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Environmental Toxicology and Chemistry, Vol. 18, No. 7, pp. 1568–1573, 1999

Printed in the USA
0730-7268/99 $9.00 + .00

LOW EXPOSURE CONCENTRATIONS OF ATRAZINE INCREASE MALE PRODUCTION IN *DAPHNIA PULICARIA*

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(*Received* 26 *May* 1998; *Accepted* 3 *November* 1998)

Abstract—Results of 24 bioassays with the water flea (*Daphnia pulicaria*) show an exposure–response relationship between *Daphnia* sex ratio and the herbicide atrazine. Exposure of *Daphnia* to atrazine during embryogenesis resulted in a shift in sex determination toward males. The shift was detectable at 0.5 ppb (nominal concentration) and pronounced at 10 ppb or more. The shift occurred in the upper part of the range of atrazine concentrations commonly found in aquatic environments (such as lakes, streams, wells, and rainwater). Our results suggest that *Daphnia* sex ratio is one to two orders of magnitude more sensitive to atrazine than are survivorship or fecundity.

Keywords—Atrazine *Daphnia* Exposure–response Sex ratio

INTRODUCTION

We developed a toxicity assay that uses *Daphnia* sex ratio as an endpoint of chemical effect [1,2]. This is a sublethal endpoint, and tests with other chemicals (e.g., nonylphenol and dieldrin) suggest that sex ratio may be more sensitive than fecundity or survivorship [1,2].

Atrazine is a widely used herbicide [3]. Atrazine has been detected (at 0.1 ppb) in 92% of reservoirs in the midwestern United States. In those reservoirs, 10% had concentrations of 5 ppb or greater [3]. Even rainwater contains atrazine at concentrations up to 3 ppb [4–6].

The lifetime-exposure safe drinking water limit was set in 1991 at 3 ppb of atrazine by the U.S. Environmental Protection Agency (EPA) [7]. Results of bioassays with *Daphnia* fecundity and survivorship endpoints suggest there is no effect below approx. 100 ppb [8–10].

Our goal in this study was to develop an exposure–response relationship for atrazine using sex ratio as an endpoint. The probable existence of such a relationship was supported by the observation by Macek et al. [9] that ''the incidence of males induced from parthenogenetic forms appeared to be higher in groups exposed to atrazine than in control groups.'' In preliminary bioassays with *D. galeata mendotae,* we confirmed that exposure to atrazine increased male production.

METHODS

The reproduction bioassay test animal was a clone of *D. pulicaria* collected in Georgia and kept in culture at the Max-Planck Institute for Limnology, Ploen, Germany. This clone routinely produces about 5 to 50% males given the appropriate environmental conditions. To produce these conditions, adult *Daphnia* females were crowded together (eight adults in 30 ml of artificial lake water) and given short days (6 h of light, 18 h of darkness). To maximize reproduction rate and fecundity, the animals were incubated at 21°C and fed abundant algae.

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Algae and *Daphnia* were both cultured in an artificial lake water called ''Combo'' medium [11]. We found that *Daphnia* survival and fecundity was maximized in double-strength Combo. This 2× Combo medium has an electrical conductance of about 490 μmho and a pH of about 8.05 (when in equilibrium with atmospheric CO_2). Double-strength Combo was used as the culture medium for *Daphnia* as well as for the algae fed to *Daphnia.*

The experimental unit was a jar containing 30 ml of Combo, eight egg-bearing *Daphnia,* and sufficient algae to maximize fecundity in the controls. Approximately equal parts of dense suspensions of the algae *Chlamydomonas* and *Selenastrum* were added to each jar to produce an initial suspension with a total of 2×10^6 cells/ml in the experimental jars. The algae sedimented out of the small (short) jars, so the *Daphnia* experienced a variation in food concentration from 2×10^6 cells/ml to near zero at the end of 3 d. Nevertheless, this is enough food for these *Daphnia* to maintain optimal reproduction.

We used a minimum of 12 jars per treatment. Treatments included (1) a control made with Combo medium but with no added chemical, (2) a control treatment including the carrier used in the bioassay (acetone) at the highest concentration used in any treatment in that experiment, and (3) chemical exposure treatments made with Combo medium, algae, and dilutions of atrazine (along with its acetone carrier). The highest acetone concentration used (45 μl acetone/L) is less than 0.01% of the 16-d growth no-observed-effect concentration [12] and less than 0.5% of the median lethal concentration (LC50) for survival or lowest-observed-effect concentration for reproduction of *D. magna* in the three-brood test [13]. A single bioassay typically contained the water and carrier (acetone) controls and up to five atrazine treatments.

