Gene expression in *Daphnia magna*: response to cyanotoxins and predators

Inaugural-Dissertation zur Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln

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„Gesellet zur Pflicht sich die Freude, dünkt Dir die Arbeit ein Spiel“

(Haupteingang Ellenrieder Gymnasium, Konstanz)

Content:

Gene expression in *Daphnia magna*: response to cyanotoxins and predators

General introduction

Daphnia is a keystone species in the energy transfer from primary producers (phytoplankton) to higher trophic levels (secondary consumers). Members of the genus *Daphnia* represent the major herbivores of algae and cyanobacteria in freshwater ecosystems and the most important food source for zooplanktivorous vertebrate and invertebrate predators. Hence, *Daphnia* abundance is controlled by bottom-up as well as by top-down factors. The effects of these bottom-up and top-down factors on *Daphnia* population dynamics show a pronounced seasonality [1]. At the end of the winter the stratification of lakes is re-established due to warmer weather, and higher resource availability and light lead to an increase in phytoplankton production. Hence, in spring, phytoplankton, the major bottom-up factor for the increase of *Daphnia* biomass, is highly available, while the pressure of predation, the major top-down factor, is low. However, in early summer, easily ingestible phytoplankton biomass decreases, while grazing resistant phytoplankton taxa, among them cyanobacteria, increase in relative abundance, leading to a decline in *Daphnia* numbers. Simultaneously due to the appearance of young-of-the-year fish and fourth-instar larvae of *Chaoborus* water midges, predation pressure on *Daphnia* is very high and remains moderate until autumn [2,3].

In summer, especially during the last few decades, cyanobacterial mass developments, so called blooms, have become wide-spread in eutrophic lakes; these blooms have been claimed to be a major factor leading to the summer-decline of *Daphnia* biomass [4,5]. Hence, in eutrophic predator-containing freshwater ecosystems the abundance of large unselective herbivores such as *Daphnia* is, to seasonally varying degrees, controlled by both, high fish predation and cyanobacteria [6].

In *Daphnia* several traits have been shown to be plastic in response to top-down control by predators: The presence of predators induces changes in a variety of morphological, life history [7-9] and behavioural [10] traits in *Daphnia*, that have demonstrated to be adaptive. Adaptive changes in the prey are indirectly induced by

predator-borne chemical cues [11] that must be termed kairomones [12]. The chemical nature of these kairomones and the physiological basis for changes of *Daphnia* are not yet understood [13]. The two only studies on the effects of predator-borne kairomones on *Daphnia* on the molecular level, have reported changes in the amount of heat shock proteins [14,15] and of actin and alpha-tubulin proteins [14] which are part of the cell-structure in *D. magna*.

Besides top-down control by predators, *Daphnia* abundances are affected by the bottom-up factors quantity and quality of phytoplankton. Cyanobacteria have been shown to be of low food quality for *Daphnia* for several reasons: cyanobacterial filaments interfere with the filtering apparatus of *Daphnia* [16,17]), cyanobacteria are lacking many essential lipids, i.e. polyunsaturated fatty acids [18] and sterols [19,20], and cyanobacteria often contain toxic secondary metabolites [21]. Profiles of secondary metabolites have been found to differ between and within cyanobacterial species [22]. Heptapeptides, especially microcystins, belong to the most extensively studied cyanobacterial secondary metabolites; microcystins inhibit protein phosphatases of *Daphnia in vitro* [23] and have been shown to reduce the fitness of *Daphnia* [24]. Cyanobacterial serine protease inhibitors belong to another group of cyanobacterial secondary metabolites (depsipeptides); protease inhibitors have been found in nearly every cyanobacterial bloom [25,26] and have been shown to reduce growth of *Daphnia* also in the presence of microcystins [27]. Cyanobacterial protease inhibitors often inhibit serine proteases, among them are trypsins and chymotrypsins, which represent the most important digestive enzymes in the gut of *D. magna* [28]. Total trypsins and chymotrypsins of *D. magna* have *in vitro* been shown to be specifically inhibited by cyanobacterial protease inhibitors [29].

Different *Daphnia* clones have shown high intra-specific variability in sensitivity to microcystins [30]. In Lake Constance, which experienced a period of high eutrophication accompanied with an increase of cyanobacterial biomass, Hairston et al. [31] have shown a decrease of clonal variability in sensitivity of *Daphnia* to a microcystin-containing cyanobacterium due to microevolution in the grazer population. Microevolution due to cyanobacterial protease inhibitors, which might lead to locally adapted *Daphnia*, is also conceivable for *Daphnia* populations. A local adaptation of a *Daphnia* population to a cyanobacterial protease inhibitor was shown by Blom et al. [32].

Local adaptation is assumed to result from positive selection of less sensitive genotypes. This positive selection should not only favour genotypes that are constitutively less sensitive, but as well genotypes, which induce responses to cope with unfavourable environmental factors. Such an inducible response might be passed on to the next generation, which should then be less sensitive. In one *D. magna* clone, adapted to a microcystin-containing cyanobacterium, tolerance to microcystin has been observed to be passed on to the next generation [33].

In *Daphnia* the underlying molecular mechanisms of differences in sensitivity to cyanotoxins and of the physiological responses to predation are not known to date.

The recent release of the *Daphnia pulex* genome database (wFleaBase: http://wFleaBase.org, JGI Genome Portal: http://www.Jgi.doe.gov/Daphnia/) offers the opportunity to analyse the physiological causes of differences in sensitivity to cyanotoxins and of the physiological responses to predator-borne kairomones of *Daphnia* under genetic aspects, e.g. the measurement of relative expression of selected genes via quantitative real-time PCR. Proteins of the cytoskeleton (actin and alpha-tubulin) have been shown to be affected by the exposure of *Daphnia* to kairomones [14]. Hence, in my thesis the genes selected for the investigation of predator-borne kairomones and dietary microcystins were genes coding for actin and alpha-tubulin and additionally genes of the basic metabolism to analyse the general effects of different stressors on *Daphnia*. For the investigation of the effects on gene-expression of *D. magna* due to dietary protease inhibitors, I chose the genes of the targets of the cyanobacterial protease inhibitors, i.e. digestive serine proteases of *D. magna.* In order to also analyse the effects of dietary protease inhibitors on proteases at the protein level, protease-activity staining of SDS-PAGEs and photometrical protease activity measurements were performed.

Part I of the thesis focuses on the general effects of cyanobacteria with or without microcystins and of predation on the expression of selected genes of the cell-structure and the basic metabolism of *D. magna*. Therefore, a quantitative real-time PCR (QPCR) set-up for *Daphnia* was adopted and applied.

In **Part II** I focused on the single effects of two types of cyanobacterial protease inhibitors, i.e. trypsin- and chymotrypsin-inhibitors, on their specific targets, i.e. the proteases trypsins and chymotrypsins and their respective genes, in a single *D. magna* clone. Liquid chromatography coupled with mass spectrometry and

subsequent database-search was applied to assign the protease genes to the active digestive trypsins and chymotrypsins in *D. magna*. A new QPCR set-up was developed for the analysis of protease expression with the establishment of specific protease primers for *D. magna*.

In **Part III** of the thesis I established a microsatellite system, to demonstrate that five different *D. magna* clones represented different genotypes. These five clones were compared for their sensitivity to either trypsin or chymotrypsin inhibitor containing cyanobacteria. A fitness related parameter (somatic growth) was compared with changes in activity of digestive proteases and of gene-expression levels of digestive proteases with the aim to identify the molecular mechanisms of differences in sensitivity to dietary protease inhibitors in *Daphnia*.

In **Part IV** of the thesis I investigated, whether changes in gene-expression in *Daphnia* due to dietary cyanobacterial protease inhibitors were passed on to the next generation. Therefore, *D. magna* mothers were pre-exposed to cyanobacteria containing protease inhibitors. Immediately after hatching of the new-born offspring, which never ingested dietary protease inhibitors themselves, I looked for maternal effects on the gene-expression of digestive proteases. The adaptive nature of putative maternal effects was tested for in somatic growth rate assays.

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Part I:

Target gene approaches: Gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free *Microcystis aeruginosa*

Abstract

Background

Two major biological stressors of freshwater zooplankton of the genus *Daphnia* are predation and fluctuations in food quality. Here we use kairomones released from a planktivorous fish (*Leucaspius delineatus*) and from an invertebrate predator (larvae of *Chaoborus flavicans*) to simulate predation pressure; a microcystin-producing culture of the cyanobacterium *Microcystis aeruginosa* and a microcystin-deficient mutant are used to investigate effects of low food quality. Real-time quantitative polymerase chain reaction (QPCR) allows quantification of the impact of biotic stressors on differential gene activity. The draft genome sequence for *Daphnia pulex* facilitates the use of candidate genes by precisely identifying orthologs to functionally characterized genes in other model species. This information is obtained by constructing phylogenetic trees of candidate genes with the knowledge that the *Daphnia* genome is composed of many expanded gene families.

Results

We evaluated seven candidate reference genes for QPCR in *Daphnia magna* after exposure to kairomones. As a robust approach, a combination normalisation factor (NF) was calculated based on the geometric mean of three of these seven reference genes: *glyceraldehyde-3-phosphate dehydrogenase, TATA-box binding protein* and s*uccinate dehydrogenase*. Using this NF, expression of the target genes *actin* and *alpha-tubulin* were revealed to be unchanged in the presence of the tested kairomones. The presence of fish kairomone up-regulated one gene (*cyclophilin*) involved in the folding of proteins, whereas *Chaoborus* kairomone down-regulated the same gene.

We evaluated the same set of candidate reference genes for QPCR in *Daphnia magna* after exposure to a microcystin-producing and a microcystin-free strain of the cyanobacterium *Microcystis aeruginosa*. The NF was calculated based on the reference genes *18S ribosomal RNA*, *alpha-tubulin* and *TATA-box binding protein*. We found *glyceraldehyde-3-phosphate dehydrogenase* and *ubiquitin conjugating enzyme* to be up-regulated in the presence of microcystins in the food of *D. magna*. These findings demonstrate that certain enzymes of glycolysis and protein catabolism are significantly up-regulated when daphnids ingest microcystins. Each differentially regulated gene is a member of an expanded gene family in the *D. pulex* genome. The *cyclophilin*, *GapDH* and *UBC* genes show moderately large sequence divergence from their closest paralogs. Yet *actin* and *alpha-tubulin* genes targeted by our study have nearly identical paralogs at the amino acid level.

Conclusions

Gene expression analysis using a normalisation factor based on three reference genes showed that transcription levels of *actin* and *alpha-tubulin* were not substantially changed by predator-borne chemical cues from fish or invertebrates, although changes in expression on the protein level were shown elsewhere. These changes in protein level could be caused by others than the investigated paralogs, showing the importance of the construction of phylogenetic trees for candidate gene approaches. However, fish kairomones caused an up-regulation, and *Chaoborus* kairomone caused a down-regulation of *cyclophylin*, which proved to be a potential target gene for further analysis of kairomone effects on the life history of daphnids. Changes in food quality required a different set of reference genes compared to the kairomone experiment. The presence of dietary microcystins led to an up-regulation of two genes involved in the basic metabolism of *D. magna*, i.e. *glyceraldehyde-3-phosphate dehydrogenase* and *ubiquitin conjugating enzyme*, which suggests that microcystins in cyanobacteria have more general effects on the metabolism of *D. magna* than previously thought. Phylogenetic trees resolving relationships among paralogs that share the same gene name are shown to be important for determining the identity of the candidate genes under investigation.

Background

Notwithstanding other so called 'model organisms', of which whole genome sequences have been obtained, the ecology of the model organism *Daphnia* sp. is outstandingly well known. Therefore it is a challenge to investigate gene/environment interactions for major ecological interactions of this cladoceran. In most freshwater lakes and ponds, *Daphnia* sp. is the major consumer of algae and cyanobacteria and is also the most important prey for predatory invertebrates and planktivorous fish. In line with its intermediate position in the food chain, *Daphnia* sp. populations can be controlled by predation (top-down) or resources (bottom-up). However, the impact of top-down and bottom-up factors on *Daphnia* sp. population dynamics shows a pronounced seasonality [1]. Predation pressure is low in spring, but peaks with the appearance of young-of-the-year fish and fourth-instar larvae of *Chaoborus* water midges in early summer and remains moderate until fall [2,3]. Bottom-up factors become a major constraint on *Daphnia* sp. population growth, particularly in eutrophic lakes in the summer when mass developments of toxic cyanobacteria lead to a suppression of *Daphnia* sp. biomass [4,5]. The low predictability of intensity and seasonality of both predation pressure and dominance of toxic cyanobacteria should lead to the evolution of plastic instead of fixed adaptations [6]. Indeed, adaptive phenotypic plasticity in *Daphnia magna* to both toxic cyanobacteria and predator-borne chemical cues has been reported [7-14]. *Daphnia magna* has been shown to reduce size at first reproduction (SFR) in response to kairomones from fish whereas chemical cues from larvae of *Chaoborus flavicans* led to increased SFR; both responses have been proven to be adaptive as fish and *Chaoborus* differ in size-selectivity of their prey [10-14]. A different kind of phenotypic plasticity constitutes the enhanced tolerance of *D. magna* against cyanobacterial toxins upon exposure to a toxin producing cyanobacterium. The recent release of the *Daphnia pulex* genome sequence (wFleaBase: http://wFleaBase.org, JGI Genome Portal: http://www.Jgi.doe.gov/Daphnia/) creates the opportunity to precisely identify candidate genes that differ in their expression in response to predator-borne chemical cues (i.e. kairomones) and to a toxic cyanobacterium as a first step to decipher the underlying molecular mechanisms of adaptive phenotypic plasticity in *D. magna*.

Toxin production is a characteristic feature of several strains of the bloom-forming freshwater cyanobacterium *Microcystis aeruginosa*. A large variety of cyclic

heptapeptides, termed microcystins (which can become a health hazard to humans and livestock [15]), have been identified in *M. aeruginosa*. Wild type *M. aeruginosa* PCC7806 produces relatively large amounts of two microcystin variants (LR and RR); experiments with a microcystin-free mutant of this strain [16] have led to the conclusion that microcystins contribute to the daphnid poisoning by *M. aeruginosa* [17,18].

The microcystins of *M. aeruginosa* PCC7806 are known to inhibit protein phosphatases 1 and 2A in warm-blooded animals and in *Daphnia* sp. [19], which suggests that the poisoning effect of microcystin-LR in *Daphnia* sp. is due to the inhibition of these two protein phosphatases. However, it remains entirely unclear which major physiological pathways in *Daphnia* sp. are affected by the binding of microcystin to protein phosphatases 1 and 2A.

For single clones of *D. magna* it has been shown that they develop tolerance against a microcystin producing cyanobacterium [20,21]. Although the physiological mechanisms have not been elucidated, this increased tolerance can be transferred to the offspring via maternal effects [20]. For a better understanding of the process of physiological adaptation of daphnids to toxic cyanobacteria, it is important to examine the genes that are differentially regulated in the presence of microcystins. To achieve this goal we quantified the expression of a set of genes involved in the basic metabolism in *D. magna* when cultured on a *M. aeruginosa* PCC7806 mutant in which the production of microcystins had been knocked out, or on the microcystin-synthesizing wild type strain. As a reference cyanobacterium, we used a strain of *Synchecoccus elongatus* which is non-toxic to daphnids [22] and easily ingested; a strain of the green alga *Scenedesmus obliquus* which is widely used as standard food for daphnids was used as reference for high quality food [23].

Much of the recent evidence for inducible defences in freshwater ecology which has contributed to the general understanding of predator-prey interactions has been derived from experimental studies with daphnids. Despite considerable progress in the understanding of inducible defences, the underlying plasticity has rarely been studied at a molecular level. Only recently has the response of *D. magna* to kairomones from fish and invertebrates been investigated on the protein level, and it has been shown that a clone of *D. magna* that was isolated from a habitat where it coexists with fish and invertebrate predators responds with a decrease in the proteins actin and alpha-tubulin [24]. We hypothesized that this decrease might be due to a

change in transcription of the *actin* and *alpha-tubulin* genes and that these genes might function as targets for predator-borne chemical cues. We therefore exposed the same clone of *D. magna* as Pijanowska & Kloc (2004) [24] to similar kairomones and quantified the transcription levels of putative target genes.

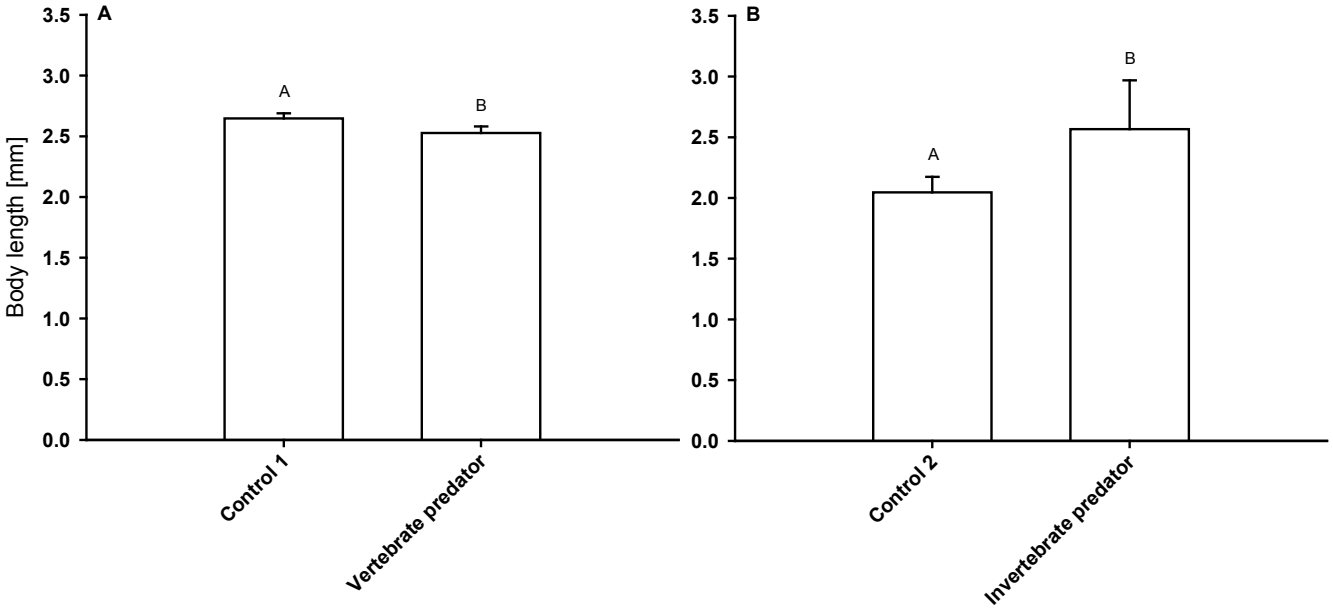

Figure 1 - Size at first reproduction of *Daphnia magna* in the kairomone experiment.
A: *D. magna* was grown either in water exposed to *Leucaspius delineatus* (sunbleak, 'Vertebrate predator') or in water without kairomone (control 1), or B: either in water exposed to larvae of *Chaoborus flavicans* ('Invertebrate predator') or in the respective kairomone-free water (control 2). Depicted is the size at first reproduction (n = 3, ± SD). Letters indicate a significant difference ($p < 0.001$) between treatments.

Results

Kairomone experiment: Effects on the life-history of *Daphnia magna*

In two different life-history experiments the size at first reproduction (SFR) of *D. magna* grown in water exposed either to sunbleaks (*Leucaspius delineatus*) or larvae of *Chaoborus flavicans* was determined. The SFR of *D. magna* grown in fish incubation water was significantly lower than SFR of the control group ($p < 0.001$, Tukey HSD after one-way ANOVA: $F_{8;\ 0.00195} = 33023.42$; Fig. 1 A), whereas SFR of *D. magna* raised in *Chaoborus* incubation water was significantly higher than SFR of *D. magna* grown in kairomone-free water ($p < 0.001$, Tukey HSD after one-way ANOVA: $F_{13;\ 0.08364} = 949.3778$; Fig 1 B).

Kairomone experiment: Identification of reference genes and normalisation factors

In the kairomone experiment, the relative expression of the six candidate reference genes in the different treatments was analysed by geNorm (see Methods) and ranked according to increasing variability (*GapDH* = *SucDH* < *TBP* < *cyclophilin* < *UBC* < *28S* < *18S*). GeNorm calculated five normalisation factors. Pair wise comparison of sequential normalisation factors showed a low level of variability between the three most stable reference genes (V2/3; Fig. 2). In accordance with the recommendation of Vandesompele et al. (2002) [25] to use a minimum number of three reference genes, the normalisation factor generated from the three least variable genes (*GapDH, SucDH, TBP*) was used for normalisation in further analyses. The normalized values of the three reference genes showed little variation across treatments, resulting in low values of SD (Tab. 1, 2).

Gene	Relative expression[a]		SD[b]
	Calibrator	Vertebrate predator	
SucDH	1.0000 ± 0.0051	1.123 ± 0.0179	0.0867
GapDH	1.0000 ± 0.0078	1.145 ± 0.0073	0.1027
TBP	1.0000 ± 0.0063	0.7778 ± 0.0164	0.1571

Table 1 - Relative gene expression of the reference genes in *D. magna* after normalisation in the fish-kairomone experiment.

D. magna was raised either in a predator-free environment ('Calibrator') or in incubation water of *Leucaspius delineatus* (sunbleak, 'Vertebrate predator').

[a]Values are mean of n = 3 replicates ± SD. Expression levels are displayed relative to the mean control level

[b]SD is the variation of one reference gene across treatments.

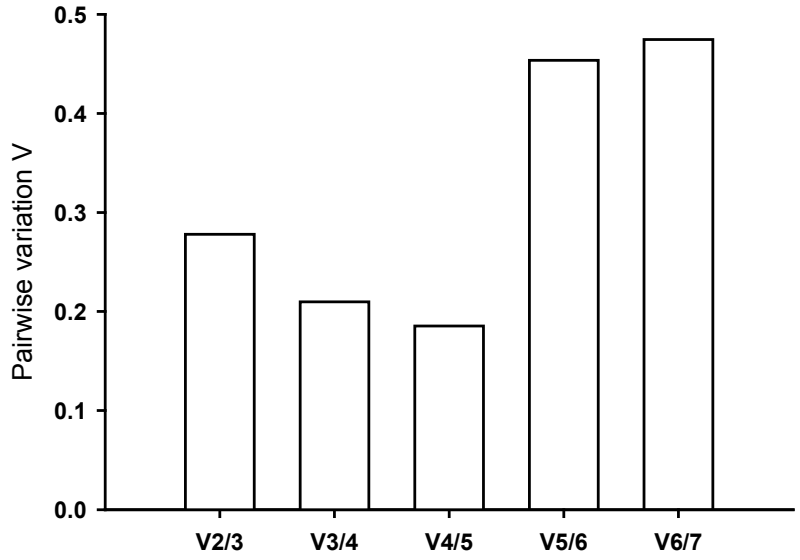

Figure 2 -Pair wise variation of sequential normalisation factors (Vn / n+1) in the kairomone experiment estimated by geNorm.
V2/3 is based on the geometric mean of the normalisation factors of *GapDH*, *TBP* and *SucDH*; V3/4 is V2/3 and *cyclophilin*; V4/5 is V3/4 and *UBC*; V5/6 is V4/5 and *28S*; V6/7 is V5/6 and 1*8S*.

Kairomone experiment: Relative normalized expression of the target genes *actin* and *alpha-tubulin*

The mean relative expression of *actin* in the fish-kairomone treatment was significantly higher than in the respective control (Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{9;0.00001} = 2037412$, $p < 0.001$; Fig. 3 A), whereas the mean relative expression of *actin* was significantly lower in the *Chaoborus*-kairomone treatment than in its respective control (Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{9;0.00001} = 2037412$, $p < 0.001$; Fig. 3 B). Chemical cues from fish led to a 1.75-fold increase in the relative expression of *actin* (Fig. 3 A), and chemical cues from *Chaoborus* larvae decreased *actin* expression 0.94-fold (Fig. 3 B). The standard deviation of the relative expression of *actin* across fish-kairomone treatments was 0.53 and was thus around four orders of magnitude higher than SD in the reference genes (Tab. 1). The standard deviation of the relative expression of *actin* across *Chaoborus*-kairomone treatments was 0.04 and had thus almost the same SD across treatments as the reference genes (Tab. 2).

Gene	Relative expression[a]		SD[b]
	Calibrator	Invertebrate predator	
SucDH	1.0000 ± 0.0024	1.4451 ± 0.0059	0.3147
GapDH	1.0000 ± 0.0035	0.8881 ± 0.0113	0.0792
TBP	1.0000 ± 0.0018	0.7792 ± 0.0058	0.1561

Table 2 - Relative gene expression of the reference genes in *D. magna* after normalisation in the *Chaoborus*-kairomone experiment.

D. magna was raised either in a predator-free environment ('Calibrator'), or in incubation water of larvae of *Chaoborus flavicans* ('Invertebrate predator').

[a]Values are mean of n = 3 replicates ± SD. Expression levels are displayed relative to the mean control level

[b]SD is the variation of one reference gene across treatments

A different response was found in the mean relative expression of *alpha-tubulin*. There was a significant 1.7-fold increase between fish-kairomone treatment and control (Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{9;0.00026} = 64420.31$, $p < 0.001$; Fig. 3C), and also a significant 1.07-fold increase of the mean relative expression of *alpha-tubulin* between control and *Chaoborus*-kairomone treatment (Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{9;0.00026} = 64420.31$, $p < 0.001$; Fig. 3 D). The standard deviation of the relative expression of *alpha-tubulin* across fish-kairomone treatments was 0.50 and was thus around four orders of magnitude higher than SD in the reference genes (Tab. 1). The standard deviation of the relative expression of *alpha-tubulin* across *Chaoborus*-kairomone treatments was 0.05 and had thus almost the same SD across treatments as the reference genes (Tab. 2).

Kairomone experiment: Relative normalized expression of non-reference genes

The non-reference genes in the fish-treatments (*28S*, *UBC*, *18S, cyclophilin*) showed values of SD across treatments (Tab. 3) that were at least four orders of magnitude higher than those of the normalised values of the three reference genes (*SucDH, GapDH*, *TBP*, SD across treatments < 0.158; Tab. 1). The same non-reference genes in the *Chaoborus*-treatment showed values of SD across treatments (Tab. 4) that were at least 1.3 orders of magnitude higher than those of the normalised values

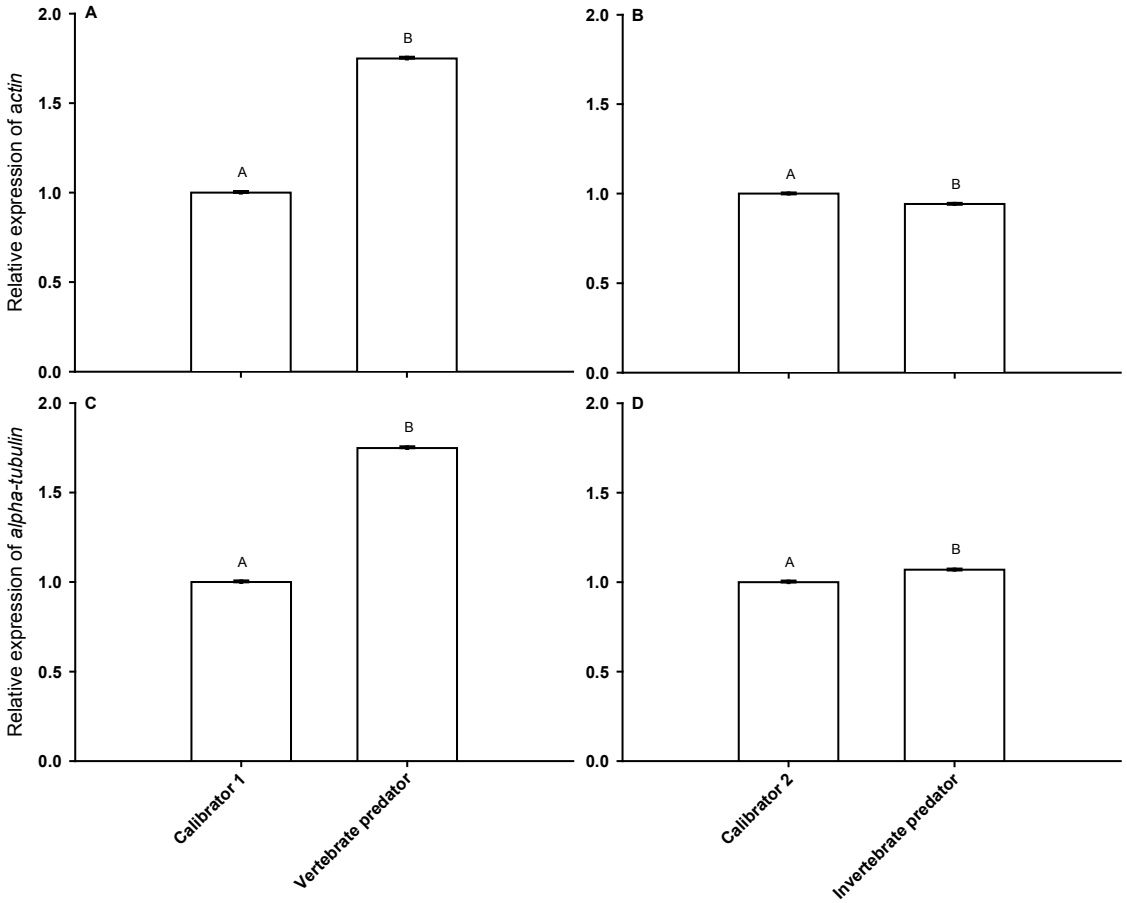

Figure 3 – Relative expression of the target genes *actin* and *alpha-tubulin* in *Daphnia magna* in the kairomone experiment.
D. magna was raised either in a vertebrate predator-free environment ('Calibrator1'), in incubation water of *Leucaspius delineatus* (sunbleak, 'Vertebrate predator'; A and C), or either in the respective calibrator ('Calibrator2') or in incubation water of larvae of *Chaoborus flavicans* ('Invertebrate predator'; B and D). Depicted is the mean relative expression (n = 3, ± SD) of *actin* or *alpha-tubulin*. Letters indicate a significant difference ($p < 0.001$) between calibrator and kairomone treatments.

of the three reference genes (SD across treatments < 0.32; Tab. 2) with one exception (*28S*, SD across treatments = 0.15; Tab. 4). These findings suggested a treatment-dependent expression. All non-reference genes showed significantly different expression between the fish-treatment (Tab. 3), the *Chaoborus* treatment and their respective controls (*28S*: $p < 0.001$, Tukey HSD after one-way ANOVA: $F_{9;\ 0.000212} = 712.2765$; *UBC*: $p < 0.001$, Tukey HSD after one-way ANOVA: $F_{9;\ 0.000012} = 30550.68$; *18S*: $p < 0.001$, Tukey HSD after one-way ANOVA: $F_{9;\ 0.000014} = 32074.06$; *cyclophilin*: $p < 0.001$, Tukey HSD after one-way ANOVA: $F_{9;\ 0.000003} = 8773865$; Tab. 4), which indicated kairomone-dependent expression. The most striking effect was the up-regulation of *cyclophilin* (2.9-fold) by fish kairomone (Tab. 3) and its down-

Gene	Relative expression[a]		SD[b]
	Calibrator	Vertebrate predator	
18S	1.0000 ± 0.0123	2.2449 ± 0.0278	0.8803
28S	1.0000 ± 0.0519	1.8555 ± 0.0873	0.6049
cyclophilin	1.0000 ± 0.0012	2.9216 ± 0.0020	1.3588
UBC	1.0000 ± 0.0046	2.0671 ± 0.0251	0.7546

Table 3 - Relative gene expression of the non-reference genes in *D. magna* after normalisation in the fish-kairomone experiment.

D. magna was raised in either a predator-free environment ('Calibrator') or in incubation water of *Leucaspius delineatus* (sunbleak, 'Vertebrate predator').

[a]Values are mean of n = 3 replicates ± SD. Expression levels are displayed relative to the mean control level

[b]SD is the variation of one reference gene across treatments

regulation (0.4-fold) by *Chaoborus* kairomone (Tab. 4). The other non-reference genes were all up-regulated in the kairomone treatments relative to their respective control, with a stronger effect of fish kairomone (1.86 – 2.25-fold; Tab. 3) than of *Chaoborus* kairomone (1.21 – 1.82-fold; Tab. 4).

Microcystin experiment: Effects on the growth of *Daphnia magna*

Four different treatments were analysed in the microcystin experiment. The green alga *S. obliquus*, a good food-alga, served as calibrator. To account for potential general cyanobacterial effects, *S. elongatus* was included in the experimental design. The aim was to be able to differentiate between the wild type and the mutant of *M. aeruginosa* PCC 7806. Growth rates of *D. magna* in the *Microcystis* treatments were significantly lower than on *S. obliquus* or *S. elongatus* (Fig. 4; $p < 0.001$, Tukey's HSD post-hoc, after one-way ANOVA: $F_{8;\ 0.002045} = 275.6914$, $p < 0.001$). However, no differences in growth on the wild type or the mutant of *M. aeruginosa* PCC 7806 were observed after four days (Fig. 4). *D. magna* on the wild type strain of *M. aeruginosa* died on day five, whereas all animals kept on the mutant survived.

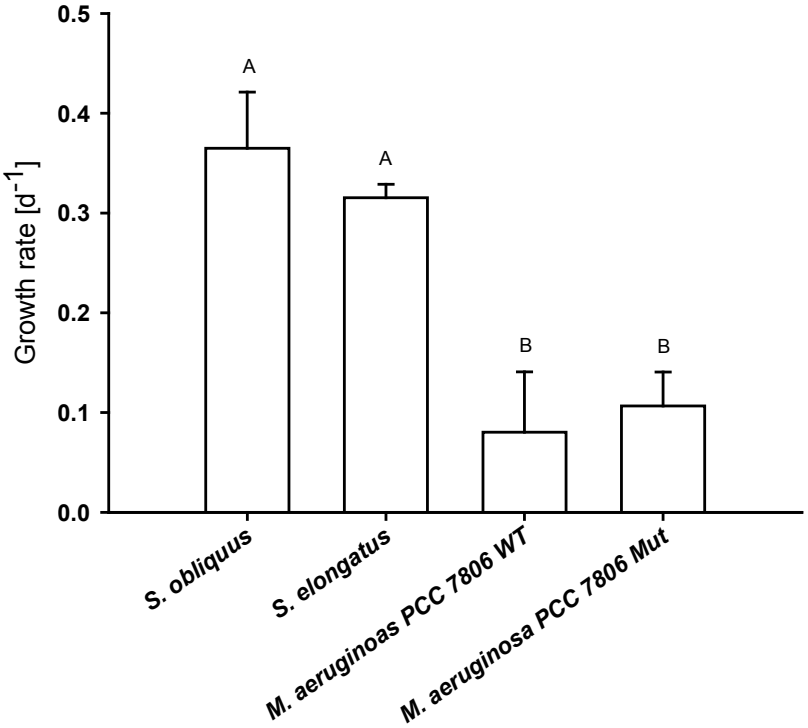

Figure 4 - Growth rates of *D. magna* in the microcystin experiment.
D. magna was raised either with pure *S. obliquus*, *S. elongatus* or the wild type (WT) or mutant (Mut) *M. aeruginosa* PCC7806. Depicted is the mean growth rate (n = 3, ± SD) of *D. magna*. Letters indicate a significant difference ($p < 0.001$) between treatments.

Microcystin experiment: Normalisation factors and identification of reference genes

The relative expression of nine candidate genes was analysed by geNorm and ranked according to increasing variability (*TBP* = *18S* < *alpha-tubulin* < *SucDH* < *actin* < *GapDH* < *cyclophilin* < *UBC* < *28S*). GeNorm calculated eight normalisation factors. Pair wise comparison of sequential normalisation factors showed a relatively high level of variability between the three least variable reference genes (V2/3; Fig. 5). The overall effect of using more reference genes was rather small. For this reason, and to simplify experimental handling, only the three least variable genes (*TBP, 18S, alpha-tubulin*) were used as reference genes.

Gene	Relative expression[a]		SD[b]
	Calibrator	Invertebrate predator	
18S	1.0000 ± 0.0033	1.8183 ± 0.0069	0.5786
28S	1.0000 ± 0.0065	1.2141 ± 0.0013	0.1514
cyclophilin	1.0000 ± 0.0012	0.3877 ± 0.0022	0.4329
UBC	1.0000 ± 0.0042	1.7394 ± 0.0042	0.5229

Table 4 - Relative gene expression of the non-reference genes in *D. magna* after normalisation in the Chaoborus kairomone experiment.

D. magna was raised in either a predator-free environment ('Calibrator'), or in incubation water of larvae of *Chaoborus flavicans* ('Invertebrate predator').

[a]Values are mean of n = 3 replicates ± SD. Expression levels are displayed relative to the mean control level

[b]SD is the variation of one reference gene across treatments

After normalisation, all genes were further analysed. The effects between feeding the wild type or the mutant strain of *M. aeruginosa* PCC 7806 were significantly different (*actin*: Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{8;\ 0.000009} = 21212.1$, $p < 0.001$; *cyclophilin*: Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{8;\ 0.000060} = 106222.7$, $p < 0.001$; *GapDH*: Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{8;\ 0.000045} = 169.04$, $p < 0.001$; *SucDH*: Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{8;\ 0.000010} = 633236.1$, $p < 0.001$; *UBC*: Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{8;\ 0.000019} = 87305.5$, $p < 0.001$; *28S*: Tukey's HSD post-hoc, $p < 0.001$ after one-way ANOVA: $F_{8;\ 0.000004} = 671320$, $p < 0.001$;) in every gene. However, the normalised values of the three reference genes showed variation across treatments in the range of 0.3 – 1.0 (Tab. 5), whereas the across-treatment variation was several times higher in three other genes: *GapDH* (4.91); *SucDH* (7.20) and *UBC* (3.79). These three genes of basic metabolism were treated as target genes, and we investigated whether their expression is regulated by the treatments.

