Population genetics, ecogenomics and physiological mechanisms of adaptation of *Daphnia* to cyanobacteria

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

Christian Küster
aus Engelskirchen

Uni-Druck, Köln

Köln 2012

Berichterstatter: Prof. Dr. Eric von Elert

Prof. Dr. Hartmut Arndt

Tag der mündlichen Prüfung: 18.01.2013

“Doppelpass alleine? Vergiss es!“

Lukas Podolski

Table of contents

Table of contents

General introduction and aim of the study

Members of the genus *Daphnia* belong to the order Cladocera, and their body sizes ranges from 0.2 mm to 5 mm in length. 150 different species of *Daphnia* occur in almost all limnic ecosystems, ranging from acidic swamps to deep permanent lakes, shallow temporary ponds and even to side arms of streams and rivers. *Daphnia* are unselective filter feeders and are typically the major herbivorous grazers of algae, (cyano)bacteria and protozoa. Depending on the *Daphnia* species, the size of the ingestible food particles ranges from 0.1 µm to 55 µm, representing the mean mesh size of the filter combs and the size of the carapace gape (Geller & Müller 1981; Gophen & Geller 1984). *Daphnia* are a major prey of planktivorous fish and invertebrate predators and thus provide an important link in the transfer of carbon and energy from primary producers (phytoplankton) to higher trophic levels. However, in eutrophic lakes this transfer is often constrained rather by food quality than by food quantity: The phytoplankton of eutrophic lakes and thus the major food source for *Daphnia* is often dominated by bloom-forming and/or filamentous cyanobacteria (Schreurs 1992), which are also associated with harmful effects to human health and livestock (Carmichael 1994). Due to the formation of colonies and filaments, the particle size of cyanobacteria often exceeds 55 µm and is therefore not an available carbon source for *Daphnia*. Especially large-bodied *Daphnia* species, such as *Daphnia magna* and *Daphnia pulex*, suffer from clogging of their filtering apparatus and increase rejection rates when feeding on filamentous cyanobacteria (DeMott *et al.* 2001). *Daphnia* have to cope with cyanobacteria especially in late summer and early fall (Sommer *et al.* 1986), when the temperature of the epilimnion reaches its maximum (Jöhnk *et al.* 2008). During such times, cyanobacterial mass developments of the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Planktothrix* or *Limnothrix* have become a wide-spread phenomenon in freshwater ecosystems (Dokulil & Teubner 2000).

The causes for the constrained carbon transfer from cyanobacteria to *Daphnia* have been extensively investigated in the past decades. At least three major properties of cyanobacteria may contribute to their relatively poor quality as a food source for *Daphnia*: As already outlined above, bloom-forming cyanobacteria often exhibit filaments or colonies that can interfere with the filtering apparatus of *Daphnia* (Porter & Mcdonough 1984), which has been suggested as a strategy to escape from zooplankton grazing in eutrophic lakes (i.e. (Ghadouani *et al.* 2003). In addition, cyanobacteria lack sterols and have a low content of polyunsaturated fatty acids. This cyanobacterial deficiency in essential lipids leads to reduced somatic and population growth of *Daphnia* due to constrained carbon assimilation

(Von Elert 2002; Von Elert *et al.* 2003; Martin-Creuzburg & Von Elert 2004; Martin-Creuzburg *et al.* 2008). Finally, many cyanobacteria produce secondary metabolites that are toxic for *Daphnia* and can thus reduce the fitness of *Daphnia* in terms of survival, growth and reproduction (Lürling & van der Grinten 2003; Rohrlack *et al.* 2003; Von Elert *et al.* 2012).

Besides microcystins, which belong to the group of heptapeptides and which are the most extensively studied class of cyanobacterial toxins (Sivonen & Jones 1999; Rohrlack *et al.* 2001), the role of cyanobacterial protease inhibitors has recently become a focus of attention. The most abundant protease inhibitors are depsipeptides that contain the modified amino acid 3-amino-6-hydroxy-2-piperidone (Von Elert *et al.* 2005). More than twenty depsipeptides, isolated from a wide range of cyanobacterial genera, have been described as protease inhibitors, specifically inhibiting the serine proteases chymotrypsin or trypsin (Gademann & Portmann 2008). Both digestive enzymes account for more than 80% of the proteolytic activity in the gut of *D. magna* (Von Elert *et al.* 2004) and are verified as targets for an inhibition by cyanobacterial inhibitors (Agrawal *et al.* 2005; Schwarzenberger *et al.* 2010). However, *D. magna* clones can respond to cyanobacterial inhibitors by remodeling their digestive enzymes and by an elevated expression of respective protease genes (Von Elert *et al.* 2012; Schwarzenberger *et al.* 2010). A study by Czarnecki *et al.* (2006) has demonstrated that the potential of natural seston to inhibit trypsins is comparable to that of pure cultures of cyanobacteria, which suggests that inhibition of *Daphnia*'s proteases is not confined to experiments with cyanobacterial cultures as food but occurs also in nature. However, Czarnecki *et al.* (2006) investigated lake seston that was sampled only at one particular date and thus this study did not account for possible seasonal and inter-annual fluctuations of cyanobacterial protease inhibition in *Daphnia*.

Chapter 1 examines the question whether the inhibitory potential of natural seston on *Daphnia*'s trypsins and chymotrypsins changes seasonally. Additionally, I have investigated whether seasonally fluctuating protease inhibition might have impacts on the genetic diversity and the tolerance to cyanobacterial protease inhibitors of a co-occurring *Daphnia* population. In order to test this, seston from a hypereutrophic pond was sampled regularly over two successive years and analyzed for its potential to inhibit proteases of a single clone of *D. magna* under standardized laboratory conditions. Simultaneously, I isolated *D. magna* clones at two times within one season, measured the clonal diversity of the sub-population via microsatellite analyses and subsequently used these established clones for growth experiments on diets differing in the amount of cyanobacterial protease inhibitors. I hypothesized that seasonally fluctuating sestonic protease inhibition had led to an increase of the mean tolerance of the *Daphnia* population to cyanobacteria with protease inhibitors.

Several studies have already shown that *Daphnia* individuals or populations are able to adapt to the presence of toxin-producing cyanobacteria (i.e. Gustafsson & Hansson 2004). Sarnelle & Wilson (2005) suggested that *D. pulicaria* populations, exposed to high cyanobacterial levels over long periods of time, can adapt in terms of being more tolerant to dietary toxic cyanobacteria. However, **chapter 1** considered for the first time the possibility of rapid adaptation of a natural *Daphnia* population to the occurrence of sestonic protease inhibition within one season. Specifically, I hypothesized that *Daphnia* clones isolated after the seasonal peak of sestonic protease inhibition would be more tolerant to cyanobacterial protease inhibitors than clones isolated before the peak.

Adaptation of *Daphnia* populations to environmental stressors is typically a result of microevolutionary processes. Concomitant with the occurrence of cyanobacteria in a lake natural selection favors those *Daphnia* genotypes that are better adapted to constraints associated with increased cyanobacterial abundances. The investigation of the origin and the persistence of genetic variation, which in itself is a precondition of evolutionary processes, provides a feasible possibility to understand how species and populations have evolved in response to selection. In *Daphnia* several target genes that are probably involved in the response to cyanobacterial toxins have been identified so far (i.e. Pflugmacher *et al.* 1998; Schwarzenberger *et al.* 2010). However, the variation of these target genes in natural *Daphnia* populations differing with respect to the coexistence with cyanobacteria has not been studied yet. The screening of target genes of whole populations is a crucial precondition to identify those functional phenotypes of expressed target genes that cause the difference between tolerant and sensitive *Daphnia* genotypes. In **chapter 2** I demonstrate that high resolution melting analysis (HRMA) is a cost-effective and powerful tool for the rapid screening and genotyping of target genes from large numbers of *Daphnia* individuals. As a proof of principle for HRMA I have analyzed digestive trypsins of two *D. magna* populations from different habitats. The trypsin genes chosen for **chapter 2** have been shown to be active in the gut of *D. magna* and thus constitute possible targets for an interference with cyanobacterial trypsin inhibitors (Schwarzenberger *et al.* 2010). Both *D. magna* populations probably differed in their experience with respect to cyanobacteria. One population originated from a pond containing toxic cyanobacteria that possibly produce protease inhibitors and the other from a pond without such cyanobacteria. I first sequenced pooled genomic PCR products of digestive trypsins genes from both populations to identify variable DNA-sequences. In a second step, those variable DNA-sequences of trypsin loci were screened for SNPs in each *D. magna* clone from both populations using HRMA. The hypothesis was

that *D. magna* clones from ponds with cyanobacteria have undergone selection by these inhibitors, which has led to different trypsin alleles.

Besides the analyses of differences of the tolerance to cyanobacterial protease inhibitors within (**chapter 1**) and among (**chapter 2**) *Daphnia* populations, **chapter 3** covers the issue of potential interspecific differences between two *Daphnia* species (*D. magna* and *D. pulex*). I assumed that *D. magna* coexist more frequently with cyanobacteria than *D. pulex* does. This putatively more frequent coexistence of cyanobacteria and *D. magna* might have resulted in an increased tolerance of *D. magna* to cyanobacterial protease inhibitors. Seven clones each of *D. magna* and *D. pulex* were isolated from different habitats in Europe and North America. In order to test for interspecific differences in tolerance to cyanobacteria, somatic and population growth rates on varying concentrations of two cyanobacterial strains were determined for each *D. magna* and *D. pulex* clone. Both cyanobacterial strains contained either chymotrypsin or trypsin inhibitors, but no microcystins. Possible differences in tolerance to cyanobacterial protease inhibitors might have several causes, for which I have tested in the **chapter 3**: (1) I hypothesized that high growth rates on cyanobacterial diets might result from high specific protease activities; therefore I determined the specific trypsin and chymotrypsin activity of each of the investigated *D. magna* and *D. pulex* clones. (2) I assumed that a higher sensitivity of *Daphnia*'s gut proteases might cause reduced somatic and population growth rates for diets with cyanobacterial protease inhibitors. Therefore I compared the sensitivity of gut chymotrypsins and trypsins to the respective cyanobacterial protease inhibitors among the different clones of each *Daphnia* species.

After considering various aspects of *Daphnia*'s tolerance to cyanobacterial protease inhibitors within (**chapter 1**) and among populations (**chapter 2**) as well as between two species (**chapter 3**), a different perspective is taken in **chapter 4**, which deals with anthropogenic long-term effects on *D. galeata* in Lake Constance. Due to intensive agriculture, phosphorus-containing detergents and the growing industry the total phosphorus (P_{tot}) concentration of Lake Constance increased more than ten-fold in the middle of the last century. Thus, Lake Constance underwent a period of eutrophication from the mid 1950s to mid 1980s leading to pronounced changes in the taxonomical composition of the phytoplankton community (Kümmerlin 1998). During the course of eutrophication, cyanobacterial genera became abundant in Lake Constance, while formerly dominating diatom species nearly vanished (Kümmerlin 1998). Due to a subsequent decline of the anthropogenic phosphorus input since 1980, concentrations of P_{tot} decreased over time (Güde *et al.* 1998). In the meantime Lake Constance has undergone the process of re-oligotrophication and has become an oligotrophic one (Kümmerlin 1998); the re-

oligotrophication went along with declining relative abundances of cyanobacteria in the phytoplankton community. Concomitant with changes in the phytoplankton community (Weider *et al.* 1997) noted significant genotypic shifts in the co-occurring *D. galeata* population in Lake Constance. Hairston *et al.* (1999, 2001) have shown that these shifts went along with an increase of the mean tolerance of the *D. galeata* population to cyanobacteria. However, it remained unclear which target genes and metabolic pathways of the *D. galeata* population were directly affected by the appearance of cyanobacteria during eutrophication. In order to identify the physiological mechanisms and target genes underlying the increased mean tolerance to cyanobacteria I have analyzed in **chapter 4** the genome of three *D. galeata* populations of Lake Constance via next-generation sequencing. *D. galeata* resting eggs were isolated from three different layers of sediment cores, representing the time before, during and after the peak of the eutrophication of Lake Constance. The extracted DNA from individual resting eggs was pooled for each population separately. The genomic DNA of each population was subsequently analyzed via next-generation sequencing to determine the genetic diversity of the *D. galeata* population and to identify those genes and pathways that were mainly influenced by these environmental changes mentioned above.

Taken together, the four chapters of the present study cover the influence of cyanobacteria on *Daphnia* populations from different perspectives. Several studies have already described differences in *Daphnia*'s tolerance to cyanobacteria, but just a few have dealt with the question, which particular genes and metabolic pathways in *Daphnia* were affected by the presence of cyanobacteria. The present study considers various aspects of *Daphnia*'s tolerance to cyanobacteria within and between populations as well as between two *Daphnia* species and tries to shed light onto the physiological mechanisms of *Daphnia*'s adaptation to cyanobacterial toxins in natural populations.

Chapter 1

Seasonal dynamics of sestonic protease inhibition: impact on *Daphnia* populations

Abstract

Daphnia populations often show rapid microevolutionary adaptation to environmental changes. Here we investigated the possibility that microevolution of *Daphnia* populations could be driven by natural sestonic protease inhibition (PI). We hypothesized that PI changes seasonally, which might lead to concomitant changes in tolerance to PI in a co-occurring *Daphnia magna* population. To test this, seston from a eutrophic pond was sampled regularly over two successive years. Extracts of these freeze-dried samples were used to determine their inhibitory potential (IP) on *D. magna* gut proteases. In the summer seston the IP against chymotrypsins exceeded that of spring seston 200-fold. To test for possible impacts on the co-existing *D. magna* population, we isolated clones before (spring) and after (fall) the peak of the IP. Microsatellite analyses revealed that the two subpopulations were genetically distinct. Individual exposure of three clones from each population to varying concentrations of a cyanobacterium that contains chymotrypsin inhibitors revealed a decrease in population and somatic growth rate for each clone, but no seasonal effects on *Daphnia*'s tolerance. To include maternal effects we conducted a multi-clonal competition experiment on various cyanobacterial concentrations. However, no evidence for seasonally increased tolerance of *D. magna* to dietary protease inhibitors could be found.

Introduction

Due to anthropogenic phosphorus loading, many lakes have undergone a period of eutrophication in the last decades (Correll 1998; Daniel *et al.* 1998). The phytoplankton of eutrophic lakes is usually dominated by bloom-forming species of cyanobacteria of the genera *Microcystis*, *Planktothrix*, *Limnothrix, Anabaena* or *Oscillatoria* (Dokulil & Teubner 2000; Schreurs 1992). A considerable accumulation of cyanobacterial biomass can be observed in late summer and early fall (Sommer *et al.* 1986), when the temperature of the epilimnion reaches its maximum (Jöhnk *et al.* 2008). Abundances of zooplankton, especially of non-selective filter feeders such as *Daphnia* (one of the main grazers of cyanobacteria), are highly influenced by the presence of cyanobacteria. The increasing dominance of cyanobacteria has been claimed to be a major factor leading to the decline in *Daphnia* abundances within and between lakes (Threlkeld 1979; Hansson *et al.* 2007). Although *Daphnia* populations and individuals can adapt to the presence of cyanobacteria by developing a higher tolerance to the occurrence of cyanobacteria (Gustafsson & Hansson 2004; Hairston *et al.* 1999; Hairston *et al.* 2001; Sarnelle & Wilson 2005), the traits underlying this adaptation have not yet been fully elucidated.

The causes for the poor assimilation of cyanobacterial carbon by *Daphnia* have been studied extensively in past decades. Three major food quality constraints have been revealed: (1) Cyanobacteria can be filamentous or colonial; both forms lead to mechanical interferences with the filtering process of daphnids (Porter & Mcdonough 1984). (2) Cyanobacteria lack sterols and have a low polyunsaturated fatty acid content; both are essential for *Daphnia* nutrition (Von Elert 2002; Von Elert *et al.* 2003). In *Daphnia*, this deficiency in lipids leads to reduced somatic growth and reproduction of daphnids due to constrained carbon assimilation (Von Elert & Wolffrom 2001; Martin-Creuzburg & Von Elert 2004; Martin-Creuzburg *et al.* 2008). (3) Many cyanobacterial strains also contain toxins such as microcystins, which have a negative effect on the fitness of *Daphnia* (Sivonen & Jones 1999; Rohrlack *et al.* 2001). However, a microcystin-free cyanobacterial strain of the genus *Microcystis* resulted in reduced growth of *Daphnia*, even though mechanical interferences and lipid deficiency could be excluded as causal factors (Lürling 2003). The reduced growth rates might be explained by the presence of other secondary metabolites in cyanobacteria that inhibit survival, growth, reproduction and/or feeding of *Daphnia* (Lürling & van der Grinten 2003; Rohrlack *et al.* 2003). Among such secondary metabolites, protease inhibitors are reported from a wide range of genera of marine and freshwater cyanobacteria. More than twenty depsipeptides which act as protease inhibitors have been described from different cyanobacteria genera (Gademann & Portmann 2008). Most of these depsipeptides

specifically inhibit the serine proteases chymotrypsin or trypsin: These two digestive enzymes are responsible for 80% of the proteolytic activity in the gut of *Daphnia magna* (Von Elert *et al.* 2004), and several studies indicate that cyanobacterial protease inhibitors directly inhibit digestive proteases of *Daphnia* (Agrawal *et al.* 2005; Schwarzenberger *et al.* 2010). A study by Czarnecki *et al.* (2006) has shown that the potential of natural seston to inhibit *Daphnia* trypsins is comparable to the inhibitory potential of various *Microcystis* strains. This makes it reasonable to assume that the interference of cyanobacterial protease inhibitors with digestive proteases of *Daphnia*, which had thus far only been demonstrated in cyanobacterial strains, occurs in nature as well. Blom *et al.* (2006) recently showed that *Daphnia* sp. coexisting with *Planktothrix rubescens*, a cyanobacterium that contains the trypsin inhibitor oscillapeptin J, are significantly less sensitive to oscillapeptin J than *Daphnia* sp. from lakes free of this cyanobacterium. Considering the finding that almost 60% of 17 water blooms of cyanobacteria contained protease inhibitors (Agrawal *et al.* 2001), it is thus reasonable to assume that increased tolerance to cyanobacteria in *Daphnia* populations may be caused by enhanced tolerance to the cyanobacterial protease inhibitors. As a consequence, the question arises as to whether this increased tolerance at the population level is caused by a constant selection pressure due to cyanobacterial protease inhibitors or by a short-term bottleneck effect due to the seasonal occurrence of cyanobacteria.

We hypothesized that, in accordance with the seasonal succession of phytoplankton, the potential of the edible fraction of a natural seston to inhibit digestive trypsins and chymotrypsins in *Daphnia* would vary with season. This seasonality would result in increased frequencies of *Daphnia* genotypes with enhanced tolerance to cyanobacterial protease inhibitors.

In the present study we investigated whether the potential of the natural seston to inhibit the proteases trypsin and chymotrypsin in *D. magna* changed seasonally. Furthermore we determined the clonal diversity of two *D. magna* samples, isolated before and after the maximum of inhibitory potential in the seston, by using microsatellites in order to detect concurrent changes in the corresponding *D. magna* population. Additionally, we conducted growth experiments with single *D. magna* clones from each of the two samples on treatments differing in the content of cyanobacteria with protease inhibitors. To investigate competitive interactions and maternal effects at different cyanobacterial protease inhibitor concentrations, we also performed a multi-clone experiment with the same clones over many generations.

Specifically, our predictions were that: (1) The potential of natural seston to inhibit *Daphnia* chymotrypsins and trypsins changes seasonally. In accordance with the seasonal

occurrence of cyanobacteria, we expect a higher potential of the seston to inhibit proteases in fall than in spring; (2) *D. magna* clones from the fall are more tolerant to cyanobacterial protease inhibitors, which results in higher growth rates on cyanobacterial diets in single-clone experiments. Potential costs of this adaption might result in lower growth rates and fitness on non-cyanobacterial diets. (3) *D. magna* clones from the fall show higher clonal frequency in multi-clonal experiments on a cyanobacterial diet containing protease inhibitors than clones from the spring.

Material and methods

Origin and cultivation of organisms

Two species of phytoplankton were used in the somatic and population growth experiments as well as in the competition experiment: The cyanobacterium *Microcystis aeruginosa* NIVA Cya 43 (Culture Collection of Algae, Norwegian Institute for Water Research) was grown in 2 l chemostates in sterile Cyano medium (Von Elert & Jüttner, 1997) at a dilution rate of 0.1 d^{-1} (20°C; illumination: 40 µmol m^{-2} s^{-1}).The green algae *Chlamydomonas* sp. (Strain 56, culture collection of the Limnological Institute at the University of Constance) was grown in 5 l semi-continuous batch cultures (20°C; illumination: 120 µmol m^{-2} s^{-1}) by replacing 20% of the culture with fresh sterile Cyano medium every Monday, Wednesday and Friday in the late exponential phase of the culture.

The *D. magna* clones were isolated from the Aachener Weiher (AaW; N50° 56' 2.40", E6° 55' 40.81"). The AaW is an urban pond of 0.04 km² and a maximum depth of 1.6 m. The AaW has a Secchi depth of less than one meter, and is therefore classified as a hypertrophic lake (Carlson 1977). Forty-seven *D. magna* clones were isolated on June 4, 2008 (spring samples) and 43 clones on September 3, 2008 (fall samples). *D. magna* clone B used in the enzyme assay originated from the *Großer Binnensee*, a lake in Germany (Lampert & Rothhaupt 1991). All clones of *D. magna* were cultured separately in aged, membrane-filtered tap water and fed with saturating concentrations of *C.* sp. for at least three generations.

Preparation of the seston samples

To test the potential of the edible fraction of seston (< 55 µm) in the AaW to inhibit proteases, samples were taken at constant intervals from spring to fall in 2007 and 2008.

Fifty to 80 l of surface water was screened with a 55 µm mesh. In the discharge the particulate fraction was then gently concentrated using a hollow-fiber filtration technique (A/G Technology Corp. Needham, CFP-1-D-6a, 0.1 µm mesh size). The final volume of approximately 0.8 l was frozen at -20°C and freeze-dried (Christ LOC-1m, LPHA 1-4). Lyophilized seston was subsequently homogenized using a mortar and pestle. The resulting powder was thoroughly mixed. 50 mg and 100 mg of freeze-dried natural seston were suspended in 500 µl of 60% methanol and sonicated for 15 min followed by centrifugation (3 min at 10^4 x g). The extract was separated from the residue, and the supernatant was used in the protease assay.

Protease assays

The activities of trypsins and chymotrypsins of *D. magna* clone B were measured as according to (Von Elert *et al.* 2004). Six-day-old individuals were each transferred to 5 µl (per animal) of cold (0°C) 0.1 M phosphate buffer (pH 7.5) containing 2 mM dithiothreitol (DTT) on ice. 2 mM DTT in the incubation buffer was used as according to Johnston *et al.* (1995). Individuals were homogenized with a Teflon pestle, the homogenate was centrifuged (3 min at 1.4 x 10^4 x g), and the supernatant was used immediately in the enzyme assay. SuccpNA (N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide, Sigma, 125 µM) was used as a substrate for chymotrypsins, while BapNA (N-R-benzoyl-DL-arginine 4-nitroanilide hydrochloride, Sigma, 1.8 mM) served as a substrate for trypsins. Trypsin and chymotrypsin assays were performed in a potassium phosphate buffer (0.1 M, pH 7.5). The absorption was measured continuously for 10 min at time intervals of 30 sec at 30°C at 390 nm with a Cary 50 photometer (Varian, Palo Alto, USA). Absorption increased linearly with time in all assays. The relevant parameter for the enzyme assays was the inhibition potential of the seston samples. To ensure comparable conditions for all protease assays, we normalized the protease activities to the same conversion rate of respective substrates prior to the addition of aliquots of the seston extracts. Five to 20 µl of different concentrations of each extracted seston biomass were tested for inhibition, whereas controls with 20 µl of 60% methanol had no effects on protease activities. The resulting protease activities were plotted as a function of extracted seston biomass. By fitting a sigmoidal dose response curve, the extracted biomass that resulted in a 50% inhibition of *Daphnia* protease activity (IC_{50}) was calculated. Low IC_{50} values of the analyzed samples indicated a high inhibitory potential.

Microsatellite analyses

The population structure of the spring and the fall *D. magna* populations were analyzed using polymerase chain reactions (PCR) of six polymorphic microsatellite loci with subsequent DNA fragment length analyses. The PCR assay for each *D. magna* clone was performed in a final volume of 50 µl containing 50 ng of genomic DNA, 0.2 µM of respective forward and reverse primer (Table 1), 0.2 mM of dNTPs, 2.5 units of Taq-DNA polymerase (5 Prime, Gaithersburg, USA) and 5 µl of corresponding 10x PCR buffer (5 Prime, Gaithersburg, USA). Cycling parameters were 95°C for 5 min to activate the DNA polymerase followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at respective primer T_a (Table 1) and 30 s elongation at 72°C. Final elongation was performed afterwards for 10 min at 72°C. After checking the success of the amplification via agarose gel electrophoresis, DNA fragment length analyses were performed with a 3730 DNA Analyzer (Applied Biosystems, Foster City, USA).

Table 1 Microsatellite loci used to genotype the *D. magna* clones, the respective forward and reverse sequences, GenBank accession number and the corresponding annealing temperatures (T_a).

Locus	Primer Sequences [5' - 3']	GenBank accession	T_a [°C]
DMA 12	**F:** AGCCAATCATCAATTCCCTC **R:** AAGGTCCGAATTGGATTACG	AF291912[1]	58
DMA 3	**F:** AAAGGAAAGCAACCGCTGC **R:** AAAAGGAAGGGGAATTACCC	AF291910[1]	58
DMA 14	**F:** GGGCAAGACACAGGTGC **R:** TGGCGGCATGCTGTCTAC	AF291913[1]	58
DP 162	**F:** CGAATCCGTTCGTCAAAAGC **R:** TGGCGGCATGCTGTCTAC	wfms0000166[2]	58
S6-38	**F:** GATGTCTTGCATCAACAGTG **R:** AGTCAAAGGTATGACTCACC	S6-38[3]	49
DMA 15	**F:** GTGTATTCTAAAATCGAATTTCG **R:** ACGTCAATGATCTTATTATACC	EU131363[1]	50

[1]developed by John Colbourne, unpublished

[2]Colbourne *et al.* (2004)

[3]Ortells *et al.* (2012)

Results

We found a seasonal variation of chymotrypsin inhibitory potential of the edible fraction of the AaW seston for 2007 and 2008 (Fig. 1A). In both years IC_{50} values decreased considerably in the summer, indicating a high inhibitory potential of the seston. From June to July 2007 a 200-fold decrease of chymotrypsin IC_{50} values was observed. In 2008 a significant decrease of IC_{50} was first measured for seston samples taken in August. The IC_{50} values remained on a comparatively low level until the end of September. Less seasonal variation was observed with regard to the inhibition of *D. magna* trypsins. A considerable decrease of trypsin IC_{50} values was only observed in 2007 (Fig. 1B). Contemporaneous with the chymotrypsins, the IC_{50} values for trypsins decreased significantly in July 2007. The IC_{50} values regarding the inhibition of trypsins remained at a low level for samples taken in 2008. However, seston samples taken in 2007 showed ten-fold lower IC_{50} values for the inhibition of *D. magna* chymotrypsins than of trypsins. The seston of 2007 showed higher overall effects on both chymotrypsins and trypsins than the seston of 2008 did. When *C.* sp. was assayed for comparison, inhibitory effects were found neither for chymotrypsins nor for trypsins (IC_{50}> 10^4 µg/ml). Additional information on all concentrations of extracted seston from 2007 and 2008 that were used for the determination of the IC_{50} values can be found in the supporting material (Fig. 4-7).

To test for potential changes in clonal diversity of both *D. magna* samples, we analyzed 47 spring and 43 fall clones by using six polymorphic microsatellite loci. We found 21 genetically distinct genotypes in the spring samples and 13 in the fall samples. Using exact p-values, allele frequencies differed significantly ($p < 0.05$) from Hardy-Weinberg equilibrium in three loci of both populations (DMA 12, DP 162 und S6-38) due to increased heterozygosity (Table 2). Contrarily, the fall population indicates a deficit of heterozygous at loci DMA 3, DMA 14 and DMA 15 ($p < 0.05$), while the spring population only deviated significantly from Hardy-Weinberg equilibrium at locus DMA 3 (Table 2). The genetic distance between *D. magna* spring and fall samples was quite low ($F_{ST} = 0.005$); however, both samples were significantly distinct ($p < 0.05$).

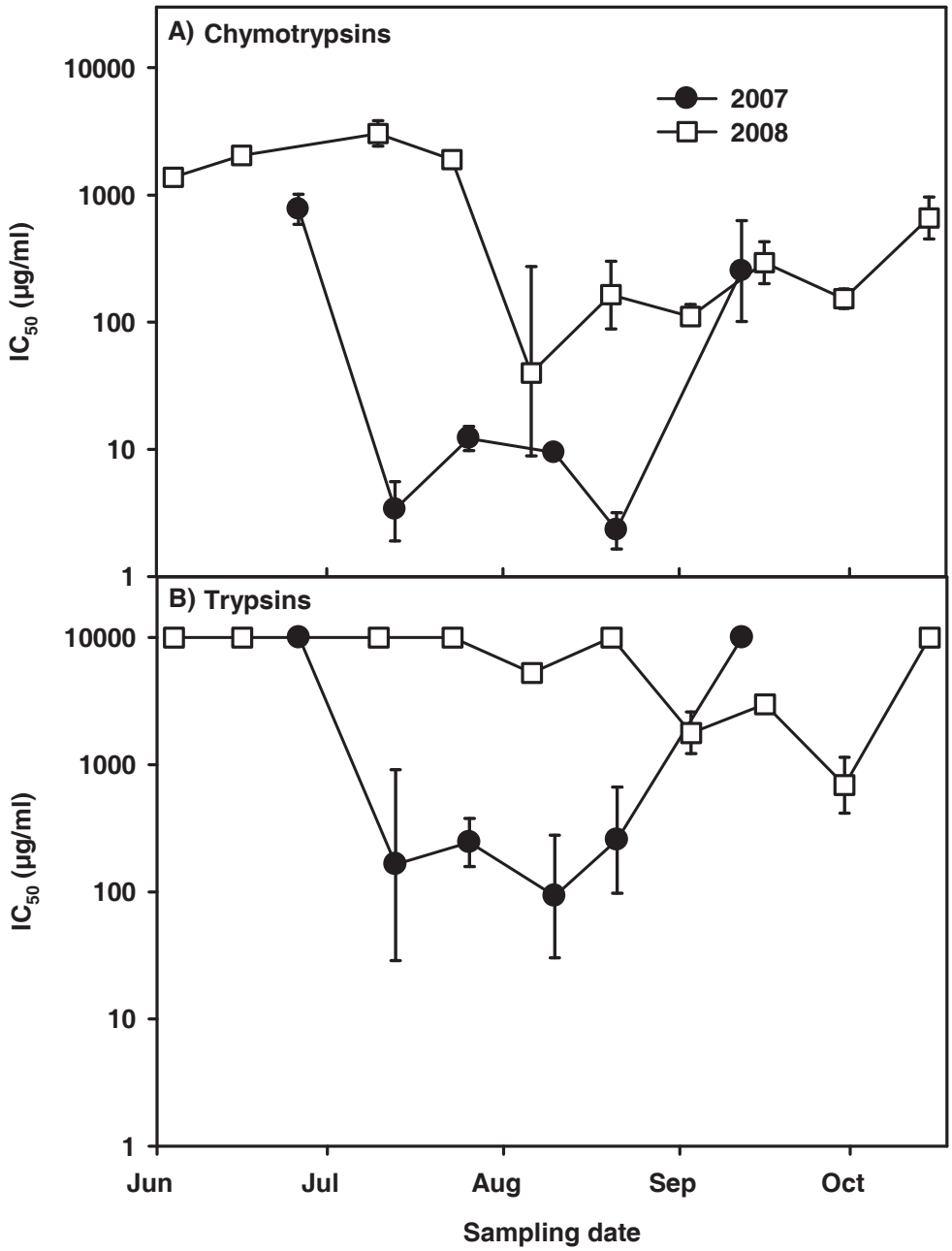

Figure 1 Inhibition of digestive proteases from homogenates of *D. magna* clone B by dry weight extracts of the edible fraction (< 55 µm) of seston of the AaW from different sampling dates. Seston samples from 2007 (black circles) and 2008 (white squares) were assessed for their effect on digestive chymotrypsins (A) and trypsins (B) of *D. magna*. Depicted are IC_{50} concentrations of extracted freeze-dried seston (± 95% CI). IC_{50} values indicate the inhibitory potential of the seston sample. Low IC_{50} values indicate a high inhibitory potential of the seston, which means that little freeze-dried seston was needed to inhibit 50% of *D. magna* chymotrypsins. Non-overlapping 95% confidences intervals (CI) among two samples were assumed as significantly different. 95% CI may appear asymmetric due to log-scale of IC_{50} values. Where error bars are not visible, the 95% CI are smaller than symbols.

Table 2 Observed (H_o) and expected (H_e) heterozygosity of the *D. magna* spring and fall samples in six polymorphic microsatellite loci, according to Hardy-Weinberg equilibrium. N: number of analyzed clones, N_a: number of alleles. Significant differences at $p < 0.05$ (*) and $p < 0.01$ (**).

Locus	Population	N	N_a	H_e	H_o	p
DMA 12	Spring	47	3	0.595	0.766	**0.00019****
	Fall	43	3	0.495	0.721	**0.00011****
DMA 3	Spring	47	2	0.595	0.085	**0.00000****
	Fall	43	2	0.477	0.000	**0.00000****
DMA 14	Spring	47	2	0.485	0.426	0.69900
	Fall	43	2	0.385	0.512	**0.04160***
DP 162	Spring	47	3	0.573	0.830	**0.00033****
	Fall	43	3	0.595	0.814	**0.00013****
S6-38	Spring	47	2	0.374	0.489	**0.04400***
	Fall	43	2	0.484	0.791	**0.00000****
DMA 15	Spring	47	2	0.446	0.425	0.0971
	Fall	43	2	0.385	0.512	**0.0415**

We performed growth experiments using three different diets to check for possible seasonal adaptation to chymotrypsin inhibition by the seston. Three genotypes of each *D. magna* sample were kept on three different diets: Animals were fed either with pure *C.* sp. or with one of two various mixtures of *C.* sp. and *M. aeruginosa*. We found an overall effect of diet on somatic growth rates of each of the *D. magna* clones (Fig. 2A; ANOVA, S2: $F_{2,6} = 678.4$; $p < 0.001$; S7: $F_{2,6} = 66.83$; $p < 0.001$; S15: $F_{2,6} = 251.8$; $p < 0.001$, F3: $F_{2,6} = 219,7$; $p < 0.001$; F11: $F_{2,6} = 137.7$; $p < 0.001$; F24: $F_{2,6} = 1052.6$; $p < 0.001$). Somatic growth rates in *M. aeruginosa* treatments were significantly lower than in the control with pure *C.* sp. With increasing *M. aeruginosa* concentration, the growth rates of all tested clones decreased significantly (Tukey's HSD, $p < 0.05$). Population growth (Fig. 2B) was also affected by diet (ANOVA, S2: $F_{2,6} = 144.5$; $p < 0.001$; S7: $F_{2,6} = 57.0$; $p < 0.001$; S15: $F_{2,6} = 7.33$; $p < 0.001$; F3: $F_{2,6} = 28.7$; $p < 0.001$; F11: $F_{2,6} = 296.3$; $p < 0.001$; F24: $F_{2,6} = 227.6$; $p < 0.001$). Similar to the somatic growth rates, the population growth rates of clones F11, F24, S2 and S7 decreased significantly with increasing *M. aeruginosa* concentration (Tukey's HSD, $p < 0.05$). With regard to clones F3 and S15, only treatments with 20% *M. aeruginosa* resulted in lower population growth rates. The factor 'season' affected neither the somatic nor the population growth rates.

