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CHOLINE REQUIREMENT OF THE MICROCRUSTACEAN *MOINA MACROCOPA*: A PURIFIED DIET FOR CONTINUOUS CULTURE

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Abstract

Under axenic culture conditions, choline is a required nutrient of the microcrustacean *Moina macrocopa*. Lecithin (phosphatidylcholine) as a component of an artificial biphasic diet serves as an efficient source of choline. *Moina* can synthesize choline efficiently via methylation of dietary ethanolamine. The animals assimilate dietary choline 10 times more efficiently from a particulate source than from a soluble one. Liver infusion, the only undefined component of the artificial medium, contains 1% choline and contributes substantially to choline's availability. The liver infusion can be adequately replaced by an increase in dietary particulate choline or soluble choline. As a result, the artificial medium for the growth and continuous reproduction of *Moina* is now completely defined. *Moina macrocopa*'s requirement for choline in a particulate form is estimated to be 750–850 mg/100 g diet at a culture temperature of 26°C.

Introduction

Lecithin (phosphatidylcholine) is an important nutrient for crustacean growth and metabolism. Lester *et al.* (1975) showed that solubilization of cholesterol was effected by N-(N-dodecanosarcosyl) taurine (DST), a model of the type of detergents synthesized by crustaceans, and that the process is more efficient in the presence of lecithin. Kanazawa *et al.* (1979) showed that certain lecithins incorporated into an artificial diet for the prawn *Penaeus japonicus* enhanced growth.

In the lobster *Homarus americanus*, Conklin *et al.* (1980) eliminated mortality associated with juvenile molting by including soy lecithin in a purified diet. D'Abramo *et al.* (1981) showed that the active ingredient of the soy lecithin was phosphatidylcholine (PC), and suggested that the lecithin molecule was associated with a lipoprotein that efficiently transported cholesterol from the hepatopancreas to the hemolymph. In a continuation of these studies, we designed the present work to analyze the effect of lecithin on the growth of the microcrustacean *Moina macrocopa* cultured axenically on an artificial diet. As choline is a general requirement of insects (Dadd, 1970), we concurrently investigated the dietary contribution of choline from lecithin and the possible choline contribution of liver infusion, the only undefined ingredient of the artifical diet used.

Materials and Methods

Moina macrocopa, a freshwater crustacean of the order Cladocera, was grown axenically on an artificial diet. The culture medium was biphasic, composed of a particulate and a soluble phase. It had only one undefined component: liver infusion (Oxoid). This diet (the control) has supported continuous growth and reproduction

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Abbreviations: PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

of several strains in our laboratory for over 4.5 years (approximately 162 generations). The diet's constituents are listed in Table I. The sources of ingredients and procedures of formulation are described elsewhere (D'Abramo, 1979). Total particulate concentration was 20.30 mg%. The egg lecithin component of the diet was associated with lipid-protein particles (particulate phase) and was provided at a concentration of 0.75 mg%. Known choline sources were the lecithin and the vitamin mix, which provided 0.14 mg% choline in the form of choline dihydrogen citrate.

Various additions, deletions, and substitutions to the control diet were made to investigate the nutrient contribution of lecithin. The nutritive quality of these various diets was evaluated by comparing biomass of populations grown from a single parthenogenetic female (strain L1) inoculant. First instar females were aseptically transferred from the control medium (Table I) to the various test media. All populations were grown at 26°C in near total darkness in 20 × 125 mm screw cap culture tubes containing 10 ml of medium. Animals were exposed to light only during the daily suspension of particles via a vortex mixer, and during determinations of the time when the original inoculant released her first brood. The initial particle concentration in the culture tubes was the same (380×10^3 ml^{-1}) for all media, since particle ingestion rates depend on ambient concentration (D'Abramo, 1980).

Initially each of three culture tubes containing an experimental diet was inoculated with a single female. To eliminate some variability, day 1 of population growth was designated as the day on which an original female inoculant released her first brood of young. When populations had developed, three animals from each tube were used as subsequent inoculants. Sequential transfers continued until 20 populations (5 harvested on each of days 7, 8, 9, and 10) were collected. The

TABLE I

Composition of control diet as per cent w or v/v. Total particulate = 20.30 mg%.

