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EXPERIMENTAL TECHNIQUES FOR PRESERVING DIATOMS USED AS FOOD FOR LARVAL *PENAEUS AZTECUS*[1]

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ABSTRACT

The diatoms Skeletonema costatum *and* Thalassiosira *sp. were freeze-dried and fed to larval brown shrimp,* Penaeus aztecus, *being reared to first stage mysis from first stage protozea. Feeding levels ranged from 100,000-1,000,000 cells/ml.*

Both diatoms were also frozen, either in the presence or absence of the protectants glycerol and dimethyl sulfoxide. Frozen Skeletonema *was suitable when frozen in the presence of protectants;* Thalassiosira *supported good growth of larval shrimp when frozen alone or with protectants.*

Although neither frozen nor freeze-dried foods were as good as live diatoms at equal concentrations, these foods may be used supplementally to eliminate complete dependence on live algal cultures.

INTRODUCTION

The shrimp culture methods practiced at the Galveston Biological Laboratory have been discussed by Cook and Murphy (1966, 1969), and recent improvements have been reported by Mock and Murphy (1971). Briefly, gravid female shrimp are obtained from offshore and brought into the laboratory for spawning. Spawning usually occurs within 24 hr and the shrimp which hatch subsist through the naupliar stages on the stored yolk in their bodies. After an additional 36 hr, however, the larvae have molted to the protozoeal stage and require algal food. Algal cultures must be started several days prior to the acquisition of the gravid shrimp so that the mass cultures reach maximum cell numbers just before the larvae begin feeding.

A number of different algae have been used successfully as food for larval shrimp. Cook and Murphy (1969) used *Skeletonema costatum, Thalassiosira* sp., *Cyclotella nana, Phaeodactylum* sp., *Tetraselmis* sp., *Gymnodinium splendens* and *Exuviella* sp. Ewald (1965) in rearing *Penaeus duorarum* fed a mixture of unicellular algae, including *Dunaliella tertiolecta, Tetraselmis* sp., *Phaeodactylum tricornatum* and *Chlorella* sp. Hudinaga (1942) working with *Penaeus japonicus* fed *S. costatum* and *Nitzschia closterium.*

Although a large number of algal species may possibly be used as larval food, only a few have been cultured in quantities and concentrations sufficient for large numbers of larvae. *Skeletonema costatum* is the species most frequently used at the Galveston Biological Laboratory. Cook and Murphy (1969), however, experienced difficulties in culturing *S. costatum* when sea water was used as the basic culture medium. Mock and Murphy (1971) indicated some of the problems in the culture of large quantities of *S. costatum* and noted the development of a more dependable culture medium consisting of tap water and Instant Ocean salts. [2, 3]

To decrease our dependence on the correct timing of algal cultures, techniques of freeze-drying and freezing are being investigated as possible methods of preserving diatoms. The freeze-drying process was applied to biological material by Shackell in 1909 and further developed as a

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[1] Contribution No. 330 from the National Marine Fisheries Service Biological Laboratory, Galveston, Texas.

[2] Instant Ocean R — Aquarium Systems, Inc., 1450 E. 289th St., Wickliffe, Ohio.

[3] Trade names referred to in this publication do not imply endorsement of commercial products.

technique by Flosdorf and Mudd in 1935 (Flosdorf, 1954). From these initial experiments, drying by sublimation has developed into the most widely used technique for preservation of biological materials such as blood plasma, blood products, micro-organisms and pharmaceuticals. Freeze-dried naked flagellates such as *Isochrysis galbana* and *Monochrysis lutheri* were successfully fed to various molluscan larvae (Loosanoff, 1962; Hidu and Ukeles, 1962).

