Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3433–3437, June 1984
Ecology

Effect of selenium deficiency on cuticle integrity in the Cladocera (Crustacea)

(limnology/trace element nutrition/zooplankton nutrition/zooplankton culture)

KATHLEEN IRWIN KEATING AND BRENDA CABALLERO DAGBUSAN

Department of Environmental Science, Cook College, and the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ 08903

Communicated by G. Evelyn Hutchinson, November 30, 1983

ABSTRACT **_Daphnia pulex_ de Geer and _Daphnia magna_ Straus populations cannot be maintained in defined (_sensu stricto_) media containing less than 0.1 part per billion (ppb) of selenium. A concentration of 1 ppb is sufficient to satisfy minimal needs in otherwise sufficient media. In the first generation with no selenium added to the medium or detected in it, media deficiency is shown by a premature cuticle deterioration visually similar to senescence, by progressive loss of distal segments of second antennae (primary swimming appendages), and by a shortened lifespan. No progeny attain reproductive maturity in the second generation. Although experimental animals in prime condition exhibit a shortened lifespan in the first generation maintained at 0.5 ppb selenium, culture lines can be maintained at 0.5 ppb for indefinite numbers of generations if established as young orthoclones. Tests in organic-rich media indicate a significant sparing effect of organic additions. This selenium requirement is reminiscent of that for the stability of feathers in domestic fowl.**

Selenium deficiency diseases in domesticated vertebrates are well known (1, 2) and a ubiquitous need for the element is suggested by its roles in the structure of glutathione peroxidase and in electron transport (3–5). No specific metabolic role for selenium in invertebrates has yet been reported.

The recent development of the cladoceran maintenance system [the MS system (6)] has made study of the trace nutrient requirements of the Cladocera practical. The MS system (Table 1) relies on wholly defined media in which crystalline vitamin B_{12} is the only organic nutrient intentionally included in animal media, and biotin and thiamin are the only additional extraneous organic compounds that could be carried into animal cultures when MS-grown algae are used to feed the Cladocera. Uncertain inorganic trace contaminants are not introduced by the addition of commercially produced organics because the cladoceran's (heterotroph's) need for organic macromolecules is met by algae (autotrophs) produced within the controlled system. Unintended additions are, thereby, limited to those accompanying the best grade inorganic salts available and those inadvertently leached from equipment that comes in contact with media.

Prior studies, which led to the successful elucidation of cladoceran organic nutrient requirements (7–10) depended on culture media containing such undefined organics as liver extracts and yeasts. Although D'Abramo and Baum (11) clearly demonstrated that a liver extract contributes a significant concentration of choline to the nutritional regime established in their *Moina macrocopa* cultures, its role as a source of trace inorganics was not explored. Since trace elements, including selenium, concentrate in the liver and kidneys of vertebrates during long-term exposure to subtoxic levels, such inclusions must at the least confound the interpretation of trace nutrient requirements. Most other organic additions to culture media are equally suspect. In light of the capacity of selenium to substitute for sulfur, especially in methionine and cysteine (12, 13), extracts of animal tissues, blood fractions, and yeasts would be particularly undesirable in studies relating to selenium requirements.

Organics not only carry unlisted trace inorganics into cultures, directly satisfying requirements, they may also ameliorate specific demands for such elements. In the case of selenium deficiency in mammals, vitamin E serves as a palliative, lessening the absolute requirement for selenium but not wholly substituting for it (1).

