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A direct test of cyanobacterial chemical defense: Variable effects of microcystin-treated food on two *Daphnia pulicaria* clones

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Abstract

To determine the direct effects of microcystin on the fitness of herbivorous zooplankton, we experimentally added microcystin-LR to freeze-dried cells of palatable *Chlorella* and fed these compound-treated cells to two clones of *Daphnia pulicaria* that had shown differing responses to a diet containing a strain of *Microcystis aeruginosa* that produces microcystin. The *Daphnia* that performed better on a diet containing live *Microcystis* showed reduced population growth when exposed to microcystin-LR-treated *Chlorella*, whereas the *Daphnia* that performed poorly on the diet containing live *Microcystis* was not affected by the experimental diet containing microcystin-LR. This is the first study to unambiguously show that some *Daphnia* strains are and some strains are not harmed by the consumption of microcystin-LR. These surprising results were not generated by interference from lipophilic secondary metabolites in *Microcystis*. When the crude lipophilic extract of *Microcystis* was added to dried *Chlorella* cells, it enhanced the fitness of both *Daphnia* clones. We hypothesize that the *Daphnia* clone more tolerant to live cells may upregulate resistance when cued by the presence of the live *Microcystis* cells, but not by microcystin-LR alone. Alternatively, the *Daphnia* clone that grew well on a diet containing live *Microcystis* may sequester compounds from *Microcystis* that defend the cyanobacteria from autotoxicity; these compounds would have been unavailable to *Daphnia* consuming freeze-dried *Chlorella* treated with microcystin-LR alone. Thus, microcystin-LR can suppress *Daphnia* fitness when consumed; however, the effects of microcystin vary across clones of herbivorous zooplankton and the consequences of this variance should not be overlooked when considering zooplankton-cyanobacteria interactions.

Blooms of cyanobacteria threaten aquatic communities and global water supplies (Paerl 1988) because they produce secondary metabolites that can harm or kill fishes, livestock, and humans (Carmichael 1992). Some of the more commonly studied cyanotoxins include cyclic peptides that target mammalian livers (e.g., microcystin and cylindrospermopsin), nerve synapses (e.g., anatoxin-*a*), and gastrointestinal tracts (e.g., lyngbyatoxin-*a*) (Carmichael 1992; Chorus and Bartram 1999; Zurawell et al. 2005). Despite a large correlative literature suggesting that cyanotoxins may deter feeding by zooplankton (Lampert 1987; Watanabe et al. 1996; Zurawell et al. 2005), the effect of cyanobacterial secondary metabolites on ecologically relevant organisms, such as herbivorous zooplankton, is equivocal (Wilson et al. 2006a). There are no direct empirical tests of how cyanobacterial secondary metabolites affect grazers consuming these compounds.

Cyanobacteria have been proposed to lower zooplankton grazing rates in three basic ways: (1) by occurring as large colonial and filamentous morphologies that clog filtering appendages or are otherwise inedible, (2) by being nutritionally deficient, and (3) by producing toxic secondary metabolites (Porter and Orcutt 1980; Lampert 1987). There are direct tests of the effects of morphology (Ferrão-Filho and Azevedo 2003) and nutrition (DeMott and Müller-Navarra 1997; von Elert and Wolffrom 2001) on zooplankton feeding, but no study has unambiguously assessed how consuming cyanobacterial secondary metabolites affects zooplankton fitness. Past experiments have compared zooplankton performance when (1) immersed in media containing dissolved cyanotoxins (Reinikainen et al. 2001) or cyanobacterial extracts (Wheeler et al. 1942), (2) fed diets containing cyanobacteria versus being starved (Arnold 1971; reviewed by Wilson et al. 2006a), (3) fed diets containing cyanobacteria versus foods supporting better growth (Arnold 1971; reviewed by Wilson et al. 2006a), (4) fed diets of two conspecific cyanobacteria that are or are not toxigenic to mammals (Smith and Gilbert 1995), and (5) fed diets consisting of a wild-type cyanobacterium containing microcystins or its mutant that lacks the ability to produce microcystins but may produce other toxic, nonmicrocystin oligopeptides (Rohrlack et al. 2005). Results from these studies show that cyanobacteria are poor food for grazers, but do not unambiguously demonstrate the effects of consuming cyanobacterial secondary metabolites (see review by Wilson et al. 2006a), because cyanotoxins are either present in unrealistic forms (dissolved in water rather than bound in cells) or are potentially confounded by other factors (i.e., cyanobacterial species or strains with and without a toxic secondary

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metabolite may differ in other respects, as well). Experiments where grazers are immersed in media containing dissolved secondary metabolites do not mimic the typical route of exposure for zooplankton (i.e., grazing) and routinely use metabolite concentrations several orders of magnitude higher (DeMott et al. 1991) than found in nature (Chorus and Bartram 1999). Furthermore, a recent meta-analysis of zooplankton–cyanobacteria interactions as a function of cyanobacterial "toxicity" (defined by effects when injected into rodents) could not detect a consistent effect of cyanobacterial toxins on herbivorous zooplankton (Wilson et al. 2006a).

Zooplankton often perform well on diets of dried algae (Naylor et al. 1993; but see Dobberfuhl and Elser 1999 for effects of freeze-dried vs. oven-dried algae on *Daphnia magna* fitness) despite changes in some biochemical constituents (Dobberfuhl and Elser 1999). We thus used freeze-dried *Chlorella* as a base food and treated these cells with specific chemicals or cyanobacterial extracts while treating control *Chlorella* with only the solvents used to apply compounds to the treatment food. This technique, the metabolite-treated dried food bioassay, is routinely used to identify chemical defenses of marine macroalgae and invertebrates (Hay et al. 1994, 1998) but has not previously been used for investigations of phytoplankton defenses (but see Ianora et al. 2004 for a similar design using live phytoplankton). Freeze-dried algae make realistic experimental foods for the types of comparisons described in this study given that the morphology and nutritional content are consistent for treatment and control foods; thus the only difference between the diets is the phytoplankter secondary chemistry of interest. Furthermore, this experimental technique is especially suited for unselective grazers, like *Daphnia*; this approach may not work with more selective grazers, like copepods (DeMott 1986) that may ignore dead, phytoplankton-based foods.

The secondary chemistry of cyanobacteria has been studied extensively (Watanabe et al. 1996; Chorus and Bartram 1999; Zurawell et al. 2005); however, many past studies have focused only on the water-soluble, cyanobacterial constituents, like the hepatotoxic phosphatase inhibitor (Carmichael 1992) microcystin. Although much less studied, lipophilic cyanobacterial compounds could also function as chemical defenses against grazing (Kurmayer and Jüttner 1999) or, alternatively, as nutrients enhancing zooplankton growth (DeMott and Müller-Navarra 1997). We used the metabolite-treated dried algal bioassay to directly test the effects of the widely studied hepatotoxin, microcystin-LR, and the crude lipophilic extract from a toxin-producing strain of *Microcystis* against two clones of the lake-dwelling cladoceran *Daphnia pulicaria* that varied in their response to a diet containing live, microcystin-producing *Microcystis* (Sarnelle and Wilson 2005).

