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Gender–Dependent Problems in Toxicity Tests with *Ceriodaphnia dubia*

G. Jayne Haynes, Arthur J. Stewart, and Bret C. Harvey
Oak Ridge National Laboratory, Environmental Sciences Division, P.O. Box 2008, Oak Ridge, Tennessee 37831-6351, USA

The 7-d, three-brood Ceriodaphnia chronic toxicity test developed by Mount and Norberg (1984) is used to assess biological quality of water and wastewaters. The test is becoming widely accepted because it involves simple procedures, uses easily measurable and biologically meaningful endpoints (survival and fecundity), and uses a microcrustacean known to be relatively sensitive to various toxicants. The test uses individual animals as true replicates, and is relatively powerful statistically.

As with other cladocerans, Ceriodaphnia is parthenogenetic under favorable environmental conditions, and males are rare. When overcrowded or when they experience changes or inadequacies in diet, photoperiod, and/or water temperature, however, cladocerans may produce males and sexual females (Hutchinson 1967; Allan and Goulden 1980; Wetzel 1983). In the sexual phase of their life cycle, female cladocerans can produce males which mate with sexual females. If fertilized, ephippial eggs produced by sexual females can give rise to parthenogenetic females, which re-establishes the parthenogenetic phase of the life cycle (Hutchinson 1967; Wetzel 1983). The factors triggering the sexual phase of Ceriodaphnia's life cycle are not yet known, and although male Ceriodaphnia are rarely observed in lakes or ponds (Frenzel 1977, Burgis 1967), laboratory populations can include parthenogenetic females, males, and sexual females (Berner 1986).

Early instar male Ceriodaphnia dubia closely resemble juvenile females and can be distinguished from parthenogenetic or sexual females only after they are 4 or 5 d old. Then, males can be identified by their more restless swimming behavior, by body shape (males have more posteriorally tapered bodies and are slightly smaller than females of the same age), and on the basis of coloration (males tend to be more reddish-pink, Berner 1986).

Because male and female Ceriodaphnia neonates cannot be readily distinguished from one another, a 7-d C. dubia test started with neonates produced by females from stressed cultures may include an initially indeterminate number of males. The presence of males (which can be ascertained with certainty only when the test is nearly complete) can affect interpretation of toxicity test results in two ways. First, males reduce the number of animals (replicates) producing offspring, which reduces the power to

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Send reprint requests to G.J. Haynes at the address above

detect differences in fecundity among treatments. Second, the interpretation of survival data from Ceriodaphnia tests that contain an indeterminate number of males can be biased if the two genders differ in sensitivity to toxicants.

In this paper the results of toxicity tests of water samples from streams near the Oak Ridge National Laboratory are used to show the extent to which the presence of male C. dubia can confound the interpretation of a 7-d Ceriodaphnia test's fecundity endpoint. We also show the results of short-term experiments suggesting that male C. dubia are more sensitive than females to cadmium and phenol, and discuss the statistical and practical implications of the problems caused by the presence of males in chronic toxicity tests with this species.

MATERIALS AND METHODS

Ceriodaphnia dubia (J. Richard 1894; identified by D. Berner, Temple University, Philadelphia, Pennsylvania) cultures producing the animals used in the tests were started with animals obtained from the Environmental Protection Agency's laboratory in Athens, Georgia. The 1-L cultures were maintained in reconstituted hard or moderately hard water (100 or 175 mg/L hardness, expressed as $CaCO_3$), were fed twice daily with a trout chow-cerophyll-yeast mixture (TCY), and were thinned and transferred to new water in clean beakers weekly. The culturing and feeding procedures followed those described by Horning and Weber (1985). The reconstituted water and the TCY were also prepared according to Horning and Weber (op. cit.).

The problem of an initially indeterminant number of males was first evident to us in tests which were being used to determine spatiotemporal patterns of ambient toxicity at 15 sites in 5 streams near the Oak Ridge National Laboratory. These tests used 10 replicates of 16 treatments (15 sites and a reconstituted hard water control). At the start of a test, the animals were 0-4 h old; they were reared at 25°C in prerinsed, disposable 20-mL polystyrene beakers, each of which contained about 17 mL of undiluted sample. The animals were visually inspected and transferred to fresh sample daily; 100 µL of TCY was added to each new beaker before the animals were transferred. Although tests as described above were conducted monthly, we summarize here only the results of five tests, four of which contained an unusually large proportion (9.6 to 38.8%) of males.

