

## Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria

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### Abstract

We quantified within-species variation in the tolerance of the large, lake-dwelling daphnid, *Daphnia pulicaria*, to toxic cyanobacteria in the diet. Juvenile growth rates on diets consisting of 100% *Ankistrodesmus falcatus* (a nutritious green alga) or 100% *Microcystis aeruginosa* (toxic) were compared for *D. pulicaria* clones isolated from lakes expected to have low and high levels of bloom-forming cyanobacteria during summer. Growth rates of clones isolated from high-nutrient lakes (range of total phosphorus, 31–235  $\mu\text{g L}^{-1}$ ) were higher, and showed less relative inhibition, on the cyanobacterial diet compared to clones isolated from low-nutrient lakes (range of total phosphorus, 9–13  $\mu\text{g L}^{-1}$ ). Our results suggest that *D. pulicaria* populations exposed to high cyanobacterial levels over long periods of time can adapt to being more tolerant of toxic cyanobacteria in the diet.

A well-established tenet in limnology holds that the taxonomic composition of summer phytoplankton assemblages shifts with phosphorus enrichment toward greater dominance by cyanobacteria (Smith 1986; Trimbee and Prepas 1987; Watson et al. 1997). It is also widely accepted that harmful species of cyanobacteria (temperate species within the genera *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Oscillatoria*) are more likely to attain bloom densities in freshwaters that are nutrient rich (Paerl 1988). One important consequence of this shift toward cyanobacteria with eutrophication is that summer phytoplankton assemblages in eutrophic lakes are relatively resistant to zooplankton grazing (Porter 1977; Lampert 1987; DeMott 1989, 1999; Sarnelle 1993). The grazing resistance of filamentous and colonial cyanobacteria, although not absolute, has been suggested as a reason for escape from grazer control of summer phytoplankton biomass in enriched lakes (Carpenter 1989; Faafeng et al. 1990; Brett and Goldman 1997; Carpenter et al. 2001; Ghadouani et al. 2003). In addition, many laboratory assays have demonstrated that various species of bloom-forming cyanobacteria can inhibit the feeding, growth, and reproduction of zooplankton, and in particular members of the genus *Daphnia*, as well as being of relatively low food quality for herbivores in general (Lampert 1982, 1987).

Limited recent observations, however, suggest that zooplankton populations may adapt to tolerate bloom-forming cyanobacteria in their diets. The seminal observation was perhaps made by Gilbert (1990), who found that a toxic

strain of *Anabaena* reduced the population growth rate of one clone of *Daphnia* to near zero while having no effect on another clone. In this case, the two clones were probably different species (*D. pulex*, a pond-dwelling species, was the sensitive clone; *D. pulicaria*, a lake-dwelling species, was the unaffected clone) because they were isolated from different habitat types (Hebert 1995). Other studies also suggest that *D. pulex* is more sensitive than *D. pulicaria* to inhibition by cyanobacteria (DeMott et al. 1991). Subsequent research has indicated considerable within-species variation in the tolerance of European *Daphnia* to the presence of toxic cyanobacteria in the diet (Hietala et al. 1997; Repka 1997, 1998) and that individual clones can adapt phenotypically to toxic cyanobacteria (Gustafsson and Hansson 2004). Two of these studies examined the hypothesis that *Daphnia* clones from more enriched lakes would be less inhibited by cyanobacteria than clones from less enriched lakes, but found no evidence to support it (Repka 1997, 1998). However, all the lakes from which *Daphnia* were isolated would be considered highly eutrophic by North American standards (total phosphorus [TP]  $\geq 100 \mu\text{g L}^{-1}$ ). Indeed, empirical models predict that summer phytoplankton assemblages will be largely dominated by bloom-forming cyanobacteria at TP levels of  $\sim 100$ – $1,000 \mu\text{g L}^{-1}$  (Trimbee and Prepas 1987; Jensen et al. 1994; Watson et al. 1997; Downing et al. 2001), so differences in selection pressure driving the evolution of cyanobacterial tolerance among such a group of lakes may be slight.

