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Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing

Julia Kubanek[1]
School of Biology and School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0230

Terry W. Snell and Christen Pirkle
School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332-0230

Abstract

In order to test whether phytoplankton such as the red tide dinoflagellate *Karenia brevis* negatively affect rotifer grazers and what mechanisms are involved in these interactions, we conducted laboratory feeding experiments using *K. brevis* cultured from Gulf of Mexico coastal waters and two species of rotifers, one co-occurring and the other from an inland sea in Russia. The co-occurring rotifer *Brachionus ibericus* did not ingest *K. brevis* as a sole diet but tolerated *K. brevis* in a diet mixed with *Rhodomonas lens*, whereas the allopatric rotifer, *Brachionus plicatilis*, rejected *K. brevis* in either diet, although *B. plicatilis* started feeding on the mixed diet after a delay of 4 days. The deterrence of *K. brevis* was mediated by its cellular organic extracts and not by exudates, with compounds other than brevetoxins PbTx-2, PbTx-3, and PbTx-9 implicated in deterrence. The finding that closely related rotifers respond differently to *K. brevis* and to its chemical defense suggests that a shared evolutionary history may have led *B. ibericus* to tolerate *K. brevis* in a mixed diet.

Marine plankton communities are unexpectedly diverse (Hutchinson 1961), with large numbers of primary producer and consumer species. The abundance of generalist grazers is expected to lead to strong selection for anti-predatory defenses by phytoplankton prey. Previous studies have shown that some phytoplankton avoid or deter grazers (Huntley et al. 1986; Teegarden 1999; Selander et al. 2006), some are nutritionally inadequate to support grazer growth or reproduction (Dam and Colin 2005; Prince et al. 2006), whereas others are toxic, causing grazer paralysis, mortality, or reproductive failure (Sykes and Huntley 1987; Miralto et al. 1999; Colin and Dam 2002*b*). Much of this research has been done with pelagic copepods, and substantial interspecies and interpopulation variance has been observed in the effectiveness of phytoplankton defenses against these zooplankters (Turner and Tester 1989; Colin and Dam 2002*a*; Colin and Dam 2005). The hypothesis that some bloom-forming phytoplankton inhibit grazing via chemical defenses has been supported using heterotrophic dinoflagellate and ciliate grazers, whose sensitivity to phytoplankton defenses varies among grazers (Wolfe 2000). It has been suggested that the failure of zooplankton to use phytoplankton biomass could contribute to the formation of harmful algal blooms in some environments (Turner et al. 1998).

[1] Corresponding author (julia.kubanek@biology.gatech.edu).

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Rotifers are common microzooplankton grazers seasonally abundant in estuarine and coastal waters as well as in inland fresh and salt lakes (Wallace and Snell 2001), with many cosmopolitan species (Dumont and Segers 1996). Several species of the rotifer genera *Brachionus* and *Synchaeta* are widespread and numerous in near-shore marine systems (Wallace et al. 2006). Although few studies on phytoplankton defenses have considered rotifers as potential grazers, their generalist feeding behavior (Starkweather 1980) and substantial role in transforming primary production into animal biomass (Stockner and Porter 1988) make rotifers appropriate models for understanding aquatic food web dynamics. The red tide dinoflagellate *Karenia brevis* (ex *Gymnodinium breve*, ex *Ptychodiscus brevis*) is an episodically abundant phytoplankter in the Gulf of Mexico that is occasionally driven onshore by currents and winds (Tester and Steidinger 1997), accumulating in dense blooms that kill fish and contaminate shellfish (Landsberg 2002). Rotifers are likely to encounter this harmful alga in coastal waters and may be important in controlling, or failing to control, *K. brevis* blooms.

There have been few previously published studies exploring the feeding behavior or physiological responses of rotifers to Gulf of Mexico phytoplankton (but *see* Buskey and Hyatt 1995). The current study was conducted to test whether sympatric and allopatric rotifers consume *K. brevis*, and if not, why not. If rotifers consumed *K. brevis*, then we asked what effect this consumption had on their reproduction and population dynamics to better understand marine food webs and the ecology of harmful algal blooms.

Materials and methods

Phytoplankton—*K. brevis* originally collected from Sarasota Bay, Florida, in the eastern Gulf of Mexico

(Provasoli-Guillard National Center for Culture of Marine Phytoplankton [CCMP] strain 2228; 510 pg carbon [C] and 95 pg nitrogen [N] per cell; dimensions 24.4 × 24.4 × 12.9 μm; cell volume 4.0 × 10^3 μm^3 [Prince et al. 2006]) and *Rhodomonas lens* originally collected from the Bahamas (CCMP strain 739; 44.3 pg C and 9.9 pg N per cell; dimensions 12.4 × 6.7 × 6.7 μm; cell volume 2.9 × 10^2 μm^3 [Prince et al. 2006]) were obtained as nonaxenic cultures from the CCMP. Strains were maintained at 22°C with a 12 h light : 12 h dark (LD) cycle in a Percival incubator with Philips F32T8/TL741 Universal/Hi-Vision fluorescent bulbs mounted vertically, producing irradiance of 100–145 μmol m^{-2} s^{-1} throughout the incubator (measurement using Biospherical Instruments light meter model QSL2100). Phytoplankters were grown in autoclaved L1 media (CCMP) made with filtered 32–35 parts-per-thousand salinity natural seawater collected in Maine. Preliminary growth curves were prepared using in vivo chlorophyll fluorescence measurements (Turner Designs TD-700 calibrated with chlorophyll *a* standard) and visual cell counts using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber. Stock cultures were consistently transferred to fresh media once stationary phase was reached.

Rotifers—*Brachionus plicatilis* (strain RUS) and *Brachionus ibericus* (strain IR2) were originally collected from the Azov Sea, Russia, and from Indian Rocks Beach, Florida, respectively, as previously described (Snell and Stelzer 2005). More than 10 generations of each rotifer species were maintained on *Tetraselmis suecica* under similar light and temperature regimes, and resting eggs were collected by filtration, air-dried, and stored at −20°C, eliminating the possibility of maternal effects in feeding experiments using rotifers hatched from these eggs. Rotifers were hatched in filtered artificial seawater at 22°C for 48 h in the incubator described above and then transferred by pipet to filtered natural seawater approximately 1 h before the start of feeding experiments.

