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RESEARCH ARTICLE

Inducible tolerance to dietary protease inhibitors in *Daphnia magna*

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SUMMARY

***Daphnia* has been shown to acquire tolerance to cyanobacterial toxins within an animals' lifetime and to transfer this tolerance to the next generation. Here we used a strain of the cyanobacterium *Microcystis aeruginosa*, which contained two chymotrypsin inhibitors (BN920 and CP954), the green alga *Scenedesmus obliquus* as reference food and a clone of *D. magna* to investigate the physiological mechanism of acquired tolerance to these cyanobacterial toxins. The intracellular concentrations of CP954 and BN920 were 1550 and 120 μmol l^{-1}, respectively. When food suspensions of the green alga contained >60% *M. aeruginosa*, growth rates of *D. magna* were reduced. When grown on the green alga, three chymotrypsins ranging in mass from 16 to 22 kDa were distinguished in *D. magna*. Purified BN920 and CP954 specifically inhibited *D. magna* chymotrypsins. Feeding with encapsulated BN920 resulted in growth depression in *D. magna* and replacement of the chymotrypsins by three chymotrypsins with smaller molecular mass. With just 20% *M. aeruginosa*, the same changes in the chymotrypsin pattern as with the pure inhibitor were observed. IC_{50} values for inhibition of chymotrypsins of *D. magna* growing on the green alga were 5.4 nmol l^{-1} (BN920) and 7.4 nmol l^{-1} (CP954). When *D. magna* was grown on 20% *M. aeruginosa*, 2.2-fold higher IC_{50} values were observed. This indicated that increased tolerance to these dietary inhibitors was acquired within an animal's lifetime by remodelling the digestive chymotrypsins, which in turn serves as an intra-generational defence against these cyanobacterial inhibitors. This mechanism might be relevant for the transfer of tolerance to the next generation through maternal effects.**

Key words: *Daphnia*, tolerance, cyanobacteria.

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INTRODUCTION

The frequency of cyanobacterial mass developments (cyanobacterial blooms) in coastal waters and lakes has increased worldwide during the last century, and increasing temperatures due to global warming will further favour cyanobacterial dominance in many aquatic ecosystems (Paerl and Huisman, 2008). Blooms of cyanobacteria are not only often associated with hazards to human health and livestock and with reduced recreational quality of water bodies (Carmichael, 1994), but also may be detrimental to herbivorous zooplankton and, through trophic interactions, the whole ecosystem (de Bernardi and Giussani, 1990; Wilson et al., 2006). The efficiency with which biomass and energy are transferred through the food web and sustain the production of higher trophic levels declines with nutrient loading and system productivity (McQueen et al., 1986; Carpenter and Kitchell, 1984), and in hypertrophic lakes biomass and energy transfer is often inhibited at the phytoplankton–zooplankton link, resulting in an accumulation of phytoplankton, i.e. frequently composed of cyanobacteria (McQueen et al., 1989). In particular, toxic cyanobacteria have been shown to contribute to shifts in zooplankton size and community composition (Hansson et al., 2007).

The aquatic herbivorous crustacean *Daphnia* often provides an important trophic link for the transfer of carbon from primary producers to higher trophic levels (e.g. Lampert, 1987). Three major causes for the low assimilation efficiency of cyanobacterial carbon by *Daphnia* have been highlighted: (1) the occurrence of cyanobacteria as large colonies or filaments that prevent ingestion (Porter and McDonough, 1984), (2) a deficiency of cyanobacteria in essential lipids such as sterols and polyunsaturated fatty acids, which prevent assimilation (Von Elert, 2002; Von Elert et al., 2003; Martin-Creuzburg et al., 2008), and (3) the production of a variety of bioactive secondary compounds, which may cause illness and even death of livestock and humans (Carmichael, 1994). If these secondary compounds are of similar toxicity to herbivorous zooplankton as to livestock and if these compounds thus protect cyanobacteria against grazing, herbivorous zooplankton may foster the dominance of cyanobacterial strains with secondary metabolites highly toxic to mammals. Hence, there is considerable value in deciphering the specific mechanisms of interaction between cyanobacteria and herbivorous zooplankton (Wilson et al., 2006).

There is strong evidence that, at the population level, *Daphnia* develop tolerances to cyanobacterial toxins in their natural environment (Hairston et al., 1999; Sarnelle and Wilson, 2005). However, experimental evidence is unfortunately too unspecific to allow for a precise identification of the underlying mechanisms, as in most cases the observed effects cannot unambiguously be ascribed to a single cyanobacterial metabolite (reviewed by Wilson et al., 2006). In general, assessing dissolved extracts does not constitute a natural way of exposure for a filter-feeding herbivore, and comparing cyanobacterial strains producing a particular toxin with another non-toxin-producing strain does not account for other unknown differences in secondary metabolites between cyanobacterial strains.

Because of their high hepatotoxicity in mammals, the most commonly studied class of cyanobacterial toxins are microcystins. Unequivocal evidence for a detrimental role of these toxins to herbivorous zooplankton has been provided by comparison of a wild-type strain (producing several microcystins) and a mutant strain (with no microcystins) of the same cyanobacterium (Lürling, 2003; Rohrlack et al., 2005a) and by supplementation of a green alga with pure microcystin-LR (Wilson and Hay, 2007). Nevertheless, the effects of the microcystin-free mutants point to the relevance of other bioactive metabolites, and it has been suggested that more attention should be paid to cyanobacterial compounds other than microcystins as causes of the poor quality of cyanobacteria for herbivorous zooplankton (Wilson et al., 2006).

As the relative abundance of cyanobacteria within a given lake shows high interannual and, in particular, high seasonal variability, selection should particularly favour inducible traits that enhance the tolerance to microcystins even within a given *Daphnia* genotype (Hansson et al., 2007). Using a single clone of *D. magna* that was isolated from a pond that was free from cyanobacterial blooms for several years, it was shown that the tolerance to microcystins is an inducible defensive mechanism developed during an individual's lifetime (Gustafsson et al., 2005). However, the physiological basis (e.g. detoxification mechanism) for the induced tolerance in this clone of *D. magna* has not been elucidated.

