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DIXENIC CULTURE OF *DAPHNIA MAGNA*, STRAUS

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Banta (1921) demonstrated that *Daphnia* could be grown agnotobiotically with relative ease. Since then, rearing was improved by replacing horse-manure infusion with unialgal cultures (Léfèvre, 1942). Large populations of *D. magna* were maintained for 18 months in artesian well water, and fed with yeast and *Scenedesmus;* these cultures were stable and supplied abundant progeny of known age, genetic homogeneity and vigor, for insecticide bio-assays (Dewey and Parker, 1964). Attempts to replace pond water with synthetic salt solutions have met with limited success (Anderson, 1945; Freeman, 1953; Boyd, 1957; Taub and Dollar, 1964). Dewey and Parker (1964) reported that under the most favorable conditions (Wesson's (1932) salt mixture in distilled water) fecundity gradually declined and reproduction ceased at the 32nd generation.

Taub and Dollar (1968) re-explored the dependence of alga-fed *Daphnia pulex* on water biologically conditioned in aquaria (BCW). They concluded that algae grown in defined media were deficient in nutritional factors needed by *Daphnia*. Presumably "BCW" contained unidentified substances, which fostered normal development and ovulation of *Daphnia*.

Since this problem seemed nutritional, we axenized *D. magna* and fed it on pure cultures of algae to define conditions for sustained culture.

Material and Methods

Crude cultures

Agnotobiotic *D. magna* strain #10 was kindly supplied by Dr. F. Taub, College of Fisheries, Seattle, Washington. Stock cultures were maintained in covered "storage dishes" (Corning #3250), 30 individuals in 200 ml of DM_2, a synthetic medium containing (w/100 ml dist. H_2O): KCl, 5 mg; $MgSO_4 \cdot 7H_2O$, 4 mg; Ca (as Cl^-), 2 mg; K_2HPO_4, 0.6 mg; KH_2PO_4, 0.6 mg; $NaNO_3$, 5 mg; $NaSiO_3 \cdot 9H_2O$, 2 mg; Fe (as Cl^-), 0.05 μg; Metals PII, [1 ml of P II metals contains: ethylenediaminetetraacetic acid (as Na_2), 1 mg; Fe (as Cl), 0.01 mg; B (as H_3BO_3), 0.2 mg; Mn (as Cl), 0.04 mg; Zn (as Cl), 5 μg; Co (as Cl), 1 μg; (Provasoli, McLaughlin and Droop, 1957)] 1 ml; Metals S II, [1 ml of S II metals contains: Br (as Na), 1 mg; Sr (as Cl), 0.2 mg; Rb (as Cl), 0.02 mg; I (as Cl), 0.02 mg; Mo (as Na salt), 0.05 mg; I (as K), 1 μg; V (as NH_4VO_3), 1 μg; (Provasoli, McLaughlin and Droop, 1957)] 1 ml; vitamin B_{12}, 1 μg; and thiamine HCl, 10 μg; adjusted to pH 7.0.

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They were fed axenically grown *Scenedesmus obliquus* (Indiana Collection No. 393) and *Chlamydomonas reinhardi,* minus strain (Indiana Collection No. 90). Continuous cultivation was kept by subculturing the first 30 newborn nauplii of each generation.

Transfer dishes were prepared 8 days previously by inoculating DM_2 with algae grown on DA medium. [DA medium: mg/100 ml: Na_3·citrate·$2H_2O$, 2.0; $MgSO_4 \cdot 7H_2O$, 2.0; K_2HPO_4, 2.0; Fe (as SO_4), 0.2; Thiotone (Baltimore Biol. Lab), 60.0; Trypticase (Baltimore Biol. Lab), 16.0; yeast extract (Difco), 5.0; pH 6.5 (DA = Medium A, Table 2, p. 840, Provasoli and Pintner, 1953).] Addition of DA to DM_2 promoted initially rapid growth of bacteria which soon subsided and was succeeded by algal growth. Mortality was high if newborn *Daphnia* were inoculated during the period of bacterial growth.

