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NUTRITIONAL AND THERAPEUTIC ROLE OF L-ASCORBIC ACID IN CRUSTACEA

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Scientists from the University of Arizona's Environmental Research Laboratory and the University of Sonora began a joint experimental shrimp culture project in 1972 near the fishing village of Puerto Peñasco, Sonora, Mexico. The village of approximately 28,000 people is located on the Northern Gulf of California some 60 miles south of the Arizona border. Previous to 1972, the experimental site had been used continuously by the universities as a research and development facility for solar energy, seawater distillation and controlled-environment agriculture (CEA) of greenhouse vegetables.

The basic principles of CEA were incorporated into the shrimp culture project at its beginning (Fig. 1). The goal of the shrimp culture project was to develop a commercially-viable technology for the intensive controlled-environment aquaculture (CEAq) of marine penaeid shrimp. Intensive CEAq of shrimp offers several advantages over less intensive or extensive culture methods, of which pond culture is an example. Intensive CEAq permits maximal growth rate, survival and stocking densities through optimization of water quality, feeding regimes and disease management. The higher densities possible in CEAq systems allow for higher productivity on less acreage. For example, it has been demonstrated that CEAq of certain species of penaeid shrimp can produce 40,000 to 60,000 pounds of shrimp tails/acre/year. This is compared to a maximum reported yearly yield of 2,500 pounds of whole shrimp per acre in North American pond culture systems.

However, CEAq of penaeid shrimp is not without its disadvantages. Besides its high capital and energy costs, its high labor and feed costs, several types of diseases are likely to occur and have a significant effect on shrimp raised in CEAq systems. In fact, the very conditions of high density per unit volume of water that make intensive culture so attractive from a commercial viewpoint, also predispose the shrimp to crowding stress and to the potential of rapid transmission of infectious disease.

Almost from the start, a particular disease syndrome, which was named black death disease in 1975 (Fig. 2), was present in California brown shrimp (*Penaeus californiensis*) reared at the Peñasco facility and in Gulf of Mexico brown shrimp (*P. aztecus*) being held at the Lab's Tucson facility. Originally the disease was thought to be a peculiar form of bacterial shell disease, which it superficially resembled, but later histopathological studies showed that black death was a distinctly different disease. Unlike shell disease, which is characterized by blackened (black pigment due to melanin deposition) eroded lesions of the shell, black death disease is expressed as melanized lesions that occur in soft connective tissues like those that occur under the shell (Fig. 3). In black death, there is no erosion of the shell proper.

In 1975, records of black death epizootics were carefully kept, and eight separate epizootics of the disease were observed in juvenile *P. californiensis* (Table 1). In every case, the affected shrimp were being reared in tanks or raceways which were housed in opaque ("non-algal") air-supported structures (Fig. 4a) so that little or no sunlight reached the tank or raceway water, and hence, no algal

growth was present. Handling and other forms of stress affecting shrimp in these systems often resulted in occurrences of the disease in otherwise healthy-appearing populations of shrimp. The disease was not observed in tanks or raceways within transparent air-supported structures receiving sunlight ("algal" systems), and hence, had marine algae, such as *Enteromorpha* sp., *Ulva* sp., and various diatom and blue-green algae species growing attached to the tank and raceway bottoms and sides (Fig. 4b).

Bacterial infections often accompany black disease. *Vibrio alginolyticus* has been the dominant organism isolated from shrimp having black death in its final stages. The use of oral antibiotics, given as medicated feed, was found to have little or no effect on daily mortality rates in populations of shrimp having black death, indicating that the bacterial infections were secondary invaders which may or may not have contributed to daily mortality rates, and that these were not the cause of the disease. The presence of the disease in non-algal systems, absence in algal systems, the secondary nature of bacterial infections accompanying the syndrome, and the similarity of lesions in shrimp with black death disease to those seen in scurvy in animals and man, indicated a possible cause and effect relationship of black death to ascorbic acid nutrition.

Addition of high levels of ascorbic acid to the diets fed to populations of shrimp with an ongoing black death epizootic resulted in a gradual reduction in daily mortality rates and an eventual alleviation of the syndrome within the affected population. In addition, initial nutritional studies have shown improved survival, 82% versus 55%, in groups of shrimp fed 4,000 mg and 59 mg added ascorbic acid per kilogram of diet, respectively.

These preliminary feeding results, coupled with histopathological findings and the observation that the black death syndrome does not occur in shrimp that have living algae available in their diet, indicated to us that ascorbic acid may act as a vitamin in penaeid shrimp.

To further study the hypothesis that ascorbic acid may act as a vitamin in penaeid shrimp, a proposal was prepared and submitted to the Office of Sea Grant in early 1976. The objectives of the studies funded as a result of that proposal were:

(1) To determine the requirement of penaeid shrimp for ascorbic acid; to establish the dynamics of tissue accumulation and depletion; and to elucidate the interactions of ascorbic acid with other essential nutrients;

(2) To determine the metabolic role of L-ascorbic acid at the subcellular level in penaeids;

(3) To determine the therapeutic role of L-ascorbic acid in wound healing, resistance to stress, and maintenance of defense mechanisms in penaeids.

Accomplishments during the first year of the proposed two-year study included:

(1) Experimental induction and description of the ascorbic acid deficiency disease syndrome;

(2) Development of a technique for evaluation of the effect of an ascorbic acid deficiency on the wound repair process in juvenile shrimp;

(3) Determination of the bacterial microflora in normal versus ascorbic acid deficient shrimp;

(4) Development of an analytical method for the determination of ascorbic acid in cold-extruded feed; and

(5) Determination of the effect of size, dietary regime and the presence of algae on the tissue concentration of ascorbic acid in juvenile shrimp. Further discussion of these accomplishments is given in the following paragraphs.

(A) A deficiency syndrome was induced experimentally in California brown shrimp (*Penaeus californiensis*) by feeding a diet devoid of added ascorbic acid. Shrimp fed this diet developed blackened (melanized) lesions after six to eight weeks in the subcuticular tissues of the general body surface, in the walls of the esophagus, stomach and hindgut, and in the gills and gill cavity. The induced syndrome was grossly and histologically identical to the black death disease syndrome which has been previously observed, often in epizootic proportions in tank and raceway-reared *P. californiensis* and *P. aztecus*.