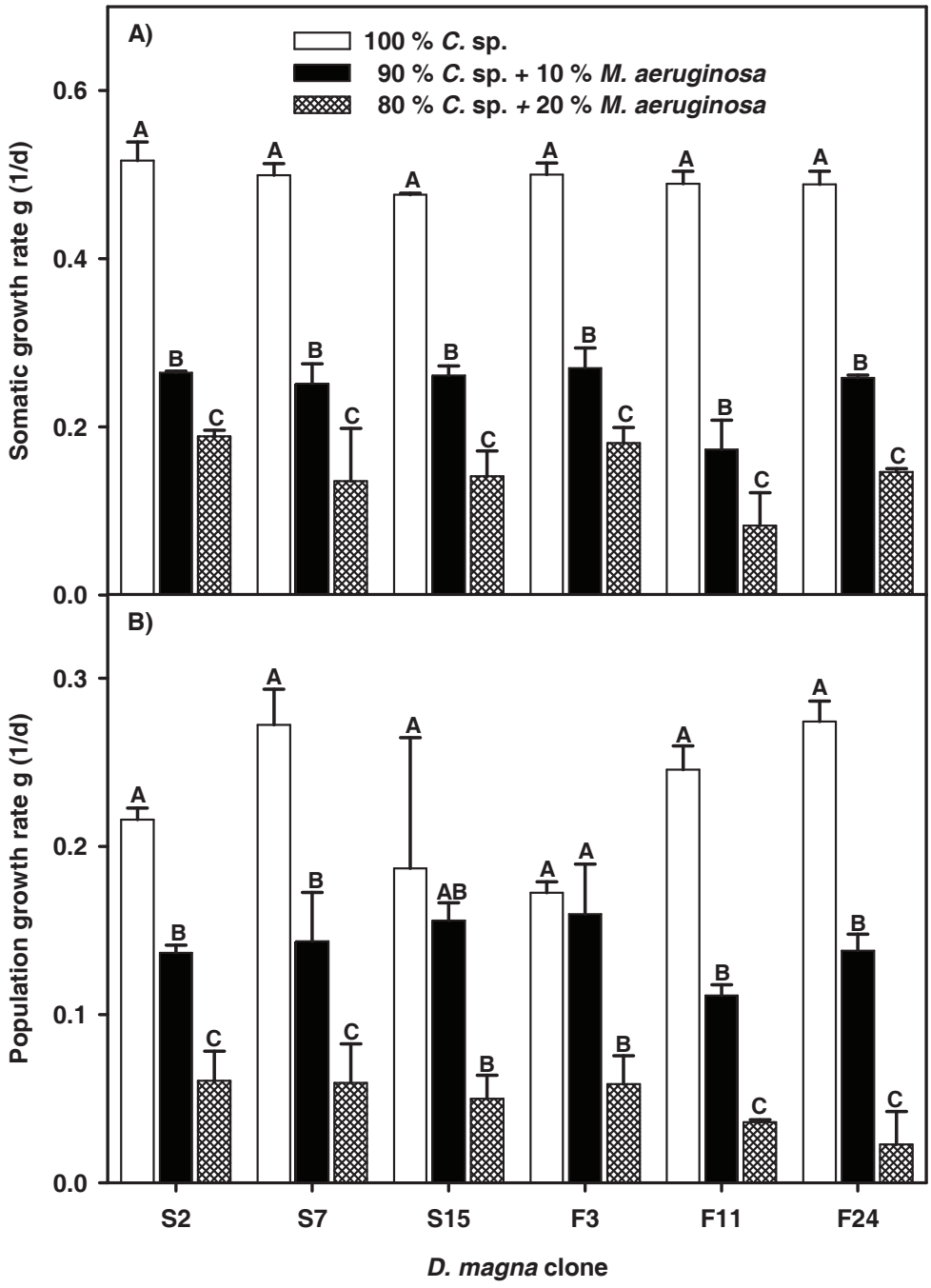

Figure 2 Somatic (A) and population (B) growth rates (mean ± SD, n=3) of *D. magna* clones from the AaW on pure *C.* sp. and on two mixtures of *C.* sp. and *M. aeruginosa*. Clones labeled with 'S' and 'F' were isolated in spring and fall, respectively. Different significance levels (one Way ANOVA, Tukey's HSD test, $p < 0.05$) within clones are indicated by different letters.

Table 3 Results of repeated-measurement analyses of variances of *D. magna* clone frequency in control and *Microcystis* treatment. Significant differences at $p < 0.05$ (*) and $p < 0.01$.

	SS	*df*	*F*	*P*
Clone S2				
Treatment	0.002178	1	0.8252	0.393861
Error	0.018475	7		
Time	0.064362	2	11.2087	**0.001241****
Time x treatment	0.000936	2	0.1631	0.851125
Error	0.040195	14		
Clone S7				
Treatment	0.143614	1	5.67122	**0.048779***
Error	0.177263	7		
Time	0.019229	2	0.73833	0.495626
Time x treatment	0.004162	2	0.15980	0.853845
Error	0.182306	14		
Clone S15				
Treatment	0.085287	1	3.67251	0.096844
Error	0.162562	7		
Time	0.016339	2	0.63204	0.546011
Time x treatment	0.004847	2	0.18750	0.831080
Error	0.180957	14		
Clone F3				
Treatment	0.004433	1	0.63738	0.450891
Error	0.048690	7		
Time	0.014072	2	2.22842	0.144476
Time x treatment	0.000985	2	0.15592	0.857095
Error	0.044204	14		
Clone F11				
Treatment	0.005287	1	1.93804	0.206508
Error	0.019096	7		
Time	0.008627	2	4.47832	**0.031372***
Time x treatment	0.001802	2	0.93546	0.415606
Error	0.013484	14		
Clone F24				
Treatment	0.016051	1	0.6264	0.454665
Error	0.179381	7		
Time	0.031368	2	0.5769	0.574439
Time x treatment	0.001483	2	0.0273	0.973145
Error	0.380612	14		

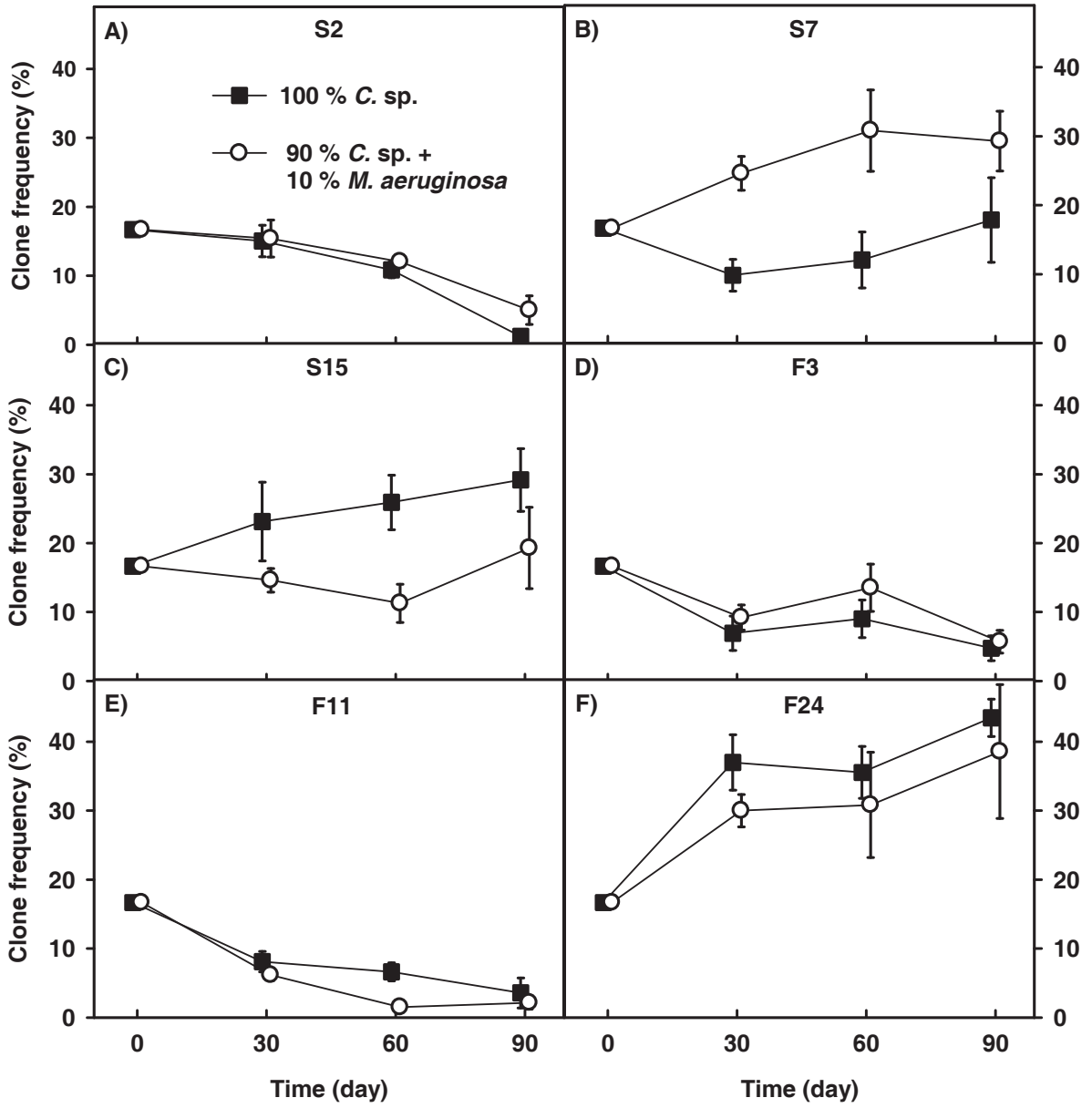

Figure 3 Mean frequency (± SD, n=5) of *D. magna* clones in the competition experiment. Clones were isolated from the AaW either in spring (A-C) or in fall (D-F) 2008. Black squares and white circles represent frequency of clones grown on pure *C.* sp. or on a mixture of 90% *C.* sp. and 10% *M. aeruginosa* after 30, 60 and 90 days.

In addition to the single growth experiments, a long-term experiment was performed in order to consider further effects due to maternal mechanisms or clonal interactions. *D. magna* clones were cultured together in two treatments differing in cyanobacteria content (Fig. 3). This competition experiment showed significant effects for factors 'time' or 'diet' on some of the six *D. magna* clones (Table 3). Clone S2 and F11 showed significantly lower relative abundances on day 90 than on day 30 on both pure *C.* sp. and on the mixture with

90% *C.* sp. and 10% *M. aeruginosa* (Fig. 3A, E). *D. magna* clone S7 was more abundant on the *M. aeruginosa* mixture than on pure *C.* sp. (Fig. 3B). None of the factors 'time', 'diet' and 'time x diet' had significant effects on clones S15, F3 and F24 (Fig. 3C, D, F) . To exclude negative effects due to food quantity, the food concentration was always kept above 0.2 mg C/l, the incipient limiting food level of *D. magna* (Porter *et al.* 1982).

Discussion

Czarnecki *et al.* (2006) have recently shown that the potential for natural lake seston to inhibit trypsins of *Daphnia* was comparable to the potential of cultures of *Microcystis* sp. to inhibit trypsins. The present study showed for the first time that natural lake seston can also inhibit *Daphnia* chymotrypsins, which, in addition to trypsins, account for the largest proportion of proteolytic activity in the gut of *D. magna* (Von Elert *et al.* 2004). With regard to the well-known seasonal succession in the phytoplankton community (Sommer *et al.* 1986), our study is the first to investigate seasonal changes of the potentials of a natural lake seston to inhibit trypsins and chymotrypsins of *D. magna*. We focused on the seston fraction smaller than 55 µm, which constitutes the edible size fraction for daphnids (Gophen & Geller, 1984; Hessen, 1985), and found seasonal changes in the potential of the AaW seston to inhibit *D. magna* chymotrypsins in each of two successive years. From the end of June to mid-July 2007, this inhibitory potential increased more than 200-fold within merely three weeks. We found a similar pattern of chymotrypsin inhibition in 2008. Due to the lack of data about the phytoplankton composition in the AaW, it remains unclear whether the increase of the inhibitory potential was a consequence of a higher relative abundance of cyanobacteria containing protease inhibitors. We cannot rule out the possibility that this increase resulted from a relatively high cellular content of protease inhibitors within an otherwise unaltered phytoplankton community. Such increases of the content of particular secondary metabolites in response to growth conditions have already been demonstrated for microcystins in various *Microcystis* strains (Long *et al.* 2001; Wiedner *et al.* 2003).

In parallel with seasonal changes in the potential of the phytoplankton to inhibit proteases, the genetic structure of the *D. magna* population of the AaW changed in 2008. Microsatellite analyses of *D. magna* clones established in the laboratory showed that the spring and fall samples were genetically distinct. We found a decline in the number of *D. magna* genotypes from spring to fall. Due to selective differences among *Daphnia* clones an erosion of clonal diversity during the parthenogenetic phase is a common phenomenon in *Daphnia*

populations (Lynch 1987; Vanoverbeke & De Meester 2010). The loss of genetic variability in the AaW coincided with an increase of the inhibitory potential on chymotrypsins in the seston and could result from natural selection due to increasing protease inhibition on *D. magna* genotypes.

Three spring clones and three fall clones of *D. magna* were assayed in order to test whether seasonal changes in genotypes might be caused by the inhibitory potential of the seston on chymotrypsins. All six *D. magna* clones showed a significant reduction of their somatic growth rate with increasing cyanobacterial abundance in the food. However, only four of the six clones exhibited a reduction in population growth when feeding on 10% *M. aeruginosa*. Two clones showed no negative effects in population growth, even at a relative food abundance of 20% *M. aeruginosa*. This supports the data of Lürling (2003), who used the same strain of *M. aeruginosa* and reported a reduction in population growth of *D. magna* clones at a concentration of ≥ 25% *M. aeruginosa*. The *M. aeruginosa* strain used here produces the chemically known chymotrypsin inhibitors Cyanopeptolin 954 and Nostopeptin BN920 (Von Elert *et al.* 2005) and contains no microcystins (Lürling 2003). The observed negative effect of 20% of this cyanobacterium on somatic and population growth of the six *D. magna* clones suggests that this reduction was caused by an interference of the cyanobacterial inhibitors with the chymotrypsins in the gut of *D. magna*. Evidence that this strain of *M. aeruginosa* interferes with *D. magna* chymotrypsins is based on several studies (Von Elert *et al.* 2005; Schwarzenberger *et al.* 2010). Schwarzenberger *et al.* (2010) reported that the activity of gut chymotrypsins of *D. magna* decreased clearly when the animals were fed with *M. aeruginosa*. Additionally, *D. magna* can respond with physiological plasticity to dietary cyanobacterial protease inhibitors by increasing the expression of the targets of these inhibitors, i.e. chymotrypsins, and by the expression of less-sensitive isoforms (Schwarzenberger *et al.* 2010, Von Elert *et al.* 2012). Clearly these regulatory responses are adaptive for *D. magna*, as they increase the capacity for protein digestion in the presence of dietary protease inhibitors. These specific responses in chymotrypsin expression strongly point at an interference of the two known chymotrypsin inhibitors with *D. magna* chymotrypsins when this strain of *M. aeruginosa* is fed.

Variances in the mean tolerance to cyanobacteria of *Daphnia* populations over time have already been demonstrated. For example, Weider *et al.*(1997) noted significant genotypic shifts in the *D. galeata* population of Lake Constance, Germany, by collecting resting eggs from lake sediments dating from the mid-1960s to mid-1990s and analyzing the genotypic structure of the hatchlings from these resting eggs. The observed shifts in genotype composition were strongly correlated with changes in the trophic state of the lake. These

genotypic shifts were related to micro-evolutionary changes within the population in their ability to cope with cyanobacteria as food (Hairston *et al.* 1999; Hairston *et al.* 2001). However, in our single-clone growth experiments we could not confirm our hypothesis that an adaptation of the *D. magna* population to cyanobacteria in the AaW occurred over the seasons; clones isolated in fall 2008 did not grow better on mixtures with *M. aeruginosa* than clones isolated in spring did.

Gustafsson *et al.* (2005) have shown that *D. magna* can acquire a tolerance to cyanobacteria within a single animal's lifetime and transfer this tolerance through maternal effects to the next generation. Such a maternal transfer is not accounted for in the single-clone growth assays described above. Maternal effects are adequately considered in multi-generational competition experiments. Such experiments have demonstrated that food quality determined which of several clones from the *D. pulex* species complex becomes dominant in an experimental population (Weider *et al.* 2005; Weider *et al.* 2008). Here we conducted such a multi-generational competition experiment, and 10% of the chymotrypsin-inhibitor-producing cyanobacterium significantly affected the clonal composition of the populations. In the competition experiment the same *D. magna* clones as used in the single-clone growth assays were cultured together for 90 days on either pure *C.* sp. or on a mixture of *C.* sp. and *M. aeruginosa* at saturating food levels. Two kinds of effects occurred at the end of the experiment: First, the relative frequency of two clones (S2, F11) decreased significantly, whereas three clones (F24, S2, S15) dominated the clonal compositions in both treatments. This is reflected in a reduction of Shannon's diversity (data not shown). Earlier studies have already demonstrated that the genetic diversity in a multi-clonal competition experiment decreased over time under constant conditions (Weider *et al.* 2008; Nelson *et al.* 2005). The single-clone growth experiments served hereby as controls for the competition experiment, as the two experiments were started simultaneously with the same cohort of newborns. Due to the fact that all *D. magna* clones had positive population growth rates on both treatments we can rule out the possibility that the decrease in frequency of clone S2 and F11 resulted from a general intolerance to the different food treatments directly from the start.

The second effect observed in the competition experiment was the significantly higher frequency of *D. magna* clone S7 in the treatment with 10% *M. aeruginosa* than on the pure green alga *C.* sp.. This suggests that clone S7 has a greater potential to dominate the *D. magna* population in the presence of *M. aeruginosa* than in its absence. In the single-clone growth experiment, the somatic and population growth of clone S7 in the presence of *M. aeruginosa* did not differ from the growth of the other clones. However, the present study underlines earlier results (Weider *et al.* 2008) showing that single clone experiments cannot

predict the outcome of a complex competition experiment. The discrepancy in growth rates between single clone and competition experiments of *D. magna* clone S7 can be explained by inducible mechanisms. Increased tolerance can be transferred to the offspring via maternal effects, a fact that has been interpreted as an inducible defense (Gustafsson *et al.* 2005). It remains unclear whether this inducible tolerance results from microevolution and hence can only be found in *D. magna* clones originating from lakes containing cyanobacteria.

We hypothesized that *D. magna* clones from the fall population would show higher clonal frequencies in the *M. aeruginosa* treatment. This could not be confirmed, and thus we could not find any evidence that the *D. magna* clones isolated in fall were better adapted to cyanobacteria containing protease inhibitors than the spring clones were. We used three distinct clones of each sample, a number which might not have been sufficient to represent the clonal *D. magna* composition of the spring and the fall samples in the AaW. Possibly existing seasonal patterns of tolerance to cyanobacteria might be detectable by using a greater number of *D. magna* clones of each sample. In contrast to the study of Weider *et al.* (2008) in which seven *Daphnia* clones from different lakes were used, we used six *D. magna* clones originating from just one habitat. It is reasonable to assume that clones originating from the same population are genetically more similar with respect to traits that mediate tolerance to cyanobacteria than clones isolated from various populations that differ with respect to the prevalence of cyanobacteria. Sarnelle & Wilson (2005) demonstrated differences in the tolerance to cyanobacteria of *D. magna* clones from different habitats, giving evidence for local adaptation to cyanobacteria. In populations coexisting with cyanobacteria, less tolerant clones might have become extinct due to natural selection caused by the cyanobacteria. Since the selection pressure exerted by protease inhibitors probably already existed over several previous seasons in the AaW, sensitive genotypes would have been under negative selection for numerous generations and thus only be present in low numbers. It remains unclear how many of the *D. magna* genotypes of the spring population originated from clones that remained in the lake during the winter or originated from hatched ephippia in spring. Ex-ephippia genotypes constitute new genotypes resulting from sexual reproduction and are not under selection by food quality until hatching. *D. magna* clones freshly hatched from ephippia might thus be less tolerant to cyanobacteria. A high proportion of freshly hatched genotypes from ephippia in the spring should result in a detectably lower fitness on cyanobacterial diet compared to clones from the fall. If more clones of the population in spring originated from *D. magna* genotypes which had persisted over the winter, the mean selection pressure on the population due to seasonally peaking

protease inhibitors could be too small to cause detectable fitness differences between spring and fall samples.

The present study shows for the first time seasonal changes in the potential of a natural lake seston to inhibit the major digestive proteases trypsin and chymotrypsin of *D. magna*. The inhibitory potential on chymotrypsins was clearly higher in fall than in spring for each of two successive years. However, in single-clone growth- and competition experiments we could not find seasonal differences in tolerance between the *D. magna* spring and fall samples to a cyanobacterium containing chymotrypsin inhibitors. Contrary to our hypothesis, *D. magna* genotypes from the fall were not better adapted to a cyanobacterial diet containing protease inhibitors than genotypes from the spring were. It remains to be seen if the coexistence with seasonally varying dietary protease inhibitors in the AaW has led to a locally adapted *D. magna* population with such a high level of tolerance to protease inhibitors that seasonally peaking protease inhibitors have no detectable effects on the tolerance of *D. magna* within this population. It will be interesting to see if a comparison among *D. magna* populations that exist in the presence or absence of dietary protease inhibitors provides evidence that seasonally occurring protease inhibitors constitute a constraint strong enough to lead to locally adapted populations.

References

Agrawal MK, Bagchi D & Bagchi SN (2001) Acute inhibition of protease and suppression of growth in zooplankter, *Moina macrocopa*, by *Microcystis* blooms collected in Central India. *Hydrobiologia*, 464, 37-44.

Agrawal MK, Zitt A, Bagchi D *et al.* (2005) Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. *Environmental Toxicology*, 20, 314-322.

Blom JF, Baumann HI, Codd GA & Juttner F (2006) Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp(3),(E)-Dhb(7)]microcystin-RR. *Archiv für Hydrobiologie*, 167, 547-559.

Carlson, R. E. (1977) Trophic state index for lakes. *Limology and Oceanography*, 22, 361-369

Colbourne JK, Robison B, Bogart K & Lynch M (2004) Five hundred and twenty-eight microsatellite markers for ecological genomic investigations using *Daphnia*. *Molecular Ecology Notes*, 4, 485-490.

Correll DL (1998) The role of phosphorus in the eutrophication of receiving waters: A review. *Journal of Environmental Quality*, 27, 261-266.

Czarnecki O, Henning M, Lippert I & Welker M (2006) Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). *Environmental Microbiology*, 8, 77-87.

Daniel TC, Sharpley AN & Lemunyon JL (1998) Agricultural phosphorus and eutrophication: A symposium overview. *Journal of Environmental Quality*, 27, 251-257.

Dokulil MT & Teubner K (2000) Cyanobacterial dominance in lakes. *Hydrobiologia*, 438, 1-12.

Excoffier L, Laval G & Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics*, 1, 47-50.

Gademann K & Portmann C (2008) Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. *Current Organic Chemistry*, 12, 326-341.

Gophen M & Geller W (1984) Filter mesh size and food particle uptake by *Daphnia*. *Oecologia*, 64, 408-412.

Guo SW & Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, 48, 361-372.

Gustafsson S & Hansson LA (2004) Development of tolerance against toxic cyanobacteria in *Daphnia*. *Aquatic Ecology*, 38, 37-44.

Gustafsson S, Rengefors K & Hansson LA (2005) Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. *Ecology*, 86, 2561-2567.

Hairston NG, Holtmeier CL, Lampert W *et al.* (2001) Natural selection for grazer resistance to toxic cyanobacteria: Evolution of phenotypic plasticity? *Evolution*, 55, 2203-2214.

Hairston NG, Lampert W, Caceres CE *et al.* (1999) Lake ecosystems - Rapid evolution revealed by dormant eggs. *Nature*, 401, 446.

Hansson LA, Gustafsson S, Rengefors K & Bomark L (2007) Cyanobacterial chemical warfare affects zooplankton community composition. *Freshwater Biology*, 52, 1290-1301.

Hessen DO (1985) Filtering structures and particle-size selection in coexisting Cladocera. *Oecologia*, 66, 368-372.

Jöhnk KD, Huisman J, Sharples J *et al.* (2008) Summer heatwaves promote blooms of harmful cyanobacteria. *Global Change Biology*, 14, 495-512.

Johnston KA, Lee MJ, Brough C *et al.* (1995) Protease activities in the larval midgut of *Heliothis virescens*: evidence for trypsin and chymotrypsin-like enzymes. *Insect Biochem.Molec.Biol.* 25, 375-383.

Lampert W & Rothhaupt KO (1991) Alternating dynamics of rotifers and *Daphnia magna* in a shallow lake. *Archiv für Hydrobiologie*, 120, 447-456.

Long BM, Jones GJ & Orr PT (2001) Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. *Applied and Environmental Microbiology*, 67, 278-283.

Lürling M (2003) *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedesmus obliquus*. *Limnology and Oceanography*, 48, 2214-2220.

Lürling M & van der Grinten E (2003) Life-history characteristics of *Daphnia* exposed to dissolved microcystin-LR and to the cyanobacterium *Microcystis aeruginosa* with and without microcystins. *Environmental Toxicology and Chemistry*, 22, 1281-1287.

Lynch M (1987) The consequences of fluctuating selection for isozyme polymorphisms in *Daphnia*. *Genetics*, 115, 657-669.

Martin-Creuzburg D & Von Elert E (2004) Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata*. *Journal of Chemical Ecology*, 30, 483-500.

Martin-Creuzburg D, Von Elert E & Hoffmann KH (2008) Nutritional constraints at the cyanobacteria-*Daphnia magna* interface: The role of sterols. *Limnology and Oceanography*, 53, 456-468.

Nelson WA, McCauley E & Wrona FJ (2005) Stage-structured cycles promote genetic diversity in a predator-prey system of *Daphnia* and algae. *Nature*, 433, 413-417.

Ortells R, Olmo C & Armengol (2012) Colonization in action: genetic characteristics of *Daphnia magna* Strauss (Crustacea, Anomopoda) in two recently restored ponds. *Hydrobiologia*, 689, 37-49.

Porter KG, Gerritsen J & Orcutt JD (1982) The effect of food concentration on swimming patterns, feeding-behavior, ingestion, assimilation, and respiration by *Daphnia*. *Limnology and Oceanography*, 27, 935-949.

Porter KG & Mcdonough R (1984) The energetic cost of response to blue-green-algal filaments by Cladocerans. *Limnology and Oceanography*, 29, 365-369.

Reynolds J, Weir BS & Cockerham CC (1983) Estimation of the co-ancestry coefficient - basis for a short-term genetic-distance. *Genetics*, 105, 767-779.

Rohrlack T, Christoffersen K, Hansen PE *et al.* (2003) Isolation, characterization, and quantitative analysis of microviridin J, a new *Microcystis* metabolite toxic to *Daphnia*. *Journal of Chemical Ecology*, 29, 1757-1770.

Rohrlack T, Dittmann E, Börner T & Christoffersen K (2001) Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. *Applied & Environmental Microbiology.* 67, 3523-3529.

Sarnelle O & Wilson AE (2005) Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, 50, 1565-1570.

Schreurs,H. (1992) Cyanobacterial dominance, relation to eutrophication and lake morphology, thesis, University of Amsterdam.

Schwarzenberger A, Zitt A, Kroth P, Mueller S & Von Elert E (2010) Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. *BMC physiology*, 10, 6.

Sivonen K &h Jones G (1999) Cyanobacterial toxins. toxic cyanobacteria in water: a guide to their public health Consequences. *Monitoring and Management*, *41-111*.

Sommer U, Gliwicz ZM, Lampert W & Duncan A (1986) The PEG-Model of seasonal succession of planktonic events in fresh waters. *Archiv für Hydrobiologie*, 106, 433-471.

Threlkeld ST (1979) Midsummer dynamics of 2 *Daphnia* species in Wintergreen Lake, Michigan. *Ecology*, 60, 165-179.

Vanoverbeke J & De Meester L (2010) Clonal erosion and genetic drift in cyclical parthenogens - the interplay between neutral and selective processes. *Journal of Evolutionary Biology*, 23, 997-1012.

Von Elert E & Jüttner F (1997) Phosporus limitation and not light controls the extracellular release of allelopathic compounds by *Trichormus dolium* (cyanobacteria). *Limnology and Oceanography*, 42, 1796-1802

Von Elert E & Wolffrom T (2001) Supplementation of cyanobacterial food with polyunsaturated fatty acids does not improve growth of *Daphnia*. *Limnology and Oceanography*, 46, 1552-1558.

Von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography*, 47, 1764-1773.

Von Elert E, Martin-Creuzburg D & Le Coz JR (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 1209-1214.

Von Elert E, Agrawal MK, Gebauer C *et al.* (2004) Protease activity in gut *Daphnia magna*: evidence for trypsin and chymotrypsin enzymes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 137, 287-296.

Von Elert E, Oberer L, Merkel P, Huhn T & Blom JF (2005) Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. *Journal of Natural Products*, 68, 1324-1327.

Von Elert E, Zitt A, Schwarzenberger A (2012) Inducible tolerance in *Daphnia magna* against dietary protease inhibitors. *Journal of Experimental Biology*, 215, 1209-1214

Wacker A & Von Elert E (2001) Polyunsaturated fatty acids: evidence for non-substitutable biochemical resources in *Daphnia galeata*. *Ecology*, 82, 2507-2520.

Weider LJ, Lampert W, Wessels M, Colbourne JK & Limburg P (1997) Long-term genetic shifts in a microcrustacean egg bank associated with anthropogenic changes in the Lake Constance ecosystem. *Proceedings of the Royal Society of London - Series B: Biological Sciences*, 264, 1613-1618.

Weider LJ, Makino W, Acharya K *et al.* (2005) Genotype x environment interactions, stoichiometric food quality effects, and clonal coexistence in *Daphnia pulex*. *Oecologia*, 143, 537-547.

Weider LJ, Jeyasingh PD & Looper KG (2008) Stoichiometric differences in food quality: impacts on genetic diversity and the coexistence of aquatic herbivores in a *Daphnia* hybrid complex. *Oecologia*, 158, 47-55.

Wiedner C, Visser PM, Fastner J *et al.* (2003) Effects of light on the microcystin content of *Microcystis* strain PCC 7806. *Applied and Environmental Microbiology*, 69, 1475-1481.

Supporting material

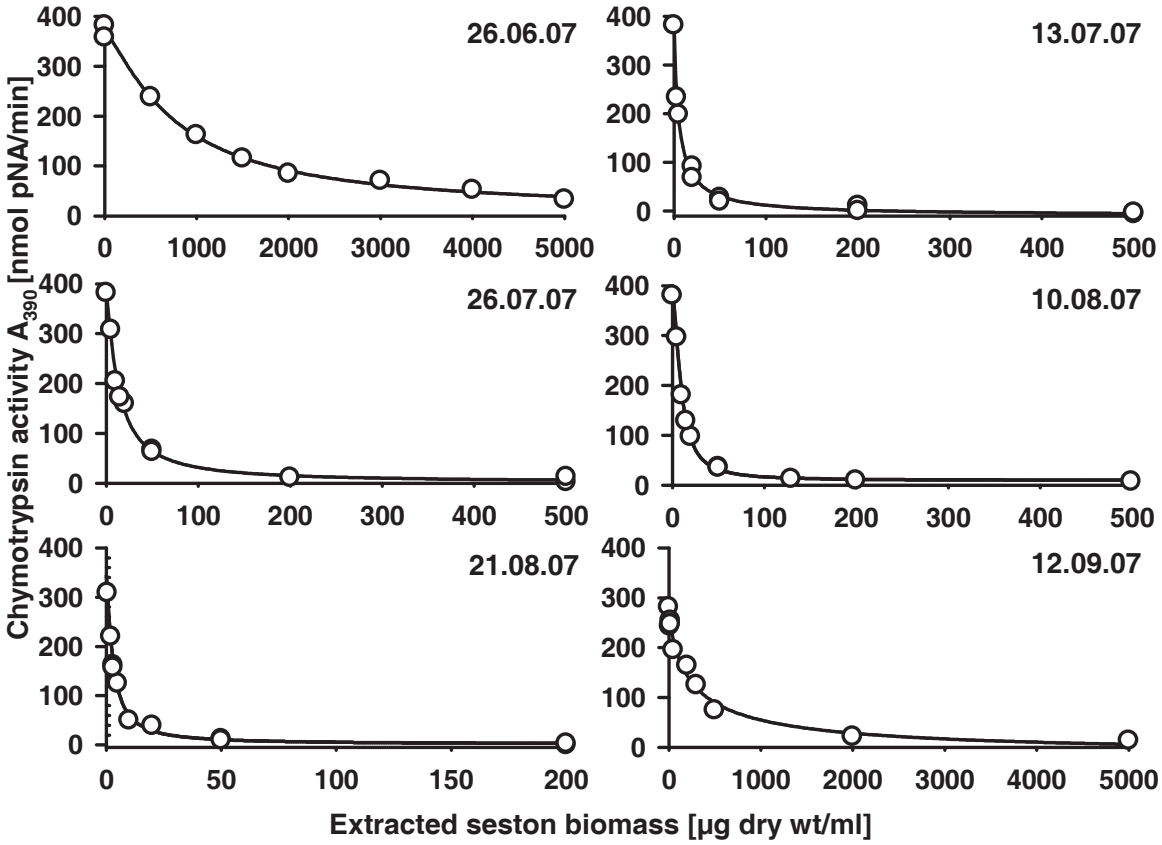

Fig. 4 *In vitro* effects of increasing concentrations of extracted biomass of each seston sample taken in 2007 on the normalized activity of chymotrypsins of *D. magna* clone B. IC_{50} values (which indicate the inhibitory potential of a seston sample and are presented in Fig. 1A as black circles) are based on sigmoid dose-response curves (solid lines). IC_{50} values with corresponding 95% confidence intervals (CI) were calculated using the software GraphPad Prism (GraphPad Software, Inc.). Note different scales of the axes.

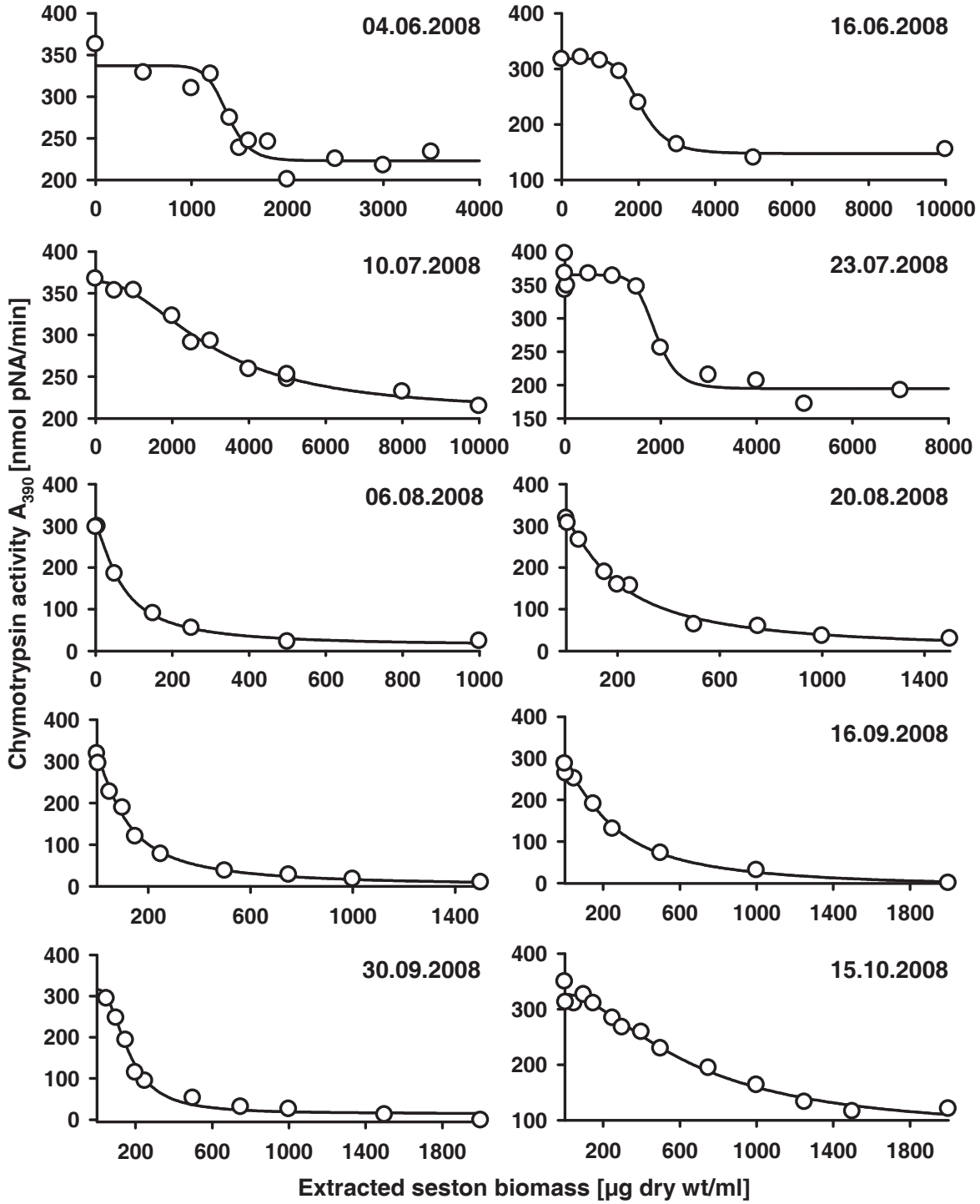

Fig. 5 *In vitro* effects of increasing concentrations of extracted biomass of each seston sample taken in 2008 on the normalized activity of chymotrypsins of *D. magna* clone B. IC_{50} values (which indicate the inhibitory potential of a seston sample and are presented in Fig. 1A as black circles) are based on sigmoid dose-response curves (solid lines). IC_{50} values with corresponding 95% confidence intervals (CI) were calculated using the software GraphPad Prism (GraphPad Software, Inc.). Note different scales of the axes.