Atrazine was purchased from ChemService (West Chester, PA, USA). Atrazine solutions were made from a refrigerated 12.5-ppb stock solution. The stock solution was kept in the dark and remade every 2 weeks. All concentrations reported in this study were nominal based on dilutions of the stock solution. We used 12 exposure concentrations, from 0.01 to 500 ppb.

Because atrazine is not very soluble in water, it was necessary to prepare the stock solution by first dissolving the atrazine in acetone and then dissolving the acetone–atrazine solution in Combo. Each atrazine bioassay included a water (Combo) control and a carrier (acetone) control. The acetone concentration in acetone controls was always the same, 45 ppm acetone, which corresponded to the amount of acetone used with the highest concentration of atrazine (500 ppb). A bioassay lasted for 6 d, with one transfer of medium after 3 d. Adult egg-carrying *Daphnia* were incubated for 3 d, about the length of one molt cycle (neonates are released each time the adult molts). At the end of the 3 d, the adults were transferred to new medium, and the first batch of young were discarded. The rational for the transfer was that the first batch of young were not all exposed to the test chemicals for the entire developmental period; it was likely that some adults molted and released young as soon as they were placed in the jars. However, any young released after the first 3 d would have spent their entire developmental period exposed to their parent's treatment solution. Moving adults to new media also allowed for renewal of the algae supply.

Three days after the transfer to new medium, the contents of each jar were poured into a petri dish, the water was removed with a pipette, and the number of adults, resting eggs, and newborn males and females were counted using a low-magnification dissecting microscope. See Dodson et al. [2] for details of morphological structures used to separate the sexes.

Acceptance criteria

Based on our experience, we adopted several criteria for accepting bioassay data for analysis. At the end of 6 d, we required that for each treatment, there were at least eight replicates containing at least three neonates each. Replicates with two or fewer neonates were not included in the statistical analysis. We also required that adult females show at least 60% survival, averaged over the two control treatments. Our observation was that normal aging of the adults produces survivorship of about 70 to 95% for the 6 d. The major loss of adults occurred due to mishandling during the transfer at the end of the first 3 d. Finally, sex ratios did not differ significantly between the two controls (using a *t* test and arcsine-transformed sex ratios).

Data analysis protocol

For each experimental unit (a single jar), at the end of the 6 d, we recorded the number of surviving adults, male and female neonates, number of ephippial eggs present, and any abnormal morphological features. As we did the bioassays, we saw no evidence of variation in morphology or the number of ephippial eggs produced per jar. Therefore, the three endpoints used in the study were sex ratio, total number of offspring, and adult female survival.

Statistical analysis

Controls. The first step in the statistical analysis was to use a *t* test [14] to determine whether we could reject the null hypothesis that the sex ratios of the two controls (plain Combo control and acetone carrier control) were significantly different. If we failed to reject the null hypothesis at the 5% level, then we combined the two controls. In our study, we performed 25 assays. A significant difference in sex ratio was observed only once, and results of this assay were not used. Thus, our data set included results from 24 assays. Combining the controls gave a better estimate of the average sex ratio within each assay.

Indices. The within-assay averages (for control treatments) for adult survival, fecundity, and sex ratio (for controls) showed considerable variation among assays (e.g., see the sex ratios of controls in Table 1). This presented the challenge of how to compare results of atrazine exposure among assays. We compensated for the variation in controls among bioassays by using indices. An index was calculated within each assay, for a specific endpoint, as the difference between the average value of the controls and the average value in a given exposure treatment. For example, for the first line of data in Table 1 (the results of the first assay), the average sex ratio of the combined controls was 0.118, and the average sex ratio of the 0.001-ppb atrazine treatment was 0.162. The sex ratio index for this example was the difference between the two values, or 0.044. The same procedure was used for adult survival and total offspring.

