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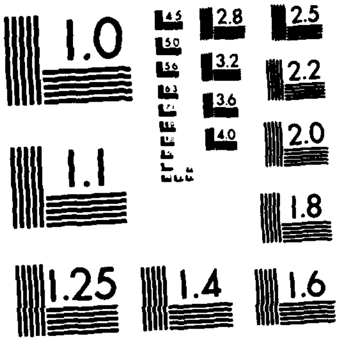

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DETERMINATION OF THE TOXICITY TO AQUATIC ORGANISMS OF HMX AND RELATED WASTEWATER CONSTITUENTS

PART 1:

THE EFFECTS OF FOOD CONCENTRATION, ANIMAL INTERACTIONS AND WATER VOLUME ON SURVIVAL, GROWTH AND REPRODUCTION OF *Daphnia magna* UNDER FLOW-THROUGH CONDITIONS

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January, 1983

Final Report

Contract No. DAMD 17-80-C-0011

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distribution unlimited

Prepared for

Health Effects Division
U.S. ARMY MEDICAL BIOENGINEERING RESEARCH AND DEVELOPMENT LABORATORY
Fort Detrick, Frederick, MD 21701

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The purpose of this study was to evaluate the effects of food concentration, animal interaction and water volume on survival, growth and reproduction of *Daphnia magna* under flow-through conditions. A response surface design was used to determine the interactive, as well as the individual, effects of the three factors. Results indicated that there were no important interactive effects of the three factors on survival, growth or reproduction of *D. magna*. Individual effects of the factors on reproduction were observed. Food concentration produced a linear trend with increasing food resulting in an increase

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in offspring production. The number of daphnids per container produced a quadratic trend with the maximum offspring production occurring in vessels containing approximately 14 daphnids. Water volume produced a slight linear trend with increasing water volume resulting in an increase in offspring production.

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EXECUTIVE SUMMARY

The purpose of this study was to evaluate the effects of food concentration, animal interaction and water volume on survival, growth and reproduction of *Daphnia magna* under flow-through conditions. A response surface design was used to determine the interactive, as well as the individual, effects of the three factors. Results indicated that there were no important interactive effects of the three factors on survival, growth or reproduction of *D. magna*. Individual effects of the factors on reproduction were observed. Food concentration produced a linear trend with increasing food resulting in an increase in offspring production. The number of daphnids per container produced a quadratic trend with the maximum offspring production occurring in vessels containing approximately 14 daphnids. Water volume produced a slight linear trend with increasing water volume resulting in an increase in offspring production.

INTRODUCTION

Chronic toxicity tests performed with water flea (generally *Daphnia magna* or *D. pulex*) have gained widespread acceptance as a means of predicting the harmful effects of potential environmental contaminants on aquatic invertebrates. Two general methodologies currently exist for performing chronic toxicity tests with daphnids, static renewal procedures (Comotto, 1981) and flow-through procedures (Adams, 1981). Static renewal procedures involve preparing solutions of the test material at a defined concentration gradient, exposing daphnids to the solutions, and periodically transferring the daphnids to freshly prepared test solutions to maintain consistent exposure levels. Flow-through procedures utilize intermittent or continuous flow apparatus (Lemke et. al., 1978) as a means of continually replenishing test solutions. A review of the literature suggests that static renewal procedures (Biesinger and Christensen, 1972; Biesinger et al., 1974; Bertram and Hart, 1979; Canton and Adema, 1978; Maki and Johnson, 1975, Parkhurst et al., 1981; Schober and Lampert, 1977, Winner and Farrell, 1976) have historically been more commonly used than flow-through procedures (Gledhill et al., 1980; Maki, 1979; Macek et al., 1976a, b; Nebeker and Puglisi, 1974). This preference can be attributed to several factors:

1. Static renewal tests can be performed in most laboratories with minimal or no added instrumentation (i.e., without a diluter apparatus).

2. Daphnids are very sensitive to water quality, water currents and the presence of low concentrations of toxic materials which are often used in the construction of diluter apparatus (i.e., rubber, brass, copper), despite continuous warnings against the use of such materials (Adams, 1981; Comotto, 1981; USEPA, 1975). Such factors can adversely affect the performance of daphnids in flow-through tests, thereby discouraging investigators from using such methods.

3. A plethora of data exist pertaining to the culturing and nurturing of daphnids under static conditions, which can be applied to static renewal techniques.

Nonetheless, the advantages to the use of a flow-through test are obvious. The system provides for the continual replacement of the test media, therefore minimizing the potential for fluxes in exposure concentrations due to volatilization, sorbtion, etc., of test materials. In addition, flow-through test systems allow for the maintenance of acceptable and consistent dissolved oxygen levels and minimize the accumulation of metabolic wastes. As toxicologists acquire a greater understanding of the biology of daphnids, as it applies to toxicological assays, flow-through tests should become more readily used.

EG&G Bionomics has completed over 40 flow-through chronic toxicity tests with D. magna from 1969 to 1981. Certain factors have been identified during the conduct of these tests which could hamper the proper interpretation of the results of a test. Three such factors are:

1. food concentrations,
2. interactive effect between animals, and
3. water volume requirements.

The purpose of this study was to assess the effects of these three parameters, individually and in combination with each other, on survival, growth and reproduction of *D. magna* under flow-through conditions.

MATERIALS AND METHODS

Experimental Design

The response surface design presented in Table 1 was used to assess the effects of the number of animals per container, test media volume per animal and food concentration on daphnid reproduction, survival and growth. The design has 15 distinct points with from two to six replicates at each point. The design is a modification of the design 8A.8 in Cochran and Cox (1957). The design proposed in Cochran and Cox has the property that the standard error of the fitted surface (which is assumed to be quadratic) is roughly equal in a unit sphere about the center of the design.