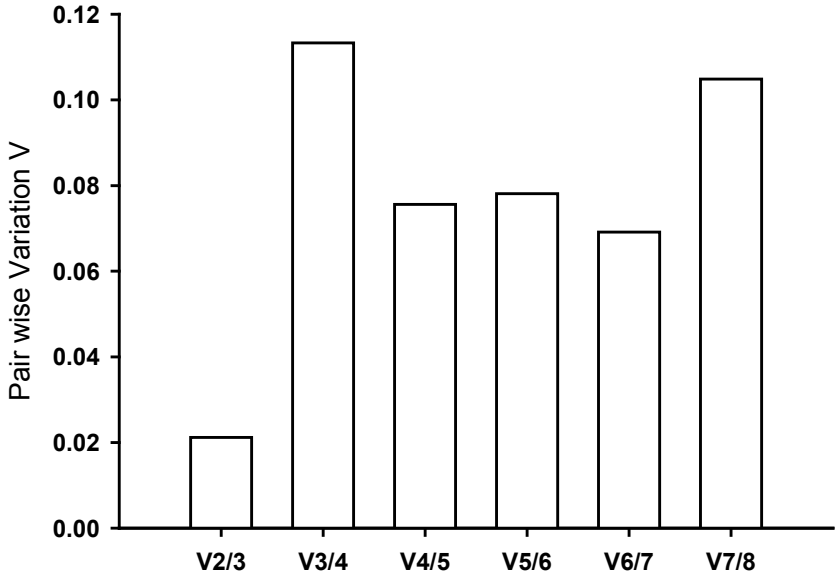

Figure 5 - Pair wise variation of sequential normalisation factors (Vn / n+1) in the microcystin experiment estimated by geNorm.

V2/3 is based on the geometric mean of the normalisation factors of *TBP*, *18S* and *alpha-tubulin*; V3/4 is V2/3 and *SucDH*; V4/5 is V3/4 and *actin*; V5/6 is V4/5 and *GapDH*; V6/7 is V5/6 and *cyclophilin*; V7/8 is V6/7 and *UBC*, V8/9 is V7/8 and *28S*.

	Relative expression[a]				
Gene	***S. obliquus***	***S. elongatus***	***M. aeruginosa* WT**	***M. aeruginosa* Mut**	**SD**[b]
alpha-tubulin	1.0000 ± 0.003	0.3937 ± 0.002	0.4077 ± 0.001	0.3028 ± 0.001	0.3194
TBP	1.0000 ± 0.009	1.0186 ± 0.006	1.3176 ± 0.008	3.0758 ± 0.221	0.9926
18S	1.0000 ± 0.002	2.4935 ± 0.002	1.8613 ± 0.007	1.0736 ± 0.0002	0.7079

Table 5 - Relative gene expression of the reference genes in *D. magna* after normalisation in the microcystin experiment.

D. magna was fed either the green alga *S. obliquus* or microcystin-free cyanobacteria (*S. elongatus* or the mutant of *M. aeruginosa* PCC 7806 [Mut]) or the microcystin-producing wild type of *M. aeruginosa* PCC 7806 (WT).

[a]Values are mean of n = 3 replicates ± SD. Expression levels are displayed relative to the mean control level

[b]SD is the variation of one reference gene across treatments

Microcystin experiment: Relative expression of the target genes *GapDH*, *SucDH* and *UBC* following normalisation

The mean relative expression of *GapDH* in the microcystin-free treatments with *S. elongatus*, *S. obliquus* and the mutant of *M. aeruginosa* PCC 7806 ranged between 0.1 and 1 (Fig. 6), whereas the treatment with the microcystin-producing strain of *M. aeruginosa* showed a relative expression of over 10. The pattern of the relative expression of *UBC* was similar. Expression in the treatments with the green alga and the microcystin-free cyanobacteria ranged between 0.01 and 1, whereas the treatment with *M. aeruginosa* wild type showed a relative expression of > 8.0 (Fig. 6). The target gene *SucDH* showed a higher relative expression in the microcystin-free mutant of *M. aeruginosa* (4.0-fold; Fig. 6) than in the microcystin-free treatments with the green alga (1) and *S. elongatus* (1.92); however, the relative expression of *SucDH* in the wild type of *M. aeruginosa* was even 16-folds higher than in the calibrator (green alga).

Resolving gene identities by homology to the *Daphnia pulex* genome

The *D. magna* candidate gene sequences were used for protein database searches for *D. pulex* homologs in the Dappu v1.1 draft genome sequence assembly (September, 2006) and annotation. Phylogenetic trees were subsequently constructed from the aligned amino acids.

Fifteen protein sequences for actin could be found. The protein sequence of the orthologous actin in the *D. pulex* sequence (Actin P = Dappu-306442) clustered with five other sequences (Fig. 7A). The highest similarity (55%; p-distance 0.7%) was found with two paralogous sequences (Dappu-228751 and Dappu-305550 (Fig. 7 B). P-distances ranged from 0% to 87.7% indicating a very high variability between all of the actin paralogs. Sixteen *D. pulex* proteins with significant sequence similarity to cyclophilin could be found. The cyclophilin protein sequence of the orthologous *D. pulex* sequence (Cyclo P = Dappu-92663) clustered with another sequence (Dappu-215551; p-distance 32%; Fig. 7D). Cyclophilin showed a very high variability with p-distances between 25.2% and 98.1%. For GapDH six significant protein sequence hits could be revealed. The GapDH protein sequence of the orthologous *D. pulex* sequence (GapDH P = Dappu-302823) clustered significantly with another sequence (NCBI GNO 531324; p-distance 34.7%; Fig. 7E). GapDH showed a very high variability with p-distances between 34.7% and 93.9%.

Twenty-three significant protein sequence hits for UBC were found. The UBC protein sequence of the orthologous *D. pulex* sequence (UBC P = Dappu-120690) clustered significantly with two other sequences (Fig. 7 F). The highest similarity showed Dappu-69870 (97%; p-distance: 19.7%). The variability between paralogs was very high with p-distances between 19.7% and 94.4%.

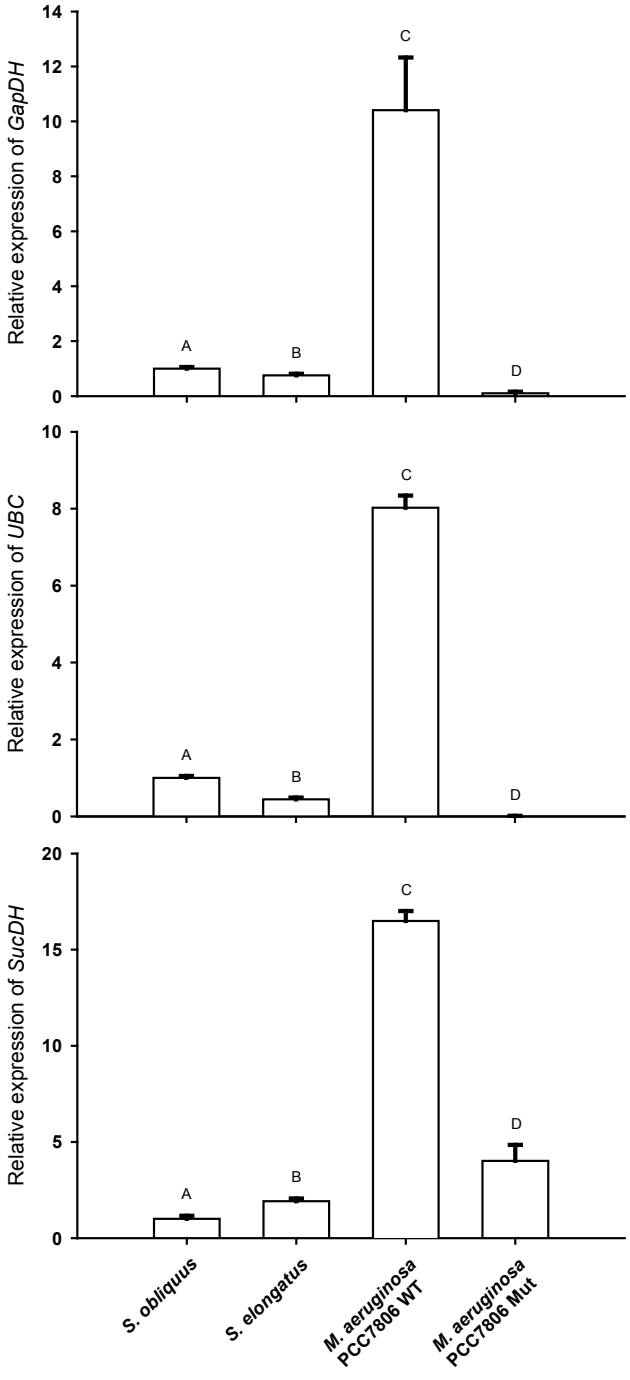

Figure 6 - Relative normalized expression of the target genes *GapDH* (top), *UBC* (middle) and *SucDH* (bottom) in *Daphnia magna* in the microcystin experiment.
D. magna was raised either on the green alga *S. obliquus* (= calibrator set at 1), on microcystin-free cyanobacteria (*S. elongatus* or the mutant of *M. aeruginosa* PCC 7806), or on the microcystin-producing *M. aeruginosa* PCC 7806 wild type. Depicted is the mean relative expression (n = 3, ± SD) of *GapDH*, *UBC* and *SucDH*. Letters indicate a significant difference ($p < 0.001$) between treatments.

Two different alpha-tubulin loci containing the orthologous DNA piece WFes0007807 were unearthed. For both loci taken together 11 significant protein sequence hits could be found. The alpha-tubulin protein sequences of the orthologous *D. pulex* sequences (alpha Tubulin P 1 = Dappu-315805 and alpha Tubulin P 2 = Dappu-301837) both clustered significantly with three other sequences (Fig. 7C). Dappu-315806 showed 91% similarity to alpha Tubulin P 1 (p-distance 2.5%), and. Dappu-220904 was similar to alpha Tubulin P 2 (50%; p-distance 3.4%). The variability of alpha-tubulin was very high (p-distances between 2.5% and 77.7%).

Discussion

The occurrence of cyclic heptapeptides, termed microcystins, is widespread in cyanobacteria and not restricted to the genus *Microcystis*; mass development of microcystin-producing cyanobacteria can constitute a high risk for intoxication of humans and livestock [27]. Numerous studies have been carried out in order to determine the ecological significance of microcystin production in cyanobacteria. The availability of the mcy$^-$ mutant of PCC7806, which is genetically identical to the wild type except for its inability to synthesize microcystins [16], made it possible to more precisely analyse the role of microcystins in daphnid poisoning. Life-table experiments with the wild type and the mcy$^-$ mutant of PCC7806 with *Daphnia galeata* have shown that the wild type was poisonous to *D. galeata*, whereas the mutant strain had no lethal effects [17,18]. These findings and similar results for *D. magna* [28] suggest that microcystins play a role in the defence of *M. aeruginosa* against zooplankton grazing. Feeding on the cyanobacterium *M. aeruginosa* led to significantly reduced growth of *D. magna* compared to animals grown on the high quality food alga *S. obliquus* [23] or the non-toxic cyanobacterium *S. elongatus* [22]. Although there was reduced growth in *D. magna* feeding on *M. aeruginosa* compared to the reference cyanobacterium, there was no difference between the wild type and the mcy- mutant treatment. However, a specific microcystin effect became evident on day five, when *D. magna* raised on the wild type strain died, whereas no mortality was observed in *D. magna* raised on the mutant strain.

In an in-vitro system, microcystin-LR has been shown to inhibit protein phosphatases 1 and 2A in crude extracts of *Daphnia* sp. [19]. However protein phosphatase 1 and

2A each comprise a family of protein serine/ threonine phosphatases with a wide range of different specificities that are mediated by different interactors [29] and regulatory subunits [29,30]. Hence it remains entirely unclear which specific physiological pathways in daphnids are affected by the binding of microcystin to protein phosphatases 1 and 2A.

Here for the first time in-situ effects of dietary microcystins on gene expression of daphnids were investigated. The experiments presented in this paper were designed to identify genes involved in the general metabolism in *D. magna* in which the expression level responds to the presence of microcystins. We therefore compared the effects of the microcystin-producing wild type *M. aeruginosa* PCC7806 and the mcy$^-$ mutant of this strain on the relative expression of genes involved in basic metabolism. We found substantial up-regulation of *GapDH* (Dappu-302823) and *UBC* (Dappu-120690) in response to the presence of microcystins in the food of *D. magna*, which demonstrates that certain enzymes of glycolysis and protein catabolism are significantly up-regulated when daphnids ingest microcystins. For the first time a specific gene regulation in response to dietary microcystins has been demonstrated in daphnids. This up-regulation might have enabled *D. magna* to avoid a microcystin-specific depression of growth until day four but could not prevent mortality on day five of the growth experiment.

Upon exposure to the microcystin-producing wild type of *M. aeruginosa* PCC7806, *D. magna* has been shown to develop a tolerance against this toxic strain within an individual's lifespan and to transfer this tolerance to the next generation through maternal effects, a fact that has been interpreted as an inducible defence against microcystin [8]. It remains to be tested which role the observed up-regulation of *GapDH* and *UBC* plays in the inducible tolerance of *D. magna* to microcystins. Furthermore, clones of *D. magna* have been shown to differ in their tolerance to *M. aeruginosa* PCC7806 [31], which suggests a genetic basis for increased toxin tolerance. It remains to be investigated whether the up-regulation of *GapDH* and *UBC* contributes to the tolerance to *M. aeruginosa* PCC7806.

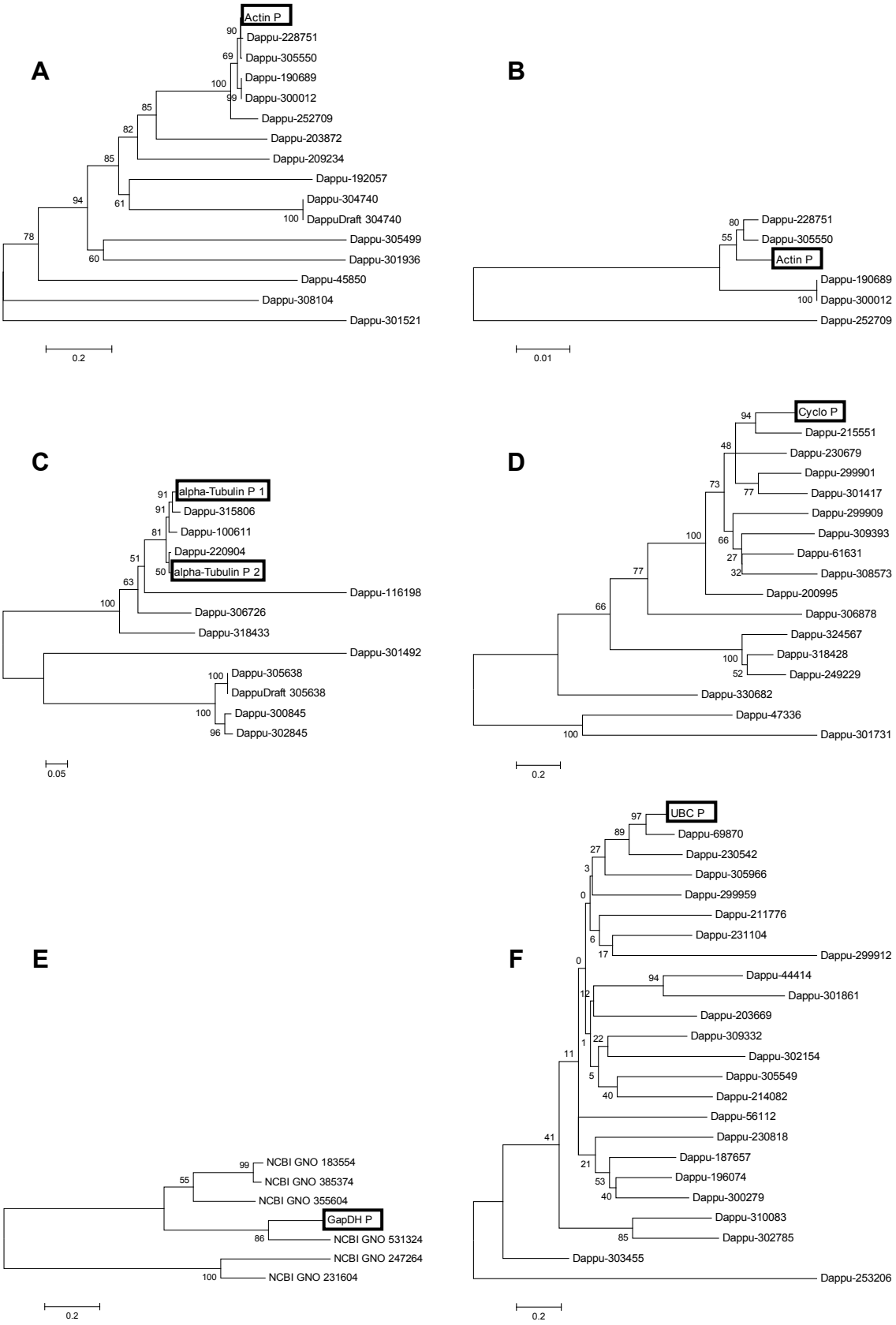

Figure 7 – Phylogenetic trees of the target genes of all experiments.

Neighbor-Joining-Trees with Bootstrap Test of Phylogeny of protein sequences of *D. pulex*. Phylogenetic trees of actin (A) and focus on the cluster of Actin P (B). Phylogenetic trees of alpha-tubulin (C), cyclophiline (D), GapDH (E) and UBC (F). The proteins of the genetic sequences of *D. pulex* equivalent to the utilized *D. magna* sequences are highlighted by putting them in boxes.

In addition to the microcystins in PCC7806 wild type, both the wild type and mcy⁻ mutant PCC7806 produce other classes of secondary metabolites of unknown biological activity [32,33]. *D. magna* feeding on either of these two strains revealed a substantial up-regulation of *SucDH*, and it remains to be seen which cyanobacterial compounds induce this up-regulation of a key enzyme of the tricarboxylic acid cycle. In order to account for possible general effects of cyanobacteria on expression of the investigated genes, we fed *Synechococcus elongatus* to *D. magna*. This cyanobacterium is easily ingested by daphnids and does not contain toxins or inhibitors [22]. The effects of *S. elongatus* on *GapDH*, *UBC* and *SucDH* were negligible compared to the afore mentioned effects of *M. aeruginosa*, which indicates that the up-regulation of the tested loci of *GapDH*, *UBC* and *SucDH* in *D. magna* is a specific and not a general response to cyanobacterial secondary metabolites. It would be interesting to see, if this holds true for all different paralogs of the affected genes or if the up-regulation is restricted to specific clusters or single paralogs of these highly variable genes (Fig. 7 E-F).

Predation is an important stressor in aquatic communities, and many studies using *Daphnia* sp. have contributed to an understanding of the adaptive value of inducible anti-predator defences in the genus *Daphnia*. Achieving a better understanding of the mechanisms and constraints of the evolution of inducible anti-predator defences requires more research on the mechanisms of inducible defences at the molecular level. Only recently has this field been started to be explored. Our work was stimulated by the paper of Pijanowska & Kloc, (2004) [24], who used a clone of *D. magna* which has been shown to be plastic with regard to life-history traits and behaviour [10,11,13,14,34] in response to kairomones from fish and *Chaoborus*. Pijanowska & Kloc (2004) [24] have shown a dramatic decrease of the proteins actin and alpha-tubulin in this clone of *D. magna* when it was exposed to kairomones from planktivorous fish or the larvae of *Chaoborus* water midges. These identical effects of vertebrate and invertebrate kairomones suggested that actin might play a major role in anti-predator responses in *D. magna* in general. Using the same clone of *D. magna*, we here demonstrate that an exposure to chemical cues from both invertebrate and vertebrate predators results in a change in *actin* expression. However, although significant, the 1.75-fold (fish) increase and 0.94-fold (invertebrate) decrease in *actin* expression was rather moderate and did not reflect the dramatic decrease of the protein actin reported by Pijanowska & Kloc [24]. The

same holds true for the weak although significant increase in the gene *alpha-tubulin* in the fish (1.71) and the *Chaoborus* treatments (1.07). Since we found two possible alpha-tubulin orthologous protein sequences in *D. pulex*, which were very similar to each other (Fig. 7 C), we concluded that the effect on the expression holds true for all paralogs in their cluster. Therefore, the substantial decrease of actin and alpha-tubulin on the protein level reported by Pijanowska & Kloc [24] could be a posttranslational process, e.g. miRNA-mediated regulation or increased degradation, as has been suggested by the authors [24]. We conclude that these loci of *actin* and *alpha-tubulin* are no strong target genes for anti-predator defences. However, construction of phylogenetic trees reveals very high variability between the different paralogs of actin and alpha-tubulin (Fig. 7 A-C). It remains to be tested if the decrease of actin and alpha-tubulin on the protein level reported by Pijanowska & Kloc [24] is caused by another paralogous sequence sharing the same gene name.

Following normalisation to NF, it turned out that the expression of *28S*, *UBC*, *18S* and *cyclophilin* was affected by the type of kairomone. Genes involved in protein biosynthesis (*18S, 28S*) and protein catabolism (*UBC*) were up-regulated by kairomone. These effects were considerably stronger for fish kairomone. The expression of *cyclophylin* (Dappu-92663), a gene involved in protein folding, was up-regulated in the presence of kairomones from vertebrate and down-regulated by kairomones from invertebrate predators. The finding that the two kairomones differ in their effect on *cyclophylin* in *D. magna* is in accord with the observation that the life-history response of this clone of *D. magna* differs between kairomones released from fish or *Chaoborus* [24]. *Cyclophilin*, could serve as a potential target gene for further analysis of kairomone effects on daphnids. It remains to be seen how *cyclophilin* is involved in mediating kairomone effects on life history of daphnids and if this is specific to the orthologous sequence and to related paralogous sequences of *cyclophilin.*

Our study is the first detailed study that investigates effects of kairomones from vertebrate and invertebrate predators and of microcystin on gene expression of genes involved in different basic metabolic processes in *D. magna*. Kairomones from both vertebrate and invertebrate predators led to the well-established adaptive shifts in SFR in *D. magna* giving evidence for biologically active incubation water from either predator. Similarly, evidence for specific effects of microcystin comes from the higher mortality of *D. magna* on the wild type strain than on the mutant of *M.*

aeruginosa PCC 7806. Calculating a combination normalisation factor based on the geometric mean of three genes for the kairomone experiment and for the growth experiment, stressor-specific regulation of some of the genes involved in basic metabolism is demonstrated.

All target genes in *Daphnia* show a surprisingly high variability between paralogs. If such a high variability holds true for other genes in *D. magna*, this could hint at a highly plastic genome, which might be adaptive for an animal that living in a very complex aquatic environment and therefore has to maintain a high potential for adaptations.

Conclusion

Three (*GapDH, TBP, SucDH*) of the seven genes investigated (*GapDH, TBP SucDH, 28S, UBC, 18S, cyclophilin*) were found to be stable across the kairomone treatments and were used as reference genes for normalization. Although significant, no substantial kairomone-dependent regulation of *actin* and *alpha-tubulin* was found, indicating that the dramatic decrease of actin and alpha-tubulin at the protein level in response to kairomones (reported earlier) was not due to a regulation of the transcription of the *actin* and *alpha-tubulin* loci investigated here. Therefore these gene loci cannot serve as target genes in the analysis of kairomone effects on *D. magna*. If this holds true for the other paralogs sharing the same gene name remains to be tested. However, the expression of other genes involved in protein biosynthesis, protein catabolism and protein folding, especially the regulation of *cyclophilin* by kairomones, indicated major effects on protein folding. These genes have the potential to serve as target genes in further analysis of kairomone effects on the life history of daphnids.

Three genes (i.e. *18S, TBP, alpha-tubulin*) proved to be stable across microcystin-free and microcystin-containing cyanobacterial food treatments and were used for normalization. Two of the candidate genes (*UBC* and *GapDH*) were shown to have toxin-specific regulation and were clearly up-regulated when microcystins were present in the food. This indicates that microcystins strongly affect protein catabolism and glycolysis in *D. magna* when the animals ingest microcystins via the natural route of exposure, i.e. uptake of microcystin-containing food items; it remains to be seen which role the observed up-regulation of *GapDH* and *UBC* plays in the inducible tolerance of *D. magna* to microcystins.

The construction of phylogenetic trees is an essential step in target gene analysis in *Daphnia* in order to account for the high variability between different paralogs. Phylogenetic trees of the different paralogs are indispensable for clustering the utilized loci with similar ones and to delineate them from others. Related paralogs might have a similar relevance within the genome. This approach is especially important in an organism like *Daphnia sp.* with p-distances showing a very high variability between different paralogs.

Methods

Test species and cultures

A clone of *Daphnia magna* originating from Lake Binnensee, Germany, inhabited by fish and various invertebrates, was cultured at 20°C in membrane-filtered tap water (conductivity: 740 µS/ l; pH 7.2; major ions: Ca^{2+} (110 mg/ l) and HCO^{3-} (270 mg/ l)). Fifteen animals per litre were kept under non-limiting food concentrations (2 mg C_{part} / l) with *Scenedesmus obliquus* (SAG-276-3a) (Stammsammlung für Algen, Göttingen, Germany) as food alga. Only third clutch neonates which had been born within 12 h were used for the experiments.

The green alga *Scenedesmus obliquus*, the cyanobacteria *Synechococcus elongatus* (SAG 89.79) and *Microcystis aeruginosa* (UTEX LB 2063 and PCC 7806), and a genetically engineered microcystin synthetase knock out mutant of *Microcystis aeruginosa* (PCC 7806 mcy^- [16]) were cultivated semi-continuously in cyanophycean medium [35] at 20°C, with half of the medium exchanged weekly. The medium consisted of 0.6mM $CaCl_2$ x 2 H_20, 0.8 mM $NaNO_3$, 0.4 mM K_2HPO_4 x 3 H_2O, 0.4 mM $MgSO_4$ x 7 H_2O, 0.01 mM NaFeEDTA, 0.8 mM KCl, 100 µM H_3BO_3 and 20 µM Na_2MoO_4 x 2 H_2O and had a pH of 8.5. Cyanobacteria were cultivated with constant light at 95 µE/ m^2/ s, *S. obliquus* at 130 µE/ m^2/ s. Carbon concentrations of the autotrophic food suspensions were estimated from photometric light extinction (800 nm) and from carbon-extinction equations previously determined.

Experimental design

Kairomone experiment

Fish-conditioned water was prepared by exposing four sunbleaks (*Leucaspius delineatus*) in 4 l of tap water at 20°C for 24 h without feeding during the whole experiment. The fish were stopped being fed for 24 h prior to the experiment; the water thus did not contain any faeces.

The *Chaoborus* incubation water was prepared by exposing 60 fourth-instar larvae of *Chaoborus flavicans* (which had previously been allowed to feed on zooplankton for 4 h each day of the experiment) in 1 l of tap water at 15°C for 20 h.

Predator-conditioned water was filtered (GFF), and in case of fish-conditioned water diluted 1:4, prior to introducing *D. magna*. From a cohort of *D. magna* neonates that had been born within 12 h from synchronized mothers, five animals each were exposed in 250 ml glass containers to predator-conditioned or control water from birth until maturity. All treatments were run in triplicate. *D. magna* were fed the green alga *S. obliquus* (4 mg Cpart / l); the media were changed daily. At the day when the 1st clutch was visible size at first reproduction (SFR) was determined as the size of the egg-bearing *D. magna*. For each replicate a mean SFR was calculated, and these mean values were used to calculate the respective mean value and the variance for the treatment.

Microcystin experiment

From a cohort of new born *D. magna*, 8-10 animals each were transferred to 1 l of tap water with a food concentration of 2 mg C_{part} / l. The animals were either fed the green alga *S. obliquus* as a control for high quality food or one of the three cyanobacteria. Each day the medium and the food were exchanged. The experiment took place under low light conditions at 20°C and lasted for four days for the real-time PCR analysis. All food treatments were run in triplicate, and somatic growth rates of *D. magna* were determined from dry weight of animals collected at the start and at day four of the experiment. according to [23].

RNA extraction and reverse transcription

At the end of the experiments the animals' RNA was extracted using the RNeasy Mini Kit (Qiagen). In order to remove any traces of genomic DNA, the RNA was treated with Desoxyribonuclease I (Fermentas) following the manufacturer's instructions. The integrity of the RNA was verified with 1.5 % agarose gel

electrophoreses. RNA concentrations were determined with a Qubit Fluorometer (Invitrogen). 1 µg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was diluted 50-fold resulting in total RNA concentrations of 1 ng / µl. The cDNA was stored at -20°C.

Quantitative real-time PCR (QPCR)

Nine different housekeeping genes recently introduced for QPCR in *D. magna* by Heckmann et al. (2001) [26] were used in QPCR analysis: *actin, alpha-tubulin, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GapDH), succinate dehydrogenase (SucDH), TATA-box binding protein (TBP), ubiquitin conjugating enzyme (UBC), 18S ribosomal RNA (18S), 28S ribosomal RNA (28S)*.

QPCR was conducted on the 7300 real time PCR system (Applied Biosystems). Each reaction contained 5 µl of cDNA template, 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2.5 µM of each primer in a final volume of 20 µl. Each reaction was conducted in triplicate. Cycling parameters were 95°C for 10 min to activate the DNA polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After the actual analysis, dissociation curves were performed to verify that no primer-dimers had been amplified. Outliers and samples diverging from the dissociation curve were omitted.

Data analysis and statistics

The raw data were analysed after QPCR. Because of the differing amplification efficiencies of the primer pairs [26], the relative expressions were calculated as quantities using the formula $W_R = (E+1)^{(C_{Tmin}-C_{Tx})}$ (a modification of the $2^{-\Delta\Delta C_T}$ Method [36]), in which W_R is a quantity for the relative expression of one sample, E is the amplification efficiency of its assay, C_{Tmin} is the lowest threshold cycle of all samples of this assay, and C_{Tx} is the threshold cycle of the analysed sample. The quantities could then be imported into geNorm version 3.4 [25], an Excel (Microsoft) based tool which calculates the minimum required number and best-suited combination from a given set of reference genes and from that generates a “normalisation factor” to be used for stable normalisation of QPCR measurements.

After determination of a normalisation factor from the set of reference genes, the raw data of the QPCR runs were imported into qBase version 1.3.5 [37], an Excel (Microsoft) based tool which calculates relative gene expression normalised using

the normalisation factor generated with geNorm. *D. magna* fed entirely with *S. obliquus* served as calibrator which was always set as 1.

The growth rates and the body length were $(x2)^{-1}$ transformed when needed to ensure homogeneity of variances and analysed with ANOVA and Tukey's honestly significant difference (HSD) for post hoc comparisons to assess differences in relative expression.

The data generated with qBase were log-transformed when needed to ensure homogeneity of variances and analysed with ANOVA and Tukey's honestly significant difference (HSD) for post hoc comparisons to assess differences in relative expression.

The statistics were performed with Statistica 6.0.

Database search and construction of phylogenetic trees

To compare the *Daphnia magna* sequences with the *Daphnia pulex* database (http://wfleabase.org) and to find out whether there was more than just one locus in the genome for the genes of interest, the *D. magna* sequences of the target genes *actin, alpha-tubulin, cyclophilin, GapDH* and *UBC* [26] were blasted (blastn) against the wFleaBase. The best hit with the highest score was taken as the ortholog for the sequence of interest in *D. pulex*. Its protein sequence was blasted (blastp; e-value cut off limit = 0.01) against the wFleaBase. All protein sequences with a significant hit were taken and aligned (BioEdit v.7.0.5.3 [38]) with the orthologous protein sequence. Using the program MEGA 4 [39] Neighbor-Joining trees with Bootstrap Test of Phylogeny were constructed and p-distances were calculated. Hypothetically the *D. pulex* trees and the *D. magna* trees are approximately identical.

Abbreviations

C_{part}	particulate organic carbon
GapDH	glyceraldehyde-3-phosphate dehydrogenase
SucDH	succinate dehydrogenase
TBP	TATA-box binding protein
UBC	ubiquitin conjugating enzyme
18S	18S ribosomal RNA
28S	28S ribosomal RNA

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Part II:

Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors

Abstract

Background

The frequency of cyanobacterial blooms has increased worldwide, and these blooms have been claimed to be a major factor leading to the summer-decline of the most important freshwater herbivores, i.e. representatives of the genus *Daphnia*. This suppression of *Daphnia* is partly attributed to the presence of biologically active secondary metabolites in cyanobacteria. Among these metabolites, protease inhibitors are found in almost every natural cyanobacterial bloom and have been shown to specifically inhibit *Daphnia*'s digestive proteases *in vitro*, but to date no physiological responses of these serine proteases to cyanobacterial protease inhibitors in *Daphnia* have been reported *in situ* at the protein and genetic levels.

Results

Nine digestive proteases were detected in *D. magna* using activity-stained SDS-PAGE. Subsequent analyses by LC-MS/MS and database search led to the identification of respective protease genes. *D. magna* responded to dietary protease inhibitors by up-regulation of the expression of these respective proteases at the RNA-level and by the induction of new and less sensitive protease isoforms at the protein level. The up-regulation in response to dietary trypsin- and chymotrypsin-inhibitors ranged from 1.4-fold to 25.6-fold. These physiological responses of *Daphnia*, i.e. up-regulation of protease expression and the induction of isoforms, took place even after feeding on 20% cyanobacterial food for only 24 h. These physiological responses proved to be independent from microcystin effects.

Conclusion

Here for the first time it was shown *in situ* that a *D. magna* clone responds physiologically to dietary cyanobacterial protease inhibitors by phenotypic plasticity of the targets of these specific inhibitors, i.e. *Daphnia* gut proteases. These regulatory responses are adaptive for *D. magna*, as they increase the capacity for protein digestion in the presence of dietary protease inhibitors. The type and extent of these responses in protease expression might determine the degree of growth reduction in

D. magna in the presence of cyanobacterial protease inhibitors. The rapid response of *Daphnia* to cyanobacterial protease inhibitors supports the assumption that dietary cyanobacterial protease inhibitors exert a strong selection pressure on *Daphnia* proteases themselves.

Background

Increasing nutrient input has led to eutrophication in many lakes, which coincides with the increasing dominance of bloom-forming cyanobacteria in the phytoplankton assemblages [1,2]. This increasing dominance of cyanobacteria has been claimed to be a major factor leading to the decline in *Daphnia* abundance across and within lakes [3-5]. These observations from the field are corroborated by laboratory studies which have demonstrated negative effects of cyanobacteria on *Daphnia* [6,7]. However, the generality of these observations has been called into question by a manipulative field study [8], and other recent studies have indicated that *Daphnia* may adapt to increasingly tolerate dietary cyanobacteria [9-11]. However, in no case have the underlying physiological and genetic mechanisms for increased tolerance been addressed. The genome of *Daphnia* has recently become available, creating the opportunity to address the interaction of cyanobacteria and *Daphnia* on the levels of gene expression and proteins more specifically.

Cyanobacteria are known to contain toxins and an array of other biologically active secondary metabolites [12,13]. Cyanobacterial protease inhibitors are among the most widely spread secondary metabolites, as they have been found in nearly every cyanobacterial bloom [13,14]. Different protease inhibitors have been isolated from different cyanobacteria genera [15] as well as from different cyanobacterial strains of the same species [16,17]. Many cyanobacterial protease inhibitors act against serine proteases (i.e. trypsins and chymotrypsins), which represent the most important digestive proteases in the gut of *Daphnia magna* [18], and of which a surprisingly high number was found in the genome of *D. pulex* [19], a closely related species of *D. magna*.

Here we tested whether *Daphnia* displays physiological plasticity in response to dietary cyanobacterial protease inhibitors, assuming that positive selection for increased plasticity might be one mechanism for the recently reported adaptation of *Daphnia* to co-occuring cyanobacteria [20]. More specifically, we investigated the physiological response of a given genotype of *D. magna* to dietary cyanobacterial

protease inhibitors, making use of the previously reported specific interaction of these inhibitors with digestive trypsins and chymotrypsins of *D. magna* [18]. By feeding them mixtures of a green alga with two different strains of the cyanobacterium *Microcystis aeruginosa*, which either contained trypsin- or chymotrypsin inhibitors, we were able to independently determine the effects of the two families of inhibitors on the expression and the activity of *D. magna*'s digestive proteases.

Results

Somatic growth rates on different food treatments

When feeding on the green alga *S. obliquus*, *D. magna* grew at 0.47 d^{-1} (Fig. 1), whereas the growth rate on 20% of the cyanobacterium *M. aeruginosa* was significantly reduced (one way ANOVA: $p < 0.05$; $F_{2,6} = 180.8$) in both cyanobacterial treatments. Growth on the mutant of PCC 7806 was significantly lower (0.15 d^{-1}) than on NIVA Cya 43 (0.33 d^{-1}).

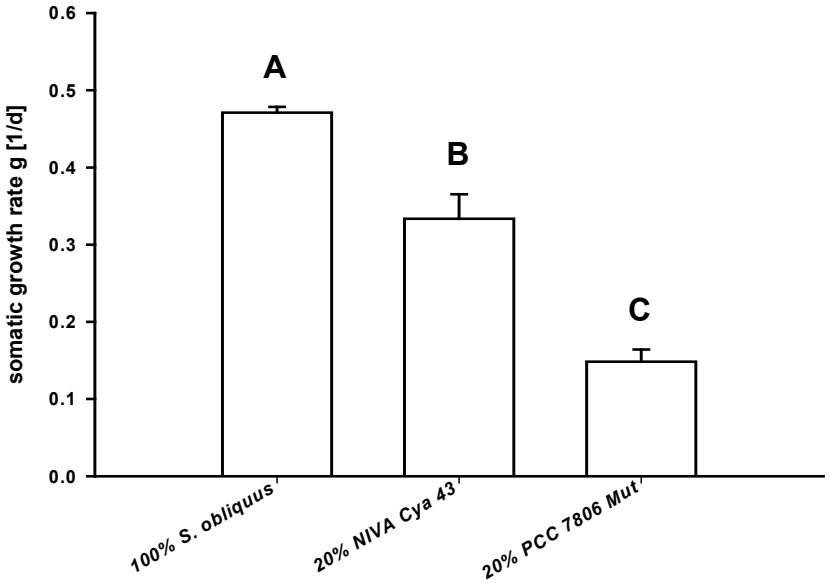

Figure 1 – somatic growth rates
Somatic growth rates of *Daphnia magna* grown on either 100% *S. obliquus*, 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* or on 20% *M. aeruginosa* PCC 7806 Mut and 80% *S. obliquus* (n = 3, ± SD). Letters indicate a significant ($p < 0.05$) difference between the treatments.