Preparation of K. brevis *cellular extracts*—Once in early stationary phase (24,000 cells mL^{-1}), 1,000 mL of *K. brevis* culture was slowly filtered with gentle stirring through a 5-μm Millipore Isopore TMTP membrane, using an Amicon ultrafiltration unit, until 10–20% of the starting volume remained as retentate. Filtered natural seawater was added to rinse cells, and the retentate volume was again reduced by filtration. Light microscopy was used to verify that *K. brevis* cells did not pass through the membrane and that cells remaining in the unit were intact. The concentrated cells were then lysed by adding 100 mL of deionized water, and organic compounds released by cell lysis were extracted using a mixture of adsorbent resins (equal proportions of Diaion HP-20 [Supelco], Amberlite XAD-7-HP [Acros Organics], Amberlite XAD-16 [Supelco], totaling approximately 150 mL of wet resin) as previously described (Prince et al. 2006). After 12 h, the resin was collected by filtration through 160-μm pore size Nitex nylon mesh followed by vacuum filtration, then rinsed with deionized water, and organic compounds were eluted from the resin with alternating methanol and acetone (each approximately 250 mL). The solvents were removed by rotary evaporation, yielding a cellular extract of *K. brevis*. An aliquot of this extract was analyzed by liquid chromatography–mass spectrometry (LC-MS) to quantify brevetoxins against a standard curve, revealing that the extract derived from 1,000 mL of *K. brevis* culture contained 420 μg PbTx-2 (brevetoxin 2 or brevetoxin B) (i.e., 420 ng mL^{-1} PbTx-2), or the equivalent of 18 pg PbTx-2 per *K. brevis* cell. Other more minor brevetoxins were also present (e.g., PbTx-3), but none at concentrations approaching PbTx-2, and so these were not quantified.

Preparation of K. brevis *extracellular extracts*—An early stationary phase culture of *K. brevis* (17,000 cells mL^{-1}) was treated with adsorbent resin as previously reported (Prince et al. 2006), which permitted extraction of extracellular organic compounds without disruption of *K. brevis* cells. Other than omission of the cell concentrating and lysis steps, all other aspects of this extraction process were the same as described above for the cellular extract. LC-MS quantification of brevetoxins from the *K. brevis* extracellular extract revealed 23 ng mL^{-1} PbTx-2, or the equivalent of 1.4 pg PbTx-2 exuded per *K. brevis* cell.

Brevetoxins used directly in feeding experiments were purified from *K. brevis* cultures as previously described (Bourdelais et al. 2004).

General procedure for feeding experiments—Feeding experiments testing the effects of different diets on rotifer feeding behavior, egg production, and population growth were conducted in 24-well sterile polystyrene plates (Corning), with 1.1–1.6-mL filtered natural seawater in each well. Rotifers freshly hatched from resting eggs and starved for 2–12 h were dispensed into experimental wells by pipet (5–12 rotifers per replicate well; n = 4–10 wells per treatment with treatments interspersed), and then the appropriate diet (or seawater for starvation controls) was introduced into each well. At approximately 24-h intervals starting 2–3 h after diets were added to experimental wells, the following data were collected by examination under an Olympus SZ61 dissecting light microscope at ×10 magnification: total number of live rotifers, number of live rotifers with food in their gut, and number of live rotifers carrying (amictic) eggs. At each time point, each well was checked visually for remaining food, and in experiments in which food appeared to have been mostly consumed, diets were added again to all treatments. At the end of the experiment (5–6 d), 10 μL of Lugol's solution was added to each well to immobilize (kill) and stain rotifers to facilitate counting. From the raw data, the proportion of live rotifers in each well with food in their gut was used to assess feeding behavior. The proportion of live rotifers carrying eggs at a time point 24–48 h from the start of the experiment (allowing sufficient time for rotifers to produce eggs but not so much time that eggs had hatched) was used to assess reproduction. We also calculated the intrinsic rate of increase (r) for rotifer populations, with $r = (\ln N_t - \ln N_o)/T$, where T was the duration of the experiment in days.

Feeding experiments comparing effects of live phytoplankton diets—Seven to ten rotifers per well (n = four replicate wells per treatment) were exposed to one of four possible treatments: (1) *K. brevis* (2,300–3,400 cells mL^{-1}), (2) *R. lens* (70,000–220,000 cells mL^{-1}), (3) *K. brevis* and *R. lens* (same amount of each as in treatments 1 and 2, resulting in a 1 : 2 ratio of *K. brevis*/*R. lens* based upon cell volume), or (4) no food (starvation control). Rotifers were fed at t = 0 h, and then again at t = 24 h (for *B. plicatilis*) or t = 48 h (for *B. ibericus*), because food appeared to be limiting at those time points.

Feeding experiments testing effects of K. brevis *cellular extracts and cellular brevetoxins*—We fed rotifers rehydrated yeast particles (Culture Selco, INVE) treated with *K. brevis* cellular extracts, combined or not combined with a live *R. lens* diet, to test for potential feeding deterrents associated with *K. brevis* cells. In preliminary experiments, we found that rotifers fed and reproduced well on Culture Selco yeast (100 μg mL^{-1}, dry weight) when mixed with *R. lens* and had a somewhat lower fitness but still persisted without significant population decline on a sole diet of Culture Selco yeast at 150 μg mL^{-1}. We calculated the appropriate amount of *K. brevis* cellular extract to add to yeast, corresponding to an amount of extract derived from a similar biomass of *K. brevis* cells. For testing of pure brevetoxins on yeast, we used a 100 : 10 : 1 ratio of PbTx-2, PbTx-3, PbTx-9 to mimic a realistic distribution of brevetoxins (Roszell et al. 1990), such that the concentration of PbTx-2 (18 pg per *K. brevis* cell) was the same in the *K. brevis* cellular extract-yeast treatment and the brevetoxin-yeast treatment. The extract or brevetoxin mixture was dissolved in a minimal amount of methanol, the dried yeast added, the mixture sonicated for 60 s, and then the solvent evaporated with a Savant Speed-Vac concentrator. Seawater was then added to hydrate the extract- or brevetoxin-treated yeast. (In a preliminary experiment with extract-treated yeast we found that after 16 h in seawater with no rotifers present, approximately 10% of PbTx-2 remained associated with yeast particles, 20% of PbTx-2 had diffused into the surrounding seawater, and 70% was lost by decomposition or adsorption to plastic wells.) Control yeast particles were prepared in the same way (including using methanol) but without the addition of extract or brevetoxins. Because adding yeast to experimental wells already containing rotifers caused the rotifers to adhere to the wells, we added the yeast to the wells first and allowed it to settle for 2 h, then added *R. lens* (200,000–290,000 cells mL^{-1}), then rotifers (5–10 rotifers per well; n = 4–6 replicate wells per treatment). For experimental wells in which yeast was tested as a sole diet (without *R. lens*), inert carmine beads (0.5 mg mL^{-1}) were added with yeast to visualize rotifer gut fullness as used in rotifer toxicological assays (Snell 2005), because yeast particles alone were invisible in rotifer guts.