In adapting this design, we decided to measure number of animals on a linear scale and water volume per animal and feeding level on log scale. We wanted the number of animals to range from 5 to 20 per aquarium, feeding rate from 7.5 to 30 mg/L and test media volume from 25 to 200 mL/daphnid. These ranges were selected because they encompass levels commonly recommended in test procedures currently employed in daphnid chronic toxicity tests. Following Cochran and Cox (page 305) we defined the variables·

$$x_{i1} = .2243 \text{ x number of animals}_i - 2.8033$$
$$x_{i2} = 1.6177 \text{ x } \log_e \text{ (volume/animal)}_i - 6.8893$$
$$x_{i3} = 2.4266 \text{ x } \log_e \text{ (feeding level)}_i - 6.5714$$

Following their design, the number of animals would be tested at levels of 5, 8, 12, 17, 20; volume at levels of 25, 38.1, 70.1, 131.2, 200 mL/daphnid and feeding at levels of 7.5, 10, 15, 23 and 30 mg/L. However, we decided to modify the number of animals and the volume per animal so that we could use tanks with standard volumes of 125, 250, 500, 1000 and 2000 mL. We would only require two odd size tanks of 357 and 1428 mL. Furthermore, we decided to replicate each outer point twice to increase the precision of the response surface estimate and to replicate the points with 5 and 20 animals four times in order to resolve whether the variation in offspring production was due to an aquarium effect or an animal effect.

Water used in this study consisted of deionized well water, reconstituted to a total hardness of 150-180 mg/L as $CaCO_3$, a pH range of 7.9-8.3, a specific conductance of 400-600 µmhos/cm and a total alkalinity of 100-130 mg/L as $CaCO_3$. The water was reconstituted in an 1800-L fiberglass tank and pumped to the test system with a FMI Model RPD laboratory metering pump. The water was filtered through an XAD-7 resin column prior to delivery to the test apparatus to remove any potential organic contaminants.

Aquaria used in this study were constructed of glass. One side of each aquarium was three centimeters shorter than the others, which resulted in a drainage area over the upper edge of the shortened side. This area was covered with 40 mesh Nitex^R screen to prevent the escape of daphnids. Appropriate volumes of water intermittently flowed to the aquaria using an apparatus similar to the water cells of a Mount and Brungs (1967) proportional diluter. Water flowed to the aquaria at rates such that each aquarium experienced five volume replacements every 24 hours.

Food used in this study consisted of a mixture of PR11 fish food (5 mg/mL) and a unicellular green alga (Ankistrodesmus sp.) suspension (5×10^6 cells/mL). The PR11 mixture was prepared by homogenizing 50 g of food pellets in 200 mL of water for two minutes with a Polytron^R homogenizer. The 200-mL mixture was then diluted to three liters in water. The three-liter mixture was filtered through fine mesh netting to remove large particles. Two 25-mL samples were then oven dried to determine the PR11 concentration. The food mixture was appropriately diluted to yield a 5.0 mg/mL suspension. The appropriate concentration of algal cells was obtained by centrifuging culture stocks. The appropriate amount of food was volumetrically added to each aquarium three times daily as recommended by Adams (1981).

The D. magna used in this study were obtained from cultures maintained at EG&G Bionomics. Water used to culture the daphnids was of the same quality as the water used in the test. Daphnids ($\leq$24 hours old) were impartially assigned to each test aquarium at the initiation of the test. Determinations of adult survival and production of offspring were made on weekdays from days 7 through 28. The offspring were removed, counted using a Fisher Count-All^R Model 600 particle counter (LeBlanc, 1979) and discarded. Survival data derived on day 28 and reproduction data derived on days 21 and 28 were statistically analyzed. The individual total length of test daphnids were determined on day 28. Daphnids were removed from the test solutions, placed on a watch glass and total length determined using a Bausch and Lomb Stereozoom 7 microscope equipped with an ocular micrometer.

Statistical Analysis of Reproduction

Let Y_{ij} be the average number of offspring per female at the jth replicate of the ith point in the design. Furthermore, let V_i be the number of animals in each tank at this point. Let ε_{ij} be a unit normal random variable. Then the 2nd order response surface model for Y_{ij} is specified by:

$$Y_{ij} = \beta_o + \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \beta_{11} X_{i1}^2 + \beta_{22} X_{i2}^2 + \beta_{33} X_{i3}^2 + \beta_{12} X_{i1} X_{i2} + \beta_{13} X_{i1} X_{i3} + \beta_{23} X_{i2} X_{i3} + \sigma^2 (\alpha + (1-\alpha)/V_i) \varepsilon_{ij}.$$

There are two questions to be answered:

1. Is the observed variation in offspring production due to biological variation in the individual daphnids or is it due to some chance factor that affects an entire aquarium. The extent to which the variation is an aquarium affect is measured by α. If $\alpha=1$, the variation between replicate tanks will not depend on the number of animals in the tanks. This would occur if the variation was an aquarium effect. On the other hand, if $\alpha=0$, the variation is proportional to $1/v_i$ which would occur if the variation is due to the variation in the fecundity of each animal.

2. How is the offspring production, growth and survival related to number of animals, water volume per animal, and feeding.

To answer the first question, we computed

$Z_i = \Sigma_j \; (Y_{ij} - \overline{Y}_i)^2/n_{i-1}$

where n_i is the number of replicates at point i.

Each Z_i is independent and distributed

$\sigma^2(\alpha+(1-\alpha)v_i) \; . \; \chi^2 n_{i-1}/n_{i-1}$

If $\alpha<1$, z_i will decrease with v_i, while if $\alpha=1$, Z_i will be unrelated to v_i. The following approximate test of trend (p=.05 one sided) can be used to test whether $\alpha=1$. If $c = \sum_{i=1}^{n} 1/v_i$ reject the null hypothesis if

$$\sum_{i=1}^{n} (1/v_i-c) \ln z_i/(\sum_{i=1}^{n} (1/v_i-c)^2 \; . \; 2/(n_i-1)^{\frac{1}{2}} > 1.645.$$

If $\alpha\neq1$, it can be estimated by maximum likelihood and in this case a weighted least squares approach must be used to estimate $\beta_o,\beta_1 \ldots.$ In our data, no relationship between z_i and v_i was observed and the test of trend was nonsignificant so we concluded that $\alpha=1$ and we used ordinary least squares to estimate $\beta_o,\beta_1,\ldots.$

Statistical Analysis of Survival and Growth

The logistic model was used with survival and growth as the dependent variables and the same independent variables as in the reproduction comparison.

Water Quality Analysis

In order to ensure that water quality remained consistent in all aquaria throughout the study, temperature, dissolved oxygen concentration and pH of the water within the test aquaria were measured daily. Temperatures were measured with a Weston dial thermometer. Dissolved oxygen concentrations were determined with a YSI Model 54BP dissolved oxygen meter and probe. The pH's were measured with an Instrumentation Laboratories Model 175 pH meter.

