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NATURAL SELECTION FOR GRAZER RESISTANCE TO TOXIC CYANOBACTERIA: EVOLUTION OF PHENOTYPIC PLASTICITY?

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Abstract.—We studied the selection response of the freshwater grazing zooplankter, *Daphnia galeata*, to increased abundance of cyanobacteria in its environment. Cyanobacteria are a poor-quality and often toxic food. Distinct genotypes of *D. galeata* were hatched from diapausing eggs extracted from three time horizons in the sediments of Lake Constance, Europe, covering the period 1962 to 1997, a time of change in both the prevalence of planktonic cyanobacteria and levels of phosphorus pollution. We assessed whether the grazers evolved to become more resistant to dietary cyanobacteria by exposing genetically distinct clones to two diets, one composed only of the nutritious green alga, *Scenedesmus obliquus* (good food), and the other a mixture of *S. obliquus* and the toxic cyanobacterium *Microcystis aeruginosa* (poor food). Genotype performance was measured as the specific rate of weight gain from neonate to maturity (g_j).

We evaluated evolutionary change in the *Daphnia* population using an analysis of reaction norms based on relative (log-transformed) changes in g_j. Log(g_j) is a measure of the proportional effect of dietary cyanobacteria on other fitness components of the *Daphnia* phenotype. For comparison, we also analyze absolute (i.e., nontransformed) changes in g_j and discuss the interpretations of the two approaches. Statistical results using a general linear model demonstrate a significant effect of genotype (showing differences in g_j among genotypes), a significant genotype × food-type interaction (showing differences in phenotypic plasticity among genotypes), and, in the case of log-transformed data, a significant sediment-genotype-age × food-type interaction. The latter shows that phenotypic plasticity evolved over the period studied.

Two constraints act on response to selection in the *D. galeata*–Lake Constance system. First, g_j on a diet containing poor food is highly correlated with g_j on a diet of good food, thus evolving resistance also meant evolving an increase in g_j on both diets. Second, because genotypes with a high g_j also grow to a large adult body size, which in turn increases *Daphnia* vulnerability to fish predation, we suggest that selection only acted to favor genotypes possessing a high potential g_j after cyanobacteria became prevalent. The presence of cyanobacteria depressed realized g_j and led to animals of small adult body size even if their genotypes had the potential for high g_j and large size. With realized g_j reduced, genotypes with an inherently high value could be selected even in the presence of predatory fish. The joint action of selection by dietary cyanobacteria and vulnerability to fish predation provides an explanation for the observed evolution of resistance to poor food through reduced phenotypic plasticity.

Key words.—*Daphnia*, developmental plasticity, egg bank, evolution of resistance, genotype-by-environment interaction, reaction norm, selection response.

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Most organisms respond phenotypically, through physiological or developmental plasticity, to changes in their environments (Schlichting and Pigliucci 1998). When individuals differ genetically in the magnitude of their responses, natural selection can cause evolutionary change in this plasticity (e.g., Via 1994; Via et al. 1995) so that phenotypes compensate, at least to some extent, for fitness variation across environments. For animals, one common type of plasticity lies in variable growth response to diet. Examples include the protuberances developed by some rotifers when their diet contains a particular vitamin (Gilbert 1976), herbivorous insects that develop distinct morphologies when feeding on different plant diets (Tauber et al. 1986; Greene 1989), and the distinct jaw morphologies developed by fish fed on differing prey types (Meyer 1987; Wimberger 1991).

Each of these examples of plasticity, and many others both diet based and not (e.g., Tollrian and Harvell 1999), depend on a specific cue in the environment to which genotypes respond. In contrast, a very common phenotypic response to altered diet is a simple change in growth rate mediated by nutritional metabolism. Although growth-rate responses of this type may not at first appear to have adaptive potential, they do if different genotypes express different levels of change in growth rate (i.e., plasticity) when food resources vary. In this case, there exists variance for phenotypic plasticity upon which selection can act. For example, like other organisms, freshwater zooplankton fed a nutritious diet typically grow more quickly and mature sooner than those consuming a more limiting diet (e.g., Twombly and Tisch 2000), and in at least some instances different genotypes fed a common diet exhibit different growth rates (e.g., Mitchell and Lampert 2000; Stibor and Lampert 2000). In the example we consider here, *Daphnia* that experienced a long-term change in the phytoplankton community on which it fed, adapted

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through genetically based changes in the mean growth-rate of somatic tissue (Hairston et al. 1999). Because different *Daphnia* genotypes also differed in the level of change in growth rate when they were fed on two distinct diets, our question is whether these observations also represent an example of evolution of reduced phenotypic plasticity.

Daphnia galeata living in Lake Constance from the mid-1960s to the mid-1970s experienced a pelagic environment undergoing increased abundance of planktonic algae and elevated proportions of cyanobacteria during summer. Cyanobacteria are known to be poor food for zooplankton because they cause mechanical interference with feeding (Haney 1987; Lampert 1987a), have low nutritional value (Ferrão-Filho et al. 2000), and contain hepatotoxins and neurotoxins (Carmichael 1994) that markedly reduce survival (Lampert 1977, 1981a,b; Ferrão-Filho et al. 2000). When cyanobacteria are present in lake plankton, strong selection may be expected to act on generalist grazers such as *Daphnia*, favoring those genotypes that are best able to survive, grow, and reproduce when cells of this poor food source are present. That is, we expect the grazers to evolve resistance to dietary cyanobacteria.

In our study of *D. galeata* in Lake Constance (Hairston et al. 1999), we found that the population possessed genetic variation in its physiological response to dietary cyanobacteria, measured as specific juvenile growth rate, g_j. This has been shown to be a sensitive indicator of genotype response to a variety of physiological conditions (Lampert and Trubetskova 1996; Tessier et al. 2000) and is linearly related to the intrinsic rate of population increase, *r*. Representatives of past populations obtained by hatching diapausing eggs from lake sediments deposited at three different time periods in the past showed evolution of mean *Daphnia* g_j between 1962 and 1997 (Hairston et al. 1999). As phosphorus pollution levels in the lake increased (Güde et al. 1998) and the prevalence of planktonic cyanobacteria climbed, the *Daphnia* evolved so that the mean g_j of genotypes fed a diet containing cyanobacteria increased. We also found, however, that the mean g_j of these same genotypes increased over this time for animals fed a standard high-quality algal diet, thus raising a question of how to interpret the evolution we observed. Because our experiment involved measuring the g_j of multiple *Daphnia* genotypes fed on two distinct food mixtures (with and without cyanobacteria) across three time periods, we evaluate reaction norms as a means of investigating the evolution of resistance to dietary cyanobacteria. This, in turn, provides an opportunity to obtain a more mechanistic understanding of the change in sensitivity to diet composition that we observed.