Methods

Daphnia *collection*—One *D. pulicaria* female was isolated from each of two small (<0.3 km^2) glacial lakes in southern Michigan in 2004. Descendents from these mothers were used in this study. The Baker Lake clone (BA2) was from a mesoeutrophic lake (Barry County, latitude +42°38′90″, longitude −85°30′20″, summer, mixed layer, total phosphorus concentration 20–41 $\mu g\ L^{-1}$, Sarnelle and Wilson 2005; maximum depth 9 m, anoxic summer hypolimnion, Cáceres and Tessier 2004). The Lake Sixteen clone (SI4) was from an oligotrophic lake (Allegan County, latitude +42°33′90″, longitude −85°36′80″, summer, mixed layer, total phosphorus concentration 9–12 $\mu g\ L^{-1}$, Sarnelle and Wilson 2005; maximum depth 27 m, persistent oxygenated summer hypolimnion; Geedey 1997). Quantitative phytoplankton data do not exist for these lakes, but observations (C. Cáceres, S. Hamilton, A. Tessier, and A. Wilson pers. comm.) suggest that Baker Lake experiences cyanobacterial blooms (e.g., *Microcystis aeruginosa* was abundant when we collected *D. pulicaria*); we observed no cyanobacteria in Lake Sixteen when we collected *Daphnia* there. Consistent with these observations, an empirical model based on summer epilimnetic phosphorus concentrations in Michigan lakes (Raikow et al. 2004) estimates that cyanobacteria should be abundant in Baker Lake (69% of total phytoplankton biovolume) but rare (<9%) in Lake Sixteen. Introduction of zebra mussels (*Dreissena polymorpha*) could confound this model by inducing cyanobacterial blooms in phosphorus-poor lakes (Vanderploeg et al. 2001; Raikow et al. 2004; Sarnelle et al. 2005), but zebra mussels had invaded neither lake at the time of *Daphnia* collection.

Daphnia *genetic analysis*—A recent study demonstrated that the somatic growth of the *Daphnia* clone from Baker Lake was significantly less inhibited by a diet of toxic *Microcystis* than was the clone from Lake Sixteen (Sarnelle and Wilson 2005). The two *Daphnia* clones also exhibited subtle phenotypic differences in culture. For example, the Baker Lake clone was darker than the Lake Sixteen clone. Moreover, the Lake Sixteen clone tended to produce dark ephippia in old, dense cultures, whereas the Baker Lake clone produced only light-colored ephippia. These differences suggested genetic divergence between the two *Daphnia* clones. To quantify this, the two *Daphnia* isolates were genetically discriminated using variation of three microsatellite loci (Dp464, Dp496, Dp502; Colbourne et al. 2004). Multiple live animals from each clone were rinsed thoroughly with distilled water to remove attached bacteria and phytoplankton, and then genomic deoxyribonucleic acid (DNA) was extracted with a Qiagen DNeasy kit following the manufacturer's protocol. Amplification of microsatellite alleles via polymerase chain reaction (PCR) was performed in 10-μL volumes (10–50 ng of DNA, 1 μL of 10× buffer [10 mmol L^{-1} Tris HCl at pH 8.3, 50 mmol L^{-1} KCl, 0.001% gelatin, and 1.5 mmol L^{-1} $MgCl_2$], 0.2 μL of deoxyribonucleotide triphosphates, 0.15 μL of forward primer with attached fluorescent label, 0.15 μL of reverse primer, and 1 unit of Taq DNA polymerase). Each locus was separately analyzed for each *Daphnia* sample (six reactions). PCR was performed on a Robocycler 40 gradient under the following conditions: 95°C for 2 min followed by 39 cycles of 95°C for 30 s, 55°C for 20 s, and 72°C for 30 s with a final extension at 72°C for 7 min. PCR products were analyzed using an ABI Prism 3100 genetic

analyzer and microsatellite nucleotide lengths were determined with Genescan 3.7 and Genotyper 3.7 software (Applied Biosystems).

Daphnia *maintenance and experimental design*—*Daphnia* clones were maintained in a temperature-controlled room (25°C) in autoclaved lake water (Lake Lanier, oligotrophic lake in north Georgia) on a mixture of green algae, including *Ankistrodesmus*, *Chlorella*, and *Chlamydomonas* grown in a nutrient-rich medium (modified BG-11 medium, Vanderploeg et al. 2001).

Three separate experiments were performed to determine the effect of: (experiment 1) a live cyanobacterium (*M. aeruginosa* UTEX 2667), (experiment 2) the cyanotoxin, microcystin-LR, and (experiment 3) the crude lipophilic extract of *M. aeruginosa* (UTEX 2667) on the somatic and population growth rates of both clones of *Daphnia*. To test the effect of a toxic *Microcystis* strain on *Daphnia* growth, *Daphnia* were fed *Chlorella* by itself (exp. 1 control diet) or a mixture of 80% *Chlorella* and 20% *M. aeruginosa* UTEX 2667 (exp. 1 treatment diet), by biovolume. The *Chlorella* strain used in all of the experiments was unicellular, averaged 8 μm (SD = 2 μm) in diameter, and is nutritionally sufficient for the long-term maintenance of *Daphnia* cultures. *M. aeruginosa* UTEX 2667 (referred to as *Microcystis* herein) grows only as single cells (average $\pm$ SD = 4 μm $\pm$ 1 μm in diameter) and produces microcystins. Live algae were prepared by growing cells in batch cultures using a nutrient-rich medium (modified BG-11 medium), concentrating live, exponentially growing cells in a centrifuge, and then resuspending the algal pellet in 0.7-μm glass fiber (Whatman GF/F) filtered lake water.

M. aeruginosa can produce multiple microcystins, but microcystin-LR is the form most commonly detected and studied, most widely available, and most toxic (Chorus and Bartram 1999; Zurawell et al. 2005), so we concentrated our efforts on this metabolite. To test the effect of microcystin-LR on *Daphnia* growth, *Daphnia* were fed freeze-dried *Chlorella* treated with dimethyl sulfoxide (DMSO) into which we had dissolved microcystin-LR (exp. 2 treatment diet) or freeze-dried *Chlorella* treated with DMSO alone (exp. 2 control diet). In both cases, the DMSO was removed using a speed vacuum concentrator and cells were stored frozen under nitrogen until use. Freeze-dried *Chlorella* was prepared by growing batch cultures, concentrating exponentially growing cells in a centrifuge, freezing the cells, and then freeze-drying the alga. After rehydration, freeze-dried cells look similar to live cells, but are slightly smaller (average $\pm$ SD = 7 μm $\pm$ 2 μm in diameter). To prepare the diets, freeze-dried *Chlorella* was treated with 0.4 mL of DMSO containing 83 μg of microcystin-LR (treatment diet; toxin purchased from Axxora) or with 0.4 mL of DMSO alone (control diet).

To determine if negative effects of microcystin-LR were due to toxins dissolving into the media from the treated cells (which would be an experimental artifact) rather than due to consumption of treated cells (the ecologically relevant treatment), we fed *Daphnia* DMSO-treated, freeze-dried *Chlorella* either without (control diet) or with (treatment diet) microcystin-LR dissolved into the media at 12 μg L^{-1}. This concentration equaled the concentration of microcystins that dissolved off cells and occurred in the media unbound to cells during our experiment with microcystin-treated, freeze-dried *Chlorella* (exp. 2).

To determine if previously uninvestigated lipophilic defenses might also be present in our strain of *Microcystis*, we fed *Daphnia* freeze-dried *Chlorella* treated with the crude lipophilic extract (twice the natural concentration on the basis of algal dry weight) from an exponentially growing *Microcystis* culture (exp. 3 treatment diet) or with only the organic solvents (exp. 3 control diet; 1 part methanol : 1 part ethyl acetate : 3 parts ether) used to dissolve this extract onto the cells. We then compared performance on the different diets. *Microcystis* extract was obtained by extracting twice the dry weight of *Microcystis* cells as the dry weight of *Chlorella* cells to which the extract was added. We used twice the natural concentration to allow for losses due to extraction inefficiencies, possible degradation of active metabolites, or compound loss on the glassware in these small-scale extractions. Dried *Microcystis* cells were extracted under sonication with a mixture of 50% water : 50% methanol (two extractions, 1 h per extraction) and a mixture of 25% methanol : 75% ethyl acetate (two extractions, 1 h per extraction). The extract was passed through a 0.7-μm filter and a water : ethyl acetate partition used to separate the water-soluble from the lipophilic compounds. The water fraction containing the microcystins was discarded. The lipophilic fraction was dried using a speed vacuum concentrator. The appropriate mass of freeze-dried *Chlorella* was added to the extract, solvent added to dissolve the extract onto the cells, and this mixture was dried using a speed vacuum concentrator.

