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Decrease in *Daphnia* egg viability at elevated pH

Abstract—The effect of high pH on the reproduction of two *Daphnia galeata* clones was experimentally investigated in the laboratory. We observed that the mortality of juveniles and adults did not increase with increasing pH in the range pH 9.0–10.5, which agrees with what is generally reported in the literature for cladoceran zooplankton and suggests that the threshold value for mortality is between pH 10.5 and 11.5. However, both egg mortality and the proportion of stillborn neonates increased at pH 10.0 and above, and the two clones differed in their sensitivities to pH. Consequently, pH already affects population growth rate markedly from pH 10.0 onward. Because pH values ≥ 10.0 are common during spring and summer in many eutrophic and hypertrophic lakes due to intense photosynthetic activity, we expect that high pH has a much larger effect on the population structure of *Daphnia* and the community composition of microcrustacean zooplankton in such water bodies than was assumed previously.

Comparison of the species composition of microcrustacean zooplankton in many Northwest European, North American, and temperate Asian lakes has shown that the number of species present is strongly correlated with pH, with species diversity highest in lakes varying in pH from 6.8 to 7.2 (Ivanova 1987). Thus, it seems that the abundance and presence of many zooplankton species are negatively affected by both low and high pH. Considerable experimental research has been done on the effects of pH on the population dynamics and community composition of microcrustacean zooplankton (e.g. Havens 1992). However, these studies were concerned with the effects of acidification, while the ecological importance of high pH has been less investigated. Information based on field and laboratory experiments suggests that most cladoceran species have an upper pH limit in the range of 10.5–11.5 (O'Brien and DeNoyelles 1972; Hansen et al. 1991). It is unclear, however, how these high pH values affect the population growth rate of cladocerans. Most previous studies have been concentrated on direct toxic effects of pH on the free-living stages. However, an elevated pH may affect the population growth rate through chronic effects on somatic growth and fecundity. In our study, we examine the response of a *Daphnia galeata* population to elevated pH. Special interest is focused on the impact of elevated pH on egg viability. The pH values tested in the experiments were chosen because spring and summer pH values of many eutrophic and hypertrophic lakes and ponds in the temperate zone fall within this range (e.g. Jeppesen et al. 1990).

The experiments were carried out with two randomly selected clones of *D. galeata,* DGD2 and DGD3, which had been collected from Tjeukemeer, a large eutrophic lake in the north of the Netherlands. Fresh food media were prepared every 2–3 d, 3 times a week. *Chlamydomonas reinhardi* was offered as a food organism in a concentration well above the incipient limiting level (100,000 cells ml^{-1}; ~2.5 mg C $liter^{-1}$). The algae were cultured axenically in a continuous culture system under constant light (150 W m^{-2}) and constant temperature (20±1°C). The composition of the algal culture medium is given in

Table 1. Composition and concentrations (in mg liter^{-1}) of salts, trace elements, and Fe-EDTA complex in the algal culture medium.

Medium composition		Trace element composition		Fe-EDTA complex solution	
KNO_3	250	H_3BO_3	0.061	Na-EDTA	8,000
$MgSO_4 \cdot 7H_2O$	125	$MnCl_2 \cdot 4H_2O$	0.198	$(NH_4)_2SO_4Fe_2(SO_4)3.24H_2O$	10,000
$(NH_4)_2HPO_4$	15	$MnSO_4 \cdot H_2O$	0.169		
$NaH_2PO_4 \cdot H_2O$	115	$ZnSO_4 \cdot 7H_2O$	0.287		
K_2HPO_4	10	$CuSO_4 \cdot 5H_2O$	0.0025		
$CaCl_2 \cdot 2H_2O$	5	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0124		
$NaHCO_3$	7.5	$Co(NO_3)_2 \cdot 6H_2O_4$	0.08		
Trace elements	0.625 ml	NH_4VO_3	0.01		
Fe-EDTA	0.625 ml				

Table 1. Before suspending the algae in the *Daphnia* medium, the algal culture medium was removed by centrifuging twice for 10 min at 2,500 rpm and by subsequently washing the algae with *Daphnia* medium. We prepared this medium according to the recipe of Lynch et al. (1986), which we slightly modified to prevent (as much as possible) pH fluctuations in the *Daphnia* medium; i.e. we used 0.5 mg liter^{-1} K_2HPO_4 (instead of 6.0) plus 6.0 mg liter^{-1} KH_2PO_4. The algae were then resuspended in a mixture (vol/vol) containing 88% artificial *Daphnia* medium supplemented with 12% fresh 0.45-μm-filtered lake water. Daphnids were acclimatized to their experimental conditions for two generations. They were kept under constant illumination at 17.5±0.2°C, and pH was kept constant at 9.0±0.1. It was not feasible to acclimatize the daphnids to the different experimental pH values because very few viable newborn were produced at the higher pH levels. As soon as the animals produced newborn, the mothers were removed. These newborn were reared to maturity and their offspring used in the experiments.

