# THE FLAGELLATE BODO LENS (O.F. MULLER) AS FOOD FOR LARVAE OF THE NATIVE PACIFIC COAST OYSTER

by

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TABLE OF CONTENTS

TABLE OF CONTENTS--continued

TABLE OF CONTENTS--continued

LIST OF TABLES

LIST OF TABLES--continued

LIST OF FIGURES

# THE FLAGELLATE *BODO LENS* (O.F. MULLER) AS FOOD FOR LARVAE OF THE NATIVE PACIFIC COAST OYSTER

## I. INTRODUCTION

### Investigation Background and Scope

This paper covers studies made at the Yaquina Bay Laboratory of the Department of Fish and Game Management of Oregon State College, figure 1, during the summer of 1950 with *Bodo lens* (O.F. Muller) as a food organism for the larvae of the Native Pacific Coast Oyster, *Ostrea lurida* Carpenter. Work was first begun by the Department of Fish and Game Management on the native oyster in 1939 when Professor Roland E. Dimick undertook a study to determine facts that might serve as an aid in the rehabilitation of the depleted native oyster fishery of Yaquina Bay. It was from this study, which lasted until 1946, that it was determined that the chief limiting factor to commercial native oyster production was probably insufficient successful natural reproduction. An investigation was then undertaken to find, if possible, a method for artificially rearing native oyster larvae which might eventually result in the production of native oyster seed on a commercial scale. Oyster investigators working on artificial rearing of the larvae have long been troubled with the problem of furnishing the proper

food organisms for the larvae. Partial successes with larval rearing obtained here have probably in part been due to the feeding of cultures of a colorless, naked flagellate, Bodo lens, to the larvae. The phase of the investigation reported here was devoted to a study of Bodo lens, its identification, its requirements, its use as a larval food, and the factors that influence its growth under laboratory conditions.

## Investigation from the Summer of 1939 until the Summer of 1950

The studies conducted by the Department of Fish and Game Management from the summer of 1939 until 1946 were concerned with the factors affecting the reproduction, survival, and growth of the native oyster in Yaquina Bay. Periodicy in spawning, survival of larvae, character of the substrate to which the larvae must eventually attach, success of attaching, or setting, of larvae, and growth of adults were all investigated. Once a larvae has attached to the substrate, or has set, it is much more able to withstand severe environmental conditions and has become a spat, the "seed oyster" of the industry. It was found from the study of the success of larval setting during various years that only approximately once every four years was the "set" large enough to sustain a

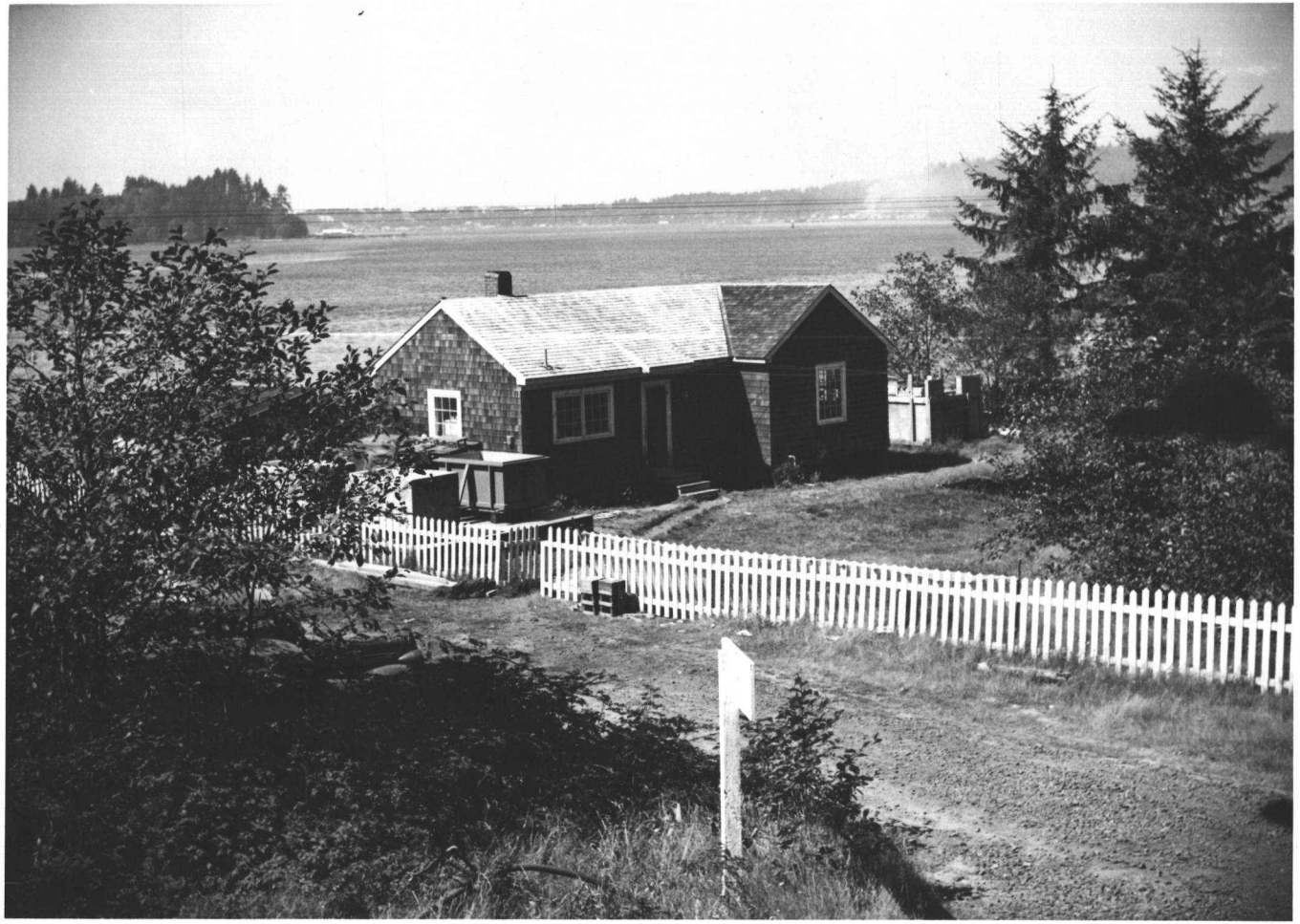

Figure 1. Yaquina Bay Laboratory of the Department of Fish and Game Management (photograph by E.P. Haydu)

commercial fishery. If the native oyster fishery of Yaquina Bay were again to attain commercial importance, a new source of seed oysters would need to be found.

Mr. Robert W. Morris, research assistant of the Oregon Agricultural Experiment Station, began work in 1946 to determine a method for artificially rearing the larvae of the native oyster from the time it is released from the adult to the time it has set and become a spat. During the next three years he was successful in keeping the larvae alive for increasing periods of time, though he did not succeed in rearing them to the spat stage. Various methods and materials were experimented with during this period, and many of these became a necessary part of later work.

The first successful rearing of native oyster larvae to the spat stage at the Yaquina laboratory was done by Mr. Eugene P. Haydu in the late summer of 1949. The following winter the success was repeated when larvae were made available through induced spawning of the adults. These successes were felt to be due in part to feeding the larvae rich cultures of small protozoa and frequently changing the water to prevent the accumulation of nitrogenous wastes.

## Investigation during the Summer of 1950

Experimentation during the summer of 1950 was designed to clarify the role of food organisms and nitrogenous wastes in the rearing of oyster larvae, and to improve methods of rearing larvae on an experimental scale. Work of the preceding summer and winter had indicated that proper food and proper control of nitrogenous wastes were the primary requisites to the successful rearing of larvae. The results of work done during the summer of 1950, however, forced a modification of this concept of the problem, and this will be discussed later.

Though food is probably not the main factor, or even one of the two factors, determining success in the rearing of oyster larvae, it doubtlessly plays an important role; and a large part of the summer was devoted to its study. Food was considered as consisting of either bacteria or protozoa or both. The algae were not carefully considered as it was hoped the rearing could be carried out under conditions of semidarkness. Mr. Gerald H. Watson conducted a study of bacteria as larval food, while this paper is a report on work done to determine the role of protozoa as larval food.

Bodo lens was the protozoa about which most of this part of the work centered, as this organism was

considered as probably being an important larval food. The experiments were selected and designed to determine the characteristics of Bodo lens, whether it is suitable as a larval food, how it can be best used for this purpose, and under what artificial rearing conditions the production of this organism is maximum. Not all of the determinations made from these experiments could be final. The investigation and experimentation involved the identification, culturing, and growth determination of Bodo lens; a study of the ingestion and assimilation of this organism by the larvae; the rearing of native oyster larvae; and a study of the factors determining the production of protozoa. These and minor studies are included in the following sections.

# II. REVIEW OF LITERATURE

## Reproduction and Development of the Native Oyster

### Reproduction

A clear understanding of reproduction in the native oyster has been brought about through the investigations of Stafford (32, pp. 131-132) and Coe (6, pp. 119-144). The native oyster is a protandric hermaphrodite (alternately male and female), and the eggs are fertilized in the mantle cavity of the female by sperm which have been released into the water by the male.

Moore understood that there was a hermaphroditic oyster on the coast of Washington (25, p. 268), but he did not realize this oyster was Ostrea lurida, which he mentioned on the previous page. Stafford found the eggs and sperm of Ostrea lurida associated in the same gonad and came to the conclusion that this oyster was a hermaphrodite (32, pp. 131-132). Coe determined that this oyster was protandric hermaphrodite and worked out the sex cycle (6, pp. 119-144). Germ cells first mature in the one-year-old oyster and it is at this time a male (6, pp. 122-124). The native oyster, from this time on, is alternately female and male; though germ cells of both sexes may be present at the same time due to the phase

change beginning before discharge of the sex products is complete (6, p. 129).

Sperm balls containing the sperm are released into the water by the male oyster. The sperm separate from these balls and finally reach the mantle cavity of the female. The eggs are released into this cavity by the female, and here fertilization takes place and development begins. (15, p. 271)

## Development

The early development of Ostrea lurida was worked out by Stafford (33, pp. 107-108) and was recently investigated by Hori (15, pp. 271-272), who was apparently unaware of Stafford's work. Hopkins was able to determine by sampling the larvae in the mantle cavities of the adults the average period of time required for larvae to reach the various stages of development (12, pp. 467-470). The drawings of the development of native oyster larvae included in figure 2 are taken from Hori (15, p. 274).

Fertilization of the egg takes place on the gills in the mantle cavity of the female native oyster. The newly formed zygote then begins dividing into macromeres and micromeres. A morula forms, figure 2 drawing 6, and then the blastula develops. The invagination

resulting in the formation of the blastopore and archenteron gives the gastrula a rather heart-shaped appearance, figure 2 drawing 7. The gastrula soon develops a prototroch which enables it to swim by ciliary action. Until this stage the embryos are white and are termed "white sick." (15, p. 271) The formation of the prototroch takes place on about the third day (12, p. 468).

Shells become apparent on the larvae, figure 2 drawing 8, on the fourth day (12, p. 468). By the fifth day the shells can enclose the larvae completely, and it has reached the straight-hinge stage (12, p. 470). The larvae darken and soon are termed "black sick", figure 2 drawing 9, at which time they are due for release from the mantle cavity (15, p. 272). They are ordinarily somewhat less than 180 mu at this time (15, p. 272) and have required an average of ten days to attain this development (12, pp. 467-470).

After their release from the mantle cavity the larvae are free-swimming for a period of about one month (12, p. 470). At the end of this time they have grown to about 320 mu, figure 2 drawing 16, and are ready to set (15, p. 273), though this size may vary some with different conditions (12, p. 471). With attachment and metamorphosis, the larvae have become spat, the "seed" of the oyster industry.

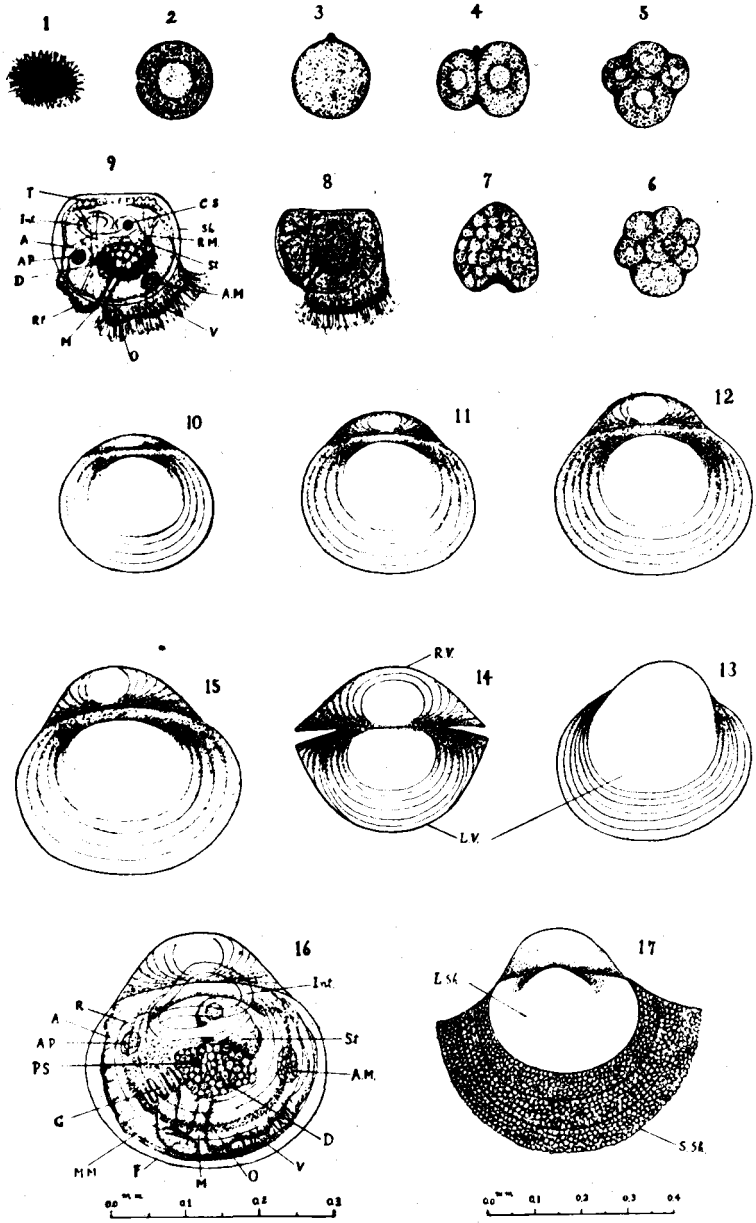


Figure 2. Development of Native Pacific Coast Oyster (Ostrea lurida) from Hori (15, p. 274). 1, Sperm-ball; 2, Ovum; 3, Ovum with polar bodies; 4, First cell division; 5, Second cell division; 6, Third cell division; 7, Gastrula stage; 8, 9, Larvae in the straight-hinge stage; 10, Shell 205 x 183 mu (umbo developing); 11, Shell 230 x 210 mu; 12, Shell 260 x 240 mu (right side); 13, Shell 260 x 240 mu (left side); 14, Shell 260 x 240 mu (umbo side); 15, Shell 300 x 280 mu (umbo prominent); 16, Larvae full grown 320 x 310 mu; 17, Spat.

## Artificial Rearing of Oyster Larvae

### Early Efforts

Efforts to rear oyster larvae under artificial conditions extend back at least into the 1880's, and the many investigations conducted since then have resulted in considerable literature. An attempt will be made to outline the progress of these investigations by reviewing what are considered some of the more important contributions.

Interest in the artificial rearing of oyster larvae developed soon after 1879 when Brooks was successful in artificially fertilizing the eggs of the American oyster, Ostrea virginica (1, p. 426). The same year he was able to keep the larvae alive for six days, or until they reached the straight-hinge stage. At this time he was aware of the difficulties of maintaining proper temperature, changing the water, and furnishing the correct food for the larvae. (2, pp. 449-451)

Ryder was possibly the first to attempt to utilize on a commercial basis the technique of artificial fertilization developed by Brooks. He proposed that the natural production of larvae in oyster spawning ponds be supplemented by larvae from artificially-fertilized eggs (30, p. 399). Not actually rearing the larvae, he would

hold them in dishes only until they reached the swimming stage; at which time he would release them in a manner described by him in the following quotation.

> It is during the flood tide that I would therefore recommend the practice of putting artificially-fertilized embryos in the swimming stage of development into the outlet of the canal to be swept back amongst the collectors toward the spawning pond. (30, p. 400)

Nelson, in 1904, was attempting to rear larvae until the spat stage in small containers of various descriptions. He did not accomplish this, though he did rear larvae to shelled stages. (28, pp. 415-434) At this time he was not trying to produce spat on a commercial basis, but his statement which follows would indicate he thought it possible and eventually hoped to do so.

> Our ultimate aim is to establish a system of oyster culture that shall be as much under control as is fish culture. (28, p. 420)

## Recent Efforts

Hori was probably among the earliest investigators to rear larvae under artificial conditions until they reached the spat stage. He was successful during 1927 in obtaining a few spat from larvae of the Japanese oyster (Ostrea gigas) by feeding cultures of a small,

unicellular green algae (Chlorella pacifica) and by making frequent water changes (14, p. 79). Six years later, Hori reared the larvae of Ostrea lurida artificially for a period of 22 days, at the end of which time the larvae became spat. He was unsuccessful, on this occasion, in rearing these larvae on Chlorella, but succeeded with larvae kept in vessels to which he added juice from the fronds of Ulva. (15, p. 273)

Cole found that he was unable to obtain satisfactory settlements of spat unless large numbers of naked flagellates were present and that large numbers of unicellular green algae were detrimental (7, p. 13). He concluded that the free-swimming larvae were unable to utilize such forms as Chlorella due to their inability to digest the cell wall (7, p. 20), and hence could utilize only naked flagellates (7, p. 24).

