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Effects of Temperature and Nutritional State on the Toxicity of Acridine to the Calanoid Copepod, *Diaptomus Clavipes* Schacht

John D. Cooney
Carl W. Gehrs
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ENVIRONMENTAL SCIENCES DIVISION
Publication No. 2098

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EFFECTS OF TEMPERATURE AND NUTRITIONAL STATE ON
THE TOXICITY OF ACRIDINE TO THE CALANOID
COPEPOD, DIAPTOMUS CLAVIPES SCHACHT¹

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ENVIRONMENTAL SCIENCES DIVISION
Publication No. 2099

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ABSTRACT

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Acute and chronic bioassays were performed on the calanoid copepod, Diaptomus clavipes, using the azaarene, acridine, as the test compound. Tests were performed at three temperatures (16°, 21°, 26°C) and over a range of nutritional conditions. Survival, growth, development, and reproduction were all affected by exposure to acridine. These effects were modified by temperature and nutritional state of the animals.

In acute bioassays, LC50's were used to estimate median lethal concentrations. Ninety-six hour LC50's for adult Diaptomus clavipes ranged from 1.64-6.70 mg/l depending upon temperature and nutritional state of the animals. LC50's were highest for well-fed animals at 16°C. As food availability decreased, sensitivity to acridine increased as temperature increased. No differences in LC50's were found between the sexes except in starved animals at 26°C where males were more sensitive than females. Acute bioassays on immature stages (NI-CI) were more sensitive than the adult stage, with LC50's ranging from 1.18-1.75 mg/l for the three temperatures. As acridine concentration increased, duration of immature stages increased and adult size decreased. These two variables were most sensitive to toxicant stress at 16°C. Metasomal lengths of first stage copepodids were unaffected by acridine concentration but decreased with increasing temperature.

Three measures of reproduction (number of eggs/clutch, number of clutches/female/day, number of eggs/female/day), were examined. These measures varied in their sensitivity depending on temperature being most sensitive at 21°C. At 16°C and 26°C, clutch size did not significantly vary among the acridine concentrations when compared to controls. At 21°C, clutch size was significantly reduced in acridine exposed animals. Rate of clutch production was a more sensitive indicator of reproductive inhibition than clutch size at all temperatures. Rate of egg production which utilizes information on clutch size and clutch production yielded results similar to the rate of clutch production.

Acute and chronic bioassays with acridine revealed that Diaptomus clavipes was more sensitive than Daphnia magna except for the adult acute test where Diaptomus clavipes was more tolerant.

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CHAPTER I

INTRODUCTION

Within the next several decades, the processes of coal gasification and liquefaction are anticipated to provide a significant fraction of the liquid fossil fuels in this country (Herbes et al. 1976). Both processes, however, will produce substantial quantities of highly contaminated wastewater. The Toxic Substances Control Act (TSCA 1976) and the Resource Conservation and Recovery Act (RCRA 1976) require that effects on ecosystems from exposure to potentially toxic materials be determined. Since these acts were enacted, bioassay procedures have received greatly increased emphasis especially for aquatic ecosystems (Little 1977).

The recommendations of Standard Methods (APHA 1976) and EPA documents on acute toxicity tests (EPA 1975, 1978) for using the most sensitive, locally important, species in a particular aquatic system provide the opportunity for examining a variety of macroinvertebrates and microinvertebrates as potential bioassay organisms. The great majority of freshwater animals are planktonic metazoans belonging to either the Rotifera or the Crustacea, represented mainly by the Cladocera in the subclass Branchiopoda and the Copepoda in the subclass Maxillopoda (Hutchinson 1967). The majority of invertebrate bioassays in the past century have used cladocerans in the genus Daphnia as test organisms. This is primarily due to their sensitivity to a variety of toxic substances (Anderson et al. 1948). Virtually all this work has dealt with a single species, Daphnia magna (Kemp et al. 1971).

Daphnia magna has a very limited geographic range in North America and even within this range it is confined to small bodies of water (Brooks 1957). Since Daphnia magna is not a frequent component of zooplankton communities, continued use of this species in research designed to formulate water quality standards can be justified only if it can be demonstrated that Daphnia magna has a sensitivity comparable with that of more widespread, important members of zooplankton communities (Winner 1976).

There is very little information in the literature concerning the use of freshwater copepods, especially calanoids, in bioassays (Bunting and Robertson 1975) due primarily to difficulties in culturing (Anderson et al. 1948, Robertson et al. 1974). In recent years, however, culturing techniques for the calanoid copepod, Diaptomus clavipes, have been improved (Robertson et al. 1974, Cooney et al. 1978) allowing for the use of this organism in toxicity testing.

Copepods differ from cladocerans in many important aspects of their life history. Copepods reproduce sexually and in some species, including Diaptomus clavipes, mating must occur before each viable clutch is produced. In cladoceran populations, reproduction is usually by parthenogenesis only. Females are present most of the year and males usually appear when the population is stressed in some manner (Pennak 1978). In cladocerans, eggs undergo a single maturation division in the ovary and are then released via the oviducts into a brood chamber where they undergo further development. The eggs hatch in the brood chamber into young similar in form to the adult and are then released to the outside. In copepods, Diaptomus clavipes

included, males transfer the sperm to the female in spermatophores and it is then stored. Fertilization occurs as the eggs leave the reproductive tract. Eggs are carried by the female in a single median egg sac until hatching. The eggs hatch into a larva called a nauplius. There are six naupliar stages and six copepodid stages in the life cycle of a copepod with the sixth copepodid stage being the adult. As metamorphosis proceeds, the larval nauplius elongates and adds appendages with a drastic change in shape as it molts into the first copepodid stage. Growth in copepods is restricted to the immature stages (determinate) whereas, in cladocerans, growth is continuous throughout life (indeterminate), being the greatest in the immature stages. Reproduction is linked to the molting process in cladocerans, but in copepods it is dependent upon a successful mating encounter.

Calanoid copepods, especially those from the genus Diaptomus, are a major component of many freshwater communities. Therefore, bioassays with calanoid copepods would yield useful information on the potential hazards of the introduction of toxicants to aquatic ecosystems by examining the sensitivity of eggs, larvae, and adults, both males and females, to a toxicant stress. Changes in reproductive parameters such as mating frequency, clutch size, and egg viability can be easily examined. Growth, since it is restricted to the immature stages, can be examined in the absence of other confounding factors such as reproduction. As a group, copepods are also much more homogeneous in their general size and structure than the Cladocera (Pennak 1978) making laboratory results obtained with Diaptomus clavipes more easily

extrapolated to copepod populations in the field. In addition, its small size, short generation time and large reproductive output makes Diaptomus clavipes an excellent choice for use in toxicity tests.

The effects of environmental factors on the population dynamics of zooplankton present complex problems which are inadequately understood due primarily to lack of knowledge concerning their general biology, environmental requirements, and interrelations within the ecosystem (Hazelwood and Parker 1961, Armitage et al. 1973). Past studies have evaluated several of these factors in an attempt to determine the more important factors affecting zooplankton populations. Temperature has been shown to affect longevity, growth, metabolism, and reproduction in aquatic poikilotherms. The degree to which these biological processes are affected is dependent upon several factors including: range of temperatures to which an organism is exposed, exposure time, developmental stage in an organism's life history, and interaction of temperature with other environmental parameters (Bunting 1974). Food supply has also been found to affect reproduction, growth, and survival of zooplankton (Edmondson 1964, 1965; Comita and Anderson 1959; Kamps 1979; Elmore 1980). Buikema et al. (1980) in a review of the methods that have been used in Daphnia toxicity testing, found that they were not as standardized or reproducible as once believed. In spite of the myriad toxicological data and experience available, Buikema felt the variation in data was a result of food supply, temperature and other environmental parameters which were not considered in the experimental design.

Goss and Bunting (1976) tested thermal tolerance of Daphnia magna and Daphnia pulex and recommended that in studies with zooplankton the following should be given more consideration: (1) use of an adequate number of test organisms, (2) consideration of and statements of acclimation conditions, (3) adequate control groups, (4) duration of tests sufficiently long to be meaningful, (5) statistical design and analysis, (6) interaction of environmental factors, (7) rate of change of the factor being evaluated, (8) effect on all major life history stages of the organisms. Many of the shortcomings of bioassay tests cited by Buikema et al. (1980) could be readily corrected by more careful experimental design, execution and analysis.

With these recommendations in mind, the objectives of this study were to evaluate the use of the calanoid copepod Diaptomus clavipes, as a test organism in bioassays. Studies were conducted over a range of temperature and food conditions, and experiments on life stage lethality, reproduction, development, and growth were performed for each combination of these variables.

The compound chosen for study was the azaarene, acridine (2, 3, 5, 6-dibenzopyridine). The azaarenes are a class of nitrogen containing polycyclic aromatic compounds associated with synthetic fuel production. The possible development of a large scale synthetic fuels industry in the United States could create the potential for release of substantial quantities of organic wastes high in nitrogenous materials to aquatic ecosystems (Gehrs 1976). Some members of this class are known mutagens (Lehninger 1970) and/or carcinogens (Arcoz et al. 1968; Christensen et al. 1975). Azaarenes have also been observed in

effluents and raw wastewaters from other industrial processes such as coking in concentrations ranging from 1 to 100 mg/l (Schmidt et al. 1974). Among the azaarenes, acridine, a three ring compound, is intermediate in molecular weight and acute toxicity to aquatic organisms (Southworth et al. 1978). In addition, toxicity tests with acridine have been performed on a variety of freshwater organisms including green algae (Selenastrum capricornutum), bluegreen algae (Microcystis aeruginosa), cladocerans (Daphnia magna, Daphnia pulex), chironomids (Chironomus tentans), fathead minnows (Pimephales promelas), bluegill sunfish (Lepomis macrochirus), rainbow trout (Salmo gairdneri), and ciliates (Tetrahymena pyriformis) (Southworth et al. 1978, Schultz et al. 1980, Giddings 1980, Parkhurst et al. 1981, Cushman and McKamey 1981, Birge and Black 1981). This data base provides useful information for comparisons of sensitivity and response similarities with the data obtained using Diaptomus clavipes.

CHAPTER II

MATERIALS AND METHODS

General Culture

Stock cultures of Diaptomus clavipes were initiated from animals gathered in 1973 from a small man-made impoundment in Cleveland County, Oklahoma (see Gehrs 1972 for description of impoundment).

Self-propagating cultures have since been maintained at $21^{\circ}\pm 1^{\circ}\text{C}$ in 38-liter capacity aquaria containing filtered well water. During the present study, new cultures were started at $16^{\circ}\pm 1^{\circ}\text{C}$ and $26^{\circ}\pm 1^{\circ}\text{C}$ from the 21°C stock animals and maintained in 38-liter capacity aquaria in Percival controlled environment chambers. An acclimation period of two months preceded testing. All animals used in testing were removed from these aquaria.

Water used for culturing and testing was obtained from wells located on the Oak Ridge Reservation and pumped directly into a 500 gallon polyethylene holding tank in the laboratory. Before entering the laboratory, the well water was passed through a small sand filter which removed the large particulate matter. The quality of the water remained relatively constant throughout the year (see Table A-1). During this study, the pH ranged from 7.0 to 8.0 while the dissolved oxygen varied from 6.5 to 10.5 parts per million (ppm). All water used in culturing was filtered through stainless steel bolting cloth (mesh size $61\ \mu\text{m}$). In addition, all water used in testing was Millipore filtered ($0.45\ \mu\text{m}$). Water was allowed to equilibrate at the desired temperature for 48 hours (h) before it was used.

The lighting regime used for all experiments was 12 hour light/12 hour dark with an intensity of 100-110 footcandles (ft-c) at the level of the beakers. Since gold fluorescent lights eliminate light of wavelengths less than 500 nanometers (nm), they were used to avoid any possible photolysis of organic polycyclic compounds.

Food material used in culturing and testing was a modification of a mixture first developed by Biesinger and Christensen (1972) for Daphnia magna and used successfully by Robertson et al. (1974) and Cooney et al. (1978) for Diaptomus clavipes.

A stock food suspension was prepared by mixing 10 grams (g) of Purina trout chow (see Tables A-2 and A-3) with one liter (1) of distilled water in a blender for five minutes and then straining the entire mixture through stainless steel bolting cloth (mesh size 61 μm). The material retained on the bolting cloth was discarded and the filtrate served as the stock suspension. This was refrigerated at 10°C when not in use so that bacterial growth and other changes were retarded. A new stock suspension was prepared weekly. This food has the advantage that it can be easily made up to the same specifications each time it is produced. This permits control of the relative amounts of food added to different cultures by changing the concentration of trout chow per unit volume of distilled water or by changing the amount of food suspension added to each culture.

Four samples for total organic carbon analysis were obtained from the stock suspension and from each of two dilutions of the stock suspension (1:1, 1:3). The method used for total organic carbon analysis was based on the standard method of the U.S. Environmental

Protection Agency (1979) using an Oceanography International carbon analyzer (model 6524). These total organic carbon values were: stock suspension, 2.81 mg/ml; 1:1 dilution, 1.38 mg/ml; 1:3 dilution, 0.68 mg/ml. Cultures received 300 milliliters (ml) of a dilute trout chow suspension (1 part of stock suspension diluted with 3 parts of distilled water) twice weekly.

Measurement Techniques

During some of the experiments, several measurements of length were obtained on adults, first stage copepodids, and eggs. These measurements were made under a compound microscope with a Vickers A.E.I. image-splitting eyepiece using either a magnification of 40X or 100X. The eyepiece was calibrated with a stage micrometer. At a magnification of 40X, one vernier unit equaled 2.5×10^{-3} millimeters (mm) while at 100X, one vernier unit equaled 8.89×10^{-4} mm.

The length of the metasome, excluding the wings, was measured on adult males and females at a magnification of 40X. First stage copepodids were measured at a magnification of 100X. Metasome length was chosen over the total length measurement, since the urosome may bend or the segments telescope or contract when immobilized (Marshall and Orr 1955).

The eggs, after they had been removed from the females, were covered with water to prevent desiccation and then measured at a magnification of 100X. The greatest and least diameters were determined. The depth of the egg was assumed to be equal to the least diameter. This was verified by measuring the diameter in various

positions on the egg. Egg volumes were calculated using the formula for a prolate spheroid:

$$V = (1/6)K^3\pi ab^2$$

where:

V = egg volume in mm³

K = 8.889 X 10⁻⁴ mm/vernier unit

a = greatest diameter in vernier units

b = least diameter in vernier units

π = 3.1416.

Toxicant Preparation

Acridine used in testing was purchased from Aldrich Chemical Company, Inc. and purified to greater than 99 percent as determined by thin layer chromatography in a chloroform:methanol:acetic acid (90:5:5) solvent system (Walton 1981). Stock solutions were prepared by dissolving 10 milligrams (mg) of acridine in one milliliter (ml) of reagent-grade methanol and then adding this to one liter (l) of filtered well water. This solution was magnetically stirred for one hour and then appropriate dilutions were made.

Using a Perkins Elmer spectrophotometer, a series of acridine dilutions were analyzed at 353 nm and a linear regression line fitted to the data. This was:

$$\text{Absorbance} = 0.00277 + 0.05464 (\text{Concentration}) \quad r^2 = 0.991 \quad .$$

This standard was compared with absorbance data obtained from three temperatures (16°, 21°, 26°C) at three time periods (0, 48, 96 hours) using an analysis of covariance (Neter and Wasserman 1974). This was done to determine if the relationship between concentration and absorbance changed over temperature and time. The results indicated that all the lines were parallel and indicated a reduction in concentration with increasing time and temperature. This reduction was slight, however. The proportions of the original concentrations at 96 h for 16°, 21°, and 26°C were 0.98, 0.96, and 0.91, respectively.

General Handling of Test Animals

Before testing, adults were removed from stock cultures by netting, separated by sex, and placed in 100 ml beakers containing 80 ml of filtered well water at a density of five adults per beaker. Each beaker received 1 ml of a 1:3 dilution of the stock food suspension (1 part stock suspension:3 parts distilled water) daily for five days. Water in the beakers was not changed during this period. At the end of the feeding period, all animals from these beakers were combined (sexes separate) in two-liter beakers and allowed to mix for one hour. This would reduce any possible bias in nutritional state or any other factor associated with the individual beakers. At this time, the animals were randomly assigned to the treatments. Since there was the possibility of mortality during this period, 20-25 percent more animals were handled in excess of those required for individual experiments. Modification of the handling procedure for individual experiments are detailed in the following sections.

Adult Acute Bioassay

In this experiment, three feeding regimes were employed as pretreatments to evaluate the effect of nutritional state on acute toxicity. The first feeding regime ("fed") was outlined in the preceding section. In the second feeding regime ("starved"), the animals were handled in the same manner except they received no food for a five day period. In the third feeding regime ("stock"), animals were removed from the stock cultures and placed directly into two-liter beakers and then tested. A five day period was chosen since at 16°C (the lowest temperature used) egg development times were approximately four days. Therefore, in the fed and starved regimes, no ovigerous females were present at the time of testing. This was not the case in the stock regime where a variable number of ovigerous females were present.

For each test, six concentrations of acridine were used ranging from 2.4-10.0 mg/l evenly spaced on a logarithmic scale. Since methanol was used as a solvent, two controls were used, a methanol control (1 ml methanol per liter of well water) and a well water control. The methanol control equaled the methanol concentration in the highest acridine concentration.

Six 100 ml beakers were used per concentration. Sixty animals (30 males and 30 females) were randomly placed in these beakers at a density of 10 animals per beaker (sexes separate). A total of 480 animals were used in each experiment. Observations on mortality

were obtained at 24 hour intervals for 96 hours. This test was done twice for each temperature and feeding regime combination for a total of 18 tests.

Reproduction Bioassay

In this experiment, at each temperature, 280 animals (1:1 sex ratio) were randomly placed in 100 ml beakers at a density of two animals/beaker (1:1 sex ratio) and 20 beakers/concentration. Five concentrations of acridine were used ranging from 0.32-3.2 mg/l evenly spaced on a logarithmic scale. Well water and methanol controls (0.32 ml methanol/l) were also employed. One half of the beakers at each concentration received food while the other half received no additional food. A 1:1 dilution of the stock food suspension was added to the various dilutions of acridine at a rate of 1 ml/100 ml water and then magnetically stirred until the food was thoroughly mixed. This suspension was then poured into the individual beakers. The concentrations were renewed every two days.

Observations were made daily for 15 days on the number of dead animals and ovigerous females. Ovigerous females were removed and measured. The eggs were removed, counted, and then placed in tissue culture plates at the same concentrations. They were observed daily until hatching at which time the percent egg hatch was noted. Dead animals were replaced. This experiment was repeated for each temperature.

Acute Egg and Naupliar Bioassays

Two experiments were performed to evaluate egg and naupliar mortality. In the first experiment, 200 animals (1:1 sex ratio) were placed in the 100 ml beakers at a density of 10 animals/beaker (1:1 sex ratio). Observations were made hourly for 12 hours for the presence of ovigerous females at which time the females were removed and individually placed in 100 ml beakers containing filtered well water. When the eggs were half-way through their development, they were removed from the females and counted. At the three temperatures used (16°, 21°, 26°C) these times were approximately 42, 26, and 18 hours, respectively. These intact egg clutches were then randomly placed in tissue culture plates at the various treatments (5 clutches/concentration). Six concentrations of acridine were used ranging from 0.32-5.6 mg/l evenly spaced on a logarithmic scale. A well water and methanol control (0.56 ml methanol/l) were also employed. Observations on percent egg hatch and naupliar mortality were made hourly until the first molts were noted. The experiment ended when all controls had molted. This test was repeated for each temperature.

A second experiment was performed to determine the survivorship of nauplii to the first copepodid stage (C1). Egg clutches were obtained in the same manner as outlined previously. After the egg clutches were removed from the females, they were placed in tissue culture plates containing filtered well water and observed hourly until hatching. Nauplii which hatched within four hours of each other were combined and then randomly assigned to the various treatments. Six concentrations

of acridine ranging from 0.32-5.6 mg/l evenly spaced on a logarithmic scale were used. A well water and methanol control (0.56 ml methanol/l) were also employed. Nauplii were individually placed in wells of the tissue culture plates which contained approximately 2 ml per well. One-half of the nauplii at each concentration (50 nauplii/concentration) received food at the same level as in the reproduction bioassay while the other half received no additional food. The concentrations were renewed every two days. Observations on molting and mortality were made at eight hour intervals until the animals molted into the first copepodid stage (C1).

Chronic Bioassay

This experiment was designed to evaluate the effects of acridine on survival, growth, development, and reproduction parameters. The design was similar to the reproduction bioassay except that all 20 beakers at each concentration received food. This food was administered at the same rate as in the reproduction bioassay. Observations on presence of ovigerous females were made hourly for the first 12 hours and then at 12 hour intervals for the next 36 hours. Clutches produced during the first 12 hours were removed when the eggs were half-way through their development.

Fifty eggs were measured at each concentration with a maximum of 10 eggs per clutch. The eggs were individually placed in tissue cultures plates at the same concentrations and food levels at which they were laid. Observations were made hourly until hatching and then extended to eight hours until they molted into copepodids. Twenty-five

first stage copepodids (CI) were then measured, if available, at each concentration. At this time, the observation interval was extended to 12 hours. Presence of molts and dead animals was noted at each interval. When the animals molted into the fifth copepodid stage (CV), they were transferred to individual eight dram shell vials containing approximately 30 ml of media. The transfers were made because it appeared that food availability might be limiting since the duration of the fourth copepodid stage (CIV) was much longer than expected.

Upon reaching adulthood (CVI) all animals were measured. Females were then combined with two males from the stock cultures and observed daily for 15 days in another reproduction experiment. Control females were observed for 20-25 days. Males were combined with two to three females from stock cultures and observed daily until a viable clutch was produced. All adults were maintained at the same acridine concentrations and food level as before.

CHAPTER III

ADULT ACUTE BIOASSAY

In the analysis of toxicity data, the survival time of an animal is a response of prime importance. For this study, the following logistic function was used to describe the survival time, T:

$$P [T < t] = p(t) = \{1 + \exp [(\mu - \ln t)/\delta]\}^{-1}$$

where μ and δ are the associated location and scale parameters, respectively, of the survival distribution. For each combination of experimental factors (concentration, temperature, food level, sex, test) the observations were summarized in the form of Table 1. The likelihood function for each combination of experimental factors is given by the multinomial expression:

$$L = \frac{n!}{n_1!n_2!n_3!n_4!n_5!} (p(t_1))^{n_1} (p(t_2)-p(t_1))^{n_2} \\ (p(t_3)-p(t_2))^{n_3} (p(t_4)-p(t_3))^{n_4} (1-p(t_4))^{n_5}$$

which is a function of the unknown parameters μ and δ . The maximum likelihood estimates of μ and δ were found by maximizing L (or equivalently, $\ln L$) as a function of μ and δ . The routine used was an iterative procedure which made use of the first and second derivatives of the likelihood function. Initial estimates were obtained graphically from a plot of:

$$\ell(p(t)) = \ln [(1-p(t))/p(t)] = \mu/\delta - 1/\delta (\ln t)$$

against $\ln t$.

Table 1. Data format and notation for observations on adult mortality in Diaptomus clavipes for use in the computation of the likelihood function (L) for each combination of experimental factors

Observation Times (Hours)	Number Alive At Start of Interval	Number Dying During Interval	Number Alive at End of Interval
t_1 (24)	n	n_1	$n - n_1$
t_2 (48)	$n - n_1$	n_2	$n - n_1 - n_2$
t_3 (72)	$n - n_1 - n_2$	n_3	$n - n_1 - n_2 - n_3$
t_4 (96)	$n - n_1 - n_2 - n_3$	n_4	$n - n_1 - n_2 - n_3 - n_4 = n_5$

A likelihood ratio statistic was used to test for significant differences in the parameter values between the two tests. This statistic was calculated from the difference of the log likelihood function when the parameter estimates were obtained separately and when the two tests were combined. This statistic can be compared with a chi square statistic with the appropriate degrees of freedom. It was found that in the majority of the cases, there were no significant differences ($P > 0.05$) between the tests. This became evident after plotting the μ values for each test against concentration for each combination of the other experimental factors (temperature, food level, sex). Therefore, it was decided to combine the data for individual tests in order to obtain a single estimate for each combination of experimental factors.

After estimates of the parameters were found, a goodness-of-fit statistic was calculated for each combination of experimental factors. The statistic makes it possible to evaluate the adequacy of the logistic survival function in describing the survival data for any group of observations. In the majority of cases, the logistic survival function appeared adequate ($P > 0.05$). This function was inadequate when the proportion of dead animals was less than 10 percent at 96 hours or when it was 100 percent at 24 hours. Therefore, those data were not used in further analysis. Figures B-1-B-18 graphically present logistic survival functions for each combination of experimental factors.

After the maximum likelihood estimates of the parameters μ and δ were obtained, plots of μ versus concentration and δ versus

concentration were examined to evaluate the effect of acridine concentration on these two parameters. The following hyperbolic function was used to describe the relation between μ (or δ) and concentration for each combination of temperature, food level, and sex:

$$y = ax^b$$

where y is μ (or δ) and x is acridine concentration. The estimates of the parameters a and b were obtained using the linearized form of the above equation:

$$\ln y = \ln a + b (\ln x)$$

In order to determine if there were any significant differences in survival times between the sexes, males and females were tested separately for each temperature, feeding regime, and concentration combination. The first comparison determined if there were any significant changes in y as a function of concentration between the sexes. This was examined by calculating the residual sums of squares for individual lines (one line for each combination of experimental factors) and comparing it with the residual sum of squares combined over sexes. The results indicated that there were significant differences between the sexes ($F = 2.56$, $df = 18/56$, $P < 0.01$) among the various experimental factors. The starved feeding regime contributed the most variation, and when the data were partitioned and reanalyzed, sex differences were found only in the starved feeding regime ($F = 4.76$, $df = 6/19$, $P < 0.01$). In the fed and stock feeding regime, there were no detectable differences between the sexes

($F = 1.69$, $df = 12/37$, $P > 0.10$). Therefore, they were combined. Two separate analyses were performed from this point, one for the starved feeding regime (sexes separate) and one for the fed and stock feeding regimes (sexes combined).

