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Evolutionary trade-off between defence against grazing and competitive ability in a simple unicellular alga, *Chlorella vulgaris*

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Trade-offs between defence and other fitness components are expected in principle, and can have major qualitative impacts on ecological dynamics. Here we show that such a trade-off exists even in the simple unicellular alga *Chlorella vulgaris*. We grew algal populations for multiple generations in either the presence ('grazed algae') or absence ('non-grazed algae') of the grazing rotifer *Brachionus calyciflorus*, and then evaluated their defence and competitive abilities. Grazed algae were better defended, yielding rotifer growth rate 32% below that of animals fed non-grazed algae, but they also had diminished competitive ability, with a growth rate under nutrient-limiting conditions 28% below that of non-grazed algae. Grazed algae also had a smaller cell size and were more concentrated in carbon and nitrogen. Thus, *C. vulgaris* genotypes vary phenotypically in their position along a trade-off curve between defence against grazing and competitive ability. This genetic variation underlies rapid algal evolution that significantly alters the ecological predator–prey cycles between rotifers and algae.

Keywords: grazing resistance; predator–prey interaction; rapid evolution; green alga; rotifer; *Brachionus calyciflorus*

1. INTRODUCTION

Because organisms must allocate limited resources among a finite set of traits, defence theory suggests that no genotype can have superior ability in defence against its natural enemies, without paying a fitness cost in some other life-history character (Herms & Mattson 1992; Mole 1994). Trade-offs between defence and other fitness components have been explored in various ecological relationships, including that between defence against herbivores and its cost in terrestrial plants. Although trade-offs have often been demonstrated (Strauss *et al.* 2002), a substantial number of empirical studies exist where none was found (Bergelson & Purrington 1996; Strauss *et al.* 2002).

Defence against herbivory is also known in planktonic algae, where mechanisms of grazer defence include morphology (size, shape, colony formation), viable gut passage, and toxic chemical production (e.g. Porter 1973; Sterner 1989; Gliwicz 1990; Lampert & Sommer 1997). In comparisons of multiple algal species, Grover (1995) and Agrawal (1998) reported trade-offs between grazing resistance and algal growth rate. In contrast to single species, trade-offs are not necessarily expected to be seen among species, because resource acquisition can differ widely, with some taxa able to allocate more total resources to all fitness traits combined than are others. Although fitness trade-offs are a key component of defence theory, only a few studies have explored their existence within a single algal species. One example is the chemical cues released by grazers that induce colony formation in the freshwater algae *Scenedesmus subspicatus* and *S. acutus* (Hessen & Van Donk 1993; Lampert *et al.* 1994), in which the colonial forms are less vulnerable to consumption, but have higher sinking rates from illuminated surface waters than single-celled forms (Hessen & Van Donk 1993; Lürling & Van Donk 2000). As with studies of terrestrial plants, not all studies of algae have found a cost of defence: Jakobsen & Tang (2002) observed that colony-formation by *Phaeocystis* was favoured under intense grazing by protists, but could detect no difference in algal growth rate between colonial and solitary forms.

In this study we investigate the trade-off between defence and its cost in the unicellular green alga *Chlorella vulgaris* Beij preyed upon by the rotifer *Brachionus calyciflorus* Pallas. This trade-off is of particular interest because we have independent evidence that algal defence changes during the course of predator–prey cycles in laboratory microcosms (chemostats: continuous flow-through culture systems), and this in turn substantially alters the period and phase relation of predator–prey cycles (Shertzer *et al.* 2002; Yoshida *et al.* 2003). Using our system, Fussmann *et al.* (2000) demonstrated that a simple model embodying only a few mechanistic assumptions can accurately predict the qualitative dynamic behaviour of this two-species planktonic community. The system showed the predicted dynamical behaviours including coexistence at equilibria, coexistence on a limit cycle (predator–prey cycles), and extinction of either the predator alone or both the predator and the prey, as a function of total nutrient concentration and the dilution rate of the culture medium. However, the model could not predict quantitative properties of population cycles: the observed cycles were too long and nearly exactly out-of-phase (rather than the expected quarter-cycle phase lag between predator and prey).

