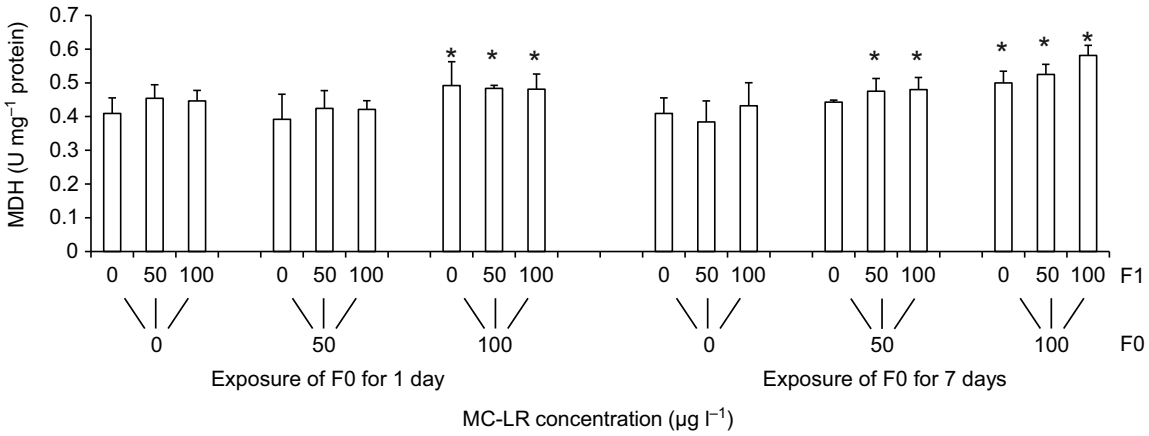

Fig. 5. Enzymatic response of malate dehydrogenase of *Daphnia magna* neonates (F1) after exposure to increasing concentrations of MC-LR (0, 50 and 100 μg l^{-1}) for 1 day. The parental generation F0 that originated these neonates was previously exposed to 0, 50 or 100 μg l^{-1} for 1 or 7 days. Asterisks denote significant differences to control (*t*-test, **P*<0.05).

of the mothers, as can be seen in neonates from mothers exposed to 50 μg l^{-1} MC-LR for 7 days. According to the three-way main effects ANOVA, the exposure concentration of the mothers was the factor causing a statistical difference ($P<0.05$). According to the Tukey's test ($P<0.001$), the activity of neonates originated from mothers exposed to 100 μg l^{-1} MC-LR for 1 and 7 days was significantly higher than the rest (Fig. 5).

Transgenerational effect of MC-LR exposure on *D. magna* progeny through 21 day toxic tests

Survival

Survival of the progeny after MC-LR exposure depends on the concentration and duration of exposure of the parental generation. When the parental generation F0 was non-exposed to MC-LR, the resulting progeny's survival was negatively affected (reduced to 60–80%) at both tested MC-LR concentrations (50 and 100 μg l^{-1}; Fig. 6A,B). Survival of the offspring from mothers exposed to 50 and 100 μg l^{-1} MC-LR for 1 day was even more reduced, down to 50% after 21 days, when neonates were exposed to 50 and 100 μg l^{-1} MC-LR (Fig. 6C,E). The decrease in survival of the non-exposed offspring (Fig. 6C,E) shows that 1 day of exposure of the F0 to 50 and 100 μg l^{-1} MC-LR causes a decrease of survival of the offspring, even if they had never contact to the toxin, suggesting the presence of transgenerational effects, which cannot be assessed in a single generation test.

The offspring from the mothers exposed to 50 and 100 μg l^{-1} MC-LR for 7 days (Fig. 6D,F) showed decreased survival when exposed to 50 or 100 μg l^{-1} MC-LR (down to 80% survival when the F1 from 50 μg l^{-1} mothers was exposed to 50 μg l^{-1} MC-LR) but not as marked as the offspring from the parental generations exposed for 1 day (down to 40% when the F1 from 50 μg l^{-1} MC-LR mothers was exposed to 50 μg l^{-1} MC-LR). It is evident from the survival data that the longer time of exposure (7 days) of the parental generation to any concentration of MC-LR (50 and 100 μg l^{-1}) induces a certain degree of tolerance that improves the ability of the offspring to survive when challenged with MC-LR, and that a short time of exposure (1 day) to the toxin at the tested concentrations does not induce tolerance in the resulting offspring.