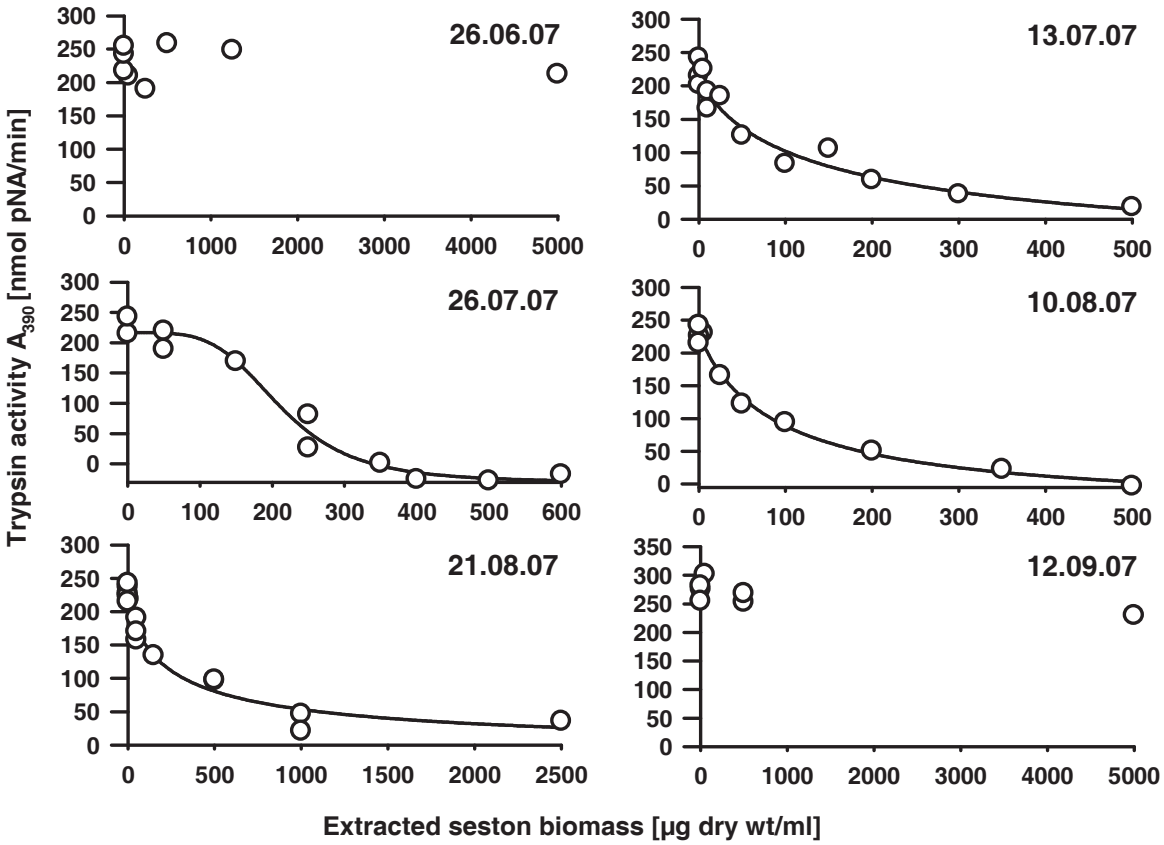

Fig. 6 *In vitro* effects of increasing concentrations of extracted biomass of each seston sample taken in 2007on the normalized activity of trypsins of *D. magna* clone B. IC_{50} values (which indicate the inhibitory potential of a seston sample and are presented in Fig. 1B as black circles) are based on sigmoid dose-response curves (solid lines). IC_{50} values with corresponding 95% confidence intervals (CI) were calculated using the software GraphPad prism (GraphPad Software, Inc.). Note different scales of axes.

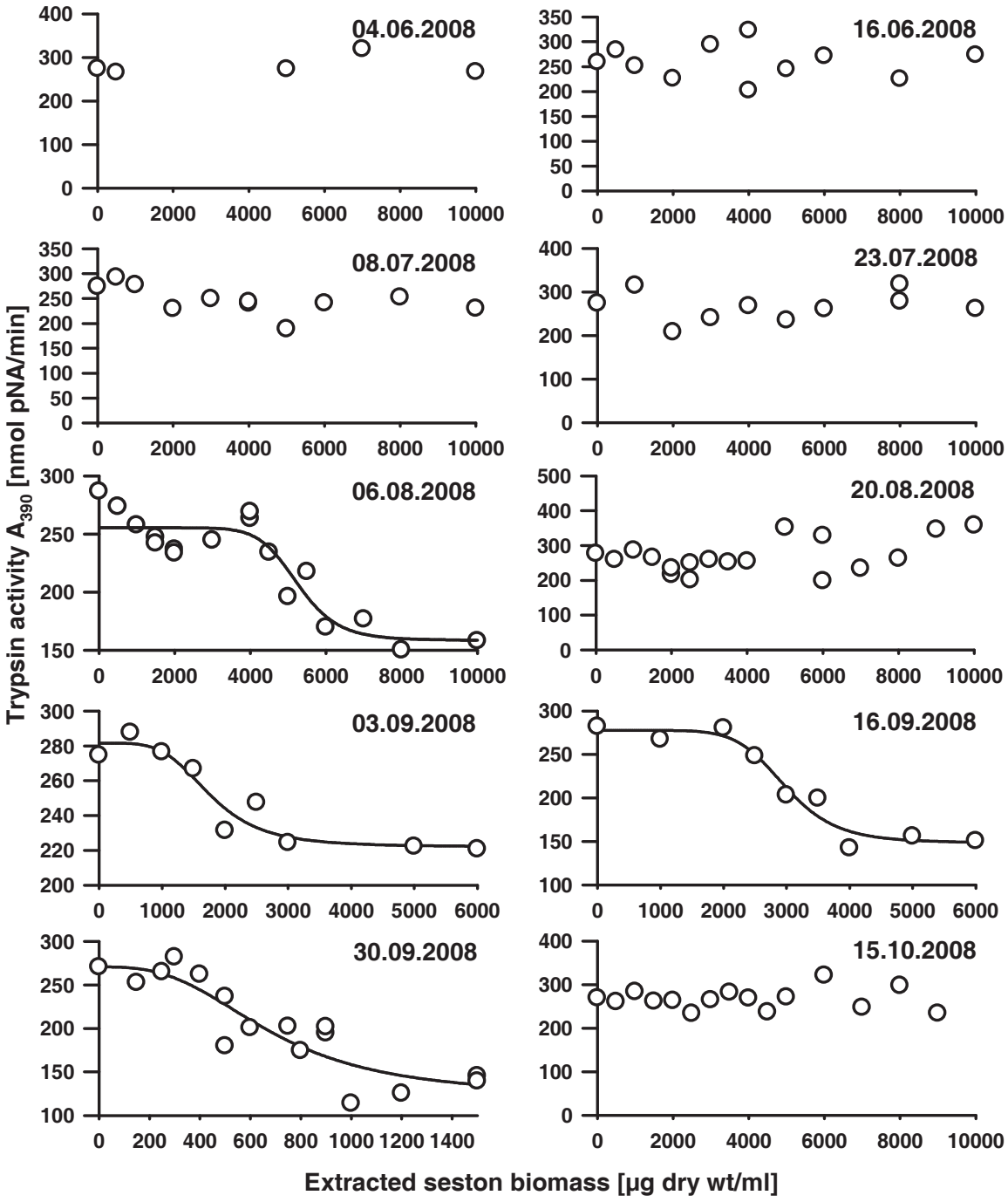

Fig. 7 *In vitro* effects of increasing concentrations of extracted biomass of each seston sample taken in 2008 on the normalized activity of trypsins of *D. magna* clone B. IC_{50} values (which indicate the inhibitory potential of a seston sample and are presented in Fig. 1B as black circles) are based on sigmoid dose-response curves (solid lines). IC_{50} values with corresponding 95% confidence intervals (CI) were calculated using the software GraphPad Prism (GraphPad Software, Inc.). Note different scales of the axes.

Chapter 2

High-resolution melting analysis: a genotyping tool for population studies on *Daphnia*

Abstract

Determining genetic variation at the DNA level within and between natural populations is important for understanding the role of natural selection on phenotypic traits, but many techniques of screening for genetic variation are either cost intensive, not sensitive enough or too labor and time-consuming. Here we demonstrate high resolution melting analysis (HRMA) as a cost-effective and powerful tool for screening variable target genes in natural populations. HRMA is based on monitoring the melting of PCR amplicons. Due to saturating concentrations of a dye that binds at high concentrations to double-stranded DNA, it is possible to genotype high numbers of samples rapidly and accurately. We analyzed digestive trypsins of two *Daphnia magna* populations as an application example for HRMA. One population originated from a pond containing toxic cyanobacteria that possibly produce protease inhibitors, the other from a pond without such cyanobacteria. The hypothesis was that *D. magna* clones from ponds with cyanobacteria have undergone selection by these inhibitors, which has led to different trypsin alleles. We first sequenced pooled genomic PCR products of trypsins from both populations to identify variable DNA sequences of active trypsins. Secondly we screened variable DNA sequences of each *D. magna* clone from both populations for single nucleotide polymorphisms (SNPs) via HRMA. The HRMA results revealed that both populations exhibited phenotypic differences in the analyzed trypsins. Our results indicate that HRMA is a powerful genotyping tool for studying the variation of target genes in response to selection within and between natural *Daphnia* populations.

Introduction

The analysis of genetic variation at the molecular level is necessary to understand the key mechanisms of adaptive evolution in response to selection. Due to the rapidly growing number of sequenced eukaryotic genomes and corresponding techniques, the possibilities for analyzing genotype-phenotype interactions has increased considerably in the last decade. In natural populations, adaptive and neutral genetic diversity have to be distinguished in this context, both between and within populations. The neutral genetic variation can be assessed relatively easy by using neutral markers such as microsatellites or allozymes. However, genetic variation and population differentiation of adaptive traits are not directly linked with neutral genetic diversity (Ohta 1992; Holderegger *et al.* 2006). For this reason, the variation in loci coding for phenotypic traits has to be determined to more properly estimate population differentiation in response to selection. Various approaches for screening of such loci have been successfully established in the past decades and can be used to analyze variation in populations. Conformational analyses, such as DGGE, SSCP or RSCA, are based on separation of PCR samples on a gel or a different matrix and require prior sample processing steps, which are time and labor consuming and increase the risk of contamination, because PCR products are exposed to the environment (Reed *et al.* 2007). Direct genotyping techniques such as chain termination sequencing or high-throughput sequencing techniques, like Next-Generation Sequencing, are indeed quite sensitive but still too costly for many large-scale population studies.

In the present study we describe a rapid, sensitive and cost-effective method for the detection of genotype variations that can be used for genotyping single individuals in populations: High-resolution melting analysis (HRMA). HRMA is a well established technique for gene scanning based on the melting curves of PCR products (Gundry *et al.* 2003), which does not require any processing, reagent addition or separations after PCR. It is based on monitoring the melting behavior of whole amplicons of up to 250 base pairs after a common PCR amplification. The characteristic dissociation (melting) behavior of each double-stranded DNA (dsDNA) depends mainly on its GC content, sequence length, nucleotide composition and strand complementarity (Ririe *et al.* 1997). HRMA is most commonly performed in the presence of a saturating concentration of a fluorescent dye that binds specifically to dsDNA (Wittwer *et al.* 2003). This binding leads to a fluorescence signal that can be detected by highly sensitive quantitative PCR instruments. Fluorescent dyes used for HRMA have higher fluorescence levels when bound to dsDNA than in an unbound state or after binding to single-stranded DNA (Wittwer *et al.* 2003). To characterize the melting curve of a DNA sample, the fluorescence is continuously monitored during the slow heating of the sample.

The temperature-dependent dissociation of the dsDNA directly results in a decrease of fluorescence. Detection and differentiation of distinct DNA samples relies on differences in the melting temperature (T_m) and the shape of the melting curve (Montgomery *et al.* 2007), so that most single nucleotide polymorphisms (SNPs) can be genotyped by HRMA. However, some homozygous SNP variants cannot be differentiated because they possess a similar or even identical melting curve shape. In particular, homozygous SNP variants of class III (C/G) and class IV (A/T) mutations, also known as neutral base pair changes (Gundry *et al.* 2008), can result in almost identical melting curves. In these cases, a known reference homozygous genotype is added to each unknown homozygous sample (Palais *et al.* 2005). The newly formed artificial heteroduplexes can subsequently be differentiated in a second melting analysis by the shape of the corresponding melting curves; this procedure ensures the complete identification of all homozygous variants.

HRMA has been applied in numerous clinical studies and for medical molecular diagnostics, and has a broad range of applications (Reed *et al.* 2007). Another important field of HRMA applications is the identification of parasitic organisms such as trematodes (Radvansky *et al.* 2011), nematodes (Areekit *et al.* 2009) and parasitic protozoa in humans (Andriantsoanirina *et al.* 2009) and in fungal pathogens (Luchi *et al.* 2011). Furthermore, HRMA has been suggested as an alternative genotyping system for variation detection in neutral markers in a population, for example in swordfish (Smith *et al.* 2010) or garden sage (Mader *et al.* 2010), for insect species identification (Winder *et al.* 2011) and for differentiating between social forms in populations of the red imported fire ant (Oakey *et al.* 2011).

HRMA has recently been reported in the ecological literature (e.g. Smith *et al.* 2010; Seeb *et al.* 2011, Henri & Mouton 2012). In the present study we used HRMA in an ecological context to determine the genetic variation of target genes between and within two natural populations of the water flea *Daphnia* magna. In contrast to *Daphnia pulex*, which has recently become a model organism (Colbourne *et al.* 2011), the reference genome data for *D. magna* is still only partially available. Members of the genus *Daphnia* are unselective filter feeders of algae and cyanobacteria in freshwater ecosystems and are often the most important food source for planktivorous vertebrate and invertebrate predators. *Daphnia* is therefore a keystone species in the energy transfer from primary producers (phytoplankton) to higher trophic levels. In eutrophic lakes this energy transfer is constrained mainly by food quality and not by food quantity (due to relatively high abundances of cyanobacteria). Three major food quality constraints have been revealed for the poor assimilation of cyanobacterial carbon by *Daphnia*: (1) Mechanical interferences with the filtering process of *Daphnia* due to

colonial or filamentous forms of the cyanobacteria (Porter & Mcdonough 1984). (2) The low content of polyunsaturated fatty acids and the lack of sterols in cyanobacteria, both of which are essential for *Daphnia* nutrition (Von Elert 2002; Von Elert *et al.* 2003). (3) Many cyanobacterial strains also produce toxins which negatively affect the fitness of *Daphnia* (Rohrlack *et al.* 2001; Lürling 2003). Protease inhibitors are very common among such cyanobacterial toxins: More than twenty depsipeptides, acting as protease inhibitors, have been found in cyanobacterial toxins (Gademann & Portmann 2008). These depsipeptides specifically inhibit the proteases chymotrypsin and trypsin, two digestive enzymes that are responsible for more than 80% of the proteolytic activity in the gut of *Daphnia* magna (Von Elert 2004). Studies by Agrawal *et al.* (2005), Schwarzenberger *et al.* (2010) and Von Elert *et al.* (2012) have shown a direct inhibition of the digestive proteases of *Daphnia* by cyanobacterial protease inhibitors. Furthermore, Czarnecki *et al.* (2006) have shown that the potential of natural phytoplankton to inhibit trypsins is comparable to the inhibitory potential of cyanobacterial strains. This strongly suggests that cyanobacterial protease inhibitors interfere with digestive proteases in *Daphnia* in nature. However, *Daphnia* populations can adapt to the presence of cyanobacteria in terms of a higher tolerance (Gustafsson & Hansson 2004; Hairston *et al.* 1999; Sarnelle & Wilson 2005). Blom *et al.* (2006) showed that *Daphnia* sp. coexisting with the cyanobacterium Planktothrix rubescens, which produces the trypsin inhibitor oscillapeptin J, are significantly more tolerant to oscillapeptin J than *Daphnia* sp. from habitats free of Planktothrix rubescens. Furthermore, Von Elert *et al.* (2012) have recently shown that a physiological plasticity at the protein level in *Daphnia* leads to higher tolerance against cyanobacterial protease inhibitors. Considering the finding that almost 60% of 17 water blooms of cyanobacteria contained protease inhibitors (Agrawal *et al.* 2001), it is reasonable to assume that increased tolerance to cyanobacteria in *Daphnia* populations may be the result of higher tolerance to the cyanobacterial protease inhibitors.

In the present study we analyzed the genetic diversity of all three digestive trypsins that have been shown to be active in the gut of *D. magna* between and within two distinct populations. The populations originated from ponds, which mainly differ in the presence and absence of toxic cyanobacteria that possibly contain protease inhibitors. The hypothesis was that the coexistence of *D. magna* with cyanobacterial trypsin inhibitors has led to selective pressure on digestive trypsins, resulting in differences in trypsin variability compared to a population existing without cyanobacteria. To test this, we established clones from each population under constant laboratory conditions. After DNA extractions and normalizations steps, we performed a polymerase chain reaction (PCR) with each clone. We subsequently pooled the genomic PCR-products of all digestive trypsin genes from each of the two

populations. The pooled PCR-products of both populations were then sequenced to distinguish between conserved and variable DNA sequences of active digestive trypsins. Then we screened the variable DNA sequences in the trypsin loci for polymorphisms by using HRMA with 15 single clones from each *D. magna* population.

Material and methods

Origin of Daphnia magna clones

Fifteen *D. magna* clones (termed as S_n clones) were isolated from Lake Bysjön (LAT 55.675448 LON 13.545070) in June 2010, cultivated as single clones at 20°C and are referred to hereafter as the Swedish population. Lake Bysjön is a hypereutrophic lake and is located in southern Sweden (Hansson *et al.* 2007). It frequently has massive cyanobacterial blooms (Gustafsson 2007).

Fifteen *D. magna* clones (termed as P_n clones) were isolated from a small pond in the Kampinoski National Park near Warsaw, Poland (LAT 52.322722 LON 20.730515) in August 2010. The clones were cultivated as single clonal lineages at 20°C and are referred to hereafter as the Polish population. The ponds in Kampinoski National Park have no recorded cyanobacterial blooms. All *D. magna* clones were cultured separately in aged, membrane-filtered tap water and fed with saturating concentration of a green alga (*Chlamydomonas* sp.).

DNA sample preparation

Genomic DNA of each single *D. magna* clone from the Swedish and the Polish population was extracted from at least ten adult individuals using the peqGOLD® Tissue DNA Mini Kit (Peqlab, Erlangen, Germany) as according to the manufacturer's standard protocol. DNA concentration of each sample was determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA) and normalized to 20 ng/µL.

Table 1 Primer sequences (5`-3`) and corresponding melting temperature (T_m) for population sequencing and subsequent HRMA of the digestive trypsins **610**, **208** and **152**.

Locus	Primer sequences (5` - 3`)	T_m (°C)
*t**610***.1	**F:** GCCCATGATTGATGATGACA	57
	R: TTTGGACAAGATGTCGCTGT	57
*t**610***.2	**F:** CATCGTTGCTGGTGAACACA	58
	R: GGTGATGGAAGTAGCTCCGTA	57
t610.3	**F:** GATGCCACTTGCCGTGAC	57
	R: ATGGGATTGTTGGCGAAA	57
***t610*.HRM**	**F:** AGCTCCCCACTCTCGTTGA	57.5
	R: AATATCGCAAAATGAATTACCG	57
*t**208***.1	**F:** AGCTGATCCCATCCAACAGG	59.5
	R: GGAGCATCCAACTACAAAATCG	59
*t**208***.2	**F:** CGACATCTCTCTGCTCTTTGT	55
	R: TTAAATCAGGAACAAGTCAGAACA	57
*t**208***.3	**F:** TCTTCCCTTCCATGATCTGC	57
	R: GCTTCATTCGACACCGTTTT	57.5
*t**208***.**HRM**	**F:** GATGCTCCATTGGACCTGAG	57
	R: TGATTTTGAGGTGCGTTTGA	57
*t**152***.1	**F:** AGCTGATCCCATCCAACAAG	57
	R: AATGAAGCTGGCAACACCAC	58
*t**152***.2	**F:** AGCGGCCTCGAACAGAAC	58
	R: CGGAGTGTGTCGGAGATGAT	57.5
*t**152***.3	**F:** TTGCTGGGTTTGATGCAGT	57
	R: TTGATAGGGATGGGGATGG	57.5
*t**152***.exon2	**F:** GAGTTGACGCTTCCATCCTC	57
	R: CCTGTAGTCCTCGTGGATAATGA	58.5
*t**152***.exon5	**F:** GCAGTCCGGTGGTATCATCT	57
	R: CGGACGAGATAAATGTTCCAA	58
*t**152***.exon6	**F:** TCAGGTGGTATCGACTCTTGC	57.5
	R: TGAAGTTGATTCTAGGCGTTTG	57.5

Sequencing and screening for SNPs

All genes chosen for the SNP screening and the HRMA coded for digestive trypsins, which are active in the gut of *D. magna* (Schwarzenberger *et al.* 2010). Genome sequences of the trypsin genes (*t610*, *t208, t152*) were obtained from an unpublished *D. magna* genome assembly. Summaries for assembly and scaffolding are reported on the *Daphnia* Genomics Consortium webpage: https://wiki.cgb.indiana.edu/display/grp/Daphnia+magna+Genome.

All primers, based on these trypsin genes for screening the population for SNPs were designed using Primer 3 software (Rozen & Skaletsky 2000). To ensure the reliability of the designed primers, all primer pairs were designed with the aim to minimize the formation of secondary structures such as hairpins, self-dimers, cross-dimers and primer mismatches. Due to repetitive regions in the introns of trypsin *t152* three additional primer pairs had to be designed to amplify and sequence the whole trypsin gene. The amplification was performed via polymerase chain reaction (PCR) in a final volume of 10 µL containing 10 ng of genomic DNA, 0.2 µmol of each primer, 10 µmol of the dNTPs, 0.25 U Taq DNA polymerase and 1 µL of corresponding PCR buffer (5 Prime, Hamburg, Germany). After checking the success of the amplification via agarose gel electrophoresis, aliquots of 1 µL PCR products of each single clone were pooled for the Swedish and Polish clones separately. The pooled PCR products from each population were then purified using a GenElute^{TM} PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, USA) and sequenced via chain termination sequencing by GATC Biotech (Konstanz, Germany) using the ABI Big Dye Terminator Mix v3.1 on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA). Subsequently, the sequenced amplicons were screened for SNPs that affect phenotypic traits in terms of relevant amino acid substitutions. Additional information on the exact location of all primer pairs and the fragment size they amplify can be found in the supporting online material.

HRM analysis

For the HRMA, primer with an amplicon size of between 100 and 200 base pairs were designed (Table 1), with the non-synonymous SNP towards the middle. The HRMA assay for each clone was performed in 96 well plates in a final volume of 25 µL containing 50 ng of genomic DNA, 0.3 µmol of each primer, 12.5 µL SensiMix HRM^{TM}(Bioline, London, Great Britain), 1 µL of EvaGreen^{TM} dye (Bioline, London, Great Britain) and 5 mmol $MgCl_2$.

The DNA amplifications and the subsequent melting curve analyses were carried out using a LightCycler 480 System® (Roche Diagnostics, Risch, Switzerland) under the following conditions: 10 min initial polymerase activation at 95°C followed by 55 cycles of amplification, each including 15 s denaturation at 95°C, 10 s annealing at 57°C and 10 s extension at 72°C. Immediately after amplification, the products were heated to 95°C for 1 min and then cooled to 40°C followed by an incremental temperature increase of 0.05°C/s to a target temperature of 95°C. The fluorescence was monitored during the entire heating process. Data analyses were carried out using LightCycler 480 Gene Scanning Software® version 1.5. The melting curve data of the amplicons were normalized to the fluorescence (Y-axis) and temperature (x-axis) and grouped according to melting curve shape. However, temperature shifting normalization removes potential discriminatory power provided by the temperature data, but compensates for well-to-well temperature variations between samples. The necessity for a temperature normalization step mainly depends on the high resolution melting instruments (Herrmann *et al.* 2007). Due to DNA mismatches at the SNP locations, the melting process of heterozygous samples proceeds inconstantly resulting in a flattened melting curve with more than one inflection point. In contrast, the melting curve shape of homozygous samples indicates a consistent melting behavior with just one inflexion point (sigmoid shape). Homozygous samples that were identified by the number of inflection points of their melting curves were mixed with a known homozygous genotype followed by an additional melting analysis. This ensured the complete identification of all homozygous variants. Subsequently, one sample from each genotype was sequenced (chain-termination sequencing) in order to determine the genotype specific nucleotide sequence.

Results

The sequence analysis of pooled PCR products of *t152* revealed that this locus was polymorphic. However, only synonymous SNPs were detected, which means that trypsin 152 was entirely conserved in terms of amino acid sequence within all clones of the Swedish and Polish *Daphnia magna* populations. With regard to trypsin 610, the sequence of pooled PCR products of the Swedish *D. magna* genotypes exhibited a single nucleotide variation coding for the amino acid position 119 (Fig. 1A). At this position the Swedish haplotypes contained the codon GAA or GCA; GAA codes for glutamic acid (Glu), GCA for alanine (Ala). Glu and Ala differ with respect to the characteristics of their side chains: Glu is characterized by a negatively charged, acidic side chain, while Ala carries a hydrophobic side chain. The Polish

D. magna genotypes exhibit GAA only at this position, so that T610 has Glu at this position in all Polish clones.

Regarding trypsin 208 the sequence of pooled PCR products of the Swedish *D. magna* genotypes exhibited a single SNP coding for the amino acid position 130 (Fig. 1B). At this position, Swedish haplotypes contained either the triplet codon ATG, which codes for methionine (Met), or ATT, which codes for isoleucine (Ile). In contrast to Ile, Met contains a sulfur atom and is more polar. The Polish *D. magna* genotypes exhibit only ATT at this position in *t208*, so that Ile shows up at position 130 in all clones. DNA primer for HRMA were designed with the specification that the resulting amplicons cover these variable nucleotide sequences in *t610* and *t208*. Subsequently, each *D. magna* clone from both populations was genotyped individually at these variable loci via HRMA.

The HRMA of the chosen variable DNA sequence of trypsin **610** of all 30 *D. magna* clones from the Swedish and the Polish populations revealed the presence of three different genotypes. From each genotype one sample was subsequently sequenced (Fig. 2A). Out of two polymorphic nucleotide sites within this 130 base pair fragment, one polymorphism (position 71) was an A/C/M variation resulting in a Glu/Ala amino acid variation, while the remaining nucleotide variation (position 39) was a synonymous substitution. Figure 2B shows the normalized melting curves of the amplified DNA fragments of all *D. magna* clones. Additional temperature shifts of the melting curves were performed in order to compensate for temperature variations between the wells of the LightCycler 480 System®. This resulted in a decrease of sensitivity, so that melting curves could mainly be discriminated by the shape of the curves. Nine heterozygous genotypes (GT3) exhibited well-defined melting curves that could be accurately distinguished from those of the homozygous genotypes. The melting curves of the homozygous genotypes (GT1, GT2) merged into one group due to almost identical shapes of the melting curves. To ensure the complete identification of all variants, all homozygous genotypes (identified by the sigmoid shape of the corresponding melting curves with just one inflexion point) were mixed with a known homozygous genotype, i.e. GT1 (Figs. 2C & 2D), generating heteroduplexes. After an additional melting analysis, the resulting melting curves had either the shape of a heterozygous or a homozygous variant (Fig. 2D). An unchanged homozygous melting curve indicated that the original genotype was

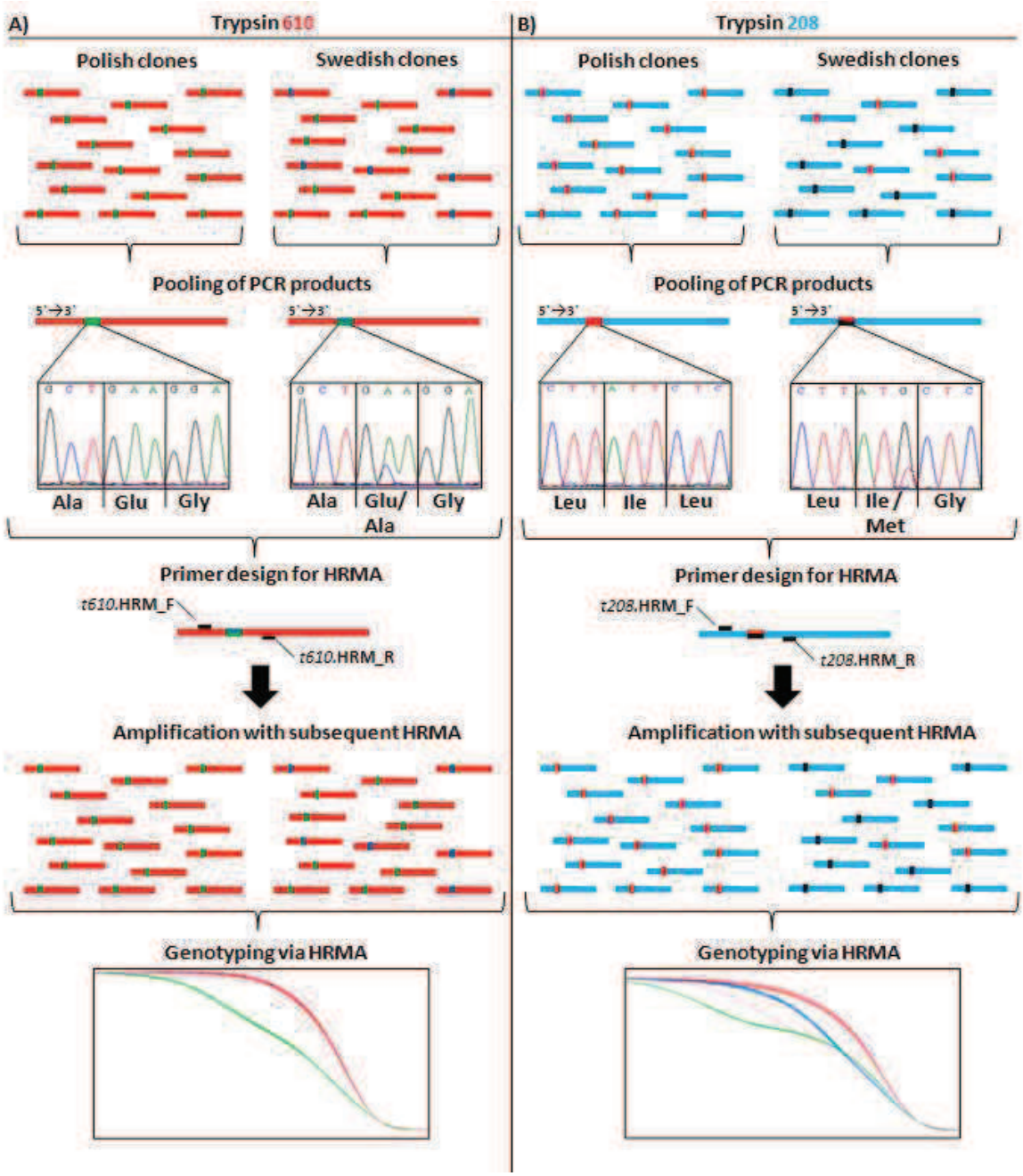

Figure 1 Procedure of determining variable parts in genes of digestive trypsins **610** (A) and **208** (B) of the Swedish and Polish populations. Genes of digestive trypsins of single clones from both populations were amplified using the PCR technique. Afterwards, the PCR products from different clones were pooled, sequenced and then screened for SNPs which affect phenotypic traits in terms of relevant amino acid substitutions (for the Swedish and Polish populations separately). Primers for the HRMA were designed such that resulting amplicons cover these variable nucleotide sequences. Subsequently, melting curves of the amplicons of each single *D. magna* clone were analyzed separately via HRMA. Trypsin **152** is not displayed due to the absence of non-synonymous nucleotide sequence variations.

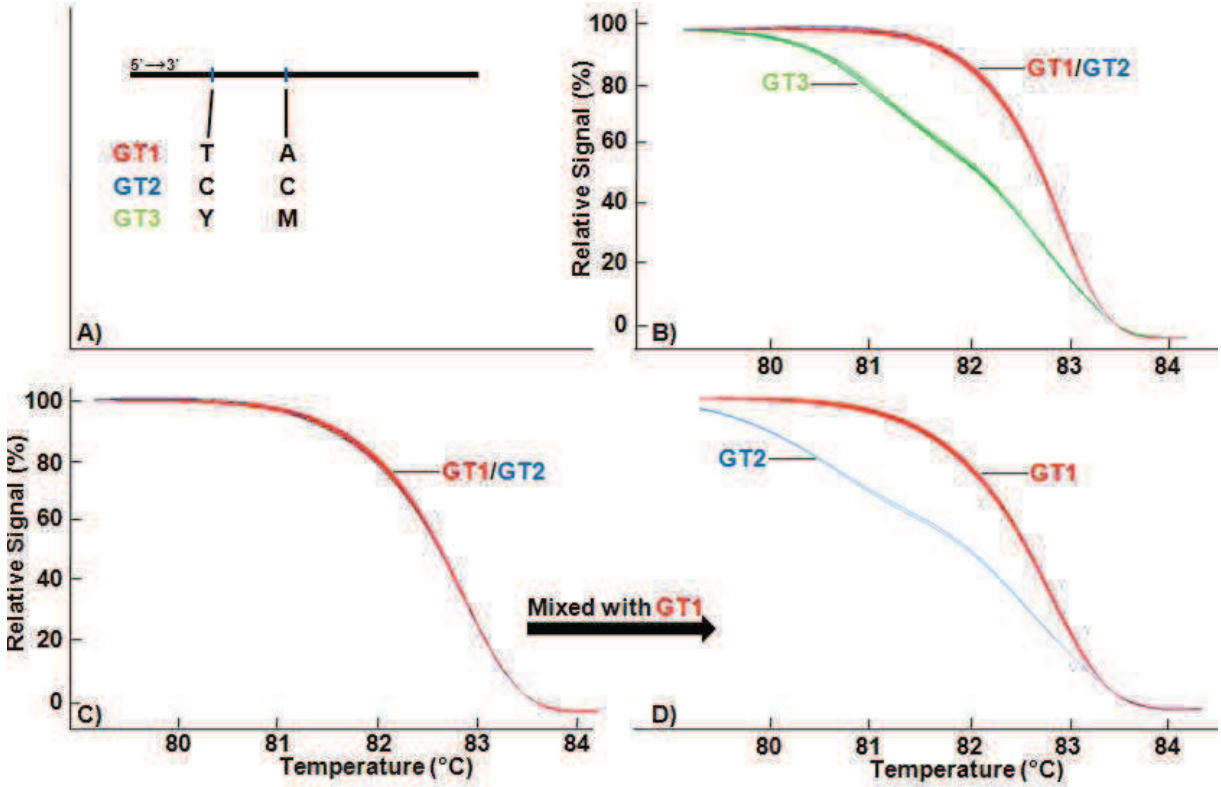

Fig. 2. Variable parts within the PCR amplicon of the digestive trypsin gene ***t610*** (A). The nucleotide variation at the second position (A/C/M) results in a phenotypic change in terms of an amino acid substitution (Glu/Ala). B) displays the melting curves of all *D. magna* clones from both populations after normalization of the fluorescence and correction for well-to-well temperature differences, revealing two homozygous (**GT1** + **GT2**) and one heterozygous (**GT3**) genotype. C) The homozygous genotypes **GT1** and **GT2** could not be distinguished by their melting curves; after mixing **GT1** and **GT2** with a known homozygous genotype (**GT1**), the two genotypes could be distinguished in subsequent melting analyses (D). The last step was performed to ensure the complete identification of all homozygous variants.

the homozygous genotype GT1, while a heterozygous melting curve pointed at an original genotype GT2.