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Table 1. Cell volume, C and N contents, and C : N atomic ratio of *Chlorella vulgaris*.
(*C. vulgaris* were selected either under intense rotifer grazing pressure (grazed) or in the absence of rotifers (non-grazed). *n* represents the number of chemostats used for each treatment. *p*-values were adjusted by Holm's sequential method to correct for multiple comparisons. C and N concentrations were computed from cell volume and cell quota, and so are not independent of the other reported comparisons.)

	grazed ($n = 4$)		non-grazed ($n = 3$)				
	mean	s.e.m.	mean	s.e.m.	d.f.	*p*-value	adjusted *p*-value
cell volume (10^{-9} mm^3)	8.073	0.766	15.857	0.707	5	0.002	0.010
cell quota of C (pg cell^{-1})	3.454	0.383	2.563	0.230	5	0.181	0.543
cell quota of N (pg cell^{-1})	0.421	0.091	0.239	0.029	5	0.216	0.543
C : N atomic ratio	10.334	0.970	12.684	0.753	5	0.186	0.543
C concentration (10^{-1} mg mm^{-3})	4.256	0.077	1.644	0.181	5	< 0.001	< 0.001
N concentration (10^{-1} mg mm^{-3})	0.502	0.058	0.154	0.022	5	0.008	0.032

This inconsistency indicated that the model lacked at least one important mechanism necessary to describe the system. Shertzer *et al.* (2002) proposed four biologically plausible hypotheses for the missing mechanism and tested them by comparing the population cycles observed by Fussmann *et al.* (2000) with mathematical simulation models incorporating each mechanism. They found algal evolution in response to rotifer predation to be the only strong candidate for explaining the novel dynamics. Their result is consistent with an earlier theoretical study by Abrams & Matsuda (1997), showing that prey evolution can lead to out-of-phase predator–prey cycles. Finally, experimental verification for the algal evolution hypothesis was obtained by Yoshida *et al.* (2003), who showed that cultures containing a single genotype (clone) of *Chlorella* had short-period cycles with a quarter-phase lag between predator and prey abundance peaks, whereas cultures with multiple algal clones had the unusual long-period cycles with predator and prey peaks out of phase. Simulation models constructed to mimic the clonal nature of *Chlorella* reproduction closely predicted the different dynamics observed.

These evolutionary models all stipulate that different algal genotypes are selected according to the intensity of rotifer grazing. At high grazer density, genotypes with high defence (low food value) and low competitive ability should predominate. However, genotypes with low defence (high food value) and high competitive ability should be favoured at low grazer densities. Here, we report experiments testing whether this is the case in our laboratory microcosms by comparing the characteristics of two algal lines selected either in the presence or in the absence of rotifers. Selection experiments of this type have been a powerful technique for identifying trade-offs in a variety of organisms (e.g. Mitchell-Olds & Bradley 1996; Kraaijeveld & Godfray 1997; Strauss *et al.* 1999; Webster & Woolhouse 1999; Koella & Boëte 2002; Fry 2003). In our earlier study showing the effects of evolutionary change on predator–prey cycles (Yoshida *et al.* 2003), we briefly described this evolutionary trade-off in order to report its consequences for ecological dynamics. Here, we fully define its characteristics as a novel example of an evolutionary trade-off in a simple organism.

2. MATERIAL AND METHODS

We studied the unicellular green alga *C. vulgaris* (UTEX no. 26), and its rotifer predator *B. calyciflorus* (taken originally from Milwaukee Harbour, Wisconsin, and provided by M. Boraas). They were cultured in 350 ml glass single-stage chemostats (continuous flow-through culture systems; Walz 1993) at 25 ± 0.3 °C under constant fluorescent illumination at 120 ± 20 μE m^{-2} s^{-1}. The culture medium contained nitrate, the limiting nutrient, at a concentration of 80 μM and non-limiting concentrations of other nutrients, trace metals and vitamins.