During the summer of 1987 we initiated studies to determine if male C. dubia were more susceptible than females to toxicants. For these experiments, males were obtained as follows: a month before the tests were to start, three 1-L cultures were stressed by not changing the water, so that the animals became overcrowded. The temperature of the cultures was lowered from 25°C to 17°C over a 6-h period; the cultures were maintained at 17°C without feeding for 18 h. The temperature was then rapidly increased to 27°C and was kept there 8 h. This procedure was repeated twice. The

cultures were then placed under continuous fluorescent light for 5 d. Each culture was then divided into two equal portions by transferring half of the animals (and the old medium) into two clean 1-L beakers. Freshly prepared reconstituted hard water was added to bring the volume of each beaker up to 1 L. For 2 wk thereafter, the six cultures were maintained at 25°C under a 16:8 (L:D) photoperiod and were given only half as much food as the normal cultures. Three and a half weeks after the stress regime was initiated, males were evident in all six cultures.

Initial comparisons of the sensitivity of male and female C. dubia to $CdCl_2$ were made to determine the concentrations needed to obtain time-to-death values. Four additional experiments with reagent-grade $CdCl_2$ were then used to evaluate gender-dependent differences in susceptibility of C. dubia to cadmium. The males and females used in these tests were collected from the same cultures to preclude possible differences in physiological state of the animals due to culture conditions. Assessment of gender was made by microscopic examination, as before. In three of the tests, 20 animals of each sex were exposed to 104, 417, or 852 μg Cd/L in reconstituted water in separate 20-mL polystyrene beakers. A final test of males and females in reconstituted hard water used 852 μg Cd/L. In this test, 17 males and 17 females were tested in separate 250-mL borosilicate glass beakers. All tests were made at 25°C. The animals were inspected at 30-min intervals during the first day of each test, and less frequently thereafter. The fraction of animals of each gender alive was plotted vs time to estimate the LT_{50} (length of time needed to kill 50% of the animals) for each cadium concentration.

Gender-specific differences in C. dubia's responses to phenol were determined using 26 replicate pairs of animals, 1 female and 1 male per pair. Each pair was exposed to 140 mg/L of reagent-grade phenol prepared in reconstituted moderately hard water. Each pair was tested in a polystyrene beaker containing about 18 mL of freshly-prepared test solution; a pair of animals was added to a test beaker with a 2-mm bore glass pipet. As a pair of animals was added to a beaker, a stopwatch was started. The animals were then observed continuously, and the time to death for each member of the pair was recorded. Data from this test were analyzed by comparing, for each pair of animals, the difference in survival time. The mean difference for the 26 pairs was then tested against zero with a one-sided Student's t-test.

RESULTS AND DISCUSSION

Results of five Ceriodaphnia tests of ambient stream waters near the Oak Ridge National Laboratory are shown in Table 1. In the first test, 150 neonates were present at the start of the test, but 39 died by the end of the test. Of the 111 animals that remained, 11 (= 9.9%) were males. In the April test, 46 of the 150 animals initially present had died by the end of the test, and we found 10 males (= 9.6%) among the 104 survivors. In the May

Table 1. Results of five 7-d static-renewal chronic toxicity tests of reconstituted hard water (= Control) and water samples from stream sites (= Exposed) near the Oak Ridge National Laboratory. Samples were tested using Ceriodaphnia dubia. In February, only 11 sites were tested; in June, 14 sites were tested. Fifteen sites were tested in the other test periods.

Test date	Number of Neonates (Day 0)	Number of Adults (Day 7)	Number of Males (Day 7)	Fecundity of females (Day 7)
February 19-26				
Control	10	8	0	13.0 ± 6.0
Exposed	110	102	0	15.7 - 26.5
March 20-27				
Control	10	9	0	22.9 ± 4.7[a]
Exposed	150	111	11	2.0 - 21.1[b]
April 17-24				
Control	10	10	1	25.4 ± 10.8
Exposed	150	104	10	0.0 - 28.5
May 22-29				
Control	10	10	2	21.1 ± 3.1
Exposed	150	121	47	0.0 - 25.4
June 19-26				
Control	10	10	4	24.8 ± 3.9
Exposed	140	97	24	0.0 - 33.0

[a] Fecundity values for females in controls are means ± 1 SD.