Soluble phase		Particulate phase	
KCl	3 mg	Cholesterol	0.6 mg
$MgSO_4 \cdot 7H_2O$	4 mg	SA gel[4]	3.0 ml
Ca (as Cl^-)	2 mg	FV particles[5]	1.0 ml
Glycylglycine	50 mg		
K_3PO_4	2 mg		
$Na_2SiO_3 \cdot 9H_2O$	2 mg		
Fe (as NH_4^+citrate)	0.05 mg		
Metal mix L[1]	1 ml		
Nucleic Acid mix V[2]	2 ml		
Oyster glycogen	10 mg		
Vitamin mix M1B[3]	2 ml		
Liver infusion	40 mg		

[1] 1 ml = $Na_2EDTA \cdot 2H_2O$, 3.81 g; Zn (as $SO_4^=$), 0.30 mg; B, 0.12 mg; Mn (as Cl^-), 0.087 mg; Fe (as $NH_4SO_4^=$), 0.06 mg; Co (as Cl^-), 0.024 mg; Cu (as $SO_4^=$), 0.024 mg; Mo (as NH_4^+), 0.036 mg.

[2] 1 ml = adenylic acid, 20 mg; guanylic acid, 10 mg; cytidylic acid, 10 mg; thymidine, 10 mg.

[3] 1 ml = thiamine HCl, 0.5 mg; nicotinamide, 1.5 mg; pyridoxine HCl, 0.2 mg; biotin, 0.06 mg; putrescine·2HCl, 0.1 mg; vitamin B_{12}, 0.002 mg; choline H_2citrate, 0.2 mg; riboflavin, 0.2 mg; folic acid, 0.1 mg; Ca pantothenate, 4 mg.

[4] 1 ml = rice starch, 10 mg; 2× crystalline egg albumin, 4.10 mg.

[5] 1 ml = 2× crystalline egg albumin, 8 mg; egg lecithin, 0.75 mg; BHT (butylated hydroxytoluene), 1 mg; ergocalciferol, 0.66 mg; retinolpalmitate, 0.25 mg; palmitic acid, (16:0) 1 mg; oleic acid (18:1), 0.3 mg; linoleic acid (18:2), 0.7 mg; linolenic acid (18:3), 1 mg.

sequential transfer procedure diminished carryover of nutrients from the original control medium to the experimental medium.

When populations were harvested, animals were immediately counted, sized, and categorized. The categories were: female instars I–IV, adult parthenogenetic females, adult gamogenetic females, and adult males. Biomass of each population was determined from a length–dry weight relationship derived previously (D'Abramo, 1979). The number of animals that had died, as indicated by body distortion, was also recorded. Diets were compared by the average biomass of all 20 populations (composite biomass) harvested during the four day period. Statistical analysis employed two-way ANOVA and multiple comparison Scheffe tests (Zar, 1974). Standard error of mean values were calculated from the error mean square value derived from the ANOVA. Sets of dietary treatments were designed after examination of previous results. As the number of treatments increased, various groups were arranged as separate experiments for ease of comparison. Experiment I evaluated the effect of the egg lecithin (70% PC, ICN Nutritional Biochemicals, Cleveland, OH) component of the particulate portion of the diet. Quantities of lecithin were 0.0, 0.25, 1.00, and 1.50 mg% (control = 0.75 mg%). In experiment II, pure phospholipids (egg PC, soy PC, soy phosphatidylinositol (PI), and egg phosphatidylethanolamine (PE) (Sigma Chemical Co., St. Louis, Mo.)) were substituted for the egg lecithin of experiment I. These phospholipids were added at 0.5 mg%, since the control diet had egg lecithin that was 70% pure. In experiment III (diets B–F, Table II), lecithin was eliminated from the diets and the combined effects of increased fat in the particles and increased choline from the vitamin mix were analyzed. To compensate adequately for the calorific contribution of the lecithin, the fatty acid component of the particles of the diet was increased from 3.0 to 4.5 mg%. The choline in the vitamin mix (soluble component) was increased from 0.14 to 0.49 to 0.70 mg%. Experiment IV (diet A, Table III) evaluated the liver infusion's contribution with both particulate and soluble sources of choline absent. Total fat content of the particles remained at 4.5 mg%.