Chlorella vulgaris populations were exposed to two different treatments: constant intense rotifer predation (four replicate chemostats, dilution rate $\delta = 0.17$–$0.19\,\mathrm{d}^{-1}$), or unselective mortality from elevated washout (three replicate chemostats, $\delta = 1.20$–$1.25\,\mathrm{d}^{-1}$). In the former conditions, rotifers and algae coexist at a stable steady state where algal density is depressed by grazing far below the limits set by nutrient supply (Fussmann *et al.* 2000). As a result, the algae experienced elevated nutrient availability. To have nearly equal nutrient availability across treatments, we set a higher dilution rate in the rotifer-free chemostat. As a result, algal carbon (C) : nitrogen (N) in the two treatments was similar (molar C : N = 10–13; table 1) and was much lower than for algae cultured under nutrient-deficient conditions (molar C : N = 20 at $\delta = 0.16\,\mathrm{d}^{-1}$ with rotifers absent), suggesting that the algae in both of our experimental treatments experienced equivalent high nutrient availability. Algae were harvested after each chemostat reached equilibrium, and then used to determine the effects of previous selection under common conditions.

The size distribution of algal cells was measured using a particle counter (CASY 1, Schärfe, Reutlingen, Germany), and their C and N contents were determined by using a CN analyser (CE Elantech, Lakewood, NY, USA). For the latter measurement, algae were filtered on precombusted (3 h at 450 °C) glass-fibre filters (Whatman GF/F) and dried at 60 °C.

We measured 'algal food value' as a proxy for the strength of algal defence against predation. Algal food value was determined by bioassay using rotifer population growth rate when fed algae at a density well above the incipient limiting concentration (Halbach & Halbach-Keup 1974). Experimental procedures followed Rothhaupt (1995). Because *C. vulgaris* responses to the presence or absence of rotifers were uniform within each treatment and distinct between the treatments (figure 1; table 1), we used algae from a single chemostat for each treatment for the sake of simplicity of this bioassay. For algae from each treatment, 20 rotifer individuals were fed 20 ml of algal suspension at 7×10^6 cells ml^{-1} in triplicate plastic vials. These vials were kept in the dark on a

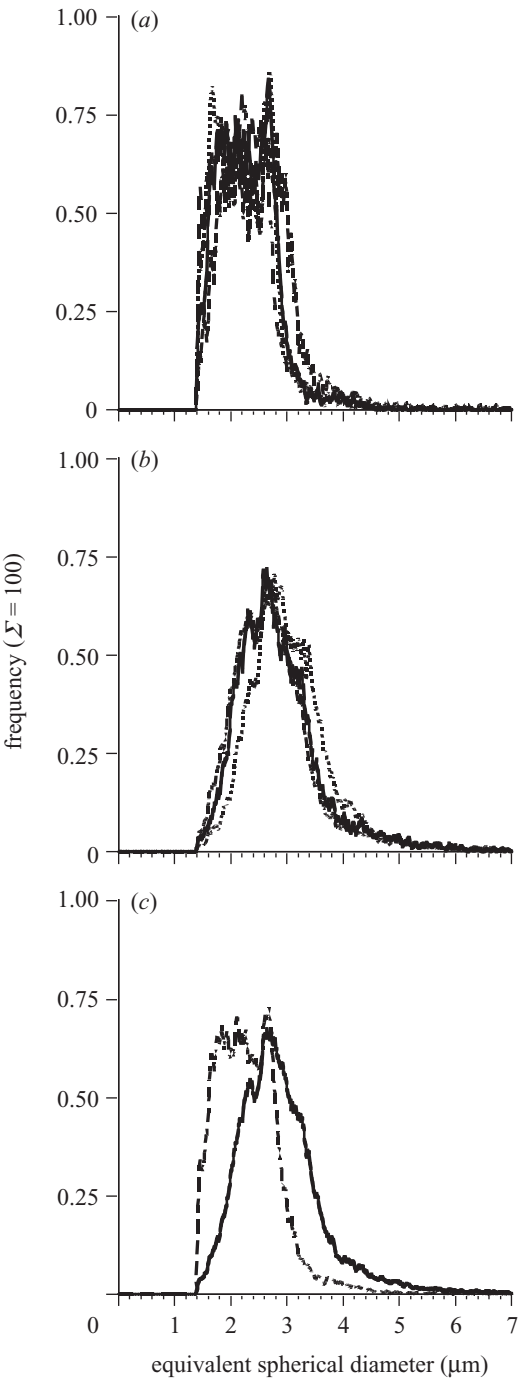

Figure 1. Comparison of size distributions of *Chlorella vulgaris* in four replicates of (*a*) the grazed, and three replicates of (*b*) the non-grazed experimental treatments. (*c*) Mean distributions of grazed (dashed line) and non-grazed (solid line) algae.