All experiments were conducted in a temperature-controlled room (25°C) under dim lighting provided by cool, white, fluorescent bulbs (18 h : 6 h light : dark) and each experiment lasted 12 d. Before the experiments, neonates from the stock cultures of each *Daphnia* clone were placed in separate vials filled with filtered lake water and fed daily with *Chlorella* (>1 mg carbon L^{-1}) until maturity. This concentration is well above the limiting concentration for *Daphnia* fed green algae raised at $\approx$20°C (i.e., 0.05 mg carbon L^{-1}, Lampert 1977). Neonates (<24 h old) from these mothers were pooled and then each experiment was initiated (day 1) by adding five or six randomly chosen neonates to five replicate 500-mL bottles per treatment (total 60 experimental bottles used for all three primary experiments for both *Daphnia* clones) filled with filtered lake water and a biovolume of particulate food equivalent to 1 mg carbon L^{-1} (6.5×10^6 μm^3 mL^{-1}; biovolume to carbon conversion from Kilham et al. [1997]). Food concentrations were determined by counting 20 fields of two replicate 0.125-mL subsamples from each treatment using a Palmer–Maloney chamber. To estimate *Daphnia* lengths at the beginning of each experiment, a random subset of neonates from both *Daphnia* clones was collected and measured.

All bottles were sealed with small ($\approx$2 mL) air bubbles and placed on a rotating plankton wheel (0.5 rotations per minute, 48-container maximum capacity [8 $\times$ 6 alignment],

bottles rotate vertically). *Daphnia* were transferred to new bottles with filtered lake water (all treatments) and new food daily, except for treatments used in the first experiment consisting of live foods where the grazers were moved to new bottles with fresh media every 2 d. Survivorship was recorded during *Daphnia* transfer. When *Daphnia* were transferred to the new bottles, *Daphnia* carrying eggs were identified, and neonates were counted and discarded. On day 5 (experiment consisting of live foods) or 6 (experiments consisting of freeze-dried foods treated with solvents and microcystin or the lipophilic extract of *Microcystis*), all live *Daphnia* were individually placed into a water droplet on a slide and measured with a compound microscope to estimate juvenile somatic growth rates (length, micrometers per day) using the formula: $(\ln L_{t1} - \ln L_{t0})/time$, where L_{t0} and L_{t1} are initial (day 1) and final (day 5 or 6) animal lengths, respectively.

Because each bottle contained more than one animal, it was not possible to determine when specific *Daphnia* carried eggs. Thus, we used the percentage of mothers carrying eggs each day as a proxy for maturation rate. Females in some of the treatments never carried eggs and we wanted to compare time to maturity across treatments, so we estimated the time for 50% of the mothers in each replicate bottle to reach maturity (50% egg sighting; ES_{50}). At the end of each experiment, we calculated the total number of live neonates produced per mother by dividing the total number of neonates in each bottle by the number of surviving mothers per day and then summing these values across the entire experiment for each bottle for each day of the experiments. Population growth rates (r) were iteratively calculated for each beaker using the Euler equation: $1 = \Sigma_{x=0}^{12} e^{-rx} l(x)m(x)$, where r is the rate of population growth (day^{-1}), x is the age class (day; 0 to 12), $l(x)$ is the probability of surviving to age x, and $m(x)$ is the number of neonates produced per *Daphnia* per bottle on day x. In case of no reproduction, r was determined from changes in abundance of *Daphnia* over time ($r = [\ln density_{t+1} - \ln density_t]/time$). One replicate (with 80% survival on day 11 for the Lake Sixteen clone [mixed live food treatment]) and one replicate (with 100% survival on day 9 for the Baker Lake clone [lipophilic extract treatment]) were spilled on days 12 or 10, respectively, so total neonates produced per mother and population growth rate for 12 d could not be calculated for these replicates.

To determine the microcystin content of treated cells and of the water in which the *Daphnia* were feeding, microcystin concentrations were quantified using enzyme-linked immunosorbent assay (ELISA; An and Carmichael 1994). Filtered samples for all treatment diets were collected on preweighed glass fiber filters (Whatman GF/F) from each experiment, dried, weighed to determine dried algal biomass, stored frozen, and subsequently extracted twice in 75% aqueous methanol before ELISA. Filtered whole water samples from the microcystin-LR addition treatment were also analyzed using ELISA to determine the concentration of microcystin-LR dissolved in the water. The ELISA method is quantitative and very sensitive, thus enabling reliable detection and quantification of small quantities of microcystins, but it does not distinguish among types of microcystins. Other quantification methods (e.g., high-performance liquid chromatography) can distinguish microcystin types, but are less sensitive and require larger samples. Our need to reliably measure small quantities of microcystins (those on remaining cells, those leached to the water, etc.) required the use of ELISA.

Statistical analyses—Differences among treatment effects were assessed via analysis of variance (ANOVA). Two-way ANOVA assessed interactions between *Daphnia* clone and treatment food types for each experiment. *t*-tests determined if growth rates were different from 0. Pearson's product moment correlations and Bonferroni adjusted probabilities determined how well juvenile somatic growth rates were related to population growth rates. Data were log-transformed as needed to conform to the assumptions of parametric statistics. All analyses were performed with Systat 11 (Systat Software, 2004).

Results

The two *Daphnia* clones exhibited different nucleotide lengths for two microsatellite primers (Dp464 [Baker Lake clone = 147, 147; Lake Sixteen clone = 148, 148] and Dp496 [Baker Lake clone = 201, 216; Lake Sixteen clone = 196, 202]), but not for the third primer (Dp502 [149, 149]).

Our treatments rarely affected *Daphnia* survivorship. For only one of our six contrasts could we detect a significant treatment effect on survivorship; coating *Chlorella* with the organic extract of *Microcystis* decreased survivorship from 88% to 70% for the Baker Lake clone ($p = 0.048$, Table 1; ANOVA). However, given that we made six contrasts and that the p-value was only marginally significant, the probability of making a type 1 error (detecting a difference when none exists) is appreciable; we are thus reluctant to attach much importance to this one statistically significant contrast. Across all treatments, survivorship averaged 76% (range = 40–100%) for the clone from Baker Lake and 79% (range = 57–100%) for the clone from Lake Sixteen.

When fed live foods (exp. 1), both *Daphnia* clones showed greater somatic growth and neonate production (ANOVA, $p \leq 0.007$; Fig. 1a,b; Tables 2 and 4), faster maturity (i.e., ES_{50}; ANOVA, $p \leq 0.001$; Fig. 2a,b,e; Table 3), and higher population growth rate (ANOVA, $p \leq 0.006$; Fig. 1c; Table 5) on the control diet of 100% *Chlorella* than on the treatment diet of 80% *Chlorella* and 20% *Microcystis*. Both somatic growth and population growth of the Baker Lake clone exceeded that of the Lake Sixteen clone on the mixed treatment diet containing cyanobacteria (ANOVA, $p \leq 0.001$; Fig. 1a,c). The Baker Lake population increased significantly on the mixed treatment diet (*t*-test, $p < 0.001$; Fig. 1c), whereas the Lake Sixteen population's change did not differ significantly from zero (*t*-test, $p = 0.548$; Fig. 1c) on the same diet. A significant interaction between diet type and *Daphnia* clone was not observed for somatic growth rate, maturation rate, neonate production, or population growth rate (two-way ANOVA, $p \geq 0.055$; Tables 2–5).