The choline in the liver infusion, the only undefined component of the maintenance medium, was analyzed quantitatively according to the method of Lim and Schall (1964). Experiment V (diets B–G, Table III) substituted for the liver infusion ingredient by increasing particulate and soluble choline sources, singly or in combination. In one sequence (diets B, C, G) the concentration of soluble choline as choline dihydrogen citrate was increased from 0.14 to 0.7 to 1.75 mg%, while the concentration of the pure soy lecithin in the particulate phase remained at 0.5 mg% (0.069 mg% choline). Soluble choline levels of 1.40 and 2.00 mg% (diets D and

TABLE II

Effect of increases in soluble choline on the composite biomass and mortality of 20 harvested populations of Moina macrocopa *grown on artificial diets. Liver infusion constant at 40 mg%.*

Diet	Fatty acid concentration	Soluble choline (mg%)	Particulate choline (mg%)	Biomass (μg) ± SD	% Mortality
A	3.0	0.0	0.075	716 ± 253	4.5
B	3.0	0.0	0.0	463 ± 238	16.1
C	4.5	0.0	0.0	619 ± 267	18.7
D	4.5	0.14	0.0	442 ± 178	18.2
E	4.5	0.49	0.0	883 ± 293	7.8
F	4.5	0.70	0.0	916 ± 102	4.2

TABLE III

Effect of no dietary choline, and substitution of the choline of the liver infusion with soluble and particulate sources of choline (singularly and in combination), upon composite biomass and mortality of 20 harvested populations of Moina macrocopa *grown on artificial diets. Liver infusion absent. Particulate source of choline was pure soy lecithin.*

Diet	Fatty acid concentration (mg%)	Soluble choline (mg%)	Particulate choline (mg%)	Biomass (μg) ± SD	% Mortality
A	4.5	0.0	0.0	387 ± 212	36.6
B	3.0	0.14	0.069	598 ± 348	30.0
C	3.0	0.70	0.069	979 ± 190	1.2
D	4.5	1.40	0.0	946 ± 188	3.0
E	2.5	0.0	0.138	742 ± 236	4.4
F	4.5	2.00	0.0	758 ± 168	2.8
G	3.0	1.75	0.069	614 ± 139	4.1

F) were substituted for the particulate sources of choline. Finally, all sources of soluble choline were removed and pure soy PC was increased to 1.0 mg% (0.138 mg% choline, diet E). The amount of free fatty acids normally added to the particles was decreased proportionate to the quantity and quality of the fatty acids introduced through the increase in soy PC.

RESULTS

In all experiments the effect of harvest day on *Moina* population biomass was significant ($P \leq 0.01$). In some cases (experiments I and II) this factor contributed to large standard deviations within the average biomass of the twenty harvested populations grown on each diet. For experiments I and II standard error of mean values for each diet were 72.8 μg and 112.9 μg respectively.

Experiment I

Eliminating the egg lecithin component from the control diet significantly reduced productivity ($P \leq 0.01$). Growth rates on the diet containing 1.5 mg% egg lecithin were significantly ($P \leq 0.01$) higher than those of the other diets. On any harvest day, biomass generally increased as the amount of egg lecithin in the diet increased from 0.0 to 1.5 mg% (Fig. 1).

Experiment II

Growth increased when the 70% pure egg lecithin of the control diet was replaced by pure phospholipid sources (egg PC, soy PC, and egg PE). The egg PC and soy PC were significantly ($P \leq 0.01$) better than the control diet. The pure soy PI substitute, however, produced poor growth and high mortality. Growth with the dietary egg PC was significantly greater ($P \leq 0.01$) than that associated with the egg PE (Fig. 2).