rotating shaker table at *ca.* 100 r.p.m. to prevent the algae from sinking. The harvested algae were washed with sterilized culture medium after being concentrated by centrifugation at 6000*g* for 20 min. The algal suspension was completely renewed daily, and the rotifers cleared less than 20% of the cells per day (usually much less). The rotifers were counted and the number of eggs per female was determined daily under a dissecting microscope. Twenty rotifers (or the remaining ones in the case of negative growth rates) were transferred into a new vial containing fresh algae, while maintaining population structure. When the per cent increase or decrease of the rotifer population had stabilized for at

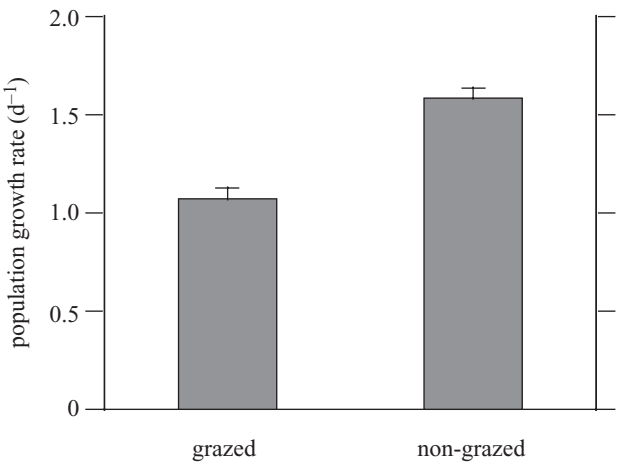

Figure 2. Mean population growth rates (per day ± 1 s.e.m.) of *Brachionus calyciflorus* fed grazed and non-grazed *Chlorella vulgaris* at a high food concentration (see § 2). Population growth rates were measured after they had stabilized (difference significant: ANOVA, $F_{1,4} = 48.6$, $p = 0.002$).

least 3 days (coefficient of variation < 0.1), the experiment was terminated. Population growth rate for daily intervals were calculated as

$$r = \ln(N_t) - \ln(N_{t1})$$

where r is the population growth rate (per day), and N_t and N_{t1} are rotifer numbers on consecutive days. We compared the algal food value using a *t*-test performed on mean population growth rates for the last 3 days of the experiment.

Algal population growth rates were measured in short-term batch cultures under three different nutrient conditions (1, 4 and 80 μM of nitrate). We used algae from a single chemostat for each treatment as we did for the bioassay above. Algae were inoculated at 500 cells ml^{-1}, grown at 25 °C and constant illumination at 120 $\mu E\, m^{-2}\, s^{-1}$ (the same as the chemostat experiments), and cell density and size distribution were monitored daily. Algal populations grew exponentially for the first 4–5 days; maximum growth rate was determined as the maximum derivative of a local polynomial curve fitted to log(algal density) versus time. Differences in growth rates between *Chlorella* populations were analysed by a *t*-test.

To assess whether or not high mortality as a result of elevated washout (as opposed to grazing) affects algal size distribution, two algal populations were exposed to either a high dilution rate (2.5 d^{-1}) or a constantly low dilution rate (0.5 d^{-1}). After both populations reached equilibrium, we measured algal size distribution as described above. Then, for the high dilution chemostat, we decreased the dilution rate to 0.5 d^{-1} to compare the size distribution under common conditions. After the algal population again reached equilibrium, its size distribution was measured.

3. RESULTS

Chlorella cultured under constant and intense rotifer grazing pressure ('grazed algae') were distinct in food value, growth rate at limiting nitrate concentrations, cell size, and tissue C : N from those grown in the absence of rotifers but with a comparable mortality rate imposed by an elevated chemostat dilution rate ('non-grazed algae'). The size-frequency distribution of the grazed algae was shifted to smaller cell diameters (mean equivalent spherical diameter (ESD) = 2.34 μm, $n = 4$) compared with non-grazed algae