Although microcystins are the best-studied cyanobacterial toxins, they are probably not the most frequently encountered secondary metabolites in cyanobacterial mass developments. In a survey of 14 natural water blooms dominated by *Microcystis* spp., the cyanobacterial biomass proved to contain microcystins in only 20% of the cases (Agrawal et al., 2006), but almost 60% of the blooms contained protease inhibitors (Agrawal et al., 2001). Out of 89 strains of the cyanobacterium *Planktothrix* isolated from diverse localities and geographic regions, 70% produced inhibitors of *Daphnia* trypsins (Rohrlack et al., 2005b). Protease inhibitors are reported from a wide range of genera of marine and freshwater cyanobacteria, and many of these protease inhibitors act against serine proteases (i.e. trypsins and chymotrypsins) (Gademann and Portmann, 2008). This indicates that protease inhibitors are a widespread class of secondary metabolites in cyanobacteria. The finding that trypsins and chymotrypsins represent the most important digestive proteases in the gut of *D. magna* (Von Elert et al., 2004) suggests that cyanobacterial protease inhibitors interfere with these digestive proteases in the gut of *Daphnia* spp. This putative mode of interference is corroborated by the finding that coexistence of *Daphnia* spp. with cyanobacteria has resulted in increased tolerance against a cyanobacterial protease inhibitor (Blom et al., 2006).

Here, we made use of a strain of *M. aeruginosa* that contains two previously identified chymotrypsin inhibitors (Von Elert et al., 2005) and investigated whether the effects on the growth and digestive proteases of *D. magna* observed from being fed the cyanobacterium can be attributed to the cyanobacterial protease inhibitors. We further tested whether *D. magna* shows an increased tolerance to cyanobacterial protease inhibitors that is developed during an individual's lifetime. We hypothesized that, because of specific interference of the inhibitors with the digestive proteases, such an increased tolerance of *D. magna* to cyanobacterial protease inhibitors would be observed on the level of the digestive proteases. This study was conducted using a single clone of *D. magna* that was isolated from a lake that had no cyanobacterial mass developments (Lampert and Rothhaupt, 1991). We chose *D. magna* from this population to assure that any potential mechanism of enhanced tolerance was not activated.

MATERIALS AND METHODS

Cultivation of organisms

A clone of *Daphnia magna* Straus 1820 that originates from Lake Binnensee, Germany (Lampert and Rothhaupt, 1991), which is not known to have had cyanobacteria at the time of isolation of the clone, was cultured in membrane-filtered tap water at 20°C and fed with the green alga *Scenedesmus obliquus* (Turpin) Kützing 1833 (SAG 276-3a, Culture Collection Göttingen, Germany) at non-limiting concentrations (2 mg C l^{-1}). *Scenedesmus obliquus* grows mainly as single cells with a mean spherical diameter of 5.9 μm that are easily ingestible by *D. magna* (Lampert et al., 1994). The freshwater cyanobacterium *Microcystis aeruginosa* Kützing 1846 [strain NIVA-Cya 43, Norwegian Institute for Water Research (NIVA) Culture Collection, Oslo, Norway] produces no microcystins and grows as single cells that are easily ingestible by *Daphnia* spp. (Lürling and Van der Grinten, 2003). *Scenedesmus obliquus* and *M. aeruginosa* were grown in 10 l bottles of Cyano medium (Von Elert and Jüttner, 1997) at 20°C and 100 μmol photons m^{-2} s^{-1}. The cultures were harvested by centrifugation (3000 ***g***) when they reached the stationary phase, and were re-suspended in fresh medium for subsequent growth experiments or frozen at –20°C and lyophilized (*M. aeruginosa*) for subsequent extraction.

Preparation of *D. magna* homogenate

Neonates of *D. magna* were grown on 2 mg C l^{-1} of either pure *S. obliquus* or a mixture of 80% *S. obliquus* and 20% *M. aeruginosa* for 6 days and were transferred to 100% *S. obliquus* during the last 4 h prior to preparation of the homogenates in order to exclude potential effects of compounds released from cyanobacteria in the gut. Preparation of *D. magna* homogenates was performed according to Von Elert et al. (Von Elert et al., 2004) as follows. Daphnids were transferred to 5 μl per animal of 0.1 mol l^{-1} phosphate buffer (pH 7.5) containing 2 mmol l^{-1} dithiothreitol and were homogenized with a pestle. The homogenate was centrifuged for 3 min at 14,000 ***g***. The protein concentration of the supernatant – the *D. magna* homogenate – was analysed using a Qubit fluorometer and the appropriate Quant-iT^{TM} Protein Assay Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's advice, and was subsequently used for measurement of protease activity and for SDS-PAGE.

Measurement of trypsin and chymotrypsin activity

It has been shown previously that ≥99% of the chymotrypsin and trypsin activity obtained from whole *D. magna* homogenate originates from gut-associated enzymes (Von Elert et al., 2004).

Chymotrypsin activity of the *D. magna* homogenate was measured photometrically according to Von Elert et al. (Von Elert et al., 2004). Briefly, 10 μl *D. magna* homogenate was mixed with 980 μl 0.1 mol l^{-1} potassium phosphate buffer, pH 7.5. The buffer contained 125 μmol l^{-1} *N*-succinyl-alanine-alanine-proline-phenylalanine-para-nitroanilide [S$(Ala)_2$ProPhepNA] and 1% dimethyl sulfoxide (DMSO). The change in absorption was measured at a wavelength of 390 nm at 25°C continuously over 10 min. The trypsin activity was measured using the artificial substrate *N*-benzoyl-arginine-para-nitroanilide (BApNA) according to Von Elert et al. (Von Elert et al., 2004). Ten microlitres of *D. magna* homogenate was mixed with 895 μl 0.1 mol l^{-1} potassium phosphate buffer, pH 6.5. The buffer contained 1.88 mmol l^{-1} BApNA and 7.5% DMSO. The change in absorption was measured at a wavelength of 390 nm at 25°C continuously over 10 min.

Preparation and fractionation of extracts of *M. aeruginosa*

One gram of lyophilized powder of *M. aeruginosa* strain NIVA Cya 43 was extracted twice with 50 ml methanol, and the extract was diluted with ultrapure water to yield a 5% methanol solution that was subsequently applied to a previously activated and equilibrated reversed-phase octadecyl silica (ODS) cartridge (10 g sorbent, Varian, Darmstadt, Germany). The retained material was eluted with 100% methanol and evaporated under vacuum; the resulting crude extract was then dissolved in 1 ml of methanol (MeOH). For the determination of dose-dependent effects of this extract on the activity of *D. magna* trypsins and chymotrypsins, five different dilutions of 1 ml of crude extract were used. Of each of these dilutions, 10 µl were added to enzyme assays of trypsin or chymotrypsin; the controls received 10 µl of MeOH. Each concentration was tested three times in independent protease assays.