A paper, *Black Death, a Disease Syndrome of Penaeid Shrimp Related to a Dietary Deficiency of Ascorbic Acid*, was published in the proceedings of the eighth annual meeting of the World Mariculture Society (see Appendix A).

(B) In order to evaluate the effect of a dietary ascorbic acid deficiency on the shrimp's ability to heal wounds, a special method for inflicting a non-lethal wound in shrimp of only a few grams in weight had to be developed.

Various materials were tested including nylon monofilaments, surgical sutures, cotton and synthetic fiber threads, etc. Sterile polyester thread, inserted through the abdomen between the first and second abdominal segments with a sterile needle, was found to result in non-lethal wounds that were accompanied by a strong wound healing response. Using this technique, groups of normal and scorbutic *P. californiensis* of 3 g average weight were wounded. The process of wound repair in these animals is to be followed histologically and comparisons between shrimp receiving a normal versus a deficient diet will be made. These studies are scheduled to be completed during the second year of the two-year study.

(C) Because a terminal bacteremia often occurs in shrimp with the black death syndrome, studies were made in which the bacterial flora of shrimp with the disease and normal shrimp from the same population were compared. Microbial isolates were made from blood, muscle and intestines of moribund shrimp with the black death syndrome and from normal shrimp. No fungi were isolated, but various salt-tolerant bacterial forms were represented. Included among these isolates were *Vibrio alginolyticus* (the most commonly isolated organism), *Acinetobacter* sp., *Aeromonas* sp., *Pseudomonas* sp. and various other *Vibrio* spp. Because members of the genus *Vibrio* were isolated as frequently from tail muscle tissue and intestines of shrimp having

black death in its terminal phase as from normal control shrimp, these bacterial pathogens are considered to be opportunistic invaders which are a normal part of the shrimp microflora.

(D) To establish the dynamics of ascorbic acid in terms of its accumulation, depletion and storage capacity in various shrimp tissues, and its half-life in prepared diets for shrimp, analytical methods for ascorbic acid had to be tested and modified to these applications. A potentiometric titration method was selected, which gave a sensitivity of 0.15 mg ascorbic acid per gram in shrimp feed and 0.05 mg ascorbic acid per gram of shrimp tissue (Fig. 5). This technique will be used during the second year of the study to determine the dynamics of ascorbic acid in shrimp tissues in relation to dietary intake.

Analysis of diets fortified with up to 4 g of ascorbic acid per kilogram of diet revealed that approximately 90% of the added ascorbic acid was lost in diet preparation. Further studies will be made in this area during the second year of the study. However, the finding that much of the vitamin is destroyed in processing of the diet prior to feeding indicates that less than 10% of the added ascorbic acid is actually available to shrimp fed the artificial diet. These findings help to explain how an ascorbic acid deficiency syndrome could develop in shrimp populations reared in non-algal tanks,

but fed a diet containing an amount of the vitamin recommended as adequate for meeting the dietary requirements of vertebrates that require the vitamin.

(E) The contribution of algae to the ascorbic acid available to shrimp was studied in a preliminary experiment. Juvenile *P. californiensis* were reared in tanks with and without an algal community. After five days, there was a significantly higher tissue ascorbic acid level in animals held in the tanks that contained algae.

Further studies planned for the second year of this study (July, 1977 through June, 1978) include:

(1) Completion of studies on the wound healing response in scorbutic versus non-scorbutic juvenile *P. californiensis* and *P. stylirostris*.

(2) Studies on the role of the vitamin in resistance to stress in penaeids.

(3) Completion of studies to determine the dietary requirement of *P. californiensis* and *P. stylirostris* for ascorbic acid.

(4) Investigations on the dynamics of tissue uptake and depletion of ascorbic acid in penaeids.

(5) Determination of the tissue distribution of ascorbic acid in penaeids.

(6) Determination of "total vitamin C" in feeds and tissue.

(7) Determination of the contribution of "in situ" algae to ascorbic acid nutrition.

(8) Determination of reaction rates of crustacean enzymes that are associated with ascorbic acid and collagen metabolism.

Table 1. Black Death Epizootics in *Penaeus californiensis* in Non-algal Systems at Puerto Peñasco in 1975

Approximate Dates of Epizootic	Tank Type and Volume	Shrimp Length (TL)*
Jan 18 - Feb 19	60 ℓ, Fiberglass	35-60mm
Feb 17 - late Mar	23000 ℓ, Raceways	60-90
Mar 25 - May 20	60 & 3000 ℓ, Fiberglass	65-100
May 29 - Jun 11	23000 ℓ, Raceways	90-105
Jun 24 - mid Jul	23000 ℓ, Raceways	100-120
Jun 16 - Jul 22	60 & 3000 ℓ, Fiberglass	45-75
Aug 25 - Oct 1	3000 ℓ, Fiberglass	30-80
Oct 27 - mid Nov	60 ℓ, Fiberglass	80-100

*TL = Total length from tip of rostrum to tip of telson.

FIGURE LEGENDS

Figure 1. Aerial view of the University of Arizona-University of Sonora experimental shrimp culture facility at Puerto Peñasco, Sonora, Mexico.

Figure 2. Juvenile California brown shrimp (*Penaeus californiensis*) with the black death disease syndrome. Research funded by a Sea Grant to the University of Arizona has shown the disease to be due to a dietary deficiency of Vitamin C.

Figure 3. Photomicrograph of a stained tissue section taken from a blackened lesion of a shrimp with black death. The black spots are aggregations of shrimp inflammatory cells that have become blackened due to the deposition of melanin.

Figure 4a. Inside view of a non-algal raceway system at Puerto Peñasco.

4b. Inside view of an algal raceway system at Puerto Peñasco.

Figure 5. Effect of feed ascorbic acid levels on ascorbic acid concentration in tissues.