Concerning trypsin **208**, the HRMA of the variable amplicon revealed the presence of five distinct *D. magna* genotypes within both populations. From each genotype one sample was subsequently sequenced (Fig. 3A). Out of four polymorphic nucleotide sites within this 152 base pair fragment, one nucleotide polymorphism (position 90) was a G/T/K variation resulting in a Met/Ile amino acid variation. The remaining nucleotide polymorphisms at positions 36, 51 and 69 were synonymous nucleotide substitutions. The melting curves of all analyzed *D. magna* clones were normalized for fluorescence and were temperature shifted (Fig. 3B). The heterozygous genotypes (GT3, GT4 and GT5) could be discriminated by the

shape of the respective melting curve from each other and from the curves of the homozygous genotypes. The sigmoidally shaped melting curves of the homozygous samples (GT1, GT2), as above, were not distinguishable and merged into one group**.** As above, the homozygous samples were mixed with a known homozygous genotype, i.e. GT1 (Figs. 3C & 3D). After additional melting analyses, the resulting melting curves had either the shape of a heterozygous or a homozygous variant (Fig. 3D). An unchanged homozygous melting curve indicated that the original genotype was the homozygous genotype GT1, while a heterozygous melting curve pointed at an original genotype GT2.

With regard to the Polish and the Swedish populations, the HRMA of the variable parts of trypsin **610** and **208** confirmed the sequence analyses of the pooled PCR products. All 15 Polish *D. magna* clones exhibited the same homozygous genotype for trypsin **610** and **208**, resulting in Glu at amino acid position 119 for trypsin **610**, and Ile at amino acid position 130 for trypsin **208** as phenotypes (Table 2). In accordance with the sequencing results of the pooled PCR product for ***t610*** and ***t208***, the genotype and respective amino acid phenotype distribution for trypsin **610** and trypsin **208** of the Swedish population was more diverse (Table 2). Combining the HRMA results of trypsin **610** and trypsin **208,** five different genotypes leading to five trypsin phenotypes were observed for the Swedish *D. magna* clones (Table 2).

Table 2 Genotype and corresponding amino acid phenotype of trypsins **610** and **208** for each single clone of the Swedish (S) and the Polish (P) populations.

	Trypsin 610		**Trypsin 208**	
***D. magna* clone**	**Genotype**	**Phenotype**	**Genotype**	**Phenotype**
S6, S7, S14, S15, S39	GT1	Glu	GT1	Met
S8	GT2	Ala	GT2	Ile
S2	GT2	Ala	GT5	Met
S16, S22, S38	GT3	Glu/Ala	GT1	Met
S3, S4, S12, S17, S24, S36	GT3	Glu/Ala	GT4	Met/Ile
P1, P3, P4, P5, P6, P13, P18, P19, P20, P22, P24, P26, P27, P30, P31	GT1	Glu	GT3	Ile

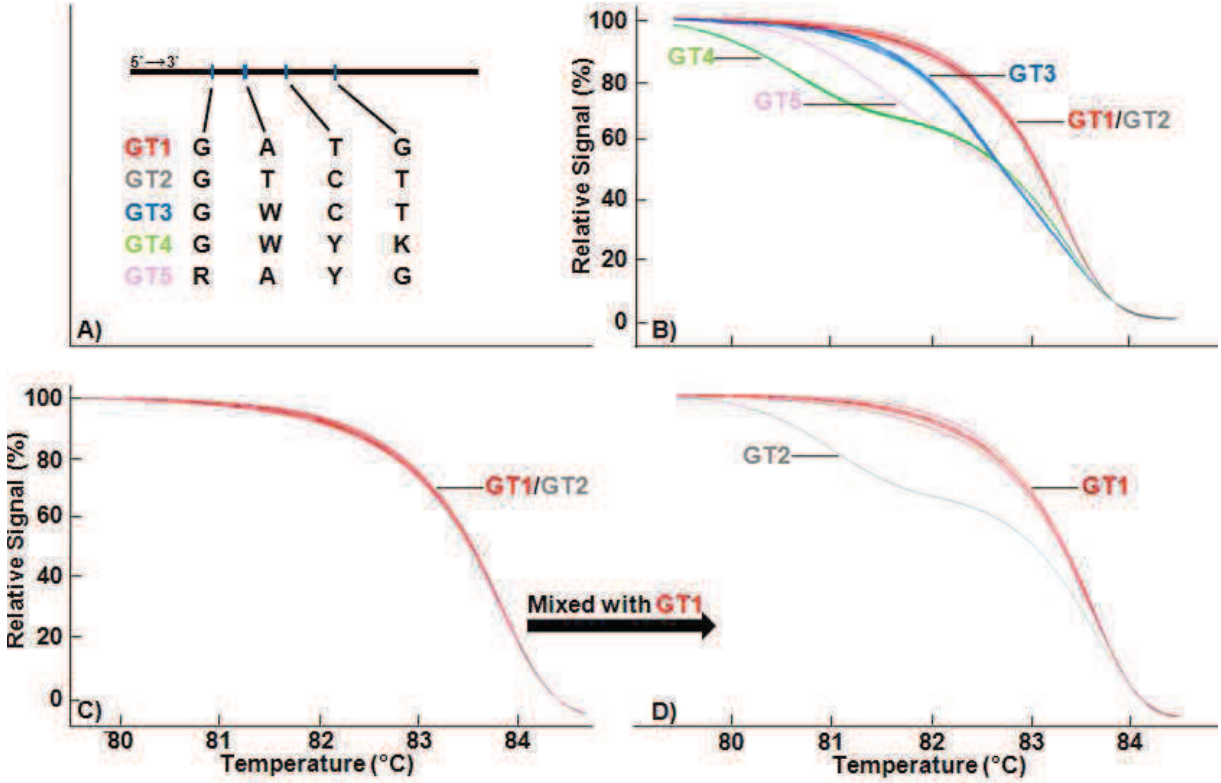

Figure 3 Variable parts within the PCR amplicon of the digestive trypsin gene **208** (***t208***) (A). The nucleotide variation at the fourth position (G/T/K) results in a phenotypic change in terms of an amino acid substitution (Ile/Met). B) displays the melting curves of all *D. magna* clones from both populations after normalization of the fluorescence and correction for well-to-well temperature differences. Due to the shape of the curves, two homozygous (**GT1** and **GT2**) and three heterozygous (**GT3, GT4** and **GT5**) genotypes could be distinguished. C) The homozygous genotypes **GT1** and **GT2** could not be discriminated by their melting curves; after mixing **GT1** and **GT2** with a known homozygous genotype (**GT1**), the two genotypes could be distinguished in subsequent melting analyses (D). The last step was performed to ensure the complete identification of all homozygous variants.

Discussion

Natural genetic variation is the fundamental basis for evolution. The study of the origin and persistence of genetic variation at the molecular level is a prerequisite to understand how species and populations have evolved in response to selection. Single nucleotide polymorphisms (SNPs), defined as a single base substitution at a specific locus, are the most abundant class of genetic variation found in eukaryotic genomes (Vignal *et al.* 2002). It is important to find reliable molecular tools for the detection of SNPs within and between natural

populations, because even a single nucleotide substitution in a gene that affects phenotypic traits can result in a complete change of the characteristics of a protein (Levan *et al.* 2001; Mandola *et al.* 2003).

The sequence of the pooled PCR products revealed that two DNA sections in trypsin 610 and 208 were polymorphic in terms of relevant, non-synonymous nucleotide variations among the Swedish and the Polish *D. magna* populations. The results of the subsequently performed HRMA showed that all heterozygous genotypes were clearly distinguishable by the shape of the corresponding melting curves, resulting in one heterozygous genotype for trypsin 610 and three heterozygous genotypes for trypsin 208. However, two homozygous genotypes could not be distinguished in each of the two trypsins. This was due to the temperature variations between the wells of the LightCycler 480 System®, which resulted in an increase of the experimental noise. Consequently, genotypes could be discriminated by the shape of the respective melting curve rather than by the melting temperature. Homozygous genotypes finally became distinguishable after generating heteroduplexes by mixing each unknown homozygous genotype with a known homozygous genotype (Palais *et al.* 2005). Subsequent HRMA yielded melting curves of the artificial heteroduplexes that were either unchanged or exhibited the melting curve shape of a heterozygous genotype, which permitted conclusions about the genotype of the sample. The generating of heteroduplexes could alternatively be performed by consistently homozygous sample mixing (Vossen *et al.* 2009).

The final results of the HRMA regarding trypsin 610 and 208 revealed that the clonal diversity in the Swedish *D. magna* population was much higher than in the Polish population. All 15 Polish clones exhibited the same genotype for each of the two trypsins. This functionally constrained monomorphism at the two trypsin loci in the Polish *D. magna* population could be a consequence of low environmental variations. In such a situation, one would expect that a few well-adapted clones would have been selected for. In addition, Matthes (2004) has demonstrated that monomorphism in a *D. pulex* population of a temporary habitat can be a consequence of founder effects. In contrast to the monomorphic Polish population, three genotypes for trypsin 610 and five genotypes for trypsin 208 were found in the Swedish *D. magna* population. The habitat of the Swedish clones, Lake Bysjön, is known to be a hypereutrophic lake with seasonally occurring cyanobacterial blooms (Hansson *et al.* 2007; Gustafsson 2007). In such hypereutrophic lakes, seasonal changes in the phytoplankton community might lead to altering selection factors for the zooplankton community, especially for non-selective filter feeders like *Daphnia* (DeMott 1989). This is especially true for bloom-forming cyanobacteria containing toxic secondary metabolites,

which inhibit *Daphnia*'s gut proteases; such cyanobacteria are more abundant in late summer and early fall (Dokulil & Teubner 2000; Schreurs 1992; Czarnecki *et al.* 2006). These changes in the phytoplankton community can be interpreted as being disturbances for the respective *Daphnia* community. Weider (1992) observed that the frequency of disturbances can have a pronounced effect on clonal diversity of a *D. pulex* population. In these experiments, a disturbance event consisted of randomly diluting the animal density in the experimental beakers. Weider (1992) showed that the highest level of clonal *D. pulex* diversity was maintained at intermediate scales of disturbances. It is therefore reasonable to assume that the higher clonal diversity in the Swedish population might be a consequence of intermediates disturbances. However, it remains unclear whether this is a consequence of the altering phytoplankton community or is caused by other abiotic or biotic factors.

With regard to trypsin 610, all Polish clones contained a Glu at amino acid position 119, while 13 out of 32 Swedish haplotypes exhibited Ala at this position. It remains undeterminable whether this phenotypic difference between the two populations was caused by natural selection or by random genetic drift. Theoretically, a substitution of Glu with Ala could decrease the sensitivity of an enzyme to a specific inhibitor. Shi *et al.* (1999) have shown that an amino acid substitution in rat's palmitoyltransferase of Glu with Ala did not affect catalytic activity, but inhibition of this mutant enzyme by a specific inhibitor was reduced 100-fold. Further experiments, such as heterologous expression and subsequent enzymatic characterization of the different isoforms, have to be performed to determine if the partial substitution of Glu with Ala in the Swedish *D. magna* population might be the consequence of adaptive evolution, possibly caused by cyanobacteria.

For trypsin 208 in the Swedish population, we found five different genotypes resulting in either Met or Ile as phenotypes at amino acid position 130, while all *D. magna* clones from the Polish population exhibited Ile at this position. A substitution of Ile with Met within an enzyme is most commonly regarded as a conservative change, because Ile and Met belong both to the same amino acid group with hydrophobic side chains (French & Robson 1983). An alignment of trypsin 208 from *D. magna* with trypsin from the narrow-fingered crayfish, of which the X-ray structure has been determined (Fodor *et al.* 2005; Fodor *et al.* 2006) (accession code: Q52V24, UniProt), revealed that the Met/Ile variability at amino acid position 130 is probably located at the surface of trypsin 208 in a beta-sheet structure. Ile is C-beta branched and is therefore slightly more predestinated in beta-sheets than Met (Betts & Russel 2003). Thus, it can be assumed that phenotypes containing an Ile at amino acid position 130 might be structurally a bit more stable than phenotypes containing a Met. However, the amino acid sequence of trypsin 208 from *D. magna* was less than 45%

identical with that of the crayfish trypsin. Additional structural comparisons were restricted by the absence of structure data from trypsins of closer related genera. Thus, we cannot rule out the possibility that the occurrence of phenotypes with Met at amino acid position 130 in trypsin 208 in the Swedish population was a response to selection caused by cyanobacterial protease inhibitors.

The present study has demonstrated the application of high-resolution melting analysis (HRMA) in population studies of *Daphnia*. In combination with pooled DNA sequencing HRMA can be used to screen for gene variants in natural populations. HRMA is a screening tool for the detection of unknown polymorphisms and for the identification of known polymorphisms in target genes of natural populations. Even single SNPs of class III (C/G) or class IV (A/T) mutations can be detected easily with HRMA. Its simplicity, sensitivity and specificity as well as its low cost could make HRMA the method of choice for screening high numbers of individuals in populations for variability in target genes.

References

Agrawal MK, Bagchi D & Bagchi SN (2001) Acute inhibition of protease and suppression of growth in zooplankter, *Moina macrocopa*, by *Microcystis* blooms collected in Central India. *Hydrobiologia*, 464, 37-44.

Agrawal MK, Zitt A, Bagchi D *et al.* (2005) Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. *Environmental Toxicology*, 20, 314-322.

Andriantsoanirina V, Lascombes V, Ratsimbasoa A *et al.* (2009) Rapid detection of point mutations in *Plasmodium falciparum* genes associated with antimalarial drugs resistance by using High-Resolution Melting analysis. *Journal of Microbiological Methods*, 78, 165-170.

Areekit S, Kanjanavas P, Pakpitchareon A *et al.* (2009) High resolution melting real-time PCR for rapid discrimination between *Brugia malayi* and *Brugia pahangi*. *Journal of the Medical Association of Thailand = Chotmaihet thangphaet*, 92 Suppl 3, S24-S28.

Betts MJ & Russel RB (2003) Amino acid properties and consequences of substitutions. *Bioinformatics for geneticists* (eds Barnes MR, Fray IC), pp. 289-316, John Wiley & Sons, Hoboken.

Blom JF, Baumann HI, Codd GA & Jüttner F (2006) Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp(3),(E)-Dhb(7)]microcystin-RR. *Archiv für Hydrobiologie*, 167, 547-559.

Colbourne JK, Pfrender ME, Gilbert D *et al.* (2011) The Ecoresponsive Genome of *Daphnia pulex*. *Science*, 331, 555-561.

Czarnecki O, Henning M, Lippert I & Welker M (2006) Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). *Environmental Microbiology*, 8, 77-87.

DeMott WR (1989) The role of competition in zooplankton succession. *Plankton ecology. Succession in plankton communities* (eds U. Sommer), pp. 195-252. Springer Verlag, Berlin.

Dokulil MT & Teubner K (2000) Cyanobacterial dominance in lakes. *Hydrobiologia*, 438, 1-12.

Fodor K, Harmat V, Hetenyi C *et al.* (2005) Extended intermolecular interactions in a serine protease-canonical inhibitor complex account for strong and highly specific inhibition. *Journal of Molecular Biology*, 350, 156-169.

Fodor K, Harmat V, Neutze R *et al.* (2006) Enzyme : Substrate hydrogen bond shortening during the acylation phase of serine protease catalysis. *Biochemistry*, 45, 2114-2121.

French S & Robson B (1983) What Is a conservative substitution? *Journal of Molecular Evolution*, 19, 171-175.

Gademann K & Portmann C (2008) Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. *Current Organic Chemistry*, 12, 326-341.

Gundry CN, Vandersteen JG, Reed GH *et al.* (2003) Amplicon melting analysis with labeled primers: A closed-tube method for differentiating homozygotes and heterozygotes. *Clinical Chemistry*, 49, 396-406.

Gundry CN, Dobrowolski SF, Martin Y *et al.* (2008) Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons. *Nucleic acids research*, 36, 3401-3408.

Gustafsson,S. (2007) *Zooplankton response to cyanotoxins*. PhD thesis, Lund University, Lund.

Gustafsson S & Hansson LA (2004) Development of tolerance against toxic cyanobacteria in *Daphnia*. *Aquatic Ecology*, 38, 37-44.

Hansson LA, Gustafsson S, Rengefors K & Bomark L (2007) Cyanobacterial chemical warfare affects zooplankton community composition. *Freshwater Biology*, 52, 1290-1301.

Hairston NG, Lampert W, Caceres CE *et al.* (1999) Lake ecosystems - Rapid evolution revealed by dormant eggs. *Nature*, 401, 446.

Herrmann MG, Durtschi JD, Wittwer CT & Voelkerding KV (2007) Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. *Clinical Chemistry*, 53, 1544-1548.

Henri H & Mouton L (2012) High-Resolution Melting technology: a new tool for studying the Wolbachia endosymbiont diversity in the field. *Molecular Ecology Resources*, 12, 75-81.

Holderegger R, Kamm U & Gugerli F (2006) Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology*, 21, 797-807.

Levan TD, Bloom JW, Bailey TJ *et al.* (2001) A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. *Journal of Immunology*, 167, 5838-5844.

Luchi N, Pratesi N, Simi L *et al.* (2011) High-Resolution Melting Analysis: a new molecular approach for the early detection of *Diplodia pinea* in Austrian pine. *Fungal Biology*, 115, 715-723.

Lürling M (2003) *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedesmus obliquus*. *Limnology and Oceanography*, 48, 2214-2220.

Mader E, Lohwasser U, Boerner A & Novak J (2010) Population structures of genebank accessions of *Salvia officinalis* L. (Lamiaceae) revealed by high resolution melting analysis. *Biochemical Systematics and Ecology*, 38, 178-186.

Mandola MV, Stoehlmacher J, Muller-Weeks S *et al.* (2003) A novel single nucleotide polymorphism within the 5 ' tandem repeat polymorphism of the Thymidylate synthase gene abolishes USF-1 binding and alters transcriptional activity. *Cancer Research*, 63, 2898-2904.

Matthes M (2004) Low genotypic diversity in a *Daphnia pulex* population in a biomanipulated lake: the lack of vertical and seasonal variability. *Hydrobiologia*, 526, 33-42.

Montgomery J, Wittwer CT, Palais R & Zhou L (2007) Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. *Nature Protocols*, 2, 59-66.

Oakey J, Harris E, Pease B, Jennings C & McCubbin K (2011) Differentiation of *Solenopsis invicta* social forms using high resolution melt PCR. *Bulletin of Entomological Research*, 101, 581-589.

Ohta T (1992) The nearly neutral theory of molecular evolution. *Annual Review of Ecology and Systematics*, 23, 263-286.

Palais RA, Liew MA & Wittwer CT (2005) Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping. *Analytical Biochemistry*, 346, 167-175.

Porter KG & Mcdonough R (1984) The energetic cost of response to blue-green-algal filaments by cladocerans. *Limnology and Oceanography*, 29, 365-369.

Radvansky J, Bazsalovicsova E, Kralova-Hromadova I, Minarik G & Kadasi L (2011) Development of high-resolution melting (HRM) analysis for population studies of *Fascioloides magna* (Trematoda: Fasciolidae), the giant liver fluke of ruminants. *Parasitology Research*, 108, 201-209.

Reed GH, Kent JO & Wittwer CT (2007) High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*, 8, 597-608.

Ririe KM, Rasmussen RP & Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry*, 245, 154-160.

Rohrlack T, Dittmann E, Börner T & Christoffersen K (2001) Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. *Applied & Environmental Microbiology.*, 67, 3523-3529.

Rozen S & Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods in molecular biology (Clifton, N.J.)*, 132, 365-386.

Sarnelle O & Wilson AE (2005) Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, 50, 1565-1570.

Schreurs,H. (1992) *Cyanobacterial dominance, relation to eutrophication and lake morphology.* PhD thesis, University of Amsterdam, Amsterdam.

Schwarzenberger A, Zitt A, Kroth P, Mueller S & Von Elert E (2010) Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. *BMC physiology*, 10, 6.

Seeb J, Pascal C, Grau E *et al.* (2011) Transcriptome sequencing and high-resolution melt analysis advance single nucleotide polymorphism discovery in duplicated salmonids. *Molecular Ecology Resources*, 11, 335-348.

Shi JY, Zhu HF, Arvidson DN & Woldegiorgis G (1999) A single amino acid change (substitution of glutamate 3 with alanine) in the N-terminal region of rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA inhibition and high affinity binding. *Journal of Biological Chemistry*, 274, 9421-9426.

Smith B, Lu C & Bremer J (2010) High-resolution melting analysis (HRMA): a highly sensitive inexpensive genotyping alternative for population studies. *Molecular Ecology Resources*, 10, 193-196.

Vignal A, Milan D, SanCristobal M & Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution*, 34, 275-305.

Von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography*, 47, 1764-1773.

Von Elert E, Martin-Creuzburg D & Le Coz JR (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 1209-1214.

Von Elert E (2004) Food quality constraints in *Daphnia*: interspecific differences in the response to the absence of a long chain polyunsaturated fatty acid in the food source. *Hydrobiologia*, 526, 187-196.

Von Elert E, Zitt A, Schwarzenberger A (2012) Inducible tolerance in *Daphnia magna* against dietary protease inhibitors. *Journal of Experimental Biology*, 215, 1209-1214

Vossen RHAM, Aten E, Roos A & den Dunnen JT (2009) High-Resolution Melting Analysis (HRMA)-More Than Just Sequence Variant Screening. *Human Mutation*, 30, 860-866.

Weider LJ (1992) Disturbance, competition and the maintenance of clonal diversity in *Daphnia pulex*. *Journal of Evolutionary Biology*, 5, 505-522.

Winder L, Phillips C, Richards N *et al.* (2011) Evaluation of DNA melting analysis as a tool for species identification. *Methods in Ecology and Evolution*, 2, 312-320.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG & Pryor RJ (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry*, 49, 853-860.

Chapter 3

Interspecific differences between *D. pulex* and *D. magna* in sensitivity to cyanobacterial protease inhibitors

Abstract

It is known that cyanobacteria negatively affect herbivores due to the production of toxins such as protease inhibitors. In the present study we investigated potential interspecific differences between two major herbivores, *Daphnia magna* and *Daphnia pulex*, in terms of their tolerance to cyanobacterial protease inhibitors. Seven clones each of *D. magna* and of *D. pulex* were isolated from different habitats in Europe and North America. To test for interspecific differences in tolerance to cyanobacteria, somatic and population growth rates on varying concentrations of two *Microcystis aeruginosa* strains were determined for each *D. magna* and *D. pulex* clone. The *M. aeruginosa* strains NIVA and PCC^- contained either chymotrypsin or trypsin inhibitors, but no microcystins. Each *Daphnia* clone showed a reduction of population and somatic growth rate with increasing concentrations of NIVA. Mean somatic and population growth rate reductions on a diet with 20% NIVA were respectively 2.7-fold and 4.9-fold higher in *D. pulex* than in *D. magna*. The reduction in population growth on 10% PCC^- was 3.2-fold higher in *D. pulex* than in *D. magna*. This indicates that *D. magna* is more tolerant to cyanobacteria with protease inhibitors than *D. pulex*. The reduction of growth rates was probably caused by an interference of cyanobacterial inhibitors with proteases in the gut of *Daphnia*, as other conceivable factors explaining the reduced growth could be excluded as causal factors. Protease assays revealed that the sensitivities of chymotrypsins and trypsins to cyanobacterial protease inhibitors did not differ between *D. magna* and *D. pulex*. However, *D. magna* exhibited a 2.3-fold higher specific chymotrypsin activity than *D. pulex*, which explains the observed higher tolerance to cyanobacterial protease inhibitors of *D. magna.* The observed higher tolerance of *D. magna* to cyanobacterial protease inhibitors compared to that of *D. pulex* might result from differences in their coexistence with cyanobacteria.

Introduction

The frequency of cyanobacterial blooms in many marine and freshwater environments has increased world-wide during the last century, partly due to increasing temperatures as a consequence of global warming (Paerl & Huisman 2008). When the temperature of the epilimnion reaches its maximum in late summer and early fall (Jöhnk *et al.* 2008), the phytoplankton of eutrophic lakes and ponds is mainly dominated by bloom-forming cyanobacterial species of the genera *Microcystis*, *Anabaena* or *Oscillatoria* (Dokulil & Teubner 2000). Blooms of cyanobacteria are often associated with harmful effects on human health and livestock (Carmichael 1994). Additionally, cyanobacteria are of poor food quality for herbivorous zooplankton in freshwater ecosystems. Bloom-forming cyanobacteria have been claimed to be a major factor for a constrained mass and energy transfer from primary producers to higher trophic levels during eutrophication (Threlkeld 1979; Hansson *et al.* 2007). The herbivorous crustacean *Daphnia* often provides an important link for the transfer from primary production, e.g. from cyanobacteria, to higher trophic levels (Lampert 1987; Persson *et al.* 2007).

Negative relationships between bloom-forming cyanobacteria and the abundance of *Daphnia* have been discussed extensively over the years, and three major quality constraints of cyanobacteria as a food source have been revealed so far: (1) The occurrence of cyanobacterial filaments and the formation of colonies hinder ingestion by interfering with the filtering apparatus of *Daphnia* (Porter & Mcdonough 1984). (2) Compared to most green algae, cyanobacteria contain low levels of essential lipids such as highly unsaturated fatty acids and sterols, which leads to reduced somatic and population growth of *Daphnia* due to constrained carbon assimilation (Von Elert 2002; Von Elert *et al.* 2003; Martin-Creuzburg & Von Elert 2004; Martin-Creuzburg *et al.* 2008). (3) Many cyanobacteria produce a variety of bioactive secondary metabolites, such as hepatotoxins like microcystins (Sivonen & Jones 1999) and/or protease inhibitors (Gademann & Portmann 2008; Agrawal *et al.* 2005; Von Elert *et al.* 2012). These compounds reduce the fitness of *Daphnia* in terms of survival, growth and reproduction (Lürling & van der Grinten 2003; Rohrlack *et al.* 2001). In addition to microcystins (which are the most extensively investigated class of cyanobacterial toxins), the role of protease inhibitors in herbivore/cyanobacteria interaction has recently also become a focus of attention. More than twenty depsipeptides have been found in different genera of marine and freshwater cyanobacteria (Gademann & Portmann 2008), specifically inhibiting the serine proteases chymotrypsin and trypsin. These two classes of proteases are the most

important digestive enzymes in the gut of *D. magna* and are responsible for more than 80% of the proteolytic activity (Von Elert *et al.* 2004).

Recent studies have indicated that protease inhibitors from cyanobacterial sources indeed interfere with *Daphnia*'s digestive proteases (Schwarzenberger *et al.* 2010; Agrawal *et al.* 2005) and lead to reduced somatic growth of *Daphnia* (Von Elert *et al.* 2012). It is known that the edible size fraction of natural phytoplankton can inhibit *Daphnia*'s trypsins and chymotrypsins (Kuster *et al.* 2012). This inhibitory potential of seston can be in the same order of magnitude as pure cyanobacterial cultures (Czarnecki *et al.* 2006). Hence, it is reasonable to assume that an interference of cyanobacterial protease inhibitors with *Daphnia*'s digestive proteases occurs in nature and is ecologically relevant.

However, several studies have also demonstrated that *Daphnia* may develop tolerances against cyanobacterial toxins at the population level (Sarnelle & Wilson 2005; Gustafsson & Hansson 2004; Hairston *et al.* 2001). Blom *et al.* (2006) have recently shown that *Daphnia* sp. coexisting with *Planktothrix rubescens*, a cyanobacterium that contains the trypsin inhibitor oscillapeptin J, is significantly more tolerant to oscillapeptin-J than *Daphnia* sp. from a lake free of this cyanobacterium. Considering the finding that almost 60% of 17 cyanobacterial blooms contained protease inhibitors (Agrawal *et al.* 2001), it is reasonable to assume that increased tolerance to cyanobacteria in *Daphnia* populations may be caused by an enhanced tolerance to the cyanobacterial protease inhibitors. It has been suggested that at least two fundamental mechanisms underlie the increased tolerance to these dietary inhibitors: (1) Colbourne *et al.* (2011) have hypothesized that the ability of *Daphnia* to cope with different environmental conditions is a consequence of an elevated rate of gene duplications resulting in tandem gene clusters. And indeed, a surprisingly high number of genes of digestive serine proteases have been found in the recently published genome of *D. pulex* (Colbourne *et al.* 2011). (2) Von Elert *et al.* (2012) have shown that a physiological plasticity at the protein level in *Daphnia* in terms of remodeling the digestive enzymes leads to increased tolerance against cyanobacterial protease inhibitors.

In the present study we test for interspecific differences between two *Daphnia* species (*D. magna* and *D. pulex*) in their tolerance to cyanobacterial protease inhibitors. *D. pulex* and *D. magna* are both large-bodied species and frequently encountered in fishless ponds, where predation risks by visually feeding fish are comparatively low (Demott & Pape 2005). Due to the availability of full-genome data (*D. pulex*, Colbourne *et al.* 2011) or EST libraries (*D. magna*, Watanabe *et al.* 2005), both *Daphnia* species are predestinated for further ecological investigations and were therefore chosen for use in the present study. To determine potential

differences between *D. pulex* and *D. magna* in their tolerance to cyanobacteria containing protease inhibitors, we performed single-clone somatic and population growth experiments in which the clones were fed with various cyanobacterial mixtures containing trypsin or chymotrypsin inhibitors. Possible differences in tolerance to cyanobacterial protease inhibitors might have several causes and are therefore tested in the present study: (1) We determined the specific trypsin and chymotrypsin activity of each of the investigated *D. magna* and *D. pulex* clones. We hypothesized that high growth rates on cyanobacterial diets might result from high specific protease activities. (2) For each *Daphnia* clone we determined the sensitivity of gut chymotrypsins and trypsins to the respective cyanobacterial protease inhibitors. We assumed that higher sensitivity values of *Daphnia*'s gut proteases might cause reduced somatic and population growth rates for diets with cyanobacterial protease inhibitors.

Materials and Methods

Origin and cultivation of organisms

Two cyanobacterial strains and one green alga were used in the single-clone growth experiments: The cyanobacterium *Microcystis aeruginosa* NIVA Cya 43 (Culture Collection of Algae, Norwegian Institute for Water Research), subsequently labeled as 'NIVA', is known to contain the chymotrypsin inhibitors Cyanopeptolin 954 and Nostopeptin 920 (Von Elert *et al.* 2005). NIVA was cultured in 2 l chemostates in sterile Cyano medium (Von Elert & Jüttner 1997) at a dilution rate of 0.1 d^{-1} (20°C; illumination: 40 µmol m^{-2} s^{-1}). *M. aeruginosa* PCC 7806 Mut contains the trypsin inhibitors Cyanopeptolin A-D (Weckesser *et al.* 1996) and was grown in 0.75 l chemostates under otherwise identical conditions as NIVA. *M. aeruginosa* PCC 7806 Mut is a genetically engineered microcystin synthetase knock-out mutant of *M. aeruginosa* PCC 7806 (Dittmann *et al.* 1997) and is subsequently labeled as 'PCC$^-$'. Neither *M. aeruginosa* strain contains microcystins. The green algae *Chlamydomonas* sp. (strain 56, culture collection of the Limnological Institute at the University of Constance) was grown in 5 l semi-continuous batch cultures (20°C; illumination: 120 µmol m^{-2} s^{-1}) by replacing 20% of the culture with sterile Cyano medium every Monday, Wednesday and Friday in the late exponential phase of the culture. *C.* sp. contains neither chymotrypsin/trypsin inhibitors nor microcystins.

Seven *D. magna* and seven *D. pulex* clones originating from different habitats (Table1) were used in the somatic and population growth experiments. All clones were cultured separately in aged, membrane-filtered tap water and fed with saturating concentrations of *C.* sp. for at least three generations prior to the experiment.

Table 1. Characterization of the *Daphnia* clones used in the experiment.

***Daphnia* spp.**	**Clone**	**Location**	**Latitude**	**Longitude**	**Reference**
D. pulex	Gerstel	Germany	N/A	N/A.	Koch *et al.* 2009
D. pulex	NFL3	USA	N39°54'	W84°55'	Schaack *et al.* 2010
D. pulex	Gräf	Germany	N50°49'04"	E10°42'02"	Matthes 2004
D. pulex	Disp14	Canada	N42°13’	W83°02'	Haag *et al.* 2009
D. pulex	Povi113	USA	N42°45’	W85°21’	Schaack *et al.* 2010
D. pulex	Giev08	Germany	N51°57’48"	E7°34'38"	Y. Reydelet[1]
D. pulex	TCO	USA	N43°49'48"	W124°08'53"	Colbourne *et al.* 2011
D. magna	F10	Germany	N50°56'02"	E6°55'41"	Kuster *et al.* 2012
D. magna	G38	Belgium	N51°04'04"	E3°46'25"	This study
D. magna	S15	Sweden	N55°40'31"	E13°32'42"	Kuster & Von Elert 2012
D. magna	P6	Poland	N52°19'21"	E20°43'49"	Kuster *et al.* 2012
D. magna	P	The Netherlands	N51°44'01"	E5°08'17"	De Meester 1994
D. magna	B	Germany	N54°19'39"	E10°37'45"	Lampert & Rothhaupt 1991
D. magna	W	Poland	N/A	N/A	Pijanowska *et al.* 1993

[1] Y. Reydelet, personal communication, 2011

Somatic and population growth assays

Each of the 14 clones was assayed in single-clone experiments for somatic and population growth. Five (*D. magna*) to seven (*D. pulex*) juveniles from the same cohort of the third clutch and not older than 24 h were kept in 0.25 l aged and filtered tap water (membrane filter of 0.45 µm pore size) under constant dim light at 20°C. The animals were fed with non-limiting food concentrations (2 mg C/L) either of 100% *C.* sp. or of various mixtures of *C.* sp. and the two *M. aeruginosa* strains: In two treatments the animals were fed either with a mixture of 80% *C.* sp and 20% NIVA or with a mixture of 50% *C.* sp. and 50% NIVA. In one further treatment the animals were fed with a mixture of 90% *C.* sp. and 10% PCC^-. Each treatment was triplicated, and animals were transferred daily into fresh water with saturating food concentrations. Somatic growth rates were calculated on day six as according to Wacker & Von Elert (2001) as

$$g = [(\ln (G_t) - \ln (G_0)]/d.$$

for which (G) is the body weight of a subsample of the animals at the beginning (G_0) and end (G_t) of the experiment. Mean individual dry weights were mean values of two individuals. Population growth rates were calculated from daily survival and fecundity of the first clutch by using Euler's equation,

$$1 = \Sigma\, l_x \bullet m_x \bullet e^{-lx}$$

in which l_x is the survival rate and m_x the size of the first clutch on day x. Population and somatic growth rates were calculated for each replicate and subsequently averaged to give the mean of the treatment.

Preparation of cyanobacterial extracts and Daphnia homogenates

Freeze-dried NIVA or PCC^- were thoroughly homogenized and mixed separately. 50 mg of the resulting powder was suspended in 500 µl of 60% methanol and sonicated for 15 min followed by centrifugation (3 min at 10^4 x g). Subsequently the supernatant was separated from the residue and used as extract in the protease assays.

Seven-day-old individuals of each of the seven *D. magna* and *D. pulex* clones grown on non-limiting food concentrations of *C.* sp. were homogenized with a Teflon pestle. The

homogenate was subsequently centrifuged (3 min at 1.4 x 10^4 x g) and the resulting supernatant was used immediately in the enzyme assays.