The next step in the analysis was to test for equality of the estimated slopes, b , for the two feeding regime groupings. In both analyses, this test for parallelism was not significant ($P > 0.05$). The common slopes were -1.79 for the fed and stock regimes and -1.48 for the starved regime, both significantly different from zero ($P < 0.0001$). The negative slopes indicate that as concentration increases, half life (μ) decreases. After these tests, an analysis of covariance was used to test for significance of the different experimental factors. In the fed and stock regimes, there were significant differences between the foods ($F = 21.07$, $df = 1/54$, $P < 0.0001$) and among the temperatures ($F = 3.30$, $df = 2/54$, $P < 0.0445$) with a significant interaction ($F = 6.65$, $df = 2/54$, $P < 0.0026$). In the starved feeding regime, there were significant differences among the temperatures ($F = 22.54$, $df = 2/24$, $P < 0.0001$). The overall sex effect was not significant ($F = 4.16$, $df = 1/24$, $P > 0.0525$), but there was a significant interaction ($F = 9.41$, $df = 2/24$, $P < 0.001$). The final adjusted means for the various experimental factors were then compared using a a posteriori test of Bonferroni (Tables 2 and 3) and displayed graphically in Figures 1 and 2. For the fed and stock feeding regimes, there were significant increases in half life at 16°C and 26°C when the animals were fed. At 21°C , however, the adjusted means were not significantly different from

Table 2. A posteriori comparisons of the adjusted means for time to 50 percent mortality (half life) in Diaptomus clavipes in the fed and stock feeding regimes using Bonferroni's multiple range test.

Temperature (°C)	16	26	21	21	16	26
Feeding Regime	fed	fed	stock	fed	stock	stock
Adjusted Means (hours) ^{a,b}	156.02	106.70	89.12	87.36	76.71	68.03

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level

^bmeans transformed to original units

Table 3. A posteriori comparisons of the adjusted means for time to 50 percent mortality (half life) in Diaptomus clavipes in the starved feeding regime for both sexes using Bonferroni's multiple range test.

Temperature (°C)	16	16	21	21	26	26
Sex	male	female	female	male	female	male
Adjusted Means (hours) ^{a, b}	81.34	62.83	48.77	46.17	44.80	19.57

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level

^bmeans transformed to original units

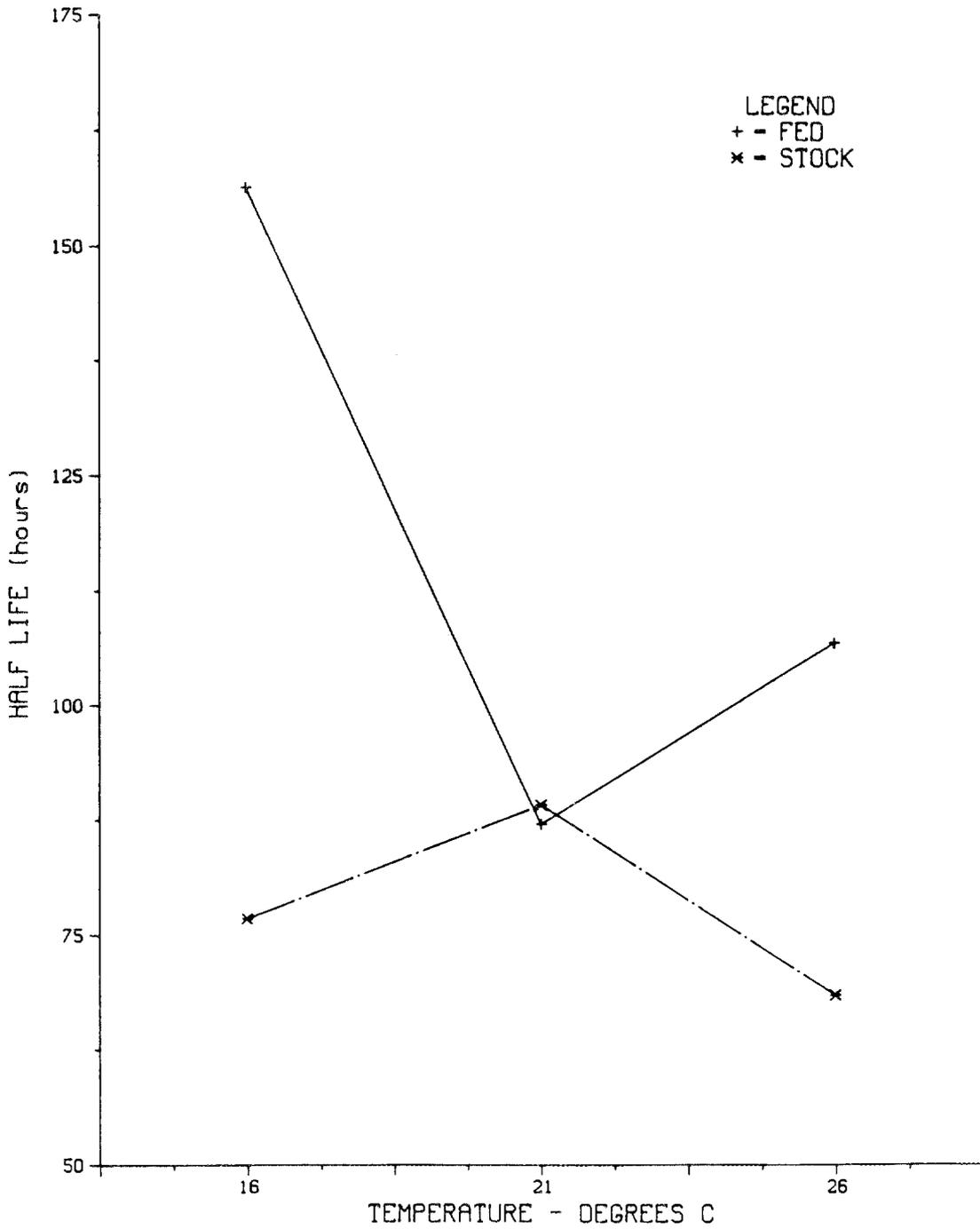


Figure 1. Adjusted means for time to 50 percent mortality (half life) in Diaptomus clavipes (sexes combined) in the fed and stock feeding regime for the adult acute bioassays.

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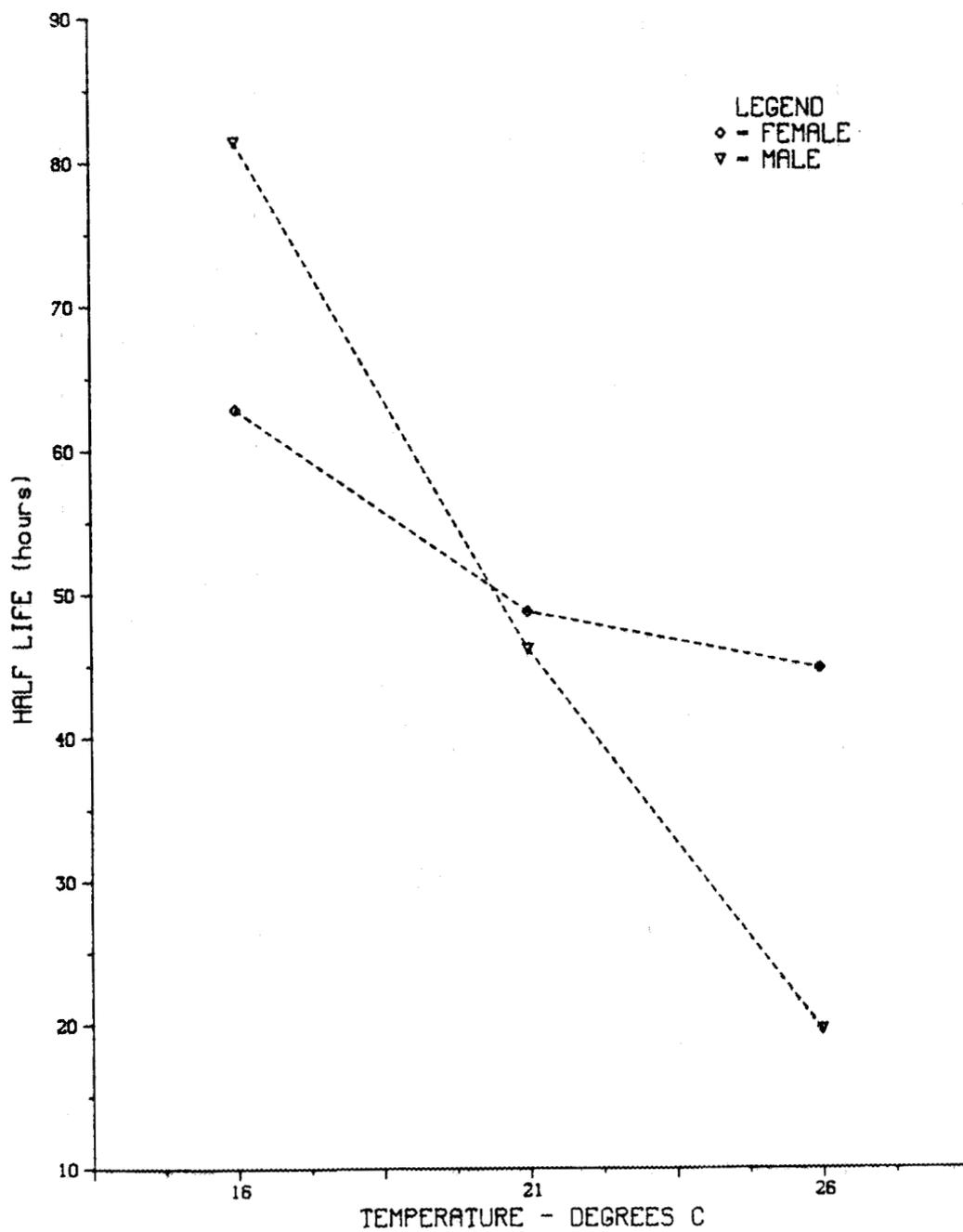


Figure 2. Adjusted means for time to 50 percent mortality (half life) in male and female Diaptomus clavipes in the starved feeding regime for the adult acute bioassays.

each other. There was no significant temperature effect in the stock animals. In the starved feeding regime, the adjusted means were generally lower than those for the fed and stock feeding regimes. Only the males at 26°C were significantly different than all other means.

Analysis of the scale parameter (δ) was also performed for the entire data set using the same regression equation as previously used for μ to estimate the relation between δ and concentration. It was of interest to determine if a single line (pooled over all experimental factors) would provide an adequate fit for these data.

This was examined by calculating the residual sum of squares for individual lines (one line for each combination of experimental factors) and comparing it with the residual sum of squares for a single line. The results indicated there were significant differences in this relationship among the various experimental factors ($F = 2.80$, $df = 34/56$, $P < 0.001$).

When the data were partitioned, there were no significant differences among experimental factors for the fed and stock feeding regimes ($F = 1.36$, $df = 22/37$, $P > 0.10$), but there were significant differences in the starved feeding regime ($F = 5.76$, $df = 10/19$, $P < 0.001$). The relation between δ and concentration for the fed and stock feeding regimes was estimated by the following regression equation:

$$\ln(\delta) = 0.11031 - 0.6294 \times \ln(\text{concentration}) \quad r^2 = 0.44$$

The estimated slope of the line was significantly different from zero ($t = 6.79$, $df = 1$, $P < 0.0001$) thus indicating a negative relationship

between δ and concentration. In the starved feeding regime, this relationship was less apparent. When individual lines were fitted for each factor, only two of six had slopes significantly different from zero ($P < 0.05$) and both were at 21°C. The two significant regression equations were:

$$\text{Females: } \ln(\delta) = 0.721990 - 1.12776 \times \ln(\text{concentration}) \quad r^2 = 0.93$$

$$\text{Males: } \ln(\delta) = 1.33317 - 1.61315 \times \ln(\text{concentration}) \quad r^2 = 0.69$$

The results of this analysis indicate that as concentration increases the scale parameter (δ) decreases. The reciprocal of δ represents the rate of change in the logistic survival function. As concentration increases, the rate of change ($1/\delta$) also increases leading to a shorter time to 50 percent mortality (μ), a result, consistent with the previous analysis.

To compare control mortality among the various experimental factors, a stepwise logistic regression was computed with the experimental factors (temperature, feeding regime, sex, test, and control type) as independent variables (Cox 1970). The cumulative mortality at 96 h was used in this analysis. The only significant experimental factors ($P < 0.05$) were temperature and feeding regime. The proportion dead at 96 h for each combination of significant factors are graphically displayed in Figure 3. It is apparent that the pattern of mortality in the starved regime is different from that of the other two regimes. This was also the only regime which exceeded 10 percent mortality at 96 h.

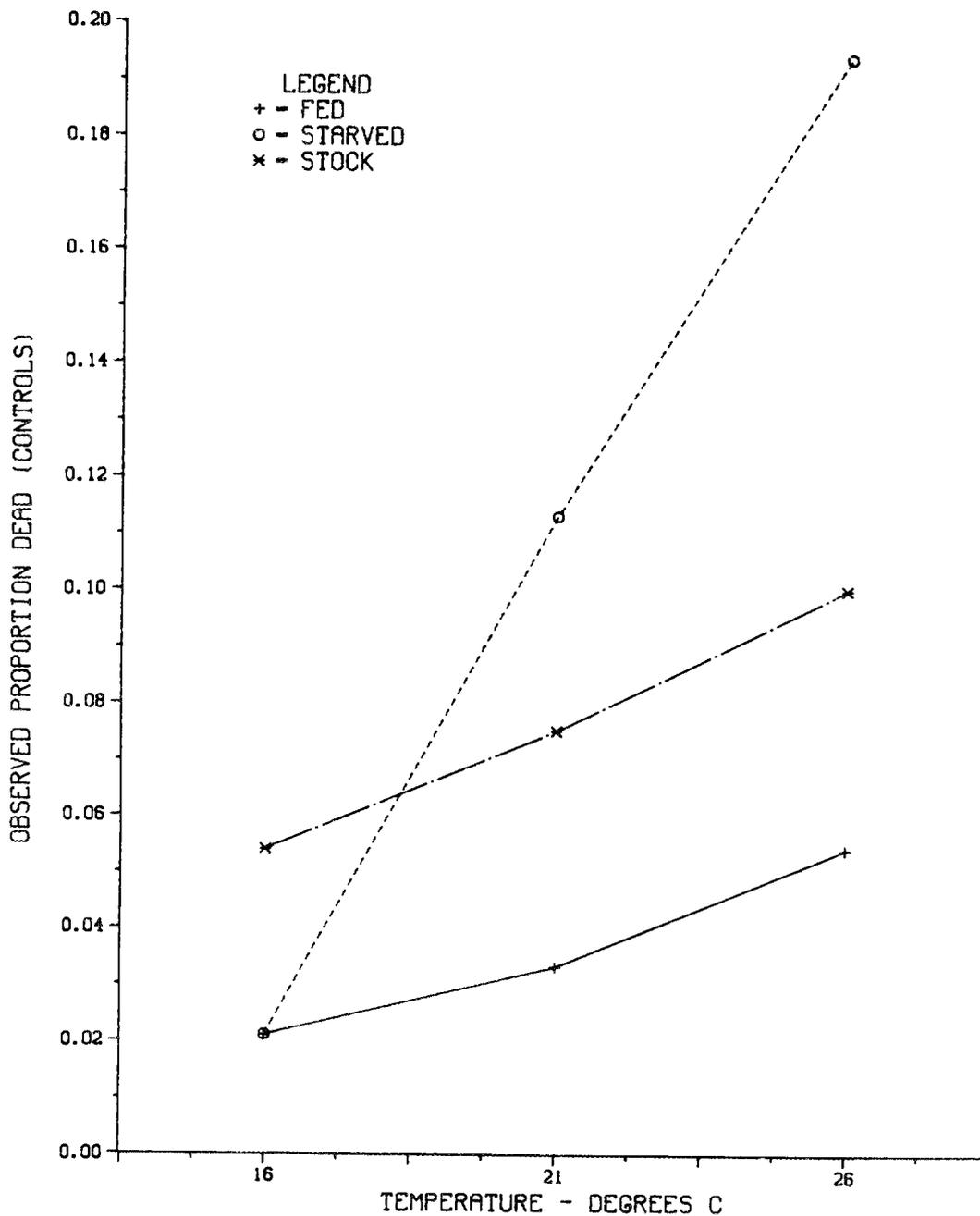


Figure 3. Control mortality at 96 hours in Diaptomus clavipes for each combination of temperature and feeding regime for the adult acute bioassays.

Estimates of 96 h LC50's were calculated from the regression equations (based on the logistic survival function) obtained for each combination of experimental factors (temperature, feeding regime, sex) using inverse prediction. Ninety-five percent fiducial limits were then calculated using Fieller's theorem (Finney 1978). Table 4 gives these LC50's with their associated fiducial limits.

For comparative purposes, an additional analysis was performed using the maximum likelihood estimates of probit analysis (Finney 1971). The cumulative mortality at 96 h was used in this analysis. The results were similar to those found for the logistic survival function. The data were partitioned in the same manner with the starved feeding regime analyzed separately from the fed and stock feeding regime. In both analyses, the probit lines were found to be parallel (equal slopes). The common slopes were 2.40 for the fed and stock regimes and 1.92 for the starved feeding regime. Therefore relative potencies could be calculated. For the fed and stock feeding regime, there were no sex differences in relative potency, therefore, the data were combined and reanalyzed. Table 4 gives the LC50 values for the fed and stock regimes and these are graphically displayed in Fig. 4. Table 5 gives the relative potency values. For the starved feeding regime, sex differences were found at 26°C; therefore the data were not combined. Table 4 also gives the LC50 values for the starved regime and these are graphically displayed in Fig. 5. The relative potency values are given in Table 6.

Since there was control mortality in the various experimental factors (Fig. 3), the data were corrected using Abbott's Formula

Table 4. Adult acute toxicity of acridine to *Diaptomus clavipes* for three feeding regimes at three temperatures based on the method of probit analysis and the logistic survival function.

Temperature	Feeding regime	Sex	96 h-LC50 (mg/l)	95% fiducial limits	
				Upper	Lower
16	Fed	---	6.70 (6.43) ^a	7.71 (7.42)	5.81 (5.59)
21		---	4.83 (4.31)	5.36 (4.94)	4.36 (3.76)
26		---	5.41 (4.96)	6.04 (5.68)	4.85 (4.33)
16	Stock	---	4.51 (4.18)	4.97 (4.79)	4.07 (3.64)
21		---	4.89 (4.92)	5.46 (5.64)	4.39 (4.30)
26		---	4.22 (3.74)	4.67 (4.30)	3.82 (3.25)
16	Starved	Female	3.61 (4.52)	4.33 (5.16)	2.94 (3.95)
16		Male	4.30 (4.59)	5.13 (5.24)	3.55 (4.02)
21		Female	3.04 (2.75)	3.63 (3.20)	2.50 (2.34)
21		Male	2.93 (2.59)	3.47 (3.02)	2.40 (2.18)
26		Female	2.87 (3.02)	3.41 (3.48)	2.35 (2.59)
26		Male	1.64 (2.36)	2.04 (2.78)	1.26 (1.97)

^aThe numbers inside the parentheses were calculated by the probit method while the numbers outside the parentheses were obtained using the logistic survival function.

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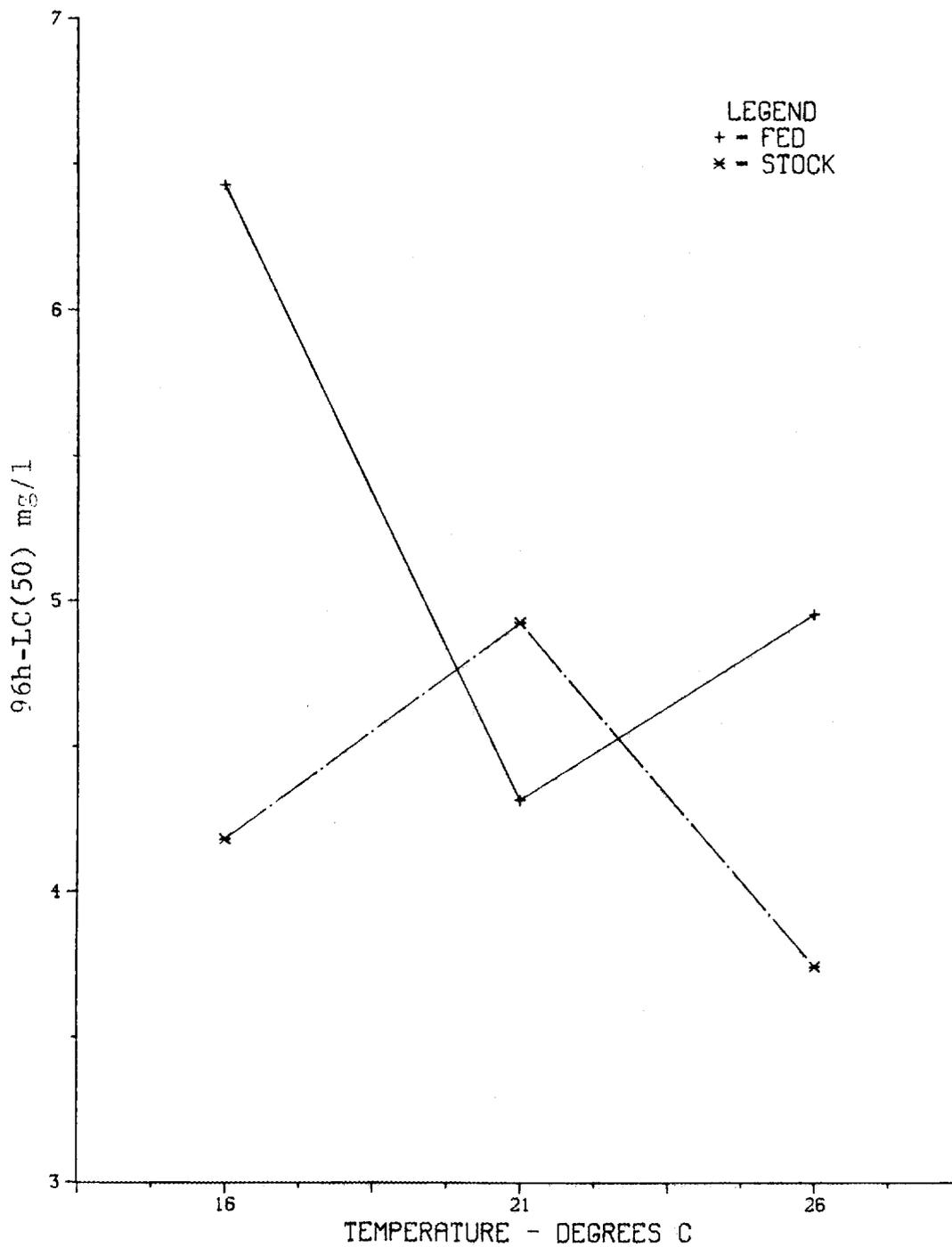


Figure 4. LC50 values at 96 hours (probit method) for *Diaptomus clavipes* (sexes combined) in the fed and stock feeding regime for the adult acute bioassays.

Table 5. Relative potency values for Diaptomus clavipes in the fed and stock feeding regimes at three temperatures based on the method of probit analysis

Temperature °C (Feeding regime)	16 Fed	16 Stock	21 Fed	21 Stock	26 Fed	26 Stock
16 (fed)	-----					
16 (stock)	0.65*	-----				
21 (fed)	0.67*	1.03 ^{ns}	-----			
21 (stock)	0.77*	1.18 ^{ns}	1.14 ^{ns}	-----		
26 (fed)	0.77*	1.18 ^{ns}	1.15 ^{ns}	1.01 ^{ns}	-----	
26 (stock)	0.58*	0.89*	0.87 ^{ns}	0.76*	0.76*	-----

*significant at the 0.05 level

^{ns}not significant at the 0.05 level

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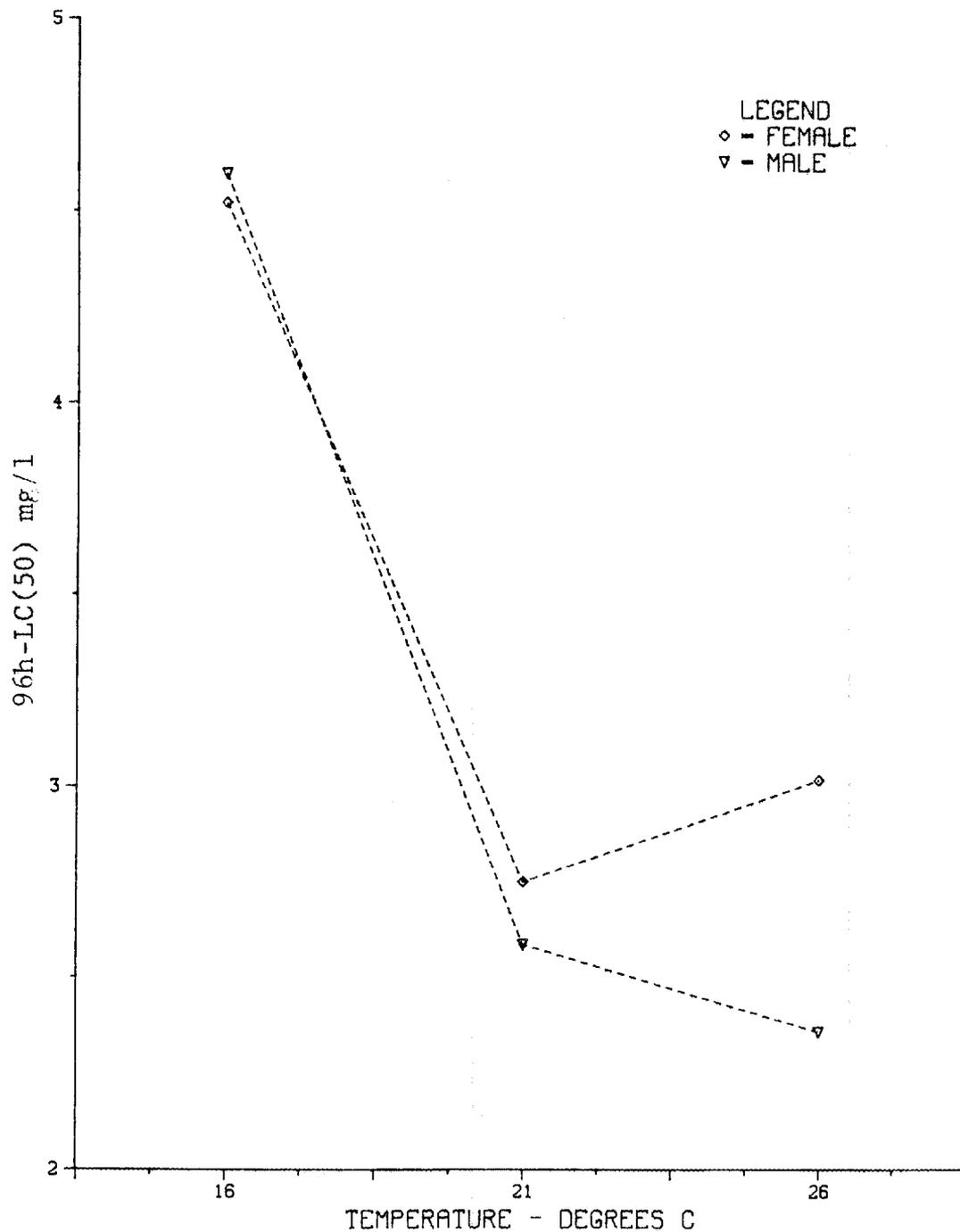


Figure 5. LC50 values at 96 hours (probit method) for male and female *Diaptomus clavipes* in the starved feeding regime for the adult acute bioassays.