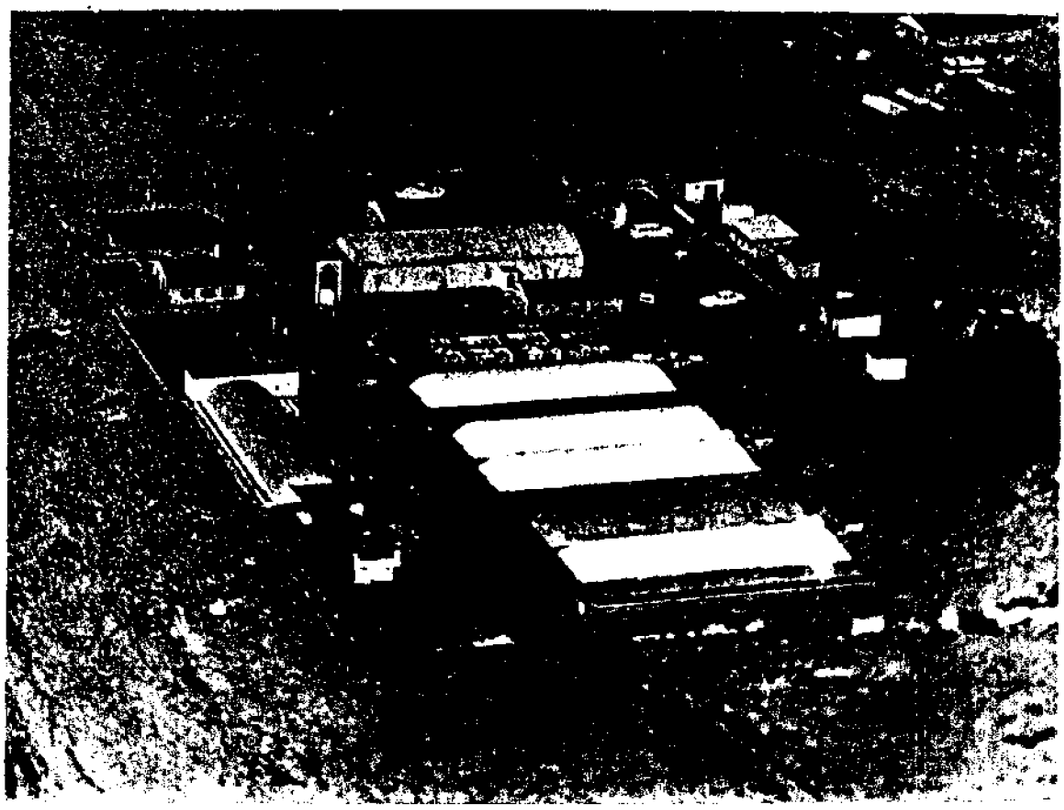
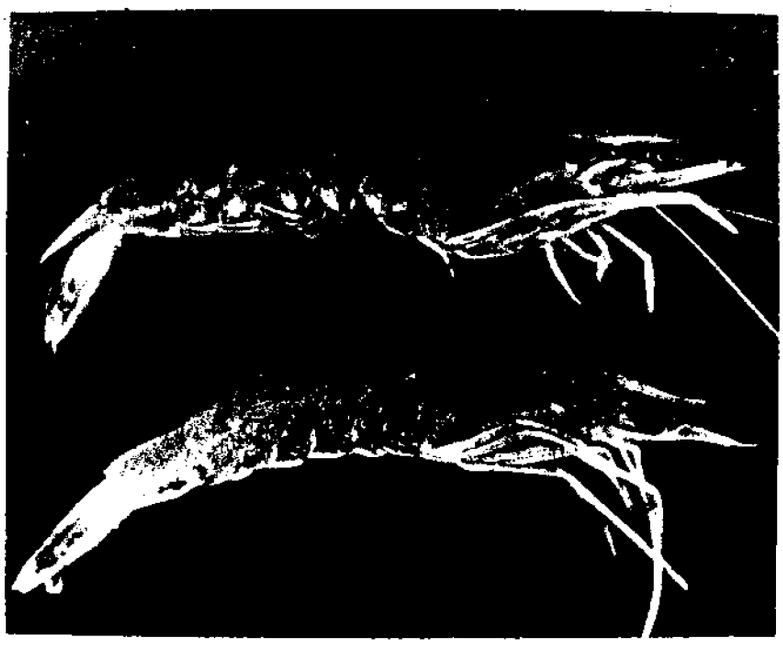


Figure 1.



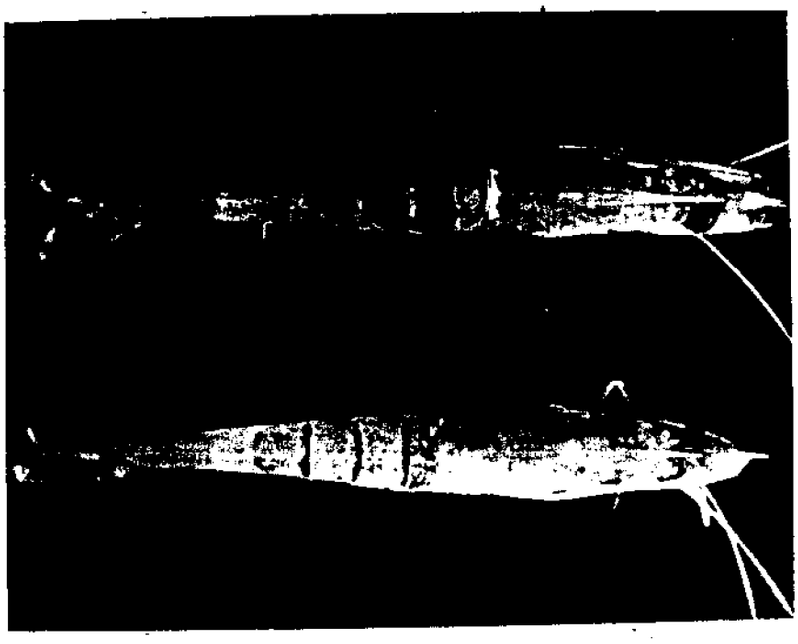


Figure 2.