[b] Fecundity values are given as ranges of the means for all sites, calculated using only females that survived all 7 d in each test. Mean fecundity was zero if all animals in water from a site died before a test was complete.

test, 121 of 150 animals survived all 7 d, but 47 (= 38.8%) were males. In the June test, 97 of 140 animals survived all 7 d, but 24 of these (= 24.7%) were males. Fecundity of females in the controls and in the water from most of the stream sites was high (>21 offspring per female) in every test, showing that the food and test conditions were good and that the animals used in the tests were "healthy".

The results of the tests to determine gender-specific susceptibility of C. dubia to Cd are shown in Table 2. The tests were conducted at different times and used different Cd concentrations,

Table 2. LT_{50} (length of time, in hours, to kill 50%) values for male and female Ceriodaphnia dubia tested in various concentrations of cadmium. Seventeen to 20 animals of each gender were used in each test.

	Cadmium concentration (μg/L)		
Gender Tested	104	417	852
Male	28.4	15.0	11.5
Female	75.0	26.5	16.4

but their outcomes were consistent: males died more quickly than females each case.

In the experiment to determine gender-specific susceptibility of C. dubia to phenol, the same trend was evident: the male died more rapidly than the female in 25 of the 26 tests, and at the same time as the female in only one test. Time to death for the males was 4.82 ± 1.70 min (mean ± 1 SD) while that for females was 8.39 ± 2.75 min. The mean difference in survival time was significantly greater than zero ($t = 7.43$; $p < 0.001$). The correlation between time-to-death for males and females ($r = 0.473$) was also significant ($n = 26$, $p = 0.015$).

Results of our tests indicate that C. dubia maintained in reconstituted water and fed only TCY may produce neonates that are unsatisfactory for chronic toxicity test purposes. Up to 38% of the neonates produced by females in such cultures can turn out to be males, even though sibling female neonates develop into "healthy" adults with high fecundity. In many other tests, by way of contrast, the proportion of males was acceptably low (0-3%). The high test-to-test variability in the proportion of males even when females in such tests have high fecundity suggests that relatively subtle changes in conditions associated with the culturing and/or isolation procedures described by Horning and Weber (1985) can significantly enhance the production of males by C. dubia.

The appearance of males in cultures of Ceriodaphnia has been reported to be both fairly common and species specific. Berner (1986), for example, found that cultures of C. reticulata produced very few males, whereas cultures of C. dubia always produced some. In Daphnia obtusa, the production of males can be triggered by rapid reductions in food supply (Slobodkin 1954). The specific factors controlling the production of male C. dubia in laboratory cultures are not yet known, but greater-than-usual numbers of males can be found in stressed cultures. Furthermore, the results

of ongoing studies with C. dubia suggest that factors relating to food or crowding are more important than those associated with temperature or photoperiod.

Cowgill (1987) argued convincingly that nutritional status of cladocerans can critically affect their sensitivity to toxicants, and recommended that cladoceran diets be supplemented with algae to ensure high nutritional status. Our findings support that recommendation for yet another reason: nutritional status of the adults in cultures of C. dubia, at least, may impart an unknown degree of bias into toxicity tests by affecting the ratio of females to males. Adding green algae to TC increases fecundity of the females and appears to reduce the frequency of occurrence of males in C. dubia. We have used Haematococcus lacustris, Selenastrum capricornutum, and Ankistrodesmus falcatus as supplements to TCY with some success. A. falcatus has proved to be particularly advantageous in that it grows rapidly and can be harvested quite easily by gentle centrifugation.

Adult male C. dubia were distinctly more sensitive than adult females to concentrations of cadmium in tests where 50% kill times ranged from 11.5 to 75 h (Table 2). Males also died much more rapidly than females in the acute tests with phenol. We found no published studies directly addressing the question of gender-specific differences in susceptibility of Ceriodaphnia to toxicants. However, Berner (1986) reported that, after anesthetizing cultures of C. reticulata with CO_2, "although all the animals resumed swimming following their return to culture water, only females survived. The males died within 12 hours...". Daphnia magna males have also been reported to die about 10% sooner than females (400 min, vs 442 min) when exposed to $HgCl_2$ at a concentration of 81.4 mg/L (Breukelman 1932). Similarly, males of D. magna died 7-29% sooner than females when exposed to KCN (MacArthur and Baillie 1929). For D. magna, at least, there appears to be a metabolic basis for gender-specific differences in sensitivity to toxicants (discussed, interestingly, by MacArthur and Baillie (1929) in a section titled "Effect of sex upon heart rate"): the respiration rate of males is 22% greater than that of females of the same age. Such studies suggest that gender-specific differences in susceptibility of daphnids to toxicants could be common.