METHODS AND MATERIALS

In most experiments a strain of *Daphnia pulex* from Nebraska, isolated by S. Schwarz and identified by C. Goulden, was used; some comparative studies were made on a strain of *D. magna* from the collection of L. Provasoli at Yale University. Bacteria-free conditions were maintained for both food and animal cultures. Temperature was 20 ± 2°C. At this temperature *D. pulex* adults molt at approximately 56-hr intervals, with a first brood on day 6 and 16–18 broods per lifespan, and *D. magna* adults molt at approximately 80-hr intervals, with a first brood on day 9 and 17–20 broods per lifespan. Both produce in excess of 300 progeny per individual mother. These figures are based on several years of both natural water-based and defined medium-based cultures. Light was 300 ± 20 footcandles (1 footcandle = 10.8 lux) from cool white fluorescent tubes. The light/dark cycle was 24/0 or 18/6 hr. There was a moderate increase in time between molts and in progeny per mother in the 18/6 cycle. The greater regularity in this cycle facilitates handling. Culture volume was 11.5–13.0 ml (including food additions) for *D. pulex*; 26.5 ml for *D. magna*. Animal medium was MS (Table 1) with various concentrations of selenium. Algal media included A-MS [essentially due to the work of Spina (14), Grafton (15), and Dagbusan]; Provasoli and Pintner's DA (16); and a natural water-based diatom medium—water collected from Linsley Pond (North Branford, CT) charcoal-stripped of organics (17) with 1.5% ESIsi macronutrient addition (18). Optical density was measured with a Bausch and Lomb Spectronic 20 at 435 nm. Selenium levels were determined with a Perkin–Elmer model 603 atomic absorption spectrometer equipped with a hydride system, MHS-10. Water employed in media and used for final rinse of glassware was distilled–deionized (organic and inorganic columns) and redistilled with a Corning Mega-Pure MP 6A still. Certified grade salts were employed for all media save that selenium was added as either (*i*) SeO_2 (Fisher AAS standard solution) or (*ii*) H_2SeO_3 (Fisher certified).

Food algae were harvested from cultures 6–16 days old. Both cells and the liquid in which they grew were added to animal cultures. Species included were *Chlamydomonas*

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: ppb, parts per billion (10^9).

Table 1. MS media components

Component	A-MS algal medium	MS animal medium
M solution		
Na_2EDTA	5 ppm	5 ppm
B (H_3BO_3)	1000 ppb	1000 ppb
Fe ($FeCl_3$)	400 ppb	400 ppb
Mn ($MnCl_2 \cdot 4H_2O$)	200 ppb	200 ppb
Li (LiCl)	100 ppb	100 ppb
Rb (RbCl)	100 ppb	100 ppb
Sr ($SrCl_2 \cdot 6H_2O$)	100 ppb	100 ppb
Br (NaBr)	50 ppb	50 ppb
Mo ($Na_2MoO_4 \cdot 2H_2O$)	50 ppb	50 ppb
Cu ($CuCl_2 \cdot 2H_2O$)	25 ppb	25 ppb
Zn ($ZnCl_2$)	25 ppb	25 ppb
Co ($CoCl_2 \cdot 6H_2O$)	5 ppb	5 ppb
I (KI)	5 ppb	5 ppb
Se (SeO_2)*	1 ppb	1 ppb
V (NH_4VO_3)	0.5 ppb	0.5 ppb
S solution		
Glycylglycine	250 ppm	250 ppm
$NaNO_3$	150 ppm	50 ppm
$CaCl_2 \cdot 2H_2O$	38 ppm	38 ppm
$MgSO_4 \cdot 7H_2O$	20 ppm	20 ppm
$Na_2SiO_3 \cdot 9H_2O$	145 ppm	10 ppm
KCl	10 ppm	10 ppm
$K_2HPO_4 \cdot 3H_2O$	10 ppm	10 ppm
KH_2PO_4	25 ppm	10 ppm
Vitamins		
Thiamin·HCl	75 ppb	
Biotin	0.75 ppb	
Vitamin B_{12}†	0.75 ppb	

M solution contributes various minor elements, especially metals; weights are for target element (source compound is within parentheses). S solution contributes major nutrients while providing suitable total dissolved solids and acceptable monovalent-to-divalent ion ratios; weight is for whole compound. ppm, parts per million; ppb, parts per billion (10^9). Note: Before autoclaving, adjust pH (NaOH or HCl, as needed) to 8.40 ± 0.05 for A-MS; or 8.00 ± 0.05 for MS. Ninety-six hours after autoclaving, the pH should be approximately the same as before autoclaving.

*Selenium was added only after the deficiency was recognized.