Bruce, Knight, and Parke, using growth and percentage of settlement of the larvae of Ostrea edulis as their criteria, attempted to determine the identity and nature of the algal cell best suited for larval food (3, pp. 337-374). They obtained percentages of successful settlement varying from 0.06% to 99.0% with the six organisms used (3, p. 371). Slight differences in the shade of algae of the same color were thought of possible importance to the larvae (3, p. 372). The following

explanation for differences in the food value to the larvae of the various algae was offered.

> It appears probable that the varying usefulness exhibited by these differently coloured organisms, depends directly upon the degree to which the included algal food reserves, glycogen, oil, etc. serve the immediate needs of the developing larvae. (3, p. 372)

By 1948 Imai was successfully rearing the larvae of Ostrea gigas to the spat stage in earthenware crocks in which he used sand-filtered water having a salinity of around 17 parts per thousand (16). Much of his success was no doubt due to his use of rich cultures of Monas (a colorless, naked flagellate) as food for the larvae. He has successfully reared larvae in concrete tanks on an experimental, seed-production basis (16). After Monas was found to be an excellent larval food, Imai and Hatanaka studies the physical and nutritional requirements of Monas in order to develop a culture medium low in nitrogenous components (17). The following quotation from their paper shows the importance of this study.

> In our experience of larval rearing, the culture medium with organic substances rich in protein content, particularly of animal origin, always gave negative results, possibly due to the harmful effects of decomposition products of organic nitrogen. (17)

Davis, working on the Atlantic coast, reared

larvae of Ostrea lurida to the spat stage in periods varying from 10 to 23 days (8, p. 591). He makes the interesting statement that larvae reared at a temperature kept between 16.0 and 18.5 degrees centigrade never set though they reached 290 mu after living 30 days; while larvae reared between 19.0 and 22.0 degrees centigrade set in 10 to 23 days when about 300 mu in size (8, p. 591).

Different investigators have worked for years in efforts to artificially rear oyster larvae, and it is only within comparatively recent years that they have met with even moderate success. The many failures they have experienced have certainly not all been due to the same factors. The complex ecology of confined water in which larval rearing attempts have been made leaves open many avenues to failure. Bruce, Knight and Parke present a brief and enlightened summary of the problem faced by investigators within this field.

> The high rate of mortality among developing larvae, which has been the experience of many workers in the field, may be considered to be attributable to two sets of factors which are inseparably associated with the fact of isolation of any small body of water. These factor-groups may be described as 'biological' and 'biophysical'--the one concerns the presence or absence of the appropriate food material and the conditions leading to the further production of that material; the other involves the delicate physical

> balance, or dissociation-equilibrium, upon which the continued usefulness of sea water as a medium for growth and development so greatly depends. (3, p. 370)

This grouping of causes for failure in larval rearing attempts allows not only for the obvious factors such as temperature, oxygen, light, salinity, and food, but for the subtle influences such as the "decomposition products of organic nitrogen" which Imai (17) considered important. There is, however, one factor which, there is reason to believe, may be of some importance and for which no provision is made in the above grouping.

Predation and disease may present an obstacle to successful larval rearing. Dodgson writes of work in which Sherwood demonstrated the predation of Noctiluca on the larvae of Ostrea edulis (10, p. 344). Hori and kusakabe offer the following as partial reason for their early failure to rear Ostrea gigas.

> In culture it is very difficult to get rid of infusoria which are very harmful. In healthy condition they (the larvae) are not injured by them, but the more unhealthy larvae become, the more infusoria multiply in water and at last they (the larvae) die away being attacked by them. (13, p. 48)

Zobell makes the following statement.

> As the causative agents of diseases of marine plants and animals fungi may be extremely important. (36, p. 133)

Taxonomy of Bodo lens (O.F. Muller)

Bodo lens was first described by O.F. Muller in 1786 as Monas lens (27, p. 4). Muller established the genus Monas in 1773 and defined its members only as punctiform organisms. He very incompletely described ten species in 1786, but two of these were later shown to be bacteria. (29, pp. 399-400)

Saville Kent in 1880 placed Muller's Monas lens in the family Heteromitidae and changed its genus to Heteromita. The genus Heteromita was described as including individuals which were plastic, possessed no differentiated cuticular investment, had one anteriorly and one posteriorly directed flagellum, and were without a distinct mouth. (18, p. 290) Kent's description (18, pp. 291-293) and illustrations of Heteromita lens were detailed and quite good.

Klebs lists this organism as being in the genus Bodo and in the family Bodonina of the order Protomastigina (19, p. 319). Kudo described the order Protomonadina as containing a heterogeneous group of protozoa, most of which are parasitic, many of which are plastic, which have no definite pellicle, one or two flagella, holozoic or saprozoic nutrition, and which as a rule reproduce by longitudinal fission (22, p. 268). Kudo described the family Bodonidae as a group of organisms having one

anteriorly and one posteriorly directed flagellum originating in the anterior end, one to several contractile vacuoles, reproduction by binary fission, and having holozoic or saprozoic nutrition (22, p. 289).

Lohmann observed an organism he described as Heteromita ionica (24, p. 50) which appears to be Bodo lens. This was the opinion of Hamburger who included the synonomy of Bodo lens (11, p. 198).

Wailes in 1939 listed Bodo lens as occurring in Departure Bay on the Canadian Pacific coast along with Bodo rostratus and Bodo caudatus (34, p. 3).

The organism that was studied during the course of this investigation was identified as Bodo lens on the basis of its similarity to Heteromita lens as described by Kent (18, pp. 291-293). This similarity is discussed in the description of Bodo lens included in section IV. The organism studied showed considerable variation in size and form. Yet, the largest individuals were usually smaller than the minimum lengths of 8 mu given for Bodo rostratus by Wailes (34, p. 3) and 9 mu given for Bodo globosus by Calkins (4, p. 425). Kent agreed with Calkins in stating that the contractile "vesicle" occupies an anterior position in Bodo globosus (18, p. 295) and (4, p. 425). As Bodo lens has a posterior vacuole, this constitutes a less variable distinguishing characteristic

than do size and shape. In general, the various forms of the organism identified here as Bodo lens coincide much more closely to the descriptions of that species than to those of either of the other two species, as given by these three workers.

# III. METHODS AND MATERIALS

## Sources of Protozoa

The cultures of protozoa utilized in descriptive and experimental work were started from two sources which apparently differed little in the species present. The culture series on which the descriptions of *Bodo lens* and other protozoa were based were begun with innoculations from crocks in which oyster larvae were being successfully reared. Many of the cultures used in later experimental work were originally innoculated with samples of bottom material and water from the native oyster bed areas in Yaquina Bay.

## Culture Media

The components of the two most important culture media used in the course of this investigation are given in table I. It will be seen from table I that medium #1 contains the same nutrients as medium #2 except for the calcium chloride in the latter. However, much smaller amounts of starch and potassium nitrate are used in medium #2.

Medium #1 was first used for culturing protozoa at the Yaquina laboratory by Mr. Robert W. Morris. It

TABLE I

Culture Media

| Components | Amounts | |
|---|---|---|
| | Medium #1 | Medium #2 |
| Sea water (salinity 17 ppm) | 1.00 l | 1.00 l |
| Starch | 1.50 g | 0.10 g |
| Potassium nitrate | 0.30 g | 0.04 g |
| Sodium acid phosphate | 0.05 g | 0.05 g |
| Calcium chloride | 0.00 g | 0.05 g |

seems probable that he developed this medium after receiving a letter from Mr. Takeo Imai (16) giving, in a general way, the nutrients in a culture medium being used in oyster rearing work in Japan. Medium #1 was used at the Yaquina laboratory for most culture work until the summer of 1950 when it was determined that medium #2 was more suited to supposed cultural needs.

Medium #2 contains the various nutrients in quantities within the optimum range for culturing Monas as determined by Imai and Hatanaka (17). An experiment conducted during the summer of 1950 (section V) indicated that growth of Bodo lens was nearly as good in medium #2 as in medium #1 in spite of the smaller amounts of some nutrients in #2. It was thought that these smaller amounts would be an advantage in larval rearing due to a decrease in nitrogenous material, and medium #2

was used during the remaining part of this investigation.

Pure Culture Technique

Cultures of protozoa "pure" in the sense that they contained but a single species of protozoa (but containing bacteria) were needed. The usual method of starting cultures of one species of protozoa by collecting a single protozoa with the aid of a capillary pipette under a microscope was found unsatisfactory. However, the following modification of this method was found to be satisfactory. A fairly rich mixed culture containing the desired species was selected. A capillary pipette was then used to place a minute drop of this culture on the corner of a clean slide. This tiny drop was next examined under a microscope on which the mechanical stage had been set so the corner of the slide would automatically fall in the center of the field. This was important as the drop would dry before it could be observed if it were necessary to search for it under the microscope. The drop was made small enough that it would little more than cover the field when using the low power objective with a 12.5 x ocular.

If large numbers of protozoa were present in the first drop, the mixed culture was diluted with sterile medium until the drops contained zero, one, two, or three

protozoa. Each new drop was placed on the corner of a clean slide. When one was observed which contained only the species of protozoa desired it was washed into a petri dish containing sterile medium to start the pure culture. It was necessary, of course, that the worker be able to recognize the desired species at the low magnification.

Intra-vitam Staining

Intra-vitam staining technique was used in determining the morphology of Bodo lens (section IV) and in determining the ability of oyster larvae to ingest certain protozoa (section VI). Azure II was used in studying the morphology of Bodo lens. A drop of a water solution of azure II was added to several drops of a good culture of Bodo lens and this was studied immediately beneath a microscope. Internal structures, inclusions, and flagella were rendered more easily visible in the protozoa; though the protozoa steadily lost vitality.

The use of intra-vitam staining made it possible to study the feeding of oyster larvae on protozoa that were alive yet were easily followed due to their stained condition. Enough methylene-blue solution was added to a culture of protozoa to give the culture a definite

light blue color. As soon as the protozoa were sufficiently stained, a drop of the culture was added to a group of oyster larvae in a hollow slide with several drops of sea water. The feeding of these larvae was then studied using both the low and high power objectives of the microscope.

Counting of Protozoa

Three different methods of counting protozoa were utilized during the investigation. The first two of these methods were used to obtain estimates of the numbers of protozoa per cubic centimeter. The third method was devised to obtain only figures representing relative abundance. As a regular counting chamber for this type of work was not available, a Spencer haemacytometer, improved Neubauer type, was substituted, with modifications made in its use.

In determining the growth curves of Bodo lens (section V), counts were made each day for nine days on four different cultures. When a count was to be made on one of these cultures, the culture was stirred and a 5 cc sample was drawn off. To this was added 0.3 cc of Noland's combination fixative-stain. The sample was then shaken to insure an even distribution of organisms, and a part of it was used to fill the chamber of the haemacytometer. This

was then placed under the microscope and the high power objective was used. The protozoa occurring in each of 40 of the smaller squares were counted and the average per square was determined. This average was multiplied by 100,000 to give the number of protozoa per cubic centimeter. Two counts were made, and the average was used.

In determining the number of Bodo lens ingested by oyster larvae (section VI), it was necessary to modify the procedure used above by taking a larger sample. The procedure followed was the same except that the protozoa occurring in each of all 25 larger squares were counted and an average per square was determined. This average was multiplied by 10,000 to give the number of protozoa per cubic centimeter. This count was made three times, rather than twice, to determine an average figure for use.

The study of the production of Bodo lens in crocks having varied conditions (section VIII) required a counting technique quite different from those used above due to the smaller numbers of organisms present. The haemacytometer could not be used, being designed to estimate larger numbers; and it was necessary to devise a technique which, while not actually determining the numbers of organisms per cubic centimenter, would serve as an indication of the relative numbers of organisms present. The aid of an appropriate counting chamber no

doubt would have made it possible to compare these smaller numbers and obtain an estimate of the number of organisms per cubic centimeter. It is regrettable this was not possible. Nevertheless, though the method of comparison is not satisfactory in all respects, it is believed that it was a fairly reliable measurement of the more important differences in protozoa production under the conditions that were tested.

In order to obtain an index of the relative abundance of protozoa in a crock, a 400 cc sample was first taken of the stirred contents. Seven different water slides were then made using two drops of the sample under a 7/8 inch square, #2, glass cover slip. Each slide was examined under the low power objective by moving the slide so that the field of view passed from the right side of the cover slip to the left side once. The number of organisms occurring on each slide in this area (the diameter of the field in width and 7/8 inch in length) were counted. The organisms counted were divided into two groups: (1) those less than 10 mu being considered Bodo lens, and (2) those greater than 10 mu being listed as such. The figures obtained by averaging the counts made on the seven slides were then used as the indices of the relative abundance of protozoa present in the crock. When slides had extremely large numbers of Bodo

lens, these were counted by estimated groups of ten so as to facilitate the work. It is not believed that any errors introduced by this would appreciably affect the final conclusions.

## Source of Native Oyster Larvae

Native oyster larvae were obtained during the summer of 1950 from groups of adult oysters collected from the beds of the Oregon Oyster Company. These adults were tonged at low tide, and, after having their valves cleaned of barnacles and other external organisms, were placed in 12-gallon stoneware crocks of aerated sea water. Larvae which were present in the mantle cavities of some of these adults were usually released into the surrounding water within a week. Larvae can sometimes be obtained at any season by holding the adults at the correct temperature during the entire period of gonadal development and spawning. This, however, involves considerable effort and is unnecessary during the summer months when the oysters are spawning naturally.

## Counting of Oyster Larvae

Frequently it was necessary to estimate numbers of oyster larvae in order to place the desired numbers in

rearing crocks and other experimental containers. The usual procedure was to fill a suitably sized beaker from the crock in which the larvae to be used were held. After the contents of this beaker were stirred to insure a uniform distribution of the larvae, ten, one cubic centimeter samples were drawn off and placed in watch glasses. The larvae in these samples were counted and an average number of larvae per cubic centimeter determined. The contents of the beaker were again stirred and the number of cubic centimeters needed to furnish the desired number of larvae were measured from the beaker into the rearing crock.

Measuring of Oyster Larvae

The measuring of oyster larvae was necessary to determine rate of growth and approximate size at time of setting. The method of measuring employed was probably a fairly good indication of these. It would have been desirable, however, if the method could have been reliable enough to determine small differences in average growth between different groups of larvae. The size of sample measured would very likely have had to be at least four times as large to have met this third need. Nevertheless, as the results are used, they are probably fairly dependable.

A sample of the larvae to be measured was taken from the rearing crock in a beaker. Five larvae were caught from this with the aid of a dropper and placed in a hollow slide. A drop of a 5% solution of urethane was added to cause the larvae to close their valves, stopping their movement and making measurement easier. The larvae were then measured parallel to the hinge at the widest point with a micrometer-type ocular. An average of the five measurements was then recorded in microns.

Chlorination of Oyster Larvae

The obtaining of protozoa-free oyster larvae for several of the experiments conducted during the course of this investigation was desirable. Different methods for obtaining these were tested, and chlorination of the larvae was selected as being the most satisfactory. This method, as used, did not completely rid the larvae of protozoa. Nevertheless, the protozoa were reduced to such a low level that for several days the larvae were free enough of protozoa for the purposes of most of the experiments conducted. The method used could, with modifications, probably entirely free oyster larvae of protozoa; and thus it would be a valuable tool for larval food studies.

The water containing the larvae to be chlorinated

was placed in a glass container in which the larvae could be observed. A solution of sodium hypochlorite was then added by small increments until the larvae closed their shells and settled to the bottom. The larvae were then protected from the full effect of the free and combined forms of the chlorine; and the sodium hypochlorite solution was added until the chlorine totaled 10 parts per million as determined by ortho-tolidine. The water was next nearly all siphoned out, care being taken not to disturb the larvae resting on the bottom. Sterile sea water was added to the larvae and remaining water, greatly diluting the chlorine and chlorine compounds present. The concentration of these was then redetermined and enough sodium thiosulfate added to neutralize them.

By removing the excess water and diluting with sterile sea water, the amount of sodium thiosulfate needed for neutralization was considerably reduced, thus reducing the amount of its possibly harmful end products. If deemed desirable, the entire procedure would be repeated again after a short interval during which the swimming larvae would be ridding themselves of protozoa previously enclosed and protected by the larval shells. This treatment apparently had no harmful effect on the larvae.