We isolated the chymotrypsin inhibitors nostopeptin BN920 (BN920) and cyanopeptolin 954 (CP954) from *M. aeruginosa* strain NIVA Cya 43 according to Von Elert et al. (Von Elert et al., 2005): material retained on the cartridge was eluted in steps with 50 ml each of 20, 40 and 60% MeOH. The fraction eluting with 60% MeOH from the cartridge was concentrated and fractionated by reversed-phase HPLC [Nucleosil 150-5 C18, 250×4 mm; Macherey-Nagel, Düren, Germany; 1 ml min^{-1} flow rate with 30:70 acetonitrile:water containing 0.05% trifluoroacetic acid (TFA)], and peaks eluting at 8.8 and 12.7 min were collected in repeated HPLC runs. BN920 and CP954 were identified by retention time and the UV spectrum of reference compounds. Fractions collected from HPLC were re-extracted with an ODS cartridge (500 mg; Varian) equilibrated with 5% MeOH, and then the cartridge was washed with 5% MeOH to neutral pH, and the inhibitors were eluted with MeOH, evaporated to dryness and dissolved in MeOH. Purity and identity of CP954 and BN920 were confirmed by high-resolution liquid chromatography–mass spectrometry (LC-MS) (Exactive, ThermoFisher Scientific, Schwerte, Germany; ESI, positive scan mode, spray voltage 4.3 kV, capillary temperature 275°C, sheath gas flow rate 35 a.u., auxiliary gas flow rate 5 a.u., log-mass calibration): CP954: m/z=955.43258; BN920: m/z=921.47138. For the determination of concentration-dependent effects of BN920 and CP954 on the chymotrypsin activity of *D. magna*, 12 aliquots of 5–30 µl of stock solutions with known concentrations of BN920 or CP954 were tested, and the respective concentration giving 50% of maximal inhibition (IC_{50}) with corresponding 95% confidence intervals was calculated fitting a sigmoid dose–response curve using the software GraphPad Prism, version 4.0 (GraphPad Software, La Jolla, CA, USA).

Quantification of inhibitor content

BN920 and CP954 were quantified by calibration curves generated with stock solutions of known concentrations of the reference compounds and peak areas based on 208 nm. For the determination of the intracellular concentrations, 50 ml (~5 mg C) of a dense culture of *M. aeruginosa* NIVA-Cya 43 were centrifugated (4000 ***g***, 5 min), and the pellet was extracted with 10 ml and then 8 ml of 80% MeOH. The pooled extracts were evaporated to dryness and redissolved in 300 µl of 33% MeOH. Aliquots of 50 µl were used for separation and quantification by HPLC. Amounts of BN920 and CP954 were normalized to the biovolume of the extracted cyanobacterial cells, which was determined from another aliquot of the dense culture by cell counting and sizing of the cells using an inverted microscope.

Microscopy of the liposomes had shown that liposomes are not stable in the presence of 30:70 acetonitrile:water. To estimate the inhibitor content of liposomes loaded with BN920, aliquots of the resuspended pellet were injected into the HPLC, and BN920 was quantified as given above. It was not possible to distinguish between internal and external BN920.

SDS-PAGE and activity staining of the *D. magna* homogenate

Daphnia magna homogenate (20 µg protein) with 5 µl 4× Laemmli buffer (Laemmli, 1970) was loaded on a 12% SDS-polyacrylamide gel and run at 200 V at 6°C. After the run, the gels were activity stained according to Von Elert et al. (Von Elert et al., 2004): the gels were washed and incubated with agitation for 30 min at 4°C and for another 90 min at 20°C in 50 mmol l^{-1} Tris-HCl (pH 9) containing 1.5% (w/v) casein Hammerstein. Gels were washed, fixed in 12% trichloracetic acid for 30 min, stained with 0.25% Coomassie Brilliant Blue in methanol:acetic acid:water (50:10:40, by volume) and destained in methanol:acetic acid:water (42:8:50, by volume). Clear zones indicate protease activity. The molecular weights of the visible proteases were compared with the peqGold Prestained Protein Marker III (peqlab, Erlangen, Germany). Effects of chymotrypsin inhibitors were stained separately by incubation of the gels at ambient temperature for 30 min in a solution of either the synthetic chymotrypsin inhibitor chymostatin (5 µmol l^{-1}) or the pure natural chymotrypsin inhibitors BN920 (55 nmol l^{-1}) or CP954 (33 nmol l^{-1}) prior to the incubation in the presence of casein. Chymostatin was added from a stock solution (0.83 mmol l^{-1} in DMSO); for the two other inhibitors, stock solutions in ethanol were used. Controls have shown that the respective concentrations of DMSO or ethanol during incubation of the gel did not affect activity of any of the proteases (data not shown).

Growth experiments

Growth experiments were conducted with juveniles born within 8 h originating from the third clutch of mothers originating from the third clutch of a single animal. The growth experiment with different mixtures of *S. obliquus* and *M. aeruginosa* was performed at 20°C with 20 animals in 1 l of aged tap water and a food concentration of 2 mg C l^{-1} and three replicates per treatment. Treatments consisted of mixtures of *S. obliquus* and *M. aeruginosa* at ratios of 100:0, 80:20, 60:40, 40:60 and 20:80 percent carbon of both strains. Animals were transferred daily into freshly prepared food suspensions. The growth experiment lasted for 6 days, and dry masses of bulks of animals taken at the beginning and the end of the experiment were determined on an electronic balance (±1 µg; CP2P, Sartorius, Göttingen, Germany); from this, individual masses were calculated. Mass-specific growth rates (g) were calculated using the formula $g=(\ln x_{te}-\ln x_{tb})/\Delta t$, where x_{te} and x_{tb} are the individual masses at the end and the start of the experiment, respectively, and Δt is the test duration, i.e. 6 days. Carbon concentrations of food suspensions were estimated from carbon extinction equations determined prior to the experiment for the cyanobacterium and the green alga. Photometric light extinction (470 nm) was determined in a photometer (MN PF 11, Macherey-Nagel, Düren, Germany), and aliquots of the suspensions were filtered onto precombusted GF-filters (Whatman, Dassel, Germany); the carbon content was subsequently determined in a C-N analyzer (Flash 2000, ThermoFisher Scientific).

