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A static-renewal, seven day test was developed and utilized to study the effects of nutrition on the sensitivity of Mysidopsis intii Holmquist to zinc. It was found that juvenile growth was the most reliable and sensitive endpoint that could also reflect the subtle effects of low zinc concentrations and diet modifications. It was shown that even slight diet modifications could significantly alter growth over seven days. It was also shown that this small change in overall growth was accompanied by a heightened sensitivity to zinc. This heightened sensitivity was evident as a lowering of the Lowest Observed Effect Concentration of zinc in diets that suppressed growth. Though this shift in response to a toxicant is subject to variability, the fact that it was observed should make researchers and regulators aware of the potential significance of dietary influences on bioassays of pollutants.

The Effects of Nutrition on the Zinc Sensitivity of *Mysidopsis intii*

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Paul Mitchell Vance, Author

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INTRODUCTION

Pollution threatens marine and freshwater resources worldwide. The sources, methods of dispersal, and types of these pollutants are extremely diverse. This diversity complicates the process of measuring existing levels and reducing their impacts. Excessive nutrient levels, which are considered pollutants in many regional areas, are caused by municipal wastes and nonpoint sources. These nutrients cause eutrophication and detrimental biological oxygen demand. Heavy metals, radionuclides, polyaromatic hydrocarbons and many other harmful compounds arise as byproducts of industrial processes and fossil fuel combustion. Compounds or elements of this type can interfere with normal physiological functions, and many are teratogens or carcinogens. Countless instances of this type of contamination have caused major kills of aquatic organisms, closure of aquacultural areas, and direct health hazards to humans. Pesticides are commonly found in aquatic systems associated with agricultural areas and these compounds can drastically reduce biodiversity and productivity of immense drainage systems. All of these problems are associated with anthropogenic wastes and are a threat to the bodies of water we so heavily rely upon.

Current pollution monitoring programs rely on two major testing procedures; chemical analysis and bioassays (NOAA, 1990). The results of these types of tests are the basis for regulation of industrial discharges and other disposal practices. Samples to be tested are usually collected from waste effluents or areas which are suspected of contamination; however, the chemical makeup of the sample is often unknown.

Chemical analysis of water samples provides information about the concentrations of specific compounds that are present in a system. This type of analysis has been in practice for decades and offers very important information. Excessive (or insufficient) concentrations of almost any compound can be identified and traced to its source. Technology has developed to the point where researchers can now measure certain compounds present at concentrations of nanograms per liter (ng/l or parts per trillion).

Regardless of the accuracy of these analyses, one must keep in mind the limitations of such information. The total concentration of a chemical in a certain sample does not always indicate which forms of the chemical are present or how they are associated with other components of the sample. Adsorption to organic compounds or sequestration by chelating agents can drastically alter the amount of toxicant that is bioavailable (Sprague, 1985). Therefore, although chemical analysis is very important in determining

the chemical composition of a sample, it does not always provide sufficient information to predict the effects of toxicants in experimental or natural situations.

To determine the responses of living organisms to potential toxicants in water, tests are conducted that expose organisms to either defined or unknown samples. Bioassays are conducted by first diluting a sample in a series with uncontaminated control water. The sample may be a complex effluent collected from the field or a specific toxicant added to uncontaminated water in the laboratory. Bioassays can also be used to test sediments, dredge spoils, or other sample types. Test organisms are exposed to each treatment for a pre-determined time under strictly controlled experimental conditions. By identifying the specific experimental treatment that causes a significant detrimental effect, one can assign a relative toxicity value to the original sample. This type of study, while not being chemically quantitative, provides information on actual biological effects of undefined samples.

Often, before bioassays are implemented, extensive preliminary tests are conducted to determine the sensitivity of an animal to "reference toxicants". These preliminary tests use known concentrations of prepared toxicants to determine the general sensitivity of a species. Series of tests are often conducted, utilizing compounds that represent the major classes of toxins.

There are many different types of aquatic toxicity tests. Exposure systems can utilize flow-through, static-renewal, or static chambers. Tests range in duration from relatively rapid tests lasting minutes to hours (acute tests), to long term (chronic) tests that follow individuals or even populations from several days to several weeks incorporating exposure to all life stages from egg to egg. Sediment tests, suspended material tests, mesocosm studies, and many other types of tests are being developed and used for regulatory purposes.

Acute tests are used to determine the effects of sudden exposures to high concentrations of toxicants. The response parameter usually measured in such tests is mortality (Nimmo and Hamaker, 1982; ASTM E729-88a, 1989). In natural settings however, the inhabitants of a community are normally exposed to varying concentrations of a mixture of compounds over longer periods of time. To study these more subtle effectors of aquatic life, long term exposures are conducted to determine how chemical pollutants alter the growth or reproductive capacities of test organisms (Ward *et al.*, 1981; Breteler *et al.*, 1982; Gentile *et al.*, 1982; DeWitt *et al.*, 1992).

The United States Environmental Protection Agency (USEPA) is responsible for designing and implementing marine pollution testing programs that are carried out in laboratories nationwide. These programs use many different types of tests to monitor the specific toxicity of

effluents, dredge spoils, or other waste products specific to an area. Many different species are used in marine tests, including: sheepshead minnow (Cyprinidon variegatus), silversides (Menidia spp.), copepods (Acartia spp.), polychaete worms (Capitella capitata and Neanthes arenaceodentata), mysid shrimp (Mysidopsis spp.), grass shrimp (Palaemonetes spp.), amphipods used for sediment tests (Rhepoxynius, Eohaustorius, Gammarus, and Leptocheirus), abalone (Haliotus rufescens), macroalgae (Macrocystis pyrifera), sea-urchins (Strongylocentrotus spp.), and many others (ASTM E729-88a, 1989; Anderson *et al.*, 1990; DeWitt *et al.*, 1992).

Before a species can be utilized in pollution monitoring tests, it must be proven to be adequate for such uses by extensive preliminary study. Ideally, a test organism should have the following characteristics:

1) Sensitivity to many pollutants so that impacts of different toxicants can be studied.
2) Representative of a large group of related organisms.
3) Short life cycle to allow for tests that include both growth and reproductive endpoints.
4) High growth rates to allow for detectable differences in body size in short time periods.
5) Adaptability to artificial culture conditions. A reliable source of large numbers of healthy animals is crucial.
6) Has an important role in the ecology of its community.

Bioassays are important tests because they can provide greater insight concerning the biological impact of pollution in natural situations. They can also provide informative guidelines or specific criteria for the regulation of municipal or industrial discharges into aquatic systems. Inaccurate results of analytical tests or bioassays could either allow excessive discharges or cause unnecessary regulation and result in higher economic costs.

In most types of aquatic bioassays, the diets of test organisms are usually strictly controlled. They may be fed defined artificial diets (Keating, 1983; Schlekat *et al.*, 1992), monocultured prey (Hatakeyama and Yasuno 1987; ASTM E1203-92, 1992; ASTM E1463-92, 1992; Cerda and Olive, 1993), or nothing at all (Ward *et al.*, 1981; Nimmo and Hamaker, 1982; ASTM E1367-92, 1992). These are regular practices for both laboratory tests and for the culturing of stock populations of animals for future tests. Controlled diets such as these, probably do not replicate the diets that these organisms would experience under natural conditions. Laboratory diets lack at least the diversity if not the predominant natural prey species. The combination of unnatural diets and the artificial environment of the laboratory could significantly alter any effects a particular toxin might have on an animal under natural conditions (Robinson *et al.*, 1988).

In order to identify cases in which differences in diet could alter an organism's response to a toxicant, a

specific test system must be thoroughly defined. This study defines such a test and utilizes it to investigate the effects of diet quality and quantity on the zinc sensitivity of the mysid shrimp, Mysidopsis intii (Holmquist, 1957).

Mysidopsis intii was chosen as a test species for this study because it fulfills all of the previously listed criteria. M. intii is native to the eastern Pacific Ocean (Holmquist, 1957) and therefore is suited for tests conducted on Pacific waters. For compounds tested on M. intii to date, it shows sensitivities that are as low as that of Mysidopsis bahia, the mysid species currently utilized by the USEPA (Shaffer and Langdon, Submitted). The life cycle of M. intii is completed in 20 days and it reproduces well in laboratory cultures. Mysids play an important role in the food web of aquatic communities worldwide (Markle and Grant, 1970; Odum and Heald, 1972; Kirn *et al.*, 1986; Loch and Miller, 1988; ASTM E1367-92, 1992). Because of their importance in food webs and their relatively high sensitivity to toxins, mysids are appropriate indicators of the general health of marine communities. Research with M. intii has shown that it would be an ideal marine crustacean for monitoring pollutants of the northeastern Pacific Ocean (Kreeger *et al.*, 1991; Wagner, 1994; Langdon *et al.*, submitted; Shaffer and Langdon, submitted).

After a standard test using Mysidopsis intii was in place, the sensitivity of the mysid was documented under specified conditions with a "Standard" diet. That "Standard" diet was then altered to test how nutrition affects the growth and zinc sensitivity of the mysid. Three different diet modifications were tested - one quantitative, and two qualitative. The quantitative change was made by adjusting the total ration fed to the mysids. The first qualitative change involved compromising the diet by eliminating one of the two prey species from the "Standard" diet. The second qualitative change was made by enriching one of the prey species for two days prior to using it as food in toxicity tests. Brine shrimp (Artemia sp.) nauplii were used as one of the food species. They were enhanced by feeding them single-celled diatoms, (Chaetoceros calcitrans), and the flagellate, Pseudoisochrysis paradoxa. This enrichment increases the total fatty acid content of the Artemia, and the proportion of omega-3 highly unsaturated fatty acids present in the lipid component (Kreeger *et al.*, 1991) and represented a biochemical modification of the mysids' diet.

The diet modifications described were used to study the effects of nutrition on both the growth and the zinc sensitivity of M. intii. The close relationship between dietary intake and an organism's growth represents a basic premise of the study of nutrition (growth is equal to food intake minus metabolic energy, tissue requirements and

waste products). "Animal nutrition has been defined as the series of processes by which an animal takes in and assimilates the various foods needed to promote growth and maintain and replace worn tissues" (Wilson, 1979).

The effect of nutrition on an animal's response to a toxicant is a complex and often indirect process which involves many complex biochemical pathways (Klaassen *et al*, 1991). An organism's resistance to any particular toxin depends on its specific defenses to that toxin and its general health. If an animal's diet is not adequate, it may not have the energy available to sufficiently chemically regulate a toxin, or the amino acids necessary to stage an intracellular sequestration. The following experiments attempt to bring to light the interaction between diet and zinc sensitivity of M. intii, and to predict the magnitude of such an interaction in a simple test situation.

MATERIALS AND METHODS

STANDARD TOXICITY TEST DEVELOPMENT:

Mysidopsis intii has been continuously cultured in the laboratory for five years using conditions (temperature, salinity, and photoperiod) determined previously (Kreeger *et al.*, 1991; Langdon *et al.*, Submitted). Test animals were collected from these cultures for all experiments. Test water temperature was maintained at 20°C (± 2°C) by

allowing it to equilibrate with ambient room temperature. Room temperature was controlled with a heating\air-conditioning unit. When necessary, salinity was adjusted to 34 ppt with concentrated brine (70 - 140 ppt). Photoperiod was controlled with a 24-hour Dayton electrical timer (16 h light : 8 h dark).

Life Cycle (Growth and Development):

Devising a test that could identify real effects of nutrition required identification of the most sensitive period of growth of M. intii. Documentation of the life cycle of M. intii was undertaken to establish key periods during which important points of growth and development occur (Experiment #1). To effectively monitor these developmental changes without altering their timing or magnitude, a sampling-without-return method was employed. A cohort of 200 juveniles released on the same day were collected and subsequently held in a 40 l glass aquaria. They were fed Artemia nauplii and the harpacticoid copepod, Tigriopus californicus,(Tig) *ad libitum*. Water was changed every other day by siphoning at least 90% of the water through a 100 μm mesh and refilling the aquarium with 0.7 μm filtered seawater. On days that did not include a water change, samples of 10 animals were taken for general observation and length measurements. Body length was defined as the distance between the anterior edge of the eyes and the tip of the uropods (see Figure 1a).