†Vitamin B_{12} is now added to MS animal media at a concentration of 1 μg/liter.

reinhardi Dangeard, GMS minus strain, OD at harvest was 800 ± 100 (approximately 4×10^6 cells per ml); *Nitzschia frustulum* Kützing, OD at harvest was 540 ± 50 (approximately 6×10^6 cells per ml); and *Ankistrodesmus convolutus* Corda, OD at harvest 1500 ± 150 (approximately 12×10^6 cells per ml). To 10-ml cultures of *D. pulex* were added 2 ml of *C. reinhardi* and 1 ml of *N. frustulum*. On occasion *D. pulex* was fed 0.25 ml of *A. convolutus*, 0.25 ml of *C. reinhardi*, and 1 ml of *N. frustulum*; on other occasions the *D. pulex* were fed 0.25 ml each of only the two green algae (*A. convolutus* and *C. reinhardi*). To 20-ml cultures of *D. magna* were added 1.5 ml of *C. reinhardi*, 4 ml of *N. frustulum*, and 1 ml of *A. convolutus*.

DETERMINATION OF SELENIUM REQUIREMENTS

During the early development of the basic macronutrient environment of the MS system, a small aliquot of natural water was included in animal cultures. This addition was in the form of a lakewater (Linsley Pond) culture medium for *N. frustulum*, the diatom employed as one of the food organisms. Diatoms were fed to the cladocerans by pipetting a portion of the algal culture (both cells and liquid) into the animal culture. In a given experiment, 8–16% (by volume) of the animal culture medium was, thus, actually natural water.

Once an acceptable macronutrient environment was established, the lakewater-based diatom medium was replaced by an early version of A-MS algal medium. The other food algae (the green algae) were grown in the high organic medium, DA. Animal cultures fed green algae from DA are considered organic-rich because the mixed organics from DA are carried into the animal culture when food algae are added.

When the natural water was eliminated from the MS system, both *D. magna* and *D. pulex* exhibited a consistent and obvious shortening of lifespan, and other cladoceran species, kept as stocks for future experimentation, required much more frequent transfer to ensure survival. Examination of dead and moribund experimental animals exposed a consistent pattern of cuticle deterioration associated with these premature deaths. This deterioration, which visually resembled a protracted senescence, appeared in *D. pulex* in five stages. *D. magna* exhibited a similar pattern. Each stage usually occupied a single molt period.

Stage 1. Softening, or surface deterioration, of the extremities of the cuticle of major swimming appendages (second antennae)—evidenced about 1 day before ecdysis by a "stickiness"; i.e., algal cells adhering to surfaces.

Stage 2. Incomplete redevelopment after molting of that portion of the cuticle covering setae (Fig. 1, segments A). Only the second antennae are so affected—even at Stage 5 other setae remain intact.

Stage 3. Incomplete redevelopment after molting of that portion of the cuticle covering rami—resulting in loss of rami (Fig. 1, segments B). Again, only the second antennae are so affected.

Stage 4. Incomplete redevelopment after molting of that section of the cuticle covering the basal portions of appendages—resulting in the loss of the remainder of the second antennae (Fig. 1, segments C). Weaker animals die at this point in the progression (Fig. 2 A–C and Fig. 3 A–C).

Stage 5. Incomplete redevelopment after molting of that portion of the cuticle covering the body of the animal—resulting in the animal's death.

Progressive degeneration began earlier in each generation. After 17 generations few animals reproduced, and none of their progeny survived to reproduce. Initially Stage 1 appeared at the 10th to 12th brood and the progression spanned five molt cycles (four molts). As lifespan shortened, the period of deterioration shortened and the stages became less clearly separated. When the last remnant of extraneous or-

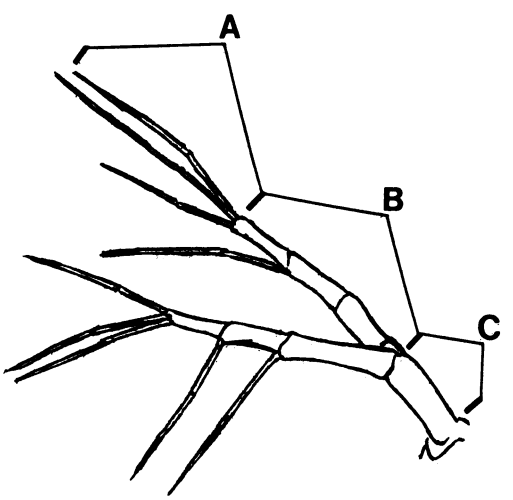

FIG. 1. Daphnid second antenna. A, Setae; B, rami; C, base of antenna.

ganics was removed from the system (discussed below), the period of deterioration was limited to two or three molt cycles.