Feeding experiments testing effects of K. brevis *extracellular extracts and extracellular brevetoxins*—An amount of extracellular extract corresponding to that produced by 1,500 cells mL^{-1} *K. brevis* (approximating a moderate *K. brevis* bloom) was dissolved in 10 μL dimethyl sulfoxide (DMSO) and added to wells containing 5–10 rotifers, and *R. lens* (200,000 cells mL^{-1}) was immediately added. For the extracellular brevetoxin treatment, PbTx-2, PbTx-3, and PbTx-9 in a 100 : 10 : 1 ratio (Roszell et al. 1990) was dissolved in 10 μL DMSO and added to each experimental well such that the total concentration of brevetoxins was 9 ng mL^{-1}, a reasonable bloom-level waterborne concentration (Cheng et al. 2005). *R. lens*–only treatments and starvation controls received 10 μL DMSO (n = 4–10 replicate wells per treatment).

Statistical analyses—Feeding behavior results were analyzed by one-way repeated measures analysis of variance (ANOVA) following arcsine transformation of proportional data. For comparisons of feeding behavior data between *B. plicatilis* versus *B. ibericus* or between newly hatched versus older rotifers, two-factor repeated measures ANOVA was used, with diet and species/age as factors. Egg production results were analyzed by one-way ANOVA followed by Tukey post-hoc test on arcsine transformed data. Rates of population increase (r) were compared by one-way ANOVA followed by Tukey post-hoc test but without arcsine transformation. All analyses were performed using Systat, and $p \leq 0.05$ was considered significant. Figures depict means ± one standard deviation.

Results

Effects of K. brevis *diet on rotifer feeding, reproduction, and population growth*—When *K. brevis* was offered as a sole diet, the feeding behavior of both rotifer species (*B. plicatilis* and *B. ibericus*) was almost completely inhibited (Fig. 1). The failure of rotifers to feed on *K. brevis* translated into a failure to produce eggs and a population decline similar to that of starved rotifers (Fig. 2). In contrast, rotifers fed *R. lens* as a sole diet had consistently full guts (until *B. plicatilis* began to run out of food after 6 d; Fig. 1) and produced eggs within 24–48 h, and rotifer populations exhibited rates of increase (r) ranging from 0.20 d^{-1} to 0.43 d^{-1}, resulting in population increases of 2–7-fold over 5–6 d (Fig. 2).

Rotifer feeding on a mixed diet of K. brevis *and* R. lens—Fewer *B. plicatilis* individuals fed on a 2 : 1 mixture of *R. lens* and *K. brevis* than on *R. lens* alone ($p < 0.001$; Fig. 1A), but the inhibitory effect of *K. brevis* appeared to wear off after approximately 4 d ($p = 0.003$ for effect of time). When this experiment was repeated adding a contrast between the feeding behavior of newly hatched *B. plicatilis* (as in the first experiment) with that of 2-day-old starved *B. plicatilis*, the inhibitory effect of *K. brevis* in a mixed diet was again observed ($p < 0.001$; df = 1; $F = 34.6$). However, in this second experiment the feeding inhibition wore off more quickly, and rotifers started to feed at high rates on the mixed diet within 36 h (data not shown). There was, however, no difference between the acclimation time for newly hatched versus 2-day-old *B. plicatilis* ($p = 0.23$; df = 1; $F = 1.47$ for effect of age by two-factor repeated measures ANOVA; data not shown).

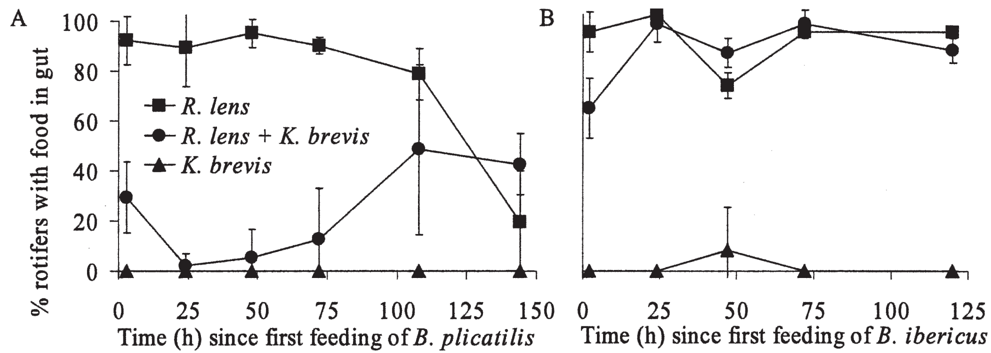

Fig. 1. Feeding behavior of (A) *B. plicatilis* and (B) *B. ibericus* exposed to single or mixed diets of *K. brevis* and *R. lens* ($n = 4$). Starved rotifers never had food in their guts (data not shown). In this and every figure, error bars indicate one standard deviation. One-way repeated measures ANOVA detected significant effects of diet, time, and time × diet interactions for both species (all $p < 0.01$; df = 2–10; $F = 4.4$–292). Exclusion of *K. brevis*-only data did not affect the statistical outcome for *B. plicatilis*, but for *B. ibericus* resulted in a loss of significance for the effect of diet ($p = 0.16$; df = 1; $F = 2.6$). Two-factor repeated measures ANOVA comparing *B. plicatilis* and *B. ibericus* data indicated significant effects of species, diet, species × diet, time × diet, time × species, time × diet × species (all $p < 0.001$; df = 1–8; $F = 4.4$–267), and time ($p = 0.019$; df = 4; $F = 3.2$).

Effects of K. brevis *deterrence on rotifer fitness*—The delayed feeding of *B. plicatilis* on a mixture of *K. brevis* and *R. lens* resulted in initially depressed rotifer egg production compared to rotifers fed a sole diet of *R. lens* ($p < 0.001$; Fig. 2A). However, once rotifers began to feed on the mixed diet they started to produce eggs (data not shown). Nevertheless, the temporary inhibition of reproduction by *K. brevis* significantly reduced *B. plicatilis* population growth (mixed diet $r = -0.01 \pm 0.12$ d^{-1} vs. *R. lens* single diet $r = 0.20 \pm 0.02$ d^{-1}; $p = 0.047$; Fig. 2C), such that *B. plicatilis* populations fed only *R. lens* increased 14× more over 6 d than rotifer populations exposed to an equal amount of *R. lens* mixed with *K. brevis*.