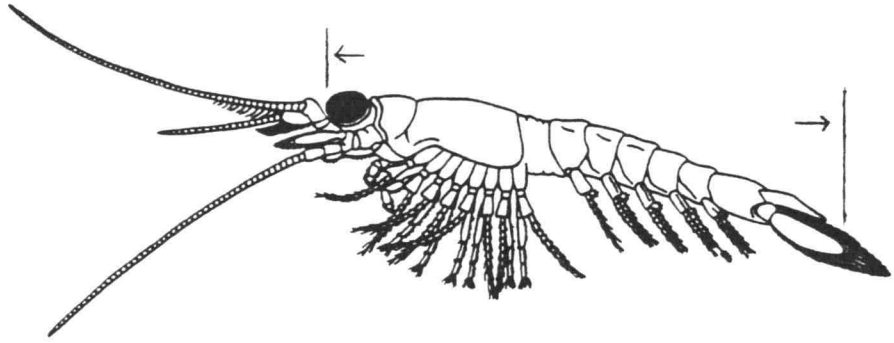

Figure 1a. Line drawing of Mysidopsis intii and body length endpoints (anterior edge of eye and posterior tip of uropods).
(Drawing reproduced from Langdon *et al.*, Submitted)

Figure 1b. Digital image of 7-day-old Mysidopsis intii and body length measurement line.

After body length was determined, the morphological characteristics of each animal were recorded. Notes were made of general morphology, coloration, secondary sexual characteristics, and other signs of development.

Growth was monitored by measuring changes in average body length over time. Measurements were taken by first immobilizing a live animal on a depression slide by reducing the amount of water surrounding it. The mysid was oriented so that it rested on its ventral surface and the slide was then placed on top of a graduated glass reticle and viewed through a dissecting microscope. The reticle had a 10 mm scale with markings every 0.1 mm. The anterior edges of the mysid's eyes were positioned so that they were on the "zero" line and the measurement was taken to the tips of the uropods. Three such readings were recorded for each animal and a mean value was calculated.

Once basic information on the life cycle and environmental needs of M. intii were obtained, pilot tests could proceed. Because of the short life cycle of M. intii, and the high growth rate exhibited in early stages, the initial two weeks of growth was chosen as a suitable time period in which to develop growth and/or reproductive tests.

Weight / Length Relationship:

An experiment was conducted that examined the relationship between the body length and the dry weight of

M. intii. A group of 45 mysids were used. The sex and length of each individual were recorded. Each animal was rinsed with 0.5 M ammonium sulfate to remove sea salts and placed on a muffled, tared, square of aluminum foil (baked at 450° C. for at least 12 hours). The mysid was wrapped in the foil and dried at 60° C. for 24 hours. The foil packet was weighed, and the tare weight was subtracted from the total weight to obtain each animal's dry weight. The cube root of the dry weight value was then compared with body length using linear regression.

Length Measurement Methods Comparison:

Shortly after the initial growth / life cycle study was completed, video image analysis equipment became available. Because of the limited level of precision of the reticle measurement method (0.1 mm), it was decided to determine if body lengths could be measured more quickly or with greater accuracy using the image analysis system. The video image analysis system consisted of a Zeiss STEMI-SR binocular dissecting microscope, a fiber optics illuminator, a Cohu video camera, a Leading Edge personal computer equipped with digital image analysis software (Java, from Jandel Co.), and a Lenco PMM-925 black and white monitor. With this system, an image could be digitized and analyzed with the software. The system was calibrated using a glass reticle with a 10 mm scale subdivided into 0.1 mm graduations. Measurements were

taken by placing a mysid on a depression slide and positioning it on it's side to obtain a profile view. The slide was placed under the microscope and adjustments were made in the animal's position and lighting in order to obtain a clear image of the entire animal, including the often transparent uropods. When the image was satisfactory, the image was "frozen" and digitized by the computer. Using a "mouse", the profile of the mysid image was traced along the dorsal side from the anterior edge of the eye to the tip of the uropods. The contour of the abdomen was followed as closely as possible using however many line segments necessary to form a smooth line (Figure 1b). Ten animals from each experimental treatment were measured. These measurements were automatically stored in a data worksheet and later imported into "Statgraphics" (Version 4.0, Statistical Graphics Corporation; Orem, UT) for analysis.

Measurements from each of the two methods were compared for variability and ease of data collection. A group of 10 animals was measured first with the reticle method and then with the image analysis system. The length of each animal was measured three separate times with each method. The variation within each set of three measurements was compared between the two methods. For each set of measurements, the coefficient of variation (CV) was calculated. A paired t-test was then used to compare the CVs between the two length-measurement methods.

Defining a "Standard" Test Diet:

Throughout the initial period of the EPA-sponsored project, the effects of diet on growth, mortality, and reproductive capacity were observed. Choosing a standard diet for all the tests required consideration of many factors. Test animal growth, health, and hardiness are very important factors when one wishes to make inferences between test results and phenomena occurring in the wild. Reliable availability of dietary components of consistent quality is crucial when comparing results from different experiments for either test development or inter-laboratory studies. Cost is another factor that cannot be ignored for obvious reasons. After careful consideration of these factors, it was decided to supply the test animals with 100 newly hatched Artemia nauplii/mysid/day together with 125 Tigriopus/beaker/day. This "Standard Diet" was used as a reference for comparison of diets throughout the rest of the study.

The Artemia were hatched from reference grade cysts (RAC II - Reference Artemia Cysts) provided by the USEPA, Narragansett, RI. This diet was chosen because Artemia nauplii provided sufficient nutrition for growth (Gentile *et al.*, 1982; Lussier *et al.*, 1985) and it included the Tigriopus supplement necessary for good reproduction (Kreeger *et al.*, 1991). This "Standard" diet also allowed for modifications that could enhance or compromise its nutritional value for experimental treatments.

Start Day and Duration:

Two experiments were designed to pinpoint the best times for test initiation and test duration. Tests were conducted using the conditions outlined in Table 1. The "Standard" diet was provided daily. Only the age of the mysids at the onset of the test (initial zinc exposure) and the duration of the tests were varied. Multiple treatment sets were prepared and water change schedules were manipulated so that different exposure durations could be compared. All duration experiments used nominal concentrations of 70 ppb zinc versus no zinc as exposure treatments. This level of toxicant was chosen because it would provide significant growth reduction. Starting with mysids less than 24 hours old, durations of 4, 5, 6, 7, and 8 days were tested. Another test with a seven-day exposure period was examined by starting with four-day-old mysids and terminating the test on day 11.

The results of these tests were compared using one-way analysis of variance (ANOVA) and Tukey's multiple range tests to determine which exposure duration resulted in the best test schedule (the start day and duration that detected differences in final lengths between control and zinc exposed animals).

Description of the Standard Seven-Day Toxicity Test:

Based on the results of experiments described in the previous section, a seven-day static-renewal test was developed. Tests were initiated on the day that newly released mysids were collected (day zero), and terminated when the mysids were seven days old. Fifteen mysids were placed in a three liter beaker containing two liters of 0.7 μm filtered seawater. Each test consisted of control beakers with no zinc added, and various numbers of other treatments containing nominal concentrations of zinc. Diet comparisons were conducted by preparing a second complete set of all control and zinc treatments and supplying the animals in this second set with the experimental diet being tested. All treatments were tested in triplicate. The endpoints measured were final body length (growth) and mortality. Food organisms were harvested, counted, and supplied to the mysids daily. Water in each test chamber was changed every other day. Physical parameters of all experiments were maintained within the values given in Table 1. The daily schedule for the standard test protocol is outlined in Table 2.

Table 1. Standard experimental test conditions. Basic design description and acceptable ranges of physical parameters.

STANDARD EXPERIMENTAL TEST CONDITIONS

Test Parameter	Value (range)
Salinity	34 ± 2 ppt
Temperature	20 ± 1°C
Photoperiod	16 h light : 8 h dark
Test Chamber	3 l Borosilicate beaker
Mysids per Beaker	15
Standard Diet	100 *Artemia*/mysid/day and 125 Tig/beaker/day
Exposure Period	Day 0 - Day 7
Number of Replicates	3
Test Water	0.7 μm filtered seawater

Table 2. Schedule for conducting standard seven-day tests.

SCHEDULE FOR STANDARD SEVEN-DAY TESTS

DAY	TASKS
Week -1	Prepare diet components Have zinc stocks ready Clean and label beakers
-1	Filter water Isolate brooding females Set out beakers Make beaker labels
0	Collect Day-0 mysids Randomize into seeding sets Recount mysids Add test water to beakers Take samples for Zn analysis Feed
1	Check Temp Feed
	Check Temp Water Change Feed
3	Check Temp Feed
4	Check Temp Water Change Feed
5	Check Temp Feed
6	Check Temp Water change Feed
7	Take down test Count survivors Chill survivors overnight
Post Test	Fix mysids Submit chemistry samples Measure fixed mysids Statistical analysis

PREPARATION FOR A TEST:

Before an experiment could be started, the following preliminary procedures had to be conducted:

Juvenile Harvest System:

The seven-day growth test was started by harvesting juveniles less than 15 hours old from the juvenile harvest system (JHS). The JHS (Figure 2) was a shallow glass aquaria (55 X 60 X 15 cm) filled with 6 cm of 0.7 μm filtered seawater. This was equivalent to approximately 20 liters. The JHS was divided into two unequal parts with a strip of 1000 μm Nitex® mesh. This size of mesh allowed only the newly released mysids to swim through. Segregation of the age classes of mysids in this way helped decrease cannibalism and allowed easier collection of the juveniles.

The JHS was set up in the afternoon or early evening the day before the test. Brooding females which appeared to be ready to release (distended brood pouches with a brownish or grayish color) were individually pipetted from the brood cultures and placed in the larger compartment of the juvenile harvest system together with Artemia and Tigriopus (greater than or equal to the standard ration).

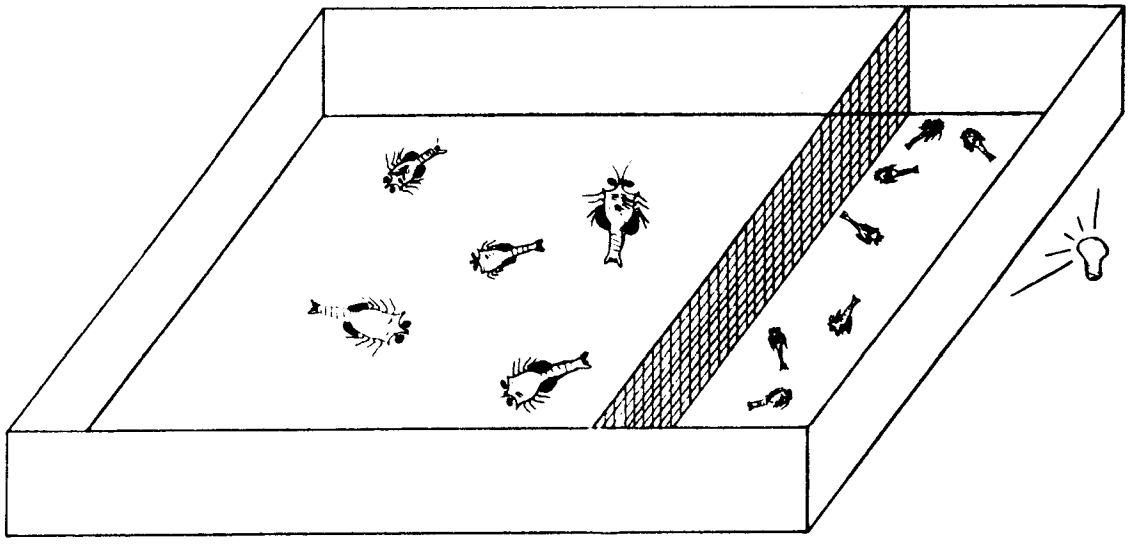

Figure 2. Diagram of the juvenile harvest system. Females with broods in large portion of tray. After release, juveniles swim through the mesh toward the light to the smaller, "safe" side of the tray.

The top of the JHS was covered with black plastic and a 40-watt incandescent lightbulb was mounted near the juvenile side of the tank. A 24-hour timer was used to turn the bulb on for two one-hour periods during the night (at 2 a.m. and 4 a.m.). This allowed time for the juveniles to move through the screen into the "safe" side. The positive phototactic behavior exhibited by M. intii in these conditions facilitated the movement of newly released juveniles to the "safe" side of the aquarium. The morning of test initiation, juveniles were counted and transferred from the JHS into 20-liter glass aquaria containing at least 15 l fresh filtered seawater. They were provided Artemia, *ad libitum*, and held in these aquaria until needed for the test (less than 4 h).

Glassware Cleaning:

All glassware was cleaned by soaking in 0.02 % sodium hypochlorite solution for at least 12 hours, scrubbing with a scouring pad, and finally, a 15 minute soak in 10 % hydrochloric acid. Thorough rinses with dechlorinated water were carried out after each washing step. Before use, any glassware involved with the test was rinsed with test water.