A buffer of NaOH-$NaHCO_3$ was used to make the different pH series. Between the different series, only the ratios between the two salts were varied, keeping total salt content and sodium concentration the same for the different series. Four constant pH treatments were applied: 9.0±0.1, 9.5±0.1, 10.0±0.1, and 10.5±0.1 (mean ± range). We kept 16 individual cultures per pH series (8 for each clone). Cultures were started with neonates collected within 24 h of birth and placed individually in 100-ml test tubes that were sealed at the top with a silicon plug to prevent loss of CO_2 from the medium. Newborn from different mothers were equally divided over the different series. Every 2–3 d, 3 times a week, the individuals were examined and then transferred to clean tubes with fresh medium. During each observation, dead individuals were noted and removed. Live animals were observed, every molt noted, and the number of eggs-embryos and of newborn recorded. Furthermore, egg mortality (i.e. egg resorption) was noted, and newborn were discarded after being examined; observations were stopped when the daphnids reached the fourth adult instar.

Table 2. Mean percentage (SD) P, C, and N of dry weight of *Chlamydomonas reinhardi* before and 48 h after suspension in *Daphnia* medium at different pH.

pH	P content	C content	N content
Before suspension			
10.0–11.0	1.06 (0.04)	40.0 (0.18)	5.30 (0.34)
48 h after suspension			
9.0	1.24 (0.02)	41.25 (1.11)	5.11 (0.07)
9.5	1.13 (0.01)	40.11 (3.46)	5.55 (0.49)
10.0	1.10 (0.02)	40.44 (0.45)	5.77 (0.03)
10.5	1.13 (0.09)	42.63 (0.36)	5.60 (0.49)

The P content of the food algae (*Chlamydomonas*) from the different pH series and the algal medium was measured according to Murphy and Riley (1962) after combusting the material at 550°C and then dissolving the P in the ash in 2 N H_2SO_4. A Carbo Erba gas analyzer was used to analyze the C-N composition of the algae.

The intrinsic rate of population increase r was computed in order to integrate the data on growth and reproduction by means of the Euler equation:

$$1 = \sum_{x=0}^{N} \mathrm{e} \exp(-rx) l_x m_x .$$

r is the per capita rate of increase for the population (d^{-1}), x is the age class in days (0, 1, . . . , N), l_x is the probability of surviving to age x, and m_x is the fecundity at age x. Because r is a population parameter, and hence only one value per treatment is available, it is not possible to compute confidence limits directly. These were computed with a jackknife method (Meyer et al 1986) and used to analyze the significance of the differences between treatments by means of t-tests with P-values corrected by a sequential Bonferonni technique (e.g. Rice 1988).

The P, N, and C contents of the algal food particles were measured just before and 48 h after their suspension in *Daphnia* medium (Table 2). The P contents of the algae remained high in the *Daphnia* medium over the whole pH range, and pH treatments caused only small, nonsignificant changes in the P, N, and C composition of the algae in the *Daphnia* cultures (P content: $F_{3,4} = 4.09$; $P = 0.10$; N content: $F_{3,4} = 1.31$; $P = 0.39$; C content: $F_{3,4} = 0.74$; $P = 0.58$). Thus, the apparent food quality of the algae was not influenced by the pH treatment.

Within the pH range 9.0–10.5, we observed no clear

Table 3. Mean number of eggs per female (±95% C.L.) of two clones (DGD2, DGD3) of *Daphnia galeata* cultured at different pH values.