There are at least two modes by which the *Daphnia* population may have evolved resistance to dietary cyanobacteria. One possibility is a change in phenotypic plasticity. Our performance measure, g_j, expressed as a reaction norm, indicates the physiological response of each genotype to a change in its food environment from one comprised purely of good food to a diet containing cyanobacteria. The slope of this reaction norm is a measure of the extent to which a genotype is impacted by change in diet. Thus, one evolved response would be a decrease in reaction-norm slope (over multiple generations), showing a reduced degree of sensitivity to cyanobacteria and hence evolution of reduced phenotypic plasticity. A second possibility for evolution of resistance would be a general increase in physiological performance in both good and poor food environments as cyanobacteria became more prevalent in the population's diet. Although this latter result could also be interpreted, in the absence of other selection forces, as a response to increasing cyanobacteria in the environment, it would raise the question of why g_j only increased after cyanobacteria became important. As we will describe here, one possible answer is that overall fitness in the field is not a monotonically increasing function of g_j. Animals that grow faster and reach a larger adult size are more vulnerable to predation by visually feeding zooplanktivorous fish.

Quantifying Phenotypic Plasticity

To interpret our data on evolution of resistance using reaction norms, we must first adopt a relevant definition of phenotypic plasticity and then decide on the appropriate way to scale the axis on which phenotypic value is plotted. Via (1994) and Schlichting and Pigliucci (1998) define phenotypic plasticity as any phenotypic change that is caused exclusively by a change in the environment. As Via et al. (1995) point out, plastic changes need not be adaptive. Although the great majority of papers discussing the existence and evolution of plasticity are concerned with adaptive phenotypic change, it is also possible for change to be neutral or even maladaptive. In the case of *Daphnia* responding to food quality, its physiological response, g_j, changes as a function of food quality. If g_j affects overall fitness, as we assume and as Lampert and Trubetskova (1996) showed for laboratory conditions, then its decline in the face of a modest drop in food quality should be considered maladaptive. As a result, we interpret a reduction in this phenotypic plasticity as an adaptive response. That is, the smaller the extent by which the g_j of any given *Daphnia* genotype is affected by the addition of cyanobacteria to its diet, the better adapted that genotype is to variation in food quality in its environment (i.e., it expresses a more generalist response). Natural selection of this kind leads to countergradient variation in which phenotypes are more, rather than less, similar across environmental conditions (Levins 1969; Conover and Schultz 1995).

Scaling Reaction Norms

The question of how to scale reaction norms is critical for interpreting data on the evolution of phenotypic plasticity, and as will be seen in our results, its answer dictates what we conclude about the evolutionary response of *Daphnia* to increases of dietary cyanobacteria in Lake Constance. Essentially, we must decide how the juvenile growth rate, $g_{j,i}$, of genotype *i* is related to its fitness, W_i. Because most studies using reaction norms, including ours, have been carried out to evaluate how a population, or group of populations, has responded to temporally or spatially varying selection, the link from phenotype to fitness is at least implicit. Ideally, a reaction norm should reflect the change in W_i associated with the alternate phenotypes induced by a change in environment. For any given genotype, we wish to know by what proportion

the W_i associated with the expressed phenotypes is affected by a change in environment. For the question addressed in this paper, we wish to know if a proportional change in $g_{j,i}$ reflects an equivalent proportional change in W_i. Thus, if W_i is a linear function of $g_{j,i}$, which is the typical implicit assumption used in studies of fitness surrogates, then it is appropriate to investigate proportional changes in W_i by carrying out graphical and statistical analyses using $\log(g_{j,i})$, which is the same as analyzing the ratios of the $g_{j,i}$. If, instead, W_i is an exponential function of $g_{j,i}$ (i.e., if $\log[W_i]$ versus $g_{j,i}$ is linear), then it is appropriate to use $g_{j,i}$ directly (i.e., without any transformation) in graphical and statistical analyses of reaction norms. This is because a proportional change in W_i in the two environments is equivalent to the difference between the values of $\log(W_i)$. For the results we present here, the answer to the question of whether the *D. galeata* population in Lake Constance underwent evolution of phenotypic plasticity depends critically on whether we carry out a log-transformation of the g_j values and, therefore, what we assume about the relationship between fitness and juvenile growth rate. We conclude that *D. galeata* in this population did evolve reduced phenotypic plasticity because we cannot think of a plausible reason why W_i should be related exponentially to $g_{j,i}$. In the Discussion, we consider this issue further in the light of our data. We present the results of both log-transformed and nontransformed data, however, as an illustration of how this decision can influence conclusions in a study of plasticity evolution.

Materials and Methods

Lake Constance, Its Eutrophication, and Long-Term Changes in the Phytoplankton

Lake Constance is a large (surface area: 541 km^2) and deep (maximum depth: 253 m), subalpine lake at the northern edge of the Alps. The lake experienced increasing eutrophication throughout the period 1920 to 1980 due to rapid human population growth within the watershed (Güde et al. 1998). A common symptom of eutrophication is an increase in planktonic cyanobacteria (Smith 1998), and this taxon appeared in Lake Constance beginning in the mid 1960s. Following international governmental action, phosphorus input to the lake declined markedly from 1978 to the end of the 20th century.

Data on phytoplankton species identity and abundance in Lake Constance are available from a sampling program that began in 1957. For the years 1957–1960, however, extremely rare phytoplankton taxa were not counted and were only noted as being either present or absent (R. Kümmerlin, pers. comm.). For these years, we interpret an absence of cyanobacteria on the list of species encountered as evidence that this group was essentially lacking. Beginning in 1961, samples for species identity and abundance were taken biweekly during the growing season at a central sampling station (Kümmerlin and Bürgi 1989; Kümmerlin 1998). Data are missing only for the years 1963, 1964, and 1983.

To express the changes in cyanobacterial abundance in Lake Constance over the period from 1961 to the present, we calculated the mean biovolume of all members of this taxon for the months July through October, as well as the mean and the maximum percentage that cyanobacteria made up of the total phytoplankton biovolume. This seasonal period is appropriate for our analysis because it includes the entire range of dates that cyanobacteria were abundant in the lake each year (Kümmerlin and Bürgi 1989; Stüber 1998) and much of the period that *D. galeata* was present each year (i.e., May to October; Straile and Geller 1998). *Daphnia galeata* resides exclusively in the epilimnion of Lake Constance, where mean summer temperatures reach 14°C (Stich and Lampert 1981).

Obtaining Daphnia Clones from Sediment Diapausing Eggs

Two species of *Daphnia*, *D. galeata* and *D. hyalina*, have coexisted in Lake Constance throughout the period of eutrophication encompassed by this study. In addition, interspecific hybrids between these two taxa have been detected (Weider and Stich 1992). To explore evolutionary changes in reaction norms, however, it was necessary to obtain genotypes from a range of periods in the past. *Daphnia galeata* overwhelmingly dominated the hatchling pool from the diapausing eggs isolated from the lake's sediments. No *D. hyalina* hatchlings were obtained, and only a very small number of hatchlings (9 of 1407; Weider et al. 1997) were *D. galeata* × *hyalina* hybrids. For this reason, our study is restricted solely to *D. galeata*.

The *D. galeata* clones were collected as diapausing eggs from a sediment core taken at a deep-water station (> 100 m deep) in Upper Lake Constance in 1997. From previous electrophoretic analysis (Weider et al. 1997) and unpublished (RAPD) DNA analysis (L. J. Weider), we know that each clone is genetically distinct. Sediments were dated by counting annual laminations and confirmed by measurements of zinc concentrations and ^{137}Cs-dating in a parallel core, a method that provides very precise sediment dating (Wessels et al. 1995; Weider et al. 1997). The presence of annual layering within these sediments shows that little or no sediment mixing (e.g., by bioturbation) has occurred in the sediments sampled. The core was sliced at 1-cm intervals and *Daphnia* diapausing-egg cases (ephippia) were isolated by sieving. Because each 1-cm slice included roughly three annual laminations, the ages of the eggs obtained are only known within this accuracy. Each egg that hatched was established as a parthenogenetic isoclonal line for at least 40 generations so that any maternal effects carried over from the diapausing eggs were minimized.