Mass

It is evident that exposure to MC-LR (50 and 100 μg l^{-1} MC-LR) caused growth impairment, as can be seen in Fig. 7. The progeny from 1-day-exposed mothers (0, 50 and 100 μg l^{-1} MC-LR) weighed 0.145 to 0.493 mg dry mass (per *D. magna*). When the parental generation was acutely (1 day) exposed to 50 and 100 μg l^{-1} MC-LR, a decrease in mass was not found. Here it is important to remark that the mass was determined only in the surviving individuals after 21 days of exposure and that the exposure of the offspring from 1-day-exposed mothers provoked up to 50% mortality.

The dry mass of the surviving individuals from 7-day-exposed mothers after the 21 day chronic exposure to 0, 50 and 100 μg l^{-1} MC-LR ranged from 0.145 to 0.482 mg (per *D. magna*). When the mothers were exposed for 7 days to 50 and 100 μg l^{-1} MC-LR, the mass was significantly diminished (*t*-test, $P<0.05$) amongst all treatments, even if the offspring were not exposed to the toxin, showing a magnification of the effects into the next generation. According to the three-way main effects ANOVA, the only significant factor ($P<0.05$) was concentration of exposure of the progeny; according to the Tukey's test ($P<0.05$), those exposed to 50 μg l^{-1} are significantly lower in mass than the other groups (Fig. 7).

DISCUSSION

Transgenerational effects of MC-LR in *D. magna* were apparent for the enzymes CAT, sGST, mGST and MDH and furthermore on the survival and growth of the F1. Moreover, transgenerational effects were related to exposure concentration and particularly exposure duration of the parental generation. The strongest response to exposure of the parental generation was observed for the sGST, which is the most important enzyme considering its participation in the biotransformation of MC-LR and thus potential involvement in tolerance. In the groups of F1 *D. magna* that originated from non-exposed mothers, sGST increased in direct relation to MC-LR concentration, confirming previous results and suggesting induction of the enzyme activity as a result of the presence of the toxin and its subsequent biotransformation (Wiegand et al., 2002; Ortiz-Rodríguez and Wiegand, 2010).

From studies in vertebrates is known that a structurally diverse range of electronegative, slightly lipophilic xenobiotics triggers induction of GST. MC-LR would meet those criteria. Induction of GST genes may occur through the antioxidant-responsive element, the xenobiotic-responsive element, the GST P enhancer 1 or the glucocorticoid-responsive element (Hayes and Pulford, 1995). Moreover, activity of the GST enzyme can be regulated by