RESULTS

The results of daily pH monitoring of the water in the individual test aquaria indicated that the pH's varied minimally between aquaria. The pH of the water in the test aquaria ranged from 7.6-8.1 throughout the test. Dissolved oxygen (DO) concentrations were highly correlated (r=0.88, p=0.01) to food concentrations (Figure 1) ranging from a low DO concentration of 6.5 mg/L measured at a feeding level of 30 mg/L to 7.7 mg/L measured at a feeding level of 7.5 mg/L. Temperature of the water ranged from 21 to 22°C throughout the test.

The cumulative offspring production on days 14, 21 and 28 in addition to average lengths and survival of daphnids after 28 days are presented in Table 2.

Analysis of Reproduction (at 28 Days)

Table 3 summarizes the offspring production at 28 days in each of the 15 groups. When the log standard deviation of the average offspring production is plotted against the number of animals in each tank, there does not appear to be any decreasing trend. This indicates that the variation in offspring production is due to an aquarium rather than an animal variation. Furthermore, a weighted least squares analysis of this data does not show a significant association between number of animals and standard deviation. Thus ordinary least squares can be used to analyze the 28-day offspring production data because the variances can be assumed to be homogeneous even though there are a different number of animals in each aquarium.

Table 4 shows the analysis of variance table for the model given by the 2nd order response surface model with $\alpha=1$. The residual sums of squares has been partitioned into the residual sum of squares due to lack of fit and the within group sum of squares. The ratio of the lack of fit mean square to the within group mean square is not significant; thus there is no evidence of lack of fit. The ratio of the regression mean square to the residual sum of squares is suggestive at $p=0.07$. Thus there is a suggestion of an effect of the variables. The regression coefficients are given in Table 5. The coefficient of number of animals, number of animals^2 and food concentration were significant.

Figure 2 shows predicted offspring production with upper and lower 90% confidence intervals by number of animals and Figure 3 shows the same graph by food concentration. The former graph shows a quadratic trend with a maximum near 14 animals while the latter shows a linear trend with increasing food availability.

However, both of these trends are not very striking when compared to the large variability of the data. The predicted offspring produced per female relative to test medium volume, although nonsignificant, does suggest a slight increase in numbers of offspring produced with increasing volumes (Figure 4). Again, this trend is not striking when compared to the large variability between replicate aquaria.

Since offspring production improved with feeding, the maximum offspring production occurred at the boundary of the region. The optimal combination was 14 daphnids per container, feeding at 30 mg/L and a volume of 200 mL per animal.

The confidence bands in Figures 2, 3 and 4 indicate the degree to which the estimated response surface can be trusted. Figure 2 (number of animals) shows a quadratic response curve; however, the decrease in offspring production with numbers greater than 14 may be due to chance variation. In Figure 3 offspring production seems to increase with feeding level. However, the wide confidence bands after 22.5 mg/L indicate that the increase after this point may be unreliable. The increased variability among the data points at the extreme ends of each graph, as indicated by the wide confidence bands, is attributed to the design of the experiment.

Analysis of Reproduction (at 21 Days)

In the 21-day offspring production data, the variances were also homogeneous. Table 6 shows an analysis of variance table for the 21-day data and Table 7 shows the regression coefficients. The data fit the model as before.

The test for an interactive effect of the variables was not significant. The coefficients for number of animals, number of $animals^2$ and food $concentrations^2$ were significant indicating that these variables may have some effect. The coefficient for media volume per animal was not significant. Figure 5 shows the effect of number of animals and Figure 6 shows the effect of food concentration. Clearly, number of animals does not have a large effect on 21-day offspring production. There was a decrease in offspring production at food concentrations greater than 18 mg/L ; however, this may be due to the low offspring production of the tanks receiving a food concentration of 30 mg/L.

Survival Analysis (28-Day)

To check for aquarium-to-aquarium heterogeneity, we used the test based on the fact that $\sin^{-1} \sqrt{p}$ is approximately normal with variance $(4n)^{-1}$ where n is the number of animals per container. The resulting statistic was nonsignificant. There were two aquaria that might be outliers. Both had 16 animals per container and both had a feeding level of 10 mg/L. One had a volume of 31.23 mL /daphnid and the other had a volume of 125 mL/daphnid. Both these aquaria had only seven survivors but their replicates had survivals of 14 and 12, respectively.

A logistic regression was run using a model similar to (1). (Replace Y_{ij} by log $(P_{ij}/(1-P_{ij}))$ where P_{ij} is the probability of survival and remove the error term.) The likelihood ratio test of whether the coefficients were zero was nonsignificant, p=.333. However, there was a significant (p=.04) interaction between feeding level and number of animals. The likelihood ratio test for a model containing number of animals, feeding level and number of animal interaction was significant at p=.06. However, when the two outlying aquaria were removed, the likelihood ratio test for the model containing these terms was not significant. In conclusion, there is a suggestion of poor survival on an aquaria-by-aquaria basis when there are a large number of animals at a low feeding level.

Length (28-Day)

There was evidence of aquarium-to-aquarium heterogeneity in the length measurements. The within aquarium standard deviation was approximately 0.1 so the standard error of the mean varies from 0.04 (5 animals) to 0.02 (20 animals) which is much less than the differences in the mean length between replicates. Analysis using model 1 indicated that the covariates tested had no effect on growth of daphnids. The coefficients had the same sign as those in the young production analysis. There was a correlation ($r^2=0.73$) between young production and length which was highly significant.

DISCUSSION

Although the data suggest that some trends exist relating to the three parameters measured, the fact that many of the data points were replicated only once resulted in high variability. The variation observed in this test tended to occur between aquaria as opposed to between individual animals. That is, all animals within a given aquarium tended to respond to certain factors, specific for that aquarium, which would affect longevity and the degree to which the daphnids would reproduce and grow. These data suggest that for the proper interpretation of the results of a chronic toxicity test with *D. magna*, the test should be replicated more than one time in order to identify aquarium-to-aquarium variability and differentiate it from the effects of the test material.