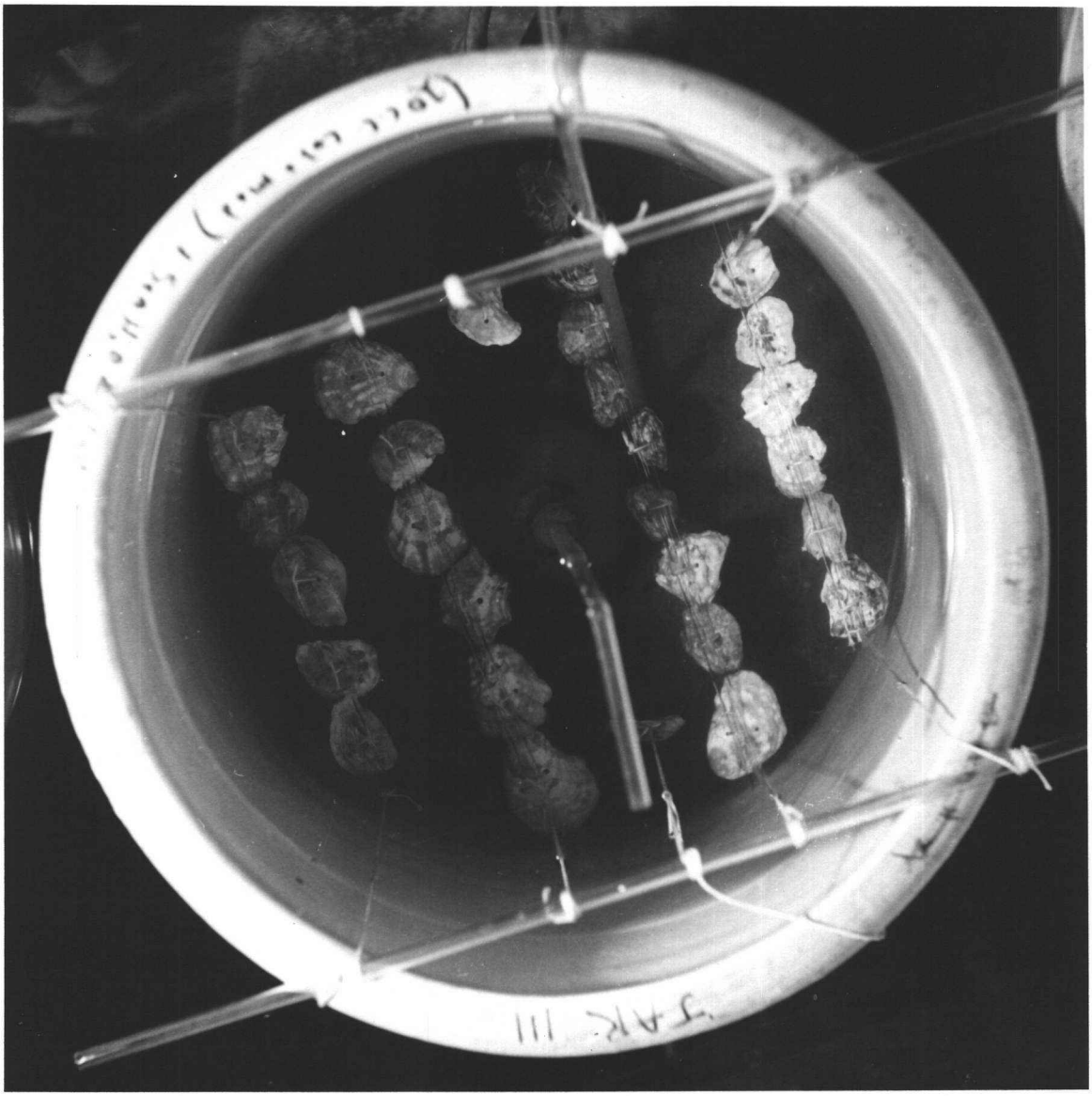

Figure 3. Rearing Crock for Oyster Larvae
(photograph by E.P. Haydu)

## Rearing Crocks used for Oyster Larvae

Two sizes of ordinary stoneware crocks, glazed on the inside, were used for rearing the larvae. The larger crocks had a total capacity of 12 gallons, while the smaller ones held 4 gallons. Figures 3, 4, and 5a show these rearing crocks. In order to change the water in the crocks without injury to or loss of larvae, a filter-type siphon such as shown in figure 5a was used in each crock. The filter portion of this siphon consisted of a bottle packed with alternate layers of glass-wool filtering fiber and sand, the bottle itself being embedded in a dish of sand. Glass tubing passing through a cork in the bottle was connected to the rubber-tubing siphon hose. Circulation and aeration of the water in the crocks was accomplished by a gentle flow of air bubbles introduced into the water through a piece of bent glass tubing and controlled with a small clamp, figure 5a. When the larvae were about ready to set, cultch consisting of oyster and clam shells suspended with strings was introduced, figure 3.

## Temperature Control

Temperature control for water in the crocks in which the various experiments were conducted was

accomplished with circulating water baths equipped with thermo-regulators and heaters. The water baths consisted of sheet-metal tanks in which the water was circulated by small electric motors with shafts and propellors, figure 4. The thermo-regulators were of the vapor-mercury type. Tube type, immersion heaters rated at 200 watts and having stainless-steel sheaths were used.

A temperature lower than that of the atmosphere was required for the experiment on the production of Bodo lens in crocks having varied conditions (section VIII). A fairly constant temperature of 15 degrees centigrade was obtained by using running water from the laboratory supply in the water bath.

## Water Filtration

The water in which the oyster larvae were reared was filtered to remove plant and animal organisms as well as detritus and silt. A diagram of the filter used is shown in figure 5b. A 12-gallon crock was one-third filled with sand, and into this sand was set a bottle containing alternate layers of glass-wool filtering fibre and sand. Tubing was passed through a rubber cork in the bottle and then into a series of five-gallon carboys. The first two of these carboys served to collect the filtered water, while the third carboy acted as a trap to catch fresh

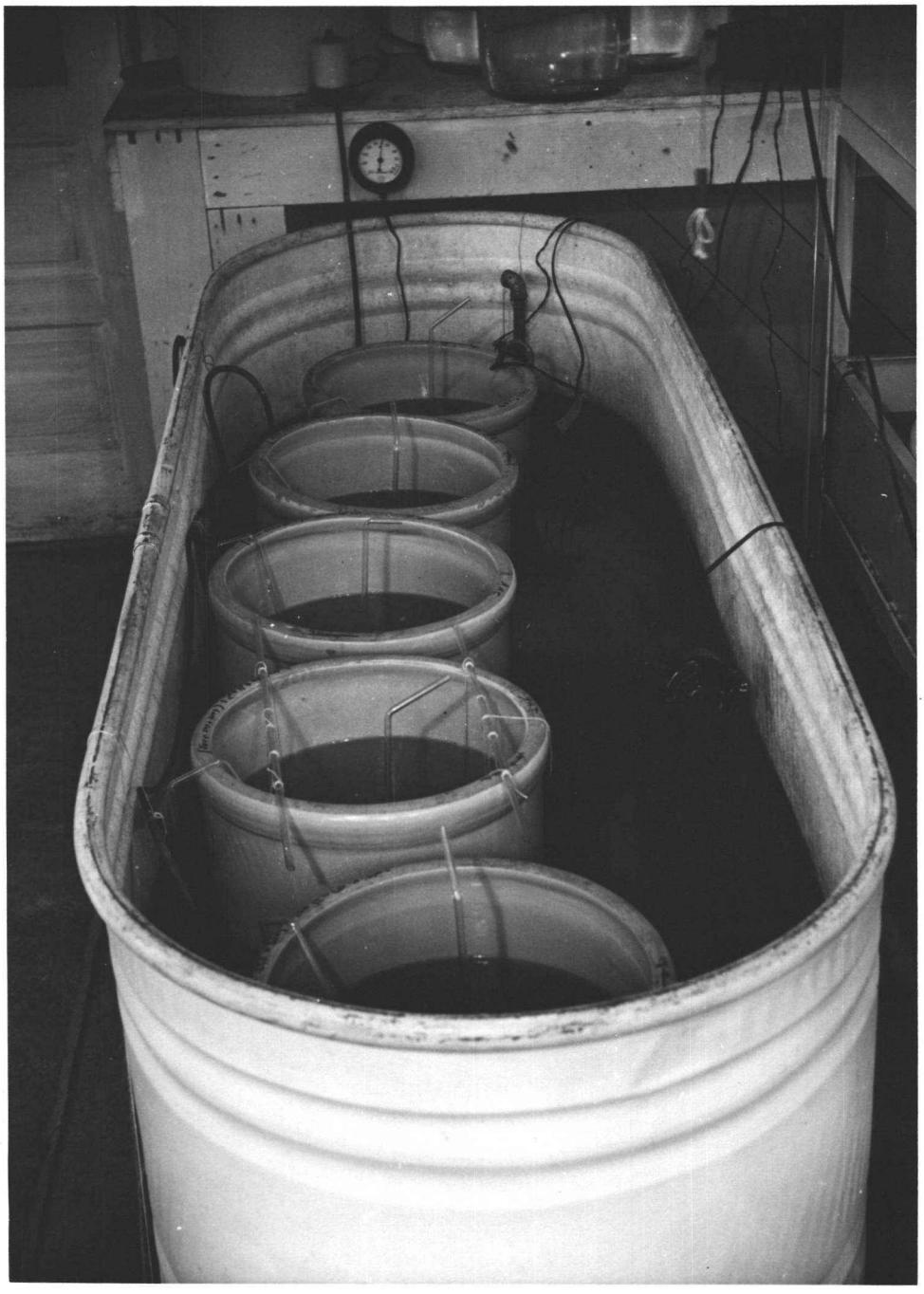

Figure 4. Circulating Water Bath for Temperature Control of Rearing Crocks (photograph by E. P. Haydu)

water from the suction pump. Mr. Robert W. Morris was the first to use a filter of this design at the Yaquina laboratory. The filter, when used in this manner, did not remove all of the microscopic organisms. It did, however, remove sufficient material to reduce organic decomposition and siltation in the crocks to low level.

The experiment on the production of Bodo lens in crocks having varied conditions (section VIII) required a degree of water filtration which would remove nearly all of the microscopic plant and animal organisms. The filtration apparatus used to accomplish this was the same one used for the lesser degree of filtration except for the bottle embedded in the sand. Whenever the higher degree of filtration was necessary, the tubing going to the carboys was connected to a bottle containing a porcelain filter candle around which glass-wool filtering fibre was tightly packed, figure 5b.

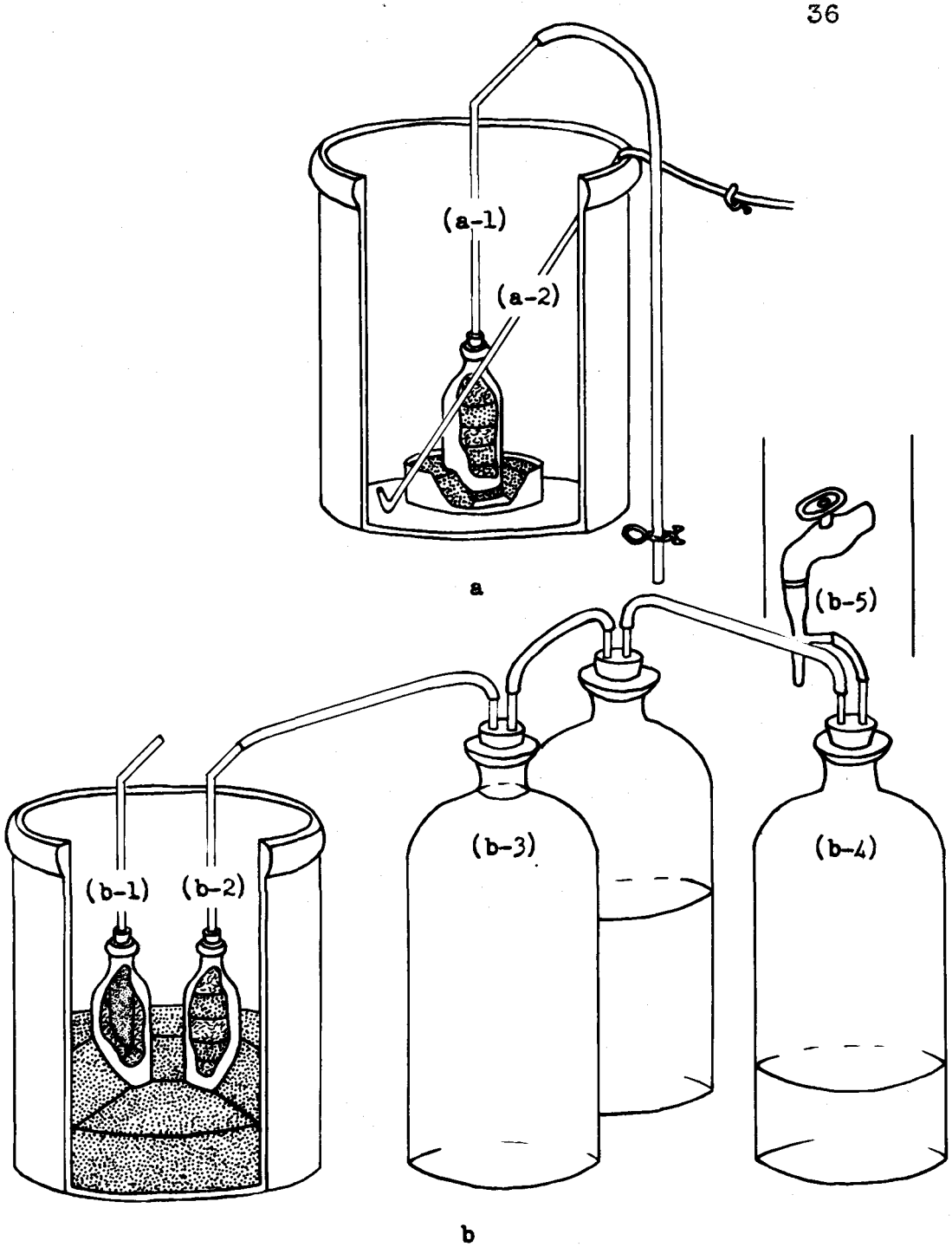


Figure 5. Rearing Crock and Water Filter. a, Rearing crock showing filter-type siphon (a-1) and tube through which air was introduced (a-2); b, Water filter showing filter-bottle equipped with porcelain candle (b-1), regular filter-bottle (b-2), collecting carboy (b-3), fresh-water trap (b-4), and suction pump (b-5).

# IV. DESCRIPTIONS OF *BODO LENS* AND ASSOCIATED PROTOZOA

## Description of *Bodo lens*

The identifying and describing of *Bodo lens* presented some difficulty due to its several growth forms. In order to determine the relationship of these growth forms, different cultures were innoculated with the different forms. These cultures clearly demonstrated that these different forms were but the various growth forms of a single species, later identified as *Bodo lens*. Kent, in his description of *Heteromita lens* (18, pp. 291-293), included drawings of its various growth forms which were like many of the forms observed; though all of the life-cycle stages he diagramed were not identified here.

*Bodo lens* is a minute, colorless, unarmored flagellate having two flagella, one of which trails, a rather plastic form, and various organellae. Kent stressed the plastic form of this organism.

> Body exceedingly soft and plastic, susceptible of considerable alteration of contour, usually subglobose, peach-shaped, or more or less ovate with a slightly narrower anterior extremity; flagella equal in size, very slender and flexible throughout, about twice the length of the body; endoplast spherical, subcentral; contractile vesicle posteriorly situated. Length 1-5000" to 1-3250". (18, p. 291)

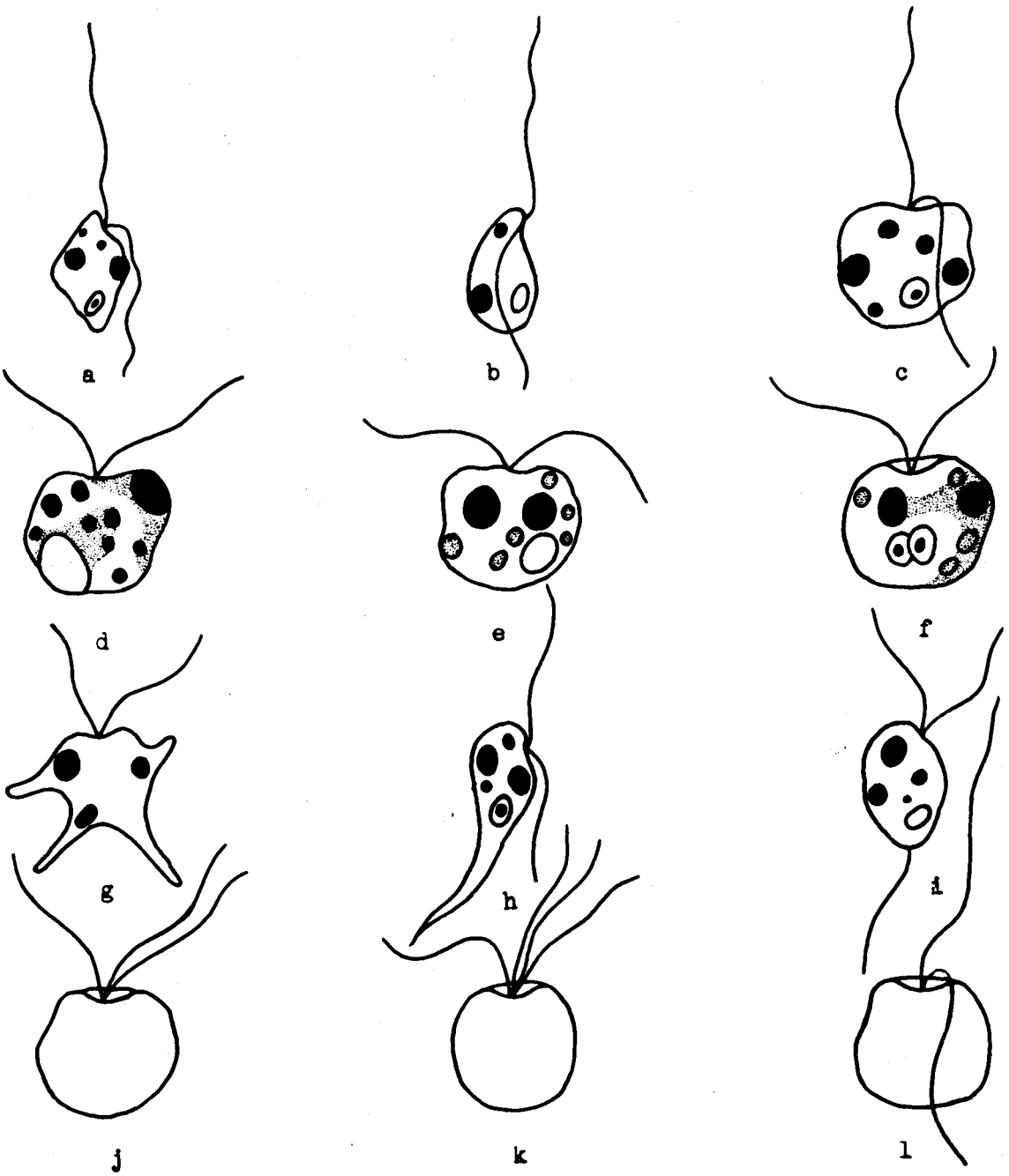


Figure 6. Bodo lens (O.F. Muller) x 4000. a, b, c, Most typical forms; d, Stained with Jenner's; e, Intra-vitam stained with azure II; f, Composite drawing; g, Amoeboid form; h, Elongate form; i, With filar pseudopodia; j, k, With 3 and 4 flagella during binary fission; l, With extended leading flagellum.