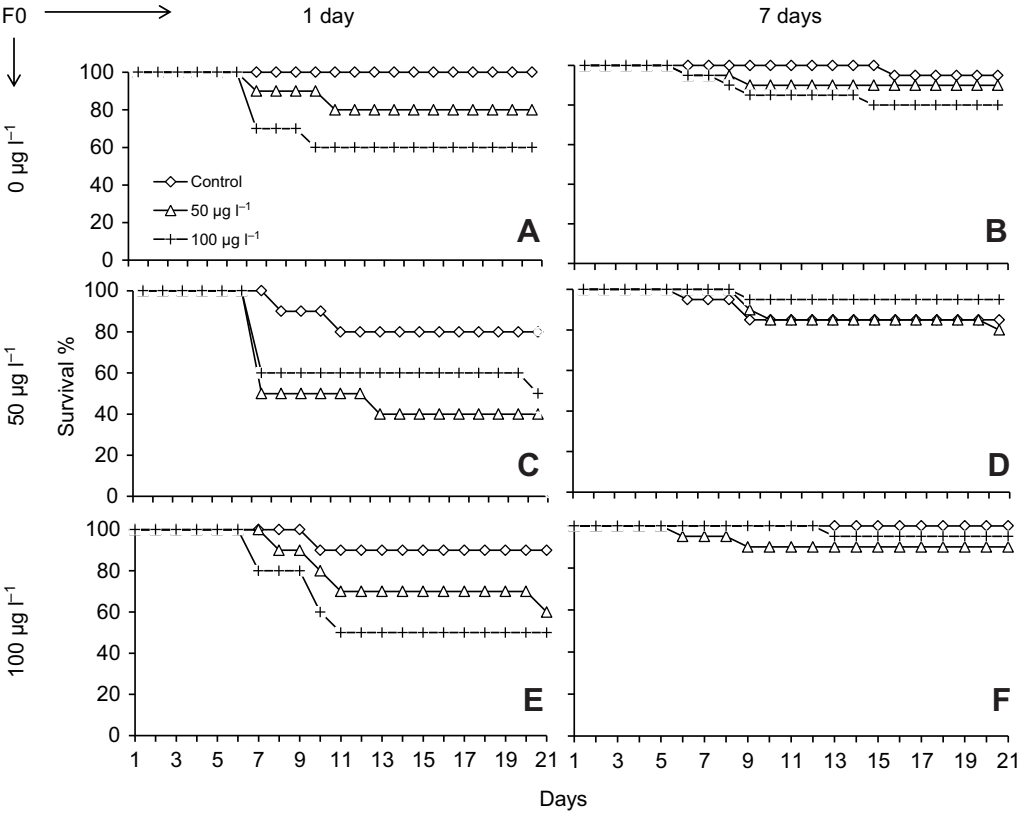

Fig. 6. Survival during the chronic (21 day) toxicity test of the progeny F1 from exposed parental generations F0 to (A,B) 0, (C,D) 50 or (E,F) 100 µg l^{-1} of MC-LR for 1 and 7 days. (A,C,E) Offspring from 1-day-exposed F0; (B,D,F) offspring from 7-day-exposed F0. The offspring were also exposed to 0, 50 or 100 µg l^{-1} MC-LR.

phosphorylation and because MCs inhibit protein phosphatases 1 and 2A, this could indirectly influence GST activity. Phosphorylation of the human sGST pi-isoform increased its activity (Okamura et al., 2009). Whether this mechanism applies to *D. magna* needs further investigation. In the experimental setup, a phosphorylation effect may be excluded, as the progeny was never exposed while developing in the mother's pouch. However, exposure of the mother to MC-LR for 1 day prevented the progeny from responding to MC-LR exposure with activation of sGST, possibly indicating an exhaustion of that mechanism.

A different situation arose after exposure of the mothers for 7 days to 50 and 100 µg l^{-1} MC-LR, where the activity of the progeny reached the highest activities, comparable or even higher than those neonates exposed to 100 µg l^{-1} MC-LR from non-exposed mothers (Fig. 1). As no increasing pattern was found within the groups from equally exposed (concentration-wise) mothers, sGST activity may have been maximally increased in these neonates. Considering the importance of this enzyme on the biotransformation of MC-LR, it is likely that an increase in enzyme activity presents a higher capacity for detoxification. This leads us to assume that 7 days exposure of the parental generation to 50 and 100 µg l^{-1} MC-LR confers a higher degree of tolerance to the offspring *via* constitutively increased sGST activity. The mechanism of the transfer from the mother to the offspring remains unclear, but cytoplasmic elements (transcription factors) that could induce a higher enzyme activity might be involved (Gallo, 2008).