Preparation and loading of liposomes

Liposomes were prepared according to Martin-Creuzburg et al. (Martin-Creuzburg et al., 2008). Briefly, 7 mg of 1-palmitoyl-2-oleoyl-phosphatidylcholin (POPC) and 3 mg of 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) were dissolved in 2 ml CH_2Cl_2 and subsequently evaporated to dryness (rotatory evaporator) in a

flask. After addition of 10 ml of buffer A (150 mmol l^{-1} NaCl, 20 mmol l^{-1} NaPi, pH 7.0), the flask was incubated on a rotary shaker (100 revolutions min^{-1}) at room temperature for 30 min, and the suspension was then sonicated in an ultrasonic bath for 1 min. This liposome stock suspension was stored at 4°C for up to 2 weeks for further use. Prior to addition to *D. magna* cultures, the stock suspension was sonicated for another 2 min.

Liposomes were passively loaded with BN920 according to Brandl and Massnig (Brandl and Massnig, 2003). Briefly, a methanolic solution of pure inhibitor, obtained after HPLC from 0.5 g of lyophilized *M. aeruginosa*, was evaporated to dryness and re-dissolved in 0.5 ml of buffer A. Subsequently, 5 ml of liposome stock solution were concentrated by centrifugation (150,000 ***g***, 90 min), and the resultant pellet was resuspended in 0.5 ml of the above-mentioned buffer containing BN920 and transferred to a vial. This vial was then incubated at 60°C (4 h) to facilitate loading of the liposomes with the inhibitor. For a control treatment, 5 ml of liposome stock solution were concentrated and treated similarly without the addition of inhibitor. Both the concentrated and the loaded vesicles were stored at 4°C for several days until use.

Growth and feeding experiment with liposomes

The growth experiment with BN920 encapsulated in liposomes was performed with five newborn *D. magna* in 250 ml of water and 2 mg C l^{-1} *S. obliquus*. Treatments consisted of *S. obliquus* either without additions (control), with addition of 20 µl of unloaded concentrated liposomes (lipos) or with addition of 20 µl of liposomes loaded with BN920 (lipos+BN920). This resulted in 2.6×10^4 liposomes ml^{-1} with a diameter of 1.9 µm, which comprised 2.4% of the biovolume of *S. obliquus*. Each treatment was triplicated. Animals were transferred daily into freshly prepared food suspensions, and mass-specific growth rates were calculated from dry masses of animals determined after 6 days as described above. We tested for effects on the protease pattern by incubation of ten 5-day-old *D. magna* individuals for 20 h in 80 ml of water either without additions (control), with the addition of 8 µl of concentrated liposomes (lipos) or with the addition of 8 µl of liposomes loaded with BN920 (lipos+BN920). The initial food concentration of 0.1 mg C l^{-1} *S. obliquus* was maintained by addition of the same amount of alga every 4 h. Subsequently, the animals were transferred into freshly prepared suspensions of *S. obliquus* (2 mg l^{-1}) for another 4 h and then stored at –20°C until SDS-PAGE and activity staining of the homogenates.

Statistical analysis

Treatment effects on growth rates and protease activity were analysed *via* one-way ANOVA and a *post hoc* Tukey's honestly significant difference (HSD) test. A Levene's test was conducted to determine the homogeneity of variances. The level of significance was $P<0.05$. Statistical analyses were conducted in Statistica 6.0 (StatSoft, Hamburg, Germany). For the determination of the effects of IC_{50} concentrations of BN920 and CP954 for the inhibition of chymotrypsin activity of *D. magna*, effects of 12 different concentrations of either inhibitor were recorded, and the respective IC_{50} with corresponding 95% confidence intervals were calculated fitting a sigmoid dose–response curve using GraphPad Prism, version 4.0.

RESULTS

In vitro effects on *D. magna* digestive proteases

In *D. magna* homogenate, the specific activity of the serine protease chymotrypsin was approximately fourfold higher than that of trypsin (Fig. 1). Addition of increasing amounts of a methanolic extract of *M. aeruginosa* led to inhibition of the digestive trypsins (one-way ANOVA, $F_{4,10}=43.04$, $P<0.0001$); addition of the same volume of methanol had no effect. Although 100 µg of extracted cyanobacteria had no effect on the trypsin activity (Tukey's HSD, $P=0.37$; Fig. 1A), an extracted cyanobacterial biomass of just 2 µg led to significant inhibition of chymotrypsin in comparison to the control (one-way ANOVA, $F_{4,10}=34.85$, $P<0.0001$; Tukey's HSD, $P<0.05$; Fig. 1B); addition of the same volume of methanol had no effect. This indicated that the strain of *M. aeruginosa* used in the present study had a substantially higher content of inhibitors against digestive chymotrypsins than inhibitors against trypins of *D. magna*.

Whole-body homogenate of *D. magna* grown on *S. obliquus*, which was subjected to SDS-PAGE and subsequently stained for activity, revealed nine distinct protease bands in a range from 16 to 73 kDa (Fig. 2). When, prior to the activity staining, the gel was incubated with chymostatin (a synthetic inhibitor of chymotrypsins), activity of three proteases ranging from 16 to 22 kDa was no longer detectable, which indicated that these three proteases were chymotrypsins (Fig. 2). The strain of *M. aeruginosa* used here has previously been shown to produce the two major chymotrypsin inhibitors BN920 and CP954 (Von Elert et al., 2005). Application of these two pure compounds resulted in inhibition, although slightly weaker, of the same three protease bands (Fig. 2), which

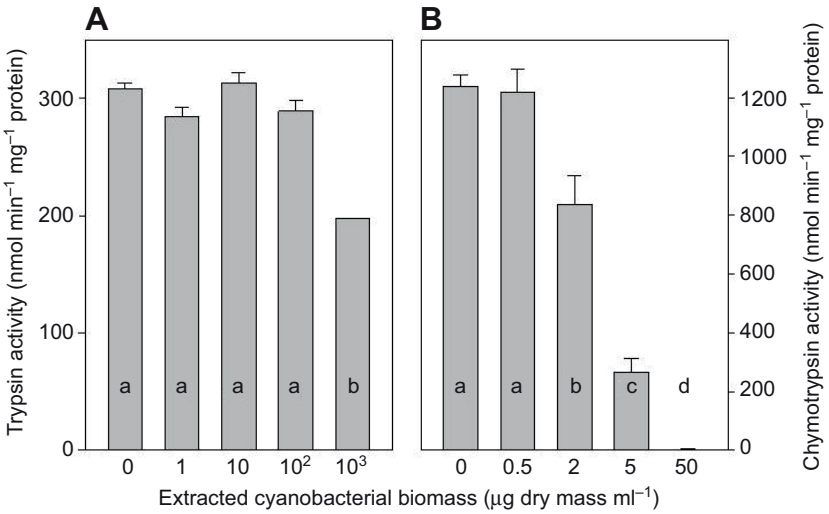

Fig. 1. *In vitro* effects of increasing concentrations of extracted biomass of *Microcystis aeruginosa* on the mean specific activity of trypsin (A) and chymotrypsin (B) from *Daphnia magna*. Error bars indicate +s.e.m. (N=3); identical letters denote non-significant differences among treatments and control (no extracted biomass) within A or B (Tukey's HSD, $P<0.05$). Note the different scales.