To date, there is evidence indicating that a zooplankton population has adapted to tolerate cyanobacteria for only one lake in Europe. Based on the performance of animals hatched from diapausing eggs deposited over several decades, a temporal correlation was uncovered between the eutrophication of Lake Constance and shifts in the cyanobacterial tolerance of *Daphnia galeata* clones (Weider et al. 1997; Hairston et al. 1999, 2001). In this paper, we seek to extend this observation by asking whether *Daphnia pulicaria* clones isolated from six North American lakes with widely varying levels of enrichment differ in their ability to tol-

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Table 1. Ranges of total phosphorus (TP) for the six lakes.

Category	Lake	Location (Lat. N, Long. W)	TP ( $\mu\text{g L}^{-1}$ )	Data sources*
Low TP	Lawrence	42°26'27", 85°21'03"	8–10	1, 2
Low TP	Sixteen	42°33'90", 85°36'80"	9–12	1, 3
Low TP	Warner	42°28'16", 85°31'30"	12–14	1, 3
High TP	Baker	42°38'90", 85°30'20"	21–40	2, 3
High TP	Wintergreen	42°23'50", 85°23'07"	50–70	3, 4
High TP	MSU Lake 1	42°40'53", 84°28'57"	170–300	5, 6

\* 1, Geedey (1997); 2, A. Tessier (unpubl. data); 3, S. Hamilton (unpubl. data); 4, Manny et al. (1994); 5, Spencer (1981); 6, O. Sarnelle (unpubl. data).

erate the presence of a toxic cyanobacterium, *Microcystis aeruginosa*, in the diet. We address this question with an assay of juvenile growth rate (Hairston et al. 2001). The latter has been shown to correlate with population growth rate (Lampert and Trubetskova 1996). The range of lake enrichment in our study extends from oligotrophic to highly eutrophic and so represents a broader range of potential cyanobacterial abundance than in previous studies.

## Methods

*Daphnia pulicaria* clones were isolated from six small (<0.3 km<sup>2</sup>) lakes in southern Michigan (Table 1). Five of the lakes are natural, and glacial in origin. The sixth, MSU Lake 1, was created as part of a project to treat secondary sewage effluent, and as a consequence has been highly eutrophic since its initial filling with treated effluent in 1973 (Spencer 1981). The six lakes were grouped into two categories based on the midpoint of ranges of summer TP concentration (Table 1): low TP (9–13  $\mu\text{g L}^{-1}$ ) and high TP (31–235  $\mu\text{g L}^{-1}$ ). Empirical models based on temperate lakes worldwide (Trimbee and Prepas 1987; Watson et al. 1997; Downing et al. 2001), and Michigan lakes in particular (Raikow et al. 2004), predict that the percentage of mean summer phytoplankton biomass comprising cyanobacteria should vary greatly across this phosphorus gradient. For example, Raikow et al.'s model for Michigan lakes predicts that percent cyanobacteria in summer should range from 7% to 17% in the low-TP lakes and from 69% to 100% in the high-TP lakes. Published phytoplankton data for Lawrence Lake, Wintergreen Lake, and MSU Lake 1 (Threlkeld 1979; Spencer 1981; Spencer and King 1984; Taylor and Wetzel 1988), and qualitative observations by the authors for Baker Lake (*Microcystis aeruginosa* was common in the lake when we collected *D. pulicaria*) are consistent with this expected difference. In addition, we saw no evidence of bloom-forming cyanobacteria in Lake Sixteen or Warner Lake when we collected *D. pulicaria*, and it is reasonable to assume, based on existing empirical models, that these lakes harbor relatively few bloom-forming cyanobacteria in general. None of the lakes have been invaded by the zebra mussel (*Dreissena polymorpha*), which is known to lead to increases in *Microcystis aeruginosa* in low-TP lakes (Vanderploeg et al. 2001; Raikow et al. 2004; Sarnelle et al. 2005).