The microsomal fraction of the GST (mGST) showed a contrary pattern. The activity of this enzyme was not changed in *D. magna* whose mothers were not exposed or exposed for 1 day, except for non-exposed neonates originated from mothers exposed to 100 µg l^{-1} MC-LR, who presented a higher enzyme activity (Fig. 2). Opposite to sGST, mGST in the progeny decreased after the mothers' exposure for 7 days. A strong decrement of the enzyme activity was found even in non-exposed neonates, suggesting that involvement of the membrane-associated GST in MC biotransformation is limited. A similar decrease of mGST activity has been seen in adult *D. magna* exposed to increasing concentrations of MC-LR for 1 day (Ortiz-Rodríguez and Wiegand, 2010). The implications for a reduced activity of GST involve less tolerance, as the ability to detoxify would be reduced. However, the total activity of mGST is lower than that of sGST, as is, presumably, its contribution to the detoxification. Similarly, in *Daphnia carinata*, decreasing GST (total) with age was associated with higher mortality and reduced tolerance towards toxic cyanobacteria (Guo and Xie, 2011).

Even though *D. magna* is a model organism for freshwater studies, it is important not to generalize into other detoxication pathways or to toxins of different molecular structure, as Kozlowski-Suzuki et al. (Kozlowski-Suzuki et al., 2009) did not find a clear relationship between GST and tolerance in copepods towards toxic phytoplankton (the dinoflagellates *Alexandrium minutum*, *A. tamarense* and *Prorocentrum lima*, and the haptophyte *Prymnesium parvum*). In their study, the phytoplankton contained nodularin, toxins causing paralytic shellfish poisoning and diarrhetic shellfish poisoning, and ichthyotoxins with haemolytic activity instead of MC. GST did not seem to be involved in detoxification of those toxins in copepods.

In *D. magna*, the activity of CAT has been linked positively with the formation of ROS originated by acute changes in temperature and oxygen availability (Becker et al., 2011), and from the presence of contaminants or stressors causing oxidative stress, such as UV (Vega and Pizarro, 2000; Kim et al., 2010), diesel oil (Zhang et al., 2004) and nanoparticles (Kim et al., 2010). In the present study, the increase in CAT activity was directly related to MC-LR

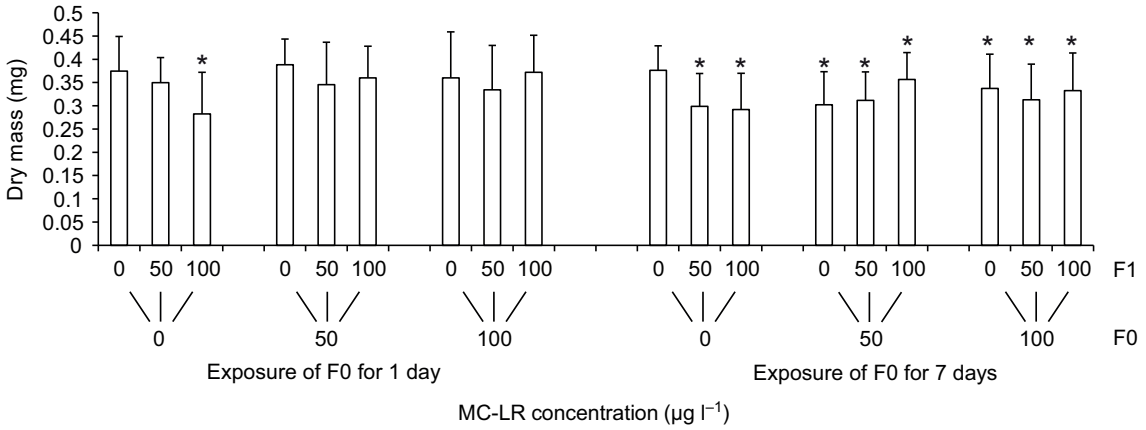

Fig. 7. Dry mass of *Daphnia magna* (21 days old) exposed to MC-LR (0, 50 or 100 µg l^{-1}). The parental generation F0 that originated these individuals was previously exposed to 0, 50 or 100 µg l^{-1} MC-LR for 1 or 7 days. Asterisks denote significant differences to control (*t*-test, **P*<0.05).

concentration (when the mothers were not exposed, Fig. 3), confirming results from previous studies, as MCs belong to oxidative-stress-generating compounds (Amado and Montserrat, 2010; Ortiz-Rodríguez and Wiegand, 2010). The changes in CAT evidenced the generation of reactive oxygen species and the consequent physiological response of the enzyme as part of the antioxidant system. Similar to sGST, exposure of the mothers for 1 day rather weakened the response in the offspring when challenged with the toxin, as no CAT response was provoked by MC-LR exposure apart from one exception of a significant increase in neonates exposed to 50 µg l^{-1} MC-LR from mothers exposed to 50 µg l^{-1} MC-LR.