Protease activity and protease inhibition assays

The activity of the proteases chymotrypsin and trypsin in homogenates of all *Daphnia* clones was measured as according to Von Elert *et al.* (2004). The protein concentration of the supernatant was analyzed using a Qubit fluorometer and the appropriate Quant-iT^{TM} Protein Assay Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's standard protocol. For the protease activity and protease inhibition assays, SuccpNA (N-succinyl-L-alanyl-L-alanyl-L-prolyl-Lphenylalanine 4-nitroanilide, Sigma, 125 µM in DMSO) was used as a substrate for chymotrypsins, while BapNA (N-R-benzoyl-DL-arginine 4-nitroanilide hydrochloride, Sigma, 1.8 mM in DMSO) served as a substrate for trypsins. Trypsin and chymotrypsin assays were performed in a potassium phosphate buffer (0.1 M, pH 7.5). The absorption change was measured continuously for 10 min at 30°C at 390 nm with a Cary 50 photometer (Varian). Specific proteolytic activity was determined as nmol *para*-nitroanilide liberated per minute and µg protein for synthetic substrates. With regard to the chymotrypsin inhibition assays, homogenates of each of the *D. magna* and *D. pulex* clones were assayed after addition of 10 – 15 different concentrations of the NIVA extract. The resulting chymotrypsin activities were plotted as a function of extracted NIVA biomass per ml assay volume. By fitting a sigmoidal dose response curve, the concentration of extracted NIVA biomass which resulted in a 50% inhibition of *Daphnia* chymotrypsin activity (IC_{50}) was calculated. The higher the IC_{50} values for the analyzed *D. magna* and *D. pulex* homogenates, the more tolerant were their respective chymotrypsins to chymotrypsin inhibitors from NIVA. Trypsin inhibition of the *D. magna* and *D. pulex* clones was assayed as above, except that different concentrations of the PCC$^-$ extract were used.

Data analysis

For the inhibition assays of each single clone of *D. magna* and *D. pulex*, the protease activities were plotted as a function of extracted NIVA or PCC$^-$ biomass. Resulting IC_{50} values were calculated by fitting a sigmoid dose-response curve using the software Graph Pad Prism (GraphPad Software, Inc.). A Student's t-test was used to analyze the effect *M. aeruginosa* had on *Daphnia*'s somatic and population growth rates as well as on protease inhibition and specific protease activity. A significance level of p = 0.05 was applied to all statistical analyses. All statistical tests were performed using SigmaPlot 11 (Systat Software, Inc.)

Results

In order to test for interspecific differences between *D. pulex* and *D. magna* with regard to their tolerance to cyanobacteria, we performed population and somatic growth experiments on four different diets. Seven clones of each species, each originating from different habitats, were fed either with pure *C.* sp. or with mixtures of *C.* sp. and either of two *M. aeruginosa* strains, NIVA and PCC$^-$.

On a mixture of 80% C. sp. and 20% NIVA, the somatic growth of *D. pulex* was reduced by 44.4% (± 6.3% SE) compared to the treatment with 100% C. sp., while *D. magna*'s somatic growth rate was reduced significantly less (16.2% ± 6.9% SE, Fig. 1A, *t*-test: t_{12}= 3.024, p<0.05). The population growth rates were similarly affected, with a reduction of 56.1% (± 11.2% SE) for *D. pulex* and 11.4% (± 10.9% SE) for *D. magna* compared to the treatment with 100% C. sp. (Fig. 1C). The reduction in population growth rate was hereby significantly greater for *D. pulex* than for *D. magna* (*t*-test: t_{12}= 3.024, p<0.05). With increasing NIVA concentration from 20% to 50%, the average somatic growth reduction increased significantly for *D. pulex* from 44.4% (± 6.3% SE) to 66.2% (± 5.4% SE; Fig. 1B; test: t_{12}= -2.63, p<0.05), while the growth rate reduction for *D. magna* also increased significantly from 16.2% (± 6.9% SE) to 45.7% (± 6.4% SE; Fig. 1B, test: t_{12}= -3.139, p<0.05). On 50% NIVA, the somatic growth rate of *D. pulex* was reduced significantly more than that of *D. magna* (Fig. 1B, *t*-test: t_{12}= 2.466, p<0.05). With regard to the population growth rate, the reduction of *D. pulex* increased from 56.1% (± 11.2% SE) on the mixture with 20% NIVA to 82.5% (± 7.4% SE) in the 50% NIVA treatment (Fig. 1D, test: t_{12}= -1.966, p=0.073). The population growth rate reduction for *D. magna* increased significantly from 11.4% (± 10.9%

SE) on 20% NIVA to 53.8% (± 8.3% SE) on 50% NIVA (Fig. 1D, *t*-test: t_{12}= -3.102, p<0.05). When comparing the effects on population growth rates of the mixture with 50% NIVA, the reduction in *D. pulex* (82.5% ± 7.4% SE) was significantly greater than in *D. magna* (53.8% ± 8.3% SE; Fig. 1D, *t*-test: t_{12}= 2.587, p<0.05).

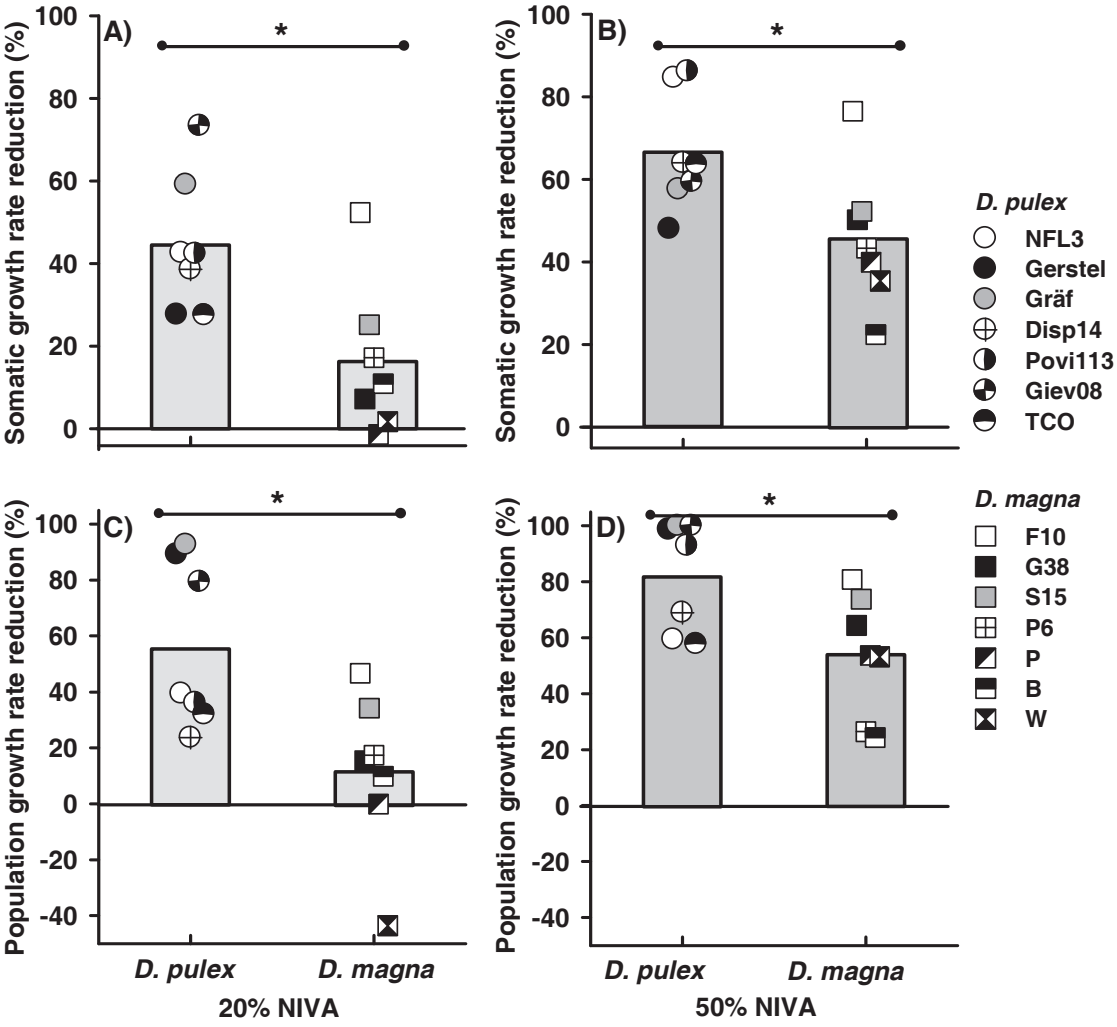

Figure 1. Reduction of relative somatic (A, B) and population (C, D) growth rates of clones of *D. pulex* (circles) and *D. magna* (squares) in response to different mixtures of *Chlamydomonas* sp. and *Microcystis aeruginosa* strain NIVA. Animals were fed either with 80% *C.* sp. and 20% NIVA (A, C) or with 50% *C.* sp. and 50% NIVA (B, D). Grey bars represent the mean values (*n*=7). Significant differences (Student's t-test, p < 0.05) between species are indicated by an asterisk.

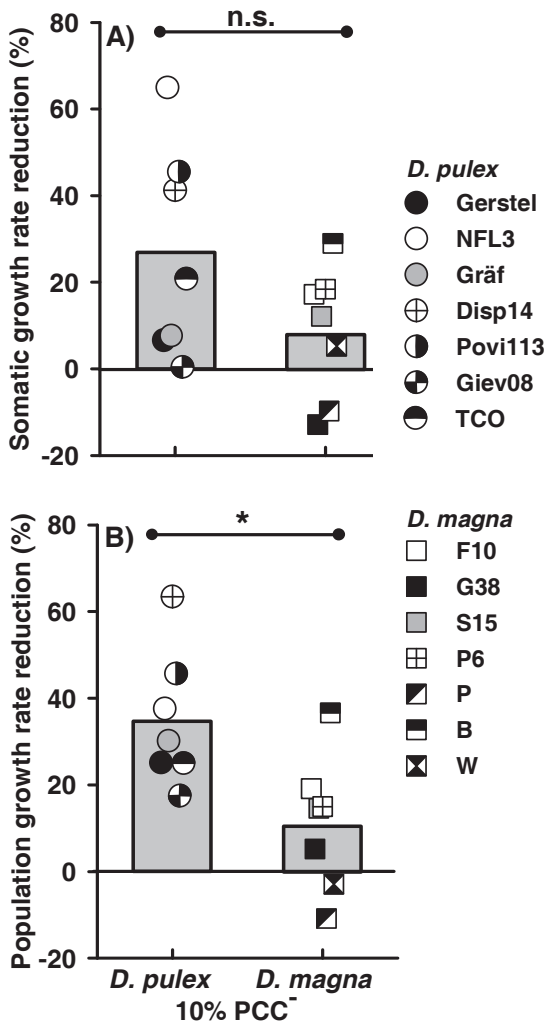

Figure 2 Reduction of relative somatic (A) and population (B) growth rates of clones of *D. pulex* (circles) and *D. magna* (squares) in response to Microcystis aeruginosa strain PCC$^-$. Animals were fed with a mixture of 90% *Chlamydomonas* sp. and 10% PCC$^-$. Grey bars represent mean relative growth rate reductions (n=7). Significant differences (Student's t-test, $p < 0.05$) between species are indicated by an asterisk, while no differences are labeled with "n.s.".

Compared to the treatment with 100% *C.* sp., the average relative somatic growth rate reduction on a mixture with 90% *C.* sp. and 10% PCC$^-$, was 26.7% (± 9.7% SE) for the *D. pulex* clones and 8.5% (± 5.8% SE) for the *D. magna* clones (Fig. 2A); however these effects were not different (*t*-test: t_{12}= 1,687, p=0.117). Considering the population growth on the same treatments, *D. pulex* exhibited significantly greater growth rate reductions (34.9% ± 5.9% SE) than *D. magna* (11% ± 5.9% SE; Fig. 2B; *t*-test: t_{12}= 2.878, p<0.05).

We furthermore quantified the specific activity of trypsins and chymotrypsins for each of the *D. magna* and *D. pulex* clones as possible causes for the observed differences in tolerance of the two species to cyanobacterial protease inhibitors. The specific chymotrypsin activity of *D. magna* (331.8 nmol/min/mg protein ± 44.4 nmol/min/mg protein SE) was dramatically higher (+ 225%) than the activity of *D. pulex* (147.4 nmol/min/mg protein ± 40.4 nmol/min/mg protein SE; Fig. 3A, *t*-test: t_{12}= -3.073, p<0.05). The specific trypsin activity for *D. pulex* was 45.2 nmol/min per mg protein (± 7.8 nmol/min/mg protein SE), whereas it was 54.4 nmol/min per mg protein (± 6.6 nmol/min/mg protein SE) for *D. magna* (Fig. 3B); however, these values were not different (*t*-test: t_{12}= -0.897, p=0.387).

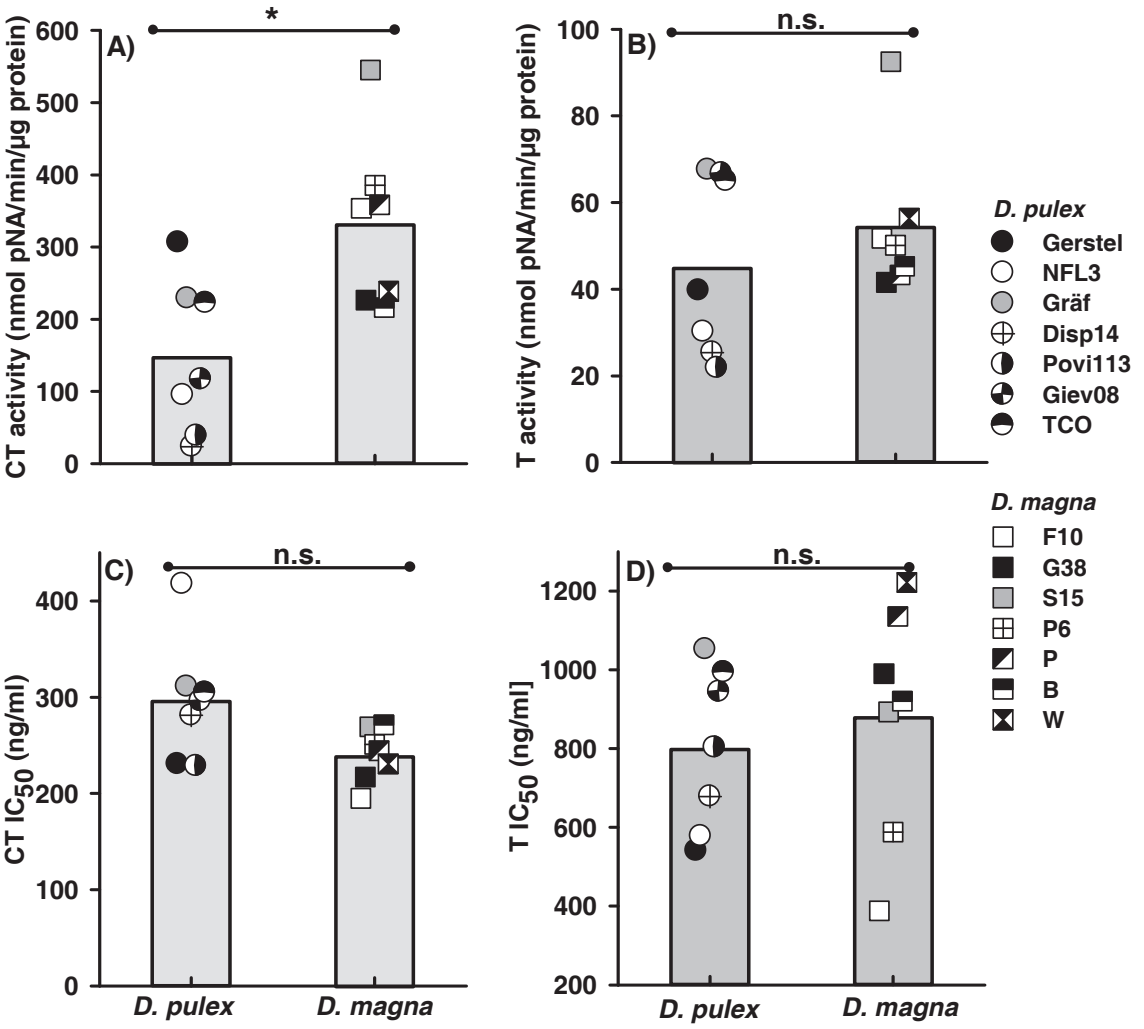

Figure 3 Two possible causes for the observed differences in the sensitivity of *D. magna* and *D. pulex* to cyanobacterial protease inhibitors were tested: (A) specific chymotrypsin (CT) and (B) trypsin (T) activity of the *D. pulex* (circles) and *D. magna* clones (squares). Inhibition of digestive proteases from homogenates of clones of *D. pulex* (circles) and *D. magna* (squares): (C) effects of extracts of *M. aeruginosa* strains NIVA on chymotrypsins, and (D) effects of extracts of *M. aeruginosa* strains PCC$^-$ on trypsins. Depicted are IC_{50} values, which represent the concentration of extracted dry weight of cyanobacterial biomass that is required to cause a 50% inhibition of respective proteases. Low IC_{50} values indicate a high sensitivity of proteases from the respective *Daphnia* clones. Grey bars represent mean values (n=7). Significant differences (Student's t-test, $p < 0.05$) among species are indicated by an asterisk; no differences are labeled with "n.s.".

In order to test for possible additional causes of the interspecific differences in their tolerance to cyanobacterial protease inhibitors, the sensitivity of *Daphnia*'s trypsins and chymotrypsins to cyanobacterial protease inhibitors was determined. This was achieved by specifying the concentration of extracted cyanobacterial biomass that was needed to inhibit

50% (IC_{50}) of either *Daphnia*'s chymotrypsins or their trypsins (Fig. 3C, D). IC_{50} values of the effects of NIVA extracts on *Daphnia*'s chymotrypsins were 295.6 ng/mL (± 24 ng/mL SE) for *D. pulex* and 239.8 ng/mL (± 10.5 ng/mL SE) for *D. magna*, although these effects were not different (Fig. 3C, *t*-test: t_{12}= 2.136, p=0.054). When specifying the IC_{50} values of effects of PCC^- extracts on *Daphnia*'s trypsins, no differences between *D. pulex* (798.7 ng/mL ± 77.7 ng/mL SE) and *D. magna* (876.8 ng/mL ± 111.6 ng/mL SE) were detected (Fig. 3D, *t*-test: t_{12}= -0.574, p=0.577).

Discussion

Cyanobacterial blooms are often associated with hazards to human health and livestock, but are also harmful to many freshwater herbivorous grazers such as *Daphnia*. Owing to the dominance of cyanobacteria in eutrophic lakes and ponds in late summer (Jöhnk *et al.* 2008), *Daphnia* genotypes from these habitats have to cope more frequently with the poor food quality of cyanobacterial carbon than genotypes from habitats free of cyanobacterial blooms. The causes for the poor assimilation of cyanobacterial carbon by *Daphnia* have been studied extensively in past decades, leading to the following observations: Firstly, colonial or filamentous cyanobacteria can mechanically interfere with *Daphnia*'s filtering apparatus (Porter & Mcdonough 1984). Secondly, cyanobacteria lack essential sterols (Von Elert *et al.* 2003) and sufficient amounts of polyunsaturated fatty acids (Martin-Creuzburg *et al.* 2008). Thirdly, the production of hepaptotoxins such as microcystins can significantly reduce the fitness of *Daphnia* (Lürling & van der Grinten 2003). In the present study, all the *D. pulex* clones and most of the *D. magna* clones showed a reduction in somatic and population growth at a concentration of 20% NIVA. The negative effects mentioned above can be ruled out as causal factors for the observed growth reductions, since the food mixtures used here consisted of saturating concentrations of the widely used high-quality reference food *C.* sp. Furthermore, both cyanobacterial strains as well as *C.* sp. were cultured as single cells and did not contain any microcystins. However, the somatic and population growth of some *D. magna* clones was reduced only at a concentration of 50% NIVA. This supports data of Lürling (2003), who used the same strain of *M. aeruginosa* (NIVA) and reported a reduction in growth of *D. magna* clones at a concentration of ≥ 25% NIVA. On the mixture with PCC^-, some *D. magna* clones and the majority of the *D. pulex* clones exhibited a reduction of population and somatic growth rate already at a concentration of 10%. Higher concentrations would probably have resulted in a significant growth reduction in all *D. magna* and *D. pulex*

clones, since several other studies (Schwarzenberger *et al.* 2010; Schwarzenberger *et al.* 2012) have reported a clear reduction in growth of daphnids at a concentration of 20% PCC$^-$.

Both *M. aeruginosa* strains (NIVA and PCC$^-$) produce exclusively either the chemically known chymotrypsin inhibitors Cyanopeptolin 954 and Nostopeptin 920 (NIVA, Von Elert *et al.* 2005) or specific Cyanopeptolins (A-D) which are known to inhibit trypsins (PCC$^-$, Weckesser *et al.* 1996). Thus, it is reasonable to assume that the observed somatic and population growth rate reduction of the *D. magna* and *D. pulex* clones in response to cyanobacteria was caused by a dietary inhibition of either *Daphnia*'s digestive chymotrypsins or trypsins. In this study somatic and population growth rates of *D. magna* and *D. pulex* served as a measure of tolerance to cyanobacteria with protease inhibitors and as an approach to test for interspecific differences. In several cases it has been demonstrated that coexistence of *Daphnia* with toxic cyanobacteria leads to local adaptation of the *Daphnia* populations, as was evidenced by increased tolerance to cyanobacteria (Hairston *et al.* 1999; Sarnelle & Wilson 2005). These findings support the notion that the presence of cyanobacteria positively selects for more tolerant *Daphnia* genotypes.

In the present study we have shown for the first time that *D. magna* exhibits a higher mean tolerance to cyanobacterial protease inhibitors than *D. pulex*. This might result from general differences in their coexistence with cyanobacteria. *D. pulex* and *D. magna* are both large-bodied *Daphnia* species, and are therefore most commonly abundant in fishless or turbid ponds, where predation risk by visually feeding fish is comparatively low (Demott & Pape 2005). In contrast to *D. magna*, which is able to tolerate elevated temperatures of up to 25°C (Wojtal-Frankiewicz 2012), the geographic distribution of *D. pulex* ranges from the temperate to the arctic zone (Colbourne *et al.* 1998). Additionally, Bengtsson (1993) has already shown that *D. pulex* usually reaches their highest densities in spring and early summer, while *D. magna* shows no clear seasonal density pattern. Cyanobacteria have, in general, higher temperature optima than other algal groups (Dokulil & Teubner 2000) and dominate the phytoplankton community mainly in late summer and early fall (Jöhnk *et al.* 2008). Thus, it is reasonable to assume that a coexistence of cyanobacteria with *D. magna* occurs more frequently than a coexistence of cyanobacteria with *D. pulex*. This putatively more frequent coexistence of *D. magna* and cyanobacteria would thus result in an increased tolerance of *D. magna* to protease inhibitors from cyanobacterial sources. Microevolutionary adaptation of *Daphnia* populations to cyanobacteria has been experimentally confirmed: Exposure of a mixed population of several *Daphnia* clones to a microcystin-producing strain of *M. aeruginosa* resulted in an enhanced tolerance in subsequent generations (Gustafsson & Hansson 2004). Such a maternally transferred increase in tolerance of *Daphnia*'s offspring

generation has also been demonstrated for the microcystin-free *M. aeruginosa* strain PCC$^-$ (Schwarzenberger & Von Elert 2012) which was also used in the present study.

With the aim of identifying the causes of the observed differences in *Daphnia*'s tolerance, we focused on interspecific differences in the tolerance to cyanobacterial protease inhibitors. Von Elert *et al.* (2012) have recently shown that *D. magna* responded to the presence of chymotrypsin inhibitors in the diet by a switch in the set of digestive chymotrypsins that resulted in more tolerant isoforms. In the present study we could not find a correlation between less sensitive proteases and higher growth rates. The protease inhibition assays revealed that the tolerance of chymotrypsins and trypsins to cyanobacterial protease inhibitors did not differ between *D. magna* and *D. pulex*. However, the mean specific chymotrypsin activity of *D. magna* was significantly higher than that of *D. pulex.* This elevated specific chymotrypsin activity of *D. magna* coincides with the less reduced somatic and population growth rates in the presence of chymotrypsin inhibitors, which might be a consequence of a more frequent coexistence of cyanobacteria and *D. magna* than of the former and *D. pulex*. Schwarzenberger *et al*. (2010) have recently shown that a clone of *D. magna* responded to dietary protease inhibitors by increased expression of protease genes. However, with regard to the significantly higher population growth rates of *D. magna* on PCC$^-$ than those of *D. pulex*, we couldn't find differences in species-specific trypsin characteristics, neither with respect to the specific activity nor to the respective sensitivity. Thus, we cannot rule out the possibility that other factors such as more efficient detoxification mechanisms in *D. magna* could be responsible for the observed higher tolerance to cyanobacterial trypsin inhibitors. One possibility of reducing toxic effects in *Daphnia* is to biotransform toxins by conjugation to glutathione, which has already been demonstrated for the cyanobacterial hepatotoxin Microcystin-LR (Pflugmacher *et al.* 1998). Although similar detoxification mechanisms of protease inhibitors in *Daphnia* have not been detected so far, they cannot be excluded as a causal factor for the higher tolerance of *D. magna* on diets with cyanobacterial trypsin inhibitors.

As a result of continuing global warming, negative effects of toxic cyanobacteria on human health, livestock and the whole ecosystem will become stronger in the near future (Paerl & Huisman 2008). Much effort is therefore invested in preventing or controlling cyanobacterial blooms. Besides reducing nutrient input into lakes and ponds, the control of cyanobacterial blooms through biological agents including organisms such as bacteria, viruses and unicellular grazers has been discussed extensively in the last decades (Sigee *et al.* 1999; Tucker & Pollard 2005). Depending on initial conditions, *Daphnia* may also control the development of bloom-forming cyanobacteria and may even suppress established

cyanobacterial blooms (Sarnelle 2007). The present study suggests that *D. magna* may control the development of cyanobacterial blooms more efficiently than *D. pulex* due to significant differences in their tolerance to cyanobacteria with protease inhibitors. In light of with increasing temperatures as a consequence of global warming, our results suggest that toxic cyanobacterial blooms and coinciding harmful effects to the ecosystem will occur more frequently in lakes with *D. pulex* than in lakes with *D. magna*.

References

Agrawal MK, Bagchi D & Bagchi SN (2001) Acute inhibition of protease and suppression of growth in zooplankter, *Moina macrocopa*, by *Microcystis* blooms collected in Central India. *Hydrobiologia*, 464, 37-44.

Agrawal MK, Zitt A, Bagchi D *et al.* (2005) Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. *Environmental Toxicology*, 20, 314-322.

Bengtsson J (1993) Interspecific competition and determinants of extinction in experimental populations of 3 rockpool *Daphnia* species. *Oikos*, 67, 451-464.

Blom JF, Baumann HI, Codd GA & Juttner F (2006) Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp(3),(E)-Dhb(7)]microcystin-RR. *Archiv für Hydrobiologie*, 167, 547-559.

Carmichael WW (1994) Toxins of cyanobacteria. *Scientific American*, 270, 78-86.

Colbourne JK, Crease TJ, Weider LJ *et al.* (1998) Phylogenetics and evolution of a circumarctic species complex (Cladocera : *Daphnia pulex*). *Biological Journal of the Linnean Society*, 65, 347-365.

Colbourne JK, Pfrender ME, Gilbert D *et al.* (2011) The ecoresponsive genome of *Daphnia pulex*. *Science*, 331, 555-561.

Czarnecki O, Henning M, Lippert I & Welker M (2006) Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). *Environmental Microbiology*, 8, 77-87.

De Meester L (1994) Life-Histories and habitat selection in *Daphnia* - Divergent life-histories of *Daphnia magna* clones differing in phototactic behavior. *Oecologia*, 97, 333-341.

Demott WR & Pape BJ (2005) Stoichiometry in an ecological context: testing for links between *Daphnia* P-content, growth rate and habitat preference. *Oecologia*, 142, 20-27.

Dittmann E, Neilan BA, Erhard M, vonDohren H & Borner T (1997) Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. *Molecular Microbiology*, 26, 779-787.

Dokulil MT & Teubner K (2000) Cyanobacterial dominance in lakes. *Hydrobiologia*, 438, 1-12.

Gademann K & Portmann C (2008) Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. *Current Organic Chemistry*, 12, 326-341.

Gustafsson S & Hansson LA (2004) Development of tolerance against toxic cyanobacteria in *Daphnia*. *Aquatic Ecology*, 38, 37-44.

Haag CR, McTaggart SJ, Didier A, Little TJ & Charlesworth D (2009) Nucleotide polymorphism and within-gene recombination in *Daphnia magna* and *D. pulex*, two cyclical parthenogens. *Genetics*, 182, 313-323.

Hairston NG, Holtmeier CL, Lampert W *et al.* (2001) Natural selection for grazer resistance to toxic cyanobacteria: Evolution of phenotypic plasticity? *Evolution*, 55, 2203-2214.

Hairston NG, Lampert W, Caceres CE *et al.* (1999) Lake ecosystems - Rapid evolution revealed by dormant eggs. *Nature*, 401, 446.

Hansson LA, Gustafsson S, Rengefors K & Bomark L (2007) Cyanobacterial chemical warfare affects zooplankton community composition. *Freshwater Biology*, 52, 1290-1301.

Jöhnk KD, Huisman J, Sharples J *et al.* (2008) Summer heatwaves promote blooms of harmful cyanobacteria. *Global Change Biology*, 14, 495-512.

Koch U, Von Elert E & Straile D (2009) Food quality triggers the reproductive mode in the cyclical parthenogen *Daphnia* (Cladocera). *Oecologia*, 159, 317-324.

Kuster CJ & Von Elert E (2012) High-resolution melting analysis: a genotyping tool for population studies on *Daphnia*. *Molecular Ecology Resources*, 12, 1048-1057.

Kuster C, Schwarzenberger A & Von Elert E (2012) Seasonal dynamics of sestonic protease inhibition: impact on *Daphnia* populations. *Hydrobiologia*, *in press* (doi: 10.1007/s10750-012-1303-x).

Lampert W (1987) Laboratory studies on zooplankton-cyanobacteria interactions. *New Zealand Journal of Marine and Freshwater Research*, 21, 483-490.

Lampert W & Rothhaupt KO (1991) Alternating dynamics of rotifers and *Daphnia magna* in a shallow lake. *Archiv für Hydrobiologie*, 120, 447-456.

Lürling M (2003) *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedesmus obliquus*. *Limnology and Oceanography*, 48, 2214-2220.

Lürling M & van der Grinten E (2003) Life-history characteristics of *Daphnia* exposed to dissolved microcystin-LR and to the cyanobacterium *Microcystis aeruginosa* with and without microcystins. *Environmental Toxicology and Chemistry*, 22, 1281-1287.

Martin-Creuzburg D & Von Elert E (2004) Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata*. *Journal of Chemical Ecology*, 30, 483-500.

Martin-Creuzburg D, Von Elert E & Hoffmann KH (2008) Nutritional constraints at the cyanobacteria-*Daphnia magna* interface: The role of sterols. *Limnology and Oceanography*, 53, 456-468.

Matthes M (2004) Low genotypic diversity in a *Daphnia pulex* population in a biomanipulated lake: the lack of vertical and seasonal variability. *Hydrobiologia*, 526, 33-42.

Paerl HW & Huisman J (2008) Climate - Blooms like it hot. *Science*, 320, 57-58.

Persson J, Brett MT, Vrede T & Ravet JL (2007) Food quantity and quality regulation of trophic transfer between primary producers and a keystone grazer (*Daphnia*) in pelagic freshwater food webs. *Oikos*, 116, 1152-1163.

Pflugmacher S, Wiegand C, Oberemm A *et al.* (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochimica et Biophysica Acta-General Subjects*, 1425, 527-533.

Pijanowska J, Weider LJ & Lampert W (1993) Predator-mediated genotypic shifts in a prey population - Experimental evidence. *Oecologia*, 96, 40-42.

Porter KG & Mcdonough R (1984) The energetic cost of response to blue-green-algal filaments by cladocerans. *Limnology and Oceanography*, 29, 365-369.

Rohrlack T, Dittmann E, Börner T & Christoffersen K (2001) Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. *Applied & Environmental Microbiology.*, 67, 3523-3529.

Sarnelle O & Wilson AE (2005) Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, 50, 1565-1570.

Sarnelle O (2007) Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. *Limnology and Oceanography*, 52, 2120-2127.

Schaack S, Choi E, Lynch M & Pritham EJ (2010) DNA transposons and the role of recombination in mutation accumulation in *Daphnia pulex*. *Genome Biology*, 11.

Schwarzenberger A, Kuster CJ & Von Elert E (2012) Molecular mechanisms of tolerance to cyanobacterial protease inhibitors revealed by clonal differences in *Daphnia magna*. *Molecular Ecology*, 21, 4898-4911.

Schwarzenberger A & Von Elert E (2012) Cyanobacterial protease inhibitors lead to maternal transfer of increased protease gene expression in *Daphnia*. *Oecologia, in press* (doi: 10.1007/s00442-012-2479-5).

Schwarzenberger A, Zitt A, Kroth P, Mueller S & Von Elert E (2010) Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. *BMC physiology*, 10, 6.

Sigee DC, Glenn R, Andrews MJ *et al.* (1999) Biological control of cyanobacteria: principles and possibilities. *Hydrobiologia*, 395, 161-172.

Sivonen K & Jones G (1999) Cyanobacterial toxins. Toxic cyanobacteria in water: A guide to their public health consequences . *Monitoring and Management: 41-111*.

Threlkeld ST (1979) Midsummer dynamics of 2 *Daphnia* species in Wintergreen Lake, Michigan. *Ecology*, 60, 165-179.

Tucker S & Pollard P (2005) Identification of cyanophage Ma-LBP and infection of the cyanobacterium *Microcystis aeruginosa* from an Australian subtropical lake by the virus. *Applied and Environmental Microbiology*, 71, 629-635.

Von Elert E & Jüttner F (1997) Phosphorus limitation and not light controls the extracellular release of allelopathic compounds by *Trichormus doliolum* (cyanobacteria). *Limnology and Oceanography*, 42, 1796-1802.

Von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography*, 47, 1764-1773.

Von Elert E, Martin-Creuzburg D & Le Coz JR (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*).

Proceedings of the Royal Society of London Series B-Biological Sciences, 270, 1209-1214.

Von Elert E, Agrawal MK, Gebauer C *et al.* (2004) Protease activity in gut *Daphnia magna*: evidence for trypsin and chymotrypsin enzymes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 137, 287-296.

Von Elert E, Oberer L, Merkel P, Huhn T & Blom JF (2005) Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. *Journal of Natural Products*, 68, 1324-1327.

Von Elert E, Zitt A & Schwarzenberger A (2012) Inducible tolerance to dietary protease inhibitors in *Daphnia magna*. *Journal of Experimental Biology*, 215, 2051-2059.

Wacker A & Von Elert E (2001) Polyunsaturated fatty acids: Evidence for non-substitutable biochemical resources in *Daphnia galeata*. *Ecology*, 82, 2507-2520.

Watanabe H, Tatarazako N, Oda S *et al.* (2005) Analysis of expressed sequence tags of the water flea *Daphnia magna*. *Genome*, 48, 606-609.

Weckesser J, Martin C & Jakobi C (1996) Cyanopeptolins, depsipeptides from cyanobacteria. *Systematic and Applied Microbiology*, 19, 133-138.

Wojtal-Frankiewicz A (2012) The effects of global warming on *Daphnia* spp. population dynamics: a review. *Aquatic Ecology*, 46, 37-53.

Chapter 4

Rapid evolution revealed by dormant eggs: physiological mechanisms

Abstract

Genetic diversity in populations of *Daphnia* is continually shaped by variation of abiotic and biotic selection factors over time. Many lakes, e.g. Lake Constance, have undergone a period of eutrophication due to anthropogenic phosphorus loading. Several studies have shown that resultant changes in the phytoplankton community have directly affected population densities of one of the main filter feeders of Lake Constance, *Daphnia galeata*. In order to investigate the influence of changing selection factors in Lake Constance on the genetic structure of the *D. galeata* population, we have isolated *D. galeata* resting eggs from four different layers of sediment cores, representing the time periods before, during and after the peak of the eutrophication and the concomitant occurrence of cyanobacteria. We have pooled genomic DNA from the different time periods and applied high-throughput sequencing with a mean coverage of 50x to each population separately. We used the data to determine the genes and metabolic pathways in the *D. galeata* population that were affected by these environmental changes. Our data suggests that cyanobacterial protease inhibitors and oxidative stress induced by microcystins from cyanobacteria have caused selective pressure on the respective target genes and have led to putatively adaptive population-wide genomic changes.