Test Water:

All seawater used in the test was filtered using a series of 200, 30, and 0.7 μm Pall cartridge filters, pumped into 200-liter Nalgene® plastic tanks, and allowed to equilibrate to room (test) temperature (20 °C). An adequate volume of test water was collected to last the entire test to maintain constant water quality. During periods of increased river flow (normally during winter months), the salinity of Yaquina Bay water could fall to 25 ppt and sometimes had to be adjusted to 34 ppt. This was accomplished by adding an appropriate volume of hypersaline brine (HSB). Brine solution was prepared by partially freezing 60 l filtered seawater overnight. The next morning, the remaining liquid (hypersaline brine, 70 - 140 ppt) was transferred to a 20-liter plastic container with a siphon. Large quantities of high salinity HSB could be prepared by freezing and refreezing multiple volumes so that one final batch could be used for several successive tests.

Zinc Solution Preparation and Analysis:

Zinc exposures consisted of nominal concentrations of zinc chloride prepared by diluting stock solutions with control water. Stock solutions were 10 or 70 mg/l zinc chloride (Sigma Chemical Co., St. Louis, MO) in deionized water. Zinc stock solutions were stored at 4°C and kept for no longer than 6 months. Zinc treatment solutions were

prepared in a two-liter graduated cylinder by initially adding the appropriate volume of zinc stock and then filling the cylinder to the two liter level. A sheet of Parafilm® was placed over the top of the cylinder and the solution was mixed by inverting the cylinder at least three times. Water samples (20 ml) were collected from the graduated cylinder before the water was poured into test beakers and stored at 4°C in acid-washed scintillation vials for zinc analysis.

Analyses of water samples for zinc were performed by American Scientific Corporation staff (contract personnel at the USEPA - Environmental Research Laboratory (Narragansett) - Pacific Marine Ecosystem branch - Newport, OR, Hatfield Marine Science Center). Samples were acidified to below pH 2.0 using trace metal grade nitric acid within 24 hours of sampling. Analysis for total zinc was performed with a Perkin Elmer 5100 Flame Atomic Absorption Spectrometer.

Culture of Food Species:

Tigriopus californicus, an harpactacoid copepod, were collected from supratidal splash pools at Yaquina Head Outstanding Natural Area and Rocky Creek State Park on the central Oregon coast. The copepods were separated from contaminating debris by repeatedly adding seawater and decanting the swimming animals. These animals were used to start cultures in fiberglass trays (130 X 95 X 15 cm)

containing approximately 150 l seawater. Cultures were maintained at 40 - 50 ppt salinity and at 20 - 25°C. T. californicus cultures were fed phytoplankton mixtures of Pseudoisochrysis paradoxa (isolate VA-12), Chaetoceros calcitrans, and Isochrysis galbana (Caribbean strain). Each copepod culture was supplied with 10 l of algae every week (at cell densities between 1 x 10^6 and 5 x 10^6 cells per ml). In addition, about 2.0 g of ground Tetramin® fish flakes were added to each culture every week. Copepods were collected from culture trays by siphoning a sufficient volume of the culture through a 100 µm mesh sieve. They were rinsed with seawater, counted, and offered to the mysids.

Artemia cysts were hydrated and hatched daily in 1.0 l glass separatory funnels. Approximately 6.0 g of Artemia cysts (Rac II reference cysts from USEPA, Narragansett, RI) were placed in the funnel together with 1.0 l filtered seawater. The cultures were gently aerated. The cysts were incubated in 30-34 ppt water at 25°C for 48 hours. After that time, aeration was removed and the nauplii were concentrated at the bottom of the funnel. This separated them from unhatched cysts and other floating debris. These animals were then collected on a 37 µm mesh sieve, rinsed with seawater and counted in preparation for feeding.

CONDUCTING A TEST:

After all necessary items were ready and at hand, a test could be started. The schedule is outlined in Table 2.

Test Animal Counting and Randomization:

After rinsing the beakers with test water, approximately 50 ml test water was placed in each beaker and the mysids were randomly distributed among the experimental beakers. Randomization was carried out by pipetting three randomly selected mysids at a time into each of the beakers until all of the beakers held 15 mysids. The mysids in each beaker were then recounted to verify the correct final number (15). Excess water (i.e. greater than 50 ml) remaining in the beaker from the randomization process was removed by pipet before the test water was added. Beakers which had no zinc added were labelled and filled with water (2.0 l) directly from the test water storage tank via a Tygon® hose.

Feeding:

Mysids were fed the "Standard" ration of 100 newly hatched Artemia/mysid/day and 125 Tigriopus/beaker/day. The ration of Artemia was adjusted according to the number of mysids remaining in the beaker. Because of the lower number of Tigriopus provided (8.33 per mysid per day, assuming 15 mysids/beaker), the density of this prey item was maintained throughout the test. Lower Tigriopus

densities might have made it difficult for the mysids to find and capture them. The rations just described were sufficient for the first 10 days of the mysid's life cycle. This "Standard" diet could be completely eaten in less than 24 hours by mysids more than 10 days old. If a test was to continue beyond 10 days, the ration would need to be increased to maintain food availability.

Toxicity Test Water Change:

The test solutions were changed on days 2, 4, and 6. This was accomplished by siphoning out at least 90% of the water along with any debris on the bottom of the beaker. The water that was removed was checked for any mysids accidentally transferred from the test chamber. Any mysids removed in the siphoning process were returned to the test beaker. The animals were then counted to document survival, and the beaker was refilled with fresh test solution. Missing mysids were counted as dead. If a mysid was inadvertently injured or lost during transfer, the initial number was reduced by one to compensate for the loss. New test solution was prepared as described earlier. Water samples for zinc analysis were collected immediately prior to adding the test solutions to the beakers.

Test Termination:

After the seventh day of zinc exposure, the test was terminated by removing and counting all obviously living

mysids from the test beaker. Any mysids that were non-motile and did not respond to gentle prodding with the end of a smooth glass rod were considered dead and were discarded. The live animals were recounted and transferred into a 20 ml plastic vial and placed in the refrigerator (4° C) overnight. This cooling process helped prevent the curling of the mysid's abdomens that could make measuring mysid body lengths more difficult. The next morning, buffered formaldehyde (pH 9.0) was added to fix the animals for storage until they could be measured.

Data Analysis:

Measured zinc concentrations for each treatment level were averaged and the standard deviation was calculated. Statistical analyses were conducted in "Statgraphics" (Version 4.0, Statistical Graphics Corp., Orem, UT). Analysis of variance tests (ANOVA) were conducted on the final average body lengths of mysids to determine the level of significance of the difference between means of the experimental treatment groups ($\alpha = 0.05$). ANOVA was also conducted between the no-zinc control beakers of the two experimental diets ($\alpha = 0.05$). Tukey's multiple range tests were conducted to identify which treatment groups had final mean body lengths significantly different from the control group within each diet treatment ($\alpha = 0.05$). The lowest zinc treatment that was associated with a group of animals that had final mean body lengths significantly

different from the control group was identified as the Lowest Observed Effect Concentration (LOEC). Survival data collected on the final day of the test was converted to a percentage, arcsine transformed, and also analyzed using ANOVA.

NUTRITION EXPERIMENTS:

Several experiments were conducted using the standard test conditions previously described (see Table 1). Only zinc concentrations and diet treatments were manipulated to determine nutrition and zinc sensitivity interactions.

Range Finder: (Experiment #6)

To determine the range of zinc concentrations in which M. intii demonstrated a reduction in growth, an initial range-finder test was conducted using the "Standard" diet only. Four zinc concentrations (18, 32, 56, and 100 ppb) and a no-zinc control were prepared.

Initial Tigriopus Elimination Test: (Experiment #7)

The first type of qualitative diet modification was made by eliminating Tigriopus from the diet. Final body lengths of mysids receiving the "Standard" diet were compared to those receiving the diet without Tigriopus. Both treatments received a full ration of Artemia each day. The zinc concentrations tested were 0, 50, and 70 ppb.

Follow-Up *Tigriopus* Elimination Test: (Experiment #8)

In an effort to substantiate and expand the results of experiment #7, the *Tigriopus* elimination experiment was repeated. The range of zinc concentrations tested was expanded to include nominal values of 0, 21, 37, 50, 70, 101, and 230 ppb.

Initial *Artemia* Enrichment Test: (Experiment #9)

The second type of qualitative diet modification (a diet enhancement) was made by feeding (enriching) the *Artemia* on phytoplankton before using them as food in a test. The *Artemia* were cultured in ten liters of a mixture of *Pseudoisochrysis paradoxa* and *Chaetoceros calcitrans*, (cell density = 1×10^6 to 5×10^6) for 48 hours before the test was initiated. The two diets included *Tigriopus* copepods and either newly hatched ("Standard") or enriched *Artemia*. Nominal zinc concentrations were 0, 50, 70, and 101 ppb.

Follow-Up *Artemia* Enrichment Test: (Experiment #10)

In an effort to substantiate and expand the results of experiment #9, the test of the effects of enrichment of *Artemia* used in the diet was repeated. The range of zinc concentrations tested was also expanded and included nominal values of 0, 37, 50, 70, 101, and 230 ppb.

Ration Test: (Experiment #11)

This experiment tested a quantitative alteration of the "Standard" diet by supplying the mysids with 100%, 50%, or 35% of the "Standard" diet ration. The percentages of the "Standard" diet were the number of both prey species. In a preliminary experiment several rations were examined which showed that the growth of M. intii was not significantly reduced until the ration was lowered to 10% of the "Standard" diet. That response is quite similar to the ration reduction and growth limitation reported by Cripe *et al.* (1989) for M. bahia. In order to initiate different levels of potential growth, the diet treatments started with very low rations for the newly released mysids and were increased in a stepwise manner until the test rations reached 35% and 50% of the standard ration. The high ration was the "Standard" ration of 100 Artemia/mysid/day and 125 Tigriopus/beaker/day. The medium ration started at 7%, on day 3 it was increased to 25%, and on day 5 it was increased to 50%. The low ration started at 4%, on day 3 it was increased to 7%, and on day 5 it was increased to 35%.

RESULTS

STANDARD TOXICITY TEST DEVELOPMENT

Life Cycle (Growth and Development):

Mysidopsis intii were released as fully functional juveniles after a seven-day brood period. Almost immediately after release, juveniles were observed swimming, orienting into water currents, and feeding on Artemia nauplii. The body length of newly released juveniles was 1.8 to 2.0 mm. Some of the highlights of the life cycle of M. intii can be seen in Figure 3. At the time of their release from the brood pouch and for some time thereafter, the two sexes of M. intii were indistinguishable. Only after a week of growth could sexes be distinguished. At that time abdominal pleopods could be seen developing on the males. The females did not yet possess these appendages, and when they did appear, they were much smaller than on the males. Another distinguishing feature was the antennal scales. The antennal scales of the male extended well past the anterior edge of the eye whereas those of the female just reached or barely surpassed that point. The antennal scale dimorphism may have been visible one or two days sooner than the occurrence of abdominal pleopods but was a much more difficult characteristic to see and/or quantify.
Day seven to day nine was the earliest that sexual dimorphism was observed; males had abdominal pleopods and

elongated antennal scales and females showed faint signs of developing brood pouches and had no abdominal pleopods. By day 12 the brood pouch was complete although still small. Quite often on day 13, females had eggs in their brood pouches. The brood usually consisted of 8 - 12 spherical, light blue eggs held in position by the two oostegites. The structure of the oostegites allowed exposure to ambient seawater and they were observed expanding and contracting slightly to possibly rearrange or aerate the developing embryos. It was not until day 17 that the small buds of the male gonads were first observed. On day 20, the females released their first brood and immediately began brooding a new batch of eggs. There was some variation in the duration of the brood period; clutches were sometimes held for as short a time as five days to as long as eight days. There were also instances of aborted broods. Though it was not thoroughly studied, broods could apparently be either released prematurely or resorbed.