The indices were normally distributed (Komolgorov-Smirnov test, $p > 0.05$) and had variances (at each concentration of atrazine) that were independent of the atrazine level. For the range between 0.5 and 500 ppb (over which atrazine had a significant effect), the average and variance of the sex ratio index for each treatment level were not correlated ($df = 8$, $p > 0.05$). The normal distribution and the lack of a correlation between index and concentration allowed us to use regression analysis and *t* tests to analyze the indices [14].

Adult survival index. Adult survival was measured as the number of living adults present in a jar at the end of the 6-d bioassay. The index was calculated as the average survival in a treatment minus average survival in the appropriate control. The adult survival indices were tested for correlation with atrazine concentration using simple linear regression [14].

Fecundity index. Fecundity was measured as the number of total offspring produced per jar divided by the number of adults that survived the duration of the bioassay. The index was the difference between average fecundity in a treatment, minus average fecundity in the appropriate control. The fecundity indices were tested for correlation with atrazine using simple linear regression [14].

Sex ratio index. We calculated the sex ratio as

$$\text{Sex ratio} = (\text{number of males})/(\text{number of males} + \text{females})$$

The advantage of this method of calculating sex ratio was that the ratio is defined even if a jar contained only males. If we had used the ratio between males and females, the ratio would be undefined when there were no females in the jar. Average sex ratio was the average of the sex ratios of the replicate jars in a treatment. Jars that included fewer than three offspring (a rare occurrence) were not included in the calculation of the average sex ratio.

The sex ratio indices were tested for statistical significance using a one-way *t* test at each concentration. At each concentration, there were between two and eight sex ratio indices. We calculated the average and variance sex ratio index at each concentration. We then calculated the 95% confidence interval for the mean, using the one-way *t* value appropriate for the number of indices at that concentration [14]. If the confidence interval did not overlap with zero (sex ratio index), we concluded the mean index was significantly different from an index of zero.

Table 1. Data for calculation of 95% confidence intervals for the average sex ratio index[a]

Atrazine (ppb)[b]	Assay	Control sex ratio	Sex ratio index	Average sex ratio index	SEM index	*n*	$t_{0.05}$ (1-tailed)	95% Confidence interval
0.01	55	0.118	0.044					
	60	0.204	0					
	62	0.092	−0.049					
	63	0.036	−0.017					
	59	0.069	0.124	0.0204	0.0299	5	2.015	0.060
0.1	50	0.104	0.063					
	52	0.050	0.08					
	55	0.118	−0.026					
	62	0.092	−0.042					
	53	0.044	0.025	0.02	0.0239	5	2.015	0.048
0.5	49	0.242	0.17					
	50	0.104	0.063					
	52	0.050	0.07					
	66	0.134	−0.011					
	53	0.044	0.02	0.0624	0.0307	5	2.015	0.062
1	48	0.081	0.258					
	49	0.092	−0.02					
	49	0.242	0.024					
	50	0.104	0.079					
	52	0.050	0.028					
	55	0.118	0.116					
	53	0.044	0.002	0.0696	0.0359	7	1.895	0.068
5	38	0.239	0.039					
	37	0.149	−0.097					
	59	0.069	0.095					
	60	0.204	0.123					
	65	0.405	0.179					
	66	0.134	−0.021					
	67	0.188	0.151					
	36	0.269	0.057	0.0658	0.0325	8	1.86	0.060
10	48	0.081	0.127					
	48b	0.092	0.207					
	62	0.092	−0.016					
	65	0.405	0.239					
	52	0.050	0.069	0.1252	0.0463	5	2.015	0.093
15	38	0.239	0.123					
	37	0.149	−0.077					
	59	0.069	0.204					
	60	0.204	0.121					
	62	0.092	0.033					
	36	0.269	0.211	0.1025	0.0447	6	1.943	0.087
25	47	0.179	−0.031					
	55	0.118	0.287					
	59	0.069	0.099					
	65	0.405	0.212					
	53	0.044	0.11	0.1354	0.0541	5	2.015	0.109
50	47	0.179	0.23					
	38	0.239	0.08					
	37	0.149	0.288					
	36	0.269	0.156					
	50	0.104	0.243					
	58	0.271	0.23					
	52	0.050	0.026	0.179	0.0376	7	1.895	0.071
100	48	0.081	0.327					
	48b	0.092	0.099					
	47	0.179	0.051					
	46	0.594	0.231					
	40	0.409	0.214					
	35	0.188	0.472					
	23	0.093	0.353					
	49	0.242	0.365	0.264	0.0821	8	1.86	0.153
250	35	0.188	0.471					
	23	0.093	0.381	0.426	0.0367	2	2.92	0.107
500	35	0.188	0.476					
	23	0.093	0.367	0.4215	0.0445	2	2.92	0.130

[a] Confidence intervals were calculated using the standard error of the mean index for each exposure concentration and the one-tailed Student's *t* distribution appropriate for the number of replicate exposures (*n*). ''Assay'' refers to the code number of each assay and is given for identification purposes.