The lakewater had been included as a source for ultratrace elements. Thus, it was quite likely that one to several essential trace nutrients, not previously included by design, were lost when lakewater was eliminated from the system. Trace nutrient additions were, therefore, tested. Of six elements (Al, Cr, F, Ni, Se, Sn), selected for their known significance to other organisms, only selenium restored cuticle integrity. A 0.5-ppb addition to the animal medium produced lifespans and reproductive patterns similar to those of broodmates fed lakewater diatoms. In Fig. 4, compare curves C, D, and E (all with added selenium) to curve A (with lakewater); contrast these with curve B (selenium deficient). If a portion of the lakewater diatom medium in which no diatoms had grown was added either to cultures fed no diatoms (Fig. 5, curve A) or to cultures fed diatoms from the new (deficient) inorganic medium (Fig. 5, curve C), lifespan was also restored. The food value of the diatom is demonstrated by a drop in number of progeny when no diatom is fed to the animals (Fig. 5, compare curves A and C). When neither selenium nor lakewater was provided, then premature cuticle deterioration and death were inevitable, whether or not diatoms were provided (Fig. 4, curve B and Fig. 5, curve B).

The detectable level of selenium in the lakewater diatom medium ranged from 0.8 to 1.1 ppb. Since 1 ml of this medium was added to 10 ml of animal cultures when diatoms were provided, approximately 0.1 ppb of selenium was added to animal cultures at feeding. This explained both the apparent sufficiency of the system when lakewater diatoms were provided, and the appearance of appendage loss when lakewater diatoms were replaced. Close examination of animals fed lakewater diatoms exposed minor, previously unrecognized, cuticle damage (Fig. 3 D–F); therefore, an addition of 0.5 ppb (instead of the 0.1 ppb associated with the lakewater diatom medium addition) to media was considered desirable. There was no concern that an excess of selenium would be introduced since in tests of selenium chronic toxicity (19) a level of 200–300 ppb produced only subtle sublethal effects on Cladocera.

Because bioconcentration of trace elements by algae is likely, the practice of providing a portion of the food algal culture (both algal cells and liquid) was adopted. This affords some control over amounts of trace elements carried into animal cultures at feeding. Unfortunately, the form in which a trace element is presented will determine its availability at any given concentration. Thus, estimates of requirements are likely to be approximate.

It was at this point that a green algal medium based on the MS inorganics was introduced to replace the high organic DA algal medium. This was a particularly meaningful change