Similar to sGST, semichronic exposure stimulated CAT activity: when the F0 were exposed to 50 and 100 µg l^{-1} MC-LR for 7 days, CAT activity in the offspring was either slightly raised without further exposure of the offspring, or increased in most cases when offspring were challenged with the toxin. Again, the duration of exposure of the parental generation to MC-LR had an effect on the ability of the progeny to increase an antioxidant response when exposed to MC-LR. Thus, 7 days of exposure of the mothers were necessary for inheritance of activity of this element of the antioxidant system to protect the progeny, but exposure of the parental generation for 1 day disabled it.

Amongst the energy-related enzymes, LDH was not altered after exposure of the progeny to any concentration of MC-LR, thus at the tested exposure concentrations or duration, energetic input *via* pyruvate was not disturbed (Fig. 4). LDH activity changes in *D. magna* evidenced impairment of the pyruvate–lactate conversion and thus energy restoration at high concentrations of zinc or MC-LR (Diamantino et al., 2001; Guilhermino et al., 1994; Ortiz-Rodríguez and Wiegand, 2010). However, at the tested concentrations, the relevance of its activity for energy supply during MC exposure may be insignificant. Moreover, transgenerational effects were not demonstrated for this enzyme.

MDH also required higher exposure concentration or duration of F0, e.g. to 100 µg l^{-1} for 1 day, for an elevated activity in the progeny compared with controls (Fig. 5). This short exposure of the F0 to a high concentration elevated the MDH activity in the progeny independent of the concentration of F1 exposure. A similar pattern was found when the F0 was exposed for 7 days, but in this case, exposure of the mothers to 50 µg l^{-1} was already sufficient to cause increased MDH activity. The activity of MDH shows a strong positive correlation with oxygen consumption rates and is considered an effective biochemical proxy for metabolic rate in aquatic invertebrates (Morgado and Soares, 1995; Dahlhoff et al., 2002; Donnelly et al., 2004). The activity of MDH follows a pattern similar to that of the sGST from the offspring of 7-day-exposed mothers. Considering the participation of this enzyme in producing energy, we can conclude that in *D. magna* challenged with MC-LR, increased biotransformation, antioxidant and other cellular processes such as repairing of damages due to toxic stress demand additional energy. A general prediction that arises from models involving graded physiological responses that are metabolically costly is that metabolic rate should increase with increasing levels of toxicant (Calow, 1991). In *D. magna*, higher exposure concentrations of the progeny confirm this statement, as well as longer exposure duration of the mothers. This upregulation of biotransformation, antioxidant and energy-producing enzymes, which the progeny inherited, could enable those individuals to detoxify the MC-LR and maintain other physiological processes (growth or reproduction) under toxic stress, given that sufficient energy resources are available for the additional metabolic expenses.

No transgenerational effect considered as potentially participating in the tolerance was found on the progeny of the 1-day-exposed mothers. A combination of concentration plus length of exposure was needed to achieve an effective transfer.

The effect of biotransformation-, antioxidant- and energy-related metabolism on enzyme activity was complemented with an assessment of survival and growth in 21 day toxicity tests. MC-LR has a detrimental effect on the survival of *D. magna*: increasing concentrations decreased the life expectancy to 60–80% after 21 days, as seen in the progeny of non-exposed mothers (Fig. 6A,B). Dao et al. found a similar effect on survival after conducting continuous exposures including the developing progeny in the brood chamber (Dao et al., 2010). Treatments with low toxin concentrations (5 µg l^{-1}) slightly affected survival, whereas at concentrations of 50 µg l^{-1} (pure MC-LR or cyanobacterial crude extract for 60 days) the survival of individuals was strongly decreased. More than 50% of mortality occurred after 35 days of exposure to 50 µg l^{-1} of pure MC-LR or crude extract in adults; moreover, reproduction was seriously affected (Dao et al., 2010). In that study, mortality started to occur after 5 or 6 days, which coincides with the time of the start of reproduction (appearance of eggs in the brood chamber), suggesting that reproduction withdraws