During the performance of flow-through chronic toxicity tests with *D. magna*, food concentration, number of animals per test vessel, and water volume within the test vessel can influence the reproduction capacity of the animals. The present study indicated that, after 28 days, the greatest reproduction would be expected to occur at a food concentration of at least 30 mg/L Food concentrations higher than 30 mg/L were not tested. The optimal number of animals per container, in terms of greatest offspring production, was approximately 14, and the optimal water volume was 200 mℓ per daphnid, or greater. Although optimal conditions were identified which resulted in the greatest number of offspring produced per female, these conditions may not always be desirable. Thirty mg/L of food consisting primarily of fish food such as PR-11 can result in a significant accumulation of food particles within the aquaria. These particles can lead to reduced water quality by stimulating the growth of microorganisms within the aquaria and can serve as a substrate for the sorption of test chemicals. Therefore, we recommend the use of 15 mg/L since this will allow for nearly the same production of offspring and provides improved water quality conditions.

It would appear that although the number of animals per container, and the food concentration both have an effect on the reproductive capacity of D. magna under flow-through conditions, the effects are small compared to the aquarium to aquarium variation in reproductive capacity. The effects of animal number and feeding level are important enough to require standardization throughout an experiment but a successful test can be performed with these parameters set at any of the values that we tested.

Conclusion

Food concentration and number of animals significantly affected offspring produced per female during flow-through chronic toxicity tests with D. magna. There does not appear to be any significant interactive effects of these two parameters, nor did the volume per animal have any significant effect at all. The recommendations published in "Proposed standard practice for conducting *Daphnia magna* chronic toxicity tests in a flow-through system" (Adams, 1981) in respect to food concentration (15 mg/L), number of animals per container (10) and test media volume (150 mL/daphnid) are all adequate for the successful performance of meaningful and sensitive tests.

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TABLE 1. RESPONSE SURFACE DESIGN USED TO ASSESS THE EFFECTS OF NUMBER OF ANIMALS PER CONTAINER, TEST MEDIA VOLUME AND FOOD CONCENTRATION ON DAPHNID REPRODUCTION, GROWTH AND SURVIVAL

Point #	Number of daphnids	Test media volume (mL) per daphnid	Test container volume (mL)	Food concentration (mg/L)	Number of replications
1	5	71.4	357	15	4
2	8	31.25	250	10	2
3	8	125	1000	10	2
4	8	31.25	250	23	2
5	8	125	1000	23	2
6	10	25	250	15	2
7	10	71.4	714	7.5	2
8	10	71.4	714	30	2
9	10	71.4	714	15	6
10	10	200	2000	15	2
11	16	31.25	500	10	2
12	16	125	2000	10	2
13	16	31.25	500	23	2
14	16	125	2000	23	2
15	20	71.4	1428	15	4

TABLE 2. CUMULATIVE OFFSPRING PRODUCTION, GROWTH AND SURVIVAL OF *D. magna*

Number of animals/ container	Volume/ animal (mL)	Food concentration (mg/L)	Offspring/ female (14 days)	Offspring/ female (21 days)	Offspring/ female (28 days)	Average length (28 days)	Survival (28 days)
5	71.4	15	9	36	50	3.9	4
5	71.4	15	8	36	51	3.8	4
5	71.4	15	13	55	73	4.2	3
5	71.4	15	8	37	52	3.8	3
8	31.25	10	22	72	103	4.0	7
8	31.25	10	5	56	106	4.4	7
8	125	10	21	60	67	3.7	5
8	125	10	38	92	115	4.2	6
8	31.25	23	21	71	94	4.2	7
8	31.25	23	22	83	128	4.0	7
8	125	23	27	95	117	4.2	7
8	125	23	25	53	92	4.2	8
10	71.4	7.5	20	67	87	4.1	9
10	71.4	7.5	19	62	73	4.0	7
10	25	15	19	62	73	3.9	9
10	25	15	42	117	157	4.5	8
10	71.4	15	34	80	97	4.0	7
10	71.4	15	22	70	86	4.0	9
10	71.4	15	32	80	105	4.0	8
10	71.4	15	17	58	91	3.9	8
10	71.4	15	39	88	115	4.1	8
10	71.4	15	30	81	93	4.0	7
10	200	15	33	81	88	4.1	7
10	200	15	19	64	78	4.2	8
10	71.4	30	22	41	78	4.1	6
10	71.4	30	15	40	104	4.3	6
16	31.25	10	15	53	72	4.0	14
16	31.25	10	9	68	79	3.9	7
16	125	10	28	61	65	3.8	7
16	125	10	26	67	98	4.2	12
16	31.25	23	17	52	83	3.9	13
16	31.25	23	31	85	139	4.3	15
16	125	23	29	69	130	4.3	13
16	125	23	24	57	104	4.1	13
20	71.4	15	23	69	77	4.0	16
20	71.4	15	29	74	110	4.1	14
20	71.4	15	25	60	67	3.8	14
20	71.4	15	27	72	35	4.0	15

TABLE 3. CUMULATIVE OFFSPRING PRODUCED PER FEMALE *D. magna* UNDER VARYING CONDITIONS OF CROWDING, TEST MEDIA VOLUME AND FOOD CONCENTRATION

Number of daphnids	Volume per daphnid	Food concentration (mg/L)	Number of replications	Average number of offspring produced per female	Standard deviation offspring production
5	71.4	15	4	56.5	11.0
8	31.25	10	2	104.5	2.1
8	125	10	2	111.0	24.0
8	31.25	23	2	91.0	33.9
8	125	23	2	104.5	17.7
10	25	15	2	115.0	59.4
10	71.4	7.5	2	80.0	9.9
10	71.4	15	6	97.8	10.6
10	71.4	30	2	91.0	18.4
10	200	15	2	83.0	7.1
16	31.25	10	2	75.5	4.9
16	31.25	23	2	111.0	39.6
16	125	10	2	81.5	23.3
16	125	23	2	117.0	18.4
20	71.4	15	4	84.8	18.4

TABLE 4. ANALYSIS OF VARIANCE TABLE FOR 28-DAY CUMULATIVE OFFSPRING PRODUCTION PER FEMALE *D. magna*

Source	Sums of Squares	DF	Mean Square
regression	848	9	942
residual	13036	28	466
lack of fit	2566	5	513
within group	10470	23	455

TABLE 5. REGRESSION COEFFICIENTS FOR OFFSPRING PRODUCTION (28-DAY)