In 1949 and 1959, respectively, the freeze-thaw protective agents glycerol and dimethyl sulfoxide (DMSO) were introduced, greatly increasing the interest in freeze preservation of cells (Sherman, 1964). The mechanisms by which these agents afford protection are not completely understood. It is certain, however, that the colligative properties, including (1) decrease in eutectic freezing point; (2) facilitation of super-cooling; and (3) salt buffering, are involved (Sherman, 1964). Protection from low temperature is also afforded to the cells by the ability of these neutral solutes to prevent excessive concentrations of electrolytes during freezing (Lovelock and Bishop, 1959). Lovelock and Bishop (1959) further reported that DMSO had certain advantages over glycerol. Smaller volumes and shorter pretreatment times were required with DMSO than with glycerol because DMSO penetrated the cells faster.

In the present paper, freeze-drying and freezing (with and without protectants) were investigated for the purpose of preserving diatoms on a short-term basis to eliminate our dependence on live algal cultures in feeding larval shrimp.

MATERIALS AND METHODS

The survival of the larval stages of the brown shrimp (*Penaeus aztecus*) from first stage protozoea to first stage mysis was used in most experiments to evaluate the usefulness of freeze-dried and frozen diatoms as larval shrimp food. With the exception noted below experiments were carried out at 27-28°C and salinity of 30‰. Larval shrimp were obtained from the hatchery at the Galveston Biological Laboratory.

The diatoms, *S. costatum* and *Thalassiosira* sp., were cultured according to the techniques developed at the Galveston Biological Laboratory by M. Alice Murphy (personal communication). Diatom cultures were harvested and concentrated by one of three methods prior to freeze-drying. The Sharples centrifuge was used to concentrate the diatom cultures when volumes of more than a few liters were involved, and either a clinical centrifuge or a vacuum filter apparatus was used to concentrate cultures of smaller volumes.

A laboratory freeze-dryer was used in the initial experiments. This freeze-dryer required the addition of dry ice and acetone throughout the operation. The diatoms were dried either in flasks or directly in the clarifier bowl of the Sharples centrifuge. In subsequent work, the use of an automatic, mechanical refrigerated freeze-dryer greatly increased our capacity to freeze-dry diatoms. The diatoms were stored frozen until they were reconstituted for feeding.

The same techniques of concentration were used when the diatoms were frozen. After concentration, diatoms were either frozen immediately, or were suspended in 1-2 ml of a 0.1% solution of glycerol or DMSO and then frozen at temperatures between -12°C and -20°C.

Experiments were conducted in three sizes of containers: 940-liter tanks simulating hatchery conditions; 1000-ml beakers aerated and held in constant temperature rooms; or 250-ml beakers held in shaking water-baths.

The ratio of number of larvae to water volume was approximately one larva per 6 ml of medium regardless of container size. Thus there were 128,000 larvae in the experimental 940-liter tank and 139,000 in the control tank. The 250-ml beakers, containing 150 ml of sea water, were stocked with 25 larvae, and the 1000-ml beakers, with 900 ml of sea water, had 150 larvae. Mortality in the tanks was estimated from daily aliquot counts. The animals in the small beakers were counted and transferred to fresh water daily, while larvae from one of the eight 1000-ml beakers prepared for each condition were removed daily and preserved for later counting.

RESULTS

Skeletonema as a Food Organism

Freeze-dried vs. live food

In a preliminary experiment simulating conditions in the shrimp hatchery, 45% of those larvae fed only freeze-dried food survived 7 days to become first stage mysis. For the first 2 days of the experiment survival of animals fed live and freeze-dried food was similar (Fig. 1). On the third day, however, larvae fed live food began to die. Deaths were attributed to a combination of reduced temperatures and the effects of large volumes of culture medium. Algal cultures at this time were being grown at temperatures of 20-22°C (6-8°C below that of the larval culture) and cell concentrations were relatively low. To feed the desired number of algal cells it was necessary to remove 250-300 liters of tank medium each day and replace it with an equal volume of the cold algal culture.