Table 6. Relative potency values for Diaptomus clavipes in the starved feeding regime at three temperatures and two sexes based on the method of probit analysis

Temperature °C (Sex)	16 Female	16 Male	21 Female	21 Male	26 Female	26 Male
16 (female)	-----					
16 (male)	1.02 ^{ns}	-----				
21 (female)	0.61*	0.60*	-----			
21 (male)	0.57*	0.56*	0.94 ^{ns}	-----		
26 (female)	0.67*	0.66*	1.10 ^{ns}	1.17 ^{ns}	-----	
26 (male)	0.52*	0.51*	0.86 ^{ns}	0.91 ^{ns}	0.78*	-----

*significant at the 0.05 level

^{ns}not significant at the 0.05 level

(Finney 1971) and reanalyzed. The 95 percent fiducial limits for the LC50 values for the corrected data encompassed the LC50 values for the uncorrected data with a mean difference of less than 6 percent. Only females at 26°C and 21°C in the starved feeding regime showed differences of 10 percent and 14 percent respectively. The only change in the relative potency came at 26°C in the starved feeding regime where the difference in LC50 between males and females went from significant ($P < 0.05$) for the uncorrected data to nonsignificant ($P > 0.05$) for the corrected data. Therefore, control mortality did not significantly affect the LC50 values until it exceeded approximately 10 percent.

Measurement of the effects of the experimental factors (temperature, food, sex) upon the acute toxicity of acridine to the adult calanoid copepod, Diaptomus clavipes yielded similar results for both the logistic survival function and the probit method. Sexual differences in acute toxicity were restricted to the starved feeding regime at 26°C. When these data were corrected for control mortality, this difference became nonsignificant. The U.S. Environmental Protection Agency (1975, 1978) suggested that if control mortality exceeded 10 percent, a toxicity test was unacceptable. The most commonly used method to correct for control mortality is the application of Abbott's Formula (Finney 1971). The use of this formula assumes that the two types of mortality operate independently, and it is rarely possible to test the assumption on which it is based. At a total response of 50 percent, application of Abbott's Formula with control mortalities of 5, 10, 15 and 20 percent would yield a reduction

in response of 3, 6, 9 and 12 percent, respectively. At responses less than 50 percent the reduction in response is less. At a total response of 100 percent, there is no change in response. In this study, control mortality did not exceed 10 percent except in the starved feeding regime. The starved feeding regime was analyzed separately to avoid pooling of data with varying percentages of control mortality. If selection of a toxicity test were based on survivorship of controls, either the fed or stock regimes would be adequate for any of the three temperatures.

Removing animals from stock cultures and feeding them before testing led to higher 96 h LC50 values at 16°C. There were no detectable differences between the two regimes at 21°C, however. This may be due to the nutritional condition of animals in the various cultures. The feeding schedule for cultures was based on tests at 21°C. Cooney et al. (1978) found that cultures at 21°C, fed at the rate specified earlier, had a high proportion of ovigerous females with clutches ranging from 15 - 20 eggs. Using these rates for other temperatures may not lead to the same nutritional conditions. Observations on population sizes (obtained during the collection of test animals) indicated that populations were much larger at 16°C than at 21°C. This was probably due to the longer life span of animals at 16°C. Large populations could not be maintained at 26°C either due to a lack of food or trying to culture an animal near the limit of its natural temperature range. Diaptomus clavipes is a multivoltine species with highest specific birth rates (m_x) in the early spring when water temperatures are less than 20°C (Gehrs 1972). Although

cultures can still be maintained at this temperature at the feeding rates specified, the animals may be somewhat stressed. This can be verified by comparing the 96 h LC50 values for 16°C and 26°C in the stock and starved feeding regimes (Table 4). This difference averaged less than 1 mg/l, thus suggesting a possible food stress in stock animals at these temperatures.

Banta (1939) stated that molting was a critical period for cladocerans and other arthropods. Anderson (1948) found that mortality in toxicity tests with Daphnia magna was highest during the time of molting. Lee and Buikema (1979) found that Daphnia pulex were most sensitive to chromate during the time of molting. They suggested that this cyclic sensitivity may affect the results of toxicity tests with Daphnia especially since cladoceran populations may be reproductively synchronous (Green 1956) and as such would not be uniformly distributed with respect to time of molting. Distinct changes in death rate with time, as reported by Sherr and Armitage (1971) and Anderson (1948), might be explained on the basis of culture synchrony. At temperatures below 20°C or under the influence of some toxicants, all animals may not molt during the usual 48 h time period for acute toxicity tests, thus yielding misleading results (Lee and Buikema 1979). In copepods, there are not molts after the adult stage is reached, therefore, there is no cyclic sensitivity to a toxicant due to physiological processes which occur during molting such as calcium deficiencies of the exoskeleton (Marshall et al. 1964). Comparison of the LC50 values at 21°C for Diaptomus clavipes (Table 4) with literature values (Table 7) indicate that adult Diaptomus are more tolerant than most of

Table 7. Acute toxicity of acridine to various freshwater algae, crustaceans, insects, snails, and fish

Test Organism	End Point	Concentration	Reference
<u>Selenastrum capricornutum</u> (green alga)	4h-EC20 ^a	1.3 mg/l	Giddings 1980
<u>Microcystis aeruginosa</u> (blue-green alga)	4h-EC20	3.6 mg/l	Giddings 1980
Natural algal community	4h-EC20	3.3 mg/l	Giddings 1980
<u>Daphnia pulex</u> (adult cladoceran)	24h-LC50	2.9 mg/l	Southworth et al. 1978
<u>Daphnia magna</u> (first instar cladoceran)	48h-LC50	2.3 mg/l	Parkhurst et al. 1981
<u>Chironomus tentans</u> (fourth instar insect)	48h-LC50	2.0 mg/l	Cushman and McKamey 1981
<u>Physa heterostropha</u> (adult snails)	48h-LC50	10.93 mg/l	R.E. Millemann (unpublished)
<u>Acheta domesticus</u> (cricket eggs)	LC50	15.10 mg/l	Walton 1980
<u>Acheta domesticus</u> (cricket eggs)	LD50	7.4 µg/g	Walton 1980
<u>Acheta domesticus</u> (cricket nymph)	48h-LD10	332 µg/g	Walton 1980
<u>Pimephales promelas</u> (adult fathead minnow)	96h-LC50	2.3 mg/l	R.E. Millemann (unpublished)
<u>Salmo gairdneri</u> (embryo-larval rainbow trout)	96h-LC50	0.32 mg/l	Birge and Black 1981
<u>Lepomis macrochirus</u> (embryo- larval bluegill sunfish)	96h-LC50	1.13 mg/l	Birge and Black 1981

^aEC20=effective concentration inhibiting photosynthesis by 20 percent

the invertebrates tested. Anderson (1948) speculated that thresholds of toxicity would be higher in adult copepods, than in cladocerans, since they cease to molt after becoming nature. Anderson's earlier work (Anderson et al. 1948), however, indicated that adult Diaptomus oregonensis were much more sensitive than Daphnia magna for all substances tested. The reason for this increased sensitivity was inadequate culturing technique which probably stressed the animals before testing. The results of this study are in agreement with Anderson's conclusions. Well-fed animals (indicative of adequate culturing techniques) led to higher LC50 values than in Daphnia magna. As the animals became stressed, due to a decrease in available food, they become more sensitive to the toxicant (Table 4).

The E.P.A. standard methods (1975, 1978) advocate the use of unfed first instar Daphnia for the 48 h acute toxicity tests. Buikema et al. (1980) stated that first instar Daphnia, without food, would not be expected to live much longer than 48 h. Therefore, it is possible that the lethality value obtained may be a result of both toxicity and starvation. Lemke and Lampert's (1975) study on Daphnia pulex indicated that starvation stress was the least for adult animals between 2.0 and 2.5 mm in length. Buikema suggested, based on Lemke and Lampert's data, that toxicity tests with Daphnia be conducted on adult organisms in this size range. Comparison of the 48 h-LC50 for first instar Daphnia magna (2.3 mg/l) with the 96 h-LC50 for the starved male and female Diaptomus clavipes (Table 4) revealed very little difference. This suggests that the greater sensitivity of first instar Daphnia may be the result of an interaction of starvation and toxicity.

The interaction of temperature and nutritional state of test animals led to a doubling in acute toxicity (Table 4) in this study depending upon the levels chosen. Temperature, over the range tested, did not strongly affect the acute toxicity of adult Diaptomus clavipes in the fed and stock regimes (Table 2). However, in the starved regime, increasing temperatures led to an increase in sensitivity (Table 3). Therefore, these factors must be taken into account when comparing results from different experiments or when trying to extrapolate from laboratory experiments to safe concentrations for natural populations.

CHAPTER IV

REPRODUCTION BIOASSAY

Reproduction was observed during a fifteen day period to evaluate toxicant effects over a range of temperature and food conditions. Three measures of reproduction were examined: number of eggs per clutch, number of clutches per female per day, and number of eggs per female per day. This last measure, rate of egg production, is not an independent measure of reproduction since it utilizes information on both clutch size and clutch production. However, comparison of this factor with the other two, allows evaluation of the relative contributions of each factor to the total egg production for any combination of experimental factors.

Several investigators have suggested that clutch size (number of eggs per clutch or clutch volume) was a function of the size of the female (Ravera and Tonelli 1956, Bayly 1962, McLaren 1965, Cole 1966, Maly 1973). This was true for females in the fed control groups for all three temperatures. An analysis of covariance (Neter and Wasserman 1974) (data for number of eggs per clutch transformed using the square root transformation) indicated that the slopes for the three lines (one line for each temperature) were equal (interaction: $F = 0.56$, $df = 2/499$, $P > 0.5688$) but they varied in position ($F = 104.53$, $df = 2/501$, $P < 0.0001$). The common slope was 3.94 which was significantly different from zero ($t = 8.71$, $df = 1$, $P < 0.0001$). The final adjusted means for the three temperatures were then compared using the a posteriori test of Bonferroni (Table 8) and this showed

Table 8. A posteriori comparisons of the adjusted means for number of eggs per clutch in Diaptomus clavipes for the fed control females at three temperatures using Bonferroni's multiple range test

Temperature (°C)	21	16	26
Adjusted Means ^{a, b}	20.98	13.26	12.92

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level

^bmeans transformed to original units

that the 21°C mean was larger than both the 16° and 26°C means. This relationship did not appear to hold in all instances. When the data for the various concentrations were examined, the majority showed no relationship. A 3 x 6 x 2 factorial analysis of variance on female lengths was computed with temperature, concentration, and feeding regime as main effects. No main factors or interactions were significant ($P > 0.05$) indicating no detectable differences in female size among the various experimental factors (Table 9). Therefore, female size was not used as a covariate to correct the reproduction data.

In the starved feeding regime, very few animals produced more than one or two clutches. Therefore, the data base was much smaller than the fed animals and was analyzed separately. No clutches were produced at a concentration of 3.2 mg/l for either fed or starved animals. Since two tests were performed, it was necessary to determine if there were any differences between the two tests. A chi square statistic was computed for each temperature and feeding regime combination based on the number of clutches produced at each concentration for each test. No test statistic was significant at the 0.10 level. Since each of the six test statistics was an independent chi square, they were combined to yield a single chi square statistic which was not significant at the 0.50 level indicating no detectable differences between the two tests in clutch production. Therefore, the data for the two tests were combined.

Percent egg hatch was calculated for all combinations of experimental factors and it was found that in most cases it exceeded 90 percent, except at the highest concentration where reproduction

Table 9. Mean metasomal lengths of female Diaptomus clavipes in the fed and starved feeding regimes for the reproduction toxicity tests

Temperature (°C)	Concentration (mg/l)	n	Feeding regime				
			Fed \bar{x}	SE ^a	n	Starved \bar{x}	SE
16	0.0(WW) ^b	20	1.45	0.02	14	1.47	0.02
	0.0(MC) ^b	21	1.46	0.02	10	1.46	0.02
	0.3	17	1.42	0.02	10	1.47	0.04
	0.6	19	1.47	0.02	8	1.47	0.03
	1.0	15	1.43	0.02	12	1.56	0.02
	1.8	6	1.50	0.03	5	1.48	0.04
21	0.0(WW)	28	1.47	0.02	22	1.48	0.01
	0.0(MC)	31	1.49	0.01	15	1.48	0.02
	0.3	26	1.47	0.01	13	1.48	0.02
	0.6	26	1.49	0.02	10	1.50	0.02
	1.0	22	1.47	0.02	14	1.52	0.02
	1.8	7	1.46	0.05	3	1.45	0.01
26	0.0(WW)	26	1.47	0.01	20	1.45	0.02
	0.0(MC)	28	1.45	0.01	17	1.48	0.02
	0.3	22	1.47	0.01	11	1.49	0.02
	0.6	21	1.47	0.02	14	1.47	0.03
	1.0	17	1.48	0.01	8	1.49	0.03
	1.8	2	1.44	0.01	6	1.43	0.02

^an=sample size, \bar{x} =means (mm), SE=standard error (mm)

^bWW=well water control, MC=methanol control

occurred (1.8 mg/l) (Table 10). Further analysis of naupliar data would yield similar results, therefore, only the egg data were used.

A 3 x 6 factorial analysis of variance was computed for number of eggs per clutch (data transformed using the square root transformation). The two fixed factors were temperature and acridine concentration. There were significant differences among the temperatures ($F = 35.40$, $df = 2/920$, $P < 0.0001$), concentrations ($F = 10.65$, $df = 5/920$, $P < 0.0001$) and in the interaction term ($F = 4.11$, $df = 10/920$, $P < 0.0001$). Comparisons of the temperature and concentration means were made using the "sum of squares simultaneous test procedure" (SS-STP) outlined in Sokal and Rohlf (1981) (Table 11). This test was used because comparisons, other than pairwise comparisons of the means, were examined. These comparisons, indicated that there were no significant differences between the well water and methanol controls for all three temperatures with the 21°C mean being significantly larger than both the 16° or 26°C. However, all concentrations except the 1.8 mg/l concentration at 21°C produced significantly smaller clutches than the 21°C control. The probable reason for the lack of difference between the controls and the 1.8 mg/l concentration was the small sample size ($n = 6$) at this concentration. The means for number of eggs per clutch are graphically displayed in Fig. 6.

The analysis of variance computed for rate of clutch production (number of clutches per female per day) yielded results similar to that of clutch size with significant differences among the temperatures

Table 10. Percent egg hatch for clutches produced by Diaptomus clavipes in the fed and starved feeding regimes at three temperatures

Temperature (°C)	Concentration (mg/l)	n	Feeding regime				
			Fed \bar{x}	SE ^a	n	Starved \bar{x}	SE
16	0.0(WW) ^b	48	0.98	0.01	13	0.99	0.01
	0.0(MC) ^b	56	0.97	0.01	11	0.99	0.01
	0.3	31	0.94	0.03	10	1.00	--
	0.6	33	0.95	0.03	8	1.00	--
	1.0	20	0.95	0.02	10	0.98	0.01
	1.8	6	0.76	0.17	5	1.00	--
21	0.0(WW)	110	0.96	0.01	21	0.91	0.04
	0.0(MC)	112	0.97	0.01	11	0.96	0.02
	0.3	88	0.97	0.01	10	0.98	0.01
	0.6	71	0.95	0.01	10	0.84	0.06
	1.0	46	0.94	0.02	10	0.85	0.05
	1.8	6	0.98	0.01	3	0.76	0.14
26	0.0(WW)	85	0.97	0.01	19	0.99	0.01
	0.0(MC)	94	0.97	0.01	15	0.96	0.02
	0.3	69	0.96	0.01	8	0.98	0.01
	0.6	33	0.95	0.02	13	0.98	0.01
	1.0	28	0.91	0.04	6	1.00	--
	1.8	2	0.67	0.33	6	0.89	0.05

^an=sample size, \bar{x} =mean(mm), SE=standard error (mm)

^bWW=well water control, MC=methanol control

Table 11. A posteriori comparisons of the mean number of eggs per clutch in Diaptomus clavipes for three temperatures in the fed feeding regime using the "sum of squares simultaneous test procedure" (SS-STP)

Comparisons	Temperature (°C)			
	All	16	21	26
Controls (MC vs. WW) ^a	ns	ns	ns	ns
Controls vs. 0.3	**	ns	***	ns
Controls vs. 0.6	***	ns	*	ns
Controls vs. 1.0	*	ns	***	ns
Controls vs. 1.8	ns	ns	ns	ns
Controls (16°C vs. 21°C vs. 26°C)	***	--	--	--
Controls (16°C vs. 21°C, 16°C vs. 26°C)	--	--	***	ns
Controls (21°C vs. 26°C)	--	--	--	***
0.3 (16°C vs. 21°C vs. 26°C)	**	--	--	--
0.3 (16°C vs. 21°, 16°C vs. 26°C)	--	--	*	ns
0.3 (21°C vs. 26°C)	--	--	--	ns
0.6 (16°C vs. 21°C vs. 26°C)	ns	--	--	--
1.0 (16°C vs. 21°C vs. 26°C)	ns	--	--	--
1.8 (16°C vs. 21°C vs. 26°C)	ns	--	--	--

^aWW=well water control, MC=methanol control

ns=not significant at the 0.05 level

*significant at the 0.05 level

**significant at the 0.01 level

***significant at the 0.001 level

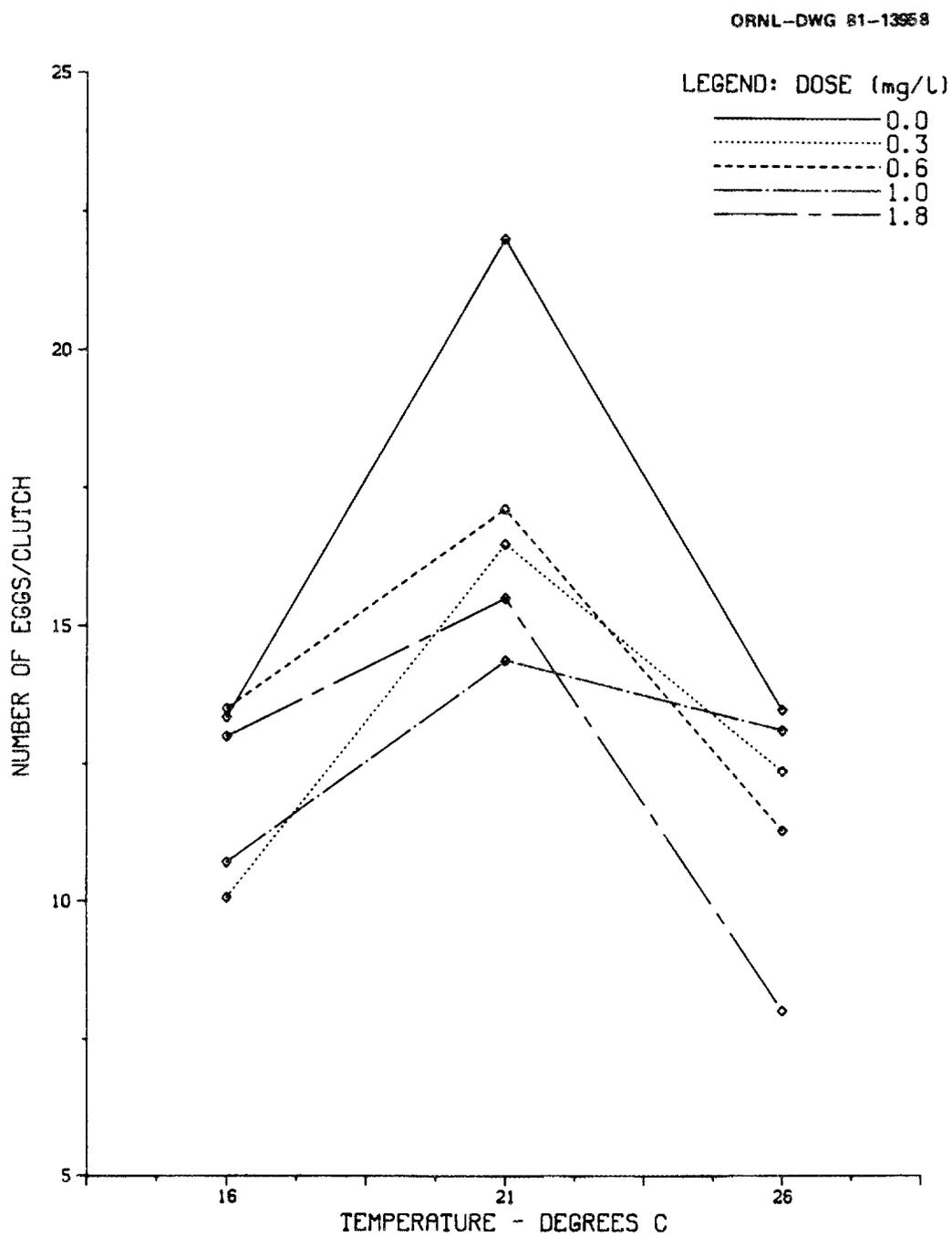


Figure 6. Means for number of eggs per clutch in Diaptomus clavipes at three temperatures in the fed feeding regime.

($F = 80.73$, $df = 2/342$, $P < 0.0001$), concentrations ($F = 104.61$, $df = 5/342$, $P < 0.0001$), and in the interaction term ($F = 6.66$, $df = 10/342$, $P < 0.0001$). Comparisons were made using the SS-STP procedure (Table 12). These comparisons again showed no differences between the well water and methanol controls. However, fewer clutches were produced at 16°C than at either 21° or 26°C in the control groups. At 16°C, only the highest concentration (1.8 mg/l) was significantly different from the control. At 21° and 26°C, there were significant reductions in clutch production at 0.6, 1.0, and 1.8 mg/l concentrations when compared with the controls. There were no differences between the 0.3 mg/l concentration and the controls for all three temperatures. The mean rates of clutch production are graphically displayed in Fig. 7.