Antibiotics

Serial washings of nauplii from crude cultures failed to eliminate bacteria. Antibiotics were tried after determining their effectiveness on the bacterial flora association with *Daphnia.* Samples of water from crude cultures were diluted with an equal volume of Dextrose Broth (Difco); 4 hours later 1-ml aliquots were spread on solidified nutrient agar (Difco). One hour later, surplus fluid was removed and antibiotic sensitivity discs (Difco) were pressed on the agar surface. Zones of inhibition, at room temperature, were recorded at 24, 48 and 72 hours. No one antibiotic completely inhibited bacterial growth. Streptomycin sulfate and chloromphenicol were the most effective; however, resistant strains developed within the zones of inhibition in all cases.

Antibiotics effective against the micro-organisms were tested for toxiciity to *D. magna.* Nauplii or adults of *Daphnia* were exposed to varying concentrations of antibiotics in 10 ml of DM (= DM_2 without thiamine and B_{12}). Organic substances were excluded to avoid excessive growth of resistant bacteria. The antibiotics were dispensed from fresh Seitz-filtered stock solutions. *Chlamydomonas reinhardi* and *Scenedesmus obliquus* were added as food. The cultures were observed twice daily. Inhibitory concentrations of antibiotics paralyzed the second antennae of the *Daphnia,* the animals settled to the bottom of the tube and soon died.

Single antibiotics permitting survival of adults and larvae for > 2 days were (μg/ml): chloramphenicol 25; kanamycin 100; nalidixin 50; neomycin 20; polymixin 7.5 (1000 μg = 7760 units) penicillin 500 (1000 μg = 1650 units); streptomycin-SO_4 100; tetracycline 10; trichomycin 50.

Several mixtures designed to suppress a wide bacterial spectrum were prepared and tested. Chloramphenicol, kanamycin, nalidixin and polymixin were omitted because few *Daphnia* exposed to them survived. The concentration of penicillin was kept below 450 μg/ml because larger doses changed the pH of the media to < 5.6—a pH not tolerated by *Daphnia* in these media.

Axenization of Daphnia magna

In crude cultures *D. magna* nauplii became females after 8 days and deposited their first parthenogenetic brood (4–6 larvae per female). Seven-day-old gravid females with eggs ready to hatch were transferred to tubes containing 10 ml of

sterile DM and 0.5 ml of antibiotic mix D [antibiotic mix D contained (μg/ml): neomycin 10; streptomycin 1500; penicillin 6000 (1000 μg = 1650 units); tetracline 400]. This concentration, although lethal to adults, allowed 2–3-day survival —an ample time for release of neonates.

To prevent fecal accumulation and overgrowth by bacteria, the females were transferred every 15 minutes to fresh tubes similarly prepared. Usually the nauplii hatched during the same working day; if not, gravid females were left overnight in DM and 0.25 ml/10 ml of antibiotic mix D. Alternatively, sublethal doses of antibiotic mixtures (0.1 ml/10 ml), were used; obviously more rinses were needed to remove by dilution the bacterial flora.

Neonates collected within minutes after deposition were washed through 10 serial baths (10 ml DM + 0.25 ml antibiotic mix D). The animals remained in each bath 10 minutes. After the 10th wash, sterility was tested by transferring them into DA liquid medium for $\frac{1}{2}$ hour [This tube was incubated in the dark at 28° C. If infected, visible turbidity appeared before algal growth could mask it. Darkfield microscopy was also used to detect infectants.]; then they were distributed singly into tubes with 10 ml of DM_2. Several food algae were added aseptically alone and in combinations to find an adequate food for axenic cultures.

Maintenance of axenic stock cultures

Bacteria-free nauplii developed into fertile females readily when fed *S. obliquus* and *C. reinhardi* grown in DA medium. To maintain dixenic cultures, filial generation nauplii were collected and washed three times in 10 ml of DA liquid medium; the last wash tube served as sterility test. The washed larvae were distributed singly in screwcap tubes (20 × 125 mm Pyrex) containing 10 ml of DM_2 and inoculated with 0.5 ml each of dense cultures of the two algae grown in liquid DA. The tubes were incubated at room temperature in racks illuminated (200 ft-c) continuously by white fluorescent lamps. This initial inoculum of algae sufficed to feed the larvae and to produce ample algae for feeding *ad libitum* the adult female for 20 days if each newborn brood was withdrawn within a day of deposition. Every 30 or so generations, records of survival, developmental time, and fecundity were made on a set of seven tubes. Survival of nauplii was 90–100%; of these, 90–95% became fecund females in 8 days. The average production was 29.7 nauplii per female life-span. After the 20th day, the algal population became too small to support *Daphnia* fertility. Over 200 generations of *Daphnia* have been obtained to date without lessening of vitality.