Activity and stability of *Daphnia* serine proteases

The chymotrypsin activity of the *Daphnia* homogenate was 0.23 µmol pNA/ min/ µg protein. There was a small, albeit significant (one way ANOVA: $p < 0.05$; $F_{1,4} = 0.0002$) decrease between the specific activity of chymotrypsin of the *Daphnia* homogenate and the same homogenate treated with 2 M urea (0.22 µmol pNA/ min/ µg protein).

The specific trypsin activity of the *Daphnia* homogenate was 0.02 µmol pNA/ min/ µg protein. There was no significant (one way ANOVA: $p = 0.06$; $F_{1,4} = 0.06$) effect of 2 M urea on trypsin activity.

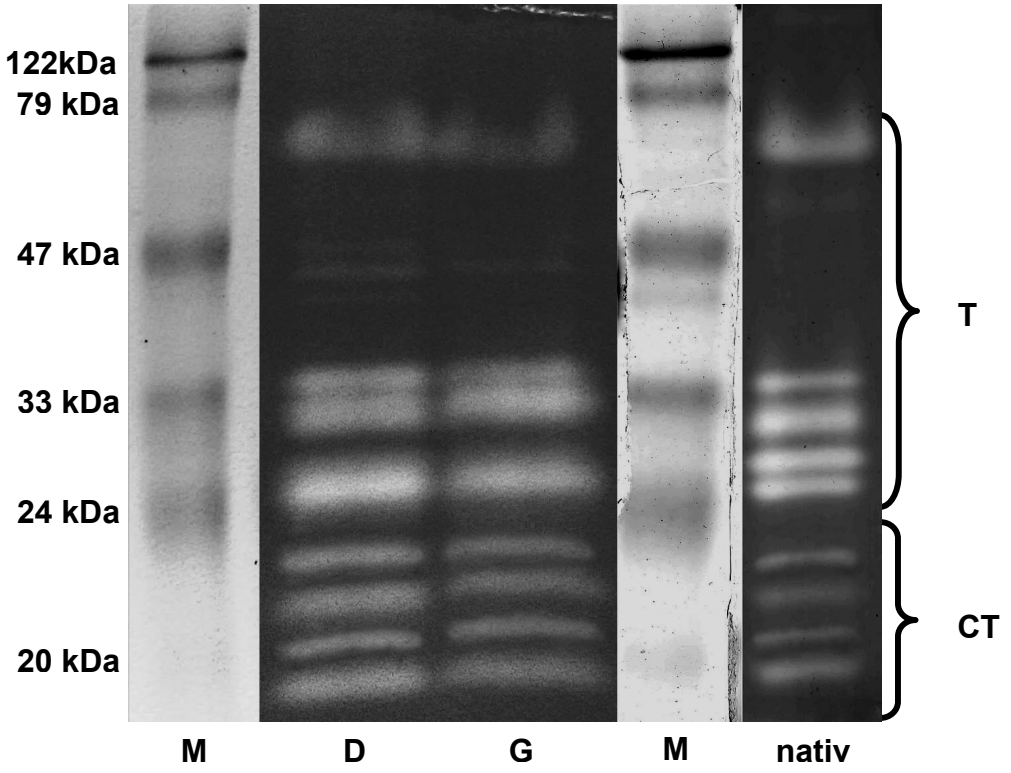

Figure 2 – SDS-PAGE and native gel of *Daphnia* homogenate
Activity stained SDS-PAGE of *Daphnia* homogenate (DH) or gut homogenate (GH) and a native PAGE of *Daphnia* homogenate. White bands indicate active proteases. Protein marker (M). Trypsins (T) and chymotrypsins (CT) are assigned according to Agrawal et al., 2005 [18].

SDS-PAGE and native PAGE of *Daphnia* homogenate

The protease pattern (Fig. 2) of the homogenate of *D. magna* grown on 100% *S. obliquus* showed five bands for trypsins (between 24 - 70 kDa; [18]) and four bands (between 18 – 23 kDa) which, based on indirect evidence, had been previously

suggested to be chymotrypsins [18]). There was no difference between the protease pattern of the *Daphnia* homogenate (whole *Daphnia*) and the gut homogenate (gut + hepatopancreas). The pattern was the same on a native PAGE (Fig. 2).

Food treatments: SDS-PAGE

The protease pattern (Fig. 3) of the homogenate of *D. magna* grown on 100% *S. obliquus* was the same as that of the homogenate of *D. magna* grown on 20% of the microcystin-free mutant of the cyanobacterium PCC 7806 (containing mostly trypsin inhibitors) [18] with regard to the number and the apparent molecular weight of the bands. However, weaker trypsin bands in the treatment with the cyanobacterium indicated reduced trypsin activity, while the intensity of the chymotrypsin bands was not affected.

In the homogenate of *D. magna* grown on 20% NIVA Cya 43 (which contains strong chymotrypsin inhibitors [16]), the band pattern of the trypsins with regard to the number and the apparent molecular weight of the bands did not change in comparison to animals grown on pure 100% *S. obliquus*. However, the intensity of the trypsin bands between 24 and 34 kDa increased; as did the chymotrypsin band at 21 kDa. A different band pattern in the chymotrypsin bands also became obvious (Fig. 3). The two visible bands between 17 and 19 kDa on the SDS-PAGE in the treatment with the cyanobacterium had a different apparent molecular weight than the 18 kDa chymotrypsin band in the treatment with only the green alga.

Amplification efficiencies of the protease primers

The amplification efficiencies (AE) of the protease QPCR-primers all had a value around 1 (Tab. 1), which means a doubling of DNA in every cycle. The amplification efficiencies were considered in the analysis of the QPCR results.

Food treatments: QPCR

The treatment with 100% *S. obliquus* served as the calibrator for the other treatments; its relative protease expression was therefore always set to 1. *Alpha-tubulin*, *SucDH* and *GapDH* served as endogenous controls. The expression of the proteases changed significantly in both cyanobacterial treatments (one way ANOVA: ***T152***: $F_{2,8}$ = 210813.08; $p < 0.05$; ***T208***: $F_{2,6}$ = 62182.09 $p < 0.05$; ***CT383***: $F_{2,6}$ =

1141713.53; $p < 0.05$; ***CT448*****:** $F_{2,6} = 229315.29$; $p < 0.05$; ***CT802*****:** $F_{2,8} = 2455.61$; $p < 0.05$) compared to the calibrator (Fig. 4).

In the treatment with 20% NIVA Cya 43, the proteases were up-regulated between 5.05-fold (*T208*) and 26.7-fold (*CT383*); in the treatment with 20% PCC 7806 Mut the relative expression was also significantly up-regulated in all proteases; however, the effects were weaker than in the treatment with NIVA Cya 43 [between 1.4-fold (*T208*) and 5.9-fold (*T152*)]; (Fig. 4).

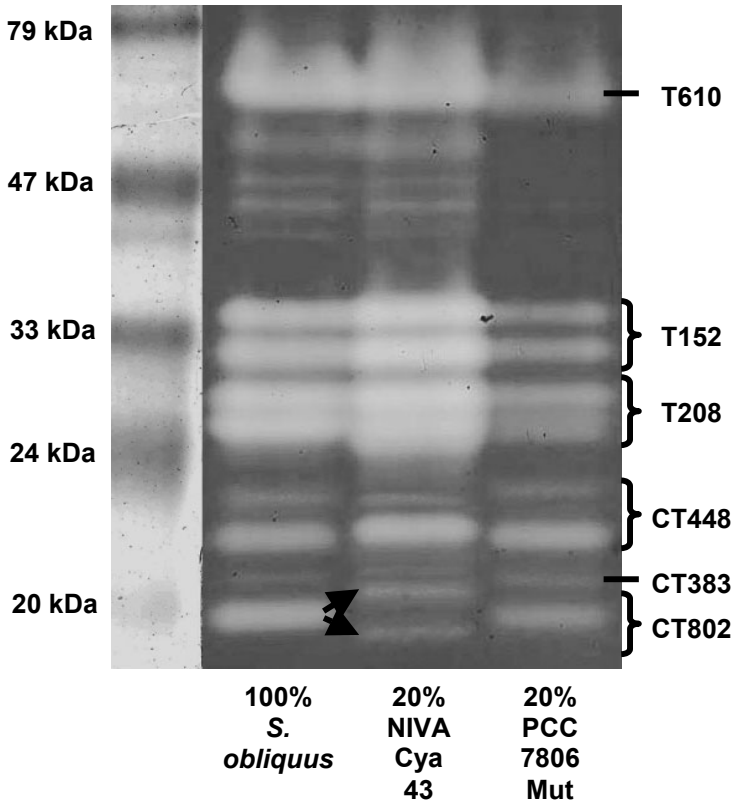

Figure 3 – SDS-PAGE of homogenate of *D. magna* grown on different food treatments
Activity stained SDS-PAGE of homogenate of *D. magna* grown on either 100% *S. obliquus*, 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or on 20% *M. aeruginosa* PCC 7806 Mut and 80% *S. obliquus*. White bands indicate active proteases. Arrows point at shifts in the protease pattern. The numbers to the right depict the results of LC-MS/MS analysis and database search of the proteases (CT = chymotrypsin, T = trypsin).

Serine protease	AE
T152	1.0472
T208	1.0001
CT383	1.0452
CT448	1.0525
CT802	1.0554

Table 1 – Amplification efficiencies
The amplification efficiencies (AE) of the primers developed for QPCR of the five serine proteases.

LC-MS/MS analysis of protease bands

The homogenate of *Daphnia* fed with 100% *S. obliquus* and the homogenate of *Daphnia* fed with NIVA Cya 43 (leading to a different protease band pattern) (Fig. 3) were subjected to LC-MS/MS analysis. Most bands of the SDS-PAGE with homogenate of *D. magna* fed with 100% *S. obliquus* could be identified via LC-MS/MS and database search using the MOWSE algorithm as implemented in the MS search engine Mascot (Matrix Science Ltd. London, UK) [21] (Tab. 2). The sequence match for the mass spectra of the bands from the 100% *S. obliquus* homogenate was low (≤ 10%). However, there were hardly any strikingly non-matching sequences. Since the proteases had very few cutting sites for trypsins, which is not surprising for they are all serine proteases, the sequence match with 7 to 10% was acceptable.

The bands of the 100% *S. obliquus* homogenate previously suggested to be chymotrypsins [18] could be assigned to the proteases 802 (18 kDa band) and 448 (22 kDa and 21 kDa bands), which had the best combination of the factors probability MOWSE score as well as the highest number of matched sequences and the highest sequence coverage (Tab. 2). All three identified proteases were chymotrypsins. Only one (20 kDa) of the suggested chymotrypsin bands could not be identified. The bands between 24 and 34 kDa, formerly specified as trypsins [18], could be assigned to the proteases 152 and 208, which both are trypsins. They could not be differentiated because they matched to the same set of sequences. However, the bands of the homogenate of *D. magna* fed with 20% NIVA Cya 43 and 80% *S. obliquus* (Fig. 3) could all be identified (Tab. 3).

Band (apparent MW)	number of matched peptides	sequence of matched peptide	sequence coverage	Probability based mowse score	hit	function
18 kDa	3	LDAADEPTR IINDVALIR LDAADEPTRVEVR	9%	143	802	chymotrypsin
21 kDa	2	ITETERLEIR TADGPGGISPTLQK	10%	105	448	chymotrypsin
	1	TADGPGGISPTLQK		86	WFes0109692	n.n.
22 kDa	2	ITETERLEIR TADGPGGISPTLQK	10%	66	448	chymotrypsin
24 kDa	2	VVAGEHSLR TDSGLEQNR	7%	109	152/ 208	trypsin
	2	VVAGEHSLR TDSGLEQNR		109	WFes0171720	n.n.
	1	AIFVDGGIHAR		56	WFes0141987	n.n.
25 kDa	2	VVAGEHSLR TDSGLEQNR	7%	114	152/ 208	trypsin
	2	VVAGEHSLR TDSGLEQNR		114	WFes0171720	n.n.
32 kDa	2	VVAGEHSLR TDSGLEQNR	7%	79	152/ 208	trypsin
	2	VVAGEHSLR TDSGLEQNR		79	WFes0171720	n.n.
34 kDa	2	VVAGEHSLR TDSGLEQNR	7%	122	152/ 208	trypsin
	2	VVAGEHSLR TDSGLEQNR		122	WFes0171720	n.n.
	1	AIVVDGGIHAR		48	WFes0128520	n.n.

Table 3 – results of LC-MS/MS analysis of homogenate of *D. magna* grown on 100% *S.obliquus*
D. magna were raised on 100% *S. obliquus*. Depicted are the results of LC-MS/MS analysis (apparent molecular weight of the cut band, number of the matched peptides, sequence of the matched peptides, sequence coverage with the hits in the database, probability based mowse score, hit in the database and the function of the hits).

The LC-MS/MS results of the bands between 24 and 34 kDa were identical to those of the respective bands in the 100% *S. obliquus* lane and were assigned to the trypsins 152 and 208. However, here the identification was clearer: the two bands at 24 and 25 kDa were trypsin 208; the two others were trypsin 152. The band at 75 kDa that was visible in both gels could be identified as protease 610 in the 20% NIVA Cya 43 homogenate, which is also a trypsin, matching the results of Agrawal et al., 2005 [18]. As also found for the gel with *D. magna* fed with 100% *S. obliquus*, the four bands between 17 and 22 kDa were also assigned to the proteases 802 and 448, although the two bands between 17 and 19 kDa (chymotrypsin 802) of the gel with *D. magna* fed with 20% NIVA Cya 43 had another apparent molecular weight.

Although no longer active in the SDS-PAGE (no hydrolytic activity), the chymotrypsin 802 at 18 kDa was still found at this position in the LC-MS/MS analysis. These three different bands from the two SDS-PAGEs of both homogenates were all assigned to CT802, and thus represent three different isoforms of the same protease. In the homogenate of *Daphnia* fed with 20% NIVA Cya 43, the bands between 21 and 22 kDa identified as protease 448 were the same as in the gel with homogenate of *Daphnia* grown on 100% *S. obliquus*. The protease at 20 kDa was identified as protease 383, another chymotrypsin. The proteases of both homogenates in the SDS-PAGE are accordingly assigned (Fig. 3).

Expression of serine proteases after 24 h

The protease pattern of *D. magna* grown on 100% *S. obliquus* was the same after 24 as after 48 h (Fig. 5). When fed with 20% NIVA Cya 43, a shift in the protease pattern of the daphnids already took place after 24 h. A subsequent transfer of the animals to 100% *S. obliquus* for another 24 h led to an intermediate pattern with active proteases from both treatments, the 20% NIVA CYA 43 and the 100% *S. obliquus* treatment (Fig. 5).

Cyclophilin, *SucDH* and *UBC* served as endogenous controls in the QPCR analysis. The relative expression of proteases had changed compared to the calibrator (24 h 100% *S. obliquus*; Fig. 6). All effects were significant (one way ANOVA: ***T152*****:** $F_{3,8}$ = 3745.94; $p < 0.01$; ***T208*****:** $F_{3,8}$ = 14892.5; $p < 0.01$; ***CT383*****:** $F_{3,8}$ = 6777.99; $p < 0.01$; ***CT448*****:** $F_{3,8}$ = 32554.5; $p < 0.01$; ***CT802*****:** $F_{3,8}$ = 10845.1 $p < 0.01$).

The proteases T152, T208 and CT448 showed consistent results. In the food treatment with 20% NIVA Cya 43 they were up-regulated after 24 h compared to the calibrator (24 h 100% *S. obliquus*; Fig. 6). *T208* and *CT448* were slightly up-regulated after 48 h on 100% *S. obliquus*, while for *T152* the expression stayed the same as after 24 h on 100% *S. obliquus*; all three proteases showed the highest (3.4 to 5.97-fold) up-regulation in the treatment with 24 h 20% NIVA Cya 43/ 24 h *S. obliquus* compared to all other treatments.

In *CT383* the results were the same except for the 24 h 20% NIVA Cya 43 treatment. Here, the expression was significantly lower than in the calibrator.

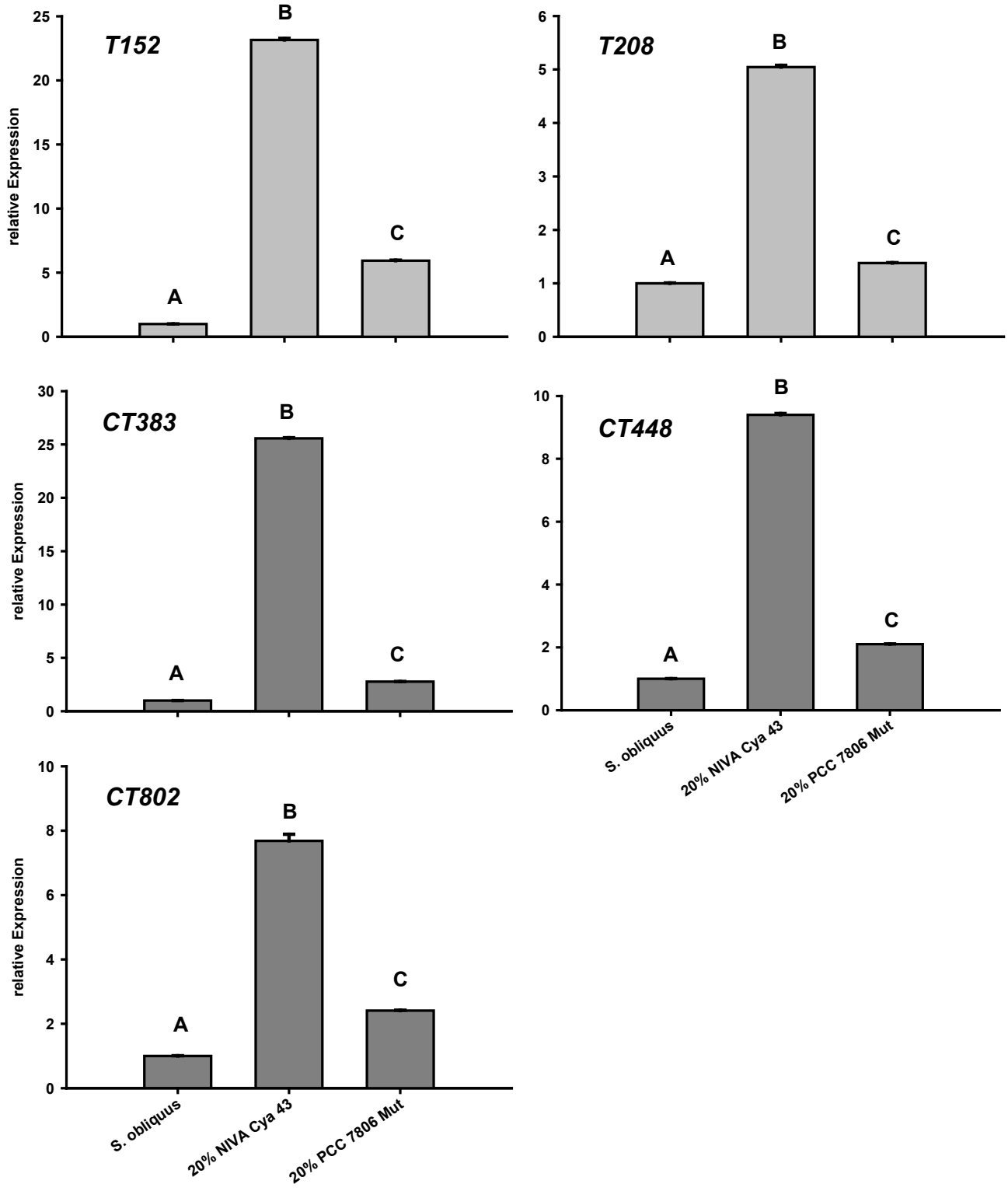

Figure 4 – relative gene expressions of proteases of *D. magna* grown on different food treatments
D. magna were grown on three different food treatments (100% *S. obliquus*, 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* or on 20% *M. aeruginosa* PCC 7806 Mut and 80% *S. obliquus*). Depicted is the mean relative expression (n = 3, ± SD) of trypsins (T152, T208) or chymotrypsins (CT448, CT383, CT802). Letters indicate a significant difference ($p < 0.05$) between calibrator (*S. obliquus*) and cyanobacterial food treatments.

The regulation of *CT802*, however, differed considerably from that of the other proteases. Here, in the treatment with 24 h 20% NIVA Cya 43 the expression was up-regulated already over 7-fold compared to the calibrator. After 48 h *S. obliquus* the expression of *CT802* increased 11.9-fold compared to 24 h *S. obliquus* and was therefore even more induced than in the treatment with 24 h 20% NIVA Cya 43. However, *CT802* showed a higher level of induction after 24 h 20% NIVA Cya 43/ 24 h *S. obliquus* compared to the calibrator (10.5-fold), but was significantly lower than on 48 h *S. obliquus*.

Expression of serine proteases in the presence of microcystin

In order to test for effects of microcystin on protease expression, *D. magna* were fed the microcystin-producing strain of PCC 7806 WT and its mutant PCC 7806 Mut, which is incapable of producing microcystin. *Actin*, *SucDH* and *alpha-tubulin* served as endogenous controls in the QPCR analysis. There were significant (one way ANOVA: ***T152*****:** $F_{2,6} = 2071.32$; $p < 0.05$; ***T208*****:** $F_{2,6} = 42016.5$; $p < 0.05$; ***CT383*****:** $F_{2,6} = 36400.9$; $p < 0.05$; ***CT448*****:** $F_{2,6} = 516$; $p < 0.05$; ***CT802*****:** $F_{2,6} = 57697$ $p < 0.05$) changes in relative expression between the treatments with 10% microcystin-free strain PCC 7806 Mut, 10% microcystin-containing PCC 7806 WT and 100% *S. obliquus* (Fig.7). These changes in relative protease expression were low (0.34 to 1.6-fold) and negligible compared to the effects of the trypsin and chymotrypsin inhibitors from the 20% cyanobacterial food treatments with PCC 7806 Mut and NIVA Cya 43.

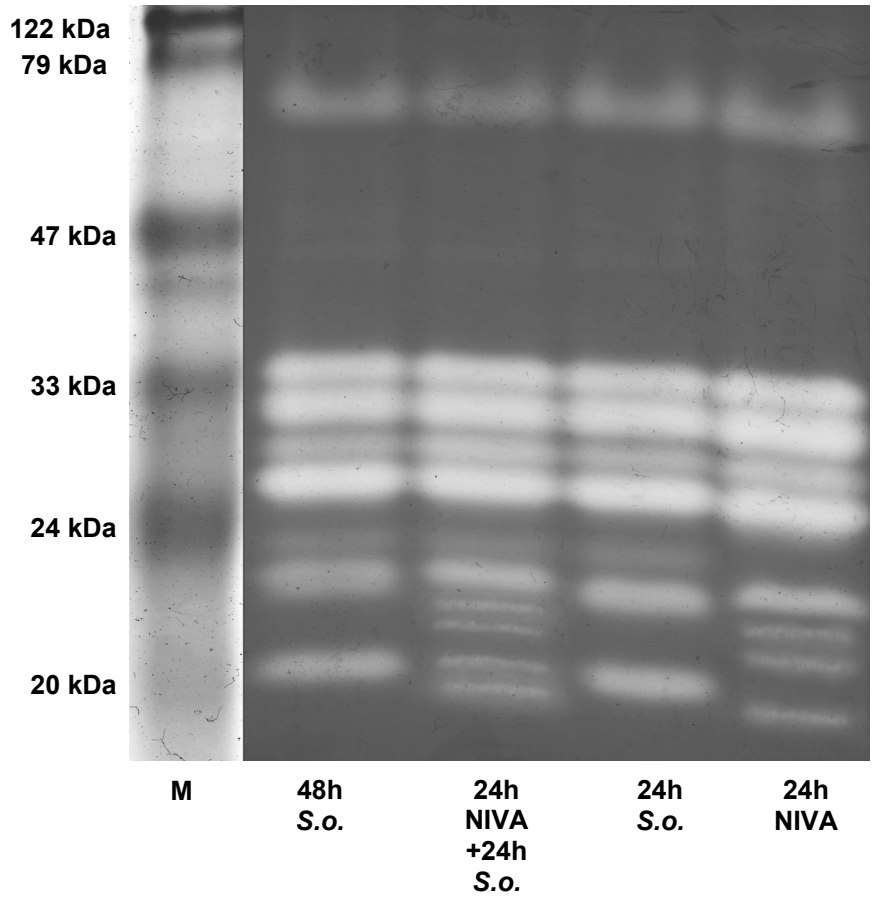

Figure 5 – SDS-PAGE of homogenate after 24 and 48 h food treatments
Activity stained SDS-PAGE of homogenate from *D. magna* grown on 100% *S. obliquus* for 24 h ("24 h *S.o.*") or 48 h ("48 h *S.o.*"), *D. magna* grown on 20% NIVA CYA 43 and 80% *S. obliquus* for 24 h ("24 h NIVA") or for another 24 h on 100% *S. obliquus* ("24 h NIVA + 24 h *S.o.*"). White bands indicate active proteases. M = marker proteins.

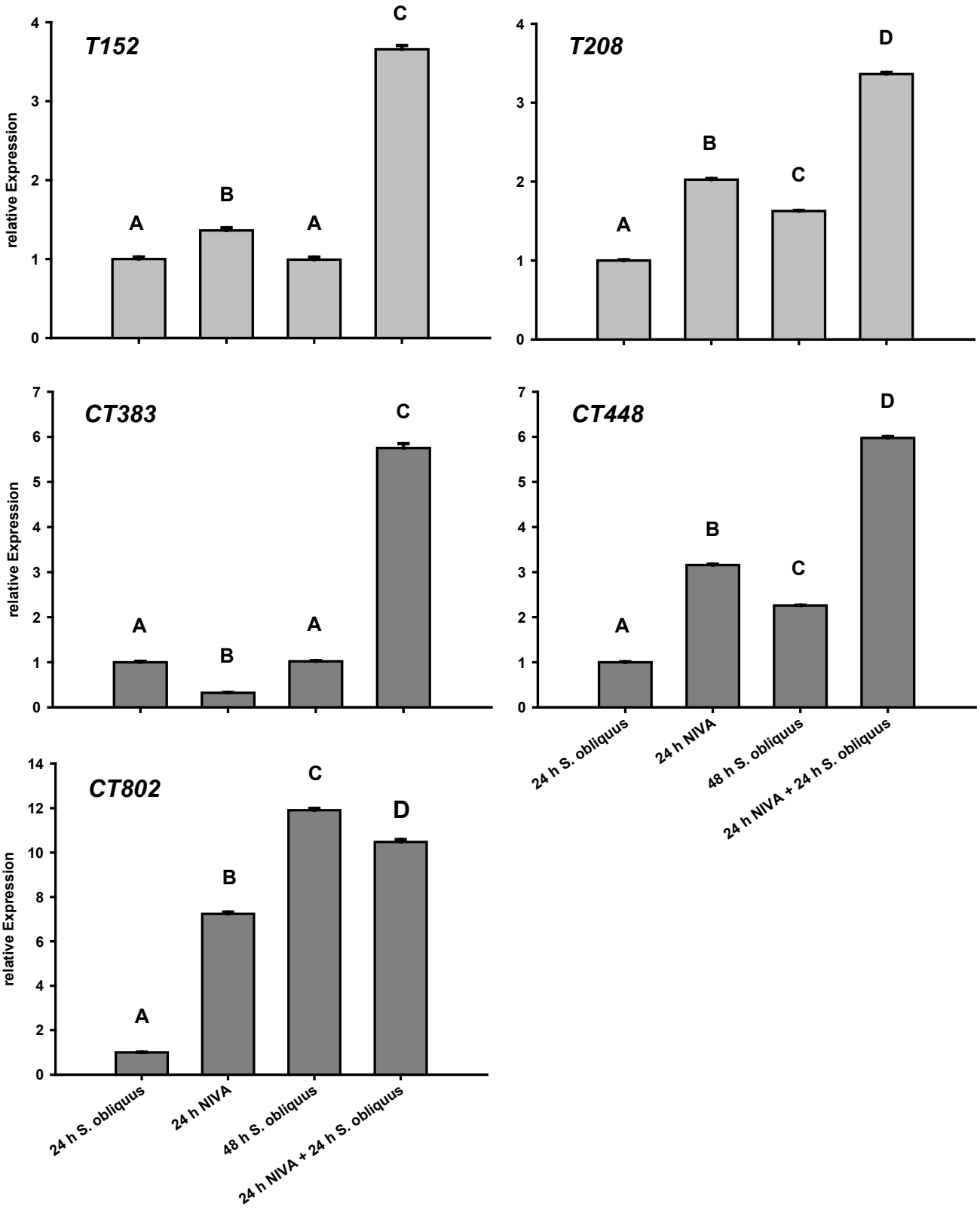

Figure 6 – relative gene expressions of proteases of *D. magna* grown on different food treatments after 24 and 48 h

Relative gene expression of proteases form *D. magna* grown on 100% *S. obliquus* for 24 h or 48 h ("24 h *S.obliquus*" or "48 h *S.obliquus*"), or grown on 20% NIVA CYA 43 and 80% *S. obliquus* for 24 h ("24 h NIVA"), or for another 24 h on 100% *S. obliquus* ("24 h NIVA + 24 h *S.obliquus*"). Depicted is the mean relative expression (n = 3, ± SD) of trypsins (T152, T208) or chymotrypsins (CT448, CT383, CT802). Letters indicate a significant difference ($p < 0.05$) between calibrator (24 h *S. obliquus*) and cyanobacterial food treatments.

Discussion

Daphnia magna fed with 100% green alga showed normal to high growth rates (Fig. 1), whereas the growth rate when fed with 20% cyanobacterial food was reduced. A reduction in growth rate or body length as an effect of cyanobacterial food has been observed previously in various *Daphnia* species: Growth reduction due to interference of filamentous cyanobacteria with the filtering apparatus [22,23] or due to a deficiency of polyunsaturated fatty acids (PUFAs) [24] or sterols [25], or because of the toxin content [26]. The *M. aeruginosa* strains used here were single-celled, small enough to be ingested, and did not contain microcystins [27,28]. Furthermore, a reduction in growth rate due to PUFA or sterol limitation can be excluded, since ≥ 80% of the dietary carbon was of eukaryotic origin, i.e. *S. obliquus* [29]. Therefore, in our case, growth rate reduction in response to cyanobacteria is probably caused by serine protease inhibitors, since the cyanobacterial strains used contain either mainly trypsin inhibitors (PCC 7806 Mut [18]) or strong chymotrypsin inhibitors (NIVA Cya 43 [16]).

The most important digestive serine proteases in *Daphnia magna* are trypsins and chymotrypsins [30]. For whole body homogenate, we found that the activity of chymotrypsins was ten times that of trypsins, corroborating findings for gut homogenate [30]. Von Elert et al. [30] observed nine protease bands in *D. magna* gut homogenate on an activity stained SDS-PAGE; the identical pattern is discernable for whole body homogenate (Fig. 2). This demonstrates that whole-body *Daphnia* homogenate can be used for further analyses, which is in accordance with the finding that whole-body activity shows only minor differences from the proteolytic activity of the gut associated activity [30]. Digestive proteases often have a compact molecular structure; this structure does not seem to be affected by SDS, as the protease pattern from native and SDS-PAGE was identical (Fig. 2). From another crustacean species, *Cancer pagurus*, it is known that trypsin and chymotrypsin activity is stable in regard to temperature and to many organic solvents [31]; our study shows that 2 M urea hardly affects trypsin and chymotrypsin activity in *Daphnia*, also indicating very stable proteases. Hence, it was not surprising that the proteases remained folded under denaturing conditions in SDS-PAGE, which resulted in a misinterpretation of the true molecular weight. A similar misinterpretation was made earlier for a trypsin from *Crangon* spp. [32]. In SDS-PAGE of *D. magna* homogenate, the proteases had apparent molecular weights between 17 and 75 kDa. Where possible, the true

molecular weights of the proteases were calculated after translation of the cDNA sequences from the EST-database (http://www.nematodes.org/Neglected Genomes/ARTHROPODA/Crustacea.html; [33]) from the start- to the stop-codon. The true molecular weights differed from the apparent molecular weights (Tab. 4).

The protease band pattern of *D. magna* grown on 20% PCC 7806 Mut was the same (Fig. 3) as on 100% *S. obliquus*. However, all trypsin bands showed reduced activity when the animals were fed with 20% PCC 7806 Mut, which is explicable by the release of cyanobacterial trypsin inhibitors from the *Microcystis* cells after ingestion by *Daphnia* during subsequent digestion in the gut. The activity of the chymotrypsins in the gel was not affected by this cyanobacterium, which is in accordance with the findings that PCC 7806 Mut mostly contains trypsin inhibitors [18]. A different effect was visible for *D. magna* fed with 20% NIVA Cya 43. This cyanobacterial strain affected both types of serine proteases. In spite of the high content of chymotrypsin inhibitors in this cyanobacterial strain [16], the chymotrypsin band at 21 kDa of the *Daphnia* homogenate slightly increased in intensity (Fig. 3). Although the protein was still detectable, the 18 kDa chymotrypsin was no longer active in the presence of dietary chymotrypsin inhibitors, leading to reduced total chymotrypsin activity in *D. magna* homogenate. This reduction of chymotrypsin activity was partly compensated for by the expression of the two new isoforms at 17 and 19 kDa. These two new isoforms proved to be active in the presence of chymotrypsin inhibitors (Fig. 3), indicating that these isoforms are less sensitive against the cyanobacterial inhibitors than the 18 kDa chymotrypsin. The additional expression of less sensitive chymotrypsins strongly suggests that this regulatory response to dietary chymotrypsin inhibitors is adaptive to *D. magna*.

Despite the expression of additional chymotrypsins, the overall activity of these proteases was reduced in the presence of dietary chymotrypsin inhibitors (Fig. 3).

Moreover, chymotrypsin inhibitors led to a strong increase in trypsin activity. This can be considered to be an indirect effect of the chymotrypsin inhibitors and might be a compensatory physiological response of *D. magna* to cope with a reduced chymotrypsin activity.

Protease	Accession number	Primer forward	Primer reverse	Apparent MW	True MW
T152	DMC00580	5'-TGG ATG CTC CAT TGG ACT TGA-3'	5'-CGG AAA CGG TGA CGA TGA TTC-3'	32-34 kDa	25.3 kDa
T208	DMC05983	5'-TGC GTT AGA GGA GTT GAC GCT-3'	5'-TGA AGC TGA CAA CAC CAC GGT-3'	24-25 kDa	
CT383	DMC00886	5'-TTG GCA CCT TCC ACC GAA T-3'	5'-TCA TCA GGA CTG GAG AAA CGC-3'	20 kDa	23.98 kDa
CT448	DMC01045	5'-TCA TCA ACG GAG CTG AGG CTA-3'	5'-AGA ACC CAC TGG TCG GAA ATC-3'	21-22 kDa	26.4 kDa
T610	DMC00622			75 kDa	24.1 kDa
CT802	DMC00275	5'-TCA GAT TGC CCA ACC CCA T-3'	5'-TCC GCT GAT GTG GAG CAT ATC-3'	17-19 kDa	24.7 kDa

Table 4 – proteases of *D. magna*
Delineated are the names and the accession numbers of the proteases of *D. magna* from the EST-database search and the established forward and reverse primers for the QPCR analyses (no primers for T610 were established). Apparent molecular weights (MW) resulted from the comparison of the molecular weight marker with the visible protease bands in the SDS-PAGE, whereas true MW resulted from the translation of the sequences (start- to stop-codon) of the EST-database http://www.nematodes.org/NeglectedGenomes/ARTHROPODA/Crustacea.html; [33]). No complete sequence was available for T208. (CT = chymotrypsin, T = trypsin).

In summary, the ingestion of the trypsin inhibitors cyanopeptolins [15,17,18] from PCC 7806 Mut only led to a decrease in trypsin activity of *D. magna*, whereas the chymotrypsin inhibitors nostopeptin BN920 and cyanopeptolin 954 [16] from NIVA Cya 43 not only affected chymotrypsin, but also led to an increase in trypsin activity as a putative compensatory response.

The previous classification of five protease bands on SDS-PAGE as trypsins was based on the inhibition by synthetic inhibitors, and the remaining active protease bands were merely postulated to be chymotrypsins [18]. Here, via LC-MS/MS and subsequent database search, all *Daphnia* proteases were clearly identified as trypsins or chymotrypsins and assigned to protease genes. The reason for two bands in the same lane in the SDS-PAGE referring to the same protease gene might be protein ripening or unspecific proteolytic degradation during the gel run. Alternatively, it might be attributed to the presence of splice variants or isoforms stemming from two different gene copies simultaneously active in *Daphnia*.

Only two homogenates showed differences in the protease band pattern: 100% *S. obliquus* and 20% NIVA Cya 43 (Fig. 3). Both were therefore analysed by LC-MS/MS (Tabs. 2, 3). The chymotrypsin bands between 17 and 19 kDa showed a different molecular weight pattern in the two homogenates (Fig. 3). However, the active chymotrypsin band at 18 kDa in the 100% *S. obliquus Daphnia* homogenate and two active bands (17 and 19 kDa) in the homogenate of *Daphnia* fed with 20% NIVA Cya 43 were all identified as CT802. Although not visible as an active protease in the 20% NIVA Cya 43 treatment on the SDS-PAGE (Fig. 3), the CT802 protein still was found at 18 kDa. This result points to total inhibition of the 18 kDa isoform of CT802 due to dietary chymotrypsin inhibitors from NIVA Cya 43. However, other isoforms of CT802 which were more resistant to the inhibitor were expressed, and it is reasonable to assume that these isoforms stem from different gene copies. The *D. pulex* genome has been shown to contain a surprisingly high number of gene duplicates, leading to lineage-specific gene family expansions, which resulted in high numbers of genes (e.g. peptidases in *D. pulex* [34]). Rapid gene family expansions in phenotypically important genes suggest scenarios wherein adaptive natural selection favours additional copies, e.g. for adaptation to increased dosage [35]. In several insects, amplification of different esterase genes was the reason for resistance to organophosphate pesticides [36-38]; resistant strains of the mosquito *Culex pipiens* even showed a 250-fold increase in copy numbers [39].