	DGD2		DGD3	
pH	Mean	*N*	Mean	*N*
9.0	11.1 (1.90)	30	8.6 (1.45)	22
9.5	9.7 (1.57)	28	7.7 (1.16)	23
10.0	9.0 (1.28)	31	6.0 (1.07)	21
10.5	7.6 (1.72)	27	7.5 (1.44)	24

relationship between mortality and pH. Furthermore, mortality rates in the culture were moderately low (i.e. only a few percent per day above what is usually observed in well kept cultures, Vijverberg 1989). We analyzed length-growth vs. instar number in relation to pH and clone type by using a two-way ANOVA with a repeated-measures design. pH effects were not significant (P = 0.068), but instar effect (P = 0.001) and interactions between instar number and pH (P = 0.037) and between clone and pH (P = 0.045) were significant.

The number of eggs per ovigerous female in the different pH series was analyzed with a two-way ANOVA; pH and clone were used as the independent variables and the number of eggs carried by the different adult instars as the dependent variables. To avoid serial correlation between the measurements of different instars of the same animal, we used a repeated-measures design to analyze the number of eggs. The mean number of eggs per adult female decreased significantly with pH (ANOVA, Tables 3, 4), but the differences were small. Also a substantial and significant increase in egg mortality occurred with increasing pH (Fig. 1; χ^2 = 491.8; df = 3; $P < 0.001$). Eggs degenerated and were resorbed before the next molt occurred. At pH 10.5, egg mortality was high, although at pH = 10.0, egg mortality also resulted in reduced fecundity. We did not find a clonal effect: egg mortality was not significantly different between the two clones (χ^2 = 1.19; df = 1; P = 0.276).

Dead and inactive neonates were frequently observed. In a minority of cases, the neonates were still alive, although in a very poor condition, as judged by their heartbeat and irregular and spasmodical movements of their

Table 4. Summary table of two-way ANOVA with clone (cl) and pH as independent variables, the square roots of the number of eggs as the dependent variable, and instar as the repeated-measures effect.

Effect	MS	df	*F*	*P*
Clone	4.6718	32	12.97	<0.001
pH	5.6250	32	15.62	<0.001
Instar	3.6628	96	12.13	<0.001
cl × pH	0.8851	32	2.46	0.081
cl × instar	0.9205	96	3.05	0.032
pH × instar	3.9803	96	13.19	<0.001
cl × pH × instar	0.4917	96	1.63	0.118

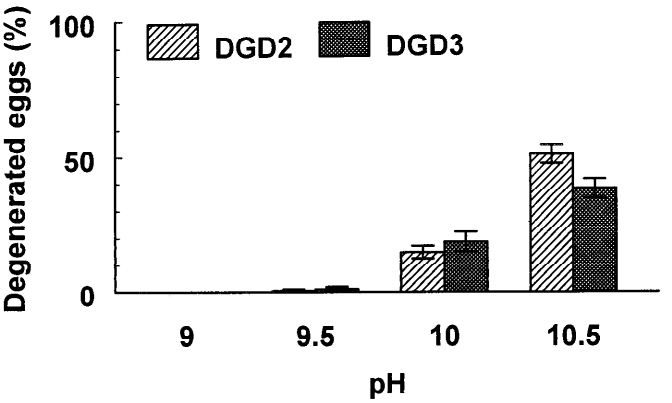

Fig. 1. Mean percentage of degenerated eggs with 95% C.L. of two clones (DGD2, DGD3) of *Daphnia galeata* cultured at different pH values; mean and confidence limits calculated according to the binomial distribution.

antennae. However, these individuals were always lying on the bottom of the Petri dish, were never observed swimming, and invariably died within 24 h after they had been seen. It is likely that most neonates were born alive but were in such a poor condition that they died soon thereafter. All these newborn were categorized as stillborn neonates. We observed a distinct and significant increase in stillborn neonates with increasing pH (Fig. 2; χ^2 = 193.2; df = 3; $P < 0.001$). At pH 10.5, almost half the neonates were stillborn, which caused a marked reduction in fecundity; newborn at pH 10.0 were also seriously affected, while the effects were smaller at the series below pH 10.0. We also found a significant clone effect (χ^2 = 11.05; df = 1; $P < 0.001$). Generally, clone DGD3 showed a markedly higher mortality of newborn than DGD2 except at pH 10.5, where the latter showed a slightly higher mortality (Fig. 2).