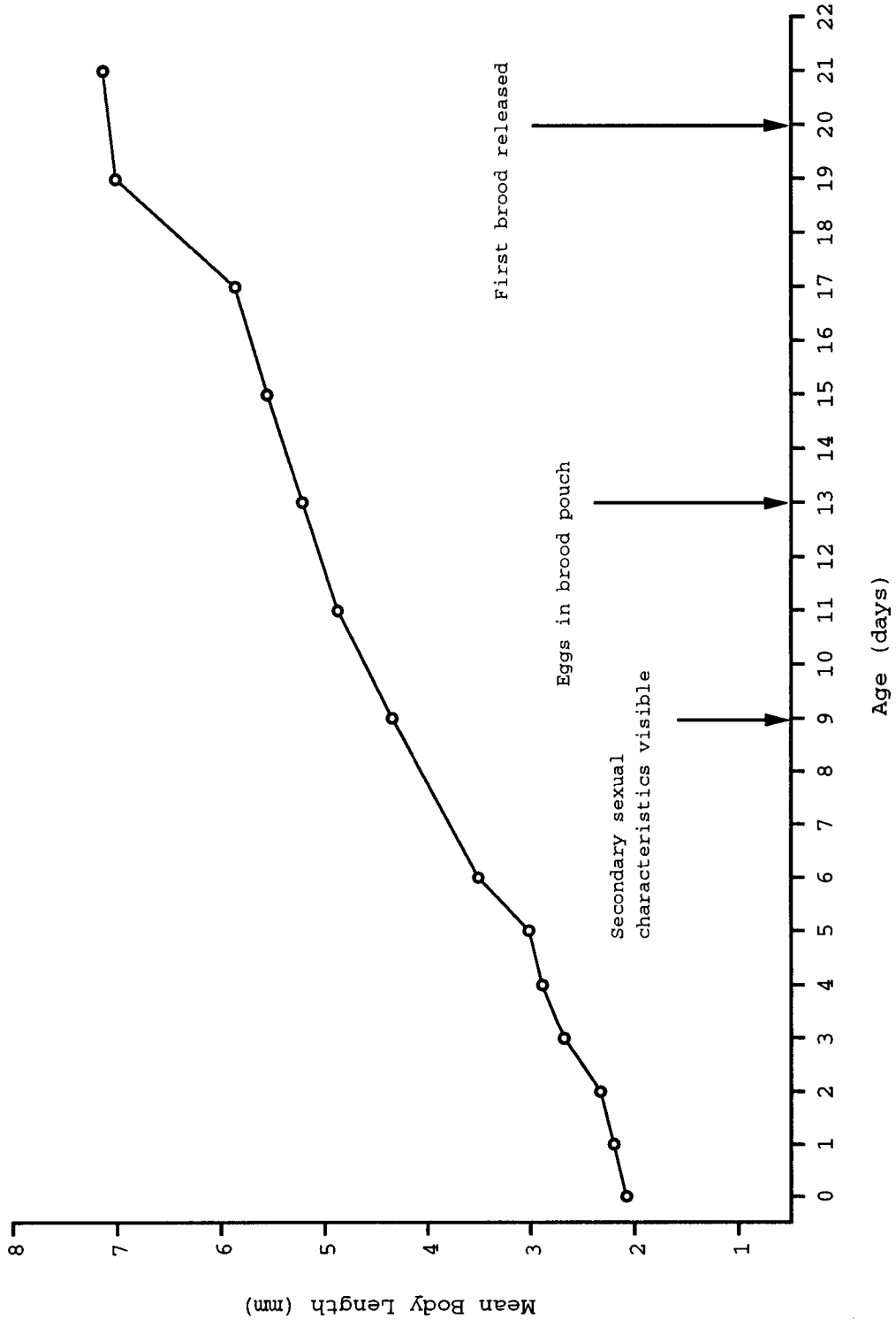

Figure 3. Growth curve of Mysidopsis intii fed the "Standard" diet of 100 Artemia/mysid/day and 125 Tigriopus/beaker/day at 20 degrees C. Each mean length value represents the lengths of 10 animals from an individual beaker. Standard errors are all less than 0.105 mm.

The growth rate of M. intii for the first two weeks was between 4% and 6% per day except for obvious increases on days three and six that could be indicators of molting events (Figure 4). After day six, the growth rate began to decrease from 6% per day to 3% per day until another spike around day 18 or 19. This event is just prior to the females' first brood release. After day 20, growth rates were minimal as most individuals attained their maximum size between 7 and 8 mm by their fourth week.

Weight / Length Relationship: (Experiment #2)

The dry weights of individual mysids ranged from 21 μg for juveniles just over 3.0 mm, to just under 1.0 mg (989 μg) for full grown adults (7.7 mm). Individual weights of animals smaller than 4.0 mm were fairly inconsistent as the values obtained (20 - 30 μg) were close to the detection limits of the balance used (Perkin-Elmer AD-2Z Autobalance). Small mysids (less than 4.0 mm) were weighed in groups of five and average weights were calculated. Average weights of animals were calculated for the following length categories (2.0 - 2.5, 2.5 - 3.0, and 3.0 - 3.5 mm). The cube root of the dry weights of 45 mysids were obtained and plotted against their lengths. A linear regression produced the function $Y = 7.485X + 0.079$ with an R^2 value of 0.983 (Fig. 5).

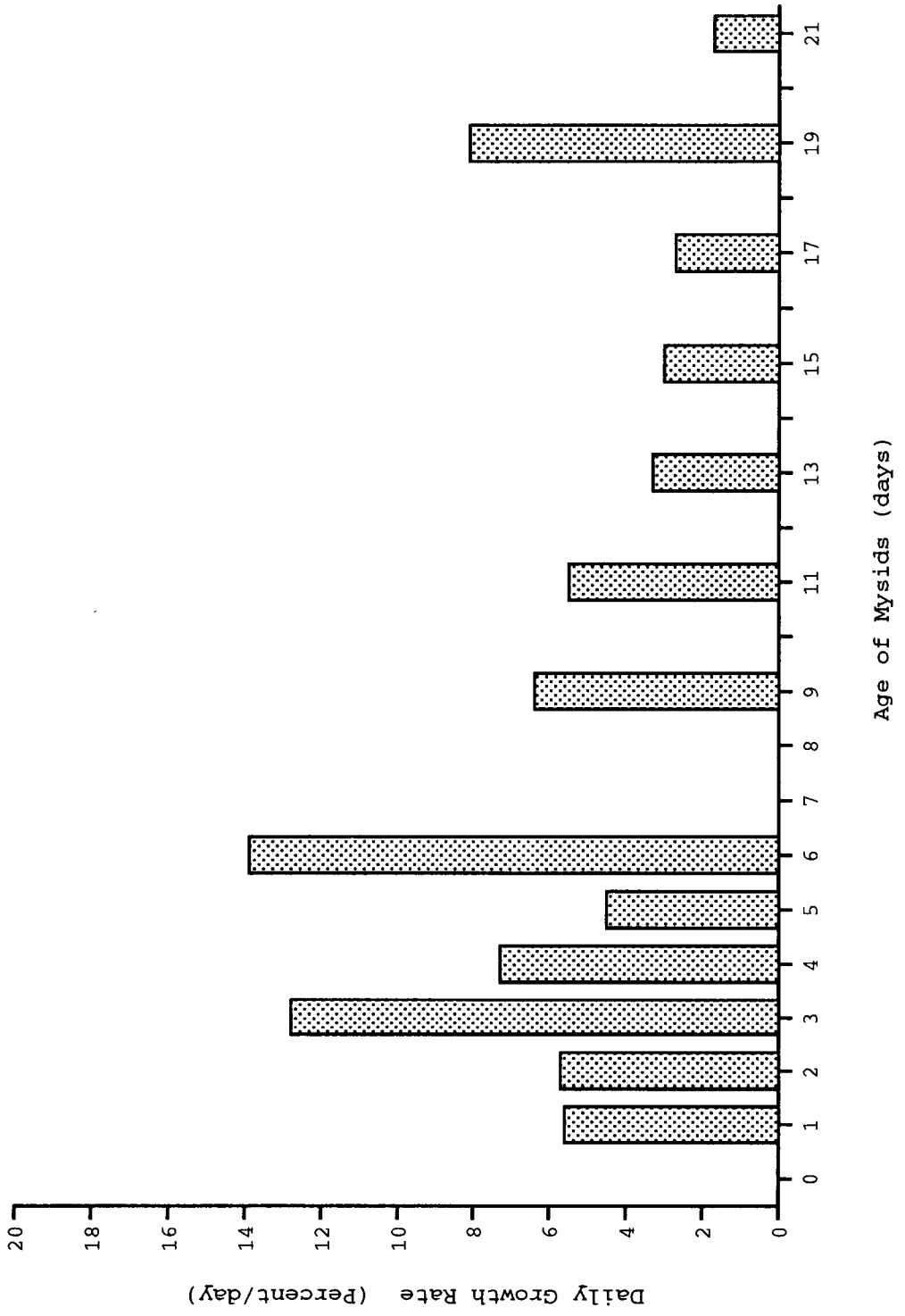

Figure 4. Daily percent growth rate of Mysidopsis intii fed the "Standard" diet. Growth peaks on days 3, 6, and 19 may correspond to molting events. For each age sampled, n = 10.

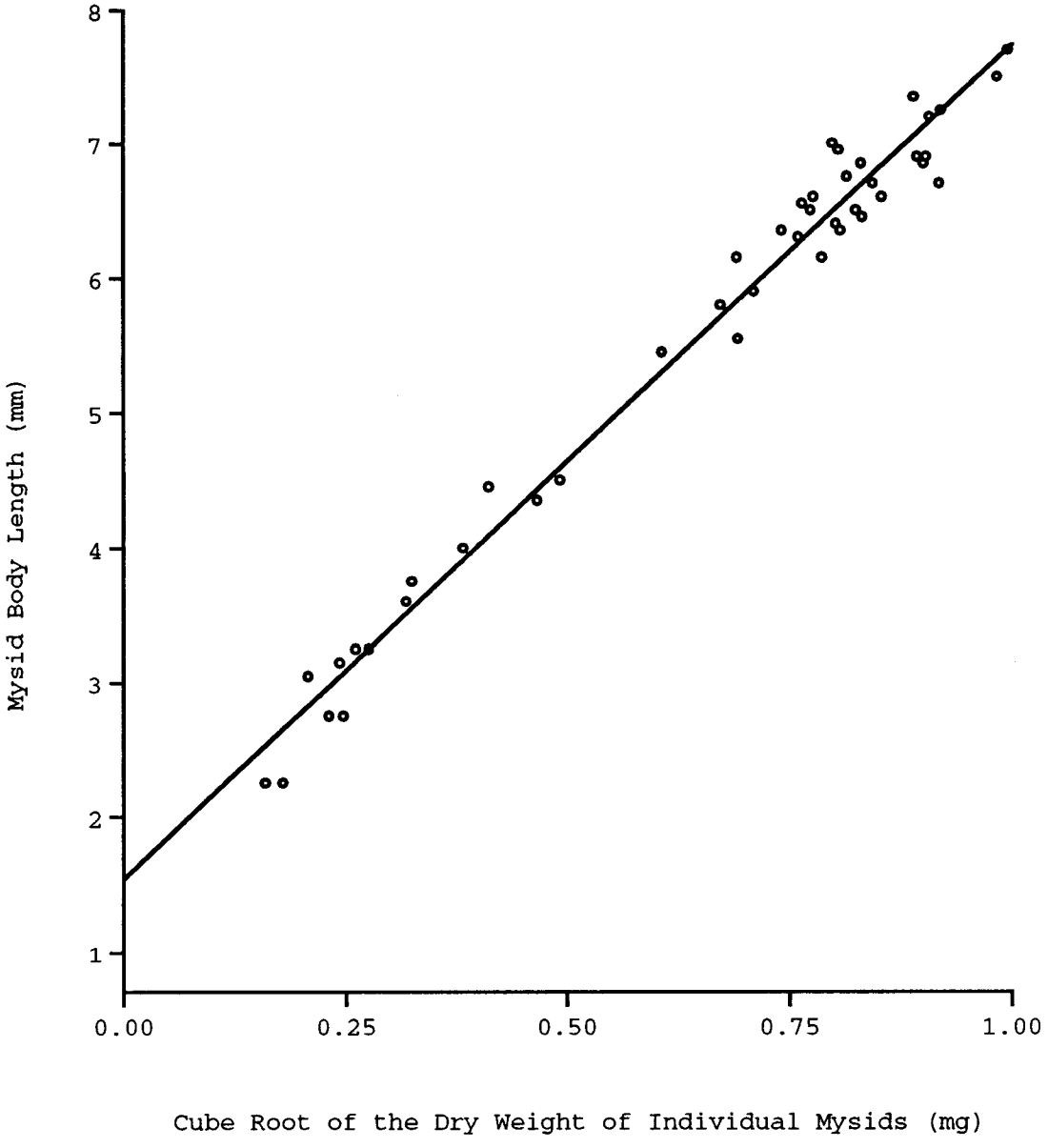

Figure 5. Linear regression of the lengths of individual mysids vs. the cube root of their dry weight (n=45).
y = 7.485x + 0.079; r2 = .983

The close relationship between length and weight values for these mysids suggest that either of the two measurements of body size would be acceptable as a representative of growth. Body length was chosen over weight because length was easier to measure and, because of the instrument limitations, weights would have had to have been pooled group averages.

Length Measurement Methods Comparison:

Body length measurements collected using the reticle method and the image analysis method were compared statistically. Results from the comparison of 10 animals are displayed in Table 3. Measurements made with the image analysis system showed wider ranges of lengths obtained from some individuals (mysids #1 and #4 show ranges of 0.33 and 0.24 mm respectively). The total mean lengths of the two groups were similar (5.399 mm for the visual method vs. 5.417 mm for the image analysis method). To compare the variability of the two techniques, coefficients of variation were calculated for each set of three measurements on the ten animals using both methods. These values were compared using a paired t-test. There was not a significant difference between the coefficients of variance of the two methods (significance level = 0.0721, t-statistic = -2.036).