Although temperatures in the control tank

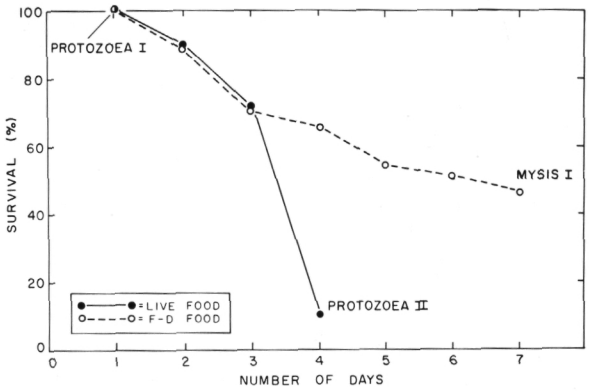

FIG. 1. *Survival of* P. aztecus *larvae fed freeze-dried (F-D) or live* Skeletonema *at 10^6 cells/ml.*

were not recorded, it is probable that at certain times, at least, it may have been below the 24°C considered necessary for larval development (Cook, 1969; Cook and Murphy, 1969).

In addition to the fact that temperatures were not comparable between control and experimental tanks, the algal culture medium is slightly toxic to larval shrimp (Z. P. Zein-Eldin, personal communication). Consequently, the addition of unusually large quantities may have been partially responsible for the mortalities. In spite of the death of the controls, the overall survival of the larvae feed only freeze-dried *Skeletonema* suggested that the preserved food might be a useful adjunct in the feeding of larval shrimp.

Because of the amount of food required and the time involved in preparation of freeze-dried food on such a large scale, additional experiments were conducted with larvae maintained in glass beakers. The concentrations of food were initially

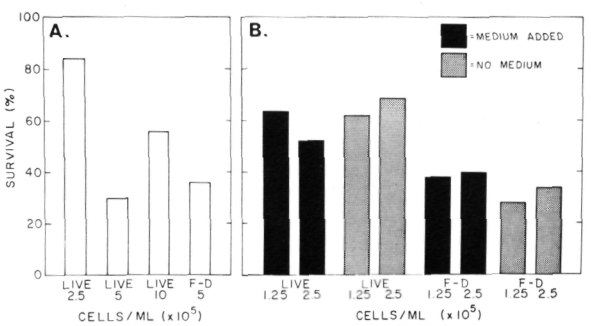

FIG. 2. *Survival to the mysis stage of* Penaeus aztecus *larvae fed live and freeze-dried (F-D)* Skeletonema, *container size 1000 ml (2A) or 250 ml (2B).*

those used in the hatchery (500,000-1,000,000 algal cells/ml). Lower concentrations (125,000 and 250,000) were also tested, however, since in previous rearing experiments larvae had been fed at rates of only 1,500/ml (Cook and Murphy, 1969). As shown in Figures 2A and B, freeze-dried food was not as satisfactory as live food; survival to the mysis stage was about half that of larvae fed live *Skeletonema*. In addition, concentrations of live food of 125,000 cells/ml were as satisfactory as higher concentrations.

To test for possible effects of the addition of small quantities of the algal culture medium, concentrated live cells and freeze-dried cells were fed to larvae held in 1-liter beakers with and without algal culture medium (Fig. 2B). The daily addition of 10-15 ml of the algal culture medium apparently did not affect the growth or survival of larvae held in the 250-ml beakers.