Experiment III

Adding free fatty acids to compensate for the calorific loss of the lecithin deletion (diet C, Table II) yielded biomass comparable to the control but resulted in 19% mortality. In diets containing 4.5 mg% total fatty acids and no lecithin, increasing

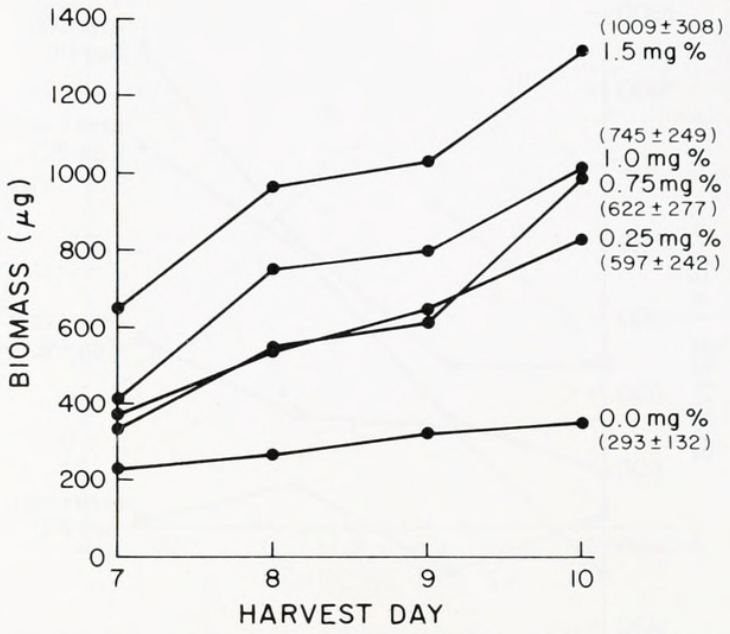

FIGURE 1. Effect of increasing dietary egg lecithin (70% pure) levels on the growth of *Moina macrocopa* fed an artificial diet. Control diet = 0.75 mg% lecithin. Liver infusion was present, soluble choline (as choline dihydrogen citrate from vitamin mix) = 0.14 mg%. Points for each day represent average biomass of five observations. (N) = average + SD biomass of all 20 observations for a particular diet. Standard error of the mean for any diet on any day = 72.8 μg. Average population mortality < 2%, except 14.3% for populations grown on diet with no lecithin.

soluble choline as choline dihydrogen citrate (diets D, E, F, Table II) further enhanced growth rates and reduced mortality. At 2 mg% choline dihydrogen citrate (0.7 mg% choline, diet F) population biomass was not significantly different ($P \leq 0.05$) than that of the pure egg lecithin diet (experiment II, Fig. 2) containing 0.069 mg% choline in the particulate form. The relative amounts of soluble and particulate sources of choline needed to achieve comparable growth rates indicate that a particulate source of choline is 10 times more efficient than a soluble source for these filter feeders.

Comparing the composite biomass data of the control diet with diet A, and that of diet C with diet D (Table II), suggests that the choline available from the vitamin mix additive (0.14 mg%) made no significant contribution to the population biomasses ($P \leq 0.01$).

Experiments IV and V

When *Moina* was fed diet A (Table III), which lacked both liver infusion and egg lecithin, growth was slow, and almost 40% of the population died. The amount of lecithin in the liver infusion was negligible (G. Holz, Suny, Upstate Medical Center, personal communication), but quantitative analysis showed that choline was 1.1% of the dry weight. A 40 mg% addition of liver infusion, therefore, provides 0.44 mg% choline, three times the level of soluble choline introduced via the vitamin mix of the control diet.

The experimental diets in which liver infusion was substituted with particulate choline, soluble choline, or a combination of the two demonstrated a growth response to choline. When 0.5 mg% soy lecithin was used as a particulate source of choline in a diet containing 3.0 mg% total fatty acids, and the amount of soluble