Introduction

Due to rapid human population growth within the watershed and concomitant anthropogenic phosphorus loading, many lakes in Europe, e.g. Lake Constance, have undergone a period of eutrophication in the last century (Güde *et al.* 1998). However, Lake Constance may certainly serve as an excellent example for a successful management of environmental problems in aquatic systems: The intensive agriculture, P-containing detergents as well as the growing industry resulted in a more than ten fold increase of the total phosphorus (P_{tot}) concentrations (7 to 87 µg L^{-1}) in Lake Constance from the beginning of the 20th century till 1980 (Fig. 1). Due to a comprehensive sewage treatment program including P-precipitation and substitution of phosphorus in detergents, the phosphorus input into Lake Constance declined steadily since 1980 (Güde *et al.* 1998). This has led to re-oligotrophication and resulted in oligotrophic conditions in Lake Constance with levels of P_{tot} as low as 7 µg L^{-1} (Kümmerlin 1998).

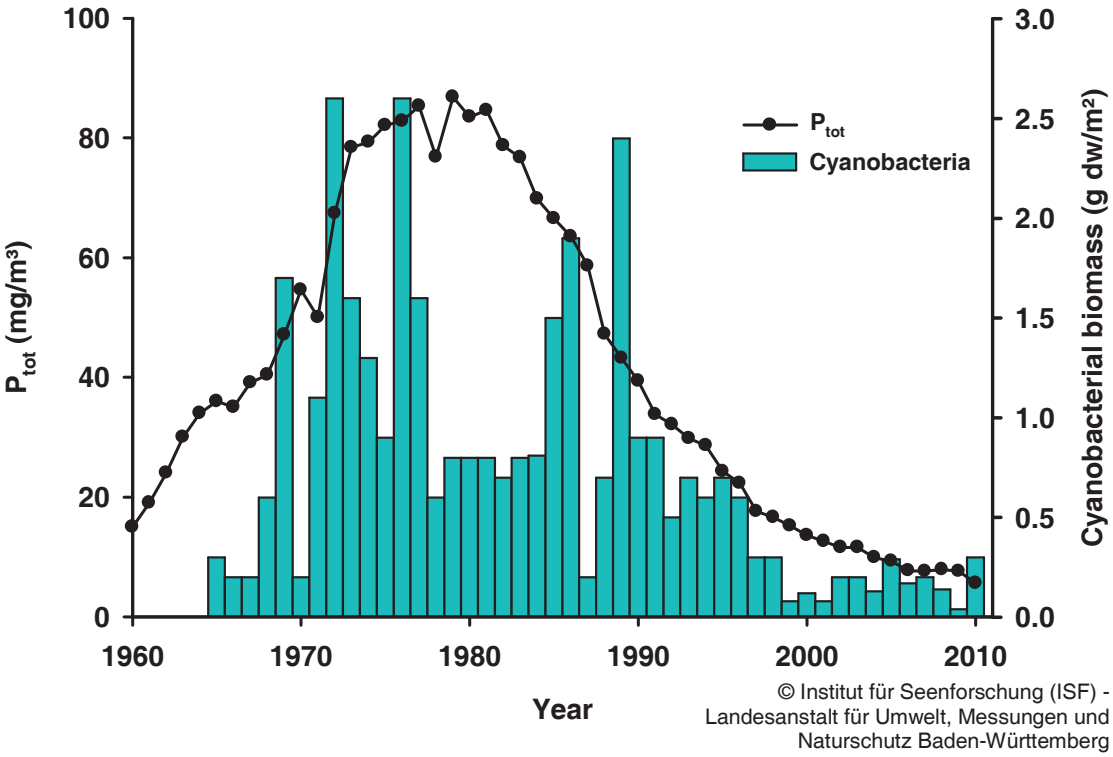

Figure 1 annual concentration of P_{tot} (left Y-axis) and cyanobacterial biomass (right Y axis) in Lake Constance from 1960 till 2010.

Concomitant with increases of the P_{tot}-concentration, the taxonomical composition of the phytoplankton community changed during the course of eutrophication. In the early 1960s the originally dominating diatoms of the genus *Cyclotella* were mainly replaced by diatoms of the genus *Stephanodiscus* (Kümmerlin 1998). At the peak of the phosphorus concentration in 1980 cyanobacterial species, such as *Anabaena planctonica* and *Aphanizomenon flos-aquae*, became also abundant in Lake Constance (Fig. 1). These species are generally regarded as indicators for eutrophic conditions in a lake (Kümmerlin & Bürgi 1989). The proportion of cyanobacteria increased during the period of highest phosphorus concentrations (1969-1979) and reached a maximum of even up to 28% (average = 13%) of the total phytoplankton biovolume during summer months (Hairston *et al.* 2001). During the first two decades of progressive re-oligotrophication in the mid 1980s and 1990s cyanobacteria became less abundant again. Ongoing increases of relative abundances of phytoplankton species that are indicative for oligotrophic conditions, such as *Cyclotella* ssp. (Bacillariophyceae) and Chrysophyceae, are recorded since 1986 (Kümmerlin 1998).

Resultant changes in the phytoplankton community of Lake Constance had also profound effects on the zooplankton community, e.g. on *Daphnia galeata,* the major herbivorous grazer in Lake Constance. For example, Weider *et al.* (1997) noted significant genotypic shifts in the *D. galeata* population in Lake Constance, by collecting resting eggs from lake sediments dating from the mid-1960s to mid-1990s and analyzing the genetic structure of the hatchlings from these resting eggs. The observed shifts in genotype composition of the *D. galeata* population were strongly correlated with changes of the total phosphorus in Lake Constance. Hairston *et al.* (1999, 2001) have shown that these shifts in genotype composition went along with an increase of the mean tolerance of *D. galeata* clones to cyanobacteria during the time of eutrophication and with the concomitant appearance of cyanobacteria. Hairston *et al.* (1999) provided strong experimental support for the reasoning that the occurrence of cyanobacteria was indeed the selective force driving the shifts in genotype frequency in the *D. galeata* population of Lake Constance observed by Weider *et al.* (1997). However, it remains unclear, which particular genes and metabolic pathways of the *D. galeata* population were directly affected by the appearance of cyanobacteria.

Cyanobacteria are, in general, known to be poor food for the major herbivorous grazer in Lake Constance, *D. galeata*. At least three major food quality constraints of cyanobacterial carbon to daphnids have been revealed so far: (1) Filamentous or colonial forms of cyanobacteria can cause mechanical interference with *Daphnia*'s filtering apparatus (Porter & Mcdonough 1984). (2) Cyanobacteria have low nutritional value in terms of a low content of polyunsaturated fatty acids and the lack of sterols; both are essential for *Daphnia* (Von Elert

2002, Von Elert *et al.* 2003). (3) Many cyanobacterial strains contain toxins, such as hepatotoxins, neurotoxins (Sivonen & Jones 1999), and/or protease inhibitors (Gademann & Portmann 2008, Agrawal *et al.* 2005, Von Elert *et al.* 2012). These secondary metabolites affect the fitness of *Daphnia* in terms of reduced survival, growth and/or reproduction (Lürling & van der Grinten 2003; Lürling *et al.* 2011, Kuster *et al.* 2012). Type and composition of these secondary metabolites differ from strain to strain and between natural blooms of cyanobacteria (Gademann & Portmann 2008, Agrawal *et al.* 2001). However, only a few of these metabolites have already been characterized in terms of their exact mode of toxicity on the one hand and the physiological response of *Daphnia* to these toxins on the other hand. In the case of cyanobacterial protease inhibitors, which interfere with *Daphnia*'s major digestive enzymes (chymotrypsin and trypsin, Von Elert 2004), the physiological response of *Daphnia* to these inhibitors has been elucidated by showing that *Daphnia* remodel their digestive enzymes and increase expression of the respective protease genes (Von Elert *et al.* 2012, Schwarzenberger *et al.* 2010). Another possibility of adaptation to cyanobacterial toxins is to reduce the toxic effects in *Daphnia* by biotransformation of the toxins by conjugation to glutathione (GSH), which has already been demonstrated in-vitro for the cyanobacterial hepatotoxin microcystin-LR (MC-LR, Pflugmacher *et al.* 1998). Besides glutathione-s-transferases (GSTs), which catalyze the conjugation of GSH to MCs, more than ten other gene families have been reported to be direct or indirect targets of this cyclic peptide at the cellular and molecular level in animal cells (reviewed in Campos & Vasconcelos 2010). Thus, the huge variety of different toxic cyanobacterial metabolites and their possible targets in *Daphnia*'s genome makes it nearly impossible to identify a priori those specific genes in a natural and locally adapted *Daphnia* population that have undergone positive selection in the presence of toxic cyanobacteria. With regard to the *D. galeata* population in Lake Constance a single-target gene approach is therefore not a reasonable way to identify the physiological mechanism of the increased tolerance to cyanobacteria.

Meanwhile, affordable sequencing (Shendure & Ji 2008) of whole genomes allows for multiple-target gene approaches. Application of next-generation sequencing at the population level has enabled the easy detection of single nucleotide polymorphisms (SNPs) in genomes population-wide. Coleman & Chishom (2010) used a population-genomic approach to compare two bacterial populations from two distinct ocean regions differing in the concentration of available phosphorus. Their results revealed that phosphorus-related genes from these two bacterial populations form phylogenetically distinct clusters, whereas house-keeping genes did not.

In the present study we have analyzed the genetic structure of three *D. galeata* populations of Lake Constance via next-generation sequencing. As according to Weider *et al.* (1997) we have isolated *D. galeata* resting eggs from three different layers of sediment cores, representing the time before, during and after the peak of the eutrophication of Lake Constance. We subsequently extracted the DNA from each resting egg, pooled the DNA for each population separately and applied next-generation sequencing with a mean coverage of 50x. We used the data to determine the genetic diversity of the *D. galeata* populations. In order to understand the physiological mechanisms underlying the adaptation to increased cyanobacterial abundances we performed a genome-wide assessment of SNPs and copy number variations of several gene families in each of the three *D. galeata* populations. Outstanding changes in SNP frequencies can be a hint for natural selection on respective genes resulting in traces of a genetic bottleneck, as has already been demonstrated for human populations (i.e. Schmegner *et al.* 2005). Additionally we hypothesized that copy numbers of possible target genes, which might be affected by the presence of cyanobacteria, might have increased in the *D. galeata* populations during the time of eutrophication, as *Daphnia* has been shown to cope with different environmental conditions by increasing the rate of gene duplications (Colbourne *et al.* 2011).

Materials & Methods

Study site

Lake Constance is a large (surface area: 571km²), deep (maximum depth: 253 m) and warm-monomictic lake situated at the borders of Germany, Switzerland and Austria on the northern edge of the Alps (Fig. 2). It consists of three water basins: the *Obersee* (upper lake) the *Untersee* (lower lake), and a connecting stretch of the river Rhine, called the *Seerhein*. Data on phytoplankton species identity and abundances in Lake Constance were collected continuously since 1957. For the years 1957-1964 no cyanobacterial species were found in the upper basin of Lake Constance (Hairston *et al.* 2001). In the following years cyanobacteria became common in Lake Constance and increased in abundance to mean summer dry weights between 1 and 2.5 g/m² (Fig. 1). Concomitant with a decrease in phosphorus the abundances of cyanobacteria decreased continuously after 1990 to values around 0.2 mg/m³.

Field collection, sediment dating and ephippia collecting

Three sediment cores were collected at 150 m depth in the upper basin of Lake Constance (*Obersee*) in late summer of 2011 (Fig. 2, N47°37'32.12" E9°26'21.96"). Sediments were dated by counting annual laminations including high-water phases with increased sedimentation. Earlier studies by Wessels *et al.* (1995) and Weider *et al.* (1997) have already shown that dating by annual laminations coincides with exact measurement of zinc concentrations and ^{137}Cs-dating and therefore provides a very precise sediment dating. Three different sediment age categories were chosen representing the time before (1954-1960), during (1974-1979) and after (1994-1999) the peak of eutrophication in Lake Constance. As according to Weider *et al.* (1997) the outer edge of each section was sliced away in order to avoid the possible inadvertent transfer of ephippia between layers. The sediment strata of all three sediment cores were subsequently sieved and screened with a 200 µm mesh to collect the ephippia, which were then sorted by hand under a dissecting microscope, counted and stored in distilled water at 4°C.

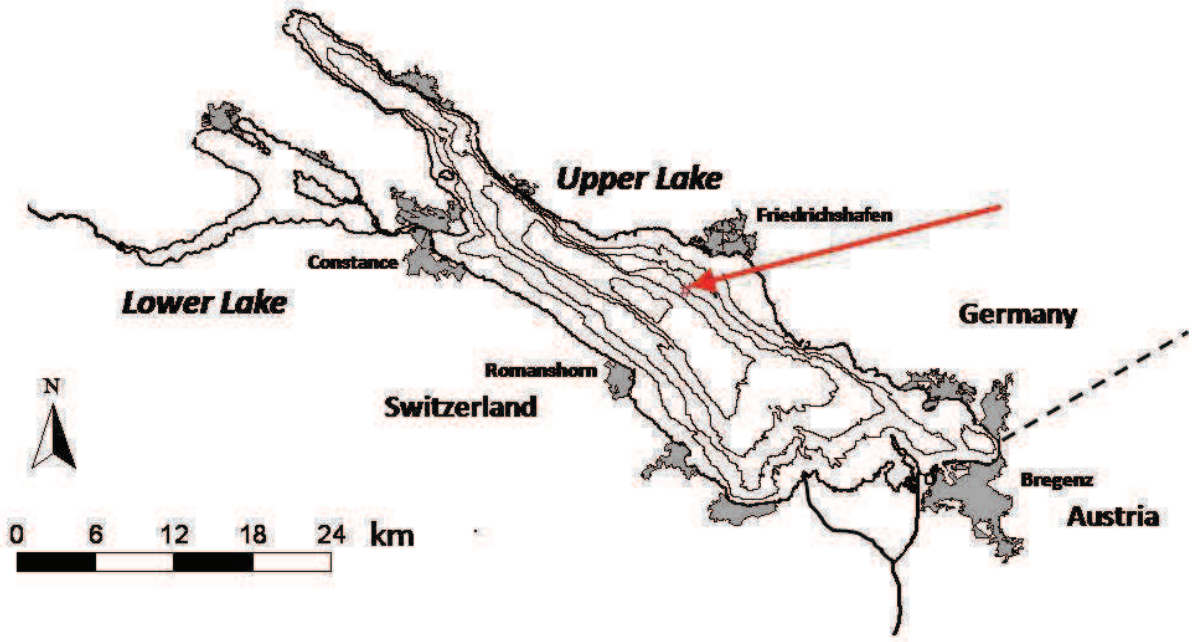

Figure 2 Map of Lake Constance, modified after Wessels (1998). The sampling site of the sediment cores are marked by an arrow

DNA extraction and high-throughput sequencing

130 diapausing eggs from each of the three sediment age categories were decapsulated, because an earlier study indicated that ephippia interfere with DNA amplification reactions (Pollard *et al.* 2003). *Daphnia* genotypes in ephippial eggs result from sexual recombination and can therefore be assumed as genetically unique. Decapsulated eggs were subsequently pooled for each sediment age separately. The resultant population DNA was extracted by using the peqGOLD® Tissue DNA Mini Kit (Peqlab, Erlangen, Germany) as according to the manufacturer's standard protocol. DNA concentration of each sample was determined using Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen AG, Carlsbad, USA). The DNA extraction efficiency and the final genomic DNA concentration varied between all samples (1954-1960: 1.9 ng/µl; 1974-1979: 8.7 ng/µl; 1994-1999: 4.6 ng/µl), but was sufficient to be normalized and to build up genome libraries for all populations using the Illumina/Solexa Genome Analyzer System. The *D. galeata* populations representing the time before (1954-1960), during (1974-1979) and after the eutrophication (1994-1999) are referred hereafter as POP_{pre}, POP_{eu} and POP_{post}, respectively.

Single-read mapping and RPKM measures

Total reads of each population were blasted against an unpublished *D. galeata* transcriptome using blastn. This reference *D. galeata* transcriptome consisted of expressed sequence tags (ESTs) derived from one inbred G100 isoclonal lineage that had been experimentally challenged under 15 different conditions. The transciptome consists of 29500 contigs with an average length of 665bp (further information on the *D. galeata* transcriptome can be found on https://wiki.cgb.indiana.edu/display/grp/Daphnia+galeata). The minimum score for a positive single read mapping onto the transcriptome assembly was set to $1E^{-30}$. In order to test for possible adaptation of the *D. galeata* populations to the appearance of cyanobacteria in Lake Constance on the level of gene copy number variations, we quantified gene levels in reads per kilobase of exon model per million mapped reads (RPKM, Mortazavi *et al.* 2008). The measurement of the RPKM values depicts the molar concentration of a gene after normalizing for length and for the total number of reads in the sample. This ensures a transparent comparison of gene levels both within and between population DNA samples. As according to Mortazavi *et al.* (2008) the RPKM values for each contig were calculated using the equation:

$$RPKM = \frac{reads}{\frac{mapped\ reads}{1000000} \times \frac{contig\ length}{1000}}$$

Genes with RPKM values ≥ 10 were considered as ecologically relevant and were therefore included in the analysis. RPKM ratios ≥ 2 of ecologically relevant genes between two populations were assumed in the present study as significantly different. If, for an ecologically relevant gene, the ratio of the RPKM values from two populations was ≥ 2, the RPKM values were assumed as significantly different.

SNP analysis

Major allele frequency (MAF) refers to the frequency at which the most common allele occurs in a population. The MAF of each single nucleotide polymorphism (SNP) in each population was determined. For each population the MAF of all SNPs in each contig was arithmetically averaged. MAFs with coverage of at least ten reads per SNP and population were considered as ecologically relevant and therefore included in the analysis.

In order to determine changes in allele frequencies (CAF), we calculated changes of MAFs from POP_{eu} and POP_{post} related to MAFs from POP_{pre} for each contig separately using the equation:

$$\sum_{i}^{n} \frac{|pi - qi|}{n}$$

In which *pi* is the MAF for SNP *i* of the POP_{pre} and *qi* the respective allele frequency in POP_{eu} or POP_{post}. “n” represents the total number of SNPs in a contig. This calculation was performed for each of the 29500 contigs and for POP_{eu} and POP_{post}, separately.

Target gene approach

In order to screen for possible target genes that might have been influenced by the elevated cyanobacterial abundance during the peak of the eutrophication of Lake Constance, several gene families were selected for the SNP analyses. We have analyzed genes that encode for proteins with functions in the processes of MC uptake, toxic effects and

biotransformation of secondary metabolites (Table 1). Target genes found in the literature were searched for homologs in *D. pulex* transcripts using the wfleabase database (Colbourne *et al.* 2005). Genes from *D. pulex* were subsequently blasted against the *D. galeata* transcriptome using blastx. The minimum score for a positive blast result was set to $1E^{-50}$. MAFs and CAFs were calculated for each gene and averaged to give the mean of each gene family.

Table 1. Characteristics of target genes used in the comparative SNP analysis

Protein/Gene	Biological function	Effects of cyanobacterial toxins	Genes in *D. galeata*	Reference
Glutathione peroxidase	lessening of formation of lipid hydroperoxides, protection from oxidative damage	Detoxification of microcystin-LR-induced lipid hydroperoxides	4	Gehringer *et al.* (2004)
Glutathione-S-transferase	Contribution to biotransformation of xenobiotics, protection from oxidative damage	Detoxification of microcystins	17	Pflugmacher *et al.* (1998)
Mitogen activated protein kinase (MAPK)	Signal transduction, cell proliferation and differentiation	Microcystin-LR induced apoptosis through activation of multiple MAPK pathways	5	Komatsu *et al.* (2007)
Protein phosphatase	Regulation of protein activity	Inhibition by microcystins	5	DeMott & Dhawale (1995)
Elastase	Protease	Inhibition by elastase inhibitors	11	Singh *et al.* (2005)
Chymotrypsin	Protease	Inhibition by chymotrypsin inhibitors	15	Von Elert *et al.* (2012)
ABC-Transporter	Membrane transporter	Increased gene expression induced by microcystin-LR	14	Ame *et al.* (2009)
NADH/NADPH oxidase	Electron transfer to superoxide	Microcystin-LR affects their phosphorylation resulting in reactive oxygen species (ROS)	5	Nong *et al.* (2007)
Trypsin	Protease	Inhibition by trypsin inhibitors	72	Agrawal *et al.(*2005)

Data analysis

Kruskal-Wallis-test was used to analyze the effect of time on the MAFs of each gene family in each population. Analysis of variance (ANOVA) could not be used due to a non-Gaussian distribution of the MAFs. A significance level of $p = 0.05$ was applied to all statistical analyses. All statistical tests were performed with SigmaPlot 11 (Systat Software, Inc.). With regard to the CAF analysis, non overlapping error bars of a gene family were assumed to be significantly different from the mean of all SNPs in each contig.

Results

With regard to the RPKM analyses median and mean values of each of the 29500 contigs in all populations (POP_{pre}, POP_{eu}, POP_{post}) were quite similar (Table 2). We therefore assumed a permission for RPKM comparisons among all populations.

Table 2 Summary of RPKM values for POP_{pre}, POP_{eu} and POP_{post}

Population	Min.	1st. Qu.	Median	Mean	3rd Qu.	Max.
POP_{pre}	0.43	19.41	26.07	53.09	32.32	164578
POP_{eu}	0.27	22.32	27.79	52.56	32.79	155855
POP_{post}	0.2	20.58	26	53.21	31.21	152337

Comparison of RPKM values between POP_{pre} and POP_{eu} revealed that 1003 ecologically relevant predicted genes differed between both populations. 318 genes were significantly more abundant in POP_{pre} than in POP_{eu}, while it was the other way around for 685 genes (Fig. 3). When comparing the RPKM values of POP_{eu} and POP_{post} overall 444 gene were significantly different, out of which 166 genes were more abundant in POP_{post} than in POP_{eu}, while 278 ecologically relevant gene models had significantly higher RPKM values in POP_{eu} than in POP_{post} (Fig. 4).

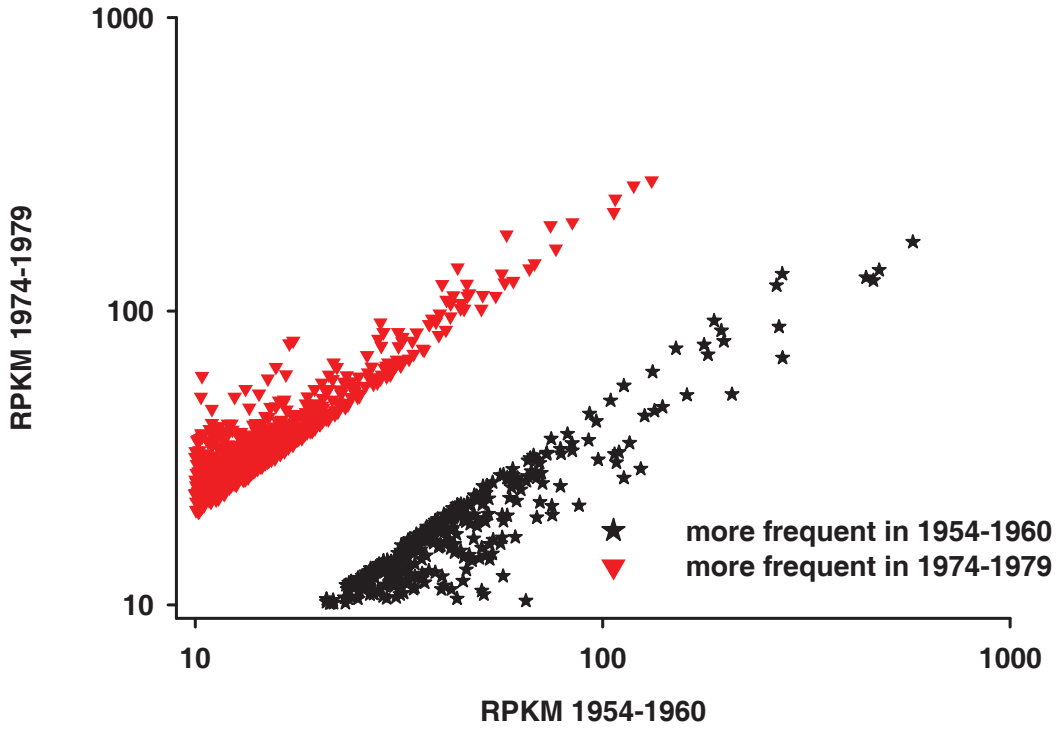

Figure 3 Comparison of RPKM values of the genomic DNA of *D. galeata* isolated from resting eggs from two different sediment layers, representing the time periods before (1954-1960, x-axis) and during (1974-1979, y-axis) the peak of the eutrophication in Lake Constance. RPKM values depict the molar concentration of a predicted gene after normalizing for length and for the total number of reads in the sample. The higher the RPKM value, the more abundant is a gene in a population. Each *red triangle* represents a gene with a significantly higher RPKM value in the population from 1974-1979 compared to the population from 1954-1960. *Black stars* represent genes that are significantly more frequent in the population from 1954-1960 compared to the population from 1974-1979. Mind the log-scale.

With regard to the comparison between POP_{post} and POP_{pre} RPKM values of 1112 gene were different: 634 genes were significantly more abundant in POP_{post} than in POP_{pre}, while it was the other way around for 478 genes (Fig. 5). One group of genes had outstandingly high RPKM values in all populations and was additionally significantly more abundant in POP_{post} than in POP_{pre} (genes in red circle, Fig. 4). Blasting these genes via blastx against the NCBI non-redundant protein sequences (http://www.ncbi.nlm.nih.gov/) revealed that each gene of this group resulted from mtDNA. The RPKM values obtained for mitochondrial genes were normalized to RPKM values of single copy genes from the same population, in order to

obtain estimates of the number of mitochondrial genes per cell. A subsequent one-way analysis of variances (ANOVA) exhibited significant differences of these normalized RPKM values of mitochondrial genes between the three populations ($F_{2,27}$=545.051; p<0.001). Normalized RPKM vales of mitochondrial genes increased significantly from 164 (± 9 SE) in POP_{pre} to 235 (± 13 SE) in POP_{eu} and further on to 334 (± 12 SE) in POP_{post} (Fig. 6, p<0.001).

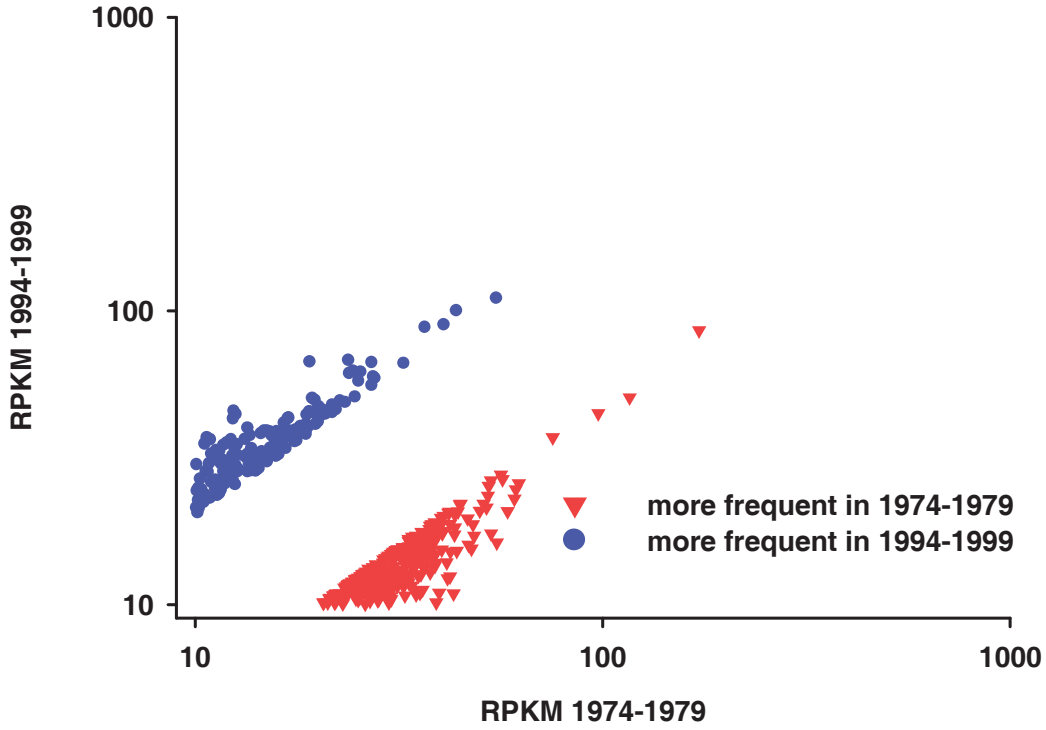

Figure 4 RPKM value comparisons between the genomic DNA of *D. galeata* DNA isolated from resting eggs from two different sediment layers, representing the time periods during (1974-1979, x-axis) and after (1994-1999, y-axis) the peak of the eutrophication in Lake Constance. RPKM values depict the molar concentration of a predicted gene after normalizing for length and for the total number of reads in the sample. The higher the RPKM value, the more abundant is a gene in a population. Each *blue circle* represents a gene with a significantly higher RPKM value in the population from 1994-1999 compared to the population from 1974-1979. With regard to the *red triangles* it is the other way around; these genes are significantly more frequent in the population from 1974-1979 compared to the population from 1994-1999. Mention the log transformation of both axes.

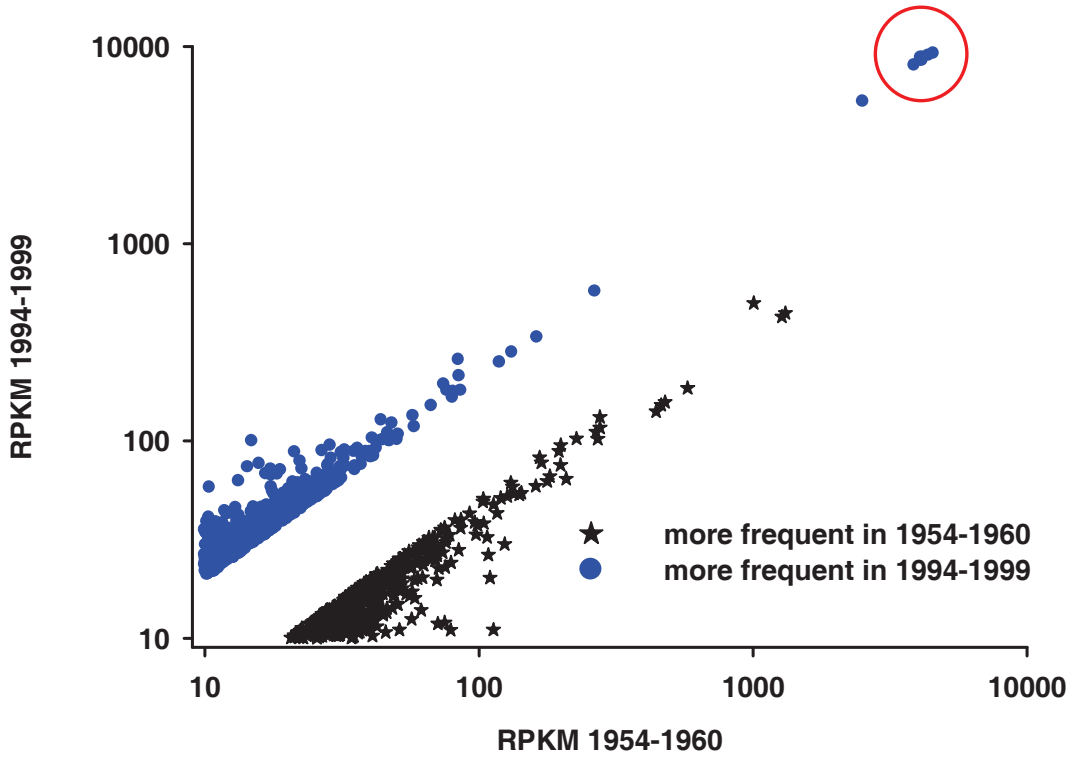

Figure 5 Comparison of RPKM values of the genomic DNA of *D. galeata* DNA isolated from resting eggs from two different sediment layers, representing the time periods before (1954-1960, x-axis) and after (1994-1999, y-axis) the peak of the eutrophication in Lake Constance. RPKM values depict the molar concentration of a predicted gene after normalizing for length and for the total number of reads in the sample. The higher the RPKM value, the more abundant is a gene in a population. Each *blue circle* represents a gene with a significantly higher RPKM value in the population from 1994-1999 compared to the population from 1954-1960. With regard to the *black stars* it is the other way around; these genes are significantly more frequent in the population from 1954-1960 compared to the population from 1994-1999. Mention the log transformation of both axes. The red circle indicates a group of predicted genes that resulted from mitochondrial DNA.

Concerning the MAF analyses, six out of ten target genes exhibited neither increases or decreases in the major allele frequencies of respective SNPs over time (Fig. 7, Kruskal-Wallis, $p > 0.05$): Elastases ($H_2=0.130$, $p = 0.937$), protein phosphatases ($H_2=1.340$, $p = 0.512$), mitogen-activated protein kinases ($H_2=2.940$, $p = 0.230$), ABC transporter($H_2=5.155$, $p = 0.076$), NADPH oxidases ($H_2=1.580$, $p = 0.454$), glutathione-peroxidases ($H_2=4.500$, $p = 0.104$). However, four other target genes exhibited significant effects regarding the major base frequencies of SNPs (Fig. 7): trypsins ($H_2=43.865$, $p \leq 0.001$), chymotrypsins ($H_2=6.855$, $p \leq 0.05$), GSTs ($H_2=22.633$, $p \leq 0.001$) and mitochondrial genes ($H_2=23.094$, $p \leq 0.05$) had significantly higher MAF values in POP_{post} than in POP_{pre}.

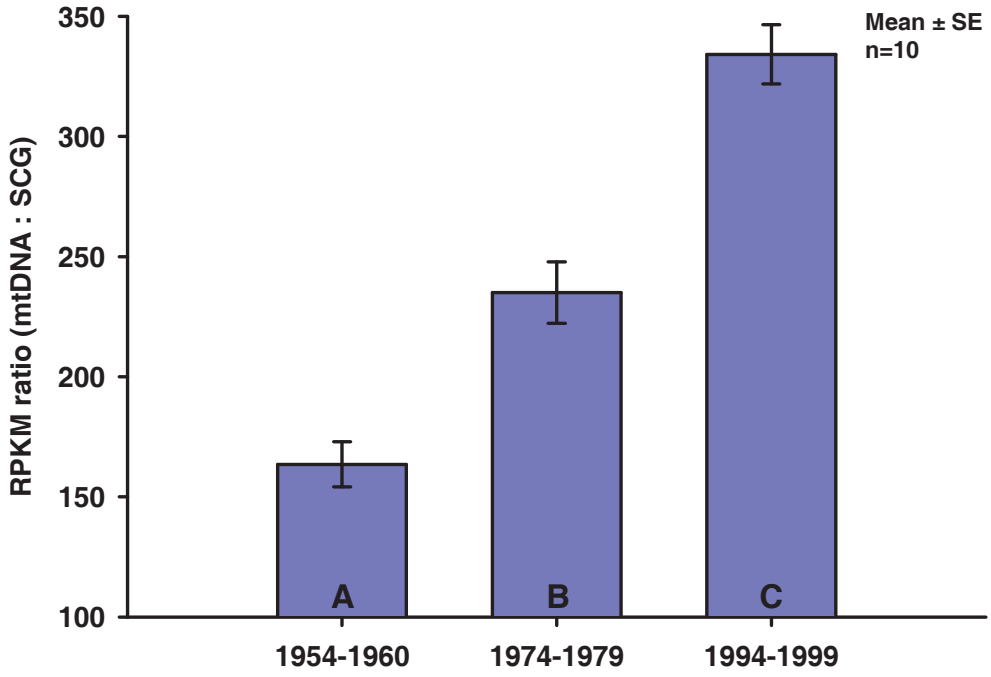

Figure 6 Estimation of the number of mitochondrial genes per cell for each of the *D. galeata* DNA from resting eggs from three different sediment layers, representing the time periods before (1954-1960), during (1974-1979) and after (1994-1999) the eutrophication peak in Lake Constance (mean ± SE, n = 10). The RPKM values obtained for mitochondrial genes were normalized to RPKM values of single copy genes from the same population. Different letters indicated significantly differences between populations (one Way ANOVA, Tukey's HSD test, $P<0.001$).