Thirty-two clones of *D. galeata* from three sediment-age categories were tested for resistance to cyanobacteria in their diets. Twelve clones were tested from sediment ages before and just after the appearance of cyanobacteria in the lake water column: two clones from 1962–1964 and 10 from 1969–1971 (only two clones were used from 1962–1964 because only two eggs hatched from this sediment layer). Ten clones were tested from 1978–1980, the time of peak eutrophication, and 10 clones were assayed from the most recent sediments (three from 1992–1994 and seven from 1995–1997). With the exception of the two clones from the 1962–1964 sediment layer, the remaining 30 clones were chosen haphazardly from a larger clone bank collection of 112 clones isolated from a range of sediment ages.

Measuring Daphnia Performance in the Presence of Cyanobacteria

The resistance of each clone to dietary cyanobacteria was measured as the impact of food environment on somatic juvenile growth rate, g_j (day^{-1}), the specific rate of mass increase during the period from neonate to maturity: $g_j = [\ln(M_m/M_n)]/t$, where M_m and M_n are the masses (mg) of the mature and neonate *Daphnia* and t is the period from neonate ($\leq$16 h old) to maturity (typically 5 or 6 days at the 20°C of our experiment). This equation models growth as an arbitrary function of time where the rate, g_j, is the mean value over the time period measured (Voronov 1991) and thus provides a suitable comparative measure of performance among clones.

Juvenile growth rate is highly correlated ($r^2 = 0.99$) in laboratory experiments with life-table estimates of the instantaneous rate of increase for *Daphnia* populations (Lampert and Trubetskova 1996) and therefore has been interpreted as a reasonable measure of fitness in an exponentially growing population without other constraints on body size such as size-selective predation. Here, however, it is used strictly as a measure of relative genotype performance, because rapid growth also leads to large body size, which can result in low fitness in the presence of zooplanktivorous fish (see below). For each clone, g_j was measured for animals fed on two different diets, one a poor food diet containing a mixture of the cyanobacterium *Microcystis aeruginosa* (20% by carbon content) with a high quality algal resource (*Scenedesmus obliquus*, 80% by carbon content), and the other a good food diet containing only the *Scenedesmus*. The *Microcystis* culture we used was originally isolated by H. Mueller from Lake Constance in 1972 during the period of eutrophication. It had previously been shown to be toxic to *Daphnia pulicaria* (Lampert 1981a,b, 1987a), had a substantial impact on reducing *D. galeata* growth rates in our preliminary experiments, and was found by HPLC analysis carried out at the time of our study to contain high concentrations of microcystin-LR, a known hepatotoxin. The *Scenedesmus* culture we used has been shown to be a good food for *Daphnia* growth and reproduction (Lampert 1987b). We fed *Microcystis* in a mixed food source with *Scenedesmus* because it is lethal to *Daphnia* in pure culture (Lampert 1981a,b) and because cyanobacteria were only a dominant group in the Lake Constance phytoplankton for brief periods in summer around the time of peak eutrophication (Stüber 1998; Gaedke 1998; Kümmerlin 1998). Both *Microcystis* and *Scenedesmus* were fed in single-celled (noncolonial) morphologies with modal cell diameters of 4.2 μm (range: 3.4–5.5 μm) and 4.3 μm (3.0–7.3 μm), respectively.

Both poor food and good food diets were supplied to the growing *Daphnia*. For each clone, $g_{j,good}$ and $g_{j,poor}$ were measured separately using replicate 250-ml flow-through culture chambers that sustained food at levels well in excess of growth-limiting concentrations (Lampert et al. 1988). Three replicate chambers, each containing five to 17 individuals, were used for each growth-rate measurement on each *Daphnia* clone. Chambers were maintained in controlled temperature baths at 20°C (±0.2°C). Fresh food suspensions of both diets at concentrations of 1 mg C/L were prepared daily from exponentially growing algal cultures and supplied to the flow-through chambers via peristaltic pumps at a rate of 1 L/day. Initial weights were determined for each clone using a minimum of seven individual neonates isolated at the time of the start of a growth trial. At the termination of a growth trial, all individuals within each chamber were isolated and weighed together. All weight measurements were made on animals dried for 24 h at 60°C. In addition, the length of each individual was measured as the distance from the top of the eye to the base of the tail spine, using an ocular micrometer.

The experimental set-up, which contained 30 flow-through chambers, permitted growth-rate measurements to be carried out on five clones at a time (two food treatments × triplicate measurements × five clones). Thus, assays of the 32 clones included in this study required seven separate runs of the set-up. To minimize potential biases that might be introduced by running trials at different times, clones from each sediment age were included in each run. All clones in a run were initiated at the same time from neonates (newly hatched animals ≤16 h old). A run was terminated when the first animals in any clone began to carry eggs. All eggs carried were included in the final weight measurements of ovigerous females. Because our performance measure is specific-weight change, it is not critical that all animals start and finish the experiment at exactly the same age or developmental stage.

Data Analysis: Defining Reaction Norms

To investigate the reaction norms of each *Daphnia* genotype from each time period, both direct (i.e., nontransformed) and log-transformed juvenile growth rates (g_j) were analyzed. For nontransformed data, the reaction norm is simply the difference between $g_{j,good}$ and $g_{j,poor}$. As explained in the introduction, the reason for log-transformation is not for normalization (in fact, the residual plots and normal probability plots were not significantly different from normal either with or without transformation), but rather to evaluate the impact of food environment on fitness when g_j is taken to be a relative measure of performance. The resistance of a genotype to dietary cyanobacteria, and thus its phenotypic plasticity with respect to food type, is then the fraction by which performance (g_j) is reduced when fed poor food in comparison with good food. In this case, the reaction norm of a genotype is a ratio (e.g., $[g_{j,good}]/[g_{j,poor}]$) that is equivalent to the difference between the log-transformed growth rates ($\log[g_{j,good}] - \log[g_{j,poor}]$). This approach is mathematically comparable to the index of resistance to dietary cyanobacteria (growth rate reduction $= 1 - [g_{j,poor}/g_{j,good}]$) used by Hairston et al. (1999), but provides a more explicit depiction of how genotypes differ.