[b] ppb = μg/L.

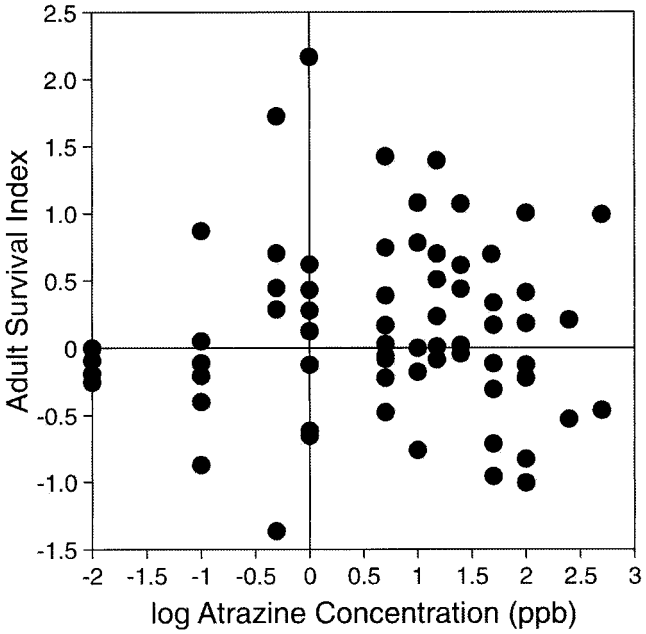

Fig. 1. Effect of low exposure concentrations of atrazine on short-term adult survival. Each dot represents the difference between a treatment and its control. A positive value indicates that survival in the atrazine treatment was greater than survival in the control for that bioassay.

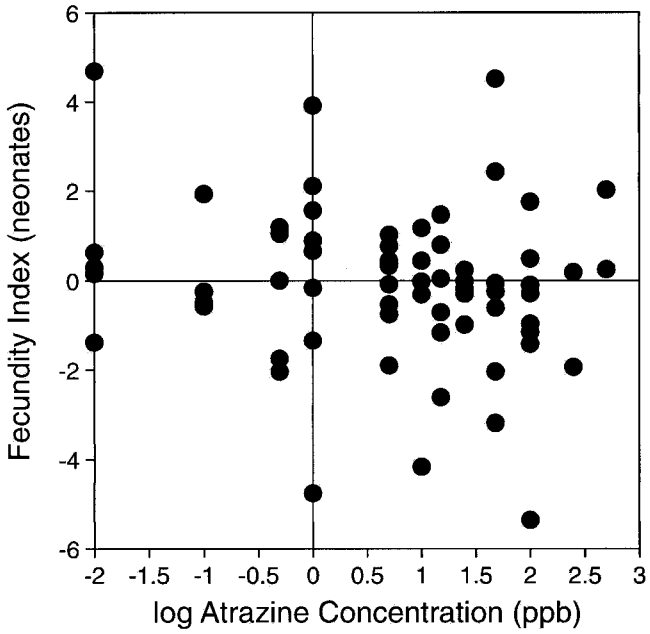

Fig. 2. Effect of low exposure concentrations of atrazine on short-term fecundity. Each dot represents the difference between a treatment and its control.

RESULTS

Controls

Within each assay, a *t* test showed no significance difference between water and carrier controls for 24 of the 25 assays. A binomial comparison of the water and carrier controls showed no significant overall trend in sex ratio index. In the 24 bioassays, the carrier control had a higher sex ratio (compared to the water control) 10 of 24 times ($p > 0.05$, binomial test).