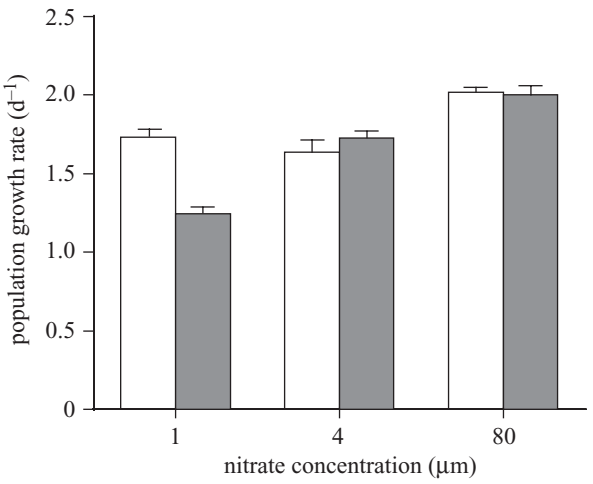

Figure 3. Mean maximum realized population growth rates (per day + 1 s.e.m.) of grazed (grey bars) and non-grazed (white bars) *Chlorella vulgaris* at three different nutrient concentrations. The difference between grazed and non-grazed *Chlorella* was significant at 1 μM nitrate (*t*-test: $t = 12.82$, $p < 0.001$) and non-significant at 4 μM nitrate ($t = 1.28$, $p = 0.27$) and 80 μM nitrate ($t = 0.31$, $p = 0.78$).

(mean ESD = 2.92 μm, $n = 3$; figure 1), with the result that grazed algae had a cell volume about half that of non-grazed algae (table 1). The difference in cell size between grazed and non-grazed lineages proved to be heritable (see below). Microscopic inspection showed that most of the *Chlorella* in both treatments occurred as single isolated cells, and thus the size difference we observed was not a result of differences in the number of autospores (a stage in *Chlorella* asexual reproduction containing multiple daughter cells in a single cell membrane) per cell. By contrast, the cell quotas (contents per cell) of C and N were not different between grazed and non-grazed algae (table 1). As a result, grazed algae had significantly higher C and N concentrations (mass per cell volume) than non-grazed ones (table 1), indicating that the cells of grazed algae were more compact in C and N.

Algal food value was estimated by bioassay using the population growth rate of rotifers in batch culture provided with algae at high density. After 5 to 8 days of growth, rotifer population age structure and growth rate stabilized. The equilibrium growth rate was significantly greater (by 47%) for rotifers fed on non-grazed algae than for those fed on grazed algae ($t = 6.97$, $p = 0.002$; figure 2).

Algae from both the grazed and non-grazed treatments exhibited similarly high growth rates when cultured on a medium containing sufficient nitrate (80 μM). At intermediate nitrate conditions (4 μM), growth rates of both groups decreased but were still similar to each other. By contrast, under severely nitrate deficient conditions (1 μM), the population growth rate of the algae originating from the grazed treatment decreased more and was significantly lower than that of algae from the non-grazed treatment ($t = 12.81$, $p < 0.001$; figure 3). Because algal growth rates were measured in cultures that had undergone many cell divisions after being isolated, the dissimilarity observed between treatments at low nitrate conditions represents a heritable difference.