Several variables that may have influenced cultures were studied. In each case nauplii were transferred consecutively into six 10-ml tubes of DM to minimize carry-over of nutrients and to eliminate the algae, before being inoculated into the experimental tubes.

Results

Experience with mineral media for lake algae indicates that they require dilute media (Chu, 1942; Rodhe, 1948; Provasoli and Pintner, 1953). Although optimal growth depends, roughly, on Ca/Mg, Na/K and divalent/monovalent ratios being optimal, planktonic algae adjust well to wide variations provided that tolerance limits in respect to total solid concentrations are not approached (Provasoli, McLaughlin

and Pintner, 1954). Chu (1942) simply diluted the old medium of Benecke, then determined the best ratios for planktonic algae. Murachi and Imai (1954) found that a slightly modified Bristol solution diluted 10 × was satisfactory for *Moina macrocopa.*

Several dilute media for freshwater algae were tried. Various modifications and combinations of the more promising media led to medium DM (= DM_2 minus vitamins), which supported good algal growth without injuring *Daphnia.* These trials were done with bacterized cultures of *Daphnia.* Under such conditions *D. magna* fed *Scenedesmus* survived only a few generations. Other food organisms were tried. The combination *S. obliquus* and *Chlamydomonas reinhardi* seemed best: it allowed 30 non-axenic generations before fertility decreased. [These two algae were grown in DM medium and inoculated in new DM a few days before transferring the newborn *Daphnia* of the next generation.]

In several cases, for lack of well-grown algal cultures, we used, as inoculum algal cultures from the culture collection which were grown on agar slants of DA medium; the organic components do not harm algal growth and permit detection of contamination. Because of the organic content of the medium, care was taken at first to remove the algae without digging into the agar since carry-over of nutrients might lead to excessive bacterial growth. On the contrary, introduction of DA agar flakes resulted in better growth of *Daphnia.* This observation became useful when fertility of *Daphnia* declined; large flakes of DA agar were added with the algal inoculum and restored fertility. Later on 0.5 ml of each algal culture grown in DA broth was used as inoculum. Thirty additional non-axenic generations were obtained before maintenance of the bacterized strain was discontinued.

Meanwhile axenic newborn specimens of *D. magna* were obtained. Several potential food organisms were tried which had been grown on DA agar. Inoculated axenically into DM_2 medium, *Chlorella vulgaris* and *C. elipsoidea* supported development to young females but not reproduction; *Navicula pelliculosa* and *Saccharomyces cerivisiae* allowed maturation of adults and egg production, but the eggs never hatched. *Scenedesmus quadricaudatum* and *Chlamydomonas moewusii* permitted only survival of larvae (1–3) days). *S. obliquus* and *C. reinhardi,* used singly, supported growth up to young females; increasing the light from 100 to 200 ft-c resulted in adult females but no reproduction. *S. obliquus* at 200 ft-c produced substances which thickened the medium; as a result, the specimens of *Daphnia* were immobilized and soon died. *D. magna* fed on the combination *S. obliquus* and *C. reinhardi* in DM_2 gave adult females at 100 ft-c and finally nauplii at 150 and 200 ft-c. Surprisingly the presence of *C. reinhardi* resulted in no gelling of the medium by *S. obliquus.*

Ten generations of *D. magna* were obtained in DM_2 with the 2 algae as food, then fertility fell off sharply. By that time we had found with bacterized cultures that the addition of DA (1 ml/10 ml medium) restored fertility. This held even under germ-free conditions. We are now a the 200th germ-free generation; mean generation time 8.5 days; mean survival of fecund females 20–22 days; mean newborn production per female life-span 30 (Table I).

Agitation and higher concentrations of nitrates and phosphates were tried to eliminate the possibility that the organic enrichment simply made good a deficiency of the medium in these nutrients. In media without vitamins (DM), increase in

total phosphates up to 4 mg% and nitrates to 40 mg% resulted in ovigerous females (as opposed to young females with lower concentrations of N and P) but the eggs generally did not hatch and the few nauplii obtained were sickly. Addition of vitamin B_{12} and thiamine (DM_2), DA or liver extract resulted in viable nauplii. Several modifications of DM_2 were then tried (Table I). Higher N and P and/or the addition of an N-containing pH buffer failed to support more generations even in the presence of B_{12} and thiamine.