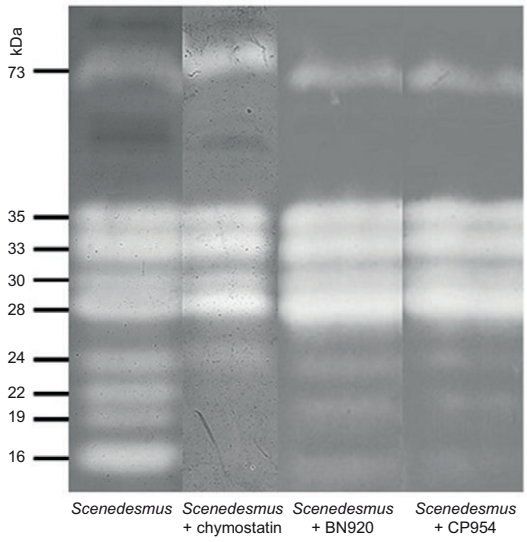

Fig. 2. Substrate-SDS-PAGE gels of whole-body homogenate of *D. magna* grown on the green alga *Scenedesmus obliquus*. Activity staining was performed with untreated homogenate as control (*Scenedesmus*) or with the same homogenate incubated either with the synthetic inhibitor chymostatin (+chymostatin) or with the natural chymotrypsin inhibitors nostopeptin BN920 (+BN920) or cyanopeptolin 954 (+CP954) prior to staining. Clear zones indicate protease activity. The molecular mass of the bands is given in kDa.

demonstrated that BN920 and CP954 specifically inhibit the chymotrypsins in the whole-body homogenate of *D. magna* and have no inhibitory effects on other non-chymotrypsin proteases.

Dietary effects of the cyanobacterium on *D. magna*

When *D. magna* was grown on saturating carbon concentrations of *S. obliquus* or on mixtures with *M. aeruginosa*, no mortality was observed. Somatic growth was significantly affected by the food composition (one-way ANOVA, $F_{5,12}$=113.7, P<0.001; Fig. 3A). However, only if more than 60% of the dietary carbon consisted of *M. aeruginosa* was a significant inhibition of growth observed (Tukey's HSD, P<0.001; Fig. 3A). Activity staining of proteases after SDS-PAGE of *D. magna* grown on pure green alga revealed nine distinct protease bands in a range from 16 to 73 kDa (Fig. 3B). The different diets clearly affected the protease pattern: on pure *S. obliquus*, three proteases in the range of 16 to 22 kDa were observed, which, in the presence of just 20% *M. aeruginosa*, were replaced by proteases with lower molecular masses (arrows in Fig. 3B). No further changes were observed with further increases in the relative concentration of *M. aeruginosa* (Fig. 3B).

The concentration of BN920 in cells of *M. aeruginosa* was 121±22 µmol l^{-1}, and CP954 had an intracellular concentration of 1550±36 µmol l^{-1} (N=3, mean ± s.e.m.).

Dietary effects of pure natural protease inhibitor on *D. magna*

To test whether the observed reduction in growth and the changes in the pattern of digestive proteases could be caused by the chymotrypsin inhibitors BN920 and CP954 present in *M. aeruginosa*, we supplemented the green alga *S. obliquus* with BN920 that had been isolated as pure compound from the same strain of *M. aeruginosa* and then been encapsulated in liposomes. Supplementation with the inhibitor resulted in 24% reduction in somatic growth of *D. magna* (one-way ANOVA, $F_{2,5}$=10.81, P<0.05; Tukey's HSD, P<0.05; Fig. 4A), which demonstrated that natural chymotrypsin inhibitors can cause growth reduction in *D. magna*. Feeding on *S. obliquus* supplemented with liposomes loaded with BN920 led to a shift in the chymotrypsin pattern (Fig. 4B) that was identical to the effect of ≥20% cyanobacterial carbon in the diet (Fig. 3B). In the suspension of loaded liposomes, BN920 had a concentration of 100±12 µmol l^{-1} (N=3, mean ± s.e.m.); however, it was not possible to distinguish between internal and external BN920.

Dietary effects of the cyanobacterium on tolerance to natural protease inhibitors

To test whether the altered pattern of chymotrypsins induced by only 20% of *M. aeruginosa* in the diet (see Fig. 3B) constituted an adaptive physiological response of *D. magna*, we investigated the tolerance of *D. magna* chymotrypsins to the dietary chymotrypsin inhibitors BN920 and CP954. As a measure of tolerance, we determined the IC_{50} values of *D. magna* homogenate raised either on the pure green alga or on a mixture of this green alga with 20% *M. aeruginosa*. Both cyanobacterial inhibitors, BN920 and CP954, showed pronounced concentration-dependent effects on chymotrypsin activity in these two different whole-body

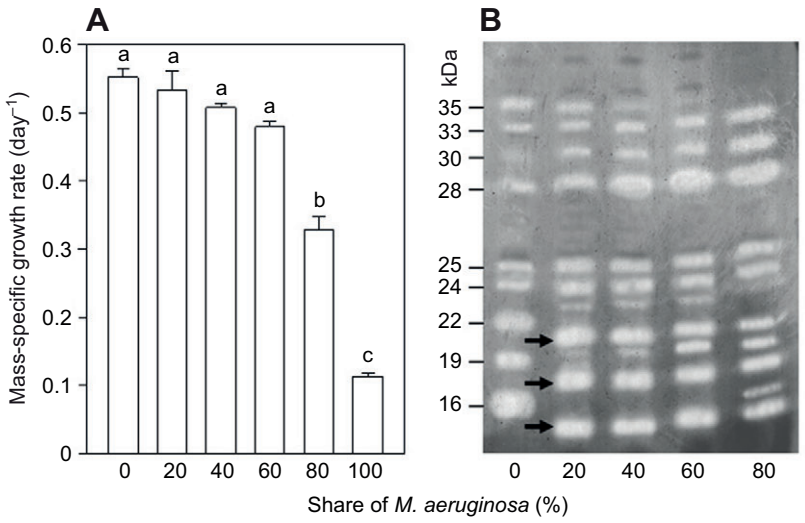

Fig. 3. Effect of mixtures of the green alga *S. obliquus* with different proportions of the cyanobacterium *M. aeruginosa* on the (A) somatic growth rate and (B) pattern of digestive proteases of *D. magna*. Error bars indicate +s.e.m. (N=3); identical letters denote non-significant differences (Tukey's HSD, P<0.05). Clear zones in B indicate protease activity. Molecular mass is given in kDa. Arrows indicate new protease bands that appeared if *M. aeruginosa* was present in the diet.