Of the 22 *D. pulicaria* clones we isolated, 16 were started from live females and 6 (3 from Lawrence Lake, 2 from

MSU Lake 1, and 1 from Wintergreen Lake) from ephippia taken from surficial sediments. Sediments were collected with an Ekman dredge and females were hatched from ephippia following the general procedures given in Cáceres (1998). Clones from Lawrence Lake and MSU Lake 1 were isolated in 2003; all other clones were isolated in 2004. Each lake was represented by 3–4 clones in the growth experiment, with a total of 12 clones from low-TP lakes and 10 clones from high-TP lakes. All clones were identified as *D. pulicaria* on the basis of the postabdomen of the male. It is currently unknown whether this identification criterion is sufficient to distinguish between *D. pulicaria* and *D. pulicaria* × *D. pulex* hybrids, creating some uncertainty about the possibility that some of our clones could be hybrids (Herbert 1995). However, extensive genetic analyses of the *Daphnia* populations in the three low-TP lakes have never detected hybrid genotypes (Dudycha 2004). Of the high-TP lakes, previous studies also suggest that hybrids do not occur in Baker Lake (Cáceres and Tessier 2004). In the Discussion, we consider ramifications of the possibility that some of the clones from the two lakes with the highest TP (Wintergreen Lake, MSU Lake 1) might be hybrids.

*Daphnia* clones were routinely cultured in the laboratory in a medium consisting of a 50:50 mixture of glass-fiber filtered aquarium water and reagent-grade deionized water. The pH of the *Daphnia* medium was 7.8–8.0. The aquarium used as the water source was initially filled with well water and equipped with an under-gravel filter covered with calcareous gravel, and contained several small fathead minnows (*Pimephales promelas*) that were fed flake food daily. *Daphnia* clones were cultured at 20°C in 500-ml flasks filled with medium and fed the green alga *Ankistrodesmus falcatus* (mean cell dimensions: 2.5  $\mu\text{m}$  × 50  $\mu\text{m}$ ). The alga was grown in semicontinuous culture on modified WC medium (Stemberger 1981), and cells were centrifuged and resuspended in deionized water before being added to *Daphnia* flasks. Cetyl alcohol powder (1–2 mg) was added to each flask to prevent animals from being trapped in the surface film (Desmarais 1997). All 22 clones were grown under common-garden conditions for four generations (~8 weeks) immediately preceding the growth experiment.

Two growth experiments were conducted, the first using *D. pulicaria* clones from three lakes (Lawrence, Wintergreen, and MSU Lake 1), the second using clones from all six lakes. Data from the two experiments were pooled for all analyses. On the day before an experiment, large fecund

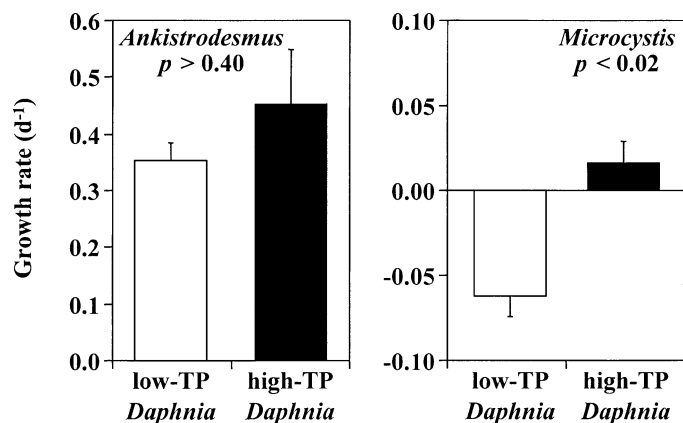


Fig. 1. Mean growth rates of *D. pulicaria* isolated from lakes with low (9–13  $\mu\text{g L}^{-1}$ ) and high (31–235  $\mu\text{g L}^{-1}$ ) total phosphorus concentrations for the two diets, 100% *Ankistrodesmus* (left panel) and 100% *Microcystis* (right panel). Error bars represent standard errors based on variation among lakes in each lake category ( $n = 3$ ). Probability values are for two-tailed  $t$ -tests of differences between means.