TABLE I

Modification of Basal media

(*food algae* S. obliquus *and* C. reinhardi)*

	Media designation (mg%; w/v)						
	DM_2	DM_3	DM_3 +DA	DM_4	DM_4 +G	DM_6	DM_6
P (as K_2HPO_4 and KH_2PO_4)	0.24	0.24	0.24	0.24	0.24	0.81	0.81
N (as $NaNO_3$)	0.82	0.82	0.82	0.82	0.82	3.3	3.3
N (in 30 mg% glycylglycine buffer)		6.3					
Thiamine (μg%, w/v)	10	10	10		6.3		10
B_{12} (μg%, w/v)	0.1	0.1	0.1				0.1
Yeast extract			0.5				
Thiotone (Baltimore Biol. Lab.)			6.0				
Trypticase (B.B.L.)			1.6				
Daphnia magna							
Generations**	12	12	>200	1	1	1	10
Generation time***	9.8	8.6	8.5 [6–11]	9	9	9	12.9
Mean new born/♀****	15.6	23	29.7 [10–66]	9	5	2	21.3

* Algae grown separately in various liquid media and fed to *Daphnia* being cultured serially in corresponding media.

** Consecutive generations obtained.

*** Days needed by a newborn nauplius to become a fertile female.

**** Average of several trials.

Since the only factors inducing sustained, if limited fertility were vitamin B_{12} and thiamine, other vitamins were tried as replacements of the organic enrichment of the DA medium. Since the growth and fertility of *Daphnia* apparently depended upon the composition of the medium in which the algae were grown, depleted algae grown serially in a medium without vitamins (medium DM) when inoculated with *Daphnia* nauplii in media with vitamins might show a substantial difference in number of F_1 nauplii. This was indeed so: the effect of various vitamins was evident in 3 weeks. The only vitamin which significantly increased the F_1 nauplii was B_{12}. Combined with B_{12} and thiamine, pantothenate increased nauplii production (Experiment A, Table II); pyridoxine was occasionally effective.

To confirm these short-term results, in Experiment B (Table II), the algae were again grown without vitamins (DM_7) and subjected to the experimental variables, reduced after the F_1 to one optimal concentration for each vitamin. The

TABLE II

*Effect of vitamins (No. nauplii/♀)**

Vitamin addition (μg%)	Exp. A	Exp. B												Exp. C†
	F_1 only	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{15}	F_{20}	
Mineral base (DM_7)	2	0.7	0											0
DM_7 + B_{12} 0.05		8.3	9	discontinued										
DM_7 + B_{12} 0.1	18	21	26	24	26	18	8	1.7	0.3	0				26
DM_7 + B_{12} 0.2		19	22	discontinued										
DM_7 + thiamine 25		0												
DM_7 + thiamine 50	2.5	4.5	0											0
DM_7 + thiamine 100		2.3	0											
DM_7 + pantothenate 12**		0												
DM_7 + pantothenate 25**	0	0												0
DM_7 + pantothenate 50**		1.3	0											
DM_7 + B_{12} 0.1 + thiamine 50	20	23	24	22	15	11.3	7	0						23
DM_7 + B_{12} 0.1 + pantothenate 25	32	25	22	17.3	3	0								31
DM_7 + B_{12} + thiamine + pantothenate 25***	44	33	29	35	32	28	36	31	31	28	31	26	28	45
DM_7 + B_{12} + thiamine + pantothenate + pyridoxine****								28	32	30	33	26	31	
DM_7 + B_{12} 0.1 + thiamine 50 + DA (1 ml/10)		29	38	41	28	37	38	36	37	31	31	31	33	

* Each value in table is the average of triplicate tubes.
** Ca·pantothenate was filter-sterilized and added aseptically.
*** The No. nauplii given for F_1–F_5 are for the aseptic addition of 25 μg% of Ca·pantothenate. From F_6 on, the values are for 250 μg% Ca·pantothenate autoclaved with the medium. The generations F_6–F_{10} were run in duplicate series (*i.e.*, these 2 concentrations of pantothenate) of triplicate tubes; the average number of nauplii per female was almost identical indicating that autoclaving resulted in a high inactivation of pantothenate. The series with aseptic addition of pantothenate was discontinued at the F_{11} which became infected.
**** This series was inoculated with nauplii, of the F_6 in B_{12} + thiamine + Ca·pantothenate.
† See text.

previous results were confirmed: only B_{12} + thiamine + pantothenate supported fertility beyond the F_8, and the average number of nauplii per female life-span was similar but consistently lower than with DM_7 + B_{12} + thiamine + DA (1 ml/10 ml)—a combination similar to DM_2 + DA which supported over 200 aseptic generations. Most of the effect of the organic enrichment could thus be replaced.