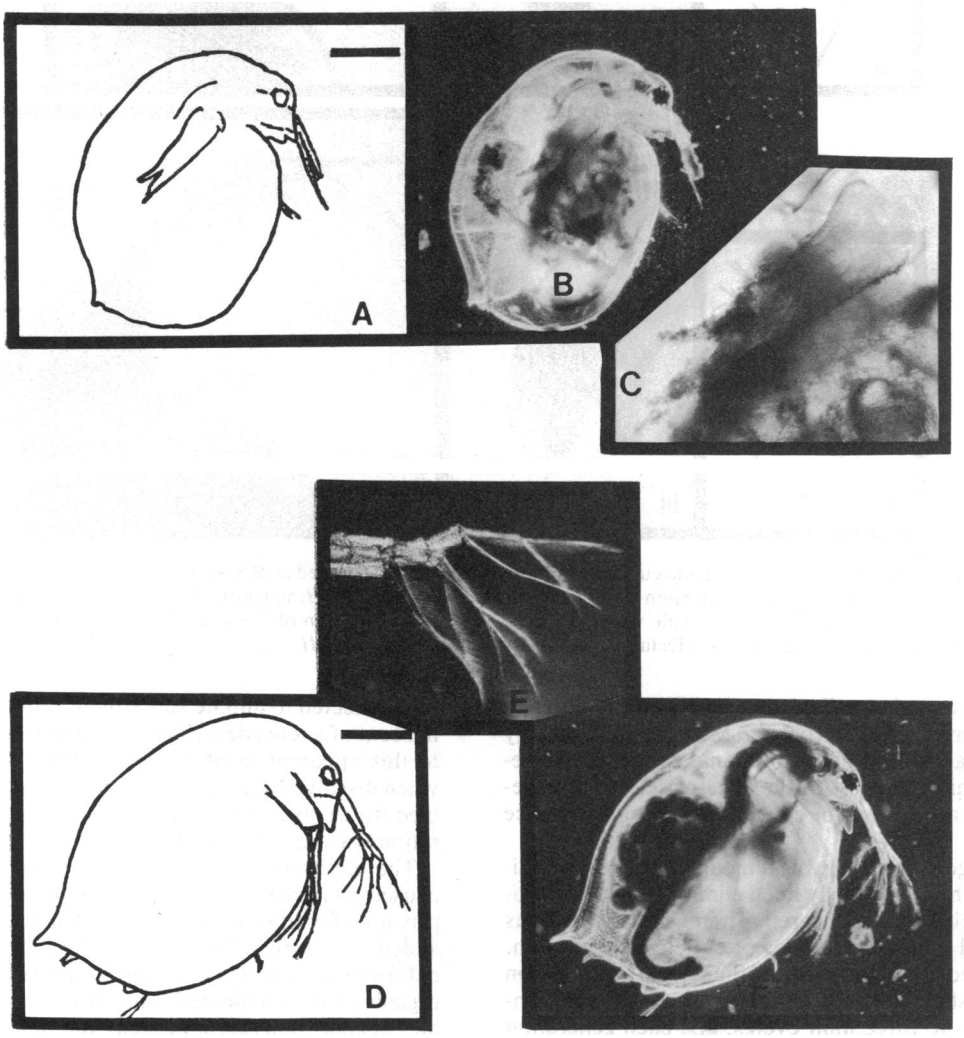

FIG. 2. *D. magna*, broodmates born in inorganic culture. (Bars = 1 mm.) (Mother reared in MS with selenium at 1.0 ppb.) (*A* and *B*) Reared in medium with less than 0.1 ppb selenium ("selenium-free"); side view. (*C*) Detail of the upper center of *B*, magnification 2⅔ × that of *B*. (*D* and *E*) Reared in medium with 1.0 ppb selenium; side view. (*F*) Detail of *E*, magnification 3 × that of *E*.