Table 1. Means, standard errors, and sample sizes for survivorship (% survival on day 12) of each *Daphnia pulicaria* clone (Baker Lake and Lake Sixteen) used in three experiments that included diets composed of 100% *Chlorella* (exp. 1 control) or 20% *Microcystis aeruginosa* UTEX 2667 and 80% *Chlorella* (exp. 1 treatment) or composed of freeze-dried *Chlorella* coated with DMSO (exp. 2 control) versus DMSO with particle-bound microcystin-LR (exp. 2 treatment) or organic solvents (exp. 3 control) versus organic solvents with the particle-bound crude lipophilic extract of *Microcystis* (exp. 3 treatment), respectively.

			Survivorship (% survival on day 12)			
Daphnia	Exp.	Diets	*n*	Mean	Std. error	*p*-value
Baker	1	100% *Chlorella*	5	96.00%	4.00%	0.347
Baker	1	20% *Microcystis*	5	100.00%	0.00%	
Sixteen	1	100% *Chlorella*	5	72.00%	8.00%	0.894
Sixteen	1	20% *Microcystis*	4	70.00%	12.91%	
Baker	2	DMSO	5	63.33%	9.72%	0.156
Baker	2	DMSO w/microcystin	5	40.00%	11.30%	
Sixteen	2	DMSO	5	56.67%	10.00%	0.083
Sixteen	2	DMSO w/microcystin	5	80.00%	6.24%	
Baker	3	organic solvent	5	88.00%	4.90%	0.048
Baker	3	org. solvent w/extract	4	70.00%	5.77%	
Sixteen	3	organic solvent	5	96.00%	4.00%	0.347
Sixteen	3	org. solvent w/extract	5	100.00%	0.00%	

Cell-bound microcystins were present in the diets containing live *Microcystis* (535 μg [g dry weight]$^{-1}$) and on freeze-dried *Chlorella* treated with DMSO and microcystin-LR (42 μg [g dry weight]$^{-1}$). The concentration of microcystin-LR in the latter diet was 8% of that in the mixed diet, but only $\approx$1% of the amount we had applied (4,400 μg [g dry weight]$^{-1}$); thus the majority of the microcystin-LR was dissolved in the treatment water (12 μg L^{-1}) and not bound to the freeze-dried alga. Microcystins were not detected in the other treatment foods, including the diet containing the lipophilic extract of *Microcystis*.

Adding microcystin-LR to freeze-dried *Chlorella* (exp. 2 treatment diet) did not significantly affect somatic growth rate (ANOVA, $p \geq 0.308$; Fig. 1a; Table 2), time to maturity (ANOVA, $p \geq 0.186$; Fig. 2c,d,e; Table 3), or neonate production (ANOVA, $p \geq 0.139$; Fig. 1b; Table 4) for either *Daphnia* clone when each of these factors was considered in isolation. However, subtle effects of the treatment diet containing particle-bound microcystin-LR on somatic growth, survivorship, and neonates produced per female combined to significantly lower population growth for the Baker Lake clone (ANOVA, $p = 0.032$; Fig. 1c; Table 5), but not for the clone from Lake Sixteen (ANOVA, $p = 0.375$; Fig. 1c; Table 5). Averaging across these diets, the Lake Sixteen clone reached maturity 0.5 d faster (two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 2c,d,e; Table 3) and produced more than three times as many live neonates per female than the Baker Lake clone (two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 1b; Table 4). Faster maturation rates and larger average clutch sizes translated into significantly higher population growth for the Lake Sixteen clone than the Baker Lake clone (two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 1c; Table 5). Population growth rates for the Lake Sixteen clone on both diet types were significantly greater than 0 (*t*-test, $p \leq 0.001$; Fig. 1c). In contrast, population growth rate for the Baker Lake clone fed foods treated with DMSO only (exp. 2 control diet) did not differ from zero (*t*-test, $p = 0.563$; Fig. 1c), while population growth rate when feeding on the DMSO and microcystin-LR treated food (exp. 2 treatment diet) was significantly below zero (*t*-test, $p = 0.047$; Fig. 1c). A significant interaction between diet type and *Daphnia* clone was observed for population growth rate (two-way ANOVA, $p = 0.014$; Table 5). A significant interaction did not occur between diet type and *Daphnia* clone for somatic growth, maturation rate, or neonate production (two-way ANOVA, $p \geq 0.312$; Tables 2–4).

Given that microcystin-LR was both on our experimental food and dissolved in the media, it was unclear whether the detrimental effect of microcystin-LR on the Baker Lake *Daphnia* was ecologically meaningful (due to consuming food treated with microcystin-LR) or only an artifact of the methodology (due to soaking in the compound, which would not typically occur in nature at such concentrations). A separate experiment (i.e., dissolved toxin experiment) demonstrated that the effects of particle-bound microcystin-LR were not artifactual. Population growth rates of both *Daphnia* clones were unaffected when fed DMSO-treated, freeze-dried *Chlorella* in media to which we had added dissolved microcystin-LR at a concentration of 12 μg L^{-1} (Baker Lake clone: ANOVA, $p = 0.887$; control, $r = 0.084 \pm 0.015$ d^{-1} [mean $\pm$ SE], treatment, $r = 0.087 \pm 0.016$ d^{-1}; Lake Sixteen clone: ANOVA, $p = 0.456$; control, $r = 0.099 \pm 0.008$ d^{-1}, treatment, $r = 0.088 \pm 0.012$ d^{-1}). Thus, negative effects of the diet containing particle-bound microcystin-LR (exp. 2 treatment diet) on the Baker Lake *Daphnia* were caused by consuming food-bound microcystin-LR.

Feeding *Daphnia* freeze-dried *Chlorella* treated with organic solvents (exp. 3 control diet) or organic solvents and the lipophilic extract of *Microcystis* (exp. 3 treatment diet) had no effect on the somatic growth of either *Daphnia* clone (ANOVA, $p \geq 0.168$; Fig. 1a; Table 2), but the