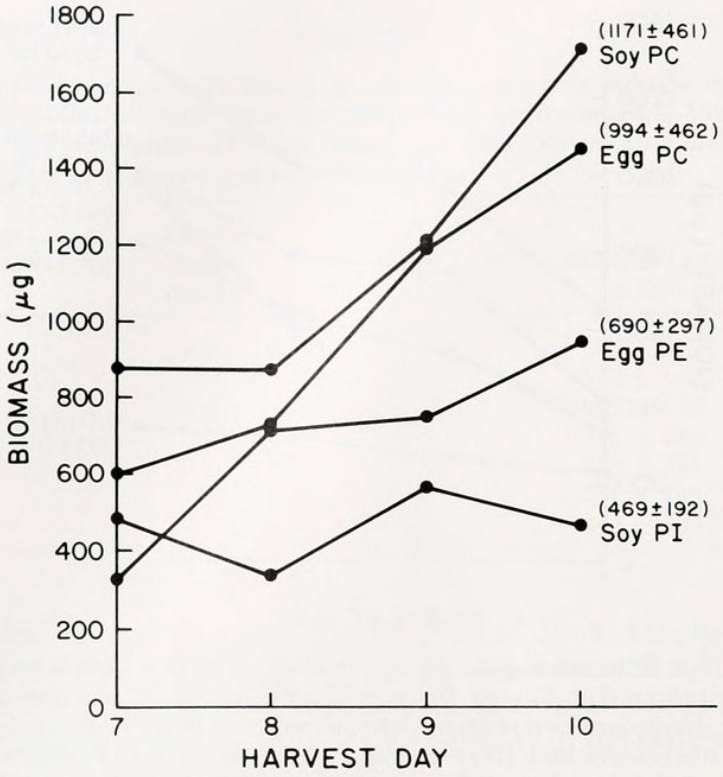

FIGURE 2. Effect of different pure phospholipids on growth of populations of *Moina macrocopa* fed an artificial diet. Phospholipid concentration was constant at 0.5 mg%. Liver infusion was present, soluble choline (as choline dihydrogen citrate in vitamin mix) = 0.14 mg%. Points for each day represent average biomass of 5 observations. (N) = average + SD biomass of all 20 observations for a particular diet. Standard error of the mean for any diet on any day = 112.95 μg. Average population mortality < 2%, except 12.8% for populations grown on diet containing PI.

choline was increased from 0.14 mg% (diet B) to 0.7 mg% (diet C), more animals survived, and biomass increased to 83.3% of that from a similar diet containing 40 mg% liver infusion and 0.14 mg% soluble choline (experiment II, soy PC, Fig. 2). Similar growth and survival occurred on diets in which the sole source of choline was soluble (1.40 mg%, diet D) or particulate (0.138 mg%, diet E). However, growth was significantly reduced from that of diets D and E ($P \leq 0.01$) by further increases in the concentration of total dietary choline, in either soluble form (2.00 mg%, diet F) or in combination (1.75 mg% soluble, 0.069 mg% particulate, diet G) (Table III). Figure 3 shows the relationship of total dietary choline (mg%) to the composite biomass of *Moina* populations grown on those diets that contained no liver infusion. Total dietary choline levels were standardized and expressed as particulate sources. Soluble choline amounts were converted to their particulate equivalent by assuming a previously described particulate-soluble nutrient source efficiency of 10 to 1. The fatty acid concentrations of these diets varied slightly from 2.5–4.5 mg%, but the growth response to choline is apparent.

Experimental diets that yielded composite biomass values that were less than 500 μg were considered nutritionally inferior. Often, the original female inoculant or her first clutch died, and many additional populations had to be started to attain some representative biomass data. This procedure introduced an element of artificial selection. We assume that under normal conditions these low biomass diets would not support continuous growth and reproduction. Population growth comparisons did not involve these nutritionally inadequate diets.