energy resources from maintenance processes, disabling the ability to withstand the toxic stress. Moreover, those offspring suffered from mortality, even if they were raised in control medium. In the present study, it is evident that exposure of the parental generation to MC-LR for 1 day (50 and 100 $\mu g l^{-1}$) did not confer any advantage to the progeny in terms of survival after 21 days (Fig. 6C,E). The survival of this progeny was equally affected by the exposure. However, in the progeny from the mothers exposed for 7 days to 50 and 100 $\mu g l^{-1}$ MC-LR, the mortality was reduced compared with the progeny from 1-day-exposed mothers, suggesting that sufficiently long exposure of the parental generation to MC-LR provides an enhanced chance of surviving the MC-LR exposure for 21 days (Fig. 6D,E), correlating to increased activity of biotransformation and antioxidant enzymes. It is important to point out that the offspring were not exposed while developing inside the brood chamber of the mother.

With these results, we show transgenerational effects, which could improve the fitness of the population. Similar results have been reported for *D. magna* by Gustafsson and colleagues (Gustafsson and Hansson, 2004; Gustafsson et al., 2005) using cyanobacterial cells as food, particularly comparing an MC-producing strain with a non-producer. In their study, the offspring, particularly the first two generations, from previously exposed mothers showed improved fitness (as intrinsic rate of population increase) when exposed to toxic cyanobacteria compared with the progeny from non-exposed mothers. A plausible explanation given by the authors is that after one generation in the toxic environment, the offspring in the second generation could be born with induced genes of detoxifying enzymes, and thus improve their performance in the toxic environment. We confirm this explanation in our study as the offspring from mothers exposed to MC for 7 days showed a constitutively higher enzyme activity of sGST (Fig. 1) Moreover, we can demonstrate that MC-LR exposure of the parental generation incited MC-LR tolerance in its offspring, as indicated by improved survival.

Besides natural toxins, some studies have shown improved survival of the progeny from exposed *Daphnia* sp. mothers to a wide variety of compounds, e.g. copper (LeBlanc, 1982), low concentrations of cadmium (Muyssen and Janssen, 2004), high concentrations of mercury (Tsui and Wang, 2005a; Tsui and Wang, 2005b) and the herbicide molinate (Sánchez et al., 2004). However, the opposite has also been shown, highlighting the importance of the mechanism of toxicity of the substance. In some cases, tolerance is not achieved or transgenerational effects are not clear, for example after exposure of F0 to the plastic additive and surfactant *p*-nonylphenol (Tanaka and Nakanishi, 2002) or the pharmaceuticals carbamazepine, diclofenac, 17α-ethinylestradiol and metroprolol (Dietrich et al., 2010). Even a magnification of the detrimental effect has been shown, as was found with lauryl sulphate (Le Blanc, 1982), the pesticides tetradifon (Villarroel et al., 2000) and diazinon (Sánchez et al., 2000), the endocrine disruptors 17β-oestradiol E2, diethylstilbestrol, bisphenol A and 4-nonylphenol (Brennan et al., 2006), uranium (Massarin et al., 2010) and the herbicide glyphosate (Papchenkova et al., 2009). It is important to remark that in this last set of studies, where a magnified effect was found in the subsequent generations, a clear effect was not found in the parental generations, highlighting the importance of multigenerational approaches in ecotoxicology to the understanding and evaluation of the impact of chemicals in the ecosystem.