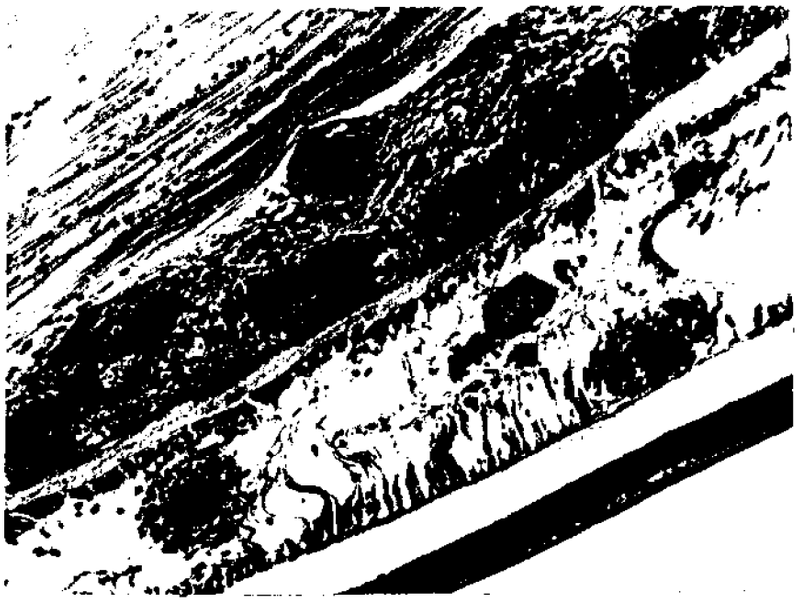


Figure 3.

Figure 4a.

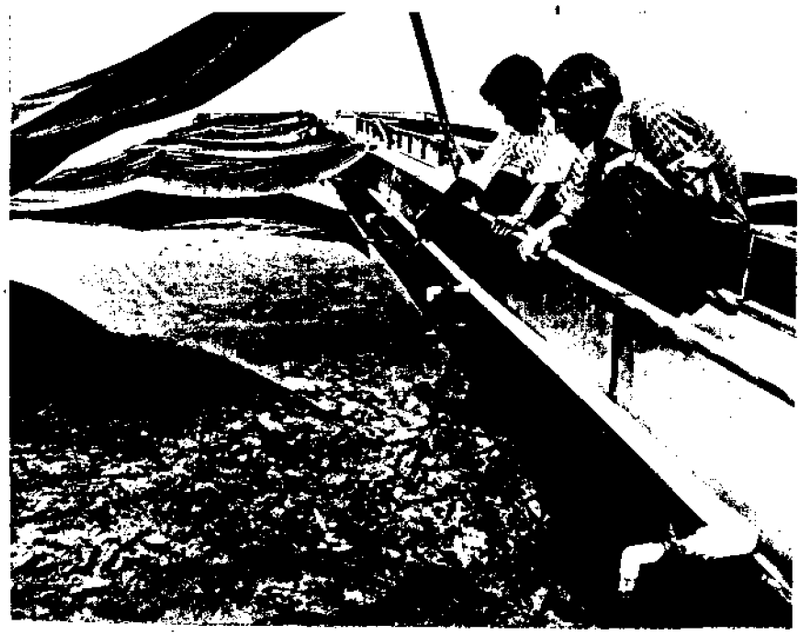


Figure 4b.