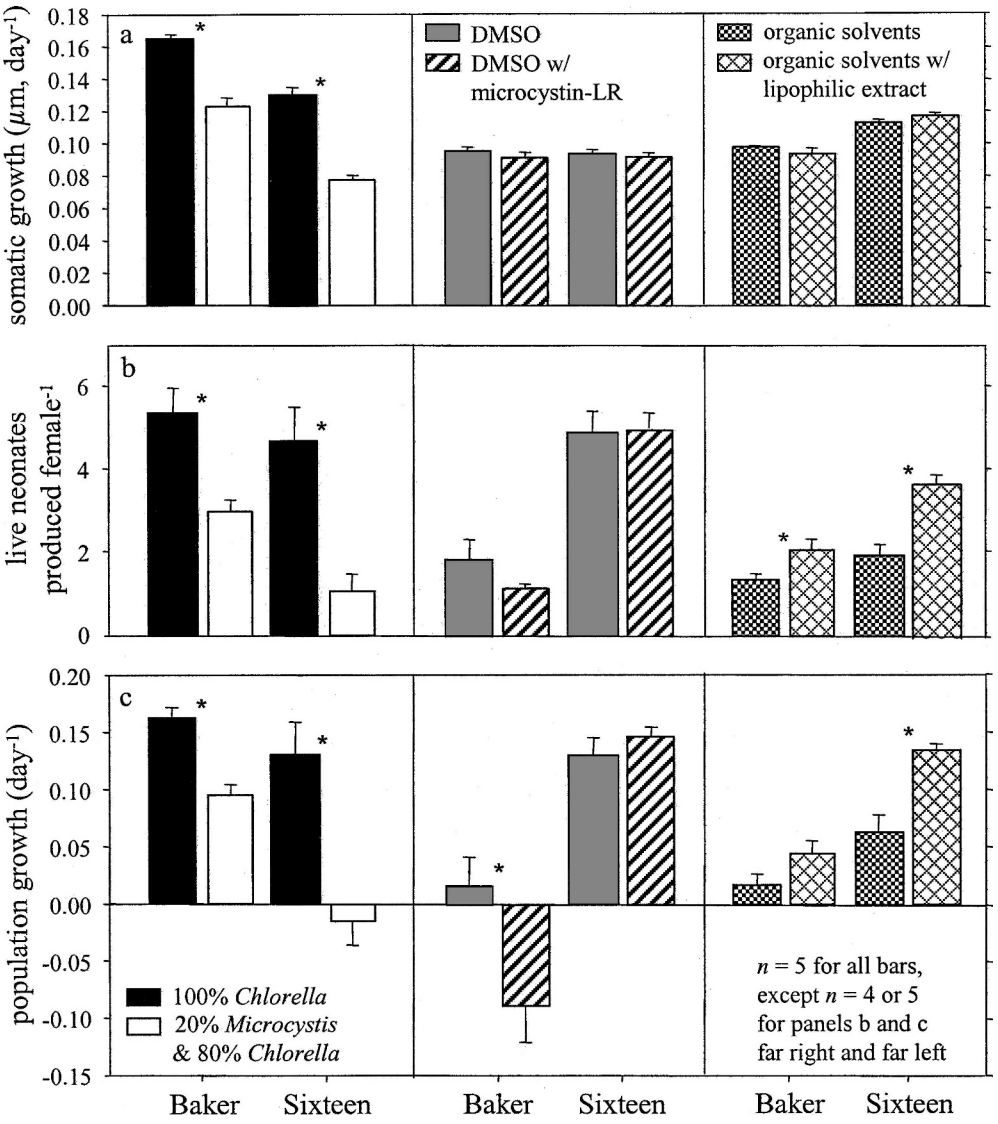

Fig. 1. (a) *Daphnia pulicaria* juvenile somatic growth rates over 5 or 6 d (length, micrometers per day), (b) number of live neonates produced per *Daphnia* over 12 d, and (c) population growth rates over 12 d (r, day^{-1}) for two *Daphnia* clones (Baker Lake and Lake Sixteen) from three experiments lasting 12 d each. Experiments included diets composed of 100% *Chlorella* (exp. 1 control) or 20% *Microcystis aeruginosa* UTEX 2667 and 80% *Chlorella* (exp. 1 treatment) or composed of freeze-dried *Chlorella* coated with DMSO (exp. 2 control) versus DMSO with particle-bound microcystin-LR (exp. 2 treatment) or organic solvents (exp. 3 control) versus organic solvents with the particle-bound crude lipophilic extract of *Microcystis* (exp. 3 treatment), respectively. * = $p < 0.05$. Error bars = 1 SE. Sample size for each treatment per *Daphnia* clone = 5, except for neonate production and population growth data (panels b and c) of the Baker Lake clone fed food treated with organic solvents with the lipophilic extract of *Microcystis* ($n = 4$) and of the Lake Sixteen clone fed the mixed live food diet because on days 10 and 12, respectively, one replicate was lost.

extract significantly increased the production of neonates per female for both clones (ANOVA, $p \leq 0.049$; Fig. 1b; Table 4). The Baker Lake clone reached maturity faster when fed lipid-supplemented food (exp. 3 treatment diet; ANOVA, $p = 0.015$; Fig. 2c,e; Table 3), but the Lake Sixteen clone exhibited similar maturation rates on both diets (ANOVA, $p = 0.277$; Fig. 2d,e; Table 3). The enhanced fecundity of *Daphnia* when consuming foods supplemented with cyanobacterial lipids (exp. 3 treatment diet) translated into higher population growth for the Lake Sixteen clone (ANOVA, $p = 0.002$; Fig. 1c; Table 5), but not for the Baker Lake clone (ANOVA, $p = 0.110$; Fig. 1c; Table 5). When the population growth rate was averaged across both diets in this experiment, the Lake Sixteen clone population grew 3.1 times faster than the Baker Lake clone (two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 1c; Table 5). A statistically significant interaction between diet type and *Daphnia* clone occurred for maturation rate (two-

Table 2. Analysis of variance results for juvenile somatic growth rate (length, micrometers per day) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment/Diets	Test	*Daphnia*	Trait	Source	df	MS	*F*-ratio	*p*-value
1	One-way	Baker	Juvenile growth rate	Food type	1	0.004	39.953	<0.001
100% *Chlorella*	ANOVA			Error	8	0.000		
20% *Microcystis* and		Sixteen		Food type	1	0.007	103.001	<0.001
80% *Chlorella*				Error	8	0.000		
	Two-way	Both clones	Juvenile growth rate	Food type	1	0.011	126.842	<0.001
	ANOVA			*Daphnia*	1	0.008	92.171	<0.001
				Interaction	1	0.000	1.863	0.191
				Error	16	0.000		
2	One-way	Baker	Juvenile growth rate	Food type	1	0.000	1.183	0.308
DMSO	ANOVA			Error	8	0.000		
DMSO		Sixteen		Food type	1	0.000	0.359	0.566
w/microcystin-LR								
				Error	8	0.000		
	Two-way	Both clones	Juvenile growth rate	Food type	1	0.000	1.462	0.244
	ANOVA			*Daphnia*	1	0.000	0.036	0.851
				Interaction	1	0.000	0.166	0.689
				Error	16	0.000		
3	One-way	Baker	Juvenile growth rate	Food type	1	0.000	1.521	0.252
Organic solvents	ANOVA			Error	8	0.000		
Organic solvents w/		Sixteen		Food type	1	0.000	2.304	0.168
lipophilic extract				Error	8	0.000		
	Two-way	Both clones	Juvenile growth rate	Food type	1	0.000	0.003	0.954
	ANOVA			*Daphnia*	1	0.002	83.149	<0.001
				Interaction	1	0.000	3.708	0.072
				Error	16	0.000		

way ANOVA, $p = 0.010$; Table 3) but not for somatic growth rate, neonate production, or population growth rate (two-way ANOVA, $p \geq 0.052$; Tables 2, 4, and 5).

Correlations between somatic and population growth rates across all studies were positive for both *Daphnia* when analyzed together (Pearson $r = 0.541$, $p < 0.001$, $n = 58$) or independently (Baker Lake clone, Pearson $r = 0.746$, $p < 0.001$, $n = 29$; Lake Sixteen clone, Pearson $r = 0.451$, $p = 0.014$, $n = 29$). Significant positive correlations were also observed for the experiments incorporating live foods (exp. 1; Pearson $r = 0.866$, $p < 0.001$, $n = 19$) and foods treated with organic solvents or organic solvents and the lipophilic extract of *Microcystis* (exp. 3; Pearson $r = 0.772$, $p < 0.001$, $n = 19$). No relation was observed for the experiment consisting of foods treated with DMSO or DMSO and microcystin-LR (exp. 2; Pearson $r = -0.051$, $p = 0.832$, $n = 20$).