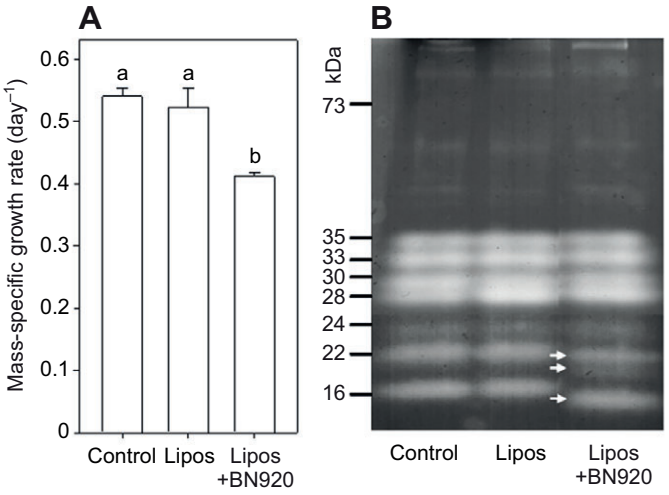

Fig. 4. Effect of pure natural chymotrypsin inhibitors on the (A) somatic growth rate and (B) pattern of digestive proteases of *D. magna*. (A) Animals were grown on the green alga *S. obliquus* without supplementation (control) or with supplementation of pure liposomes (lipos) or liposomes loaded with the natural chymotrypsin inhibitor nostopeptin BN920 (lipos+BN920). Error bars indicate +s.e.m. (N=3); identical letters denote non-significant differences (Tukey's HSD, P<0.05). (B) Protease pattern of *D. magna* grown on the green alga *S. obliquus* and incubated for 24 h without further additions (control), with pure liposomes (lipos) or with liposomes loaded with nostopeptin BN920 (lipos+BN920). Molecular mass is given in kDa; clear zones indicate protease activity.

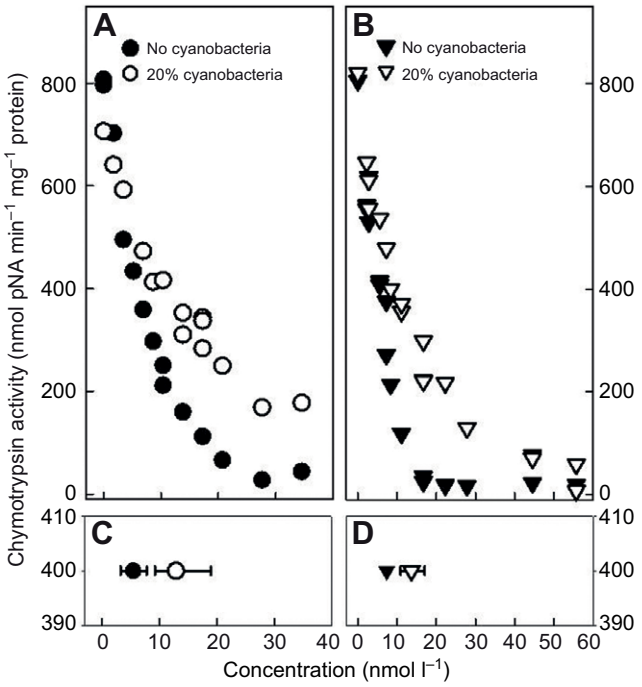

Fig. 5. *In vitro* effect of the natural chymotrypsin inhibitors (A) nostopeptin BN920 (BN920, circles) and (B) cyanopeptolin 954 (CP954, triangles) on chymotrypsin activity in whole-body homogenate of *D. magna* in response to diet. *Daphnia magna* was grown either on the pure green alga *S. obliquus* (no cyanobacteria, closed symbols) or on a mixture of the green alga and 20% *M. aeruginosa* (20% cyanobacteria, open symbols). (C,D) Mean IC_{50} values (error bars denote 95% confidence intervals) obtained for BN920 (C) and CP954 (D).

homogenates of *D. magna* (Fig. 5A,B). Homogenates from animals grown on 20% *M. aeruginosa* required higher concentrations of either inhibitor than homogenates from animals grown in the absence of the cyanobacterium to obtain the same inhibitory effect on chymotrypsins (Fig. 5A,B). Fifty percent inhibition of *D. magna* chymotrypsins by pure BN920 required twofold higher concentrations if animals had been grown on 20% *M. aeruginosa* (IC_{50}=12.9 nmol l^{-1}) than when they had been grown on pure green alga (IC_{50}=5.4 nmol l^{-1}; Fig. 5C). Similarly for CP954, growth in the presence *versus* absence of the cyanobacterium led to twofold higher IC_{50} values (IC_{50}=13.7 *vs* 7.4 nmol l^{-1}; Fig. 5D). In both cases, the 95% confidence intervals of the IC_{50} values for animals grown with and without the cyanobacterium did not overlap (Fig. 5C,D), which indicated that the presence of *M. aeruginosa* in the diet induced chymotrypsins in *D. magna* that were more tolerant against the chymotrypsin inhibitors of this cyanobacterium.

DISCUSSION

Our results demonstrated that two chymotrypsin inhibitors of a strain of the cyanobacterium *M. aeruginosa* specifically inhibit the chymotrypsins and do not interfere with other digestive proteases in *D. magna*. By using an encapsulated pure chymotrypsin inhibitor of *M. aeruginosa*, we have shown that cyanobacterial chymotrypsin inhibitors can cause growth depression in *D. magna* and induce changes in the pattern of digestive chymotrypsins in this important species in the food web of pelagic freshwater ecosystems. The same specific changes were induced by a strain of *M. aeruginosa*, which produces these inhibitors, already at a relative abundance as low as 20% of dietary carbon, which results in digestive chymotrypsins that are more tolerant to the cyanobacterial inhibitors. We will discuss the evidence that this inducible enhanced tolerance of the digestive proteases in *D. magna* is adaptive.