Table 3. Body lengths of 10 individual mysids using two measurement methods; the reticle method and the image analysis (Java) method. Triplicate measurements, means, standard deviations, and coeffecients of variation are shown. A paired t-test of the coefficients of variation showed no significant difference in variation between the two methods.

Comparison of the Reticle and Image Analysis measurement methods

Animal #	Reticle Method	Mean Length (mm)	S. D. (mm)	C. V. (%)	Java Method	Mean Length (mm)	S. D. (mm)	C. V. (%)
1	5.90	5.93	0.029	0.487	5.52	5.64	0.185	3.2800
1	5.95				5.54			
1	5.95				5.85			
2	6.05	6.07	0.029	0.476	6.06	6.09	0.042	0.6831
2	6.05				6.14			
2	6.10				6.08			
3	6.70	6.70	0.050	0.746	6.51	6.61	0.091	1.4670
3	6.75				6.69			
3	6.65				6.62			
4	6.50	6.48	0.029	0.446	6.52	6.53	0.120	1.8390
4	6.45				6.65			
4	6.50				6.41			
5	4.30	4.30	0.050	0.116	4.13	4.10	0.079	1.9370
5	4.35				4.16			
5	4.25				4.01			
6	6.55	6.53	0.029	0.443	6.57	6.60	0.031	0.4636
6	6.50				6.63			
6	6.55				6.61			
7	1.70	1.73	0.029	1.671	1.75	1.75	0.010	0.5714
7	1.75				1.74			
7	1.75				1.76			
8	5.70	5.70	0.050	0.887	5.83	5.80	0.038	0.6534
8	5.75				5.76			
8	5.65				5.82			
9	6.10	6.15	0.050	0.813	6.30	6.30	0.035	0.5571
9	6.15				6.27			
9	6.20				6.34			
10	4.60	4.58	0.029	0.631	4.58	4.57	0.050	1.1000
10	4.55				4.62			
10	4.60				4.52			
Means:		**5.42**		0.672		**5.40**		1.255
S. D.s		1.52		0.416		1.54		0.897

Defining a "Standard" Test Diet:

Previous experimentation conducted under the cooperative agreement between the EPA and OSU produced data that showed no significant differences in growth or survival when using newly hatched vs. two-day enriched Artemia as a food source (Kreeger *et al.*, 1991). The same study did however demonstrate that it was necessary to include Tigriopus in the diet to allow for consistent mysid reproduction. It is thought that the copepods provide a source of fatty acids necessary for germinal tissue growth. These results, along with information concerning food preparation and presentation, led us to choose the "Standard" diet described earlier (100 Artemia/mysid/day and 125 Tig/beaker/day). Even though reproductive endpoints were not considered in the nutrition/zinc sensitivity experiments, the copepod component in the diet allowed for more diet manipulations.

Start Day and Duration:

Experiments #4 and #5 were carried out to pinpoint the best time to begin and end the test. Since response to toxicants varies throughout the lives of many animals (USEPA, 1987), the most sensitive stage needed to be identified. Test "start days" (age of test animals at first exposure) examined were day zero and day four. Termination days (age of animals at the end of the test) examined were day four, day five, day six, day seven, day

eight, and day eleven. Tests that were started on day 0 and ended on day 7 consistently resulted in significant differences in final body length between control and zinc treatments (Table 4). This schedule was used for all subsequent tests.

ZINC EXPOSURE EXPERIMENTS:

Range Finder: (Experiment #6)

Prior to beginning dietary experiments, an experiment was conducted to determine the general sensitivity of M. intii to zinc. It included a no-zinc control and four experimental zinc concentrations (18, 32, 56, and 100 ppb zinc). Analysis of test water samples taken immediately prior to adding the animals showed measured values of total zinc concentrations that were somewhat higher than the nominal concentrations. This deviation from experimental concentrations occurred to some extent in every test. The source of the discrepancy has not been determined, and no consistent pattern exists throughout all the analyses.

Table 4. Comparison of different test durations conducted with Mysidopsis intii. Start day, duration, and termination day were varied. All animals received the "Standard" diet. Zinc concentrations were 0 or 70 ppb (nominal). Analysis of variance was used to check for significant differences between final body lengths of the 0 ppb and 70 ppb zinc treatments ($\alpha = 0.05$).

TEST DURATION EXPERIMENTS

Start Day - End Day	Test Duration (Days)	Treatment Lengths (mm) 0 ppb - 70 ppb (Difference)	p - Value
Experiment # 4			
0 - 4	4	2.69 - 2.63 (0.06)	0.1211
0 - 5	5	2.90 - 2.82 (0.08)	0.0344
0 - 6	6	3.33 - 3.18 (0.15)	0.0285
0 - 7	7	3.68 - 3.53 (0.15)	0.0127
0 - 8	8	4.62 - 4.20 (0.42)	<0.0001
Experiment # 5			
0 - 7	7	3.49 - 3.12 (0.37)	<0.0001
0 - 8	8	4.01 - 3.63 (0.38)	<0.0001
4 - 11	7	4.78 - 4.84 (-0.06)	0.5392

For the initial test, the average measured values (and standard deviations) corresponding to the 0, 18, 32, 56, 100 ppb nominal zinc concentrations were 16.9 (±13), 36.9 (±7.7), 51.4 (±6.8), 65.8 (±1.5), and 112.2 (±7.1) ppb respectively. The differences between measured and nominal zinc concentrations in this experiment were 11 - 18 ppb higher than expected.

Analysis of variance of mysid final mean body lengths detected differences between zinc treatment groups ($p \leq 0.0001$). Final mean body lengths were significantly smaller in both the 65 and the 112 ppb treatment groups when compared to the controls using Tukey's multiple range test (Figure 6). The average body length of mysids in the 112 ppb treatment (3.10 mm) was 8% less than that of the control animals (3.35 mm). The LOEC in this experiment was 65 ppb Zn. Zinc concentrations that bracketed the LOEC (50 and 70 ppb) were chosen to explore how diet modifications could influence the sensitivity of M. intii to zinc in other experiments.

There were no significant differences in mortality between the control animals and any of the treatment groups ($p = 0.4074$). Table 5 summarizes the mortality data for all subsequent experiments.

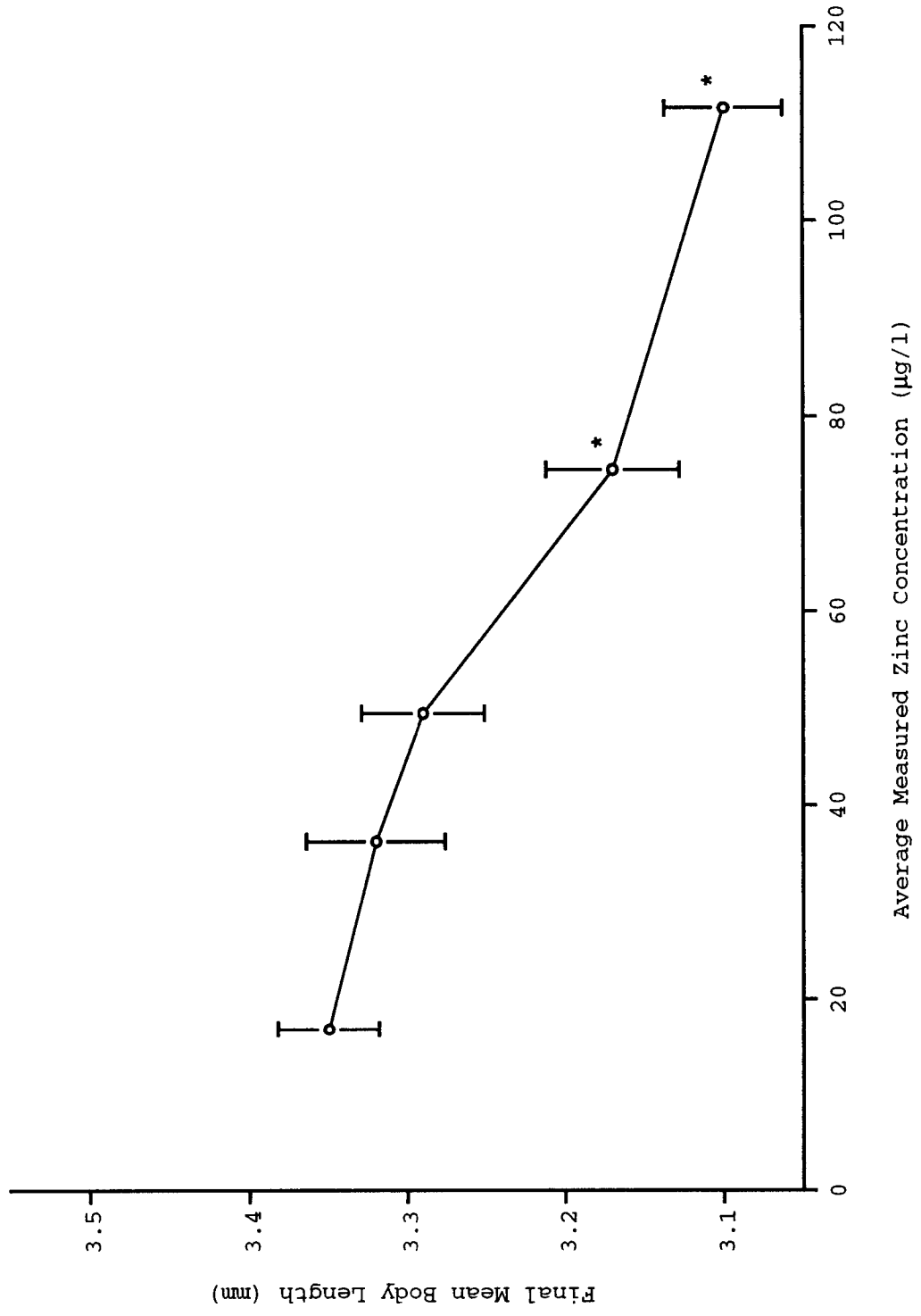

Figure 6. Range finder toxicity test for zinc. Determination of the Lowest Observed Effect Concentration (LOEC) of zinc on the length of Mysidopsis intii. Error bars denote one standard error. Asterisks represent significant difference between treatment group and the control ($p<0.05$).

Table 5. Survival data for experiments 6 through 11. Nominal zinc concentrations are reported along with arcsine transformed survival percentages. Asterisks represent significant differences between a zinc treatment and the control within a diet treatment.

SUMMARY OF SURVIVAL FOR EXPERIMENTS 6 - 11

Experiment # 6

Nominal Zn conc. (ppb)	0	18	32	56	100
Survival	70.77	85	75	66.08	74

ANOVA p = 0.4074

Experiment # 7

Nominal Zn conc. (ppb)	0	50	70
Survival ("STD" diet)	90	90	75.0 *
Survival (No Tig)	90	90	75.7 *

ANOVA p = 0.0003 (by conc.)
ANOVA p = 0.9262 (by diet)

Experiment # 8

Nominal Zn conc. (ppb)	0	21	37	50	70	101	230
Survival ("STD" diet)	85	85	90	80	85	75	60.7 *
Survival (No Tig)	82.8	80	82.8	85	85	72.8	71.1 *

ANOVA p = 0.0006 (by conc.)
ANOVA p = 0.9634 (by diet)

Experiment # 9

Nominal Zn conc. (ppb)	0	50	70	101
Survival ("STD" diet)	69.77	82.84	90	90
Survival (Enriched)	76.14	61.77	73.99	77.84

ANOVA p = 0.3038 (by conc.)
ANOVA p = 0.0566 (by diet)

Experiment # 10

Nominal Zn conc. (ppb)	0	37	50	70	101	230
Survival ("STD" diet)	69.22	67.35	73.39	75.05	69.2	65.02
Survival (Enriched)	82.3	71.56	84.63	71.24	69.7	76.9

ANOVA p = 0.7483 (by conc.)
ANOVA p = 0.1361 (by diet)

Experiment # 11

Nominal Zn conc. (ppb)	0	50	70
Survival ("STD" diet)	66.83	85	75.69
Survival (Medium)	77.84	64.37	62.1
Survival (Low)	68.99	82.84	72.84

ANOVA p = 0.1987 (by conc.)
ANOVA p = 0.1547 (by diet)

Initial *Tigriopus* Elimination Test: (Experiment #7)

The first nutrition related experiment introduced a qualitative alteration of the diet. One half of the experimental treatments did not receive the *Tigriopus* copepods that were part of the "Standard" diet. The second half of the test organisms were supplied with the "Standard" diet which did include the copepods. Each of the two diet treatment sets included nominal zinc concentrations of 0, 50, and 70 ppb. The measured zinc concentrations (and standard deviations) of these test water samples were 3.5 ($\pm$2.5), 46.2 ($\pm$8.9) and 60.9 ($\pm$5.9) ppb zinc.