Differences between rotifer species in feeding behavior and fitness consequences—In contrast to *B. plicatilis*, *B. ibericus* experienced no obvious aversion to *K. brevis* when mixed with *R. lens*, even though *B. ibericus* appeared to avoid consuming *K. brevis* as a sole diet (Fig. 1B). Not only was the effect of diet absent when analyzing the feeding behavior of *B. ibericus* on *R. lens*-only versus mixed diets ($p = 0.16$), but there was a significant effect of species ($p < 0.001$) when

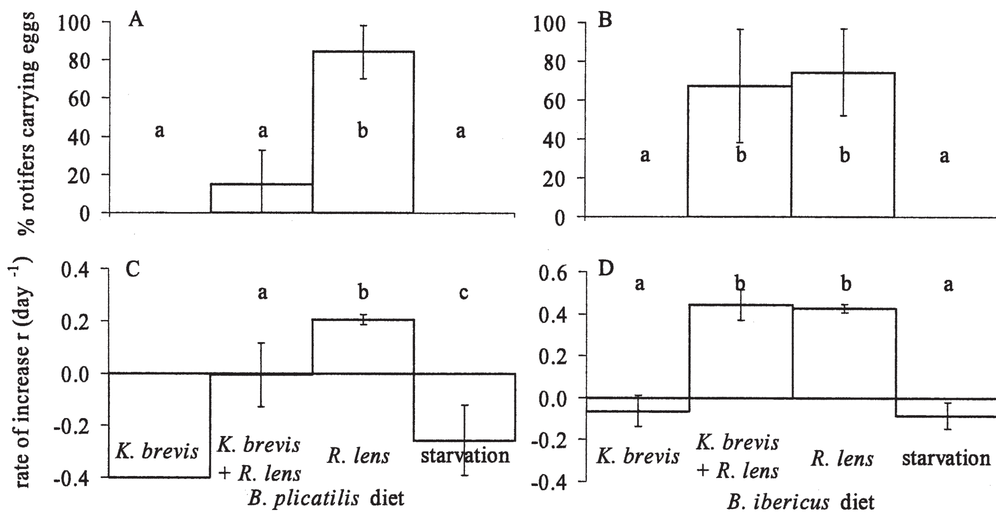

Fig. 2. Egg production of (A) *B. plicatilis* after 48 h and (B) *B. ibericus* after 24 h, and rate of population increase (r) for (C) *B. plicatilis* over 6 d and (D) *B. ibericus* over 5 d, when exposed to single or mixed diets of *K. brevis* and *R. lens* ($n = 4$). Different letters indicate treatments that significantly differed by one-way ANOVA (all $p \leq 0.001$; df = 2–3; $F = 11.5$–90.6) followed by Tukey post-hoc test (all $p < 0.05$). For *B. plicatilis* fed a sole diet of *K. brevis*, the population increase could not be statistically compared with other treatments because the final population was zero across all replicates, resulting in a nonmeasurable r (which, for visual purposes, is represented as -0.4 d^{-1}, the value that r would have been if even one rotifer had remained after 6 d).

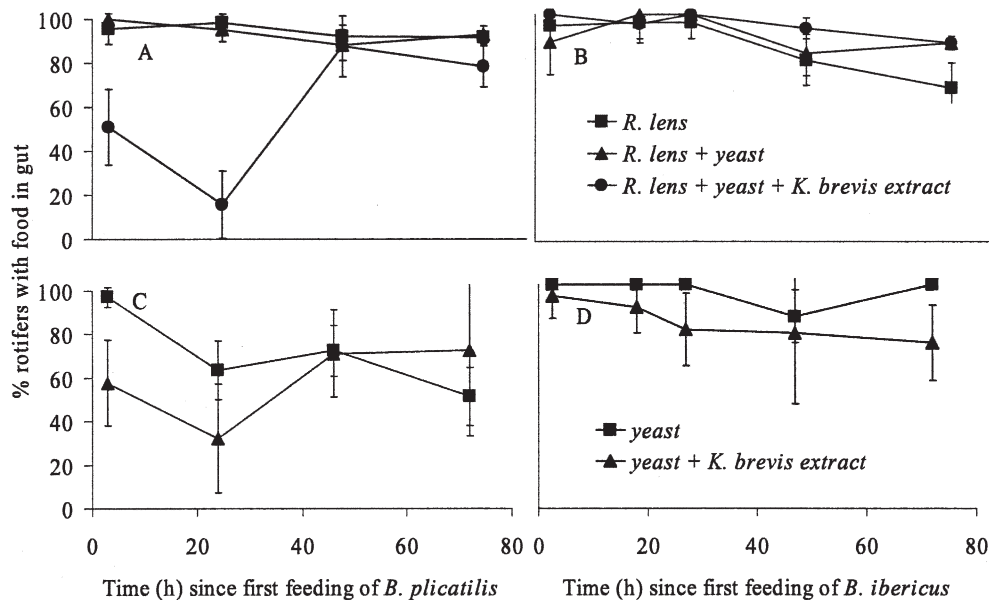

Fig. 3. Feeding behavior of *B. plicatilis* and *B. ibericus* exposed to (A,B) diets of *R. lens* and yeast treated with *K. brevis* cellular extracts, or (C,D) yeast treated with *K. brevis* cellular extracts as a sole diet ($n = 6$ for *B. plicatilis*; $n = 4$ for *B. ibericus*). Starved rotifers never had food in their guts (data not shown). One-way repeated measures ANOVA detected: (A) for *B. plicatilis*, a significant effect of diet ($p < 0.001$; df = 2; $F = 85.3$), a marginal effect of time ($p = 0.055$; df = 3; $F = 2.73$), and a time × diet interaction ($p < 0.001$; df = 6; $F = 17.0$); (B) for *B. ibericus*, no effect of diet ($p = 0.096$; df = 2; $F = 3.08$), a significant effect of time ($p < 0.001$; df = 4; $F = 19.5$), and no time × diet interaction ($p = 0.15$; df = 8; $F = 1.62$). Two-factor repeated measures ANOVA comparing (A) *B. plicatilis* and (B) *B. ibericus* data indicated significant effects of species, diet, species × diet, time × diet, time × species, time × die × species (all $p \leq 0.002$; df = 1–24; $F = 6.8$–54.8). One-way repeated measures ANOVA detected: (C) for *B. plicatilis*, significant effects of diet ($p = 0.022$; df = 1; $F = 7.28$), time ($p = 0.013$; df = 3; $F = 4.25$), and a time × diet interaction ($p = 0.001$; df = 3; $F = 7.03$); (D) for *B. ibericus*, a significant effect of diet ($p = 0.001$; df = 1, $F = 33.0$), and no effects of time or time × diet interaction ($p = 0.17$-0.22; df = 4; $F = 1.57$-1.78). Two-factor repeated measures ANOVA comparing (C) *B. plicatilis* and (D) *B. ibericus* data indicated significant effects of species and diet (both $p < 0.001$; df = 1; $F = 22.4$–78.5), a time effect ($p = 0.015$; df = 3; $F = 3.83$), a time × species × diet interaction ($p = 0.002$; df = 3; $F = 5.73$), and no time × diet or time × species interactions ($p = 0.077$–0.16; df = 3; $F = 1.80$–2.43).

comparing the *B. plicatilis* and *B. ibericus* datasets (Fig. 1). In addition, *B. ibericus* fed mixed *K. brevis* and *R. lens* reproduced at similar rates to conspecifics fed only *R. lens* ($p = 0.86$; Fig. 2B), leading to similar population growth over 5 d among these two treatments ($p = 0.99$; Fig. 2D).