To test if the observed changes in activity of the proteases on the SDS-PAGEs are caused by a change in gene expression, the relative expression of two trypsin genes (*T152, T208*) and of three chymotrypsin genes (*CT383, CT448, CT802*) in *D. magna* fed with three different food treatments were analysed with QPCR (Fig. 4). In the treatment with 20% NIVA Cya 43, both trypsins and *CT448* were up-regulated by a factor of between 5 and 23. These results are reflected in the increased activity that was visible on the SDS-PAGE (Fig. 3). However, although the relative expression of *CT383* and *CT802* also increased 8 to 26-fold (Fig. 4), only low activity of CT383 and of the two newly expressed isoforms of CT802 was observed on the SDS-PAGE (Fig. 3). One explanation for the low activity of CT802 might be that the new isoforms are not as active as CT802 in *D. magna* fed with green alga, which could also explain why these isoforms are not permanently induced when feeding on high quality food. CT383, of which no isoforms were detectable, might be more sensitive to the chymotrypsin inhibitors, so that the higher expression of *CT383* might have been insufficient to compensate for simultaneous inhibition of the CT383 protein. Hence, no elevated activity was visible on the SDS-PAGE (Fig. 3).

The 1.4 to 6-fold increase of the relative trypsin expression in *D. magna* fed with 20% PCC 7806 Mut was not visible as increased activity in the gel (Fig. 3); the same is true for the increase in chymotrypsin expression (2- to 3-fold; Fig. 3). SDS-PAGE is not sensitive enough to quantify protease activity, as doubling the amount of *Daphnia* homogenate in SDS-PAGE did not lead to an apparent increase in protease activity on the gels (data not shown). Hence, it is not surprising that the change in expression of trypsins and chymotrypsins of *Daphnia* from up to 5-fold was not visible as an increase in activity in SDS-PAGE.

Effects of different food treatments on the activity of digestive proteases in *D. magna* were already observed after 24 hours, which means that only a short period is required for *D. magna* to respond to the occurrence of dietary protease inhibitors. Such a rapid physiological response seems to be highly adaptive, since newborn *Daphnia* have to establish an optimal protease pattern quickly after birth to be able to initiate digestion.

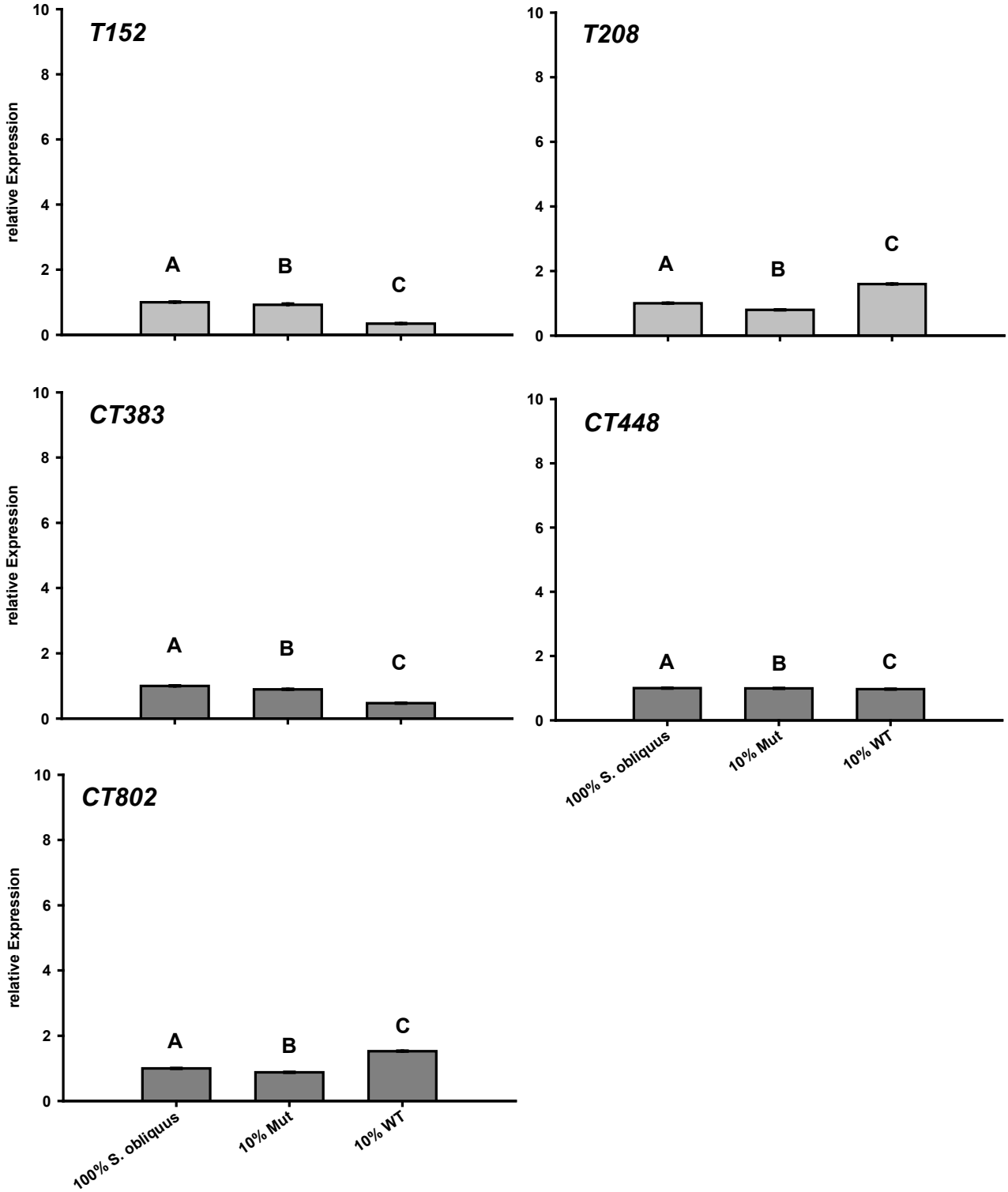

Figure 7 – relative expressions of proteases of *D. magna* grown in the presence or absence of microcystin

D. magna were grown on three different food treatments (100% *S. obliquus*, 10% *M. aeruginosa* PCC 7806 Mut and 90% *S. obliquus*, or on 10% *M. aeruginosa* PCC 7806 WT and 90% *S. obliquus*). Depicted is the mean relative expression (n = 3, ± SD) of trypsins (T152, T208) or chymotrypsins (CT448, CT383, CT802). Letters indicate a significant difference ($p < 0.05$) between calibrator (*S. obliquus*) and cyanobacterial food treatments. The y-axis is scaled to 10 to allow comparison with Fig. 4.

Nevertheless, the additional expression of isoforms and the observed up-regulation of protease expression of *Daphnia* fed with cyanobacteria are allocating additional resources to these proteins and can be assumed to be costly. To render the induction of proteases an evolutionary stable strategy, the level of expression should be tightly linked to the level of dietary protease inhibitors. However, such an immediate down-regulation of protease expression after sudden removal of dietary protease inhibitors was not observed, and an intermediate band pattern was visible in SDS-PAGE (Fig. 5). These observations can easily be explained by the fact that cyanobacterial mass developments in nutrient-rich lakes usually last for several months in summer so that the disappearance of cyanobacteria is a gradual process that can take several weeks [4]. Hence, the disappearance of cyanobacterial protease inhibitors will be considerably slower under field conditions than under our experimental conditions, which means that *Daphnia* are fully capable of adjusting the expression level of proteases to the presence of protease inhibitors in the natural diet.

As CT802 was the only protease showing a completely different response to cyanobacterial protease inhibitors in QPCR analyses, it has been concluded that *CT802* must be regulated in a manner different from the other proteases. Interestingly, CT802 is the only protease which expresses new isoforms after *Daphnia* had been fed with dietary cyanobacterial food (Fig. 3 and 5). Further investigation of the regulation and of the processing leading to these new isoforms is needed. However, protease inhibitors are obviously a strong trigger for up-regulation of protease expression and for an induction of new isoforms. These protease inhibitors might also exert a strong selection pressure on *Daphnia* proteases themselves.

Numerous studies have focused on microcystins as the only reason for decreased fitness in *Daphnia* due to cyanobacteria, since microcystin LR is known to inhibit protein phosphatases of *Daphnia* in-vitro [40]. However, other secondary metabolites also have proved to have adverse effects on *Daphnia* [41,42]. The most widespread group of cyanobacterial secondary metabolites are protease inhibitors, which appear in nearly all cyanobacterial blooms [13,14], whereas this is not the case for microcystins. Von Elert et al. [30] have shown that the most important group of digestive enzymes in *Daphnia* are trypsins and chymotrypsins; these enzymes are

indeed inhibited *in vitro* by specific cyanobacterial inhibitors [18]. By differentiating the effects of PCC 7806 WT and its microcystin-deficient mutant on *Daphnia*, both of which are known to contain strong trypsin inhibitors [18], the negative effects of microcystin-producing cyanobacteria on *Daphnia* could only be assigned to this compound to a limited degree [43,44]. QPCR results showed no difference in the regulation of proteases in the gut of *D. magna* between PCC 7806 WT and Mut, which clearly demonstrates that the interaction of cyanobacterial protease inhibitors with digestive proteases in *Daphnia* is not affected by microcystins. Protease inhibitors should affect gut proteases of *Daphnia* immediately after ingestion of the cyanobacterial food particles and the subsequent release of inhibitors during digestion, before microcystins come in contact with their targets, i.e. protein phosphatases I and II. This suggests that the tolerance of digestive proteases against dietary protease inhibitors in *Daphnia* coexisting with cyanobacteria should be under strong positive selection, even in the presence of other cyanobacterial inhibitors.

Daphnia serine proteases have been shown in-vitro to be inhibited by specific cyanobacterial inhibitors [18]. Here for the first time it was shown that a *D. magna* clone in-situ physiologically responds to dietary cyanobacterial protease inhibitors by phenotypic plasticity of the targets of these specific inhibitors, i.e. *Daphnia* gut proteases. The finding that *D. magna* responds to dietary protease inhibitors by up-regulation of protease expression on the RNA-level and by the expression of new and less-sensitive protease isoforms on the protein level strongly suggest that the observed phenotypic plasticity is adaptive.

Conclusion

To our knowledge this is the first report on physiological plasticity in *D. magna* in response to the most widely spread cyanobacterial inhibitors, i.e., protease inhibitors. We have been able to show distinct physiological responses to dietary trypsin and chymotrypsin inhibitors. These physiological responses involve increased expression of the targets of these inhibitors, digestive trypsins and chymotrypsins, and the expression of less-sensitive isoforms. Clearly these regulatory responses are adaptive for *D. magna* as they increase the capacity for protein digestion in the presence of dietary protease inhibitors. It is therefore reasonable to assume that the kind and extent of these responses in protease expression determine the degree of

growth rate reduction in *D. magna* in the presence of cyanobacteria with protease inhibitors. These physiological responses proved to be independent from microcystin effects, as there was no difference between protease expression of *D. magna* fed with *M. aeruginosa* PCC7806 WT and its microcystin-free mutant form. *Daphnia* neonates respond very quickly to cyanobacterial food (24 h), which supports the assumption that dietary cyanobacterial protease inhibitors exert a strong selection pressure on *Daphnia* proteases themselves.

Methods

Cultivation of *Daphnia magna*

The *Daphnia magna* clone 'Binnensee' [45] was cultivated in 1 l filtered (0.2 µm) water from a nearby pond (Aachener Weiher in Cologne) and fed daily with a saturating concentration of *Scenedesmus obliquus* SAG 276-3a. The water and the food were exchanged every two days. Neonates from the 3rd clutch which were no more than twelve hours old were used for the experiments.

Cultivation of algae and cyanobacteria

Scenedesmus obliquus SAG 276-3a was grown in sterile 5 l semi-continuous batch cultures on cyanophycean-medium [46] at 20°C and constant light (150 µE/ m^2/ s). Every day 1 l of algal suspension was exchanged with fresh medium.

The cyanobacteria *Microcystis aeruginosa* NIVA Cya 43, a microcystin-free strain [27], the wild-type *M. aeruginosa* PCC 7806 WT and its mutant *M. aeruginosa* PCC 7806 Mut [28] (further referred to as NIVA Cya 43, PCC 7806 WT and PCC 7806 Mut) were cultivated in chemostats on cyanophycean-medium at 20°C and constant light (50 µE/ m^2/ s). The dilution rate was 0.23 d^{-1}.

Somatic growth on different food treatments

Growth experiments were performed in 250 ml of 0.2 µm filtered pond water for six days with five neonates per replicate and a food concentration of 2 mg C/ l. The treatments were either 100% *Scenedesmus obliquus*, 20% NIVA Cya 43 and 80% *S. obliquus* or 20% PCC 7806 Mut and 80% *S. obliquus*. Each treatment was run in triplicate. Water and food were exchanged daily. The dry weight of the animals was used to calculate the somatic growth rate (g, d^{-1}) of each treatment according to Wacker et al., 2001 [47] using the formula $g = (\ln x_{te} - \ln x_{tb})/ \Delta t$, for which x_{te} is the

weight after six days, x_{tb} is the weight at the start of the experiment and Δt is the test duration, i.e. six days.

Preparation of *Daphnia* and gut homogenates

Neonates of *Daphnia magna* grown on 2 mg C/ l of *S. obliquus* for six days were transferred to 5 µl 2 mM DTT per animal and were homogenized with a pestle. The homogenate was centrifuged for 3 min at 14,000 x g. The protein concentration of the supernatant – the *Daphnia*-homogenate – was analyzed using a Qubit fluorometer and the appropriate Quant-iT™ Protein Assay Kit (Invitrogen) as according to the manufacturer's advice.

Guts including the hepatopancreases of *D. magna* grown on 2 mg C/ l of *S. obliquus* for six days were separated and transferred to 5 µl 2 mM DTT per gut, as according to Agrawal et al., 2005 [18] and treated in the same way as the *Daphnia*-homogenate.

Activity and stability of serine proteases of *D. magna*

Chymotrypsin activity of the *Daphnia*-homogenate was measured photometrically using the artificial substrate N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-*para*-Nitroanilide ($S(Ala)_2ProPhepNA$; Sigma; [30]). 10 µl *Daphnia*-homogenate was mixed with 980 µl 0.1 M potassium-phosphate-buffer, pH 6.5. The buffer contained 125 µM $S(Ala)_2ProPhepNA$ and 1% DMSO. The change in absorption was measured at a wavelength of 390 nm at 30° C continuously over 10 min. The trypsin activity was measured using the artificial substrate N-Benzoyl-Arginine-*para*-Nitroanilide (BApNA; Sigma; [30]). 10 µl *Daphnia*-homogenate was mixed with 895 µl 0.1 M potassium-phosphate-buffer, pH 6.5. The buffer contained 1.88 mM BApNA and 7.5% DMSO. The change in absorption was measured at a wavelength of 390 nm at 30° C continuously over 10 min. To test the stability of proteases, the buffers with *Daphnia*-homogenate were incubated for 2 min with 2 M urea before the kinetic analysis; activity was compared to the control without urea. Protein concentrations were analysed with the Qubit fluorometer.

SDS-PAGE and native gel of *Daphnia*-homogenate

Daphnia-homogenate (20 µg protein) with 5µl 4x Laemmli-buffer [48] was loaded on a 12% SDS-polyacrylamide gel and run at 200 V. *Daphnia*-homogenate (20 µg protein) with 5µl 4x Laemmli-buffer without SDS was loaded on a native (no SDS)

12% polyacrylamide gel and run at 200 V with SDS-free running buffer. After the run the gels were activity stained as according to Von Elert et al., 2004 [30]. The molecular weights of the visible proteases were compared between the two methods. The marker on all PAGEs was the peqGold Prestained Protein Marker III (peqlab).

LC/ MS-MS analysis of protease bands

Proteases were subjected to purification prior to LC-/MSMS analysis as follows:
200 live *D. magna* grown on 100% *S. obliquus* or 20% *M. aeruginosa* and 80% *S. obliquus* were homogenized and centrifuged as described above. 500 µl of the supernatant were mixed with 500 µl ultrapure water and were loaded onto a strong anion-exchanger column (SAX; Varian, No. 1210-2044). The proteases bound to the column were eluted with one bed volume of 0.9 M NaCl and dialysed at 4°C for 24 h in 1 l of 10 mM imidazole-buffer, pH 6.9. Subsequently the proteases were precipitated with ice-cold 70% acetone. The pellet (centrifugation: 10 min, 14,000 x g at 4°C) was lyophilized and resuspended in 40 µl of ultrapure water. It was mixed with 10 µl 4x Laemmli-buffer, loaded onto a 12% SDS-PAGE and Coomassie-stained after electrophoresis.
Coomassie-stained protein bands were excised from the gel, chopped into cubes and washed three times with acetonitrile-water (1:1). The gel pieces were shrunk with neat acetonitrile, allowed to rehydrate in 50 mM NH_4HCO_3 and dried in a speedvac. 10 mM DTT in 50 mM NH_4HCO_3 were added to the dried gel pieces, and proteins were reduced for 45 min at 56°C. To alkylate reduced cysteine residues, the remaining liquid was removed, and an equal volume of 50 mM iodoacetamide in 50 mM NH_4HCO_3 was added. The reaction was allowed to proceed for 30 min in the dark. Prior to in-gel digestion, the gel pieces were washed and dried as above. The gel pieces were allowed to rehydrate in an ice-cold solution of 12.5 ng/ µl semiTrypsin (for homogenate of *Daphnia* fed with 20% NIVA Cya 43) or Trypsin (for 100% *S. obliquus* homogenate; sequencing grade, Promega) in 10 mM NH_4HCO_3. After 45 min on ice, excessive enzyme solution was replaced by 5 - 20 µl of buffer without enzyme, and proteins were digested at 37°C overnight. The digestion was stopped by the addition of 5 – 20 µl 1% TFA, and peptides were extracted for 30 min at 37°C.
LC-MS/MS data for the 100% *S. obliquus* homogenate were acquired according to Hanisch et al., 2009 [49]. For the homogenate of *Daphnia* fed with 20% NIVA Cya

43, LC-MS/MS data were acquired on a HCT ETD II iontrap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nano ESI source (Bruker Daltonics, Bremen, Germany). Samples were introduced by an easy nano LC system (Proxeon, Odense, Denmark) using a vented column setup comprising a 0.1-mm-by-20-mm trapping column and a 0.075-by-100-mm analytical column, both self packed with ReproSil-Pur C18-AQ, 5 µm (Dr. Maisch, Ammerbuch, Germany). 5 µl to 18 µl of sample were aspirated into the sample loop, and a total of 25 µl was loaded onto the trap column at a flow rate of 6 µl/ min. Loading pump buffer was 0.1% formic acid (FA). Peptides were eluted with a gradient of 0% to 35% acetonitrile (ACN) in 0.1% FA over 20 min and a column flow rate of 300 nl/ min. Subsequently the ACN content was raised to 100% over 2 min, and the column was regenerated in 100% ACN for additional 8 min.

Data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Compass 3.0 software. MS1 scans were acquired in standard enhanced mode. Five single scans in the mass range from *m/z* 400 to *m/z* 1400 were combined for one survey scan. Up to three doubly and triply charged ions rising above a given threshold were selected for MS/MS experiments. Ultrascan mode was used for the acquisition of MS2 scans in the mass range from m/z 100 m/z 1600, and three single scans were added up. The ion charge control value was set to 250000 for all scan types. Peaklists in mascot generic format (mgf) were generated from the raw data by using the Data Analysis software module (Bruker Daltonics, Bremen, Germany).

Proteins were identified by using a local installation of MASCOT 2.2 (Matrix Science Ltd, London, UK). All serine proteases (13) from a *D. magna* EST-database (http://www.nematodes.org/NeglectedGenomes/ARTHROPODA/Crustacea.html; [33]) and a complete *D. pulex* database (http://wfleabase.org/; [19]; release: July 2007) were used. The database search could be reduced to serine proteases because Agrawal et al. [18] assigned all visible protease bands in SDS-PAGE to serine proteases. Searches were submitted via Proteinscape 2.0 (Bruker Daltonics, Bremen, Germany) with the following parameter settings: enzyme “semiTrypsin”, fixed modifications “carbamidomethyl”, optional modifications “Methionine oxidation” and missed cleavages “1”. The mass tolerance was set to 0.4 Da for peptide and fragment spectra. The most probable hits for the bands of the SDS-PAGEs were determined by the number of matched peptides, the percent-wise sequence coverage, and the probability MOWSE score.

Food treatments: SDS-PAGE and quantitative real-time PCR (QPCR)

15 neonates of *D. magna* clone Binnensee were grown on 2 mg C/ l in 1 l filtered pond water on either 100% *S. obliquus*, 20% NIVA Cya 43 and 80% *S. obliquus* or on 20% PCC 7806 Mut and 80% *S. obliquus*. Each treatment was run in triplicate. The water and the food were exchanged daily. The experiment was stopped after six days, after which half of the animals were used for 12% SDS-PAGE followed by activity staining; RNA was extracted from the other half using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was purified with DNase I (Fermentas) and reverse transcribed with High-capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems).

Nine different housekeeping genes recently introduced for QPCR in *D. magna* by Heckmann et al., 2006 [50] were used in QPCR analysis: *actin, alpha-tubulin, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GapDH), succinate dehydrogenase (SucDH), TATA-box binding protein (TBP), ubiquitin conjugating enzyme (UBC), 18S ribosomal RNA (18S), and 28S ribosomal RNA (28S)*. A normalisation factor was calculated based on the endogenous controls assessed by geNorm [51] according to Schwarzenberger et al., 2009 [52].

Forward and reverse primers for QPCR were established from the EST-database for five proteases that were found in the SDS-PAGEs (http://www.nematodes.org/NeglectedGenomes/ARTHROPODA/Crustacea.html [33]; Tab. 4). Real Time PCR with different concentrations of cDNA from six-day-old *D. magna* grown on *S. obliquus* was performed, and amplification efficiencies for the protease primers were calculated as according to Livak et al., 2001 [53] using the formula AE = 10^(-1/ slope), where AE is the amplification efficiency. qRT- PCR was performed as according to Schwarzenberger et al., 2009 [52]. *D. magna* fed with 100% *S. obliquus* served as calibrator, which was always set as 1.

Microcystin effect on the expression of serine proteases

To investigate the effect of microcystin on *Daphnia* gut proteases, 15 neonates of *Daphnia magna* Binnensee were grown on 2 mg C/ l in 1 l filtered pond water on either 100% *S. obliquus*, 10% PCC 7806 WT and 90% *S. obliquus* or on 20% PCC 7806 Mut and 90% *S. obliquus* for six days. Since the mortality after this time on 20% of the microcystin-containing WT was too high, *D. magna* were grown in the presence of 10% of either cyanobacterial strain. Each treatment was run in triplicate. The medium was exchanged daily. QPCR was conducted following Schwarzenberger et al., 2009 [52].

Expression of serine proteases after 24 h

15 neonates of *Daphnia magna* Binnensee were grown on 2 mg C/ l in 1 l filtered pond water on either 100% *S. obliquus* or on 20% NIVA Cya 43 and 80% *S. obliquus*. Each treatment was run in triplicate. RNA and proteins were extracted from half of the animals after 24 hours. Thereafter the remaining animals grown on the mixture with cyanobacteria and the animals grown on the green alga were further cultivated for another 24 hours on 100% *S. obliquus*. Again proteins and RNA were extracted, and Real Time PCR was conducted. The proteases were activity stained after SDS-PAGE.

Statistics

The statistics were conducted with the program Statistica 6.0. The data were analysed via one-way ANOVA and a post-hoc analysis (Tukey HSD). A Levene's Test was conducted to ensure homogenous variances. The data were ln (x+1) transformed when needed. The level of significance was $p < 0.05$.

Table 2 – results of LC-MS/MS analysis of *Daphnia* homogenate
D. magna were raised on 80% *S. obliquus* and 20% *M. aeruginosa* NIVA Cya 43. Depicted are the results of LC-MS/MS analysis (apparent molecular weight of the cut band, number of the matched peptides, sequence of the matched peptides, sequence coverage with the hits in the database, probability based mowse score, hit in the database and the function of the hits).

Band (apparent MW)	number of matched peptides	sequence of matched peptide	sequence coverage	Probability based mowse score	hit	function
75 kDa	6	LSSPLSLNTK	22%	33	610	trypsin
		TEHEAYSSR		[39]		
		TEHEAYSSR		46		
		SIDSGDEQYSDILSK		105		
		IVGGTQASPNEFPYQISLR		[54]		
		IVGGTQASPNEFPYQISLR		84		
	2	ISDTLR	6%	31	152	trypsin
		TDSGLEQNR		34		
34 kDa	20	AGEHSLR	45%	68	152	trypsin
		VVAGEHSL		40		
		GVDASILR		54		
		VAGEHSLR		66		
		VVAGEHSLR		88		
		IVGGTTVEPN		36		
		LDWIAANRG		42		
		TDSGLEQNR		55		
		AYGGTAANPEVYPS		35		
		AYGGTAANPEVYPSML		48		
		SGWGTTSSGGVISDTLR		[68]		
		SGWGTTSSGGVISDTLR		86		
		VSGWGTTSSGGVISDTLR		104		
		VVAGEHSLRTDSGLEQNR		30		
		IVGGTTVEPNSLPFQISLQ		79		
		IVGGTTVEPNSLPFQISLQ		[29]		
		IVGGTTVEPNSLPFQISLQR		94		
		GVASIIIHEDYRPLTFEND		49		
		GVASIIIHEDYRPLTFENDISL		22		
		GVASIIIHEDYRPLTFENDISLL		45		
	3	VSGLEQNR	7%	58	79	trypsin
		VVAGEHDLS		22		
		VVAGEHDLSQVSGLEQNR		57		
	1	TADGPGGISPTLQK	5%	73	448	chymotrypsin
32 kDa	22	AGEHSLR	42%	[27]	152	trypsin
		AGEHSLR		64		
		AGEHSLR		[28]		
		VVAGEHSL		37		
		GVDASILR		56		
		VAGEHSLR		62		
		LDWIAANR		51		
		VVAGEHSLR		67		
		IVGGTTVEPN		26		
		LDWIAANRG		43		
		TDSGLEQNR		69		
		RVVAGEHSLR		25		
		AYGGTAANPEVYPS		53		

		SFFLDWIAANRG		60		
		SVDVPVVDDDTCNR		22		
		GWGTTSSGGVISDTLR		97		
		IVGGTTVEPNSLPFQI		46		
		SGWGTTSSGGVISDTLR		101		
		SGWGTTSSGGVISDTLR		[61]		
		GEHSLRTDSGLEQNR		30		
		VVAGEHSLRTDSGLEQNR		49		
		IVGGTTVEPNSLPFQISLQR		72		
	12	AGEHSLR	18%	[27]	208	trypsin
		AGEHSLR		64		
		AGEHSLR		[28]		
		VVAGEHSL		37		
		GVDATILR		50		
		VAGEHSLR		62		
		VVAGEHSLR		67		
		TDSGLEQNR		69		
		RVVAGEHSLR		25		
		GEHSLRTDSGLEQNR		30		
		VVAGEHSLRTDSGLEQNR		49		
		IVGGTVVEPNSLPFQISLQR		45		
	6	LSVPSAK	21%	22	79	trypsin
		VSGLEQNR		58		
		VVAGEHDLS		25		
		LDWIAANR		51		
		IVGGTEVVPN		35		
		IVGGTEVVPNSLPFQISLQR		69		
	3	TADGPGGISPTLQK	13%	107	448	chymotrypsin
		YIHPDWNPNTLTGDVAL		32		
		YIHPDWNPNTLTGDVALIK		42		
	5	GANIDNLR	37%	47	239	serinprotease
		IVGGVEVVPN		38		
		SLPFQVSLQR		50		
		DAAHCIAGANIDNLR		37		
		DAAHCIAGANIDNLR		[27]		
	2	LDAADEPTR	5%	53	802	chymotrypsin
		LDAADEPTRVEVR		31		
	1	ITLGAHDR	3%	26	383	chymotrypsin
26 kDa	19	AGEHSLR	39%	64	208	trypsin
		GVDATILR		56		
		VVAGEHSLR		75		
		VVAGEHSLR		[70]		
		IVGGTVVEPN		37		
		LDWIATNR		45		
		PLDGNAAEAR		57		
		TDSGLEQNR		58		
		LDWIATNRV		47		
		SYFLDWIATNR		79		
		IVGGTVVEPNSLPF		35		
		IHEEYRPLTFEND		62		
		IHEEYRPLTFEND		[39]		
		IVGGTVVEPNSLPFQI		34		
		SGWGTTSSGGIISDELR		70		
		VVAGEHSLRTDSGLEQNR		70		

		IVGGTVVEPNSLPFQISLQR		71		
		IVGGTVVEPNSLPFQISLQR		[59]		
		IVGGTVVEPNSLPFQISLQR		[27]		
	8	AGEHSLR	23%	64	152	trypsin
		GVDASILR		50		
		VVAGEHSLR		75		
		VVAGEHSLR		[70]		
		TDSGLEQNR		58		
		SVDVPVVDDDTCNR		26		
		SGWGTTSSGGVISDTLR		40		
		VVAGEHSLRTDSGLEQNR		70		
	5	AWLYR	21%	23	149	serinprotease
		YAWLYR		25		
		IDYDYSEER		77		
		SETCDPAEPSVFTR		84		
		TDVETTPMGTFMGWGATVAGGGFSPR		43		
	4	LDAADEPTR	14%	42	802	chymotrypsin
		IINDVALIR		69		
		PSDDAAGISPVLR		66		
		LDAADEPTRVEVR		26		
	6	VSGLEQNR	17%	54	79	trypsin
		LDLSVPSAK		34		
		IYLTTPLDLSVPSAK		42		
		IVGGTEVVPNSLPFQISLQR		[44]		
		IVGGTEVVPNSLPFQISLQR		44		
		IVGGTEVVPNSLPFQISLQR		[25]		
	3	ITETERLEIR	16%	29	448	chymotrypsin
		TADGPGGISPTLQK		90		
		LSSYLSWISSITGL		33		
	1	TANEPSQVTVSTT	5%	46	383	chymotrypsin
24 kDa	47	AGEHSLR	58%	[44]	208	trypsin
		AGEHSLR		51		
		AGEHSLR		[20]		
		SGLEQNR		27		
		VVAGEHSL		31		
		GVDATILR		59		
		VAGEHSLR		[58]		
		VAGEHSLR		67		
		VVAGEHSLR		82		
		IVGGTVVEPN		69		
		LDWIATNR		50		
		TDSGLEQNR		55		
		TDSGLEQNR		[53]		
		IHEEYRPL		27		
		TDSGLEQNRG		39		
		LDWIATNRV		37		
		FLDWIATNR		55		
		IHEEYRPLT		38		
		YFLDWIATNR		61		
		SLRTDSGLEQNR		31		
		SYFLDWIATNR		87		
		SYFLDWIATNR		[28]		
		YFLDWIATNRV		36		
		SYFLDWIATNR		[57]		

		SYFLDWIATNR		[62]		
		IVGGTVVEPNSLPF		[26]		
		IVGGTVVEPNSLPF		[41]		
		IVGGTVVEPNSLPF		44		
		IHEEYRPLTFE		30		
		SYFLDWIATNRV		47		
		IHEEYRPLTFEN		52		
		IVGGTVVEPNSLPFQ		55		
		IVGGTVVEPNSLPFQ		[33]		
		TDSGLEQNRGVVSFK		31		
		IHEEYRPLTFEND		35		
		IVGGTVVEPNSLPFQI		51		
		SGWGTTSSGGIISDELR		102		
		SGWGTTSSGGIISDELR		[25]		
		IVGGTVVEPNSLPFQIS		62		
		SGGPLFVLPLDGNAAEAR		55		
		IHEEYRPLTFENDIS		69		
		SGWGTTSSGGIISDELRR		33		
		IVGGTVVEPNSLPFQISLQR		70		
		IVGGTVVEPNSLPFQISLQR		[44]		
		IHEEYRPLTFENDISLLF		44		
		IHEEYRPLTFENDISLLF		[22]		
		SALGSYSQSCGGSILDANVIIDAAHCVR		81		
	14	IINGAEATPH	41%	68	448	chymotrypsin
		LSSYLSWISS		35		
		DVYLGAHNVR		47		
		ITETERLEIR		40		
		TADGPGGISPTLQK		83		
		LPAPVDISGNNVR		54		
		LSSYLSWISSITGL		44		
		LPAPVDISGNNVRPI		[47]		
		LPAPVDISGNNVRPI		77		
		IINGAEATPHEFPW		61		
		IINGAEATPHEFPWVT		43		
		YIHPDWNPNTLTGDVA		40		
		IINGAEATPHEFPWVTA		48		
		YIHPDWNPNTLTGDVALIK		74		
	12	AWLYR	29%	23	149	serinprotease
		AGGGFSPR		42		
		KLDGVLR		29		
		VAGGGFSPR		61		
		YAWLYR		32		
		DYDYSEER		40		
		IDYDYSEER		66		
		SETCDPAEPSVFTR		71		
		GQVNLIDYDYSEER		98		
		AGQVNLIDYDYSEER		113		
		VLGELVVVAGQVNLIDYDYSEER		26		
		TDVETTPMGTFMGWGATVAGGGFSPR		63		
	15	AGEHSLR	19%	[44]	152	trypsin
		AGEHSLR		51		
		AGEHSLR		[20]		
		SGLEQNR		27		
		VVAGEHSL		31		
		VAGEHSLR		[58]		

		VAGEHSLR		67		
		VVAGEHSLR		82		
		TDSGLEQNR		55		
		TDSGLEQNR		[53]		
		TDSGLEQNRG		39		
		SFFLDWIAANR		28		
		SLRTDSGLEQNR		31		
		SFFLDWIAANRG		66		
		SGWGTTSSGGVISDTLR		58		
	12	SGLEQNR	20%	27	79	trypsin
		VSGLEQNR		51		
		IVGGTEVVPN		47		
		SYFLDWIAANR		80		
		IVGGTEVVPNSLPF		21		
		IVGGTEVVPNSLPFQ		35		
		IVGGTEVVPNSLPFQI		24		
		IVGGTEVVPNSLPFQIS		34		
		VVAGEHDLSQVSGLEQNR		[51]		
		VVAGEHDLSQVSGLEQNR		110		
		IVGGTEVVPNSLPFQISLQR		41		
		IVGGTEVVPNSLPFQISLQR		[33]		
	3	LDAADEPTR	9%	45	802	chymotrypsin
		IINDVALIR		57		
		LDAADEPTRVEVR		31		
	3	IVGGVEAVPH	13%	30	383	chymotrypsin
		FSITLGAHDR		27		
		TADGILEGVSPV		31		
22 kDa	33	TGDVALIK	49%	54	448	chymotrypsin
		LSSYLSW		25		
		YLGAHNVR		39		
		LSSYLSWI		34		
		IINGAEATPH		66		
		TLTGDVALIK		42		
		LSSYLSWIS		30		
		ITETERLEI		52		
		LSSYLSWISS		47		
		DVYLGAHNVR		61		
		TETERLEIR		33		
		TADGPGGISPTLQ		34		
		LSSYLSWISSI		53		
		ITETERLEIR		[32]		
		ITETERLEIR		35		
		TADGPGGISPTLQK		62		
		LPAPVDISGNNVR		75		
		YIHPDWNPNTL		33		
		SSAGCESGNPDGYAR		98		
		SSAGCESGNPDGYAR		[27]		
		LPAPVDISGNNVRPI		[60]		
		LPAPVDISGNNVRPI		74		
		LYFDVYLGAHNVR		21		
		YIHPDWNPNTLTGDV		75		
		IINGAEATPHEFPWVT		55		
		YIHPDWNPNTLTGDVA		51		
		YIHPDWNPNTLTGDVA		[34]		
		IINGAEATPHEFPWVTA		53		

		YIHPDWNPNTLTGDVAL		45		
		YIHPDWNPNTLTGDVAL		[44]		
		YIHPDWNPNTLTGDVALIK		116		
		YIHPDWNPNTLTGDVALIK		[23]		
		ANEKYIHPDWNPNTLTGDVALI		22		
	8	AGEHSLR	13%	51	208	trypsin
		VVAGEHSL		29		
		VAGEHSLR		[22]		
		VAGEHSLR		58		
		VVAGEHSLR		79		
		TDSGLEQNR		80		
		IVGGTVVEPNSLPFQI		39		
		IVGGTVVEPNSLPFQI		[20]		
	7	WIAANR	9%	24	152	trypsin
		AGEHSLR		51		
		VVAGEHSL		29		
		VAGEHSLR		[22]		
		VAGEHSLR		58		
		VVAGEHSLR		79		
		TDSGLEQNR		80		
	2	IVGGVEAVPHEFPWQVA	16%	30	383	chymotrypsin
		TYTVHPGWNPSTLADDIALIR		45		
	2	WIAANR	5%	24	79	trypsin
		VSGLEQNR		43		
	2	LDAADEPTR	5%	21	802	chymotrypsin
		LDAADEPTRVEVR		22		
	1	DYDYSEER	3%	34	149	trypsin
21 kDa	34	ITETERL	45%	29	448	chymotrypsin
		RITETER		33		
		IINGAEATPH		75		
		LSSYLSWIS		32		
		LSSYLSWISS		51		
		TETERLEIR		32		
		TADGPGGISPTLQ		65		
		LSSYLSWISSI		73		
		ITETERLEIR		[24]		
		ITETERLEIR		34		
		ITETERLEIR		[27]		
		ITETERLEIR		[24]		
		TADGPGGISPTLQK		71		
		TADGPGGISPTLQK		[50]		
		LPAPVDISGNNVR		85		
		LPAPVDISGNNVR		[43]		
		AGCESGNPDGYAR		47		
		YIHPDWNPNTL		30		
		SSAGCESGNPDGYAR		94		
		LPAPVDISGNNVRPI		83		
		LPAPVDISGNNVRPI		[58]		
		VSSAGCESGNPDGYAR		91		
		IINGAEATPHEFPWVT		55		
		YIHPDWNPNTLTGDVA		59		
		YIHPDWNPNTLTGDVA		[37]		
		IINGAEATPHEFPWVTA		66		
		SFVSSAGCESGNPDGYAR		110		