The analysis of changes in major allele frequency (CAFs) of POP_{eu} and POP_{post} compared to POP_{pre} revealed a mean CAF of about 10.4% concerning all 29500 genes for both populations (Fig. 8). CAFs of gluthathione-peroxidases, NADH-oxidases, elastases and chymotrypsins were assumed as not to differ from the mean in both populations due to overlapping error bars. However, CAFs of GSTs (POP_{eu}: 14.5% ± 0.96% SE; POP_{post}: 14.6% ± 0.95% SE), ABC-transporter (POP_{eu}: 11.4% ± 0.75% SE; POP_{post}: 12.3% ± 0.68% SE) and trypsins (POP_{eu}: 11.96% ± 0.46% SE; POP_{post}: 12.06% ± 0.45% SE) were in POP_{eu} as well as in POP_{post} significantly higher than the mean, whereas major allele frequencies of protein-phosphatases (POP_{eu}: 7.9% ± 1.05% SE; POP_{post}: 8.6% ± 1.35% SE) and mitochondrial genes (POP_{eu}: 1.48% ± 0.41% SE; POP_{post}: 1.57% ± 0.51% SE) changed significantly less than the mean of all genes. Mitogen-activated protein kinases exhibited significant lower CAFs for POP_{eu} (7.9% ± 2.1%), while they were not different from the mean in POP_{post} (9.04% ± 1.56%).

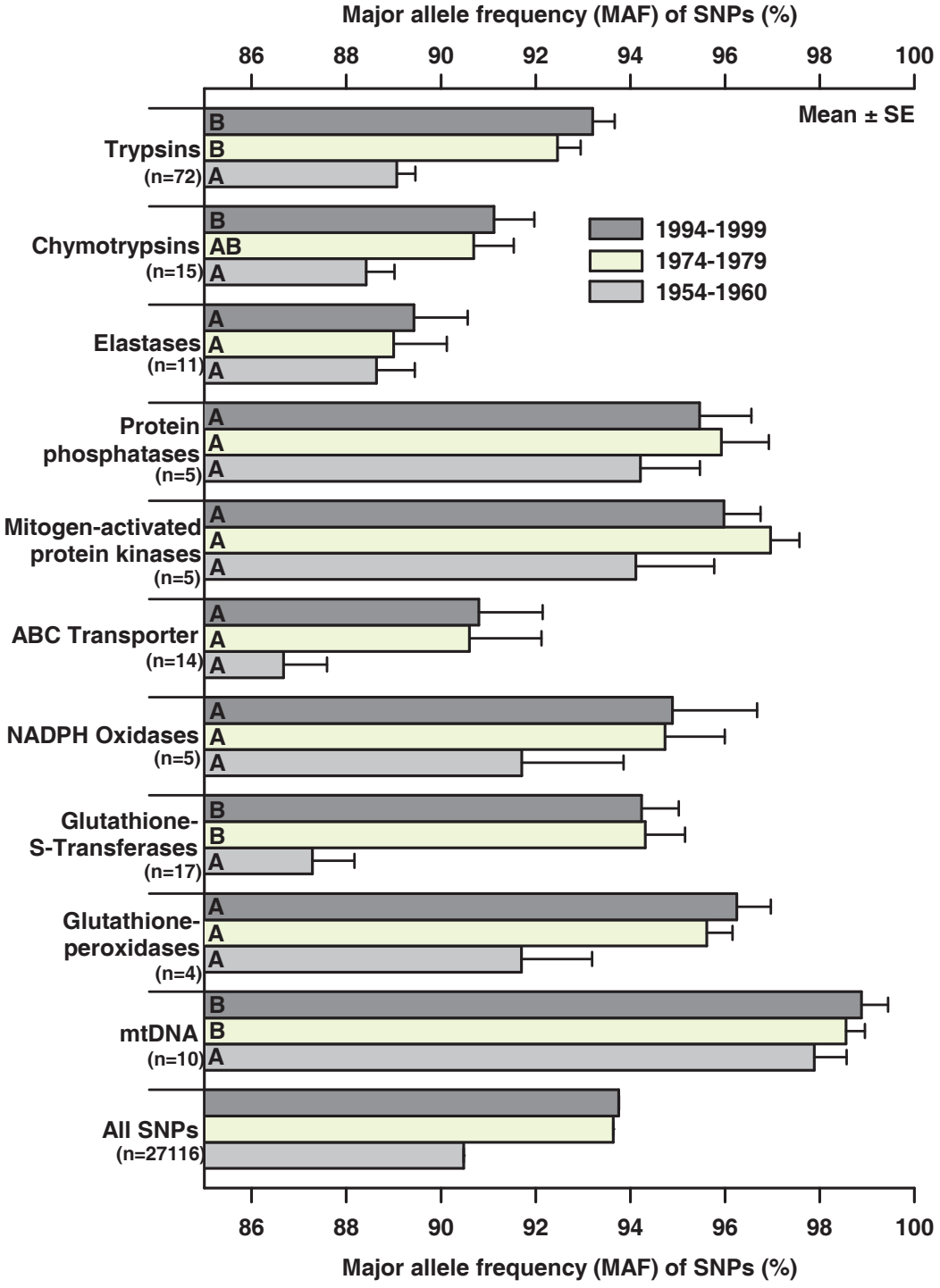

Figure 7 Major allele frequencies (MAFs) of SNPs within target gene families from *D. galeata* DNA isolated from resting eggs from three different sediment layers, representing the time periods before (1954-1960), during (1974-1979) and after (1994-1999) the eutrophication peak in Lake Constance (mean ± SE, various n). Target gene families were selected on the basis of that interference of cyanobacterial secondary metabolites with the gene products has been reported elsewhere. High mean frequencies of major alleles indicate less variability within a gene family. Different letters within target gene families indicated significantly differences between populations (one Way ANOVA, Tukey's HSD test, $P<0.001$).

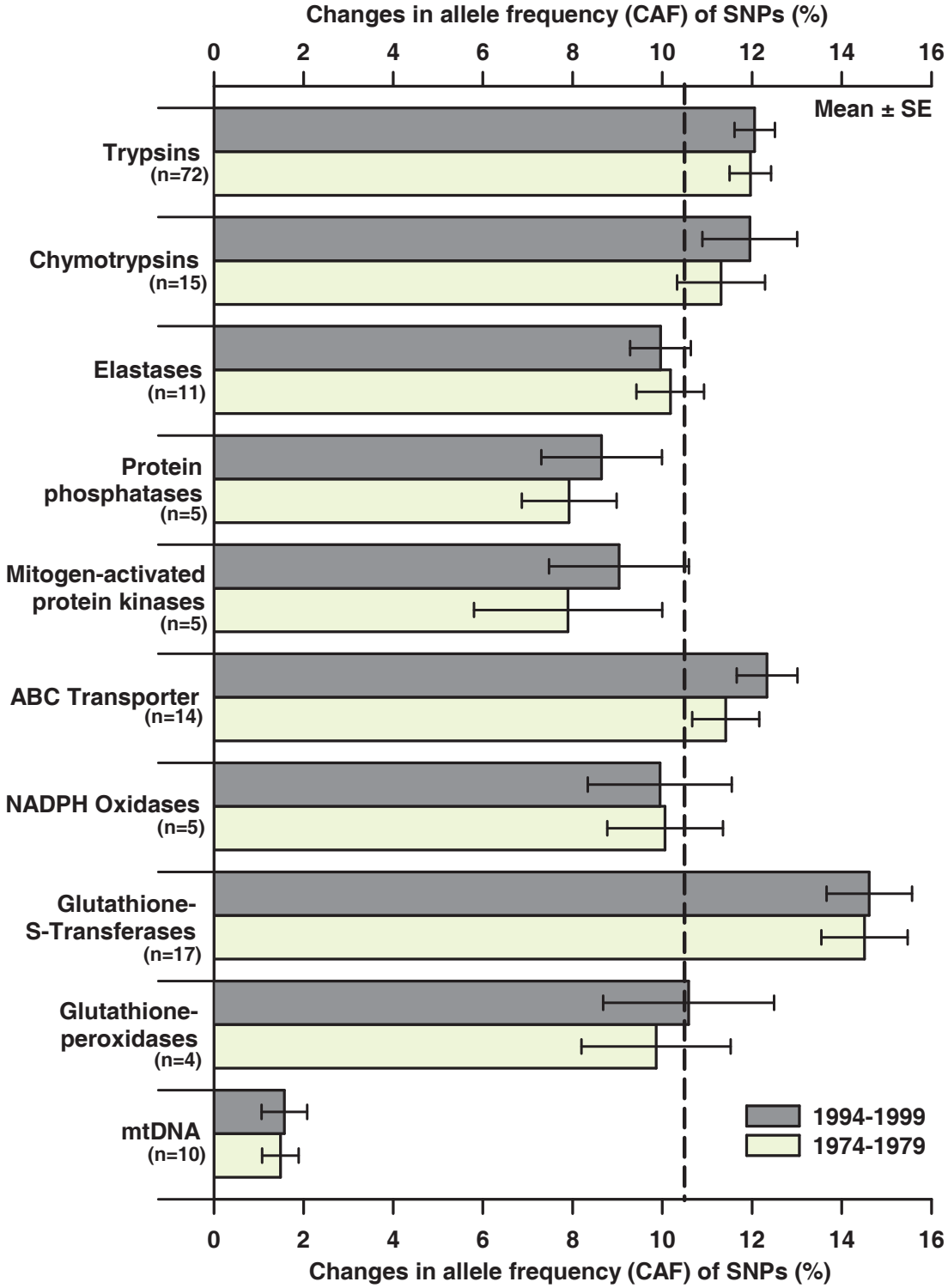

Figure 8 Changes of the major allele frequencies (CAFs) of SNPs within target gene families from *D. galeata* DNA isolated from resting eggs from two different sediment layers, representing the time periods during (1974-1979) and after (1994-1999) the eutrophication peak in Lake Constance compared to resting eggs from a sediment layer, representing the time period before (1954-1960) the eutrophication peak (mean ± SE, various n). The dotted line represents the mean frequency changes of all SNPs. Values with error bars not-overlapping the dotted line were assumed as significantly different from the mean.

Discussion

Anthropogenic phosphorus loading in the past century has led to an enhancement of eutrophication in many lakes in Europe (Güde *et al.* 1998). In Lake Constance these changes in the trophic state resulted in shifts in the taxonomical compositions of the phytoplankton favoring the abundance of cyanobacterial genera (Kümmerlin 1998). Concomitant with the altered phytoplankton community in Lake Constance, Weider *et al.* (1997) noted significant genotypic shifts in the *D. galeata* population by collecting resting eggs from lake sediments. In the present study we verified these genotypic population shifts by comparing RPKM values and SNP frequencies of the *D. galeata* populations before, during and after the time of eutrophication in Lake Constance. With regard to the RPKM-values more than twice as many genes changed significantly from POP_{pre} to POP_{eu} than from POP_{eu} to POP_{post}, while the temporal distance of adjacent populations was the same (~ 20 years). Additionally, the higher mean MAFs of all genes in POP_{eu} than in POP_{pre} lead to the conclusion that the genotypic composition of the *D. galeata* population changed substantially as a result of changing environmental conditions in Lake Constance during the time of eutrophication.

It has been suggested that these changes in the genetic structure of the *D. galeata* population were related to micro-evolutionary changes which reflected differences of the clones in their ability to cope with cyanobacteria as food (Hairston *et al.* 1999, 2001). Cyanobacteria are capable of producing a wide range of toxic secondary metabolites including hepatotoxins, neurotoxins and protease inhibitors (Wiegand & Pflugmacher 2005). During the time of eutrophication the species *Anabaena planktonica* and *Aphanizomenon flos-aquae* became abundant in Lake Constance (Kümmerlin 1998). Members of the genus *Aphanizomenon* are known to produce neurotoxins and cyanotoxins, such as cyclindrospermopsin (Banker *et al.* 1997), while *Anabaena* produces the neurotoxins anatoxin-a (Bruno *et al.* 1994) and MICs (Sivonen & Jones, 1999). Furthermore, Hairston *et al.* (1999) used in their study a microcystin producing strain of the genus *Microcystis*, which was originally isolated from Lake Constance in 1972, which leads to the suggestion that members of the genus *Microcystis* were also abundant in Lake Constance during the time of eutrophication. Therefore it is reasonable to assume that the *D. galeata* population of Lake Constance had to struggle with microcystins and other toxins from cyanobacterial sources during the time of eutrophication. Consequently, natural selection favored those *D. galeata* genotypes that were better adapted to increasing cyanobacterial toxin concentrations leading to an increase of the mean tolerance of the *D. galeata* population to cyanobacteria, which has already been observed by Hairston *et al.* (1999, 2001). However, the respective genes

and metabolic pathways of the *D. galeata* population, which were affected by changes of the phytoplankton community and the occurrence of cyanobacteria, remained unclear in the mentioned studies. Here we found evidence that the occurrence of cyanobacterial toxins were contributing to the genotypic shift in the *D. galeata* population of Lake Constance during the course of eutrophication.

The most frequent cyanotoxins in freshwater ecosystem are the cyclic peptide toxins of MCs and nodularins (Chorus & Bartram 1999). MCs are potent hepatotoxins produced by a variety of cyanobacterial genera, such as *Anabaena*, *Oscillatoria*, *Planktothrix* and *Microcystis* (Chorus & Bartram) and are known to irreversibly inhibit the protein phosphatases PP1 and PP2A of *Daphnia* (DeMott & Dhawale 1995). Additionally, several recent reports have argued that an exposure to MCs causes the production of ROS [e.g. hydrogen peroxide (H_20_2), superoxide anion (O_2^-), hydroxyl (OH•)] which leads to oxidative stress within animal cells resulting in lipid peroxidation and DNA damage (Ding *et al.* 2001, Amado & Monserrat 2010). Oxidative degradation of lipids and fatty acids as a result of oxidative stress lead to the formation of hydroperoxides, representing the end product of lipid peroxidation (Sharma *et al.* 2004). The termination of ROS-induced lipid peroxidation and the detoxification of hydroperoxides are therefore crucial to protect cells from oxidative stress (Sharma *et al.* 2004). Alpha class GSTs are known to play an even more important role in protection against lipid peroxidation than glutathione-peroxidases (Yang *et al.* 2002). GSTs can interrupt the autocatalytic chain of lipid peroxidation by reducing hydroperoxides through the conjugation to GSH (i.e. Yang *et al.* 2001).

GSTs probably catalyze the biotransformation of microcystins in *Daphnia* by conjugation to GSH, which has already been shown in vitro (Pflugmacher *et al.* 1998). The resultant conjugate of MC and GSH is less toxic than MC alone and has been suggested as a first step of the cellular detoxification mechanism (Amado & Monserrat 2010).

Several studies have indicated that exposure to MCs leads to higher GST activity and increased transcription of GST genes in animal cells (i.e. Cazenave *et al.* 2005, Gehringer *et al.* 2004). In the present study we demonstrated that major allele frequencies of GSTs changed significantly stronger during the course of eutrophication in Lake Constance than the mean of all other genes. Our data suggest that MICs from cyanobacterial sources led to elevated levels of oxidative stress in *Daphnia* cells during the time of eutrophication and increased inhibition of the protein phosphatases PP1 and PP2a. Thus it is reasonable to assume that the selection pressure on *Daphnia*'s detoxifying enzymes, such as GSTs, increased concomitant with the occurrence of cyanobacteria that contained toxins. As a

consequence, natural selection should have favored those *D. galeata* genotypes that exhibited more efficient GSTs. In addition, we observed an overall increase of the major allele frequencies of all genes, which indicates a decrease of the genetic diversity of the *D galeata* population in Lake Constance. Our data suggests that the loss of genetic variability as well as the even significantly stronger decrease in GST diversity of POP_{post} was a consequence of the occurrence of MCs from cyanobacterial sources.

The assumption that an increase in the abundance of cyanobacteria constitutes the major selection factor for the *D. galeata* population during the time of eutrophication, is further corroborated by the finding that the mtDNA in the *D. galeata* population increased concomitantly with the occurrence of cyanobacteria in Lake Constance. Several studies have demonstrated that MCs from cyanobacteria interact with mitochondrial components, leading to dysfunction of the organelle and cell apoptosis (Campos & Vasconcelos 2010, Amado & Monserrat 2010). In *Daphnia* the exposure of cells to MC-LR finally leads to broken and blurry mitochondria in the alimentary canal and epidermis (Chen *et al.* 2005). In more detail, MCs were suggested to disrupt the mitochondrial electron transport chain, which then favors the generation of ROS (Ding *et al.* 2002). High ROS levels coincide with dysfunctional mitochondria due to depolarization of the membrane potential and increased mitochondrial membrane permeability (Ding *et al.* 2000a; Ding & Ong 2003). Additionally, Mikhailov *et al.* (2003) have shown that MC-LR can bind to the beta subunit of ATP-synthase, which finally contributes to mitochondrial membrane depolarization, disruption of mitochondrial electron transport chain and the generation of ROS. The depolarization of the mitochondrial membrane potential finally leads to the release of cytochrome c and subsequently to apoptosis of the cell (i.e. Zhang *et al.* 2007). The observed significant higher amount of mtDNA in POP_{eu} than in $POP_{prä}$ is possibly a physiological response to cyanobacterial toxins and may result from two different mechanisms: Firstly, the number of mitochondria per cell increased in the *D. galeata* population during the time of eutrophication. Lee *et al.* (2000) have shown that oxidative stress induced by H_2O_2 results in an increase of functional mitochondria and mtDNA in human fibroblasts. Our data suggest that the observed increase might constitute a compensatory response to the enhanced disruption of mitochondria caused by the increased exposure to cyanobacterial toxins during the time of eutrophication. Secondly, the number of mtDNA copies per mitochondrium increased, while the number of mitochondria per cell remained constant. Several studies have indicated that exposure to MCs induces DNA damage in vitro and in vivo by the oxidation of purines (Juhel *et al.* 2007, Zegura *et al.* 2004). Liu *et al.* (2003) and Wang *et al.* (2011) have demonstrated that copy number changes of human mtDNA are correlated with oxidative stress. Thus, we suggest

that an increased mtDNA copy number might compensate for deletions in the mtDNA caused by increased oxidative stress. Further comparisons have to be performed to elucidate whether the increased ratio of mtDNA to single copy genes indicates an increase of the number of mitochondria per cell or an increase of mtDNA copies per mitochondrium. However, in POP_{post} the number of mtDNA per cell was the highest, even higher than in POP_{eu}. *D. galeata* resting eggs for POP_{eu} were isolated from the sediment layers representing the time period from 1974-1979. We suggest that the selection pressure on *Daphnia*'s mitochondria caused by cyanobacterial microcystins maintained till the end of the 1980s, since the last cyanobacterial peak was recorded in 1988 (Fig. 1). We assumed that the maintenance of microcystins in the 1980s led to a successive linear increase of mtDNA in the *D. galeata*. Thus, the finding of highest mtDNA copy number in POP_{post} it not very surprising, since the recovery to the status of pre-eutrophication in terms of neutral evolution is much slower than adaptive evolution assuming that the increased amount of mitochondria has no disadvantage for the *D. galeata* population.

Besides significant increases of major allele frequencies (MAF) in GSTs and copy numbers of mtDNA, MAFs of *Daphnia*'s serine proteases trypsin and chymotrypsin also increased significantly from $POP_{prä}$ to POP_{post}. Trypsins and chymotrypsins are responsible for 80% of the proteolytic activity in the gut of *Daphnia* (Von Elert *et al.* 2004). Several studies indicate that cyanobacterial protease inhibitors directly inhibit these digestive proteases of *Daphnia* (Agrawal *et al.* 2005; Schwarzenberger *et al.* 2010) and lead to reduced somatic growth of *Daphnia* (Von Elert *et al.* 2012). More than twenty depsipeptides were found in different genera of marine and freshwater cyanobacteria (Gademann & Portmann 2008), specifically inhibiting the serine proteases chymotrypsin and trypsin. A recent study by Kuster *et al.* (2012) has shown that the edible size fraction of natural seston can inhibit *Daphnia*'s trypsins and chymotrypsins, and this inhibitory potential can be in the same order of magnitude as pure cyanobacterial cultures (Czarnecki *et al.* 2006). Hence, it is reasonable to assume that an interference of cyanobacterial protease inhibitors with *Daphnia*'s digestive proteases occurs in nature and is ecologically relevant. In the *D. galeata* population of Lake Constance chymotrypsins and trypsins exhibited significantly higher relative changes in their major allele frequency than the mean of all SNPs. The loss of genetic variability in protease genes coincided with increasing abundances of cyanobacteria in the course of Lake Constance's eutrophication. We suggest that this loss could result from natural selection due to increasing cyanobacterial protease inhibition and the extinction of less tolerant *D. galeata* genotypes.

In conclusion, the here presented population genome approach renders evidence that at least two independent cellular mechanisms caused by cyanobacterial toxins are responsible for the changes of the genetic structure of the *D. galeata* population in Lake Constance: (1) MICs from cyanobacterial sources have caused increased levels of ROS, the major causes for oxidative stress in cells and their mitochondria. (2) Cyanobacterial inhibitors interfered with *Daphnia*'s proteases leading to an inhibition of their digestive enzymes. Life-history experiments with ex-ephippia hatchlings demonstrated that eutrophication in Lake Constance resulted in more cyanobacteria-tolerant *D. galeata* populations (Hairston *et al.* 1999). Our data reveals that at least two types of cyanobacterial inhibitors, protease inhibitors and MCs were major selective forces driving the microevolutionary changes towards a more cyanobacteria-tolerant *D. galeata* population during the time of eutrophication.

References

Agrawal MK, Bagchi D & Bagchi SN (2001) Acute inhibition of protease and suppression of growth in zooplankter, *Moina macrocopa*, by *Microcystis* blooms collected in Central India. *Hydrobiologia*, 464, 37-44.

Agrawal MK, Zitt A, Bagchi D *et al.* (2005) Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. *Environmental Toxicology*, 20, 314-322.

Amado L & Monserrat J (2010) Oxidative stress generation by microcystins in aquatic animals: Why and how. *Environment International*, 36, 226-235.

Ame MV, Baroni MV, Galanti LN, Bocco JL & Wunderlin DA (2009) Effects of microcystin-LR on the expression of P-glycoprotein in Jenynsia multidentata. *Chemosphere*, 74, 1179-1186.

Banker R, Carmeli S, Hadas O *et al.* (1997) Identification of cylindrospermopsin in Aphanizomenon ovalisporum (Cyanophyceae) isolated from Lake Kinneret, Israel. *Journal of Phycology*, 33, 613-616.

Bruno M, Barbini DA, Pierdominici E, Serse AP & Ioppolo A (1994) Anatoxin-a and a previously unknown toxin in *Anabaena planctonica* from blooms found in Lake Mulargia (Italy). *Toxicon*, 32, 369-373.

Campos A & Vasconcelos V (2010) Molecular mechanisms of microcystin toxicity in animal cells. *International Journal of Molecular Sciences*, 11, 268-287.

Cazenave J, Wunderlin DA, Bistoni MDL *et al.* (2005) Uptake, tissue distribution and accumulation of microcystin-RR in Corydoras paleatus, Jenynsia multidentata and Odontesthes bonariensis - A field and laboratory study. *Aquatic Toxicology*, 75, 178-190.

Chen W, Song LR, Ou DY & Gan NQ (2005) Chronic toxicity and responses of several important enzymes in *Daphnia magna* on exposure to sublethal microcystin-LR. *Environmental Toxicology*, 20, 323-330.

Chorus,I. & Bartram,J. (1999) Toxic Cyanobacteria in Water: a Guide to Public Health Significance. *Monitoring and Management*: 416pp

Colbourne JK, Singan VR & Gilbert DG (2005) wFleaBase: The Daphnia genome database. *BMC Bioinformatics*, 6.

Colbourne JK, Pfrender ME, Gilbert D *et al.* (2011) The ecoresponsive genome of *Daphnia pulex*. *Science*, 331, 555-561.

Coleman ML & Chisholm SW (2010) Ecosystem-specific selection pressures revealed through comparative population genomics. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 18634-18639.

Czarnecki O, Henning M, Lippert I & Welker M (2006) Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). *Environmental Microbiology*, 8, 77-87.

Demott WR & Dhawale S (1995) Inhibition of in-vitro protein phosphatase-activity in 3 zooplankton species by microcystin-lr, a toxin from cyanobacteria. *Archiv für Hydrobiologie*, 134, 417-424.

Ding WX & Ong CN (2003) Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *Fems Microbiology Letters*, 220, 1-7.

Ding WX, Shen HM & Ong CN (2000) Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. *Hepatology*, 32, 547-555.

Ding WX, Shen HM & Ong CN (2001) Critical role of reactive oxygen species formation in microcystin-induced cytoskeleton disruption in primary cultured hepatocytes. *Journal of Toxicology and Environmental Health-Part A*, 64, 507-519.

Ding WX, Shen HM & Ong CN (2002) Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes. *Biochemical and Biophysical Research Communications*, 291, 321-331.

Gademann K & Portmann C (2008) Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. *Current Organic Chemistry*, 12, 326-341.

Gehringer MM, Shephard EG, Downing TG, Wiegand C & Neilan BA (2004) An investigation into the detoxification of microcystin-LR by the glutathione pathway in Balb/c mice. *International Journal of Biochemistry & Cell Biology*, 36, 931-941.

Güde H, Rossknecht H & Wagner G (1998) Anthropogenic impacts on the trophic state of Lake COnstance during the 20th century. *Archiv für Hydrobiologie*, 53, 85-108.

Hairston NG, Holtmeier CL, Lampert W *et al.* (2001) Natural selection for grazer resistance to toxic cyanobacteria: Evolution of phenotypic plasticity? *Evolution*, 55, 2203-2214.

Hairston NG, Lampert W, Caceres CE *et al.* (1999) Lake ecosystems - Rapid evolution revealed by dormant eggs. *Nature*, 401, 446.

Juhel G, O'Halloran J, Culloty SC *et al.* (2007) In vivo exposure to microcystins induces DNA damage in the haemocytes of the zebra mussel, Dreissena polymorpha, as measured with the comet assay. *Environmental and Molecular Mutagenesis*, 48, 22-29.

Komatsu M, Furukawa T, Ikeda R *et al.* (2007) Involvement of mitogen-activated protein kinase signaling pathways in microcystin-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. *Toxicological Sciences*, 97, 407-416.

Kümmerlin RE (1998) Taxonomical response of the phytoplankton community of Upper Lake Constance (Bondesee-Obersee) to eutrophication and re-oligotrophication. *Archiv für Hydrobiologie*, 53, 109-117.

Kümmerlin RE & Bürgi H (1989) Die langjährige Entwicklung des Phytoplanktons im Bodensee (1961–1986). *Berichte der Internationalen Gewässerschutzkommission für den Bodensee*, 39.

Kuster C, Schwarzenberger A & Von Elert E (2012) Seasonal dynamics of sestonic protease inhibition: impact on *Daphnia* populations. *Hydrobiologia*, 1-14.

Lee HC, Yin PH, Lu CY, Chi CW & Wei YH (2000) Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochemical Journal*, 348, 425-432.

Liu CS, Tsai CS, Kuo CL *et al.* (2003) Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. *Free Radical Research*, 37, 1307-1317.

Lürling M & van der Grinten E (2003) Life-history characteristics of *Daphnia* exposed to dissolved microcystin-LR and to the cyanobacterium *Microcystis aeruginosa* with and without microcystins. *Environmental Toxicology and Chemistry*, 22, 1281-1287.

Lürling M, Faassen EJ & Van Eenennaam JS (2011) Effects of the cyanobacterial neurotoxin beta-N-methylamino-L-alanine (BMAA) on the survival, mobility and reproduction of *Daphnia magna*. *Journal of Plankton Research*, 33, 333-342.

Mikhailov A, Harmala-Brasken AS, Hellman J, Meriluoto J & Eriksson JE (2003) Identification of ATP-synthase as a novel intracellular target for microcystin-LR. *Chemico-Biological Interactions*, 142, 223-237.

Mortazavi A, Williams BA, McCue K, Schaeffer L & Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods*, 5, 621-628.

Nong Q, Komatsu M, Izumo K *et al.* (2007) Involvement of reactive oxygen species in microcystin-LR-induced cytogenotoxicity. *Free Radical Research*, 41, 1326-1337.

Pflugmacher S, Wiegand C, Oberemm A *et al.* (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochimica et Biophysica Acta-General Subjects*, 1425, 527-533.

Pollard HG, Colbourne JK & Keller W (2003) Reconstruction of centuries-old Daphnia communities in a lake recovering from acidification and metal contamination. *Ambio*, 32, 214-218.

Porter KG & Mcdonough R (1984) The energetic cost of response to blue-green-algal filaments by cladocerans. *Limnology and Oceanography*, 29, 365-369.

Schmegner C, Hoegel J, Vogel W & Assum G (2005) Genetic variability in a genomic region with long-range linkage disequilibrium reveals traces of a bottleneck in the history of the European population. *Human Genetics*, 118, 276-286.

Schwarzenberger A, Zitt A, Kroth P, Mueller S & Von Elert E (2010) Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. *BMC physiology*, 10, 6.

Sharma R, Yang Y, Sharma A, Awasthi S & Awasthi YC (2004) Antioxidant role of glutathione S-transferases: Protection against oxidant toxicity and regulation of stress-mediated apoptosis. *Antioxidants & Redox Signaling*, 6, 289-300.

Shendure J & Ji H (2008) Next-generation DNA sequencing. *Nature Biotechnology*, 26, 1135-1145.

Sivonen K & Jones G (1999) Cyanobacterial toxins. Toxic cyanobacteria in water: A guide to their public health consequences. *Monitoring and Management: 41-111*.

Von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography*, 47, 1764-1773.

Von Elert E (2004) Food quality constraints in *Daphnia*: interspecific differences in the response to the absence of a long chain polyunsaturated fatty acid in the food source. *Hydrobiologia*, 526, 187-196.

Von Elert E, Agrawal MK, Gebauer C *et al.* (2004) Protease activity in gut *Daphnia magna*: evidence for trypsin and chymotrypsin enzymes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 137, 287-296.

Von Elert E, Martin-Creuzburg D & Le Coz JR (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 1209-1214.

Von Elert E, Zitt A & Schwarzenberger A (2012) Inducible tolerance to dietary protease inhibitors in *Daphnia magna*. *Journal of Experimental Biology*, 215, 2051-2059.

Wang YC, Lee WC, Liao SC *et al.* (2011) Mitochondrial DNA copy number correlates with oxidative stress and predicts mortality in nondiabetic hemodialysis patients. *Journal of Nephrology*, 24, 351-358.

Weider LJ, Lampert W, Wessels M, Colbourne JK & Limburg P (1997) Long-term genetic shifts in a microcrustacean egg bank associated with anthropogenic changes in the Lake Constance ecosystem. *Proceedings of the Royal Society of London - Series B: Biological Sciences*, 264, 1613-1618.

Wessels M, Lenhard A, Giovanoli F & Bollhofer A (1995) High resolution time series of lead and zinc in sediments of Lake Constance. *Aquatic Sciences*, 57, 291-304.

Wiegand C & Pflugmacher S (2005) Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicology and Applied Pharmacology*, 203, 201-218.

Yang Y, Cheng JZ, Singhal SS *et al.* (2001) Role of glutathione S-transferases in protection against lipid peroxidation - Overexpression of hgsta2-2 in k562 cells protects against hydrogen peroxide-induced apoptosis and inhibits JNK and caspase 3 activation. *Journal of Biological Chemistry*, 276, 19220-19230.

Yang YS, Sharma R, Zimniak P & Awasthi YC (2002) Role of alpha class glutathione S-transferases as antioxidant enzymes in rodent tissues. *Toxicology and Applied Pharmacology*, 182, 105-115.

Zegura B, Lah TT & Filipic M (2004) The role of reactive oxygen species in microcystin-LR-induced DNA damage. *Toxicology*, 200, 59-68.

Zhang H, Zhang J, Chen Y & Zhu Y (2007) Influence of intracellular Ca2+ mitochondria membrane potential, reactive oxygen species, and intracellular ATP on the mechanism of Microcystin-LR induced apoptosis in carassius auratus lymphocytes in vitro. *Environmental Toxicology*, 22, 559-564.

Concluding remarks and perspectives

Cyanobacteria are prokaryotic photoautotrophs and belong to the most successful and oldest life forms on earth (Gademann & Portmann 2008). They include unicellular and colonial species and inhabit almost all aquatic environments as planktonic cells or bentic biofilms. Besides the well known association with hazards to human health and livestock (Carmichael 1994) cyanobacterial blooms cause substantial reductions of the fitness of freshwater zooplankton. Cyanobacteria have been claimed to be a major factor for a constrained mass and energy transfer from primary producers to primary consumers (i.e. daphnids, copepods) and even higher trophic levels, such as insect larvae or planktivorous fish. *Daphnia*, which is an unselective filter feeder and important herbivorous grazer in many lakes and ponds, has to struggle with the poor food quality of cyanobacteria due to multiple factors. One of these factors is the morphology of cyanobacterial cells. For example, large cyanobacterial filaments can resist ingestion by interfering with the filtering apparatus of *Daphnia* (DeMott *et al.* 2001). Additionally cyanobacteria lack sterols and contain only small amounts of polyunsaturated fatty acids. Such lipids are essentials for *Daphnia*'s nutrition, since daphnids cannot synthesize these compounds in sufficient amounts by itself (i.e. Von Elert *et al.* 2003, Von Elert 2002). Thirdly, cyanobacteria produce a variety of secondary metabolites, such as microcystins and protease inhibitors (Sivonen & Jones 1999, Gademann & Portmann 2008). These compounds lead to significantly reduced growth in *Daphnia* (i.e. Von Elert *et al.* 2012). In the growth experiments of **chapter 1** and **chapter 3** biochemical lipid deficiencies and negative effects of microcystins can be ruled out as causal factors for the observed growth reduction of *Daphnia* on cyanobacterial diets, since microcystin-free cyanobacteria were fed in mixtures with a green algal ensuring sufficient supply of essential lipids. Additionally, all cyanobacterial strains as well as the green alga were cultured as single cells and were such well ingestible. The cyanobacterial strains used here produced exclusively either the chemically known chymotrypsin inhibitors Cyanopeptolin 954 and Nostopeptin BN920 (Von Elert *et al.* 2005) or specific cyanopeptolins (A-D), which are known to inhibit trypsins (Weckesser *et al.* 1996). Von Elert *et al.* (2012) have recently shown that the concentration of chymotrypsin inhibitors in cyanobacterial cells were four to five orders of magnitude higher than the concentration, which is needed to inhibit 50 % of *Daphnia*'s chymotrypsin activity. Thus, it is reasonable to assume that the observed negative effects on population and somatic growth were caused by an interference of the cyanobacterial protease inhibitors with trypsins and chymotrypsins in the gut of *Daphnia*. Both digestive enzymes are responsible for more than 80 % of the proteolytic activity in the

gut of *D. magna* (Von Elert *et al.* 2004). Several studies have demonstrated a direct inhibition of *Daphnia*'s trypsins and chymotrypsins by cyanobacterial inhibitors (Von Elert *et al.* 2005, Schwarzenberger *et al.* 2010, Agrawal *et al.* 2005). The data presented in **chapter 1** corroborate the finding of Czarnecki *et al.* (2006) that an inhibition of *Daphnia*'s trypsins occurs in nature and might therefore be ecologically relevant. Furthermore, the data in **chapter 1** show for the first time that the seston fraction smaller than 55 µm, which constitutes the edible size fraction for *Daphnia* (Gophen & Geller 1984, Hessen 1985), can also inhibit *Daphnia*'s chymotrypsins. Besides trypsins, chymotrypsins account for the largest proportion of proteolytic activity in the gut of *D. magna* (Von Elert *et al.* 2004). With regard to the well-known seasonal succession of the phytoplankton community (Sommer *et al.* 1986) **chapter 1** describes for the first time seasonal changes of the potential of natural lake seston to inhibit both types of proteases in *D. magna*. The potential of the edible seston to inhibit chymotrypsins was up to 100-300 times higher in fall than in spring for each of the successive years that were analyzed, which strongly suggests that the proportion of cyanobacteria with protease inhibitors in the phytoplankton community of this lake increased significantly within each season. This coincides with the finding that cyanobacteria mass developments occur more frequently at the end of the season, when the temperature of the epilimnion reaches its maximum (Sommer *et al.* 1986, Jöhnk *et al.* 2008).