daphnids from the stock cultures were pipetted into 40-ml glass vials (~10 daphnids per vial) filled with glass-fiber-filtered lake water (Lake Lanier, an oligotrophic lake in north Georgia) to which *Ankistrodesmus* was added as food. The following day (day 0), neonates (<24 h old) of each clone were transferred individually into 100-ml glass beakers filled with 80 ml of glass-fiber-filtered lake water, and a random subset of neonates was blotted and transferred individually to a dried and tared weighing tin for initial mass estimates (day 0). Standard deviations for initial mass were typically low (average = 0.66  $\mu\text{g}$ ). In most cases, five beakers per clone were employed for each diet, depending on the availability of neonates. Neonates were fed either *Ankistrodesmus falcatus* or a single-celled toxic strain of *Microcystis aeruginosa* (UTEX2667; mean cell diameter 5  $\mu\text{m}$ , cultured in BG-11 medium) at growth-saturating concentrations (1.5 mg C L<sup>-1</sup>) (Lampert 1977). Cells of both species were harvested from exponentially growing batch cultures, centrifuged, and resuspended in filtered lake water before adding to experimental beakers. Beakers were incubated at 25°C with a 16:8 light:dark cycle, and neonates were transferred to new beakers with fresh medium and food daily.

On day 3 of the experiment, each animal was blotted and transferred individually to a dried and tared weighing tin. Tins with animals were then dried at 55°C and weighed to the nearest 0.1  $\mu\text{g}$  on a Cahn C-31 Microbalance. Toxin (microcystin) quota for the *Microcystis* used in the experiments was determined via enzyme linked immunosorbent assay (ELISA) (An and Carmichael 1994). Centrifuged and resuspended cells were collected on glass-fiber filters, stored frozen, and subsequently extracted in 75% aqueous methanol before ELISA.

Instantaneous somatic growth rate ( $g$ , d<sup>-1</sup>) was calculated for each beaker as  $(\ln W_f - \ln W_i)/3$ , where  $W_i$  and  $W_f$  are animal mass on initial (day 0) and final (day 3), respectively. We calculated a relative index of growth inhibition by *Microcystis* for each clone as:  $(g_a - g_m)/g_a$  where  $g_a$  is growth

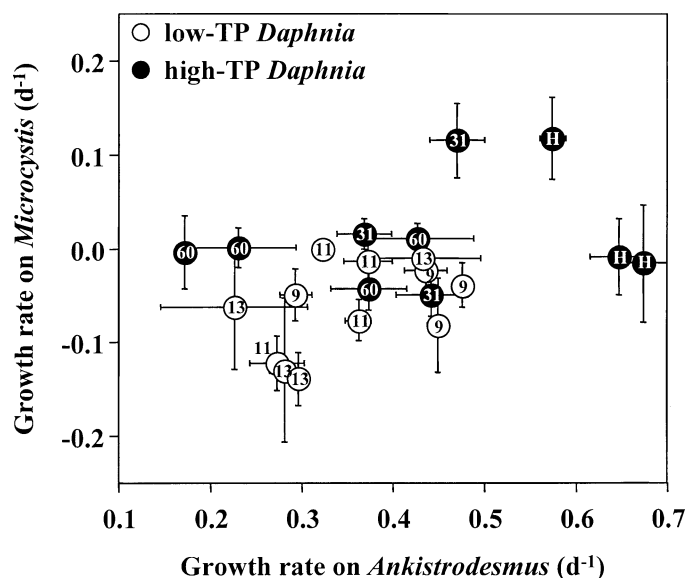


Fig. 2. Growth rates on the two diets (100% *Ankistrodesmus* and 100% *Microcystis*) for each *D. pulicaria* clone. Numbers within each symbol denote total phosphorus concentrations ( $\mu\text{g L}^{-1}$ ) for each of the six source lakes (H = 235). Error bars represent standard errors based on variation among experimental beakers.

rate on *Ankistrodesmus* and  $g_m$  is growth rate on *Microcystis* (Hairston et al. 1999). A larger value of this index indicates greater inhibition of growth rate by *Microcystis* relative to *Ankistrodesmus*. Values greater than 1 can occur if animals gain weight on a diet of *Ankistrodesmus* and lose weight on a diet of *Microcystis*. Growth responses were averaged across clones for each lake, and differences between low-TP and high-TP lakes were assessed via two-tailed  $t$ -tests with lakes as replicates ( $N = 6$ ).