Variable	Coefficient	Std. Error	p Value
Intercept	101.52		
X1 = number of animals - 11.37	1.99	0.98	0.05
X2 = Log (vol/animal) - 1.83	-14.91	14.47	0.31
X3 = Log (food conc.) - 3.18	46.79	22.98	0.05
$(X1)^2$	- 0.52	0.22	0.03
$X1 \cdot X2$	5.56	4.25	0.20
$X1 \cdot X3$	9.88	7.10	0.17
$(X2)^2$	33.63	59.90	0.57
$X2 \cdot X3$	10.62	98.39	0.91
$(X3)^2$	-65.53	142.53	0.64

TABLE 6. ANALYSIS OF VARIANCE TABLE FOR 21-DAY CUMULATIVE OFFSPRING PRODUCTION PER FEMALE

Source	Sum of Squares	DF	Mean Square
regression	3195.96	9	355.11
residual	8381.94	28	299.36

TABLE 7. REGRESSION COEFFICIENTS FOR OFFSPRING PRODUCTION (21-DAY)

Variable	Coefficient	Std. Error	P (2 tail)
intercept	77.91953		
X1	1.55170	0.787	0.059
X2	-2.29210	11.612	0.845
X3	-11.11953	18.535	0.553
$(X1)^2$	-0.39801	0.183	0.038
X1•X2	0.81335	3.411	0.813
X1•X3	1.62267	5.738	0.779
$(X2)^2$	19.44482	48.088	0.689
X2•X3	-67.40557	79.486	0.404
$(X3)^3$	-226.08815	114.689	0.059

Figure 1. Correlation between food concentration and dissolved oxygen concentrations of test water during the 28-day flow-through test with D. magna.

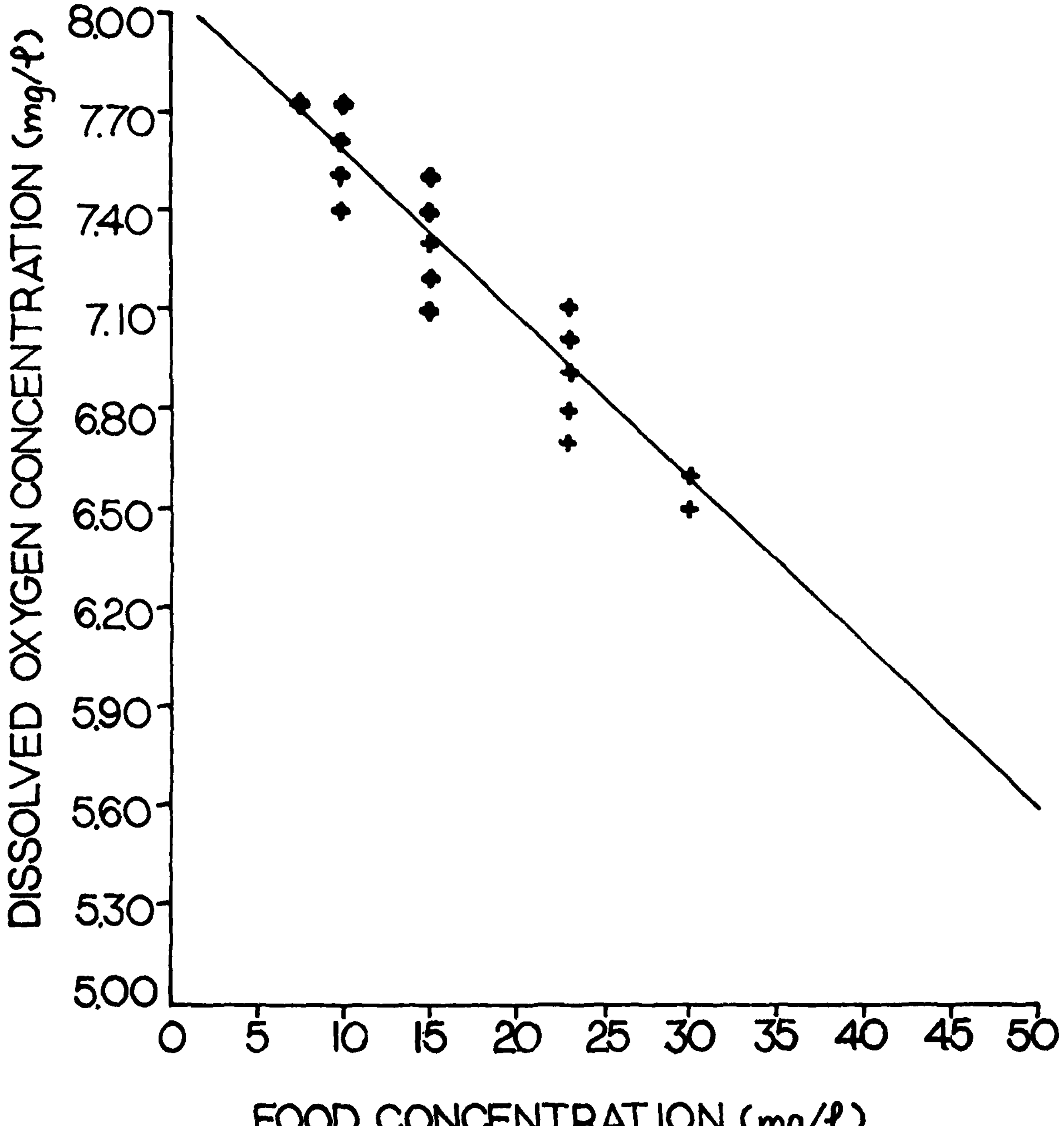


Figure 2. Predicted offspring per female vs. number of daphnids after 28 days with effects relating to food concentration and water volume held constant (90% confidence intervals).