Frozen vs. live food

In a single experiment in which protectants

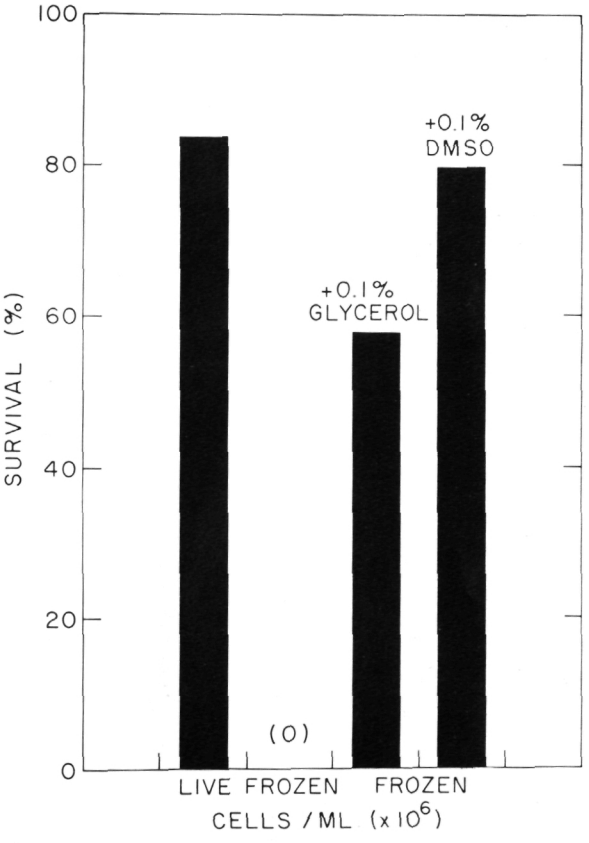

FIG. 3. *Survival after 7 days of* P. aztecus *larvae fed live and frozen* Skeletonema *at 10^6 cells/ml with and without protectants.*

were tested, all larvae fed the frozen *Skeletonema* without a protectant died (Fig. 3). Larvae fed the frozen diatom treated with either 0.1% glycerol or 0.1% DMSO survived almost as well as larvae fed live food. This suggests that these protectants may improve the food quality of frozen *Skeletonema*.

Thalassiosira as a food organism

Freeze-dried vs. live

Cook and Murphy (1969) suggested on the basis of limited feeding tests that *Thalassiosira* was more satisfactory as a food for larval shrimp than *Skeletonema*, and that *Thalassiosira* should be fed at lower cell densities. As with *Skeletonema*, however, survival to the mysis stage of larvae fed freeze-dried *Thalassiosira* was less than that of larvae fed live *Thalassiosira* at equal concentrations or at double concentration (Fig. 4).

Frozen vs. live food

During this study survival on frozen *Thalassiosira* was better than on frozen *Skeletonema*.

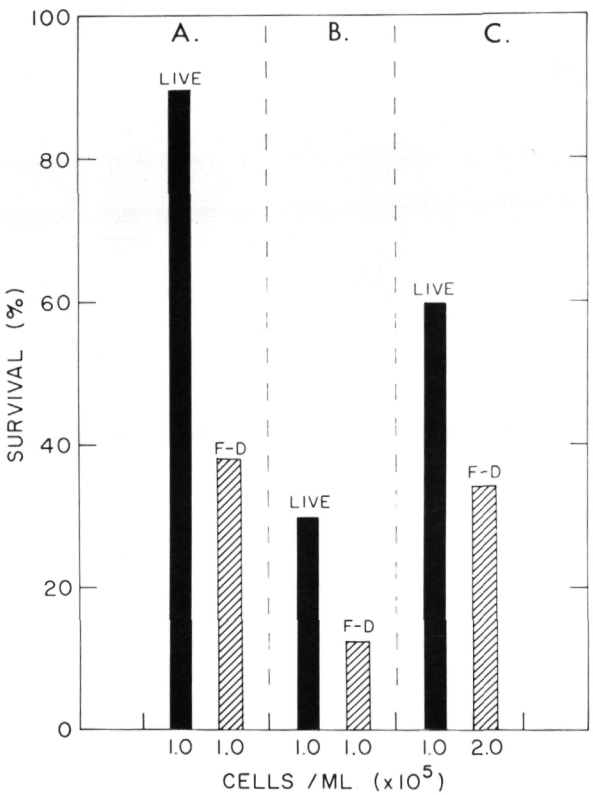

FIG. 4. *Survival to the mysis stage of* P. aztecus *larvae fed live and freeze-dried (F-D)* Thalassiosira *in three experiments, A. (1000 ml) B. (1000 ml) C. (250 ml).*