## Results

Survivorship over the three-day experiment was high for all *D. pulicaria* clones on both diets (mean = 92% for *Ankistrodesmus* diet, 80% for *Microcystis* diet), and there were no significant differences in survivorship between *D. pulicaria* from the two lake categories for either diet ( $t$ -tests,  $p > 0.45$ ). *Daphnia* clones generally grew well on a diet of *Ankistrodesmus*, and there was no significant difference in growth on this diet between lake categories (Fig. 1), despite substantial overall variation in growth rate (Fig. 2). Both the slowest- and the fastest-growing clones on the *Ankistrodesmus* diet were from high-TP lakes (Fig. 2).

As expected, all clones grew poorly on the *Microcystis* diet. More importantly, *D. pulicaria* from high-TP lakes grew significantly better, on average, than *D. pulicaria* from low-TP lakes on the *Microcystis* diet (Fig. 1). On average, *D. pulicaria* from low-TP lakes lost weight when fed *Microcystis* (mean growth rate significantly less than 0,  $t$ -test,  $p < 0.04$ ,  $n = 3$ ), while *D. pulicaria* from high-TP lakes did not ( $t$ -test,  $p > 0.30$ ,  $n = 3$ ). Over all lakes, there was a marginally significant positive relationship between *D. pulicaria* growth rate on the *Microcystis* diet ( $y$ ) and lake TP ( $x$ ) (log-log relationship:  $y = -0.05 + 0.03x$ ,  $R^2 = 0.63$ ,  $p$

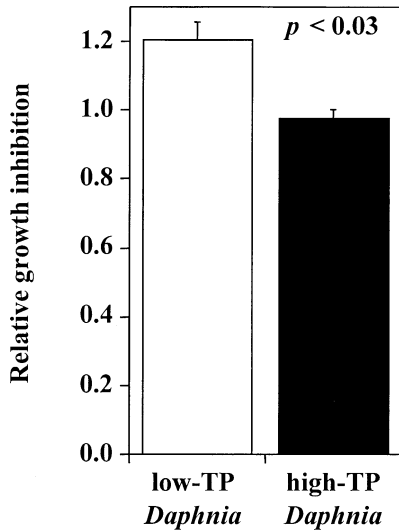


Fig. 3. Mean growth inhibition by *Microcystis* relative to *Ankistrodesmus* for *D. pulicaria* isolated from lakes with low (9–13  $\mu\text{g L}^{-1}$ ) and high (31–235  $\mu\text{g L}^{-1}$ ) total phosphorus concentrations. Error bars represent standard errors based on variation among lakes in each lake category ( $n = 3$ ). Probability value is for two-tailed  $t$ -test of difference between means.

< 0.06,  $n = 6$ ). Given the results for the two diets, it was not surprising that growth inhibition by *Microcystis* relative to *Ankistrodesmus* was higher for *D. pulicaria* from low-TP lakes than from high-TP lakes (significantly different,  $t$ -test,  $p < 0.03$ ; Fig. 3).

Toxin (microcystin) quota for the *Microcystis* used in the experiment was 36  $\mu\text{g mg}^{-1}$  C (standard error = 0.01). This translates to a microcystin concentration in the experimental beakers of 54  $\mu\text{g L}^{-1}$ . The latter should be considered a minimal estimate of exposure level for the *Daphnia* in the experiment because we did not measure dissolved toxin that may have leaked out of cells during the centrifugation and resuspension process.