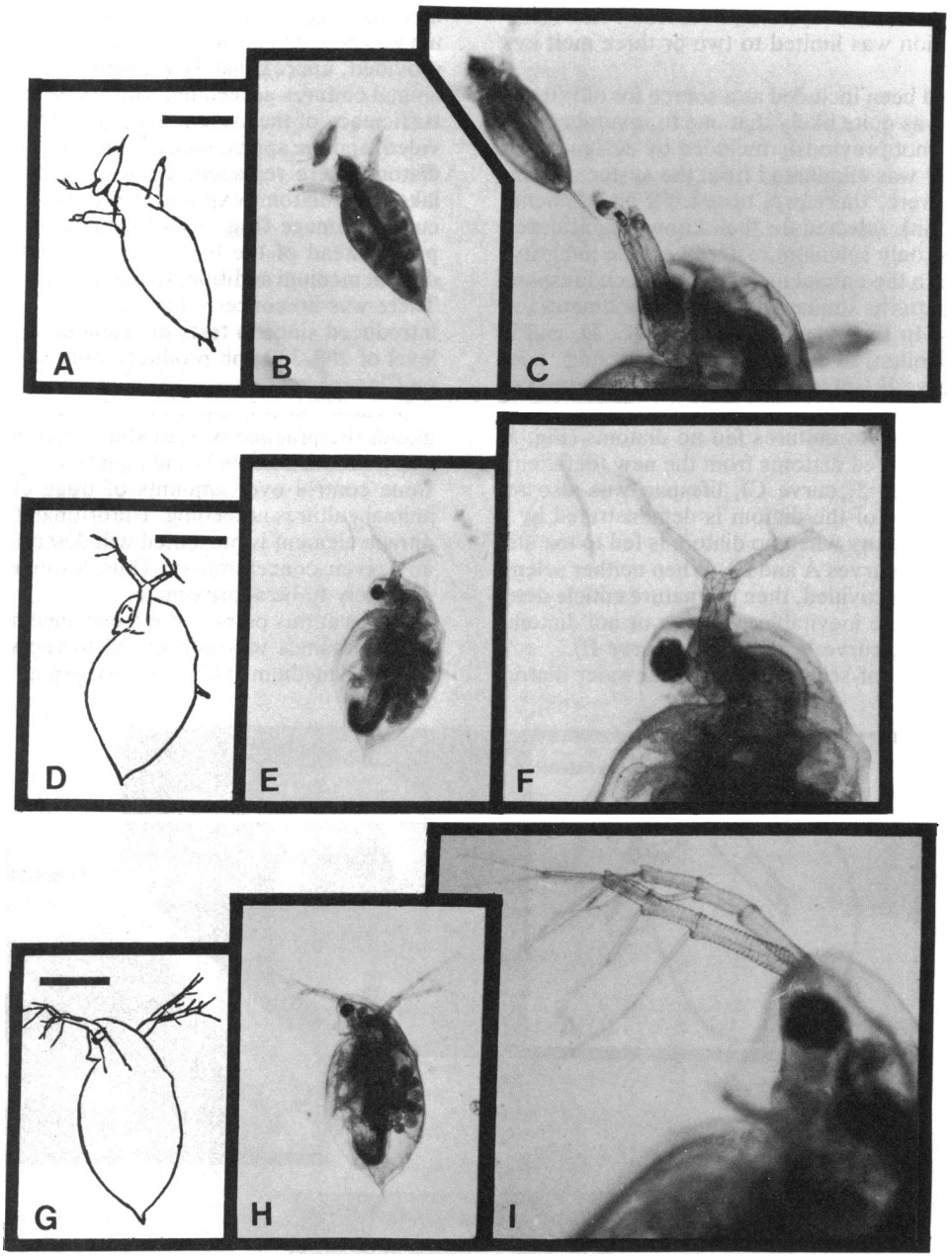

FIG. 3. *D. pulex*, broodmates born in inorganic culture. (Bars = 1 mm.) (Mother reared in MS with selenium at 1.0 ppb.) (*A* and *B*) Reared in medium with less than 0.1 ppb selenium ("selenium-free"); dorsal view. (*C*) Detail of *B*, magnification 3½ × that of *B*. (*D* and *E*) Reared in medium with approximately 0.1 ppb selenium; side view. (*F*) Detail of *E*, magnification of 3 × that of *E*. (*G* and *H*) Reared in medium with approximately 1.0 ppb selenium; side view. (*I*) Detail of *H*, magnification 4 × that of *H*.

because it represented the final step in the elimination of poorly defined organics (20) from the MS system. Not only is selenium contamination of such organic components likely, but also certain organics (in the case of selenium, especially vitamin E) are known to reduce requirements for trace inorganics.

With the change in the green algal medium the loss of cuticle integrity that had previously been eliminated by the addition of 0.5 ppb selenium reappeared. There were distinctions between this and prior occurrences of cuticle deterioration. Damage appeared in much older animals, the progression through the five stages was foreshortened so that it was completed within two to three molt cycles, and each generation repeated a similar pattern of premature senescence; i.e., there appeared to be no progressive shortening of lifespan. Animals could be maintained for an indefinite number of generations in 0.5 ppb selenium if progeny from early broods were selected to initiate subsequent generations. This is reminiscent of events described by Lansing (21, 22). In response to this apparent need by older animals for more selenium when dissolved organics were eliminated, selenium was doubled (to 1 ppb), and the early onset of visible cuticle deterioration was again eliminated.

The elimination of the organics associated with DA also caused a change in the response of animals to selenium deprivation (conditions in which no selenium was intentionally added to cultures). The number of generations that occurred before extinction when organics were present was 18. When organics were eliminated, only two generations were possible. Second-generation progeny did not reproduce.