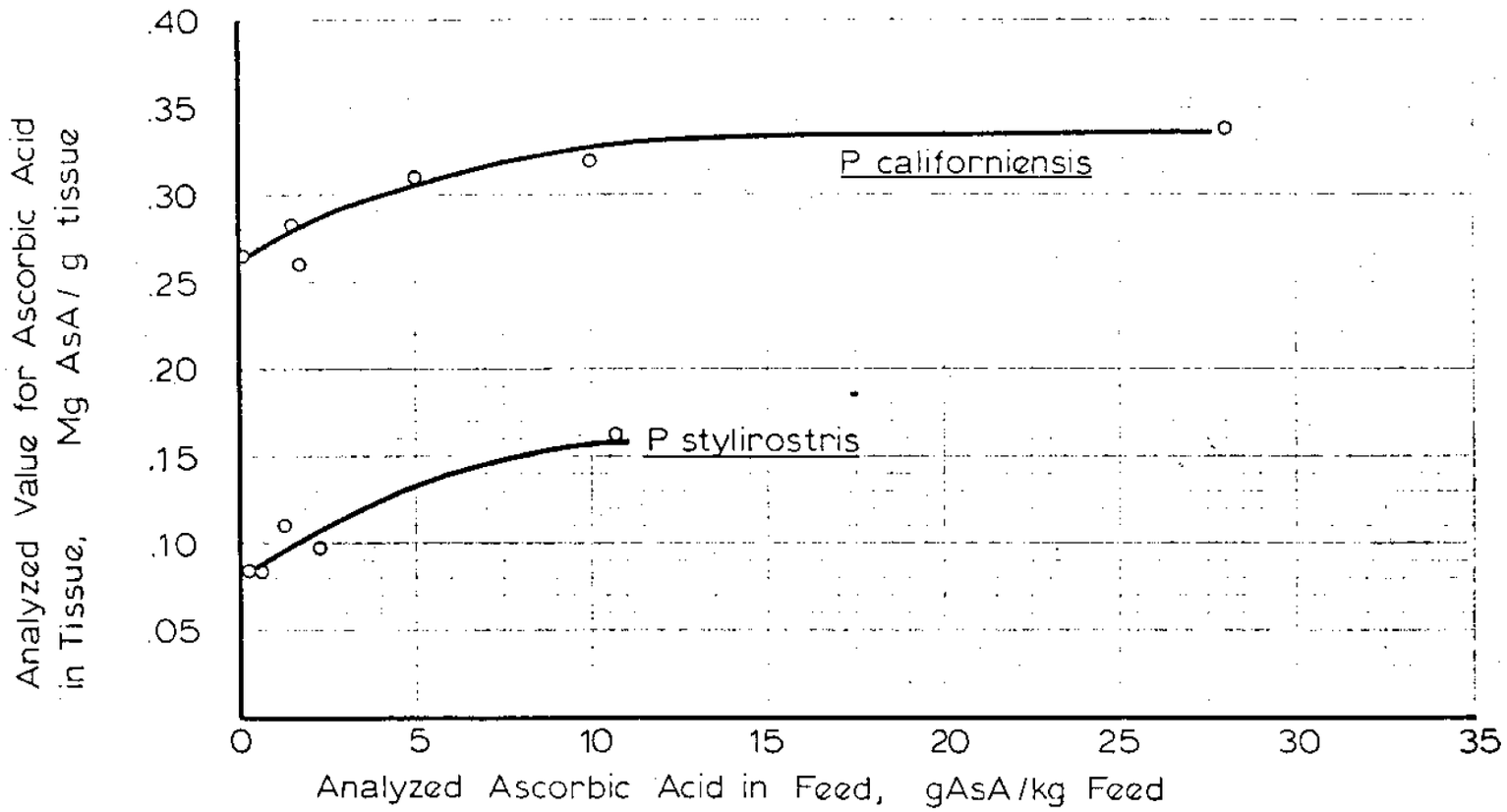


fig. 5 Effect of Feed Ascorbic Acid Levels on Ascorbic Acid Concentrations in Tissues

figure 5

(APPENDIX A)

Black Death, a Disease Syndrome of Penaeid Shrimp Related to a Dietary Deficiency of Ascorbic Acid

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ABSTRACT

California brown shrimp (*Penaeus californiensis*) fed a diet low in or without added L-ascorbic acid, developed blackened lesions after 6 to 8 weeks in the subcuticular tissues of the general body surface, in the walls of the esophagus, stomach and hindgut, and in the gills and gill cavity. The condition has been previously observed in tank and raceway-reared *P. californiensis* and *P. aztecus* often in epizootic proportions, and has been named black death disease.

The disease has only been observed in postlarval and juvenile shrimp fed alginate bound artificial diets in tanks or raceways that received no sunlight and contained no plant material. Handling and other stress factors often precipitated epizootics in apparently healthy groups of shrimp. Once clinical signs of the disease became apparent, affected shrimp ceased feeding, and death usually followed within 24 to 72 hours. Opportunistic bacteria such as *Vibrio* spp. and *Pseudomonas* spp., often, but not always, produced a terminal septicemia in affected shrimp. Mortalities of 1 to 5% per day due to this disease were observed on several occasions in tank and raceway-reared *P. californiensis* in 1974 and 1975 at Puerto Peñasco, Mexico.

Histopathology showed the blackened lesions to be composed of masses of hemocytes and necrotic tissue. The black pigmentation of the lesions was due to melanin deposited in areas of heavy hemocyte infiltration, particularly near the centers of developing hemocyte nodules. No parasites or bacteria could be demonstrated in the centers of these nodules.

INTRODUCTION

The feeding of prawns and shrimp has been investigated by several laboratories (for review see Forster, 1975), but the nutritional requirements of those crustaceans remain only vaguely defined. Although a vitamin pre-mix is included in the formulation of many of tne artificial diets of shrimps and prawns, almost nothing is known about their vitamin requirements. Most workers have added to crustacean diets a vitamin package prepared for vertebrate diets, such as those intended for meeting the requirements of teleost fishes (Havler *et al.*, 1969), on the assumption that the vitamins are required.

Vitamin C (L-ascorbic acid) nutrition in shrimps and prawns has received some attention. Kitabayashi *et al.*, (1971; cited by Forster, 1975) observed that the addition of glucose to their standard diet was only beneficial when added together with Vitamin C. Deshimaru and Kuroki (1976) studied the requirements of inositol and ascorbic acid in *Penaeus japonicus*. They tested dietary levels of ascorbic acid within the range of 0 to 1.0% and observed the best growth in groups receiving the base diet with no ascorbic acid. The amount of food ingested by the prawn

decreased with increasing ascorbic acid in the food. However, the diets lacking or containing 0.1% ascorbic acid produced high mortalities ($\geq$ 70% of test population after 11 weeks) at a water temperature of 28° ~ 30°C and shrimp fed those diets showed particular signs, such as, decolorization and development of a greyish-white color on the margin of the carapace, the lower part of the abdomen, and on the tips of walking legs. Adequate levels of ascorbic acid in the test diet was assumed to be about 0.3%.

Presented in the present paper are: a) a description of black death disease, a disease syndrome of *P. californiensis* and *P. aztecus*, and b) experimental evidence that the disease is related to a dietary deficiency of ascorbic acid.

MATERIALS AND METHODS

Source of Experimental Animals

Most of the shrimp for these studies were *Penaeus californiensis* hatched from spawn obtained from wild parent stock collected from the Northern Gulf of California and reared in tanks and raceways at the University of Arizona - University of Sonora shrimp culture research station at Puerto Peñasco, Sonora, Mexico. A few *P. aztecus* from the National Marine Fisheries Service experimental shrimp culture facility at Galveston, Texas were also included in this study.

Histological Methods

Shrimp with signs of black death disease from naturally occurring epizootics in tank and raceway reared populations of

California brown shrimp (*P. californiensis*) at Puerto Peñasco and shrimp from experimental groups receiving ascorbic acid deficient diets were preserved for histological examination using Davidson's fixative. Embedding and sectioning were accomplished using routine histological methods. Tissue sections of lesions typical of the black death disease syndrome were routinely stained with hematoxylin and eosin. Special stains used in an attempt to determine the etiology of the lesions included periodic acid-Schiff, Masson's trichrome, Wolbach's giemsa, Ziehl-Neelsen, Brown-Brenn Gram stain, and the Schmorl's reaction for melanin on $KMnO_4$ bleached and on unbleached sections (Thompson, 1966).

Ascorbic Acid Feeding Experiment

California brown shrimp (*P. californiensis*) of 0.5 g average initial weight were fed artificial diets with 0, 100, and 2000 mg of L-ascorbic acid added per kg of base diet. A total of 450 shrimp were divided into 3 experimental groups with 6 replicates of 25 shrimp each per replicate. Each experimental group consisted of 150 shrimp. Shrimp were held in 60 liter flow-through fiber-glass tanks with water exchange rates of 5 to 10 times per day and were fed the experimental diets for 8 weeks. Water temperatures were 22.5 to 23.3°C during the experimental period. The base diet consisted of wheat (49%), soybean meal (5%), shrimp meal (32%), menhaden meal (10%), liquid fish solubles (1%), a vitamin pre-mix (1%, but without ascorbic acid), and alginate binders (2%). Diet A was the base diet with no added L-ascorbic acid, diet B was the

same diet with 100 mg of L-ascorbic acid/kg of diet mix, and diet C had 2000 mg of L-ascorbic acid/kg of diet mix.