Effects of K. brevis *cellular extracts on rotifers*—When yeast was treated with *K. brevis* cellular extracts and offered to *B. plicatilis* (as part of a mixed diet with *R. lens*), rotifers were significantly deterred from feeding on the mixture for at least 24 h ($p < 0.001$ for effect of diet; Fig. 3A). By 48 h, rotifers appeared to have acclimated to this food mixture and consumed the mixture of extract-treated yeast and *R. lens* at similar rates to rotifers fed only *R. lens* or *R. lens* mixed with untreated yeast ($p = 0.055$ for effect of time).

Egg production was initially 98% lower for *B. plicatilis* fed *R. lens* and yeast treated with *K. brevis* cellular extracts than for conspecifics fed either only *R. lens* or *R. lens* mixed with untreated yeast ($p < 0.001$ for both contrasts; Fig. 4A). Rotifers fed *R. lens* and extract-treated yeast started to produce eggs after 2 d, and by 3–5 d were producing eggs at similar rates to *R. lens* and *R. lens* + untreated yeast (data not shown). The population increase rate for *B. plicatilis* fed extract-treated yeast with *R. lens* was marginally lower than that of *B. plicatilis* fed untreated yeast with *R. lens* ($p = 0.15$), and indistinguishable from *B. plicatilis* fed only *R. lens* ($p = 0.89$; Fig. 4B). After 5 d of exposure to yeast treated with *K. brevis* cellular extract, the *B. plicatilis* population size was 37% lower than for conspecifics fed *R. lens* and untreated yeast ($p < 0.001$; df = 3; $F = 176$; data not shown).

When yeast treated with *K. brevis* cellular extracts was fed to rotifers as a sole diet, feeding deterrence was also observed for both *B. plicatilis* and *B. ibericus* (effect of diet $p = 0.001$–0.022; Fig. 3C,D), although the magnitude of this effect was much less than the almost 100% inhibitory effect of live *K. brevis* as a sole diet (Fig. 1). As seen with live diets, *B. plicatilis* appeared to be somewhat more inhibited by *K. brevis* extracts than was *B. ibericus*, especially in the first half of the feeding experiment ($p < 0.001$ for effect of species by two-factor repeated measures ANOVA; Fig. 3C,D).

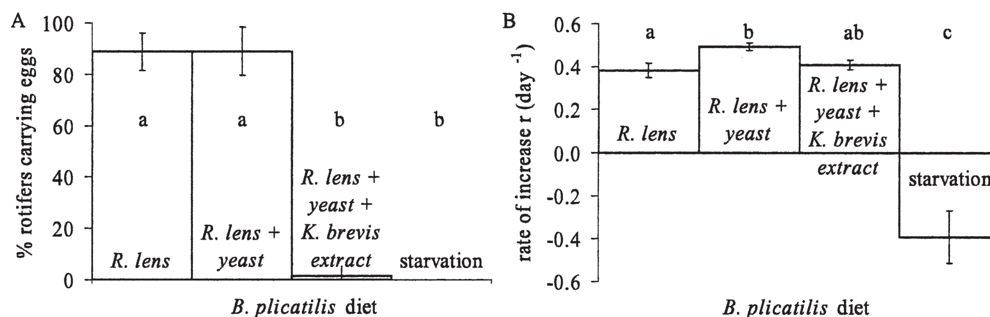

Fig. 4. (A) *B. plicatilis* egg production after 28 h and (B) rate of population increase (r) over 5 d when exposed to diets of *R. lens* and yeast treated with *K. brevis* cellular extracts ($n = 6$). Different letters indicate treatments that significantly differed by one-way ANOVA ($p < 0.001$; df $= 3$; $F = 71.8$–245) followed by Tukey post-hoc test (all $p < 0.05$).

Consistent with the finding that *B. ibericus* was not inhibited from feeding on a mixed diet of *K. brevis* and *R. lens*, yeast treated with *K. brevis* cellular extracts and offered as part of a mixed diet with *R. lens* did not deter the feeding behavior of *B. ibericus* (Fig. 3B). When these data for *B. ibericus* were analyzed by repeated measures ANOVA, there was a marginal effect of diet trending toward the *K. brevis* extract being stimulatory ($p = 0.096$ comparing rotifers fed *R. lens*, *R. lens* + untreated yeast, and *R. lens* + extract-treated yeast). When compared with data for *B. plicatilis*, significant effects of species, diet, time, and all possible species/diet/time interactions (all $p \leq 0.002$) were detected by two-factor repeated measures ANOVA (Fig. 3A,B).

Given that *B. ibericus* fed actively on yeast coated with *K. brevis* cellular extracts when mixed with *R. lens*, it was not surprising that these rotifers reproduced and their populations grew at rates indistinguishable from conspecifics fed untreated yeast and *R. lens* ($p = 0.96$ for egg production; $p = 0.90$ for rate of population increase; data not shown). The rates of increase (r) for *B. ibericus* fed each of these three diets were 0.35 ± 0.01 d^{-1} (*R. lens*-only diet), 0.49 ± 0.07 d^{-1} (*R. lens* + untreated yeast), and 0.51 ± 0.05 d^{-1} (*R. lens* + extract-treated yeast).

Effects of brevetoxins and K. brevis *extracellular extracts on rotifers*—To test whether brevetoxins associated with *K. brevis* cells were responsible for deterring *B. plicatilis* feeding behavior, rotifers were offered a mixture of *R. lens* and yeast treated with brevetoxins (using PbTx-2, PbTx-3, and PbTx-9 at concentrations mimicking that found in *K. brevis* cells, *see* Materials and methods). There was no apparent deterrent effect of cellular brevetoxins in this experiment, since 80–100% of rotifers exposed to *R. lens*, *R. lens* + untreated yeast, or *R. lens* + brevetoxin-treated yeast had full guts throughout the 5-d experiment (effect of diet $p = 0.56$; Fig. 5A).