Adult survival

We found no indication of a relationship between atrazine concentration and adult survival (Fig. 1; linear regression, $r^2 = 0.002$, $p > 0.05$). That is, adult survival index neither increased nor decreased with atrazine concentration. However, adult survival was slightly higher in exposure treatments than in controls. Using all concentrations, average adult survival index in exposure treatments was 0.144 adults per jar, which is significantly greater than zero (*t* test, $n = 67$, $p < 0.05$).

Fecundity

No significant relationship was observed between atrazine concentration and the fecundity index (Fig. 2; $r^2 = 0.022$, $p > 0.05$). Over all concentrations, the average fecundity index was −0.087 offspring per adult, which is not significantly different from zero (*t* test, $n = 67$, $p \gg 0.05$).

Sex ratio

The average standard deviation of the sex ratio index, over 12 exposure concentrations, was 0.0968. This suggests a resolution of about 5 percentage points, given four to nine replicates. Atrazine strongly influenced sex ratio (Table 1 and Fig. 3). At concentrations between 10 and 500 ppb, sex ratio was greatly increased (more males were produced relative to controls). The effect of atrazine decreased with decreasing atrazine concentration. The lowest concentrations at which we observed a significant effect was 0.5 ppb.

DISCUSSION

Adult survival did not show an exposure–response relation to atrazine concentrations between 0.01 and 500 ppb in our 6-d exposures (Fig. 1). The unexpected increase in the survival index of 0.144 adults per jar in exposure treatments is a small difference given that the trials started with eight adults per jar.

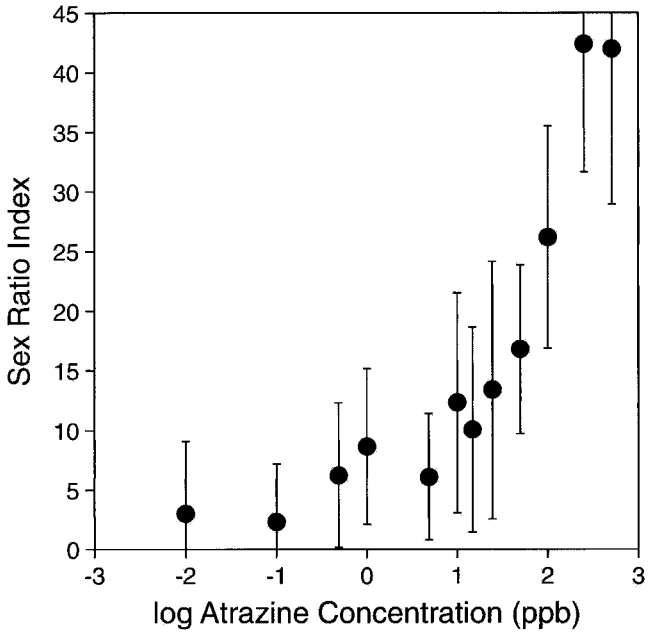

Fig. 3. Effect of low exposure concentrations of atrazine on sex ratio. Dots represent the average difference between treatments and control values. Bars are 95% error bars based on one-tail probabilities. Number of replicate observations used to estimate each ratio are given just below the graph.

Adult survival

Previous studies reported that short-term exposure to atrazine reduces *Daphnia* survival at concentrations of 1,150 ppb or higher [8,9]. Our results and the results of previous studies allow us to conclude that we were looking at sublethal effects of atrazine and that any change in sex ratio was due to a developmental shift in the sex determination mechanism rather than a mortality or fecundity effect.

Neonate production

Neonate production (average clutch size, a measure of *Daphnia* fecundity) in the 6-d bioassays was not affected by atrazine concentrations between 0.01 and 500 ppb. Others have reported that atrazine reduces *D. magna* fecundity at about 200 ppb or higher after long-term exposure [8], although this may be the result of bioaccumulation rather than acute toxicity. A change in sex ratio due to mortality could result if atrazine kills more females than males during development. However, if this mortality were in effect, we would expect to see fewer total neonates, especially at higher concentrations. No such trend in fecundity was observed (Fig. 2), so we concluded that the observed change in sex ratio was due to a developmental shift in the sex determination mechanism rather than a mortality effect. The developmental shift from females to males occurs during either oogenesis (before the eggs are placed in the brood chamber) or embryogenesis (after the eggs begin developing). The shift probably does not occur after the neonates are released from the brood chamber, because late-stage embryos in the brood chamber can be identified as male or female by the morphological features of the head.