Shrimp were fed twice daily, and dead, moribund, or obviously distressed shrimp were removed daily and examined for signs of black death disease.

Microbiology

Hemolymph from shrimp displaying clinical signs of black death disease was obtained by cardiac puncture from shrimp of 4 to 5 grams average weight or larger, or from abdominal muscle tissue fluid and/or homogenate from shrimp smaller than 4 to 5 grams average weight, and was cultured for potential bacterial pathogens on Trypticase Soy Agar, Marine Agar 2216, and TCBS Agar (Difco). Cultures were incubated at 26°C, and isolates obtained were identified when possible to genus and species using the method of Lewis (1973) and the API-20 bacterial identification system (Analytab Products Inc., 200 Express St., Plainview, N.Y. 11803).

RESULTS

History of Naturally Occurring Black Death Epizootics at Puerto Peñasco

In 1975, eight separate epizootics of black death disease were observed in juvenile (30 mm to 105 mm total length) *P. californiensis*. Black death epizootics, or at least occasional shrimp with the disease, were observed during every month except November and December of 1975. In every case the affected shrimp were being reared in tanks or raceways (Mahler, *et al.*, 1974),

which were housed in opaque ("non-algal") structures so that little or no sunlight reached the tank or raceway water, and hence, no algal growth was present. Handling and other stress factors affecting shrimp in these systems often precipitated an epizootic in apparently healthy groups of shrimp. The disease was not observed in tanks or raceways that received sunlight and hence, had marine algae, such as, *Enteromorpha* sp., *Ulva* sp., and several diatom species growing attached to the tank and raceway bottoms and sides. Observations of shrimp in these "algal" systems indicated that the shrimp consumed some of the algae, but appeared to preferentially feed upon copepods, amphipods, nematodes, protozoans, etc., that were present as epifauna on the algae.

Attempts to establish a bacterial etiology of black death disease in *P. californiensis* proved fruitless, despite my earlier opinion that this disease was a form of shell or brow spot disease (Lightner, 1975). However, subsequent microbiological and histopathological findings reported here have not supported that earlier view.

Cultures from hemolymph were prepared from shrimp showing early signs of black death disease and from moribund and/or fresh dead shrimp from 6 of the 7 epizootics at Puerto Peñasco in 1975. Opportunistic bacteria, such as *Vibrio alginolyticus*, *V. anguillarum*, and *Pseudomonas* spp. were often, but not always isolated from these hemolymph samples. Based upon the antibiotic sensitivities of the predominate organisms isolated, the antibiotics Furanace, Gallimycin (Erythromycin), Tylosin, and Terramycin were administered

on various occasions to affected populations by either direct addition of the antibiotic to the water or by addition of the antibiotic to the food. No beneficial effects of antibiotic therapy were noted. However, when a population of severely affected *P. californiensis* (50-80 mm TL) was moved from a non-algal 3000 liter fiberglass tank to a algal tank of the same size, gradual remission of the disease was observed during the following 2 weeks and mortality rates dropped to near zero.

Gross Signs of Black Death Disease

In *Penaeus californiensis* blackened areas develop under the exoskeleton on the abdomen, on the carapace, in the gills, and in the foregut and hindgut (Figs. 1-3). This disease is distinguished from shell or brown spot disease, which it superficially resembles by the lack of eroded areas in the exoskeleton. The blackened tissues lie under the exoskeleton in black death disease. Once signs of the disease become apparent, affected animals cease feeding, and death usually follows within 24 to 72 hours. In naturally occurring epizootics of black death in Puerto Peñasco in 1975, mortality rates of 1 to 5% per day were observed.

The most common grossly visible lesion in affected populations of *P. californiensis* was a blackened hindgut, followed by (in order) shrimp with blackened hindguts and stomachs, and shrimp with blackened "saddle" lesions under the dorsal portions of the abdominal pleura and blackening of the dorsal portion of the branchial cavity. The regularity of gross appearance of shrimp with black death disease was striking. The appearance of *P.*

californiensis with black death disease is identical to the appearance of specimens of *Penaeus aztecus* (also from "non-algal" tanks and given to me by Z.P. Zean-Eldin, NMFS, Galveston, Texas). Histopathological examination of the lesions in *P. aztecus* proved them to be identical to those typically seen in *P. californiensis* (Fig. 2).

Histopathology of Black Death Disease

Histological examination of the blackened areas in *P. californiensis* and *P. aztecus* with black death disease showed the lesions typically to be present in loose connective tissues, such as those found in the subepithelial tissues of the cuticular hypodermis (Fig. 4), of the serosal layer of the stomach wall (Fig. 5), and in the serosal, glandular and epithelial layers of the hindgut (Fig. 6). Scattered blackened lesions were occasionally found in the compound eye basal to the ommatida, in the muscle fascia, in thorasic and abdominal striated muscles, and within the gill processes and gill filaments.

Lesions were composed of masses of hemocytes and tissue debris. Many of the homocyte accumulations and nodules, particularly the larger ones, were blackened by an insoluble brownish (in histological sections) pigment that was demonstrated by histochemical means to be melanin (Lightner and Redman, "in press"). Routine and special histochemical stains failed to show any evidence of parasites, bacteria, or fungi within or associated with the lesions. The cuticle of the exoskeleton, stomach and hindgut linings was not directly affected in this disease. No cuticular erosions, hemocytic infiltration or melanization of the cuticle like those observed in

shell disease (Rosen, 1970; Cook and Lofton, 1973), were observed in shrimp with black death disease.