The three most typical forms of Bodo lens observed are shown in figure 6, drawings a, b, and c, though even these forms were subject to some variation. The largest individuals usually assumed a globose form, figure 6c, while the smaller ones might be irregular, figure 6a, tear-drop, figure 6b, or globose in shape. Kent considered the globose form the characteristic adult state and gave the following descriptions of its two main variations.

> An ovoid form with a somewhat narrower anterior extremity (Fig. 2), on the symetrical side, and an irregular, almost amoebiform contour (Fig. 3) on the unsymetrical one, represent the most constant departures from the typical subspheroidal shape that have to be recorded. (18, p. 292)

The variations recorded by Kent are much the same as those observed here, his figure 2 being nearly identical with drawing b, and his figure 3 being not unlike drawing a.

Bodo lens typically varied in size from 2 by 2.5 mu to 6 by 7.5 mu. A new culture offering good growth conditions usually contained individuals of the smaller sizes due to the rapidity with which binary fission took place. An older culture, presenting less favorable conditions, usually contained individuals approaching the larger sizes. Under conditions unsuited to growth and reproduction, individuals as large as 6 by 10 mu have been observed.

Bodo lens has two flagella and, when this organism is swimming, one of these is extended anteriorly while the other trails. These flagella, as observed when the organism was resting, were somewhat longer than the body; however, when the organism was swimming, the leading flagellum stretched to twice its resting length, figure 61. The only detail of flagellar attachment that was observed under oil immersion was an anterior, cup-like depression of the body wall, figure 6f. Bodo lens was observed with three and then four flagella when going through the stages of binary fission, figure 6j,k.

Many dark bodies were apparent in the living Bodo lens, but their various identities were not clearly established. No difference in the staining reaction of these bodies was detected when Jenner's stain was used after fixing with methyl alcohol; although different regions of the cytoplasm varied in staining reaction, figure 6d. When azure II was used as an intra-vitam stain (on the living organisms), usually two of the larger bodies were darkly stained while the other bodies took the stain little if at all, figure 6e. Most of these non-staining bodies were probably bacteria on which the Bodo lens were actively feeding. The two dark-staining bodies were very likely nuclei, either newly divided or about to fuse in some life-cycle stage attendant with

reproduction. Kent apparently observed individuals possessing two nuclei which he attributed to the coalescence of two individuals preceding the formation of a sporocyst (18, p. 292).

One and sometimes two vacuoles were observed situated posteriorly, figure 6f; but whether either of these were contractile, as stated by Kent (18, p. 291), was not demonstrated. At least one of these was thought to be a food vacuole because of a small dark particle frequently observed in the lumen.

Bodo lens showed a marked tendency to assume forms, structures, and numbers of flagella that could be considered neither typical nor normal. Kent observed this.

> Sometimes the normal spheroidal or ovate contour is retained for a considerable interval, but more usually the peripheral wall appears to lose its customary more firm consistence, and the whole body-sarcode becomes projected in various directions, after the manner of ragged and irregularly developed pseudopodia. (18, p. 292)

He felt that these irregular forms were due to a lack of oxygen and stated that when the oxygen supply was replenished, the organisms regained their typical forms (18, p. 292).

The greatest irregularities in the form of Bodo lens were observed here in cultures grown in a medium

using sea water of a salinity of 27 rather than the 17 parts per thousand of the cultures from which the innoculations were made. In these cultures some of the Bodo lens assumed an amoeboid form, figure 6g, and others a carrot-like shape, figure 6h. Filar pseudopodia were observed on many of the Bodo lens. Several filar pseudopodia might be present, but usually there was only a trailing one, figure 6i. The amoeboid shapes were at times formed by cytoplasm passing into and expanding the filar pseudopodia. Bodo lens, under these conditions, was observed with numbers of flagella as high as eight. This was probably caused by the higher salinity not being suited to the normal cell division and reproduction of organisms originally reared at a lower salinity. The change of salinity was also the most likely cause of the other irregularities.

## Descriptions of Associated Protozoa

### Protozoa A

Three protozoa in addition to Bodo lens were of immediate concern to the investigation. Since it was impossible to devote the time necessary to definitely identify these organisms, for purposes of discussions included here they were designated as Protozoa A,

Protozoa B, and Protozoa C.

Protozoa A is shown in figure 7a. It was tentatively identified as Oxyrrhis marina Dujardin, though it was not studied with sufficient care to make this determination definite. This species was considered by Kofoid and Swezy to be in the family Protodiniferidae of the order Dinoflagellata (20, pp. 117-119). Protozoa A was tentatively placed here mainly on the basis of its subovoidal body form and asymetrical posterior recess. The body length was about 25 mu. Only a trailing flagellum was observed. An irregular, centrally-located nucleus was stained when methylene blue was used as an intra-vitam stain. There were several small vacuoles anteriorly.

## Protozoa B

Protozoa B is shown in figure 7b. It is a ciliate which was placed in the order Holotricha and the suborder Hymenostomata. It was not studied sufficiently for further identification. Protozoa B usually measured 13 by 28 mu. The cilia were rather long and there was a single, long caudal cilium. The nucleus was located near the anterior end; the cytostome was near the middle. There was one contractile vacuole in a posterior position.

## Protozoa C

Protozoa C is shown in figure 7c. This ciliate was placed in the order Spirotricha and the suborder Hypotricha but was identified no further. It measured 50 to 80 mu in length. Protozoa C had a prominent adoral zone. Only a single row of marginal cirri was observed. There were about six anal cirri. Other identifying cirri were not observed, probably due to insufficient study.

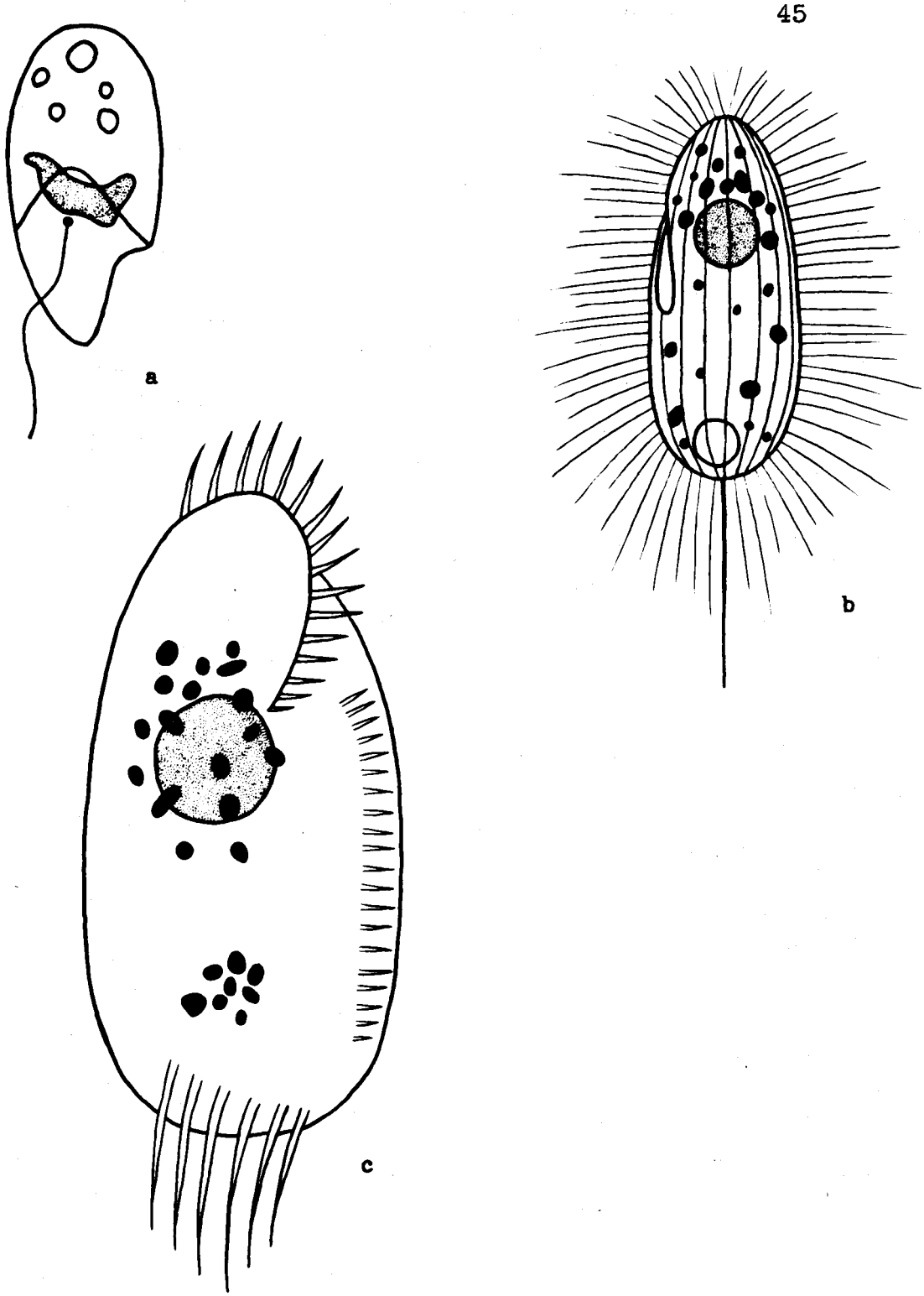


Figure 7. Associated Protozoa x 2000. a, Protozoa A; b, Protozoa B; c, Protozoa C.

# V. SELECTION OF IDEAL MEDIUM FOR CULTURING *BODO LENS* FOR OYSTER LARVAE FOOD

## Requirements of Medium Used for Culturing *Bodo lens* for Oyster Larvae Food

A good medium for culturing *Bodo lens* for oyster larvae food, it was thought, must satisfy two basic requirements. The first of these was self-evident; the culture medium selected must furnish a good growth medium for *Bodo lens*. The second requirement was that the medium, which would be added daily to the rearing crocks, be not harmful to the oyster larvae. Nitrogenous wastes, resulting from the eventual breakdown of nitrates in the medium, were considered as the products most apt to be harmful to the larvae. Thus, the desired medium should produce good cultures of *Bodo lens* and be low in nitrates.

Medium #1, table I, had been used earlier in successfully rearing oyster larvae and contained relatively small quantities of nutrients including nitrate. However, the work of Imai and Hatanaka suggested that even smaller quantities of nutrients than were being used in medium #1 would suffice (17). These workers determined the cultural requirements of a species of *Monas*; and, as *Monas* and *Bodo lens* are in closely related families having similar forms of nutrition, it was believed that the

TABLE II

Growth of Bodo lens in Medium #1 and Medium #2
(Recorded as Number per Cubic Centimeter)

| Day | Medium #1 | | | Medium #2 | | |
|---|---|---|---|---|---|---|
| | Culture 1 | Culture 2 | Mean | Culture 1 | Culture 2 | Mean |
| 1 | 100,000 | 450,000 | 275,000 | 400,000 | 300,000 | 350,000 |
| 2 | 500,000 | 1,400,000 | 950,000 | 550,000 | 400,000 | 475,000 |
| 3 | 3,170,000 | 2,700,000 | 2,935,000 | 1,650,000 | 2,200,000 | 1,925,000 |
| 4 | 2,450,000 | 3,000,000 | 2,725,000 | 1,650,000 | 1,950,000 | 1,800,000 |
| 5 | 3,150,000 | 2,600,000 | 2,875,000 | 1,650,000 | 1,350,000 | 1,500,000 |
| 6 | 2,500,000 | 2,300,000 | 2,400,000 | 1,600,000 | 1,300,000 | 1,450,000 |
| 7 | 1,600,000 | 1,850,000 | 1,725,000 | 2,100,000 | 1,350,000 | 1,725,000 |
| 8 | 2,150,000 | 1,600,000 | 1,875,000 | 1,450,000 | 1,100,000 | 1,275,000 |
| 9 | 1,500,000 | 1,150,000 | 1,325,000 | 1,100,000 | 1,100,000 | 1,100,000 |

cultural requirements of these two organisms would be similar. The composition of medium #2, table I, was thus based on the requirements of Monas.

Imai and Hatanaka summarized the optimum conditions for the culturing of Monas as follows:

> salinity, about 30-50% sea water
> glucose, about 100 mg/liter
> potassium nitrate, 40-400 mg/liter
> sodium phosphate, 5-50 mg/liter
> calcium chloride, 5-50 mg/liter
> temperature of incubation, 15-20 degrees centigrade. (17)

The salinity of the sea water used in medium #2 was thus the same as in medium #1. The starch and potassium nitrate in medium #2 were reduced to the minimums, a small fraction of what they were in medium #1. The maximum quantities of sodium phosphate and calcium chloride were used as there was no apparent reason to fear the end-products of these two nutrients. The problem was then to determine whether medium #2, with its greatly reduced capacity for producing toxic end-products, would serve as well as medium #1 for culturing Bodo lens.

## Growth of Bodo lens in Medium #1 and Medium #2

The growth of Bodo lens in medium #1 and medium #2 was determined from four cultures. These cultures were made in 500 cc erlenmeyer flasks; two of the flasks

receiving 250 cc each of medium #1, while the other two each received 250 cc of medium #2. Each of the four cultures was then innoculated with 4 cc of a stock culture, and the flasks were stoppered with cotton. The temperature of the cultures during the nine days of incubation ranged from 18.0 to 23.0 degrees centigrade.

Counts to determine the number of Bodo lens per cubic centimeter in each of the four cultures were made once a day, and the results are presented in table II and figure 8. The mean for the two cultures made with medium #1 and the mean for the two cultures made with medium #2 were calculated for each day, and these means are the values discussed here and graphed in figure 8. The numbers of Bodo lens per cubic centimeter in the individual cultures may be obtained from table II.

The numbers of Bodo lens per cubic centimeter in both medium #1 and medium #2 increased rapidly, the means for both media reaching a peak on the third day. The peak of 2,935,000 reached in medium #1 was higher than the 1,925,000 reached in medium #2. From these peaks there was a general decline in both media, though a secondary peak of the numbers in medium #1 occurred on the fifth day. Medium #2 had a secondary peak on the seventh day. By the ninth day, the number of Bodo lens in medium #1 had declined to 1,325,000 while in medium #2 it had declined to

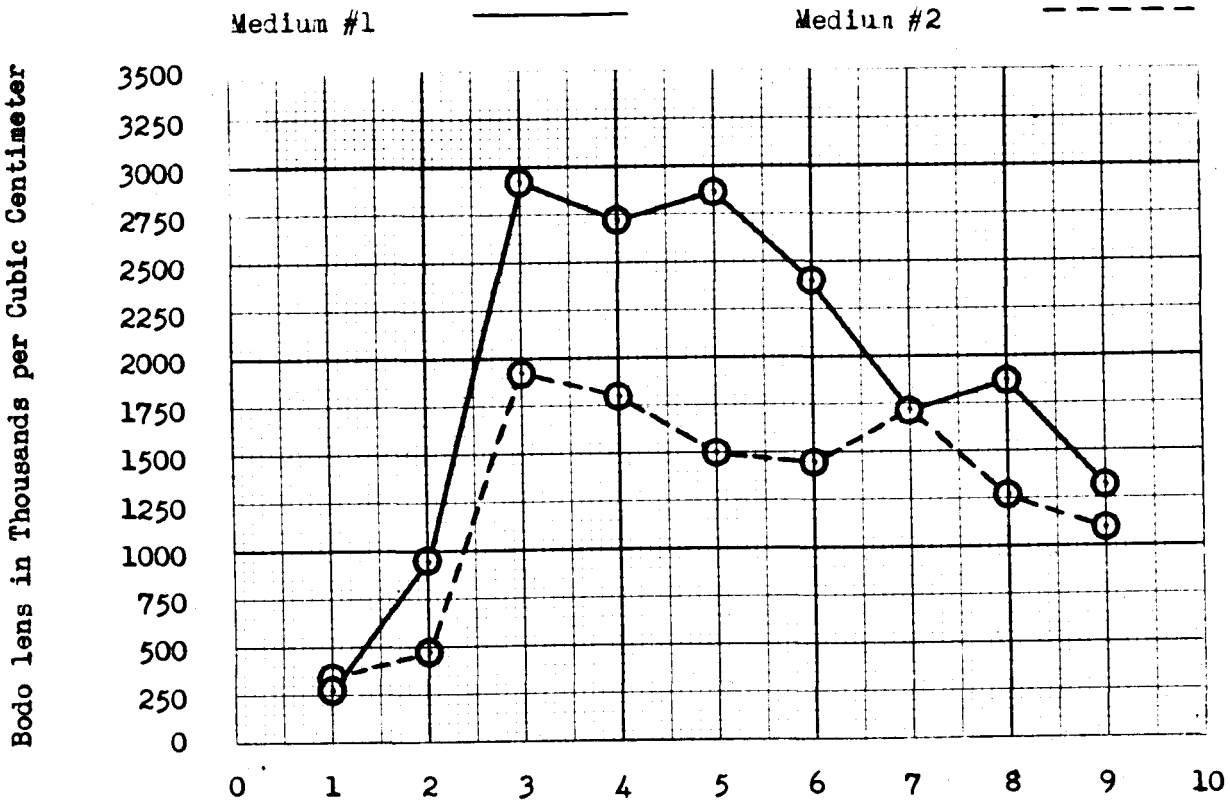


Figure 8. Growth of Bodo lens in Medium #1 and Medium #2

1,100,000 per cubic centimeter. Bodo lens maintained a higher concentration in medium #1 than in medium #2 throughout the nine days except for the first and seventh days when the concentrations were about equal.

From these results it was determined that even though higher concentrations of Bodo lens could be obtained in medium #1, medium #2 was more suitable for making cultures for feeding oyster larvae. While a concentration of nearly 3,000,000 Bodo lens per cubic centimeter was reached, medium #1, nearly 2,000,000 was reached in medium #2. Yet, medium #2 contained only one-fifteenth as much starch and less than one-seventh as much potassium nitrate as medium #1. Thus, four times as much medium #2 culture as medium #1 culture could be used in feeding the larvae, and still be adding only about one-third as much starch and one-half as much potassium nitrate to the rearing crocks. In this way medium #2 made it possible to feed the oyster larvae larger numbers of Bodo lens while using smaller amounts of nutrients having potentially dangerous end-products.