Neonate sex ratio

Carrier control. Because the sex ratio in the carrier control was not significantly different from that of the control, we concluded that acetone, at the concentration used in our studies (45 ppm), had no effect on *Daphnia* sex ratio. The lack of an acetone effect makes interpretation of the atrazine effect more straightforward.

Exposure–response curve. The neonate sex ratio was shifted toward male production in atrazine treatments. A plot of the log average atrazine concentration versus sex ratio index (Fig. 3) showed a significant increase in sex ratio from 500 to as low as 0.5 ppb (using *t* tests on the sex ratio index values at each atrazine concentration). The overall pattern was for an increase in sex ratio index with increasing atrazine concentration, from 0.5 to 500 ppb. Below 0.5 ppb, we did not find a significant effect. However, it is possible that this lack of effect was due to the low power of the statistical tests rather than a lack of effect. For example, the mean sex ratio index observed at 0.01 ppb had a variance of about 0.00286 (see Table 1). To detect a 0.02 difference between control and treatment using a *t* test, we would have needed a minimum of 22 replicate treatments. This level of replication was beyond the scope of our study.

Sensitivity. The lowest concentration at which a statistically significant sex ratio effect was observed (0.5 ppb) is much lower than previously reported for survivorship or fecundity endpoints [8–10]. The sex ratio effect occurs at atrazine concentrations that are about two orders of magnitude below those previously known to affect *Daphnia* survival or fecundity. Therefore, sex ratio is a much more sensitive endpoint than survival or fecundity when *Daphnia* is exposed to atrazine.

Daphnia sex ratio is significantly affected at concentrations that are below the U.S. EPA safe drinking water level for lifetime exposure (3 ppb) and within the range of drinking water consumed (often between 0.1 and 1.0 ppb) in the agricultural midwest [10; J. Janczy, personal communication].

Ecological implications of increased sex ratio

The *Daphnia* reproductive strategy is called ''cyclic [or facultative] parthenogenesis.'' In nature, the population ordinarily reproduces asexually, producing genetically identical female offspring. Males are produced in response to environmental signals, such as short days, a change in food concentration, and chemicals produced in crowded populations [15]. Males typically appear during only one generation at a specific time of year, usually the fall. At peak male production, the sex ratio is typically below 50% [16]. The advantage of asexual reproduction is a maximal rate of population growth [1]. Fast reproduction is one of the major defenses *Daphnia* have against their predators. The advantage of sexual reproduction is genetic recombination, but the cost is a decreased population growth rate. Ordinarily, the lower population growth rate is not a problem, because the environmental cues that induce male production are also correlated with the appearance of a suboptimal environment in the near future. However, production of males at an ecologically inappropriate time of year (as when induced by agricultural chemicals) would reduce population growth at a time when the environment is optimal and the population needs to grow as rapidly as possible to persist in the face of intense predation.

Any decrease in *Daphnia* numbers due to chemical induction is of concern because of the important position *Daphnia* occupy in aquatic food webs. *Daphnia* are a major item in the diet of fish and invertebrate predators and, through their feeding on algae, play a major role in the control of water quality [16]. *Daphnia* survival and fecundity are strongly influenced by natural chemical signals in the water [17]. These chemicals, produced by their food, predators, and competitors, directly affect *Daphnia* morphology, life history, and behavior. Atrazine and other manmade chemicals [1,2,16,18,19] mimic natural chemicals by affecting *Daphnia* morphology, life history, and/or behavior. Because *Daphnia* are not adapted to the manmade chemicals, it is likely that the changes induced by these chemicals are detrimental. Given the concentrations of atrazine often observed in the environment, in the range of 0.1 to 1ppb in surface water [3], it is probably a common occurrence for *Daphnia* populations to be expressing a maladapted life history strategy because of producing males at the wrong time or in excessive numbers.

In vertebrates, atrazine is an indirect endocrine disruptor [20] that increases activity of the aromatase enzyme that converts testosterone into estrogen [21]. Identifying mechanisms by which atrazine affects *Daphnia* sex determination will give insights into invertebrate hormone systems and may even contribute to greater understanding of vertebrate endocrine systems, sex determination, and the role of atrazine as an endocrine disruptor.