K. brevis extracellular extracts and pure brevetoxins (PbTx-2, PbTx-3, and PbTx-9 totaling 9 ng mL^{-1}) added directly to seawater along with *R. lens* did not deter feeding by *B. plicatilis*, suggesting that compounds exuded from *K. brevis* were not involved in deterrence (effect of treatment $p = 0.40$; Fig. 5B). Additionally, rotifer egg production and population growth were indistinguishable among the three treatments (*R. lens*, *R. lens* + *K. brevis* extracellular extract, *R. lens* + extracellular brevetoxins) ($p = 0.070$–0.99, trending toward the exudates being stimulatory rather than deterrent; data not shown). A similar feeding experiment with *K. brevis* extracellular

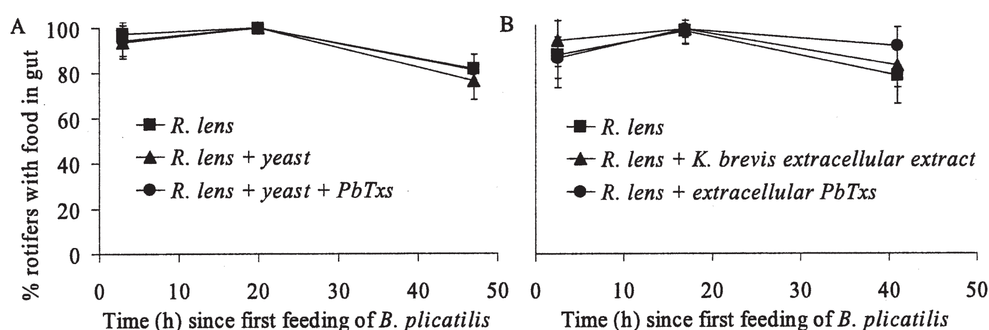

Fig. 5. Feeding behavior of *B. plicatilis* exposed to diets of (A) *R. lens* and yeast treated with brevetoxins ($n = 4$), or (B) *R. lens* and *K. brevis* extracellular extracts or extracellular brevetoxins ($n = 10$). Starved rotifers never had food in their guts (data not shown). One-way repeated measures ANOVA detected: (A) no effect of diet ($p = 0.56$; df $= 2$; $F = 0.61$), an effect of time ($p < 0.001$; df $= 2$, $F = 41.5$), and no time $\times$ diet interaction ($p = 0.91$; df $= 4$; $F = 0.24$); (B) no effect of treatment ($p = 0.40$; df $= 2$; $F = 0.96$), an effect of time ($p < 0.001$; df $= 2$; $F = 27.1$), and a time $\times$ treatment interaction ($p = 0.017$; df $= 4$; $F = 3.29$).

extracts and *R. lens* was conducted using *B. ibericus*, with a similar outcome: *K. brevis* exudates had no effect on *B. ibericus* feeding behavior, egg production, or population growth, compared with rotifers fed only *R. lens* (all $p > 0.50$; data not shown).

Discussion

K. brevis *is unpalatable to brachionid rotifers*—*K. brevis* was found to be a poor food for *B. plicatilis* and *B. ibericus*, because these rotifers avoided consuming a sole diet of *K. brevis* compared to a sole diet of a known palatable food, *R. lens* (Fig. 1). The failure of rotifers to feed on a sole diet of *K. brevis* had a direct negative consequence on rotifer fitness, with zero egg production and negative population growth during the course of 5–6 d, indistinguishable from starved controls (Fig. 2). The lack of rotifer grazing on *K. brevis*-only diets (at $2.3–3.4 \times 10^6$ cells L^{-1} in our experiments) may play a role in facilitating the maintenance of *K. brevis* red tides, because in the Gulf of Mexico *K. brevis* can bloom nearly monospecifically with thousands to millions of cells per liter (Tester and Steidinger 1997), which would be expected to lead to low rotifer grazing rates and rotifer population declines. However, other components of the microzooplankton community such as ciliates and heterotrophic dinoflagellates, as well as metazoan grazers, are likely to be important predators of bloom-forming phytoplankton (Turner et al. 1998), such that the ecosystem implications of reduced grazing on *K. brevis* by rotifers remain of interest for future investigation.

Shared evolutionary history may predispose B. ibericus *to tolerate* K. brevis *in a mixed diet*—When rotifers were exposed to *K. brevis* mixed with *R. lens*, *B. ibericus* appeared to consume the mixed diet (Fig. 1B), and its egg production and population growth was similar to conspecifics fed only *R. lens* (Fig. 2B,D). In contrast, *B. plicatilis* fed on the mixed diet only after a delay of 4 d, resulting in reduced egg production and population growth (Figs. 1A, 2A, 2C). The recovery in *B. plicatilis* feeding rates appeared to be driven by acclimation to *K. brevis* with time rather than by near-starvation, since there was no difference in the onset time of feeding on mixed diets among newly-hatched vs. 2-day-old *B. plicatilis* (*see* Results). The observation that individual rotifers can become acclimated to an unpalatable food has not been previously reported. However, because our feeding behavior data relied on rotifer gut fullness rather than on cell clearance rates, we cannot exclude the possibility that rotifers favored *R. lens* over *K. brevis* when exposed to the mixed diet. Nevertheless, *B. plicatilis* and *B. ibericus* differed in their responses to mixed diets that included *K. brevis*: the presence of *K. brevis* did not deter *B. ibericus* from actively filtering food and deriving adequate nutritional benefit, whereas *B. plicatilis* was deterred by *K. brevis* in a mixed diet and suffered reduced fitness as a result (Figs. 1, 2). Environmental differences between *B. ibericus* and *B. plicatilis*, including maternal effects, cannot explain the observed pattern, since rotifers of both species were hatched under identical conditions from resting eggs, whose ancestors (for at least 10 generations) were reared on identical diets that did not include either *K. brevis* or *R. lens*. Thus, it appears most likely that genetic differences between the *B. ibericus* and *B. plicatilis* populations used in the current study account for the different feeding responses of rotifers to *K. brevis*.