For both the log-transformed and nontransformed data, phenotypic plasticity was investigated using a mixed general linear model (SAS Institute 1998). The model included sediment age, food, and sediment-genotype-age × food-type as fixed effects. Genotype, nested within sediment age, and genotype × food-type were modeled as random effects and tested using likelihood ratio tests (Littell et al. 1996). Statistical analyses were performed using SAS (1998) PROC MIXED and Data Desk (Velleman 1997). A significant effect

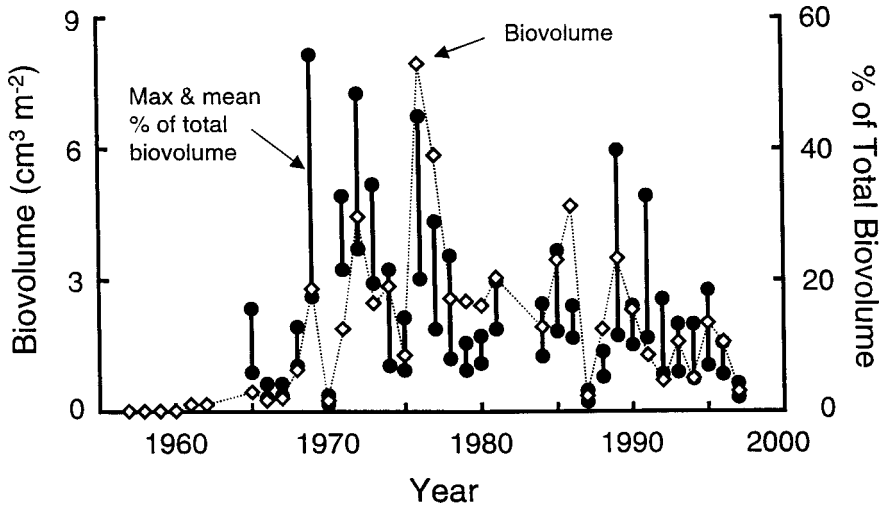

FIG. 1. The density and percent composition of cyanobacteria in the water column of Lake Constance during July through October for the period 1957 to 1997. Density (open diamonds) expressed as biovolume ($cm^3\ m^{-2}$). Percent composition, as biovolume of cyanobacteria relative to the total biovolume of all phytoplankton, is presented (filled circles connected by a bar) both for the mean percentage (bottom circle) during July through October of each year and the maximum percentage (top circle) recorded during this period.

of genotype alone would indicate that the clones differ in juvenile growth rate. Significance of the genotype by food-type (i.e., genotype × environment; G × E) interaction term would indicate that genotypes differ in phenotypic plasticity for physiological response to food type and significance of the sediment-genotype-age by food-type (i.e., age × environment; A × E) interaction term would indicate that this phenotypic plasticity evolved over the period of time encompassed by our study.

RESULTS

Eutrophication and Long-Term Changes in the Phytoplankton

Cyanobacteria increased in abundance in the main basin of the lake from undetectable levels prior to 1961 to mean summer values of between 1.3 and 8.0 cm^3/m^2 for the years 1969 to 1979, with 1970 as the only exception (Fig. 1). During this period, total phosphorus concentration in the lake at the time of winter mixing in the water column increased from 15 to more than 85 μg/L (Güde et al. 1998). A lake management program brought about reductions in phosphorus concentrations beginning in 1980, so that by 1997 water column total phosphorus had declined to 18 μg/L. Summer cyanobacterial biovolume also declined somewhat in the 1980s and 1990s, but showed considerable interannual variation (Fig. 1). Although cyanobacteria were never a dominant component of the phytoplankton for an extended period in any year (Sommer et al. 1993; Gaedke 1998; Kümmerlin 1998), from the late 1960s through the 1970s they increased in density and also made up an increasing percentage of the phytoplankton during summer months (Fig. 1). At the end of the period of phosphorus increase in the lake (1969–1979), cyanobacteria averaged 13% (range = 1–25%) of mean summer phytoplankton biovolume and attained maxima that averaged 28% (range = 2–54%) of total phytoplankton biovolume. This brackets the 20% of carbon content that cyanobacteria made up in our experimental poor-food treatment.

The Effect of Dietary Cyanobacteria on Daphnia Length, Weight, and Growth

Consistent with earlier investigations of the effects of cyanobacteria on *Daphnia* physiology (Lampert 1981a, 1987a,b), all *D. galeata* clones in our study experienced decreased juvenile growth rate when fed a mixture of *Microcystis* and *Scenedesmus* in comparison with pure *Scenedesmus* (Fig. 2; Table 1, food effect). Across all sediment ages, growth rate on poor food was between 12% and 42% lower than that on good food, depending upon the clone.

The effect that cyanobacteria had on *Daphnia* growth resulted in diminished body weight for a given length (Fig. 3). Clones fed on poor food had a significantly lower intercept for their log(length) versus log(weight) regression than the same set of clones fed good food (ANCOVA, df = 1, F = 31.0, $P \leq 0.0001$), although the slopes were virtually identical (ANCOVA interaction term, df = 1, F = 0.17, P = 0.68). In addition, clutch size was significantly lower when clones were fed the diet containing cyanobacteria (ANOVA, df = 1, F = 18.3, P = 0.0003). Finally, our experience in this study strongly suggests that time to maturity was substantially longer for animals given poor food, but we did not collect the necessary data from this experiment to assess this statistically. Despite these differences, the poor-food diet was adequate for survival to maturity; individual mortality was very low in all experimental chambers in both food treatments.

Reaction Norms: Data Not Transformed

The reaction norms using g_j data without transformation for each of the genotypes (clones) are illustrated in Figure 2. In addition to the effect of food type on juvenile growth rate, there is also a significant difference among genotypes in overall g_j (Table 1; $P < 0.005$). There is a significant effect of sediment-genotype-age on g_j (Table 1; P = 0.0086) showing that the mean growth rate of the *Daphnia* genotypes in-

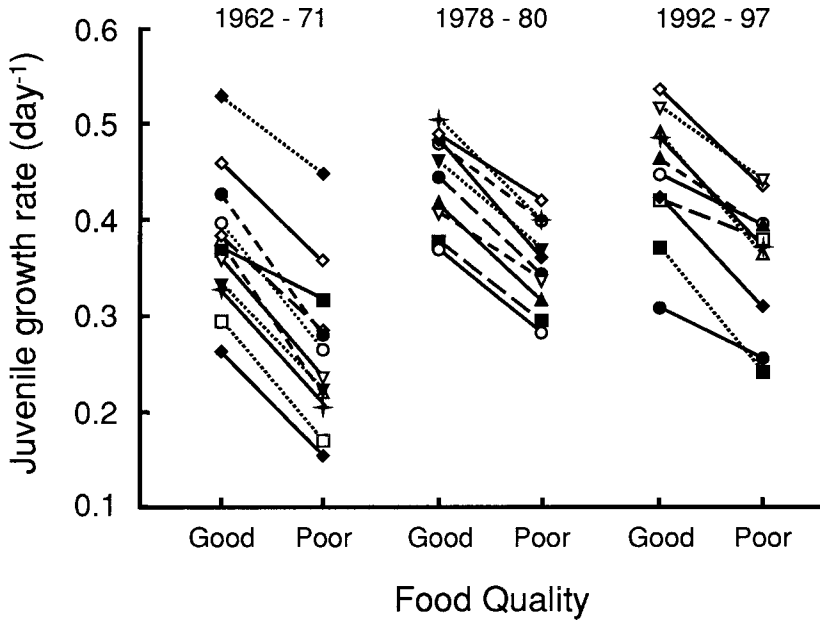

FIG. 2. Norms of reaction for *Daphnia galeata* juvenile growth rate (g_j, scale not transformed) as a function of two food types: good food is 100% *Scenedesmus obliquus* and poor food is 80% *S. obliquus* and 20% *Microcystis aeruginosa* (a toxic cyanobacterium isolated from Lake Constance). Each line represents results for a genetically distinct clone cultured from a diapausing egg hatched from sediments laid down during one of three time periods (see Fig. 1): early in the appearance of cyanobacteria in the phytoplankton (1962–1964 and 1969–1971), the period of peak cyanobacteria density (1978–1980), and recent sediments (1992–1994 and 1995–97). Across all time periods, the slopes of the lines vary showing genetic variation for resistance. That is, there is a significant effect of genotype on g_j (i.e., a significant G × E, genotype × food-type interaction). However, the A × E (sediment-genotype-age × food-type) interaction is not significant, suggesting that there was no genetic change in resistance to dietary cyanobacteria, and thus no evolution of reduced phenotypic plasticity. But, compare this conclusion with Figure 4.