Protease inhibitors

Protease inhibitors are widespread in terrestrial plants and are well known for their contribution to the defence against herbivores (Zavala et al., 2004). The putative mode of action is a disruption of the protein digestion in the gut of the herbivore, and thus ingestion but not assimilation of the protease inhibitor by the herbivore is required to affect protein digestion in the animal (Jongsma and Bolter, 1997). Protease inhibitors are widespread in cyanobacteria. Trypsin and chymotrypsin inhibitors have both been reported to occur in the genera *Microcystis* (Weckesser et al., 1996; Von Elert et al., 2005; Bister et al., 2004), *Planktothrix* (Rohrlack et al., 2004; Blom et al., 2003; Baumann et al., 2007) and *Oscillatoria* (Sano and Kaya, 1996; Sano and Kaya, 1995). However, the occurrence of these types of inhibitors is not restricted to these cyanobacterial genera, suggesting that their production is adaptive for cyanobacteria. Because cyanobacteria are phylogenetically much older than any crustacean, the adaptive value for the original evolution of inhibitors of trypsins and chymotrypsins in cyanobacteria was not the interference with the digestion of herbivores. This, however, does not preclude that the adaptive value of a secondary metabolite has changed during evolution, so that protease inhibitors may serve as anti-herbivore defence as it is the case with protease inhibitors in terrestrial plants (Glawe et al., 2003). From an ecological perspective, cyanobacterial protease inhibitors are a particularly interesting group of secondary metabolites; notwithstanding other cyanobacterial secondary metabolites that interfere with the performance of herbivores, the target of interference in the herbivore is well known, i.e. digestive proteases

in *Daphnia*. This allows deciphering physiological mechanisms of susceptibility and acquired tolerance in the herbivore.

Effects of cyanobacterial protease inhibitors on *D. magna*

Extracts of *Microcystis* sp. have been shown to inhibit trypsin activity in crude extracts of the cladocerans *Moina macrocopa* (Agrawal et al., 2001), *D. magna* (Rohrlack et al., 2003; Agrawal et al., 2005) and *Daphnia* sp. (Blom et al., 2006). From the strain of *M. aeruginosa* used in this study, two chymotrypsin inhibitors that were inhibitory against bovine chymotrypsins have been isolated in a bioassay-guided fractionation (Von Elert et al., 2005). Here, the same two compounds, BN920 and CP954, were shown to specifically inhibit *D. magna* chymotrypsins and not to interfere with *D. magna* trypsins.

It has been demonstrated that trypsins and chymotrypsins constitute the two most important classes of digestive proteases in the guts of *D. magna* (Von Elert et al., 2004). This suggests that cyanobacterial trypsin and chymotrypsin inhibitors constitute an anti-herbivore defence that interferes with major digestive proteases in *D. magna*. In *D. magna* grown on the green alga *S. obliquus*, nine different proteases have been distinguished using zymograms (Von Elert et al., 2004), and five proteases ranging from 22 to 75 kDa were identified as trypsins through the use of synthetic inhibitors (Agrawal et al., 2005). Here, effects of the synthetic inhibitor chymostatin and the cyanobacterial chymotrypsin inhibitors cyanopeptolin 954 and nostopeptin BN920 reveal that the remaining three proteases in *D. magna* with a molecular mass ranging from 16 to 24 kDa are chymotrypsins.

Based on the finding that the strain of *M. aeruginosa* used in this study contains a significantly stronger inhibitory activity against *D. magna* chymotrypsins than against trypsins, it is reasonable to assume that ingestion of this cyanobacterial strain by *D. magna* leads primarily to interference with the animal's chymotrypsins and not with its trypsins. Accordingly, *D. magna* responded to the presence of chymotrypsin inhibitors in the diet by changes in the set of digestive chymotrypsins only. At a relative abundance of 20% of *M. aeruginosa*, a change in apparent molecular mass for three of the chymotrypsins and a more than twofold enhanced tolerance to the cyanobacterial chymotrypsin inhibitors measured as IC_{50} values was observed. This suggests that these qualitative changes in digestive chymotrypsins are adaptive for the herbivore, as it makes the animals less susceptible to the dietary inhibitors, so that even higher concentrations of chymotrypsin inhibitors in the diet (up to 60% *M. aeruginosa*) do not decrease growth of *D. magna*. However, it is not possible to experimentally prove that without this altered set of chymotrypsins growth depression of *D. magna* would have occurred at ≤60% *M. aeruginosa*. A comparable adaptive qualitative remodelling of the digestive protease complement in response to dietary protease inhibitors has been reported for a terrestrial herbivorous beetle that thereby became tolerant to the plant defensive protease inhibitor (Zhu-Salzman et al., 2003).

The protease families of trypsins and chymotrypsins are characterized by a highly conserved reaction centre (Beynon and Bond, 2001). Although the variety of trypsin and chymotrypsin inhibitors in cyanobacteria is huge (Gademann and Portmann, 2008), all these inhibitors interfere with the binding of substrate to the enzymes' reaction centre. Hence it is reasonable to assume that any chymotrypsin with a slightly more hindered access for one inhibitor will also be a more tolerant protease with regard to other chymotrypsin inhibitors. This suggests that enhanced tolerance of *D. magna* chymotrypsins to BN920 and CP954 also provides a higher tolerance to other chymotrypsin inhibitors.

Although a remodelling of the digestive protease complement was observed with just 20% of cyanobacterial carbon, only at >60% of *M. aeruginosa* in the diet did the inhibitory effects become so strong that a depression of growth occurred. When instead of a stationary batch culture a chemostat culture of *M. aeruginosa* NIVA Cya 43 with a dilution rate of 0.23 day^{-1} was used, a diet including just 20% cyanobacterial carbon significantly reduced the growth of the same *D. magna* clone as was used in this study (Schwarzenberger et al., 2010). This strongly suggests that the content of protease inhibitors is affected by growth conditions of the cyanobacterium, as has been shown for other secondary metabolites in cyanobacteria (Jähnichen et al., 2001; Tonk et al., 2009; Van de Waal et al., 2009).