DISCUSSION

The pattern of cuticle deterioration exhibited by selenium-deprived animals is visually quite similar to that observed in

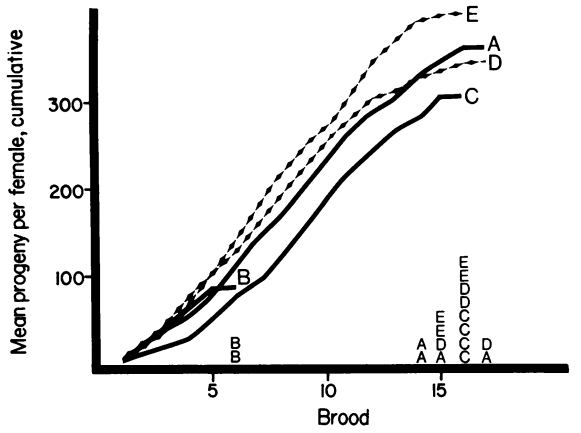

FIG. 4. Lifespan and reproduction in deprived and nondeprived cultures of *D. pulex*. Curves A, B, and C represent progeny of broodmates. Curves D and E are from a later generation, for comparison. Deaths of individual animals are recorded along the horizontal axis; letter indicates group to which dead animal belongs. Curve A, animals fed diatoms from lakewater medium. Curve B, animals fed diatoms from A-MS with no selenium (deficient). Curve C, animals fed diatoms from A-MS with no selenium but with 0.5 ppb selenium added to animal medium (first generation). Curve D, animals fed diatoms from A-MS with no selenium but with 0.5 ppb selenium added to animal medium (third generation). Curve E, animals fed diatoms and green algae from A-MS with 1.0 ppb selenium and with 1.0 ppb selenium added to animal medium. This is the second generation of *inorganic* animals. The animals represented by curves A–D were reared in organic-rich circumstances in which 0.5 ppb of selenium was sufficient. Animals represented by E required more selenium.

nondeprived animals as they pass through the last stages of life. It is also consistent with the description of senescence in insects offered by Rockstein and Miquel (23). "Normal" senescence in *D. pulex* and *D. magna* is usually signaled by minor damage to the most distal segments of the major swimming appendages, accompanied by cuticle deterioration—the cuticle becomes irregularly deformed in the final molt and evidence of an aborted ecdysis is commonly present at death. The cuticle does not, however, become "sticky" (as suggested by adherence of algal cells to its surface) until after the animal is dead.

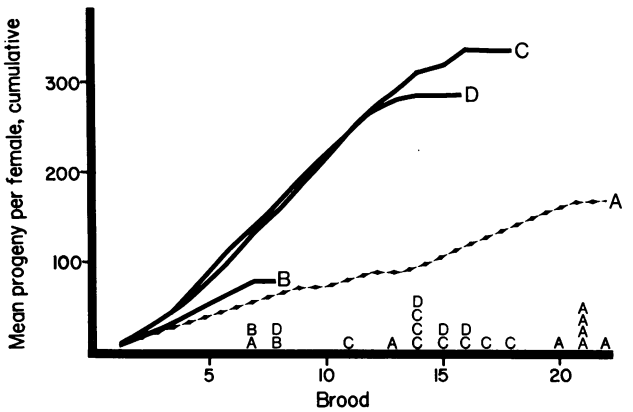

FIG. 5. *N. frustulum* vs. lakewater as the source for selenium when diatoms were grown in lakewater; curves represent progeny of *D. pulex* broodmates. Deaths of individual animals are recorded along the horizontal axis; letter indicates group to which dead animal belongs. Curve A, no diatoms with 1 ml of lakewater added. Curve B, 1 ml of diatoms grown in Se-deficient A-MS medium with no lakewater added. Curve C, 1 ml of diatoms grown in Se-deficient A-MS medium with 1 ml of lakewater added. Curve D, 1 ml of diatoms grown in lakewater medium. Lakewater was carried into animal cultures when diatoms were fed to the animals. This represents procedures prior to elimination of lakewater from the MS system.

The premature "senescence" resulting from selenium deficiency that is reported herein may not be related to true senescence, being instead a breakdown in the metabolic pathways associated solely with molting and/or reestablishment of the cuticle. It is possible that at the molecular level the specific deficiency-related malfunction may be in a metabolic pathway that is not peculiar to the Crustacea. The structural breakdown of the stalk of feathers in selenium-deficient fowl (24, 25) is a similar phenomenon involving keratin, which is structurally related to the sclerotin of those harder parts of the crustacean cuticle that first exhibit premature deterioration.