In parallel with seasonal changes in the potential of the natural phytoplankton to inhibit proteases, the genetic structure of the *D. magna* population changed significantly. Microsatellite analyses revealed that *D. magna* clones isolated in spring were genetically distinct from those isolated in fall. Additionally, the genetic diversity of the *D. magna* population decreased within season. This loss of genetic variability coincided with an increase of the inhibitory potential on chymotrypsins in the seston and might thus result from natural selection due to increasing protease inhibition on *D. magna* genotypes. I assumed that this would consequently lead to an increased mean tolerance of *Daphnia* to cyanobacterial protease inhibitors by the end of the season. Blom *et al.* (2006) showed that *Daphnia* sp. coexisting with *Planktothrix rubescens*, a cyanobacterium that contains the trypsin inhibitor oscillapeptin J, were significantly more tolerant to this trypsin inhibitor than *Daphnia* sp. from lakes free of this cyanobacterium. However, neither in single-clone growth experiments nor in competition experiments I found evidence that *D. magna* genotypes from the fall were better adapted to cyanobacteria containing protease inhibitors in terms of higher growth rates than genotypes from the spring (**chapter 1**). It remains unclear whether seasonally peaking sestonic protease inhibition over several years had already led to a locally adapted *D. magna* population. This would result in a comparably high level of

tolerance to cyanobacterial protease inhibitors throughout the season as has been observed in **chapter 1**. Comparisons of the tolerance of *Daphnia* populations that exist in the absence or the presence of dietary protease inhibitors could provide evidence for such a local adaptation of a *Daphnia* population to cyanobacteria, as has already been shown earlier (Sarnelle *et al.* 2005).

Depending on initial conditions *Daphnia* may not only control the development of bloom-forming cyanobacteria but as well suppress already established cyanobacterial blooms (Sarnelle 2007). The data presented in **chapter 3** suggest that *D. magna* may control the development of cyanobacterial blooms more efficiently than *D. pulex* due to significant differences in their tolerance to cyanobacteria. In this case I deliberately chose cyanobacteria producing protease inhibitors but no microcystins, as protease inhibitors are among the most widespread cyanobacterial secondary metabolites and as I specifically wanted to compare *Daphnia*'s tolerance to this class of inhibitors. Protease assays revealed that the sensitivities of chymotrypsins and trypsins to cyanobacterial protease inhibitors did not differ between *D. magna* and *D. pulex*. However, *D. magna* exhibited a significantly higher specific chymotrypsin activity than *D. pulex*, which explains the observed higher tolerance of *D. magna* to cyanobacterial protease inhibitors. The differences in growth and chymotrypsin activity might result from general differences in their coexistence with cyanobacteria: Compared to other algal groups cyanobacteria have high temperature optima (Dokulil & Teubner 2000) and dominate the phytoplankton community in eutrophic lakes mainly in late summer and early fall (Jöhnk *et al.* 2008). Their important herbivorous grazers, *D. magna* and *D. pulex*, are both most commonly abundant in fishless or turbid ponds (DeMott & Pape 2005). *D. magna* is able to tolerate elevated temperatures up to 25 °C (Wojtal-Frankiewicz 2012) and is able to increase in abundance upon nutrient enrichment and associated eutrophication (Verreydt *et al.* 2012). In contrast to *D. magna* the geographic distribution of *D. pulex* ranges rather from the temperate to the arctic zone (Colbourne *et al.* 1998). In addition, *D. pulex* usually reaches its highest densities in spring an early summer, while *D. magna* shows no clear seasonal density pattern (Bengtsson 1993). Following this line of reasoning, coexistence of *D. magna* and cyanobacteria would thus occur more frequently than a coexistence of cyanobacteria with *D. pulex*. Due to microevolutionary adaptation a putatively more frequent coexistence would consequently result in an increased tolerance of *D. magna* to cyanobacterial protease inhibitors. Although there is evidence that the observed higher mean tolerance of *D. magna* to cyanobacteria with protease inhibitors results indeed from a more frequent coexistence with cyanobacteria, a correlation has still to be established in extended field studies. However, as a result of continuing global warming the appearance

of toxic cyanobacterial blooms and their negative effects on whole ecosystems will become even stronger in the near future (Paerl & Huisman 2008). The data in **chapter 3** suggests that *D. magna* may control the increase of cyanobacterial genera more efficiently than *D. pulex* due to significant differences in their tolerance to cyanobacteria with protease inhibitors. Additionally, I suggest that toxic cyanobacterial blooms and coinciding harmful effects to the ecosystem will occur more frequently in lakes with *D. pulex* than in lakes with *D. magna*.

The detection and identification of possible target genes in natural *Daphnia* populations that might have undergone selection by the presence of cyanobacterial protease is covered by **chapter 2**. Here I analyzed the genetic diversity of three digestive trypsins of *D. magna* clones from different habitats via high resolution melting analysis (HRMA), which I introduced as a cost-effective and specific genotyping tool for population studies on *Daphnia*. The chosen trypsins are known to be active in the gut of *Daphnia* and therefore provide a possible target for cyanobacterial protease inhibitors (Schwarzenberger *et al.* 2010). One *Daphnia* population originated from a pond containing toxic cyanobacteria that possibly produce trypsin inhibitors and the other from a pond without such cyanobacteria. The final results in **chapter 2** revealed that the clonal diversity of two trypsin genes was much higher in the *D. magna* population that already had experience with cyanobacteria than in the *D. magna* population without such experience. In addition, the HRMA-results revealed that both *Daphnia* populations exhibited phenotypic differences in the analyzed trypsins. Further growth experiments on cyanobacterial diets of several clones from both populations have to be performed in order to investigate whether the observed phenotypic difference of active trypsins are indeed a microevolutionary response to the presence of trypsin inhibitors. However, in **chapter 2** I successfully demonstrated that even single nucleotide polymorphisms (SNPs) of class III (C/G) or class IV (A/T) can be easily detected with HRMA allowing a reliable genotyping of the analyzed *Daphnia* individuals. Thus, HRMA provides an excellent choice for screening high numbers of individuals in population studies.

Besides positive correlation of increasing water temperatures and the abundance of cyanobacteria as a result of global warming (Paerl & Huisman 2008), which has already discussed in **chapter 3**, the eutrophication of lakes caused by anthropogenic phosphorus loading also favors the occurrence of cyanobacterial mass developments (Jöhnk *et al.* 2008). In Lake Constance an increase of the total phosphorus (P_{tot}) resulted in shifts of the taxonomical composition of the phytoplankton favoring the abundance of cyanobacterial genera (Kümmerlin 1998). Concomitant with the altered phytoplankton community in Lake Constance the genetic structure of the co-occurring *D. galeata* population changed

significantly (Weider *et al.* 1997). It has been suggested that these changes were related to the ability of the *D. galeata* population to cope with cyanobacteria as food (Hairston *et al.* 1999, 2001). However, the physiological mechanisms underlying the increased tolerance of the *D. galeata* population to cyanobacteria after the peak of eutrophication in Lake Constance remained unclear in the mentioned studies. With the aim to identify these physiological mechanisms and genes, which were affected in *Daphnia* by changes of the phytoplankton community and the occurrence of cyanobacteria, I performed in **chapter 4** a population genome approach in terms of sequencing genomic DNA of the D*. galeata* population before, during and after the time of eutrophication. As outlined in **chapter 4** I achieved evidence that selection, caused by cyanobacterial toxins, on at least two traits in *Daphnia* was responsible for the alteration of the genetic structure of the *D. galeata* population in Lake Constance: The increased ingestion of the hepatotoxin microcystin from cyanobacterial sources, have led to increased generation of reactive oxygen species (ROS) in *Daphnia* cells. ROS are known to be the major causes for oxidative stress in animal cells leading to peroxidation of lipids and fatty acids, DNA damaging as well as to dysfunctional mitochondria (Amado & Monserrat 2010). Alpha class glutathione-S-transferases (GST) are known to play an important role in protection against microcystins and oxidative stress (Yang *et al.* 2002). GSTs catalyze the biotransformation of microcystins in *Daphnia* by conjugation to glutathione (Pflugmacher *et al.* 1998). The resultant conjugate is less toxic and it has been suggested that this constitutes the first step of cellular detoxification (Amado & Moserrat 2010). In addition, GSTs can interrupt the autocatalytic chain of lipid peroxidation, which is a result of oxidative stress (Yang *et al.* 2001). The next-generation sequencing data in **chapter 4** revealed that the major allele frequencies of glutathione-S-transferases in the *D. galeata* population changed significantly stronger during the time of eutrophication in Lake Constance than the mean of all other genes. This observation supports the assumption that, as a response to oxidative stress, caused by an increased proportion of cyanobacteria with microcystins in the phytoplankton community, natural selection favored those *D. galeata* genotypes in Lake Constance that exhibited more efficient GSTs. Additionally, the amount of mitochondrial DNA increased in the *D. galeata* population during the time of eutrophication, which possibly indicates a compensation of the enhanced disruption of mitochondria due to increased exposure to cyanobacterial microcystins. Besides the impact of cyanobacterial microcystins on the genome of the *D. galeata* population, also cyanobacterial protease inhibitors probably had significant impacts on *Daphnia*'s genomic structure during the time of eutrophication, because genes of trypsins and chymotrypsins in *Daphnia* exhibited significantly higher relative changes than the mean of all genes. Taken together the data in **chapter 4** make it reasonable to assume that protease inhibitors and oxidative stress

induced by microcystins were the selective forces driving the genotypic shift towards a more cyanobacteria-tolerant *D. galeata* population during the time of eutrophication.

Daphnia is a keystone species in the functioning of lake ecosystems and is known for its rapid evolutionary response to dynamic environmental stressors (Miner *et al.* 2012). Since *Daphnia* has recently become another model organism due to the availability of full-genome data (Colbourne *et al.* 2011) the linkage between genomics and ecology of *Daphnia* has become a focus of attention. The identification of specific genes that were related to i.e. climate change, predator avoidance, parasite- and toxin resistance is a prerequisite to understand the physiological mechanisms underlying *Daphnia*'s adaptation. The present study considers various intra- and interspecific aspects of *Daphnia*'s tolerance to cyanobacteria. This dissertation covers not only more classical ecological approaches, such as growth and competition experiments, but as well focuses on the analysis of specific target genes of *Daphnia* that are related to secondary metabolites of cyanobacteria. The introduction of high-resolution melting analysis as a cost-effective tool for genotyping a large number of *Daphnia* individuals as well as population-wide genome sequencings connected with pronounced bioinformatical statistics hopefully helps to elucidate some aspects in the linkage between genome/gene evolution and the ecology of *Daphnia*.

General references

Agrawal MK, Zitt A, Bagchi D *et al.* (2005) Characterization of proteases in guts of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC 7806. *Environmental Toxicology*, 20, 314-322.

Amado L & Monserrat J (2010) Oxidative stress generation by microcystins in aquatic animals: Why and how. *Environment International*, 36, 226-235.

Bengtsson J (1993) Interspecific competition and determinants of extinction in experimental populations of 3 rockpool *Daphnia* species. *Oikos*, 67, 451-464.

Blom JF, Baumann HI, Codd GA & Juttner F (2006) Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp(3),(E)-Dhb(7)]microcystin-RR. *Archiv für Hydrobiologie*, 167, 547-559.

Carmichael WW (1994) Toxins of cyanobacteria. *Scientific American*, 270, 78-86.

Colbourne JK, Crease TJ, Weider LJ *et al.* (1998) Phylogenetics and evolution of a circumarctic species complex (Cladocera: *Daphnia pulex*). *Biological Journal of the Linnean Society*, 65, 347-365.

Colbourne JK, Pfrender ME, Gilbert D *et al.* (2011) The ecoresponsive genome of *Daphnia pulex*. *Science*, 331, 555-561.

Czarnecki O, Henning M, Lippert I & Welker M (2006) Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). *Environmental Microbiology*, 8, 77-87.

DeMott WR, Gulati RD & Van Donk E (2001) *Daphnia* food limitation in three hypereutrophic Dutch lakes: Evidence for exclusion of large-bodied species by interfering filaments of cyanobacteria. *Limnology and Oceanography*, 46, 2054-2060.

Demott WR & Pape BJ (2005) Stoichiometry in an ecological context: testing for links between *Daphnia* P-content, growth rate and habitat preference. *Oecologia*, 142, 20-27.

Dokulil MT & Teubner K (2000) Cyanobacterial dominance in lakes. *Hydrobiologia*, 438, 1-12.

Gademann K & Portmann C (2008) Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. *Current Organic Chemistry*, 12, 326-341.

Geller W & Müller H (1981) The filtration apparatus of Cladocera - filter mesh-sizes and their implications on food selectivity. *Oecologia*, 49, 316-321.

Ghadouani A, Pinel-Alloul B & Prepas EE (2003) Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. *Freshwater Biology*, 48, 363-381.

Gophen M & Geller W (1984) Filter mesh size and food particle uptake by *Daphnia*. *Oecologia*, 64, 408-412.

Güde H, Rossknecht H & Wagner G (1998) Anthropogenic impacts on the trophic state of Lake COnstance during the 20th century. *Archiv für Hydrobiologie*, 53, 85-108.

Gustafsson S & Hansson LA (2004) Development of tolerance against toxic cyanobacteria in *Daphnia*. *Aquatic Ecology*, 38, 37-44.

Hairston NG, Lampert W, Caceres CE *et al.* (1999) Lake ecosystems - Rapid evolution revealed by dormant eggs. *Nature*, 401, 446.

Hairston NG, Holtmeier CL, Lampert W *et al.* (2001) Natural selection for grazer resistance to toxic cyanobacteria: Evolution of phenotypic plasticity? *Evolution*, 55, 2203-2214.

Hessen DO (1985) Filtering structures and particle-size selection in coexisting Cladocera. *Oecologia*, 66, 368-372.

Jöhnk KD, Huisman J, Sharples J *et al.* (2008) Summer heatwaves promote blooms of harmful cyanobacteria. *Global Change Biology*, 14, 495-512.

Kümmerlin RE (1998) Taxonomical response of the phytoplankton community of Upper Lake Constance (Bondesee-Obersee) to eutrophication and re-oligotrophication. *Archiv für Hydrobiologie*, 53, 109-117.

Lürling M & van der Grinten E (2003) Life-history characteristics of *Daphnia* exposed to dissolved microcystin-LR and to the cyanobacterium *Microcystis aeruginosa* with and without microcystins. *Environmental Toxicology and Chemistry*, 22, 1281-1287.

Martin-Creuzburg D & Von Elert E (2004) Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata*. *Journal of Chemical Ecology*, 30, 483-500.

Martin-Creuzburg D, Von Elert E & Hoffmann KH (2008) Nutritional constraints at the cyanobacteria-*Daphnia magna* interface: The role of sterols. *Limnology and Oceanography*, 53, 456-468.

Miner BE, De Meester L, Pfrender ME, Lampert W & Hairston NG (2012) Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society B-Biological Sciences*, 279, 1873-1882.

Paerl HW & Huisman J (2008) Climate - Blooms like it hot. *Science*, 320, 57-58.

Pflugmacher S, Wiegand C, Oberemm A *et al.* (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochimica et Biophysica Acta-General Subjects*, 1425, 527-533.

Porter KG & Mcdonough R (1984) The energetic cost of response to blue-green-algal filaments by cladocerans. *Limnology and Oceanography*, 29, 365-369.

Rohrlack T, Christoffersen K, Hansen PE *et al.* (2003) Isolation, characterization, and quantitative analysis of microviridin J, a new *Microcystis* metabolite toxic to *Daphnia*. *Journal of Chemical Ecology*, 29, 1757-1770.

Rohrlack T, Dittmann E, Börner T & Christoffersen K (2001) Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. *Applied & Environmental Microbiology.*, 67, 3523-3529.

Sarnelle O & Wilson AE (2005) Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, 50, 1565-1570.

Sarnelle O (2007) Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. *Limnology and Oceanography*, 52, 2120-2127.

Schreurs,H. (1992) *Cyanobacterial dominance, relation to eutrophication and lake morphology.* PhD thesis, University of Amsterdam, Amsterdam.

Schwarzenberger A, Zitt A, Kroth P, Mueller S & von Elert E (2010) Gene expression and activity of digestive proteases in *Daphnia*: effects of cyanobacterial protease inhibitors. *BMC physiology*, 10, 6.

Sivonen K & Jones G (1999) Cyanobacterial toxins. Toxic cyanobacteria in water: A guide to their public health consequences . *Monitoring and Management: 41-111*.

Sommer U, Gliwicz ZM, Lampert W & Duncan A (1986) The PEG-model of seasonal succession of planktonic events in fresh waters. *Archiv für Hydrobiologie*, 106, 433-471.

Verreydt D, De Meester L, Decaestecker E *et al.* (2012) Dispersal-mediated trophic interactions can generate apparent patterns of dispersal limitation in aquatic metacommunities. *Ecology Letters*, 15, 218-226.

Von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography*, 47, 1764-1773.

Von Elert E, Martin-Creuzburg D & Le Coz JR (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 1209-1214.

Von Elert E, Agrawal MK, Gebauer C *et al.* (2004) Protease activity in gut *Daphnia magna*: evidence for trypsin and chymotrypsin enzymes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 137, 287-296.

Von Elert E, Oberer L, Merkel P, Huhn T & Blom JF (2005) Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. *Journal of Natural Products*, 68, 1324-1327.

von Elert E, Zitt A & Schwarzenberger A (2012) Inducible tolerance to dietary protease inhibitors in *Daphnia magna*. *Journal of Experimental Biology*, 215, 2051-2059.

Weckesser J, Martin C & Jakobi C (1996) Cyanopeptolins, depsipeptides from cyanobacteria. *Systematic and Applied Microbiology*, 19, 133-138.

Weider LJ, Lampert W, Wessels M, Colbourne JK & Limburg P (1997) Long-term genetic shifts in a microcrustacean egg bank associated with anthropogenic changes in the Lake Constance ecosystem. *Proceedings of the Royal Society of London - Series B: Biological Sciences*, 264, 1613-1618.

Wojtal-Frankiewicz A (2012) The effects of global warming on *Daphnia* spp. population dynamics: a review. *Aquatic Ecology*, 46, 37-53.

Yang Y, Cheng JZ, Singhal SS *et al.* (2001) Role of glutathione S-transferases in protection against lipid peroxidation - Overexpression of hgsta2-2 in k562 cells protects against hydrogen peroxide-induced apoptosis and inhibits JNK and caspase 3 activation. *Journal of Biological Chemistry*, 276, 19220-19230.

Yang YS, Sharma R, Zimniak P & Awasthi YC (2002) Role of alpha class glutathione S-transferases as antioxidant enzymes in rodent tissues. *Toxicology and Applied Pharmacology*, 182, 105-115.

Abstract

In many freshwater ecosystems *Daphnia* represent both, an important herbivorous grazer of phytoplankton and a major prey of planktivorous fish and invertebrate predators. Thus, *Daphnia* provide an important link for the transfer of energy and carbon from primary producers to higher trophic levels. In eutrophic lakes this transfer is often reduced by the occurrence of cyanobacteria that are known for their low food quality for *Daphnia*: Cyanobacteria lack essential sterols and polyunsaturated fatty acids, are often filamentous or colonial and contain even toxins like microcystins and protease inhibitors. Although *Daphnia* populations are able to adapt to the presence of cyanobacteria, little is known about the physiological mechanisms and the particular genes and metabolic pathways underlying this adaptation.

The present study considers various intra- and interspecific aspects of *Daphnia*'s tolerance to cyanobacteria. Besides classical ecological approaches, such as growth and competition experiments, the focus of the present dissertation was the analysis of specific target genes of *Daphnia* that were related to the response to secondary metabolites of cyanobacteria, such as protease inhibitors and microcystins. I found here for the first time evidence that cyanobacterial inhibitors, interfering with *Daphnia*'s major gut proteases (chymotrypsin and trypsin), occurred in the edible fraction of natural lake seston and varied significantly within season. In parallel with seasonal changes in the potential of the phytoplankton to inhibit proteases, the genetic structure of the co-occurring *D. magna* population changed significantly, which might result from natural selection due to the increased content of cyanobacterial protease inhibitors in the seston.

Depending on initial conditions *Daphnia* may also control the development of bloom-forming cyanobacteria with protease inhibitors and may even suppress already established cyanobacterial blooms. In life-table experiments with cyanobacteria containing inhibitors of chymotrypsin and trypsin I investigated the tolerance of various clones of *D. magna* and *D. pulex* with different geographic origins. I showed that *D. magna* is more tolerant to cyanobacteria with protease inhibitors and thus may control the development of cyanobacterial blooms more efficiently than *D. pulex*. Protease assays revealed that the sensitivities of chymotrypsins and trypsins to cyanobacterial inhibitors did not differ between *D. magna* and *D. pulex*. However, *D. magna* exhibited a more than two times higher specific chymotrypsin activity than *D. pulex*, which explains the observed higher tolerance to cyanobacterial protease inhibitors of *D. magna*. The higher tolerance of *D. magna* to

cyanobacterial protease inhibitors compared to that of *D. pulex* might result from differences in their coexistence with cyanobacteria.

Adaptation of *Daphnia* populations to environmental stressors is typically a result of microevolutionary processes leading to natural selection. Determining genetic variation at the DNA level within and between natural *Daphnia* population is important for understanding the role of natural selection on phenotypic traits. In the present study I demonstrate that high-resolution melting analysis (HRMA) is a cost effective and sensitive tool for screening variable target genes in natural *Daphnia* populations. HRMA is based on monitoring the melting of PCR-amplicons and has usually been applied in clinical studies and medical molecular diagnostics. As a proof of principle for the application of HRMA in *Daphnia* population genetics I have analyzed the genetic variability of digestive trypsins in two *D. magna* populations. Both populations differed in their experience with respect to cyanobacterial trypsin inhibitors. I hypothesized that *D. magna* clones from ponds with cyanobacteria have undergone selection by these inhibitors, which has led to different trypsin alleles. My results revealed that both populations exhibited phenotypic difference in the analyzed trypsins and that HRMA is a powerful genotyping tool for screening high numbers of individuals in population studies.

The last part of my thesis considers anthropogenic long-term effects on *D. galeata* in Lake Constance, which underwent periods of eutrophication and re-oligotrophication in the last century. In order to investigate the influence of changing selection factors in Lake Constance on the genomic structure of the *D. galeata* population, I have isolated and pooled *D. galeata* resting eggs from four different layers of sediment cores, representing the time periods before, during and after the peak of the eutrophication. The extracted genomic population DNA samples from the different time periods were sequenced with a mean coverage of 50x via next-generation sequencing. After mapping the total reads of each population on predicted genes of *D. galeata*, I performed a genome-wide assessment of single nucleotide polymorphisms (SNPs) and copy number variations of several gene families in each of the three *D. galeata* populations. The data were used to determine the genetic diversity of the *D. galeata* populations and to indentify particular target gene families and metabolic pathways that were influenced by the environmental changes mentioned above. The data suggest that cyanobacterial protease inhibitors and oxidative stress induced by microcystins from cyanobacteria were the selective forces contributing to the genotypic shift towards a more cyanobacteria-tolerant *D. galeata* population during the time of eutrophication.

Zusammenfassung

In vielen Seen sind Daphnien als unselektive Filtrierer einer der Hauptkonsumenten von pelagischen Phytoplanktern und dienen des Weiteren als Nahrungsquelle von planktivoren Fischen und invertebraten Räubern. Dadurch nehmen Daphnien eine Schlüsselstellung im Transfer von Energie und Kohlenstoff von Primärproduzenten zu höheren trophischen Ebenen ein. Dieser Transfer ist in eutrophen Gewässern häufig durch die Anwesenheit von Cyanobakterien gestört, da diese ein Futter von geringer Qualität für Daphnien darstellen. Cyanobakterien besitzen die Fähigkeit, Kolonien oder Filamente zu bilden, womit sie ab einer gewissen Größe nicht mehr von Daphnien ingestiert werden können. Des Weiteren fehlen Cyanobakterien Sterole und ausreichende Mengen an mehrfach ungesättigten Fettsäuren. Darüber hinaus können Cyanobakterien für Daphnien toxische Sekundärmetabolite, wie zum Beispiel Proteaseinhibitoren oder Microcystine, enthalten. Daphnienpopulationen sind in der Lage sich an die Anwesenheit von Cyanobakterien anzupassen; allerdings sind sowohl die physiologischen Mechanismen, die dieser Anpassung zugrunde liegen, als auch die verantwortlichen Gene und Stoffwechselwege bisher nur wenig erforscht.

In der vorliegenden Arbeit werden verschiedene intra- und interspezifische Aspekte untersucht, die sich mit der Anpassung von Daphnien an Cyanobakterien beschäftigen. Neben klassischen Wachstums- und Konkurrenzversuchen liegt ein Schwerpunkt dieser Arbeit in der Identifizierung und Analyse spezieller Zielgene aus Daphnien, von denen vermutet wird, dass toxische Sekundärmetabolite aus Cyanobakterien einen Selektiondruck auf diese Gene ausüben können. Zu diesen toxischen Sekundärmetaboliten gehören neben hepatotoxisch wirkenden Microcystinen auch cyanobakterielle Inhibitoren von Trypsinen und Chymotrypsinen. Diese beiden Proteasen sind zusammen für mehr als 80% der proteolytischen Aktivität im Darm von Daphnien verantwortlich. In Kapitel 1 konnte ich zeigen, dass das die für Daphnien freßbare Größenfraktion des Sestons eines natürlichen Gewässers Trypsine und Chymotrypsine inhibiert, und dass die Stärke der Inhibition starken saisonalen Schwankungen unterliegt. Des Weiteren konnte ich zeigen, dass sich parallel mit den jahreszeitlichen Schwankungen des Inhibitionsvermögens des Sestons auch die genetische Struktur der coexistierende *D. magna* Population signifikant veränderte, was eine Folge von natürlicher Selektion sein könnte.

Je nach Ausgangsbedingungen sind Daphnien in der Lage, die Entstehung cyanobakterieller Massenentwicklungen zu unterdrücken. In Wachstumsversuchen mit Cyanobakterien, die Inhibitoren gegenüber Trypsinen und Chymotrypsinen enthielten, habe

ich die Toleranz verschiedener *D. magna* und *D. pulex* Klone aus unterschiedlichen Ursprungshabitaten untersucht. Ich konnte zeigen, dass *D. magna* eine höhere Toleranz gegenüber Cyanobakterien aufweist als *D. pulex*. Dies lässt die Vermutung zu, dass *D. magna* die Entstehung von Cyanobakterienblüten effizienter kontrollieren kann als *D. pulex*. Enzymatische Untersuchungen ergaben, dass sich weder Chymotrypsine noch Trypsine aus *D. magna* und *D. pulex* hinsichtlich ihrer Sensitivitäten gegenüber cyanobakteriellen Inhibitoren unterschieden. Es konnte jedoch gezeigt werden, dass *D. magna* eine doppelt so hohe spezifische Chymotrypsinaktivität besitzt als *D. pulex*. Dies könnte ein Grund für die beobachtete höhere Toleranz gegenüber Cyanobakterien von *D. magna* im Vergleich zu *D. pulex* sein.

Eine Anpassung von Daphnienpopulation an veränderte Umweltbedingungen ist häufig eine Konsequenz von natürlicher Selektion aufgrund von Mikroevolution. Die genaue Bestimmung genetischer Variabilität innerhalb und zwischen Daphnienpopulation ist von entscheidender Bedeutung, um natürliche Selektion und deren Auswirkung auf phäntoypische Merkmale besser verstehen zu können. In der vorliegenden Arbeit wird die Methode der hochauflösenden Schmelzkurvenanalyse (high-resolution melting analysis, HRMA) als günstige und sensitive Alternative zu herkömmlichen Sequenzierungsmethoden genutzt, um genetische Variabiltät von Zielgenen effektiv analysieren zu können. HRMA basiert auf der exakten Analyse des Schmelzverhaltens kompletter PCR Produkte und wird vornehmlich in klinischen Studien sowie in der molekularen Diagnostik verwendet. Als Anwendungsbeispiel der HRMA wurde die genetische Variabilität verschiedener Trypsingene aus zwei *D. magna* Populationen untersucht. Eine der beiden Populationen stammte aus einem Gewässer ohne Cyanobakterien, während die andere Population aus einem Gewässer mit jährlich auftretenden Cyanobakterienblüten entstammt. Die Hypothese war, dass *D. magna* Klone aus cyanobakterienreichen Gewässern aufgrund von natürlicher Selektion durch Proteaseinhibitoren andere Trypsinallele aufweisen als Klone aus cyanobakterienfreien Gewässern. Die Ergebnisse ergaben Unterschiede in der Aminosäuresequenz der analysierten Trypsinen zwischen beiden *D. magna* Population. Im Allgemeinen konnte ich zeigen, dass sich die Methode der hochauflösenden Schmelzkurvenanalyse ideal zur Genotypisierung einer großen Anzahl von Individuen in Populationsanalysen eignet.

Der letzte Abschnitt meiner Dissertation behandelt den Einfluss anthropogener Langzeiteffekte auf die im Bodensee existierende *D. galeata* Population. Durch erhöhten Phosphoreintrag und nachfolgend eingeleitete Sanierungsmaßnahmen durchlief der Bodensee eine Phase der Eutrophierung und anschließenden Re-Oligotrophierung in der

zweiten Häfte des letzen Jahrhunderts. Um die Einflüsse dieser schwankenden Umweltbedingungen auf die genetische Struktur der *D. galeata* Population zu analysieren, wurden aus verschiedenen Schichten mehrerer Bohrkerne aus dem Sediment des Bodensees Dauereier von *D. galeata* isoliert. Die Schichten, aus denen die Dauereier separat isoliert wurden, entsprachen der Zeit vor, während und nach dem Höhepunkt der Eutrophierung des Bodensees. Anschließend wurde die DNA aus jeder Population separat extrahiert, mittels ‚Next-Generation Sequencing' analysiert und anschließend mit vorhandenen Transkriptomdaten von *D. galeata* abgeglichen. Dies ermöglichte es mir einen genomweiten Vergleich einzelner Nukleotidpolymorphismen (SNPs) sowie Unterschiede in der Anzahl der Kopien bestimmter Zielgene zwischen den einzelnen Populationen zu untersuchen. Die so gewonnen Daten dienten zum einen dazu, die genetische Diversität der *D. galeata* Population vor, während und nach der Eutrophierung zu messen. Des Weiteren sollten Zielgene und Stoffwechselwege identifiziert werden, die sich möglichweise durch sich wechselnden Umweltbedingungen des Bodensees in der *D. galeata* Population verändert haben. Die Analyse der Daten stützt die Vermutung, dass ein Anstieg von Proteaseinhibitoren sowie oxidativer Stress durch Mikrocystine, bedingt durch das im Zuge der Eutrophierung vermehrte Auftreten von Cyanobakterien, wichtige Selektionfaktoren darstellten, die zu den gefundenen genetischen Veränderungen der *D. galeata* Populationen beigetragen haben.

Record of achievement

Chapter 1: Seasonal dynamics of sestonic protease inhibition: impact on *Daphnia* populations

The isolation of the *D. magna* fall population, microsatellite analyses, all single-clone growth and competition experiments and the IC_{50} determination of the seston extracts from 2008 were exclusively performed by me or under my direct supervision. The isolation of the *D. magna* spring population and the IC_{50} determination of the seston extracts from 2007 were performed by A. Schwarzenberger.

Chapter 2: High-resolution melting analysis: a genotyping tool for population studies on *Daphnia*

Results described in this chapter were exclusively performed by me.

Chapter 3: Interspecific differences between *D. pulex* and *D. magna* in sensitivity to cyanobacterial protease inhibitors

Results described in this chapter were exclusively performed by me or under my direct supervision.

Chapter 4: Rapid evolution revealed by dormant eggs: physiological mechanisms

I performed the field sampling and sample processing with the help of M. Wessels from the Institut für Seenforschung Langenargen (ISF). The next-generation sequencing analysis as well as the single-read mapping on the *D. galeata* transcriptome was performed in the lab of John Colbourne at the Indiana University, USA. I contributed to bioinformatical analysis and exclusively analyzed the collected data.

Köln, 07.11.2012

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.

_____

Christian Küster

Acknowledgements

Hiermit möchte ich mich ganz herzlich bedanken bei

- meinem Betreuer **Prof. Dr. Eric von Elert** für die Vergabe und die hervorragende Betreuung meiner Doktorarbeit. Seine spezielle Mischung aus Fördern und Fordern hat bei mir genau den richtigen Nerv getroffen. Durch ihn habe ich gelernt selbständig wissenschaftlich zu arbeiten und vor allem Ergebnisse kritisch zu hinterfragen. Ich hatte jederzeit die Möglichkeit meine Ideen und Ergebnisse persönlich und umfangreich mit ihm zu besprechen. Vielen Dank!

- **Prof. Dr. Arndt** für die Erstellung des Zweitgutachtens.

- meinen Doktorbrüdern und -schwestern **Christoph, Thomas, Marc, Patrick, Marian und Jana** mit denen ich stets verrückte wissenschaftliche Hypothesen austauschen konnte und die man nie lange um ein zünftiges Feierabendbier bitten musste. Insbesondere danke ich **Chris1** für die letzten fünf Jahre, in der er meine Launen stets zu ertragen und zu kontern wusste.

- **Gesa Heinichen, Laura Schäfer** und **Lea von Ganski** für die Unterstützung im Laboralltag.

- **Jennifer Kuhn** für die Einweisung in das LightCylcer System.

- **Martin Wessels** für die Unterstützung beim Stechen der Bodenseebohrkerne.

- **John Colbourne** für die Next-generation-sequencing Analysen sowie Hilfestellung in bioinformatischen Fragestellungen.

- **Katja Preuß** und **Hannelore Krisch** als gute Seelen der Ag von Elert.

- allen restlichen Mitgliedern der Ag von Elert.

- den Jungs aus der Werkstatt, die meine abstrusen Ideen immer perfekt umzusetzen wussten.

- meiner Freundin **Sabina**, die mich in jeglicher Hinsicht während meiner Doktorarbeit moralisch unterstützt hat. Eine tollere Freundin kann es auf der Welt nicht geben ☺

- meiner **Familie** für die Unterstützung während meiner Doktorarbeit.

- allen, die ich hier vergessen habe.

Curriculum vitae

Name: Christian Küster

Geburtsdatum: 24.09.1982 in Engelskirchen

Familienstand: ledig

Nationalität: deutsch

Beruflicher Werdegang:

1989-1993 Gemeinschaftsgrundschule Heerstraße, Troisdorf

1993-2002 Gymnasium zum Altenforst, Troisdorf

2002-2003 Zivildienst im St. Johannes Krankenhaus, Troisdorf

2003-2009 Biologiestudium, Universität zu Köln

2009 Abschluss des Biologiestudiums and der Universität zu Köln. Diplomarbeit über „Saisonale Veränderung der Sensitivität von *Daphnia magna* gegenüber Cyanobakterien“

2009-2012 Promotion an der Universität zu Köln

Bisherige Publikationen im Peer-Review Verfahren

[1]**Küster, C.J.**, Schwarzenberger, A. & Von Elert, E. (2012) Seasonal dynamics of sestonic protease inhibition: Impact on *Daphnia* populations. *Hydrobiologia*, *in press* (doi: 10.1007/s10750-012-1303-x).

[2]**Küster, C.J.** & Von Elert, E. (2012) High-resolution melting analysis: A genotyping tool for population studies on *Daphnia*. *Molecular Ecology Resources* 12: 1048-1057 (doi: 10.1111/j.1755-0998.2012.03177.x).

[3]**Küster, C.J.** & Von Elert, E. (2012) Interspecific differences between *D. pulex* and *D. magna* in sensitivity to cyanobacterial protease inhibitors. *PLoS ONE*, *submitted*

Schwarzenberger, A., **Küster, C.J.** & Von Elert, E. (2012) Molecular mechanisms of tolerance to cyanobacterial protease inhibitors revealed by clonal differences in *Daphnia magna*. *Molecular Ecology* 21 (19): 4898-4911 (doi: 10.1111/j.1365-294X.2012.05753.x).

[1] entspricht chapter 1

[2] entspricht chapter 2

[3] entspricht chapter 3