## Discussion

Adaptation in *D. pulicaria* toward decreased inhibition by toxic cyanobacteria in the diet was assessed by comparing juvenile growth rates on a diet comprising 100% toxic *Microcystis* and by comparing growth inhibition by *Microcystis* relative to a good-quality food (*Ankistrodesmus*). Both analyses indicated that *D. pulicaria* isolated from lakes with high TP concentrations were less inhibited by toxic cyanobacteria than *D. pulicaria* isolated from lakes with low TP concentrations (Figs. 1–3). Given that cyanobacteria are well known to be more prevalent at high TP than at low TP (including the lakes in this study—see Methods), our results suggest that a positive spatial association exists between tolerance of cyanobacteria in *D. pulicaria* and prevalence of cyanobacteria in the environment. This conclusion echoes previous work showing such an association over time in a single lake (Hairston et al. 1999, 2001).

The criterion we used to identify clones to species may or may not be sufficient to distinguish between *D. pulicaria*

and *D. pulicaria*  $\times$  *D. pulex* hybrids; thus it is possible that some of our clones could be hybrids (Hebert 1995). Previous studies have failed to find hybrids in four of the lakes (Cáceres and Tessier 2004; Dudycha 2004), so this uncertainty should only apply to the two lakes with the highest TP, Wintergreen Lake and MSU Lake 1 (Table 1). Given existing evidence that *D. pulex* is more sensitive to inhibition by cyanobacteria than *D. pulicaria* (DeMott et al. 1991), it is reasonable to expect that, if anything, the growth of hybrids should be more negatively affected by cyanobacteria than that of *D. pulicaria*. Thus, if some of the clones from Wintergreen Lake and MSU Lake 1 were hybrids, this would tend to reduce differences in growth response between high-TP and low-TP lakes, making our finding of a difference conservative.

Determining the mechanism by which *D. pulicaria* from high-TP lakes were better able to tolerate cyanobacteria was beyond the scope of this study, and we recognize that the ability to tolerate the toxin microcystin may or may not have played a role. The toxicity of the *Microcystis* strain we used (36  $\mu\text{g microcystin mg}^{-1}$  C) was above the median for strains reported in the literature (Chorus and Bartram 1999), but microcystin concentrations in the experimental beakers (54  $\mu\text{g L}^{-1}$ ) were far below 48-h  $\text{LC}_{50}$  values for *D. pulicaria* (DeMott et al. 1991). It is possible that the differences in tolerance we observed were a function of interactions between the nutritional composition of the *Microcystis* and the abilities of the daphnids to assimilate and/or synthesize compounds needed for growth (Von Elert et al. 2002).

Although there were relatively small differences in both mean growth rate on cyanobacteria (<0.1  $\text{d}^{-1}$ ; Fig. 1) and relative growth inhibition (19%; Fig. 3) between lake categories, it should be noted that these differences were manifest in just 3 d. We kept the experiment short to minimize handling and mortality, which simplified the analysis. Mean *D. pulicaria* growth rate on the *Microcystis* diet was significantly negative for the low-TP lakes but not for the high-TP lakes, which may lead to differences in survivorship over longer time scales. Juvenile growth rate has been shown to be a close correlate of population growth rate and fitness (Lampert and Trubetskova 1996), but we note that this correlation has only been demonstrated across diets differing in quantity. It remains to be seen whether this correlation also holds across diets that differ in quality and toxicity, so we refrain from suggesting that our results are indicative of a 19% difference in the inhibition of population growth rate.

Our results are congruent with those for *D. galeata* populations sampled over time in Lake Constance (Hairston et al. 1999, 2001), but contrast with the results of Repka (1997, 1998), who did not find evidence of higher *Daphnia* tolerance of cyanobacteria in lakes with higher TP. This contrast may be related to the fact that all the lakes examined by Repka (1997, 1998) were highly eutrophic (TP: 100–1,000  $\mu\text{g L}^{-1}$ ) and likely dominated by cyanobacteria in the summer (Trimbee and Prepas 1987; Watson et al. 1997; Downing et al. 2001). Indeed, cyanobacterial dominance may even decrease at TP concentrations >1,000  $\mu\text{g L}^{-1}$  (Jensen et al. 1994). Thus, the gradient in selection pressure driving evolution of adaptation to cyanobacteria may have been much less pronounced across these highly enriched lakes, relative

to gradients in Lake Constance (Hairston et al. 2001) and the lakes in this study (Table 1).