		SFVSSAGCESGNPDGYAR		[71]		
		YIHPDWNPNTLTGDVAL		63		
		YIHPDWNPNTLTGDVAL		[40]		
		YIHPDWNPNTLTGDVALI		58		
		YIHPDWNPNTLTGDVALI		[28]		
		YIHPDWNPNTLTGDVALIK		31		
		IINGAEATPHEFPWVTALFI		35		
	15	IVGGVEAVPH	40%	58	383	chymotrypsin
		IVGGVEAVPH		[29]		
		TANEPSQVTV		55		
		TLADDIALIR		77		
		TANEPSQVTVS		50		
		TADGILEGVSPV		41		
		AGCADGFPAGFTR		87		
		TANEPSQVTVSTT		57		
		VSSYSQWIADTTGL		103		
		TADGILEGVSPVLMK		35		
		TANEPSQVTVSTTTY		65		
		VSSYSQWIADTTGLI		89		
		IVGGVEAVPHEFPWQV		44		
		IVGGVEAVPHEFPWQVA		46		
		TYTVHPGWNPSTLADDIALIR		72		
	7	LDAADEPTR	25%	60	802	chymotrypsin
		IINDVALIR		77		
		VSYFADWISSV		49		
		PSDDAAGISPVLR		75		
		LDAADEPTRVEVR		[26]		
		LDAADEPTRVEVR		27		
		STEYTVHPDWGPVR		33		
	4	WIAANR	9%	32	152	trypsin
		VAGEHSLR		39		
		VVAGEHSLR		52		
		TDSGLEQNR		48		
	3	FDQYEATTQK	16%	83	638	chymotrypsin
		IVGWGATFEGGAPATR		[47]		
		IVGWGATFEGGAPATR		75		
20 kDa	26	IALIR	61%	37	383	chymotrypsin
		TLGAHDR		52		
		ADDIALIR		71		
		SITLGAHDR		62		
		IVGGVEAVPH		70		
		TANEPSQVTV		54		
		TLADDIALIR		77		
		FSITLGAHDR		[31]		
		FSITLGAHDR		61		
		TANEPSQVTVS		55		
		TADGILEGVSPV		41		
		AGCADGFPAGFTR		77		
		TANEPSQVTVSTT		45		
		TANEPSQVTVSTTT		53		
		VSSYSQWIADTTGL		103		
		TADGILEGVSPVLMK		62		
		TADGILEGVSPVLMK		[58]		
		TANEPSQVTVSTTTY		70		

		VSSYSQWIADTTGLI		88		
		TANEPSQVTVSTTTYT		54		
		IVGGVEAVPHEFPWQVA		40		
		IVGGVEAVPHEFPWQVA		[28]		
		GSCNGDSGGPLSFDNAGVY		63		
		TVHPGWNPSTLADDIALIR		80		
		CLAPSTESNHVGDTLLVSGWGK		55		
		TYTVHPGWNPSTLADDIALIR		38		
	25	ITETERL	43%	36	448	chymotrypsin
		IINGAEATPH		84		
		TLTGDVALIK		22		
		DVYLGAHNVR		58		
		TETERLEIR		23		
		TADGPGGISPTLQ		43		
		ITETERLEIR		54		
		GCESGNPDGYAR		33		
		TADGPGGISPTLQK		73		
		TADGPGGISPTLQK		[50]		
		LPAPVDISGNNVR		83		
		AGCESGNPDGYAR		30		
		YIHPDWNPNTL		30		
		SSAGCESGNPDGYAR		85		
		LPAPVDISGNNVRPI		79		
		VSSAGCESGNPDGYAR		86		
		IINGAEATPHEFPWVT		37		
		YIHPDWNPNTLTGDVA		21		
		IINGAEATPHEFPWVTA		55		
		SFVSSAGCESGNPDGYAR		110		
		SFVSSAGCESGNPDGYAR		[70]		
		YIHPDWNPNTLTGDVALI		[37]		
		YIHPDWNPNTLTGDVALI		41		
		YIHPDWNPNTLTGDVALIK		86		
		YIHPDWNPNTLTGDVALIK		[51]		
	9	IVGGTEAVPH	29%	72	802	chymotrypsin
		IINDVALIR		67		
		AADEPTRVEVR		21		
		VSYFADWISSV		56		
		PSDDAAGISPVLR		91		
		LDAADEPTRVEVR		34		
		LDAADEPTRVEVR		[24]		
		STEYTVHPDWGPVR		[32]		
		STEYTVHPDWGPVR		50		
	5	FDQYEATTQK	35%	84	638	chymotrypsin
		GWGATFEGGAPATR		86		
		IVGWGATFEGGAPATR		85		
		QDQHICGGFIYNDR		26		
		RYDEIDELAQPWEAK		37		
	1	IVGGTEAVPN	4%	45	460	trypsin
19 kDa	29	LGSHNVR	65%	22	802	chymotrypsin
		NDVALIR		38		
		IINDVALI		41		
		LLGSHNVR		52		
		LDAADEPTR		60		
		VSYFADWI		41		

		IINDVALIR		[30]		
		IINDVALIR		71		
		IINDVALIR		[24]		
		STEYTVHPD		27		
		VSYFADWIS		36		
		NILLGSHNVR		55		
		NILLGSHNVR		[27]		
		GSCNGDSGGPLSF		67		
		VSYFADWISSV		68		
		PSDDAAGISPVLR		94		
		PSDDAAGISPVLR		[43]		
		LDAADEPTRVEVR		47		
		SSAGCEVGLPAGFAR		77		
		GKPSDDAAGISPVLR		[25]		
		GKPSDDAAGISPVLR		31		
		GSSAGCEVGLPAGFAR		83		
		LPNPIEFTPEIQPI		58		
		STEYTVHPDWGPVR		56		
		STEYTVHPDWGPVR		[32]		
		VSYFADWISSVTGLV		80		
		IVGGTEAVPHSAPWQVA		38		
		SFGSSAGCEVGLPAGFAR		61		
		EVDVPCISNAECADTY		50		
	19	TLGAHDR	58%	40	383	chymotrypsin
		SITLGAHDR		63		
		IVGGVEAVPH		84		
		FSITLGAHDR		71		
		TANEPSQVTVS		39		
		GSCNGDSGGPLSF		67		
		AGCADGFPAGFTR		68		
		TANEPSQVTVSTT		61		
		LPSPVAFTPEIAPI		47		
		VSSYSQWIADTTGL		90		
		TADGILEGVSPVLMK		[71]		
		TADGILEGVSPVLMK		86		
		TADGILEGVSPVLMK		[54]		
		TANEPSQVTVSTTTY		73		
		TANEPSQVTVSTTTYT		66		
		IVGGVEAVPHEFPWQVA		38		
		GSCNGDSGGPLSFDNAGVY		50		
		TVHPGWNPSTLADDIALIR		[53]		
		TVHPGWNPSTLADDIALIR		97		
	5	IINGAEATPH	27%	57	448	chymotrypsin
		TADGPGGISPTLQK		88		
		LPAPVDISGNNVRPI		67		
		IINGAEATPHEFPWVTA		50		
		YIHPDWNPNTLTGDVALIK		50		
	1	IVGGTEAVPN	4%	63	460	trypsin
18 kDa	47	IINDVALI	62%	28	802	chymotrypsin
		AAGISPVLR		40		
		LLGSHNVR		[45]		
		LLGSHNVR		64		
		LDAADEPTR		53		
		LDAADEPTR		[30]		
		LDAADEPTR		[21]		

		LDAADEPTR		[49]		
		DAAGISPVLR		56		
		VSYFADWI		57		
		IINDVALIR		[73]		
		IINDVALIR		[35]		
		IINDVALIR		[70]		
		IINDVALIR		[26]		
		IINDVALIR		80		
		IINDVALIR		[62]		
		IINDVALIR		[60]		
		STEYTVHPD		44		
		GNICVDTTGGK		63		
		NILLGSHNVR		34		
		NILLGSHNVR		[29]		
		TVHPDWGPVR		35		
		AADEPTRVEVR		25		
		AADEPTRVEVR		[21]		
		GSCNGDSGGPLSF		83		
		VSYFADWISSV		72		
		PSDDAAGISPVLR		96		
		PSDDAAGISPVLR		[62]		
		LDAADEPTRVEVR		39		
		LDAADEPTRVEVR		[31]		
		SSAGCEVGLPAGFAR		105		
		GKPSDDAAGISPVLR		71		
		GSSAGCEVGLPAGFAR		79		
		VSYFADWISSVTGL		[47]		
		VSYFADWISSVTGL		[50]		
		VSYFADWISSVTGL		70		
		LPNPIEFTPEIQPI		66		
		STEYTVHPDWGPVR		[20]		
		STEYTVHPDWGPVR		43		
		VSYFADWISSVTGLV		88		
		VSYFADWISSVTGLV		[28]		
		VSYFADWISSVTGLV		[72]		
		IVGGTEAVPHSAPWQV		50		
		VSYFADWISSVTGLV		[39]		
		VSYFADWISSVTGLV		[74]		
		STEYTVHPDWGPVR		[31]		
		IVGGTEAVPHSAPWQVA		58		
	6	GSCNGDSGGPLSF	32%	83	383	chymotrypsin
		VSSYSQWIADTTGL		87		
		TADGILEGVSPVLMK		71		
		TADGILEGVSPVLMK		[60]		
		TANEPSQVTVSTTTY		76		
		TVHPGWNPSTLADDIALIR		29		
	2	TADGPGGISPTLQK	13%	88	448	chymotrypsin
		YIHPDWNPNTLTGDVALIK		33		
	1	IVGGTEAVPN	4%	33	460	trypsin
17 kDa	20	LLGSHNVR	43%	55	802	chymotrypsin
		LDAADEPTR		[40]		
		LDAADEPTR		53		
		LDAADEPTR		[25]		
		IINDVALIR		71		
		IINDVALIR		[65]		

		NILLGSHNVR		21		
		GSCNGDSGGPLSF		41		
		PSDDAAGISPVLR		[48]		
		PSDDAAGISPVLR		90		
		PSDDAAGISPVLR		[50]		
		LDAADEPTRVEVR		[32]		
		LDAADEPTRVEVR		40		
		GSSAGCEVGLPAGFAR		78		
		VSYFADWISSVTGL		[52]		
		VSYFADWISSVTGL		77		
		STEYTVHPDWGPVR		37		
		VSYFADWISSVTGLV		[70]		
		VSYFADWISSVTGLV		[75]		
		VSYFADWISSVTGLV		105		
	3	FSITLGAHDR	16%	22	383	chymotrypsin
		GSCNGDSGGPLSF		41		
		TADGILEGVSPVLMK		37		
	1	TADGPGGISPTLQK	5%	58	448	chymotrypsin

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Part III:

Response of *Daphnia* to cyanobacterial protease inhibitors: intra-specific differences in digestive target proteases

Abstract

Background

Cyanobacterial blooms have been claimed to be a major factor leading to the summer-decline of the most important freshwater herbivores, i.e. representatives of the genus *Daphnia*. In nearly every cyanobacterial bloom protease inhibitors have been found; among them are trypsin- and chymotrypsin-inhibitors, that have been shown to specifically inhibit *Daphnia*'s digestive proteases *in vitro* and *in situ.* One *D. magna* genotype has been shown to specifically respond to dietary cyanobacteria, which contain protease inhibitors, with the increase of trypsin and chymotrypsin activity, with the up-regulation of protease gene-expression and with the induction of protease isoforms. Here we investigated whether these responses and the sensitivity to inhibitors differ between different *D. magna* genotypes. Variability in sensitivity to cyanobacterial protease inhibitors between clones would support the suggestion that this variability also exists in natural populations. Variability in populations would then constitute the basis for a positive selection due to protease inhibitors to less sensitive *Daphnia* clones.

Results

Five different *D. magna* clones showed different relative growth rate reductions due to protease-inhibitor-containing cyanobacterial food indicating different sensitivities of the five *D. magna* clones to two strains of *M. aeruginosa*, which either contained trypsin- or chymotrypsin-inhibitors. Four clones were more sensitive to the cyanobacterium with trypsin inhibitors than to the one with chymotrypsin inhibitors. In the fifth *D. magna* genotype, however, an inverse pattern of sensitivity compared to the other clones was observed. The *D. magna* clones showed diverse physiological changes after exposure to two types of dietary protease inhibitors: In all *D. magna* clones increased protease activity was measured, quantification by real-time PCR demonstrated changes in protease gene-expression, and the induction of protease isoforms was revealed by activity-stained SDS-PAGEs. These physiological changes

differed between the *D. magna* clones. Also the amino acid sequence of two trypsins showed intra-specific differences.

Conclusions

Strong intra-specific differences in sensitivity of five *D. magna* clones to two dietary protease inhibitor types, i.e. trypsin and chymotrypsin inhibitors, were found. The degrees of sensitivity depended on the type of protease inhibitor as well as on the *Daphnia magna* clone exposed to these inhibitors. All *D. magna* clones investigated showed specific physiological responses to dietary trypsin and chymotrypsin inhibitors. The kind and extent of these changes in protease expression probably determined the differences in relative growth rate reductions in the five *D. magna* clones in the presence of the dietary cyanobacterial protease inhibitors. Evidence suggests that the relative growth rate reduction depended on the residual activity of the inhibited protease type. The five *D. magna* clones differed in the amino acid sequence of two trypsins and in their protease band pattern in SDS-PAGE with clone P showing a unique protease or protease isoform. A similarly high variability in sensitivity to protease inhibitors, as was found here, within a natural population, holds the potential for positive selection to less sensitive *Daphnia* due to protease inhibitors in nature.

Background

Due to increasing nutrient input [1,2] cyanobacterial mass developments have become a common phenomenon in lakes. These mass developments are often associated with a summer-decline in *Daphnia* biomass. The summer-decline of *Daphnia*, the major herbivore in lakes, due to the increase in cyanobacterial biomass has been shown in several field studies [3,4]. Laboratory studies, which have demonstrated negative effects of cyanobacteria on *Daphnia* [5,6], have supported these field observations. However, the generality of this negative correlation between cyanobacterial and *Daphnia* biomass has recently been questioned in an experimental [7] and in field studies [8-10] that demonstrated that *Daphnia* have the potential to adapt to increasingly tolerate dietary cyanobacteria.

Cyanobacteria are known to contain toxins and many other biologically active secondary metabolites [11,12]. One important group of these metabolites are cyanobacterial protease inhibitors, which have been found in nearly every cyanobacterial bloom [12,13] and are thus among the most widely spread secondary

metabolites of cyanobacteria. Different protease inhibitors have been isolated from different cyanobacterial genera [14] as well as from different cyanobacterial strains of the same species [15,16] indicating that the synthesis of protease inhibitors is wide-spread in cyanobacteria. These protease inhibitors act against serine proteases like trypsins and chymotrypsins [Dissertation Part II,17], which are the most important digestive proteases in the gut of *Daphnia magna* [18]. A surprisingly high number of trypsins and chymotrypsins was found in the genome of *D. pulex* [19], a closely related species of *D. magna*. In *D. magna* these trypsins and chymotrypsins have been shown to be specifically inhibited by cyanobacterial protease inhibitors [17]. Cyanobacterial protease inhibitors thus putatively are the first defence of cyanobacteria against *Daphnia*: Protease inhibitors should affect gut proteases of *Daphnia* immediately after ingestion of the cyanobacterial food particles and the subsequent release of inhibitors during digestion, before other secondary metabolites, e.g. microcystins, come into contact with their targets. The effects of the specific interference of cyanobacterial inhibitors with digestive proteases of *D. magna* have been investigated so far only in a single *D. magna* clone [Dissertation Part II]. This clone has been shown to specifically respond to dietary cyanobacteria, which contain protease inhibitors, with the increase of trypsin and chymotrypsin activity, with the up-regulation of protease gene-expression and with the induction of protease isoforms [Dissertation Part II], which should increase *Daphnia*'s ability to digest cyanobacteria.

However, different *Daphnia* clones, i.e. genotypes, have been shown to differ in survival [20], growth [21] and reproduction [22,23] after exposure to cyanobacteria. Unfortunately, the underlying mechanisms of these clonal differences haven't been addressed until now.

Many cyanobacterial secondary metabolites have been shown to result in growth reduction in *Daphnia* [24,25]. In nature the protease inhibitors are among the most frequent cyanobacterial secondary metabolites and have been found in nearly every cyanobacterial bloom [12,13]. Here we tested the effects of dietary cyanobacterial protease inhibitors on five different *D. magna* genotypes, which were fed mixtures of a green alga with two different strains of the cyanobacterium *Microcystis aeruginosa* that either contained trypsin- or chymotrypsin inhibitors. Thus we were able to independently determine the effects of the two types of inhibitors on the different *D. magna* clones. We investigated the effects of cyanobacterial trypsin and

chymotrypsin-inhibitors on proteases of the *D. magna* clones by activity stained SDS-PAGE and by measuring specific trypsin and chymotrypsin activities. Since only recently the active trypsins and chymotrypsins of *D. magna* have been assigned to protease genes via LC-MS/MS and subsequent database search [Dissertation Part II], we could also quantify the gene-expression of single protease genes. Clonal differences in sensitivity to cyanobacterial protease inhibitors were related to changes in protease activity and gene-expression of *Daphnia* proteases, with the aim to identify the physiological mechanisms of differences in *Daphnia* sensitivity.

Results

Microsatellites

The six tested microsatellite loci proved to be suitable for the genetic differentiation of the five tested *D. magna* clones (Tab. 1). At least in two loci all clones were distinguished from each other so that the five *D. magna* clones represent five different genotypes.

Growth rates and relative growth rate reductions

Five *D. magna* clones were grown on three different food treatments; either on the reference food 100% *S. obliquus*, or on 20% *M. aeruginosa* NIVA Cya 43 that contains strong chymotrypsin inhibitors [15] and 80% *S. obliquus*, or on 20% *M. aeruginosa* PCC7806 Mut that contains trypsin inhibitors [17] and 80% *S. obliquus*.

A two-way-ANOVA showed that there was a significant effect of food treatment, a significant clone effect and a significant effect of interaction of the factors “food” and “clone” (Tab. 2), which demonstrates that food effects were genotype dependent.

Growth on the reference food *S. obliquus* did not differ between the clones A, B, C and P and growth of clone P and clone W were not different from each other (Fig. 1a; $p > 0.16$; Tukey HSD after one-way ANOVA: $F_{4,10} = 7.28$). However, clone W differed significantly from clones A, B and C ($p < 0.05$; Tukey HSD after one-way ANOVA). Due to these subtle differences in growth on the reference food, the effects of cyanobacterial food were calculated as relative growth rate reductions.

Significant differences in sensitivity to the chymotrypsin-inhibitor-containing *M. aeruginosa* NIVA Cya 43 (one-way ANOVA: $F_{4,10} = 5.64$; $p < 0.05$), as well upon feeding on the trypsin-inhibitor containing *M. aeruginosa* PCC7806 Mut (one-way ANOVA: $F_{4,10} = 54.27$; $p < 0.05$) were observed between the genotypes (Fig. 1b).

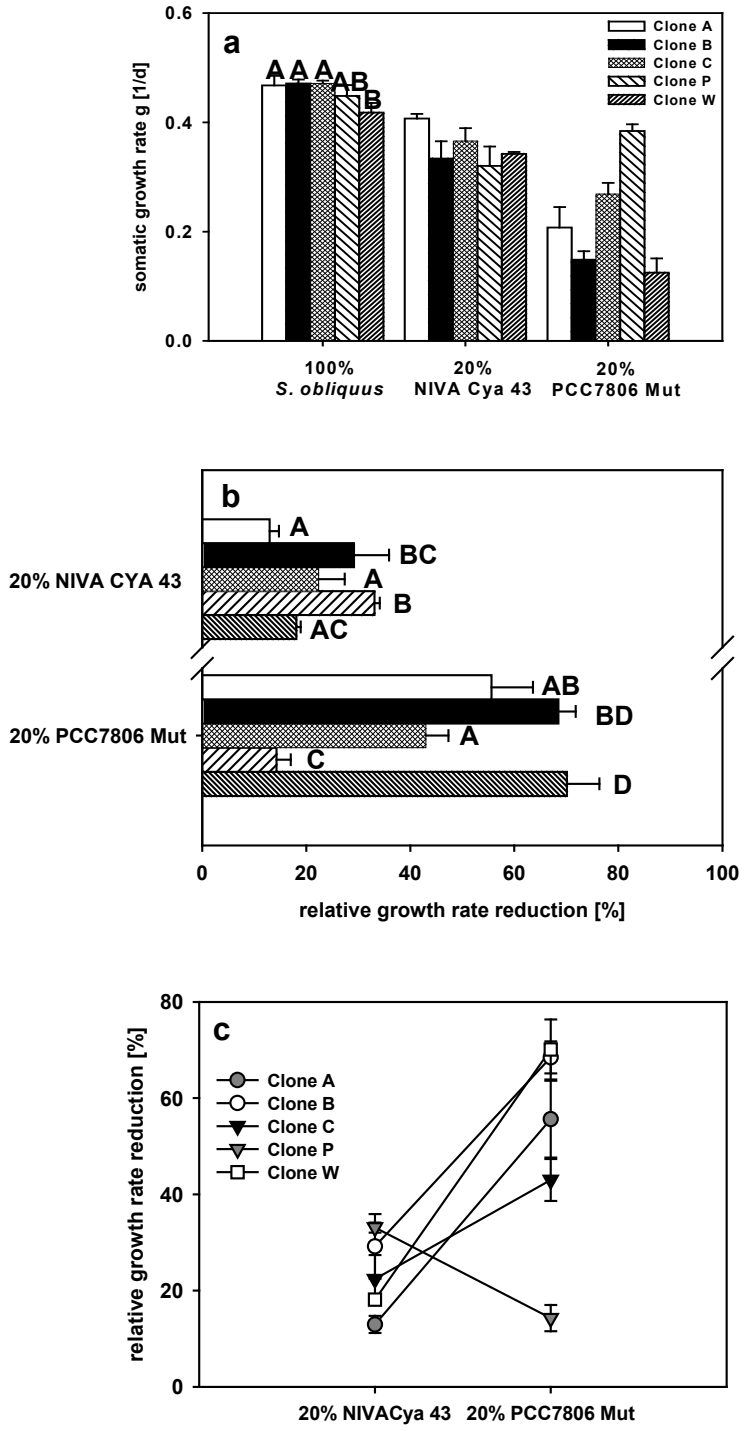


Figure 1 - Somatic growth and growth rate reduction of five *D. magna* clones exposed to 3 different food treatments

a) Somatic growth rates (mean + SD, n = 3) of five *D. magna* clones fed with either 100% *S. obliquus*, or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus*; different letters indicate a significant difference ($p < 0.05$; Tukey HSD after one-way ANOVA) between clones fed with 100% *S. obliquus*.; b/ c) Relative growth rate reductions (mean + SD, n = 3) of the same clones due to growth on the mixtures with *M. aeruginosa* in relation to the growth on 100% *S. obliquuus*. b) Different letters indicate a significant difference ($p < 0.05$; Tukey HSD after one-way ANOVA) between clones fed 20% *M. aeruginosa* NIVA Cya 43 ("20% NIVA Cya 43"), or between clones fed 20% *M. aeruginosa* PCC7806 Mut ("20% PCC7806 Mut"). c) Lines represent reaction norms of single clones (mean ± SD, n = 3) between the relative growth rate reductions due to either cyanobacterial food. Crossed lines between *D. magna* clones indicate an inverse sensitivity of these clones to the two different dietary *M. aeruginosa* strains.

A two-way ANOVA revealed significantly different effects of each of the two cyanobacterial strains on the relative growth rate reductions and that these effects depended on the *D. magna* genotype investigated (Tab. 3). The clones A, B, C and W had a significantly higher growth rate reduction on the trypsin-inhibitor containing strain *M. aeruginosa* PCC7806 Mut than on the chymotrypsin-inhibitor containing strain *M. aeruginosa* NIVA Cya 43 (Tukey HSD after two-way ANOVA; $p < 0.05$), whereas clone P showed a much lower relative growth rate reduction on *M. aeruginosa* PCC7806 Mut than on *M. aeruginosa* NIVA Cya 43 (Tukey HSD after two-way ANOVA; $p < 0.05$); indicating substantially lower sensitivity of clone P against trypsin inhibitors than against chymotrypsin inhibitors.

Interestingly the strikingly low sensitivity against *M. aeruginosa* PCC7806 of clone P was not associated with high sensitivity of *D. magna* clone P to the chymotrypsin-inhibitor containing strain *M. aeruginosa* NIVA Cya 43.

Nevertheless, Clone P showed an inverse pattern of sensitivity to the cyanobacterial strains compared to the other clones (Fig. 1c): while the relative growth rate reductions of *D. magna* clones A, B, C and W due to *M. aeruginosa* PCC7806 Mut lay in a higher range than those due to *M. aeruginosa* NIVA Cya 43, the contrary was true for clone P; hence the reaction norm of clone P crossed the reaction norms of clones A, B, C and W (Fig. 1c).

Activity of digestive proteases from the five *D. magna* clones in three different food treatments

The chymotrypsin activity of the five clones fed the reference food *S. obliquus* ranged between 60 and 218 µmol pNA/ min/ µg protein (Fig. 2a). The trypsin activity of the five clones grown on the same food ranged between 9 and 26 µmol pNA/ min/ µg protein (Fig. 2b). Nevertheless, the effects of cyanobacterial food treatments (20% *M. aeruginosa* NIVA Cya 43, or 20% *M. aeruginosa* PCC7806 Mut) were comparable between all *D. magna* clones: Chymotrypsin and trypsin activities of the *D. magna* gut proteases all were significantly different in the three food treatments within each single clone (specific chymotrypsin activities, Fig. 2a: clone A: Tukey HSD after one-way ANOVA: $F_{2,6} = 16080.3$; $p < 0.05$; clone B: Tukey HSD after one-way ANOVA: $F_{2,6} = 2302.98$; $p < 0.05$; clone C: Tukey HSD after one-way ANOVA: $F_{2,6} = 1831.7$; $p < 0.05$; clone P: Tukey HSD after one-way ANOVA: $F_{2,6} = 1304.1$; $p < 0.05$; clone W: Tukey HSD after one-way ANOVA: $F_{2,6} = 365.4$; $p < 0.05$; specific trypsin activities, Fig. 2b: clone A: Tukey HSD after one-way ANOVA: $F_{2,6} = 971.2$; $p < 0.05$; clone B:

Name of primer-pair	Dma 3R	Dma 12	Dma 14	166	S6-38	Dma15
Accession number	AF291910	AF291912	AF291913	wfms0000166		EU131363
Database	NCBI	NCBI	NCBI	wFleabase		NCBI
Annealing temperatures	58°C	58°C	58°C	58°C	49°C	50°C
Clone A (size of alleles)	186/186	136/136		224/224	110/110	130/132
Clone B (size of alleles)	182/186	144/144	200/200	224/226	110/112	130/132
Clone C (size of alleles)	186/186	136/ 144	198/200	222/226	110/110	130/132
Clone P (size of alleles)	186/189	138/142	198/198	224/224	110/110	130/130
Clone W (size of alleles)	182/189	144/144	198/200	224/226	110/112	130/132

Table 1 - Microsatellite analysis of the five *Daphnia magna* clones investigated.
Microsatellite loci with their accession numbers applied for five different *D. magna* clones. Figures separated by a slash indicate the size of the two alleles per microsatellite in each clone. Databases: NCBI (http://www.ncbi.nlm.nih.gov/), wFleabase (http://www.wfleabase.org/ [19]).

Tukey HSD after one-way ANOVA: $F_{2,6} = 981.96$; $p < 0.05$; clone C: Tukey HSD after one-way ANOVA: $F_{2,6} = 482.3$; $p < 0.05$; clone P: Tukey HSD after one-way ANOVA: $F_{2,6} = 1524.7$; $p < 0.05$; clone W: Tukey HSD after one-way ANOVA: $F_{2,6} = 2176.7$; $p < 0.05$).

In the treatment with 20% *M. aeruginosa* NIVA Cya 43, that contains strong chymotrypsin inhibitors [15], the chymotrypsin activity of digestive proteases of all clones decreased in comparison to the reference food (Fig. 2a), whereas the trypsin activity increased (Fig. 2b).

In the treatment with 20% *M. aeruginosa* PCC7806 Mut, that contains trypsin inhibitors [17], the trypsin activity decreased in all clones and was not even measureable in clone B (Fig. 2b). Simultaneously, the chymotrypsin activity of clones A, B, C and P increased (Fig. 2a). However, in clone P the chymotrypsin activity in the 20% PCC7806 Mut decreased slightly but significantly (0.8-fold; Fig. 2a).

Relation of relative growth rate reductions with specific protease activity

To investigate, whether the differences in relative growth rate reduction in the five *D. magna* clones indicating variability in sensitivity were the result of actual enzymatic activity of trypsins and chymotrypsins in *Daphnia,* both variables were related with each other.

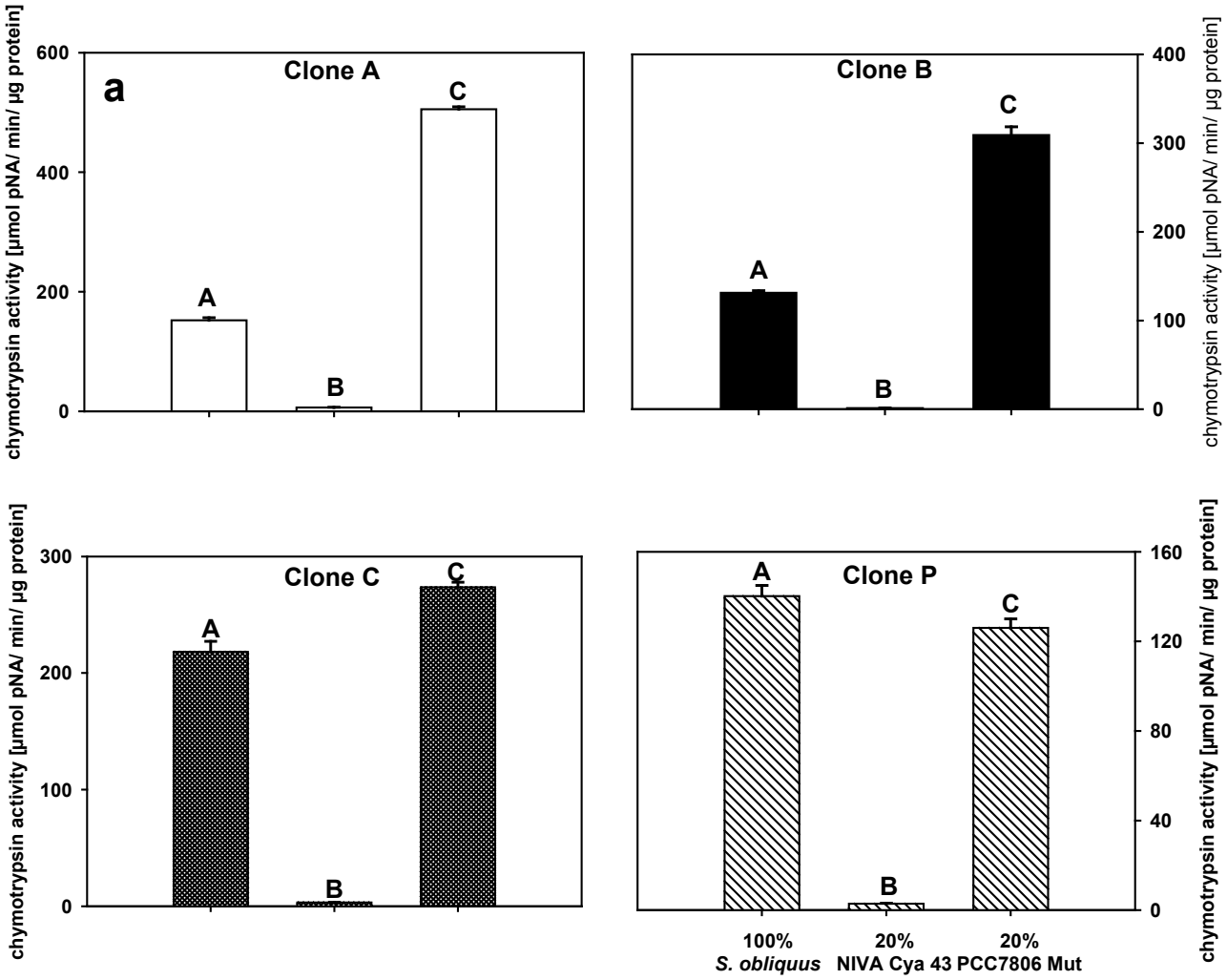
The relative growth rate reduction of the five *D. magna* clones grown on 20% *M. aeruginosa* NIVA Cya 43 showed a conspicuous but not significant negative trend (y

= -0.1315x + 5.9235; $F_{1,3}$ = 1.05, p = 0.38, R^2 = 0.26) with the specific chymotrypsin activity of the respective clones (Fig. 3a). The relative growth rate reduction observed in response to dietary chymotrypsin-inhibitors from 20% *M. aeruginosa* NIVA Cya 43 could not be explained by the trypsin activity of the *D. magna* clones ($F_{1,3}$ = 0.44, p = 0.55, R^2 = 0.13; Fig. 3b). When grown on the trypsin-inhibitor-containing strain *M. aeruginosa* PCC7806, the relative growth rate reduction of the five *D. magna* clones could not be explained by the specific chymotrypsin activity ($F_{1,3}$ = 0.25, p = 0.7, R^2 = 0.008; Fig. 3c). However, a conspicuous negative trend was observed for the relation of growth rate reduction with specific trypsin activity; since clone P differed phenotypically from the other four clones, as it had a unique protease at 55 kDa and was the only *D. magna* clone that showed a decreased chymotrypsin activity in the presence of dietary trypsin inhibitors, clone P was excluded from the linear correlation, which then became significant ($F_{1,2}$ = 23.65, p = 0.04, R^2 = 0.92; y = -0.0322x + 0.7009; Fig. 3d). However, this linear correlation was then only valid for clones without the above described characteristics of clone P.

SDS-PAGEs of five *D. magna* clones grown on different food treatments

With SDS-PAGE followed by activity staining, active protease bands of all *D. magna* clones became visible as white bands on the gels. Using LC-MS/MS the bands between 24 and 75 kDa have previously been identified as trypsins, the bands between 17 and 23 kDa as chymotrypsins [17,Dissertation Part II]. According to the protease band patterns in the 100% *S. obliquus* food treatment, the distribution of proteases of the homogenates of the five *D. magna* clones can be grouped into three different patterns (Fig. 4): The pattern of *D. magna* clone A matched with that of clone C, and the pattern of clone B matched with that of clone W. Clone P had a unique band pattern with one band at 55 kDa, which was absent in the other clones.

In the 20% *M. aeruginosa* NIVA Cya 43 treatment (Fig. 4A), the visible activity of the trypsin bands in general increased, while the activity of the chymotrypsin bands in general decreased. The chymotrypsin band at 22 kDa was an exception, as it increased in activity (Fig. 4A). In addition to changes in activity, a change in the band pattern of chymotrypsins due to 20% *M. aeruginosa* NIVA Cya 43, similar to the one described before for clone B [Dissertation Part II], was observed in *D. magna* clone B and W (Fig. 4A). This shift of the chymotrypsin band pattern is indicated by the lower position of asterisks (clone B) and arrows (clone W) in the SDS-PAGE of animals fed


a
Clone A
Clone B
Clone C
Clone P
Clone W
chymotrypsin activity [µmol pNA/ min/ µg protein]
A
B
C
100%
20%
20%
S. obliquus
NIVA Cya 43 PCC7806 Mut

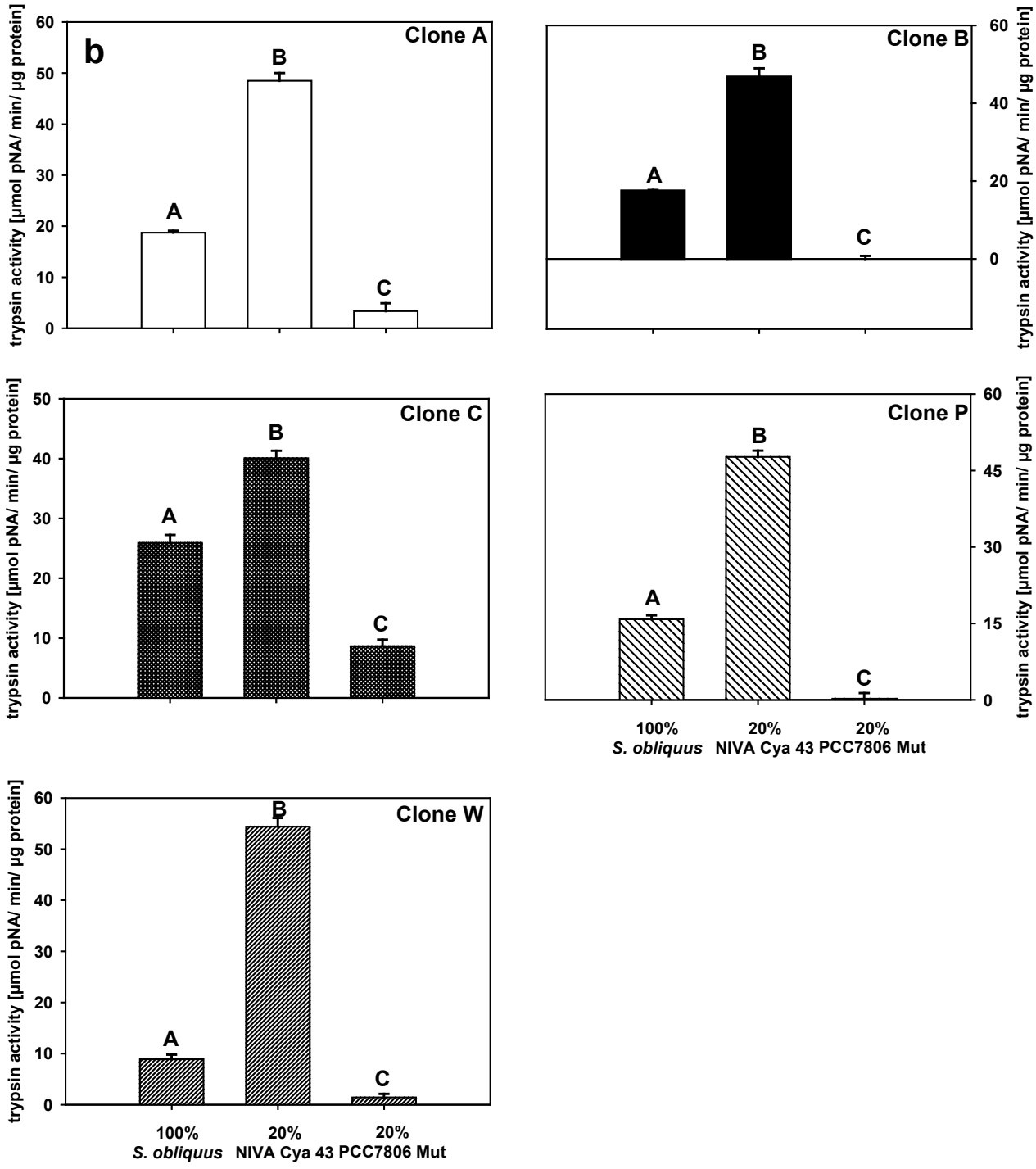


Figure 2 - Specific trypsin and chymotrypsin activities of the homogenates of five *D. magna* clones exposed to 3 different food treatments

a) Specific chymotrypsin activities of the different *D. magna* clones (page 92). b) Specific trypsin activities of the different *D. magna* clones. The five *D. magna* clones were fed either 100% *S. obliquus*, or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus*. Different letters indicate a significant difference ($p < 0.05$; Tukey HSD after one-way ANOVA) within activities of a single clone. (mean + SD, n = 3)

20% *M. aeruginosa* NIVA Cya 43 in comparison to animals fed the reference food (Fig. 4A). In the 20% *M. aeruginosa* PCC7806 Mut treatment (Fig. 4B), the activity of the trypsin bands slightly decreased, while the chymotrypsin activity slightly increased in all *D. magna* clones.