creased from one time period to the next with significant increases between both the first (1962–1970) and second (1978–1980) and the first and third (1992–1997) sediment-genotype ages (preplanned contrasts: df = 1, 29; $F = 7.70$; $P = 0.0096$; and df = 1, 29; $F = 8.68$; $P = 0.0063$, respectively) but not between the second and third ages (df = 1, 29; $F = 0.03$; $P = 0.8717$). Within each time period there is a significant genotype × food-type interaction (Table 1; $P < 0.05$) indicating that there are genetic differences in phenotypic plasticity for growth response to diet (G × E). There is, however, no significant sediment-genotype-age × food-type (A × E) interaction (Table 1; $P = 0.0624$), although the *P*-value does not greatly exceed 0.05. Under the assumptions that accompany not transforming the g_j data, this result suggests that phenotypic plasticity did not change over time.

TABLE 1. Results from a mixed general linear model using the untransformed data. Fixed effects were tested with approximate *F*-tests; ndf, numerator degrees of freedom; ddf, denominator degrees of freedom. Random effects were tested with likelihood-ratio tests (LR test). Likelihood ratio test = −2 (maximum likelihood from the test's full model − maximum likelihood from a reduced model with the random parameter removed). LR test has a chi-squared distribution with degrees of freedom equal to the difference of parameters between the full and reduced models.

Fixed sources	ndf	ddf	Type III *F*	*P*
Sediment age	2	29	5.640	0.0086
Food	1	29	399.9	0.0001
Sediment age × food	2	124	2.840	0.0624
Random sources	**df**		**LR test statistic**	***P***
Genotype	1		238.7	<0.005
Genotype × food	1		5.0	<0.05

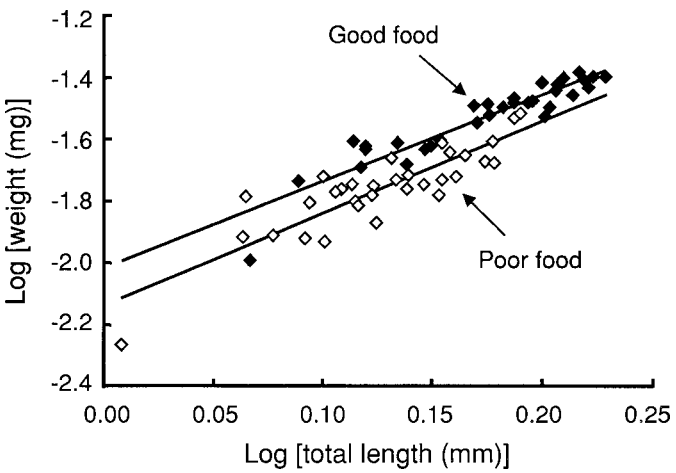

FIG. 3. Length-weight relationships (log scales) between *Daphnia galeata* adult weight and adult length (at first reproduction) for animals reared on poor food containing a mixture of cyanobacteria and green algae and those reared on good food containing only green algae. The lines have significantly different intercepts but not different slopes (see Results), showing that the poor food diet causes animals to mature at a smaller body mass in comparison to their length than does the good food diet.

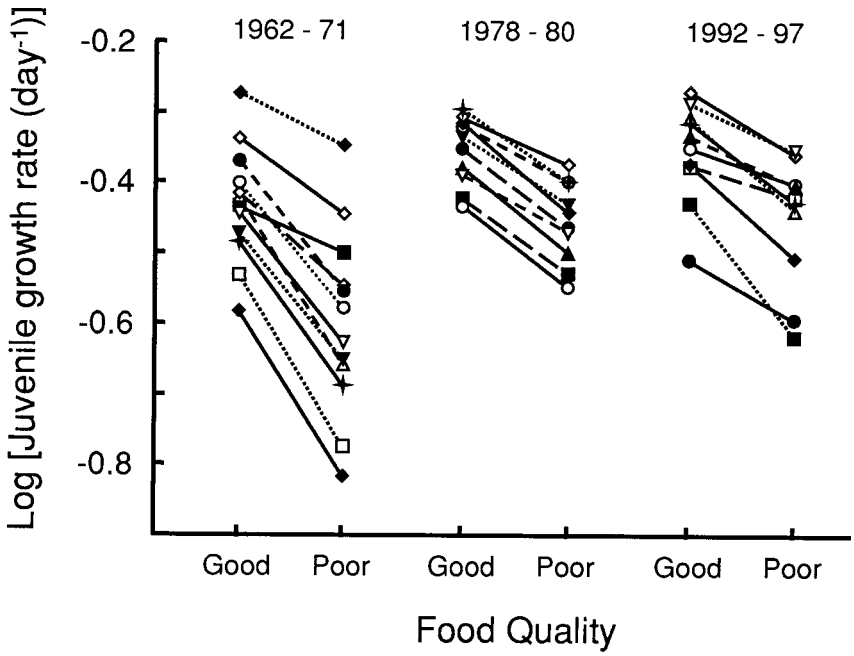

FIG. 4. Norms of reaction for *Daphnia galeata* juvenile growth rate, g_j, as in Figure 2, but with g_j plotted on a log-transformed scale. In addition to the significant effect of genotype on g_j and the significant G × E (genotype × food-type) interaction seen in Figure 2, there is also a significant A × E (sediment-genotype-age × food-type) interaction representing a genetic change in resistance to dietary cyanobacteria and thus the evolution of reduced phenotypic plasticity.

Reaction Norms: Data Log-Transformed

The reaction norms using log-transformed g_j data ($\log_{10}$ throughout) for each of the genotypes (clones) are illustrated in Figure 4. As is the case for the nontransformed data, there is a significant difference among genotypes in overall g_j (Table 2; $P < 0.005$). Within each time period, there is a significant genotype × food-type interaction (Table 2; $P < 0.005$) again indicating that there are genetic differences in phenotypic plasticity for growth response to diet (G × E). Most interestingly, there is a significant sediment-genotype-age × food-type (A × E) interaction (Table 2; $P = 0.0006$) showing that this phenotypic plasticity changed over time. This result is the opposite of that reached in our analysis of the nontransformed g_j data. Further, planned contrasts reveal that the A × E interactions are significant (i.e., the reaction-norm slopes differ) between the first and second (df = 1, 124; $F = 10.36$; $P = 0.0016$) and the first and third sediment-genotype ages (df = 1, 124; $F = 12.29$; $P = 0.0006$), but not between the second and third sediment-genotype ages (df = 1, 124; $F = 0.07$; $P = 0.79$).