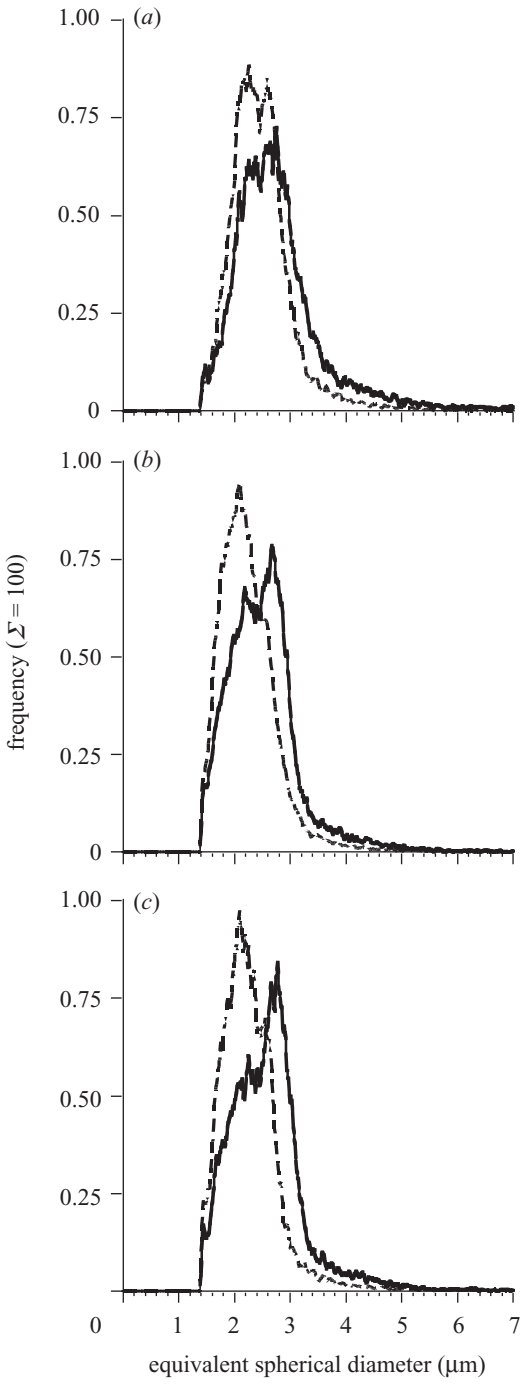

Figure 4. Size distributions of populations derived from grazed (dashed lines) and non-grazed (solid lines) *Chlorella vulgaris* that were then allowed to grow for 6 days at different nutrient levels: (*a*) 80 μM nitrate, (*b*) 4 μM nitrate and (*c*) 1 μM nitrate.

In these growth rate experiments, the difference in algal size distributions between the grazed and non-grazed algae that existed initially (figure 1) persisted, even after their populations grew to equilibrium density (figure 4). Although the grazed algae grown at 80 μM were slightly larger on average than those grown at 4 μM or 1 μM, the absolute difference in mean volume between grazed and non-grazed lineages at the end of the growth experiment was essentially identical in the three treatments (mean ESD = 2.45 and 2.75 for grazed versus non-grazed algae at 80 μM, 2.24 and 2.51 at 4 μM, and 2.26 and 2.57 at 1 μM;

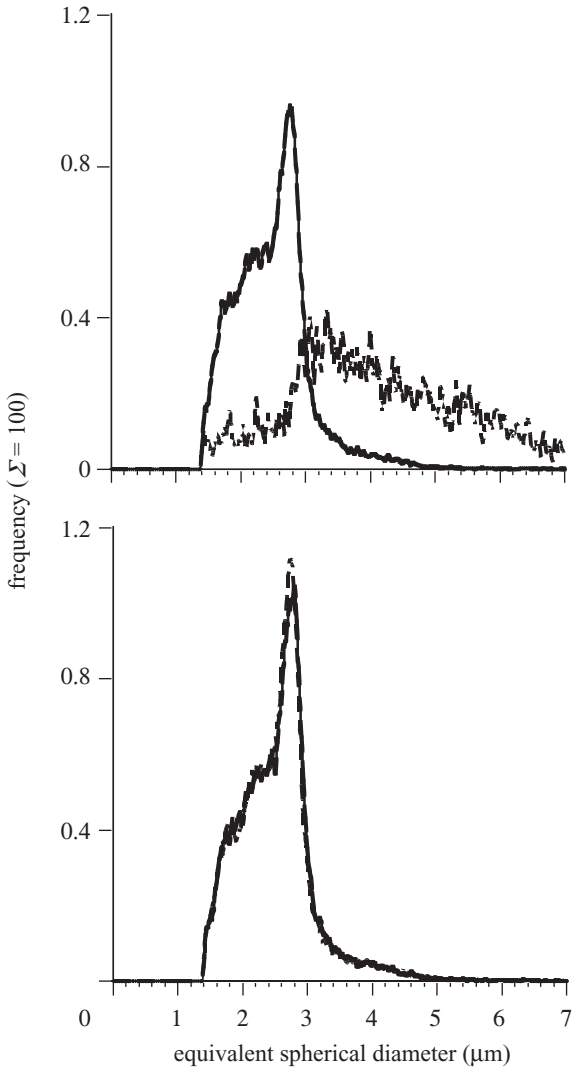

Figure 5. Size distributions of *Chlorella vulgaris* at steady state in chemostats without rotifer grazing. (*a*) Chemostats with dilution rates (δ) of $0.5\,d^{-1}$ (solid line) and $2.5\,d^{-1}$ (dashed line). (*b*) The same chemostats, after the high-δ chemostat was reduced to $\delta = 0.5\,d^{-1}$ and allowed to reach steady state again. (Dashed line, $\delta = 2.5 \rightarrow 0.5$; solid line, $\delta = 0.5 \rightarrow 0.5$.)