Relative expression of digestive proteases of five *D. magna* clones grown on different food treatments

The expression of the digestive proteases in the five *D. magna* clones due to feeding on the three different food treatments was measured with QPCR (Fig. 5). In every *D. magna* clone the food treatments had a significant effect on protease expression (one-way ANOVA Tab. 4). Different kinds of response to the *M. aeruginosa* strains were observed: **i)** slight up-regulation (clone A: 1.4 to 4.7-fold) or **ii)** high up-regulation (clone W: 16.4 to 810.3-fold) of all four protease genes due to both dietary cyanobacteria; **iii)** slight (1.4 to 7.7-fold) or no up-regulation of proteases due to *M. aeruginosa* PCC7806 Mut and simultaneously high (9.4 to 235.5-fold) up-regulation of proteases due to *M. aeruginosa* NIVA Cya 43 (clones B and P), **iv)** high (7.6 to 25.1-fold) up-regulation due to *M. aeruginosa* PCC7806 Mut and simultaneously no regulation or slight (1.1 to 3.3-fold) down-regulation due to *M. aeruginosa* NIVA Cya 43 (clone C).

	Degrees of freedom	MS	F	p
Food	2	0.196531	429.42	**p < 0.00001**
D. magna clone	4	0.012570	27.47	**p < 0.00001**
Food x *D. magna* clone	8	0.012530	27.38	**p < 0.00001**
Error	30	0.000458		

Table 2 - Two-way ANOVA for the growth experiment of five *D. magna* clones

The somatic growth rates were analysed for the effects of the factors food (100% *S. obliquus*, or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus*) and *D. magna* clone. Significant differences are indicated by bold p-values. MS = mean square

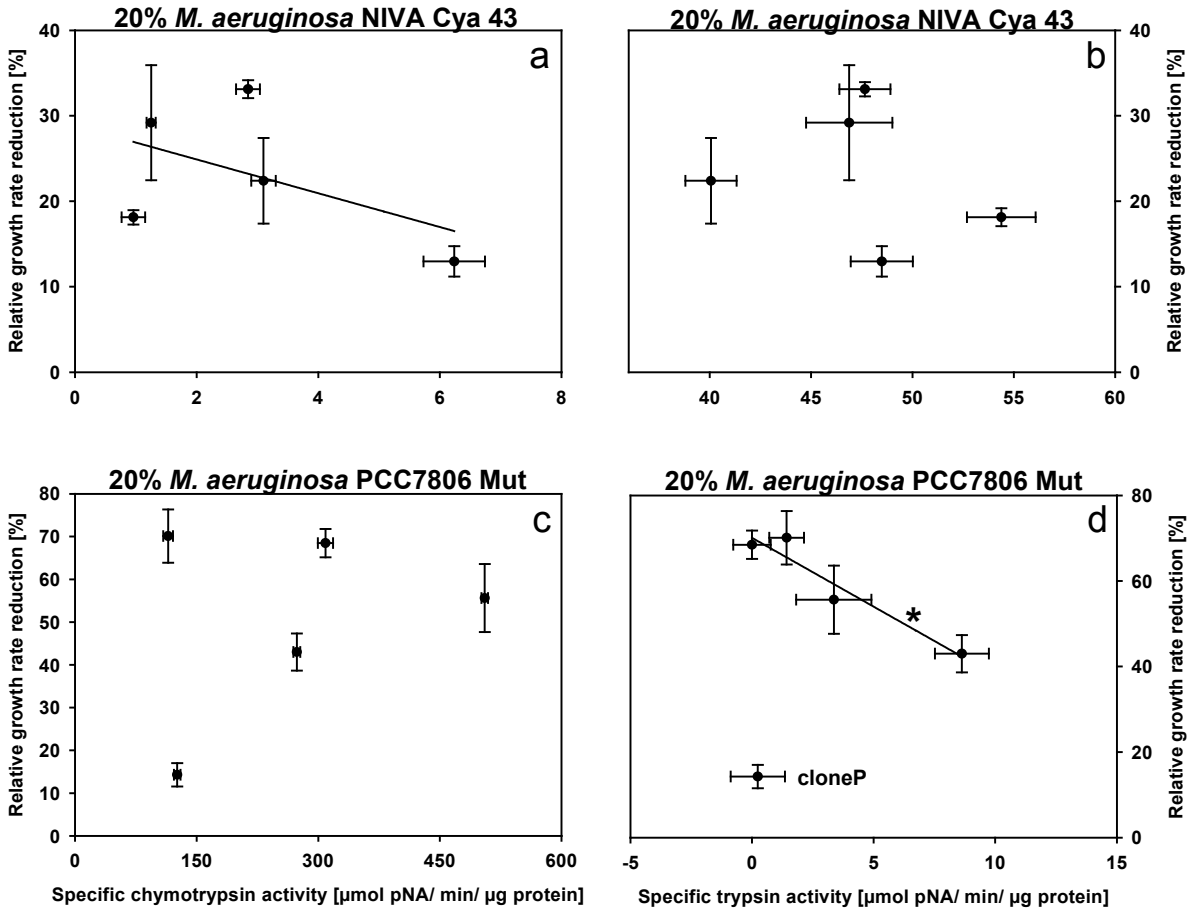


Figure 3 - Relation between relative growth rate reduction and protease activity of the five *D. magna* clones

Relative growth rate reduction as a function of the activity of digestive chymotrypsin (a) or trypsin (b) in five *D. magna* clones exposed to 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*; relation of relative growth rate reduction and c) chymotrypsin or d) trypsin activity in five *D. magna* clones exposed to 20% *M. aeruginosa* PCC7806 and 80% *S. obliquus*. Lines show linear correlations between relative growth rate reduction and protease activity; in d, clone P (depicted in the graph) was excluded from the linear correlation. Significance is indicated by an asterisk. (mean + SD, n = 3).

Phylogenetic tree of *D. magna* trypsins

The phylogenetic tree (Fig. 6) of the two *D. magna* trypsin genes revealed different alleles of the genes across the five clones (for sequences: see Appendix). In *T152* the p-distance of the alleles was smallest between clones A and C (0.001) and between clones B and W (0.002). Clone P had a p-distance of 0.006 from clones B and W and a p-distance of 0.009 to 0.01 from clones C and A. In *T208* the p-distance of the alleles between clones P and C was < 0.001, between clones B and W < 0.001, between clones P and A 0.005, and between clones A and C 0.005. Clones A, C and P had a p-distance of > 0.006 from clones B and W. In the alignment between

genomic DNA sequences of the five clones, base-pair exchanges were detected; some of these base-pair exchanges were synonymous exchanges. However, also non-synonymous mutations were detected in all clones (Tab. 5). These non-synonymous exchanges led to differences in the amino acid sequence of the trypsins between all five *D. magna* clones.

	Degrees of freedom	MS	F	p
Food	1	0.526403	232.87	**p < 0.00001**
D. magna clone	4	0.053667	23.74	**p < 0.00001**
Food x *D. magna* clone	4	0.10205	45.15	**p < 0.00001**
Error	19	0.002261		

Table 3 - Two-way ANOVA for the relative growth rate reductions of five *D. magna* clones grown on different food treatments

The relative growth rate reductions were analysed for the effects of the factors food (20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus*) and *D. magna* clone. Significant differences are indicated by bold p-values. MS = mean square.

Discussion

Five *D. magna* clones from four different lakes in Middle Europe were fed mixtures of the widely used good reference food *S. obliquus* and one of two cyanobacterial strains of *M. aeruginosa*, which either contain trypsin or chymotrypsin inhibitors. All five *D. magna* clones fed with 100% *S. obliquus* showed growth between 0.42 and 0.47/ d (Fig. 1a), which was comparable to somatic growth rates from other studies [37,38], confirming this strain as a good food source for *D. magna*. The growth rates of all clones fed with 20% cyanobacterial food decreased in comparison to growth on 100% *S. obliquus*. The clones differed significantly in growth on the reference food alga; hence, relative growth rate reductions as measurement of the sensitivity of the five clones were calculated (Fig 1b and c). A reduction in growth rate or body length as an effect of cyanobacterial food has been observed earlier in various *Daphnia* species. Several reasons for growth reductions have been identified: i) interference of filamentous cyanobacteria with the filtering apparatus [37,39], ii) deficiency of essential lipids (polyunsaturated fatty acids [40] or sterols [41]) and iii) the content of

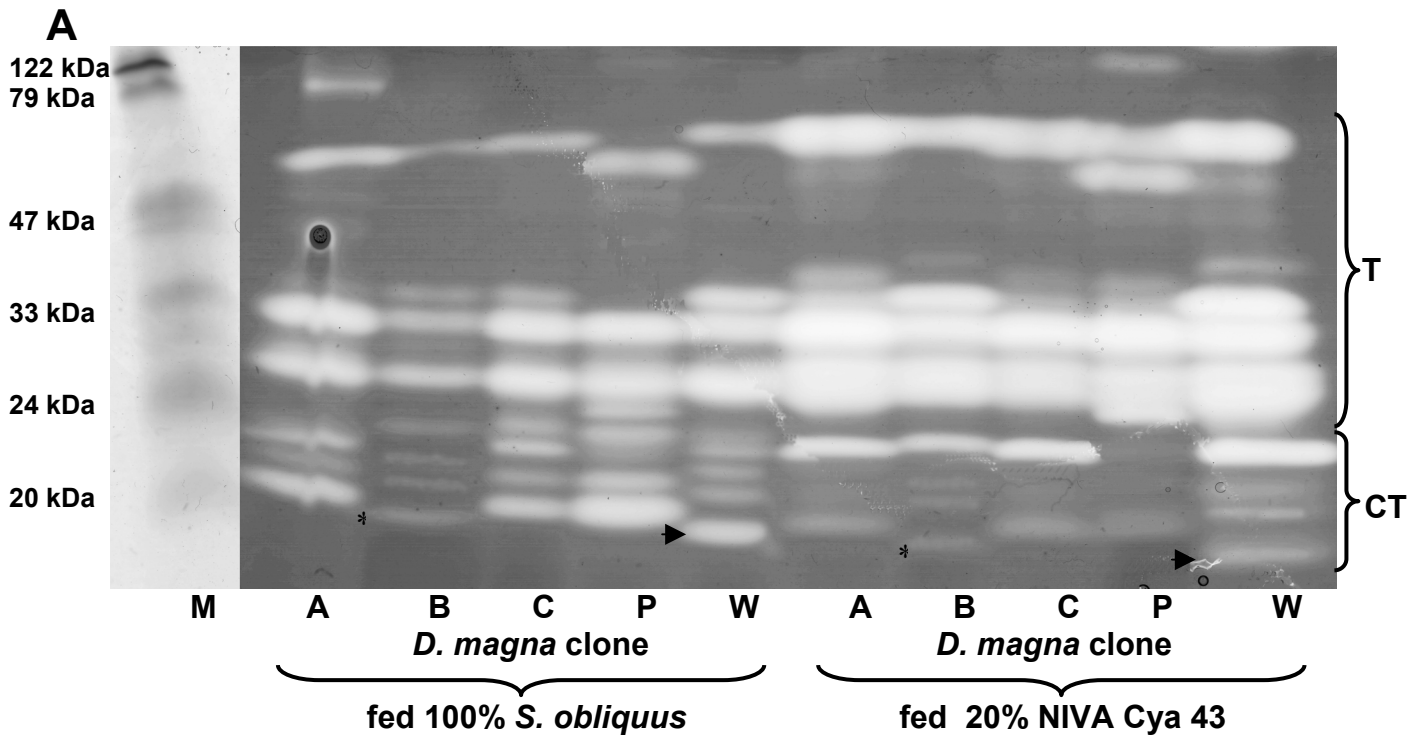
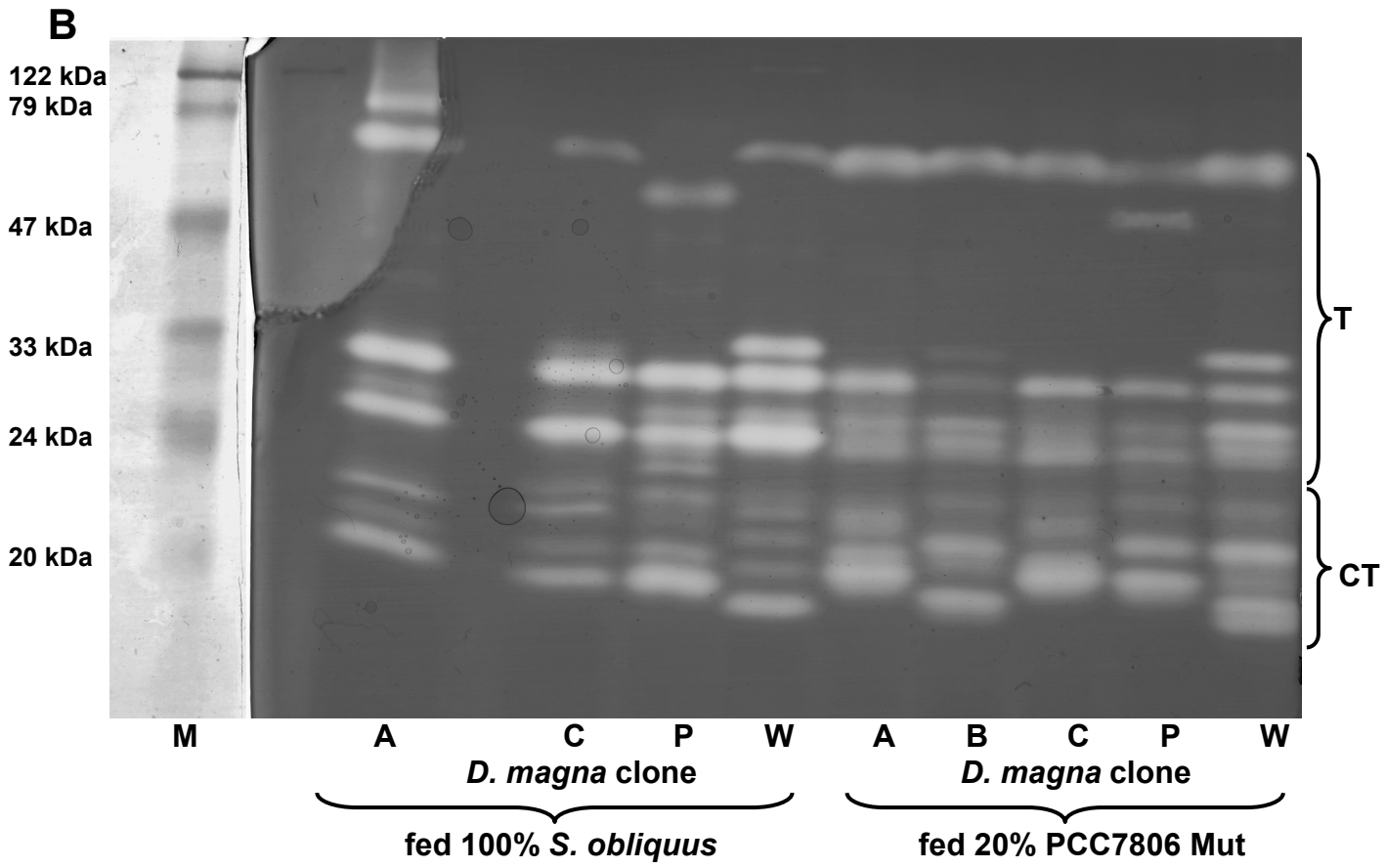


Figure 4 - Activity stained SDS-PAGEs of the homogenates of five *D. magna* clones exposed to three different food treatments

The five *D. magna* clones were fed either 100% *S. obliquus* (both gels), or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* (A), or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus* (B; page 98). Identical amounts of protein were loaded onto each lane. White bands indicate active proteases, asterisks and arrows show the shift in the chymotrypsin band pattern in *D. magna* clones B and W due to 20% *M. aeruginosa* NIVA Cya 43 in the food. Brackets show the molecular weight range of trypsins (T) and chymotrypsins (CT [17]). M = molecular weight marker (kDa).



toxins [24]. The *M. aeruginosa* strains used here were single-celled, small enough to be ingested and did not contain microcystins [29,30]. Furthermore, a reduction in growth rate due to polyunsaturated fatty acids or sterol limitation can be excluded, since ≥ 80% of the dietary carbon was of eukaryotic origin, i.e. *S. obliquus* [42]. Therefore, in our case, growth rate reduction in response to cyanobacteria is probably caused by serine protease inhibitors, since the cyanobacterial strains used contain either mainly trypsin inhibitors (*M. aeruginosa* PCC 7806 Mut [17]) or strong chymotrypsin inhibitors (*M. aeruginosa* NIVA Cya 43 [15]), which are strongly inhibiting [17,Dissertation Part II] the most important group of digestive enzymes in *D. magna*, i.e. trypsins and chymotrypsins [18].

The degree of growth depression due to cyanobacterial food differed between the five *D. magna* clones, as has been shown earlier for clones of other *Daphnia* species [20,21]. Within a *D. magna* clone the relative growth rate reduction also differed when fed the two different dietary *M. aeruginosa* strains. Hence, a high sensitivity to one protease inhibitor was not coupled with a strong growth reduction by to the other one.

	Protease genes			
D. magna	*T152*	*T208*	*CT448*	*CT802*
Clone A	$F_{2,6}$ = 5118.63	$F_{2,6}$ = 3107.18	$F_{2,6}$ = 8057.84	$F_{2,6}$ = 14972.47
Clone B	$F_{2,8}$ = 210813.08	$F_{2,6}$ = 62182.09	$F_{2,6}$ = 229315.29	$F_{2,8}$ = 2455.61
Clone C	$F_{2,6}$ = 19609.36	$F_{2,6}$ = 9093.94	$F_{2,6}$ = 172.19	$F_{2,6}$ = 10710.08
Clone P	$F_{2,6}$ = 38624.12	$F_{2,6}$ = 797224.65	$F_{2,6}$ = 1584062.71	$F_{2,6}$ = 324885.40
Clone W	$F_{2,5}$ = 13440.58	$F_{2,6}$ = 35929.87	$F_{2,4}$ = 47188.24	$F_{2,6}$ = 9887.23

Table 4 - Relative protease expression of the five *Daphnia magna* clones investigated.
Results of one-way ANOVA ($p < 0.05$) for relative gene-expression of every protease in each of the five *D. magna* clones grown on three different food types (100% *S. obliquus*, or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus*). Lower figures after F-values indicate the degrees of freedom of the variable “food type” and of the error.

In comparison to the four other clones, clone P strikingly showed an inverse pattern of sensitivity to the two different *M. aeruginosa* strains (Fig. 1c). While clone P had a similar sensitivity to *M. aeruginosa* NIVA Cya 43 as the other *D. magna* clones, it was less sensitive to *M. aeruginosa* PCC7806 Mut. We assumed that the differences in sensitivity between the five *D. magna* genotypes are the result of differences in the underlying physiology.

In order to more closely investigate the physiology of the interaction of cyanobacterial protease inhibitors with the *D. magna* clones, the targets of these inhibitors, i.e. the trypsins and chymotrypsins of all *D. magna* clones, were analysed by activity measurements of these two types of proteases under different food conditions. By making use of different strains of the cyanobacterium *Microcystis aeruginosa*, which either contained trypsin- or chymotrypsin inhibitors, it was possible to independently determine the effects of the two types of inhibitors on the specific protease activity of the *D. magna* clones.

With food containing either trypsin or chymotrypsin inhibitors the corresponding protease type decreased in activity in all *D. magna* clones (Fig. 2), confirming specific effects of these inhibitors on the respective type of proteases as has been shown earlier [17,Dissertation Part II]. Simultaneously, the other *D. magna* protease type increased in activity (Fig. 2). Hence, all *D. magna* clones showed a putatively compensatory physiological response by increasing the activity of the protease type that was not inhibited by the dietary protease inhibitors.

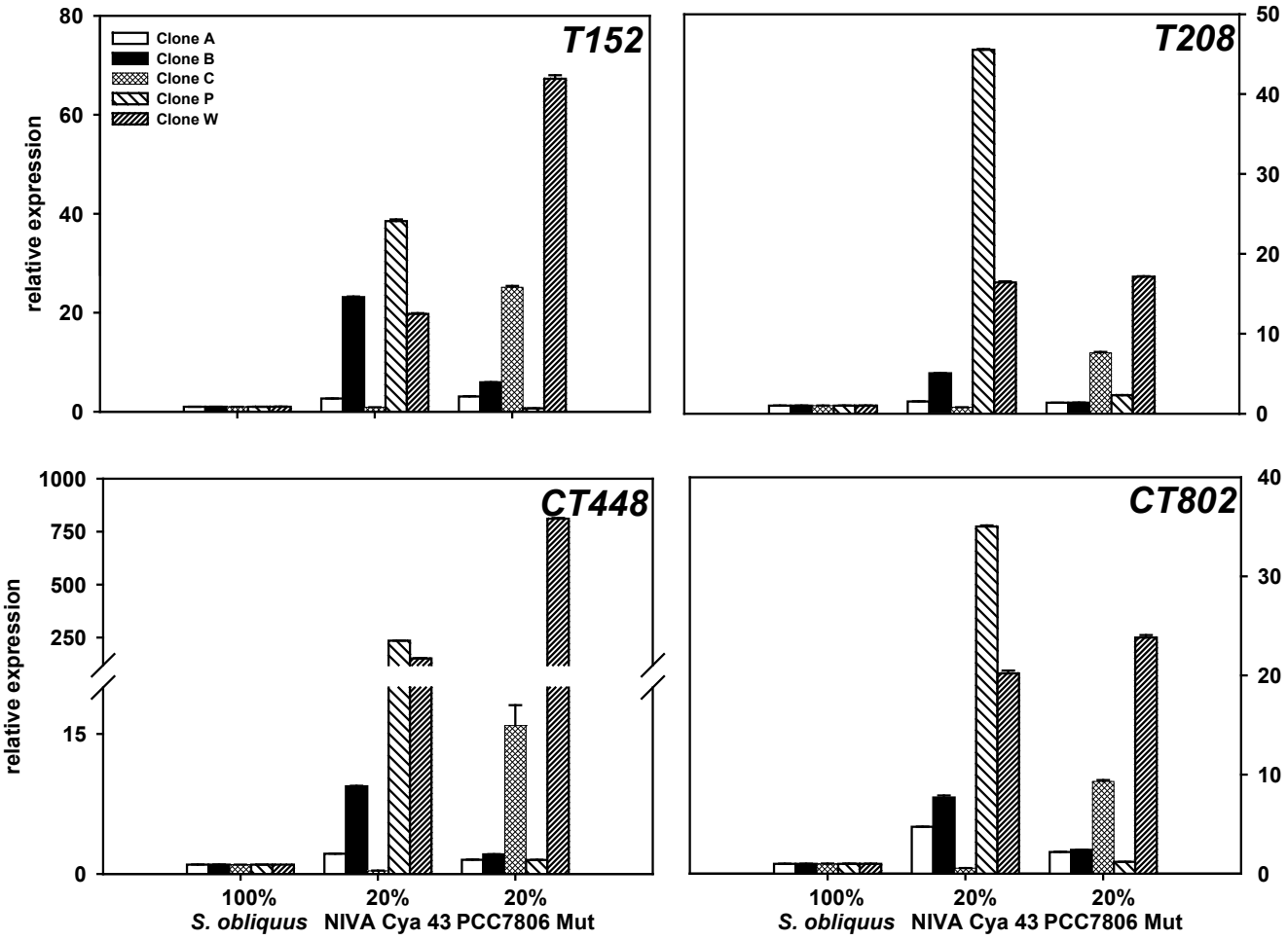


Figure 5 - Relative expression of four digestive proteases of five *D. magna* clones exposed to three different food treatments

Relative expression of two trypsins (*T152*, *T208*) and two chymotrypsins (*CT448*, *CT802*). The five *D. magna* clones were fed either 100% *S. obliquus* (calibrator: relative expression set to 1), or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*, or 20% *M. aeruginosa* PCC7806 Mut and 80% *S. obliquus*. (mean + SD, n = 3)

In order to analyse if this physiological response was indeed compensatory, the relation between relative growth rate depression and protease activity of the *D. magna* clones was investigated. Due to the different cleavage sites of chymotrypsins and trypsins it is not surprising that, although increased, trypsin activity obviously did not compensate the effect of chymotrypsin inhibition caused by *M. aeruginosa* NIVA Cya 43. Hence, in the presence of dietary chymotrypsin inhibitors, the variability in growth depression tended not significantly to be due to the clonal variability in chymotrypsin activity. The increase in trypsin activity could not be proven to be compensatory and adaptive. Similarly, in the presence of dietary trypsin inhibitors, the clonal activity of trypsins suggested, although not significantly, to determine the

	T152						T208				
position	137 bp	705 bp	831 bp	836 bp	1152 bp	1182 bp	324 bp	544 bp	1070 bp	1088 bp	1152 bp	1179 bp
Clone A	C	A	G	G	C	G	C	G	G	T	T	A
Clone B	A	A	G	A	T	A	T	T	A	C	T	G
Clone C	A	A	G	G	C	G	C	G	G	T	A	A
Clone P	A	A	C	G	T	A	C	G	A	C	T	G
Clone W	A	C	G	G	T	A	T	T	A	C	T	G

Table 5 - Non-synonymous mutations in the trypsin genes of the five *D. magna* clones

Depicted are the positions in the DNA-sequences of every *D. magna* clone of all non-synonymous base pair exchanges for two trypsins genes (*T152* and *T208*). A = arginine, C = cytosine, G = guanine, T = thymine.

degree of growth depression, whereas the observed increase in chymotrypsin activity had no compensatory effect.

In order to investigate which digestive trypsins and chymotrypsins were affected by and responded to dietary protease inihibitors, proteases were visualized on activity stained SDS-PAGEs. Whole body homogenate of *D. magna* was used for SDS-PAGE, as it showed the same protease band pattern on SDS-PAGE as gut homogenate of *D. magna* [Dissertation Part II]. In SDS-PAGEs of *D. magna* homogenate nine serine protease bands were active, which recently have been assigned to trypsins and chymotrypsins [17,Dissertation Part II]. Hitherto only the protease band pattern of *D. magna* clone B fed 100% *S. obliquus* was known [17,18,Dissertation Part II]. Surprisingly, the different *D. magna* clones showed different protease band patterns when feeding on 100% *S. obliquus* (Fig. 4 A), revealing diverse protease isoforms under the same food conditions.

However, a change in protease activity was visible in activity stained SDS-PAGEs of all clones fed 20% *M. aeruginosa* NIVA Cya 43. Surprisingly the activity of one chymotrypsin band (identified as *CT448* in a preceding study [Dissertation Part II]) increased in four *D. magna* clones when fed *M. aeruginosa* NIVA Cya 43 (Fig. 4A). Hence, the overall decrease in specific chymotrypsin activity seemed only to be valid for the other chymotrypsins. Eventually the amount of the residual chymotrypsin activity, which tended to be negatively related with relative growth rate reduction, might mainly be due to the activity of CT448 in these four *D. magna* clones. However, *CT448* in clone P seemed not to increase in activity at all. This might be the reason why clone P showed one of the highest relative growth rate reductions after feeding on *M. aeruginosa* NIVA Cya 43 compared to the other *D. magna* clones (Fig. 1b).

Interestingly, clone B and W, which had an identical protease band pattern after feeding on 100% *S. obliquus*, showed the same shift to the protease isoforms of chymotrypsin *CT802* as reported before for clone B (Fig. 4A [Dissertation Part II]). The overall adaptive effect of using isoforms could not be demonstrated, since clone B showed a high sensitivity to 20% *M. aeruginosa* NIVA Cya 43 compared to the other *D. magna* clones, which was not the case for clone W; however, without the establishment of new protease isoforms, the clones B and W might have been even more sensitive to the dietary chymotrypsin inhibitors.

When instead of cyanobacterial chymotrypsin inhibitors dietary trypsins inhibitors were provided, only minor changes in the pattern of digestive proteases in SDS-PAGE became obvious (Fig. 4B). Hence, the ability of clone P to better cope with the dietary trypsin inhibitors than other *D. magna* clones could not be explained by high protease activity; clone P indeed had a very low total chymotrypsin and trypsin activity compared to the other clones, and the clone's relative growth rate reduction was not related to residual trypsin activity (Fig. 3). A different cause must have led to the ability of clone P to cope with dietary trypsin-inhibitors; conspicuous differences between clone P and the other *D. magna* clones were the 55 kDa protease (Fig. 4B), that was exclusively present in clone P, and synonymous and non-synonymous base pair exchanges in the trypsin gene sequence (Tab. 5; Fig. 6). It is reasonable to assume that the 55 kDa protease and the isoforms of the other trypsins are less sensitive to dietary trypsin inhibitors than the trypsins of the other *D. magna* clones, which leads to an overall comparatively low sensitivity of clone P to dietary trypsin inhibitors.

The five *D. magna* clones showed two different responses to dietary protease inhibitors: an increase of protease activity and an induction of protease isoforms. The underlying mechanism for these different responses on the protein level might be differences in protease gene-expression. Since the active proteases visible on SDS-PAGE have recently been assigned to trypsin and chymotrypsin genes by LC-MS/MS[Dissertation Part II], the relative protease expression of the single genes under different food conditions was investigated.

The *D. magna* clones showed diverse intra-specific differences in gene-expression of trypsins and chymotrypsins in response to both types of dietary cyanobacterial protease inhibitors (Fig. 5). In all five *D. magna* clones exposure to one type of protease inhibitor led to an overall increase of activity of the non-inhibited protease type (Fig. 2). This elevated activity could partly be related to an increase in gene-expression. Nevertheless, this was not true in every case, since some genes in some of the five *D. magna* clones were only slightly up-regulated or not regulated at all. However, two more digestive protease genes that were not investigated here have been identified in *D. magna* [Dissertation Part II], which might be involved in the increase of protease activity. Another possibility for the increase of protease activity without increased gene-expression might be the induction of protease isoforms within a single clone, which eventually might have a higher substrate turnover. Isoforms

might result from splice variants, post-translational modifications of the proteases, or the activation of different gene copies. Since the *D. pulex* genome has been shown to contain a surprisingly high number of gene duplicates (e.g. peptidases in *D. pulex* [43]) it is reasonable to assume that the activation of different gene copies leading to isoforms of proteases is a probable scenario to cope with dietary protease inhibitors.

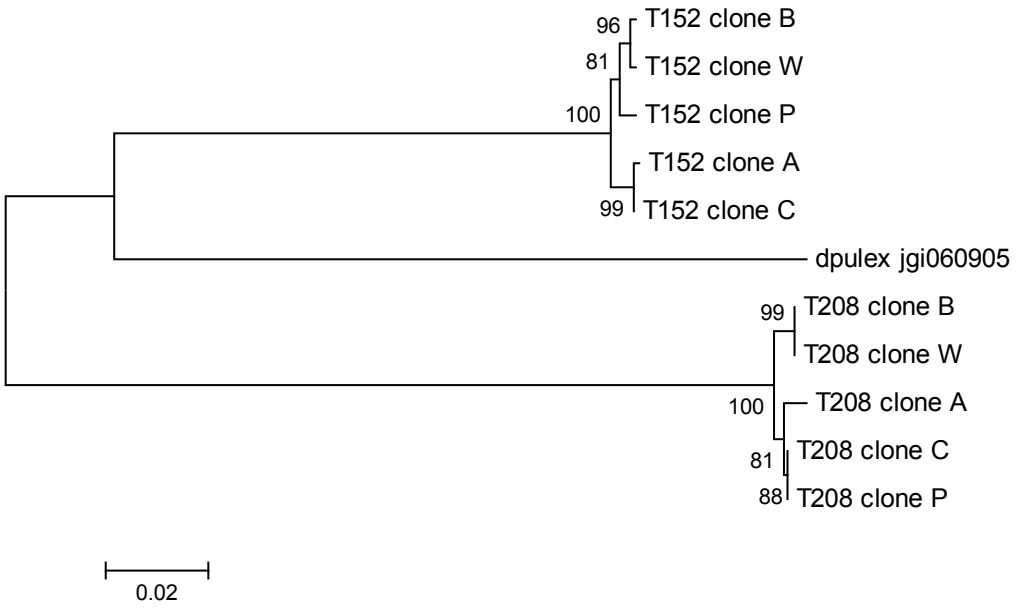


Figure 6 - Phylogenetic tree of two *D. magna* trypsin genes
A neighbour-joining tree of DNA sequences of two trypsin genes (*T152* and *T208*) of five *D. magna* genotypes (clone A, B, C, P and W). A genomic trypsin sequence of *D. pulex* (duplex jgi060905) served as outgroup.

It was shown, that rapid gene family expansions in phenotypically important genes suggest scenarios wherein adaptive natural selection favours additional copies, e.g. for adaptation to increased dosage [44]. In several insects, amplification of different esterase genes was the reason for resistance to organophosphate pesticides [45-47]; resistant strains of the mosquito *Culex pipiens* even showed a 250-fold increase in copy numbers [48].

In all five *D. magna* clones exposure to one type of protease inhibitor led to an overall decrease in activity due to inhibition of the corresponding protease type (Fig. 2), although protease gene-expression was often up-regulated. However, it remains to be tested, if after exposure to less inhibitor-containing cyanobacteria the same up-regulation might then compensate for the inhibited proteases.

To investigate whether differences in sensitivity of the *D. magna* clones might be due to a difference in protease gene sequences, a phylogenetic tree of two trypsin genes was constructed (Fig. 6). In both trypsin genes, the protease sequences of the five *D. magna* clones proved to be different alleles of the same gene. The different p-distances between the clones were not only caused by synonymous base pair exchanges but also by several non-synonymous exchanges (Tab. 5), which lead to exchanges of amino acids. A changed amino acid sequence might have consequences for the protein structure and the protease's sensitivity to inhibitors. A less sensitive protease might then lead to less sensitive *Daphnia*.

The *D. magna* clones A and C showed identical sensitivity to the trypsin-inhibitor containing *M. aeruginosa* PCC7806 Mut; so did clones B and W. Interestingly, the trypsin genes within each of these two clone pairs were closely related. This indicates that closely related trypsin genes are associated with similar sensitivity to dietary trypsin inhibitors and suggests that the observed non-synonymous base-pair exchanges in *D. magna* trypsins have affected the clonal sensitivity to trypsin inhibitors.

The five *D. magna* clones investigated in this study showed two inverse patterns of sensitivity to the two cyanobacterial strains that contained either trypsin or chymotrypsin inhibitors. All clones showed a variety of physiological responses to these inhibitors: higher protease activity, a change in protease gene-expression and the induction of protease isoforms. Linear correlations tentatively suggest that the overall activity of the target protease determines the clonal sensitivity. Hence, increases in gene-expression and the induction of isoforms that lead to higher activity of the target protease should be adaptive.

Local causes for differences in sensitivity to dietary protease inhibitors between the investigated clones of *D. magna* cannot be excluded. Sarnelle et al. [10] have documented local adaptation of *Daphnia* to toxic cyanobacteria. Positive selection of less sensitive *Daphnia* can lead to microevolution of *Daphnia* as has been shown by Hairston et al.[49], who has found decreased sensitivity of *Daphnia* to a microcystin-containing *M. aeruginosa* after a period of eutrophication in Lake Constance. Local adaptation due to microevolution of *Daphnia*, which are less sensitive to cyanobacterial protease inhibitors, might even be of more importance for *Daphnia* than local adaptation to microcystins, since protease inhibitors have been found in nearly every cyanobacterial bloom [12,13], which is not the case for microcystins. In

line with microevolutionary adaptation to protease inhibitors, *Daphnia* originating from a lake with cyanobacteria have shown lower mortality after direct exposure to a cyanobacterial protease inhibitor than *Daphnia* from a lake without cyanobacteria [50]. It remains to be tested, if local adaptation of *Daphnia* to protease inhibitors, can also be measured as a lower growth depression of *Daphnia* upon exposure to cyanobacteria that contain protease inhibitors rather than dissolved inhibitors.

As the five *D. magna* clones differed in their protease band pattern in SDS-PAGE, and as clone P had a unique protease or protease isofom, it is reasonable to expect high allelic variability in protease genes in natural populations. The wide-spread occurrence of cyanobacterial protease inhibitors in natural blooms makes it reasonable to assume that these inhibitors exert a strong selection pressure on *Daphnia* genotypes with different trypsin and chymotrypsin alleles in natural populations. This suggestion is supported by the finding that protease genes of the five clones differ in amino acid sequence.

Conclusion

This study has revealed strong intra-specific differences of the effects of dietary cyanobacterial protease inhibitors on somatic growth and hence sensitivity of *D. magna*. The strength of these effects on somatic growth depends on the type of protease inhibitor as well as on the *Daphnia magna* clone exposed to these inhibitors.