TABLE 2. Results from a mixed general linear model on the log-transformed data. See Table 1 for details.

Fixed sources	ndf	ddf	Type III *F*	*P*
Sediment age	2	29	6.290	0.0054
Food	1	29	222.9	<0.0001
Sediment age × food	2	124	7.800	0.0006
Random sources	df		LR test statistic	*P*
Genotype	1		202.1	<0.005
Genotype × food	1		13.6	<0.005

Correlates of Juvenile Growth Rate Phenotypes

Within the general increase in mean juvenile growth rate of the *Daphnia* genotypes discussed above, there is not only a significant increase in $g_{j,poor}$ over time (Fig. 4, ANOVA, df = 2, $F = 7.31$, $P = 0.0027$) , as might be expected from selection imposed by the appearance of cyanobacteria in the diet, but an accompanying increase in $g_{j,good}$ (Fig. 4, ANOVA, df = 2, $F = 4.23$, $P = 0.024$). Important for understanding this result is the fact that $g_{j,good}$ is highly correlated with $g_{j,poor}$ (Fig. 5, df = 30, $r = 0.936$, $P < 0.001$) so that selection response by one necessitates a response by the other.

Finally, in understanding how g_j influences overall fitness of individual *Daphnia* in the lake, it is important to note that the genotypes that grow the most quickly are also the ones that grow to the largest size at maturity (Fig. 6, ANCOVA for effect of log[weight] on log[g_j], df = 1, $F = 30.5$, $P < 0.001$). As will be discussed below, large body size can be a disadvantage in the presence of visually orienting zooplanktivorous fish.

DISCUSSION

The weight-specific juvenile growth rate, g_j, of the *D. galeata* population in Lake Constance evolved from a low mean value in the 1960s to higher means by the end of the 1970s, a change that persisted through the 1990s. Concluding whether the phenotypic plasticity of g_j also evolved depends on how these phenotypic values are considered to influence overall fitness of individuals living in the lake. The microevolutionary changes that occurred in the *Daphnia* population took place over the same time period as an observed increase in cyanobacterial abundance in the water column and in-

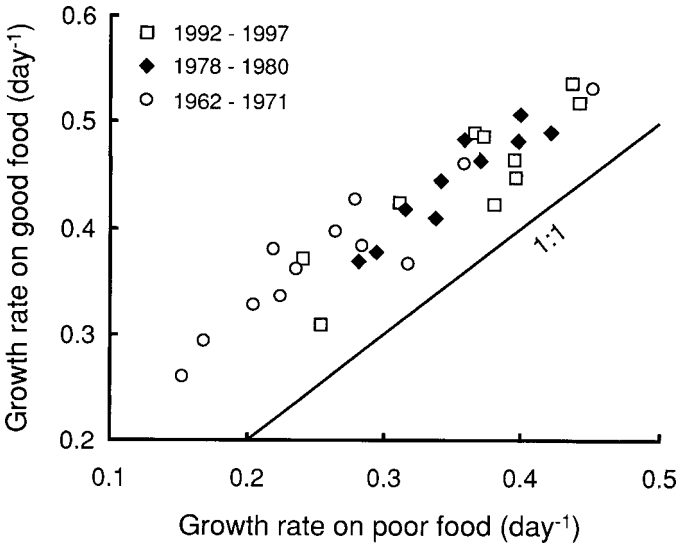

FIG. 5. Juvenile growth rates (g_j) of *Daphnia galeata* fed on good food versus that of animals fed on poor food. Each datapoint represents one of 32 genotypes hatched from diapausing eggs taken from the sediments of Lake Constance dated to one of three different time periods. Growth rates in the two food environments are highly significantly correlated (see Results), meaning that natural selection for genotypes with a high g_j on poor food must also select for genotypes with a high g_j on good food. Comparison of the data with the 1:1 line shows that $g_{j,good}$ is greater than $g_{j,poor}$ for all genotypes.

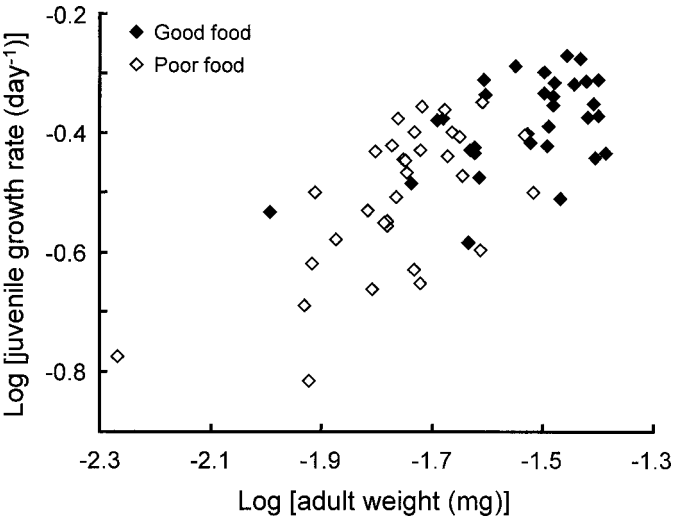

FIG. 6. The relationship between juvenile growth rate (g_j) and adult (at first reproduction) body mass for 32 genotypes of *Daphnia galeata* reared on both good (filled diamond) and poor (open diamond) food conditions. The data on both axes are log transformed. The statistically significant relationship shows that *Daphnia* that grow more quickly do so in part by growing bigger. This means that high g_j is not equivalent to fitness in the wild because large-bodied animals are often selectively consumed by visually orienting zooplanktivorous fish.

creased phosphorus concentrations in the lake due to cultural eutrophication. We conclude that cyanobacteria, which are known to be poor-quality food and are in many cases toxic, acted as the selection force to which the *Daphnia* population responded. These selection responses are strikingly rapid, occurring within the time span of little more than a decade. *Daphnia*, which are cyclical parthenogens, have approximately one sexual generation per year, in which diapausing eggs are produced. The intervening bouts of parthenogenetic reproduction, however, can produce a new generation every one to three weeks, depending on water temperature, with about 20 generations produced over the course of a summer (Geller 1989). Interestingly, over the long term (i.e., multiple bouts of sexual reproduction) cyclical parthenogenesis is expected to have little influence on the rate of response to directional natural selection (Lynch and Gabriel 1983; Lynch and Deng 1994). Below we discuss the basis for our conclusion that we have observed evolution and raise some additional considerations concerning the changes in the lake environment that accompanied its eutrophication.

If *D. galeata* g_j is directly proportional to the logarithm of fitness, then the relative decline in the fitness of a genotype reared in good versus poor food environments is appropriately expressed as the difference between the nontransformed $g_{j,i}$ values. Analysis of reaction norms under this assumption (Fig. 2), while clearly showing evolution of g_j, only hints at an evolution of phenotypic plasticity (A × E not quite significant at $P = 0.0624$, see Results). If, instead, the $g_{j,i}$ of a *Daphnia* genotype is directly related to its fitness, then a relative change in fitness should be evaluated as the proportional change in $g_{j,i}$ (i.e., the ratio of $g_{j,i}$ values) or equivalently as the difference between the logarithms of $g_{j,i}$ on the two food types. Analysis of reaction norms in this case (Fig. 4), shows, in addition to evolution of g_j, a highly significant reduction in the slope of the mean reaction norm of $\log(g_{j,i})$ over the time periods from which the *Daphnia* genotypes were obtained (A × E significant at $P = 0.0006$, see Results). This would indicate that the mean resistance (Fig. 7) of the *Daphnia* population increased after cyanobacteria became prevalent. The most logical explanation for this observation would be that increased cyanobacterial densities acted on the *Daphnia* population as an important selection agent.