The rate of egg production (number of eggs per female per day) utilizes information on both clutch size and rate of clutch production. The analysis of variance again showed significant differences among the temperatures ($F = 118.28$, $df = 2/342$, $P < 0.0001$), concentrations ($F = 75.78$, $df = 5/342$, $P < 0.0001$), and in the interaction term ($F = 10.83$, $df = 10/342$, $P < 0.0001$). Comparisons of the means were made using the SS-STP procedure (Table 13). No differences were detected between the well water and methanol control groups at all three temperatures. The rate of egg production at 21°C in the control group was significantly higher than either the 16° or 26°C means. At 16°C, there were no detectable differences in egg production among the various concentrations when compared to the controls. At 21°C, all

Table 12. A posteriori comparisons of the mean rate of clutch production in Diaptomus clavipes for three temperatures in the fed feeding regime using the "sum of squares simultaneous test procedure" (SS-STP)

Comparisons	Temperature (°C)			
	All	16	21	26
Controls (MC vs. WW) ^a	ns	ns	ns	ns
Controls vs. 0.3	*	ns	ns	ns
Controls vs. 0.6	***	ns	**	***
Controls vs. 1.0	***	ns	***	***
Controls vs. 1.8	***	***	***	***
Controls (16°C vs. 21°C vs. 26°C)	***	--	--	--
Controls (16°C vs. 21°C, 16°C vs. 26°C)	--	--	***	***
Controls (21°C vs. 26°C)	--	--	--	ns
0.3 (16°C vs. 21°C vs. 26°C)	***	--	--	--
0.3 (16°C vs. 21°, 16°C vs. 26°C)	--	--	***	ns
0.3 (21°C vs. 26°C)	--	--	--	ns
0.6 (16°C vs. 21°C vs. 26°C)	*	--	--	--
0.6 (16°C vs. 21°C, 16°C vs. 26°C)	--	--	ns	ns
0.6 (21°C vs. 26°C)	--	--	--	ns
1.0 (16°C vs. 21°C vs. 26°C)	ns	--	--	--
1.8 (16°C vs. 21°C vs. 26°C)	ns	--	--	--

^aWW=well water control, MC=methanol control

ns=not significant at the 0.05 level

*significant at the 0.05 level

**significant at the 0.01 level

***significant at the 0.001 level

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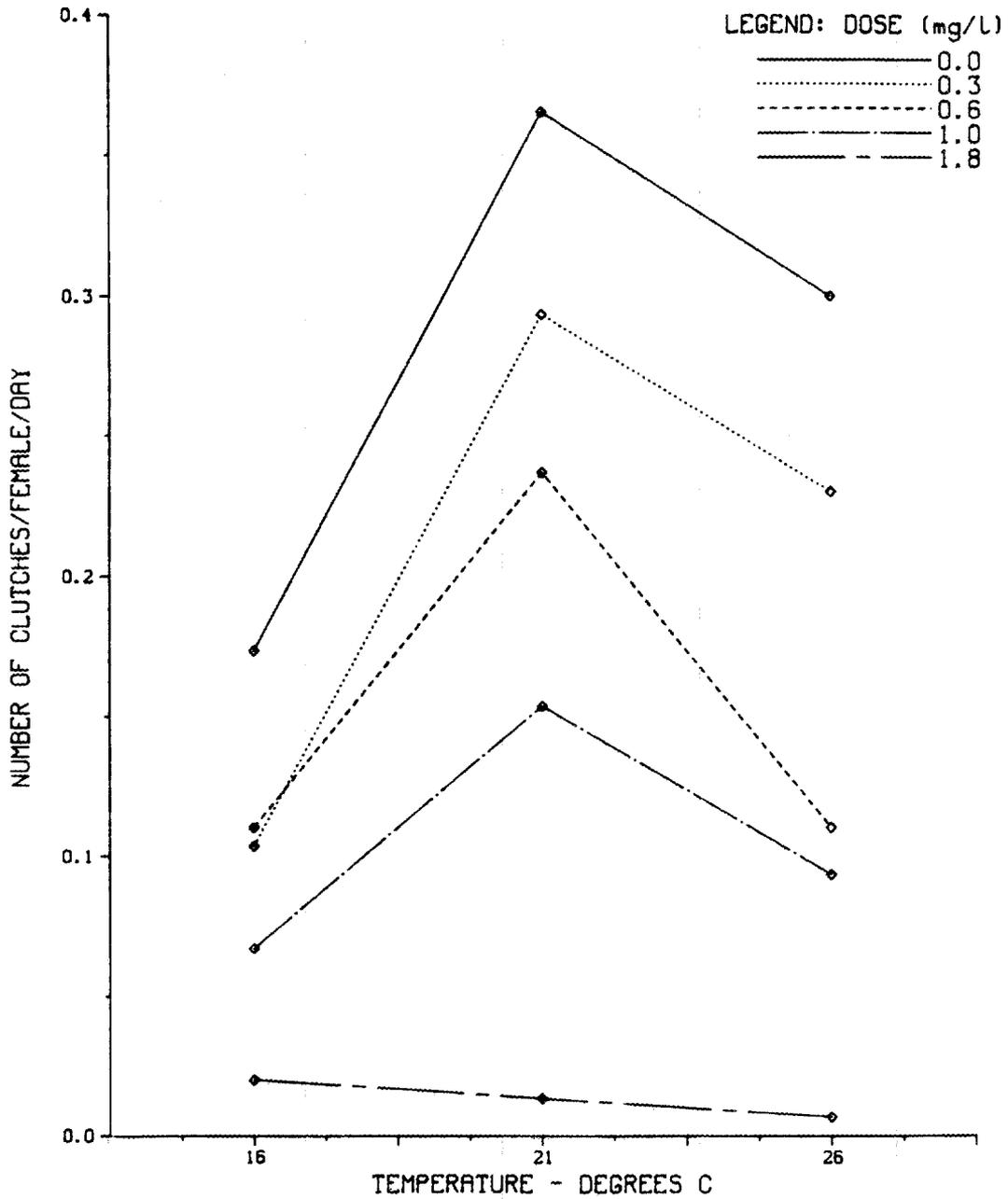


Figure 7. Means for rate of clutch production in Diaptomus clavipes at three temperatures in the fed feeding regime.

Table 13. A posteriori comparisons of the mean rate of egg production in Diaptomus clavipes for three temperatures in the fed feeding regime using the "sum of squares simultaneous test procedure" (SS-STP)

Comparisons	Temperature (°C)			
	All	16	21	26
Controls (MC vs. WW) ^a	ns	ns	ns	ns
Controls vs. 0.3	***	ns	***	ns
Controls vs. 0.6	***	ns	***	**
Controls vs. 1.0	***	ns	***	**
Controls vs. 1.8	***	ns	***	***
Controls (16°C vs. 21°C vs. 26°C)	***	--	--	--
Controls (16°C vs. 21°C, 16°C vs. 26°C)	--	--	***	ns
Controls (21°C vs. 26°C)	--	--	--	***
0.3 (16°C vs. 21°C vs. 26°C)	***	--	--	--
0.3 (16°C vs. 21°C, 16°C vs. 26°C)	--	--	***	ns
0.3 (21°C vs. 26°C)	--	--	--	ns
0.6 (16°C vs. 21°C vs. 26°C)	*	--	--	--
0.6 (16°C vs. 21°C, 16°C vs. 26°C)	--	--	ns	ns
0.6 (21°C vs. 26°C)	--	--	--	ns
1.0 (16°C vs. 21°C vs. 26°C)	ns	--	--	--
1.8 (16°C vs. 21°C vs. 26°C)	ns	--	--	--

^aWW=well water control, MC=methanol control

ns=not significant at the 0.05 level

*significant at the 0.05 level

**significant at the 0.01 level

***significant at the 0.001 level

concentrations differed from their controls. At 26°C, all concentrations except 0.3 mg/l were significantly different from the controls. The mean rates of egg production are graphically displayed in Fig. 8.

In the starved feeding regime, few females produced more than two clutches and many produced only one or none. The analysis of variance for number of eggs per clutch (data transformed using the square root transformation) indicated significant differences among the temperatures ($F = 3.29$, $df = 2/171$, $P < 0.0395$). There were no significant differences among the concentrations ($F = 0.18$, $df = 5/171$, $P > 0.0962$) or in the interaction term ($F = 1.47$, $df = 10/171$, $P > 0.1554$). The three temperature means were compared using Duncan's new multiple range test (Table 14), and showed a mean difference among the groups of about three eggs.

The rate of clutch production in the starved feeding regime was also greatly reduced. The analysis of variance indicated significant differences among the concentrations ($F = 9.23$, $df = 5/342$, $P < 0.0001$) but no differences among the temperatures ($F = 1.16$, $df = 5/342$, $P > 0.3593$), or in the interaction term ($F = 1.16$, $df = 10/342$, $P > 0.3176$). The concentration means were compared using Duncan's new multiple range test (Table 15) and showed that the well water control produced more clutches than all other concentrations and the 1.8 mg/l concentration produced the fewest.

The analysis of variance for rate of egg production revealed results similar to that of the clutch data with the only significant

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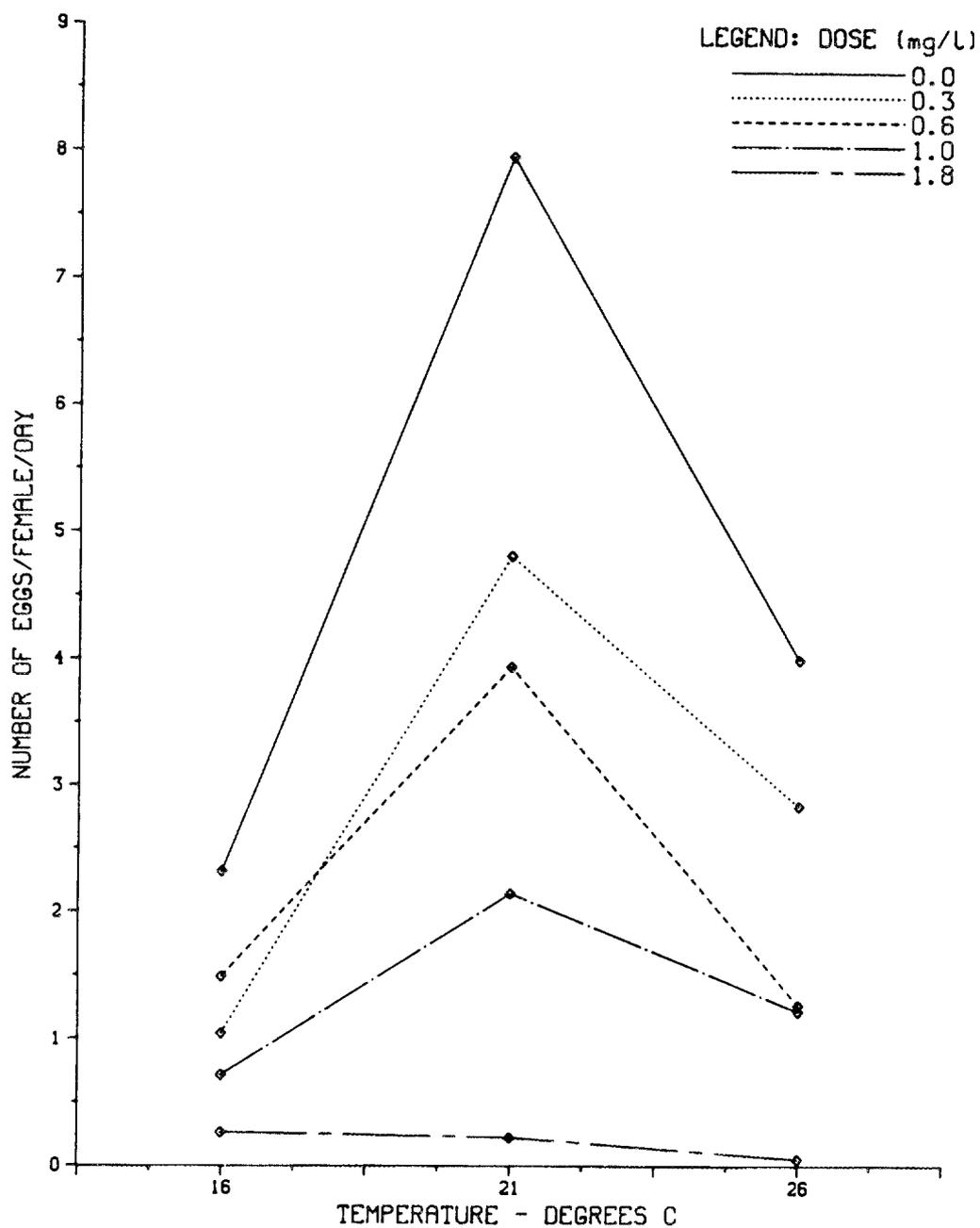


Figure 8. Means for rate of egg production in Diaptomus clavipes at three temperatures in the fed feeding regime.

Table 14. A posteriori comparisons of the mean number of eggs per clutch in Diaptomus clavipes for the starved feeding regime at three temperatures using Duncan's new multiple range test

Temperature (°C)	16	26	21
Sample size	57	67	65
Means ^{a,b}	13.65	11.55	11.08

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level

^bmeans transformed to original units

Table 15. A posteriori comparisons of the mean rate of clutch production in Diaptomus clavipes for the starved feeding regime for four acridine concentrations and two control groups using Duncan's new multiple range test.

Concentration (mg/l)	0.0(WW) ^a	0.0(MC) ^a	1.0	0.6	0.3	1.8
Means ^{b,c}	0.0611	0.0411	0.0311	0.0322	0.0311	0.0156

^aWW=well water control, MC=methanol control

^bmeans enclosed by the range of any one line are not significantly different at the 0.05 level

^csample size=60

differences found among the concentrations ($F = 7.60$, $df = 5/342$, $P < 0.0001$). The comparisons of the means were also similar to the clutch production data (Table 16).

Reproduction in all concentrations at all temperatures was markedly reduced in the starved animals (Table 17) when compared to the fed animals (Table 18). Most control animals only produced, on the average, about one clutch during the fifteen day experimental period. At the higher concentrations most females did not reproduce at all even though they were in the same nutritional state as the controls at the beginning of the study.

The results of this study indicate that the upper level for reproduction at the three temperatures tested was between 1.8 mg/l and 3.2 mg/l acridine concentration. There was no apparent effect on the viability of the eggs produced in the various concentrations of acridine except at 1.8 mg/l, the highest concentration where reproduction occurred. Percent egg hatch at this concentration was less than 90 percent. In most cases, egg viability exceeded 95 percent. Parkhurst et al. (1981) found that first instar Daphnia magna did not survive to the primiparous instar at an acridine concentration of 3.2 mg/l at a temperature of 20°C. However, at all concentrations below this, animals survived to the primiparous instar and reproduced, although the time to reach this instar was increased at the higher concentrations.

The three measures of reproduction used in this study, clutch size, rate of clutch production, and rate of egg production, differed in their sensitivity at the three temperatures. Clutch size did not

Table 16. A posteriori comparisons of the mean rate of egg production in Diaptomus clavipes for the starved feeding regime for four acridine concentrations and two control groups using Duncan's new multiple range test

Concentration (mg/l)	0.0(WW) ^a	0.0(MC) ^a	1.0	0.6	0.3	1.8
Means ^{b,c}	0.7778	0.5233	0.5078	0.4200	0.3744	0.1844

^aWW=well water control, MC=methanol control

^bmeans enclosed by the range of any one line are not significantly different at the 0.05 level of significance

^csample size=60

Table 17. Means for number of eggs per clutch, rate of clutch production, and rate of egg production, in *Diaptomus clavipes* in the starved feeding regime for the reproduction toxicity test

Temperature (°C)	Concentration (mg/l)	Clutch Size			Rate of Clutch Production		Rate of Egg Production	
		n	\bar{x}	SE ^a	\bar{x}^b	SE	\bar{x}^b	SE
16	0.0(WW) ^c	13	12.92	1.42	0.0467	0.0070	0.69	0.09
	0.0(MC) ^c	11	14.18	1.68	0.0367	0.0090	0.53	0.14
	0.3	10	13.00	3.39	0.0333	0.0090	0.43	0.14
	0.6	8	11.50	1.48	0.0233	0.0100	0.31	0.12
	1.0	10	20.10	1.90	0.0333	0.0090	0.91	0.19
	1.8	5	14.20	1.71	0.0167	0.0066	0.24	0.10
21	0.0(WW)	21	12.10	1.60	0.0700	0.0102	0.78	0.15
	0.0(MC)	11	13.27	1.24	0.0367	0.0076	0.47	0.11
	0.3	10	10.40	1.34	0.0333	0.0090	0.34	0.12
	0.6	10	12.50	1.76	0.0333	0.0076	0.36	0.10
	1.0	10	10.50	1.53	0.0433	0.0087	0.41	0.11
	1.8	3	8.00	1.15	0.0100	0.0055	0.06	0.03
26	0.0(WW)	19	13.00	1.96	0.0667	0.0108	0.86	0.15
	0.0(MC)	15	11.47	1.78	0.0500	0.0082	0.57	0.12
	0.3	8	13.25	1.92	0.0267	0.0089	0.35	0.13
	0.6	13	13.54	1.71	0.0433	0.0087	0.59	0.14
	1.0	6	10.00	2.32	0.0200	0.0070	0.20	0.08
	1.8	6	12.83	2.68	0.0200	0.0085	0.26	0.11

^an=sample size, \bar{x} =mean, SE=standard error

^bsample size=20

^cWW=well water control, MC=methanol control

Table 18. Means for number of eggs per clutch, rate of clutch production, and rate of egg production in *Diatomus clavipes* in the fed feeding regime for the reproduction toxicity test

Temperature (°C)	Concentration (mg/l)	Clutch Size			Rate of Clutch Production		Rate of Egg Production	
		n	\bar{x}	SE ^a	\bar{x}^b	SE	\bar{x}^b	SE
16	0.0(WW) ^c	48	14.23	0.69	0.1600	0.0089	2.28	0.15
	0.0(MC) ^c	56	12.59	0.66	0.1867	0.0104	2.35	0.22
	0.3	31	10.06	0.73	0.1033	0.0164	1.04	0.17
	0.6	33	13.52	0.83	0.1100	0.0176	1.49	0.26
	1.0	20	10.70	0.84	0.0667	0.0108	0.71	0.12
	1.8	6	13.00	1.06	0.0200	0.0070	0.26	0.09
21	0.0(WW)	110	21.15	0.70	0.3633	0.0280	7.63	0.57
	0.0(MC)	112	22.80	0.89	0.3667	0.0219	8.28	0.81
	0.3	88	16.48	0.68	0.2933	0.0170	4.81	0.44
	0.6	71	17.11	0.81	0.2367	0.0244	3.95	0.44
	1.0	46	14.37	0.85	0.1533	0.0188	2.14	0.28
	1.8	6	15.50	1.73	0.0133	0.0078	0.22	0.12
26	0.0(WW)	85	13.89	0.63	0.2867	0.0252	3.90	0.63
	0.0(MC)	94	13.10	0.51	0.3133	0.0200	4.11	0.34
	0.3	69	12.36	0.57	0.2300	0.0220	2.84	0.34
	0.6	33	11.27	0.81	0.1100	0.0147	1.27	0.20
	1.0	28	13.11	0.84	0.0933	0.0189	1.22	0.27
	1.8	2	8.00	2.00	0.0067	0.0067	0.05	0.05

^an=sample size, \bar{x} =mean, SE=standard error

^bsample size=20

^cWW=well water control, MC=methanol control

vary among the concentrations at two of the three temperatures (16° and 26°C). At 21°C all clutches produced in the various acridine concentrations showed significant reductions when compared to the control. Parkhurst et al. (1981) found a significant increase in clutch size and number of clutches per female (approximately 15-20 percent) in acridine concentrations ranging from 0.1 to 0.4 mg/l when compared to the controls. Reductions in clutch size occurred at higher concentrations. There was no stimulatory effect on clutch size or rate of clutch production in this study.

Rate of clutch production was a more sensitive indicator of toxicant stress than clutch size. The rate of egg production is not an independent measure of reproduction since it utilizes both the data on clutch size and clutch production. In fact, it yielded essentially the same results as the clutch production data. The only major difference was at 16°C in the 1.8 mg/l concentration where there was a significant reduction in clutch production but not in egg production.

The general pattern of reproduction in this study was an increase from 16°C to 21°C followed by a decrease from 21°C to 26°C. The values for the reproduction parameters for controls at 21°C were similar to the optimum values found by Cooney et al. (1978) in a laboratory study of reproduction in Diaptomus clavipes. Hardin (1972) found difficulty culturing an Oklahoma population of Diaptomus clavipes at 14°C due to lack of reproduction. Hardin also found that over a temperature range of 14°-31°C, long-term culturing was most successful at 21°C. This conclusion was based on total estimated production of nauplii per female which was higher at 21°C than all other temperatures. Gehrs

(1972) found in a field study in the same population of Diaptomus clavipes that the smallest clutches occurred in the summer when the temperatures averaged 25°C and the largest clutches were found at lower temperatures. In an Arizona population of Diaptomus clavipes, Kamps (1979) found that clutch size decreased at temperatures above 25°C regardless of food conditions. Goss (1978) found maximums for clutch size in the cladocerans Daphnia magna and Daphnia pulex at approximately 20°C with significant reductions above and below this temperature. Gehrs (1972) found a similar pattern in specific birth rate values (m_x) for Diaptomus clavipes. Values rose from February to April and then declined rapidly in May. The m_x values fluctuated irregularly below the yearly average ($m_x^- = 2.78$ eggs/adult/day) throughout the remainder of the year. The water temperature was below 20°C during the entire ascending phase.

Increased temperature usually means increased metabolic rates and increased sensitivity to toxicants (Buikema et al. 1980). Exceptions do occur, however (Cairns et al. 1975a,b, 1978). Reproduction in this study was not affected by acridine at 16°C whereas at 21° and 26°C, rates of egg and clutch production were significantly reduced. There were no significant increases in toxicity from 21° and 26°C.

CHAPTER V

ACUTE EGG AND NAUPLIAR BIOASSAYS

During this study, two experiments were performed to evaluate the effects of a toxicant stress on the mortality and development times of eggs and nauplii at the three temperatures tested. In the first experiment, eggs which had been produced in well water and then exposed to various acridine concentrations were allowed to hatch and molt into the second naupliar stage (NII) before the experiment ended. A chi square statistic was computed for each temperature, in order to determine if the relative proportions of clutch sizes varied among the acridine concentrations. There were no significant differences ($P > 0.05$) among the concentrations for any of the temperatures. A one way analysis of variance was then computed for number of eggs per clutch (data transformed using the square root transformation) with the data pooled over concentrations. The results indicated that there were significant differences among the temperatures ($F = 33.41$, $df = 2/237$, $P < 0.0001$). Table 19 shows the a posteriori comparisons of the mean number of eggs per clutch for the three temperatures. All three means were significantly different from each other with the largest clutches produced at 21°C. These results are similar to the control data in the reproduction bioassay.

A 3 x 8 factorial analysis of variance for egg development time revealed similar results. There were significant differences among the temperatures ($F = 3723.03$, $df = 2/216$, $P < 0.0001$) but no differences among the concentrations ($F = 1.41$, $df = 7/216$, $P > 0.2009$) or in the

Table 19. A posteriori comparisons of the mean number of eggs per clutch produced by Diaptomus clavipes at three temperatures using Duncan's new multiple range test

Temperature (°C)	21	26	16
Means ^{a,b,c}	19.65	13.97	10.79

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level.

^bmeans transformed to original units

^csample size=80

interaction term ($F = 0.69$, $df = 14/216$, $P > 0.7805$). This indicates that egg development times were not affected by the various concentrations. Table 20 shows the a posteriori comparisons of the mean egg development times with the data combined over the eight concentrations. All three means were significantly different from each other with development times increasing with decreasing temperatures.

A 3 x 7 factorial analysis of variance was computed for development time from the first naupliar stage (NI) to the second naupliar stage (NII) (data transformed using a logarithmic transformation). There were significant differences among the temperatures ($F = 563.52$, $df = 2/182$, $P < 0.0001$) and concentrations ($F = 3.73$, $df = 6/182$, $P < 0.0016$) but no significant interaction ($F = 1.28$, $df = 12/182$, $P > 0.2362$). No nauplii molted into the second naupliar stage at an acridine concentrations of 5.6 mg/l, therefore, this concentration was not included in the analysis. Table 21 shows the a posteriori comparisons of the mean naupliar (NI-NII) development times. All three temperatures were significantly different from each other with development times increasing with decreasing temperature. At 16°C the controls (methanol and well water) had significantly shorter development times than the five acridine concentrations. There were no detectable differences among the concentrations for either 21°C or 26°C.

Egg viability exceeded 90 percent for all temperature and concentration combinations. Table 22 gives means for percent egg hatch, egg development times and naupliar (NI-NII) development times.

Table 20. A posteriori comparisons of the mean development times of eggs produced by Diaptomus clavipes at three temperatures using Duncan's new multiple range test

Temperature (°C)	16	21	26
Means (hours) ^{a,b}	84.65	52.35	36.39

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level

^bsample size=80

Table 21. A posteriori comparisons of mean development time from the first naupliar stage to the second naupliar stage in Diaptomus clavipes at three temperatures subjected to seven treatments

Temperature (°C)	Concentration (mg/l)	Sample size	Means ^a (hours)
16	1.8	10	16.93
16	3.2	4	16.88
16	0.6	10	16.33
16	1.0	10	15.80
16	0.3	10	15.65
16	0.0(WW) ^b	10	14.13
16	0.0(MC) ^b	10	13.50
21	1.8	10	10.75
21	3.2	9	10.61
21	1.0	10	10.55
21	0.3	10	10.45
21	0.0(WW)	10	10.10
21	0.6	10	9.95
21	0.0(MC)	10	9.95
26	1.8	10	7.85
26	1.0	10	7.25
26	0.0(MC)	10	7.05
26	0.3	10	7.00
26	0.6	10	6.85
26	3.2	10	6.80
26	0.0(WW)	10	6.65

^aMeans enclosed by the range of any one line are not significantly different at the 0.05 level

^bWW=well water control, MC=methanol control

Table 22. Means for percent egg hatch, egg development time, and naupliar (NI-NII) development time for clutches produced by Diaptomus clavipes in the acute egg bioassay (sample size=10 except where noted)

Concentration (mg/l)	Percent Egg Hatch		Egg development Time (hours)		Naupliar development Time (hours)	
	\bar{x}	SE ^a	\bar{x}	SE	\bar{x}	SE
16°C						
0.0 (WW) ^b	0.94	0.04	83.23	1.57	14.13	0.63
0.0 (MC) ^b	0.94	0.03	83.83	1.35	13.50	0.57
0.3	0.96	0.04	81.88	1.55	15.65	0.17
0.6	0.88	0.06	84.48	1.38	16.33	0.19
1.0	0.92	0.04	85.68	1.64	15.80	0.29
1.8	0.94	0.03	87.23	1.85	16.93	0.44
3.2	0.91	0.04	85.43	0.99	16.88	0.43 ^c
5.6	0.93	0.05	85.50	1.03	--	--
21°C						
0.0 (WW)	0.98	0.01	51.33	1.35	10.10	0.18
0.0 (MC)	0.97	0.02	51.78	1.27	9.95	0.24
0.3	0.99	0.01	51.98	0.91	10.45	0.40
0.6	0.99	0.01	51.78	1.11	9.95	0.16
1.0	0.95	0.03	53.85	1.56	10.55	0.30
1.8	0.95	0.04	52.35	1.29	10.75	0.26
3.2	0.95	0.02	52.55	1.05	10.61	0.44 ^d
5.6	0.93	0.02	53.20	1.34	--	--
26°C						
0.0 (WW)	0.97	0.02	36.05	0.52	6.65	0.27
0.0 (MC)	0.93	0.02	36.77	0.59	7.05	0.32
0.3	0.95	0.02	36.40	0.59	7.00	0.33
0.6	0.95	0.02	36.35	0.34	6.85	0.61
1.0	0.98	0.02	37.05	0.52	7.25	0.47
1.8	0.98	0.02	36.13	0.49	7.85	0.56
3.2	0.99	0.01	35.83	0.47	6.80	0.37
5.6	0.96	0.02	36.58	0.35	--	--

^a \bar{x} =mean, SE=standard error

^bWW=well water control, MC=methanol control

^csample size=4

^dsample size=6

Estimates of LC50's were obtained using the maximum likelihood estimates of probit analysis and the Spearman-Kärber method (Finney 1978) (Table 23). Estimates were obtained for each test and then a single estimate for each temperature based on a combined data set. The cumulative egg and naupliar mortality was used in this analysis. All six probit regression lines (two lines for each temperature) were parallel (equal slopes). There were no significant differences in relative potency ($P > 0.05$) between the two tests for each temperature, therefore the data for the tests were combined and reanalyzed. Table 23 also gives the relative potency values.