We show specific physiological responses to dietary trypsin and chymotrypsin inhibitors in all *D. magna* clones investigated. All five clones differ in sensitivity to the two dietary protease inhibitor types, i.e. trypsin and chymotrypsin inhibitors. Different types of physiological response were observed in the five *D. magna* clones: i) Higher protease activity, ii) an altered expression of the protease genes, and iii) the induction of chymotrypsin isoforms in two clones (B and W) due to chymotrypsin inhibitors in the food. These phenotypic responses might be adaptive for *D. magna* as they could improve the capacity for protein digestion in the presence of dietary protease inhibitors. The kind and extent of these responses in protease expression probably determines the differences in relative growth rate reductions in the five *D. magna* clones in the presence of dietary cyanobacterial protease inhibitors. As the five *D. magna* clones differ in their protease band pattern in SDS-PAGE, and as clone P has a unique protease or protease isofom, it is reasonable to expect high

allelic variability in protease genes in natural populations. The wide-spread occurrence of cyanobacterial protease inhibitors in natural blooms makes it reasonable to assume that these inhibitors exert a strong selection pressure on *Daphnia* genotypes with different trypsin and chymotrypsin alleles in natural populations. This suggestion is supported by the finding that protease genes of the five clones differ in amino acid sequence.

Methods

Test species and cultures

Five clones of *Daphnia magna* originating from four different lakes in Middle Europe (clone A and C: lake Bysjön, Sweden [23]; clone B: lake Binnensee, Germany [26]; clone P: pond, Driehoek, The Netherlands [27], clone W: pond near Warzaw, Poland [26]) were cultivated at 20°C in membrane-filtered (0.2 µm) tap water.

Fifteen animals per litre were kept under non-limiting food concentrations (2 mg C/ l) with *Scenedesmus obliquus* (SAG-276-3a; SAG, Göttingen, Germany) as food alga. Only new-borne *D. magna* from the third clutch, which had been born within 24 h, were used for the experiments.

The green alga *Scenedesmus obliquus* was cultivated semi-continuously in cyanophycean medium [28] at 20°C at 130 µE/ m^2/ s, with 20% of the medium exchanged daily. The cyanobacterial strains *Microcystis aeruginosa* NIVA Cya 43, a microcystin-free strain [29], and *Microcystis aeruginosa* PCC7806 Mut, a genetically engineered microcystin synthetase knock-out mutant of PCC 7806 [30] were cultivated in chemostats on cyanophycean-medium at 20°C and constant light (50 µE/ m^2/ s). The dilution rate was 0.23 d^{-1}. Carbon concentrations of the autotrophic food suspensions were estimated from photometric light extinction (470 nm) and from carbon-extinction equations previously determined. NIVA Cya 43 is known to contain strong chymotrypsin inhibitors [15], whereas PCC7806 mostly contains trypsin inhibitors [17].

Microsatellites

Six microsatellite primer pairs were chosen for the analysis of genetic differentiation of the five *D. magna* clones (Tab. 1). The primer sequences for S6-38 were kindly provided by DeMeester, Belgium. DNA from the five *D. magna* clones was extracted

using the peqGold Tissue DNA Mini Kit (peqlab) after manufacturer's advice. Each subsequent PCR reaction contained 1 µl of DNA template, 5 µl 10 x Taq Buffer advanced (5Prime), 0.2 µM dNTPs, 2.5 mM of each primer (fluorescence-labelled forward primers) and 2.5 U Taq-Polymerase in a final volume of 50 µl. Cycling parameters were 95°C for 15 min to activate the DNA polymerase, followed by 30 cycles of 94°C for 30 s, the specific annealing temperature for the microsatellites (Tab. 1) for 30 s and 72°C for 10 min. 6 µl of PCR product was mixed with 9µl of a 1:300 dilution of Gene Scan 500 Rox Size Standard (ABI). The allele sizes were measured with the ABI 48-capillary 3730 DNA Analyzer and analysed with the software GeneMarker 1.8 (SoftGenetics).

Somatic growth on different food treatments and growth rate reductions as a measure of sensitivity of the *D. magna* clones

From a cohort of new born *D. magna*, four to five animals each were transferred to 250 ml of aged tap water with a food concentration of 2 mg C/ l. The animals were either fed the green alga *S. obliquus* as a control for high quality food or 20% of one of the two *M. aeruginosa* strains and 80% *S. obliquus*. Each day the medium and the food were exchanged. The experiment was performed under low light conditions at 20°C and lasted for six days. All food treatments were run in triplicate, and somatic growth rates of *D. magna* were determined from dry weight of animals collected at the start and at day six of the experiment, according to [31]. As a measure of sensitivity to dietary protease inhibitors the relative growth rate reduction (rgr) was calculated using the formula rgr [%] = 1 – (g_S/ g_M) * 100, for which g_S is the arithmetic mean of the growth rates of *D. magna* grown on 100% *S. obliquus* and g_M is the growth rate of the single replicates of *D. magna* grown on 20% *M. aeruginosa*.

Protease activity of homogenate of *D. magna* grown on different food treatments

Neonates of *Daphnia magna* grown for six days on 2 mg C/ l of 100% *S. obliquus* or 20% of either *M. aeruginosa* strain and 80% *S. obliquus* were transferred to 5 µl 2 mM DTT per animal and were homogenized with a pestle on ice. The homogenate was centrifuged for 3 min at 14,000 x g. The protein concentration of the supernatant – the *Daphnia*-homogenate – was analyzed using a Qubit fluorometer and the appropriate Quant-iT™ Protein Assay Kit (Invitrogen) according to the manufacturer's advice.

Chymotrypsin activity of the *Daphnia*-homogenate was measured photometrically using the artificial substrate N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-*para*-Nitroanilide ($S(Ala)_2ProPhepNA$; Sigma; [18]). 10 µl *Daphnia*-homogenate was mixed with 980 µl 0.1 M potassium-phosphate-buffer, pH 6.5. The buffer contained 125 µM $S(Ala)_2ProPhepNA$ and 1% DMSO. The change in absorption was measured at a wavelength of 390 nm at 30° C continuously over 10 min. The trypsin activity was measured using the artificial substrate N-Benzoyl-Arginine-*para*-Nitroanilide (BApNA; Sigma; [18]). 10 µl *Daphnia*-homogenate was mixed with 895 µl 0.1 M potassium-phosphate-buffer, pH 6.5. The buffer contained 1.88 mM BApNA and 7.5% DMSO. The change in absorption was measured at a wavelength of 390 nm at 30° C continuously over 10 min. Specific proteolytic activity was determined as µmol *para*-Nitroanilide liberated per minute per µg protein for synthetic substrates.

SDS-PAGE and native gel of *Daphnia*-homogenate

Daphnia-homogenates of the five *D. magna* clones (20 µg protein) with 5µl 4x Laemmli-buffer [32] was loaded on a 12% SDS-polyacrylamide gel (8 x 7 x 0.075 cm) and run at 200 V. After the run the gels were activity stained for protease according to Von Elert et al., 2004 [18]. The marker on all SDS-PAGEs was the peqGold Prestained Protein Marker III (peqlab).

Relative expression of four proteases of five *D. magna* clones grown on different food treatments

RNA was extracted from the *D. magna* using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was purified with DNase I (Fermentas) and reverse transcribed with High-capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ABI).

Four different endogenous controls (*alpha-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GapDH), succinate dehydrogenase (SucDH), TATA-box binding protein (TBP)* [33]) were used in QPCR analysis. A normalisation factor was calculated based on the endogenous controls according to Schwarzenberger et al. [34]. Two primer pairs for trypsins (*T152* and *T208* [Dissertation Part II]) and three for chymotrypsins (*CT383*, *CT448* and *CT802* [Dissertation Part II]) were used in quantitative real-time PCR (QPCR) analysis.

QPCR was performed according to Schwarzenberger et al. [34]. *D. magna* fed with 100% *S. obliquus* served as calibrator, which was always set as 1.

Sequencing of *D. magna* proteases and construction of a phylogenetic tree

Two trypsins genes (*T152*: accession number: DMC00580; *T208*: accession number: DMC05983), which have been assigned to active protease bands of *D. magna* homogenate on SDS-PAGE [Dissertation Part II], were sequenced for the five *D. magna* clones investigated. Forward (F) and reverse (R) primers were established for the whole genomic sequences of the five *D. magna* clones and for the protein-coding cDNA sequence of clone B for two trypsin genes; *T152*: F/R: 5'-TAT ACA ATC CAA GAT GAA GTT CAT CG-3'/ 5'-ATC GAT GAC GTT TAA CCT CGG-3'; *T208*: F/R: 5'-AGA TGA AGT TCA TCG TTC TTG CAG CCC-3'/ 5'-GTC TCC ATT GCT TCA TTC GAC ACC G-3'). DNA from the five *D. magna* clones was extracted using the peqGold Tissue DNA Mini Kit (peqlab) after manufacturer's advice. Each subsequent PCR reaction contained 1 µl of DNA or cDNA template, 5 µl 10 x Taq Buffer advanced (5Prime), 0.2 µM dNTPs, 2.5 mM of each primer (fluorescence-labelled forward primers) and 2.5 U Taq-Polymerase in a final volume of 50 µl. Cycling parameters were 95°C for 10 min to activate the DNA polymerase followed by 35 cycles of 93°C for 30 s, 58°C (*T152*) or 69.5°C (*T208*) for 40 s, 72°C for 2 min. Two subsequent PCR reactions were conducted with the Big Dye Terminator Cycle Sequencer v 3.1 (ABI) with 1 µl of PCR-product, 2 µl Big Dye, 1 µl 5 x Big Dye buffer, 4.4 µl H_2O and 1.6 µl of either primer (1 µM). Cycling parameters were 96°C for 1.3 min to activate the DNA polymerase followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The genes and the cDNA were sequenced on an ABI 48-capillary 3730 DNA Analyzer.

The sequences of *D. magna* clone B were blasted (blastn) against the wFleabase from *D. pulex*. The best hit with the highest score was taken as the out-group sequence for the phylogenetic tree. Since dpulex_jgi060905 was the best hit for both *D. magna* trypsin genes, all sequences from both trypsins were aligned (BioEdit v.7.0.5.3 [35]) with the *D. pulex* sequence. Using the program MEGA 4 [36] a Neighbor-Joining tree with Bootstrap Test of Phylogeny was constructed, and p-distances were calculated.

The cDNA-sequence of clone B and the genomic sequences of the five clones were aligned to search for exchanges of base-pairs in the DNA sequences of exons between clones.

Statistics

Statistical analyses were conducted with the program Statistica 6.0. The data were analysed via one-way or two-way ANOVA and a post-hoc analysis (Tukey HSD). A Levene's Test was conducted to ensure homogenous variances. To compare relative growth rate reductions of the five *D. magna* clones with specific protease activity, linear correlations were calculated. The level of significance was $p < 0.05$.

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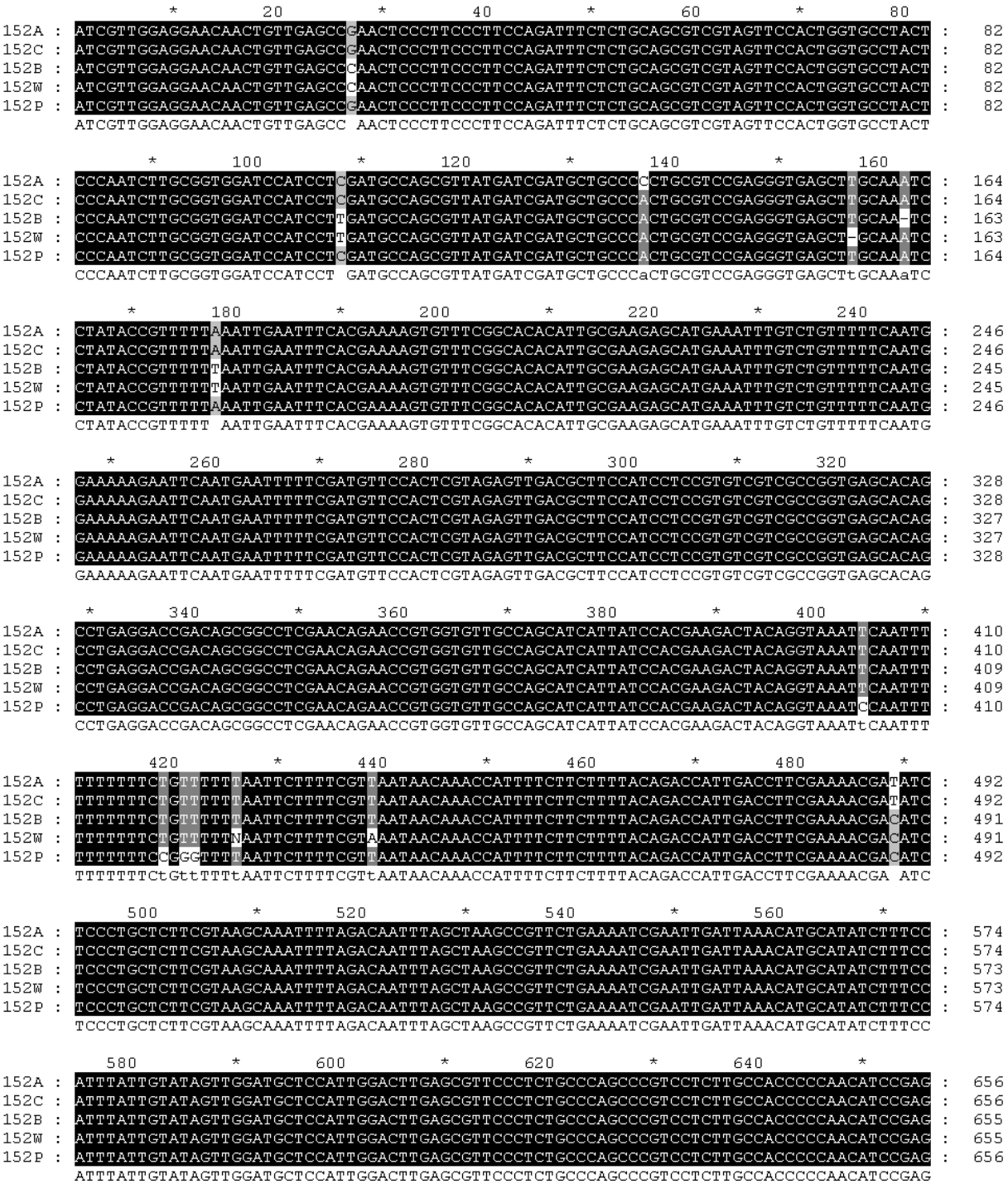
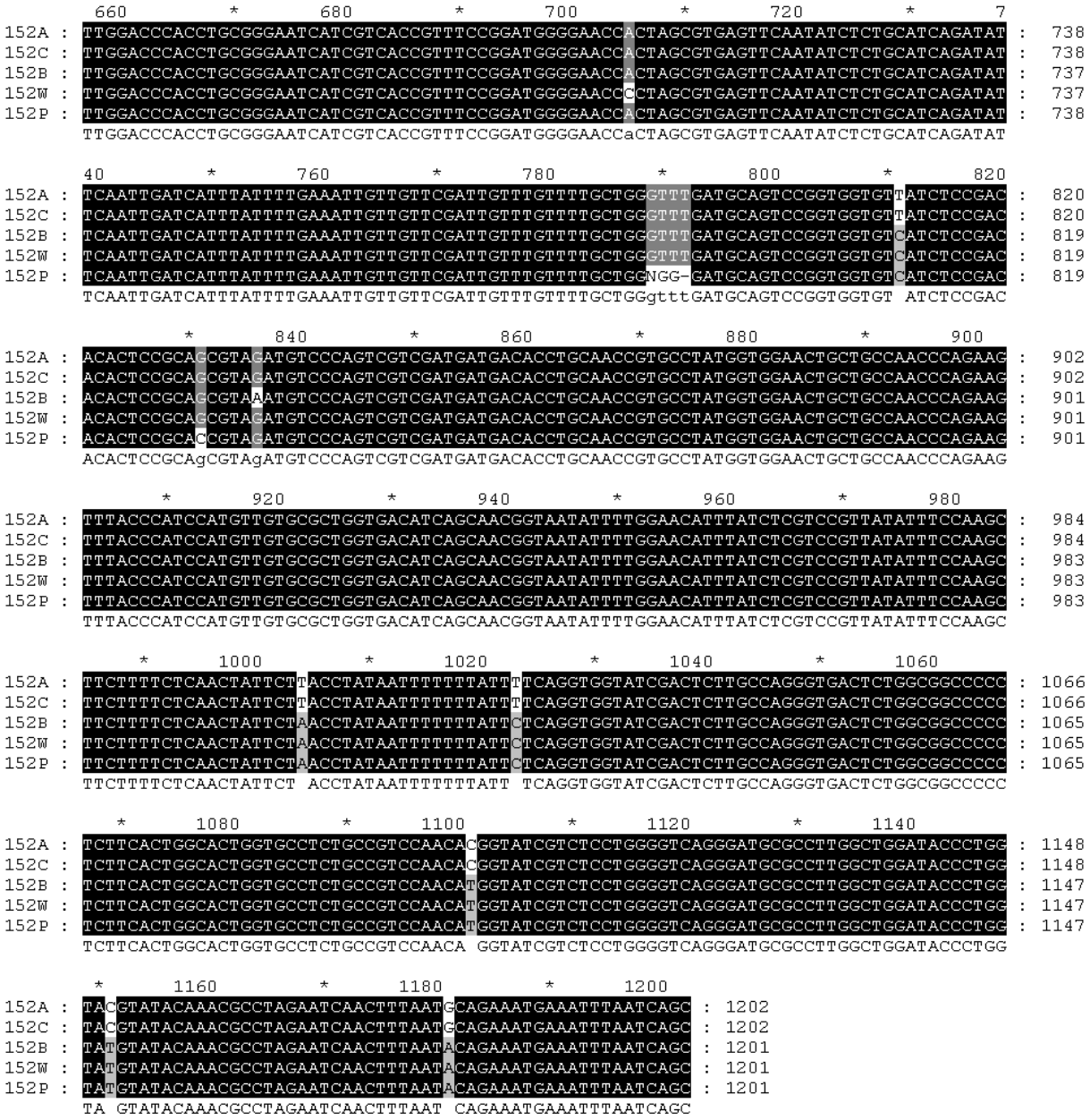
Appendix

Gene sequences of the five *D. magna* clones for the trypsin genes *T152* and *T208*.

T152:

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152C : TTGGACCCACCTGCGGGAATCATCGTCACCGTTTCCGGATGGGGAACCACTAGCGTGAGTTCAATATCTCTGCATCAGATAT : 738
152B : TTGGACCCACCTGCGGGAATCATCGTCACCGTTTCCGGATGGGGAACCACTAGCGTGAGTTCAATATCTCTGCATCAGATAT : 737
152W : TTGGACCCACCTGCGGGAATCATCGTCACCGTTTCCGGATGGGGAACCCCTAGCGTGAGTTCAATATCTCTGCATCAGATAT : 737
152P : TTGGACCCACCTGCGGGAATCATCGTCACCGTTTCCGGATGGGGAACCACTAGCGTGAGTTCAATATCTCTGCATCAGATAT : 738
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40 * 760 * 780 * 800 * 820
152A : TCAATTGATCATTTATTTTGAAATTGTTGTTCGATTGTTTGTTTTGCTGGGTTTGATGCAGTCCGGTGGTGTTATCTCCGAC : 820
152C : TCAATTGATCATTTATTTTGAAATTGTTGTTCGATTGTTTGTTTTGCTGGGTTTGATGCAGTCCGGTGGTGTTATCTCCGAC : 820
152B : TCAATTGATCATTTATTTTGAAATTGTTGTTCGATTGTTTGTTTTGCTGGGTTTGATGCAGTCCGGTGGTGTCATCTCCGAC : 819
152W : TCAATTGATCATTTATTTTGAAATTGTTGTTCGATTGTTTGTTTTGCTGGGTTTGATGCAGTCCGGTGGTGTCATCTCCGAC : 819
152P : TCAATTGATCATTTATTTTGAAATTGTTGTTCGATTGTTTGTTTTGCTGGNGG-GATGCAGTCCGGTGGTGTCATCTCCGAC : 819
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* 840 * 860 * 880 * 900
152A : ACACTCCGCAGCGTAGATGTCCCAGTCGTCGATGATGACACCTGCAACCGTGCCTATGGTGGAACTGCTGCCAACCCAGAAG : 902
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152W : ACACTCCGCAGCGTAGATGTCCCAGTCGTCGATGATGACACCTGCAACCGTGCCTATGGTGGAACTGCTGCCAACCCAGAAG : 901
152P : ACACTCCGCACCGTAGATGTCCCAGTCGTCGATGATGACACCTGCAACCGTGCCTATGGTGGAACTGCTGCCAACCCAGAAG : 901
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* 920 * 940 * 960 * 980
152A : TTTACCCATCCATGTTGTGCGCTGGTGACATCAGCAACGGTAATATTTTGGAACATTTATCTCGTCCGTTATATTTCCAAGC : 984
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152W : TTTACCCATCCATGTTGTGCGCTGGTGACATCAGCAACGGTAATATTTTGGAACATTTATCTCGTCCGTTATATTTCCAAGC : 983
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152P : TATGTATACAAACGCCTAGAATCAACTTTAATACAGAAATGAAATTTAATCAGC : 1201
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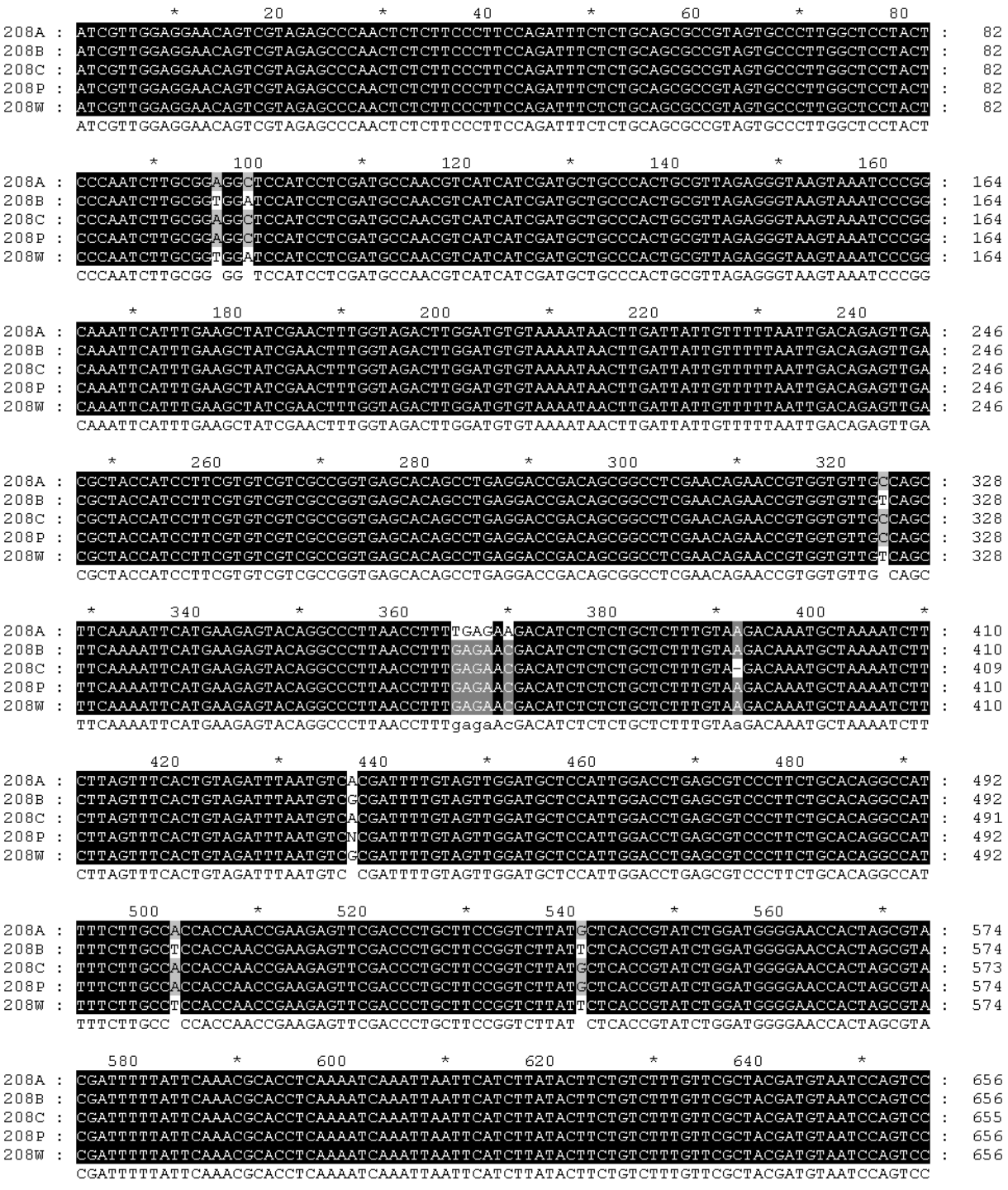
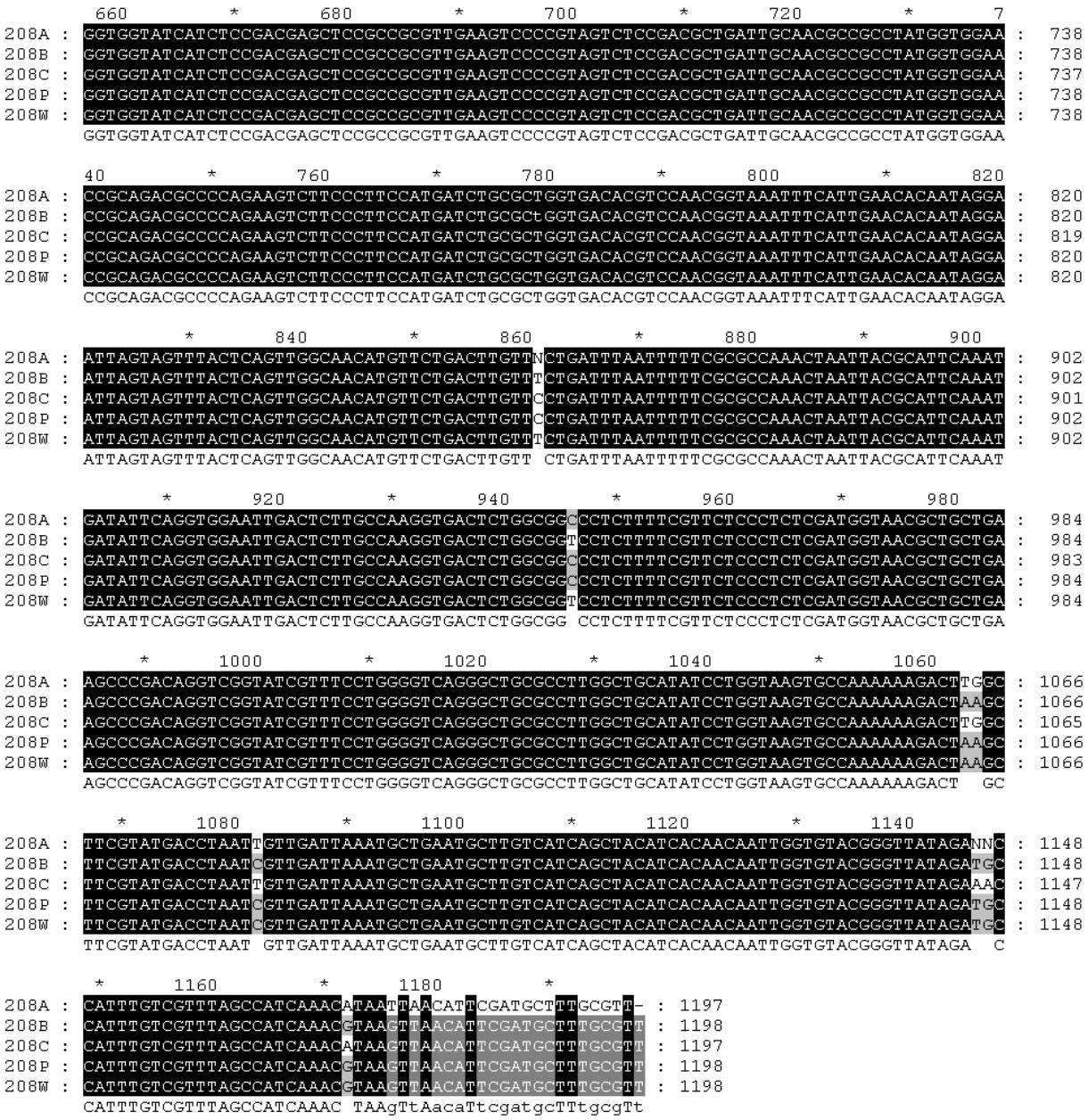
T208:





Part IV:
Cyanobacterial protease inhibitors as a trigger of maternal effects in *Daphnia*

Abstract

Background

During the last decades cyanobacterial blooms have occurred more frequently in freshwater ecosystems, which was accompanied by a summer-decline of the major herbivore, i.e. *Daphnia*. Cyanobacteria often contain protease inhibitors. These protease inhibitors have *in vitro* and *in situ* been shown to inhibit digestive proteases of *D. magna*. Different *D. magna* clones have been shown to specifically respond to dietary protease inhibitors by the induction of protease gene-expression. Induced responses might be passed on from mothers to offspring. Such a maternal transfer of adaptive responses to unfavourable environmental circumstances is a well-known phenomenon in *Daphnia*. Here we investigate, whether increased protease gene-expression caused by dietary protease inhibitors can be transferred to the offspring of *D. magna*, which should be accompanied by increased fitness of the offspring.

Results

D. magna were grown on a reference food alga (naïve mothers) or in the presence of dietary chymotrypsin inhibitors (experienced mothers). In experienced mothers an up-regulation of the gene-expression of trypsins (2.6 to 4-fold) and chymotrypsins (1.5 to 3-fold) was observed. Offspring from experienced mothers showed a similarly elevated expression of trypsins and chymotrypsins immediately after hatching although no food was ingested, which demonstrates the maternal transfer of up-regulated expression of digestive proteases. No effect on the fitness of the offspring, measured as juvenile somatic growth rate, due to increased gene-expression was detected. Also the clutch size did not differ between the offspring of experienced and naïve mothers. Interestingly, growth depression due to dietary chymotrypsin inhibitors was not detectable during the first two days after hatching, which points at the importance of maternally transferred proteins.

Conclusion

Dietary protease inhibitors led to increased protease gene-expression in mothers and, via maternal transfer, in the offspring. The adaptive value of this elevated expression of proteases could not be demonstrated. However, a proposed maternal transfer of storage proteins to the offspring of naïve and experienced mothers allowed to temporarily compensate for the presence of protease inhibitors in the diet. To our knowledge the results presented here are the first report on the adaptive nature of maternal protein allocation to eggs and a maternal transfer of elevated gene-expression in functionally relevant genes.

Background

One mechanism for adaptive phenotypic responses to environmental heterogeneity has been claimed to be maternal effects, which have been shaped by the action of natural selection [1]. These maternal effects have been observed in plants and also in animals [2] and are a well-known phenomenon in *Daphnia*. The transfer of phenotypic changes of *Daphnia* to the next generation is always triggered by an environmental factor. These environmental factors are for example infochemicals released from predators, so called kairomones, which trigger a variety of inducible morphological, life-history and behavioural responses in *Daphnia* (e.g. [3-9]). Other environmental factors leading to changes in life-history in *Daphnia* are photoperiod [10], the presence of pathogens [11] and the exposure to low-quality food, e.g. cyanobacterial strains that contain microcystins have been demonstrated to affect the growth [12,13] and survival [14] of *Daphnia*.

Many of the responses of *Daphnia* triggered by environmental factors have also been shown to be passed on to offspring of *Daphnia*, e.g. predator-induced helmet enlargement in *Daphnia* [2], fish-kairomone induced decrease [15] and chaoborus-kairomone mediated increase [16] of *Daphnia* body size, and resistance to a pathogen [17]. Maternal transfer of information on photoperiod has been demonstrated to result in production of resting eggs in the next generation [18], and only recently has the maternal transfer of cyanobacterial microcystin-tolerance to offspring been shown in *D. magna* [19]. This general observation, that information about fluctuating environmental parameters is transferred to the offspring via maternal effects suggests that the responses, which are triggered due to this information, are adaptive.

Protease inhibitors have been found in nearly every cyanobacterial bloom [20,21], which is not the case for microcystins; protease inhibitors are thus among the most wide-spread secondary metabolites of cyanobacteria. Cyanobacterial strains containing protease inhibitors, have been shown to negatively affect *Daphnia* by reducing growth and decreasing ingestion rates [13,14,Dissertation Part II and III].

Cyanobacterial protease inhibitors often inhibit serine proteases, among them are trypsins and chymotrypsins, which represent the most important digestive enzymes in the gut of *D. magna* [22]. Total trypsins and chymotrypsins of *D. magna* have *in vitro* been shown to be specifically inhibited by cyanobacterial protease inhibitors [23].

Von Elert et al. [22] observed nine protease bands in *D. magna* gut homogenate on an activity stained SDS-PAGE. These proteases have *in situ* been differently inhibited by dietary protease inhibitors [Dissertation Part II and III], which suggests that these proteases from *D. magna* differ in sensitivity to protease inhibitors. Cyanobacterial protease inhibitors putatively represent the first defence of cyanobacteria against *Daphnia*: the digestive proteases of *D. magna* have *in vitro* and *in situ* been inhibited by cyanobacterial protease inhibitors. It is therefore reasonable to assume that directly after ingestion of the cyanobacterial food particles, the digestive proteases in *Daphnia* will be affected by released protease inhibitors before other secondary metabolites, e.g. microcystins, come in contact with their targets.

However, cyanobacteria that contain protease inhibitors have been shown to trigger specific physiological responses in *Daphnia*, i.e. changes in the activity of digestive proteases, changes in protease gene-expression and induction of protease isoforms [Dissertation Part II and III]. An increase of protease gene-expression and the induction of isoforms due to dietary protease inhibitors have been shown to occur already after 24 hours [Dissertation Part II]. Since the sensitivity of the *D. magna* genotype has been shown to be apparently influenced by induced changes in digestive proteases [Dissertation Part II], it seems obvious that it also should be adaptive for *Daphnia* to transfer these changes to the subsequent generation, which should decrease the offspring's sensitivity to dietary protease inhibitors.

Evidence suggests that the sensitivity of *Daphnia* is determined by the activity of the respective target-protease of the cyanobacterial inhibitors [Dissertation Part II]. As this increase in activity of proteases seems to be partly due to the up-regulation of the target genes, we here investigated whether changes in protease gene-expression

in *D. magna*, due to dietary protease inhibitors, are maternally transferred to the offspring. We quantified the expression of protease genes of *D. magna* mothers, which had or had not experienced dietary chymotrypsin inhibitors, and compared these protease expressions with the protease expression in the offspring of these mothers. In order to investigate, whether the offspring of experienced mothers had an advantage over offspring of naïve mothers, we also determined growth rates of offspring grown on food with and without chymotrypsin-inhibitors. We hypothesized that in the presence of chymotrypsin inhibitors we would observe a higher growth rate of the offspring of experienced mothers, since the expected up-regulation of protease gene-expression passed on from the mothers, should be adaptive.

Results

Relative expression of digestive proteases in the S-line and the M-line of the F_0 and the F_1 generation of *D. magna*

The expression of the digestive proteases in the S-line and the M-line in the F_0 and in the F_1 generation of *D. magna* was measured with QPCR (Fig. 1). The pre-exposure to the two food treatments, 100% *S. obliquus* (S-line) or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* (M-line), had a significant effect on the expression of each protease in the maternal generation (one way ANOVA: ***T152*****:** $F_{3,8} = 1281.2$; $p < 0.05$; ***T208*****:** $F_{3,8} = 6132.97$; $p < 0.05$; ***CT383*****:** $F_{3,8} = 233.1$; $p < 0.05$; ***CT448*****:** $F_{3,8} = 16985.1$; $p < 0.05$; ***CT802*****:** $F_{3,8} = 6388.2$; $p < 0.05$; Fig. 1). Within the maternal generation all proteases of the M-line were significantly (Tukey HSD after one-way ANOVA; $p < 0.05$) up-regulated compared to the calibrator (F_0 generation of the S-line), indicating that 48 h of exposure to dietary chymotrypsin inhibitors led to elevated expression of trypsins and chymotrypsins in the mothers. Within the offspring of the M-line the protease genes were up-regulated as well (Fig. 1), compared to the proteases of the F_1 generation of the S-line, indicating that even in the absence of a dietary trigger (no food available) increased expression of digestive proteases was found in the M-line.

Somatic growth rates of the F_1 generation of the S-line and the M-line

All animals from the F_1 generation from the S-line and the M-line were born within 24 hours. The weight of freshly born *D. magna* from the F_1 generation did not differ between the S-line and the M-line (one-way ANOVA: $F_{1,2} = 0.199$, $p = 0.7$). After two days the somatic growth rates of all animals of the F_1 generation of the S-line and the M-line did not differ from each other (one-way ANOVA: $F_{3,8} = 0.81$; $p = 0.52$; Fig. 2). However, at day 3 and at the day of first reproduction food quality differences became obvious as was evidenced by the significantly higher growth rates on 100% *S. obliquus* than on 20% *M. aeruginosa* NIVA Cya 43 (day 3: Tukey HSD after one-way ANOVA: $F_{3,8} = 22.31$; $p < 0.05$; day of first reproduction: Tukey HSD after one-way ANOVA: $F_{3,8} = 143.98$; $p < 0.05$; Fig. 2). Nevertheless, there were no difference in growth in the presence of dietary chymotrypsin inhibitors between animals of the S-line and animals of the M-line (day 3: Tukey HSD after one-way ANOVA; $p = 0.17$; day of first reproduction: Tukey HSD after one-way ANOVA; $p = 0.97$; Fig. 2).

Clutch sizes of the F_1 generation of the S-line and the M-line

In the S-line and in the M-line first reproduction was reached at day 6 in the 100% *S. obliquus* treatment and at day 7 in the treatment with 20% *M. aeruginosa* NIVA Cya 43.

Clutch sizes differed significantly (one-way ANOVA: $F_{3,9} = 21.2$; $p < 0.05$) between the treatments but not between the S-line and the M-line (Tukey HSD after one-way ANOVA: $F_{3,9} = 21.2$; $p = 0.8$; Fig. 2).