Why do we reach different conclusions about the evolution of phenotypic plasticity depending upon whether the data are log-transformed? The answer lies in how relative changes in the $g_{j,i}$ values covary with their absolute values. Although we detected genetic variation in phenotypic plasticity (significant G × E) in the nontransformed data, the slopes of the reaction norms do not differ greatly among genotypes (Fig. 2). This means that the genotypes with the lowest mean $g_{j,i}$ values are necessarily the ones that show the greatest proportional change in $g_{j,i}$ as a function of food quality. That is, any given difference between $g_{j,good}$ and $g_{j,poor}$ comprises a larger fraction of a small $g_{j,good}$ than it does of a large $g_{j,good}$. As a result of this correlation, the evolution of phenotypic plasticity that we observe using log-transformed data can be interpreted in two different ways. On one hand, the evolution of an increase in mean g_j may have simply led to the apparent evolution of reduced phenotypic plasticity. On the other, the evolution of reduced phenotypic plasticity may have necessarily resulted in the evolution of increased mean g_j because genotypes with large mean g_j happen to be the ones with the greatest resistance. We note, however, that the two effects need not be mutually exclusive and that the selection effect

FIG. 7. Mean norms of reaction (±1 SE) of juvenile growth rates (g_j) under two different food conditions for *Daphnia galeata* genotypes hatched from three different sediment-age categories. Isoclonal lines of each of 32 genotypes were reared under both good food (no cyanobacteria) and poor food (with cyanobacteria) conditions (see Materials and Methods). Twelve clones came from hatching diapausing eggs deposited in the sediments of Lake Constance during some of the years (1962–1964 and 1969–1971) that cyanobacteria were just beginning to become prevalent; 10 clones came at the end of the decade that cyanobacteria were most abundant in the plankton (1978–1980); 10 clones came from recent sediments (1992–1994 and 1995–1997). The mean g_j under good food conditions for the clones originating from the 1960s is very similar to (and not statistically different from) g_j under poor food conditions for the clones originating from the late 1970s and the 1990s (see Discussion).

of increasing cyanobacteria in the environment might have acted to favor those genotypes that possessed both greater growth rates and a reduced sensitivity to cells of this taxon in their diet. These correlations are explored in more detail below.

Diapausing Eggs as a Historical Record

Our study of microevolution in *D. galeata* makes use of its long-lived diapausing egg bank in Lake Constance sediments. The approach makes at least two assumptions. The first is that the increase in mean g_j we see through time (i.e., a decline in g_j as a function of mean sediment-genotype age) is not simply a result of senescence of the eggs as they sit in the sediments. One argument against this concern is that the maximum g_j remains essentially constant over the period studied; that is, one would have to postulate further that not all eggs senesce. Another argument against it is that mean g_j did not change linearly with time but only changed significantly between the first and second sediment-genotype ages (i.e., between 1962–1971 and 1978–1980 and not between 1978–1980 and 1992–1997). One would have to postulate, ad hoc, that it is purely a coincidence that this change occurred at the time that cyanobacteria increased most dramatically in abundance. The second assumption is that the eggs that we hatched from different sediment depths are representative of the genotypes present in the water column at the time that they were deposited. For example, it might be that there is differential loss from the egg bank of particular genotypes (e.g., those with the highest g_j or the greatest plasticity of g_j) through time. Weider et al. (1997) found, however, good concordance between allele frequencies in the *D. galeata* population in the water column (averaged across the year for 1989–1990) and those frequencies present in the corresponding egg bank (1989–1990). Furthermore, it is difficult to explain by a hypothesized mechanism of egg aging how variance in either plasticity or g_j after decreasing from the 1960s to the late 1970s then increased again significantly by the 1990s. In contrast, microevolution combined with the reintroduction of genotypes from the past (via the egg bank) can explain all of our observations. Indeed, the egg bank provides a singular means of investigating microevolutionary processes by studying living fossils not only in Lake Constance, but in other lakes as well (e.g., Ellner et al. 1999; Cousyn et al. 2001). Furthermore, the egg bank can act as an important source of genetic variation (e.g., Ellner and Hairston 1994; Hairston et al. 1996; Ellner et al. 1999) as distinct genotypes disperse temporally from one time period to another.

Correlates of Juvenile Growth Rate

If selection in the lake acted on g_j through the presence of a poor-food environment, it is easy to understand why mean $g_{j,poor}$ increased. But why did mean $g_{j,good}$ also increase? We propose that the explanation for the increase in $g_{j,good}$ lies in constraints on the way that selection acts on this population. One constraint, internal to the physiology of the *Daphnia*, is that $g_{j,poor}$ is highly correlated with $g_{j,good}$ (Fig. 5), which means that selection response resulting in an increase in the $g_{j,poor}$ must as a consequence also result in an increase in the $g_{j,good}$. As cyanobacteria increased in prevalence in the summer phytoplankton of the lake, genotypes that were better able to grow and reproduce in this environment were favored. Because of the existing trait correlation in this population, these genotypes also possessed higher growth rates on good food. It might seem axiomatic that a genetic disposition to high juvenile growth rate on one diet should carry with it a tendency to high growth on another type of food, but this would not necessarily be true if one of the diets contains a toxic species such as the *M. aeruginosa* used here. The influence of the toxin on *Daphnia* physiology and its amelioration by some genotypes could easily affect some fitness component other than growth rate (e.g., survival). Thus, it is nontrivial that the g_j values on good and poor food turn out to be highly correlated.

Our data showing that the *Daphnia* population present prior to the appearance of significant cyanobacterial densities possessed high variation in juvenile growth rate raises the question of why selection had not previously resulted in a population dominated by genotypes with the greatest g_j values. Two possible answers postulate the existence of one or more additional selection forces acting so that high g_j does not imply high fitness under all conditions in the wild. The first possibility lies in the fact that genotypes with high g_j are also, on average, those that mature at large adult body size (Fig. 6), and large size is not advantageous in the presence of visually orienting zooplanktivorous fish. The selective

feeding by fish on larger-bodied prey is well established (e.g., Brooks and Dodson 1965; Zaret 1980; Lampert and Sommer 1997). In Lake Constance, *D. galeata* is restricted in distribution to the surface waters of the lake (primarily between 5 m and 10 m) during the day where it is maximally exposed to fish predation (Stich and Lampert 1981). The species is present in the lake plankton only during the warmer months of spring and summer when fish feeding activity is likely to be greatest (e.g., Hairston 1988), and stable isotope data for the Lake Constance food web (i.e., $\delta^{15}N$; D. Straile, pers. comm.) show that the primary zooplanktivorous fish in the lake, whitefish and perch, feed at the trophic level directly above the *Daphnia*. If size-selective fish predation was important in the lake from the 1960s to the 1990s, then during the period when cyanobacteria was rare or absent (prior to mid-1960s), selection against large body size by zooplanktivorous fish would have also selected against the genotypes with the highest growth rates. However, as cyanobacteria increased in the water column and in the diet of *Daphnia*, the realized growth rate of all genotypes would have been reduced (Fig. 2), as would have been their adult body size (Fig. 6). This in turn would have permitted the fastest-growing genotypes to become dominant, because during summer the maximum body size obtained by these animals was smaller than it would have been in the absence of cyanobacteria. In fact, the mean growth rate of the genotypes present in 1962–1971, when fed good food, is virtually identical to the mean growth rate of the genotypes present in 1978–1980 (and 1992–1997), when fed on poor food (compare circles on good food with squares and triangles on poor food in Fig. 7). If correct, our hypothesis here would suggest that the animals in the lake attained roughly the same adult body size during the periods before and after cyanobacteria became prevalent.