The estimates of LC50's obtained from both the probit analysis and Spearman-Kärber method were within 0.66 mg/l of each other. The 95 percent fiducial limits did vary however with the probit method producing the larger fiducial limits. The reason for these large limits is due to the heterogeneity of the data and lack of fit of the data to the probit model. In this case, the variances were multiplied by a heterogeneity factor (Finney 1978) before calculating the limits.

The Spearman-Kärber method is model free and therefore does not have to be corrected for heterogeneity thus yielding smaller fiducial limits (Hamilton et al. 1977). Stephen (1977) suggested that if a reliable estimate of an LC50 was to be obtained using parametric methods such as the probit or logit, it was necessary to have at least one partial kill reasonably close to 50 percent, such as between 16 and 84 percent. At 16°C, three concentrations were within this range. At 21° and 26°C, only one concentration was within this range. However,

Table 23. Summary of LC50 values and relative potency values using Diaptomus clavipes for acute egg and naupliar tests estimated by the method of probit analysis and the Spearman-Kärber method

Test	LC50 (mg/l)	Probit Method 95% Fiducial Limits		LC50 (mg/l)	Spearman-Kärber Method 95% Fiducial Limits	
		Upper	Lower		Upper	Lower
16°C						
1	1.43	3.29	0.62	1.46	1.68	1.28
2	1.80	4.31	0.83	1.70	1.98	1.47
Pooled	1.61	3.28	0.86	1.59	1.75	1.44
21°C						
1	4.25	9.91	2.12	3.60	3.96	3.26
2	3.94	7.73	2.36	3.30	3.50	3.10
Pooled	4.07	7.98	2.55	3.44	3.62	3.26
26°C						
1	5.34	13.70	2.64	4.27	4.73	3.86
2	3.20	7.75	1.50	3.00	3.44	2.62
Pooled	4.26	9.62	2.40	3.60	3.91	3.31
Relative Potency (LC50 Ratios)						
	21/16=2.52*		26/16=2.64*		26/21=1.05 ^{ns}	

*significant at the 0.05 level

^{ns}not significant at the 0.05 level

the probit method did accurately predict the LC50's when compared to the Spearman-Kärber method or to the observed proportion dead at each concentration in the raw data.

In the second experiment, survivorship to the first copepodid stage (CI) was measured under fed and starved feeding conditions. Under the starved feeding regime, survivorship to the first copepodid stage was low with usually, only the control group animals surviving (Table 24). Survival under fed conditions was higher with the 16°C and 21°C controls exceeding 90 percent (Table 25). Control survivorship to 96 hours in both fed and starved feeding regimes at 16°C and 21°C was 94 percent, however at 26°C it was only 70 percent.

In the starved feeding regime, median naupliar survival time was more than twice the average time for a fed animal to reach the first copepodid stage indicating a slowing in development. This was also indicated when the development time to CI for controls were compared between the fed and the starved feeding regimes especially at 16°C.

Few animals in either feeding regime survived to CI at concentrations of 1.8 mg/l or higher. Those animals that survived to CI at these concentrations died within 24 h and did not molt to the second copepodid stage (CII).

Estimates of LC50's based on the Spearman-Kärber method were obtained for the fed group with the data corrected for control mortality using Abbott's formula (Finney 1971). Cumulative mortality to CI was the observation. All values were within the range of 1.0 to 2.0 mg/l (Table 26). Due to the high mortality in the starved group, no estimates of lethality were made.

Table 24. Median survival time, development time (NI-CI), and survivorship (NI-CI) for Diaptomus clavipes in the starved feeding regime

Concentration (mg/l)	Median Survival Time (Days)	Development time (NI-CI) (Days)			Survivorship (NI-CI)
		n	\bar{x}	SE ^a	
16°C					
0.0(WW) ^b	14	6	15.67	0.25	0.24 ^c
0.0(MC) ^b	16	7	10.57	1.41	0.28 ^c
0.3	10	1	13.00	--	0.04 ^c
0.6	13	--	--	--	0.00
1.0	13	1	11.00	--	0.04 ^c
1.8	13	--	--	--	0.00
3.2	11	--	--	--	0.00
5.6	1	--	--	--	0.00
21°C					
0.0(WW)	7	5	5.60	0.40	0.20 ^c
0.0(MC)	9	12	7.08	0.08	0.48 ^c
0.3	8	--	--	--	0.00
0.6	8	--	--	--	0.00
1.0	8	--	--	--	0.00
1.8	7	--	--	--	0.00
3.2	8	--	--	--	0.00
5.6	3	--	--	--	0.00
26°C					
0.0(WW)	5	3	7.00	0.29	0.12 ^c
0.0(MC)	6	--	--	--	0.00
0.3	6	1	6.00	--	0.04 ^c
0.6	6	--	--	--	0.00
1.0	2	--	--	--	0.00
1.8	5	--	--	--	0.00
3.2	5	--	--	--	0.00
5.6	3	--	--	--	0.00

^an=sample size, \bar{x} =mean, SE=standard error

^bWW=well water control, MC=methanol control

^cno animals molted to CII

Table 25. Median survival time, development time (NI-CI), and survivorship (NI-CI) for *Diaptomus clavipes* in the fed feeding regime

Concentration (mg/l)	Median Survival Time (Days)	Development time (NI-CI) (Days)			Survivorship (NI-CI)
		n	\bar{x}	SE ^a	
16°C					
0.0(WW) ^b	--	25	7.56	0.68	1.00
0.0(MC) ^b	--	21	7.24	0.72	0.84
0.3	--	22	7.73	0.26	0.88
0.6	--	25	8.28	0.40	1.00
1.0	--	24	7.79	0.34	0.96
1.8	16	7	8.86	1.14	0.28 ^c
3.2	12	--	--	--	0.00
5.6	2	--	--	--	0.00
21°C					
0.0(WW)	--	23	4.48	0.15	0.92
0.0(MC)	--	22	4.64	0.17	0.88
0.3	--	23	4.43	0.12	0.92
0.6	--	23	4.65	0.18	0.92
1.0	--	21	4.57	0.16	0.84
1.8	8	10	5.80	0.44	0.40 ^c
3.2	5	2	7.00	0.00	0.08 ^c
5.6	7	--	--	--	0.00
26°C					
0.0(WW)	--	16	4.06	0.30	0.64
0.0(MC)	--	16	4.94	0.30	0.64
0.3	--	15	4.27	0.25	0.60
0.6	10	11	5.91	0.53	0.44
1.0	6	7	5.57	0.43	0.28
1.8	6	11	5.55	0.21	0.44 ^c
3.2	6	3	3.00	0.00	0.12 ^c
5.6	2	--	--	--	0.00

^an=sample size, \bar{x} =mean, SE=standard error

^bWW=well water control, MC=methanol control

^cno animals molted to CII

Table 26. Summary of LC50 values based on mortality from NI to CI in Diaptomus clavipes estimated by the Spearman-Kärber method for the fed feeding regime at three temperatures

Temperature (°C)	LC50 (mg/l)	95% Fiducial Upper	Limits Lower
16	1.55	1.74	1.38
21	1.75	2.02	1.52
26	1.18	1.47	0.95

The data collected in these two experiments indicate that naupliar development was affected by exposure to acridine. Egg development times were not affected by acridine exposure but were strongly affected by temperature with increasing development times associated with decreasing temperature. This relationship in calanoid copepods has been cited by several authors (e.g., Hutchinson 1967, Hardin 1972, Bunting 1974, Kamps 1979, Elmore 1980). This trend was also apparent in the development from NI-NII and NI-CI. At 16°C, however, acridine concentration appears to interact with temperature to produce longer naupliar development times at the higher concentrations.

Development times from NI-CI were found to be prolonged in the starved feeding regime. Coker (1934) cites experiments which showed a prolonging of development in Cyclops vernalis to five or six times the 'normal' rate by starving the animals. One animal in Coker's study survived for many weeks in the fourth copepodid stage. This was similar to the situation in this study where some nauplii at 16°C survived for 17 days without molting to the CI stage. If an animal did survive to CI in the starved group, it invariably died without molting into the second copepodid stage (CII).

Egg viability was unaffected by acridine concentration. Hatching in freshwater calanoid copepods is similar to that of freshwater cyclopoids (Davis 1959) and marine copepods (Marshall and Orr 1954). Most copepods hatch by the osmotic swelling of a non-living inner egg membrane. Davis (1959) found that in Diaptomus, the inner membrane changes permeability over a short period of time, whereupon water enters osmotically. The pressure exerted by the swelling of the inner

membrane is sufficient to rupture the thicker outer membrane (chorion) and the former, enclosing the still inactive nauplius, slips out of the chorion. Ultimately the inner membrane also bursts from the internal pressure and the nauplius is thrown free. This usually takes less than 10 minutes. The eggs are impermeable to water before this time. At the 5.6 mg/l concentration, some nauplii were found dead, still within the inner membrane although in most cases they were able to break free of the membrane. Initial experiments were performed at the 7.5 and 10 mg/l concentrations. Development proceeded normally until the outer chorion was ruptured and the inner membrane surrounding the nauplius was extruded. At this point mortality was very high. If an animal succeeded in complete hatching it usually died within five minutes. If the eggs were allowed to hatch in well water and then placed in these concentrations, some animals survived for 24-30 hours. This was also noted in the 5.6 mg/l concentration. Nauplii which hatched in the toxicant did not molt into NII and most were dead or immobile within a few hours. However, 0-4 hour old NI, when placed in this concentration lived for a much longer time, with some nauplii, surviving for 11 days. Although, there was greater than 90 percent egg hatch, the hatching nauplii may have been sufficiently stressed to the point of affecting their further development. Walton (1980) reached similar conclusions on the effects of acridine on egg viability in chronic studies with the cricket, Acheta domesticus. Acute tests indicated that eggs were more sensitive than either nymphs or adults (Table 7).

Eggs were placed in the toxicant after approximately one-half of their development and at the stage where they were impermeable to water. This would coincide with the stage after gastrulation when the limb rudiments are forming (Marshall and Orr 1955). No aberrations were observed in egg development, suggesting no toxic effects up till the time of hatching. Birge and Black (1981) found that embryo-larval stages of rainbow trout (Salmo gairdneri) and bluegill sunfish (Lepomis macrochirus) were very sensitive to a variety of coal derived organic compounds, including acridine (reagent grade) (Table 7). Teratogenesis was observed in rainbow trout at 0.41 mg/l (21 percent of hatched population) whereas, in the bluegill sunfish, significant embryopathic effects occurred at a concentration of 10.1 mg/l. Davis et al. (1981) also found that acridine had teratogenic effects on embryos (mid-blastula) of the amphibian Xenopus laevis (96 h-EC50 = 2.4 mg/l). Walton (1981) found that impurities in reagent grade acridine produced teratogenic activity in cricket embryos (Acheta domesticus). Purified acridine resulted in complete loss of teratogenic activity. Since Birge and Black (1981) and Davis et al. (1981) used reagent grade acridine, from the same chemical company (Aldrich) as in Walton's study, it is possible that the teratogenic activity they observed was due to the impurities in the acridine.

Another critical period in egg development is the time when the eggs are fertilized and extruded into the egg sac. Grobben (1881, cited by Marshall and Orr 1955) noted that newly laid eggs in the marine calanoid copepod, Calanus sp., had no membranes but both

membranes developed after the egg became spherical, within five minutes. Ramult (1925) exposed eggs of Daphnia pulex to a variety of salt solutions immediately after they were produced and before the egg membranes were formed. Ramult found that development was suppressed if eggs were exposed before the membranes were formed. However, after the egg membranes were formed, exposed eggs would develop normally till hatching. Davis (1959) found that eggs of Diaptomus ashlandi maintained at 21°C were impermeable to water within two hours after laying. Cooney and Gehrs (1980) found that egg sacs of Diaptomus clavipes could not be removed from females for at least four hours after laying because the chorion was not sufficiently hardened and the eggs would disintegrate when touched. Eggs produced in the toxicant were examined in the next section.

CHAPTER VI

CHRONIC BIOASSAYS

In this experiment, eggs from clutches produced by females in various concentrations of acridine were measured (see Materials and Methods for measurement techniques) and then allowed to develop into adults. A subsample of the developing animals was measured when they had molted to the first copepodid stage. No egg clutches were produced at an acridine concentration of 3.2 mg/l for any of the three temperatures tested. At 1.8 mg/l acridine concentration, egg clutches were produced only at 26°C.

Development times for egg clutches produced at the three temperatures in various concentrations of acridine yielded results similar to those of the acute egg bioassay. Mean development times within a temperature varied by less than two hours, thus indicating no relationship between egg development time and acridine concentration (Table 27).

A subsample of first stage copepodids (CI) was measured at the various temperatures and acridine concentrations. Stepwise multiple regression techniques were used to determine if there were any relationships between copepodid size and egg size (maximum egg length or egg volume), temperature or acridine concentration. The results indicate that there was a significant relationship between copepodid size and egg size ($P < 0.0001$) and temperature ($P < 0.0001$) but not with acridine concentration ($P > 0.15$). Therefore, acridine

Table 27. Means for number of eggs per clutch, and egg development times for clutches produced by Diaptomus clavipes in four concentrations of acridine and two controls at three temperatures in the chronic tests

Concentration (mg/l)	n	Clutch Size \bar{x}	SE ^a	Egg Development Times (hours) \bar{x}	SE
16°C					
0.0(WW) ^b	6	11.00	2.14	85.33	0.83
0.0(MC) ^b	6	9.17	1.19	85.33	1.13
0.3	6	9.00	1.00	84.29	2.04
0.6	5	16.60	2.98	84.40	1.08
1.0	5	21.60	3.09	83.15	0.75
21°C					
0.0(WW)	5	25.20	1.46	52.40	1.53
0.0(MC)	5	18.40	1.29	53.90	1.01
0.3	5	16.20	1.96	52.50	0.95
0.6	7	15.85	2.26	52.89	1.17
1.0	5	18.20	1.59	52.00	1.26
26°C					
0.0(WW)	7	9.00	2.22	37.07	0.24
0.0(MC)	7	8.14	1.14	37.00	0.65
0.3	6	9.50	0.76	37.50	0.94
0.6	7	10.29	1.67	36.43	0.49
1.0	5	16.60	4.87	37.60	0.62
1.8	7	13.57	2.52	36.68	0.49

^an=sample size, \bar{x} =mean, SE=standard error

^bWW=well water control, MC=methanol control

concentration was dropped as a factor. The resulting multiple regression equations are:

$$\text{Copepodid length} = 0.3110 + 0.8002 \times (\text{egg length})$$

$$-0.0016 \times (\text{temperature}) \quad R^2 = 0.35$$

$$\text{Copepodid length} = 0.3918 + 23.6227 \times (\text{egg volume})$$

$$-0.0015 \times (\text{temperature}) \quad R^2 = 0.36$$

The results of this analysis suggests that there is a direct relationship between copepodid size and both measures of egg size (maximum egg length and egg volume) and an inverse relationship with temperature. The means for egg size, copepodid size, and adult size can be found in Table 28.

Survivorship to the first copepodid stage (CI) and the adult stage (CVI) are given in Table 29. At 26°C, mortality was high at all concentrations with only approximately 10 percent of the controls surviving to the adult stage. All surviving adults were males. Since there were no survivors in any of the acridine concentrations, the data for this temperature were not included in this analysis. Control mortality at both 16° and 21°C was less than 20 percent.

Comparisons of naupliar (NI-NVI) and copepodid (CI-CVI) development times were made between the controls and the various acridine treated animals for each temperature. At 16°C, the mean difference was less than 2 percent for the naupliar stages and 20 percent for the copepodid stages. At 21°C, the mean differences in development times between controls and treated animals were greater. The naupliar stages were 21 percent longer for the treated animals

Table 28. Summary of means for maximum egg length, egg volume, and metasomal length of first stage copepodids (CI) and adult males and females (CVI) for Diaptomus clavipes at three temperatures, pooled over the acridine concentrations and control groups.

Temperature (°C)	Maximum egg length (mm)			Egg volume (mm) ³			CI metasomal length (mm)			CVI metasomal length (mm)					
	n	\bar{x}	SE ^a	n	$\bar{x}(x10^{-3})$	SE($x10^{-3}$)	n	\bar{x}	SE	Males			Females		
										n	\bar{x}	SE	n	\bar{x}	SE
16	130	0.1653	0.0017	130	2.1310	0.0001	130	0.4200	0.0027	91	1.30	0.004	103	1.46	0.005
21	132	0.1586	0.0020	132	1.8431	0.0001	132	0.4011	0.0031	75	1.26	0.005	81	1.43	0.008
26	120	0.1592	0.0025	120	1.8969	0.0001	120	0.3991	0.0041	9	1.19	0.010	-	-	-

^an = sample size, \bar{x} = mean, SE = standard error.

Table 29. Cumulative survivorship of Diaptomus clavipes to the first copepodid stage (CI) and to the adult stage (CVI) for four concentrations of acridine and two controls at three temperatures

Concentration (mg/l)	Temperature (°C)					
	16		21		26	
	CI	CVI	CI	CVI	CI	CVI
0.0(WW) ^a	1.00	0.94	0.96	0.82	0.56	0.10
0.0(MC) ^a	0.98	0.88	1.00	0.92	0.60	0.08
0.3	0.96	0.86	0.90	0.54	0.34	0.00
0.6	1.00	0.90	0.88	0.52	0.64	0.00
1.0	0.88	0.30	0.82	0.32	0.24	0.00
1.8	--	--	--	--	0.18	0.00

^aWW=well water control, MC=methanol control

while the copepodid stages in treated animals averaged 61 percent longer than the controls. This indicated that the time spent in the various immature stages was increased with exposure to acridine. The copepodid stages were more affected than the naupliar stages, especially at 21°C.

A 2 x 5 factorial analysis of variance was computed for each temperature using development times (NI-CVI) as the observations (data transformed using a logarithmic transformation). The two fixed factors were sex and acridine concentration. At 16°C, there were significant differences between the sexes ($F = 10.28$, $df = 1/184$, $P < 0.0016$) and among the acridine concentrations ($F = 144.16$, $df = 4/184$, $P < 0.0001$) but no significant interaction ($F = 1.89$, $df = 4/184$, $P > 0.1137$). At 21°C, similar results were reached with significant differences between the sexes ($F = 7.37$, $df = 1/146$, $P < 0.0074$) and among the acridine concentrations ($F = 108.47$, $df = 4/146$, $P < 0.0001$) but no interaction ($F = 0.55$, $df = 4/146$, $P > 0.6969$). The a posteriori comparisons of the mean development times (NI-CVI) for both 16° and 21°C are given in Table 30. No significant differences could be found between the sexes at any concentrations except for the control data at 16°C. Males had significantly shorter development times than females for both control groups. The controls, as a group, had faster development times than acridine treated animals at both temperatures. This was most apparent in the fourth and fifth copepodid stages (CIV and CV) (Figures 9 and 10). During this experiment, animals were transferred from the tissue culture plates to much larger shell vials when they had molted from the CIV stage to the CV stage. A

Table 30. A posteriori comparisons of mean development times (NI-CVI) for Diaptomus clavipes at 16°C and 21°C using Duncan's new multiple range test

Concentration (mg/l)	Sex	Sample Size	Mean ^{a,b} (Days)
16°C			
1.0	Female	8	58.05
0.6	Female	25	57.92
1.0	Male	7	57.35
0.6	Male	20	57.00
0.3	Female	24	55.39
0.3	Male	19	55.31
0.0 (WW) ^c	Female	25	50.02
0.0 (MC) ^c	Female	21	49.14
0.0 (WW)	Male	22	48.28
0.0 (MC)	Male	23	46.74
21°C			
1.0	Female	7	49.75
0.6	Female	14	47.28
0.3	Female	17	47.18
0.3	Male	10	46.34
0.6	Male	12	46.20
1.0	Male	9	44.16
0.0 (MC)	Female	25	32.27
0.0 (WW)	Female	18	29.87
0.0 (MC)	Male	21	29.85
0.0 (WW)	Male	23	27.84

^ameans closed by the range of any one line are not significantly different at the 0.05 level

^bmeans transformed to original units

^cWW=well control, MC=methanol control

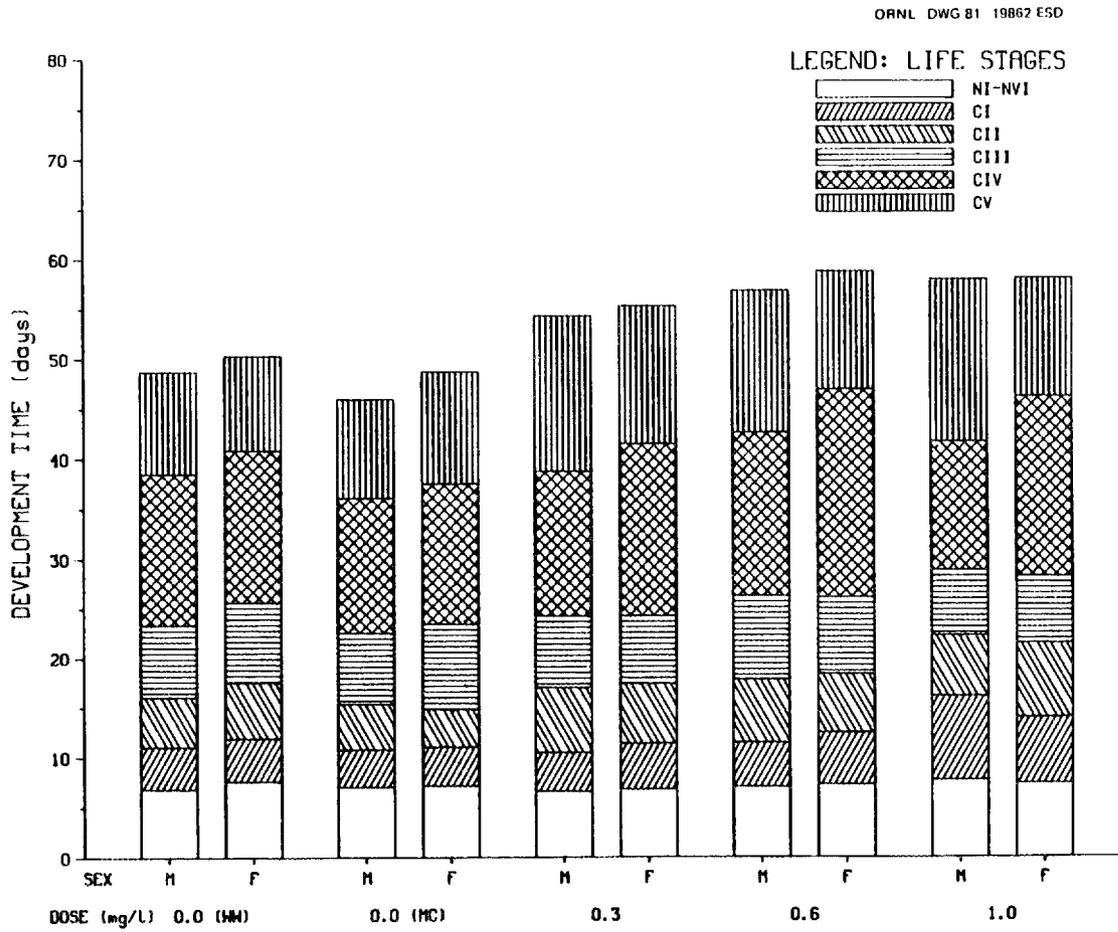


Figure 9. Development times for the immature life stages of *Diaptomus clavipes* at 16°C.