Discussion

Cyanobacteria–zooplankton interactions can significantly affect aquatic community structure by influencing the frequency and magnitude of toxic cyanobacterial blooms (Burns 1987; Haney 1987; Sarnelle 1993). Consequently, many laboratory experiments have examined mechanisms mediating these biological interactions including nutrient deficiencies, buoyancy regulation, colonial and filamentous growth habits, and intracellular secondary metabolites (reviewed by Porter and Orcutt 1980; Lampert 1987; Wilson et al. 2006a). The ecological role and mode of action of cyanobacterial secondary metabolites, commonly referred to as cyanotoxins, are ambiguous. Past studies provide little evidence that cyanobacterial secondary metabolites consistently suppress zooplankton populations more than cyanobacterial strains lacking these compounds (Wilson et al. 2006a). Moreover, direct tests of how cyanobacterial secondary metabolites affect grazers consuming these compounds are lacking. This is the first study to unambiguously test the effect of a cyanotoxin or the lipophilic extract of a toxic strain of *Microcystis* on the fitness of herbivorous zooplankton.

The two *Daphnia* clones used in this study showed contrasting responses to a diet containing a mixture of palatable *Chlorella* and a single-celled strain of *Microcystis* that produces microcystins (exp. 1 treatment diet), although both *Daphnia* performed worse on this diet than when fed only *Chlorella* (exp. 1 control diet), showing that the *Microcystis* strain was a food that suppressed consumer fitness. Interestingly, the Baker Lake clone maintained positive population growth on the mixture of live foods containing cyanotoxin-producing *Microcystis*, while the *Daphnia* from Lake Sixteen performed poorly on the same diet (Fig. 1). Studies showing considerable intraspecific variation in how diets containing cyanotoxins affect grazer fitness are not uncommon (Hietala et al. 1995; Hairston et

Table 3. Analysis of variance results for time to maturity (ES_{50}, time in days for 50% of mothers to begin carrying eggs) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment/Diets	Test	*Daphnia*	Trait	Source	df	MS	*F*-ratio	*p*-value
1	One-way	Baker	Time to maturity	Food type	1	21.025	63.558	<0.001
100% *Chlorella*	ANOVA		(ES_{50})	Error	8	0.331		
20% *Microcystis* and		Sixteen		Food type	1	50.445	26.758	<0.001
80% *Chlorella*				Error	8	1.885		
	Two-way	Both clones	Time to maturity	Food type	1	68.302	61.644	<0.001
	ANOVA		(ES_{50})	*Daphnia*	1	2.650	2.392	0.142
				Interaction	1	3.168	2.859	0.110
				Error	16	1.108		
2	One-way	Baker	Time to maturity	Food type	1	1.136	2.095	0.186
DMSO	ANOVA		(ES_{50})	Error	8	0.542		
DMSO		Sixteen		Food type	1	0.146	1.175	0.310
w/microcystin-LR								
				Error	8	0.125		
	Two-way	Both clones	Time to maturity	Food type	1	1.049	3.146	0.095
	ANOVA		(ES_{50})	*Daphnia*	1	8.633	25.896	<0.001
				Interaction	1	0.233	0.700	0.415
				Error	16	0.333		
3	One-way	Baker	Time to maturity	Food type	1	0.671	9.461	0.015
Organic solvents	ANOVA		(ES_{50})	Error	8	0.071		
Organic solvents w/		Sixteen		Food type	1	0.125	1.359	0.277
lipophilic extract				Error	8	0.092		
	Two-way	Both clones	Time to maturity	Food type	1	0.108	1.324	0.267
	ANOVA		(ES_{50})	*Daphnia*	1	5.377	65.885	<0.001
				Interaction	1	0.688	8.433	0.010
				Error	16	0.082		

al. 2001; Sarnelle and Wilson 2005) and could explain how zooplankton frequently control cyanobacterial blooms in nature (Sarnelle 1993).

Our assay using the compound-treated dried algae showed that particle-bound microcystin-LR, within the natural concentration range found in lakes, negatively affected the population growth rate of the Baker Lake *Daphnia* clone, but did not affect the fitness of the clone from Lake Sixteen. The effect of microcystin-LR in the diet occurred in the absence of other covarying factors (e.g., food size, shape, nutritional differences, etc.). The concentration of microcystin-LR on our *Chlorella* diet was 42 μg (g dry weight)$^{-1}$. The average summer concentration of microcystins for cyanobacterial populations in three additional Michigan lakes measured 6 μg (g dry weight)$^{-1}$ (unpubl. data). Thus, our test concentration of microcystin-LR was at about seven times the mean summer microcystin concentration found in lakes similar in origin and productivity to those lakes where our study *Daphnia* were isolated. In other, more productive lakes, microcystin concentrations of up to 7,300 μg (g dry weight)$^{-1}$ have been reported (Chorus and Bartram 1999). Our test concentration was less than 1% of this concentration, making our assays well within the range of microcystin concentrations occurring in nature. Effects of these maximal microcystin concentrations might considerably exceed the effects demonstrated here.

The lipophilic extract of the *Microcystis* strain used in the live foods experiment (exp. 1) did not suppress the fitness of either *Daphnia* clone, but, in fact, enhanced the fitness of both clones (Fig. 1). These results suggest an absence of lipophilic, chemical defenses for this *Microcystis* strain, and further support our hypothesis that particle-bound microcystin-LR is responsible for the negative fitness effect observed for the Baker Lake *Daphnia*. Cyanobacteria are commonly deficient in lipophilic constituents, such as highly unsaturated fatty acids, polyunsaturated fatty acids, and sterols (Stanier et al. 1971; von Elert and Wolffrom 2001). When supplemented in the diet, lipophilic compounds have been shown to promote both increased somatic (DeMott and Müller-Navarra 1997) and population (Martin-Creuzburg et al. 2005) growth of zooplankton. Thus, our finding that lipids from *Microcystis* were beneficial for *Daphnia* growth is not surprising.

The *Daphnia* clones we assayed differed in their genetic structure (i.e., microsatellite lengths) as well as in their response to the experimental diets containing live *Microcystis* or cyanobacterial compounds. This genetic and ecological variance may be associated with differences in historic exposures to cyanobacterial blooms that could cause adaptations to grazing on toxic prey (Sarnelle and Wilson 2005). For example, the Baker Lake clone that was collected from a mesoeutrophic lake that experiences frequent cyanobacterial blooms tolerated a live, mixed diet containing *Microcystis* (535 μg microcystin [g dry weight]$^{-1}$) better than the Lake Sixteen clone (Fig. 1c) that was collected from a deep, oligotrophic lake that rarely experiences cyanobacterial blooms. Moreover, the Lake

Table 4. Analysis of variance results for the number of live neonates produced per mother (over 12 d) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment/Diets	Test	*Daphnia*	Trait	Source	Df	MS	*F*-ratio	*p*-value
1	One-way	Baker	No. of live neonates	Food type	1	14.042	12.901	0.007
100% *Chlorella*	ANOVA		produced per mother	Error	8	1.089		
20% *Microcystis* and		Sixteen		Food type	1	30.559	14.548	0.007
80% *Chlorella*				Error	7	2.101		
	Two-way	Both clones	No. of live neonates	Food type	1	43.466	27.848	<0.001
	ANOVA		produced per mother	*Daphnia*	1	7.821	5.011	0.041
				Interaction	1	2.107	1.350	0.263
				Error	15	1.561		
2	One-way	Baker	No. of live neonates	Food type	1	1.163	2.700	0.139
DMSO	ANOVA		produced per mother	Error	8	0.598		
DMSO		Sixteen		Food type	1	0.003	0.003	0.956
w/microcystin-LR								
				Error	8	1.019		
	Two-way	Both clones	No. of live neonates	Food type	1	0.735	0.909	0.355
	ANOVA		produced per mother	*Daphnia*	1	57.800	71.515	<0.001
				Interaction	1	0.882	1.091	0.312
				Error	16	0.808		
3	One-way	Baker	No. of live neonates	Food type	1	1.165	5.639	0.049
Organic solvents	ANOVA		produced per mother	Error	7	0.207		
Organic solvents w/		Sixteen		Food type	1	7.310	24.510	0.001
lipophilic extract				Error	8	0.298		
	Two-way	Both clones	No. of live neonates	Food type	1	6.971	27.283	<0.001
	ANOVA		produced per mother	*Daphnia*	1	5.519	21.599	<0.001
				Interaction	1	1.143	4.475	0.052
				Error	15	0.256		