The fact that in these experiments the algae and *Daphnia* were grown in the same medium made it difficult to discern whether the organic enrichment (or the vitamins) acted directly on the crustacea or *via* the algae. To rule out the possibility that *Daphnia* fertility depended upon direct uptake of organics from the medium, the algae were grown separately on agar media to avoid carry-over of enrichment, then fed to *Daphnia* in a completely mineral medium (DM). The original experiment (Table I) of comparing DM_2 and DM_2 + DA was repeated: *C. reinhardi* and *S. obliquus* were grown seperately on DM_2 and in DA solidified with 2% agar. When grown, a loopful of each alga was scraped from the agar surface, avoiding removal of agar pieces, and inoculated with one *Daphnia* nauplius in a tube of DM (Experiment C, Table II). Additional loopfuls of algae were added if needed. The line fed on algae grown in DM_2 failed to reproduce beyond the 11th generation. The line fed on algae grown in DA showed undiminished fertility (the experiment was discontinued at the 20th generation), duplicating the results obtained when liquid DA was added directly to the algae-crustacean culture.

TABLE III

Effects of algal preconditioning

Experiment	Medium used for culturing				Average results*		
	algal inoculum		algae + *Daphnia*		Nauplii/♀	Generation time (days)	No. generations averaged
	Mineral part	Enrichment	Mineral	Enrichment			
B	DM_7 liquid (no vitamins)	0	DM_7 +	B_{12} + thia. + pantoth.	31	12.6	15
			DM_7 +	B_{12} + thia. + DA medium	34	13.7	15
			DM_7	0	0.7	17	1
C	DM agar†	B_{12} + thia.	DM	0	18.4	8.5	11
	0	DA agar†	DM	0	19.5	8.6	20
Culture maintenance	0	DA liquid	DM	B_{12} + thia. + 1 ml/10 DA	29.7	8.5	50

* Average of data from one or more generations; for each generation the results of triplicate tubes were also averaged.

† Algal cells only were removed from agar and served as inoculum.

DISCUSSION

Numerous attempts to replace pond water with defined salt solutions for continuous cultures of daphnids have failed (at best only 30 consecutive generations of *D. magna* were obtained by Dewey and Parker). We experienced a similar failure in DM medium. Later results show that for continuous cultures of *D. magna*, choice of food organisms and, especially, provision of an organic enrichment are far more important than the composition of the mineral medium; our medium DM does not differ very much from medium 37 of Taub and Dollars (1968).

Fourteen species of Cladocera are now in monoxenic continuous culture with *C. reinhardi* as food organism in a unique medium consisting of Ca acetate, albumin, water-soluble vitamins, trace metals, and distilled water (Murphy, 1970); no advantage was found in adding other mineral salts! An explanation of this astonishing feat is simply that *C. reinhardi* is a collector of essential minerals (see composition in table 5, page 615, Taub and Dollar, 1968).

It is not surprising, therefore, that other daphnids thrive on DM_2 enriched with pantothenate; many bacterized generations of *D. galeata mendotae* (J. S. Suffern, Biology Dept., Yale University, personal communication) and >10 bacteria-free generations of *D. pulex* and *S. mucronata* (D. E. Conklin, Haskins Labs., personal communication) were obtained without loss of fertility when fed in this medium on *C. reinhardi* and *S. obliquus*.