# VI. INGESTION AND ASSIMILATION OF BODO LENS BY NATIVE OYSTER LARVAE

## Ability of Native Oyster Larvae to Ingest Bodo Lens

The ability of oyster larvae to ingest certain food organisms is obviously dependent on the anatomy of the larvae and their manner of feeding, and these will be considered first. Yonge has made an excellent study of the anatomy of the digestive tract and the manner of feeding of larvae of Ostrea edulis, and figure 9 showing these is taken from his work (35, p. 317). Larvae of the native oyster, Ostrea lurida, are very similar to those of Ostrea edulis, and work done by Hori (15, pp. 272-274) and studies made here leave no reason to suspect the two species of larvae differ appreciably either in anatomy or in manner of feeding.

The velum by means of which the larvae are able to swim, figure 9, also aids in collecting food. Food organisms and other particles are thrown by the large cilia of the velum onto the ciliated tract and are there carried to the base of the velum. The arrows in figure 9 show the direction in which the cilia pass matter. At the base of the velum the organisms and other material are embedded in mucous which cilia force back toward the mouth. Excess mucous and material and organisms too large

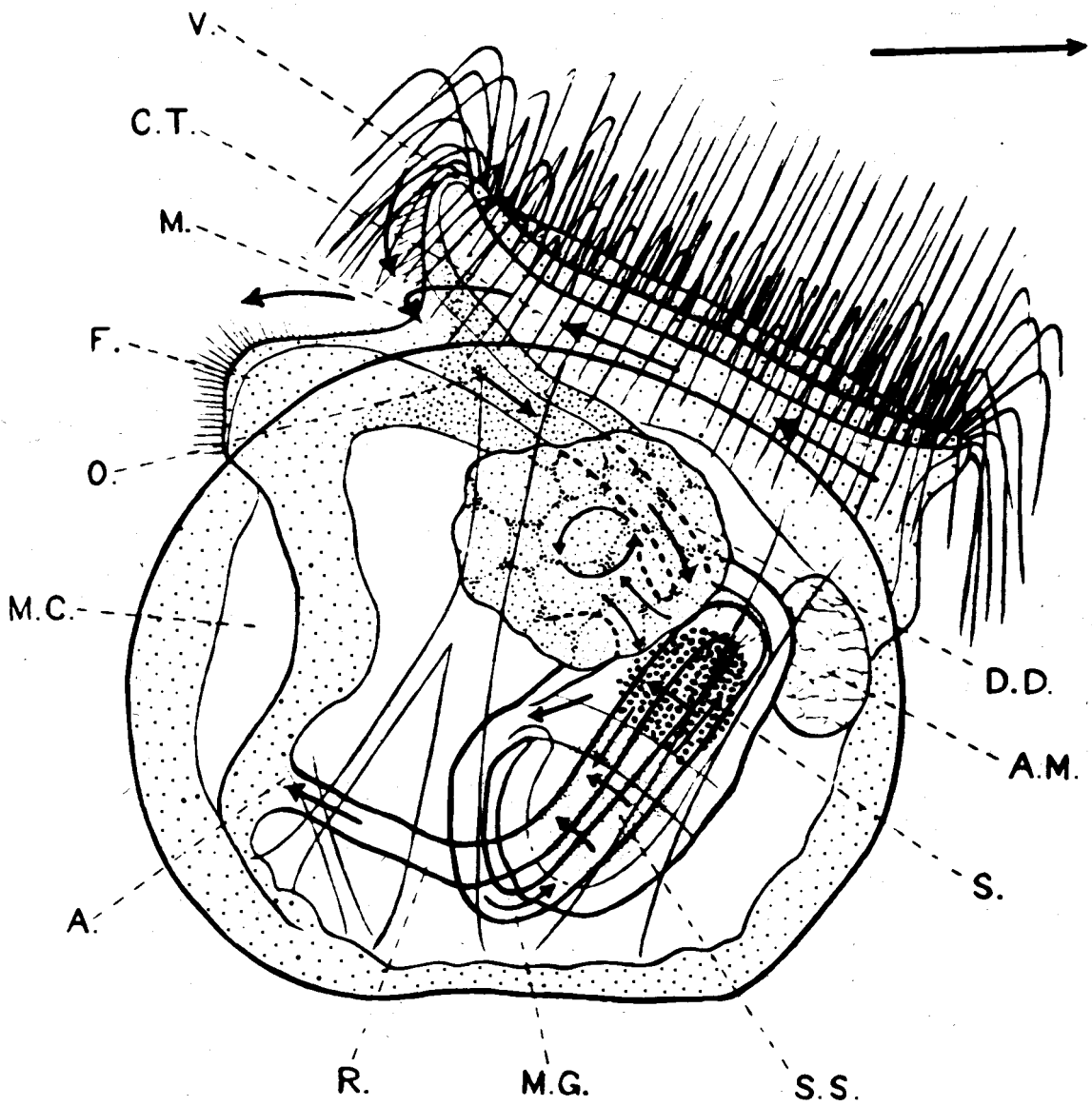


Figure 9. Anatomy of Larvae of European Oyster (Ostrea edulis) from Yonge (35, p. 317). Large arrow shows direction of larval movement; small arrows show ciliary feeding currents. A, Anus; AM, Adductor muscle; CT, Ciliated tract at base of velum; DD, Digestive diverticula; F, Foot; M, Mouth; MC, Mantle cavity; MG, Mid-gut; O, Oesophagus; R, Rectum; S, Stomach; SS, Style-sac with contained style; V, Velum.

to enter the mouth are passed off by the cilia present on the foot. (35, p. 337) The material which enters the mouth reaches the stomach through the oesophagus and eventually goes through the midgut by means of cilia which are present in all of these regions, though they are shown only in the anterior end of the stomach, figure 9. In a larvae at the shown stage of development, the external measurement of the oesophagus was 20 mu, while the stomach was 46 mu and the midgut was 12 mu. (35, p. 318)

Mucous, food organisms, and other material are constantly rotated in the stomach by cilia at a rate varying from 30 to 90 revolutions per minute. Some of this material is constantly being thrown into the digestive diverticula where it is also rotated. (35, p. 338) It is in the cells of the walls of the digestive diverticula that the assimilation of the products of extra-cellular digestion takes place, and where intra-cellular digestion occurs (35, pp. 353-354).

Several investigators have expressed opinions on the maximum size of food organisms that the larvae of Ostrea edulis are able to ingest. Their work seems somewhat inconclusive, and no work of this nature for the native oyster, Ostrea lurida, was found. In spite of the inconclusiveness of the published work, the maximum size of organism utilizable by the larvae of Ostrea edulis as

expressed by some of these workers probably offers a reasonable upper limit of food organism size to use in larval food studies. And, as larvae of Ostrea edulis and Ostrea lurida are similar in size and anatomy, such an upper limit should serve for work with larvae of the latter species until additional information is available.

Sparck observed that the larvae of Ostrea edulis were unable to ingest the diatom Nitzschia minutissima and came to the conclusion food organisms for these larvae must be in the size category of 2 to 3 mu (31, p. 51). Since this diatom is of a rigid nature and its various varieties measure 5 mu in width and from 20 to 106 mu in length, a maximum size limit of ingestion based solely on the width of such a long organism does not seem valid. It is not as though the organism measured 5 by 5 mu, and the larvae were unable to accomplish its ingestion. According to Korringa, Hagmier considered these larvae as being unable to ingest food particles larger than 8 to 10 mu (21, p. 105). Lewis, in search for seaweeds whose spores would be suitable as food for larvae of Ostrea edulis, worked on the assumption the larvae could ingest nothing larger than 10 mu (23, p. 615). Bruce and his co-workers, while rearing larvae of Ostrea edulis on algae, selected 10 mu as the upper size limit of organisms the larvae could ingest (3, p.348). A size of 10 mu is not likely to

be too great for potential larval food organisms, if their shape and pellicle are suitable.

The feeding of native oyster larvae was studied using intra-vitam stained protozoa during the investigation conducted here. The feeding of these larvae was observed under the low and high-power objectives of a microscope, this rendering the protozoa, the feeding, and the digestive tract of the larvae readily visible. Protozoa caught in the current created by the large cilia on the velum were carried into the mass of mucous around the velum as described earlier for Ostrea edulis. Once entangled in the mucous, the protozoa were usually unable to escape, and some of them were thus forced into the mouth with the mucous along with the other entangled particles.

The Bodo lens were readily entangled in the mucous, once caught in the current created by the cilia, and usually assumed a spheroidal shape. Some of the Bodo lens seemed to be broken up after being caught in the mucous. This was no doubt due to the fragile nature of this protozoa and to the manner in which the larvae "worked" the mucous mass collected in the mouth region. When this mass became large, the larvae would seemingly exert pressure on it by working the foot against the velum base and by opening and closing the shells. This may have been an attempt by the larvae to rid itself of excess

mucous or an attempt to force some of the material into the mouth. This material was eventually visible in the stomach where it was constantly rotated. No intact Bodo lens were observed in the stomach though their particles must unavoidably have been carried there along with the mucous. Stained particles thought to be nuclei of Bodo lens were observed in the stomach. It seems probable that the Bodo lens not broken up when entangled in the mucous, were broken in passing through the mouth and oesophagus into the stomach.

Cultures of Protozoa B were intra-vitam stained and the feeding of the larvae was observed when this organism was present. When this medium-sized ciliate was entangled in the mucous, it assumed a spheroidal shape as had the flagellate Bodo lens. Since this organism was not observed in the larval digestive tract, and since it did not seem to break up when entangled in the mucous, it is unlikely the larvae were able to accomplish its ingestion. Protozoa B being about 28 mu long is certainly larger than the 10 mu maximum size other workers have thought oyster larvae could ingest.

Because of its small size, and because of its extremely fragile character, Bodo lens can no doubt be ingested by larvae of the native oyster. Whether or not larger organisms can be ingested by these larvae probably

depends as much on the character of their pellicle as on their size. Small, very rigid organisms may be more difficult for the larvae to ingest than plastic, fragile, but much larger organisms. It is possible that larvae can ingest organisms larger than 10 mu when these organisms are either fragile or plastic enough for the larvae to ingest them as part of the mucous mass.

## Number of Bodo lens Ingested by Native Oyster Larvae

That some idea of the magnitude of the number of Bodo lens ingested by native oyster larvae be determined was desirable. Without at least general information on this subject, efforts to adjust the use of food cultures to the actual need of the oyster larvae were seriously handicapped. Bruce, Knight, and Parke, while rearing Ostrea edulis on an algal diet, found by checking against a control that food organisms in a rearing vessel containing larvae disappeared "at an average rate of 24,000 per larvae per day" (3, p. 353). This was the type of information needed for food studies of native oyster larvae, and an experiment was designed to determine approximately how many Bodo lens were ingested by these larvae in a day.

This experiment was designed to make it possible to compare the numbers of Bodo lens in one set of jars to the numbers in another set of jars in which there were

native oyster larvae. It was decided to add a certain amount of Bodo lens culture to each jar and then to determine the number of this protozoa present by counts. A known number of oyster larvae was added to the jars in one set. After 24 hours, counts of the Bodo lens in the jars were made again. The average number of Bodo lens in the jars having oyster larvae was compared to the average number in the jars where no larvae were present so as to determine the effect of the larvae on the number of Bodo lens.

The four jars that were used for the experiment were made of brown glass and had a capacity of about 650 cc. Sterile sea water was used and 500 cc was put into each jar. The water in the jars was aerated and gently circulated by air slowly bubbled through glass tubes. The temperature of the water during the course of the experiment ranged from 18.5 to 23.5 degrees centigrade.

A stock culture of Bodo lens made with medium #1 was used, and 5 cc of this was placed in each jar. The counting of the protozoa was done with the aid of a haemacytometer as described in section III. The oyster larvae that were used were chlorinated so as to introduce into the jars only a very minimum of other species of protozoa. The oyster larvae were counted directly, and 1,000 of them were placed in each of two of the jars.

A sample count of the larvae in these two jars was made at the conclusion of the experiment to determine if there had been any marked mortality. Twenty samples of one cubic centimeter were drawn from each of the two jars. The mean number of larvae per cubic centimeter in one jar was 1.95, while in the other jar it was 2.00. This would indicate that the number of larvae had remained constant at 1,000 per jar during the course of the experiment.

Table III gives the number of Bodo lens per cubic centimeter in each of the four jars at the beginning of the experiment and at the conclusion, 24 hours later. In one of the jars without larvae, the Bodo lens increase during this time was 10,000 per cubic centimeter, while in the other it was 48,000 per cubic centimeter. The mean increase of Bodo lens per cubic centimeter in the jars not having larvae was thus 29,000. The other two jars had two oyster larvae per cubic centimeter of sea water. In one of these the number of Bodo lens decreased 3,000 per cubic centimeter during the 24 hours. The other jar with larvae had an increase of 12,000 Bodo lens per cubic centimeter. This meant the mean increase of Bodo lens per cubic centimeter in jars having larvae was thus 24,500 per cubic centimeter less than in the jars where larvae were not present. Since there were two larvae per cubic centimeter in the jars with the lesser mean increase, the

TABLE III

Number of *Bodo lens* Ingested by Native Oyster Larvae

| Treatment | Repetition | Bodo lens per Cubic Centimeter | | | | | Treatment Difference per Larvae |
|---|---|---|---|---|---|---|---|
| | | 0 hours | 24 hours | Difference | Mean Difference | Treatment Difference | |
| No Larvae | 1 | 90,000 | 100,000 | +10,000 | | | |
| | | | | | 29,000 | | |
| | 2 | 55,000 | 103,000 | +48,000 | | | |
| | | | | | | 24,500 | 12,250 |
| Two Larvae per cc | 1 | 80,000 | 77,000 | - 3,000 | | | |
| | | | | | 4,500 | | |
| | 2 | 75,000 | 87,000 | +12,000 | | | |

difference in increase was 12,250 Bodo lens per larvae. If the smaller number of Bodo lens when larvae were present is considered due to the ingesting of the Bodo lens by the larvae, then a single native oyster larvae may ingest 12,250 Bodo lens in 24 hours.

The number of Bodo lens ingested by a native oyster larvae arrived at here should be considered as no more than a tentative estimate. As can be seen from the figures through which this number was obtained, the variation was great and many more repetitions would be necessary before the experimental results would be reliable. Even if the results were reliable numerically, the lower numbers of Bodo lens might have in part been due to a decrease in this protozoa's bacterial food supply caused by the larvae. The number of Bodo lens ingested by the larvae might depend on the concentration of this protozoa, and the high concentrations used for the experiment could introduce a third possible error. These potential errors could have been removed by more elaborate experimentation than was feasible at the time. Though the results obtained were liable to include error, it was thought they might furnish an estimate that could be used in further food studies. The 12,250 Bodo lens per larvae per day determined here and the 24,000 food organisms per larvae per day determined by Bruce and his co-workers are

certainly of the same magnitude.

## Ability of Native Oyster Larvae to Assimilate Bodo lens

The ability of oyster larvae to assimilate a food organism must be considered, even though it has been determined the larvae are able to ingest this organism. Certain organisms may have a cell wall that the enzymes of the larvae are unable to break down or penetrate. There is, for instance, some doubt whether the green algae Chlorella pacifica can be digested by oyster larvae; though it can be readily ingested (21, p. 105). There seems to be little reason to suspect, however, that native oyster larvae cannot assimilate Bodo lens. The delicacy of this protozoa and its lack of a definite pellicle would render it quite susceptible to digestive action in the larvae. Imai and Hatanaka were unable to observe intact specimens of Monas in the gut of oyster larvae due to the rapidity with which this organism broke down (17), and Bodo lens is structurally similar to the members of the genus Monas. These workers considered Monas a valuable larval food because it converted nutrients present in bacteria to a form more readily available to the larvae (17). It was still thought desirable to demonstrate conclusively, if possible, that native oyster larvae can assimilate Bodo lens. The experimental evidence that is presented here

did not do this, but it does illustrate a method of experimentation that may be of value in future larval food studies.

The experiment was designed so as to make use of a radio-active material in determining whether certain organisms are assimilated by native oyster larvae. Two bacteria, A and B, and the protozoa Bodo lens were considered as being potentially good larval food organisms. These three organisms were cultured in a medium containing a radio-active material and were afterward fed to oyster larvae. It was hoped that whether or not the larvae were utilizing the organisms could be determined by the amount of radio activity gained by the larvae under different feeding conditions.

The experiment was designed so that one series of larvae received sterile radio-active medium in which neither bacteria nor protozoa were present. The next series of larvae were fed radio-active medium in which Bacteria A had been cultured; while the third series of larvae received similar medium in which both Bacteria A and Bacteria B were cultured. The culture which the last series of larvae was fed was made with the radio-active medium and included not only Bacteria A and Bacteria B, but also the protozoa Bodo lens. It was hoped that differences in the amount of radio activity gained by

the series of larvae fed the sterile medium and those fed the cultures would indicate whether or not the larvae were gaining the radio activity directly from the medium. It was further hoped that differences in the radio activity gained by the series of larvae fed the different cultures would indicate which of the organisms the larvae could assimilate. In order to demonstrate whether or not the larvae were showing radio activity due solely to the presence of radio-active organisms in the digestive tract, different groups of larvae were allowed to feed for different lengths of time. It was thought that if the larvae were assimilating a certain organism, their radio activity would continue to increase even after sufficient time had elapsed for the digestive tract to be filled.

The various cultures of food organisms used for the experiment were made in each case by innoculating 150 cc of medium #1 with the desired organisms. When these cultures were 7 days old, and 24 hours before the feeding experiment was to begin, 1.0 milli-curie of radio-active phosphoric acid was added to each culture. It was thought that 24 hours would allow adequate time for the organisms to utilize and incorporate enough of the radio-active material.