The data we present here for gender-dependent differences in C. dubia's susceptibility to cadmium and phenol were from relatively short-term exposures (minutes for phenol, hours for cadmium), and so may not accurately reflect the outcomes of tests in which exposure periods to sublethal concentrations of toxicants are longer. The results of the acute tests with phenol must also be interpreted cautiously; with C. dubia it is virtually impossible to establish the relative contributions of differences in body size, age, and gender to the animals' responses to toxicants. When they are 7 d old, males are typically 0.50-0.75 mm in length, whereas fecund females can vary from about 0.60 to 1.20 mm, depending upon the type food that is used (unpublished data). The

animals do not become much larger than they are by day 7, however, so that there is actually little overlap in size of the animals by the time the sexes can be distinguished. The significant correlation between time-to-death values for the two genders in the acute tests with phenol may therefore reflect either batch-to-batch differences in concentration of phenol in our tests or an inverse relationship between LT_{50} and animal size, for the animals in this test were, to the best of our ability, matched with respect to size.

In either event, the occurrence of an initially indeterminate number of males in a chronic Ceriodaphnia test can diminish an investigator's ability to interpret survival data if any mortality occurs before the gender of the test animals can be determined. The magnitude of this possible error will increase with the proportion of males in a test and will be greater in cases where males are more sensitive than females to a particular toxicant. In our tests with cadmium, the magnitude of the time-to-death differences between the males and females also differed with concentration (Table 2); others have shown that gender-specific differences in susceptibility of D. magna to KCN were affected strongly by temperature (MacArthur and Baillie 1929). Such considerations may further complicate interpretation of tests involving concentrations of effluents which kill some, but not all, of the test animals. Finally, it should be noted that the tests compared the sensitivity of adult males to that of adult females, because of the difficulty in distinguishing the gender of pre-adult animals. We therefore do not know if pre-adults of the two genders differ from adult animals with respect to their sensitivity to toxicants.

Males also compromise the detection of differences in fecundity of the females simply by reducing the number of replicates available for statistical analyses. Based on the average of the mean square errors from ANOVAs of 12 monthly tests, each of which evaluated fecundity of C. dubia in controls and in water from 15 ambient stream sites, a power analysis showed that the ability to detect, at $p = 0.05$, a site in which fecundity differed from the other sites by 5.0 offspring per female would be about 45% if all 10 replicates survived and were females. If sets of 10 replicates contained on average 4 males, the power to detect a difference in fecundity of 5.0 offspring per female declines to only 22%. In effluent tests, where the number of treatments (= concentrations) are often less, the magnitude of the problem of males is similar: assuming 8 treatments with 10 replicates each, one can statistically detect, at $p = 0.05$, a treatment in which average fecundity differs by 5.0 offspring with a power of about 60% (using the average mean square error from the monthly tests). If 40% of all animals in a test are males, however, the statistical power declines to about 35%.

Until the factors triggering the production of males by Ceriodaphnia in culture are identified and controlled, it is likely that tests conducted with C. dubia (and perhaps other species of

Ceriodaphnia) are likely to suffer at least occassionally from the presence of males. In such cases, additional replicates, both of controls and of the treatments, can salvage a test's fecundity data but may not help much with respect to interpreting patterns of mortality. If the production of males is in fact triggered more by food-related factors than by changes in temperature or photoperiod, males may be expected to be more common in tests in which females have a less-than-optimum fecundity, for fecundity of daphnids is in general closely linked to diet (Weglenska 1971; Lynch 1978, 1989; Goulden et al. 1982). Any test in which mean fecundity of females is below 15 (computed using only offspring produced by animals surviving all 7 d of the test) should be considered suspect, for animals 0-4 h of age at the start of a test are physiologically capable of producing 25-30 offspring per female at 25°C in ambient waters that contain natural assemblages of algae and bacteria, based on dozens of tests we have conducted with various ambient waters. Finally, those who use Ceriodaphnia must be able to distinguish males from females, so that if males do occur, they are not scored as females producing no offspring.

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