As seen in Figure 7, mysids that did not receive copepods in their diet exhibited significant growth reduction at a lower zinc concentration than those that were fed the "Standard" diet. This effect appears in conjunction with that of overall growth reduction in the test animals receiving the compromised diet. In the control beakers of this experiment, mysids that were fed the modified diet only grew to 94.8% of the size of the animals receiving the "Standard" diet (this 5.2% reduction was significant when tested with ANOVA, $p<0.0001$). This phenomenon is common to all experiments performed in this study in that treatments receiving a qualitatively or quantitatively reduced diet exhibited reduced growth.

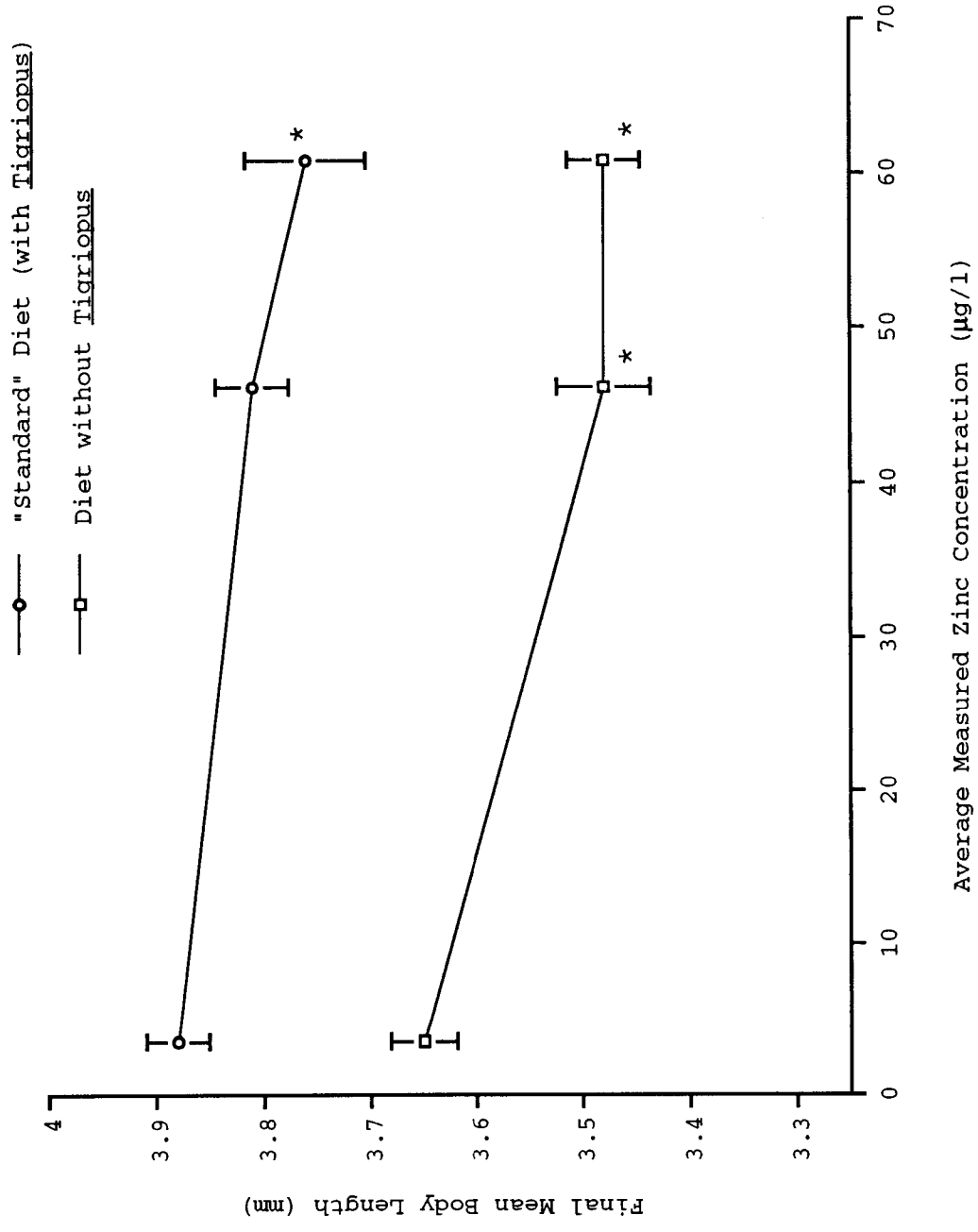

Figure 7. The effects of diet quality and zinc concentration on the final length of Mysidopsis intii (Initial Tigriopus elimination experiment). Diet treatments are with or without Tigriopus. Error bars denote one standard error. Asterisks represent significant difference between the zinc treatments and the control within each diet treatment ($p < 0.05$).

The response curve depicting the final body length of the mysids fed the substandard diet (Figure 7) shows a significant difference in final body lengths at 46 ppb whereas the animals fed the "Standard" diet did not show significantly reduced growth until the zinc concentration reached 61 ppb.

To allow for better discrimination of subtle effects of the diet on the zinc response curves, the Tigriopus elimination diet modification experiment was repeated with the nominal zinc concentration series expanded to include 0, 21, 37, 50, 70, 101, and 230 ppb zinc.

Follow-Up Tigriopus Elimination Test: (Experiment #8)

The results of the follow-up Tigriopus elimination experiment (Figure 8) demonstrated a sharp response to zinc and a significant difference in growth between the two diets ($p<0.0001$). There was not however, any evidence of the reduced diet shifting the response curve to a lower LOEC. Each of the two diets showed significant reductions in growth at the same concentrations of zinc. Measured values (and standard deviations) of zinc concentrations for experiment #8 were 15.6 ($\pm$9.1), 26.4 ($\pm$13.3), 38.7 ($\pm$13.3), 49.1 ($\pm$13.9), 68.4 ($\pm$11.3), 89.9 ($\pm$11.5), and 189.6 ($\pm$7.7) ppb.

The mean length of the mysids used in this experiment on day zero was 1.97 mm $\pm$ 0.17 mm.

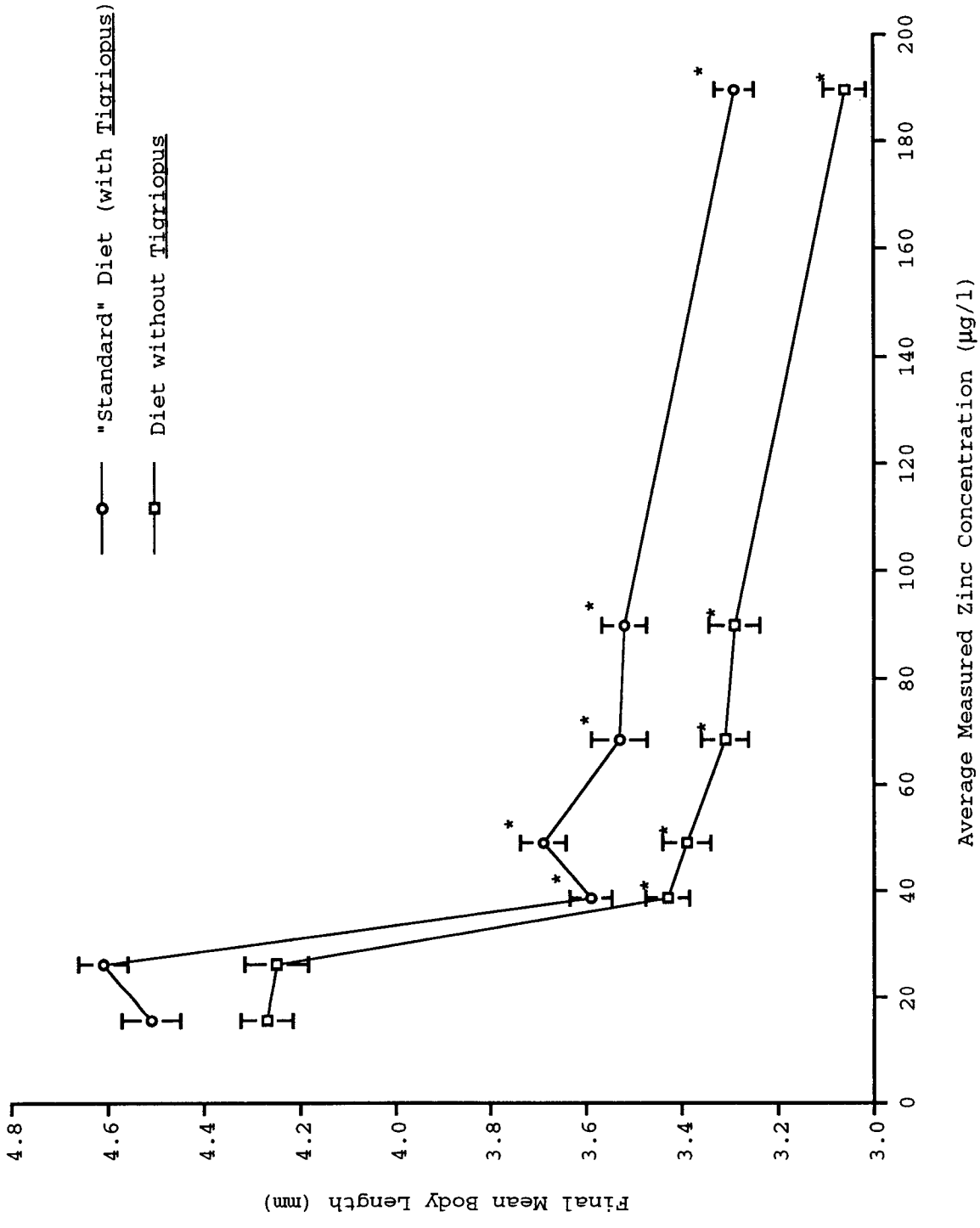

Figure 8. The effects of diet quality and zinc concentration on the final length of Mysidopsis intii (Follow-up Tigriopus elimination experiment). Diet treatments are with or without Tigriopus. Error bars denote one standard error. Asterisks represent significant difference between the zinc treatments and the control within each diet treatment ($p < 0.05$).

Initial *Artemia* Enrichment Test: (Experiment #9)

This experiment examined another qualitative change of the diet by producing nutritionally enhanced prey items. *Artemia* were enriched by allowing them to feed on phytoplankton (*Pseudoisochrysis paradoxa*, and *Chaetoceros calcitrans*) for two days before using them as food for the mysids. This experiment compared mysid growth in a nominal zinc concentration series of 0, 50, 70, and 101 ppb. The corresponding measured values (and standard deviations) were 0 (±0.0), 35.5 (±2.5), 50.5 (±4.4), and 79.4 (±3.1)ppb. The two diet treatment groups were fed either the "Standard" diet or the diet that contained the enhanced *Artemia*. Figure 9 shows that again, the overall growth was higher in the animals feeding on the enhanced diet. In this experiment, the control animals fed the "Standard" diet grew to 3.63 mm and those fed the enhanced diet grew to 3.65 mm. This slight growth difference was not significant, yet it does follow the trend seen in previous tests. Also seen in Figure 9, is that while mysids receiving the "Standard" diet (in this experiment, the lower quality diet) exhibited significantly lower growth at 79.4 ppb zinc, the mysids receiving the enhanced diet did not show significant growth reduction in any of the zinc treatments.