Since the population of *B. ibericus* used in this experiment was originally collected from the west coast of Florida where *K. brevis* blooms on a near-annual basis, it seems plausible that our *B. ibericus* population adapted to tolerate *K. brevis*, at least as part of a mixed diet, similar to copepods exposed to toxic *Alexandrium* spp. dinoflagellates (Colin and Dam 2002*a*). In contrast, the *B. plicatilis* population from an inland sea in Russia probably had no recent exposure to *K. brevis*, so these rotifers have experienced little selection pressure to adapt to feeding on *K. brevis*. Historically *B. ibericus* and *B. plicatilis* were thought to be members of the same species (*B. plicatilis*); however, recent molecular genetic studies suggest divergence may have occurred many million years ago, despite relatively few morphological differences among members of this species complex (Ciros-Perez et al. 2001). It would be useful to repeat the mixed diet feeding experiments with a *B. ibericus* population that has no experience with *K. brevis* and/or a *B. plicatilis* population that has been exposed to *K. brevis* to test whether traits related to tolerance of *K. brevis* in a mixed diet differ at the species or population levels. Additionally, the finding that rotifers can acclimate, during individual lifetimes, to an unpalatable food suggests that one possible mechanism by which *B. ibericus* could have adapted to tolerate *K. brevis* over evolutionary time is via gradual shortening of the acclimation period.

The active feeding behavior of *B. ibericus* when exposed to a phytoplankton mixture that included *K. brevis* (containing approximately 18 pg cell^{-1} brevetoxins) could provide a previously unrecognized mechanism by which rotifers serve as vectors of brevetoxins into food webs, as has been described for copepods (Tester et al. 2000). Similarly, saxitoxin-resistant clams have recently been highlighted as posing an increased risk of paralytic shellfish poisoning for humans due to an adaptive mutation that enables clams to accumulate more saxitoxins in their tissues than clams that do not share the mutation and thus cannot feed on as much toxic *Alexandrium* spp. (Bricelj et al. 2005). In the case of *B. ibericus*, it is not yet clear what traits separate it from *B. plicatilis*, although it is unlikely to be tolerance for brevetoxins, since rotifers of both species fed successfully on yeast diets containing pure PbTx-2, PbTx-3, and PbTx-9 (Fig. 5A and Results).

Unselective grazers?—Rotifers have been reported to be unselective in their feeding behavior (Lionard et al. 2005), or selective based primarily upon cell size or food value (Hotos 2002). One possible interpretation of the data in Fig. 1 is that *B. plicatilis* and *B. ibericus* may have been unable to consume *K. brevis* because of its size or morphology. However, previous studies (Hotos 2002; Wang et al. 2005) have shown that *B. plicatilis* can consume phytoplankters with diameters of 16–20 μm and

25–35 μm, respectively, including armored dinoflagellates (*K. brevis* is an unarmored dinoflagellate, and therefore should be even less likely to deter grazers because of morphology). *K. brevis* used in the current study had dimensions of $24 \times 24 \times 13$ μm, well within the acceptable range for *B. plicatilis*. Preliminary experiments also indicated that our populations of *B. plicatilis* and *B. ibericus* readily consumed another, larger dinoflagellate, *Peridinium* sp., with dimensions of $25 \times 22 \times 22$ μm (Kubanek and Pirkle, unpubl. data). Our results further support the idea that brachionid rotifers show some feeding selectivity, since *B. plicatilis* ceased feeding, at least for a period of days, when faced with an undesirable food such as *K. brevis* (Fig. 1A).

As discussed above, a sole diet of *K. brevis* was rejected by both rotifer species, leading to zero fitness (Figs. 1, 2). Although complete rejection of a phytoplankton food by rotifers appears to be unusual, rotifers including *B. plicatilis* are known to reduce feeding rates when faced with toxicant stress, such as dissolved pollutants (Snell 2005). Thus, following the experiments reported in Figs. 1 and 2, we hypothesized that the unpalatable nature of *K. brevis* was caused by deterrent chemical compounds produced by *K. brevis*. Had *K. brevis* been a source of chronic toxins, we would not have expected the high population growth rates observed for *B. ibericus* on the mixed diet (Fig. 2B,D) nor would we have expected to see a shift from rejection to acceptance of mixed diets by *B. plicatilis* (Fig. 1A).

Rejection of K. brevis *by rotifers is chemically mediated, but not caused by brevetoxins PbTx-2, PbTx-3, or PbTx-9*— The delay in feeding by *B. plicatilis* on yeast treated with *K. brevis* cellular extracts, offered with a diet of live *R. lens*, indicated that *K. brevis* is chemically defended against rotifer grazing (Fig. 3A). In contrast, *B. plicatilis* readily consumed *R. lens* alone or with untreated yeast. *B. ibericus*, however, promptly consumed all treatments, including *R. lens* with extract-treated yeast (Fig. 3B). The deterrence of *K. brevis* cellular extracts toward *B. plicatilis* but not *B. ibericus* (Fig. 3A,B) was consistent with the deterrence of live *K. brevis* cells toward *B. plicatilis* but not *B. ibericus* (Fig. 1A,B). The 24-h delay of *B. plicatilis* feeding on extract-treated yeast and live *R. lens* was shorter than the 4-d delay observed for rotifers feeding on live mixed diets (Fig. 3A vs. Fig. 1A). This difference may be explained by variance in the feeding experiments, since repetition of the experiment depicted in Fig. 1A yielded feeding deterrence that wore off more quickly (data not shown). Also potentially suggestive of experimental variation, repetition of the experiment represented by Fig. 3A with *K. brevis* extract-treated yeast resulted in *B. plicatilis* feeding deterrence lasting more than 2 d (data not shown). In addition to variance among feeding experiments, deterrent compounds could have been gradually lost from yeast particles. In a preliminary experiment, only 10% of PbTx-2 remained associated with yeast particles after 16 hours (*see* Materials and methods), suggesting that other organic compounds could also diffuse from extract-treated yeast into seawater or decompose during the course of a rotifer feeding experiment, potentially resulting in an accelerated return to active feeding by *B. plicatilis*. This limitation of the yeast-based diet probably means that the deterrence of *K. brevis* cellular extracts that we observed in separate experiments using two independently prepared batches of *K. brevis* extract represents a conservative assessment of *K. brevis* chemical defenses.

The nearly complete refusal by rotifers to feed on *K. brevis*-only diets (Fig. 1) could be only partially explained by a hypothesis of chemical defense involving cellular extracts of *K. brevis*. Although, in the absence of *R. lens*, extract-treated yeast was consumed by significantly fewer rotifers than was untreated yeast, the magnitude of this deterrence (up to 40% reduction in feeding; Fig. 3C,D) was not as strong as the almost 100% rejection of live *K. brevis* by both rotifer species (Fig. 1). The deterrence of *K. brevis* cellular extracts in the yeast-only treatments appeared stronger for *B. plicatilis* than for *B. ibericus* (Fig. 3C,D), analogous to *B. ibericus* being more tolerant of *K. brevis* cells and extracts in mixed diets (Figs. 1, 3A, 3B). In general, rotifers fed sole diets of yeast (whether treated or untreated) had lower population growth rates than rotifers fed mixed diets of yeast and *R. lens* (data not shown), and so it could be that rotifers in the yeast-only treatments were stressed and therefore did not respond as intensely to *K. brevis* extracts as to live *K. brevis*. Alternatively, it is possible that the rejection of live *K. brevis*-only diets by both *B. plicatilis* and *B. ibericus* has both a chemical defense and a nutritional basis.