There is a significant sparing effect associated with the organic-rich algal medium, DA. Adding as little as 4% by volume to animal cultures will ameliorate the effects of insufficient selenium. Our atomic absorption spectrometer/hydride system indicates no measurable quantity of selenium in DA. As estimated on the basis of the system's limit for accurate selenium measurement of 0.2 ppb, the 4% addition provides less than 0.008 ppb of selenium. Since our intentional addition of an extra 0.05 ppb of selenium had no detectable effect, this sparing cannot be wholly attributed to additional, unmeasured, selenium from DA; therefore, the sparing is likely to be at least in part due to one, or more, of the organics of DA. This warrants further study.

Our thanks are due to G. E. Hutchinson and L. Provasoli for continuing guidance, to U. Cowgill and L. Williams for sharing problems and solutions, and to G. Dozsa for technical assistance with selenium measurements. This work was supported by National Science Foundation Grant DEB 7823258, Ecology Program, and by the New Jersey Agricultural Experiment Station. This is New Jersey Agricultural Experiment Station publication no. D-07511283.

1. National Research Council (1976) *Selenium*, Medical and Biological Effects of Environmental Pollutants (National Academy of Sciences, Washington, DC).
2. National Research Council (1971) *Selenium in Nutrition* (National Academy of Sciences, Washington, DC).
3. Flohe, L., Günzler, W. & Schock, H. (1973) *Fed. Eur. Biochem. Soc. Lett.* **32**, 132–134.
4. Levander, O., Morris, V. & Higgs, D. (1973) *Biochemistry* **12**, 4591–4595.
5. Rotruck, J., Pope, A., Ganther, H., Swanson, A., Hafeman, D. & Hoekstra, W. (1973) *Science* **179**, 588–590.
6. Keating, K. (1984) *Water Res.*, in press.
7. Conklin, D. & Provasoli, L. (1978) *Biol. Bull.* **154**, 47–54.
8. Conklin, D. & Provasoli, L. (1977) *Biol. Bull.* **152**, 337–350.
9. Murphy, J. (1970) *Biol. Bull.* **139**, 321–332.
10. D'Agostino, A. & Provasoli, L. (1970) *Biol. Bull.* **139**, 485–494.
11. D'Abramo, L. & Baum, N. (1981) *Biol. Bull.* **161**, 357–365.
12. Blau, M. (1961) *Biochim. Biophys. Acta* **49**, 389–390.
13. Weiss, K., Ayres, J. & Kraft, A. (1965) *J. Bacteriol.* **90**, 857–862.
14. Spina, R. (1982) M.S. Thesis (Rutgers Univ., New Brunswick, NJ).
15. Grafton, J. (1981) M.S. Thesis (Rutgers Univ., New Brunswick, NJ).
16. Provasoli, L. & Pintner, I. (1953) *Ann. N.Y. Acad. Sci.* **56**, 839–851.
17. Keating, K. (1975) Dissertation (Yale Univ., New Haven, CT).
18. Provasoli, L. & Pintner, I. (1968) in *Cultures and Collections of Algae*, eds. Watanabe, A. & Hattori, A. (Jpn. Soc. Plant Physiol., Hakone).
19. Reading, J. (1979) M.S. Thesis (Virginia Polytechnic Inst. and State Univ., Blacksburg, VA).
20. Rohde, P. (1975) *BBL Manual of Products and Laboratory Procedures* (Becton Dickinson, Baltimore, MD).
21. Lansing, A. (1947) *J. Gerontol.* **2**, 228–239.
22. Lansing, A. (1942) *J. Exp. Zool.* **91**, 195–211.
23. Rockstein, M. & Miquel, J. (1973) *The Physiology of Insecta*, ed. Rockstein, M. (Academic, New York), pp. 371–478.
24. Supplee, W. (1966) *Poult. Sci.* **45**, 852–854.
25. Thompson, J. & Scott, M. (1970) *J. Nutr.* **100**, 797–809.