Discussion

In nature protease inhibitors belong to the most frequent cyanobacterial secondary metabolites and have been found in nearly every cyanobacterial bloom [20,21]. Cyanobacterial blooms have become a common phenomenon in lakes due to increasing nutrient input [31,32], so that *Daphnia* are more frequently co-occurring with cyanobacteria and thus with protease inhibitors. *Daphnia* have been demonstrated to be affected by protease inhibitors in the food; due to these dietary inhibitors, *Daphnia* have shown reduced growth and reduced ingestion rates [13,14,Dissertation Part II]. *Daphnia* from a lake with annual mass developments of *Planktothrix rubescens* that produces the protease inhibitor oscillapeptin J have been shown to be less sensitive when exposed to this inhibitor than *Daphnia* from a lake

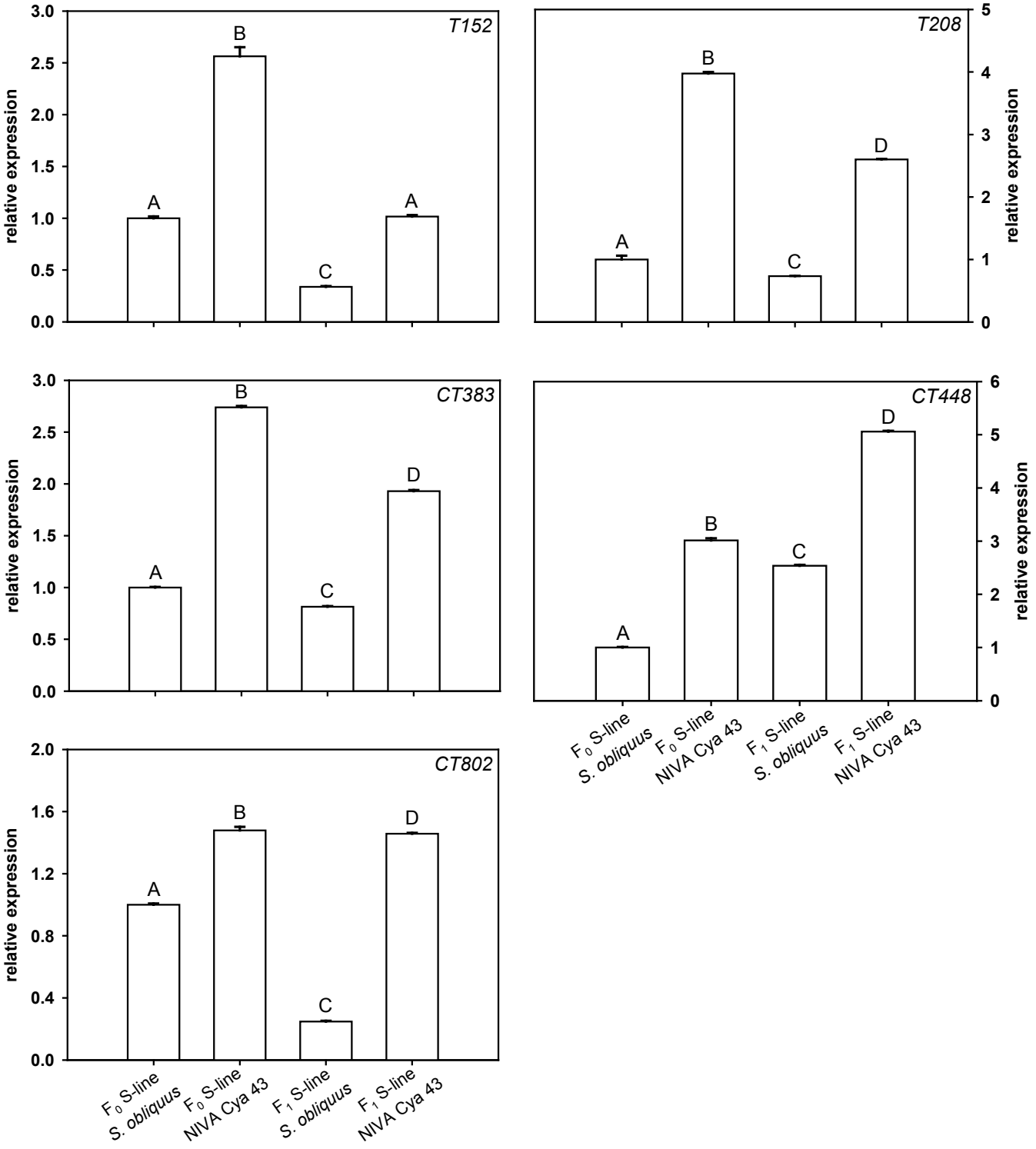


Figure 1 – relative gene-expressions of proteases of *D. magna* of the S-line and the M-line

Mean relative expression (n = 3, + SD) of trypsins (*T152*, *T208*) and chymotrypsins (*CT448*, *CT383*, *CT802*) of the F_0 generation of *D. magna* fed 100% *S. obliquus* (S-line) or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* (M-line) and of the new born offspring of both lines. Significance is indicated by letters (Tukey HSD after one-way ANOVA, $p < 0.05$). The calibrator (F_0, S-line) is set to 1.

without *P. rubescens* [33]. This reduced sensitivity to the cyanobacterial protease inhibitor suggests local adaptation of the *Daphnia* population. Local adaptation is assumed to result from positive selection of less sensitive genotypes by protease inhibitors during cyanobacterial blooms. This positive selection should not only favour genotypes that are constitutively less sensitive, but as well genotypes which respond to protease inhibitors by increased expression of protease genes. This inducible response might be passed on to the next *Daphnia* generation, which should then be less sensitive to dietary protease inhibitors.

Specific inducible responses to dietary cyanobacterial protease inhibitors have been shown for *D. magna* clone Binnensee (clone B); these responses comprise elevated activity of trypsins and chymotrypsins, increased expression of protease genes and, in the case of chymotrypsin inhibitors in the food, induction of protease isoforms [Dissertation Part II], which might increase *Daphnia*'s ability to cope with cyanobacterial protease inhibitors.

In order to investigate, whether the increased protease gene-expression is transferred to the offspring generation, protease gene-expression of mothers that had or had not experienced dietary protease inhibitors was quantified and compared to the protease gene-expression of the offspring.

The maternal F_0 generation of *D. magna* clone B showed the same up-regulation of protease genes in response to dietary cyanobacterial chymotrypsin inhibitors as was demonstrated before [Dissertation Part II and III]: the expression of trypsins and chymotrypsins was significantly up-regulated in animals of the M-line (*D. magna* fed 20% *M. aeruginosa* NIVA Cya 43) in comparison to the gene-expression in the S-line animals (*D. magna* fed 100% *S. obliquus*). This response could also be found in the subsequent F_1 generation, i.e. new born progeny that never had been exposed to cyanobacterial protease inhibitors themselves: New-born M-line animals had a higher protease gene-expression than S-line animals (Fig. 1). Hence, a maternal effect indeed occurred in response to dietary chymotrypsin inhibitors.

Juvenile somatic growth rates are a measure of fitness in *Daphnia* [34]. Hence, if a maternal effect is advantageous and thus leads to higher fitness in offspring of experienced mothers, this should become visible as an increase of somatic growth rate in the offspring generation compared to the somatic growth rate of progeny of naïve mothers.

In a six day growth experiment a mixture of 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* led to reduction of somatic growth of 30% in clone B [Dissertation Part II], which was attributed to the presence of chymotrypsin inhibitors in this strain of *M. aeruginosa*. This reduction of growth was corroborated here with 38% reduction in somatic growth on 20% *M. aeruginosa* NIVA Cya 43 after 6 respective 7 days (Fig. 2). However, here a growth depression due to the cyanobacterium became visible only from day 3 onwards, which pointed at the ability of the experimental animals to compensate for the inhibition of dietary chymotrypsins for the first 48 h of the experiment but not thereafter. This suggests that storage proteins in the yolk of the eggs have enabled the experimental animals to grow equally well regardless of the absence or presence of chymotrypsin inhibitors in the diet. Since the utilization of yolk storage proteins does not proceed via digestion in the gut, the metabolism of the storage proteins should not be affected by chymotrypsin inhibitors in the lumen of the gut. Thus, storage proteins from the yolk might compensate a reduced provision of amino acids from food digestion for a limited span of time. Since there was no difference in the growth rate between the offspring of naïve and of experienced *D. magna*, the storage proteins must have been of equal quality although experienced mothers had ingested cyanobacterial protease inhibitors. Hence, experienced mothers in comparison to naïve mothers might either have been able to cope with dietary protease inhibitors due to the up-regulation of protease gene-expression, or have invested more in the storage of proteins for the offspring than in somatic growth, which can be regarded as a maternal effect [1].

The effect of cyanobacterial chymotrypsin inhibitors on growth rate reduction became obvious at day three; nevertheless no difference was visible between the S-line and the M-line after three days. Age at first reproduction did not differ between animals from the S and the M-line within each food treatment, so that the F_1 generation of experienced mothers did not show higher fitness than the F_1 offspring of naïve mothers in the presence of dietary chymotrypsin inhibitors.

It has been shown, that tolerance to a microcystin containing cyanobacterium was transferred from *Daphnia* mothers to offspring [19]. This maternal effect went along with a shorter time to reach maturity and with an increased number of offspring. No such effects were found here between the F_1 generation of experienced and of naïve mothers (Fig. 2) when growing in the presence of protease inhibitors.

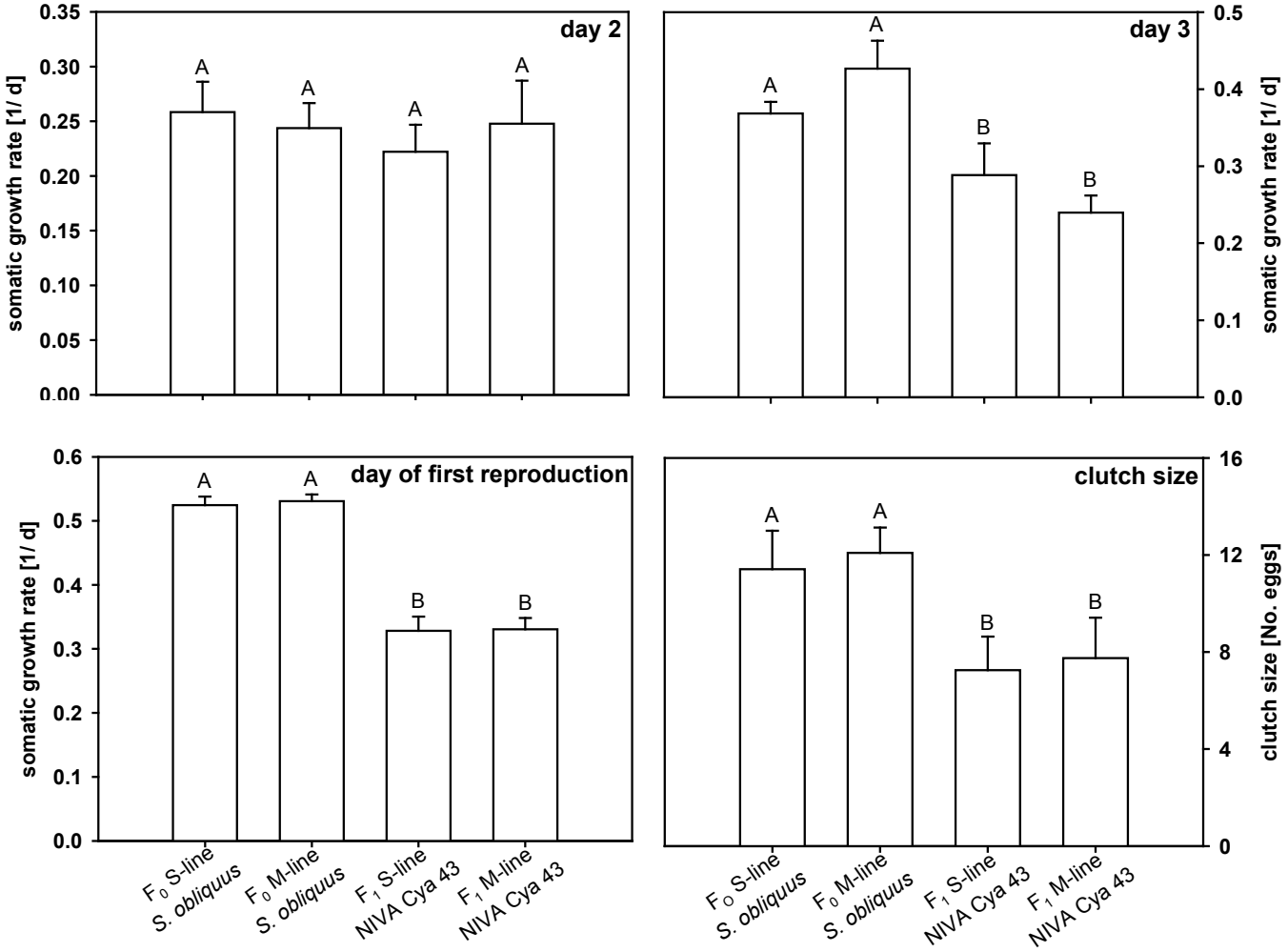


Figure 2 – somatic growth rates and clutch sizes of the F_1 generation of *D. magna* of the S and the M-line
Mean somatic growth rates and clutch sizes (n = 3, + SD) of the F_1 generation of *D. magna* fed 100% *S. obliquus* (S-line) or 20% *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* (M-line) after two and three days and at the day of first reproduction (numbers on columns depict the day of first reproduction of the S and the M-line animals for each treatment). Significance is indicated by letters (Tukey HSD after one-way ANOVA, $p < 0.05$).

A maternal effect was visible as an up-regulation of protease expression, even although the experienced mothers had only been exposed to cyanobacterial food for two days. However, no increase in fitness in offspring associated with this maternal effect from experienced mothers was detectable. Up-regulation of the gene-expression of proteases in response to dietary chymotrypsin inhibitors of new born *D. magna* occurs already after an exposure of 24 hours [Dissertation Part II]. This suggests that the F_1 generation of the naïve mothers probably was able to establish

the protease pattern, which is necessary for the digestion of *M. aeruginosa* NIVA Cya 43 in the food very shortly after hatching. Exposure of *Daphnia* to higher percentages of cyanobacteria with protease inhibitors should lead to a stronger inhibition of protein digestion of new-born *Daphnia* without up-regulated protease gene-expression; these *Daphnia* will then have consumed all storage proteins earlier. In new-born *Daphnia* from experienced mothers, on the other hand, protein digestion should be less inhibited due to the increased expression of proteases. These *Daphnia* should therefore consume storage proteins at a lower rate, such that the maternally provided storage proteins can compensate the effects of dietary protease inhibitors for a longer span of time than in the offspring from naïve mothers. Hence, it remains to be seen if the adaptive nature of maternally transferred increased expression of proteases can be demonstrated in the presence of higher levels of dietary protease inhibitors than in the experiments reported her. Exposure to higher levels of dietary protease inhibitors is well imaginable, since phytoplankton biomass has been shown to consist of up to 80% of prokaryotes during cyanobacterial blooms [35].

In the case of lipids as a major determinant of food quality for *Daphnia*, maternal allocation of lipids into eggs has been demonstrated to be adaptive as it allows for enhanced growth of new-born *Daphnia* under limitation by polyunsaturated fatty acids [36] or sterols [37].

Here the proposed allocation of storage proteins into eggs appears to be adaptive for the offspring of naïve and experienced mothers, as was evidenced by equal growth rates of the offspring grown on the reference food alga and on the chymotrypsin-inhibitor containing cyanobacterium. In the absence of maternally transferred storage proteins, depression of growth after exposure to dietary protease inhibitors would have occurred earlier.

Despite the up-regulation of gene-expression of digestive proteases in *D. magna* in response to dietary protease inhibitors, a depression of growth in *Daphnia* is detectable, which strongly suggests that growth of the animals is limited by a low provision of amino acids. A maternal transfer of proteins into eggs may be assumed to be adaptive mainly when the provision of amino acids to *Daphnia* is low. Due to the wide-spread occurrence of cyanobacterial protease inhibitors in nature, amino acid limitation of *Daphnia* is probably frequently encountered, which emphasizes the importance of maternal proteins allocated to the offspring.

In a population of *Daphnia* living in an environment with frequently occurring cyanobacteria with protease inhibitors, these inhibitors putatively exert a positive selection pressure on *Daphnia* showing a higher fitness. If this fitness resulted from maternal storage of proteins, this maternal effect would be adaptive and might lead to *Daphnia* which are less sensitive to protease inhibitors due to microevolution. Such an adaptation due to microevolution of *Daphnia* to a microcystin-containing cyanobacteria has been shown by Hairston et al. [38]. An adaptation to cyanobacterial protease inhibitors, might even be of more importance for *Daphnia* than local adaptation to microcystins, since protease inhibitors have been found in nearly every cyanobacterial bloom [20,21], which is not the case for microcystins.

Conclusion

A maternal effect on the digestive proteases of *D. magna* has been observed in the offspring as an up-regulation of gene-expression, due to exposure of the mothers to dietary protease inhibitors. The adaptive value of this observation could not be demonstrated. However, a proposed maternal transfer of storage proteins to the offspring of mothers, which had or had not experienced dietary protease inhibitors, allowed to temporarily compensate for the presence of protease inhibitors in the diet. To our knowledge the results presented here are the first report on the adaptive nature of maternal protein allocation to eggs.

Methods

Test species and cultures

Daphnia magna clone Binnensee, Germany [24] was cultivated at 20°C in membrane-filtered (0.2 µm) aged tap water. Fifteen animals per litre were kept under non-limiting food concentrations (2 mg C/ l) with *Scenedesmus obliquus* (SAG-276-3a, SAG, Göttingen, Germany) as food alga. New-born *D. magna* from the third clutch, which had been born within 24 h, were used for the experiments.

The green alga *S. obliquus* was cultivated semi-continuously in cyanophycean medium [25] at 20°C and 130 µE/ m^2/ s, with 20% of the medium exchanged daily. The cyanobacterial strain *Microcystis aeruginosa* NIVA Cya 43, a microcystin-free strain [26], that contains strong chymotrypsin inhibitors [27], was cultivated in a chemostat on cyanophycean-medium at 20°C and constant light (50 µE/ m^2/ s). The

dilution rate was 0.23 d^{-1}. Carbon concentrations of the autotrophic food suspensions were estimated from photometric light extinction (470 nm) and from carbon-extinction equations previously determined.

Experimental design

When a cohort of 8 d old *D. magna* (F_0 generation) had first delivered their second clutch to the brood pouch, fifteen animals each were transferred to 1 l of tap water with a food concentration of 2 mg C/ l. For two days, the animals were either fed the green alga *S. obliquus* (S-line animals) as a control for high quality food or 20% of *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus* (M-line). Each day the medium and the food were exchanged until the second clutch was born and the third clutch became visible in the brood chamber. Then the F_0 generation was transferred to 1 l water without any food, so that, upon release from the eggs, the third clutch animals (F_1 generation) could not ingest food. Five new born animals of the F_1 generation of the S-line or the M-line each were transferred to 250 ml of tap water with a food concentration of 2 mg C/ l. The animals of the F_1 generation were either fed 100% *S. obliquus* or 20% of *M. aeruginosa* NIVA Cya 43 and 80% *S. obliquus*. Each day the medium and the food were exchanged. The experiment was performed under low light conditions at 20°C and lasted for six days. All food treatments were run in triplicate, and somatic growth rates of *D. magna* were determined from dry weight of animals collected at the start, at day two, three and the date of first reproduction of the experiment, according to [28]. The date of first reproduction was determined as the first day when eggs were visible in the brood pouch; the clutch size was measured by counting these eggs.

Relative expression of five digestive proteases of *D. magna*

RNA was extracted from the F_0 generation immediately after birth of the third clutch using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions; RNA was also extracted from the new-born F_1 generations of *D. magna* of the S-line and the M-line. RNA was purified with DNase I (Fermentas) and reverse transcribed with High-capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ABI).

Five different endogenous controls (*alpha-tubulin, actin, glyceraldehyde-3-phosphate dehydrogenase (GapDH), succinate dehydrogenase (SucDH), ubiquitin conjugating enzyme (UBC)* [29]) were used in QPCR analysis. A normalisation factor was calculated based on these endogenous controls according to Schwarzenberger et al.

[30]. Two trypsin (*T152* and *T208* [Dissertation Part II]) and three chymotrypsin primer pairs (*CT448, CT383* and *CT802* [Dissertation Part II]) were used in quantitative real-time PCR (QPCR) analysis.
QPCR was performed according to Schwarzenberger et al. [30]. *D. magna* of the F_0 generation from the S-line fed with 100% *S. obliquus* served as calibrator, which was always set to 1.

Statistics

The statistical analyses were conducted with the program Statistica 6.0. The data were analysed via one-way ANOVA and a post-hoc analysis (Tukey HSD). A Levene's Test was conducted to ensure homogenous variances. The level of significance was $p < 0.05$.

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Abstract

Daphnia is a keystone species in the energy transfer from phytoplankton to higher trophic levels. As *Daphnia* represents the major herbivore of phytoplankton and cyanobacteria and is the most important food source for zooplanktivorous predators, *Daphnia* are controlled by two factors: predation and food quality. In *Daphnia* several phenotypic traits have been shown to be plastic in response to chemical signals from predators. However, little is known about the underlying molecular basis of this plasticity of *Daphnia*.

In an earlier study, changes in the level of actin and alpha-tubulin proteins were shown in one *D. magna* clone that was exposed to predator-borne kairomones. In the first part of the thesis I took up these findings by investigating the same *D. magna* clone. In order to analyse the level of gene expression of the actin and alpha-tubulin proteins and of several other selected genes of the basic metabolism of *Daphnia* after exposure to kairomones, real-time PCR analyses were conducted. Here, kairomones released from a planktivorous fish (*Leucaspius delineatus*) and from an invertebrate predator (larvae of *Chaoborus flavicans*) did not substantially change the transcription levels of *actin* and *alpha-tubulin*, indicating that the changes in protein level could be caused by others than the investigated gene copies. This seemed reasonable, since several paralogs of *actin* and *alpha-tubulin* were revealed by database searching of the *Daphnia* genome. However, kairomones of the vertebrate and the invertebrate predator caused changes in gene-expression of *cyclophylin*, indicating major effects on protein folding.

Over the last decades, in summer cyanobacterial mass-developments, so called blooms, have become a common phenomenon. These blooms have been claimed to be a major factor leading to the summer-decline of *Daphnia* biomass, because of the low food quality of cyanobacteria, e.g. due to cyanotoxins. The most-investigated group of these cyanotoxins are microcystins.

When a microcystin-containing strain of *M. aeruginosa* and its microcystin-free mutant were fed to *D. magna*, the same set of selected genes was investigated. The presence of dietary microcystins led to an up-regulation of *glyceraldehyde-3-phosphate dehydrogenase* and *ubiquitin conjugating enzyme*, which both are involved in the basic metabolism of *D. magna*. This suggests that microcystins in cyanobacteria have more general effects on the metabolism of *D. magna* than previously thought.

Protease inhibitors represent another important group of cyanotoxins that is more wide-spread than microcystins. In the second part of the thesis I focused on specific targets of cyanobacterial protease inhibitors in *Daphnia*, i.e. digestive proteases (trypsins and chymotrypsins). Feeding on mixtures of a reference food alga and one of two cyanobacterial strains, that either contained trypsin or chymotrypsin inhibitors, led to reduced somatic growth of *D. magna.* The reduction in growth was probably due to the inhibition of digestive proteases by cyanobacterial protease inhibitors, which putatively led to amino acid limitation of *Daphnia*. Either of the dietary protease inhibitor types had pronounced effects on digestive proteases of *D. magna* at the protein level. An *in situ* inhibition of the respective protease was observed, as well as an increase in protease activity of the non-inhibited protease type. In the case of dietary chymotrypsin inhibitors, also new protease isoforms were established already after 24 hours. The digestive proteases, which were visible on activity stained protein gels, were assigned to six different protease genes via liquid chromatography coupled with mass spectrometry and subsequent database-search. Real-time PCR analysis with primers established from these genes revealed an increase in gene-expression due to dietary protease inhibitors. The here observed physiological responses to dietary cyanobacterial protease inhibitors should be adaptive for *D. magna*, as they might increase the capacity for protein digestion in the presence of dietary protease inhibitors.

In the third part of the thesis I investigated intra-specific differences of five *D. magna* clones after exposure of either trypsin or chymotrypsin inhibitors. These *D. magna* clones were demonstrated to represent different genotypes via microsatellite analysis. Strong intra-specific differences in sensitivity, measured as relative growth rate reduction, of the *D. magna* clones to two dietary protease inhibitor types were revealed. The degree of sensitivity depended on the type of protease inhibitor as well as on the *D. magna* clone exposed to these inhibitors. The five different *D. magna* clones showed physiological responses to dietary protease inhibitors: The activity of the inhibited protease type decrease, while the non-inhibited protease type increased in activity. Linear correlations suggested that the extent of relative growth rate reduction was due to the residual activity of the inhibited protease type. Higher residual protease activity that apparently led to lower sensitivity of the *D. magna* clone seemed partly to emerge from increased protease gene-expression of the corresponding protease. However, other factors might be involved, e.g. the

establishment of more active protease isoforms. One *D. magna* clone, which had a conspicuously lower sensitivity to dietary trypsin inhibitors than the other clones, proved to have a unique protease or protease isoform and differed in amino acid sequences of two trypsins from the other *D. magna* clones investigated.

In the fourth part of the thesis I investigated, whether the increased gene expression of proteases was transferred from mothers that had experienced dietary chymotrypsin inhibitors to their offspring. The offspring of experienced mothers showed increased protease gene-expression in comparison to progenies of naïve mothers. Unfortunately, the adaptive value of this maternal effect could not be demonstrated here. However, a here proposed maternal transfer of storage proteins to the offspring of naïve and experienced mothers allowed to temporarily compensate for the presence of protease inhibitors in the diet and thus for a limitation in amino acids. To our knowledge this is also the first report on the adaptive nature of maternal protein allocation to eggs and a maternal transfer of enhanced gene-expression in functionally relevant genes.

A high variability in sensitivity to protease inhibitors due to specific physiological responses within a population holds the potential for positive selection to less sensitive *Daphnia* by these inhibitors. The assumption that dietary cyanobacterial protease inhibitors exert a strong selection pressure on their targets, i.e. *Daphnia* proteases, is supported by the finding that digestive protease genes of *D. magna* differ intra-specifically in amino acid sequences, that the described physiological changes of *Daphnia* to cyanobacterial protease inhibitors are switched on rapidly, and that increased protease gene-expression is maternally transferred to the offspring. It remains to be tested if this putative strong selection pressure has led to local adaptation in digestive proteases in natural populations of *Daphnia*.

Zusammenfassung

Daphnien sind ein wichtiges Bindeglied im Energietransfer von Phytoplankton zu höheren trophischen Ebenen. Da Daphnien die Hauptherbivoren von Phytoplankton und Cyanobakterien darstellen und die wichtigste Nahrungsquelle für zooplanktivore Prädatoren sind, werden Daphnien durch zwei Faktoren kontrolliert: Prädation und Futterqualität. Als Antwort auf chemische Signale, die von Prädatoren abgegeben werden, zeigen Daphnien in mehreren phänotypischen Merkmalen Plastizität. Es ist jedoch wenig über die molekulare Basis bekannt, die dieser Plastizität von Daphnien zugrunde liegt.

In einer früheren Veröffentlichung wurden Veränderungen in der Menge an Protein von Aktin und alpha-Tubulin in einem *Daphnia magna* Klon beschrieben, der Kairomonen, die von Prädatoren abgegeben wurden, ausgesetzt wurde. Im ersten Teil der Dissertation nahm ich auf diese Veröffentlichung Bezug. Mit Real-Time PCR analysierte ich das Niveau der Genexpression von Aktin, alpha-Tubulin und von mehreren ausgewählten Genen des Grundmetabolismus von Daphnien, nachdem ich denselben *D. magna* Klon Kairomonen aussetzte. Das Transkriptionsniveau von Aktin und alpha-Tubulin wurde nicht grundlegend durch die Kairomone eines planktivoren Fisches (Moderlieschen, *Leucaspius delineatus*) oder eines invertebraten Prädators (Larven von *Chaoborus flavicans*) verändert. Es kann also angenommen werden, dass die Veränderungen im Proteinniveau von Aktin und alpha-Tubulin durch die Transkription anderer Genkopien hervorgerufen wurden, wofür auch spricht, dass mehrere Paraloge von Aktin und alpha-Tubulin im Daphniengenom nachweisbar waren. Die Anwesenheit von Kairomonen der vertebraten und der invertebraten Prädatoren verursachten allerdings Veränderungen in der Genexpression von Cyclophilin, was starke Auswirkungen auf die Proteinfaltung in Daphnien haben könnte.

In den letzten Jahrzehnten haben sommerliche Massenentwicklungen von Cyanobakterien, sogenannte Cyanobakterienblüten, in ihrer Häufigkeit zugenommen. Mit den Cyanobakterienblüten geht im Sommer beobachtete Abnahme der Daphniendichte einher. Der Grund dafür ist die schlechte Futterqualität der Cyanobakterien, verursacht z.B. durch Cyanotoxine. Die Gruppe der Cyanotoxine, die am häufigsten untersucht wurde, ist die der Microcystine.

Dieselben Gene, die im Kairomonexperiment analysiert wurden, wurden ebenfalls auf Microcystineffekte hin untersucht, indem ein Stamm von *Microcystis aeruginosa*,

der Microcystine enthält, und dessen microcystinfreie Mutante an *D. magna* verfüttert wurden. Die in der Nahrung enthaltenen Microcystine führten zu einer Hochregulation der Gene der Glyceraldehyd-3-Phosphat-Dehydrogenase und des Ubiquitin-conjugating Enzyms, die beide am Grundstoffwechsel von Daphnien beteiligt sind. Dies führt zu der Vermutung, dass cyanobakterielle Microcystine grundlegendere Auswirkungen auf den Metabolismus von Daphnien haben als ursprünglich angenommen.

Proteaseinhibitoren stellen eine andere wichtige Gruppe der Cyanotoxine dar, die weiter verbreitet ist als Microcystine. Deshalb konzentrierte ich mich im zweiten Teil der Dissertation auf die Targets dieser cyanobakteriellen Inhibitoren, d.h. auf Verdauungsproteasen (Trypsine und Chymotrypsine) von Daphnien. Die Verfütterung von Mischungen aus einer qualitativ hochwertigen Grünalge und einem von zwei Cyanobakterienstämmen, die entweder Trypsin- oder Chymotrypsininhibitoren enthielten, führte zu reduziertem somatischen Wachstum von *D. magna*. Diese Wachstumsreduktion wurde wahrscheinlich durch die Hemmung der Verdauungsenzyme durch cyanobakterielle Proteaseinhibitoren verursacht, die vermutlich zu einer Aminosäurelimitation der Daphnien führte. Jeder einzelne der beiden im Futter enthaltenen Proteaseinhibitoren hatte auf Proteinebene deutliche Auswirkungen auf die Verdauungsproteasen der Daphnien. *In situ* zeigten sich eine Hemmung der betreffende Proteasen und ebenso eine Zunahme der Proteaseaktivität des nicht-inhibierten Proteasetyps. Als Antwort auf die mit dem Futter zugeführten Chymotrypsininhibitoren wurden neue Protease-Isoformen gebildet. Die Verdauungsproteasen der Daphnien wurden auf aktivitätsgefärbten Proteingelen sichtbar gemacht und mittels LC-MS/MS und anschließender Datenbanksuche sechs verschiedenen Proteasegenen zugeordnet. Für die Real-Time PCR Analysen wurden aus den Sequenzen dieser Gene Primer etabliert. Es wurde eine starke Zunahme der Genexpression gemessen, die durch die im Futter enthaltenen Proteaseinhibitoren ausgelöst wurde. Die physiologischen Reaktionen, die hier in Daphnien beobachtet wurden, müssten adaptiv sein, da sie die Verdauung von Proteinen in Gegenwart von Proteaseinhibitoren verbessern sollten.

Im dritten Teil der Dissertation untersuchte ich intraspezifische Unterschiede von fünf *D. magna* Klonen. Diese Unterschiede zeigten sich bei der Verfütterung von Cyanobakterien, die entweder Trypsin- oder Chymotrypsininhibitoren enthielten. Mit Hilfe von Mikrosatellitenuntersuchungen wurden die fünf Klone als fünf verschiedene

Genotypen identifiziert. Es zeigten sich starke intraspezifische Unterschiede in der Sensitivität gegenüber den im Futter enthaltenen Proteaseinhibitoren; diese Sensitivität wurde als relative Wachstumsreduktionen gemessen. Die Stärke der Sensitivität hing vom Proteaseinhibitortyp und vom betroffenen *D. magna* Klon ab. Alle fünf Klone zeigten physiologische Reaktionen als Antwort auf die in den verfütterten Cyanobakterien enthaltenen Proteaseinhibitoren: die Aktivität des inhibierten Proteasetyps nahm ab, während die des anderen Typs zunahm. Das Ausmaß der relativen Wachstumsreduktion schien, wenn auch nicht signifikant, mit der Restaktivität des inhibierten Proteasetyps linear zu korrelieren. Höhere Restaktivität, die anscheinend zu niedriger Sensitivität des *D. magna* Klons führte, konnte teilweise auf die Zunahme der Proteasegenexpression zurückgeführt werden. Jedoch können auch andere Faktoren daran beteiligt gewesen sein, wie z.B. die Etablierung aktiver Proteaseisoformen. Einer der Daphnienklone, der eine auffällig niedrige Sensitivität gegenüber Trypsininhibitoren zeigte, besaß eine Protease oder eine Proteaseisoform, die in den anderen Klonen nicht gefunden wurde. Unterschiede gegenüber den vier anderen Klonen zeigten sich auch in den Aminosäuresequenzen zweier Trypsine.

Im vierten Teil der Dissertation untersuchte ich, ob die beobachtete Zunahme der Genexpression der Proteasen von erfahrenen Müttern, an die Chymotrypsin-inhibitoren enthaltende Cyanobakterien verfüttert wurden, an die Nachkommen weitergegeben wurde. Die Töchter dieser Mütter zeigten eine höhere Proteasegenexpression als die Nachkommen naiver Mütter. Bedauerlicherweise konnte der Adaptivwert dieses maternalen Effekts hier nicht gezeigt werden. Der hier vorgeschlagene maternale Transfer von Speicherproteinen auf die Nachkommenschaft von naiven und erfahrenen Müttern führte zu einer zeitweiligen Kompensation der Aminosäurelimitierung, die sich durch die Anwesenheit von Proteaseinhibitoren im Futter der Nachkommen ergab. Unseres Wissens ist dies das erste Mal, dass für Daphnien gezeigt wurde, dass maternale Proteineinlagerungen in Eier potentiell adaptiv sind, und dass erhöhte Genexpression funktionell wichtiger Gene maternal weitergegeben wurde.

Eine hohe Variablitität in der Sensitivität gegenüber Proteaseinhibitoren, die sich durch spezifische physiologische Reaktionen der Daphnien ergibt, bildet innerhalb einer Population die Grundlage für die Selektion hin zu weniger sensitiven Daphnien. Die Vermutung, dass cyanobakterielle Proteaseinhibitoren einen starken

Selektionsdruck auf ihre Targets, d.h. auf Daphnienproteasen, ausüben, wird dadurch gestützt, dass sich Verdauungsproteasen von Daphnien intraspezifisch in ihrer Aminosäuresequenz unterscheiden, dass die physiologischen Antworten der Daphnien auf cyanobakterielle Proteaseinhibitoren schnell vonstatten gehen und dass erhöhte Proteasegenexpression maternal auf die Nachkommenschaft übertragen wird. Ob dieser vermutete starke Selektionsdruck zu lokalen Adaptationen von Verdauungsproteasen in natürlichen Daphnienpopulationen geführt hat, ist noch zu untersuchen.

Abgrenzung der Eigenleistung

Part I: Target gene approaches: Gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free *Microcystis aeruginosa*

Das Konzept für diesen Teil der Dissertation wurde von Prof. Dr. Eric von Elert und mir erarbeitet. Alle Arbeiten dieses wurden von mir durchgeführt unter der Anleitung von Prof. Dr. von Elert und Dr. Cornelius Courts. Dr. John Colbourne und Dr. Frank Nitsche halfen mir bei der Konstruktion der Phylogenetischen Bäume.

Part II: Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors

Die Idee für diesen Teil der Dissertation stammt von Prof. Dr. Eric von Elert, Dr. Anja Zitt und mir. Prof. Dr. Peter Kroth half mit nützlichen Ratschlägen. Zusammen mit Dr. Zitt entwickelte ich die Proteaseprimer für die Real-Time PCR Analysen. Die meisten Arbeiten für diesen Teil der Dissertation wurden von mir durchgeführt. Von Lino Parlow stammen die Wachstumsraten des Daphnienklons, die er im Rahmen seiner Bachelorarbeit unter Anleitung von mir und Aufsucht von Prof. Dr. von Elert ermittelte. Die Proteinaufreinigung wurde unter meiner Anleitung von Christoph Effertz durchgeführt, die LC-MS/MS-Analysen und die anschließende Datenbankrecherche stammen von Dr. Stefan Müller.

Part III: Response of *Daphnia* to cyanobacterial protease inhibitors: intraspecific differences in digestive target proteases

Das Konzept für diesen Teil der Dissertation wurde von Prof. Dr. Eric von Elert und mir erarbeitet. Die meisten Arbeiten wurden von mir durchgeführt. Von Lino Parlow stammen die Wachstumsraten der Daphnienklone, die er im Rahmen seiner Bachelorarbeit unter Anleitung von mir und Aufsicht von Prof. Dr. von Elert ermittelte; ebenso die Proteingele. Prof. Dr. Luc DeMeester stellte uns freundlicherweise die Sequenzen einiger Mikrosatellitenprimer zur Verfügung. Die genomischen Proteaseprimer wurden von Dr. Zitt entwickelt.

Part IV: Cyanobacterial protease inhibitors as a trigger of maternal effects in *Daphnia*

Das Konzept für diesen Teil der Dissertation wurde von mir unter Aufsicht von Prof. Dr. von Elert entwickelt. Alle Arbeiten wurden von mir durchgeführt.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken in Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Eric von Elert betreut worden.

Curriculum vitae

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1987 bis 1991 Grundschule im Wallgut, Konstanz

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Segelbacher G, Sacher T, Schwarzenberger A, Woitsch S, Bairlein F, Coppack T (2007) Eight microsatellite loci characterised in the European blackbird, *Turdus merula.* Journal of Ornithology

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[1] entspricht Part I der Dissertation

[2] entspricht Part II der Dissertation