Addition of vitamins to inadequate algal food had also been beneficial for *Tigriopus japonicus* (Shiraishi and Provasoli, 1959) but these findings were based on only a few generations. The results of Murphy attest that addition of vitamins is essential in supplementing the nutritive value of one alga for several daphnids. As in our experiments, vitamin B_{12} and Ca pantothenate had a decisive effect on female fertility and viability of nauplii, and permitted, with the addition of thiamine (= his basal medium), over 40 generations of *Daphnia pulex, Scapholeberis mucro-*

nata and *Simocephalus serrulatus* fed on *C. reinhardi* and *S. obliquus*. However this medium failed to support continuous culture of other daphnids until a mixture of 8 additional vitamins was added. This addition improved also the number of nauplii produced in the 1st brood of the above 3 species. In other experiments *C. reinhardi* could be employed as the sole food organism for 14 species by increasing the concentration of choline, pyridoxal, inositol, riboflavin, and nicotinamide. In this medium, *D. magna, S. serrulatus* and *D. retrocurva* were the most difficult to maintain, indicating that some daphnids may be nutritionally more exacting than others.

Previous experiments on *Artemia* (D'Agostino and Provasoli, 1968; Provasoli and D'Agostino, 1969) gave circumstantial evidence that organic enrichment influenced the fertility of *Artemia* (*i.e.*, number of generations) not directly but *via* the algae. The results with *D. magna* grown in mineral media and fed with algae which had been grown separately on organic enrichments or vitamins (Experiment C) seem to finally prove this hypothesis.

Experiments B and C support another hypothesis, *i.e.*, that the enrichments modify the nutritive value of the algae for *Daphnia*. Generation time (*i.e.*, days needed for a nauplius to become a fertile female) depended upon the kind of medium in which the algae used as inoculum were grown (Table III).

If the 2 algae were pregrown for several transfers in mineral media and inoculated in a rich medium with one *Daphnia* nauplius (Experiment B), the *Daphnia* generation time was long (12–13 days) but the number of nauplii per female was high (30–34). Conversely, if the algae were pregrown in organic media (DA or DM_2) and inoculated in mineral media (Experiment C) the generation time was short (8.5 d) and nauplii production per female was low (18–20).

The preconditioned algal inoculum, once transferred in a new medium with one *Daphnia* nauplius, starts to reproduce logarithmically; the medium greens. Simultaneously, the nauplius as it grows to a fertile female, grazes more algae. The algae, as they divide in the new medium, change in physiology and storage products, either losing gradually their nutritional value (if preconditioned in organics and inoculated in mineral media) or gaining nutritional value in the converse experiment. As it happened, the generation time should therefore be influenced by the type of medium in which the algae were pregrown, and production of nauplii by the type of medium in which the algae have been inoculated with the *Daphnia* nauplius. Since the difference in generation time between experiments B and C was of 4.5 days, the size of algal inoculum and/or rate of division of the algae were probably high. A closer equilibrium between grazing rate and algal division rate—grazing rate tending to nullify the positive or negative nutrient effect of algal division—should result in a much smaller differential in generation time. Naturally, the differential may also indicate that the medium-mediated physiological changes in the algae leading to a different cell composition are slow. The short generation time and high nauplii production obtained for 200 generations when the algae were pregrown (DA) and inoculated (DM_2 + DA) in organic enriched media confirms experiments B and C.

That the enrichment acts *via* the algae and not directly on the crustacea is further substantiated by the recent demonstration that crustacea are extremely inefficient in the uptake of solutes (Anderson and Stephens, 1969) and that because

of this, *Artemia salina,* when grown ***in vitro*** on artificial food, is unable to grow unless the major nutrients are presented as particles (Provasoli and D'Agostino, 1969).

Since the results of Murphy (1970), Lewis (1967) and of our group indicate that widely differing genera of freshwater, euryhaline and hyperhaline filter-feeding crustacea behave similarly, it seems probable that addition of vitamins to 2- or 3-membered algae-crustacea cultures might permit continuous cultivation of crustacea which so far have proved difficult to grow.

SUMMARY

1. *Daphnia magna* can be grown for at least 200 generations, axenically or in crude cultures, in a defined mineral medium, enriched with vitamin B_{12} and thiamine and 1 ml/100 of a dilute organic medium, when fed with *Chlamydomonas reinhardi* and *Scenedesmus obliquus.*

2. The organic enrichment is essential for maintaining continued fertility of *D. magna.*

3. The organic enrichment can be replaced by the addition of pantothenic acid to vitamin B_{12} and thiamine without lowering the fertility of *D. magna.*

4. The organic enrichment (or the vitamin mixture) does not act directly on *D. magna* but *via* the algae by changing their nutritional value for *Daphina.*

5. Addition of vitamins to the medium in which the algal food is grown with crustacea may allow continuous cultures of herbivorous crustacea which are considered difficult to grow.

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