all differences significant at $p < 0.05$, Kolmogorov–Smirnov test). These differences in size resulted in non-grazed algal cells being *ca.* 30% smaller in volume than grazed algae. Based on the typical algal division process that we observed (approximately four autospores per individual) and the increase in population size, roughly four to six algal generations were required for the population increases observed during the growth experiments. The differences in final size distributions therefore demonstrate that the change in cell size under selection by grazing (figure 1) is heritable.

By contrast, changes in cell size because of non-selective mortality were not heritable. Substantial differences in size distribution (figure 5*a*) occurred between algal populations grown (in the absence of rotifers) at different dilution rates (algae at $\delta = 2.5\,d^{-1}$ being larger than those at $\delta = 0.5\,day^{-1}$, presumably as a result of the higher nutrient availability). However, after the dilution rates were equalized at $0.5\,d^{-1}$, and the populations allowed to reach new equilibrium densities, the difference in size distribution disappeared (figure 5*b*, Kolmogorov–Smirnov test, not significant). Thus, differences in cell size created in chemostat cultures are not necessarily heritable. Rather, it is consumption by rotifers *per se* that produced a genetically based difference in algal size.

4. DISCUSSION

Algal prey selected under exposure to rotifer grazing showed a correlated evolved response of low food value and low growth rate under nitrate deficient conditions. The low food value of grazed algae supports the hypothesis that better defended, less edible, algae evolve under intense rotifer grazing. Because algal cells compete for nitrate, the limiting nutrient in our experimental system, the low growth rate we observed under nitrate-deficient conditions represents a lower competitive ability of grazed algae. Thus, this is a cost of defence. Ideally, the food value of grazed and non-grazed lineages would have been compared using descendants of the algae exposed to selection, but this was not feasible because we had to feed rotifers at a sufficiently high algal density to measure algal food value (see § 2). However, phenotypic characters strongly associated with reduced food value (cell size and algal growth rate) both proved to be heritable (figures 2–4), implying that the low food value algae in the grazed treatment represent a distinct set of algal genotypes from those in the non-grazed treatment, especially in these obligately asexually reproducing algae.

By comparing the effects of selective mortality (rotifer grazing) and unselective mortality (elevated washout rate), we infer that the difference in size between grazed and non-grazed algal cells (figure 1) resulted from selective grazing by rotifers. Predation depressed algal density down to *ca.* 1×10^6 cells ml^{-1} in the grazed treatment compared with an equilibrium density of *ca.* 3×10^6 cells ml^{-1} in the non-grazed treatment. Because lower algal density leads to improved nutrient availability, and hence higher intrinsic population growth, we infer that the average total mortality rate at steady state was higher in the grazed treatment. In that case, the higher mortality led to decreased cell size. By contrast, increasing the unselective morality ($\delta = 0.5$ versus $2.5\,d^{-1}$; figures 1 and 5) produced a larger mean cell size. Thus, the smaller cell sizes in the grazed treatment (figure 1) was primarily a result of natural selection imposed by rotifer consumption rather than the result of high mortality alone or of other changes in conditions (e.g. improved nutrient availability) resulting from high mortality *per se*.

How might grazed algae benefit from being small and compact? Our rotifer, *B. calyciflorus*, is known to be a size-selective feeder (Gilbert & Starkweather 1978; Rothhaupt 1990), but it is not clear whether rotifers can discriminate between the small size differences we observed between grazed and non-grazed algae (figure 1) or whether the observed differences in cell size could result in the difference in food value (i.e. rotifer growth rate) that we observed (figure 2). Preliminary measurements of rotifer feeding rates showed no significant differences between cells from the grazed and non-grazed treatments. The mean feeding rate at a high algal concentration (5.5–7.0×10^6 cells ml^{-1}) was 3.71×10^3 cells $rotifer^{-1}$ h^{-1} ($\pm1.21\times10^3$ 95% CI) for non-grazed algae and