Sixteen 250 cc beakers were used to hold the larvae for the various treatments and repetitions of the

experiment. Two-hundred native oyster larvae were placed in 200 cc of sterile sea water in each beaker. The beakers of larvae were arranged into four equal groups; and to each of the beakers in one of these groups, 4 cc of sterile medium #1 containing radio-active phosphoric acid was added. The beakers in the other three groups each received 4 cc of the appropriate, radio-active food culture. The beakers containing the larvae were left at room temperature.

The larvae were allowed to feed for the desired length of time and were then washed free of radio-active material. This was done by first adding a little sodium hypochlorite solution to each beaker, causing the larvae to close their shells and settle to the bottom. Most of the liquid was then carefully siphoned out of the beaker and sterile sea water was added. The larvae would soon begin swimming again, further freeing themselves of the radio-active material. They were then for the second time caused to settle by adding the hypochlorite solution. The liquid was again siphoned off and replaced with sterile sea water. This washing process was repeated five times, and no radio-active material could be detected in the final wash water. The desired number of larvae were picked from the bottom of the beaker and placed in a small labeled dish. After the moisture had been removed by a

heat lamp, the larvae in the dish were ready to be placed in a radio-activity counter. The radio-activity counter which was used was a Tracer Lab, 64 Scaler, having a mica window which weighed 1.5 milligrams per square centimeter.

The radio activity of the larvae after the different periods of time and after feeding on the different food organisms is recorded as counts per minute in table IV. Total, background, and sample counts per minute are given for the measurements on the larvae from each beaker; but the sample figures are the only ones with which this discussion need be concerned. Each of the sample figures given for the group of larvae that fed for 2 hours and the group that fed for 5 hours is the average counts per minute determined from 10 minutes of counting. The figures given for the two groups of larvae that fed for 24 hours are averages determined from 20 minutes of counting. The counts for the groups of larvae feeding for 2 and 5 hours were done on 25 larvae taken from the 200 in each beaker. The different counts for the two groups of larvae feeding 24 hours were made on 50 larvae from each beaker. The results given for the 5-hour group are not dependable due to faulty technique. The results given for the 2-hour group are somewhat less dependable than those given for the 24-hour groups.

Table IV shows that the radio-activity of the

TABLE IV

Radio Activity Gained by Native
Oyster Larvae from Various Marked Food Organisms
(Recorded as counts per minute)

| Hours Larvae Allowed to Feed | Source of Radio Activity | Treatment No. / Type of Radio Active Food Culture | 1 Medium #1 Only | 2 Medium #1 Bacteria A | 3 Medium #1 Bacteria A,B | 4 Medium #1 Bacteria A,B, Bodo lens |
|---|---|---|---|---|---|---|
| 2 | Total | | 33.7 | 40.9 | 39.1 | 51.5 |
| | Background | | 34.0 | 34.0 | 31.5 | 34.0 |
| | Sample | | 0.0 | 6.9 | 7.6 | 17.5 |
| 5 | Total | | 37.0 | 106.5 | 71.0 | 47.5 |
| | Background | | 32.0 | 32.0 | 32.0 | 32.0 |
| | Sample | | 5.0 | 74.5 | 39.0 | 15.5 |
| 24 | Total | | 87.1 | 93.0 | 129.0 | 64.8 |
| | Background | | 27.0 | 27.0 | 32.0 | 32.0 |
| | Sample | | 60.1 | 66.0 | 97.0 | 32.8 |
| 24 | Total | | 34.2 | 77.1 | 120.0 | 43.4 |
| | Background | | 32.0 | 32.0 | 32.0 | 32.0 |
| | Sample | | 2.2 | 45.1 | 88.0 | 11.4 |

larvae receiving the sterile medium (Treatment 1) was in only one case high enough to be anything but negligible. The readings 0.0, 5.0, and 2.2 counts per minute would indicate that the reading of 60.1 counts per minute for Treatment 1 of the first 24-hour group of larvae is due to some error. It seems safe to assume from the results of Treatment 1 that the radio activity demonstrated in larvae receiving the other three treatments was gained from the food organisms and not directly from the radio-active medium.

The purpose of allowing the oyster larvae to feed for the different lengths of time was to demonstrate whether the radio activity present in the larvae was due to their assimilating the radio-active food organisms or due to their merely having them present in the digestive tract. If the results for the 2-hour group and the 5-hour were as reliable as those for the 24-hour groups, the results might be fairly conclusive. They are not conclusive due to the unreliability of the 5-hour group. It was thought that the larvae would fill their digestive tracts with the radio-active organisms in from 2 to 5 hours, and, if they were not assimilating these organisms, there would be no further increase in the radio activity of the larvae with the increase in time. If, however, the larvae were assimilating the organisms, the radio activity

should increase for an undetermined length of time after the digestive tracts were filled. The low level of radio activity shown in Treatment 2 and Treatment 3 of the 2-hour group as compared to the much higher level shown in the same treatments of the 24-hour groups would be strong indication the organisms were being assimilated. It is unfortunate the results for the 5-hour group were not sufficiently reliable to substantiate such a conclusion.

Assuming the larvae do not gain appreciable radio activity directly from the medium, and assuming the radio activity demonstrated in the two 24-hour groups is not due solely to organisms held in the larval digestive tracts, the results of Treatments 2 and 3 indicate the larvae were able to assimilate both Bacteria A and Bacteria B. When the 24-hour groups in table IV are considered, the increase in radio activity from Treatment 1 to Treatment 2 indicates Bacteria A can be assimilated; and the increase from Treatment 2 to Treatment 3 indicates Bacteria B can be assimilated.

The results of Treatment 4, in which not only Bacteria A and Bacteria B were present but also the protozoa Bodo lens, are very difficult to interpret. The radio activity of the larvae receiving Treatment 4 in the 24-hour groups is markedly lower than that of the larvae receiving either Treatment 2 or Treatment 3. The obvious

explanation for this decrease in radio activity would be that the larvae were unable to assimilate Bodo lens. However, the presence of many other possible causes for the decrease, and there being no adequate explanation for larval inability to assimilate Bodo lens, make the obvious explanation very improbable. If the Bodo lens could not incorporate the radio-active phosphate, or if they could incorporate only a limited amount, the decrease in larval radio activity in Treatment 4 would take place. Additional experimentation would be necessary before any conclusions could be drawn from the results of Treatment 4.

This experiment was designed in the hope of demonstrating whether or not native oyster larvae could assimilate two different bacteria, A and B, and a protozoa, Bodo lens. The evidence indicates Bacteria A and Bacteria B may be assimilated. The evidence did not demonstrate that native oyster larvae could assimilate Bodo lens. Additional experimentation might be able to do this. The results of this experiment were, however, sufficiently encouraging to demonstrate the possible value of radio-active materials in future food studies of oyster larvae.

# VII. REARING OF NATIVE OYSTER LARVAE ON DIFFERENT FOOD CULTURES

## Rearing Experiments Failing Before Completion

### First Rearing Experiment

The first native oyster larvae rearing experiment started during the summer of 1950 was designed in a manner which was hoped would furnish needed information on three basic factors. It was hoped the type of food, amount of food, and frequency of water change would result in the best larval growth and spat settlement could be determined. The experiment was designed to test three different types of food, three different amounts of food, and three different frequencies of water change. A Latin Square statistical design, which is fully explained in section VIII, was used to make it possible to obtain three repetitions of each of the nine treatments mentioned and to do this using only nine rearing crocks.

The three treatments concerned with the type of food were applied to the nine crocks of larvae by dividing the crocks into three equal groups. One of these groups was fed only bacteria cultures, another bacteria and Bodo lens cultures, and the third was fed bacteria, mixed protozoa and algae cultures. The three treatments on

amount of food consisted of the same crocks divided into a different three groups, one to receive 10 cc, another 20 cc, and the third, 30 cc of food culture each day. The last three treatments were on the frequency of water change; and here again the nine crocks were divided into still a different three groups. One group had the water changed every day, another every third day, and the last group every fifth day.

Twelve liters of sea water was placed in each of the 4-gallon, stoneware rearing crocks, and this water was kept at approximately 20 degrees centigrade by the water bath. The sand-filtered water had a salinity of 32 parts per thousand and was sterilized by boiling. It was hoped the sterilization would make it possible to keep protozoa out of the crocks of larvae being fed only bacteria, and keep species of protozoa other than Bodo lens out of the crocks of larvae being fed that species. Though the 8,000 larvae added to each crock had been chlorinated to remove protozoa, this procedure was not perfected enough to keep protozoa from appearing in all of the crocks. The food cultures were all made using medium #2.

The oyster larvae began to die on the second day and were soon present only in negligible numbers. This was not understood, though there were several factors that might possibly have had some bearing. The ammonia on the

second day was 6 parts per million, and it was thought that this large amount was due to the decomposition of the organisms killed by the boiling of the sea water. The salinity of 32 parts per thousand may have been too high. The large number of larvae used or their chlorination may have had some effect. The high ammonia reading could have been an index of conditions that resulted in the loss of larvae, but neither this nor the other possibilities presented an entirely adequate explanation. An attempt was made in the second rearing experiment, however, to remove some of these possible causes of larval loss.

## Second Rearing Experiment

The design of the second rearing experiment was altered slightly from that of the first by neither boiling the sea water nor chlorinating the oyster larvae. The water for the second experiment was candle filtered which probably removed most of the protozoa. It was hoped that by using the three types of food cultures different food conditions could be created in the various crocks even though protozoa would be present in all of them. The number of oyster larvae per crock was increased to 16,000. Other than for these modifications, the design was the same as that used in the first experiment. The three different types of food cultures were grown in medium #2;

the amounts fed were again 10, 20, and 30 cc per day; and the frequencies of water change remained every day, every third, and every fifth day. The temperature was approximately 20 degrees centigrade, and the salinity was 32 parts per thousand.

The ammonia present in the rearing crocks was negligible during this second experiment, probably due to the nearly complete removal of organic matter from the water by the candle filtration. As was expected, protozoa appeared in all of the crocks; but in no crocks were they plentiful, most likely due to the lack of organic matter, the small amounts of cultures fed, and the large number of larvae.

Most of the oyster larvae were dead on the fifth day. Two possible causes of the loss in the first experiment, nitrogenous end-products (indicated by high ammonia) and chlorination, could be eliminated with some certainty as the causes of this second loss. The numbers of protozoa were low, but it was extremely doubtful the larvae starved in such a short period of time. High salinity was again a possible cause.

The large number of oyster larvae confined in such a small volume of water was considered as conceivably creating conditions bringing about the failure. Such failure might be caused by some toxic waste given off

by the larvae or by the increased opportunity for disease or predation to take their toll. These possibilities were but theories, however, and no explanation could be offered for the loss of larvae.

## Third Rearing Experiment

The third rearing experiment followed the same basic design used in the first and the second. Most of the modifications of the second experiment were continued and a few new ones were made. The water for the third experiment was not boiled and received only sand filtration; the larvae were again not chlorinated. The salinity of the sea water, which in the previous two experiments had been 32 parts per thousand, was reduced to 25 parts per thousand. The temperature remained at about 20 degrees centigrade. The number of larvae placed in each rearing crock was reduced to only 2,000.

Because of work done on the growth of Bodo lens in medium #2 (section V), and because of work done on the number of Bodo lens ingested by native oyster larvae (section VI), in the third experiment it was possible to change the amounts of food cultures fed the larvae to amounts probably more closely approaching those actually required by the larvae. The minimum amount of food culture that should be fed was determined by considering an

oyster larvae to ingest 12,000 Bodo lens each 24 hours. The standard food cultures of Bodo lens in medium #2 were considered to have 1,500,000 of these organisms per cubic centimeter. On the basis of these figures, the minimum amount of food culture required by 2,000 oyster larvae would be 16 cc every 24 hours. The amounts of food culture given to the larvae receiving the bacteria cultures and the larvae receiving the mixed cultures were the same as the amounts determined necessary to furnish the desired numbers of Bodo lens to the larvae in the other crocks. The larvae in the three rearing crocks which were to receive the minimum amount of food culture were thus given 20 cc every day. The larvae in the next group of crocks were given 40 cc, while those in the last group were given 60 cc of food culture each day. It was hoped the optimum amount of food culture to be fed would be somewhere within this range.

Only a few larvae remained alive in the rearing crocks by the sixth day, and the third rearing experiment was stopped. This failure occurred after toxic nitrogenous wastes, chlorination of larvae, lack of food, high salinity, and crowding of larvae had all been eliminated as likely causes. Again, only unsubstantiated theories were available to explain the failure. It should be noted here that a group of larvae released from the adults the

day after this experiment was begun did not decrease in number during the experiment. This entire group of larvae was left in a single 12-gallon rearing crock without aeration, feeding, or water change, and under the most crowded conditions conceivable. Still, these larvae were doing well at the end of a period which proved fatal to larvae kept under conditions that were considered to be nearly normal. Instances such as this had occurred before and seemed to present some indication that in some cases differences in the larvae themselves rather than in the rearing conditions were the cause of success or failure. Premature release of the larvae from the adults might have caused failures; but in the rearing experiments, only larvae reaching the 175 mu size considered normal at release were used. The possibility that genetic differences, or that differences in the early development of the larvae while in the adult mantle cavity, could affect the hardiness or viability of the free-swimming larvae was worth consideration and possible future investigation.

The possibility that 4-gallon rearing crocks were too small for successful rearing of larvae was considered. Conditions were no doubt more critical in 4-gallon than in 12-gallon rearing crocks due to the greater difficulty in controlling these conditions. That the difference in crock size as such was responsible for

the failures was doubtful. Larvae from the same group used in the third experiment were reared under similar conditions in 12-gallon crocks and perished during the same period of time. On the other hand, one group of larvae was held with little loss in number for a period of 10 days in 3 liters of sea water. There was no satisfactory explanation for the failures.

Rearing Experiment Reaching Completion

The design of the fourth rearing experiment was considerably simpler than the Latin Square design used in the first three experiments. The results that were finally obtained from this fourth experiment were thus of less value than the results of any of the first three experiments might have been. Only three rearing crocks were used for the fourth experiment, and these all received the same amount of food culture and the same number of water changes. The larvae in the first crock were fed bacterial cultures, those in the second crock were fed bacteria and Bodo lens cultures, and those in the third crock received mixed cultures of bacteria, protozoa and algae.

The 12-gallon rearing crocks were used, and 5,000 oyster larvae were placed in each crock. The water in all three of the rearing crocks was changed every two

days, a change of every day being thought unnecessary and there being some doubt about changes less frequent than every two days. The salinity of the sand-filtered sea water used was kept at approximately 25 parts per thousand. The temperature was about 20 degrees centigrade. Each rearing crock of larvae received 150 cc of the appropriate food culture every 24 hours. This amount was nearly four times the minimum amount of 40 cc of food culture for 5,000 larvae as determined by the method used in the third experiment. The standard food cultures used were made with medium #2 and were used only on the third and fourth days of incubation.

During the course of the fourth rearing experiment two protozoa other than Bodo lens became sufficiently numerous to have possibly had some effect on the experiment. Protozoa C (section IV) became very numerous on the twelfth day of the experiment in the rearing crock receiving the Bodo lens food cultures. Protozoa C was not apparent in the other two crocks. This large protozoa when present in sufficient numbers would certainly compete for the available food with the larvae; and, in some instances, it might even be a predator on the larvae. Protozoa C, during an experiment being conducted in the spring of 1950, became exceedingly abundant in a rearing crock coincident with a sudden decrease in larval numbers.

Though in the earlier instance there was reason to suspect predation, it was unlikely appreciable predation occurred during the fourth rearing experiment.

Protozoa A (section IV) appeared in large numbers on the twenty-second day of the experiment in the rearing crock receiving the Bodo lens food cultures. It appeared in greater numbers than had any other protozoa including Bodo lens, which was being added to the rearing crock daily. Protozoa A deserved consideration because it no doubt competed with the larvae for food; but more important, its increase in numbers presented a basic problem in protozoa culture, and hence, a problem in oyster culture. This increase in numbers might indicate it was more effective to create conditions exactly suited to the growth of the desired form than to add the desired food cultures each day. This problem became the basis of the study which is included in section VIII.

The native oyster larvae that were placed in the rearing crocks for the fourth rearing experiment were considered to have been released from the adults at a normal size. These larvae were in the straight-hinge stage and measured 179.5 mu parallel to the hinge. The native oyster larvae used by Hori measured 178 mu (15, p. 273) while Davis found newly released native oyster larvae usually measured from 169 to 185 mu (8, p. 591).

Figure 10a is a photomicrograph of native oyster larvae in the straight-hinge stage.

The average sizes of samples of larvae taken from the three rearing crocks at intervals are given in table V. Due to the inadequate number of larvae measured on each occasion, apparent differences in the sizes of larvae receiving different food cultures are not reliable; and only a general trend of growth may be determined. There was a steady increase of size of larvae receiving all three types of food cultures for the first twelve days. The fact that the larvae averaged larger first in one rearing crock and then in another during this period was an indication of nothing except the inadequate size of the sample measured. During these first twelve days, the larvae probably grew between 3 and 4 mu a day in all three rearing crocks.