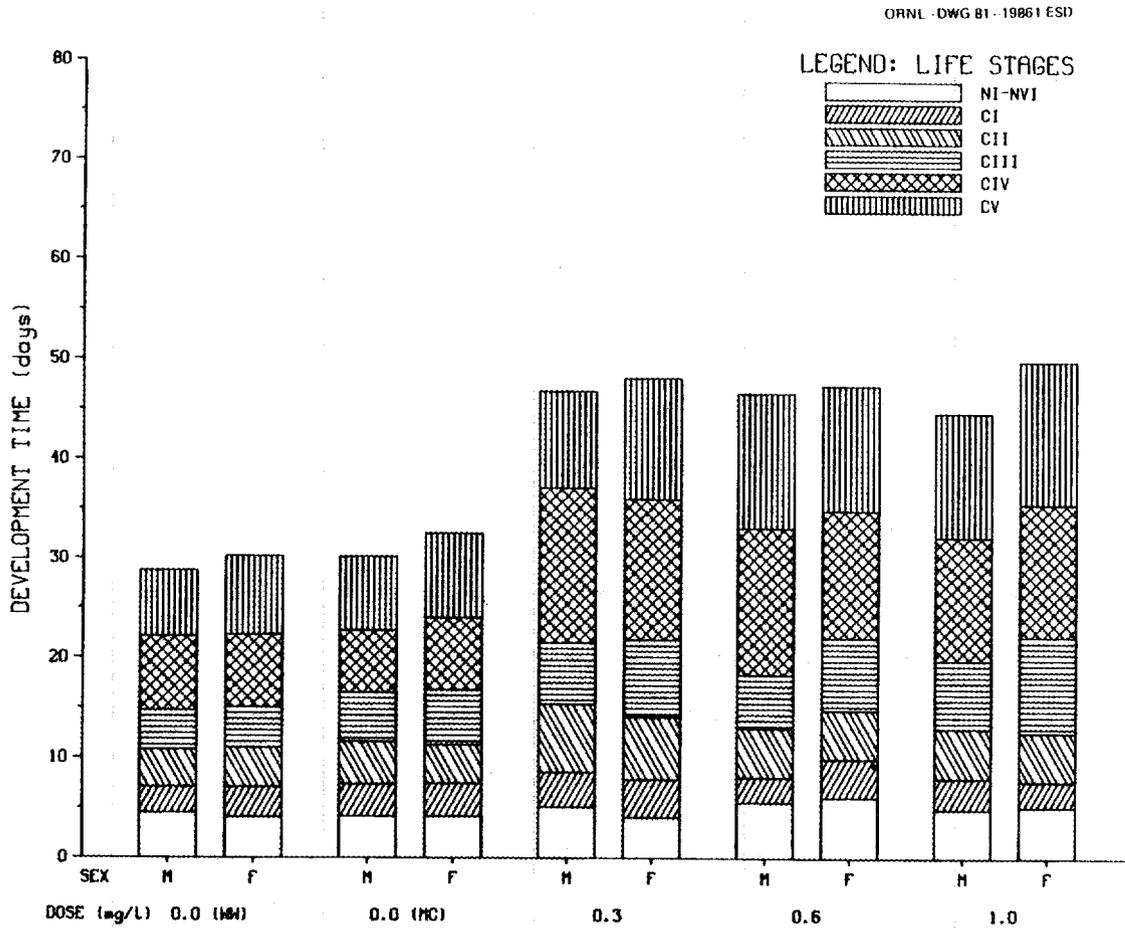


Figure 10. Development times for the immature stages of Diaptomus clavipes at 21°C.

possible reduction in food in the tissue culture plates could have lengthened this stage (CIV). This was not evident at 21°C where the duration of the CIV and CV stages was approximately equal in the controls. At 16°C, however, the CIV stage was approximately 1.5 times as long as the CV stage in the controls. This possible reduction in food might have had a synergistic effect on the development of these animals. However, development in the naupliar stages and early copepodid stages (NI-CIII) was still depressed in the acridine treated animals, when food was probably not a factor. The duration of the various immature stages at 16° and 21°C from hatching till the attainment of the adult stage are given in Tables 31 and 32. Data for the naupliar stages are pooled.

Sex ratios of adults were compared for the various concentrations at each temperature. These were compared using a chi square test to determine if there was any differential sex mortality among the various acridine concentrations within a temperature. There were no significant differences in sex ratio among the concentrations at either 16°C ($X^2 = 0.747$, $df = 4$, $P > 0.50$) or at 21°C ($X^2 = 3.101$, $df = 4$, $P > 0.50$). The pooled sex ratio (male:female) was 0.88 at 16°C and 0.93 at 21°C, only slightly different from a 1:1 ratio in favor of females.

A 2 x 5 analysis of variance test was computed for each sex with metasomal length of the adult as the observation. The fixed factors were temperature and acridine concentration. The results for males showed differences between the temperatures ($F = 24.44$, $df = 1/156$, $P < 0.0001$) and among the acridine concentrations ($F = 3.92$,

Table 31. Development times for the immature life stages of Diaptomus clavipes at 21°C

Concentration (mg/l)	Sex	n ^a	NI-NVI		CI		CII		Life Stages CIII		CIV		CV		NI-CV	
			\bar{x}	SE ^b	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
0.0(WW) ^c	Male	23	4.48	0.67	2.61	0.30	3.70	0.41	3.91	0.38	7.43	0.55	6.65	0.36	28.09	0.81
0.0(WW)	Female	18	4.06	0.94	3.00	0.36	3.94	0.50	4.00	0.51	7.28	0.58	7.94	0.85	30.22	1.11
0.0(MC) ^c	Male	21	4.14	0.48	3.24	0.37	4.24	0.36	4.90	0.48	6.19	0.60	7.48	0.59	30.24	1.04
0.0(MC)	Female	25	4.16	0.11	3.32	0.33	3.84	0.21	5.48	0.46	7.20	0.71	8.52	0.38	32.48	1.03
0.3	Male	10	5.10	0.41	3.50	0.41	6.80	0.87	6.10	0.55	15.60	1.33	9.70	0.67	46.80	2.09
0.3	Female	17	4.06	0.18	3.82	0.23	6.23	0.50	7.71	0.50	14.18	1.09	12.12	0.67	47.35	0.95
0.6	Male	12	5.58	0.29	2.50	0.15	4.92	0.36	5.33	0.53	14.75	1.62	13.50	1.99	46.25	0.66
0.6	Female	14	6.07	0.24	3.86	0.25	4.86	0.42	7.21	0.53	12.86	1.30	12.50	1.21	47.36	0.52
1.0	Male	9	4.89	0.26	3.11	0.26	5.00	0.67	6.78	0.88	12.44	1.31	12.44	1.28	44.66	2.20
1.0	Female	7	5.14	0.14	2.57	0.20	4.86	1.06	9.57	1.19	13.43	1.09	14.29	0.89	49.86	1.18

^an=sample size

^b \bar{x} =mean, SE=standard error

^cWW=well water control, MC=methanol control

Table 32. Development times for the immature life stages of *Diaptomus clavipes* at 16°C

Concentration (mg/l)	Sex	n ^a	NI-NVI		CI		CII		CIII		CIV		CV		NI-CV	
			\bar{x}	SE ^b	\bar{x}	SE										
0.0(WW) ^c	Male	22	6.82	0.20	4.23	0.28	5.00	0.43	7.27	0.54	15.18	0.75	10.23	0.67	48.36	0.61
0.0(WW)	Female	25	7.60	0.63	4.32	0.23	5.64	0.39	8.08	0.50	15.20	0.56	9.48	0.46	50.08	0.50
0.0(MC) ^c	Male	23	7.04	0.19	3.74	0.16	4.56	0.29	7.17	0.48	13.56	0.52	9.87	0.46	46.78	0.41
0.0(MC)	Female	21	7.14	0.16	3.90	0.19	3.76	0.29	8.62	0.52	14.09	1.27	11.19	1.15	49.19	0.48
0.3	Male	19	6.58	0.14	3.89	0.25	6.53	0.44	7.21	0.41	14.53	1.35	15.58	0.78	55.37	0.61
0.3	Female	24	6.79	0.12	4.58	0.26	6.00	0.36	6.88	0.45	17.25	0.89	13.83	0.76	55.42	0.34
0.6	Male	20	7.00	0.16	4.45	0.18	6.35	0.42	8.45	0.64	16.40	1.04	14.25	0.98	57.05	0.58
0.6	Female	25	7.28	0.11	5.20	0.79	5.88	0.30	7.76	0.38	20.88	1.06	11.84	0.94	57.96	0.43
1.0	Male	7	7.71	0.76	8.43	0.48	6.14	0.80	6.57	0.81	12.86	2.17	16.28	2.20	57.43	1.25
1.0	Female	8	7.37	0.32	6.62	1.03	7.50	0.42	6.75	0.67	18.00	0.78	11.88	0.99	58.12	1.14

^an=sample size^b \bar{x} =mean, SE=standard error^cWW=well water control, MC=methanol control

df = 4/156, $P < 0.0046$). The interaction term was also significant ($F = 2.50$, df = 4/156, $P < 0.0446$). The test for females yielded similar results with significant differences between the temperatures ($F = 20.64$, df = 1/174, $P < 0.0001$) and among the acridine concentrations ($F = 12.92$, df = 4/174, $P < 0.0001$) with a significant interaction ($F = 4.75$, df = 4/174, $P < 0.0012$).

The a posteriori comparisons of mean metasomal lengths for males and females are given in Table 33. At 16°C, control males were significantly larger than all acridine treated males. There were no differences in male size at 21°C. At 16°C, females were significantly larger than the two higher acridine concentration (0.6, 1.0 mg/l) but were similar in size to the lower concentration (0.3 mg/l). At 21°C, control females were significantly larger than the 1.0 mg/l concentration. There were no differences in size between the control groups (methanol and well water) for either sex or temperature.

Reproduction was observed at both temperatures and yielded results similar to those in the Reproduction section (Table 34). Time to the production of the first egg clutch was observed for both males and females (Table 35). It is apparent that as concentration increases within a temperature, so does time to the production of the first clutch. At 1.0 mg/l acridine, very few animals produced viable clutches.

It was of interest to compare egg production within a female over time in order to determine if clutch size changed in successive clutches. A t-test for paired comparisons was made for each temperature by comparing the size of the first clutch with the mean of

Table 33. A posteriori comparisons of mean metasomal lengths for male and female Diaptomus clavipes at 16°C and 21°C using Duncan's new multiple range test

Concentration (mg/l)	Temperature (°C)	Sample size	Means ^a (mm)
Males			
0.0(WW) ^b	16	22	1.32
0.0(MC) ^b	16	23	1.32
0.3	16	19	1.29
0.6	16	20	1.28
0.3	21	10	1.27
0.0(WW)	21	23	1.27
1.0	16	7	1.26
0.6	21	12	1.25
0.0(MC)	21	21	1.25
1.0	21	9	1.25
Females			
0.0(WW)	16	25	1.51
0.0(MC)	16	21	1.48
0.3	16	24	1.46
0.3	21	17	1.46
0.6	16	25	1.44
0.0(WW)	21	18	1.44
0.6	21	14	1.43
0.0(MC)	21	25	1.42
1.0	16	8	1.38
1.0	21	7	1.35

^ameans enclosed by the range of any one line are not significantly different at the 0.05 level

^bWW=well water control, MC=methanol control

Table 34. Mean clutch size (number of eggs per clutch), rate of clutch production (number of clutches per female per day), and rate of egg production (number of eggs per female per day) of Diaptomus clavipes at two temperatures produced during the chronic reproduction experiment.

Temperature (°C)	Concentration (mg/l)	Clutch size			Rate of clutch production			Rate of egg production		
		n	\bar{x}	SE ^a	n	\bar{x}	SE	n	\bar{x}	SE
16	0.0(WW) ^b	64	18.67	0.82	25	0.1707	0.0102	25	3.19	0.26
	0.0(MC) ^b	50	19.72	0.85	21	0.1587	0.0086	21	3.13	0.27
	0.3	46	15.57	0.76	24	0.1278	0.0165	24	1.99	0.27
	0.6	50	13.68	0.64	25	0.1333	0.0094	25	1.82	0.22
	1.0	6	9.83	0.83	8	0.0500	0.0167	8	0.49	0.17
21	0.0(WW)	76	16.12	0.71	18	0.2815	0.0102	18	4.54	0.34
	0.0(MC)	95	16.69	0.64	25	0.2533	0.0169	25	4.23	0.41
	0.3	41	13.20	1.16	17	0.1608	0.0172	17	2.12	0.42
	0.6	31	13.84	1.10	14	0.1476	0.0263	14	2.04	0.41
	1.0	4	6.50	1.66	7	0.0381	0.0381	7	0.25	0.25

^a n = sample size, \bar{x} = mean, SE = standard error.

^b WW = well water control, MC = methanol control.

Table 35. Mean time (days) to the production of the first subitaneous egg clutch for male and female Diaptomus clavipes at two temperatures in the chronic reproduction experiment.

Temperature (°C)	Concentration (mg/l)	Males			Female		
		n	\bar{x}	SE ^a	n	\bar{x}	SE
16	0.0(WW) ^b	22	4.50	0.43	25	7.12	0.56
	0.0(MC) ^b	23	4.96	0.49	21	7.52	0.39
	0.3	17	5.00	0.34	19	6.58	0.28
	0.6	20	6.30	0.51	25	8.84	0.46
	1.0	2	11.50	2.50	5	12.40	1.69
21	0.0(WW)	23	3.48	0.31	18	4.83	0.23
	0.0(MC)	21	4.10	0.28	25	5.48	0.26
	0.3	10	4.30	0.50	16	6.25	0.66
	0.6	12	5.92	0.69	13	5.85	0.53
	1.0	3	5.67	0.33	2	11.00	4.00

^an = sample size, \bar{x} = mean, SE = standard error.

^bWW = well water control, MC = methanol control.

subsequent clutches. The differences in clutch size was the observation. A second t-test for each temperature was computed and compared with the mean clutch size for the first 2-3 clutches with the mean of the next 2-3 clutches. At 16°C, females which had produced four clutches during the experimental period were used. At 21°C, more clutches were produced, therefore females which had produced six clutches were used. Table 36 gives a summary of the results. The first clutch was significantly larger than the mean of subsequent clutches at both temperatures. However, the mean difference was only about three eggs. This could be accounted for by the longer period of time for the production of the first clutch when compared to the interval between subsequent clutches. At 21°C, no significant differences were found when the means for the first three clutches were compared with the means for the next three clutches.

The results of this experiment indicate that survival, growth, development, and reproduction were all affected by temperature and acridine concentration. No viable egg clutches were produced at concentrations greater than 1.0 mg/l except at 26°C, where viable clutches were produced at 1.8 mg/l. Gehrs et al. (1975) found that ovigerous females of Diaptomus clavipes dropped their egg clutches when exposed to the stress of acute ionizing radiation. Dislodged egg clutches usually had lower percent egg hatch than clutches retained by females until hatching. This was also observed in the acute adult bioassays in the stock feeding regime (greater than 4.2 mg/l) when ovigerous females were present. In several instances in this experiment, a clutch was observed at one time period but not at the

Table 36. Comparisons of number of eggs per clutch within a female for control female *Diaptomus clavipes* at two temperatures in the chronic reproduction experiment.

Comparison	Mean Difference	n	T-value	PR > T
16°C				
$X_1 - \bar{X}_{2,3,4}^a$	2.92	29	3.14	0.0040
$\bar{X}_{1,2} - \bar{X}_{3,4}$	3.79	29	4.55	0.0001
21°C				
$X_1 - \bar{X}_{2,3,4,5,6}$	2.78	35	3.34	0.0020
$\bar{X}_{1,2,3} - \bar{X}_{4,5,6}$	1.04	35	1.55	0.1305

^asubscripts (1,2,3,4,5,6) stand for clutch number

next time period. Examination of the beakers revealed no dislodged clutches or loose eggs on the bottom, suggesting that the clutch was nonviable and disintegrated. Percent egg hatch of clutches removed and measured exceeded 90 percent at all temperatures and acridine concentrations. Thus, it appears that if an egg survives the first few minutes before the egg membranes form and becomes impermeable to water, the egg will develop normally and hatch.

Survival to the adult stage (CVI) was reduced at the 1.0 mg/l concentration at 16°C and at the 0.3, 0.6, and 1.0 mg/l concentrations at 21°C. At 26°C, survival was reduced at all concentrations including the controls, suggesting some stress other than acridine was operating at this temperature. Hardin (1972) and Pennak (1978) recommended that temperate calanoid copepods should be maintained at temperature less than 21°C. At higher temperatures, mortality increased.

There were no detectable differences in egg development times within any one temperature. Development times decreased with increasing temperature. This is a common phenomenon in many aquatic poikilotherms (Hutchingson 1967). Development from hatching (NI) to the adult stage (CVI) was affected by both temperature and acridine concentration, especially in the copepodid stages. Development times decreased with increasing temperature. However, presence of acridine significantly depressed development (Table 30). Although males generally matured slightly faster than females, there were no significant differences between them except in the controls at 16°C. The durations of the life stages at 21°C were similar to those reported in the literature for Diaptomus clavipes (Robertson et al. 1974).

There were no significant effects of acridine concentration on growth to the first copepodid stage (CI) in Diaptomus clavipes. However, adult size (male and female metasomal length) was significantly reduced at the higher concentrations for both temperatures. This is reasonable, since approximately 77 percent of total growth is from CI-CVI (Gurney 1929). In this study, percent of total growth from CI-CVI ranged from 68-72 percent.

Reproduction parameters which were measured in this study (Table 34) were similar to those in the reproduction bioassay (Table 18). However, reproduction at 1.0 mg/l was severely inhibited at both temperatures. An additional parameter, time to first viable clutch, was also examined in this experiment. As acridine concentration increased, time to the production of the first clutch also increased. In the reproduction bioassay, control females produced a clutch every 5.75 days at 16°C and 2.75 days at 21°C. This is a considerably shorter time interval than recorded here (Table 35). This suggests that either the gametes are not fully developed when the animals molted into CVI or food availability was lower in the shell vials when compared with the beakers.

Successive clutch sizes were also compared using control females. Although the first clutch was significantly larger than the mean for successive clutches, this difference averaged approximately three eggs for both temperatures. This small difference is probably not of ecological significance. Hardin (1972) also found no significant changes in clutch size with age in Diaptomus clavipes.

CHAPTER VII

SUMMARY AND CONCLUSIONS

The results of this study indicate that survival, growth, development and reproduction were all affected by temperature and exposure to acridine. In the acute bioassays, LC50's were used to estimate median lethal concentrations. In the chronic bioassays, the effective concentration estimated for each criteria was the no-observed-effects concentration (NOEC). The NOEC has been defined by Maki (1979) as the highest test concentration at which no statistically significant effects are observed compared with the control organisms. The NOEC is the lower limit of the concentration range within which the chronic toxicity threshold lies. The next highest concentration, the lowest concentration at which significant effects are observed, is the upper limit of the range in which the toxicity threshold lies and is defined as the lowest-observed-effects concentration (LOEC) (Parkhurst et al. 1981). The NOEC is based on the non-rejection of the hypothesis, no toxic effect, while scientific methods stress conclusions based on the rejection of a hypothesis (Skalski 1981). Failure to reject this hypothesis may be a consequence of either the concentration being "safe" or the insensitivity of the experimental protocol to detect a toxic response. Skalski (1981) recommended the LOEC which he termed LRCT (lowest rejected concentration tested) as an experimental endpoint because the rate of misclassification is known and equal to α . Table 37 gives LC50's for the acute bioassays and NOEC's and LOEC's for chronic bioassays for each temperature tested.

Table 37. Summary of estimated median lethal concentrations (LC50), estimated no-observed-effects concentrations (NOECs) and lowest-observed-effects concentrations (LOECs) for the toxicity of acridine to Diaptomus clavipes for each of the measured toxicity criteria.

Toxicity Criteria	Temperature (°C)	LC50 (mg/l)	NOEC (mg/l)	LOEC (mg/l)
<u>Survival</u>				
Adult (Fed)	16	6.42	--	--
	21	4.31	--	--
	26	4.96	--	--
Egg-NII	16	1.59	--	--
	21	3.44	--	--
	26	3.60	--	--
NI-CI (Fed)	16	1.55	--	--
	21	1.75	--	--
	26	1.18	--	--
NI-CVI	16	--	0.6	1.0
	21	--	<0.3	0.3
<u>Development</u>				
NI-NII	16	--	<0.3	0.3
	21	--	3.2	5.6
	26	--	3.2	5.6
NI-CVI	16	--	<0.3	0.3
	21	--	<0.3	0.3
<u>Growth</u>				
Adult Males	16	--	<0.3	0.3
	21	--	1.0	>1.0
Adult Females	16	--	0.3	0.6
	21	--	0.6	1.0
<u>Reproduction</u>				
#eggs/clutch	16	--	1.8	3.2
	21	--	<0.3	0.3
	26	--	1.8	3.2
Rate of egg production	16	--	1.8	3.2
	21	--	<0.3	0.3
	26	--	0.3	0.6
Rate of clutch production	16	--	1.0	1.8
	21	--	0.3	0.6
	26	--	0.3	0.6

Survival of adults was more strongly affected by feeding regime than by temperature. As food availability decreased, sensitivity to acridine increased. The 96 h LC50's for fed adults at 21°C was almost twice the 48 h LC50 for first instar Daphnia magna. This higher tolerance is probably due to the cessation of molting at maturity for copepods. When comparisons are made with the LC50 values for the immature stages where numerous molts occur, Diaptomus clavipes was more sensitive than Daphnia magna.

Development time from NI to NII showed little variation at 21°C and 26°C. However, at 16°C, development times were significantly increased at the lowest concentration tested (0.3 mg/l). This was also true for development times from NI-CVI.

The three measures of reproduction (number of eggs/clutch, number of clutches/female/day, number of eggs/female/day) had varying degrees of sensitivity depending on temperature. At 16°C and 26°C, clutch size did not significantly vary among the acridine concentrations when compared to the controls. At 21°C, clutch size was significantly reduced in all acridine exposed animals. At all temperatures, clutch size did not vary at the highest concentration where reproduction occurred (1.8 mg/l). Above this concentration, no viable clutches were produced. Rate of clutch production was a more sensitive indicator of reproductive inhibition than clutch size. Rate of egg production, yielded results similar to the rate of clutch production. Reproduction parameters were more sensitive indicators of toxic stress at 21°C. Growth was also affected by acridine concentration for both males and

females. Metasomal length decreased as acridine concentration increased. This parameter was more sensitive at 16°C than at 21°C.

Bioassays with Diaptomus clavipes should be performed at either 16° or 21°C depending on the criteria that would be used in evaluating toxic effects. Growth and development were more sensitive indicators of toxicant stress at 16°C. Reproduction and survival were more sensitive at 21°C. Bioassays should not be done at 26°C due to high mortality of the cultured animals which may have resulted from inadequate food or trying to culture and test animals at temperatures near the maximum found in natural populations which is about 28°C (Gehrs 1972).

Recommendations for acute tests would be the acute naupliar tests (NI-CI) which would take approximately 4-5 days at 21°C. This time period would include six molts and a drastic change in shape (surface area:volume ratio) at the molt from NVI-CI. The fifteen day reproduction bioassay at 21°C yielded essentially the same information as the reproduction experiment in the chronic study. As a quick test for chronic effects, inhibition of clutch production in "ripe" females (females with dark eggs in the ovaries and oviducts) could be used. Ripe control females usually produced viable clutches within a few hours after being combined with males, therefore, time to the production of a viable clutch could be used as a response. This was found to be a sensitive indicator of acridine stress and may involve behavioral modifications in the mating act or increased sensitivity of newly laid eggs.

The results obtained in this study on the toxicity of acridine to Diaptomus clavipes will be helpful in determining the potential environmental hazard to aquatic biota of releases of acridine and other organic bases in effluents from advanced fossil fuel facilities. In addition, insight was gained on the interaction of temperature and food availability on acute and chronic toxicity of acridine to Diaptomus clavipes.

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APPENDIX A

TABLES

Table A-1. Analysis of Well Water.^a

Determination	Mean	Concentration	
			Standard Error
Calcium	31.50	ppm ^b	1.13
Cadmium	0.04	ppb ^b	0.01
Chromium	0.44	ppb	0.06
Lead	0.32	ppb	0.09
Magnesium	9.73	ppm	0.56
Mercury	0.01	ppb	0.00
Nitrogen (nitrate)	0.16	ppm	0.05
Phosphorus	0.01	ppm	0.00
Potassium	0.68	ppm	0.05
Sulfate	4.88	ppm	0.39
Zinc	0.66	ppb	0.19
Alkalinity	127.93	ppm	5.16
Conductivity	2.4×10^{-4}	mhos	0.14×10^{-4}
Hardness	120.82	ppm	4.86
Free CO ₂	5.63	ppm	0.41
Dissolved organic carbon	0.59	ppm	0.05
Dissolved oxygen	9.05	ppm	0.31
Suspended solids	1.17	ppm	0.22

^aWell water analysis performed by the Analytical Chemistry Division on samples from April 1978 to February 1981 (n=28). Analyses based on U.S. Environmental Protection Agency Standard Method (1979).

^bppm = parts per million, ppb = parts per billion

Table A.2. Guaranteed analysis of Purina trout chow
(size #00-Fry), Ralston Purina Company.

---Analysis---

Crude protein not less than -----48.0 percent
 Crude fat not less than-----14.0 percent
 Crude fiber not less than-----4.0 percent
 Ash not more than-----10.0 percent
 added minerals not more than-----1.0 percent

---Ingredients---

Fish meal, soybean flour, soybean oil, wheat middlings,
 dried whey, condensed fish solubles, dried yeast, corn
 gluten meal, blood meal, calcium carbonate, ground yellow
 corn, ethoxyquin (a preservative), vitamin A supplement,
 Deactivated animal sterol (source of vitamin D-3), mena-
 dione sodium bisulfite (source of vitamin K activity),
 vitamin E supplement, DL methionine, vitamin B-12 supple-
 ment, ascorbic acid, biotin, choline chloride, folic acid,
 pyridoxine hydrochloride, thiamin, niacin, calcium panto-
 thenate, riboflavin supplement, copper sulfate, anganous
 oxide, ferrous carbonate, zinc sulfate.

Table A-3. Semi-quantitative spectrographic analysis of Purina trout chow (size #00-Fry), Ralston Purina Company.