Acknowledgement—Thanks to J.V. Cartwright, L. Larscheid, K.B. Lois, J. Schroeder, V. Shmidov, and J. Zahringer, who assisted with the laboratory work. Joe Janczy, Matt Brewer, and Chris Tracy provided valuable comments on earlier drafts. This research was supported by the University–Industrial Relations Program of the University of Wisconsin–Madison.

REFERENCES

1. Shurin J, Dodson SI. 1997. Sublethal toxic effects of cyanobacteria and nonylphenol on environmental sex determination and development in *Daphnia*. *Environ Toxicol Chem* 16:1269–1276.
2. Dodson SI, Merritt CM, Torrentera L, Winter KM, Tornehl CK, Girvin K. 1998. Dieldrin reduces male production and sex ratio in *Daphnia galeata*. *Toxicol Ind Health* 15:192–199.
3. Solomon KR, et al. 1996. Ecological risk assessment of atrazine in North American surface waters. *Environ Toxicol Chem* 15:31–76.
4. Trevisan M, Montepiani C, Ragozza L, Bartoletti C, Ioannilli E, Del Re AAM. 1993. Pesticides in rainfall and air in Italy. *Environ Pollut* 80:31–39.
5. Dankwardt A, Thurman EM, Hock B. 1997. Terbuthylazine and deethylterbuthylazine in rain and surface water: Determination by enzyme immunoassay and gas chromatography/mass spectrometry. *Acta Hydrochim Hydrobiol* 25:5–10.
6. Buser HR. 1990. Atrazine and other 5-triazine herbicides in lakes and in rain in Switzerland. *Environ Sci Technol* 24:1049–1058.
7. Keith LH. 1997. *Environmental Endocrine Disruptors*. Wiley-Interscience, New York, NY, USA.
8. Kaushik NK, Solomon R, Stephenson G, Day K. 1985. Assessment of sublethal effects of atrazine on zooplankton. *Can Tech Rep Fish Aquat Sci* 1368:377–379.
9. Macek KJ, Buxton KS, Sauter S, Gnilka S, Dean JW. 1976. Chronic toxicity of atrazine to selected aquatic invertebrates and fish. EPA 600/3-76-047, PB 255 439. U.S. Environmental Protection Agency, Springfield, VA.
10. Eisler R. 1989. Atrazine hazards to fish, wildlife, and invertebrates: A synoptic review. *US Fish Wildl Serv Biol Rep* 85:1.18.
11. Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. 1998. COMBO: A defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* 377:147–159.
12. Hermens J, Canton H, Steyger N, Wegman R. 1985. Quantitative structure–activity relationships and mixture toxicity studies of alcohols and chlorohydrocarbons: Effects on growth of *Daphnia magna*. *Aquat Toxicol* 6:209–217.
13. Cowgill UM, Milazzo DP. 1991. The sensitivity of *Ceriodaphnia dubia* and *Daphnia magna* to seven chemicals utilizing the three-brood test. *Arch Environ Contam Toxicol* 20:211–217.
14. Sokal RR, Rohlf FJ. 1981. *Biometry*, 2nd ed. Freeman, New York, NY, USA.
15. Kleiven OT, Larsson P, Hobaek A. 1992. Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* 65:197–206.
16. Dodson SI, Hanazato T. 1995. Commentary on effects of anthropogenic and natural organic chemicals on the development, swimming behavior, and reproduction of *Daphnia*, a key member of aquatic ecosystems. *Environ Health Perspect* 103:7–11.
17. Larsson P, Dodson SI. 1993. Invited review: Chemical communication in planktonic animals. *Arch Hydrobiol* 129:129–155.
18. Barry MJ. 1998. Endosulfan-enhanced crest induction in *Daphnia longicephala*: Evidence for cholinergic innervation of kairomone receptors. *J Plankton Res* 20:1219–1231.
19. Lampert W, Fleckner W, Pott E, Schober U, Störkel K-U. 1989. Herbicide effects on planktonic systems of different complexity. *Hydrobiologia* 188/189:415–424.
20. Crisp TM, et al. 1997. Special report on environmental endocrine disruption: An effects assessment and analysis. EPA/630/R-96/012. U.S. Environmental Protection Agency, Springfield, VA.
21. Crain DA, Guillette LJ Jr, Rooney AA, Pickford DB. 1997. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. *Environ Health Perspect* 105:528–533.