The average measurements included in table V had a tendency to level off and even decrease after the twelfth day. This did not mean that the growth rate of the larvae had changed; but that between the twelfth and the seventeenth day the larger larvae began settling out and becoming spat. These larger larvae were thus not measured and the average size remained nearly the same or decreased. The native oyster larvae with the prominent umbos shown in figure 10b would have soon settled out and

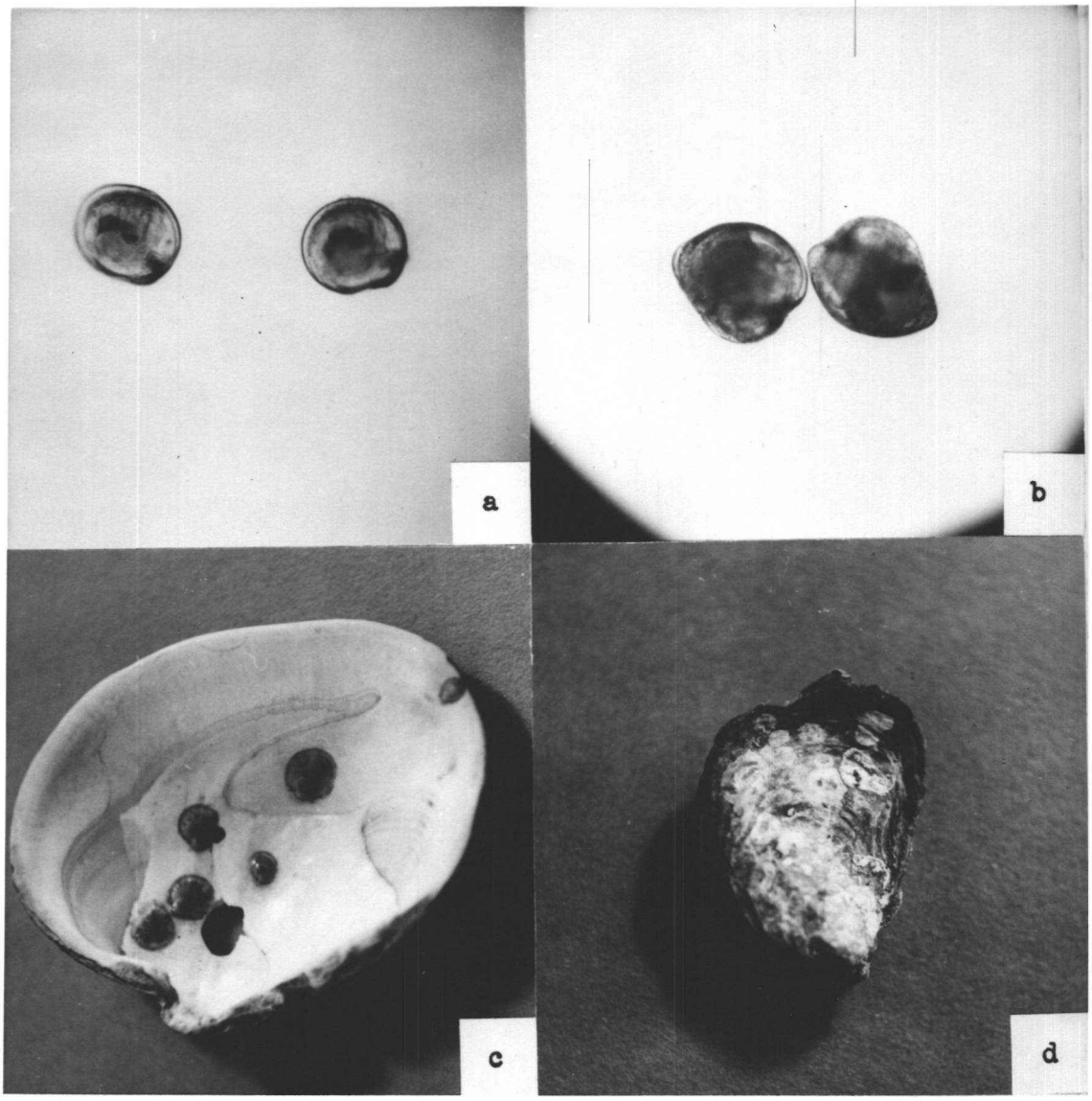


Figure 10. Native Pacific Coast Oyster (Ostrea lurida). a, Larvae in straight-hinge stage x 80; b, Larvae with prominent umbos x 80; c, Oysters in spat stage x 0.8; d, Adult oyster x 0.8. (photographs by E. P. Haydu)

become spat.

Spat settlements no doubt began before the seventeenth day on the sides of the rearing crocks, but cultch was not put in until the nineteenth day. Spat were observed on this cultch on the twenty-first day. One of these spat measured 233 mu. The largest free-swimming larvae that was measured in any of the rearing crocks was 246 mu in length. This probably meant that the larvae settled out and became spat when they reached sizes from 233 to 246 mu. This is considerably smaller than the 300 mu at which Davis found native oyster larvae to metamorphose (8, p. 591) and the 322 mu found by Hori (15, p. 273). However, spat settlement beginning between the twelfth and the seventeenth day and carrying through the twenty-second day is very much in line with what these workers found (15, p. 273) and (8, p. 591). Figure 10c shows spat that were several months old, and figure 10d shows an adult Native Pacific Coast Oyster.

The fourth rearing experiment, it was hoped, would furnish information as to which of the three types of food culture used was most suited for rearing oyster larvae. It did not do this, first of all, because the size of the samples of larvae measured was too small to detect slight differences in average growth. However, even if the sample size had been large enough to detect small

TABLE V

Average Size of Native Oyster Larvae in Rearing Crocks Receiving Different Food Cultures (Recorded in microns)

| Day | Food Cultures Used | | |
|---|---|---|---|
| | Bacteria | Bodo lens | Bact-Protozoa-Algae |
| 0 | 179.5 | 179.5 | 179.5 |
| 6 | 198.9 | 193.6 | 202.4 |
| 12 | 209.4 | 228.8 | 223.5 |
| 17 | 221.8 | 202.4 | 225.3 |
| 22 | 220.0 | 216.5 | 221.8 |

differences, it is quite unlikely any difference in growth between the three groups of larvae would have been found. The reason for this was that using three different types of food cultures apparently did not create different food conditions in the three rearing crocks.

Table VI shows the relative numbers of Bodo lens and of protozoa greater than 10 mu present in the rearing crocks on the twenty-second day of the experiment. These relative numbers were obtained in the same manner as were those which are used in section VIII except that here five rather than seven slides were used for each count. Whether cultures of bacteria, Bodo lens, or mixed organisms were added to the rearing crocks, the numbers of Bodo lens

TABLE VI

Relative Numbers of Protozoa Present in Crocks at Conclusion of Rearing Experiment

| Protozoa Present | Food Cultures Used | | |
|---|---|---|---|
| | Bacteria | Bodo lens | Bact-Protozoa-Algae |
| Bodo lens | 0.0 | 0.2 | 0.2 |
| Protozoa greater than 10 mu | 0.8 | 2.6 | 1.0 |

present were equally low (there being no practical difference between the figure 0 in the crock receiving only bacteria and the figure 0.2 in the other two crocks). It should be noted that in all three instances the protozoa greater than 10 mu were more plentiful than the Bodo lens. These larger protozoa may have been partially responsible for the low numbers of Bodo lens while they themselves were less usable by the larvae for food.

This presented a basic problem to the successful artificial rearing of oyster larvae. It seemed likely that certain food conditions would be most suitable for the larvae. Yet, if the food conditions in the rearing crocks could not be controlled by feeding certain food cultures, how were the desired conditions to be obtained? A study of the factors affecting protozoa production under

artificial conditions is included in section VIII.

Finally, why was the fourth attempt to rear native oyster larvae successful when the first three attempts during the summer of 1950 failed? Why, for that matter, had the larvae been reared successfully during the summer of 1949 and again the following winter when attempts the next summer were to fail? Many possible causes and factors have been presented and discussed. Very likely no two failures were caused by the same set of factors. This may be the reason no satisfactory explanation can be given for the many failures, or, for the successes.

# VIII. PRODUCTION OF *BODO LENS* IN CROCKS HAVING VARIED CONDITIONS

## Need of Information on Factors Affecting Production of *Bodo lens*

Once a food organism has been found that oyster larvae are able to ingest, assimilate, and grow on, it is then necessary to learn how to make that organism available to the larvae in large numbers; or it will be of no practical value to artificial larval rearing. If *Bodo lens* were considered to meet the first three requirements on the basis of work done here, it was then necessary to determine the factors affecting its abundance. When the other problems hindering the artificial rearing of oyster larvae have been solved, the most successful results will be obtained when the greatest number of food organisms are furnished under suitable conditions for the larvae.

The addition of cultures of desired protozoa to the rearing crocks had, with some reason, been considered a satisfactory means of furnishing oyster larvae suitable food organisms in some of the earlier rearing successes here. The rearing experiments described in section VII, however, indicated that the addition of cultures of *Bodo lens* as compared to the additions of bacteria or mixed cultures caused no appreciably higher numbers of *Bodo lens*,

in the rearing crocks. On the other hand, Protozoa A became extremely abundant in one of these crocks though no effort was made to encourage its growth. If the addition of the desired organisms would not assure their presence in large numbers, would the ideal conditions for the growth of these organisms do this; and if so, what were these ideal conditions?

The following two experiments were designed to furnish information on the factors determining the abundance of Bodo lens under conditions that would be suitable for the rearing of native oyster larvae. The first experiment was concerned with salinity, the effect of adding various amounts of Bodo lens culture, and the effect of using water having received different degrees of filtration. Filtration, in the final analysis, made possible a study of predatory relationships between protozoa. The second experiment tested different temperatures, no enrichment as compared to enrichment, and filtration again.

These two experiments furnished some indication of the relative importance of these various factors, but the experiments were inadequate for reasons that are discussed later. Experiments similar to these will be necessary before food production in the artificial rearing of oyster larvae is put on a controlled basis.

Improvements on the design of these experiments are suggested in the following discussion.

## Latin Square Statistical Design

A Latin Square statistical design was used for the two experiments on the production of *Bodo lens* in crocks having varied conditions; and what this design is, its applications, its limitations, and a design that might be more suited for future experiments of this type will be discussed here.

The Latin Square statistical design applies analysis of variance to a group of observations having three variables of classification. Analysis of variance is a method of testing two or more series of observations in order to determine whether these observations come from the same or different populations. In other words, it is a method of testing to find if the sample means of these series of observations come from populations which have equal or unequal means. This is done by making two different estimates of variance from these observations. The first of these estimates includes not only the variance of the observations, but the variance of the sample means of the different series. The second estimate includes only the variance of the observations. If the sample means of the different series are equal, then their

variance is equal to zero; and the two estimates should be no more different than would be expected from sample distribution. To find if the first estimate is no more larger than the second than sample distribution would cause, the "F" test is used. If the first estimate divided by the second estimate is nearly equal to one, the means may be assumed to be equal. If this dividend is larger than a selected level of the F distribution, variance due to the means must be included in the first estimate, and the means may be assumed to be unequal. Analysis of variance may be applied to many different statistical designs including the Latin Square.

Latin Square is a statistical design having three variables of classification. A single variable of classification is a single kind of factor that may be tested at different levels, such as testing several different salinities. Latin Square makes it possible to test three variables of classification such as different levels of salinity, different levels of food, and different levels of filtration at the same time. This was why the Latin Square design was thought to be particularly suited to a preliminary study of the factors affecting the production of Bodo lens. This design made it possible to test different levels of three different factors in a single experiment. Where facilities were limited, and

where the factors potentially affecting Bodo lens were many, this was a considerable advantage.

Certain assumptions are necessary before any statistical design can be applied to a problem, and this is the case with Latin Square. Of the five assumptions necessary before Latin Square can be applied to a problem, only one would be apt to cause the results to be misleading if it were not satisfied. Interaction must not be present between the factors if the Latin Square design is used. Interaction may be defined as being present if the effect of two factors applied simultaneously is not equal to the sum of their effects when they are applied separately. In an ecological problem such as this one, interaction might be expected. This is the weakest part of the design as it was used here, and an improvement to correct this will be suggested.

A second weakness showed itself during the course of these experiments. This was not so much in the design as in too small a sample size. Large differences in the numbers of Bodo lens obtained under different conditions could not be declared significant because of the low number of degrees of freedom due to the three repetitions of each treatment (sample size only 3). In future work of this type, a much larger sample size must be used so as to increase the degrees of freedom, making the obtaining of

significant results possible.

A "2 x 2 x 2" Factorial Experiment having three repetitions would make it possible to determine if and where interaction occurs. It would also increase the sample size from 3 to 12 and the degrees of freedom from 2 to 11. This experimental design would eliminate the weakness of the possibility of interaction being present, and it would increase the degrees of freedom to a level where significant results should be obtained. It would not be necessary to do all three repetitions at the same time, thus making limited facilities no serious handicap to the use of this design. Additional information on the Factorial Experiment can be obtained from the excellent work on experimental designs by Cochran and Cox (5, pp. 122-153).

## First Experiment

The first experiment was designed to determine the effect of different levels of salinity, culture addition, and water filtration on the production of *Bodo lens* under conditions suitable for the rearing of oyster larvae. Nine, 4-gallon stoneware crocks were used, and 12 liters of sea water of the desired salinity was placed in each crock. This sea water was aerated and gently circulated by air bubbles introduced through glass tubes. The

TABLE VII

Observations for First Experiment on Production of *Bodo lens* in Crocks having Varied Conditions

(Recorded as relative numbers of *Bodo lens*; observations in parenthesis other protozoa, greater than 10 mu)

| Culture Amount | Salinity in Parts per Thousand 17 | 25 | 33 |
|---|---|---|---|
| | | *One Day* | |
| 60 cc | P 22 ( 0 ) | S 4 ( 1 ) | N 8 (1.9) |
| 120 cc | S 12 (1.6) | N 7 ( 3 ) | P 125 ( 0 ) |
| 180 cc | N 13 ( 1 ) | P 87 ( 0 ) | S 21 (1.1) |
| | | *Two Days* | |
| 60 cc | P 26 ( 0 ) | S 7 (1.4) | N 12 (5.1) |
| 120 cc | S 23 (2.9) | N 13 (4.3) | P 79 ( 0 ) |
| 180 cc | N 16 (1.3) | P 41 ( 0 ) | S 17 (0.7) |
| | | *Six Days* | |
| 60 cc | P 9 ( 1 ) | S 4 (3.7) | N 2 (1.3) |
| 120 cc | S 5 (3.3) | N 1 (0.9) | P 6 ( 0 ) |
| 180 cc | N 7 (1.3) | P 2 (0.4) | S 6 (0.4) |

Filtration: P, porcelain; S, sand; N, none.

temperature of all the crocks was maintained at 20.0 degrees centigrade throughout the experiment by equipment described in section III, except for a drop in temperature to 15.6 degrees on the sixth day due to equipment failure.

The nine crocks were arranged in a manner similar to the way in which the observations are presented for the various days of the experiment in table VII. The nine crocks were divided into three groups of three each, illustrated by the columns in table VII, for the various levels of salinity tested. The nine crocks were divided into a different three groups of three each, illustrated by the rows in table VII, for the various amounts of Bodo lens culture tested. These same crocks were divided into still another three groups of three each, illustrated by the position of P's, S's, and N's in table VII, for the various kinds of filtration used. Thus, no two crocks received the same treatments, but groups of three crocks each could be selected which varied only in salinity, or only in culture amount, or only in filtration. This made it possible to test the effect of the various levels of each of these factors. This is the manner in which the Latin Square statistical design in its simplest form was used on these two experiments.

Salinity was thought to be a potentially important factor in protozoa production; and salinities of 17,

25, and 33 parts per thousand were tested. The amount of food culture of Bodo lens added to the crocks each day was thought to be apt to affect the numbers of protozoa in the crocks; and the crocks in one group received 60 cc of culture each, while the crocks in the other two groups each received 120 cc and 180 cc of culture every day. The amount of filtration of sea water used in the crocks received was considered a factor which might cause differences in protozoa production. Such differences might be due to the removal of organic matter with thorough filtration or due to the removal of forms apt to compete with or prey upon the Bodo lens. Three levels of filtration were used. One group of crocks received water which was filtered with a porcelain candle, and observations from crocks receiving this treatment are preceded with a P in table VII. The water in a second group of crocks was filtered with sand, and observations from these crocks are preceded with an S. In the third group of crocks, water which had received no filtration was used, and these observations are preceded with an N.

The relative numbers of Bodo lens present in the various crocks under the different conditions were determined in a manner described in section III. Since the rearing of oyster larvae requires from two to three weeks, what occurred in the crocks over a period of about

TABLE VIII

Statistics for First Experiment on Production of *Bodo lens* in Crocks having Varied Conditions

| Levels and Variables | One Day | Two Days | Six Days |
|---|---|---|---|
| **Levels** | **Sample Means as Relative Numbers of *Bodo lens*** | | |
| Salinity | | | |
| 17 ppm | 15.67 | 21.67 | 7.00 |
| 25 ppm | 32.67 | 20.33 | 2.33 |
| 33 ppm | 51.33 | 36.00 | 4.67 |
| Culture Amount | | | |
| 60 cc | 11.33 | 15.00 | 5.00 |
| 120 cc | 48.00 | 38.33 | 4.00 |
| 180 cc | 40.33 | 24.67 | 5.00 |
| Filtration | | | |
| Porcelain | 78.00 | 48.67 | 5.67 |
| Sand | 12.33 | 15.67 | 5.00 |
| None | 9.33 | 13.67 | 3.33 |
| **Variables** | **Calculated F Values with 2 and 2 Degrees of Freedom** | | |
| Salinity | 1.33 | 1.28 | 2.58 |
| Culture Amount | 1.56 | 2.33 | 0.16 |
| Filtration | 6.29 | 6.54 | 0.68 |

a week was important. Accordingly, determinations on protozoa numbers were made at the end of the first day, the second day, and the sixth day. Since predatory relationships between the larger protozoa and Bodo lens were thought of possible importance, the relative numbers of protozoa greater than 10 mu were determined; and these are recorded in parenthesis after the numbers of Bodo lens in table VII.

The statistics for the first experiment on Bodo lens production are given in table VIII. The sample means are given for the nine different treatments and the calculated F values (with 2 and 2 degrees of freedom) are given for the three different variables. These statistics were calculated and are given for the first day, second day, and sixth day, table VIII. The calculated F values for salinity and culture amount for the first, second, and sixth days are close enough to one to make it safe to accept the hypothesis that all of the sample means come from populations having equal means; or, in other words, there are no significant differences in the sample means for the various salinities or for the various amounts of culture tested. The F values for filtration for the first and second days are 6.29 and 6.54 respectively with 2 and 2 degrees of freedom, table VIII. The 5% point of the F distribution with 2 and 2 degrees of freedom is 19.0.