Element	Concentration
Aluminum	1,000 ppm ^a
Antimony	less than 50 ppm
Barium	20 ppm
Beryllium	less than 1 ppm
Bismuth	less than 50 ppm
Boron	8 ppm
Cadmium	less than 50 ppm
Calcium	8,000 ppm
Cesium	less than 100 ppm
Chromium	less than 50 ppm
Cobalt	less than 50 ppm
Copper	20 ppm
Gallium	less than 50 ppm
Germanium	less than 50 ppm
Gold	less than 50 ppm
Indium	less than 20 ppm
Iron	700 ppm
Lead	less than 50 ppm
Lithium	50 ppm
Magnesium	6,000 ppm
Manganese	200 ppm
Mercury	less than 50 ppm
Molybdenum	less than 5 ppm
Nickel	less than 50 ppm
Niobium	less than 50 ppm
Platinum	less than 50 ppm
Potassium	approximately 10,000 ppm
Rubidium	70 ppm
Silicon	700 ppm
Silver	5 ppm
Sodium	5,000 ppm
Strontium	300 ppm
Tantalum	less than 50 ppm
Tellurium	less than 100 ppm
Tin	less than 50 ppm
Titanium	40 ppm
Tungsten	less than 50 ppm
Uranium	less than 100 ppm
Vanadium	less than 50 ppm
Zinc	50 ppm
Zirconium	less than 50 ppm

^appm = parts per million

APPENDIX B

FIGURES

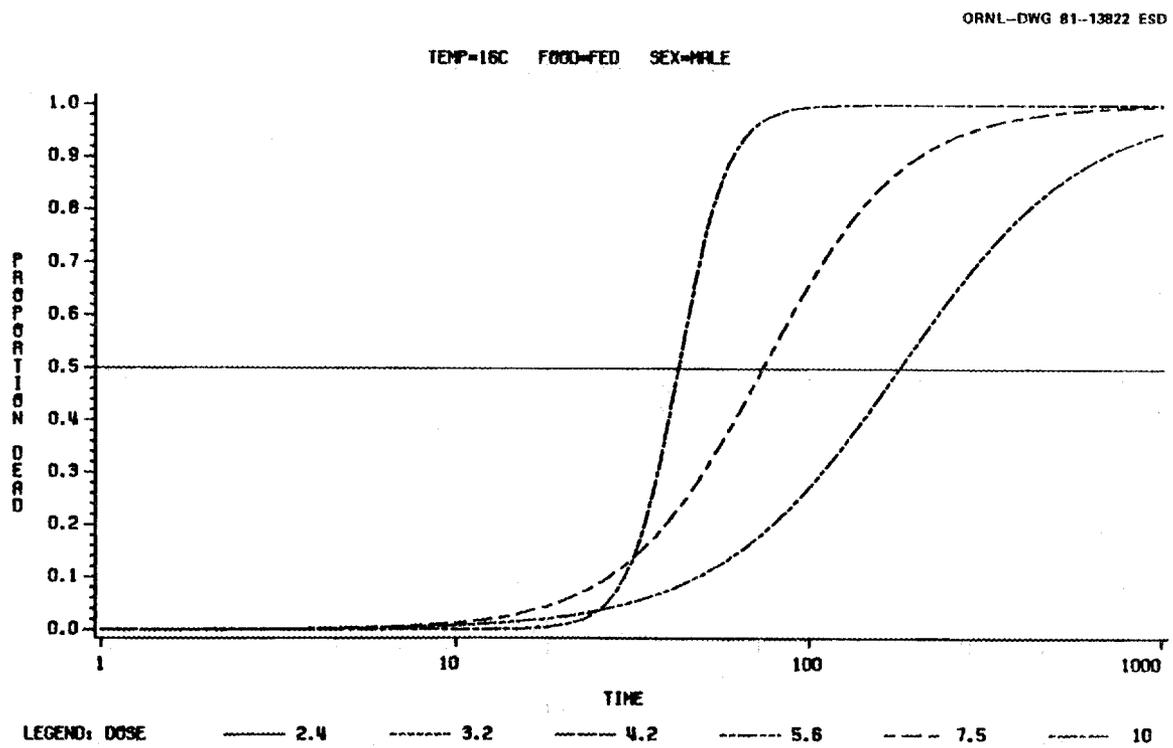


Figure B-1. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 16°C in the fed feeding regime

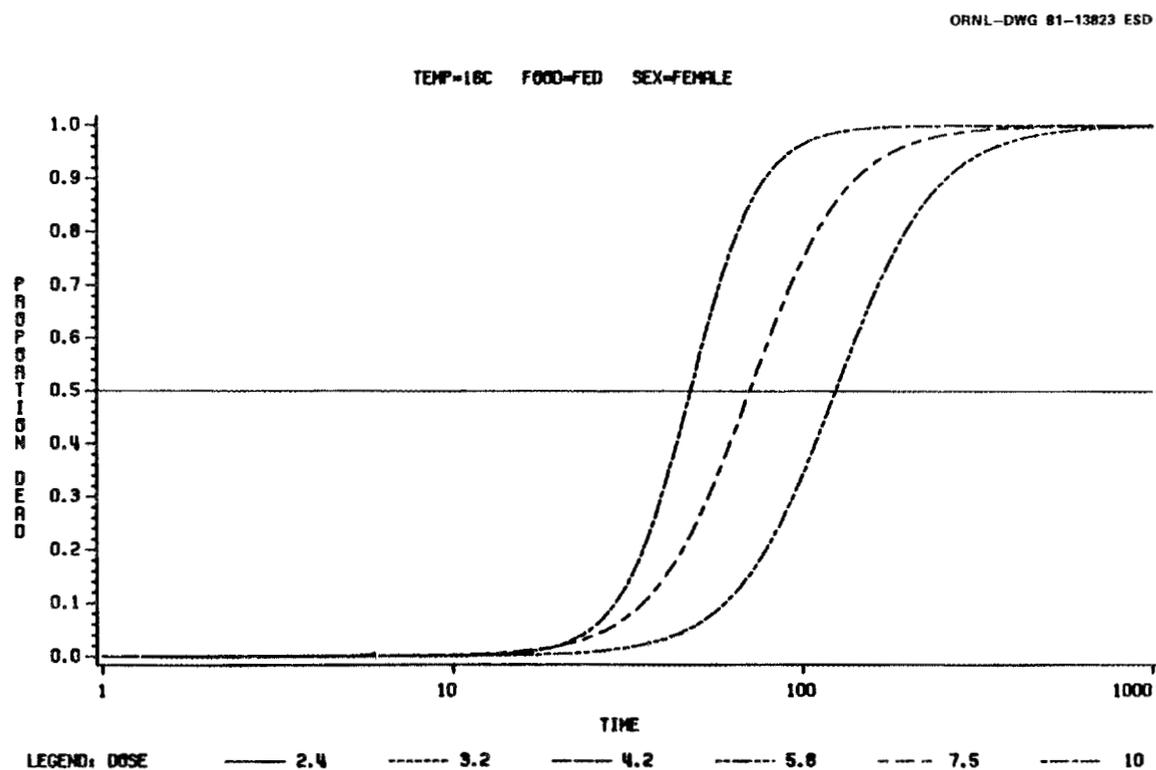


Figure B-2. Logistic survival functions of female *Diaptomus clavipes* for each acridine concentration at 16°C in the fed feeding regime

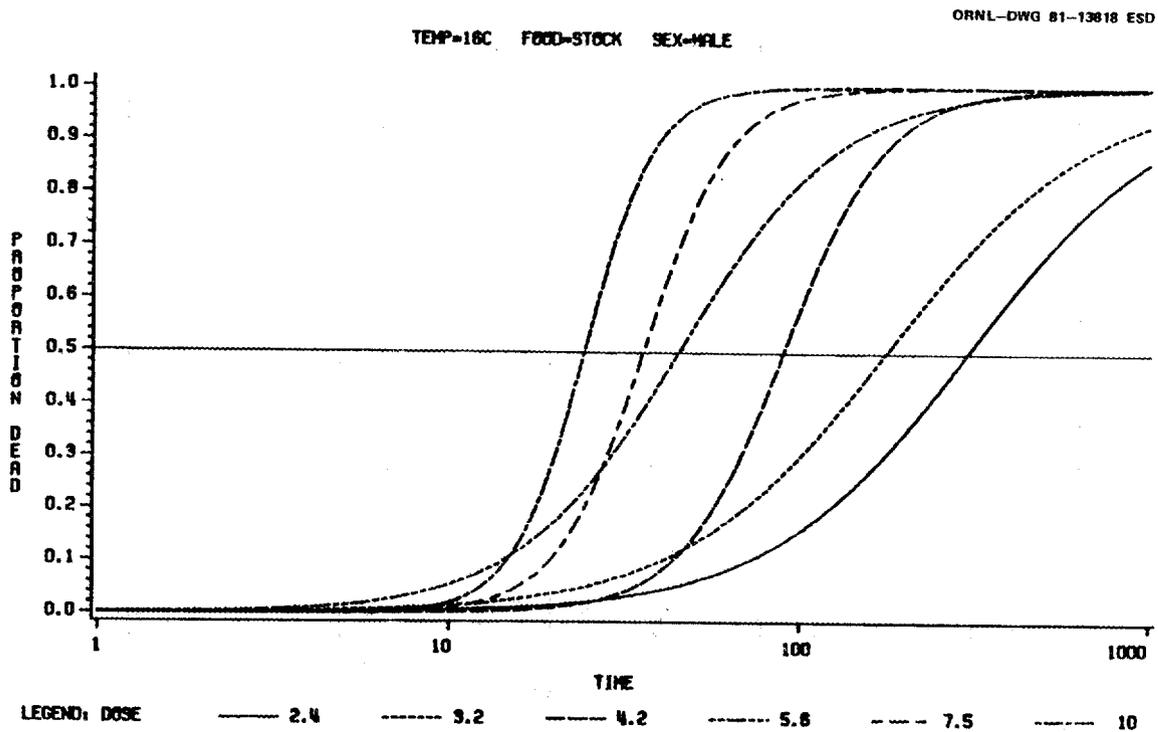


Figure B-3. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 16°C in the stock feeding regime

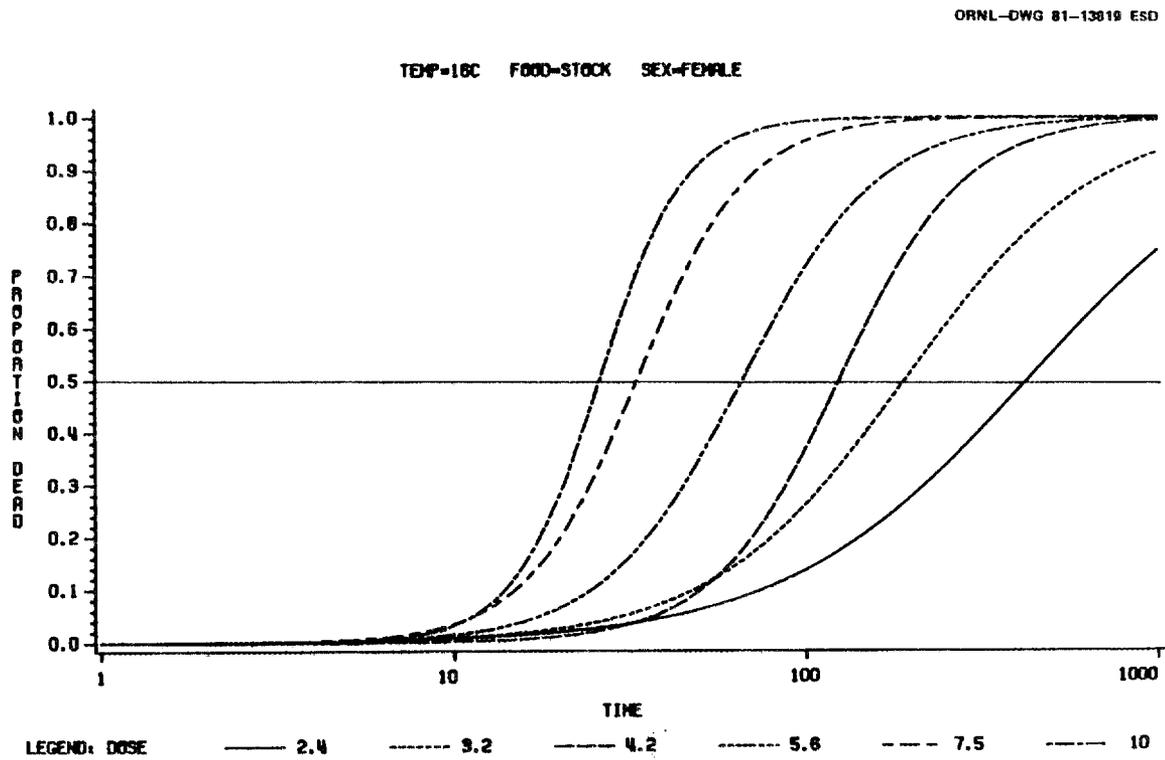


Figure B-4. Logistic survival functions of female *Diantomus clavipes* for each acridine concentration at 16°C in the stock feeding regime

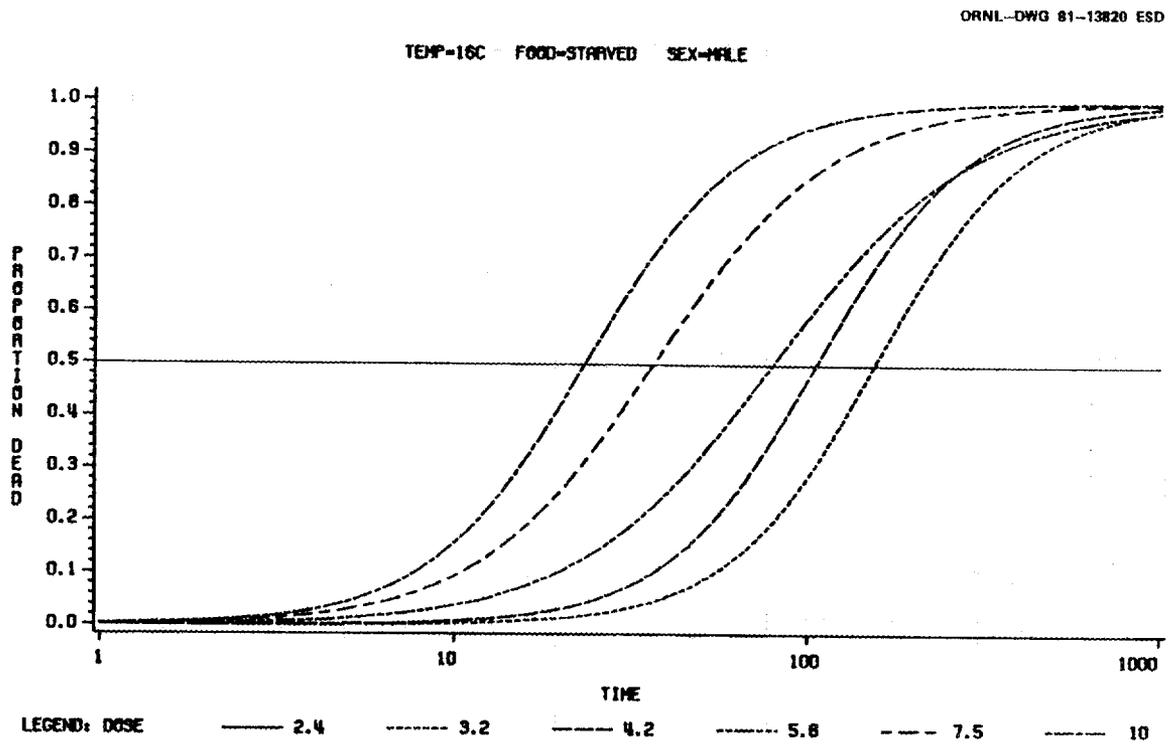


Figure B-5. Logistic survival functions of male *Diatomus clavipes* for each acridine concentration at 16°C in the starved feeding regime

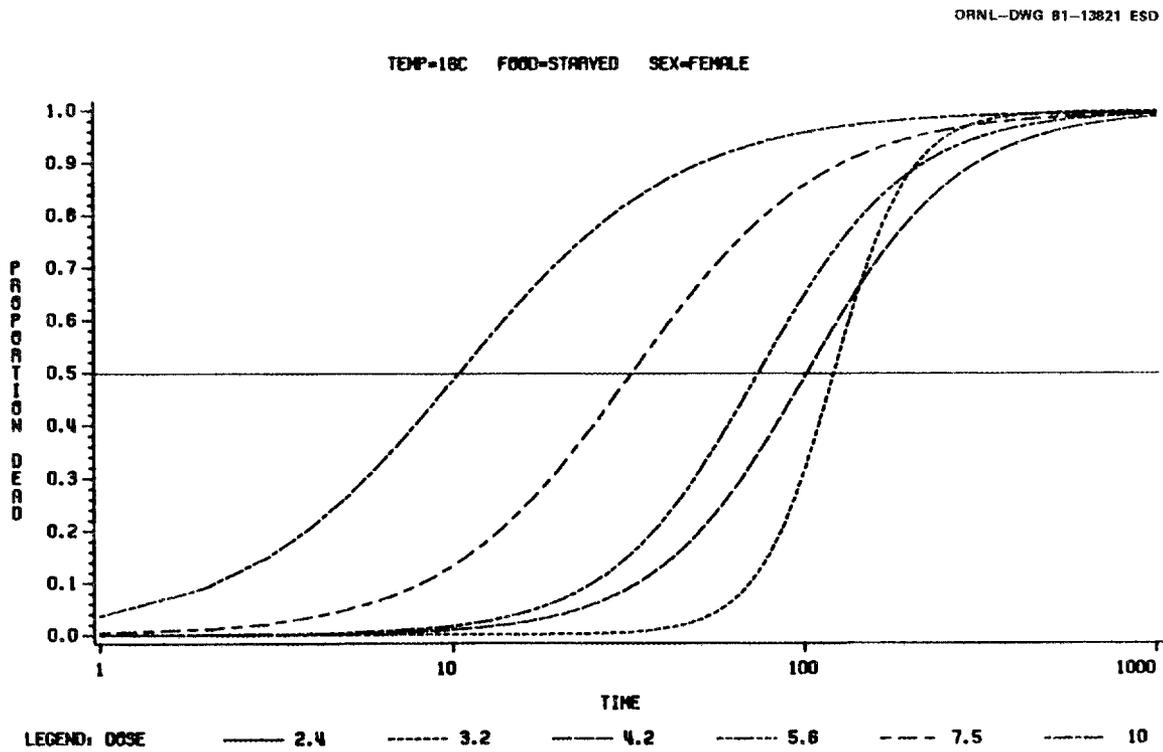


Figure B-6. Logistic survival functions of female *Diaptomus clavipes* for each acridine concentration at 16°C in the starved feeding regime

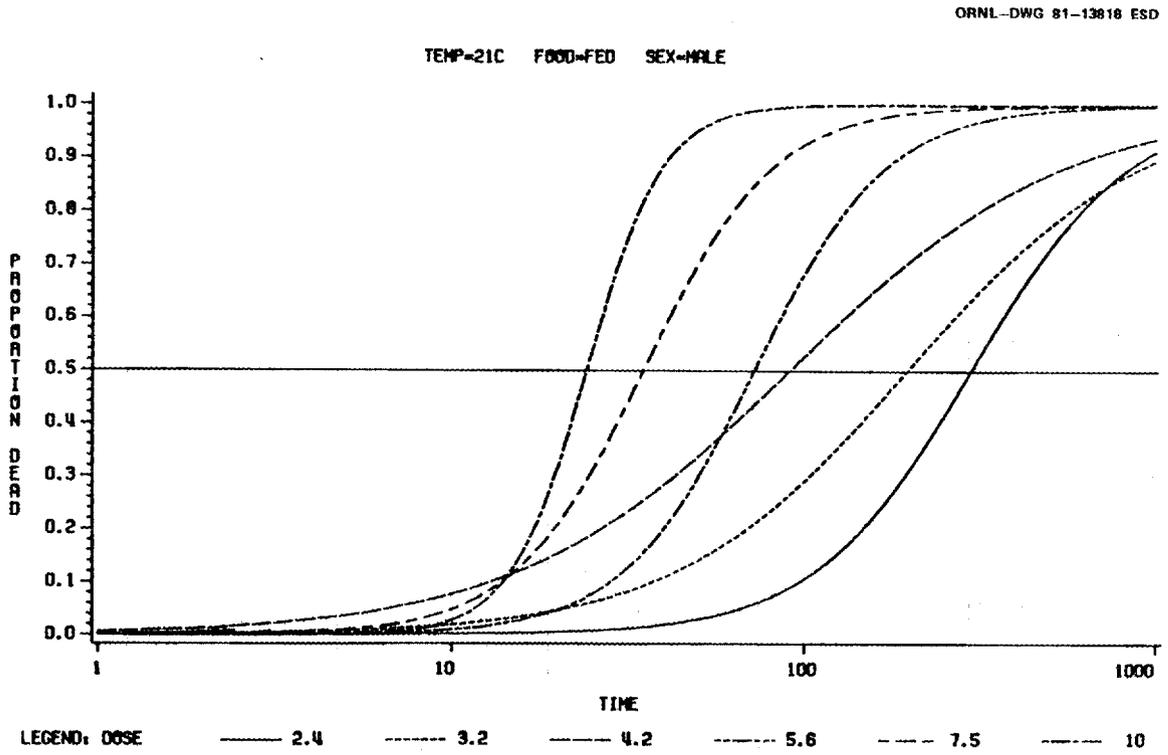


Figure B-7. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 21°C in the Fed feeding regime

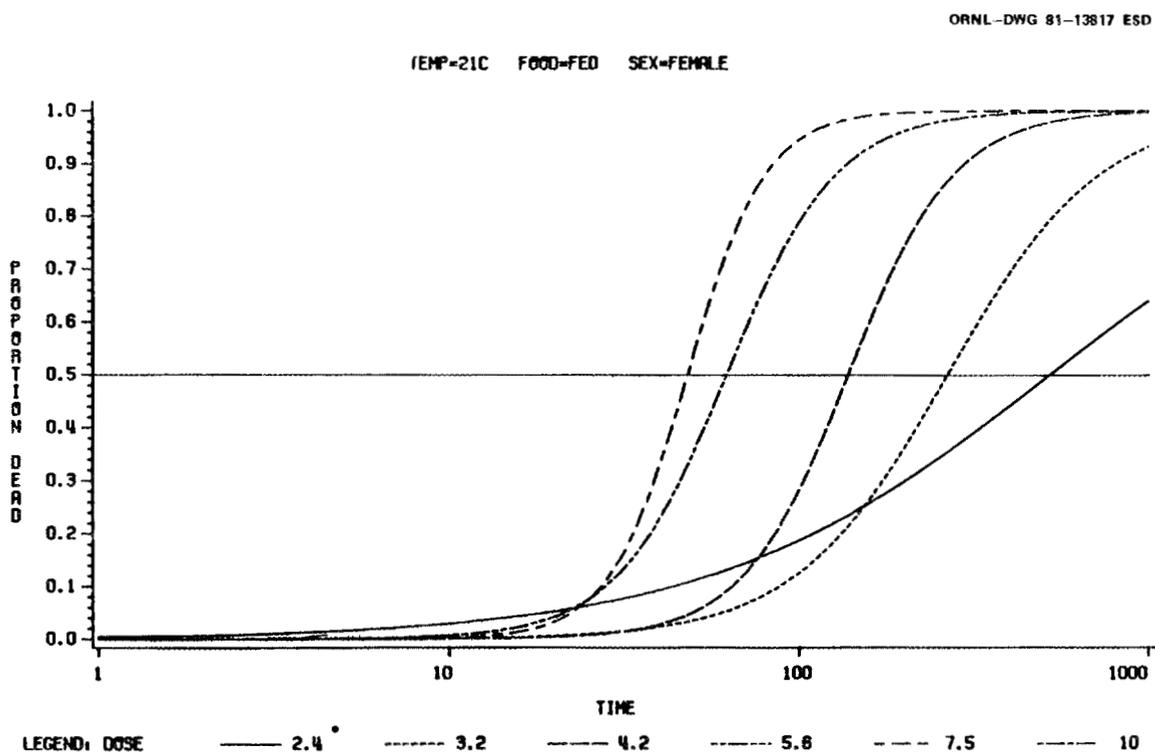


Figure B-8. Logistic survival functions of female *Diaptomus clavipes* for each acridine concentration at 21°C in the fed feeding regime

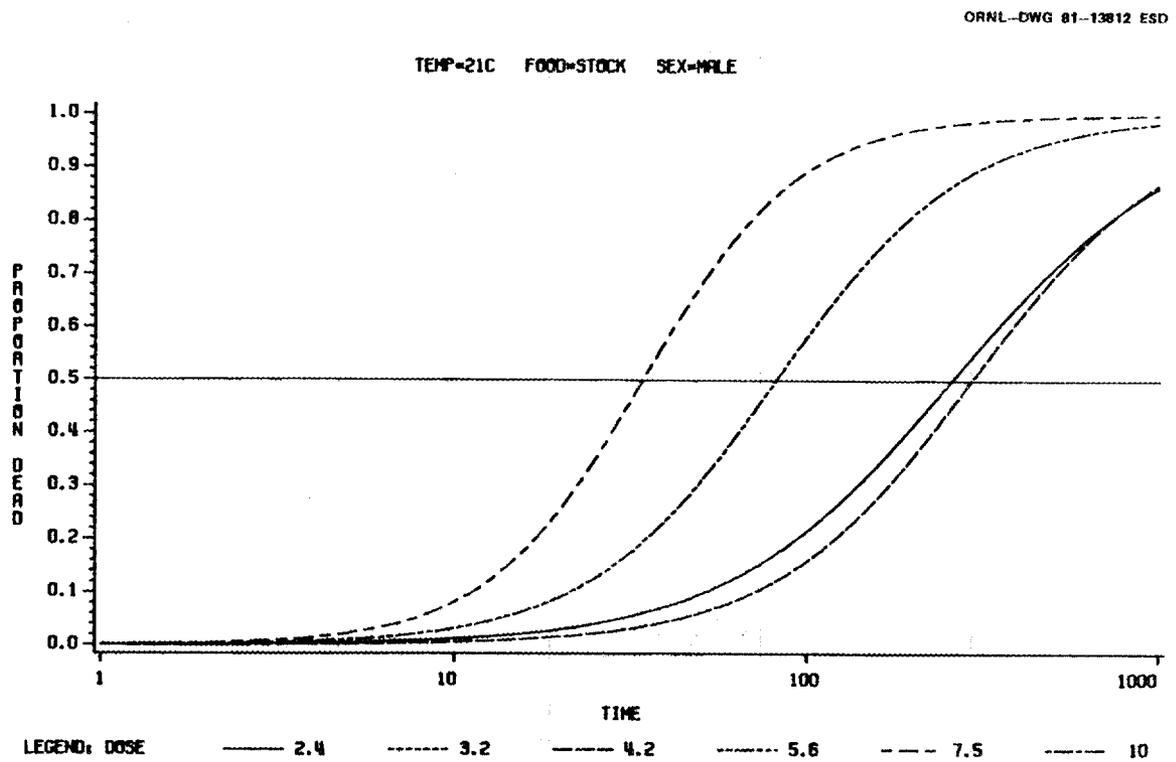


Figure B-9. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 21°C in the stock feeding regime