Sixteen clone tolerated ingestion of microcystin-LR better than the Baker Lake strain, and the fitness of both clones was enhanced by the lipophilic extract of *Microcystis*. The greater tolerance of the Baker Lake clone for live *Microcystis* did not appear to be toward microcystin-LR alone given that the Baker Lake clone performed poorly (Fig. 1c) on the experimental diet containing 8% of the microcystin-LR (42 μg microcystin [g dry weight]$^{-1}$) present in the live, mixed diet. In contrast to the Baker Lake *Daphnia*, the Lake Sixteen clone showed positive population growth (Fig. 1c) on the same experimental diet containing particle-bound microcystin-LR (Fig. 1c). Effects of microcystin-LR on *Daphnia* population growth were due to consumption of microcystin-LR, not due to effects of the metabolite leaching from cells and dissolving into the media. When we dissolved microcystin-LR into the media at the same concentration that occurred in the media during the experiment where *Daphnia* were fed microcystin-treated *Chlorella* (exp. 2), we detected no effects on the population growth of either *Daphnia* clone. Thus, although recent studies show that zooplankton can adapt to grazing on microcystin-containing cyanobacteria (Hairston et al. 2001; Sarnelle and Wilson 2005), our results suggest that these adaptations may be to live cyanobacteria in general, and not simply to microcystins (but see Gustafsson and Hansson 2004; Gustafsson et al. 2005).

Moreover, our data highlighting variation in how different *Daphnia* clones are affected by microcystins also suggest that responses by *Daphnia* to particle-bound microcystins may be influenced by the quality of the food in which microcystins occur. For example, the Baker Lake clone performed poorly on freeze-dried *Chlorella* in general (Fig. 1c), and exhibited significant positive population growth for only one diet in experiments 2 and 3 (Fig. 1c). Synergistic effects of the poor food quality of the freeze-dried algal diet and the addition of microcystin-LR could have negatively affected the Baker Lake clone. The Lake Sixteen *Daphnia* grew well on all diets containing freeze-dried *Chlorella* (Fig. 1a,c; Tables 2 and 4), and subsequently did not show any negative effects of microcystin-LR on growth, maturation rates, and fitness (Fig. 1a,c, 2d,e; Tables 2, 3, and 5). Previous work with larger consumers and prey has shown that secondary metabolites are more potent defenses when defending low-quality prey rather than when defending higher-quality prey (Duffy and Paul 1992, Hay et al. 1994, Cruz-Rivera and Hay 2003); similar effects may be occurring between blue-green algae and herbivorous grazers given that cyanobacteria are poor food for zooplankton (Wilson et al. 2006a). Future studies examining interactions between food quality (e.g., variation in nitrogen and phosphorus content) and secondary chemistry could provide valuable information about the importance of these factors for zooplankton–cyanobacteria interactions.

Why the *Daphnia* clone from Baker Lake performed best on live *Microcystis* but poorly on a diet treated with

Table 5. Analysis of variance results for population growth (r, day^{-1}) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment/Diets	Test	*Daphnia*	Trait	Source	df	MS	*F*-ratio	*p*-value
1	One-way	Baker	r (population	Food type	1	0.011	30.969	0.001
100% *Chlorella*	ANOVA		growth rate)	Error	8	0.000		
20% *Microcystis* and		Sixteen		Food type	1	0.046	15.109	0.006
80% *Chlorella*				Error	7	0.003		
	Two-way	Both clones	r (population	Food type	1	0.053	32.382	<0.001
	ANOVA		growth rate)	*Daphnia*	1	0.024	14.716	0.002
				Interaction	1	0.007	4.327	0.055
				Error	15	0.002		
2	One-way	Baker	r (population	Food type	1	0.027	6.689	0.032
DMSO	ANOVA		growth rate)	Error	8	0.004		
DMSO		Sixteen		Food type	1	0.001	0.883	0.375
w/microcystin-LR								
				Error	8	0.001		
	Two-way	Both clones	r (population	Food type	1	0.010	4.026	0.062
	ANOVA		growth rate)	*Daphnia*	1	0.152	63.120	<0.001
				Interaction	1	0.018	7.548	0.014
				Error	16	0.002		
3	One-way	Baker	r (population	Food type	1	0.001	3.356	0.110
Organic solvents	ANOVA		growth rate)	Error	7	0.003		
Organic solvents w/		Sixteen		Food type	1	0.013	19.977	0.002
lipophilic extract				Error	8	0.001		
	Two-way	Both clones	r (population	Food type	1	0.011	20.354	<0.001
	ANOVA		growth rate)	*Daphnia*	1	0.023	42.051	<0.001
				Interaction	1	0.002	4.472	0.052
				Error	15	0.001		

microcystin-LR (Fig. 1c) is unclear. However, when exposed to toxin-producing cyanobacteria or pure cyanotoxins, zooplankton may upregulate greater resistance to the effects of these foods (Ghadouani et al. 2004), so it is possible that the Baker Lake clone can upregulate resistance when cued by the live cyanobacteria, but not when cued only by microcystin-LR. The molecular basis of *Daphnia* tolerance of toxic cyanobacteria is currently being investigated (J. Colbourne pers. comm.). Behavioral rather than physiological adaptation to harmful foods may also occur. For example, when fed a microcystin-containing, single-celled strain of *Microcystis,* or this *Microcystis* in combination with the green alga *Scenedesmus*, *D. pulicaria* decreased mandibular movement rates and increased labral rejection rates (Ghadouani et al. 2004), both of which may reduce the ingestion of food, and consequently, particle-bound microcystins. Furthermore, when fed 100% *Scenedesmus*, *D. pulicaria* changed their behavior only when exposed to a high concentration (5,000 μg L^{-1}) of microcystin-LR, but not lower concentrations (50–500 μg L^{-1}; Ghadouani et al. 2004). Another explanation for the poor fitness of Baker Lake *Daphnia* when consuming a diet containing microcystin-LR could be that this *Daphnia* clone sequesters compounds from live *Microcystis* cells that the cyanobacterium uses as a defense against autotoxicity. Such compounds would have been unavailable to *Daphnia* in our assays incorporating freeze-dried *Chlorella* treated with microcystin-LR. No studies have addressed this possibility for cyanobacteria–zooplankton interactions, but autotoxicity studies have been conducted on other microbes (Kainz and Strack 1980; Mai-Prochnow et al. 2004).