Assuming all eggs are viable and result in living newborn, t-tests with the sequential Bonferonni corrections showed no significant differences in the rate of population increase (r) between treatments (Table 5a, Fig. 3). However, the combined effects of egg mortality and stillborn neonates resulted in strong and significant reductions in

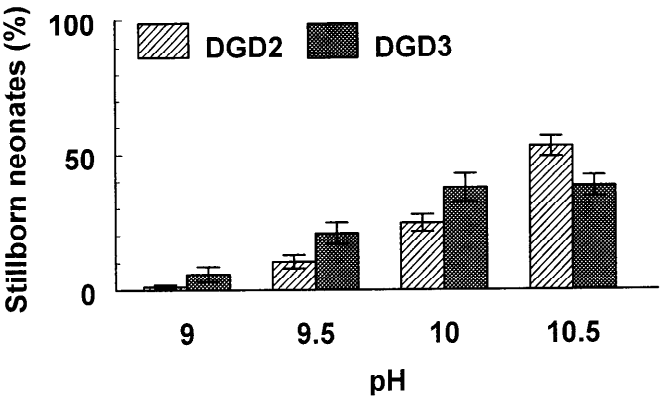

Fig. 2. As Fig. 1, but of stillborn neonates relative to the total number of neonates released.

Table 5. Summary table of t-values of test for the significance of differences in r between different treatments: a—assuming that all eggs are viable; b—after correction for egg mortality and stillborn juveniles for clone DGD2 (above diagonal) and clone DGD3 (below diagonal). Asterisks: *—significantly different at the 0.05 level after sequential Bonferonni corrections (number of tests equals 12).

pH	9.0	9.5	10.0	10.5
	a. All eggs viable			
9.0		0.87	0.59	2.56
9.5	−0.39		−0.44	1.52
10.0	2.17	2.25		2.44
10.5	1.44	1.64	−0.88	
	b. Corrected for mortality			
9.0		1.59	2.51	7.49*
9.5	−0.21		0.51	5.35*
10.0	2.43	2.46		5.47*
10.5	3.04*	3.05*	0.71	

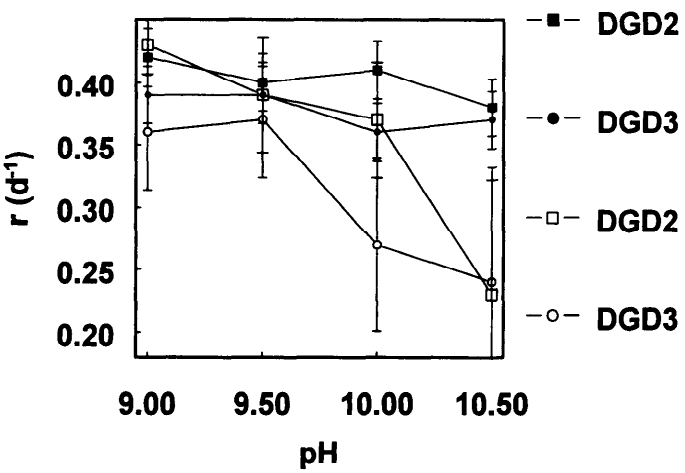

Fig. 3. Mean values with 95% C.L. of the rate of population increase (r) of two clones (DGD2, DGD3) of *Daphnia galeata* at different pH: ■, ●—assuming all eggs produced are viable and result in living newborn; □, ○—including effects of egg mortality and stillborn neonates. Confidence limits calculated with the jackknife method.

r. The r-value of clone DGD2 is significantly reduced at pH 10.5 as compared with pH 9.0, 9.5, and 10.0 (Table 5b, Fig. 3), whereas the differences among pH 9.0, 9.5, and 10.0 are nonsignificant. In contrast, the r-value of clone DGD3 shows a more general decrease over the pH range 9.0–10.5, a significant difference between pH 9.0 and 10.5 and between pH 9.5 and 10.5, but no significant differences between pH 9.5 and 10.0 or between pH 10.0 and 10.5. Both clones reached similar low r-values at pH 10.5.