Since 6.29 and 6.54 are not greater than 19.0, the differences in the sample means for filtration are not significant. The F value for filtration for the sixth day is close to one and no significant differences due to filtration were present at that time. It is unfortunate that the degrees of freedom were not higher as it is likely actual differences due to filtration existed on the first and second days. The small F values for salinity and culture amount for the first and second days and the small F values for all three variables for the sixth day tend to make the F values for filtration for the first two days impressively large, table VIII.

Not forgetting that none of the differences were shown to be significant, it might be well to discuss the higher F values present on the first two days for filtration. These higher F values were due to a greater variance in the sample means for filtration, table VIII. On the first two days when porcelain candle filtration was used rather than sand filtration or no filtration, the sample means were considerably higher. This might be explained by the fact that the porcelain filtration removed most of the protozoa larger than 10 mu. Observation of table VII will show that where the water had been porcelain filtered, not a single protozoa larger than 10 mu was recorded for the first two days. This complete lack of potentially

predatory forms could explain the larger sample means when porcelain filtration was used. On the other hand, by the sixth day, protozoa larger than 10 mu were present in two of the three crocks with porcelain-filtered water. The presence of these larger forms in sufficient numbers to be observable by the sixth day was no doubt due to the multiplying of the few to pass through the porcelain filter or enter these crocks by contamination. The fact that the differences in sample means for filtration were much less by the sixth day might be explained by the fact that these larger protozoa were no longer absent. If the degrees of freedom had been sufficiently high to obtain significant differences for filtration, Analysis of Covariance could have been used to determine if these differences were due to the absence or presence of protozoa larger than 10 mu.

Finally, from the work done here, it seems likely future investigation will show that under the conditions tested, salinity and the amount of food culture used are not so important in the production of Bodo lens as are the predatory relationships caused by the presence of larger organisms. It may be that considerable attention to the controlling of the forms present will be necessary to obtain maximum production of Bodo lens for oyster larvae food.

Second Experiment

The second experiment was designed to determine the effect of different temperatures, different kinds of crock enrichment, and, again, different kinds of water filtration on the production of *Bodo lens* under conditions suitable for the rearing of oyster larvae. In the first experiment, neither different salinities nor the use of different amounts of *Bodo lens* cultures had produced appreciable differences in the numbers of *Bodo lens* in the crocks. Different levels of water filtration produced results that were thought to be possibly different, though these differences were not significant due to an insufficient number of degrees of freedom. It was thus decided that different levels of water filtration would be tested again along with two new variables, temperature and kind of crock enrichment.

The Latin Square statistical design was used in the same manner in the second experiment as it was in the first experiment. Temperatures of 15, 20, and 23 degrees centigrade were tested, and these were obtained in the three series of crocks by using three different water baths (section III). As for the different kinds of crock enrichment, one series of crocks received no enrichment, the second series of crocks received 120 cc of sterile medium #2 each day, and the third series of crocks received 120 cc

TABLE IX

Observations for Second Experiment on Production of *Bodo lens* in Crocks having Varied Conditions

(Recorded as relative numbers of *Bodo lens*; observations in parenthesis other protozoa, greater than 10 mu)

| Crock Enrichment | Temperature in Degrees Centigrade 15 | 20 | 23 |
|---|---|---|---|
| | | One Day | |
| None | P 0 ( 0 ) | S 32 (0.6) | N 26 (1.3) |
| 120 cc Medium | S 7 (0.4) | N 22 (2.1) | P 27 ( 0 ) |
| 120 cc Culture | N 40 (3.4) | P 36 ( 0 ) | S 63 (0.3) |
| | | Two Days | |
| None | P 11 ( 0 ) | S 57 (0.4) | N 25 (2.6) |
| 120 cc Medium | S 85 (0.4) | N 39 ( 5 ) | P 127 ( 0 ) |
| 120 cc Culture | N 74 (1.4) | P 167 ( 0 ) | S 91 (0.4) |
| | | Five Days | |
| None | P 8 (1.4) | S 6 ( 1 ) | N 6 (1.1) |
| 120 cc Medium | S 17 (1.9) | N 13 (1.6) | P 18 (0.9) |
| 120 cc Culture | N 56 (1.3) | P 55 (0.4) | S 14 (0.9) |

Filtration: P, porcelain; S, sand; N, none.

of Bodo lens culture (made in medium #2) each day. It was hoped this would help to determine if mere fertilization of the crocks would have the same effect as adding grown cultures; and, if either or both of these procedures produced any more Bodo lens in the crocks than would be produced if nothing were added. The three kinds of water filtration were again porcelain, sand, and none.

The nine crocks used in the second experiment were the same crocks as those used in the first experiment, and the water in these crocks was aerated and circulated in the same manner. Twelve liters of sea water having a salinity of 25 parts per thousand was placed in each crock. At the beginning of the experiment, 60 cc of Bodo lens culture was placed in each crock to insure the presence of this organism in all crocks. After nine hours, the three series of crocks testing enrichment received their different enrichments, and thereafter received these enrichments every day during the course of the experiment.

Observations were made on the numbers of Bodo lens and the numbers of protozoa larger than 10 mu present in the various crocks at the conclusions of the first, second, and fifth days. These observations were made in the same manner as were those of the first experiment and are recorded as the relative numbers of Bodo lens and protozoa larger than 10 mu present in the various crocks.

TABLE X

Statistics for Second Experiment on Production of *Bodo lens* in Crocks having Varied Conditions

| Levels and Variables | One Day | Two Days | Five Days |
|---|---|---|---|
| Levels | Sample Means as Relative Numbers of *Bodo lens* | | |
| Temperature | | | |
| 15 C | 15.67 | 56.67 | 27.00 |
| 20 C | 30.00 | 87.67 | 24.67 |
| 23 C | 38.67 | 81.00 | 12.67 |
| Enrichment | | | |
| None | 19.33 | 31.00 | 6.67 |
| 120 cc Medium | 18.67 | 83.67 | 16.00 |
| 120 cc Culture | 46.33 | 110.67 | 41.67 |
| Filtration | | | |
| Porcelain | 21.00 | 101.67 | 27.00 |
| Sand | 34.00 | 77.67 | 12.33 |
| None | 29.33 | 46.00 | 25.00 |
| Variables | Calculated F Values with 2 and 2 Degrees of Freedom | | |
| Temperature | 5.39 | 0.44 | 0.82 |
| Enrichment | 9.95 | 2.72 | 4.57 |
| Filtration | 1.73 | 1.29 | 0.88 |

These observations are presented in table IX which illustrates (under any single day) the Latin Square design used.

The statistics for the second experiment on the production of Bodo lens are presented in table X. It will be seen from table X that the means for the various variables are high on the second day as compared to the first day, but that by the fifth day the means have decreased to a level similar to that of the first day. This initial productivity demonstrated by the high means on the second day followed by a decline demonstrated by the low means on the fifth day is the reason a study of this type must be over a period at least as long as the one used here. Conditions resulting in a sustained higher production of food are the only conditions that will benefit the larvae. A changing ecology in larval rearing crocks may decrease the productivity over a period of days unless steps are taken to control the factors operating against the production of the desired food organisms.

The highest calculated F values for the second experiment were obtained at the conclusion of the first day with the variables of temperature and enrichment, table X. This meant that the greatest variance or difference in sample means was obtained here. The calculated F values (with 2 and 2 degrees of freedom) of 5.39 for temperature and 9.95 for enrichment on the first day are

not greater than 19 which is the 5% level of the F distribution with 2 and 2 degrees of freedom; and thus the differences in means measured by these F values are not significant. It is likely that actual differences due to these two variables existed at this time, but the low number of degrees of freedom again made it impossible to declare these differences significant. If actual differences due to temperature and enrichment were assumed to be present at this time, since the F values for temperature and enrichment declined markedly by the second day, it seems likely the first day differences were due to the initial boost given production in the crocks having the higher temperatures and receiving cultures. By the second day, the natural productivity of the crocks may have masked differences created by this initial boost in certain crocks.

The calculated F values for temperature on the second day and on the fifth day, table X, are close enough to one to make it reasonably safe to assume no differences in the numbers of Bodo lens present in the crocks due to different temperatures were present at these times. The calculated F values (with 2 and 2 degrees of freedom) for filtration are close enough to one on all days to make it fairly safe to assume that no differences in means due to different kinds of filtration were present. It may be

seen from table IX that for the first and second days no protozoa larger than 10 mu were observed in any of the crocks having water receiving porcelain filtration. This would indicate actual differences in conditions present in the crocks were created by filtration (as in the first experiment) but that filtration was not an important factor when the crocks were not all receiving cultures (as in the second experiment). These different conditions gave indication of producing differences in Bodo lens production in the first experiment. That these different conditions did not produce difference in Bodo lens in the second experiment may indicate the presence of interaction between the different factors being tested in the two experiments. Such interaction might be that filtration was an important factor when all of the crocks were receiving cultures (as in the second experiment). Here again the need for a Factorial Experiment which would test for interaction and would have a larger number of degrees of freedom was shown.

Enrichment was the one variable that seemed to have a continuing effect on the numbers of Bodo lens present in the various crocks during the period tested in the second experiment. The sample means for the crocks receiving cultures rather than nothing or sterile medium were higher on every day observations were made, table X. That differences in these sample means were not significant is shown by the calculated F values of 9.95, 2.72,

and 4.57 for the first, second, and fifth days respectively, table X. A higher number of degrees of freedom would very likely have shown actual differences in protozoa production to exist on the first and fifth days due to differences in enrichment. On the fifth day the sample mean for crocks receiving cultures is higher than the sample mean for crocks receiving any other treatment; and the calculated F value of 4.57 for enrichment seems rather high when compared to the 0.82 for temperature and the 0.88 for filtration.

Actual differences in the production of Bodo lens under conditions suitable for rearing oyster larvae were probably brought about by some of the different variables studied in the first and second experiments. Improvements in the experimental design such as have been suggested will be necessary before adequate information on such differences can be made available to improve methods being used in rearing oyster larvae. These two experiments have shown the factors affecting protozoa production under larval rearing conditions to be very interrelated and complex; and control of these factors may well be necessary before the optimum conditions for the rearing of oyster larvae can be obtained.

# IX. SUMMARY

This paper covers studies made on the colorless flagellate Bodo lens (O.F. Muller) as food for larvae of the Native Pacific Coast Oyster, Ostrea lurida Carpenter. Studies designed to lead to the rehabilitation of the depleted native oyster fishery of Yaquina Bay were first begun by the Department of Fish and Game Management, Oregon State College, in 1939 under the direction of Professor Roland E. Dimick. Information gained from these studies indicated insufficient successful reproduction was a factor leading to the depletion of the fishery; and in 1946 an investigation was undertaken to determine a method of artificially rearing the larvae of the native oyster to the spat stage. This was first accomplished during the summer of 1949, and the investigation during the summer of 1950 was designed to clarify the role of food organisms and nitrogenous wastes in larval rearing. Bodo lens was selected as being an organism likely to be suitable as food for oyster larvae. The investigation and experimentation with Bodo lens as an oyster larvae food involved the identification, culturing, and growth determination of Bodo lens; a study of the ingestion and assimilation of this organism by larvae; the rearing of native oyster larvae; and a study of the production of protozoa under

conditions suitable for rearing oyster larvae.

Some of the methods and materials used for the investigation had been developed through work done here during the past several years; while some new ones were needed and developed for the particular studies made during the summer of 1950. Work was done with culture media, pure culturing, intra-vitam staining, protozoa counting, larvae counting and measuring, and larvae chlorination. The rearing crocks, temperature control equipment, and water filtration equipment were the same a s had been used here in the past. The larvae were reared in 4 and 12-gallon stoneware crocks having filter-type water siphons for changing the water. The water was gently circulated and aerated by air introduced through glass tubes. The water used was generally filtered through sand.

The identifying and describing of Bodo lens presented some difficulty due to the variability in size and form of this protozoa. Bodo lens has two flagella, one of which trails, an exceedingly plastic form, and one or two posteriorly located vacuoles. It usually varied in size from 2 by 2.5 mu to 6 by 7.5 mu. A flagellate and two ciliates were involved in the studies, and though descriptions of them have been included, they were not identified with certainty.

A study of the growth of Bodo lens in two culture

media was made in order to select the ideal medium for culturing this protozoa for oyster larvae food. The ideal medium was considered to be one which would produce good cultures of Bodo lens while being low in nitrates and other substances which might have end products harmful to the larvae. Two media were tested, medium #1 being higher in starch and nitrate than medium #2. A peak of 2,935,000 Bodo lens per cubic centimeter was reached in medium #1 on the third day, while in medium #2 a peak of 1,925,000 was reached the same day. In spite of the higher peak in medium #1, medium #2 was selected as being the most suited for growing cultures of Bodo lens for oyster larvae food. This was because medium #2 had so much less starch and nitrate than medium #1 that much more medium #2 could be added to the rearing crocks.

The feeding of native oyster larvae on intra-vitam stained protozoa was studied in order to determine whether or not the larvae were able to ingest them. Bodo lens was readily entangled in mucous used by the larvae in feeding; and due to this protozoa's fragile character it was apparently broken up before ingestion, being then taken in as a part of the mucous mass. No intact Bodo lens were observed in the gut of the larvae, but stained particles thought to be the nuclei were. Bodo lens, because of its small size and fragile character, can no

doubt be ingested by native oyster larvae. Whether larger protozoa can be ingested by these larvae may be as dependent on the character of their pellicle as on their size.

Having some idea of the number of Bodo lens ingested by native oyster larvae in a day was desirable so as to be able to adjust the amounts of food cultures used to amounts in line with the actual requirements of the larvae. The change of numbers of Bodo lens in two jars in which there were no larvae was compared to the change of numbers of Bodo lens in two jars in which larvae were present. It was determined in this manner that a native oyster larvae may ingest 12,250 Bodo lens every 24 hours. Due to the nature of this study, this figure can be considered only a rough estimate, but it does provide a working basis for larval rearing investigations until additional information is available.

There was little reason to suspect oyster larvae could not assimilate Bodo lens as this organism is fragile and lacks a protective covering against the digestive enzymes of the larvae. Nevertheless, it was thought desirable to definitely establish the assimilation of this protozoa if possible; and an experiment making use of a radio-active tracer was designed. The experiment failed to demonstrate the assimilation of Bodo lens due to the need for supporting experiments. This experiment did

demonstrate that radio-active materials could be of considerable value in future larval food studies.

Four separate artificial rearing experiments with larvae of the native oyster were conducted during the summer of 1950. The first three experiments failed for various undetermined reasons, while the oyster larvae were reared to the spat stage in the fourth experiment. The first three experiments utilized a Latin Square statistical design to make possible a study of three factors: kind of food culture used, amount of food culture used, and frequency of water change. Four-gallon, stoneware rearing crocks were used for the first three experiments; and though conditions in these crocks were no doubt critical due to their small size, size as such was probably not the cause of these failures.

The fourth experiment in which the oyster larvae were reared to the spat stage was carried out in three, 12-gallon crocks. The only variable studied in the fourth experiment was kind of food culture used, the amount of food culture and the water change being kept constant. One crock received 150 cc of bacteria culture each day, another crock received 150 cc of <u>Bodo lens</u> culture each day, while the third crock received 150 cc of a mixed culture containing bacteria, protozoa, and algae each day.

Five thousand larvae in the straight-hinge stage measuring 179.5 mu were placed in each crock. These larvae grew at a rate of 3 to 4 mu a day in all crocks. Spat settlement began between the twelfth and seventeenth day and continued through the twenty-second day. The larvae settled and became spat when they reached sizes from 233 to 246 mu. No difference in growth between the larvae in the three crocks could be detected, and though the sample of larvae measured was too small to detect slight differences, it is unlikely that differences existed. The reason for this was that the use of three different kinds of food culture apparently failed to create different food conditions in the three crocks. Counts of the relative numbers of protozoa in the three crocks showed the numbers of Bodo lens to be equally low; while the numbers of protozoa larger than 10 mu were more plentiful than Bodo lens in all crocks.

No adequate explanation can be presented for the failure of the first three rearing experiments, nor can reasons be given for the success of the fourth experiment. Many factors that might be detrimental to the larvae become more critical under the artificial conditions in small vessels, and here these factors may be more difficult to control. Nitrogenous wastes, improper salinities, chlorination and other handling of the larvae, predation, and disease were all considered as being potential causes

of failure. Crowding of the larvae resulting in loss due to toxic wastes, increased opportunity for disease, and more competition for food might at times cause failure. Differences in the larvae themselves due to genetics or pre-swarming environment might mean success or failure. Probably no two failures were caused by the same set of factors and this is no doubt the main reason no adequate explanation can be given for either the failures or the successes.

After an organism has been found suitable as food for oyster larvae, it is necessary to be able to produce that organism in large numbers under conditions suitable for the larvae or it will not benefit artificial oyster larvae rearing. The larval rearing experiments indicated that merely adding cultures of Bodo lens to the crocks would not insure its presence in large numbers. A Latin Square statistical design was used in two experiments to determine the factors affecting the production of Bodo lens. Salinity, amount of food culture used, and type of water filtration were tested in the first experiment. Temperature, kind of crock enrichment, and water filtration, again, were tested in the second experiment.

Due to interaction and insufficient degrees of freedom (sample size too small), the results of the two experiments were not significant, though actual differences

caused by some of the various treatments probably existed. The design of future experiments of this type could be considerably improved through the use of a Factorial Experiment which would detect interaction and increase the degrees of freedom to a level where significant results should be obtained.

These experiments indicated that salinity and the amount of Bodo lens culture used might not be too important in the production of Bodo lens. Water filtration, removing larger predatory protozoa, might be important under some conditions. Temperature over a period of days may not be so important as it is when cultures are first added to the rearing crocks. The addition of cultures of Bodo lens to the rearing crocks may help to maintain a higher level of abundance of this organism than would fertilization of the crocks. The factors determining the production of protozoa are complex, and additional studies of this type will be necessary until sufficient information can be obtained to make it possible to control these factors so as to produce optimum food conditions for oyster larvae.

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