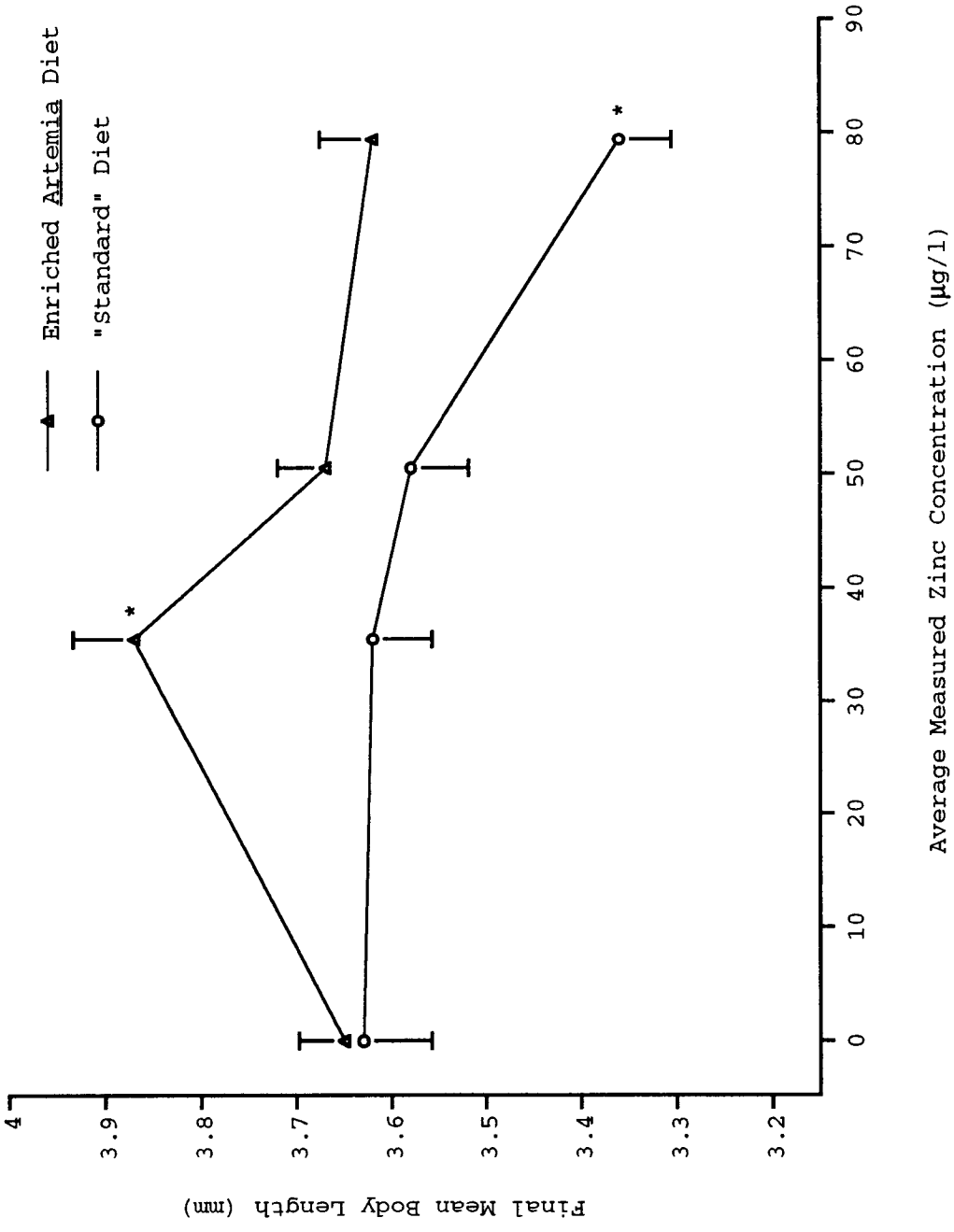

Figure 9. The effects of diet quality and zinc concentration on the final length of Mysidopsis intii (Initial Artemia enrichment experiment). Diet treatments are either newly hatched Artemia with Tigriopus ("Standard" diet) or 2-day enriched Artemia with Tigriopus. Error bars denote one standard error. Asterisks represent significant difference between the zinc treatments and the control within each diet treatment ($p < 0.05$).

The significant increase in size seen at 35.5 ppb zinc in the enhanced diet is a conspicuous and interesting result that is probably evidence of hormesis and will be discussed later.

Follow-Up *Artemia* Enrichment Test: (Experiment #10)

When the enriched *Artemia* vs. newly-hatched *Artemia* experiment was repeated, ANOVA detected a significant difference between final body lengths of the two diet treatments ($p < 0.0001$). There were also significant differences between zinc treatment groups and the control set within each diet treatment ($p < 0.0001$) (see figure 10). There was not, however, any interaction between diet and zinc exposure (i. e., the zinc LOECs for the two diet treatments were the same, 46.3 ppb). This experiment had also been expanded to include a wider range of nominal zinc concentrations (0, 37, 50, 70, 101, and 230 ppb zinc). The corresponding average measured values (and standard deviations) were 20.6 ($\pm$9.6) 46.3 ($\pm$13.3) ,77.0 ($\pm$19.6), 61.6 ($\pm$7.3), 73.5 ($\pm$2.7), and 158.8 ($\pm$6.2) ppb zinc.

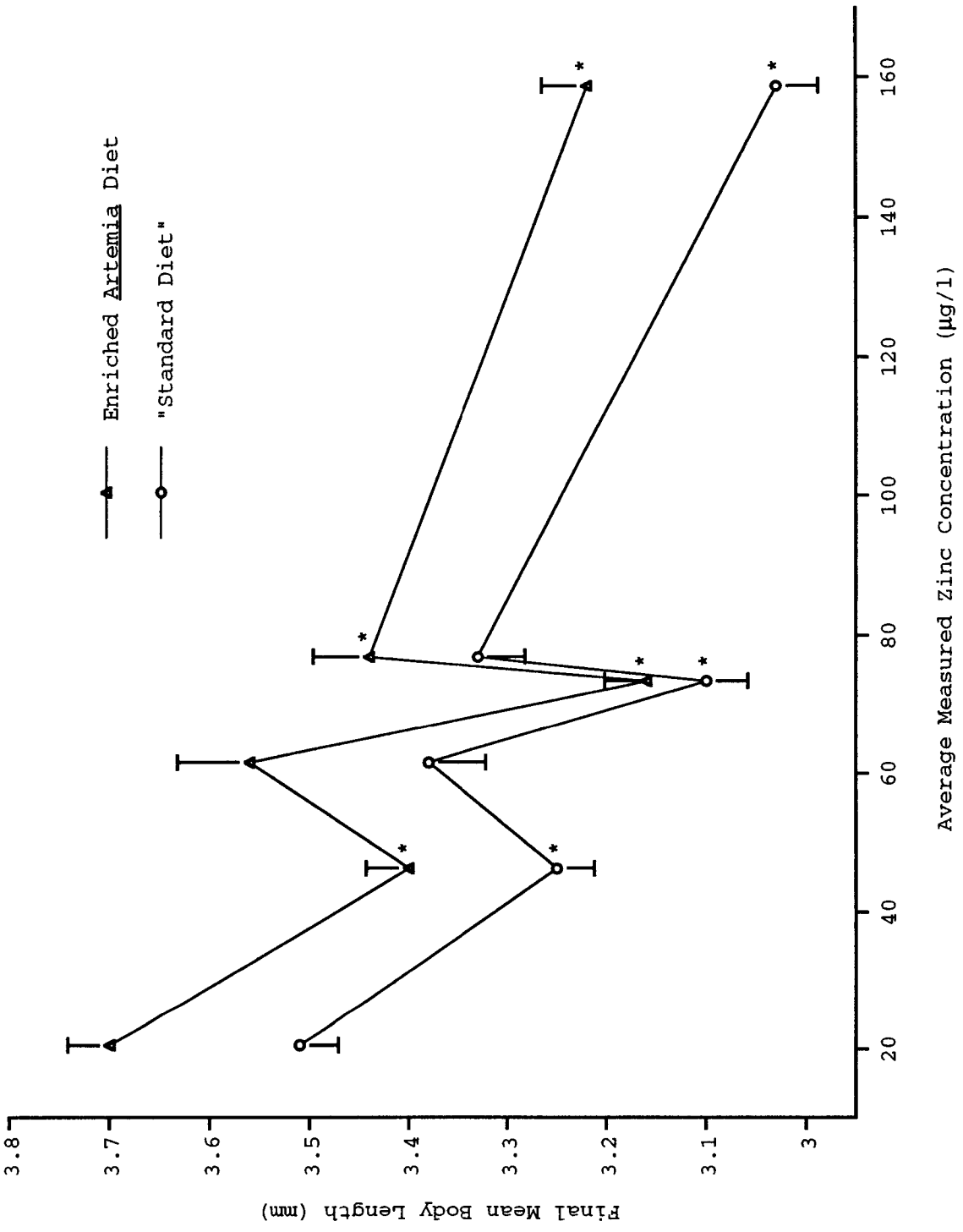

Figure 10. The effects of diet quality and zinc concentration on the final length of Mysidopsis intii (Follow-up Artemia enrichment experiment). Diet treatments are either newly hatched Artemia with Tigriopus ("Standard" diet) or enriched Artemia with Tigriopus. Error bars denote one standard error. Asterisks represent significant difference between the zinc treatments and the control within each diet treatment ($p < 0.05$).

Diet Ration: (Experiment #11)

The other type of nutrition-related toxicity test conducted was a quantitative alteration of the diet. Instead of changing the type of food, the amount of food provided was varied. For each of the three selected rations (100%, 50%, and 35% of the numbers of prey items in the "Standard" diet), juvenile mysids were exposed to nominal zinc concentrations of 0, 50, or 70 ppb. Zinc analysis of the samples from this experiment resulted in average measured values (and standard deviations) of 0.56 ($\pm$0.9), 32.6 ($\pm$0.8), and 49.1 ($\pm$0.5) ppb. Ration reduction resulted in significantly less growth in the no-zinc controls ($p < 0.0001$) of each of the two experimental diets as compared to the "Standard" diet (Figure 11). The low ration diet resulted in very little growth (69% of that achieved with the full ration). The medium ration diet resulted in 84% of the mean body length of the full ration control animals. Mysids in both the medium and the full ("Standard") diet treatments responded to 32.6 ppb zinc with reduced final body lengths that were significantly lower than the controls. However, at 49.1 ppb zinc, the mean final lengths for both of these diet treatments were not significantly lower than those in the controls. The control animals in the full ration group (the "Standard" diet), had final body lengths of 3.57 mm, which was within the range of growth observed in previous experiments.

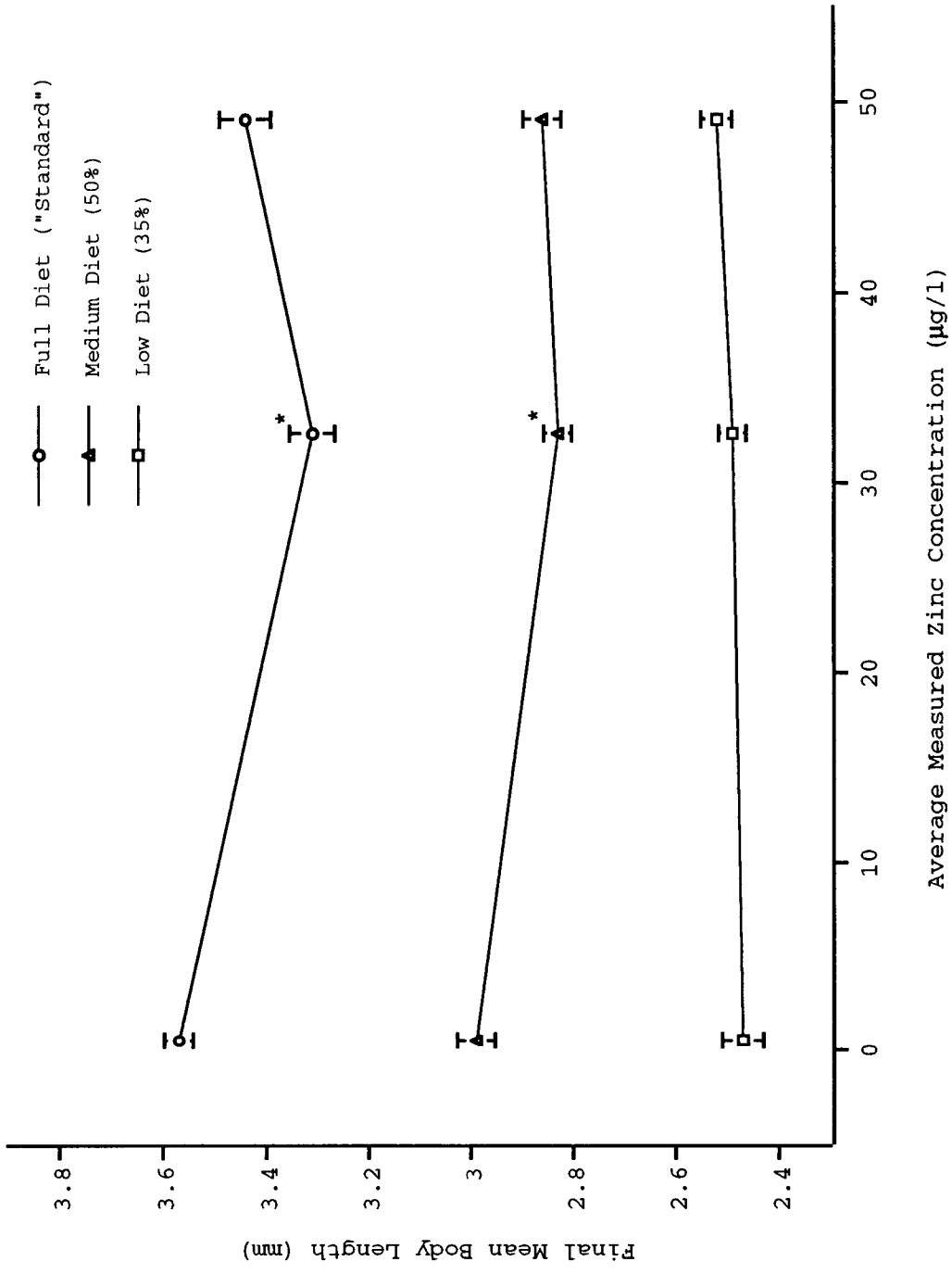

Figure 11. The effects of low, medium, and full ("Standard") rations and zinc exposure on final body length of Mysidopsis intii. Error bars denote one standard error. Asterisks represent significant difference between the zinc treatments and the control within each ration treatment ($p < 0.05$).