We observed a strong effect of high pH on reproduction, but the question arises whether this pH effect acts directly (as a stress factor) or indirectly via variations in the food quality. Direct effects that may have played a role at high pH are toxic effect of un-ionized ammonia (NH_3) on *Daphnia* and disruption of ion exchange in *Daphnia*. An indirect effect may have been the change of algal food conditions for *Daphnia* as result of pH shock undergone by the algae. *Chlamydomonas* used as food for the daphnids was cultured in continuous cultures at pH 10.0–11.0 and then suspended in *Daphnia* medium varying in pH from 9.0 to 11.0. Thus at the lower pH levels, algae endured a pH shock of 1.0–2.0 pH units. We know that changes in food quality as a result of the variation in pH are less likely because nutrient status of the algae, judged by C : P and C : N ratios, showed no pH effect (Table 2). Furthermore, the P content of the algae was high (>1%) in all four pH treatments, which is an indication that the food quality was probably high (Sterner 1993). Additionally, an indirect effect would have resulted in reduced somatic growth and a reduced number of larger eggs; larger eggs contain more yolk and will have a higher viability (Tessier and Consolatti 1989). Because we did not observe a reduced growth rate in relation to elevated pH, egg viability did not increase but decreased, and because the observed reduction in number of eggs produced was small and did not contribute to the observed overall reduction in r (see Fig. 3), the high degeneration and stillborn rates at elevated pH are likely due to direct effects.

The two direct effects on *Daphnia* that may have played a role are the toxic effect of un-ionized ammonia and the disruption of ion exchange. The equilibrium between un-ionized and ionized ammonia is strongly influenced by pH (i.e. more of the toxic NH_3 will be present at high pH). It is known that un-ionized ammonia is toxic for a number of organisms, including cladocerans. Chronic values of NH_3 for cladocerans range from 0.30 mg liter^{-1} in *Ceriodaphnia* to 1.2 mg liter^{-1} in *Daphnia magna* (U.S. EPA 1985). Of the *Daphnia* medium we used, the largest part (88% by volume) was based on distilled water and contained no ammonia at all. Only a small proportion (12%) of filtered lake water was added that contained ~0.2 mg liter^{-1} ammonia-N; thus the concentration of ammonia in freshly prepared *Daphnia* medium was ~0.02 mg liter^{-1}, which is too low to have any detrimental effect on *Daphnia*. Furthermore, because we cultured the daphnids individually in relatively large volumes (~80 ml daphnid^{-1}) and renewed the medium frequently, the effect of the accumulation of ammonium as a result of the excretion of the daphnids would have been negligible.

In some cyclopoid, copepod, and cladoceran species, the physiological effect of high pH has been shown to be caused by a pH effect on the sodium balance (Potts and Fryer 1979; Nilssen et al. 1984). Copepods usually showed a good sodium balance up to ~pH 9.5, but above this pH they showed a net sodium loss (Nilssen et al. 1984). In our study, we used a $NaHCO_3$-NaOH buffer that resulted in a concentration of Na^+ in the *Daphnia* medium of ~3.4 mmol liter^{-1}, which is high compared with natural lake water and makes it less likely that sodium became limited at higher pH levels.

It is conceivable, however, that other metals became

limited at high pH levels. Results of culture experiments by Elendt and Bias (1990) suggest that selenium deficiency in culture media may cause egg abortions and neonate mortality in *Daphnia*. We observed exactly the same phenomena at high pH so it is tempting to regard Se limitation as the possible causal factor. However, it is not likely that this was the case. First, because we used 12% filtered lake water from a hypertrophic lake for our *Daphnia* medium, the Se concentration in this medium was ~0.1 μg liter^{-1}, which is high enough for successful reproduction and low neonate mortality (Elendt and Bias 1990). Second, within the pH range studied, Se speciation did not change (W. Verwey pers. comm.) (i.e. availability of Se was not inhibited at high pH).

Deleterious effects of important abiotic influences such as pH or toxic substances are often stronger at low food levels because they usually act via the inability of the organism to keep food intake and assimilation high enough to pay for increased respiration (Reinikainen et al. 1994). We found the number of neonates produced was reduced by 50–80% due to egg degeneration and stillbirth in the pH range of 10.0–10.5. Because we cultured the daphnids at high food levels—well above the incipient limiting level—and because even in eutrophic lakes daphnids may be food limited as a consequence of prevailing low food quality (Boersma and Vijverberg 1994), pH effects on egg and neonate viability in the natural habitat may be even larger than we observed.

Several studies have reported the presence of degenerated eggs in populations of cladocerans under natural conditions (e.g. Threlkeld 1985; Boersma and Vijverberg 1995), but in none of these studies was high pH considered a possible factor for this mortality. Our study demonstrates that high pH can substantially reduce the egg viability and fitness of microcrustacean zooplankton. A pH value >10.0 is commonly found in many eutrophic and hypertrophic lakes. Therefore, the effect of high pH on the population dynamics and community composition of microcrustacean zooplankton is probably much more important than has been assumed.