Although our results are, in the main, congruent with those of Hairston et al. (2001), there are some notable differences between the studies. First, Hairston et al. (2001) reported a strong positive correlation between growth rate on good-quality food and growth rate on poor-quality food ( $r = 0.94$ ,  $p < 0.001$ ,  $n = 32$ ), a relationship that was not obvious in our data (Fig. 2;  $r = 0.41$ ,  $p > 0.05$ ,  $n = 22$ ). This contrast could be the result of differences in the nature of the poor-quality diet between studies. The poor-quality diet employed by Hairston et al. (2001) comprised 20% *Microcystis* and 80% *Scenedesmus*, while ours was 100% *Microcystis*. One might expect a stronger correlation in growth rates between diets comprising 80% and 100% of one food species, relative to diets comprising 100% of different species. The lack of strong relationship between growth rates on the two diets in our data suggests that tolerance of cyanobacteria among the *D. pulicaria* we examined may be somewhat independent of the ability to grow fast at saturating levels of good-quality food. Thus, tolerance of cyanobacteria may be independent of traits that confer fitness under the general condition of high food. This is an interesting possibility given that total phytoplankton biomass and percent cyanobacteria increase simultaneously as lakes become enriched.

The second notable contrast between our results and those of Hairston et al. (2001) concerns the degree of variation in cyanobacterial inhibition within each *Daphnia* population. Hairston et al. (2001) reported a decline in variation as cyanobacteria increased in Lake Constance, concomitant with a loss of maladapted (highly inhibited) genotypes. In essence, the distribution of adaptation values was truncated at the low-adaptation end. In contrast, we found fairly similar variation across lake types both in simple growth rates on *Microcystis* (Figs. 1, 2) and relative inhibition by *Microcystis* (Fig. 3). This contrast might relate to differences in the eutrophication history of the lakes involved in the two studies. In Lake Constance, the period of most severe eutrophication lasted about a decade and maximum cyanobacterial dominance was at most about 50% in any year (Hairston et al. 2001). Among the eutrophic lakes we studied, Wintergreen (TP  $\sim 61 \mu\text{g L}^{-1}$ ) has been highly eutrophic for at least 30 yr (Wetzel 1983), and MSU Lake 1 (TP  $\sim 235 \mu\text{g L}^{-1}$ ) was highly eutrophic when first created 30+ yr ago (Spencer 1981) and commonly harbors near 100% cyanobacteria during the growth season (O. Sarnelle, unpubl. data). Perhaps a longer period of high cyanobacteria in these lakes (relative to Lake Constance) may have allowed for colonization by, or in situ evolution of, new genotypes that are better adapted to high cyanobacteria, which could make up for variation lost via the loss of poorly adapted genotypes.

Many laboratory studies have documented that bloom-forming cyanobacteria are a poor food source for zooplankton in general and may differentially inhibit the population growth of large daphnids (Lampert 1982, 1987). However, in many of these studies the source lake of the *Daphnia* clones employed was not specified. Accumulating evidence (Gilbert 1990; Hairston et al. 2001; this study) suggests that the source lake may be critically important—daphnids from

low-nutrient lakes are likely to show greater inhibition than daphnids of the same species from high-nutrient lakes with high concentrations of bloom-forming cyanobacteria. Thus, the presumed impact of cyanobacteria in driving seasonal declines of large daphnids (Sommer et al. 1986) may need to be reappraised in light of this new information. In addition, the evolution of tolerance to poor-quality and potentially toxic cyanobacteria by large species of *Daphnia* may have important consequences for food-web interactions and the response of lakes to eutrophication (Hairston et al. 2001). For example, such adaptation might help to explain why large daphnids are able to increase dramatically and strongly suppress cyanobacterial biomass in highly eutrophic lakes after large reductions in zooplanktivorous fish abundance (Shapiro and Wright 1984; Reinertsen et al. 1990; Sarnelle 1993).

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