4.84×10^3 cells rotifer^{-1} h^{-1} ($\pm 0.81 \times 10^3$ 95% CI) for grazed algae (T. Yoshida, unpublished data). If ingestion rates on the two cell types do not differ, the difference we observed in food quality might instead be related to the fact that the grazed algae have more compact cells (table 1), and thus may be more likely to pass through rotifer guts undamaged and undigested (e.g. Porter 1973). If this defence against digestion by rotifers were the case, we might have expected survival during passage through the gut to be linked to some morphological trait of the algae such as a thickened cell wall (Porter 1975). However, we found no apparent difference in cell wall morphology or other ultrastructural characters in transmission electron micrographs of sectioned cells from the grazed and non-grazed treatments (T. Yoshida, unpublished data). As a result, the mechanism underlying the difference in food value between the grazed and non-grazed algal types remains to be worked out.

Trade-offs between defence against grazing and other fitness components have been predicted for algal–zooplankton interactions (Grover 1995), but so far there are few published studies in which such trade-offs have been explored. Agrawal (1998) compared the growth rates of grazer-resistant and grazer-susceptible algal species (classified quite roughly), and found a lower growth rate on average in defended species. However, this correlation between defence and growth rate across species is fundamentally different from the within-species trade-off that we have demonstrated here. Until now, the two clearest intraspecific examples have involved induction of algal colony formation by grazers (Lürling & Van Donk 1996; Jakobsen & Tang 2002) where coloniality inhibits consumption. In both cases, the investigators explored costs of defence, and one case found that colonies sink faster than single cells (Lürling & Van Donk 2000). In both studies, the authors looked for, but failed to find, reduced algal population growth rate as a potential cost of coloniality. Both, however, only measured algal growth under high nutrient concentrations (Lürling & Van Donk 2000; Jakobsen & Tang 2002) where effects on competitive ability are least likely to be manifest. Our study is thus distinct in showing a physiological cost of defence expressed as reduced cell division rate under nutrient limiting conditions, and in finding a trade-off among distinct genotypes, rather than among induced phenotypes.

Trade-offs between defence and other fitness components are important for understanding how genetic variation in defensive capability might be maintained (e.g. Kraaijeveld & Godray 1997; Webster & Woolhouse 1999), and are key to understanding how the rapid evolution of prey in response to predation can impact the dynamics of predator–prey cycles (Abrams & Matsuda 1997). The trade-off between algal food value and competitive ability reported here is a major determinant of the long-term qualitative properties of predator–prey cycles in this system (Yoshida *et al.* 2003). Models based on this trade-off exhibit reciprocal changes in genotype frequencies over a population cycle. Under intense rotifer grazing, the algal population becomes dominated by grazing-resistant genotypes. When most algae have become resistant, rotifers remain at a low density even though algal abundance recovers. The low grazing pressure that accompanies low rotifer abundance allows undefended algal genotypes to outcompete the defended ones, so that rotifers can increase again leading to another cycle. Although this evolutionary cycle should occur regardless of the specific details of the trade-off (Yoshida *et al.* 2003), the shape of the trade-off curve can affect the period of population cycles (Yoshida *et al.* 2003). Furthermore, its shape is also important in predicting which genotypes will survive at a steady state (Jones & Ellner 2004). This means that greater definition of the relationship between algal food value and competitive ability among algal genotypes is needed, not only to understand our specific laboratory system, but also to begin to extend its implications for population fluctuations in nature.

More generally, our results reveal that subtle evolutionary changes in prey phenotype, some of them only manifested under extreme conditions (figure 3), nonetheless had large impacts on their predators (figure 2) and on major features of the long-term food web dynamics (Yoshida *et al.* 2003). These findings suggest that ecologically significant trait-mediated interactions (Bolker *et al.* 2003; Schmitz *et al.* 2003) may be widespread even in the simplest organisms, but could easily go unrecognized unless efforts are specifically directed towards identifying the traits mediating interspecific interactions and monitoring their dynamics. Our results also add to the accumulating evidence that population and trait dynamics, either evolutionary change or induced responses, frequently interact reciprocally, so that neither can be fully understood in isolation from the other.

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