Individual mass from the surviving animals after 21 days correlated with the pattern seen in survival and biochemical variables. MC-LR had a detrimental effect on mass gain in the surviving offspring F1 from non-exposed mothers (Fig. 7). The additional energetic expenses for maintenance in the presence of the toxin caused a shift in the distribution of energy, withdrawing resources from growth and perhaps reproduction. If the parental generation was exposed to MC-LR for 1 day, the surviving offspring did not suffer from reduced growth (mass); however, survival was substantially decreased. A slight but significant loss of mass was seen in the offspring from mothers exposed for 7 days to both concentrations of MC-LR (50 and 100 $\mu g l^{-1}$). Interestingly, the mass of the offspring of exposed mothers did not follow the same concentration-dependent decreasing pattern that was found in the offspring of non-exposed mothers. In the offspring exposed to 100 $\mu g l^{-1}$ MC-LR from mothers exposed to 50 $\mu g l^{-1}$ for 7 days, mass was not affected. A 1 day exposure of the parental generation prevented the fewer surviving progeny from growing less when challenged with the toxin, and 7 days of exposure prevented the better-surviving progeny from gaining the same mass as the control individuals. It is important to remark that only surviving individuals were weighed, and mortality of the progeny from 1-day-exposed mothers was close to 50% after 21 days, whereas the progeny from 7 days exposure suffered only 20% mortality at most. Again, increased survival occurred at the expense of growth, hence energy had been allocated to maintain or repair cellular functions in the presence of the toxin. Similar decrements in mass (17–35%) have been reported when feeding *D. magna* with 25–100% of *Microcystis* cells containing the variants MC-LR, MC-YR and MC-RR (Trubetskova and Haney, 2006).

In the more environmentally relevant scenario, exposing both the mothers and the progeny during their embryonic development, offspring suffered reduced growth, independent of whether they were raised in toxin-free or toxic medium (Dao et al., 2010). As maturation is related to size, they started reproduction later. Hence, the facilitation of the observed population increase may be higher offspring rates, which would need further investigation.

CONCLUSIONS

Although 1 day exposure of the mothers did not confer any significant increase in the activity of the enzymes GST, CAT or MDH in the progeny, 7 days exposure of the mothers did, suggesting that activation factors are transferred through maternal inheritance. The elevated sGST in the progeny hinted at elevated biotransformation. The reacting CAT in the progeny enabled them to buffer oxidative stress caused by the toxin. MDH activity indicated amplified energy turnover, presumably because of the energy requirement of biotransformation, antioxidant and repair processes. As a consequence, the progeny from 7-day-exposed mothers survived better when exposed to the toxin than the progeny from 1-day-exposed mothers under the same conditions. It may be concluded that increased activity of the enzymes sGST, CAT and MDH supports survival of the daphnids. However, survival was at the expense of growth as a result of increased energy allocation into maintenance processes to cope with MC-LR, thus leading to a reduction in the energy available for growth.

These results show the detrimental effect of MC-LR on *D. magna*'s physiology and the biochemical processes affected; in addition, they evidence transgenerational or maternal effects provoked by the toxin. They evidence biotransformation, oxidative stress defense and increased energy requirements behind the observed acquirement of enhanced tolerance towards toxic cyanobacteria over generations. In addition, they show that MC-LR itself is able to induce this response.

LIST OF ABBREVIATIONS

CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
DM	dry mass
GST	glutathione S transferase
LDH	lactate dehydrogenase
MC	microcystin
MDH	malate dehydrogenase
mGST	microsomal glutathione S transferase
ROS	reactive oxygen species
sGST	soluble glutathione S transferase
TFA	trifluoroacetic acid

ACKNOWLEDGEMENTS

We are greatly thankful to Prof. Werner Kloas from the department V (IGB) for his support throughout this work, V. Viehmann (IGB) for extensive laboratory assistance and the two anonymous reviewers, whose comments improved the quality of this manuscript.

FUNDING

Funding for this research was provided by the German Academic Exchange Service, the Leibniz Institute of Freshwater Ecology and Inland Fisheries, the Secretariat of Public Education of México, the Mexican Government [to R.O.-R.] and the FAZIT Foundation, Germany [to D.T.S.].

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