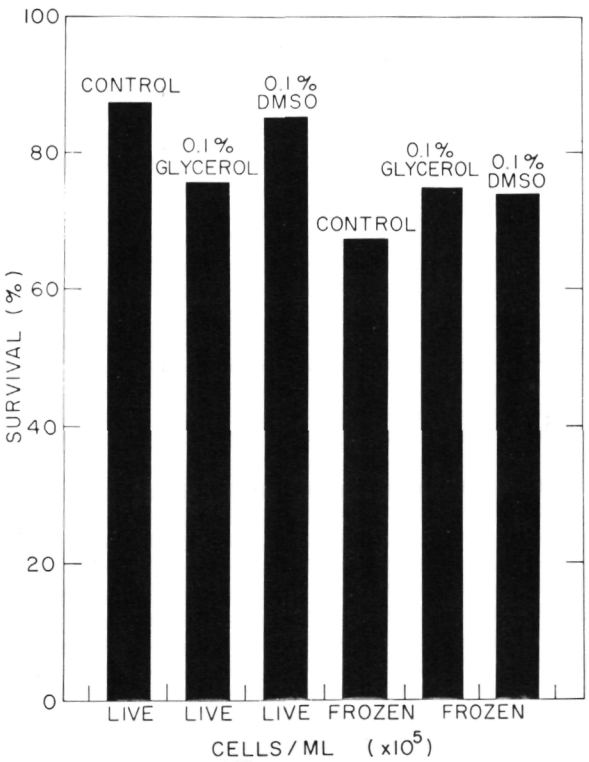

FIG. 5. *Survival for 7 days of* P. aztecus *larvae fed live or frozen* Thalassiosira *at a concentration of 10^5 cells/ml with and without protectants.*

The addition of protectants did not significantly improve the survival of larvae fed frozen *Thalassiosira* (Fig. 5). To test the effects of the protectants themselves, protectants were added to live algal cultures in concentrations equal to that present in the reconstituted suspension fed to larvae. There was no indication of effects resulting from use of the protectants (Fig. 5).

DISCUSSION

The difference in survival between groups, (see Fig. 4A, B and C) of shrimp treated identically is a fundamental difficulty in experiments of the type reported here. The researcher is limited by the periodic availability of larvae and the inherent difference between groups of eggs from females obtained at different seasons, under varying conditions at sea, etc. For these reasons it is only possible to make comparisons between groups of animals tested simultaneously. As shown in Figures 1 and 2, survival of larvae fed live *Skeletonema* varied from 0 to 84%; that of animals fed live *Thalassiosira* between 0 and 90%. In spite

of this variability, certain conclusions can be made.

Neither of the freeze-dried diatoms was completely adequate as a substitute for live food. Nevertheless, the ability of the freeze-dried food to support at least half the survival observed with the animals fed live diatoms suggests that algae preserved using this technique could be used for supplementary feeding, reducing the requirement for such carefully timed algal production.

Simple freezing of the diatoms has advantages over freeze-drying in both cost and simplicity. Much work remains, however, in elucidating effects of both techniques upon the algae themselves as well as upon the larval shrimp. For example, Holm-Hansen (1963) found differences in survival of algae depending upon freezing rate. Although no data are presently available concerning the nutritive value of *Skeletonema* and *Thalassiosira* frozen at different temperatures or at different rates, there are indications that a small percentage of these frozen diatoms may survive (C. R. Mock and B. R. Salser, personal communication). The use of protectants in freezing as well as freeze-drying also needs further study, since these agents appeared to affect the two algal species in different ways.

The long-range physiological effects upon larval shrimp of added agents such as protectants also need more detailed evaluation. Larvae fed *Thalassiosira* frozen in the presence of protectants survived at rates of 75% to third stage protozoea, but less than 10% were able to survive the molt to mysis. In contrast nearly all surviving protozoeae fed frozen *Skeletonema* in the presence of protectants were able to complete development to mysis stage.

The interactions of these factors — effects of freezing on the algae, the effects of the food upon the physiological condition of the larvae, and the value of various algal species as larval foods — are presently being evaluated in the shrimp hatchery at the Galveston Biological Laboratory.

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