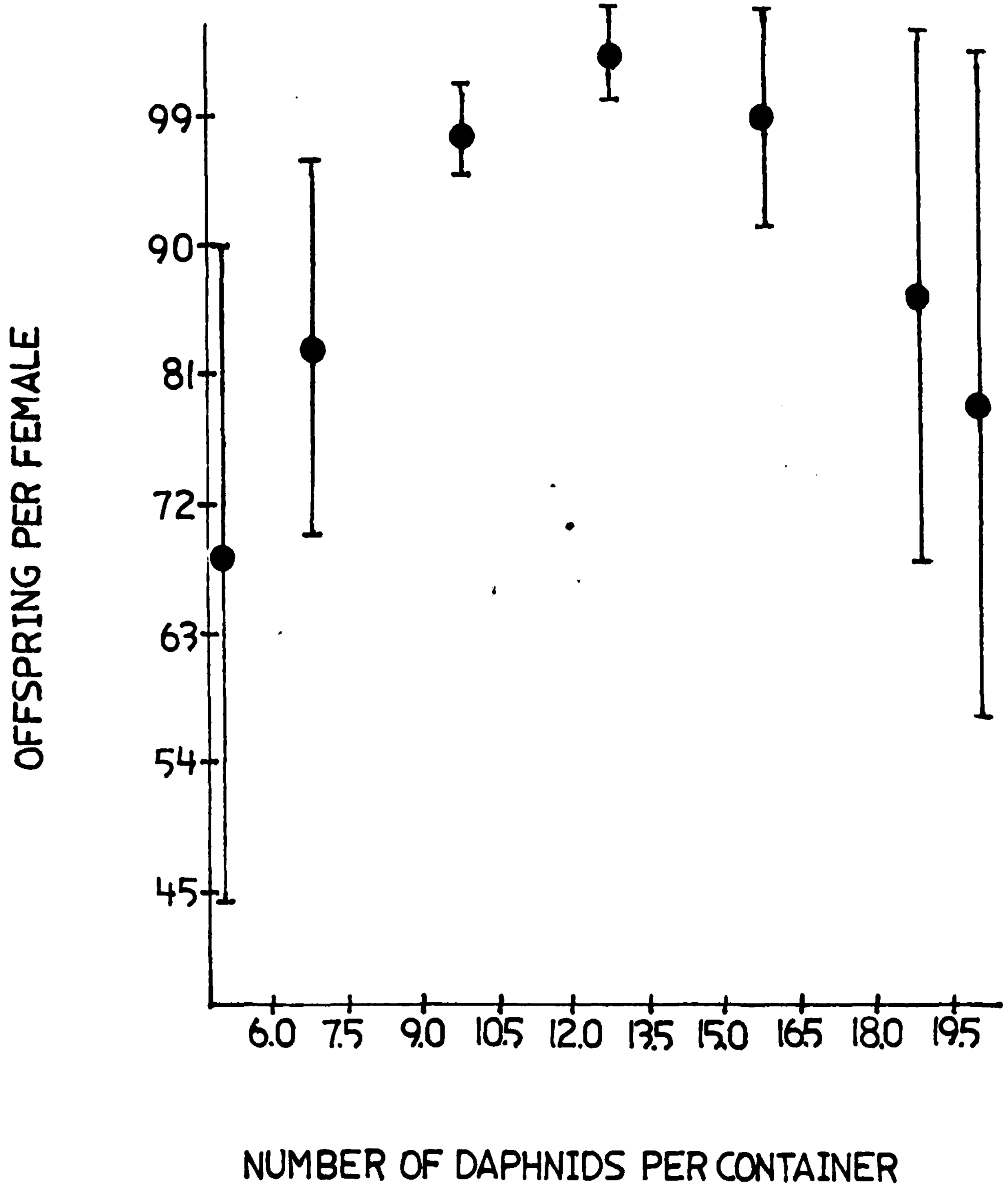


Figure 3. Predicted offspring per female D. magna vs. feeding level after 28 days with effects relating to water volume and number of daphnids per container held constant (90% confidence interval).

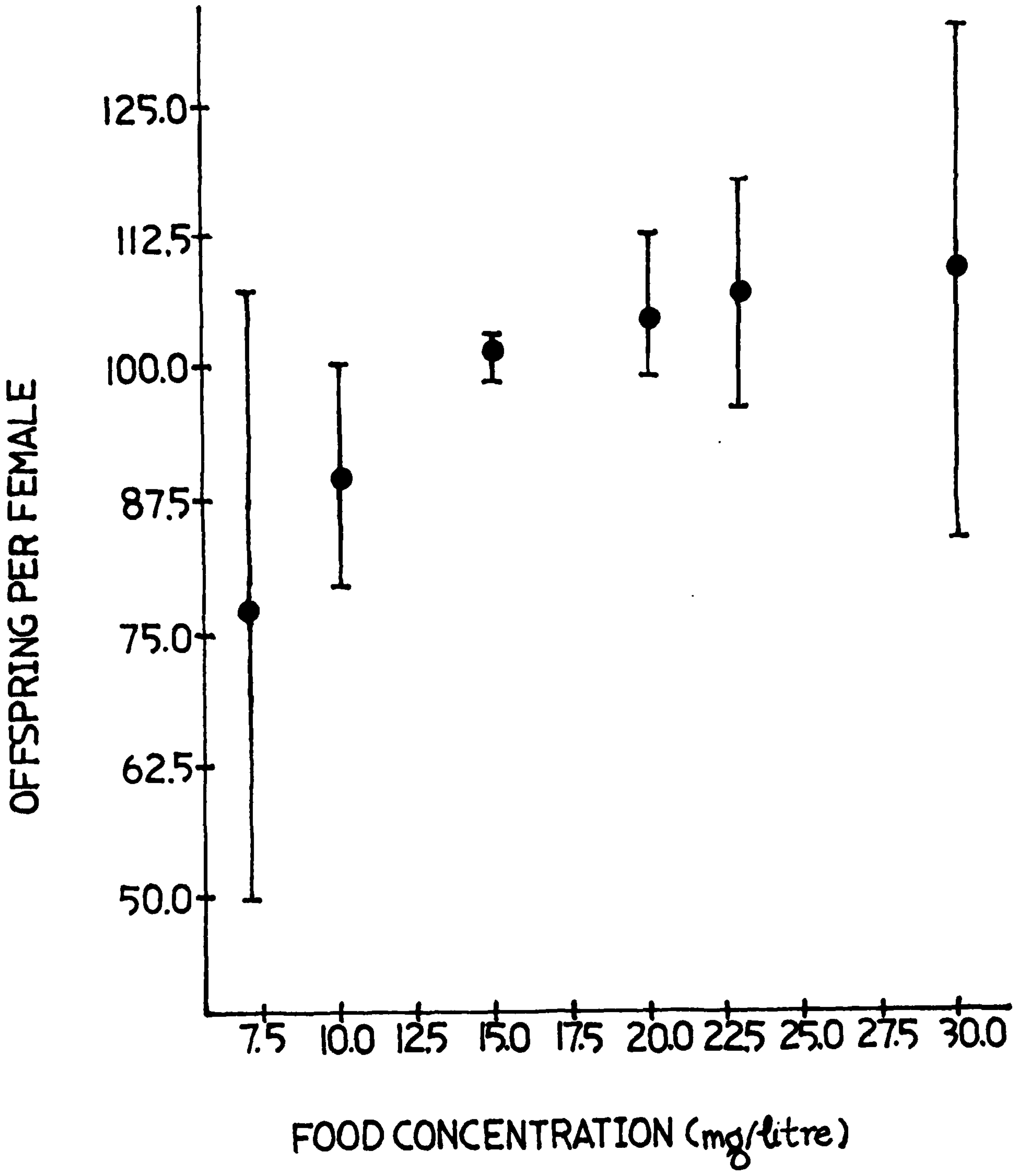


Figure 4. Predicted offspring per female D. magna vs. water volume per animal after 28 days with effects relating to food concentration and number of daphnids per container held constant (90% confidence interval).