Results obtained by feeding whole cyanobacterial cells must not be attributed to single toxins unless additional experiments can rule out that other, as yet unknown, cyanobacterial compounds are causative. As no mutant is available for the *M. aeruginosa* strain used here and as exposure to dissolved inhibitors is not the natural way of exposure for daphnids, supplementation of the purified chymotrypsin inhibitors encapsulated in liposomes was the only way to test for the relevance of the chymotrypsin inhibitors. The pronounced reduction of somatic growth in *D. magna* and the remodelling of the digestive chymotrypsins caused by encapsulated pure BN920 demonstrate that protease inhibitors in cyanobacteria are a potential cause for the low food quality of cyanobacteria for *D. magna*. Similarly clear evidence for negative effects of cyanobacterial secondary metabolites on *Daphnia* has so far only been provided for microcystins (Lürling, 2003; Rohrlack et al., 2005a; Wilson and Hay, 2007). Similar to feeding on encapsulated BN920, feeding on ≥20% *M. aeruginosa* caused a reduction of somatic growth and a remodelling of chymotrypsins in *D. magna*, which strongly suggests that these effects of feeding on intact cyanobacterial cells are also due to the two known chymotrypsin inhibitors in this cyanobacterial strain (Von Elert et al., 2005). We did not investigate the effects of encapsulated CP954, as this compound is chemically identical to BN920 except that it contains one atom of chlorine, which makes it plausible to assume identical effects of pure CP954 and BN920.

The concentrations of CP954 and BN920 in the cyanobacterial cells were 1550 and 120 µmol l^{-1}, respectively. These values exceeded the IC_{50} values for inhibition of *D. magna* chymotrypsins (~10 nmol l^{-1}) by four to five orders of magnitude. This suggests that it is reasonable to assume that upon lysis of cyanobacterial cells among tightly packed phytoplankton cells during digestion in the gut of *D. magna*, the local concentrations of each of the cyanobacterial protease inhibitors CP954 and BN920 will be high enough to interfere with *D. magna* chymotrypsins and thus lead to a reduction of growth and a remodelling of the digestive protease complement. Although the concentration of BN920 in the suspension of the liposomes (100 µmol l^{-1}) was in the same range as those determined for the cyanobacterial cells, the interpretation of this value is less clear, as it was not possible to distinguish between internal and external BN920.

The effects of pure BN920 further demonstrate that the natural way of exposure to cyanobacterial protease inhibitors leads to *in situ* changes in the pattern and activity of digestive proteases in *D. magna*, confirming that digestive chymotrypsins are targets of dietary cyanobacterial protease inhibitors in *Daphnia*. The finding that these more tolerant chymotrypsins are not produced constitutively strongly suggests that this phenotype is associated with costs that are balanced by benefits only in the presence of dietary chymotrypsin inhibitors. Currently it can only be speculated that the more tolerant chymotrypsins are inferior to the more susceptible

chymotrypsins with respect to enzymatic parameters such as the specific maximum rate of proteolysis.

Effects of cyanobacteria on *Daphnia* populations

It has repeatedly been demonstrated that *Daphnia* spp. can suppress cyanobacteria if they are able to achieve high densities before cyanobacteria become dominant (Paterson et al., 2002; Christoffersen et al., 1993), and only recently have *Daphnia* spp. been shown to be capable of suppressing even an established bloom of cyanobacteria (Sarnelle, 2007). The finding that more tolerant chymotrypsins are induced by relative cyanobacterial abundances as low as 20% suggests that this fast intra-generational switch to more tolerant chymotrypsins contributes to the capability of *Daphnia* populations to cope with cyanobacteria even at an early stage of a developing cyanobacterial bloom.

In several cases it has been demonstrated that coexistence of *Daphnia* with toxic cyanobacteria leads to local adaptation of the *Daphnia* population, as was evidenced by increased tolerance to cyanobacteria (Hairston et al., 1999; Sarnelle and Wilson, 2005). These findings support the notion that the presence of cyanobacteria positively selects for more tolerant *Daphnia* genotypes. This was experimentally confirmed, as exposure of a mixed population of several *Daphnia* clones to a microcystin-producing strain of *M. aeruginosa* resulted in an enhanced tolerance in subsequent generations (Gustafsson and Hansson, 2004). However, the physiological mechanism of increased tolerance was not elucidated in these aforementioned studies. The selection for more tolerant *Daphnia* genotypes might have been caused by cyanobacterial protease inhibitors. The IC_{50} values for *D. magna* chymotrypsins reported here are approximately threefold lower than those reported for bovine chymotrypsins (Von Elert et al., 2005), which corroborates the notion of a high specificity of cyanobacterial protease inhibitors for *Daphnia* proteases. The widespread occurrence of protease inhibitors in planktonic cyanobacteria makes it reasonable to assume that these inhibitors cause selective pressure on digestive proteases of *Daphnia* in populations coexisting with cyanobacteria. In line with this, it has been demonstrated that the coexistence of *Daphnia* with cyanobacteria results in increased tolerance against a cyanobacterial protease inhibitor (Blom et al., 2006).

The clone of *D. magna* used here is not known to have coexisted with cyanobacteria. Tolerance to toxic cyanobacteria has been shown to vary within *Daphnia* populations (Gustafsson et al., 2005; Wilson and Hay, 2007; Sarnelle et al., 2010), and it is not clear how representative the clone of *D. magna* used here is of the original population. The fact that even in *Daphnia* populations existing without cyanobacteria the capability of switching to more tolerant chymotrypsins is not lost suggests either that maintenance of the respective set of genes is not costly or that in natural phytoplankton the occurrence of dietary chymotrypsin inhibitors is not restricted to cyanobacteria. We are not aware of reports on chymotrypsin inhibitors in freshwater algae; however, it is unclear how much this lack of evidence is due to the lack of relevant research. As *Daphnia* do not seem to loose the capability to switch to more tolerant digestive chymotrypsins even in the absence of cyanobacteria, this capability is putatively widespread, so that the switching to more tolerant chymotrypsins is probably part of the physiological response to cyanobacterial protease inhibitors regardless of whether the animals exist in the presence or absence of cyanobacteria. It remains to be tested whether this remodelling of the digestive protease complement explains the transfer of enhanced tolerance from mothers to their offspring (Gustafsson et al., 2005).

Here, for the first time, it is demonstrated at the protein level that a physiological plasticity in *Daphnia* leads to higher tolerance against cyanobacterial protease inhibitors. Building on this and making use of the recent identification of the genes that encode the digestive proteases in *D. magna* (Schwarzenberger et al., 2010) will allow investigation of whether local adaptation of *Daphnia* populations to cyanobacteria can be explained by selection that has acted on the chymotrypsin genes of *Daphnia*.

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