Brevetoxins are commonly found associated with *K. brevis* cells, and to a lesser extent are found in seawater during blooms, especially toward the end of blooms when *K. brevis* cells lyse (Pierce et al. 2001; Pierce et al. 2005). Therefore, it was reasonable to hypothesize that the deterrence of *K. brevis* could be attributable to intracellular or extracellular brevetoxins, which can kill fish, shellfish, seabirds, and marine mammals (Tiffany and Heyl 1979; Flewelling et al. 2005). Yet, brevetoxins (PbTx-2, PbTx-3, and PbTx-9) had no effect on rotifer feeding behavior or reproduction when fed to *B. plicatilis* as part of a yeast diet at concentrations similar to those measured in *K. brevis* cells (Fig. 5A). A loss of brevetoxins from yeast-based diets during feeding experiments cannot account for this lack of deterrence, because this loss would be expected to occur at similar rates whether brevetoxins were part of a *K. brevis* cellular extract added to yeast and fed to rotifers (Fig. 3A) or pure brevetoxins were added to yeast and fed to rotifers (Fig. 5A). Yet, despite the partial loss of brevetoxins from yeast diets, cellular *K. brevis* extracts were deterrent to *B. plicatilis* (Fig. 3A), whereas pure brevetoxins were not (Fig. 5A). Because substantial quantities of brevetoxins leached from yeast particles during feeding assays (*see* Materials and methods), one could posit that the observed deterrence of *K. brevis* extracts could be due to waterborne brevetoxins. However, waterborne brevetoxins at a bloom-like concentration of 9 ng mL^{-1} (Cheng et al. 2005) had no effect on rotifer feeding (Fig. 5B) or on population growth of *B. plicatilis* (*see* Results). Finally, extracellular extracts of *K. brevis* (containing 22 ng mL^{-1} PbTx-2) added to seawater with *R. lens* led to no significant feeding deterrence

or fitness costs for *B. plicatilis* or *B. ibericus*, confirming that no extracellular compounds from *K. brevis* contributed to the chemical defense of *K. brevis* (Fig. 5B). Instead, we conclude that molecules associated with *K. brevis* cells other than PbTx-2, PbTx-3, or PbTx-9 (the major brevetoxins found in food webs) are responsible for the feeding deterrence of *K. brevis* cells. Isolation and identification of these deterrent compounds is ongoing in our lab.

Rotifers can be susceptible to phytoplankton toxins—Our finding that *K. brevis* is unpalatable to *B. plicatilis* because of cell-associated deterrent compounds, but that known toxins of *K. brevis* play no apparent role in this chemical defense, differs from some previous rotifer feeding studies with other toxic phytoplankton. Despite strong hemolytic activity associated with *Prymnesium parvum* filtrates, *P. parvum* was consumed by *B. plicatilis*; however rotifer population growth on this diet was low, possibly because of other, nonhemolytic toxins associated with *P. parvum* cells (Barreiro et al. 2005). The bloom-forming dinoflagellate *Heterocapsa circularisquama* was found to kill *B. plicatilis* within hours (Kim et al. 2000). A partial structure determination has been reported for a compound from *H. circularisquama* that kills *B. plicatilis* when exposed via seawater (Miyazaki et al. 2005), but since supernatant and cell lysates of *H. circularisquama* did not kill rotifers, it will be necessary to test this toxin as part of a diet rather than as a waterborne cue to establish its role in rotifer-phytoplankton feeding interactions. *B. plicatilis* was found to survive and grow on several strains of *Alexandrium* spp., but three other *Alexandrium* strains caused *B. plicatilis* populations to decline in a dose-dependent manner, faster than starvation controls (Wang et al. 2005). This suggested that some *Alexandrium* strains were toxic to rotifers, however unidentified compounds other than saxitoxins were implicated. In contrast, the Texas brown tide (*Aureoumbra lagunensis*) was shown to be nutritionally inadequate (and possibly deterrent) to *B. plicatilis* (Buskey and Hyatt 1995). Occasionally, the failure of rotifers to thrive on a phytoplankton diet has been attributed to toxicity, especially if survivorship is lower on a phytoplankton diet than for starved controls (Boenigk and Stadler 2004), when in fact feeding deterrence, nutritional inadequacy, and/or physiological stress associated with experimental conditions could have played roles. Overall, it seems that several mechanisms can contribute to the ability of some phytoplankton to reduce rotifer grazing pressure.

Interactions between K. brevis *and other members of the marine plankton are complex*—The neurotoxic effects of brevetoxins on large marine animals, especially vertebrates, have been well documented (Baden 1989). A handful of studies performed during the last 20 years have shown that neurotoxicity is not the only effect of *K. brevis* on plankton community members. *K. brevis* was found to exude compounds suppressing the growth of at least six co-occurring phytoplankton species in the Gulf of Mexico, although brevetoxins were only partially responsible for allelopathy against one competitor (Kubanek et al. 2005). *K. brevis* was shown to be discriminated against by grazing copepods (Huntley et al. 1986; Turner and Tester 1989) and by one rotifer species, but tolerated by a second, closely related rotifer species (this study). *K. brevis* appears to be nutritionally inadequate rather than toxic to one copepod species (Prince et al. 2006) and to a ciliate grazer (Buskey, unpubl. data) while being toxic to other copepods (Sykes and Huntley 1987). In fact, strains of individual phytoplankton species can have very different effects on grazers and competitors (Wolfe 2000; Tillmann and John 2002; Wang et al. 2005) suggesting that generalizing about the effects of a phytoplankton species may be inappropriate, especially when clonal populations (with near-zero genotypic variation) are typically used in laboratory experiments, as in the current study. In addition, recent studies have uncovered significant variation in toxin resistance and tolerance of phytoplankton diets among populations of single grazer species (Colin and Dam 2002*a*; Bricelj et al. 2005). In laboratory experiments, copepod resistance to toxicity was recently shown to evolve within 3–5 generations, suggesting that resistant phenotypes are present within wild populations (Colin and Dam 2005). The current study also points to the likelihood that resistance can evolve within grazer populations coincident with exposure to harmful algae. Thus, it is clear that understanding predator-prey interactions in the plankton requires the consideration of variation and specificity of responses from genotype to community levels.

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