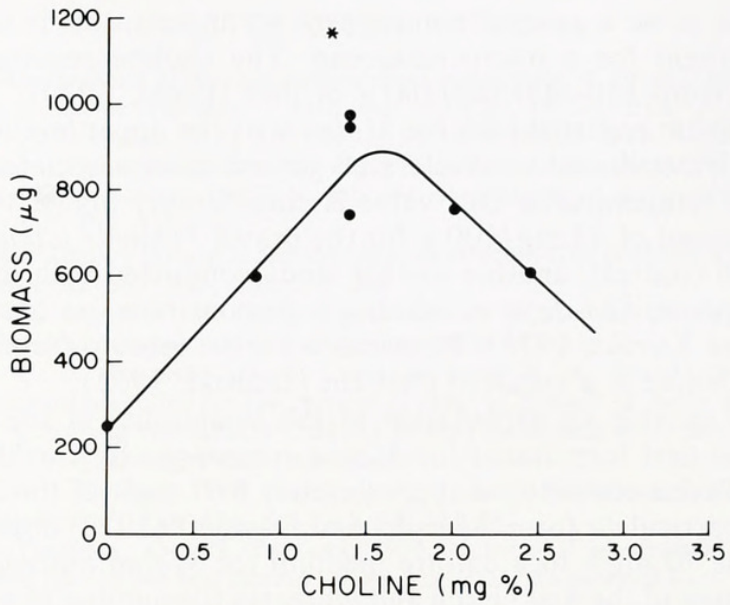

FIGURE 3. Relationship between the level of dietary choline and the average biomass of harvested populations of *Moina macrocopa* grown on artificial diets. Liver infusion was absent. Each point represents an average of 20 observations. * = average biomass of populations grown on soy PC diet (Experiment II).

DISCUSSION

Choline clearly is a required nutrient for growth and survival of the microcrustacean *Moina macrocopa*. In our artificial diets, this nutrient made its major contribution via the lecithin in the particulate portion of the biphasic media. Dietary lecithin, as a source of lipid, probably also contributes to increased growth rates. D'Abramo (1979) found that increasing dietary fatty acids from 3.0 to 4.5 mg% enhances growth of *Moina macrocopa*. Our observation of 10-fold greater effectiveness of a particulate rather than a soluble source of choline is lower than the 60-fold greater effectiveness of particulates estimated by Provasoli and D'Agostino (1969) for brine shrimp, *Artemia salina*, grown on artificial media. Provasoli and D'Agostino, however, based their estimates on growth with soluble amino acids versus particulate egg albumin. In such a situation, the inefficiency of soluble nutrient sources may be compounded by the crustacean's inability to utilize protein as singular amino acids rather than as polypeptides.

The population growth achieved by replacing the egg lecithin of the control diet with pure egg PE revealed that *Moina macrocopa* can efficiently synthesize choline via methylation of ethanolamine. This biosynthetic pathway is also described for the lobster *Homarus americanus* (Shieh, 1969). Use of this pathway alone, however, reduces population growth of *Moina* by 30%.

The greater nutritional value of pure soy PC versus pure egg PC was probably related to the different constituent fatty acids of the two ingredients. The qualitative effect of dietary fatty acids on the productivity of *Moina* has been discussed previously (D'Abramo, 1979). The poor quality of the diet containing pure soy phosphatidylinositol indicated that the requirement for lecithin is not a phospholipid requirement *per se*.

From the results obtained in experiment IV and the demonstrated increased effectiveness of particulate choline (10×), we estimate that the choline requirement for *Moina macrocopa* is 750–850 mg/100 g of particulate diet. Although choline

has been shown to be a general requirement for insects, this is the first report of such a requirement for a microcrustacean. The choline requirement of several insects ranges from 150–900 mg/100 g of diet (Dadd, 1970). The quantitative choline requirement reported here for *Moina* is at the upper level of this range and may be partially attributed to accelerated growth rates associated with the warm (26°C) culture temperatures. Our value is considerably higher than the reported choline requirement of 45 mg/100 g for the prawn *Penaeus japonicus* (Kanazawa *et al.*, 1976). In contrast, another dietary study conducted with this prawn but for a longer time period (84 days vs. 40 days), demonstrates no choline requirement (Deshimaru and Kuroki, 1979). Research with the lobster *Homarus americanus* suggests that choline is a required nutrient (Bilinski, 1962).

Our results provide an explanation of the essentiality of the liver infusion in the artifical diet first formulated for *Moina macrocopa* by Conklin and Provasoli (1977): The infusion contributed approximately 0.07 mg% of the essential nutrient choline in the particulate form. Murphy and Davidoff (1972) observed that adding liver infusion at 70 mg% to a culture medium for *Moina macrocopa* reduces the time to production of the first clutch and increases the number of young per female. Most likely, their results principally reflect the effects of increased choline, since the amount of liver infusion added equals 0.77 mg% soluble choline, a 54% increase over the amount contained in the original medium (0.5 mg%).

The ability to replace the liver infusion component of the artificial diet with choline results in a completely defined medium for continuous culture of *Moina macrocopa*. Such substitution, however, was not complete because the highest composite biomass attained on a diet without liver infusion was less than that of a similar diet containing liver infusion. Although the liver infusion contained 14% neutral lipid, previous experiments (D'Abramo, 1979) show that defatted liver infusion is equally effective. The possible contribution of other water soluble vitamins such as riboflavin and folic acid remains to be investigated.