Feeding Experiments

After 42 days of feeding, California brown shrimp (*P. californiensis*) fed diet A (no added ascorbic acid) and diet B (with 100 mg ascorbic acid/kg diet) developed gross signs of black death disease (Fig. 3). No signs of the disease were observed in shrimp being fed diet C (with 2000 mg ascorbic acid/kg diet). During the next 14 days (days 42 to 56 of the experiment), shrimp with black death disease were observed almost daily in the groups receiving diets A and B. At termination of the feeding experiment (on day 56), the shrimp receiving diet C showed 85% survival and no instances of black death disease. Shrimp on diets A had 61% survival and 9 confirmed cases of black death, while those on diet B had 65% survival and 10 confirmed cases of black death (Table 1).

DISCUSSION

Disease conditions resulting from a nutritional deficiency of ascorbic acid have been reported in fishes, such as, the coho salmon and the rainbow trout (Halver, *et al.*, 1969), and in the channel catfish (Lovell, 1975). The requirement for ascorbic acid in the culture of a number of terrestrial arthropods has also been shown (Florkin and Scheer, 1970). In cultured crustaceans, disease due to a dietary deficiency of ascorbic acid has not previously been reported. However, Deshimaru and Kuroki (1976) reported poor survival and an unusual coloration of *P. japonicus*, fed diets containing less than 0.1% L-ascorbic acid.

A disease condition in cultured *Macrobrachium rosenbergii* called "black nodule" by Delves-Broughton and Poupard (1976) was similar in gross and histological appearance to black death disease of *P. californiensis* and *P. aztecus* described in the present paper. Furthermore, although a number of bacteria were isolated from affected prawns, Koch's postulates could not be demonstrated in infectivity trials, nor was antibiotic therapy with Furanace effective in controlling the disease in the affected *M. rosenbergii*.

As of this writing, black death disease, or a similar condition, has not been induced experimentally in a white shrimp (nongrooved) species. Blue shrimp (*P. stylirostris*) and white shrimp (*P. vannamei*) have been fed identical diets and reared under similar conditions as have California brown shrimp (*P. californiensis*) at Puerto Peñasco, and *P. stylirostris* has, on occasion, developed an epizootic disease condition characterized by moderate to high mortality and by hemocytic lesions in the gills and in the fascia of striated muscle. Whether or not this syndrome is equivalent to the black death disease syndrome of brown (grooved shrimp), and moreover, if it is a function of a dietary L-ascorbic acid deficiency, remains to be demonstrated.

The results of ascorbic acid feeding experiments in this study indicate that *P. californiensis* requires dietary L-ascorbic acid. In culture situations, the vitamin must either be supplied by addition to artificial diets, or by inclusion of natural sources of the vitamin, such as living algae, in the diet. The data from the present study do not indicate the minimum daily requirement for the vitamin for *P. californiensis*, but do show that black death disease

can be prevented by feeding a diet containing 2000 mg ascorbic acid/kg of diet. This amount of vitamin C is 0.2% of the diet and is comparable to the 0.3% level suggested as adequate for meeting the dietary requirements for the vitamin in *P. japonicus* (Deshimaru and Kuroki, 1976).

Incorporation of L-ascorbic acid at 2000 mg/kg of diet does not necessarily mean that 2000 mg of the vitamin will be present in the finished diet. Ascorbic acid analyses of diet ingredients and the finished diet indicated that greater than 90% of the ascorbic acid present in the diet ingredients had been destroyed in processing. The chemist performing the analyses suggested that the high pH (pH8.3) of the feed reached in the process of binding the ration with alginate probably was responsible for the destruction of the vitamin (Park, G.L., Personnal Communication, Sunkist Research Center, Ontario, Calif.). Hence, the actual daily requirement for L-ascorbic acid in *P. californiensis* must be considerably less than 0.2% of the diet, probably even less than 0.01% of the diet.

Despite the fact that the actual daily requirement for ascorbic acid in shrimp remains to be established, we do know that a dietary deficiency of the vitamin can result in a deficiency disease in *P. californiensis* and *P. aztecus*, and we have named the condition black death disease.

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Table 1a - Results of experiments in which California brown shrimp *(Penaeus californiensis)* were fed diets containing various levels of ascorbic acid for eight weeks.

Date	Diet A, no ascorbic acid		Diet B (100mg ascorbic acid/kg diet)		Diet C (2000mg ascorbic acid/kg diet)	
April 6	150	100%	150	100%	150	100%
May 5	132	88	130	86	147	98
May 17	124	83	124	83	139	93
May 25	92	61	97	65	128	85

Table 1b - Dates on which California brown shrimp (*P. californiensis*) with black death disease were observed.

Date	Diet A	Diet B
May 17	5	2
19	1	2
23	1	3
25	2	1
26	0	2
TOTAL	9	10

FIGURE LEGENDS

Figure 1. California brown shrimp *(Penaeus californiensis)* with black death disease. Large melanized areas are visible in the soft tissues under the exoskeleton, which is not eroded. 1a - side view; 1b - top view.

Figure 2. A brown shrimp *(P. aztecus)* with black death disease. The melanized lesions shown are identical in appearance and histopathology to those in *P. californiensis*.

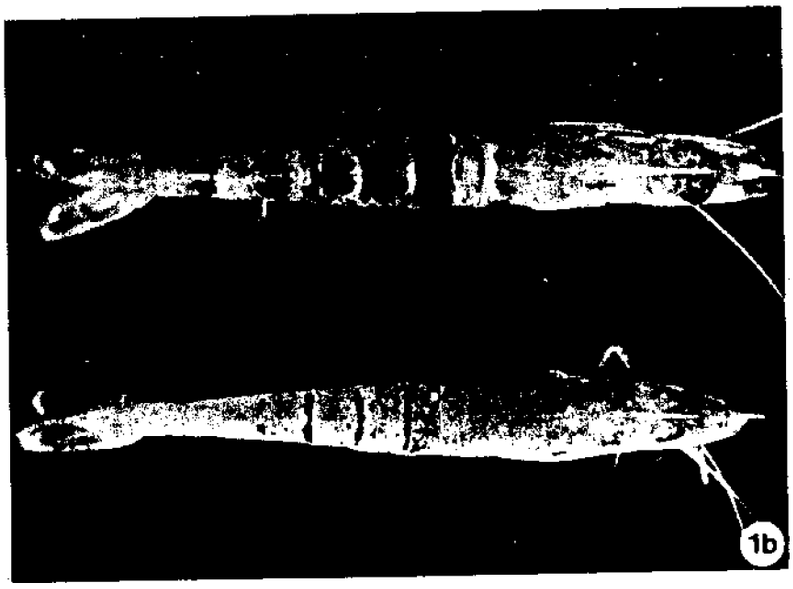
Figure 3. California brown shrimp *(P. californiensis)* with black death disease induced experimentally by a deficiency of dietary L-ascorbic acid. Arrow indicates blackened hindguts.