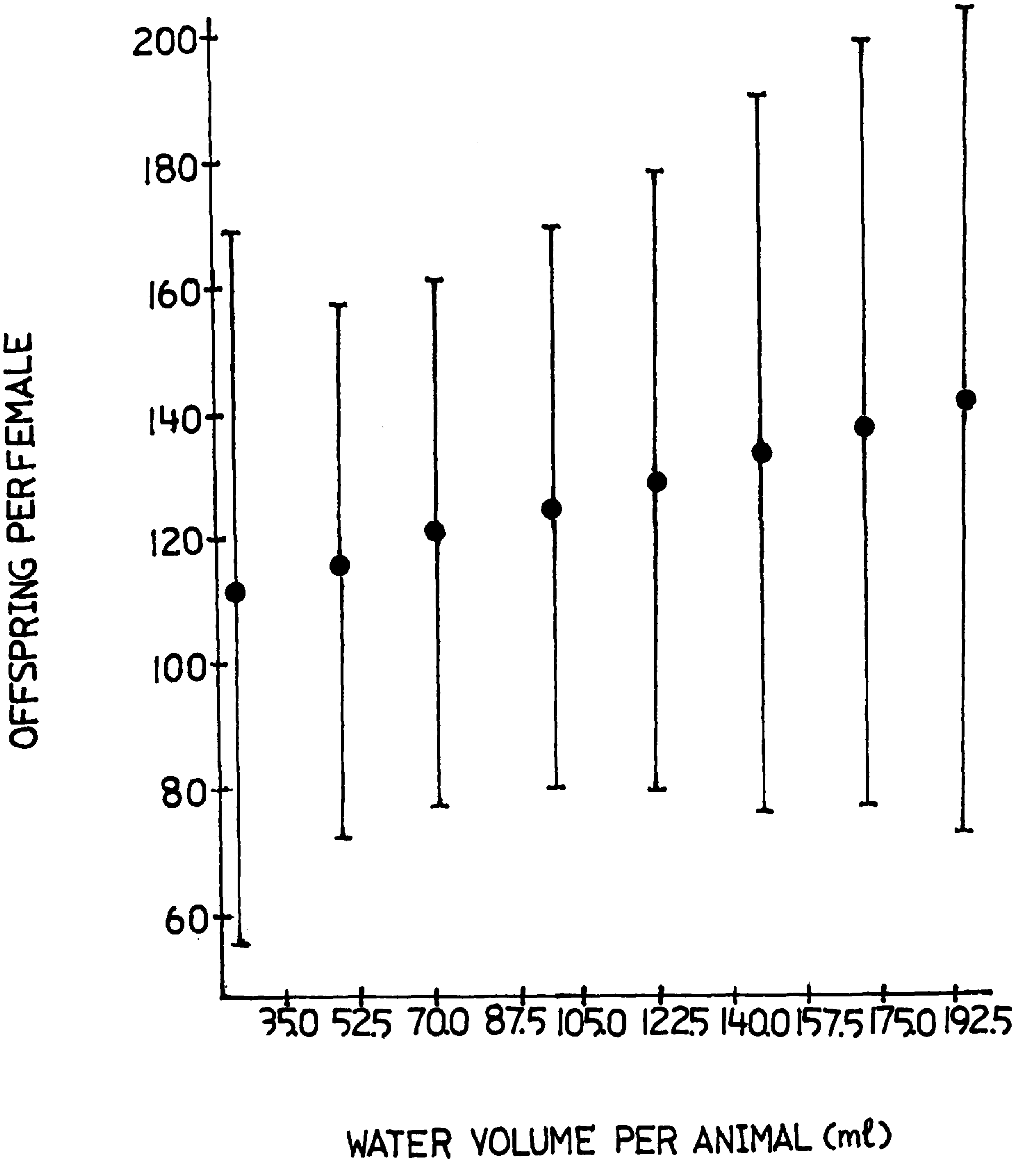


Figure 5. Predicted offspring per female vs. number of daphnids after 21 days with effects relating to food concentration and water volume held constant (90% confidence interval).