Although the population growth of the Baker Lake *Daphnia* was suppressed by microcystin-LR placed on freeze-dried *Chlorella* particles, the metabolite-treated dried algal bioassay has considerable limitations when testing water-soluble compounds such as the microcystins. After rehydration, the algal particles in this study retained ≈1% of the total microcystin-LR added. A similar retention rate was seen by Ianora et al. (2004) when live cells were coated with water-soluble compounds. However, the food-bound microcystin-LR concentration we achieved was ecologically realistic and similar to particulate toxin concentrations measured in some lakes (Watanabe et al. 1996; Chorus and Bartram 1999; Zurawell et al. 2005) and did negatively affect one of our test clones when they consumed these cells (Fig. 1). Although not perfect, the metabolite-treated dried algal bioassay technique should be useful for understanding how phytoplankton secondary metabolites affect grazers consuming these compounds. The effectiveness of the toxic-treated dried algal bioassay technique will be influenced by the ability to get compounds to stick to the dried algal diets, as well as the types of grazers used in experiments. Strategies to increase the retention of water-soluble compounds coated onto dried particles immersed in water include retreating dried cells with a lipophilic algal extract that may form a barrier around each algal particle or using structurally different algae as base foods. Lipophilic

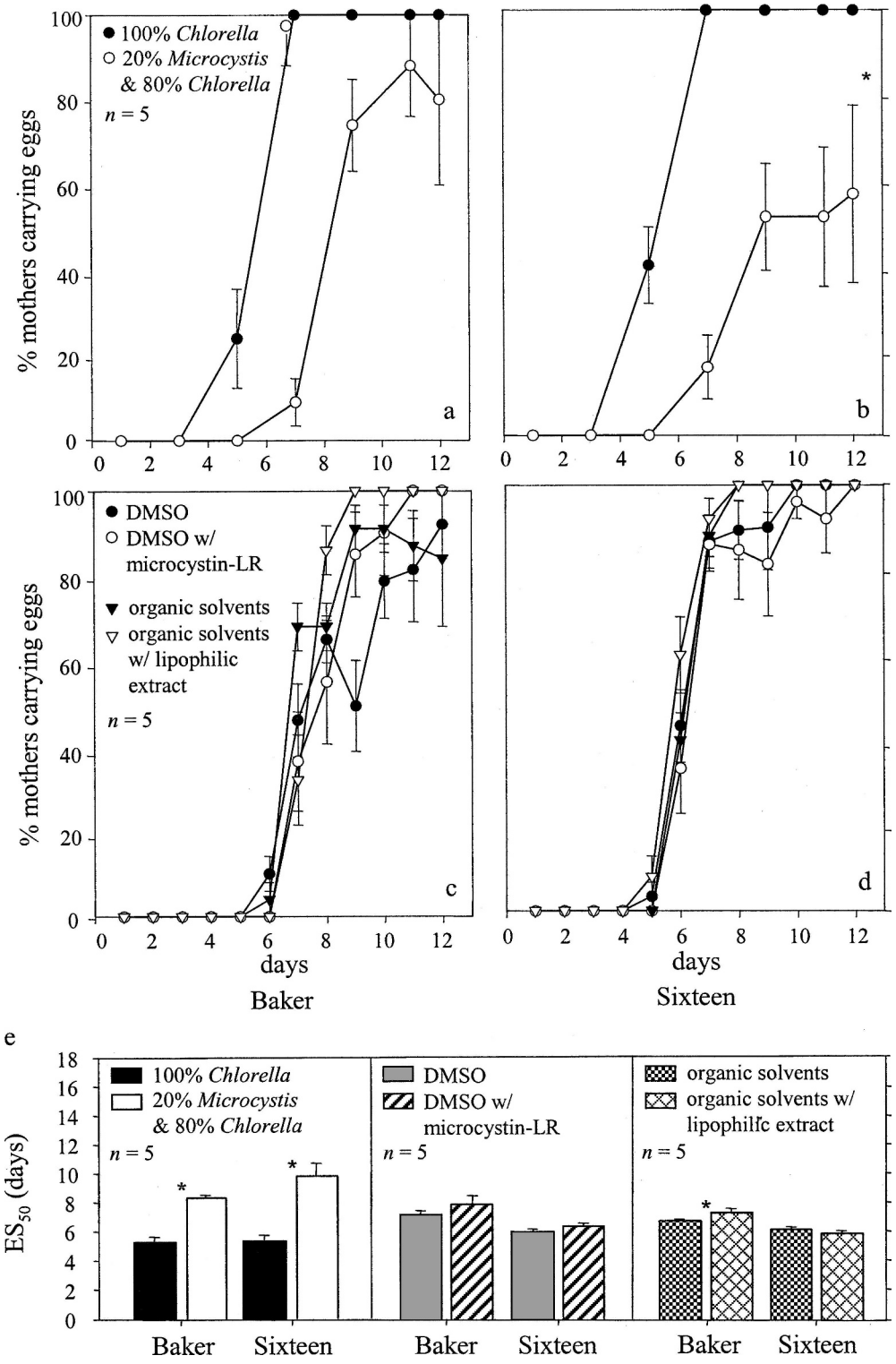

Fig. 2. Percentage of mothers carrying eggs for two *Daphnia pulicaria* clones ([a,c] Baker Lake and [b,d] Lake Sixteen) from three experiments lasting 12 d each. (e) Mean time for 50% of the *D. pulicaria* (Baker and Sixteen) mothers to begin carrying eggs (ES_{50}). Experiments included diets composed of 100% *Chlorella* (exp. 1 control) or 20% *Microcystis aeruginosa* UTEX 2667 and 80% *Chlorella* (exp. 1 treatment; panels a, b, and e) or composed of freeze-dried *Chlorella* coated with DMSO (exp. 2 control) versus DMSO with particle-bound microcystin-LR (exp. 2 treatment; panels c, d, and e) or organic solvents (exp. 3 control) versus organic solvents with the particle-bound lipophilic extract of *Microcystis* (exp. 3 treatment; panels c, d, and e), respectively. * = $p < 0.05$. Error bars = 1 SE. Sample size for each treatment per *Daphnia* clone = 5.

compounds repel water and will stick to dried foods when they are rehydrated, so this assay method should work for assessing the effects of lipid-soluble chemical defenses (Hay et al. 1998). Unselective grazers, such as *Daphnia*, are well-suited model organisms for the metabolite-treated dried algal bioassay technique; more selective grazers, such as copepods, may be less well suited because they tend to avoid consuming dead phytoplankton (DeMott 1986).

Our findings of variation in how microcystin-LR affects zooplankton clones is not surprising given that qualitative (Burns 1987; Lampert 1987; Haney 1987) and quantitative (Wilson et al. 2006a) reviews of freshwater zooplankton–cyanobacteria interactions provide little consensus on the role of cyanobacterial secondary metabolites in affecting zooplankton fitness. In addition, significant interspecific and intraspecific variation exists in the ability of zooplankton to feed and reproduce on different cyanobacterial species and genotypes (Hietala et al. 1995; Nandini and Rao 1998). Furthermore, the patchy nature of freshwater habitats and the clonal nature of many zooplankton and all cyanobacteria provide ample opportunity for selection of both deterrence and tolerance in grazers and prey, thus producing considerable variance within and between sites (Sarnelle and Wilson 2005; Wilson et al. 2005). Together, our findings that particle-bound microcystin-LR suppresses fitness of one *Daphnia* clone but not another clone suggest that ecological, physiological, chemical, and genetic variation among different cyanobacterial and herbivore genera, species, and genotypes (Sarnelle and Wilson 2005; Wilson et al. 2005, 2006b) should not be overlooked when considering the effects of specific compounds on ecologically relevant grazers. Finally, the compound-treated dried algal bioassay should allow broader tests for the presence and effects of cyanobacterial chemical defenses against consumers, as well as assessments of the relative importance of chemical, morphological, and nutritional defenses as mechanisms explaining the poor food value of cyanobacteria for herbivorous grayers.

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