Figure 4. Histological section through a subcuticular lesion in a California brown shrimp *(P. californiensis)* with black death disease. Hemocytic nodules, many with melanized centers, are present in hypodermis and subepithelial connective tissues. Note that there is no involvement or erosion of the cuticle, which distinguishes this disease from shell disease, H & E staining. X700.

Figure 5. Sagittal section through the stomach of a California brown shrimp *(P. californiensis)*. Melanized hemocytic lesions are present in the submucosa, muscularis, and serosal layers of the stomach wall. Special staining revealed no parasites, bacteria, or fungi within or associated with the lesions. H & E staining. X140.

Figure 6. Cross section of the hindgut from a California brown shrimp *(P. californiensis)* fed a diet deficient in ascorbic acid. Masses of hemocytes stained brown to black by melanin are present on the apical surfaces of the muscosal folds that project into the hindgut lumen. H & E staining. X200.


1a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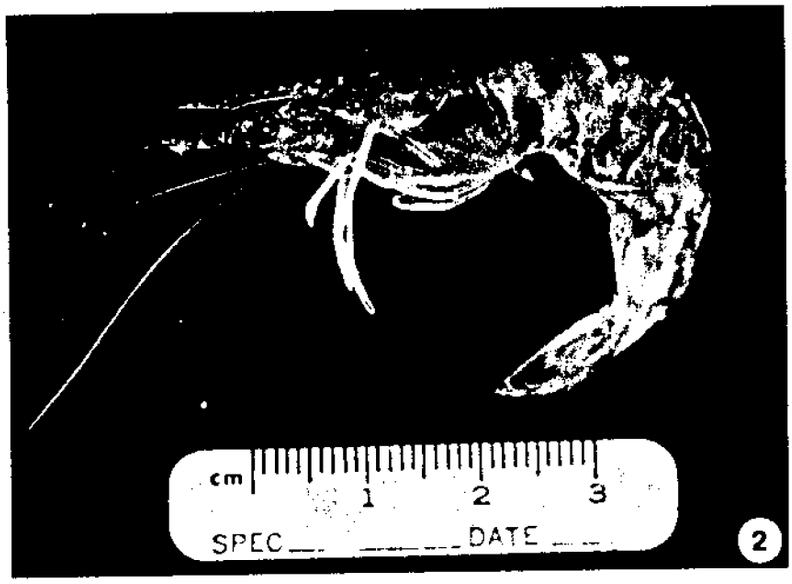
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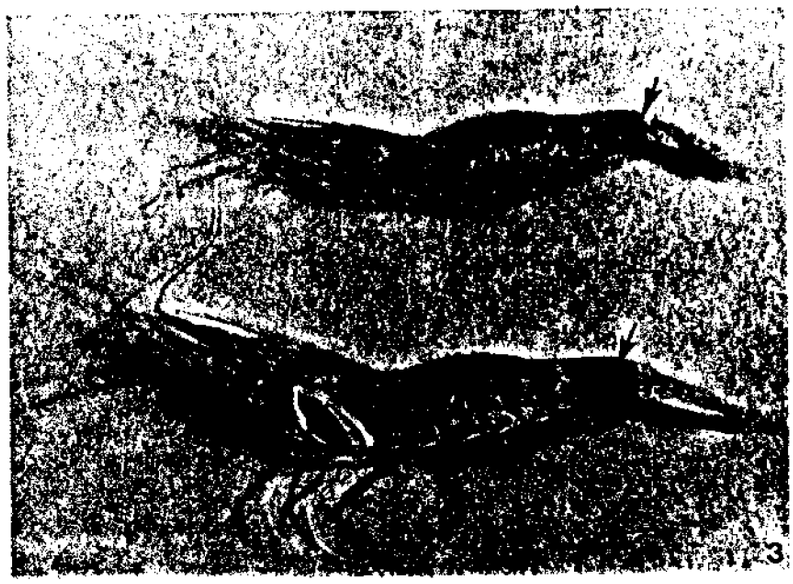
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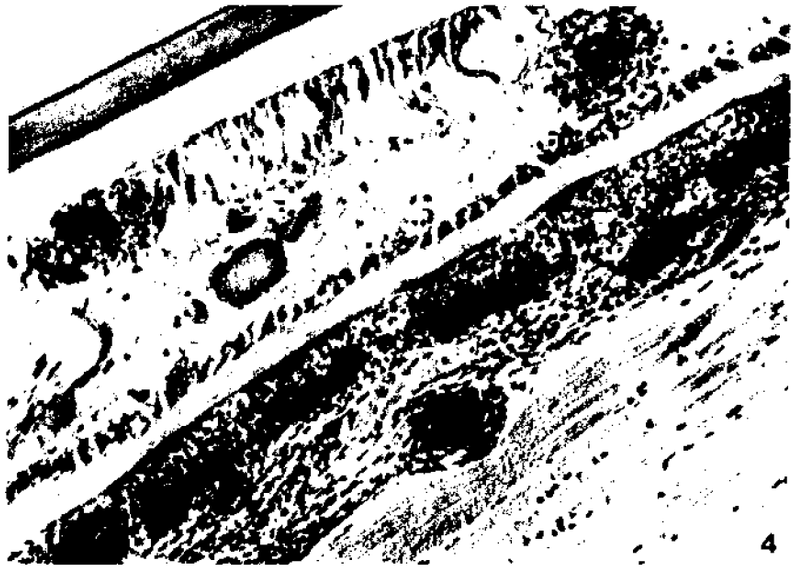

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