This predation-based explanation holds provided that the selection effect of fish predation was both sufficiently strong and roughly constant over the period in question. Annual estimates of zooplanktivorous fish density in Lake Constance between 1956 and 1997, based on fisheries yields, show only modest variation of between about 700 and 1800 metric tons and no clear long-term trend (Eckmann and Rösch 1998). The importance of fish predation on *D. galeata* remains to be clarified, however. Mass-balanced flow models of the Lake Constance food web (Gaedke and Straile 1994; Straile 1998) suggest that predatory invertebrates (*Bythotrephes* and *Leptodora*), which selectively consume intermediate-sized *Daphnia*, may be the primary consumers of *D. galeata*. In addition, analyses of whitefish stomach contents suggest a diet composed primarily of *Bythotrephes* (Becker and Echmann 1992), a result in conflict with Straile's (1998) stable isotope results discussed above.

A second possible reason why slower-growing genotypes were present during the earliest time period of our study is that individuals with low g_j may be better at surviving conditions of low food abundance. Tessier et al. (2000) documented such a trade-off among *Daphnia* species from North America. As Lake Constance became increasingly eutrophied, the mean biomass of small readily consumed phytoplankton (i.e., nanoplankton <30 μm diameter) in the lake during the primary *Daphnia* growing season (May–September) increased from about 3–4 cm^3/m^2 during the mid-1960s to nearly 20 cm^3/m^2 by the late 1970s (U. Gaedke, unpubl. data). If there were a negative relationship among genotypes between performance at low food availability and g_j assessed at high food density (as it was in our study), then slower-growing genotypes would have had a selective advantage during the 1960s and earlier. According to this hypothesis, as food availability increased through the 1960s and 1970s, faster-growing genotypes would have been selected, thus providing a second possible explanation for the general increase in g_j that we observed in the clones we studied.

Data Transformation: Observing Evolution of Plasticity

Which is a more appropriate way to treat the phenotypic ($g_{j,i}$) data, with or without log-transformation? As pointed out in the introduction, not transforming would be appropriate only if $g_{j,i}$ were directly proportional to the logarithm of fitness. Although it is true that g_j is calculated as a logarithmic function of change in mass from neonate, M_n, to maturity, M_m, over development time, t (i.e., $g_j = [\ln(M_m/M_n)]/t$; see Methods), this is not a reason to conclude that it is also a logarithmic function of fitness. Indeed, if this were the case, it would indicate that fitness should be a linear function of final body mass. We know this is not the case, and that dietary cyanobacteria actually affects *Daphnia* fitness in a complex way. Not only is mass at maturity reduced, but so are the ratio of adult mass to adult body length (Fig. 3) and the time to maturity. In addition, body mass not only influences mean clutch size of the animals, but also their vulnerability to predation and presumably other physiologically based fitness components such as swimming speed. Thus, it is more appropriate to view g_j as an integrated measure of how physiological performance is impacted by food environment. It is a means of assessing the fraction, $g_{j,poor}/g_{j,good}$, by which overall fitness, W_i, (including all other fitness components) is decremented when cyanobacteria are present in the diet. This ratio-based interpretation means that log-transforming $g_{j,i}$ is appropriate because it implies that $g_{j,i}$ is linearly related to W_i. In reality the relationship between W_i and any given fitness surrogate is much more likely to be a saturating function in which increases in character value lead to progressively smaller increases in W_i. This is likely the case for juvenile growth rate, because incremental changes at low values of g_j are likely to have a much greater impact on fitness than changes of similar magnitude at high values of g_j. In this case, the effect that log-transforming g_j has in leading to a conclusion that plasticity evolved would be accentuated so that decreases in mean plasticity over genotype age are even greater. For these reasons we conclude that resistance evolved as a decrease in phenotypic plasticity as well as an increase in $g_{j,poor}$.

If this interpretation is correct, then our results suggest that the genotypes with the highest g_j are also the ones that in general are most resistant to dietary cyanobacteria, a result that might at first appear counter intuitive because it implies that there may be no cost to resistance. Huey and Hertz (1984) obtained a similar result for animal performance as a function of environmental temperature: in general, individuals with the highest performance at one temperature also had the highest performance at all temperatures tested. They proposed,

as we have, that traits that promote maximal performance in one environment could do the same in another environment and that, if a trade-off existed, it might be with some other (unmeasured) character. For example, in the *D. galeata* population we studied, the trade-off for having a high g_j might lie in a reduced resistance to starvation (e.g., Tessier et al. 2000), as we discussed in the previous section.

By using the log-transformed data, we can further see the effect of natural selection on the population variance in plasticity. Early during the period of eutrophication that we studied (1960s and early 1970s), when cyanobacteria were just increasing in the plankton, a diversity of g_j genotypes existed in the lake with a range of levels of plasticity (Fig. 4). Some of the clones we cultured from the sediments were significantly more sensitive (in terms of g_j) to changes in food quality than were others. By the period 1978–1980, after a decade of relatively high cyanobacterial densities, the more greatly impacted genotypes (i.e., those with steeper reaction norms) had disappeared and only the least impacted ones remained. This loss of variance accompanying the evolution of reduced plasticity can be seen as a significant genotype $\times$ food-type interaction for the animals from 1962–1971 (likelihood-ratio test, df = 1, LR test statistic = 12.4, $P < 0.005$), whereas this interaction is absent for the years 1978–1980 (likelihood-ratio test, df = 1, LR test statistic = 0, $P > 0.995$). This reduction in variance at the same time that mean performance changed is a classic natural selection response (e.g., Lynch and Walsh 1998). Animals hatched from the most recently produced diapausing eggs (1992–1994 and 1995–1997) again show a significant genotype $\times$ food-type interaction (likelihood-ratio test, df = 1, LR test statistic = 20.7, $P < 0.005$), suggesting that some variance has been reintroduced to the population following a relaxation in the selection imposed by dietary cyanobacteria. It may well be that this reemergence of variance in plasticity represents temporal dispersal of genotypes via the hatching of diapausing eggs from earlier periods, although we do not have direct evidence for this. Finally, also potentially acting as selection forces influencing the g_j of *D. galeata* in Lake Constance were both size-selective predation by fish and general changes in phytoplankton biomass that would have influenced food availability.

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