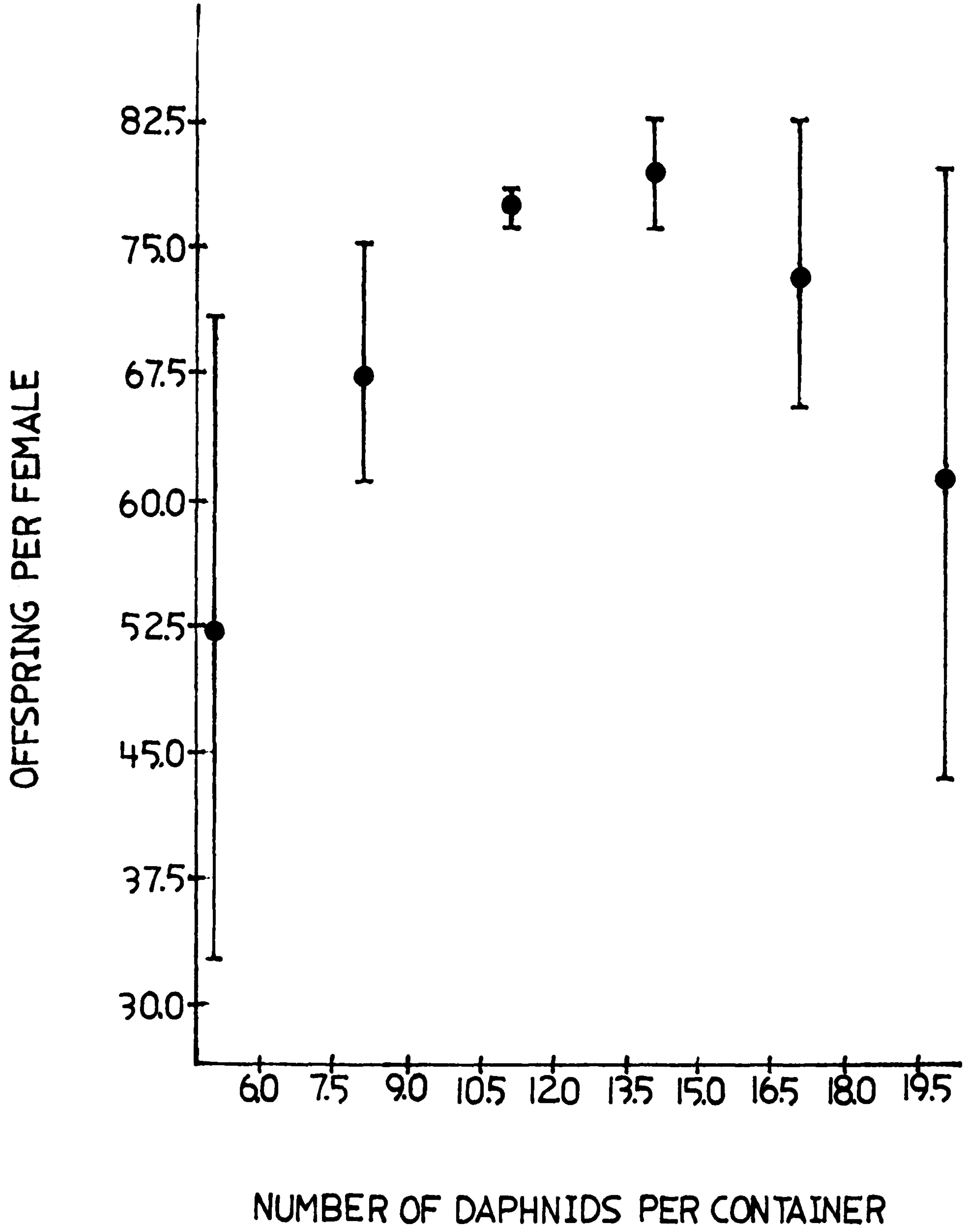
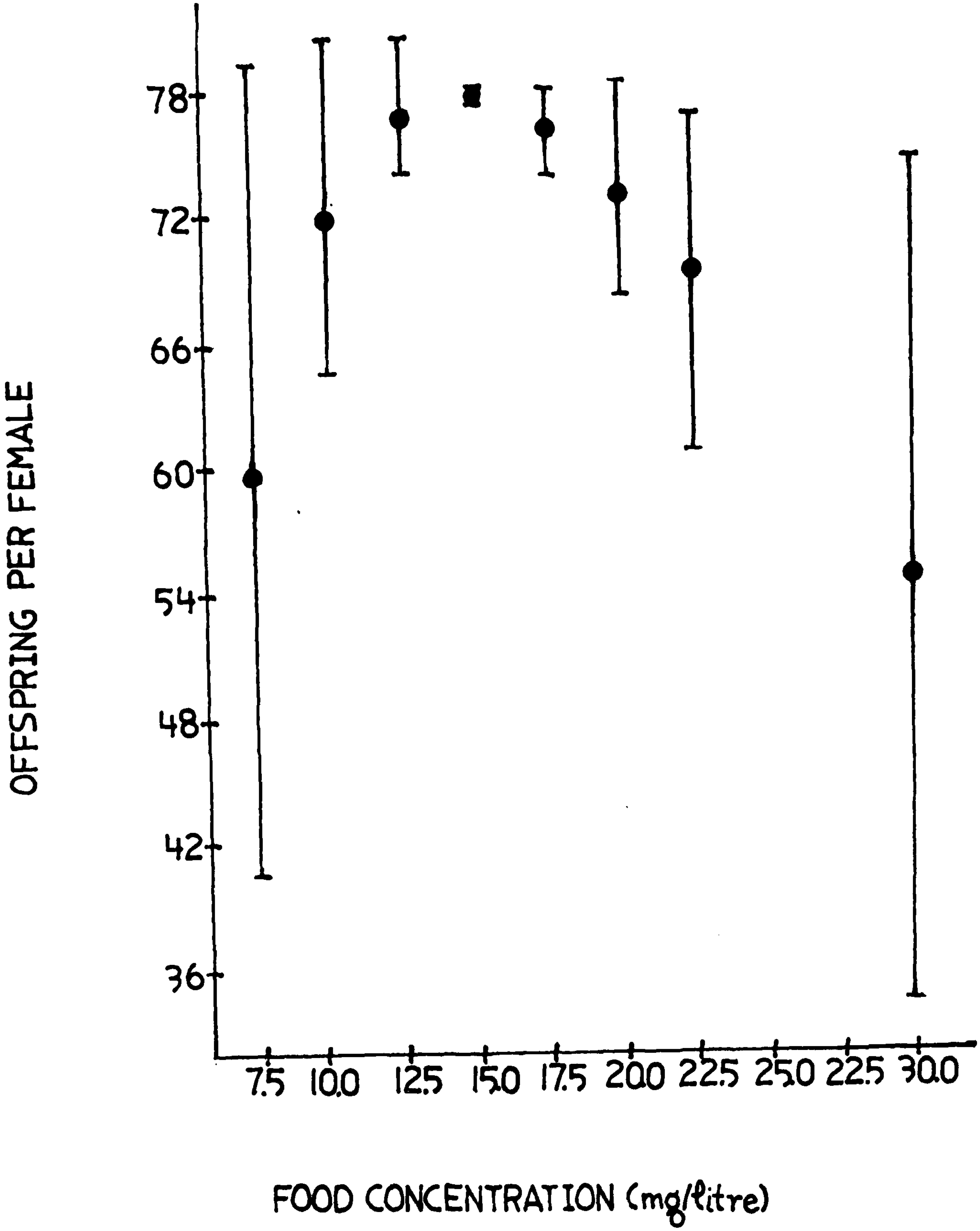


Figure 6. Predicted offspring per female vs. feeding level after 21 days with effects relating to water volume and number of animals per container held constant (90% confidence intervals).



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