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Avoidance by *Daphnia magna* of fish and macrophytes: Chemical cues and predator-mediated use of macrophyte habitat

Abstract—Recent biomanipulation studies suggest that macrophytes are an important refuge from fish predation for large pelagic zooplankton. We conducted two laboratory experiments that tested the behavioral responses of *Daphnia magna* to a macrophyte (*Myriophyllum exalbescens* L.) and a sunfish (*Lepomis cyanellus* Rafinesque) and whether responses were chemically (for fish) or structurally (for macrophytes) mediated. In the first experiment, we measured *Daphnia* response to four treatments in separate 38-liter tanks. In controls without macrophytes and fish, ~15% of the daphnids were found in the central zone (~50% of the tank area); the others were found around the tank perimeter (especially in the corners). With macrophytes present, 80% of the daphnids were found in the central zone (unvegetated in all treatments). When fish or fish odor alone were present ~35% and ~45%, respectively, of the *Daphnia* occupied the central zone. Thus, chemically mediated avoidance of *Lepomis* caused *Daphnia* to increase its occupation of macrophytes. In the second experiment, we tested whether the repellent effect of *Myriophyllum* resulted from structural characteristics of the macrophyte; the results suggest that both chemical and structural cues contributed to *Daphnia* avoidance of the macrophyte. Overall, our results are consistent with the suggestion that large pelagic zooplankton may use macrophytes as a refuge in shallow lakes where vertical migration is restricted.

Macrophytes fill multiple roles in ecosystem function (Carpenter and Lodge 1986) and in the mediation of predator-prey interactions involving fish and macroinvertebrates (Crowder and Cooper 1982; Savino and Stein 1982). Investigators have suggested that macrophytes provide a refuge to *Daphnia* from fish predation and thus contribute to biomanipulation efforts to reduce phytoplankton standing stock (Timms and Moss 1984; Jeppesen et al. 1991). Scheffer et al. (1993) suggested that macrophyte refuges for *Daphnia* contribute significantly to the stability of the high *Daphnia*-low phytoplankton-high macrophyte state in shallow lakes.

Recent documentation of diel horizontal migration of zooplankton in shallow lakes, in which large zooplankton congregate in or near macrophytes and other littoral zone structure during the day, are consistent with this hypothesis (e.g. Davies 1985; Paterson 1993). The assumption is that macrophytes in shallow lakes, as in the metalimnion and hypolimnion in deeper lakes (e.g. Gliwicz 1986; Ringelberg 1991; Lampert 1993), offer large zooplankton a refuge from fish predation. (Timms and Moss 1984; Jeppesen et al. 1991). However, we know of no direct test of the hypothesis that zooplankton movement into macrophytes is a response to fish. The use of macrophyte habitats by pelagic zooplankton seemingly contradicts earlier field (Hasler and Jones 1949; Pennak 1966) and laboratory (Pennak 1973) results that suggest that daphnids are repelled by macrophytes. We hypothesized that when faced with a choice of remaining in the presence of fish or moving into macrophytes, predation-vulnerable zooplankton seek refuge in macrophytes despite their repellency. We conducted laboratory experiments to test whether macrophytes repel *Daphnia* in the absence of fish, whether *Daphnia* avoids fish, and whether behavioral avoidance of fish overrides avoidance of macrophytes. We also tested whether the stimuli for avoidance behavior were structural for macrophytes and chemical for fish.

We conducted two experiments in August–September 1994. The first experiment tested the behavioral response of *Daphnia magna* Straus to combinations of the presence of a macrophyte (*Myriophyllum exalbescens* L.), green sunfish (*Lepomis cyanellus* Rafinesque) and associated odor, and sunfish odor alone. The second experiment tested whether the stimulus for avoidance of *Myriophyllum* was structural (visual, tactile, etc.). *Daphnia* was purchased from Ward's Natural Science Establishment and kept in fish-free laboratory aquaria. In experiments, we only used *Daphnia* >2 mm long so that the animals were easily observable and could not enter fish enclosures (*see below*). We collected aboveground portions of apparently vigorous *Myriophyllum* from Stone Lake (Cass County, southern lower Michigan) and held them in fish-free laboratory aquaria for no more than 2 d before experiments. We used dipnets to collect *Lepomis* (4–5-cm total length) from St. Joseph Lake (campus of the University of Notre Dame. Indiana) and kept them in laboratory aquaria. Young green sunfish eat zooplankton, such as large cla-