The present study shows that the absence or presence of a suboptimal amount of a micronutrient can significantly affect the survival, growth, and reproduction of *Moina macrocopa*. This implies that this culture technique could be used effectively for bioassays.

Our results also emphasize the possible shortcomings involved in estimating productivity of herbivorous zooplankton populations in terms of calorie, chlorophyll, or carbon content of coexisting phytoplankton populations. Moreover, the population dynamics of a zooplankton community may not only be regulated by factors such as temperature and predation. For example, competitive interactions may be based on the availability of a particular micronutrient and the ability of a species to store or use alternative biosynthetic pathways for production of that micronutrient. Indeed, seasonal succession of zooplankton species may be partially determined by their differing nutritional requirements.

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LITERATURE CITED

BILINSKI, E. 1962. Biosynthesis of trimethylammonium compounds in aquatic animals. III. Choline metabolism in marine crustacea. *J. Fish. Res. Bd. Can.* **19:** 505–510.

CONKLIN, D. E., L. R. D'ABRAMO, C. E. BORDNER, AND N. A. BAUM. 1980. A successful purified diet for the culture of juvenile lobsters: the effect of lecithin. *Aquaculture* **21:** 243–249.

CONKLIN, D. E., AND L. PROVASOLI. 1977. Nutritional requirements of the water flea, *Moina macrocopa. Biol. Bull.* **152:** 337–350.

D'ABRAMO, L. R. 1979. Dietary fatty acid and temperature effects on the productivity of the Cladoceran, *Moina macrocopa. Biol. Bull.* **157:** 234–248.

D'ABRAMO, L. R. 1980. Ingestion rate decrease as a stimulus for sexuality in populations of the Cladoceran, *Moina macrocopa. Limnol. Oceanogr.* **25(3):** 422–429.

D'ABRAMO, L. R., C. E. BORDNER, D. E. CONKLIN, AND N. A. BAUM. 1981. Essentiality of dietary phosphatidylcholine for the survival of juvenile lobsters. *J. Nutr.* **111:** 63–69.

DADD, R. H. 1970. Arthropod Nutrition. Pp. 35–95 in M. Florkin and B. T. Scheer, Eds., *Chemical zoology*, Vol. V. Academic Press, New York.

DESHIMARU, O., AND K. KUROKI. 1979. Requirement of prawn for dietary thiamin, pyridoxine and choline chloride. *Bull. Jpn. Soc. Sci. Fish.* **45:** 363–367.

KANAZAWA, A., S. TESHIMA, AND N. TANAKA. 1976. Nutritional requirements of the prawn. V. Requirements for choline and inositol. *Mem. Fac. Fish. Kagoshima Univ.* **25(1):** 47–51.

KANAZAWA, A., S. TESHIMA, S. TOKIWA, M. ENDO, AND F. ABDEL RAZEK. 1979. Effects of short-necked clam phospholipids on the growth of the prawn. *Bull. Jpn. Soc. Sci. Fish.* **45(8):** 961–965.

LESTER, R., M. C. CAREY, J. M. LITTLE, L. A. COOPERSTEIN, AND S. R. DOWD. 1975. Crustacean intestinal detergent promotes sterol solubilization. *Science* **189:** 1098–1100.

LIM, F., AND E. D. SCHALL. 1964. Determination of choline in feeds. *J. Assoc. Off. Agric. Chem.* **47(3):** 501–503.

MURPHY, J. S., AND M. DAVIDOFF. 1972. The result of improved nutrition on the Lansing effect in *Moina macrocopa. Biol. Bull.* **142:** 302–309.

PROVASOLI, L., AND A. S. D'AGOSTINO. 1969. Development of artificial media for *Artemia salina. Biol. Bull.* **136:** 434–453.

SHIEH, M. S. 1969. The biosynthesis of phospholipids in the lobster, *Homarus americanus. Comp. Biochem. Physiol.* **30:** 679–684.

ZAR, J. H. 1974. *Biostatistical analysis.* Prentice-Hall Inc., Englewood Cliffs, N. J. 620 pp.