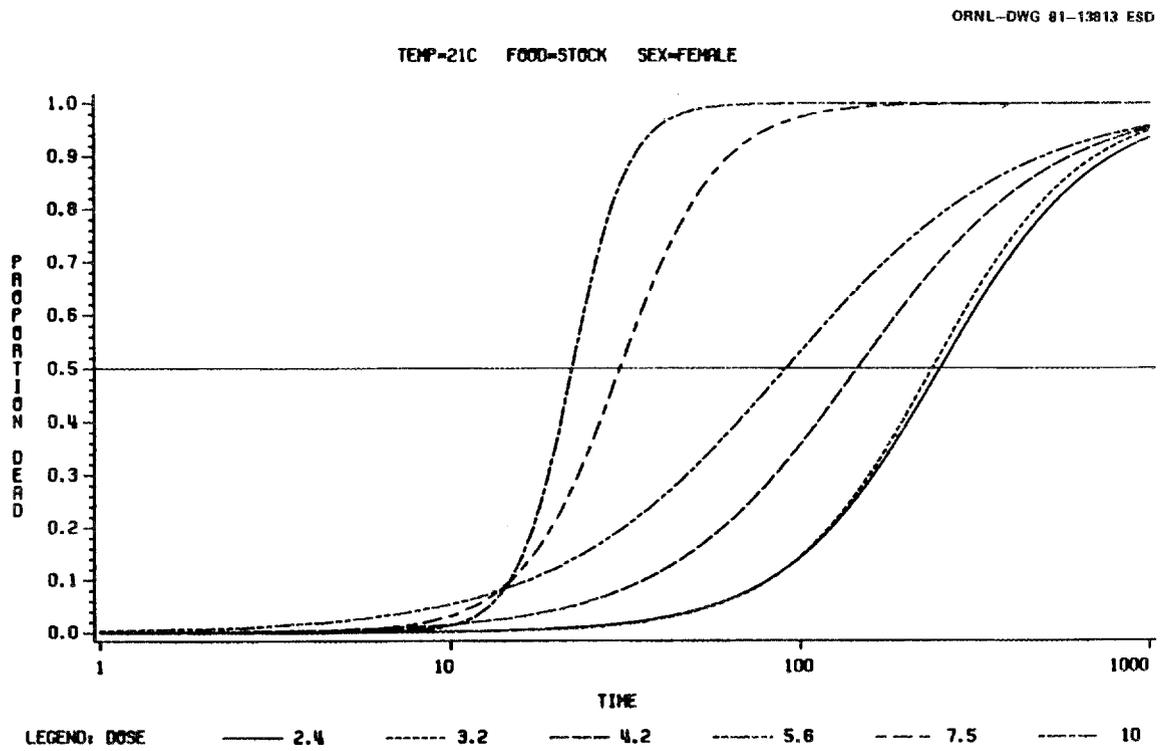


Figure B-10. Logistic survival functions of female *Diantomus clavipes* for each acridine concentration at 21°C in the stock feeding regime

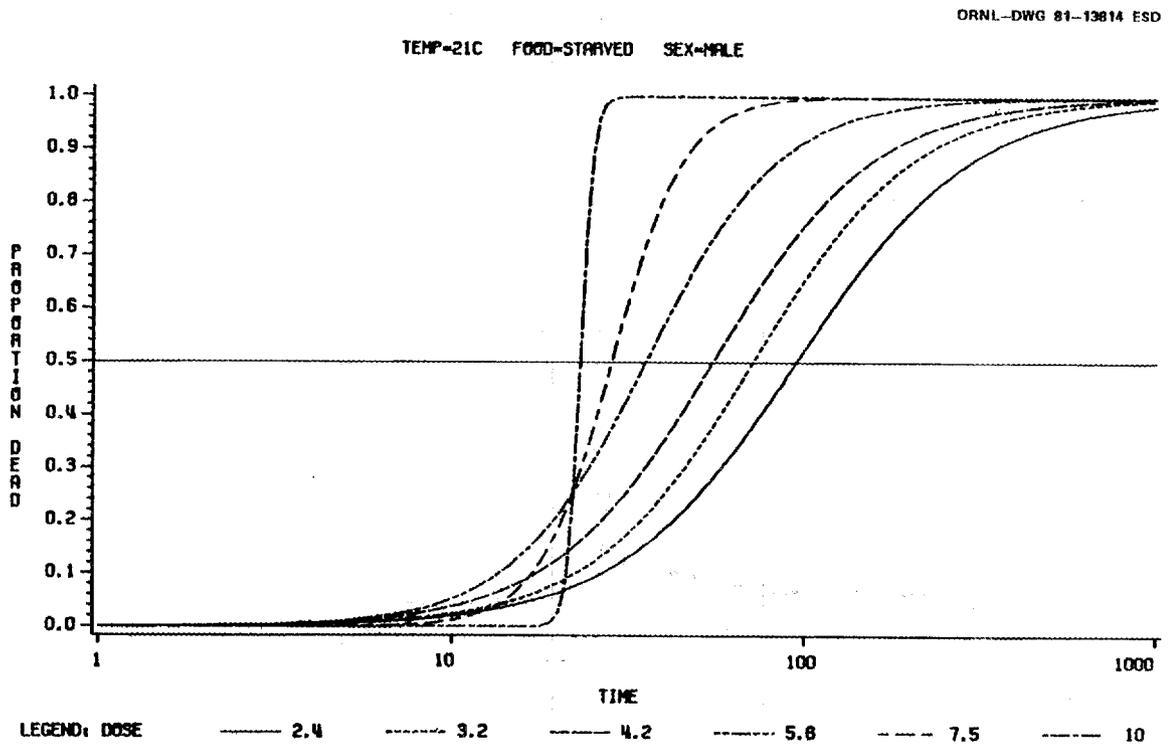


Figure B-11. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 21°C in the starved feeding regime

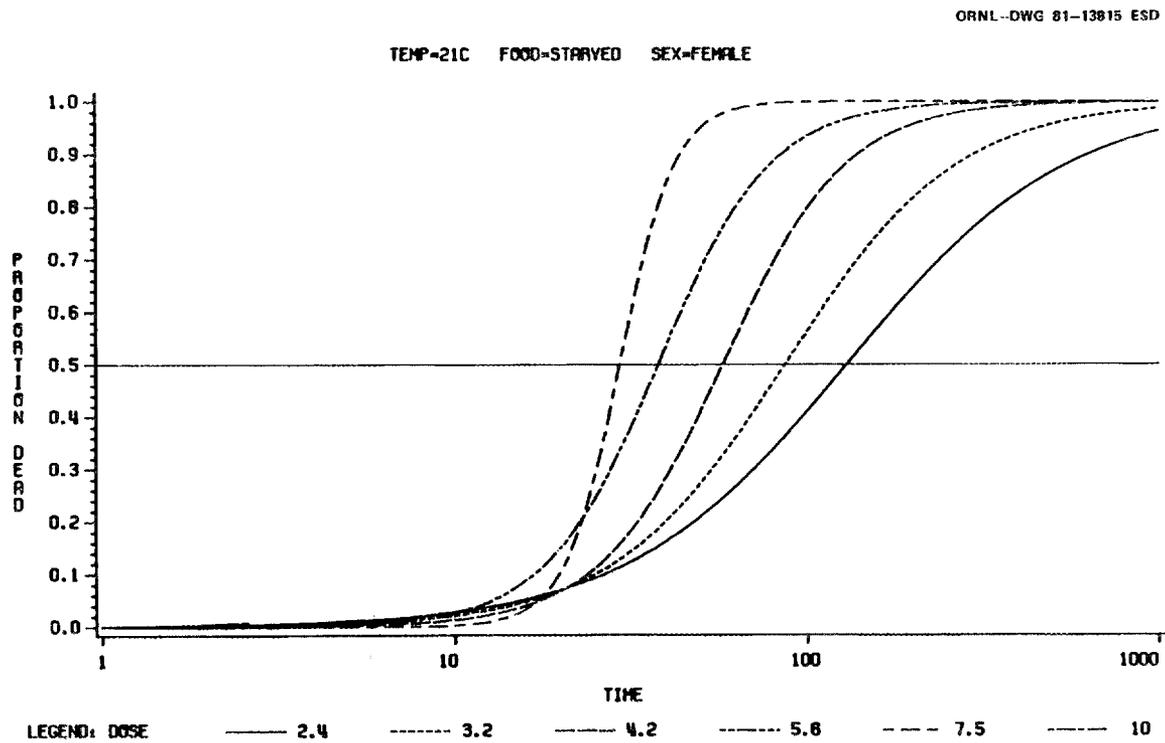


Figure B-12. Logistic survival functions of female *Diaptomus clavipes* for each acridine concentration at 21°C in the starved feeding regime

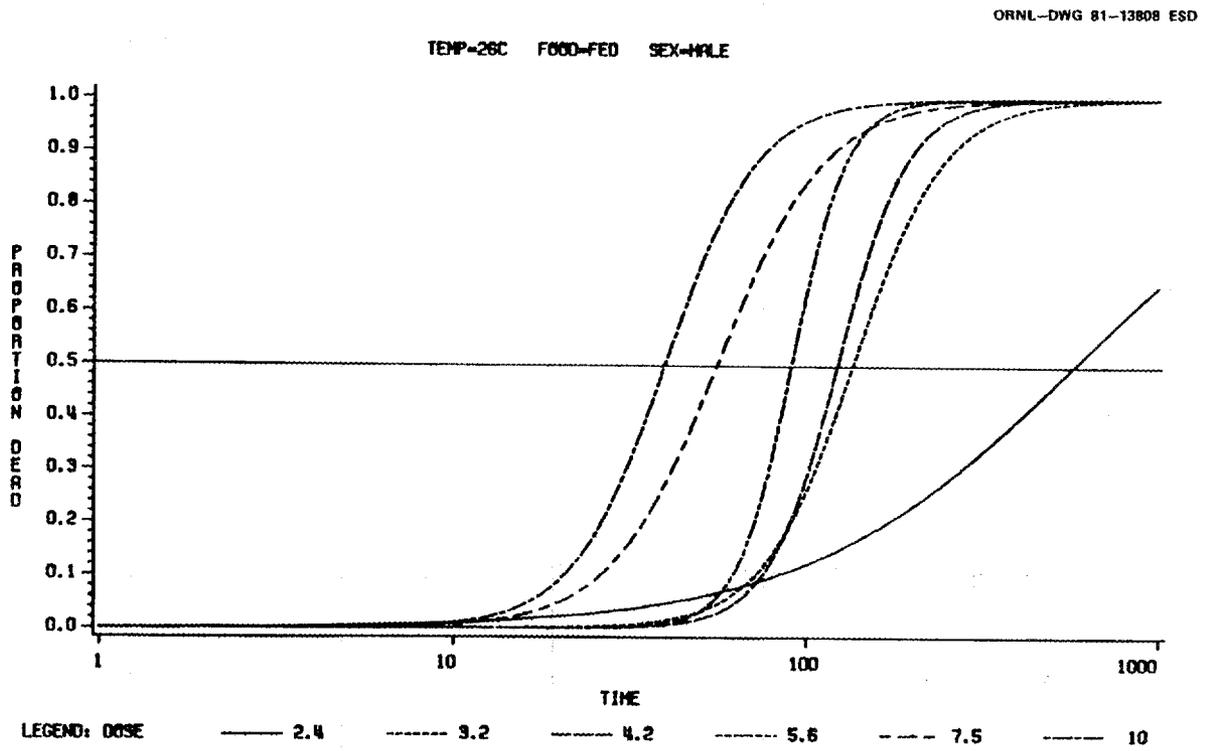


Figure B-13. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 26°C in the fed feeding regime

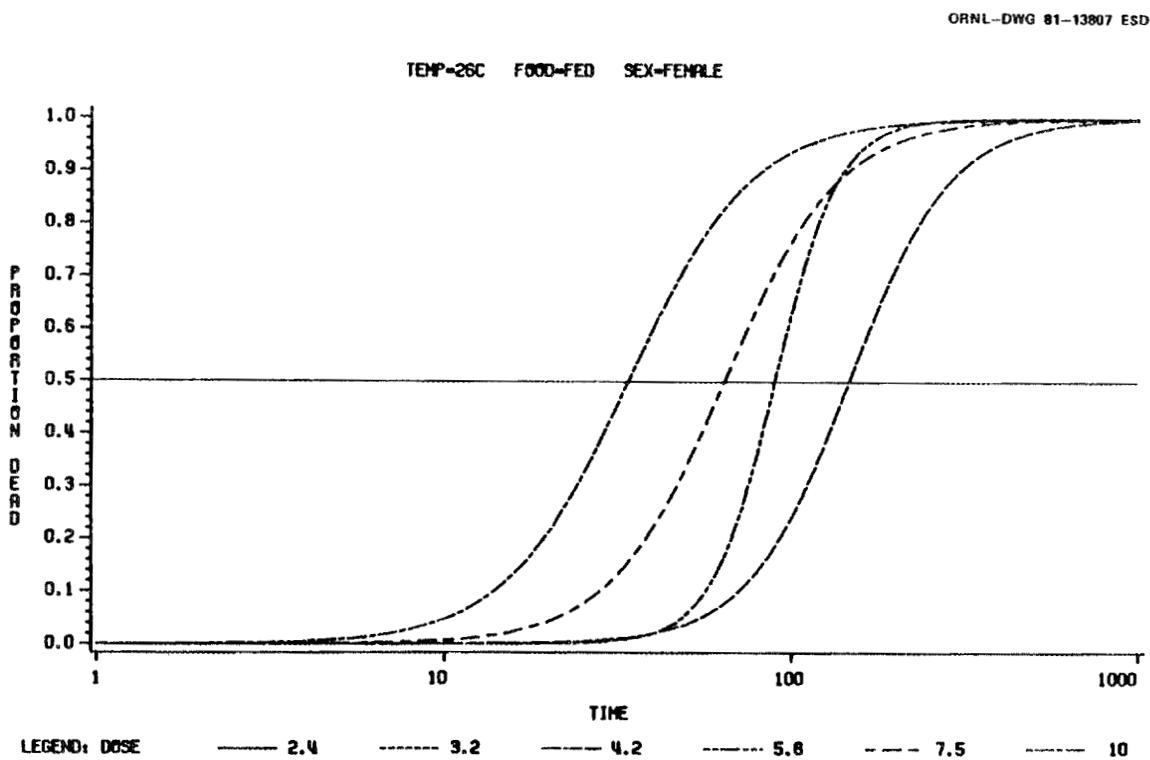


Figure B-14. Logistic survival functions of female *Diaptomus clavipes* for each acridine concentration at 26°C in fed feeding regime

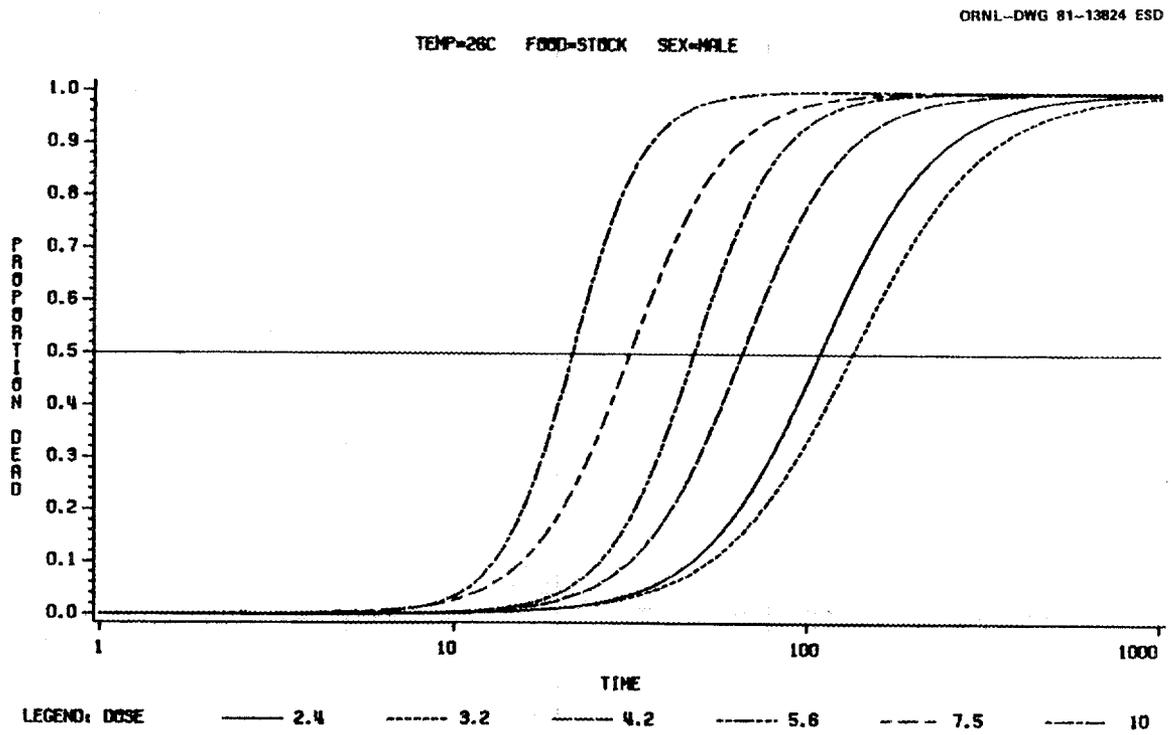


Figure B-15. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 26°C in the stock feeding regime

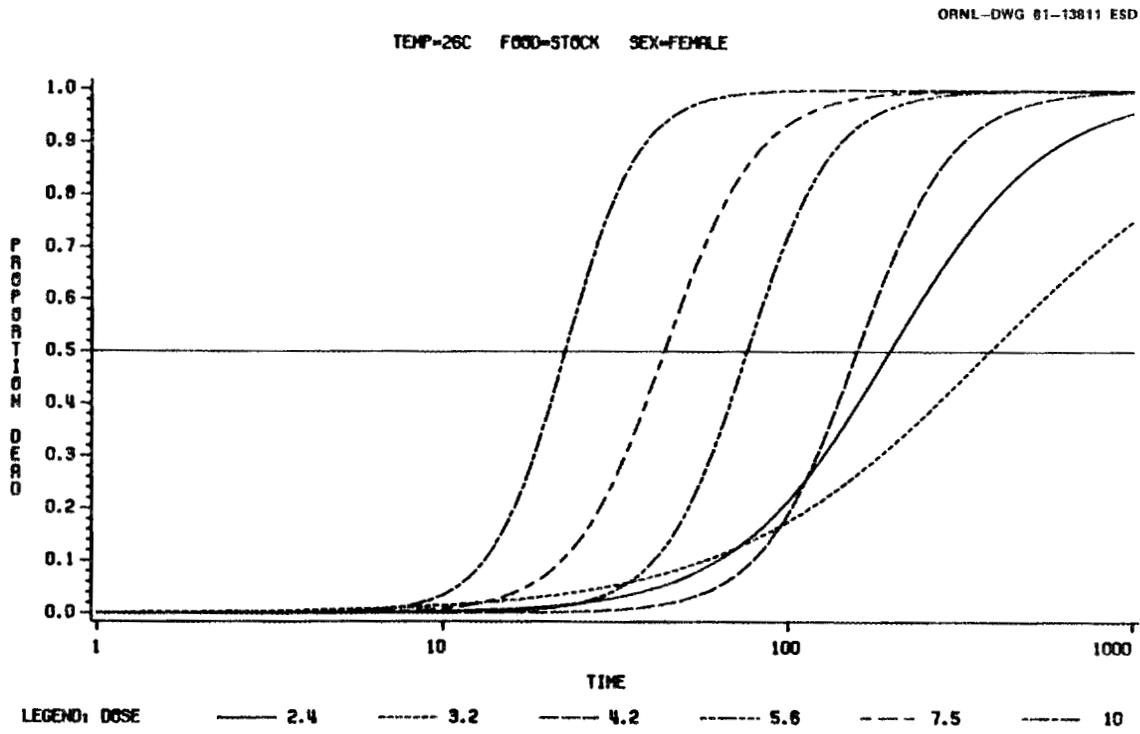


Figure B-16. Logistic survival functions of female *Diaptomus clavipes* for each acridine concentration at 26°C in the stock feeding regime

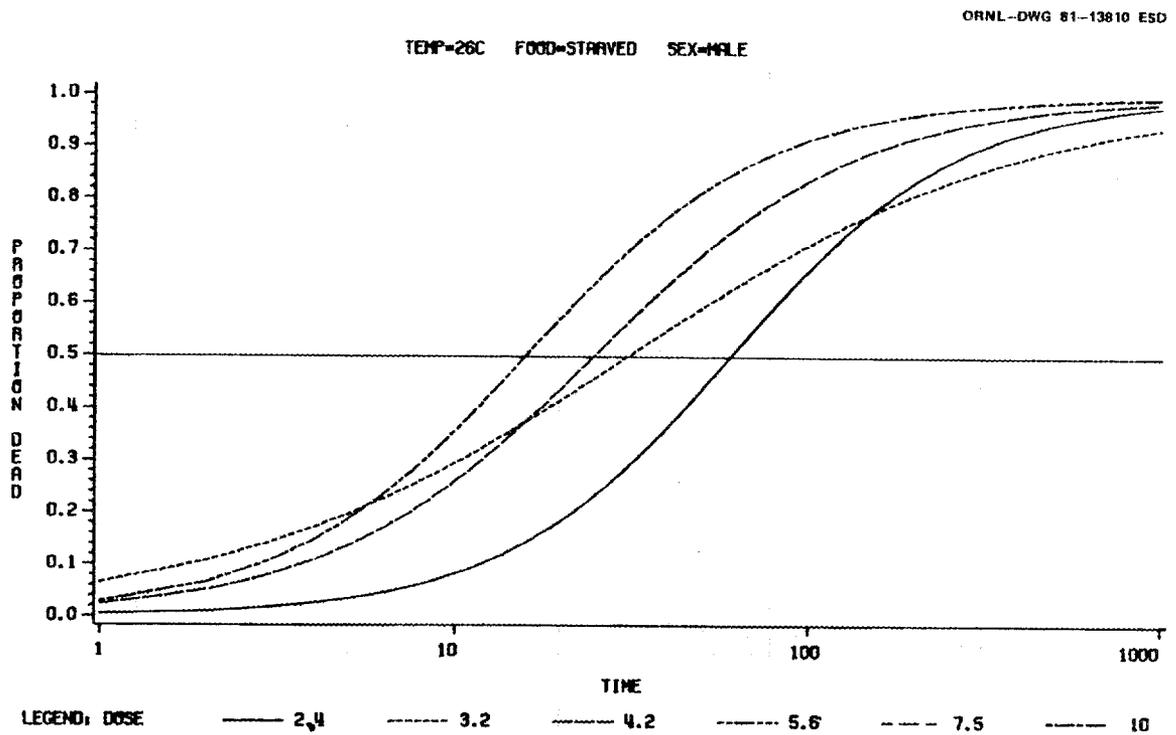


Figure B-17. Logistic survival functions of male *Diaptomus clavipes* for each acridine concentration at 26°C in starved feeding regime

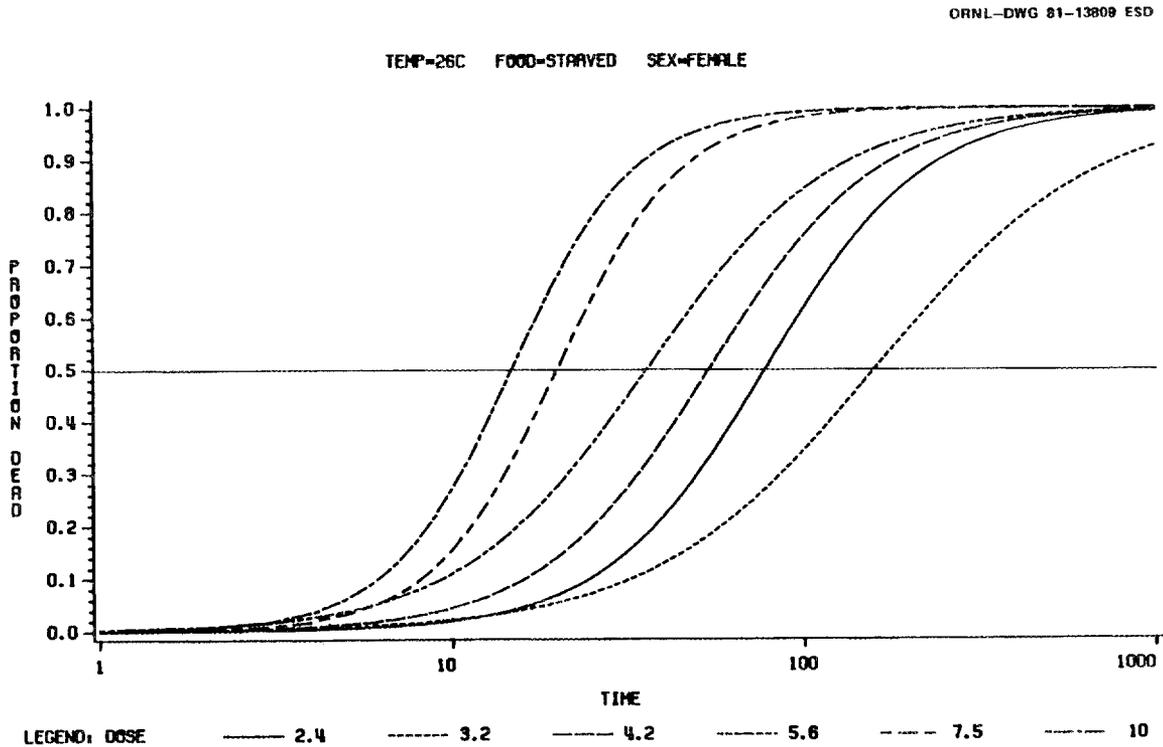


Figure B-18. Logistic survival functions of female *Diatomus clavipes* for each acridine concentration at 26°C in the starved feeding regime

INTERNAL DISTRIBUTION

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