DISCUSSION

This series of experiments demonstrates that Mysidopsis intii used in a seven-day growth test is a very good test organism for toxicological research. Its sensitivity to toxins is equal to or greater than many other marine crustaceans currently used as test species (Shaffer and Langdon, submitted; USEPA, 1987). M. intii exhibited a significant reduction in growth compared to that of controls when exposed to zinc concentrations as low as 34 ppb (Figure 9). Its short life cycle (20 days at 20°C) makes it amenable to reproductive tests and aids in laboratory culture. Its acceptance of different food sources and high juvenile growth rate will enhance further investigations on the nutritional aspects of toxin sensitivity.

The results of this study have shown that even subtle changes in diet can have significant effects on the growth of M. intii. Inhibition or stimulation of juvenile growth by the manipulation of diet under test conditions has been demonstrated with all of the tested feeding regimes. Growth was reduced by more than 5 percent with only slight qualitative changes in diet composition (Figure 8). Reducing the ration supplied to the test animals had an even more drastic affect. When compared to those that received the full ration, the final body size of control animals in the diet quantity experiment was reduced 16

percent with the medium ration and 31 percent with the low ration (Figure 11). This type of response to quantitative modifications in diet has been demonstrated with many species used in toxicity tests (Johns *et al.*, 1981; Hokanson and Lien, 1986; Helm and Laing, 1987, Cripe *et al.*, 1989).

Beyond this basic effect of diet on growth, there was also evidence of nutrition influencing zinc sensitivity. In two experiments, slight modifications in diet quality not only affected growth, they also shifted the dose response curve to exhibit a measurably lower or higher LOEC. Other studies have demonstrated a similar relationship between diet and toxin sensitivity (Biesinger and Christensen, 1972; Cerda and Olive 1993; Cowgill, 1987). The LOEC depression was less than 20 ppb in the one experiment in which it could be quantified (Figure 9). In other experiments, the shift is evident, but because there were not enough zinc concentrations bracketing the LOEC, the magnitude of the shift could not be determined.

The difficulty in picking a zinc concentration series that would clearly quantify LOEC depression was related to the fact that the LOEC varied from experiment to experiment. Sometimes the mysids showed reduced growth when exposed to 39 ppb zinc (Figure 9), and sometimes concentrations as high as 80 ppb had no effect (Figure 7).

The growth response of M. intii to zinc was altered in both types of qualitative diet modifications. Diet

enrichment (whether by means of algal enhancement or species makeup of the diet) increased the resistance of M. intii to zinc. The response to zinc in the quantitative diet modification experiment was not strong enough to show diet-related LOEC shifts. The lack of zinc response in the animals receiving lower diet rations could suggest that if growth is reduced below a certain point, then any effect that zinc would have on growth could be overshadowed.

The zinc concentrations used in this study were not high enough to cause mortality high enough to calculate LC_{50}s. It would be very interesting, however, to conduct a study similar to this one that used sufficiently high zinc concentrations to test whether diet could influence the zinc LC_{50}s of M. intii.

The linked graphs in Figure 12 help demonstrate the relationship between growth reduction and LOEC depression. When a change in diet lowers the mysids growth rate, the slope of the growth curve is reduced (Figure 12a). Those two different growth rates are the basis for the shift on the y-axis of the two dose-response curves (the difference in growth due to nutrition). The deficient diet dose-response curve may also be shifted along the x-axis to exhibit a LOEC at a lower zinc concentration (Figure 12b).

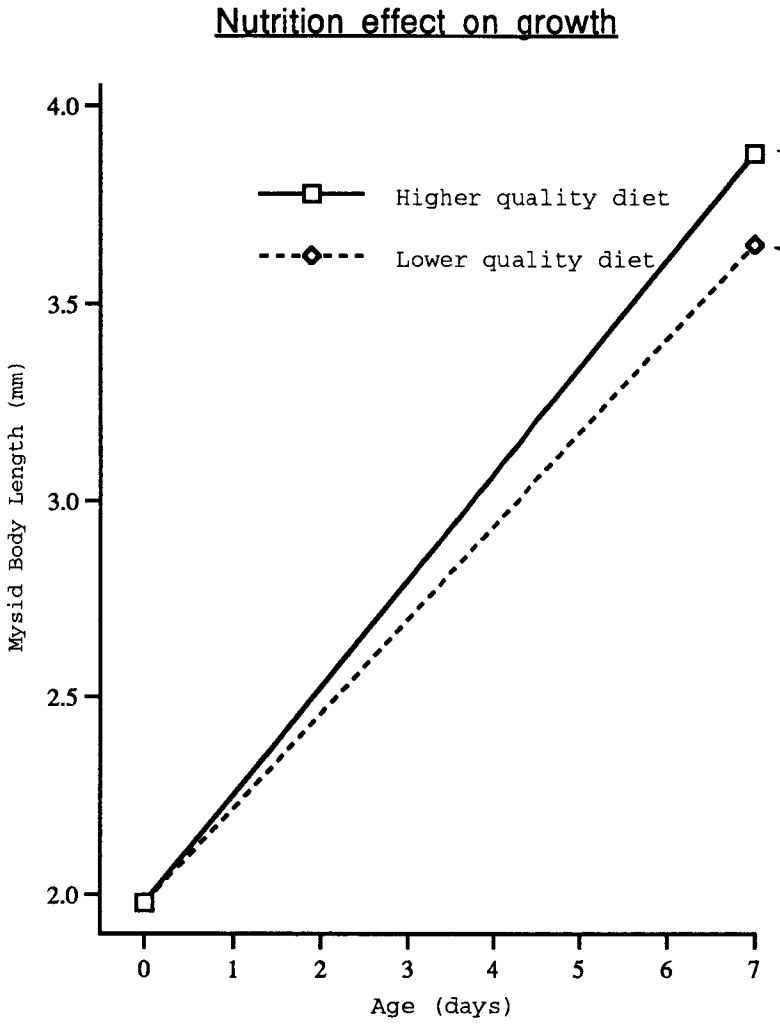

Figure 12a. Seven-day growth curves resulting from two different diets (data from experiment #9). The dashed line (lower quality diet) depicts slower growth.

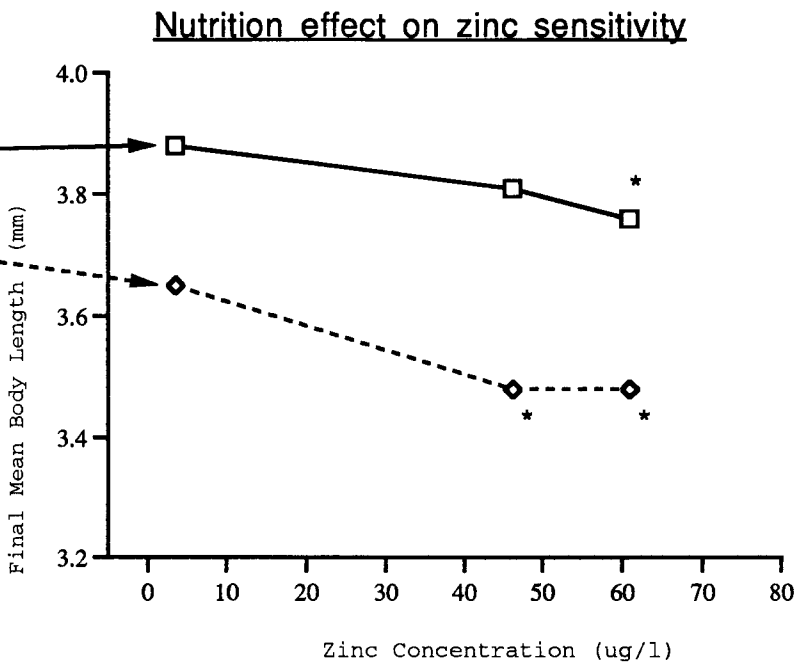

Figure 12b. Seven-day toxicity test Dose-Response curves of Mysidopsis intii to zinc using two different diets (data from experiment #9). The lines connecting the two graphs show the relationship between nutrition, growth, and zinc sensitivity. Asterisks denote significant difference between the zinc treatments and the control within each diet treatment.

However small this increase in zinc sensitivity is, it could have repercussions either in laboratory settings or in populations of animals in the wild. In field settings, exposure to a chronic toxicant may initiate cyclical trends which escalate with time. If an animal was weakened by a toxicant and subsequently could not feed as effectively, then the continued effect of the original concentration of toxicant could be exacerbated. Along the same line, if a chronically present toxicant reduces the population of a prey species, then the toxicant will have an indirect, diet-related deleterious effect on any predators that depended on that prey as well as a direct exposure effect. Chronic toxicants also cause growth-related reproductive effects. If growth is reduced, then animals may not reach their reproductive potential before seasonal or other habitat related restrictions occur (i. e. temperature changes, adequate nutrients for reproduction, tidal cycles, etc.).

The effect of dose-response shift due to diet alteration could also have an impact on the interpretation of laboratory tests (Dave, 1991; Cerda, 1993). If the diets provided to two "identical" tests carried out in separate labs (Round Robin studies) are slightly different (e.g. different strains of Artemia or other more subtle differences), then there is potential for different conclusions being drawn. The magnitude of LOEC depression documented in this study are small when compared to zinc

responses in other tests (acute test LC_{50}s are 499 µg/l - M. bahia, 294 µg/l - Acartia tonsa, 233 µg/l - C. gigas, from USEPA, 1987), but if long term implications are kept in mind, it becomes evident that even these small effects should not be dismissed.

When studying interactions between nutrition and toxin sensitivity, the endpoint used must be able to clearly reflect the organism's response to both of the two treatments being tested. Body length, the endpoint employed here, proved to be a useful tool because it did respond to both diet alterations and zinc.

The experiments conducted comparing the different methods of measuring juvenile growth all produced results that suggested no significant differences between methods. Those results allowed the decision of which method to use to be based on ease and convenience of data collection and manipulation. The choice to use body length instead of body weight was based on the facts that individual body lengths could be collected more easily than individual body weights (and probably with better accuracy), and mysids could be preserved and measured at a later date. Body length was used to detect statistically significant differences between treatments when there was only a 5% difference between the means. Juvenile growth was utilized as an endpoint because of its relation to nutrition and its sensitivity to toxins. The synergistic effect of nutrition and zinc on growth highlights the close relationship of the

two factors. Juvenile growth is also important because of its relation with other life cycle parameters. Juvenile growth can reflect similar responses in later endpoints such as reproduction, mortality and even population responses (Breteler, *et al.* 1982; Johns, 1989). In a study conducted by Breteler, *et al.* (1982), body length was found to be the most consistent endpoint.

Zinc effects on growth were measured at very low concentrations (routinely $\leq$ 50 ppb). This high degree of test sensitivity is desirable for comparing sensitivities of different animals but it also has its drawbacks. Variability surrounding both growth and measured toxin concentrations can result in some interpretation problems. Inter-experiment LOEC variation will probably not disappear, but if enough zinc concentrations are included around the LOEC range, interpretation difficulties could be minimized.

Evidence of hormesis was revealed in two of the individual experiments (Figures 6 and 7). This phenomena is the reflection of overcompensation to stress (in this case, chemical stress) and is often recognized in laboratory test situations (Stebbing, 1982; Sanders and Jenkins, 1984; Johns, *et al.*, 1989; Dave, *et al.*, 1991). This interesting phenomenon could also be better quantified and therefore possibly better understood by testing a more complete toxicant concentration series.

Further research in this area could reveal specific interactions between nutrition and the response to many types of stress. While growth is an easily measured endpoint reflective of the sum of physiological processes, a lethal endpoint measure, such as LC_{50} may alleviate some of the variance observed here. In other words, an endpoint not quite so close to the detection limits of the toxicant analysis may make shifts in toxin sensitivity more discernable. More complex test systems such as controlled mesocosms could help answer questions that cannot be addressed in a static, single predator-single prey system. Population level studies could shed light on the long term interactions between nutrition and the detrimental effects of pollution. Food chain dynamics such as biomagnification could possibly separate food-borne contaminant effects from water column effects. In whatever directions that nutrition related toxicity test research continues, the importance of carefully controlled diets (both quantitatively and qualitatively) should be considered.

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